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Branched-chain amino acid biosynthesis inhibitors: Herbicide efficacy is associated with an induced carbon–nitrogen imbalance

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SUMMARY

Acetolactate synthase (ALS; EC 4.1.3.18) and ketol-acid reductoisomerase (KARI; EC 1.1.1.86) are two consecutive enzymes in the biosynthesis of branched-chain amino acids. Several commercial herbicides inhibit ALS as their primary site of action. KARI has also attracted attention as a potential target for herbicides. Although potent and selective inhibitors of KARI have been discovered, these inhibitors display less herbicidal activity than ALS-inhibiting herbicides. To obtain a better understanding of these findings, we have compared the physiological effects induced in pea plants after KARI or ALS inhibition. Although, both types of inhibitors induce growth arrest and photosynthesis inhibition, plant death occurs more rapidly under ALS inhibition than KARI inhibition. Carbohydrates accumulated in the leaves and roots following treatments with both inhibitors. The carbohydrate accumulation in the leaves occurred as a consequence of a decrease in sink strength. In contrast, the free amino acid content was only affected through ALS inhibition. These results indicate that although KARI and ALS inhibition block the same biosynthetic pathway and exert common effects on carbon metabolism, nitrogen metabolism is more affected via ALS than KARI inhibition. Thus, metabolic alterations in nitrogen metabolism induced through ALS inhibitors might contribute to the increased efficacy of these chemicals as herbicides.

Introduction

Valine, leucine and isoleucine form the small group of branched-chain amino acids (BCAAs). Bacteria, archaea, fungi and plants synthesise BCAAs, while animals, including humans, are not able to synthesise BCAAs de novo and have to acquire these amino acids through their diets.

A unique feature of BCAA biosynthesis is that valine and isoleucine are synthesised in two parallel pathways through a single set of four enzymes, acetolactate synthase or acetohydroxy acid synthase (ALS; EC 4.1.3.18), ketol-acid reductoisomerase or acetohydroxy acid isomeroreductase (KARI; EC 1.1.1.86), dihydroxy-acid dehydratase and branched-chain aminotransferase, which catalyse the formation of these two amino acids using different substrates. ALS catalyses the condensation of either two molecules of pyruvate to form acetolactate or one molecule of pyruvate and one molecule of 2-ketobutyrate to form 2-aceto-2-hydroxybutyrate (Singh, 1999). KARI catalyses the reductive isomerisation of AL to 2,3-dihydroxy-3-isovalerate or the conversion of 2-aceto-2-hydroxybutyrate to 2,3-dihydroxy-3-methylvalerate (Durner et al., 1993).ALS is the best-studied enzyme involved in BCAA metabolism because it is the target for commercially successful herbicides. There are five different chemical classes of herbicides that inhibit ALS: sulfonylureas, imidazolinones, triazolopyrimidines, sulfonylaminocarbonyl triazolinones and pyrimidinyl-oxy-benzoates. These chemicals have emerged since the 1980s and have been demonstrated as potent, selective, broad-spectrum herbicides and inhibitors of plant growth. Due to their high efficacy, these inhibitors are used at low field application rates in the range of grams per hectare, while other herbicides are applied in the range of kilograms per hectare. The efficacy and potency of ALS inhibitors have ensured the continued success of these herbicides, which have rapidly challenged, and in some instances replaced, traditional products, particularly in cereals and soybeans (Cobb and Reade, 2010). Currently, ALS inhibitors represent the second largest class of active herbicidal products and are traditionally used in weed control for many non-transgenic crops. The combination of the widespread usage of ALS-inhibiting herbicides and the development of resistance to these herbicides has resulted in the evolution of 127 ALS inhibitor-resistant weed species (Heap, 2012). In most cases, where the underlying resistance mechanism has been investigated, resistance occurs through point mutation(s) in the ALS gene that reduce the sensitivity of the enzyme to herbicides (Powles and Yu, 2010). The characteristic attributes of ALS inhibitors (i.e., their low toxicity and high efficacy)

Abbreviations: ALS, acetolactate synthase; BCAA, branched-chain amino acid; CPCA, cyclopropane-1,1-dicaboxylic acid; KARI, ketol-acid reductoisomerase; IM, imazethapyr.

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indicate that the inhibition of BCAA biosynthesis is a suitable target for herbicidal action; however, the rapid evolution of weed resistance to ALS inhibitors limits the applicability of these compounds and represents a significant challenge. New chemistry and novel herbicides with unique modes of action are needed to manage the evolution of resistance of weeds to existing herbicides (Gerwick, 2010; Duke, 2012).

A major research effort has emerged for the development of new products to inhibit the synthesis of BCAAs through the inhibition of another enzyme in the same pathway, thereby targeting inhibitors that might behave as herbicides, similar to ALS inhibitors, but function via a new mode of action, thereby avoiding the selection pressure on ALS. Thus, efficient inhibitors of all the enzymes in the branched-chain amino acid pathway, with similar *in vitro* Ki values have been identified, but only inhibitors of ALS have been commercialised (Wittenbach and Abell, 1999; Duke, 2012). Compounds such as 2-dimethylphosphinoyl-2-hydroxyacetic acid (Hoe 704) and N-hydroxy-N-isopropyloxamate (IpOHA) are potent and selective competitive inhibitors of KARI (Schulz et al., 1988; Aulabaugh and Schloss, 1990) but only display minor herbicidal activity. Two explanations have been suggested for their lack of potency in the field: these compounds exhibit slow binding inhibition of KARI in solution and act as competitive inhibitors that prevent the optimal effects of the inhibitor through the accumulation of substrates (Leung and Guddat, 2009). Cyclopropane-1,1-dicaboxylic acid (CPCA) acts as a KARI inhibitor in *Escherichia coli in vitro* and in some weeds *in vivo* (leaf disks) (Gerwick et al., 1993). This inhibition has been enzymatically characterised in rice, showing that CPCA mimics the transition state of the reaction. Nevertheless, this inhibition is substantially less potent *in vivo* than suggested by the effects on KARI *in vitro* (Lee et al., 2005), even when different substitutions are tested (Liu et al., 2007, 2011).

After an inhibitor reaches its primary target, several physiological effects are triggered within the plant. Although the biochemical mechanism underlying the blocking of ALS activity through ALS inhibitors has been extensively studied, little is known regarding the physiological process underlying plant death resulting from inactivated ALS.

In general, after the target of an inhibitor has been affected, death occurs as a result of various causes. First, death could be associated with the lack of end products generated from the inhibited pathway. Second, plant death could result from an accumulation or increased availability of the substrates of the inhibited enzymatic pathway. Third, lethality could be associated with several side reactions triggered after the inhibition of the target. Thus, the differences in herbicidal efficacy detected between ALS and KARI inhibitors might reflect the physiological processes leading to plant death. The two types of inhibitors should have similar effects if lethality results from the lack of end products (i.e., valine, leucine and isoleucine), but these compounds would be expected to perform differently if plant death is more associated with substrate accumulation or side effects.

An impairment of carbon and nitrogen metabolism has been reported in treated plants following ALS inhibition (Zabalza et al., 2004, 2005, 2006). In contrast, although several aspects of the physiological response after KARI inhibition have been reported (Wittenbach and Abell, 1999), no exhaustive studies on the effects of these inhibitors on carbon or nitrogen metabolism have been performed.

The aim of this study was to characterise the effects of a KARI inhibitor on carbon and nitrogen metabolism compared with an ALS inhibitor. To this end, pea plants were treated with imazethapyr (IM, an ALS inhibitor) or CPCA (a KARI inhibitor), and the effects of these compounds on carbohydrate and amino acid contents were compared, as these metabolic indicators are altered following ALS inhibition. CPCA was supplied at two different concentrations to determine whether the physiological responses were dose dependent.

Materials and methods

Plant material and treatment application

Pisum sativum L. cv. Snap Sugar Boys were grown in an aerated hydroponic culture in a growth chamber (Zabalza et al., 2005). When the plants were 12 days old, the tanks were divided into four groups: one control group and three inhibitor-treated groups. The Acetolactate synthase (ALS)-inhibiting herbicide imazethapyr (IM) (commercial formula, Pursuit 10, BASF Española SA Barcelona, Spain) was applied to the nutrient solution at a concentration of 69 μM (20 mg active ingredient L^{-1}). Cyclopropane-1,1-dicaboxylic acid (CPCA) (1,1-cyclopropanedicarboxylic acid), a ketol-acid reductoisomerase (KARI) inhibitor, was applied to the nutrient solution at concentrations of 200 or 500 μM (26 and 65 mg L^{-1}, respectively). The nutrient solution was replaced every 3 days to maintain constant inhibitor concentrations in the nutrient solution throughout the experiment. The experiments were repeated in two independent series, with three replicate tanks per treatment.

The pea growth was determined using root and shoot lengths as the best indicators of growth inhibition. For metabolite analyses, leaf and root samples were collected at 0, 1, 3, 7, 10 and 15 days after treatment. Plants were also harvested at 22 days after both CPCA treatments. At harvest, leaf and root samples were collected, immediately frozen in liquid nitrogen and stored at −80 °C until further analysis.

Gas exchange measurements

The net CO_2 assimilation rate was measured in the youngest fully expanded leaf of intact plants using the portable ADC-LCi Infrared Gas Analyzer System (ADC BioScientific Ltd., Herts England). The leaf area was determined using the Li-3000 system (Li-Cor, Lincoln, Nebraska, USA).

Determination of metabolite contents

Acetolactate was extracted from the leaves and roots and analysed as previously described (Zabalza et al., 2005). The extraction and quantification of quinate through ion chromatography and amino acids via capillary electrophoresis was performed according to Orcaray et al. (2010). The glucose, fructose and sucrose contents were determined in ethanol-soluble extracts, and the ethanol-insoluble residue was extracted for starch analysis. The concentrations of starch and soluble sugars were determined using capillary electrophoresis according to the methods of Zabalza et al. (2004).

Statistical analysis

The mean values were calculated using the samples obtained from individual plants as replicates. The results were subjected to a separate one-way ANOVA for each day of treatment SPSS 16.0). The means were separated using the least significant difference method ($p < 0.05$, Fisher protected). Significant differences between each treatment and the control plants (not-treated plants) are highlighted in the figures using a different symbol for each treatment. When the obtained values were percentages, a prior transformation to arcsine $\sqrt{(x/100)}$ was applied.