Consequences on Nitrogen Metabolism in Soybean (*Glycine max* L.) as a Result of Imazethapyr Action on Acetohydroxy Acid Synthase

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The extractable activity of acetohydroxy acid synthase (AHAS; EC 4.1.3.18) was found to be severely decreased in soybean (*Glycine max* L.) shoots for a period of 120 h after treatment with imazethapyr. Inhibition did not take place when a mixture of valine, leucine, and isoleucine (VLI mixture) was added to the herbicide treatment. As a consequence of this inhibition valine, leucine, and isoleucine decreased and most of the other amino acids increased. Also a decrease in total soluble proteins was found with respect to the untreated controls. This did not exceed 25% and was prevented by the addition of the VLI mixture to the herbicide treatment. The increases in most of the free amino acids seem to be attributable to a change in the protein turnover rate of pre-existing proteins rather than to new amino acid synthesis. This behavior has been hypothesized to be a result of the demand for ammonia in connection with the decrease in branched-chain amino acids deriving from AHAS inhibition. The reduction of nitrite reductase (NiR; EC 1.7.7.1), glutamine synthetase (GS; EC 6.3.1.2), and glutamate synthase (GOGAT; EC 1.4.1.14) activities in imazethapyr-treated samples and the recovery in the imazethapyr plus VLI mixture treated samples of GS and GOGAT activities after a 24 h period following NiR recovery support this hypothesis.

Keywords: *Imazethapyr; AHAS; branched-chain amino acids; nitrogen metabolism*

INTRODUCTION

Imazethapyr [(*RS*)-5-ethyl-2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl) nicotinic acid] belongs to the group of imidazolinones, a class of herbicides active against annual and perennial monocotyledonous and dicotyledonous plants. These compounds are known to have acetohydroxy acid synthase (AHAS) as a target site. This is the first enzyme common to the biosynthetic pathway of three branched-chain amino acids, i.e., valine, leucine, and isoleucine and therefore its inhibition leads to a decrease in the levels of these amino acids (Shaner et al., 1984).

Several biochemical and physiological effects have been shown to be secondary consequences of the primary action of imidazolinones. Changes in the amounts of free amino acids and soluble protein, inhibition of photosynthate transport, and accumulation of neutral sugars have been found in *Zea mays* treated with AC 243,997 (Shaner and Reider, 1986). Inhibition of mitosis and of DNA synthesis has been shown in *Avena fatua* and *Alopecurus myosuroides* treated with AC 222,-293 (Pillmoor and Caseley, 1987). Inhibition of transpiration has been shown to occur in *Imperata cylindrica* treated with imazapyr and in *Xanthium strumarium* treated with imazaquin and imazethapyr (Shaner, 1988, 1991).

Imazethapyr is a selective imidazolinone herbicide used to control a wide spectrum of broad-leaved weeds and grasses in soybean. Soybean tolerance to this compound can result from its high level of AHAS activity (Singh et al., 1988), as well as its capacity to rapidly detoxify the herbicide, as recently demonstrated by Tecle et al. (1993). However, the half-life of the parent compound in soybean has been found to be 31 h, which is approximately the same length of time as that of corn, which is relatively sensitive (Shaner and Mallipudi, 1991). Therefore, the persistence of herbicide action on the AHAS activity of soybean could be enough to provoke some changes in plant metabolism even if without lethal consequences.

The aim of this study was to ascertain the secondary consequences on some aspects of nitrogen metabolism deriving from imazethapyr action on soybean AHAS. Hence, the trends of the contents of free amino acids and soluble proteins as well as of the activity of some enzyme systems involved in ammonia availability and assimilation into organic forms were investigated in relationship to the persistence of imazethapyr action on the AHAS activity of soybean shoots.

MATERIALS AND METHODS

Chemicals. Analytical grade imazethapyr (99.9%) was supplied by Cyanamid; standard amino acids, methyl viologen, hydroxylamine hydrochloride, γ-glutamylhydroxamate, triethylamine (TEA), and phenyl isothiocyanate (PITC) were obtained from Sigma Chemical Co. (St. Louis, MO). Brilliant Blue G was obtained from Aldrich (Steinheim, Germany), and acetonitrile (HPLC grade) and water (HPLC grade) were purchased from BDH (Poole, England). Phenylmethanesulfonyl fluoride (PMSF) was obtained from ICN Biochemicals Inc. (Costa Mesa, CA). All other reagents were of ACS grade.

Apparatus. A Perkin-Elmer HPLC instrument was assembled from the following modular components: Series 410 LC pumps, a Rheodyne Model 7125-075 injector, a Model 235 diode array detector interfaced with an Omega 2 analytical chromatographic workstation (version 2.50 software) and an Omega 235 software upgrade kit (PE Nelson), an LC-18 DB column Supelcosil, 25 cm × 4.6 mm i.d. (5 μm particle size), protected with a Supelguard precolumn (3 cm long) having exactly the same characteristics (Supelco Inc., Bellefonte, PA).

A Varian Model Cary 210 double-beam grating spectrophotometer was used.

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Plant Material and Growth Conditions. Soybean (*Glycine max* L. lyra 1+) seeds obtained from ICI Seed-SES, Italy, were used. Seeds were surface sterilized with two 3-min rinses in full strength commercial bleach (5.25% NaOCl) followed by a rinse in sterile distilled water. The seeds were germinated in sand quartz (prewashed with hydrochloric acid) in plastic pots (40 × 20 × 10 cm). Each pot contained 100 germinating seeds spaced 5.0 cm apart in 5.0 cm adjacent rows. The pots were kept in controlled conditions in the growth chamber at 24/16 °C day/night, with a 14-h photoperiod, 300 μeinstein m^{-2} s^{-1} photon flux density, and 75–80% relative humidity. Distilled water (50 mL) was applied daily to each pot.

When the seedlings were 10 days old (generally at the third trifoliolate leaf stage), one-fourth strength Hoagland solution (Hoagland and Arnon, 1950) was used instead of distilled water, and the pots were divided into three groups. One group was left as control, one was treated only with imazethapyr at 280 μg/pot (corresponding to 35 g ha^{-1}), and the third was treated with the same amount of herbicide coupled with a mixture of valine, leucine, and isoleucine (VLI mixture), each at 100-fold the imazethapyr concentration. To apply the required concentration of the herbicide, the rate per hectare was calculated according to the surface area per pot and then the herbicide, alone or in addition to the VLI mixture, was solubilized in a suitable amount of distilled water. The solutions, after the addition of Tween 20 (0.25% v/v), were applied to the respective pot twice with a mechanical sprayer, in one direction and crosswise. Shoots were collected just before herbicide application (zero time) and 12, 24, 36, 48, 60, 72, 96, and 120 h after treatment. At harvest, the entire shoots were rinsed with water and then washed with copious amounts of tap water to get rid of any adsorbed herbicide or amino acids. The shoots were dried by blotting with paper towels before the subsequent analytical procedures.

Analysis of Amino Acid Pools. Amino acids were extracted from entire shoots (5 g) with methyl alcohol (3 × 25 mL), and the extracts were collected for amino acid determination following the procedure of Rhodes et al. (1987).

A standard solution of amino acids was prepared by dissolving them to a final concentration of 1.0 mM each in 0.1 N HCl and storing it at −30 °C until needed. The standard solution and the sample extracts were dried and the amino acids were derivatized to their PTC derivatives according to the method of Bidlingmeyer et al. (1987). Subsequently, 500 μL of sodium phosphate (5 mM, pH 7.6), containing 5% acetonitrile, was added as diluent and 6.0 μL was injected into the HPLC system.

Protein Extraction and Determination. Entire shoots (15 g) were cut into pieces and homogenized by sonication with chilled acetone (75 mL) for 1 min. The crude homogenates were filtered through a Büchner funnel and washed with chilled acetone. The residues were spread on filter paper and allowed to dry at room temperature to obtain the acetone powders (Harborne, 1988). For complete protein extraction, aliquots of powders (0.5 g) were covered with buffer solution (25 mL; Tris-HCl, 0.05 M, pH 9.0) and allowed to stand for 10 min at 4 °C, and then the mixtures were centrifuged at 48200*g* for 15 min at 4 °C. Total protein content of the supernatant was determined spectrophotometrically by reaction with Brilliant Blue G according to the Bradford procedure (1976).

Assay of Acetohydroxy Acid Synthase Activity. According to the procedures of Singh et al. (1988), entire shoots were collected and samples corresponding to about 200 mg of dry matter were powdered in liquid nitrogen and used for the enzyme extraction. The activity of AHAS was measured in 0.5 mL of reaction mixture containing 0.1 mL of enzyme extract by colorimetric estimation of acetolactate, after its conversion to acetoin by decarboxylation in acidic condition. Appropriate checks of direct acetoin formation during the enzyme assay were made by measuring and subtracting the absorbance in the absence of the enzyme.

Assay of NADH-Nitrate Reductase Activity. Extraction and assay procedures of NADH-nitrate reductase activity (NR; EC 1.6.6.1) were carried out according to the method of Nakagawa et al. (1985). All steps in the enzyme extraction were conducted at 0–4 °C. Entire shoots were collected, and samples corresponding to about 200 mg of dry matter were powdered in liquid nitrogen and used for the enzyme extraction. The enzyme activity was determined in 1 mL of reaction mixture containing 0.2 mL of enzyme extract by the reduction of nitrate to nitrite and subsequent colorimetric measurement of the nitrite produced.

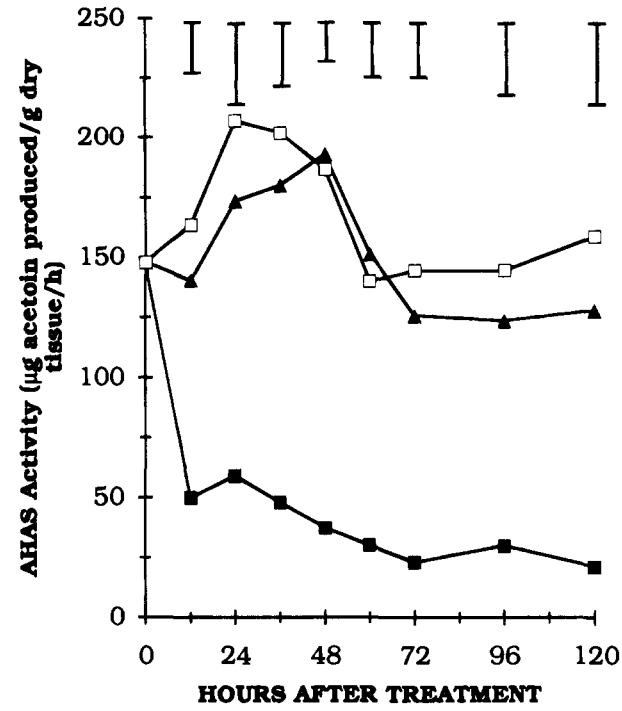

Figure 1. Activity of acetohydroxy acid synthase in soybean seedlings: (□) control; (■) imazethapyr; (▲) imazethapyr plus VLI mixture. Each value is the mean of three determinations. Vertical bars represent lsd at $p < 0.05$.

Assay of Nitrite Reductase (NiR) Activity. The extraction procedure was carried out at 0 −4 °C according to the method of Nagaoka et al. (1984). Entire shoots were collected, and samples corresponding to about 200 mg of dry matter were powdered in liquid nitrogen and used for the enzyme extraction. The activity of NiR was assayed according to the method of Wray and Filner (1970) in 1 mL of reaction mixture containing 0.1 mL of enzyme extract.

Assay of Glutamine Synthetase (GS) Activity. The procedures of extraction and assay of GS were performed according to the method of Lea et al. (1990). Entire shoots were collected, and samples corresponding to about 200 mg of dry matter were powdered in liquid nitrogen and used for the enzyme extraction. The determination of GS activity was measured in 0.5 mL of reaction mixture containing 0.2 mL of enzyme extract by colorimetric estimation of γ-glutamylhydroxamate formed after reaction of glutamate (50 mM) with hydroxylamine hydrochloride (5 mM). "Blank" tubes comprising the enzyme extract and all of the reagents except ATP were also incubated.

Assay of Glutamate Synthase (GOGAT) Activity. Entire shoots were collected, and samples corresponding to about 200 mg of dry matter were powdered in liquid nitrogen and used for the enzyme extraction according to the procedure of Marquez et al. (1988). According to the procedure of Hecht et al. (1988), the assay of GOGAT activity was performed in 1 mL of reaction mixture containing 0.2 mL of enzyme extract by continuous spectrophotometric monitoring of the consumption of NADH. For each measurement, two controls were performed with glutamine and 2-oxoglutarate being left out of the reaction medium in turn.

Each plant extraction procedure and subsequent determination of amino acids, protein content, and enzyme activities were replicated three times. All data were statistically analyzed using the least significant differences (lsd) method (Snedecor and Cochran, 1980).

Table 1. Concentration of Free Amino Acids in Soybean Seedlings as Percent of the Total Pool[a]

	hours after treatment								
		12		24		48		120	
	0	C	T	C	T	C	T	C	T
Asp	10.45	10.95	12.19 *	12.23	12.95 *	12.45	14.21 *	12.23	14.22 *
Glu	8.83	8.66	9.49 *	8.79	10.02 *	9.25	10.25 *	8.61	10.14 *
Asn	11.12	11.58	11.81	10.97	12.30 *	10.82	12.36 *	11.62	12.32 *
Ser	6.44	5.86	6.86	6.31	7.00 *	6.48	7.10 *	5.96	7.38 *
Gln	11.00	10.80	11.22 *	10.66	11.37 *	10.24	11.14 *	10.65	11.29 *
Cys	4.09	4.36	4.44	3.96	4.60 *	3.96	3.91	3.78	4.26 *
Gly	2.54	2.62	2.46	2.51	2.55	2.50	2.71 *	2.64	2.58
His	7.53	7.73	7.81	7.37	8.13 *	7.17	8.48 *	7.31	8.53 *
Thr	2.21	2.34	2.68	2.30	2.80 *	2.35	2.88 *	2.32	2.95 *
Ala	4.51	4.27	4.83 *	4.17	5.04 *	4.33	5.15 *	4.21	5.38 *
Pro	1.66	1.59	1.58	1.66	1.30 *	1.66	1.13 *	1.68	0.95 *
Tyr	3.61	3.38	3.17	3.32	2.77 *	3.46	2.67 *	3.29	2.54 *
Val	4.49	4.15	2.89 *	4.43	2.01 *	4.41	1.89 *	4.48	1.79 *
Ile	5.40	5.11	3.86 *	5.20	2.98 *	4.89	3.07 *	5.03	3.07 *
Leu	3.19	3.57	2.41 *	3.54	1.89 *	3.40	1.59 *	3.32	1.52 *
Phe	4.39	4.44	3.90 *	4.24	3.70 *	4.22	3.43 *	4.29	3.34 *
Try	7.17	7.04	6.58	6.78	6.51	6.75	6.40	6.89	6.25
Lys	1.37	1.55	1.80 *	1.58	2.09 *	1.65	1.54	1.66	1.48 *

a Values of treated samples (T) followed by an asterisk are significantly different at $p < 5\%$ with respect to untreated controls (C).

RESULTS AND DISCUSSION

Imazethapyr Effect on AHAS Activity of Soybean. As imidazolinone herbicides are known to have AHAS as a target site, the primary effect of imazethapyr on the extractable activity of this enzyme in soybean shoots was assayed. Figure 1 shows the behavior of AHAS activity during a 5-day period after treatment with imazethapyr alone and coupled with the VLI mixture. In the imazethapyr-treated shoots an initial sharp decrease in AHAS activity was observed with respect to the control, with the difference reaching an almost constant level of about 83% of the control 72 h after treatment. The stop in the decrease of the enzyme activity could be due to the detoxification of most of the absorbed imazethapyr. In fact, in a growth chamber experiment, Perucci et al. (1994) found that imazethapyr residues in soybean treated with a dosage equivalent to 45 g ha^{-1} were 30, 16, and 10 ppb 24, 72, and 120 h after treatment, respectively. The results presented in Figure 1 indicate a loss of extractable AHAS activity in the tolerant soybean of similar magnitude as that found by Shaner et al. (1990) in susceptible corn. No visual symptoms of toxicity were observed; only decreases in dry matter of treated seedlings, ranging from 9.4 to 13.0% with respect to the untreated control, were found during the entire treatment period (unpublished data). Soybean survival, despite the immediate reduction in AHAS activity, suggests the possibility of its adaptation to this situation, at the whole plant level. The experiments performed by Singh et al. (1990) on several plant species, including soybean, treated with imazapyr and imazethapyr confirm that AHAS does not significantly contribute to the natural tolerance to the imidazolinones at the whole plant level. No inhibition of the extractable activity of AHAS was found when the VLI mixture was also added to the herbicidal treatment. This seems to be in contrast to the well-known effect of AHAS feedback inhibition by valine, leucine, and isoleucine, which are the end products of the pathway (Stidham and Singh, 1991). On the contrary, in our experiment, the branched-chain amino acids seemed to protect soybean AHAS from imazethapyr action. Another example of lower feedback sensitivity to valine, leucine, and isoleucine was shown by Rathinasabapathi et al. (1990) in some variants of *Datura innoxia* treated with another AHAS inhibitor, chlorsulfuron. One key to the interpretation of this behavior could be that as the inhibition of AHAS causes a reduction in the synthesis of valine, leucine, and isoleucine, their external addition resulted in a compensatory effect on their content. Therefore, the concentration level capable of determining a feedback inhibition was not reached. In addition, the syntheses of proteins and, among these, the *de novo* synthesis of AHAS might be permitted. The true mechanism of the safening effect of the VLI mixture on the activity of AHAS in imazethapyr-treated soybean needs further experimental support to be clearly elucidated.

Table 2. Sum of Total Free Amino Acids in Soybean Seedlings

hours after treatment	nmol of amino acid pool/g of fresh tissue ± SD	
	untreated	treated
0	1102.09 ± 102.44	
12	1117.53 ± 111.75	1078.93 ± 123.81
24	1179.43 ± 109.21	1077.56 ± 121.49
48	1203.81 ± 122.68	1076.92 ± 115.33
120	1140.73 ± 104.65	1055.57 ± 129.55

Imazethapyr Effect on the Free Amino Acid and Soluble Protein Pools. To evaluate the effect of AHAS inhibition on the synthesis of free amino acids, their content in treated and untreated shoots was determined (Table 1). As expected, the more remarkable changes in treated shoots, with respect to untreated controls, resulted in decreases in valine, leucine, and isoleucine. In particular, their decreases ranged from 33, 35, and 27% to 63, 58, and 48%, respectively, during the experimental period. These decreases were accompanied by decreases, to a lesser extent, of proline, tyrosine, and phenylalanine and by increases of most other amino acids. The absolute values of total amino acids were slightly lower in treated samples (from 3 to 11%), but the decreases were not statistically significant on the basis of SD (Table 2). Studies performed on susceptible and tolerant corn lines treated with imazapyr demonstrated that the changes in amino acid pools are a secondary consequence of the inhibition of AHAS and are not attributable to other sites of the action of imidazolinones (Shaner, 1991).

The data reported in Figure 2 show a disturbance in protein synthesis in treated soybean but also confirm the ability of soybean to adapt itself to the reduction of

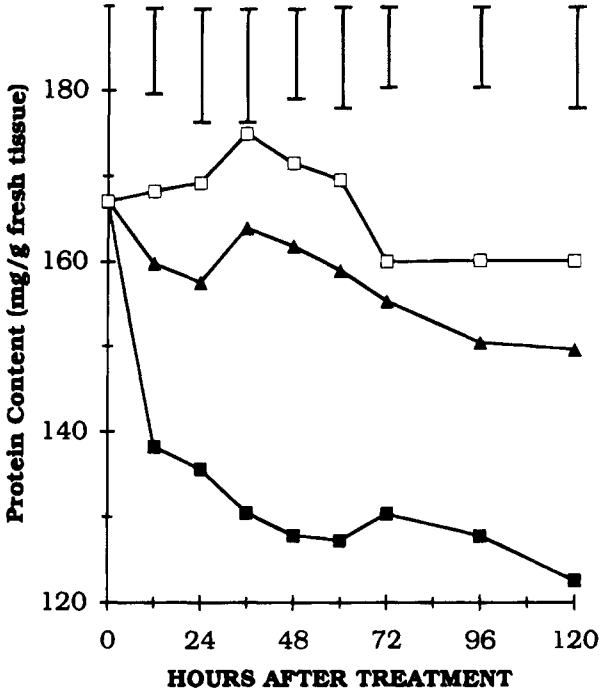

Figure 2. Protein content in soybean seedlings: (□) control; (■) imazethapyr; (▲) imazethapyr plus VLI mixture. Each value is the mean of three determinations. Vertical bars represent lsd at $p < 0.05$.

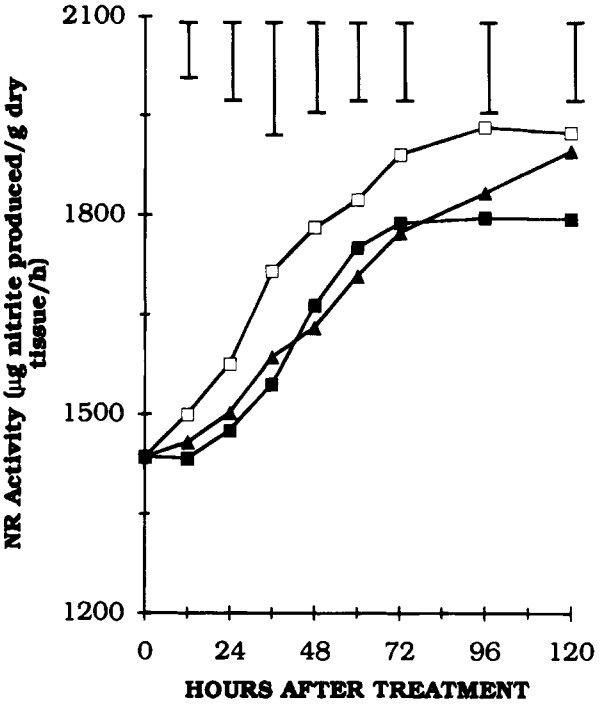

Figure 3. Activity of nitrate reductase in soybean seedlings: (□) control; (■) imazethapyr; (▲) imazethapyr plus VLI mixture. Each value is the mean of three determinations. Vertical bars represent lsd at $p < 0.05$.

AHAS activity. In fact, even though a sharp decline in protein content occurred during the first 48 h after herbicide treatment, the decreases did not exceed 26% with respect to the untreated controls, and an average decrease of 23% was maintained after 48 h for the entire subsequent experimental period. As a nonsignificant effect was observed in the protein content of shoots simultaneously treated with imazethapyr and the VLI mixture, the addition of valine, leucine, and isoleucine gave a possibility of overcoming, to a great extent, the decrease in protein synthesis.

Shaner and Reider (1986) and Rhodes et al. (1987) also observed increases in the free amino acid pools and decreases in the soluble protein pools in *Z. mays* and in *Lemna minor* treated with other AHAS inhibitors, imazapyr and chlorsulfuron, respectively. Therefore, they hypothesized that the increases in free amino acid levels were due to disruption of protein synthesis caused by changes in the turnover rate of pre-existing proteins rather than by new amino acid synthesis. The decreases of valine, leucine, and isoleucine coupled with that of proteins as well as the stopping of these trends after 48 h in the more mature shoots support this hypothesis for imazethapyr-treated soybean, also. Therefore, the ability of soybean to scavenge amino acids from pre-existing proteins represents a reasonable explanation for its survival despite the reduction of AHAS activity and the disturbance in the amino acid metabolism.

Imazethapyr Interference on Ammonia Availability and Assimilation. Since ammonia availability and its assimilation into organic forms may be considered a common prerequisite for amino acid formation, the activities of the NR–NiR system, as parameters of ammonia production, as well as of the GS–GOGAT system, as parameters of ammonia assimilation, were checked.

Figures 3 and 4 show that the activities of both NR and NiR increased during the experimental period in

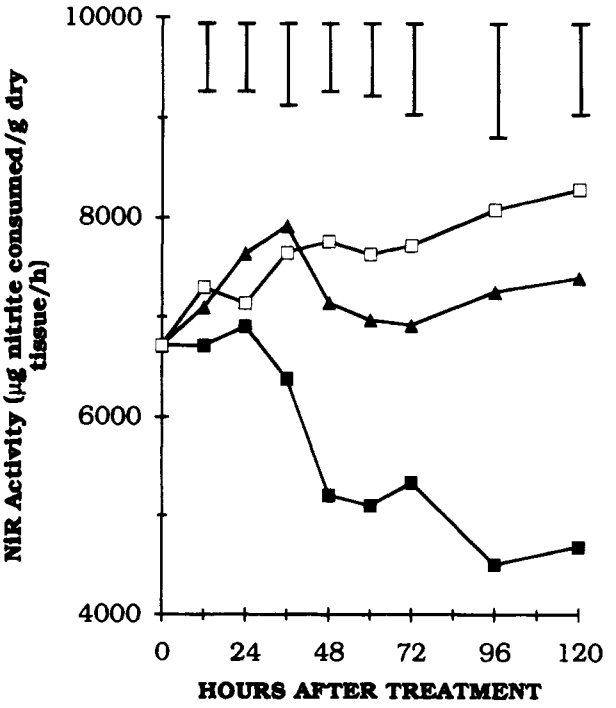

Figure 4. Activity of nitrite reductase in soybean seedlings: (□) control; (■) imazethapyr; (▲) imazethapyr plus VLI mixture. Each value is the mean of three determinations. Vertical bars represent lsd at $p < 0.05$.

the untreated shoots. The imazethapyr treatment did not affect the NR activity but significantly depressed the NiR activity after the first 24-h period. This appears to be a consequence of the loss of AHAS inhibition because addition of the VLI mixture to the herbicide treatment resulted in a prevention of NiR inhibition. Nevertheless, the inhibition of NiR activity indicates the eventual demand for ammonia as a secondary consequence of imazethapyr treatment to soybean shoots. A

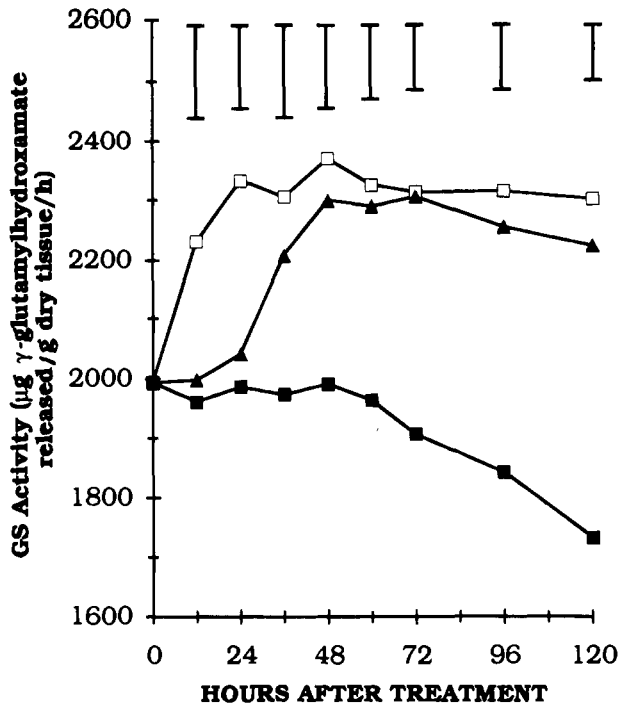

Figure 5. Activity of glutamine synthetase in soybean seedlings: (□) control; (■) imazethapyr; (▲) imazethapyr plus VLI mixture. Each value is the mean of three determinations. Vertical bars represent lsd at $p < 0.05$.

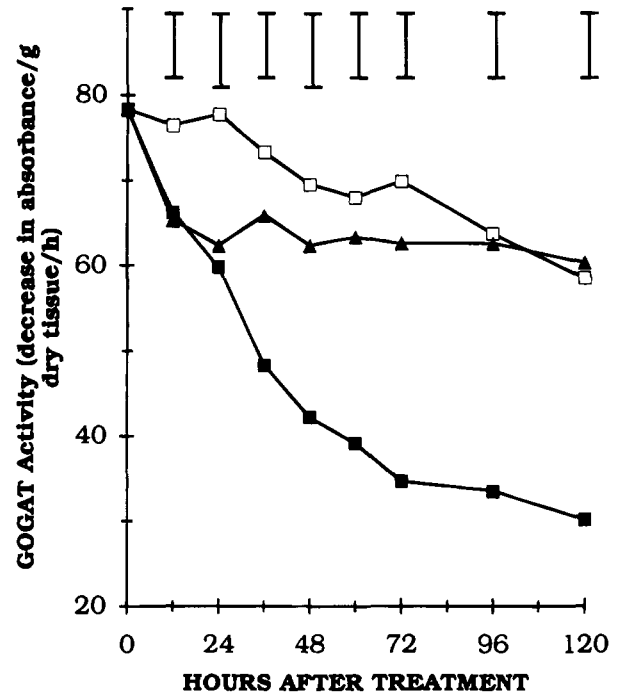

Figure 6. Activity of glutamate synthase in soybean seedlings: (□) control; (■) imazethapyr; (▲) imazethapyr plus VLI mixture. Each value is the mean of three determinations. Vertical bars represent lsd at $p < 0.05$.

relationship between changes in amino acid metabolism, following imidazolinone treatment, and nitrogen starvation was hypothesized by Shaner (1991) from his studies on imazapyr-treated corn. He concluded that the decrease of branched-chain amino acids may signal the plant that nitrogen starvation has occurred and that this signal causes an increase in the rate of protein turnover in response. This picture might also describe the situation in the shoots of soybean treated with imazethapyr.

The GS–GOGAT system provides nitrogen assimilation into glutamate which, in turn, acts as a nitrogen source for the synthesis of other amino acids. In Figures 5 and 6 the behaviors of GS and GOGAT are represented, respectively. The GS activity in the untreated controls increased during the first 24-h period, after which time an almost constant trend was observed. Instead, in the imazethapyr-treated samples a constant trend was found until 60 h from treatment, after which time a decreasing trend occurred. The GOGAT activity decreased in both the control and the imazethapyr-treated shoots, with the latter being much more pronounced. Therefore, GS and GOGAT activities in the herbicide-treated samples were significantly lowered in comparison with those of the respective untreated controls. Recoveries of both GS and GOGAT activities were found 24 h after treatment in the imazethapyr plus VLI mixture treated samples. Taking into account the delay of recoveries of GS and GOGAT activities, they do not seem to be directly due to the safening effect of the VLI mixture. Instead, if this delay, in connection with that of the starting NiR inhibition is considered, the reduction of GS and GOGAT activities seems to be attributable to the need for ammonia rather than to a direct consequence of AHAS inhibition.

In conclusion, in soybean shoots treated with imazethapyr, the hypothesis is confirmed of an ammonia demand in connection with a decreased synthesis of branched-chain amino acids which, in turn, determines an acceleration of protein turnover.

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