Growth Response and Changes in Starch Formation as a Result of Imazethapyr Treatment of Soybean (*Glycine max* L.)

Luciano Scarponi,* Luca Martinetti, and Mamdouh M. Nemat Alla†

Centro di Studio sulla Chimica e Biochimica dei Fitofarmaci del CNR, Istituto di Chimica Agraria, Università di Perugia, Borgo XX Giugno 72, 06121 Perugia, Italy

Decreased dry weight, increased fresh weight, and increased phenylalanine ammonia-lyase (PAL; EC 4.3.1.15) and tyrosine ammonia-lyase (TAL; EC 4.3.1) activities were found in imazethapyr-treated roots and shoots of soybean compared to untreated controls. In the treated seedlings, glucose content increased while starch decreased, giving rise to a decline in the sum of free and allocated glucose. Moreover, ribulosebisphosphate carboxylase activity (RUBISCO; EC 4.1.1.39) decreased in the treated shoots. The exogenous application of a mixture of valine, leucine, and isoleucine with the herbicide treatment prevented the effects on glucose and starch contents and on RUBISCO activity, but not on fresh and dry weight or PAL and TAL activities. These findings suggest that the former are a consequence of the herbicide inhibition of the synthesis of the branched-chain amino acids, while the latter could be stress symptoms.

Keywords: *Imazethapyr; Glycine max; carbohydrates; stress symptoms*

INTRODUCTION

Imazethapyr [(*R,S*)-5-ethyl-2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)] nicotinic acid is an imidazolinone herbicide used on soybean to control a wide variety of broadleaf weeds and grasses. It inhibits acetohydroxy acid synthase (AHAS; EC 4.1.3.18), a key enzyme in the biosynthesis of the branched-chain amino acids valine, leucine, and isoleucine (Shaner et al., 1984). It was demonstrated that soybean tolerance to this compound is due to its high level of AHAS activity as well as to its capacity to rapidly detoxify the herbicide (Singh et al., 1988; Tecle et al., 1993). The half-life of imazethapyr in soybean proved to be 31 h (Shaner and Mallipudi, 1991). Therefore, an interference of the herbicide on soybean AHAS for a period of at least 3 days after the treatment can occur.

In a previous study, changes in free amino acid content and total proteins as well as in the activities of some enzymes involved in ammonia availability and assimilation were shown to be secondary consequences of imazethapyr action on soybean AHAS (Scarponi et al., 1995). Shaner and Reider (1986) found increased neutral sugar levels in corn leaves following treatment with the imidazolinone imazapyr. This effect was attributed to the capacity of imidazolinones, and other AHAS inhibitors, to delay photosynthate translocation (Devine, 1989). As this effect was reversed by the exogenous application of branched-chain amino acids to the plant, the lack of photosynthate transport was considered to be in some way linked to the inhibition of synthesis of the branched-chain amino acids (Devine et al., 1990).

This study was carried out to ascertain whether any interference of carbohydrate formation, causing growth disturbance, occurs in soybean following imazethapyr treatment. A further aim of the study was to clarify whether the disturbance of carbohydrate formation is a consequence of the imazethapyr inhibition of branched-chain amino acid synthesis or due to a stress status induced by the herbicide.

Fresh and dry weights, starch and glucose contents, and phenylalanine ammonia-lyase (PAL), tyrosine ammonia-lyase (TAL), and ribulosebisphosphate carboxylase (RUBISCO) activities were checked in tissues of soybean seedlings treated with imazethapyr with and without the exogenous addition of valine, leucine, and isoleucine.

MATERIALS AND METHODS

Chemicals and Apparatus. Analytical grade imazethapyr was supplied by Cyanamid (Italy); glucose oxidase, peroxidase, *o*-dianisidine, α-amylase, and amyloglucosidase were obtained from Sigma Chemical Co. (St. Louis, MO); *trans*-cinnamic acid and *p*-coumaric acid were obtained from Fluka Chemie AC (Buchs, Switzerland). All other reagents were of ACS grade.

Determinations were carried out using a Varian Model Cary 210 double-beam grating spectrophotometer.

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 a 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 trifoliate leaf stage), one-fourth-strength Hoagland solution (Hoagland and Arnon, 1950)—pH corrected to 6.0—was used instead of distilled water, and the pots were divided into three groups. One group was used 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, each at 100-fold the imazethapyr concentration (VLI mixture). 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 combination with the VLI

* Author to whom correspondence should be addressed [telephone (39) (75) 5856231; fax (39) (75) 5856239; e-mail AGROCHIM@IPGUNIV.UNIPG.IT].

† Permanent address: Botany Department, Faculty of Science, Mansoura University, Damietta, Egypt.

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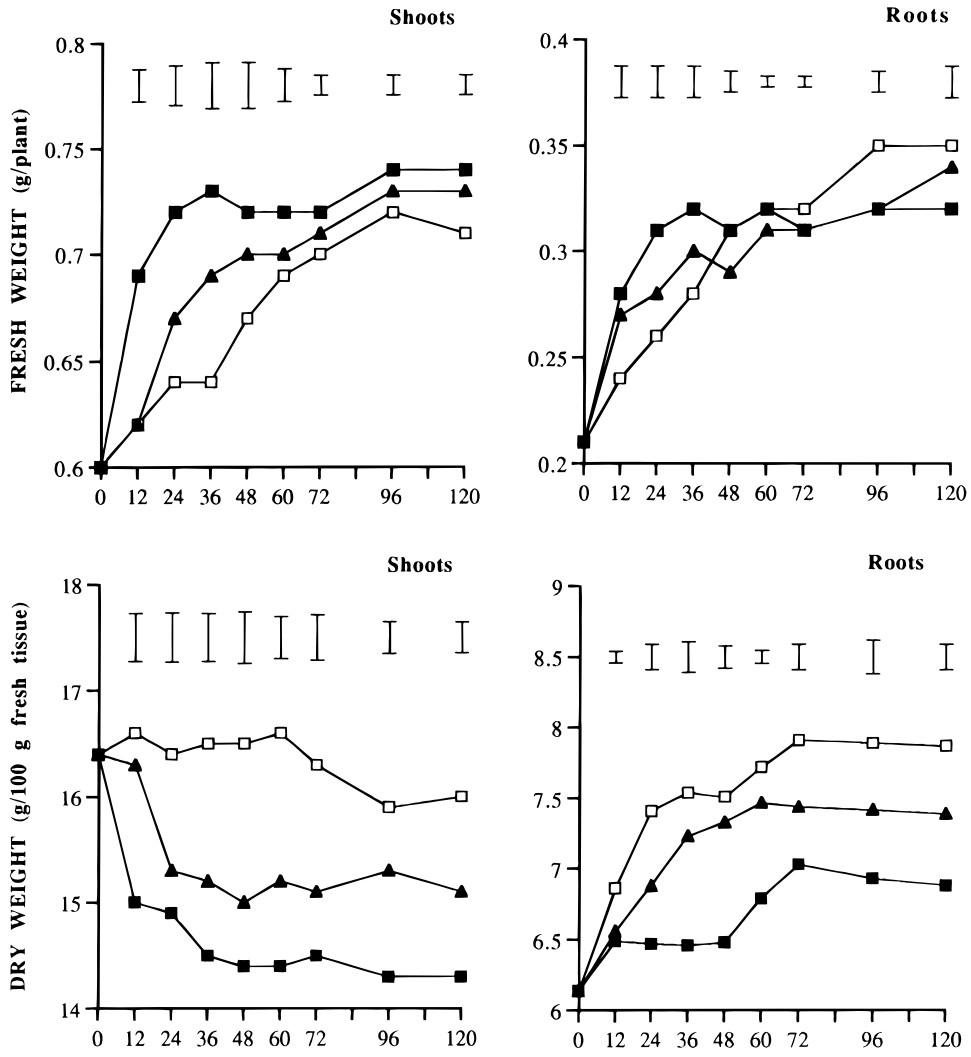

Figure 1. Fresh and dry weight in soybean shoots and roots: (□) control; (■) imazethapyr; and (▲) imazethapyr plus VLI mixture. Each value is the mean of three determinations. Vertical bars represent lsd at $p < 0.05$.

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 pots by mechanical spraying at right angles. Seedlings were collected just before herbicide application (zero time) and at 12, 24, 36, 48, 60, 72, 96, and 120 h after treatment. At harvest, shoots and roots were rinsed and then washed with copious amounts of tap water to get rid of any adsorbed herbicide or amino acids. Both shoots and roots were blotted free of water using paper towels before subsequent analytical procedures.

Determination of Glucose and Starch. Soluble sugars were separated from the total pool of carbohydrates by treating dry tissues with methanol/chloroform/water solution (12/5/3 v/v/v) (Dickson, 1979; Haissig and Dickson, 1979, 1982). The supernatants were collected and evaporated to completely remove methanol and chloroform according to the procedure of Palmer and Barker (1973). The content of glucose was then colorimetrically assayed with a mixture of glucose oxidase/peroxidase/*o*-dianisidine in a volume corresponding to 10 g of fresh tissue (Ebell, 1969; Cooper and McDaniel, 1970). To determine starch, the insoluble residues were submitted to further hydrolysis to glucose by a purified mixture of α-amylase/amyloglucosidase (Ebell, 1969; Dickson, 1979; Haissig and Dickson, 1979, 1982).

Assay of PAL and TAL Activities. Extraction and assay procedures of PAL and TAL activities were carried out according to the methods by Beaudoin-Egan and Thorpe (1985). All steps in the enzyme extraction were conducted at 0−4 °C. Samples of treated and untreated shoots and roots were collected, powdered in liquid nitrogen, and used for enzyme extraction. The enzyme activity, measured in 1 mL of reaction mixture containing either 6 μmol of L-phenylalanine for PAL or 5.5 μmol of L-tyrosine for TAL plus 100 μL of enzyme extract corresponding to 200 mg of dry weight, was determined by spectrophotometric estimation of the amounts of *trans*-cinnamic and *p*-coumaric acids formed from L-phenylalanine and L-tyrosine, respectively.

Assay of RUBISCO Activity. Extraction and assay of RUBISCO were performed according to the procedures of Keys and Parry (1990). Entire shoots were collected, ground to a fine powder with liquid nitrogen, and then used for the enzyme extraction. RUBISCO activity was assayed in 1 mL of reaction mixture containing 0.2 mL of enzyme extract corresponding to about 200 mg of dry weight and was determined by continuous spectrophotometric monitoring of the decrease in absorbance at 340 nm.

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

RESULTS AND DISCUSSION

Growth Response and Stress Symptoms. In a previous study performed under the same experimental

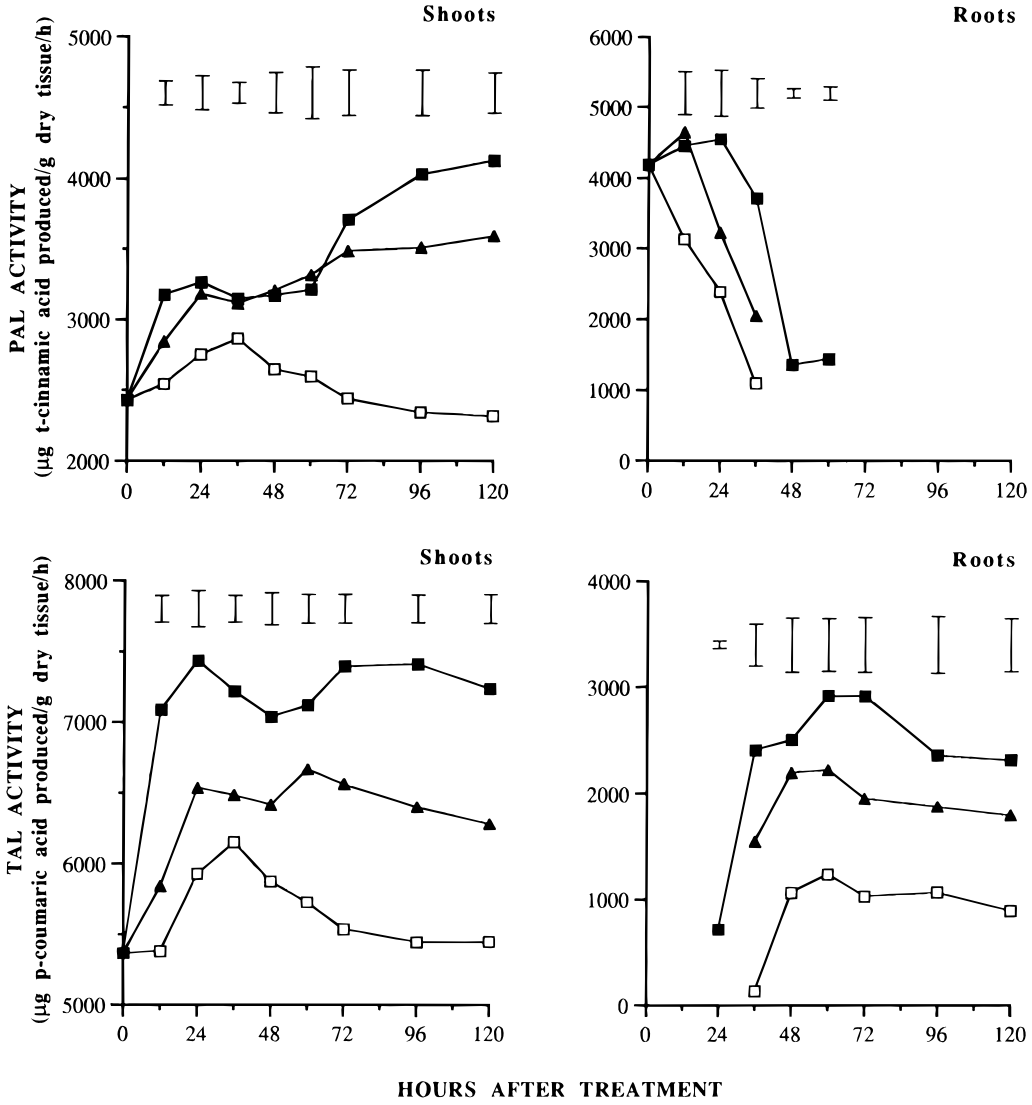

Figure 2. Activities of PAL and TAL in soybean shoots and roots: (□) control; (■) imazethapyr; and (▲) imazethapyr plus VLI mixture. Each value is the mean of three determinations. Vertical bars represent lsd at $p < 0.05$.

conditions, imazethapyr residues in soybean treated with a dosage as high as 45 g ha^{-1} were almost negligible 120 h after treatment (Perucci et al., 1994). Therefore, this was the choice of experimental period for our investigations.

Fresh and dry weights of soybean seedlings are shown in Figure 1. Compared to untreated controls, imazethapyr treatment generally resulted in significant increases of fresh weight in both shoots and roots. Conversely, the treatment significantly decreased the dry weight in shoots and roots throughout the entire experimental period. Fresh and dry weight data indicate that there is a higher level of water retention in treated shoots and roots. A similar behavior was found in *Xanthium strumarium* treated with imazaquin and imazethapyr (Risley, 1986) and in *Imperata cylindrica* treated with imazaquin (Shaner, 1988). These effects were attributed to an inhibition in transpiration which, on a leaf-area basis, indicates that the stomata are also affected by imidazolinone treatments (Shaner, 1991). In a previous study, the addition of VLI mixture in combination with herbicide treatment was able to compensate the primary consequence of AHAS inhibition (Scarponi et al., 1995); however, here it did not prevent fresh and dry weight changes. Therefore, the changes in fresh and dry weight do not appear to be a direct consequence of the inhibition of AHAS. To have further evidence of induced stress, PAL and TAL activities were investigated as they are key enzymes in the biosynthesis of phytoalexins, which are usually produced as a natural response to biotic and abiotic infections (Baily, 1982; Komives and Casida, 1983). Figure 2 shows the extractable activities of PAL and TAL in the shoots and roots. In the treated shoots, PAL and TAL activities were significantly higher than in untreated controls throughout the entire experimental period. In roots, PAL activity decreased to a nondetectable level at 48 and 72 h in the untreated and treated samples, respectively, while TAL activity was not detectable until after 12 and 24 h in the treated and untreated samples, respectively. However, when detectable, the activities of both PAL and TAL in the roots were significantly higher in treated than in untreated seedlings.

The increases in PAL and TAL activities have also been found in soybean after treatment with metribuzin, norflurazon, and fenuron (Hoagland and Duke, 1981), with atrazine (Hoagland, 1989), and with alachlor and metolachlor (Scarponi et al., 1991, 1992). All of these responses were attributed to stress symptoms. The addition of VLI mixture to the herbicide treatment did not prevent increases in PAL and TAL activities; therefore, these effects, like the changes in fresh and

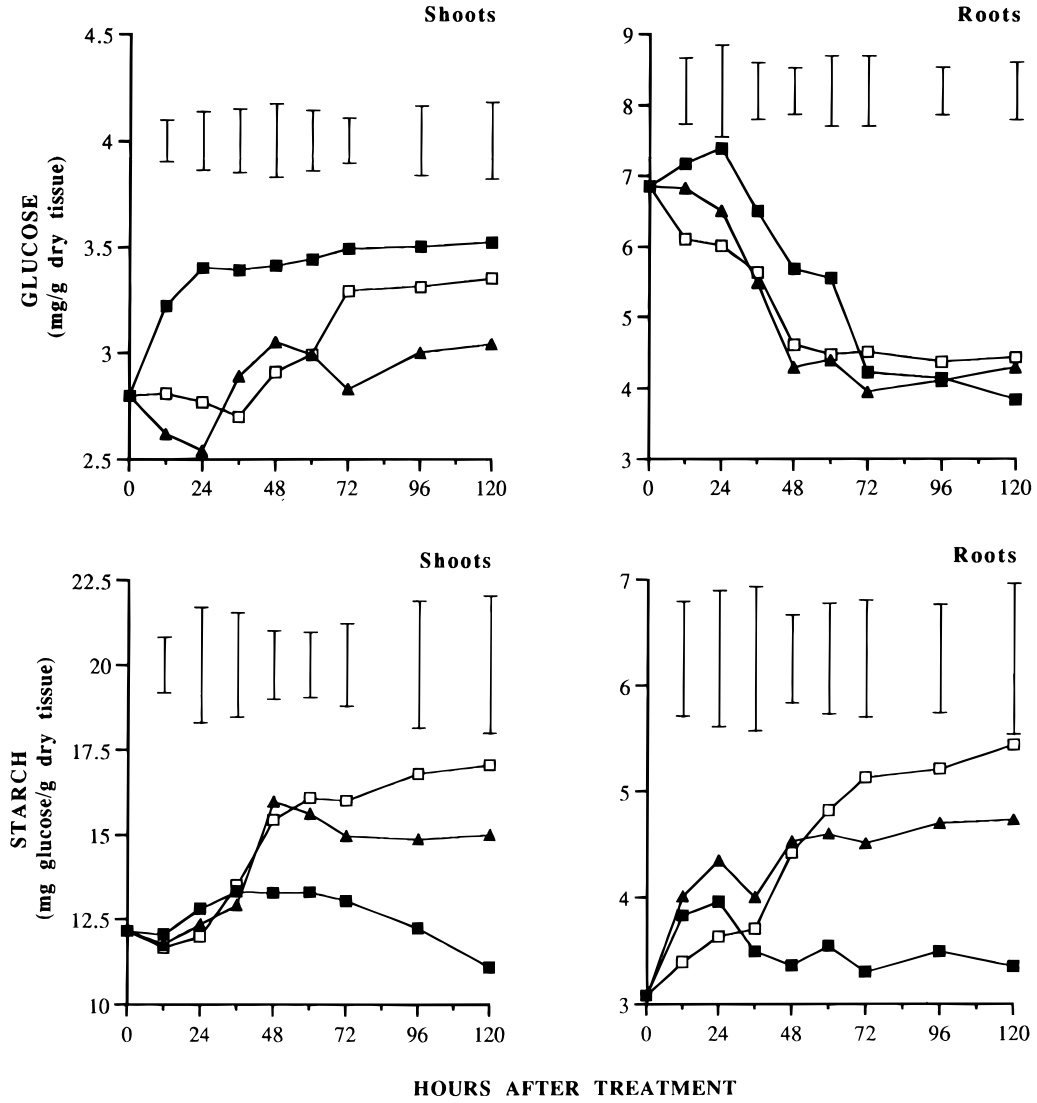

Figure 3. Contents of starch and glucose in soybean shoots and roots: (□) control; (■) imazethapyr; and (▲) imazethapyr plus VLI mixture. Each value is the mean of three determinations. Vertical bars represent lsd at $p < 0.05$.

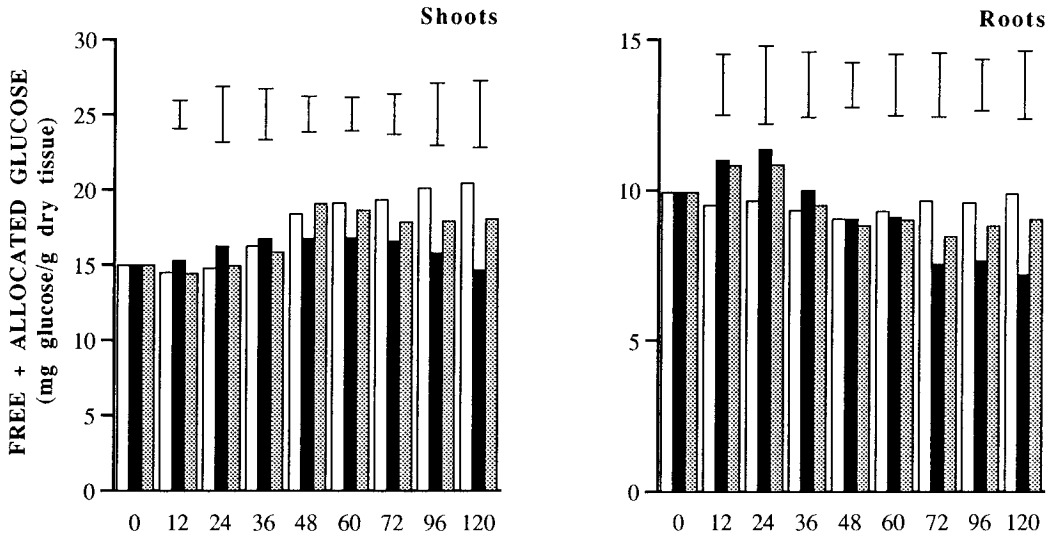

Figure 4. Sum of free and starch-allocated glucose in soybean shoots and roots: (□) control; (■) imazethapyr; and (lightly shaded bars) imazethapyr plus VLI mixture. Each value is the mean of three determinations. Vertical bars represent lsd at $p < 0.05$.

dry weight, were not a direct consequence of the imazethapyr action on AHAS activity.

Starch and Glucose Formation. Figure 3 shows that imazethapyr treatment did not significantly affect

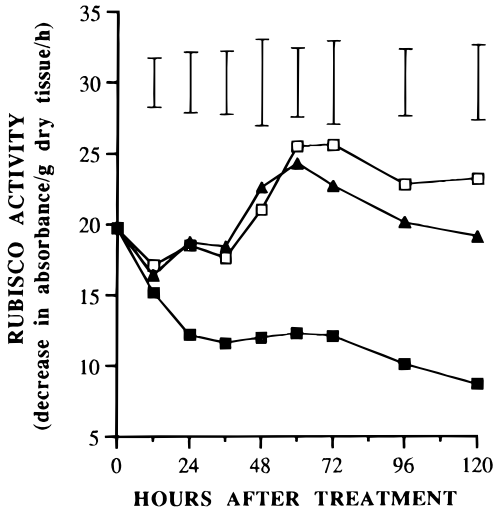

Figure 5. Activity of RUBISCO in soybean shoots: (□) control; (■) imazethapyr; and (▲) imazethapyr plus VLI mixture. Each value is the mean of three determinations. Vertical bars represent lsd at $p < 0.05$.

the starch content in soybean shoots and roots until 36 h after treatment. Thereafter, significant decreases were observed compared to untreated controls. These differences increased in time, reaching 34.9% and 38.4% in shoots and roots, respectively. Moreover, significant increases in the content of glucose were observed until 60 h after treatment in shoots and in roots ranging from 14.6% to 25.5% and from 15.4% to 24.2%, respectively.

It is known that imidazolinones and other AHAS inhibitors do not inhibit photosynthate formation, while they do inhibit photosynthate transport out of source leaves (Shaner, 1991). Therefore, the observed glucose increase in the treated soybean tissues could be due to less transport of soluble carbohydrates following imazethapyr treatment. Support for this hypothesis may be given by data for soybean tissues treated with imazethapyr in combination with VLI mixture, for which both the decreased starch and increased glucose were, to a large extent, prevented.

It is noteworthy that in the treated seedlings the glucose increases did not counterbalance starch decreases. The sums of free glucose and allocated glucose into starch in the treated tissues were lower than in untreated tissues 60 h after treatment in the shoots and 72 h after treatment in the roots (Figure 4). To ascertain any possible indirect consequence of imazethapyr treatment on CO_2 assimilation, the RUBISCO activity was measured in shoots (Figure 5). RUBISCO activity, compared to untreated controls, was less in the imazethapyr-treated tissues 24 h after treatment. As a protein depletion in soybean was found to occur immediately after imazethapyr treatment (Scarponi et al., 1995), here the delayed decrease in RUBISCO activity is unlikely to be attributable to a decrease in RUBISCO content due to protein depletion. However, the sharp increase in glucose after treatment suggests that the inhibition of RUBISCO activity may be due to the inhibition of transport of soluble carbohydrate, which, in turn, would inhibit CO_2 fixation via a feedback mechanism. This hypothesis was also put forward by Devine et al. (1990) to explain a similar behavior observed in *Fagopyrum tartaricum* treated with the AHAS inhibitor chlorsulfuron. This feedback effect would explain the concomitant increase in free glucose and decrease in the sum of free and starch-allocated glucose in the treated soybean tissues. This is further supported by the fact that the addition of VLI mixture to the herbicide treatment prevented any inhibition of RUBISCO activity.

In conclusion, the exogenous application of VLI mixture, which is capable of preventing the primary consequence of AHAS inhibition, appeared to be capable of preventing changes in glucose and starch content as well as in the RUBISCO activity. Therefore, while the above changes appear to be indirect consequences of the herbicide action on the AHAS enzyme, the changes in fresh and dry weight as well as in PAL and TAL activities appear to be mere stress symptoms since they were not prevented by the addition of VLI mixture.

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