Journal of Plant Physiology 168 (2011) 1568–1575

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.de/jplph

Unraveling the role of fermentation in the mode of action of acetolactate synthase inhibitors by metabolic profiling

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ARTICLE INFO

Article history:
Received 24 January 2011
Received in revised form 16 February 2011
Accepted 22 February 2011

Keywords:
Acetolactate synthase inhibitors
Fermentation
Imidazolinone
Hypoxia
Pyruvate
Metabolic profiling

SUMMARY

Herbicides that inhibit branched chain amino acid biosynthesis induce aerobic fermentation. The role of fermentation in the mode of action of these herbicides is not known, nor is the importance of this physiological response in the growth inhibition and the lethality caused by them. Metabolic profiling was used to compare the effects of the herbicide imazethapyr (IM) on pea plants with two other treatments that also induce fermentation: hypoxia and the exogenous supply pyruvate for seven days. While hypoxic roots did not show internal anoxia, feeding pyruvate or applying IM to the roots led to internal anoxia, probably related to the respiratory burst detected. The three treatments induced ethanol fermentation, but fermentation induced following herbicide treatment was earlier than that following pyruvate supply and was not associated with a decrease in the energy status. No striking changes were detected in the metabolic profiling of hypoxic roots, indicating that metabolism was only slightly impaired. Feeding pyruvate resulted in marked succinate accumulation and a general amino acid accumulation. IM-treated roots showed a general accumulation of glycolytic metabolites upstream of pyruvate, a decrease in some TCA intermediates and an increase in the free amino acid pool sizes. All treatments caused GABA and putrescine accumulation. Our results indicate that IM supply impairs carbon/nitrogen metabolism and this impaired metabolism is likely to be related to the growth arrest detected. As growth is arrested, carbohydrates and glycolytic intermediates accumulate and energy becomes more available.

Introduction

The mechanism of action of a group of five chemical classes of herbicides is the inhibition of the enzyme acetohydroxyacid synthase (E.C.4.1.3.18), also known as acetolactate synthase (ALS). This enzyme uses pyruvate as its major substrate and catalyses the first common reaction in the biosynthetic pathway of branched-chain amino acids (BCAA; valine, leucine and isoleucine) (Singh, 1999).

Although the mode of action of these herbicides has not been completely clarified, several physiological effects following their application have been reported. Following inhibition of ALS, plants respond quickly by increasing protein turnover to renew BCAAs, so that the BCAA pool does not decline to a level that would affect protein synthesis (Wittenbach and Abell, 1999; Royuela et al., 2000), leading to an increase in the total free amino acid pool (Shaner and Reider, 1986; Orcaray et al., 2010). A rapid increase in the level of carbohydrates in leaves of plants treated with ALS-inhibitors has been reported (Shaner and Reider, 1986), and this effect was related to decreased photoassimilate translocation to sink tissues (Bestman et al., 1990) due to a decreased sink strength (Zabalza et al., 2004). Furthermore, BCAA inhibitors have been described to elicit aerobic fermentation (Gaston et al., 2002; Zabalza et al., 2005).

While fermentation is usually related to low oxygen conditions, ethanol fermentation was induced in roots of pea plants treated with BCAA inhibitors under normoxic conditions. This induction has been proposed to be related to pyruvate, which is the common substrate of both the first enzyme of ethanolic fermentation (pyruvate decarboxylase, PDC) and ALS (Gaston et al., 2002; Zabalza et al., 2005). Inhibition of the flux through the BCAA pathway by the herbicides could lead to the diversion of carbon into the fermentation pathway, leading to the accumulation of ethanol. Alternatively, the induction of fermentation following ALS inhibition can be regarded as a response to abiotic stress, as has been reported for other

Abbreviations: ADH, alcohol dehydrogenase; ALS, acetolactate synthase; BCAA, branched-chain amino acid; GABA, γ-aminobutyric acid; IM, imazethapyr; PDC, pyruvate decarboxylase.

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doi:10.1016/j.jplph.2011.02.015

stresses not related to the lack of oxygen (Dolferus et al., 1994; Kürsteiner et al., 2003).

Despite the fact that fermentative induction following the inhibition of BCAA biosynthesis has been well established (as has been reported by different chemical classes of herbicides in Zabalza et al., 2005), the physiological role of such induction in the toxicity caused by the herbicides remains unclear. Ethanol fermentation induction following ALS inhibition can be related to an increased availability of pyruvate, the common substrate of PDC and ALS. Indeed, induction of fermentation has been reported following feeding pyruvate (Zabalza et al., 2009). Nevertheless, that study revealed that PDC induction cannot simply be explained by increased substrate availability. Feeding pyruvate to the roots led to an increase of the oxygen consumption rate after 24 h, which ultimately led to anoxia. That said, fermentative metabolism was only activated one day later, when the energy charge of the tissue was decreased, indicating that alcohol fermentation appeared to be primarily induced by a drop in the energy status of the tissue rather than by a low oxygen concentration (Zabalza et al., 2009).

It is unknown whether induction of fermentation following ALS inhibition shares a common pattern with fermentation induction following pyruvate supply. Moreover, induction of fermentation following ALS inhibition could be more similar to hypoxic stress, where fermentation induction is not preceded by internal anoxia. In this sense, it is only known that ALS inhibitors cause a respiratory burst after three days of treatment (Gaston et al., 2003), although the effect of these herbicides on internal oxygen availability and energy charge is unknown. Comparison of hypoxia and feeding pyruvate with ALS inhibition may help to elucidate the physiological role of the induction of fermentation in the mode of action of BCAA biosynthesis inhibiting herbicides. In this respect, metabolic profiling via gas chromatography–mass spectroscopy (GC–MS) was performed as a tool to evaluate and compare metabolic changes induced by the various treatments.

The aim of this study was to evaluate the role of fermentation in the mode of action of the inhibitors of BCAA biosynthesis by comparing the effects of supplying the herbicide imazethapyr (IM; an ALS inhibitor) with two other treatments that also induce fermentation: hypoxia and exogenous pyruvate. To achieve this purpose, internal oxygen content, respiration, redox status, energy charge, ethanolic fermentation and the levels of some metabolites (detected by gas chromatography–mass spectrometry) were assessed in roots of pea plants.

Materials and methods

Plant material

Pisum sativum L. (cv. Snap Sugar Boys) was grown in hydroponic culture as described in Zabalza et al. (2005). When plants were 12 days old, the tanks were divided into four groups. One group was left as the control with no treatment and the other groups were treated with IM herbicide, hypoxia or pyruvate as follows. IM herbicide (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}). Hypoxia was applied by aerating the nutrient solution with an oxygen–nitrogen mixture in order to achieve an oxygen concentration in the nutrient solution that is in equilibrium with air containing 4% oxygen (v/v) only (25% of air saturation). Pyruvate was supplied to the nutrient solution at a final concentration of 8 mM and replenished every two days. The nutrient solution of control, IM and pyruvate-treated plants was continuously aerated. The nutrient solution of control, IM and hypoxia treatments was replaced every four days.

Roots were harvested one, three, and seven days after treatments, and when indicated, also after two days from onset of the treatment. Samples were taken in the middle of the light period, immediately frozen in liquid nitrogen and stored at −80 °C.

Oxygen and respiration measurements

The oxygen concentration inside the roots was measured with a fine oxygen sensitive optode with a tip diameter of approximately 30 μM, and connected to a fiber optic oxygen meter (MicroxTX, Presens, Regensburg, Germany) as in van Dongen et al. (2003). Respiratory oxygen consumption was measured using Clark-type electrodes (Hansatech Oxygraph, H.Saur Laborbedarf, Reutlingen, Germany) as described in Zabalza et al. (2009).

Metabolite and fermentative enzymes

Redox status (expressed as NADH/NAD^+ ratio) was calculated after measuring pyridine nucleotide contents as follows: grounded root samples (0.1 g) were homogenized in either 5% tricloroacetic acid (acid extract) or 0.1 M NaOH (alkaline extract). Extracts were boiled for 6 min and centrifuged at 20,000 × *g* for 30 min. Supernatants were neutralized by addition of small amounts of 1 M NaOH or 1M HCl, respectively. The oxidized forms were determined in the acid extract and the reduced forms were determined in the alkaline extract by the enzymatic cycling method according to Matsumura and Miyachi (1980).

Adenylate energy charge (AEC) was calculated as (ATP + 0.5 ADP)/(ATP + ADP + AMP) after measuring AMP, ADP and ATP content by capillary electrophoresis as described in Galvez et al. (2005). Starch and sucrose content was analyzed as described in Zabalza et al. (2004).

PDC (E.C.4.1.1.1) and alcohol dehydrogenase (ADH, E.C.1.1.1.1) activities were assayed in desalted extracts as described by Gaston et al. (2002) and protein gel blot analysis were performed as described in Zabalza et al. (2009).

Metabolite analysis (glucose-6P, fructose-6P, 3-PGA, pyruvate, citrate, 2-ketoglutarate, succinate, malate and amino acids) by GC–MS was essentially carried out as described by Roessner et al. (2001) and Steinfath et al. (2010).

Statistical analysis

Each mean value was calculated using samples from different single plants as replications. Results shown in this paper were subjected to separate one-way ANOVA analysis for each day. Means were separated using the least significant difference (LSD Fisher protected, $p < 0.05$). Significant differences between each treatment and control plants (not-treated plants) are highlighted in the figures with different symbols for each treatment.

For metabolite measurements by GC–MS, the variation between mean values of the difference between control and treatment was evaluated using the Student's *t*-test and confirmed to be significant when $p < 0.05$.

Results

Growth, lethality and root oxygen concentrations

The three treatments studied arrested root elongation after one day from the onset of the treatments, while shoot growth inhibition was only caused by IM and pyruvate treatments (data not shown). IM and pyruvate supplied to the nutrient solution caused plant death after three weeks of treatment. The hypoxic treatment had no effect on the survival of the pea plants.

Oxygen concentrations were measured through a transect of the root from the outside to the centre of the root, in control and treated roots after one, three, and seven days of treatment (Fig. 1). One day