Changes in Mitochondrial Electron Partitioning in Response to Herbicides Inhibiting Branched-Chain Amino Acid Biosynthesis in Soybean[1]

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The adaptation of the respiratory metabolism in roots of soybean (*Glycine max* L. Merr. cv Ransom) treated with herbicides that inhibit the enzyme acetolactate synthase (ALS) was analyzed. A new gas phase dual-inlet mass spectrometry system for simultaneous measurement of $^{34}O_2$ to $^{32}O_2$ and O_2 to N_2 ratios has been developed. This system is more accurate than previously described systems, allows measurements of much smaller oxygen gradients, and, as a consequence, works with tissues that have lower respiration rates. ALS inhibition caused an increase of the alternative oxidase (AOX) protein and an accumulation of pyruvate. The combination of these two effects is likely to induce the activation of the alternative pathway and its participation in the total respiration. Moreover, the start of the alternative pathway activation and the increase of AOX protein were before the decline in the activity of cytochrome pathway. The possible role of AOX under ALS inhibition is discussed.

There are four main classes of herbicides whose first mechanism of action is the inhibition of the enzyme acetolactate synthase (ALS; EC 4.1.3.18, also known as acetohydroxyacid synthase): imidazolinones, sulfonylureas, triazolopyrimidines, and pyrimidinylsalicilyc acids, with imidazolinones and sulfonylureas the first to be commercialized. ALS is the first common enzyme in the biosynthesis of branched-chain amino acids (BCAAs): Val, Leu, and Ile. This enzyme catalyzes the condensation of either two molecules of pyruvate to form acetolactate in the Leu and Val pathway or one molecule of pyruvate with one molecule of 2-ketobutyrate to form 2-aceto-2-hydroxybutyrate as the first step in the Ile biosynthesis (Singh, 1999). These herbicides cause a significant growth inhibition that is due more to a slower cell division than to an inhibition of cell expansion, although plants stay green for several weeks before death (Wittenbach and Abell, 1999). However, the precise mechanisms that link ALS inhibition with plant death have not been clarified yet. Plants respond quickly to ALS inhibitors by increasing protein turnover to renew BCAAs, and even the critical BCAA pool does not decline to a level that would affect protein synthesis (Wittenbach and Abell, 1999; Royuela et al., 2000). Carbohydrate accumulation in leaves and roots is one of the main symptoms of ALS-inhibiting herbicides in plants treated with imazethapyr (IM), an imidazolinone (Shaner, 1991; Royuela et al., 2000). Gaston et al. (2002) also demonstrated that the increase of soluble carbohydrates in roots can even precede that of starch in leaves, supporting the hypothesis that sugar accumulation in leaves can be due to a decrease in sink strength. In this context, it is surprising to notice that despite the cessation of plant growth and the accumulation of carbohydrates in roots, total root respiration rate (V_t) is unaffected or slightly affected (Ray, 1982; Gaston et al., 2002), indicating the possible occurrence of an impaired and/or regulatory mechanism of respiration in plants treated with ALS inhibitors. Thus, an induction of aerobic fermentation in pea (*Pisum sativum*) plants treated with IM has been described recently (Gaston et al., 2002). Aubert et al. (1997) showed that these herbicides induced the alternative oxidase (AOX) capacity in sycamore (*Acer pseudoplatanus*) suspension cells. They concluded that the induction of AOX synthesis was an indirect effect likely to be due to the effects of herbicides on cell metabo-

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lism. However, the relationship between AOX synthesis and ALS inhibition and its physiological significance in a whole plant system remain unclear.

AOX is an ubiquinol oxidase, alternative to the cytochrome oxidase, found in plant mitochondria and in some fungi and protists. Electron transfer from ubiquinol to the cytochrome oxidase is coupled to two sites of proton translocation source of ATP production, whereas the alternative pathway is effectively non-phosphorylating and releases energy as heat. Among the several factors known to regulate the activity of AOX (redox state of the enzyme, reduction state of the ubiquinol pool, and level of AOX protein), a remarkable feature is that the reduced form of the enzyme is further activated by α-ketoacids, pyruvate in particular (Millar et al., 1993; Umbach and Siedow, 1996). The physiological role of this pathway remains to be clarified except in the specialized case of promoting thermogenesis during flowering in aroid spadices (Meeuse, 1975; Moore and Siedow, 1991). However, several hints on the function of AOX have been achieved during the last decade and tend to indicate that AOX might have a function related to stress situations; for example, cold (Purvis and Shewfelt, 1993; Gonzàlez-Meler et al., 1999), oxygen radicals (Purvis, 1997; Yip and Vanlerberghe, 2001), phosphate starvation (Parsons et al., 1999; Gonzàlez-Meler et al., 2001), pathogen infection (Simons et al., 1999), and also in fruit ripening (Sluse and Jarmuszkiewicz, 2000; Considine et al., 2001) and hypersensitive response after pathogen infection (Xie and Chen, 2000; Ordog et al., 2002). Recently, Vanlerberghe et al. (2002) and Robson and Vanlerberghe (2002), using different death-inducing compounds, suggested a possible role of AOX in programmed cell death, either preventing or attenuating whole plant death. These studies may indicate that AOX alleviates the effect of stresses on plant performance by avoiding the over-reduction of the electron transfer chain preventing the generation of reactive oxygen species or allowing a limited synthesis of ATP when the cytochrome pathway is restricted.

Given that respiration measured as O_2 uptake is scarcely altered by ALS inhibitor herbicides and that pyruvate is the main substrate of ALS, there is a possibility that the accumulation and diversion of this metabolite from biosynthetic pathways to respiratory pathway is causing the continuation of the respiration rate. Moreover, pyruvate could 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 been studied yet in depth.

Because AOX can operate in parallel with the cytochrome pathway and compete for electrons with an unsaturated cytochrome pathway (Guy et al., 1989; Hoefnagel et al., 1995; Ribas-Carbo et al., 1995), the only reliable method to quantitatively measure the activities of the respiratory pathways is by the use of oxygen isotope fractionation (Δ_n) in which inhibitors are only required to determine the end points of the Δ_n by each pathway (Robinson et al., 1995; Day et al., 1996). An accurate measurement of O_2 concentration and its isotopic composition is essential for the correct determination of the Δ_n. In this paper, we report the development of a gas phase dual-inlet system for simultaneous measurements of $^{34}O_2$ to $^{32}O_2$ and O_2 to N_2 ratios with intact plant tissues, similar to the one used for studies on marine organisms (Kiddon et al., 1993). This method increases the precision of measurement that, in turn, allows measurements to be made with much smaller oxygen gradients and, as a consequence, with tissues that have lower respiration rates.

We have applied this technique to further investigate the mode of action of ALS inhibitor herbicides in soybean (*Glycine max* L. Merr. cv Ransom): the possible accumulation of pyruvate and its effect on the electron transfer chain, in particular on AOX activity.

RESULTS

ALS Inhibition and Herbicide Symptoms. Plant Growth and Carbohydrate Content

IM and chlorsulfuron (CS) supply at the selected concentrations (51.75 μM and 11.18 nM, respectively) caused similar effects on soybean. Plant growth was significantly inhibited by both herbicides (Fig. 1A). Whole plant dry weight represented 80% and 40% of control plants by d 3 and 7, respectively. Root growth was immediately halted in both treatments (Fig. 1B). IM treatment caused the inhibition of ALS activity (over 50%) by d 3 and stayed over 75% and 65% for days 5 and 7, respectively (Fig. 1A). Although this degree of ALS inhibition produces a lethal phenotype, the measurements presented in this study were carried out at the initial phase of toxicity, in which the plant viability was not compromised.

Figure 2 shows a significant increase in starch and Suc contents in roots of treated plants after d 3. It is remarkable that although starch content was very low in control roots, it significantly increased in roots of herbicide-treated plants. The occurrence of this increased concentration of carbohydrates is an indication of the sustained photosynthesis and photoassimilate transport, which ensure the viability of plants during the time course of the experiment.

Pyruvate Content

The supply of herbicides that inhibits ALS activity caused an increase of root pyruvate concentration, which is the main substrate of ALS activity (Fig. 3). The pattern of this increase was very similar in both herbicide treatments, although it was earlier in IM-treated plants. With IM, there was a significant in-

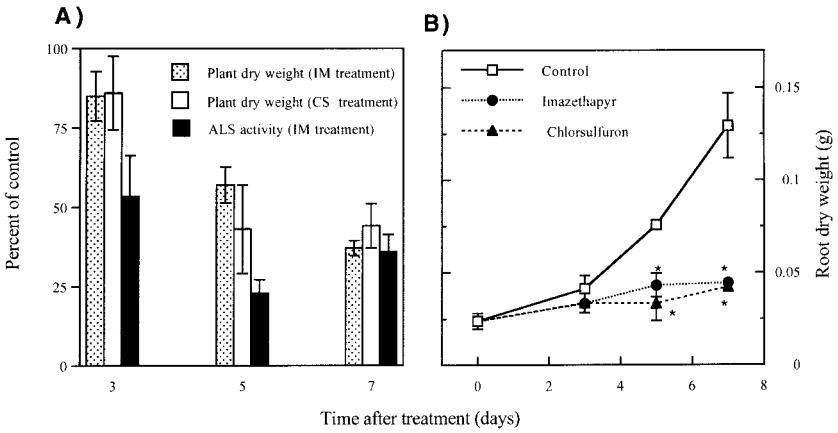

Figure 1. A, Effect of ALS-inhibiting herbicides on soybean ALS activity (IM treatment) and total dry weight relative to control plants. Mean ± SE (n = 5). Data, means, and SEs are given as percentages of control plants. The control values for plant dry weight were: 187.5 ± 30.7, 375.8 ± 10.8, and 610.7 ± 55.9 mg at d 3, 5, and 7, respectively, and for ALS activity 379 ± 23, 564 ± 114, and 288 ± 35 nmol acetoin g^{-1} dry weight h^{-1} at d 3, 5, and 7, respectively. B, Effect of ALS-inhibiting herbicides on soybean root dry weight. Mean ± SE (n = 5). *, Significant differences from the corresponding control ($P \leq 0.05$).

crease in pyruvate level at d 3, whereas with CS, it was significant from d 5. Pyruvate content increased from 414 nmol g^{-1} dry weight at the beginning of the experiment to 865 and 729 nmol g^{-1} dry weight by d 7 for IM- and CS-treated plants, respectively, whereas it remained fairly constant in control plants (Fig. 3).

Respiratory Changes Caused by Herbicide Treatments

V_t of herbicide-treated plants was higher than that of control plants at d 3 and then decreased to values similar to those of control plants at d 7 (Fig. 4A).

Δ_n measured in the absence of inhibitors increased during the experimental time course in treated plants and was significantly higher than in control plants: CS-treated plants on d 5 and 7 were 20.36‰ and 21.30‰, respectively, and IM-treated plants on d 7 were 19.87‰ (Fig. 4B). However, Δ_n decreased in control plants throughout the experimental period from 18.56‰ on d 0 to 16.90‰ on d 7. The calculation of the electron partitioning between the cytochrome and alternative pathways (τ_a) requires each pathway's fractionation to be quantified. The Δ_n by the

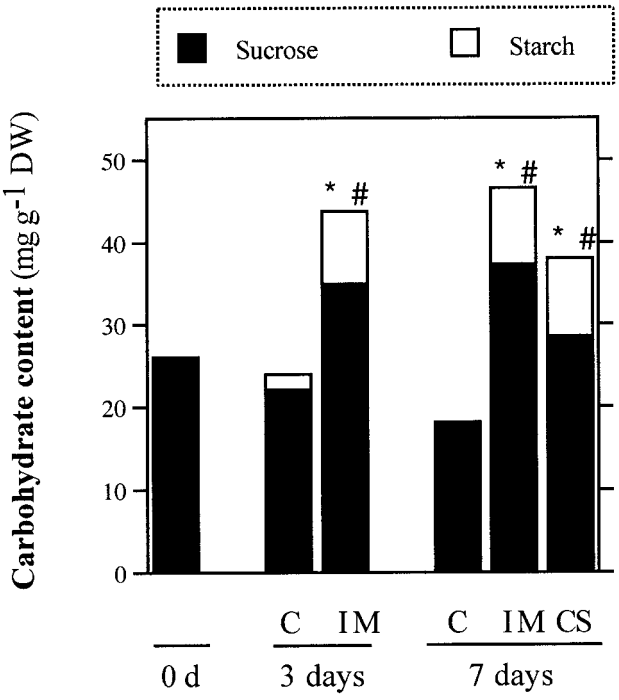

Figure 2. Effect of ALS-inhibiting herbicides on soybean root carbohydrate content: Suc, expressed as milligrams of Suc per gram dry weight; and starch, expressed as milligrams of Glc per gram dry weight. * and #, Significant differences in Suc and starch content, respectively, from the corresponding control ($P \leq 0.05$).

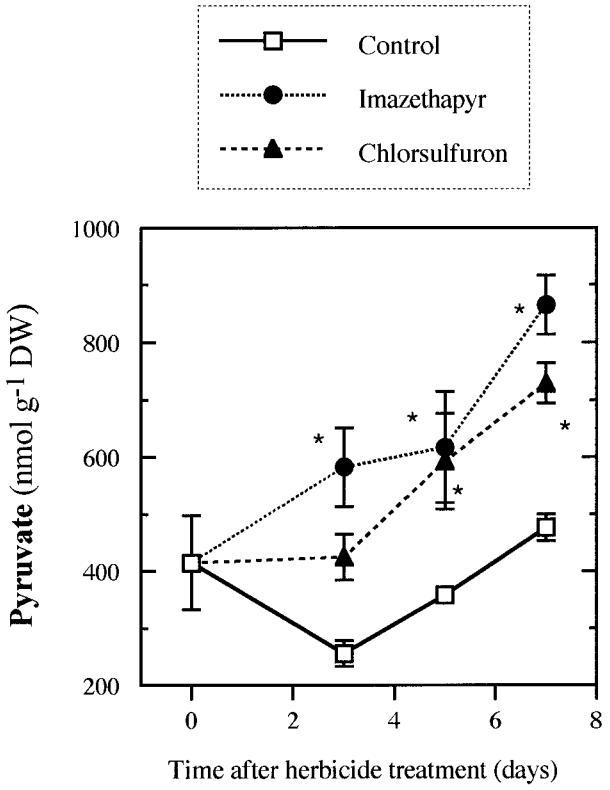

Figure 3. Pyruvate content in roots of soybean treated with ALS-inhibiting herbicides. Mean ± SE (n = 5). *, Significant differences from the corresponding control ($P \leq 0.05$).

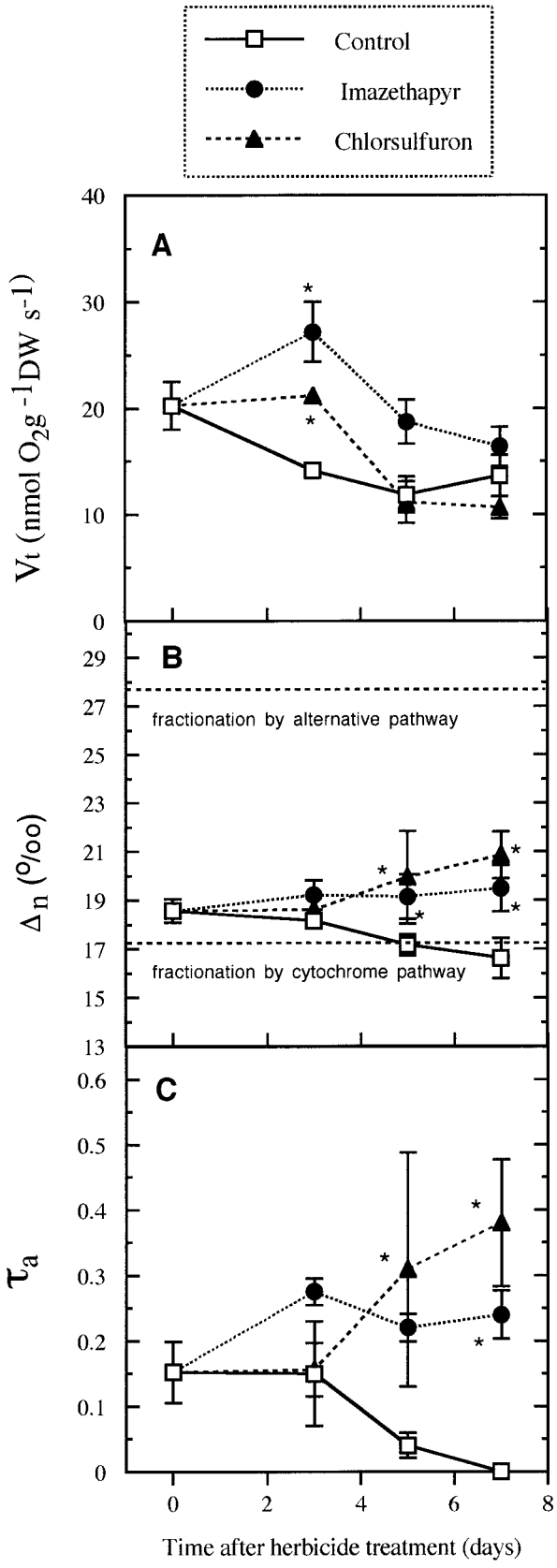

Figure 4. V_t (A), Δ_n (B), and τ_a through the alternative pathway (C), in roots of soybean treated with ALS-inhibiting herbicides. Mean ± SE (n = 4–5). *, Significant differences from the corresponding control ($P \leq 0.05$). Fractionation values for soybean roots were 17.35‰ ± 0.51‰ for the cytochrome pathway and 27.81‰ ± 0.02‰ for the alternative pathway.

cytochrome (with salicylhydroxamic acid [SHAM]) and alternative (with potassium cyanide [KCN]) pathways in roots was determined to be 17.35‰ ± 0.51‰ (Δ_c) and 27.81‰ ± 0.02‰ (Δ_a), respectively. These values were used to calculate τ_a. Electron partitioning to the alternative pathway increased during the experiment in herbicide-treated plants: CS-treated roots maintained their electron partitioning through the alternative pathway at 0.15 during the first 3 d and then increased up to 0.38 at d 7 (Fig. 4C) and in IM-treated roots peaked at d 3 at 0.27, remaining constant thereafter (Fig. 4C). In control plants, τ_a decreased from 0.15 at d 3 to virtually zero by d 7 (Fig. 4C).

The activities of each respiratory pathway were also affected by herbicide treatments. The activity of the alternative pathway (v_{alt}) increased in treated plants throughout the course of the experiment. The pattern of activation was similar for both treatments, except for the marked increase in v_{alt} by d 3 in IM-treated plants (Fig. 5, B and D). In addition, in herbicide-treated plants, the activity of the cytochrome pathway (v_{cyt}) was higher over the first 3 d than in control plants and then declined (Fig. 5, A and C). On the other hand, the initial decrease of V_t in control plants was associated with a decrease in both pathways: v_{cyt} decreased during the first 3 d and slightly increased thereafter, and v_{alt} steadily declined throughout the experiment (Fig. 5).

AOX Protein

To study the profile of reduced and oxidized forms of AOX, Umbach and Siedow (1997) proposed the use of whole tissues instead of mitochondrial extracts because AOX protein undergoes oxidation of the sulfhydryl-disulfide system in the absence of sulfhydryl reagents in the latter. In this study, dithiothreitol was used as a reducer to obtain a single reduced form of the protein and, as a consequence, to enable the measurement of the total amount of protein by immunoblotting. A single AOX band of approximately 34 kD was obtained, which is in agreement with previous determinations of the apparent molecular size of soybean AOX species in the range of 33 to 39 kD (Finnegan et al., 1997; Fig. 6). The total amount of AOX protein in roots of herbicide-treated plants is significantly higher than in control plants throughout the experiment (Fig. 6). ALS inhibitors caused a subtle increase of the total amount of AOX protein by d 3 (approximately 1.6-fold for CS-treated plants and 2.5-fold for IM-treated plants), and this increase was substantially larger by d 5 (over 4-fold) and 7 (3-fold; Fig. 6).

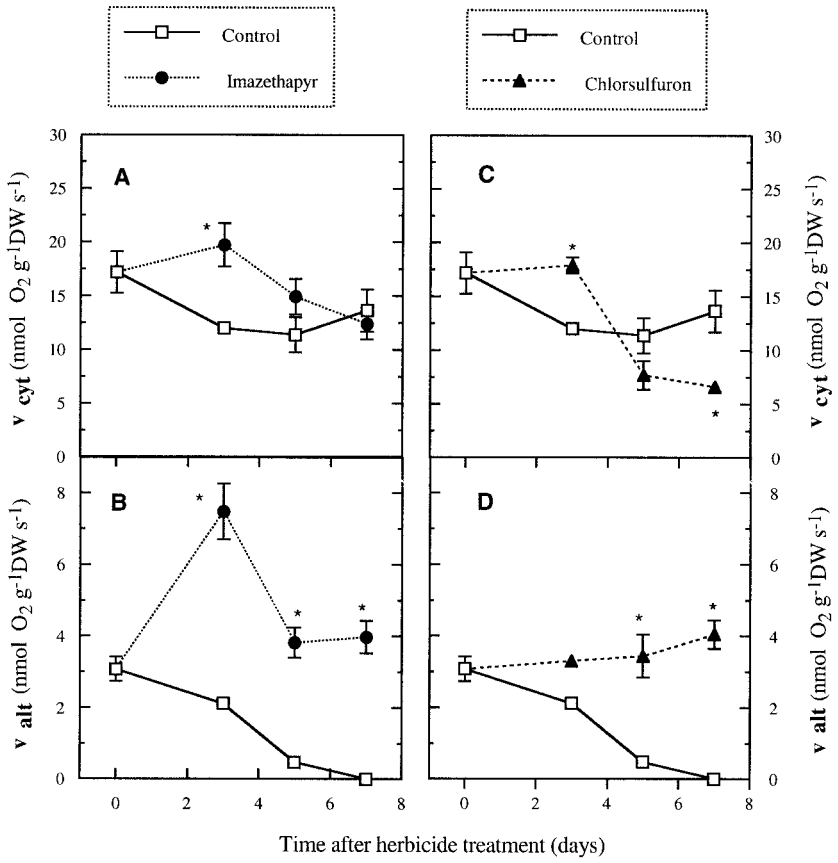

Figure 5. v_{cyt} and v_{alt} of soybean roots treated with ALS-inhibiting herbicides: A and B for IM treatments and C and D for CS treatments. Mean ± SE (n = 4–5). *, Significant differences from the corresponding control ($P \leq 0.05$).

DISCUSSION

This report shows, for the first time to our knowledge, the development of a gas phase dual-inlet mass spectrometry system to measure the activities of respiratory pathways in plants with more precision and much smaller oxygen gradients and, as a consequence, with tissues that have lower respiration rates.

This approach permitted the observation that both herbicides caused an increase in the participation of v_{alt} in total respiration (Fig. 5). These results are consistent with previous observations in sycamore suspension cells treated with ALS-inhibiting herbicides, in which an increase of AOX capacity without modification of the cytochrome pathway was observed by polarographically monitoring O_2 uptake (Aubert et al., 1997). The change in the partitioning of the electron transfer chain in soybean roots may have important implications in the adaptive response of plants to herbicides. Under ALS inhibition, there is an increase in the protein turnover that, in turn, causes an increase in the free amino acid pool (Shaner and Reider, 1986; Rhodes et al., 1987). In non-stressed conditions, protein turnover is generally considered as one of the most important maintenance processes that requires energy for protein synthesis. Under conditions in which energy requirement is strongly reduced for growth and ion uptake (which would be the case for ALS inhibitor-treated plants), ATP requirements for protein turnover increases and the maintenance of biomass becomes the most important energy requiring process in roots (Van der Werf et al., 1992). As such, the effect of ALS inhibitors should certainly alter the amount of energy allocated to growth and maintenance. The accumulation of carbohydrates is likely to be the ground for a sustained respiration rate in treated plants, through both cytochrome and alternative pathways during the first 3 d (Fig. 5). Afterward, as growth inhibition increases and maintenance respiration becomes more important, the alternative pathway increases its participation in the total respiration (Fig. 5, B and D).

The first indication that the inhibition of ALS activity could affect the alternative pathway was that herbicides caused an increase in AOX synthesis (Fig. 6; Aubert et al., 1997). It is well known that the total amount of AOX protein increases under several types of stress and, inherently, the total capacity of the pathway. However, an increase in the total amount of AOX protein does not always imply an increase of its activity (Lennon et al., 1997). In this study, the total amount of AOX content in the roots of control plants was also seen to increase throughout the experiment (Fig. 6), whereas the activity of the pathway decreased (Fig. 5). Several stress conditions induce

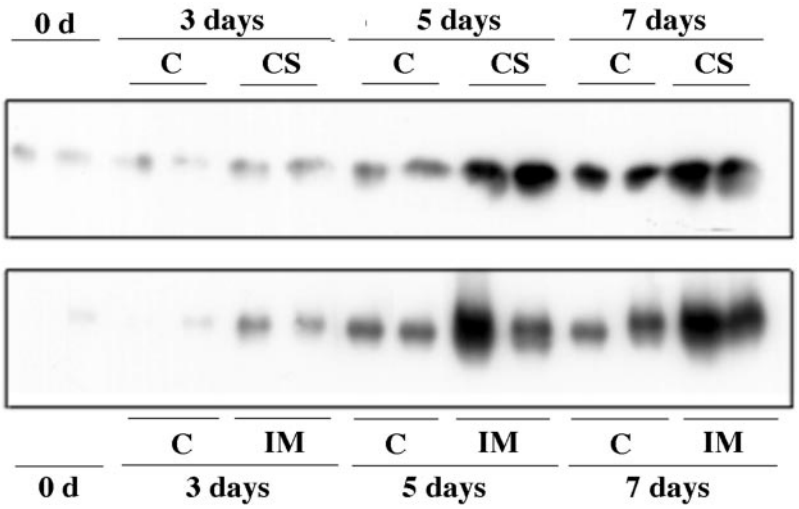

Figure 6. Immunoblots of AOX protein from whole-root tissue extracts from soybean at 0, 3, 5, and 7 d from the onset of herbicide treatment (C, control). Two lanes corresponding to two independent samples for each treatment are shown.

AOX capacity in response to a cytochrome pathway restriction (chemical inhibitors, phosphate deficiency, anoxia, and senescence; Vanlerberghe et al., 1997; Parsons et al., 1999; Amor et al., 2000; Vanlerberghe et al., 2002). However in the present case, the induction of v_{alt} and the increase of AOX protein content were before the decline in v_{cyt} in treated plants, indicating that alternative pathway does not always act as an electron bypass in response to the downstream restriction of the cytochrome pathway.

Because pyruvate is the main substrate of ALS activity, it would be expected that in plants treated with these ALS inhibitor herbicides, pyruvate concentrations might be increased, as observed in Figure 3. Furthermore, other ALS inhibitor herbicides have been shown to increase pyruvate levels in *Salmonella typhimurium* (Epelbaum et al., 1996) and in maize (*Zea mays*; Hwang et al., 1997). On the other hand, there is much in vitro evidence that suggests the importance of pyruvate as an activator of AOX in mitochondrial extracts or partially purified AOX enzyme (Day et al., 1994; Umbach et al., 1994). Pyruvate stimulation of AOX activity results from an increase in the amount of AOX protein in the reduced form (active form; (Hoefnagel et al., 1997; Millar et al., 1997). Nevertheless, there is no conclusive evidence for the role of pyruvate in vivo (Millar et al., 1998; Millenaar et al., 1998). Several stresses cause pyruvate accumulation, usually by limiting the electron transfer chain (anaerobiosis, phosphate starvation, and chemical inhibitors; Good and Muench, 1993; Vanlerberghe et al., 1997; Juszczuk and Rychter, 2002). However, conversely to these stresses, in the present study, pyruvate increase is caused by ALS inhibition. As a consequence, there is a combination of two effects: (a) an upsurge in the synthesis of AOX protein (Fig. 6), which increases the total capacity of the alternative pathway; and (b) a higher pyruvate concentration in the herbicide-treated roots (Fig. 3) that favors the participation of the alternative pathway.

This regulatory feature reinforces the possible role of AOX as a protective enzyme, preventing fermentation of accumulated pyruvate (Day et al., 1995). Previous work showed that in IM-treated pea roots, a species with low AOX activity, aerobic fermentation was elicited (Gaston et al., 2002). It is likely that the low activity of AOX in peas could not prevent the engagement of these fermentative activities by the accumulation of pyruvate. The protective role of AOX has been described previously as a response to environmental stress or in the mitochondria-dependent programmed cell death (Vanlerberghe et al., 2002). To further investigate how much AOX enables the plant to cope with ALS-inhibiting herbicides will require further experiments with plants that cannot express AOX.

To summarize, we add a new insight into the mode of action of ALS-inhibiting herbicides related to changes in mitochondrial electron partitioning, especially engaging the alternative pathway. Whether the increase in the AOX pathway enhances the adaptive response to ALS inhibition or is directly involved in the death process remains to be clarified.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Soybean (*Glycine max* L. Merr. cv Ransom) seeds were treated with 0.5% (v/v) NaOCl for 10 min and swelled in distilled water for 2 h with continuous bubbling of air. Seeds were germinated in a 1:1 (w/v) mixture of sand:perlite in plastic trays and placed in a greenhouse under controlled conditions. Water was applied daily. Nine-day-old seedlings were transferred to hydroponic tanks filled with nutrient solution (Rigaud and Puppo, 1975; supplemented with 10 mM KNO_3) and placed in a growth chamber. Growth conditions were 30°C/24°C on a 16-/8-h (light/dark) regime at 300 μmol photons m^{-2} s^{-1}. Plants were acclimatized to the new growth conditions 4 d, and then they were divided into three groups: one control and two herbicide treatments. ALS inhibitors were: IM, an imidazolinone herbicide; and CS, a sulfonylurea herbicide. They were applied to the nutrient solution

at concentrations of 51.75 μM and 11.18 nM for IM and CS, respectively. These concentrations were chosen after observing the similar effects on soybean growth. The treatment lasted 7 d, with plants being harvested at days 0, 3, 5, and 7. Length and dry weight of roots and shoots were measured.

ALS Activity

ALS activity was measured in vivo following the method of Lee and Owen (2000). Leaves from the apical meristem were excised under water to avoid cavitation and petioles were immediately immersed in 6 mL of 800 μM 1,1-cyclopropanedicarboxylic acid (CPCA). Leaves were allowed to transpire and absorb the feeding solution for 4 h in light (300 $\mu mol\ m^{-2}\ s^{-1}$). CPCA inhibits the activity of keto-acid reductoisomerase (EC 1.1.1.86) leading to acetolactate accumulation. A parallel set of leaves was fed with water to correct the basal level of acetolactate. Leaves were removed from the feeding solution and immediately frozen in liquid nitrogen and stored at −80°C. Acetolactate was extracted three times with 80% (v/v) methanol at 80°C for 20 min each and 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 Westerfeld (1945). The acetolactate produced during the 4 h of incubation with CPCA was corrected with the acetolactate content of the tissue when no inhibitor was used (feeding with water). ALS activity is expressed as nanomoles acetolactate per gram dry weight.

Starch and Suc Determinations

Frozen samples of roots (0.2 g fresh weight) were extracted in boiling 80% (v/v) ethanol. Ethanol soluble extracts were dried in a Turbovap LV evaporator (Zymark Corporation, Hopkinton, MA), and soluble compounds were redissolved with 4 mL of distilled water, mixed, and centrifuged at 2,300*g* for 10 min. The ethanol insoluble residue was extracted for starch as in MacRae (1971). Glc produced from starch and Suc were determined by high-performance capillary electrophoresis in a PACE system 5500 (Beckman Instruments, Fullerton, CA). The background buffer was 10 mM benzoate (pH 12.0) containing 0.5 mM tetradecyl-trimethyl-ammonium bromide. The applied potential was −15 kV, and the capillary tubing had an internal diameter of 50 μm and was 31.4/38.4 cm long. The indirect UV detection wavelength was set at 225 nm.

Pyruvate Extraction and Determination

Samples of roots (0.4 g fresh weight) were harvested and rapidly frozen by addition of 0.6 N trichloroacetic acid (TCA) in diethyl-ether at −100°C and stored at −80°C until extraction. Samples were homogenized with 1.5 mL of cold 5% (w/v) TCA and centrifuged at 2,000*g* for 10 min. To remove TCA, the supernatant was washed three times with diethyl-ether saturated with water. The extracts were filtered through a microfilter (0.22 μm, Millex-GV, Millipore, Bedford, MA). Pyruvate was determined by ion chromatography in a DX-500 system (Dionex Corporation, Sunnyvale, CA) in a Dionex IonPac AG11+AS11 column by gradient separation (0.2–15 mM NaOH in 15 min).

Respiratory Measurements on Intact Tissues with a Gas Phase Dual-Inlet System

V_t, v_{cyt}, and v_{alt} in roots were determined using a closed gas phase system connected to a dual-inlet mass spectrometer as described below. The measuring system consisted of a 3-mL closed cuvette where the plant tissue was placed and from which 200 μL of air was sequentially withdrawn and fed into the mass spectrometer sample bellows. Both the $^{34}O_2$ to $^{32}O_2$ and O_2 to N_2 ratios from the air analyzed were directly obtained from an isotope ratio mass spectrometer (Finnigan Delta S, Thermo Finnigan, San Jose CA) operating in dual-inlet mode and by comparison with a standard air sample. The stainless steel cuvette was equipped with two inlets: one connected to a 1-mL air-tight syringe and the other to the mass spectrometer sample bellows through a capillary tube (127-μm i.d.) with a pneumatically controlled on-off microneedle valve. The sampled air went through a liquid N_2 trap for water and CO_2 removal. To avoid any drop in the cuvette's pressure during the experiment, the air was well mixed using the air-tight syringe, which was left with 1 mL of air. Throughout the experiment, the syringe was used to mix the air. At the beginning of every measurement, 200 μL of the syringe was placed in the cuvette to maintain its atmospheric pressure. The system was regularly tested for leaks by filling the cuvette with He and measuring samples over three times the experimental time span. No oxygen signals were observed. The time between successive samples was 20 min, and the length of a full experiment would vary between 90 and 120 min. The system was previously tested using alfalfa (*Medicago sativa*) seedlings (purchased alfalfa sprouts) in the presence of KCN or SHAM, which gave values of 25.4‰ (Δ_a) and 19.7‰ (Δ_c). These values are very similar to the values observed with previous systems (Robinson et al., 1995).

Root samples (0.2–0.3 g fresh weight) were placed in the 3-mL stainless steel closed cuvette. Roots were carefully surface dried before measurements so as to minimize diffusion resistance to tissue gas exchange. All experiments were carried out at controlled room temperature (23°C). During inhibitory treatments to measure fractionation values through each pathway, either 1.0 mM KCN (in 1 mM TES, pH 8) or 10 mM SHAM (in water from a 1.0 M stock solution in dimethylsulphoxide) were applied by sandwiching the plant tissues between medical wipes soaked with the corresponding inhibitor. No recovery from inhibitor treatment was observed because respiratory rates remained constant throughout the experiment. All stocks were freshly prepared before measurement. In addition, for KCN experiments, a piece of tissue wetted with KCN was present in the cuvette (Gonzàlez-Meler et al., 2001). Calculations of the isotopic fractionation were made as described by Guy et al. (1989) and Ribas-Carbo et al. (1995) without Ar correction. The electron partitioning between the two pathways in the absence of inhibitors was calculated as described by Guy et al. (1989) without forcing the slope to intercept the origin. Over the course of the experiment, the root samples consumed at least 10% of the initial oxygen (21%). r^2 Values of all unconstrained linear regressions between −lnf and $\ln(R/R_o)$ (with at least five data points) were al least 0.995, corresponding to an error in the estimation of less than 0.5‰ (Ribas-Carbo et al., 2000).

Isolation, Blotting, and Immunodetection of the AOX Protein

One hundred to 150 mg fresh weight frozen soybean roots were ground in liquid nitrogen using a mortar and pestle in presence of 1 mL of extraction buffer (50 mM Trizma, 5 mM EDTA, 1% [w/v] SDS, and protease inhibitors [Sigma, St. Louis]). The mixture was centrifuged at 20,000*g* for 15 min. The protein concentration of the supernatant was estimated by the method of Lowry et al. (1951), and no difference among treatments or over the course of the experiment was found. As such, the gels were loaded on a protein content basis. The samples were mixed with 3× sample buffer (0.5 M phosphate buffer [pH 7], 30% [w/v] glycerol, 7.5% [w/v] SDS, 100 mM dithiothreitol, and 0.75 mM Bromphenol blue) before loading 75 μg of protein to a 10% (w/v) SDS-PAGE gel (Laemmli, 1970). After separation by electrophoresis, proteins were transferred to Immobilon PVDF transfer membranes (Millipore Corporation, Bedford, MA). AOX proteins were tagged with AOX-recognizing monoclonal antibodies (Elthon et al., 1989) at a dilution of 1:500 (w/v), followed by a horseradish peroxidase-conjugated goat anti-mouse secondary antibody at a dilution of 1:1,000 (w/v; DAKO, Boehringer Mannheim/Roche, Basel), and detected by chemiluminescence. Total protein was quantified by scanning densitometry (Phoretix 1D International Ltd., Newcastle-upon-Tyne, UK) using Ponceau staining as loading control.

Statistical Analysis

Data are reported as the mean ± SE of at least four replications in two independent experiments for each parameter, as described in the figure legends. The results were compared statistically by using a Fisher's test, and differences were considered significant when P values were ≤0.05.

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