

Phytotoxicity of Acetohydroxyacid Synthase Inhibitors Is Not Due to Accumulation of 2-Ketobutyrate and/or 2-Aminobutyrate

Dale L. Shaner and Bijay K. Singh*

American Cyanamid Company, Princeton, New Jersey 08543–0400

Acetohydroxyacid synthase (AHAS) is the site of action of herbicides of different chemical classes, such as imidazolinones, sulfonylureas, and triazolopyrimidines. Inhibition of AHAS causes the accumulation of 2-ketobutyrate (2-KB) and 2-aminobutyrate (2-AB) (the transamination product of 2-KB), and it has been proposed that the phytotoxicity of these inhibitors is due to this accumulation. Experiments were done to determine the relationship between accumulation of 2-KB and 2-AB and the phytotoxicity of imazaquin to maize (*Zea mays*). Imazaquin concentrations that inhibit growth of maize plants also cause the accumulation of 2-KB and 2-AB in the shoots. Supplementation of imazaquin-treated plants with isoleucine reduced the pools of 2-KB and 2-AB in the plant but did not protect plants from the growth inhibitory effects of imazaquin. Conversely, feeding 2-AB to maize plants increased 2-KB and 2-AB pools to much higher levels than those observed in imazaquin-treated plants, yet such high pools of 2-KB and 2-AB in the plant had no significant effect on growth. These results conclusively demonstrate that growth inhibition following imazaquin treatment is not due to accumulation of 2-KB and/or 2-AB in plants. Changes in the amino acid profiles after treatment with imazaquin suggest that starvation for the branched-chain amino acids may be the primary cause of growth retardation of maize.

Imidazolinones, sulfonylureas, and triazolopyrimidines kill plants by inhibiting AHAS (also known as acetolactate synthase; EC 4.1.3.18; Stidham, 1992). AHAS catalyzes the condensation of 2 mol of pyruvate to produce acetolactate or 1 mol of pyruvate and 1 mol of 2-KB to produce acetohydroxybutyrate in the pathways leading to the biosynthesis of Val, Leu, and Ile (Fig. 1). These herbicides are extremely potent, killing plants at rates of g ha⁻¹. There has been considerable debate regarding the reason for this high potency, particularly in light of the fact that inhibitors of other enzymes in the branched-chain amino acid pathway require much higher rates to kill plants (Wittenbach et al., 1991; Shaner and Singh, 1992). Herbicidal effects of these AHAS inhibitors may result from a direct depletion of the end products, from depletion of intermediates of the pathway for some critical processes, or from a buildup of a toxic substrate. Any or all of these mechanisms may be responsible for the plant death.

It was discovered in *Salmonella typhimurium* that inhibition of AHAS leads to accumulation of 2-KB, one of the substrates

of AHAS (LaRossa et al., 1987). A similar observation was made in *Lemna minor*, a higher plant, in which chlorsulfuron treatment led to the accumulation of 2-AB, the transamination product of 2-KB (Rhodes et al., 1987). Determinations of 2-KB were not made in the latter study. High levels of 2-KB are toxic to *S. typhimurium*. 2-AB has also been shown to disrupt cell division in *Allium* (Lazzagorta et al., 1988) and *Hordeum* root tips (Reid et al., 1985). Therefore, it was concluded that inhibition of AHAS leads to accumulation of toxic levels of 2-KB and 2-AB and that this buildup is what kills the microorganism or plant (Van Dyk and La Rossa, 1986, 1987; LaRossa and Van Dyk, 1987; LaRossa et al., 1987, 1989; Rhodes et al., 1987; Van Dyk et al., 1987; Schloss, 1989; Schloss and Aulabaugh, 1989).

Threonine deaminase is responsible for the production of 2-KB from Thr. This enzyme is feedback inhibited by Ile in plants and microbes (Umbarger, 1969; Sharma and Mazumder, 1970). It was found that Ile supplementation in combination with Val alleviated the toxic effects of chlorsulfuron in *S. typhimurium*, presumably because Ile prevented accumulation of 2-KB (LaRossa et al., 1987). Because threonine deaminase in plants is also feedback regulated by Ile, supplementation with Ile should prevent the herbicidal effects of the AHAS-inhibiting herbicides. Conversely, treatments other than AHAS-inhibiting herbicides that would cause accumulation of 2-KB and/or 2-AB in the plant should also result in plant death. Experiments conducted to test these hypotheses reveal that accumulation of 2-KB and/or 2-AB is not the primary cause of plant death following AHAS inhibition. These studies also demonstrate that disruption of the synthesis of the branched-chain amino acids causes profound effects in the levels of other amino acids.

MATERIALS AND METHODS

Plant Material

Plants were grown in a growth chamber at 30/20°C day/night temperature and 16-h daylength. Maize (*Zea mays*) seeds were germinated on wet paper, and then 5-d-old seedlings were transferred to 50-mL plastic tubes covered with aluminum foil to eliminate light and containing 35 mL of a complete nutrient solution (Miyasake et al., 1988), which was changed daily. Plants were treated with a solution of

* Corresponding author; fax 1–609–799–1842.

inhibitor and amino acid when the fourth leaf began to emerge from the whorl and remained in the presence of this solution throughout the experiment. The concentrations of inhibitor and amino acids are described later for each experiment. Growth measurements were made on this emerging leaf. The leaf sheath/shoot meristem region was extracted to measure the effects of treatments on ketoacids and amino acids. At least two replications with two to five plants per replication were used for various measurements. The experiments were conducted several times; however, the data for one example of each representative experiment are presented.

Growth Measurements

Elongation of the newest emerging leaf (fourth leaf) was measured by determining the distance from the top of the container to the tip of the leaf to the nearest millimeter. Measurements began at the time of treatment, and subsequent measurements were made every 6 to 24 h.

Extraction of Ketoacids and Amino Acids

The plant material was pulverized in liquid nitrogen and then further ground in 0.25 N HCl containing 0.1 mg mL⁻¹ 2-oxopentanoate (internal standard for ketoacid analysis) and 500 nmol mL⁻¹ of L- α -amino- β -guanadinopropionic acid (internal standard for amino acid analysis). Extraction solution (2 mL) was used for each g of tissue fresh weight. The extract was centrifuged at 25,000g for 15 min. An aliquot of the supernatant (0.25 mL) was loaded on a cation exchange column (AG 50W-X8 from Bio-Rad, Richmond, CA; resin bed volume = 4 mL) preequilibrated with 0.01 N HCl. The

column was washed with 1.5 mL of 0.01 N HCl, and then the ketoacids were eluted in 2 mL of 0.01 N HCl. Amino acids bind to this resin and were eluted with four 4-mL aliquots of 9 N ammonium hydroxide.

Derivatization of Ketoacids

A 5 mM solution of 1,2-diamino-4,5-methylene-dioxybenzene was prepared in a fresh solution of 1.5 N HCl containing 20 mM sodium dithionite and 1 mM β -mercaptoethanol. A 250- μ L aliquot of the solution containing the ketoacids was mixed with an equal aliquot of 1,2-diamino-4,5-methylene-dioxybenzene solution. The mixture was vortexed and then heated in a boiling water bath for 45 min. The derivatized ketoacid solution was diluted in the HPLC running buffer for analysis.

HPLC of Ketoacids

The HPLC system consisted of a Beckman 112 solvent delivery module (Beckman, Fullerton, CA), a DYNAMAX model FL-1 fluorescence detector (Rainin, Woburn, MA), a WISP 710B automatic sampler, and a Waters 840 data integration system (Waters Associates, Milford, MA). A radial-PAK cartridge C₁₈ reversed-phase column (5- μ m particle size; 100- \times 8-mm i.d.) was used and was connected with a stainless steel guard column packed with C₁₈ resin. The mobile phase (acetonitrile:methanol:40 mM phosphate [pH 7], 12:13:25, v/v/v) was run at a flow rate of 1.5 mL min⁻¹. For the fluorometric analysis, the excitation and emission wavelengths were 367 and 446 nm, respectively.

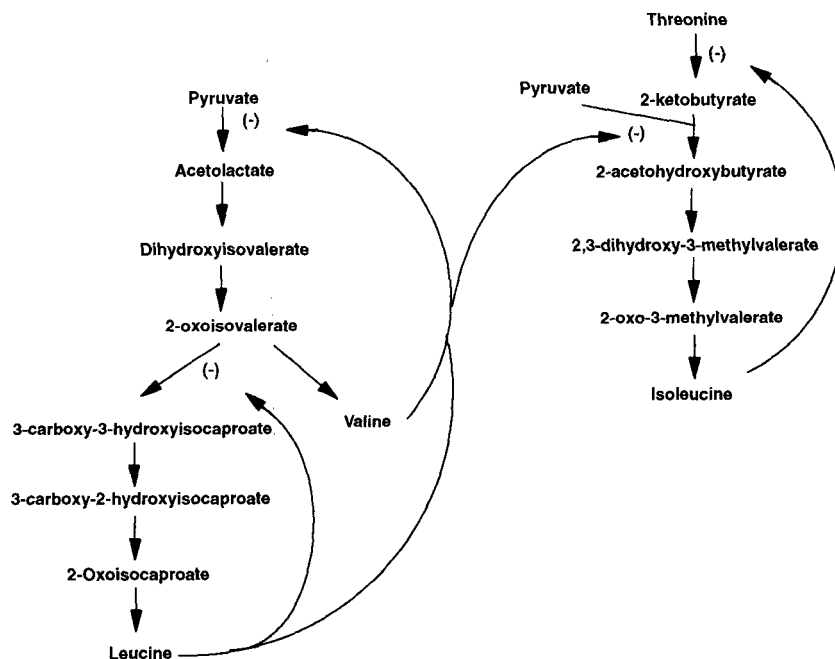


Figure 1. Biosynthesis of branched-chain amino acids in plants. AHAS catalyzes the parallel reactions that produce acetolactate and acetohydroxybutyrate. The negative signs indicate the sites of feedback regulation by the end products. Ile inhibits threonine dehydratase. Val and Leu inhibit AHAS. Leu also inhibits isopropylmalate synthase.

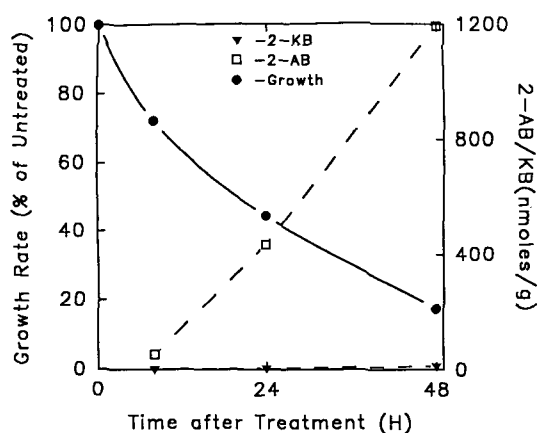


Figure 2. Effects of $10 \mu\text{M}$ imazaquin leaf elongation, 2-AB, and 2-KB levels in hydroponically grown, 10-d-old maize seedlings. Growth rates were determined by measuring the rate of leaf elongation of the fourth leaf between 8, 24, and 48 h after treatment initiation. The growth rate of untreated seedlings during this time period was $2.11 \pm 0.3 \text{ mm h}^{-1}$. The levels of 2-KB and 2-AB in untreated tissue were $<0.5 \text{ nmol g}^{-1}$ fresh weight and $<5 \text{ nmol g}^{-1}$ fresh weight, respectively.

Amino Acid Analysis

Amino acids eluted from the cation exchange column were freeze dried and then dissolved in Na-S buffer (Beckman). The solution was filtered to remove the particulate matter, and the amino acid composition was determined on a Beckman 7300 amino acid analyzer.

RESULTS

Accumulation of 2-KB and/or 2-AB in Plants

Imazaquin at $10 \mu\text{M}$ inhibited leaf elongation of the fourth leaf of 10-d-old maize seedlings, and the degree of inhibition increased with time (Fig. 2). Imazaquin treatment also caused accumulation of both 2-KB and 2-AB, which increased progressively with time. These results show a direct correlation between accumulation of 2-KB and/or 2-AB and growth inhibition following imazaquin treatment and lend support to the hypothesis that the inhibitory effects of imazaquin are related to this accumulation.

It is interesting that accumulation of 2-AB was about 100-fold greater than accumulation of 2-KB. This ratio of 2-KB and 2-AB was seen in all experiments. Because 2-AB is the transamination product of 2-KB, this result suggests that the two compounds stay in equilibrium *in vivo*, the equilibrium being in favor of 2-AB. Because 2-AB is the major component of the two, and the accumulation trend for the two compounds was the same, only the data for 2-AB will be presented hereafter.

Decrease in the Level of 2-KB and/or 2-AB

If AHAS inhibitors kill plants because of the toxic accumulation of 2-KB and/or 2-AB, then Ile should protect plants from the herbicidal effects of AHAS inhibitors because inhi-

bition of threonine deaminase by Ile should prevent accumulation of 2-KB and/or 2-AB. To test this hypothesis, intact maize plants were treated with imazaquin, Val, Leu, and Ile, either singly or in various combinations via the root system. Imazaquin inhibited leaf elongation of maize seedlings in the expected fashion (Fig. 3A). Ile, Val plus Ile, and Val plus Leu plus Ile did not have a significant effect on leaf elongation. Val alone caused growth inhibition but not to the same extent as the inhibition observed with imazaquin treatment. Amino acid treatments in combination with imazaquin are shown in Figure 3B. Ile or Val alone did not reverse the herbicidal effects of imazaquin. A combination of Val plus Ile caused a significant reversal of growth inhibition by imazaquin. However, a combination of Val plus Leu plus Ile gave the best and nearly complete reversal of the inhibitory effects of imazaquin on plant growth.

Analysis of the free amino acid levels in these plants showed that imazaquin treatment caused an increase of about 250-fold in the concentration of 2-AB (Fig. 4). When Ile was included with imazaquin, 2-AB levels were reduced to about 10% of the level present in the imazaquin-treated plants, yet Ile did not protect plants from the inhibitory effects of imazaquin (Fig. 4). It is interesting that 2-AB accumulation was 7-fold higher in Val-treated plants compared to the imazaquin-treated plants. However, Val did not reduce growth to the same extent as did the imazaquin treatment. These results

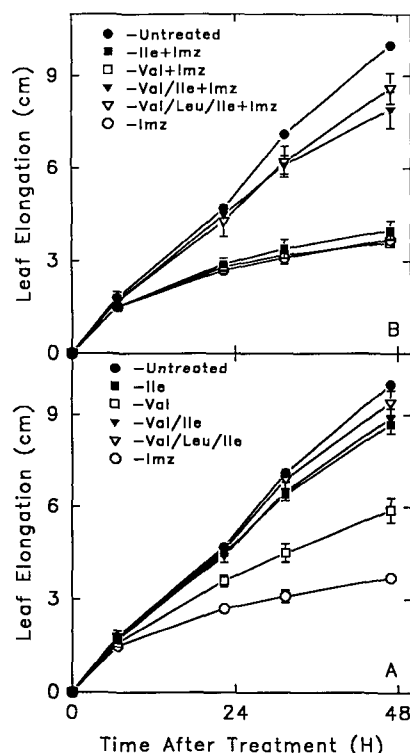


Figure 3. Effects of imazaquin (Imz) in various combinations with Val, Leu, and Ile on elongation of the fourth leaf of hydroponically grown, 10-d-old maize seedlings. Imazaquin ($10 \mu\text{M}$) and amino acid (2 mM each) treatments were supplied continuously in the nutrient solution. Vertical bars indicated $\pm 1 \text{ sd}$.

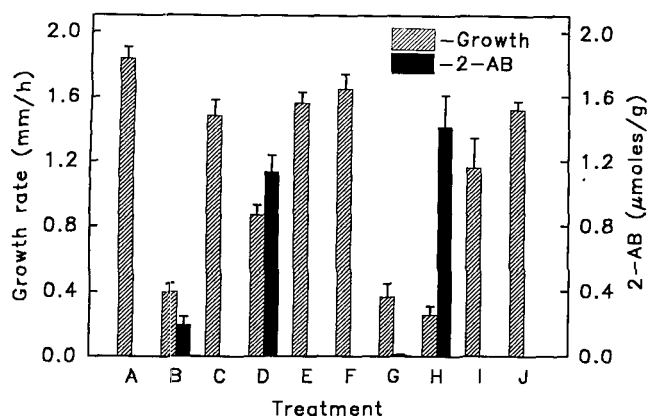


Figure 4. Effects of imazaquin (Imz) in various combinations with Ile, Val, and Leu on growth rate and 2-AB levels in hydroponically grown, 10-d-old maize seedlings. Treatments were: A, untreated; B, 10 μM imazaquin; C, 2 mM Ile; D, 2 mM Val; E, 2 mM Ile and 2 mM Val; F, 2 mM Ile, 2 mM Val, and 2 mM Leu; G, 10 μM imazaquin plus 2 mM Ile; H, 10 μM imazaquin plus 2 mM Val; I, 10 μM imazaquin plus 2 mM Ile and 2 mM Val; J, 10 μM imazaquin plus 2 mM Ile, 2 mM Val, and 2 mM Leu. Vertical bars indicated ± 1 sd.

show that there is no correlation between the levels of 2-AB in plant and growth inhibition.

Increase in the Level of 2-KB and/or 2-AB

If accumulation of 2-KB and/or 2-AB is toxic to plants, then feeding 2-KB and/or 2-AB to plants should cause accumulation of these compounds in plants and result in growth inhibition and, eventually, plant death. Because we have already shown that 2-KB and 2-AB are in equilibrium *in vivo*, feeding either of these compounds should give the same effect. Because 2-KB is not taken up by plant roots (our unpublished data), different concentrations of 2-AB or imazaquin were fed through the root system. Root-applied imazaquin inhibited growth in a dose-dependent manner (Fig. 5A) However, up to 2 mM 2-AB did not inhibit leaf elongation of maize plants (Fig. 5B). Analysis of the levels of 2-AB in these plants revealed that even the lowest concentration of 2-AB (0.5 mM) caused a nearly 2-fold greater accumulation of 2-AB within the shoot meristem region than the highest concentration of imazaquin (10 μM) (Table I), yet there was no significant effect of this level of 2-AB treatment on plant growth. Feeding 2 mM 2-AB resulted in a buildup of 2-AB *in vivo* to 1978 nmol g^{-1} fresh weight; however, even this high internal concentration of 2-AB did not significantly affect the plant growth. A similar lack of correlation was found between growth inhibition and 2-KB accumulation (data not shown). Amino acid profile data show that 2-AB feeding resulted in accumulation of Ile (Table I), which shows that 2-AB taken up by the root system must be transported to the chloroplast, where it is converted to Ile by the enzymes of this pathway. Therefore, the exogenously supplied 2-AB is going to the chloroplast, where it is supposed to be produced in the imidazolinone-treated plants. There was a correlation between the degree of inhibition of growth caused by imazaquin

and the decrease in the levels of Val and Leu in the shoot meristem region (Table I).

DISCUSSION

The results presented here show no correlation between accumulation of 2-KB and/or 2-AB and growth inhibition due to imazaquin treatment, although a comparison between untreated and imazaquin-treated plants shows a positive correlation between the accumulation of 2-KB and/or 2-AB and inhibition of plant growth (Fig. 2). However, this correlation is circumstantial. If 2-KB and/or 2-AB were the causal agent of the activity of AHAS-inhibiting herbicides, plants would be protected by supplementing with Ile, but Ile alone had no reversal effect on the growth-inhibiting activity of imazaquin either in this study (Fig. 3B) or in any other system (Rost and Reynolds, 1985). A combination of Val, Leu, and Ile was needed to provide the highest protection (Fig. 3B). Analysis of free amino acid pools in these plants showed that both treatments, Ile alone and a combination of Val, Leu, and Ile, prevented the accumulation of 2-KB and/or 2-AB. In the same experiment, Val alone caused a greater accumulation of 2-AB than imazaquin treatment, yet imazaquin inhibited growth more than Val. Similar results were also obtained in other species (e.g. cocklebur, lima bean, tomato; data not presented). These results indicated that accumulation of 2-

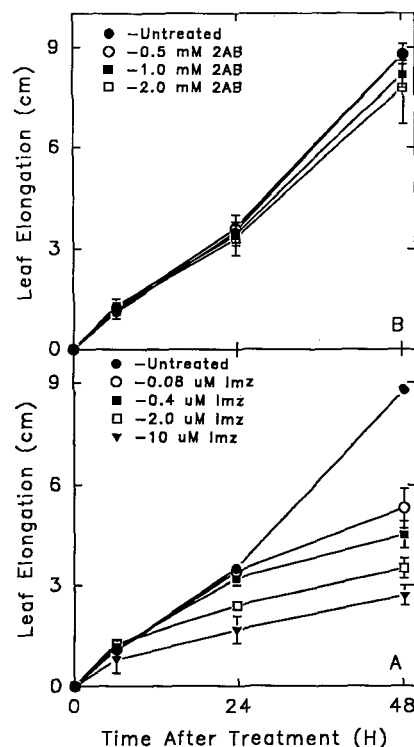


Figure 5. Effects of various concentrations of imazaquin or 2-AB on elongation of the fourth leaf of hydroponically grown, 10-d-old maize seedlings. Treatments were applied continuously during the time course of the experiment via the nutrient solution. Vertical bars indicated ± 1 sd.

Table I. Effects of different concentrations of imazaquin and 2-AB on leaf elongation rates, 2-AB accumulation, and free branched-chain amino acid levels in hydroponically grown, 10-d-old maize seedlings 24 h after initiation of treatment

Treatment	Concentration	Growth Rate ^a	2-AB	Ile	Val	Leu
		mm h ⁻¹	nmol g ⁻¹	nmol g ⁻¹	nmol g ⁻¹	nmol g ⁻¹
Control		2.06 ± 0.02	1	64	175	67
2-AB	0.5 mM	2.04 ± 0.12	517	831	265	89
	1.0 mM	1.90 ± 0.06	1042	1908	303	105
	2.0 mM	1.77 ± 0.24	1978	3431	408	119
Imazaquin	0.08 μM	0.73 ± 0.20	20	122	156	66
	0.4 μM	0.53 ± 0.07	46	139	101	61
	2.0 μM	0.43 ± 0.07	178	293	74	52
	10 μM	0.37 ± 0.07	270	214	77	48

^a Growth rate is a measure of the rate of leaf elongation between 8 and 24 h after initiation of treatment. Treatments were applied continuously via the nutrient solution.

KB and/or 2-AB cannot account for the herbicidal activity of imazaquin.

Further supporting evidence came from the next experiment in which internal concentrations of 2-KB and 2-AB were forced to increase by treating plants with high concentrations of 2-AB. Feeding 2-AB caused much greater accumulation of 2-KB (data not presented) and 2-AB than did imazaquin treatment (Fig. 5) but did not inhibit growth. A potential reason for the lack of effect of exogenous applications of 2-AB on growth is that the compound is not accumulating at the same site as it does when the plant is treated with imazaquin. However, 2-AB fed to the maize roots was going to the chloroplasts, the location where 2-AB is predicted to be produced in the imazaquin-treated plants, because it caused a large increase in the concentration of Ile.

Therefore, there is no correlation between the pools of 2-KB and/or 2-AB and plant growth. Our conclusion is also supported by a previous observation that growth inhibition preceded accumulation of 2-AB in chlorsulfuron-treated *L. minor* (Rhodes et al., 1987). However, some of the recent work on *S. typhimurium* provides even stronger support for our conclusion. Sulfometuron methyl treatment does not inhibit growth of wild-type *S. typhimurium* even though it leads to accumulation of high levels of 2-KB (Epelbaum et al., 1992). Furthermore, *S. typhimurium* TV108, expressing only AHAS isozyme I (low affinity for 2-KB), grows better with supplemental 2-KB even though this supplementation causes a buildup of 2-KB in the cells. Our results, taken together with observations in *L. minor* and *S. typhimurium*, conclusively demonstrate that accumulation of 2-AB/2-KB is not accountable for the herbicidal activity of imazaquin.

Why, then, do plants die after inhibition of AHAS and why are AHAS inhibitors such potent herbicides? To answer the first question it appears that there is a high correlation between the pool sizes of Val and Leu and the amount of growth inhibition caused by imazaquin treatment (Fig. 5, Table I). These results suggest that growth inhibition is the result of depletion of these two amino acids. It appears that AHAS-inhibiting herbicides rapidly disrupt protein synthesis and cell division. Rhodes et al. (1987) showed that the increase in amino acids following chlorsulfuron treatment is due to protein hydrolysis, which is consistent with a rapid

decrease in the levels of soluble protein (Anderson and Hibberd, 1985). This increase in the pools of amino acids was seen in all amino acids except Val and Leu (Anderson and Hibberd, 1985; Table I). The pools of amino acids measured are made up of the newly synthesized amino acids and the amino acids derived from degraded proteins. Therefore, pools of newly synthesized Val and Leu in imazaquin-treated plants may be reduced to a much greater extent than indicated from the numbers presented in Table I. It is also possible that newly synthesized amino acids may be more readily available for growth than the amino acids derived from the degraded proteins.

DNA synthesis and mitosis decrease within a few hours after application of AHAS inhibitors (Reynolds, 1986; Shaner and Reider, 1986; Rost et al., 1990). This effect could be prevented and even reversed by exogenous supplementation with Val, Leu, and Ile. Although the connection between mitosis and the branched-chain amino acid pathway is not understood, prevention of cell division at the growing point would have a negative effect on the status of the plant and could lead to death. In addition, inhibition of AHAS also causes a rapid decrease in the translocation of photosynthate to the growing points of the plant (Devine, 1989). Thus, the meristematic tissue is not only deprived of the branched-chain amino acids but is also starved for carbon. Indeed, the symptoms of AHAS-inhibiting herbicides are first observed in the meristematic region with cessation of growth of the growing point and then its ultimate death. Therefore, a disruption of the pools of the branched-chain amino acids and photosynthate translocation may lead to plant death.

In summary, high levels of 2-KB and 2-AB accumulate in imazaquin-treated plants. However, initial growth inhibition is not due to accumulation of 2-KB and 2-AB. Starvation for the branched-chain amino acids may be the primary cause of growth inhibition following AHAS-inhibiting herbicide treatment of maize plants.

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