JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Nitrogen Assimilation Studies Using ^{15}N in Soybean Plants Treated with Imazethapyr, an Inhibitor of Branched-Chain Amino Acid Biosynthesis

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The pattern of nitrogen assimilation in soybean plants treated with a herbicide that inhibits branched-chain amino acid biosynthesis was evaluated by ^{15}N isotopic analysis. The herbicide imazethapyr caused a strong decrease in nitrate uptake by roots, partly due to a reduced stomatal conductance. The inhibition of ^{15}N uptake was accompanied by a decrease in the ^{15}N content in the plant and, concomitantly, an inhibition of translocation to the shoot. Imazethapyr inhibited nitrate reductase activity in leaves and roots. Among all parameters studied, "de novo" synthesis of proteins was the first parameter of the N assimilation metabolism affected by the herbicide. These results show that this class of herbicides totally damages N metabolism and indicates a regulatory effect on N uptake and translocation that would be mediated by the increase in free amino acid pool provoked by the inhibition of branched-chain amino acid biosynthesis.

KEYWORDS: Acetolactate synthase; branched-chain amino acid; nitrate assimilation; nitrate reductase; isotopic analysis

INTRODUCTION

Utilization of nitrate, the most abundant form of inorganic nitrogen (N) available to crops, by higher plants involves several basic processes of uptake, translocation, and reduction. Acquisition and assimilation of nitrate limit plant growth and development. Moreover, nitrate acts as a signal controlling N metabolism and development of plants (*1*, *2*). In plants, nitrate is reduced to ammonium and then is incorporated into carbon skeletons to produce glutamate and glutamine, which translocate organic N from the source to the sink. Nitrate reductase (NR; EC. 1.7.1.1), the rate-limiting enzyme in nitrate assimilation, is regulated in a complex manner. Transcription of NR genes is stimulated by nitrate and light and reduced by low levels of carbohydrates, glutamic acid, and high glutamine/2-oxoglutarate ratio (*3*). At the post-transcriptional level, NR activity is rapidly and reversibly modulated by phosphorylation (*4*).

Imazethapyr (IM) is an imidazolinone herbicide which acts through the inhibition of acetolactate synthase (ALS; EC: 4.1.3.18, also known as acetohydroxyacid synthase), the first common enzyme in the biosynthetic pathway of branched-chain amino acids (BCAAs): isoleucine, leucine, and valine. Although these herbicides affect N assimilation into proteins, their precise effects on N metabolism is unknown. Several studies showed that protein synthesis was not altered in plants treated with inhibitors of BCAA biosynthesis (*5*, *6*). After inhibition of ALS, plants respond quickly by increasing its protein turnover to renew BCAAs, so that the BCAA pool does not decline to a level that would affect protein synthesis (*7*, *8*). As a consequence of this increase in protein turnover, total free amino acid pool is increased (*5*) and soluble protein content either marginally decreases (*9*) or decreases only after several days of treatment (*10*) or is not affected (*6*).

The alteration of the assimilatory pathway of N after ALS inhibition has also been studied, but results are not conclusive. Some studies dealing with the persistence of ALS-inhibitors activity in tolerant crops showed that ammonium assimilation enzymatic activities are more affected than nitrate assimilation activities (*9*) and that nitrate uptake is stimulated (*11*). Nevertheless, other studies dealing with lethal doses of ALS-inhibitors observed a reduction in the nitrate uptake and content in soybean seedlings (*12*) and a decrease in the activity of both nitrite reductase and GS-GOGAT in *Vicia faba* (*13*). However, given the fact that the different processes of N assimilation have been studied independently, the sequence of alterations in N metabolism after ALS inhibition remains unclear.

In order to study this sequence, several N assimilation parameters were studied during a short-term treatment (3 days) in soybean plants treated with the ALS-inhibitor IM. In order to assess the effects more precisely, plants were exposed to

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10.1021/jf0618224 CCC: $33.50
Published on Web 10/26/2006

labeled ^{15}N-nitrate. NR activity and its regulation under the effect of an ALS-inhibitor were also studied.

MATERIALS AND METHODS

Materials and Apparatus. Commercial IM (Pursuit 10) was supplied by BASF Española SA (Barcelona, Spain). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

A portable infrared gas analyzer (system Li-6200, Li-Cor, Lincoln, NE) was used to determine CO_2 assimilation rate and internal CO_2 concentration. Leaf area was determined using a Li-3000 system (Li-Cor, Lincoln, NE). Stomatal conductance was determined by a porometer (Delta T Device Ltd., Cambridge, U.K.). $^{15}N/^{14}N$ isotope ratios were analyzed with a mass spectrometer (Delta Plus, TERMOQUEST, Finnigan). Capillary electrophoresis was carried out using a P/ACE system 5500 (Beckman Instruments, Fullerton, CA). All spectrophotometric determinations were carried out in a double-beam spectrophotometer (Lambda 3B spectrophotometer, Perkin-Elmer, Norwalk, CT).

Plant Material and Treatment Application. Soybean seeds (*Glycine max* (L.) Merr., cv. Oxumi) were treated with 0.5% NaOCl for 10 min and imbibed in distilled water for 2 h with continuous bubbling of air. Seeds were germinated in moist vermiculite in plastic trays for 4 days at 28 °C under controlled conditions. After germination, seedlings were transferred to hydroponic tanks filled with nutrient solution (*14*; supplemented with 10 mM KNO_3) and placed in a growth chamber (Heraeus HPS-500). Growth conditions were 30/24 °C on a 16/8 h (light/dark) regime, 60/80% relative humidity (day/night), at 300 μmol photons m^{-2} s^{-1}. Plants were acclimated to the new growth conditions for 5 days, after which they were divided in two groups: a control and the herbicide treatment. The ALS-inhibitor used was IM, and it was applied to the nutrient solution at a concentration of 52 μM. The treatment lasted 3 days with plants being harvested at days 0, 1, 2, and 3.

Gas Exchange Measurements. Net CO_2 assimilation rate was measured in the first fully expanded leaf at 300 μE m^{-2} s^{-1}. Stomatal conductance was determined in the two first expanded leaves with a portable porometer.

^{15}N Feeding Experiments and Harvest. The experiment began on day 5 after transplant into the hydroponic system. Starting at the beginning of the light period, sets of plants were exposed to solutions containing $^{15}NO_3^-$ for 12 h intervals over the following 3 days in normal light period. Preliminary experiments showed that this 12 h exposure to $^{15}NO_3^-$ was sufficient time for adequate incorporation of ^{15}N into all N fractions of the root and shoot for analytical accuracy, while the time was sufficiently short to minimize cycling of soluble reduced-^{15}N from the shoot to the root. Six randomly chosen plants were removed from their growing solutions and placed into a separate solution (also in a similar hydroponic system) containing an identical nutrient composition; however, in these new solutions 1.0 mM $^{15}NO_3^-$ (10 atom % ^{15}N) was used instead of 1.0 mM $^{14}NO_3^-$, with a 1% enrichment of ^{15}N. At the end of each 12 h exposure period, plants were harvested and rapidly dissected into the both organs, leaves, and roots. These were immediately frozen at −80 °C for later ^{15}N analysis in both whole tissue and protein extract. Prior to this, roots were rinsed extensively in order to remove the external NO_3^- medium. $^{15}N/^{14}N$ isotope ratios were analyzed.

Tissue and Protein Extract Analysis. For whole tissue analysis, samples (leaves and roots) were dried for 24 h at 60 °C. Then each sample was ground to a fine powder in a mortar, 2−3 mg were placed in caps (0.12 mL, Lüdiag, CH-9230, Flawil) to determine total N and ^{15}N enrichment. Tissue samples (0.1 fresh weight) for analysis in protein extract were extracted with 1 mL of 8% perchloric acid and placed in ice for 24 h to precipitate total proteins. After precipitation, each homogenate was centrifuged at 13000*g* for 10 min, and the pellet (total proteins) was washed two times with H_2Od to remove the remaining perchloric acid. Protein extracts were placed in caps and dehydrated for 24 h by an argon stream (140 mL min^{-1}). The weight of each extract was approximately 2−3 mg.

Total N was determined in dried plant material with a NC 2500 elemental analyzer (CE Instruments, Milan, Italy) using Dumas combustion. The sample was energetically oxidized yielding a gas mixture in which N was detected by a thermoconductivity detector. $^{15}N/^{14}N$ isotope ratios were analyzed.

The sum of excess of ^{15}N in leaves and roots, calculated per root dry weight and hour, was used to measure NO_3^- uptake rate. From the total ^{15}N incorporated, the ratio between ^{15}N in leaves and ^{15}N in roots was used as an indicator for NO_3^- translocation to the shoot (expressed as a percentage). The excess of ^{15}N in protein extract was used as an indicator of "de novo" protein synthesis.

Ethanol/Water Extraction. Frozen samples of leaves and roots (0.2 g FW), stored at −80 °C, were ground to a fine powder in a mortar which had been precooled with liquid N and exhaustively 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 (2.5 mL for NO_3^- determination) of distilled water at 2300*g* for 10 min. The ethanol soluble extracts were kept at −20 °C until further use, and the insoluble residues were dried at −60 °C for 24 h.

Determination of Nitrogenous Compounds (Amino Acids, NO_3^-, and Soluble Protein). For NO_3^- and amino acid contents, ethanol soluble extracts were used. Amino acid content was determined as described in ref *15*. NO_3^- was measured by high performance capillary electrophoresis. The buffer (Fluka 82619) was at pH 7.0. The applied potential was −25 kV, and the capillary tubing had an internal diameter of 50 μm and was 50/57 cm long. The indirect UV detection wavelength was set at 254 nm. Total N was determined as described above. For soluble protein content, frozen samples of leaves and roots (0.2 g FW) were homogenized with a mortar and pestle with 100 mM phosphate buffer, pH 7. The extract was then centrifuged for 20 min at 20000*g* at 4 °C. Protein was determined in the supernatant according to ref *16*.

NR Activity. NR activity in the absence or presence of 20 mM Mg^{2+} was determined as in ref *17* with either 10 mM EDTA or 20 mM magnesium chloride in the assay buffer in frozen samples. The activation state of NR is given by the ratio of its activity in the presence (actual activity) and absence of (maximum activity) Mg^{2+}, multiplied by 100% (*18*).

Statistical Analysis. Data were obtained from two different experiments. Results were examined by one-way analysis of variance. In figures, an asterisk (*) indicates significant difference between control and herbicide plants ($p \leq 0.05$) at a given day of treatment in Fisher's test.

RESULTS

Stomatal Conductance and Net Photosynthesis. ^{15}N Uptake and Translocation to the Shoot. The IM dose applied to soybean was high enough to inhibit ALS activity and to arrest plant growth (*19*). The treated plants did not show any signs of recovery at any stage and died after 3 weeks.

Stomatal conductance was inhibited in treated plants from day 1 and remained at very low values until the end of the experiment (**Figure 1A**). Photosynthesis was significantly inhibited by IM from day 1, and the level of inhibition (40%) kept fairly constant (around 3.9 μmol CO_2 m^{-2} s^{-1}) throughout the experiment (**Figure 1B**). $^{15}NO_3^-$ uptake was rapidly inhibited by IM by 1 day after treatment (**Figure 1C**).

IM decreased ^{15}N content in leaves and roots due to its effect on $^{15}NO_3$ uptake (**Figure 2A,B**). In control plants, the ^{15}N distribution pattern between root and shoot was constant over the course of the experiment. In untreated plants 45−55% of total ^{15}N translocated from the roots to shoots whereas in IM-treated plants only 35−45% of ^{15}N translocated form roots to shoots (**Figure 2C**,**D**).

Nitrate Reduction. Nitrate reductase activity was detected in both organs of control soybean plants, with the activity in roots being 3 fold higher than in leaves (**Figure 3**). IM affected NR activity in both organs but in a different manner. In leaves, NR activity (actual and maximum) decreased from day 2 of treatment during the experiment to almost zero. In roots NR

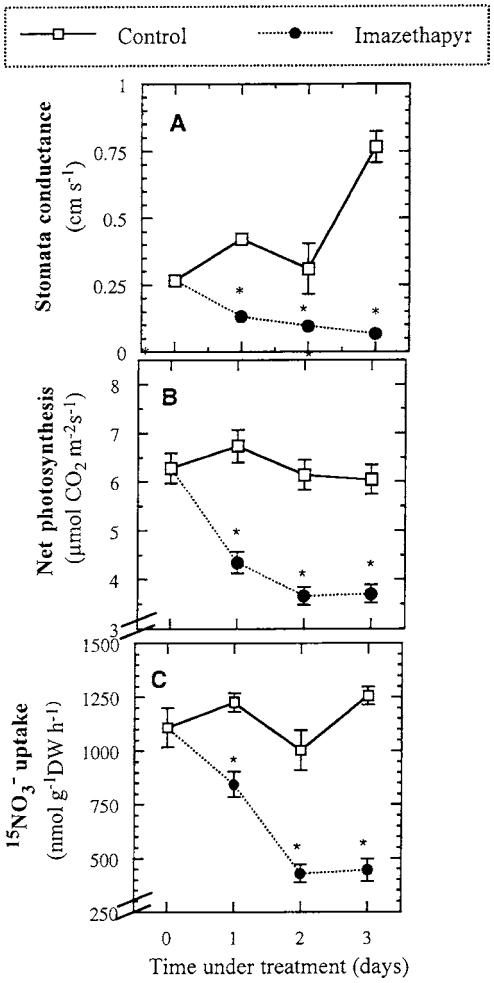

Figure 1. Stomatal conductance (**A**), net photosynthesis (**B**), and nitrate uptake (**C**) of control and IM-treated soybean plants. Data represent means ± SE ($n = 4$–6). The asterisk (*) indicates significant difference between control and IM ($p \leq 0.05$) at a given day.

activity decreased at day 1 but then the activity increased to those of control plants at day 3.

Activation state of NR in IM-treated leaves oscillated around control values during the treatment. In treated roots, the activation state was rather similar to control values and only lower by the end of the experiment (**Figure 3**).

Nitrogen Status. Total nitrogen, nitrate, total amino acid pool, and soluble protein contents were used to determine the effect of IM on N status. In leaves, N status was very similar in control and IM-treated plants (**Figure 4**). There was a transitory decrease in nitrate content by day 2 (**Figure 4C**) and the free amino acid pool increased (**Figure 4B**) while there was no effect on total N or soluble protein compared to the control plants (**Figure 4A**,**D**). In roots, total N content decreased by day 2 after IM treatment (**Figure 5A**). Other parameters related to N status showed the same pattern as in leaves (increase in free amino acid pool and no change in protein content), with the exception of a transitory increase in nitrate content by day 2 (**Figure 5C**).

Incorporation of ^{15}N in Proteins ("de Novo" Synthesis of Proteins). The incorporation of ^{15}N in proteins, which indicates the de novo synthesis of proteins, is clearly separated in time between leaves and roots. In control plants, de novo synthesis was initially mostly observed in leaves. This pattern changed and incorporation of ^{15}N was mostly observed in roots after the third day (**Figure 6**, open symbols). De novo protein

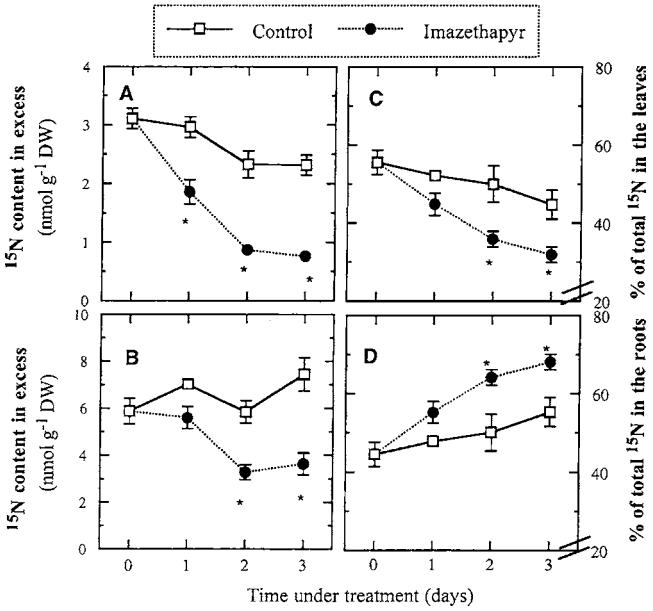

Figure 2. ^{15}N in excess in leaves (**A**) and in roots (**B**) of control and IM-treated soybean plants. Translocation of ^{15}N to the shoot expressed in terms of percentage of total ^{15}N in the leaves (**C**) and in the roots (**D**) of control and IM-treated soybean plants. Data represent means ± SE ($n = 6$). The asterisk (*) indicates significant difference between control and IM ($p \leq 0.05$) at a given day.

synthesis was immediately arrested by IM in both leaves and roots (**Figure 6**, closed symbols).

DISCUSSION

The application of 52 μM IM to the nutrient solution of soybean plants caused an inhibition of plant growth, as has been reported previously (*19*). Although this herbicide dose 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. This time course study lasted 3 days, allowing a short-term evaluation of effects of ALS-inhibitors on N metabolism.

Inhibition of photosynthesis by IM paralleled the decrease in stomatal conductance (**Figure 1A**), suggesting that stomatal conductance limited photosynthesis, which has been shown for other ALS inhibitors (*20*, *21*). It has been shown that herbicides that inhibit amino acid biosynthesis induce stomatal closure (*22*) and can affect nutritional status (*23*) and NO_3^- uptake by inhibiting transpirational water flow from roots to the leaves. IM also inhibited ^{15}N uptake but this inhibition was only partially due to a decrease in stomatal closure because transpiration decreased by day 1 and then remained constant, while ^{15}N uptake dropped immediately after treatment and decreased further until day 2 (**Figure 1**). Apart from stomatal conductance, other parameters that could inhibit ^{15}N uptake could be a direct effect of the herbicide on the transport system of NO_3^- or a reduced NO_3^- demand caused by the increased pool of free amino acids (**Figures 4B** and **5B**).

The inhibition of ^{15}N uptake by IM was accompanied by a decrease in the ^{15}N content in roots and leaves (**Figure 2A**,**B**). Interestingly, IM also affected the ^{15}N distribution within the plant, by inhibiting translocation to the leaves and resulting in accumulation in roots (**Figure 2C**,**D**). This inhibition of the ^{15}N transport from the roots to the leaves could be explained by the same reasoning as for the ^{15}N uptake. In IM-treated plants, inhibition of NO_3^- uptake and a different translocation pattern

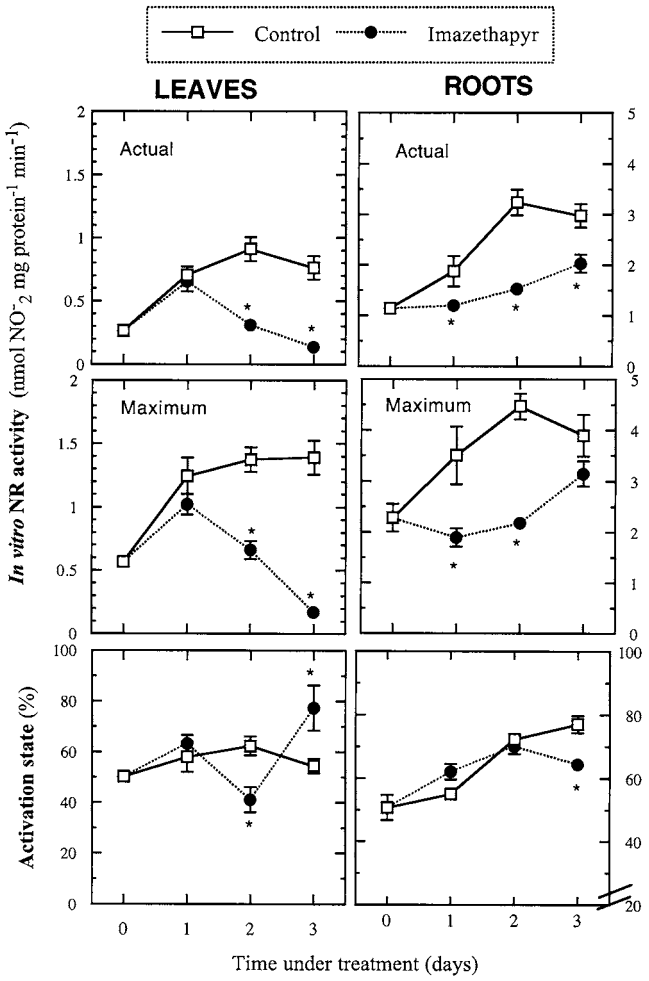

Figure 3. Nitrate reductase: actual and maximum in vitro activity and activation state in leaves (left) and roots (right) of control and IM-treated soybean plants. Data represent means ± SE ($n = 4$). The asterisk (*) indicates significant difference between control and IM ($p \leq 0.05$) at a given day.

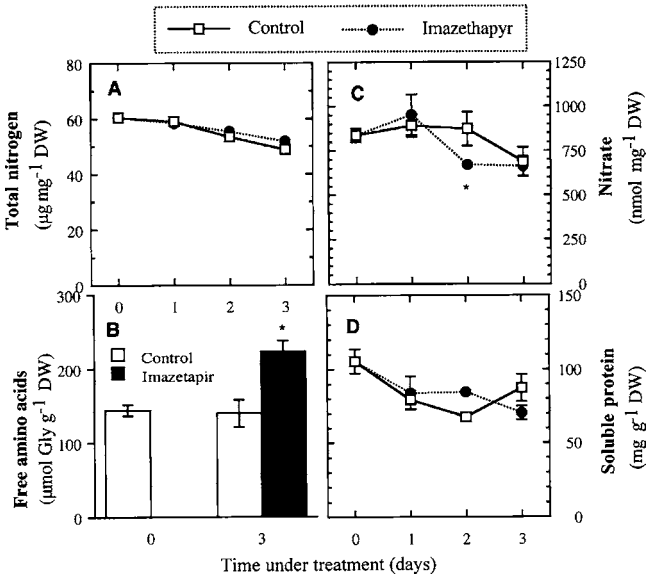

Figure 4. Total nitrogen (**A**), free amino acid (**B**), nitrate (**C**), and soluble protein (**D**) contents in leaves of control and IM-treated soybean plants. Data represent means ± SE ($n = 4$–6). The asterisk (*) indicates significant difference between control and IM ($p \leq 0.05$) at a given day.

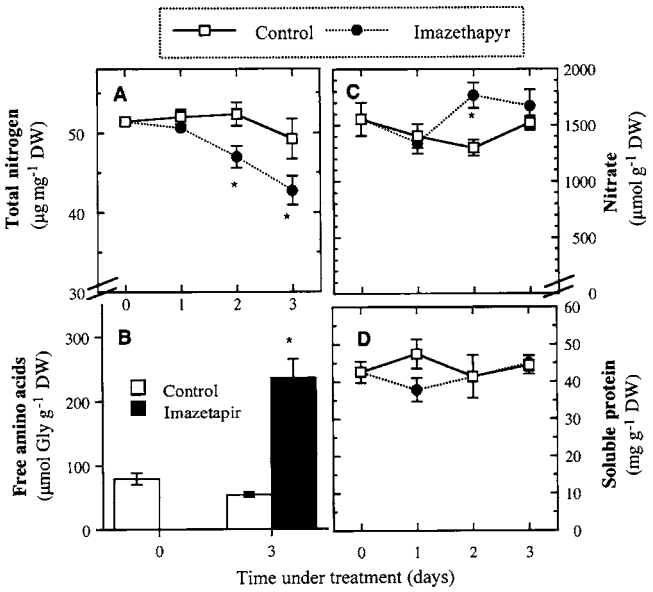

Figure 5. Total nitrogen (**A**), free amino acid (**B**), nitrate (**C**), and soluble protein (**D**) contents in roots of control and IM-treated soybean plants. Data represent means ± SE ($n = 4$–6). The asterisk (*) indicates significant difference between control and IM ($p \leq 0.05$) at a given day.

did not only cause growth inhibition but also total N was diminished in roots although not in leaves. (**Figures 4A** and **5A**). This decrease was not detected in the other N status parameters (nitrate, amino acid pool, and soluble protein content) because the amino acid pool increased and the nitrate and soluble protein content did not change. Other nitrogenous components, such as total protein content, could be responsible for this decrease.

IM inhibited NR activity in leaves and roots (**Figure 3**). The lower NR activity of treated plants coupled to the inhibition of nitrate uptake (**Figure 1C**) of treated plants could explain why total nitrate content did not decrease either in leaves or in roots (**Figures 4C** and **5C**). The increase of NR activity during the time-course of the experiment in IM-treated roots is not in accordance with the inhibition of "de novo" synthesis of proteins (**Figure 6**). These results suggest that the enzymes after NR are inhibited. NiR and the GS-GOGAT are inhibited by ALS inhibitors in *V. faba* (*13*). The inhibition of NR activity paralleled the disruption of ^{15}N distribution in the plant: a reduction of the transport of NO_3 to the leaves where NR decreased and an accumulation of N was noted in the roots, where NR increased. NR activity was 2−18 times higher in roots than in leaves of treated plants, reflecting the different distribution of N within the plant and suggesting that nitrate assimilation takes place mainly in roots.

It could have been expected that total nitrate content would accumulate because NR activity was inhibited. But, it is necessary to consider that $^{15}NO_3^-$ uptake was inhibited and its availability was limited because NO_3^- usually accumulates into the vacuole and it is not available for the NR, which is citosolic (*24*). As such, NR activity does not have to correlate to total NO_3^- content. Therefore, NR activity is more dependent on the NO_3^- flow than on the total NO_3^- content in plants treated with these herbicides. Reference *25* described that gene expression on NR is determined by the NO_3^- flow that goes from the xylem to the reduction place. It has been reported (*26*) that low rates of carbon fixation inhibit NR transcription, so the decline in photosynthesis detected in IM-treated plants could also contribute to the inhibition of NR activity.

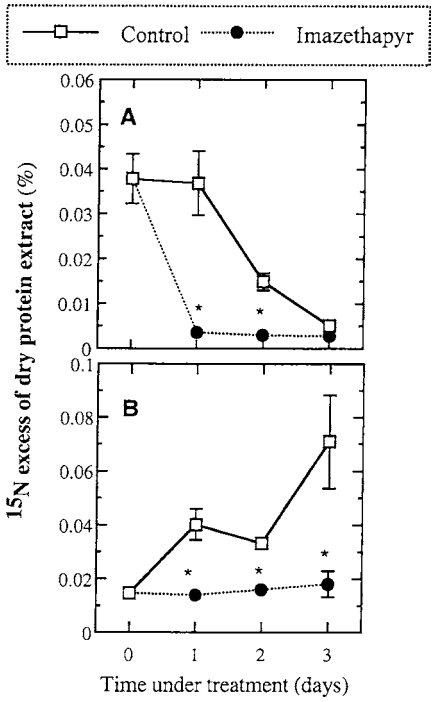

Figure 6. ^{15}N excess of dry protein extract, expressed in terms of percentage, in leaves (**A**) and roots (**B**) of control and IM-treated soybean plants. Data represent means ± SE ($n = 4–6$). The asterisk (*) indicates significant difference between control and IM ($p \leq 0.05$) at a given day.

The increase in free amino acid pool is a typical effect of ALS inhibitors (*5*, *27*), and in the present work it has been shown to take place in leaves and roots (**Figures 4B** and **5B**). It was proposed that this increased free amino acid pool came from an increase in the protein turnover mainly studying the ^{14}leucine incorporation into proteins and showing that the protein synthesis continued (*5*, *28*). In this study, soluble protein content of IM-treated plants did not decrease (**Figures 4D** and **5D**) and "de novo" synthesis of proteins was inhibited (**Figure 6**) in treated plants, showing that there would be protein synthesis but from amino acids scavenged mainly from protein turnover.

The results shown in this paper assess the effects of ALS inhibitors on "de novo" synthesis of proteins by isotopic studies for the first time. Biosynthesis of proteins, as measured by ^{15}N labeling, was inhibited by 1 day after treatment. The effect was stronger in leaves and preceded the inhibition of NR activity, while in roots the "de novo" synthesis of proteins and the NR activity were inhibited 1 day after treatment. These data indicate that "de novo" synthesis of proteins is the first step in N assimilation that is affected by the inhibition of ALS and indicates a rapid response of the plant to inhibition of the biosynthesis of BCAAs. Although "de novo" synthesis of proteins was completely abolished in IM-treated plants, an increase in the amount of alternative oxidase protein (*19*) or pyruvate decarboxylase (*29*) has been reported, suggesting that these enzymes may be induced in response to ALS inhibition.

Our results show that ALS inhibitors totally modifies N metabolism in treated plants. Nevertheless, although the disruption of nitrate assimilation can be detected within 1 day after treatment, the plant can survive for a long time as these herbicides cause a slow death (*7*). On the basis of these observations, it is possible to suggest a mechanism of regulation of N metabolism after ALS inhibition. The first process affected is the "de novo" synthesis of proteins because of the transitory lack of BCAAs, although protein synthesis can continue using amino acids scavenged from protein turnover. The lower "de novo" synthesis of proteins detected in treated plants is in accordance with the inhibition of the nitrate uptake. This effect and the related inhibition of translocation could be related to different aspects such as inhibition of stomatal conductance, lower nutrient requirements of the plant (because the growth is inhibited), and even an effect on nitrate transporters. The lower flow of nitrate triggers the inhibition of NR activity in treated plants, although the nitrate that it is not translocated to the leaves and which is accumulated in roots enhances NR activity with time.

ABBREVIATIONS USED

ALS, acetolactate synthase; BCAA, branched-chain amino acid; IM, imazethapyr; NR nitrate reductase.

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Received for review June 28, 2006. Revised manuscript received September 12, 2006. Accepted September 19, 2006. This work was supported by the CICYT-AGL 2004-03784 grant (from the Ministerio de Educación y Ciencia, Spain). L.O. and M.I. acknowledge their fellowships from the Ministerio de Educación and Universidad Pública de Navarra, respectively.

JF0618224