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Fermentative Metabolism Is Induced by Inhibiting Different Enzymes of the Branched-Chain Amino Acid Biosynthesis Pathway in Pea Plants

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The inhibition of branched-chain amino acid (BCAA) biosynthesis was evaluated in pea plants in relation to the ability for induction of fermentative metabolism under aerobic conditions. Chlorsulfuron and imazethapyr (inhibitors of acetolactate synthase, ALS, EC 4.1.3.18) produced a strong induction of pyruvate decarboxylase (PDC, EC 4.1.1.1) and alcohol dehydrogenase (ADH, EC 1.1.1.1) activities and a lesser induction of lactate dehydrogenase (LDH, EC 1.1.1.27) and alanine aminotransferase (AlaAT, EC 2.6.1.2) activities in roots. Inhibition of the second enzyme of the BCAA biosynthesis (ketol-acid reductoisomerase, KARI, EC 1.1.1.86) by Hoe 704 (2-dimethylphosphinoyl-2-hydroxyacetic acid) and CPCA (1,1-cyclopropanedicarboxylic acid) enhanced fermentative enzyme activities including PDC, ADH, and AlaAT. Fermentative metabolism induction occurring with ALS- and KARI-inhibitors was related to a higher expression of PDC. In the case of KARI inhibition, it is proposed that fermentation induction is due to an inhibition of ALS activity resulted from an increase in acetolactate concentration. Fermentative metabolism induction in roots, or at least ethanolic fermentation, appeared to be a general physiological response to the BCAA biosynthesis inhibition.

KEYWORDS: Acetolactate synthase; ketol-acid reductoisomerase; aerobic fermentation; branched-chain amino acid

INTRODUCTION

Acetolactate synthase or acetohydroxyacid synthase (ALS; EC 4.1.3.18) is the first enzyme of the biosynthetic pathway of branched-chain amino acids (BCAAs; valine, leucine, and isoleucine). ALS catalyzes the condensation of either two molecules of pyruvate to form acetolactate (AL) or one molecule of pyruvate with one molecule of 2-ketobutyrate to form 2-aceto-2-hydroxybutyrate (*1*). The second enzyme in the BCAA biosynthesis is ketol-acid reductoisomerase (KARI; EC 1.1.1.86) which catalyzes the reductive isomerization of AL to 2,3-dihydroxy-3-isovalerate or the conversion of 2-aceto-2-hydroxybutyrate to 2,3-dihydroxy-3-methylvalerate (*2*).

Imazethapyr (IM), which belongs to the imidazolinone class of herbicides, and chlorsulfuron (CS), which belongs to the sulfonylurea class, share the same mechanism of action: inhibition of ALS activity. The plant death resulting from ALS inhibition is very slow. It can take several weeks for plants to die from the appearance of first symptoms (growth inhibition) (*3*). To explain growth inhibition and plant death induced by ALS-inhibitors, several studies have been carried out in recent years (*4*−*7*). Nevertheless, the precise mechanism still has not been elucidated.

Previous studies showed an enhancement of fermentative enzyme activities, including pyruvate decarboxylase (PDC), alcohol dehydrogenase (ADH), lactate dehydrogenase (LDH), and alanine aminotransferase (AlaAT) in plants, after IM application in aerobic conditions (*5*). The remarkable increase in specific activities of these enzymes detected in roots of IM-treated plants was not caused by a mitochondrial respiration inhibition, as oxygen uptake was only slightly affected (*5*, *6*). It has been proposed that the increase of these activities may help to metabolize pyruvate, which may be accumulated because of ALS inhibition. The physiological implications of the induction of fermentative metabolism are unknown. It is possible that fermentative metabolism might help the plant to cope with the herbicide. On the other hand, since ethanol and lactate are toxic to plants, fermentative metabolism may contribute to a slow plant death (*8*). It remains to be evaluated whether fermentation induction in roots is a physiological effect of all classes of ALS-inhibitors or just IM.

Furthermore, the inhibition of BCAA biosynthesis at the level of ALS enzyme as the target of herbicides raised the issue of whether another enzyme of the same pathway might be the target for inhibitors that, therefore, may behave as herbicides. Experimental compounds such as 2-dimethylphosphinoyl-2-hydroxy-acetic acid (Hoe 704) and *N*-hydroxy-*N*-isopropyloxamate

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(IpOHA) are very potent and selective inhibitors of KARI (*9*, *10*). However, these compounds show a poor herbicidal activity compared to ALS-inhibitors. This has been attributed to the type and reversibility of the enzyme−inhibitor binding and the quantity of KARI enzyme in plants compared to ALS (*2*, *10*−*12*). Thus, it remains to be determined whether the inhibition of this biosynthetic pathway at the level of different enzymes may lead to the same physiological consequences.

The objectives of this study were to evaluate the likelihood of fermentative metabolism as a general effect of ALS-inhibitors and to assess whether this effect is shared by KARI inhibitors. To achieve these purposes, two ALS-inhibitors (IM and CS) and two KARI-inhibitors (Hoe 704 and CPCA) were applied to pea plants and their effects on ADH, PDC, LDH, and AlaAT activities were assessed.

MATERIALS AND METHODS

Chemicals and Apparatus. Commercial IM (Pursuit) was supplied by BASF SA (Barcelona, Spain) and commercial CS (Glean) was supplied by Dupont (Dupont Ibérica SA, Barcelona, Spain). Hoe 704 was a generous gift from Dr. R. Dumas. All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Polyacrylamide gels were run using the Phast System (Pharmacia LKB, Biotechnology AB, Uppsala, Sweden) or the Mini-Protean 3 (Bio-Rad, Hercules, U.S.). All spectrophotometric determinations were carried out in a double-beam spectrophotometer (Lambda 3B Spectrophotometer, Perkin-Elmer, Norwalk, CT).

Plant Material and Treatment Application. Pea seeds (*Pisum sativum* L. cv. Snap Sugar Boys) were surface sterilized as described in ref *13* prior to germination. Seeds were placed in vermiculite for 96 h at 26 °C in darkness, and then, homogeneously germinated seeds were transferred to hydroponic tanks (2.7 L) and were placed in a controlled environment (300 μmol m^{-2} s^{-1} PPF; 14 h photoperiod, 25/18 °C and 60/70% RH day/night). The nutrient solution (*14*) was supplemented with 10 mM KNO_3, was aerated continuously (700 mL $tank^{-1}$ min^{-1}), and was replaced every 3 days. ALS-inhibitors or KARI-inhibitors were applied to the nutrient solution when plants were 12-days old.

To compare the physiological responses to different inhibitors, it is essential to use doses that lead to similar growth arrest. Thus, the effect of ALS-inhibitors (IM and CS) and KARI-inhibitors (Hoe 704 and CPCA) on pea growth was evaluated using root and shoot lengths as indicators of growth inhibition.

ALS-Inhibitors. IM was applied at 69 μM (20 mg active ingredient L^{-1}). CS was applied at 5.6, 14, and 28 nM (2, 5, and 10 μg active ingredient L^{-1}, respectively).

KARI-Inhibitors. Preliminary studies were conducted to determine dose effect of KARI-inhibitors on growth similar to those described after the supply of ALS-inhibitors (*7*). Hoe 704 was applied at 65 μM (10 mg L^{-1}) and 195 μM (30 mg L^{-1}). CPCA (1,1-cyclopropanedicarboxylic acid) was applied at 50, 100, and 200 μM (6.5, 13, and 26 mg L^{-1}, respectively).

To determine if the different treatments actually lead to plant death, plants were grown under the corresponding treatment for 3 weeks, but the fundamental study was carried out during the first 7 days of treatment, with harvest at 0, 1, 3, and 7 days, and when indicated also at 5 days or 14 days. Root samples were harvested and were immediately frozen in liquid nitrogen and were stored at −80 °C for enzyme analysis. In CPCA-treated plants, both leaf and root samples were taken for AL analysis.

Enzyme Activities. Root samples (0.4 g) were homogenized, and the desalted extract was used to assay the following activities: pyruvate decarboxylase (PDC; EC 4.1.1.1), lactate dehydrogenase (LDH; EC 1.1.1.27), and alanine aminotransferase (AlaAT; EC 2.6.1.2) as described in ref *5*. Alcohol dehydrogenase (ADH; EC 1.1.1.1) was assayed as described in ref *15*. Protein concentration was determined using the method described in ref *16*.

Polyacrylamide Gel Electrophoresis and Enzymatic Activities Staining. Root samples were homogenized using a similar procedure described in ref *5* with minor modifications. Roots were extracted in 2.5 volumes of buffer. Samples for PDC and LDH activity analysis were concentrated by 8 times in a Savant speed vacuum concentrator (Savant Vac, Famingdale, NY).

PDC activity was visualized in native polyacrylamide gels following the procedure described by ref *17* using a Mini-Protean 3 system. The running gel was 5−15% (w/v) acrylamide gradient and the stacking gel contained 4% (w/v) acrylamide. PDC activity was visualized within the gel by following the method described in ref *18* with minor modifications. Gels were immersed in 0.2 M sodium phosphate (pH 6.2) containing 5 mM TPP, 5 mM $MgCl_2$, 13 mM sodium pyruvate, and 2% β-mercaptoethanol for 1 h. Activity bands appeared after incubation overnight in the dark in the same mixture containing 0.5% pararosaniline (basic red).

Native electrophoresis for ADH detection was run in a 12.5% polyacrylamide gel (Phast-System). ADH specific staining was accomplished by a modification of the procedure described by ref *19*. Activity bands appeared after incubation of the gel in the dark for 15 min in a solution composed of 25 mM TrisCl (pH 8), 0.8% (v/ v) ethanol, 0.144 mM nitro blue tetrazolium, 0.65 mM phenazine methosulfate, and 0.24 mM NAD^+.

LDH activity staining was carried out using 12.5% polyacrylamide (Phast-system). LDH bands were stained by following the method described in ref *20*. Gels were incubated overnight in the dark in a solution containing 0.15 M Tris-Cl (pH 8), 208 mM lithium L(+)-lactate, 3 mM NAD, 2 mM $MgCl_2$, 2.5 mM nitro blue tetrazolium, 0.16 mM phenazine methosulfate, and 3 mM pyrazole (added to inhibit trace staining of ADH bands).

In all native gel electrophoresis, separate lanes were run and stained for total protein using Coomasie G-250 (Pierce, Rockford, U.S.) to confirm equal loading. A lane containing a native molecular weight marker kit (HMW calibration kit, Amersham Biosciences, U.K.) was run under the same conditions to estimate the native molecular weight of PDC, ADH, and LDH.

PDC Western Immunoblotting. Root protein extracts were prepared for 7.5% SDS polyacrylamide gel electrophoresis (*21*), blotting onto PVDF membranes and treated with PDC antibodies kindly provided by Dr. König (Martin Luther University). The PDC antibody was raised in rabbits against PDC enzyme purified from germinating pea seeds (*22*). In all cases, protein concentrations were determined according to the method of Bradford (*16*) to ensure equal loading of samples. The membranes were incubated overnight in PDC antibody (dilution 1:2000). Antirabbit IgG peroxidase (Sigma A0545; dilution, 1:200 000) was used as secondary antibody and visualization of bands was performed using ECL plus Chemiluminescence Kit (Amersham Biosciences) according to the manufacturer's instructions.

AL Determination. AL was extracted according to the procedure described in ref *9* with minor modifications. Frozen material was extracted three times with distilled water at 80 °C for 20 min each. AL content was determined after its conversion to acetoin by incubation with 0.6 N H_2SO_4 at 60 °C for 15 min. Acetoin was determined according to the procedure described in ref *23*.

Statistical Analysis. For each inhibitor, results presented here are the mean of two independent experiments with four replicates (four different tanks per treatment). For each sample, different single plants were selected. Data were subjected to one-way ANOVA analyzing separately each day, and means were separated using the least significant difference ($p < 0.05$, Fisher protected). Significant differences between each treatment and control plants (not-treated plants) are highlighted in the figures.

RESULTS

Effects of BCAA-Inhibitors on Pea Growth. The supply of 69 μM IM to the nutrient solution produced about 25% root growth inhibition after 3 days and about 50% after 7 days (**Figure 1**). Shoot growth was less affected than roots by IM treatment, showing about 25% inhibition after 7 days of treatment (**Figure 2**). This was used as the reference to set up the concentration of the rest of the inhibitors. Plants treated with

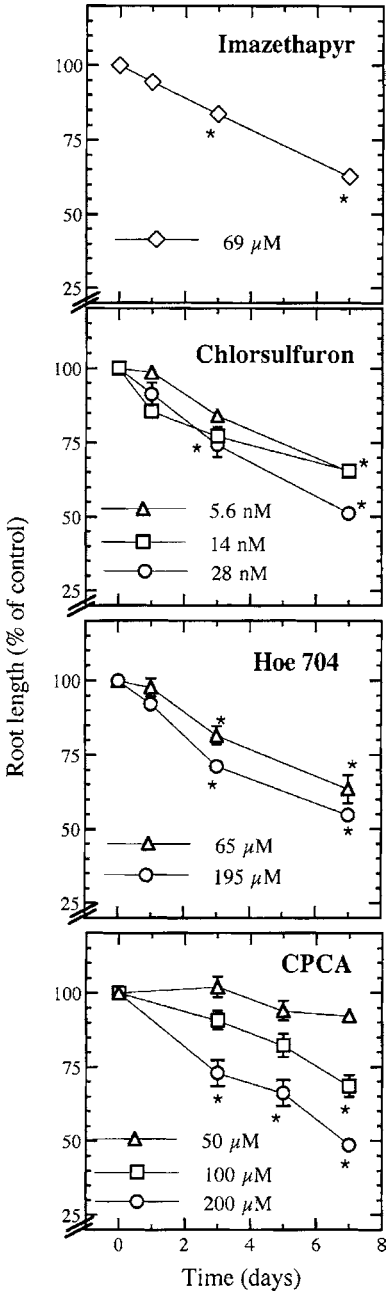

Figure 1. Effect of different ALS- and KARI-inhibitors on pea root length, expressed as the percentage relative to the control without inhibitors. Means ± SE (n = 10). * close to each symbol denotes significant difference from the control at a given day.

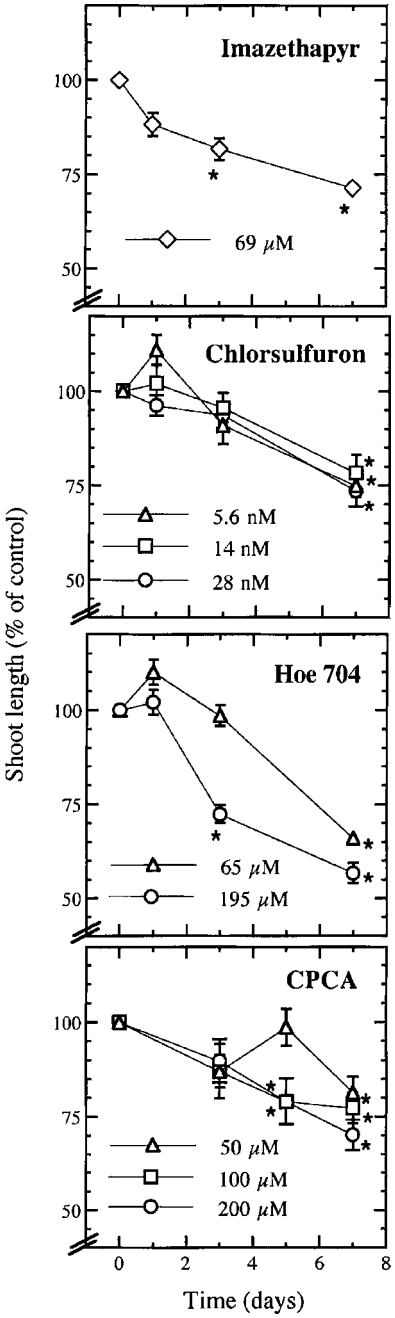

Figure 2. Effect of different ALS- and KARI-inhibitors on pea shoot length, expressed as the percentage relative to the control without inhibitors. Means ± SE (n = 10). * close to each symbol denotes significant difference from the control at a given day.

5.6, 14, and 28 nM CS presented a growth inhibition comparable to 69 μM IM (**Figures 1** and **2**). The highest CS concentration used (28 nM) and 69 μM IM caused plant death after 22−23 days from the onset of treatment (data not shown).

The KARI-inhibitor Hoe-704 caused a similar root growth inhibition at the doses 65 and 195 μM. These effects were halfway between those caused by 69 μm IM and 28 nM CS (**Figure 1**). The KARI-inhibitor CPCA at higher doses (200 μM) was more comparable to 69 μM IM and 28 nM CS (**Figures 1** and **2**).

Inhibition of ALS and Fermentative Metabolism. The time course of root fermentative activities of control and IM or CS treated plants is shown in **Figure 3**. Specific activities of ethanolic fermentative enzymes PDC and ADH were significantly increased by IM treatment. PDC activity was enhanced within 1 day and increased up to 5.4 times after 7 days. The lower CS concentration (5.6 nM) was unable to induce ethanolic fermentation, but 14 and 28 nM CS induced ethanolic fermentative activities similarly to IM treatment. PDC and ADH activities were increased up to 3 times of the levels of control plants by 28 nM CS. The specific activity of LDH was also increased by IM after 7 days of treatment. Among the CS doses studied, only the highest caused an increase of LDH activity. The activity of AlaAT, that uses pyruvate as substrate like PDC and LDH, was also significantly increased by IM treatment. CS at all tested concentrations enhanced AlaAT activity after 3 days even though the enzyme activity declined with time in control plants.

The effects of 69 μM IM and 28 nM CS in fermentative enzymatic activities of roots were compared by activity staining after native PAGE electrophoresis of crude extracts. Electrophoresis showed a single enzymatic band of PDC, with an

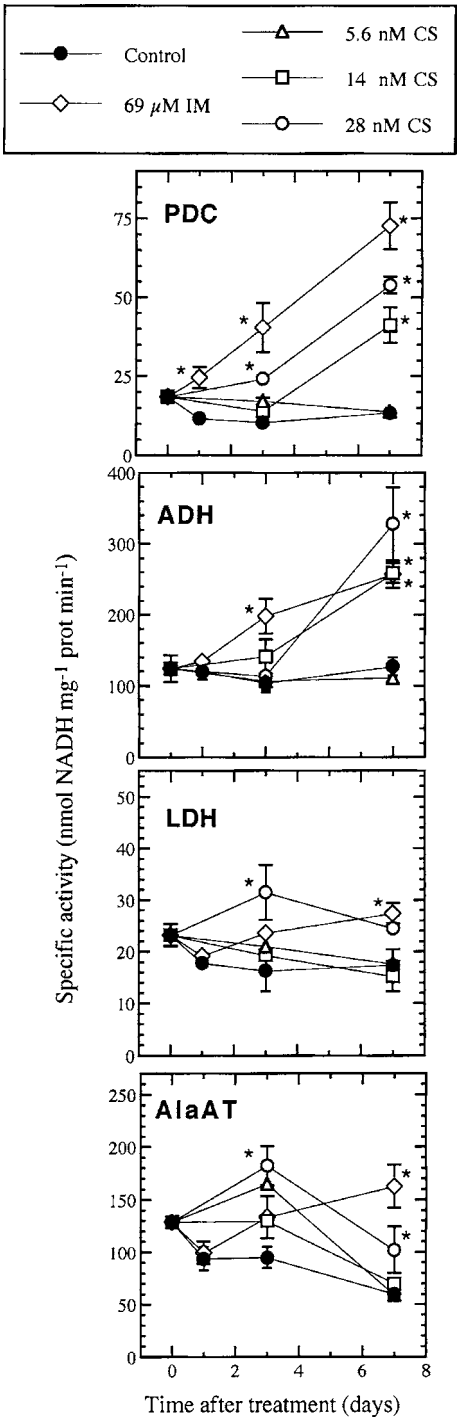

Figure 3. Effect of imazethapyr (IM) and chlorsulfuron (CS) on specific activities of pyruvate decarboxylase (PDC), alcohol dehydrogenase (ADH), lactate dehydrogenase (LDH), and alanine aminotransferase (AlaAT) in pea roots. Means ± SE ($n = 4$). * close to each symbol denotes significant difference from the control at a given day.

estimated native molecular weight of 260 kD (**Figure 4**) that increased in intensity with IM or CS treatment. This PDC induction by IM and CS at 7 days after treatment was in good accordance to the spectrophotometric determination (**Figure 3**).

Three ADH isozymes equally spaced were detected in pea roots (**Figure 4**). All of them presented an estimated native molecular weight of around 100 kD. The two slowest migration bands showed a more prominent intensity also 3 days after the onset of IM or CS treatment, showing an ADH activation that was also evident in the spectrophotometric assay for IM but not for CS (**Figure 3**). All isozyme activities increased strongly 7 days after IM or CS treatment (**Figure 4**).

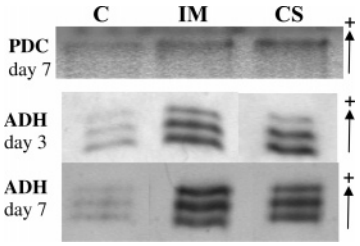

Figure 4. Native PAGE of root PDC and ADH activities 3 and 7 days after treatment. Pea plants were not treated (C) or were treated with imazethapyr (IM) or chlorsulfuron (CS 28 nM). For PDC, each lane contained 171 μg protein. For ADH, each lane contained 3 μg protein (day 3) or 2.05 μg protein (day 7).

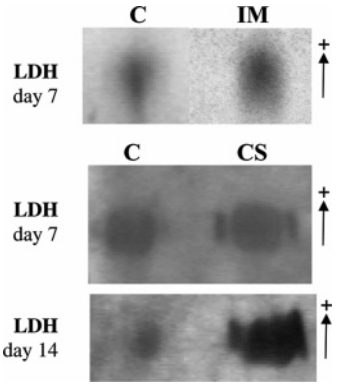

Figure 5. Native PAGE of root LDH activity. Pea plants were not treated (C) or were treated with imazethapyr (IM) or chlorsulfuron (CS 28 nM). Lanes contained 31 μg protein.

Native electrophoresis staining revealed a single LDH band with an estimated molecular weight of around 160 kD (**Figure 5**). LDH band showed a slight intensity increase 7 days after IM treatment but no changes 7 days after CS treatment, which is consistent with activities measured spectrophotometrically (**Figure 3**), but an increase 14 days after CS treatment was observed (**Figure 5**).

Inhibition of KARI and Fermentative Metabolism. Induction of PDC, ADH, and AlaAT activities was detected 7 days after the supply of Hoe 704 to the nutrient solution. However, no changes in LDH activity were observed (**Figure 6**).

The effectiveness of CPCA as KARI-inhibitor in vivo was confirmed since the supply of 200 μM CPCA to the nutrient solution led to a dramatic increase of AL (the substrate of KARI) content in leaves and roots (**Figure 7**). It is remarkable that AL content was not detectable in control leaves and roots, but it significantly increased within 3 days after CPCA treatment. Moreover, although CPCA is absorbed by roots, AL accumulation was more prominent in leaves than in roots.

CPCA effects on PDC, ADH, and AlaAT activities were observed within 7 days after the treatment. Both ethanolic fermentative activities (PDC and ADH) were induced significantly within 3 days from the onset of CPCA treatment (**Figure 8**), and these activities increased up to 2.8- and 2.1-fold 7 days after the treatment.

CPCA-treated roots showed no changes in LDH activity during the time course of the experiment (**Figure 8**). Moreover, no lactic fermentation induction after KARI inhibition was observed 9 days after 500 μM CPCA was supplied (data not shown). The AlaAT activity also was modified by the CPCA

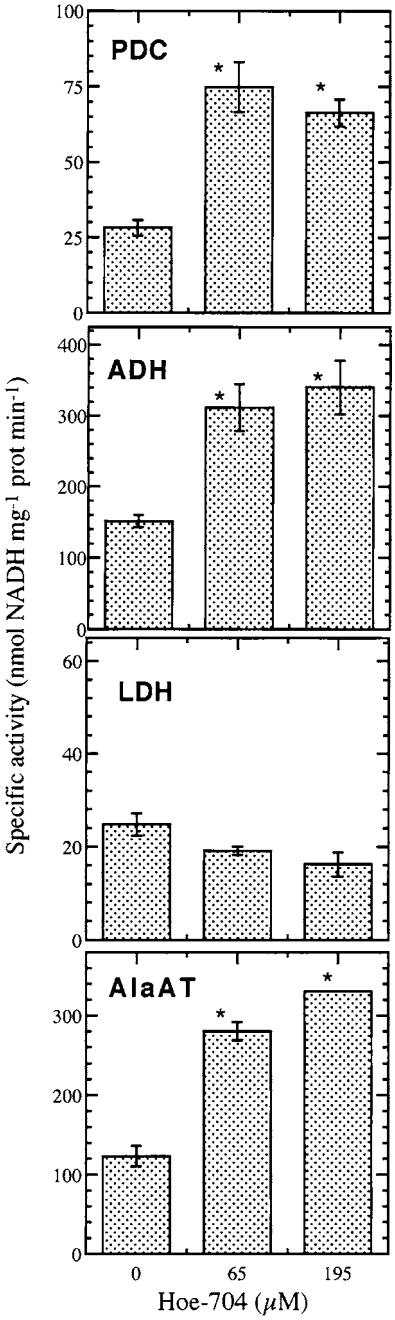

Figure 6. Effect of different Hoe 704 concentrations on the root specific activities of pyruvate decarboxylase (PDC), alcohol dehydrogenase (ADH), lactate dehydrogenase (LDH), and alanine aminotransferase (AlaAT) 7 days after treatment of pea plants. Means ± SE ($n = 4$). * denotes significant difference from the corresponding control..

treatment with only a slight increase 3 or 5 days after CPCA treatment, but a significant increase 7 days after the treatment (**Figure 8**).

Activity staining after native PAGE electrophoresis confirmed the induction of PDC and ADH (**Figure 9**) in roots of CPCA treated plants 7 days after the treatment. ADH profile (**Figure 9**) showed less induction of the most anodic isozyme, similarly to that described for IM and CS 3 days after the treatment (**Figure 4**).

Molecular mass of the two PDC subunits immunodetected by SDS gel **(Figure 10)** were around 65 kD, according to the tetrameric form in the native gel activity (260 kD) (**Figures 4** and **9**). Moreover, PDC activity enhancement observed with ALS- **(Figures 3** and **4)** and KARI-inhibitors **(Figures 6**, **8**, and **9)** was due to an induction of PCD protein expression provoked by all herbicide treatments **(Figure 10)**.

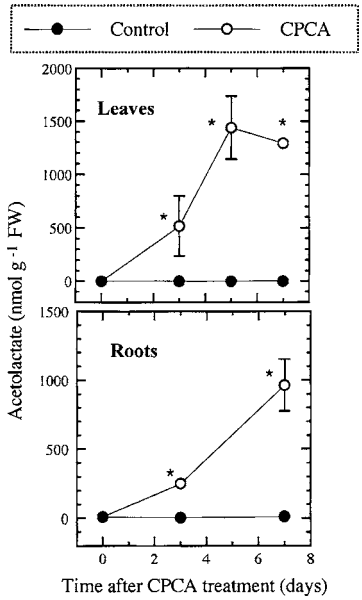

Figure 7. Effect of 200 μM CPCA on acetolactate content in leaves and roots of pea plants. Means ± SE ($n = 4$). * close to each symbol denotes significant difference from the control at a given day.

DISCUSSION

The induction of aerobic fermentation in pea plants after the supply of the ALS-inhibitor IM, an imidazolinone herbicide, has been recently described (*5*). The present study shows similar fermentative metabolism induction after the supply of another chemical class of ALS-inhibitors, the sulfonylurea CS. Ethanolic fermentation (ADH and PDC activities) was stimulated within 3 days after CS supply. Lactic fermentation (LDH) was induced by 28 nM CS at day 3 and the activity AlaAT was induced 3 days after the treatment. Hence, the fermentation induction in roots under aerobic conditions seems to be a physiological effect of both IM and CS rather than just IM.

CPCA was first reported to affect KARI in ref *24*. CPCA displays a strong KARI inhibition even though CPCA is less active than Hoe 704 in vitro (*24*). The inhibition of KARI by CPCA was confirmed in this study from the accumulation of AL in roots and leaves after CPCA supply to the nutrient solution. A similar AL accumulation was described in maize after Hoe 704 treatment (*9*) and in *Abutilon theophrasti* treated with CPCA (*24*).

The physiological response of plants treated with KARI-inhibitors was very similar to that described for ALS-inhibitors (*25*). Pea growth was inhibited after ALS inhibition by IM or CS (**Figures 1** and **2**; *7*) and after KARI inhibition by Hoe 704 or CPCA (**Figures 1** and **2**). This is the first study reporting the induction of ethanolic fermentation (ADH and PDC) after KARI activity inhibition. Moreover, this induction was evident after KARI inhibition by two different compounds, Hoe 704 and CPCA.

These results show that ethanolic fermentation activities, PDC and ADH, were predominantly induced after the inhibition of BCAA biosynthesis. Although the activation of these enzymes is usually detected mainly under hypoxia (*8*), it has been suggested that fermentation could also have a function in aerobic metabolism under several stresses (*26*). Under conditions that induce fermentative metabolism, each plant species displays a characteristic pattern with a sequential or specific induction of

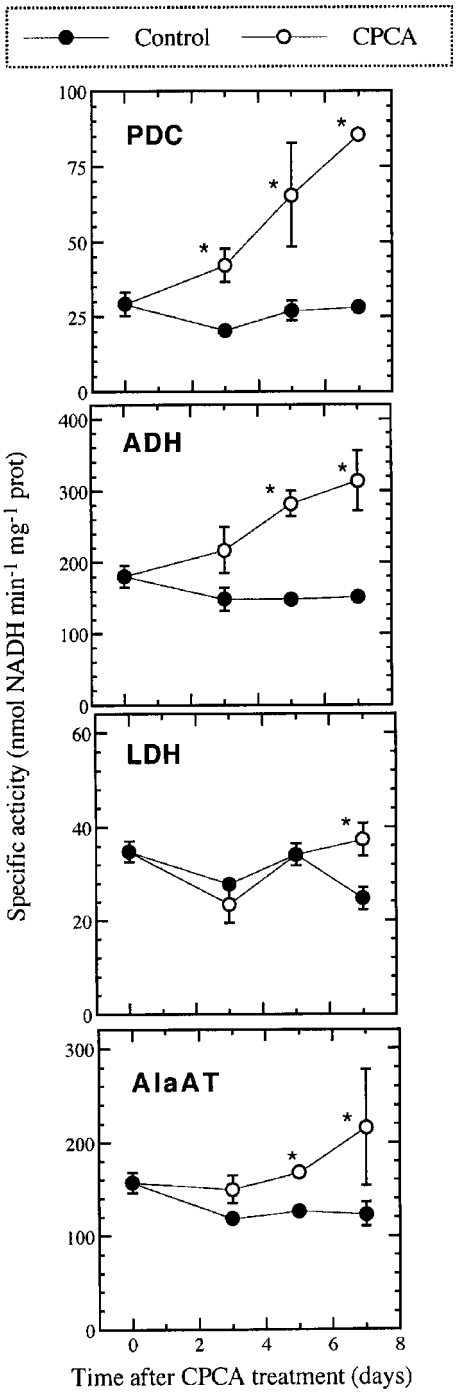

Figure 8. Effect of 200 μM CPCA on the root specific activities of pyruvate decarboxylase (PDC), alcohol dehydrogenase (ADH), lactate dehydrogenase (LDH), and alanine aminotransferase (AlaAT) in pea plants. Means ± SE ($n = 5$). * close to each symbol denotes significant difference from the control at a given day.

fermentative activities. In the case of pea, carbohydrate metabolism under anaerobic conditions is largely restricted to alcoholic fermentation (*27*, *28*).

After BCAA biosynthesis inhibition, both by ALS- or KARI-inhibitors, the remarkable increase in PDC activity was detected within 1 or 3 days, and isoenzyme activity was also enhanced. PDC activity was also enhanced in pea that was subjected to anaerobiosis, and an accumulation of ethanol was rapidly detected (*27*, *28*). Two PDC subunits near 65 kDa were immunodetected in this study, similarly to those described in pea (*22*), wheat, maize, and rice (*29*). In rice, it has been shown

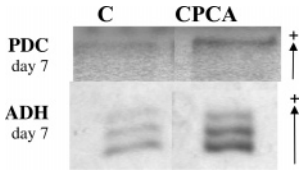

Figure 9. Native PAGE of root ADH and PDC activities 7 days after treatment. Pea plants were not treated (C) or were treated with 200 μM CPCA. Each lane contained 171 μg protein for PDC and 3.9 μg protein for ADH.

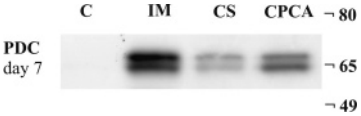

Figure 10. Western immunoblots of root PDC protein 7 days after treatment. Pea plants were not treated (C) or were treated with the ALS-inhibitors imazethapyr (IM) or chlorsulfuron (CS 28nM) or were treated with 200 μM CPCA (KARI-inhibitor). Each lane contained 39 μg protein. The running position of molecular mass markers is indicated on the right.

that the level of both PDC subunits (64 and 62 kDa) was induced by anoxia, although the 64 kDa subunit was preferentially synthesized at the onset of anoxia (*17*). In the present study, for each treatment the increase of the two subunits was similar after 7 days (**Figure 10**). The higher amount of both types of PDC subunits observed in IM and CS (ALS-inhibitors) and in CPCA (KARI-inhibitor) treated plants showed that an overexpression of the PDC protein occurs after BCAA inhibition. Therefore, the induction of catalytic activity of the PDC is an effect of overexpression of the PDC protein.

ADH has been widely studied among higher plants, especially maize. Two unlinked *Adh* loci express three dimeric isozymes (*30*). The ADH isozyme pattern detected in pea roots was very similar to the three isozyme pattern described in maize and pea epicotyls (*31*). The induction of all ADH isozymes after 7 days of ALS-inhibition was similar to the increase described in roots of pea under anaerobic conditions (*31*). The anodic isozyme showed less induction than the other two bands after 3 days of IM or CS-treatment or 7 days after CPCA treatment (**Figures 4** and **9**). Different induction pattern has been described for anaerobically stressed cotton, from which the most anodic isozyme (ADH2-ADH2) was induced more than the other two (*32*).

Some species, such as maize and rice, show an induction of lactic fermentation which precedes the ethanolic fermentation enhancement under hypoxia (*8*). However, lactic fermentation was not detected in pea plants under hypoxia (*27*). On the contrary, ALS-inhibitors caused an increase of LDH activity, faster with IM than CS, and such increase was similar to what was detected under hypoxia in barley, maize, and rice (*20*, *33*, *34*). Lactic fermentation does not seem to be an important response to ALS inhibition because change of LDH activity was slight and slow after treatment. Indeed, this was not a common physiological effect of BCAA biosynthesis inhibition, because the inhibition of the second common enzyme of the pathway, KARI, also did not increase LDH activity.

Pyruvate is converted to alanine in plant cells by AlaAT, and alanine is produced in relative large amounts in most plant roots that are exposed to hypoxia or anoxia. In the case of pea roots, alanine was accumulated under anaerobic conditions (*28*). This study shows that AlaAT activity was induced by ALS- and KARI-inhibitors.

Abiotic stresses other than hypoxia (cold, salinity, and wounding) induce the expression of ADH and PDC by an unknown process (*35*, *36*). Reference *5* reported the increase of ADH, PDC, LDH, and AlaAT activities after ALS inhibition, and the increase was related to the diversion of pyruvate that might be accumulated because of the flux shortage through BCAA pathway. Pyruvate concentration would regulate PDC activity, since PDC has a K_m for pyruvate higher than pyruvate dehydrogenase (*37*). Additionally, authors in ref *38* showed that metabolic conditions resulting in the accumulation of pyruvate had the potential to activate fermentation or to activate the alternative oxidase enzyme in the mitochondrial electron transport chain, resulting in a modulation of pyruvate level. Moreover, activation of the alternative pathway and an increased content of the alternative oxidase protein was detected in soybean after ALS-inhibition (*6*), suggesting that pyruvate might be involved in the induction of fermentative processes and the regulation of electron allocation to the cytochrome and alternative respiratory pathways.

Because KARI uses AL or 2-aceto-2-hydroxybutyrate as substrates, KARI inhibition, in contrast to ALS inhibition, does not affect directly the pyruvate levels. Data from this study suggest that after KARI inhibition, AL accumulation inhibits ALS activity which would trigger fermentative enzymatic activities. This is supported by the dramatic increase in AL content detected in plants after KARI inhibition (**Figure 6**; *9*, *24*) and by the rise of ketobutyrate (ALS substrate) levels detected in *Salmonella typhimurium* cells after KARI-inhibition (*39*). Testing this hypothesis, several experimental approaches were developed; first, the effect of AL on ALS activity in vitro was evaluated, however, being that AL is the product of ALS activity, it occurred to be technically impossible to monitor its effect as inhibitor. Second, the effect of AL on pea growth and fermentative metabolism was evaluated. However, the low stability of AL and the technical difficulties to achieve AL incorporation to plants led to inconclusive results.

In conclusion, the results presented here establish that, in pea plants, fermentative metabolism is induced after inhibiting the BCAA-biosynthetic pathway at the level of the first and the second enzymes of the pathway and confirm the similarity between the physiological effects triggered by ALS or KARI inhibition.

ABBREVIATIONS USED

ADH, alcohol dehydrogenase; AL, acetolactate; AlaAT, alanine aminotransferase; ALS, acetolactate synthase; BCAA, branched-chain amino acid; CPCA, 1,1-cyclopropanedicarboxylic acid; CS, chlorsulfuron; Hoe 704, 2- dimethylphosphinoyl-2-hydroxyacetic acid; IM, imazethapyr; KARI, ketol-acid reductoisomerase; LDH, lactate dehydrogenase; PDC, pyruvate decarboxylase.

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