Effect of Imazamethabenz-methyl on Nitrate Uptake in Wheat (*Triticum durum* L.)

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The effect of the herbicide imazamethabenz-methyl (IMZM), a mixture of the two isomers methyl (±)-2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-imidazol-2-yl]-4-methylbenzoate (para isomer) and methyl (±)-2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-imidazol-2-yl]-5-methylbenzoate (meta isomer), on the uptake of nitrate by wheat grown hydroponically was studied. IMZM stimulates the nitrate uptake in both "induced" (NO_3^--pretreated) and "uninduced" (NO_3^--starved) seedlings, most likely as a response to a plant stress. The decrease in acetohydroxy acid synthase (AHAS; EC 4.1.3.18) activity and in protein content of IMZM-treated roots supports this hypothesis. The presence of valine, leucine, isoleucine, and IMZM prevents the effects of the herbicide treatment in both induced and uninduced plants. The addition of IMZM to humic acid enhances the nitrate uptake, although to a lower extent than with the herbicide alone. Possible traces of imazamethabenz acid (IMZA) in growing units do not seem to be responsible for the greater N demand observed.

Keywords: *Nitrate uptake; imazamethabenz-methyl; herbicides; humic acid; adsorption*

INTRODUCTION

Imazamethabenz-methyl (IMZM) is an isomeric mixture of *p*- and *m*-methyl 2-imidazolinone toluate. This relatively new imidazolinone herbicide was developed for the control of wild oats (*Avena fatua*) in corn and wheat (Hedlund and Andersson, 1987). IMZM acts through the inhibition of acetohydroxy acid synthase (EC 4.1.3.18), the first enzyme in the biosynthetic pathway of the branched-chain amino acids (Pillmoor and Caseley, 1987). The herbicide in the estereal form is a very weak inhibitor of the enzyme, in contrast to the potent inhibition caused by the free acid, suggesting that its action is dependent on a de-esterification process due to plant esterases (Brown et al., 1987). Whereas the free acid does not exhibit selectivity, the selectivity mechanism of IMZM depends on the metabolic pathways of the herbicide in the different species. In susceptible species, the parent compound is de-esterified to the active acid form that is able to undergo translocation. Only traces of the active acid form are formed in tolerant species. The latter species hydroxylate rapidly the methyl substituent on the benzene ring, which is then conjugated to a glucose moiety (Shaner and Mallipudi, 1991). Both of these steps result in the loss of herbicidal activity.

No data concerning the effect of IMZM on the ion uptake in tolerant species have been reported. Therefore, the present work was undertaken to study the effect of IMZM on nitrate uptake. Experiments were carried out on wheat grown hydroponically.

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MATERIALS AND METHODS

Materials. Imazamethabenz-methyl ($C_{16}H_{20}N_2O_3$), a para- and meta-isomer mixture of IMZM (96.4% purity), was supplied by American Cyanamid Co. Princeton, NJ. *p*-IMZM and *m*-IMZM are white solids with solubilities in water of 857 and 1370 mg L^{-1} at 25 °C, respectively (Wauchope et al., 1992). Imazamethabenz acid (IMZA) was prepared by alkaline hydrolysis of IMZM according to the following procedure: 4 mL of 1 N NaOH solution were added to 0.1 g of IMZM. The suspension was stirred at room temperature until clear (∼5 h). After the solution was washed with chloroform, 1 N HCl was added until a pH of 6 was reached and the acid precipitated. It was filtered and recrystallized from ethanol, giving white crystals.

Humic acid (HA) was obtained from a clay loam Andosol from Macomer (Sardinia) according to the procedure of Stevenson (1972). After precipitation, it was centrifuged, redissolved and precipitated three times, dialyzed against distilled water until salt-free, and finally freeze-dried.

Adsorption Measurement by HA. Duplicate samples of 100 mg of humic acid were equilibrated in centrifuge polyallomer tubes with 20 mL of IMZM solution (2.59 mM). The tubes were shaken for 12 h at 25 ± 2 °C, and then the suspension was centrifuged at 30000*g* for 15 min. The supernatant was pipetted off and analyzed immediately by high-performance liquid chromatography (HPLC). The amount adsorbed by HA was calculated from the difference between the initial and final concentrations of IMZM in solution. The effect of varying pH was examined by adding $Ca(OH)_2$.

HPLC Analyses. The concentrations of IMZM and IMZA were determined by HPLC (Pusino et al., 1995). A Waters 510 liquid chromatograph, equipped with a 250 × 4 mm i.d. μBondapack C_{18} (10 μm) analytical column, a multiwavelength Waters 490 programmable detector operating at 238 nm, and a Waters Baseline 810 chromatography work-station, was used. The mobile phase (1 mL min^{-1}) was composed of acetonitrile plus water (65 + 35 by volume, pH 3). In these conditions the retention times of IMZM and of IMZA were 4.5 and 3.6 min, respectively. The detection limit for both IMZM

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and IMZA was 0.05 mg L^{-1} or 0.173 μM, as calculated from the concentration of herbicide giving a detector response approximately twice the background signal.

Plant Material and Growth Conditions. Wheat (*Triticum durum* L. cv. Appulo) seeds were surface sterilized for 20 min in 20% sodium hypoclorite and then washed with distilled water. The seeds were germinated at 25 °C in darkness. After 36 h, the seedlings were transferred into a growing unit containing 11 L of one-fourth-strength Hoagland solution (Hoagland and Arnon, 1950) lacking of nitrogen. The medium was aerated, and the pH was adjusted to 6.0 with 0.1 N NaOH and readjusted daily. Sixty germinating seeds per growing unit were placed into a growth chamber at 25 °C with a 14 h photoperiod, 300 μeinstein m^{-2} s^{-1} photon flux density, and 70% relative humidity. After 4 days, the nutrient solution was replaced. When the seedlings were 5 days old, KNO_3 and/or HA and/or IMZM and/or a valine, leucine, and isoleucine (VLI) mixture were added to nutrient solution to a final concentrations of 50 μM, 5 mg L^{-1}, 1.70 μM, 170 μM, respectively, to achieve the various conditions of our experiments. A further experiment was carried out, in the above-mentioned conditions, by adding 0.17 μM IMZA alone to the nutrient solution.

The concentration of the herbicide applied in hydroponical growing units was chosen according to the field application rates [500 g of active ingredient (ai)/ha or 1.73 mol of ai/ha] diluted 1 million times to simulate an average natural dilution from soil water. The concentration of VLI mixture was 100-fold that of IMZM, similar to that in other literature reports (Scarponi et al., 1995).

Nitrate Uptake. After exposure to different media for 2, 4, 8, 24, and 48 h, the 5-day-old roots were rinsed carefully with a nutrient solution and transferred into the 100 μM KNO_3 uptake solution. Samples were taken at 5 min intervals over a 30 min period, and the nitrate concentration was determined by spectrophotometric measurements at 210 nm (Goldsmith et al., 1973; Albuzio et al., 1986) with a UV−vis Shimadzu model 2100 spectrophotometer. The rate of nitrate uptake was calculated from the linear phase of the nitrate consumption curve and expressed as micromoles of NO_3^- adsorbed per hour per gram of fresh weight root. All procedures were performed in triplicate under axenic conditions. The solutions were aerated continuously.

AHAS Activity Assay. According to the procedure of Singh et al. (1988), 1 g of fresh weight of wheat roots was powdered in liquid nitrogen and used for enzyme extraction. The activity of AHAS was measured in 1 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.

Protein Extraction and Determination. Wheat roots (1 g of fresh weight) were ground in liquid nitrogen in a mortar with pestle. The powder was suspended in 2 mL of buffer containing 100 mM Tris-HCl, 5 mM β-mercaptoethanol, 10 mM $MgCl_2$, and 1 mM EDTA (pH 8.5), and, finally, 0.1 g of polyvinylpolypyrrolidone was added. The samples were homogenized and then centrifuged at 25000*g* for 20 min at 4 °C. Total protein content was determined colorimetrically according to the Bradford (1976) procedure.

Data Analysis. The distribution coefficient K_d was used to measure the adsorption extent. This coefficient is the ratio c_s/c_e, where c_s (μmol 100 g^{-1}) is the amount of adsorbed herbicide and c_e (μM) the equilibrium concentration in solution. Data were tested by analysis of variance and the significant differences between means were compared by the least significant difference (lsd) test criterion with a confidence level of 95% (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Roots exhibit at least two mechanisms for nitrate uptake (Hole et al., 1990; Siddiqi et al., 1990). Plants not previously exposed to nitrate, "uninduced plants", show a low, constitutive level of nitrate uptake rate.

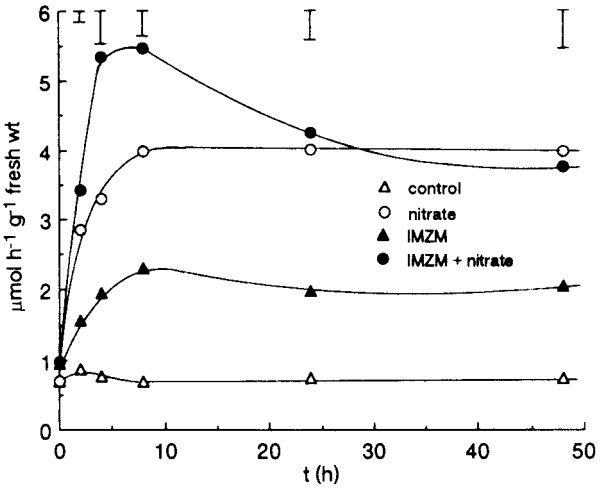

Figure 1. Nitrate uptake rates by roots in the different systems: uninduced (control), induced (nitrate), uninduced + herbicide (IMZM), and induced + herbicide (IMZM + nitrate). Each value is the mean of three determinations. Vertical bars represent lsd at $p < 0.05$.

Upon exposure to nitrate, "induced plants", the uptake rates increase; therefore, nitrate stimulates its own uptake. The trend of nitrate absorption by induced and uninduced wheat plants, over a 48 h period, is shown in Figure 1. The uptake by wheat roots that had not been previously exposed to nitrate (uninduced control) was lower than that by seedlings treated with nitrate (induced control). Eight hours of contact with 50 μM nitrate was enough to achieve the complete induction of nitrate uptake (+472%, nitrate curve in Figure 1). This finding is in agreement with the results of MacKown and McClure (1988), who found that the nitrate uptake is maximal from 6 to 8 h after nitrate contact. The herbicide treatment increases the rate of nitrate uptake for either uninduced or induced plants by about 230 and 36%, respectively. Both systems reached the highest rate of uptake after 8 h of contact with the herbicide. However, in the case of IMZM + nitrate system, it decreased thereafter to about the value characteristic of nitrate only (Figure 1). These findings suggest that the IMZM herbicide stimulates the nitrate uptake, most likely as a response to a plant stress.

Studies on the mode of action of imidazolinone herbicides indicate that the only mechanism involved is the inhibition of acetohydroxy acid synthase (AHAS) in the biosynthetic pathway of the branched amino acids valine, isoleucine, and leucine (Stidham and Singh, 1991). Therefore, the effect of the VLI mixture on the nitrate uptake in the presence or absence of the herbicide was tested. The amino acids alone had a negligible influence on the nitrate uptake by either uninduced or induced seedlings (Figure 2). It is well-known that valine, leucine, and isoleucine do not affect nitrate uptake or stimulate it only slightly (Muller and Touraine, 1992; Imsande and Touraine, 1994). When the herbicide was added to a VLI mixture, the nitrate uptake was no more stimulated (Figure 2). These findings seem to substantiate that branched amino acids contrast the effect of IMZM on nitrate uptake. The inhibitory activity on AHAS by IMZM was also assayed. The modification of AHAS activity, expressed as percentage of control, in wheat roots following the treatment with IMZM and IMZM coupled with the VLI mixture, respectively, is shown in Table 1. A decrease

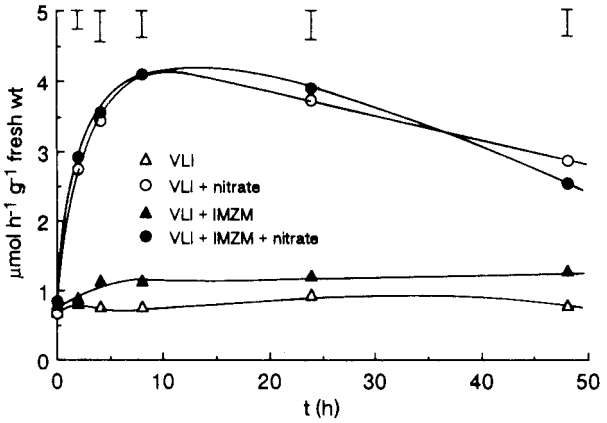

Figure 2. Nitrate uptake rates by roots in the different systems with added VLI mixture. Each value is the mean of three determinations. Vertical bars represent lsd at $p < 0.05$.

Table 1. Effect of IMZM on Activity of Acetohydroxy Acid Synthase in Wheat Roots

	activity[a] (% of control)	
h	IMZM	IMZM + VLI
0	100	100
2	80	98
4	67	93
8	49	83
24	44	84
48	38	88

[a] Each activity value is the mean of triplicate determinations.

Table 2. Protein Content[a] in Wheat Roots

h after treatment	mg/g of fresh weight		
	control	IMZM	IMZM + VLI
0	2.10	2.10	2.10
2	2.12	1.68*	1.90
4	2.20	1.51*	2.00
8	1.92	1.38*	1.80
24	2.10	1.48*	1.92
48	2.10	1.50*	1.90

[a] Each value is the mean of triplicate determinations. Values of treated samples followed by an asterisk are significantly different at $p < 0.05$ with respect to control.

Table 3. K_d Values for Adsorption of IMZM on HA at Different pH Values

pH	K_d
3.5	61.11
4.6	25.21
6.0	7.29

of enzyme activity to 62% after 48 h was observed in the IMZM-treated roots system. On the contrary, no significant inhibition of the extractable activity of AHAS was noticed owing to the addition of the VLI mixture to herbicide treatment. To evaluate the effect of AHAS inhibition on protein synthesis, the protein content in herbicide-treated and untreated roots was determined. The data reported in Table 2 show a decrease in protein content in treated wheat roots. In particular, a decrease of ~62%, with respect to control, was measured after 48 h. Again, the addition of VLI mixture to herbicide treatment resulted in a compensatory effect (Table 2). A similar behavior was observed in a previous study on the effects of imazethapyr, a herbicide structurally correlated to IMZM, on nitrogen metabolism in soybean (Scarponi et al., 1995). In this study it was found that a decrease in the protein content, due to the inhibition of activity of AHAS, does not take place after addition

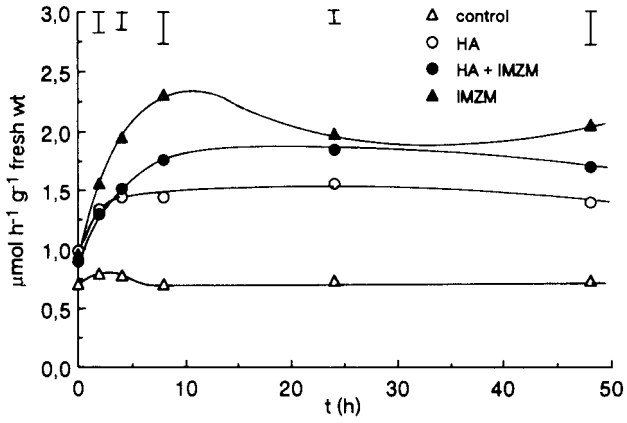

Figure 3. Nitrate uptake rates by roots in the different systems: uninduced (control), uninduced + herbicide (IMZM), uninduced + humic acid (HA), and uninduced + humic acid + herbicide (HA + IMZM). Each value is the mean of three determinations. Vertical bars represent lsd at $p < 0.05$.

of a VLI mixture to the herbicide solution. However, the availability of branched amino acids seems to remedy their failure due to reduction of AHAS activity.

The unexpected behavior of the tolerant plant could be due to a stress produced by the free acid form of herbicide, which is not expected to form in tolerant species except in very little amount (Shaner and Mallipudi, 1991).

To verify if the hydrolysis of IMZM to IMZA takes place in the solution of the growing units, a Hoagland solution at pH 6 containing only the herbicide was analyzed for the free acid after an interval of 48 h. The hydrolysis products of IMZM were not found. Since root exudates can lower the pH value of the solution surrounding the roots by ~2 units (Sposito, 1989), an experiment like the previous one was carried out at pH 4. Also in this case, the presence of the herbicide free acid was not detected.

In principle, traces of free acid, in concentration not detectable by HPLC, could be responsible for the greater N demand in the presence of the herbicide. In field conditions, the bioavailability of herbicides is governed, to a large extent, by their distribution between solid and liquid phases. Organic matter behaves as the most active soil component for the adsorption of pesticides. In particular, the adsorption of imidazolinone herbicides in soil is positively correlated with the organic carbon content and negatively with pH (Mangels, 1991). The extent of the herbicide adsorption on humic acids at different pH values was measured by the distribution coefficient (Table 3). Humic acids were more effective in the adsorption of IMZM with decreasing pH values, confirming that pH influences the adsorption of IMZM. The nitrate uptake was studied also in the presence of HA to determine if the interaction between IMZM and organic matter modifies the effect of IMZM on nitrate uptake. The presence of only HA enhanced the uptake by 105% (Figure 3). This is consistent with previous studies which reported that soil humic substances can stimulate the ion uptake by roots (Albuzio et al., 1986; Dell'Agnola and Nardi, 1987). The effect is explainable in terms of an acceleration of the synthesis of protein carriers (Vaughan and MacDonald, 1976). The addition of IMZM to HA increased the nitrate uptake (+166%), although this does not reach the value obtained with the herbicide only (+230%). The trend substantiates that the overall stimulation of the nitrate uptake by the

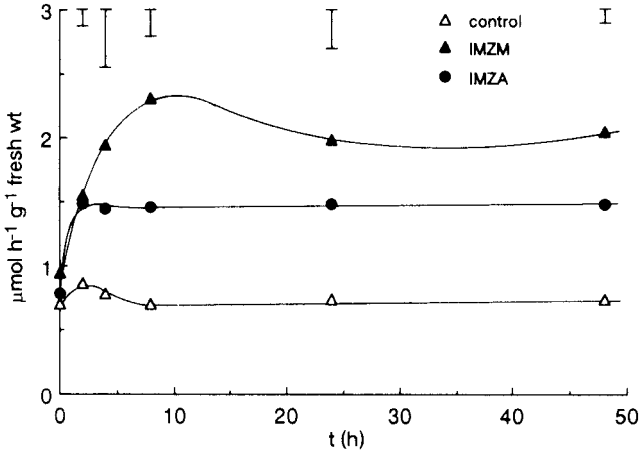

Figure 4. Nitrate uptake rates by roots in the different systems: uninduced (control), uninduced + herbicide (IMZM), and uninduced + acid (IMZA). Each value is the mean of three determinations. Vertical bars represent lsd at $p < 0.05$.

simultaneous presence of organic matter and herbicide is not the result of additive effects. More likely, it is due to the loss of bioavailability of free IMZM due to the sorption on HA. Under the experimental conditions (pH 6), traces of free acid IMZA [pK_a in the range of 3−4 according to Wepplo (1991)] should exist in anionic form, likewise humic acids with pK_a values around 5 [according to Stevenson (1972)]. However, anionic IMZA, which cannot be adsorbed by the negatively charged surfaces of HA, is available for the herbicidal action. To confirm this hypothesis, the nitrate uptake was tested in the presence of small but detectable amounts of IMZA (see Materials and Methods) on uninduced plants (Figure 4). The IMZA seedling treatment increased the nitrate uptake to a maximum of ∼108% after 4 h of contact with IMZA, and the value remained constant thereafter. The increase observed in the presence of IMZA alone was smaller than that observed on IMZM treatment (+230%). This result rules out the hypothesis that undetectable traces of free acid present in the working solution could be responsible for the observed effects on nitrate uptake. On the contrary, the estereal herbicide form, apparently resistant to chemical hydrolysis, may be translocated in roots and partially biodegraded therein to produce seedling stress. In conclusion, the exposure of wheat seedlings to IMZM herbicide determines a nitrogen starvation that stimulates the nitrate uptake. In induced seedlings, this effect seems transient, in contrast to uninduced plants.

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