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The dependence of nitrate reductase activity on the level of soluble sugars in wheat and cucumber roots growing in the presence of simazine, in light or in darkness

JÓZEF BUCZEK, JACEK BORKOWSKI, IRENA JARZYŃSKA

Department of Plant Physiology, Institute of Botany, Wrocław University, Kanonia 6/8, 50-328 Wrocław, Poland

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Abstract

At concentrations of 25 and 5 μM, simazine inhibited nitrate reductase activity in wheat and cucumber roots, respectively. It also lowered the content of soluble sugars and decreased the activities of NADH malate dehydrogenase and $NADP^+$ glucose-6-phosphate dehydrogenase. The inclusion of 50 mM glucose into the medium partially reversed the inhibitory effect of simazine on the activity of nitrate reductase in cucumber roots and slightly increased the activity of this enzyme in wheat roots. These results suggest a complex influence of the herbicide on the activity of nitrate reductase: simazine lowers the level of soluble sugars in roots and decreases the activity of the dehydrogenases supplying the reduced nucleotides indispensable for reduction of nitrates.

Key words: Triticum aestivum, Cucumis sativus, nitrate reductase, simazine

INTRODUCTION

In our last paper (Jarzyńska and Buczek 1984), it was shown that when simazine was introduced into the medium, the activity of nitrate reductase (NR) was inhibited after about 24 hrs. both in the green tissues and roots of the cucumber. Simazine, a herbicide from the triazine group, is known to be an inhibitor of electron transport in photosynthesis (Moreland 1980, Rensen 1982). Therefore, its indirect influence on the reduction of NO_3^- in plant green parts is highly probable in view of the dependence of the enzymes reducing nitrates on the products of photosynthesis (Aslam

and Huffaker 1982, 1984, Rufty et al. 1984) and the sources of reducing equivalents (Klepper 1975, House and Anderson 1980). In the roots, however, where the metabolic processes are dependent on the supply of carbohydrates, the effect of simazine on the reduction of NO_3^- can come from either lowering the level of photosynthesis products due to inhibition of this process, or through the effect of simazine on the redox processes (Vainilo et al. 1967, Ashton and Crafts 1973, Moreland 1980) supplying energy and reducing equivalents. The purpose of this paper was to examine the effect of simazine on the activity of NR in plant roots in light of the changes in the level of soluble sugars in the root tissues of plants growing in light or darkness.

MATERIAL AND METHODS

Preparation of plant material. Wheat grains (*Triticum aestivum* L. var. Luna) and cucumber seeds (*Cucumis sativus* L. var. Monastyrski) were placed after germination in 1 dm^3 jars filled with a salt solution diluted three-fold, containing (in mM per dm^3): K_2SO_4 — 3, $Ca(H_2PO_4) \cdot H_2O$ — 1, $CaSO_4$ — 2, $MgSO_4 \cdot 7H_2O$ — 1, Fe-$C_6H_5O_7$ — 0.09 and the basic microelements in the amounts given previously (Jarzyńska and Buczek 1984). The pH of the solution was brought to 6.5. The jars were placed in a photothermostat under conditions of 18 hrs. light (15.3 $W \times m^2$ and 25°C) and 6 hrs. darkness (19-20°C) for 4 days. Simazine at a concentration of 25 μM for wheat and 5 μM for cucumbers was dissolved in 2% ethanol (w/v) and in each of the experiments, introduced into the medium after 2 days of growth. An equivalent amount of ethanol, not exceeding 1 cm^3 per dm^3 was added at the same time to the control mediums. The method of treating the plants with simazine used here was adopted after preliminary experiments using various simazine concentrations and times of exposure of wheat and cucumber seedlings. The concentrations and times of action that did not cause pathological symptoms, were chosen. After 4 days of growth on the nitrogen-free medium, the plants were transferred to Hoagland's medium containing 15 mM NO_3^- (Jarzyńska and Buczek 1984) enriched with the basic microelements, with or without the addition of simazine, and left under the same growth conditions for 24 hrs. Wheat was cultivated in a full medium containing 15 mM NO_3^- while cucumbers in a diluted medium (three-fold) containing 5 mM NO_3^-. Next, part of the plants with the fresh nitrogen medium, with or without simazine, was placed under illumination under identical conditions as previously (at the time marked T = 0 on the figures), whereas the other part was placed in complete darkness. Identical experiments were performed with the addition of 50 mM glucose in the medium,

with the exception that the plants were grown for only 9 hours. At the times marked on the figures as T = 0 and after 6, 9 and 24 hrs., some of the plants were collected and NR activity and the sugar content in the fresh weight of the roots determined. In the experiment in which the effect of simazine on the induction of NR was studied (Fig. 1), the plants grew for 5 days in a nitrogen-free medium before they were placed in the nitrogen-containing one, whereas simazine and glucose were added at the same time as before.

Enzyme activity. The activity of nitrate reductase was determined in root extracts after thoroughly washing and then homogenizing them in porcelain mortar in a solution composed of: 50 mM K-phosphate buffer (pH 7.5), 1 mM cysteine, 3 mM EDTA and 1% casein taking 5 cm^3 of this solution for every 1 g of tissue. The homogenate was centrifuged for 15 minutes at 20000 × g and the supernatant used to determine NR activity. All of the above steps were done at a temperature of 0-4 C. NR activity was determined according to Hageman and Flesher (1960) measuring the NADH-dependent production of nitrites. The enzyme activity was expressed in µmoles NO_2^- per g fresh weight of tissues, produced during 1 hr. Extracts for malate dehydrogenase (dependent on NADH) and glucose-6-phosphate dehydrogenase (dependent on $NADP^+$) were made by homogenizing root tissues in a solution containing 50 mM K-phosphate buffer (pH 7.5), 5 mM cysteine, 3 mM EDTA and 1% bovine serum albumin, at a ratio of 10:1. The supernatant obtained after centrifugation of the homogenized tissue at 20000 × g for 15 min. was used in the enzyme activity assays. The activity of glucose-6-phosphate dehydrogenase was determined according to the method described by Devlin and Galloway (1968), measuring spectroscopically at 340 nm (VSU 2-P, Carl Zeiss) the rate of $NADP^+$ reduction. The activity of malate dehydrogenase was assayed according to the method of Wakiuchi et al. (1971), measuring at 340 nm the rate of NADH oxidation. The activity of the enzymes was expressed in µmoles of substrate or product per min and g fresh weight of root tissues. The results of the enzymatic assays are the mean values from 3 repetitions for each combination. Each experiment was repeated at least 3 times.

Determination of sugar content. Soluble sugars were extracted from roots after grinding 1 g of tissue in a mortar with 80% v/v ethanol. The extract was filtered through Whatman No. 1 filter paper. The filtrate was condensed under reduced pressure at 40°C, then diluted to 25 cm^3. After hydrolysis in 5% HCl at a proportion of 1 cm^3 of acid to 10 cm^3 of extract, reducing sugars and total soluble sugars were determined by the Somogyi method (Nelson 1944). For each combination, 3 independent root samples were taken.

Reagents. Simazine (2-chloro-4,6-bis(ethylamino)-1,3,5-triazine) was ob-

tained in the form of a pure substance from the Institute of Organic and Industrial Chemistry, Warsaw. NADH and $NADP^+$ from Reanal (Hungary). Casein was from Fluka. The remaining reagents were obtained from POCh — Poland, and were chemically pure.

RESULTS

Figure 1 shows the influence of simazine and glucose on the induction of NR in wheat and cucumber roots. The treatment of plants with simazine for 3 days prior to transferring them to the nitrogen-containing medium,

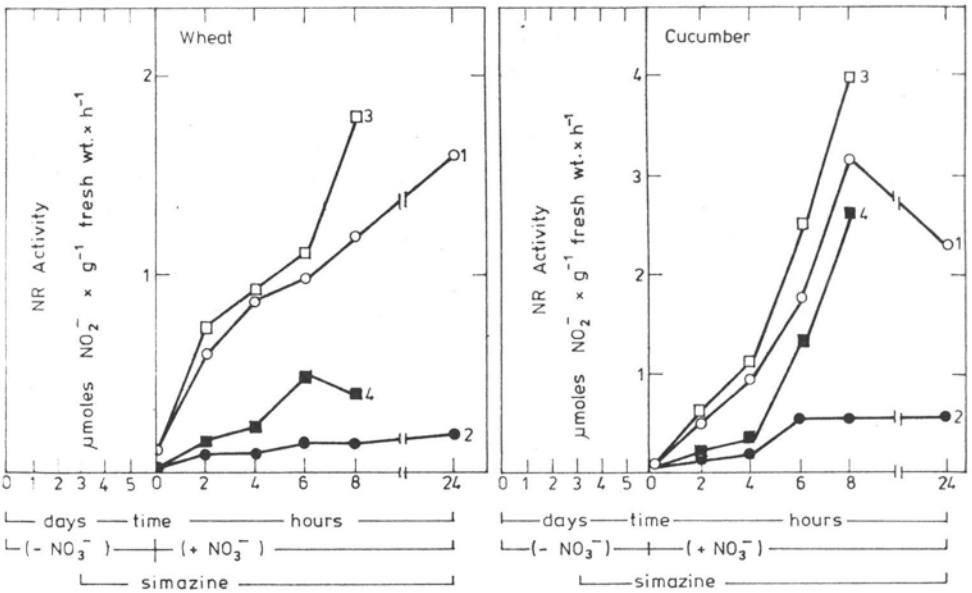

Fig. 1. The effect of simazine and glucose on the induction of NR in wheat and cucumber roots. The plants were grown for 5 days in a photothermostat (16 hrs. illumination and 8 hrs. of darkness) on a nitrogen-free medium. Simazine (25 μM for wheat and 5 μM for cucumber) was added 3 days prior to assay. At T = 0 the plants were transferred to the nitrogen-containing mediums with (2) or without (1) simazine, with 50 mM glucose (3) or with simazine and glucose (4)

clearly lowered their ability to induce NR in the roots. Whereas the introduction of glucose into the medium simultaneously with NO_3^- (T = 0), clearly stimulated the induction or activation of NR in the presence of simazine. The influence of glucose was especially clear in the case of the cucumber.

Figure 2, A and B, presents the influence of simazine and glucose on changes in NR activity in wheat roots exhibiting high NR activity; the plants

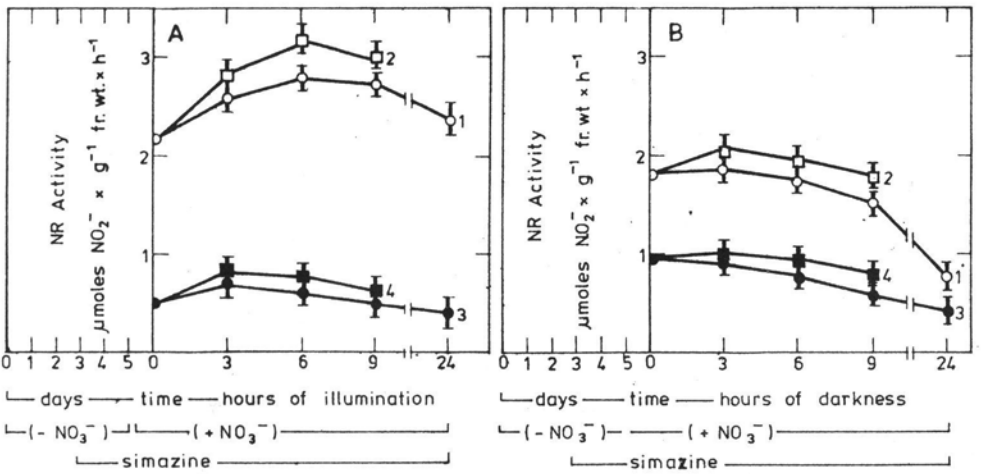

Fig. 2. The effect of simazine (25 μM) and glucose (50 mM) on NR activity in wheat roots growing under conditions of light (A) or darkness (B). The plants were grown for 4 days under conditions of 16 hrs. light and 8 hrs. darkness on a nitrogen-free medium, next for 1 day under the same lighting conditions in a medium containing NO_3^-. At T = 0 they were transferred either to constant illumination (A) or darkness (B). Simazine was administered 3 days prior to T = 0, glucose at T = 0. 1 — Control, 2 — control + glucose, 3 — simazine, 4 — simazine + glucose

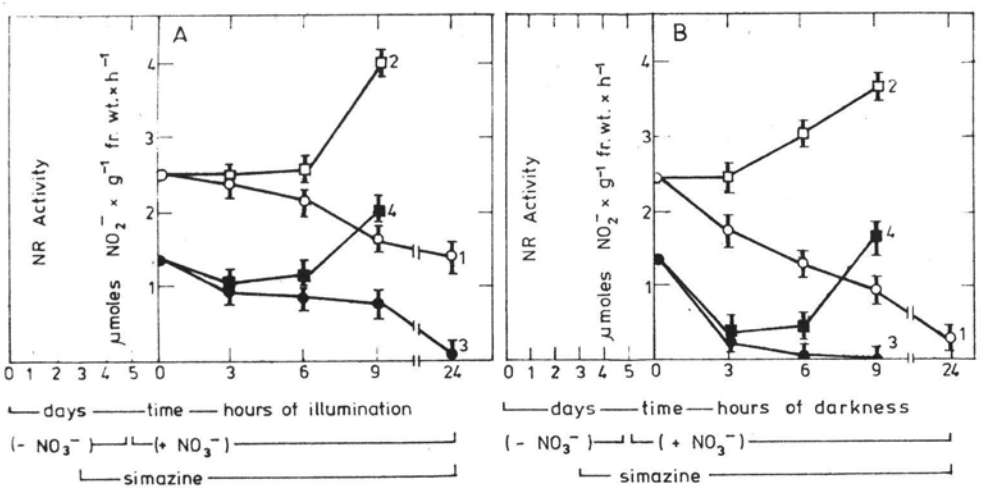

Fig. 3. The effect of simazine (5 μM) and glucose (50 mM) on NR activity in the roots of cucumbers growing under conditions of illumination (A) or darkness (B). Remaining explanations as in Fig. 2

grew for 24 hrs. preceding assay in a NO_3^- containing medium. Preliminary treatment of the plants with simazine (for 3 days preceding assay) clearly decreased the level of NR activity in wheat roots. However, complete in-

hibition of NR after a further 24 hr, period of growth in the presence of simazine, both in the light and dark, was not observed. The introduction of glucose into the medium at the moment the plants were transferred to darkness or left in constant light, did not have a significant effect on the level of NR activity, either in the plants treated with simazine, or in the controls.

The activity of NR in cucumber roots of plants growing in the dark for 24 hrs. (Fig. 3B) in the presence of simazine was already clearly decreased after 3 hrs; after 6 hrs., only traces of enzymatic activity were found. Whereas, in the light, complete loss of NR activity in the presence of simazine was found only after 24 hrs (Fig. 3A). The addition of glucose to the medium containing simazine, clearly increased the activity of the enzyme in the roots of cucumbers growing both in the light and in darkness, after only 6 hrs.

Initial treatment of plants with simazine (3 days before assay) decreased the level of soluble sugars in the roots of wheat seedlings (Fig. 4) and

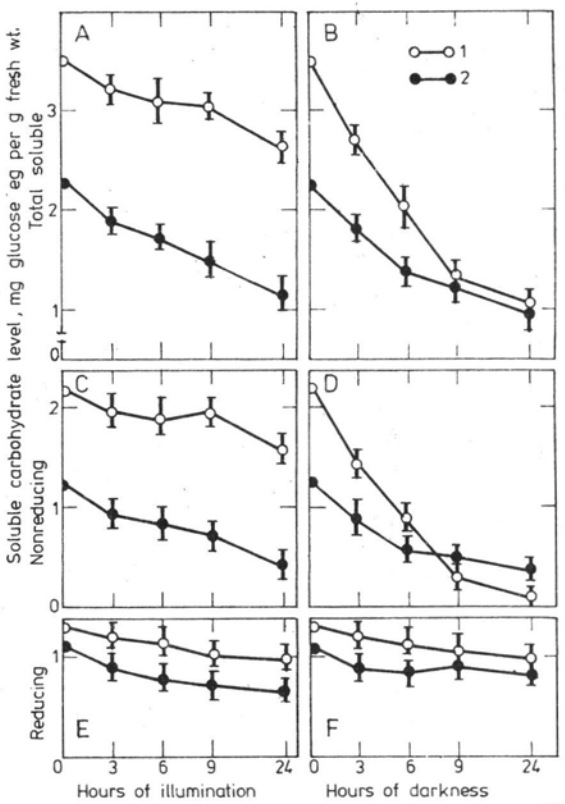

Fig. 4. The effect of simazine (25 μM) on changes in the content of soluble sugars in wheat roots growing under illumination (A, C, E) or darkness (B, D, F). Conditions of growth were as given in Material and Methods and Fig. 2. 1 — Control, 2 — simazine

cucumbers (Fig. 5). The continuation of growth of wheat seedlings for 24 hrs. under conditions of constant illumination and in the presence of simazine (Fig. 4A) was correlated with a further decrease in the soluble sugar content.

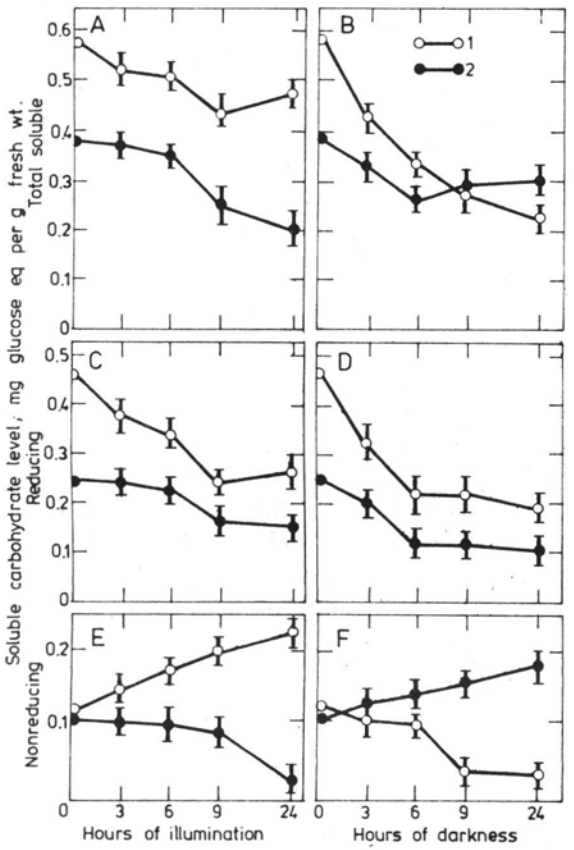

Fig. 5. The effect of simazine (5 μM) on changes in the content of soluble sugars in the roots of cucumbers growing in the light (A, C, E) and in darkness (B, D, F). The remaining explanations as in Fig. 2

After 24 hrs. their amount fell to 50% of the starting level in respect to T = 0. In control roots, however, the soluble sugar content fell only by 35%. A very similar decrease in the content of soluble sugars was found in the roots of wheat plants growing in the dark in the presence of simazine. However, a drastic fall in the level of soluble sugars was found in the roots of control plants growing in the dark. Changes in the levels of non-reducing sugars followed a similar pattern (Fig. 4C and D), while the changes in reducing sugars (Fig. 4E and F) were small at the individual assay times, and similar in all combinations.

The changes in the content of soluble sugars in cucumber roots depended

on the conditions of growth. Under illumination (Fig. 5A), simazine already clearly lowered the level of soluble sugars after 6 hrs. whereas under conditions of darkness, (Fig. 5B), an initial fall and then a rise in the soluble sugar content was observed. The probable reason for the gradual increase in the level of soluble sugars was the increase in the amount of non-reducing sugars in the roots of the cucumber plants growing in the dark in the presence of simazine (Fig. 5F). In the light, however, simazine caused a constant fall in the content of non-reducing sugars (Fig. 5E). At the same

Table 1

The in vivo effect of simazine on the activity of malate dehydrogenase and glucose-6-phosphate dehydrogenase

Plant	Time of assay, h	Activity of malate dehydrogenase, μmoles NADH × g^{-1} fresh weight × min^{-1}			Activity of glucose-6-phosphate dehydrogenase, μmoles NADP$^+$ × g^{-1} fresh weigh × min^{-1}		
		control	simazine	%	control	simazine	%
Wheat	0	19.42	13.54	70	0.45	0.35	78
	24	15.46	10.59	68	0.58	0.32	55
Cucumber	0	18.35	10.63	58	0.24	0.22	85
	24	13.56	7.73	57	0.19	0.11	58

During the entire duration of the experiment the plants grew at a photoperiod of 18 hours illumination and 6 hours darkness. For the first 4 days they grew in a nitrogen free medium, next in a NO_3^- containing medium. The first assay (T = 0) was done after 24 hours of growth in the presence of NO_3^-. Simazine at a concentration of 25 μM for wheat and 5 μM for cucumber, was added 3 days prior to the first assay.

Table 2

The in vitro effect of simazine on the activity of nitrate reductase (NR), malate dehydrogenase (MDH) and glucose-6-phosphate dehydrogenase (G-6-PDH)

Plant	Simazine concentration, μM	Activity		
		NR, μmoles NO_2^- × g^{-1} fresh weight × h^{-1}	MDH, μmoles NADH × g^{-1} fresh weight × min^{-1}	G-6-PDH, μmoles NADP$^+$ × g^{-1} fresh weight × min^{-1}
Wheat	0	0.35	3.33	0.67
	2.5	0.36	2.73^x	0.58
	25.0	0.35	2.77^x	0.48^x
	250.0	0.35	2.63^x	0.29^x
Cucumber	0	1.02	3.27	0.56
	2.5	1.00	2.85	0.56
	25.0	1.03	2.81	0.47^x
	250.0	1.02	2.44^x	0.19^x

The extracts were made from the roots of 5 day old plants growing for 24 hours before extraction in a nitrate medium. The results are the mean values of 5 replications for each combination. Statistical analysis was caused out using Students t-test; ^{x} denotes a significant difference in respect to the control

time, the content of reducing sugars in the roots of cucumbers growing in the presence of simazine, both under illumination (Fig. 5C) and in darkness (Fig. 5D) fell by one-half after 9 and 6 hrs., respectively, after which it reached a constant level.

Table 1 presents the results of the assays of malate dehydrogenase and glucose-6-phosphate dehydrogenase activities in the roots of the studied plants growing in the presence of simazine. The results show that the activity of both dehydrogenases fell by 30 to 40% in comparison with the control. Similar results were obtained from the roots of plants growing in the dark, or in the presence of glucose, both in light and in darkness. The in vitro effect of simazine on the activity of both enzymes is presented in Table 2. Simazine inhibited the activity of malate dehydrogenase, and a bit more strongly, of glucose-6-phosphate dehydrogenase in crude tissue extracts of these enzymes, with a concentration effect being clearly visible. It is worth mentioning that none of the concentrations of simazine used here inhibited NR activity in vitro.

DISCUSSION

The inhibition of NR activity in the roots of plants growing in the presence of simazine, found in this study, along with the simultaneous lowering of the soluble sugar level and of the activity of certain dehydrogenases, suggests at least two pathways by which this herbicide affects the reduction of nitrates in roots. One of them is connected with the effect of simazine on the activity of NR through the inhibition of photosynthesis, which leads to a decreased supply of carbohydrates in roots. The other probably is related to the effect of simazine on the root metabolic processes supplying reduced nucleotides.

The first possible pathway of simazine action is supported by the following results of this study: 1) Treatment of plants for 3 days with simazine caused a significant decrease in the content of soluble sugars (Figs. 4 and 5) with a simultaneous distinct fall in NR activity (Figs. 2 and 3). 2) Further, 24 hr. treatment of plants with simazine caused a fall in the level of soluble sugars in the roots of plants growing in the dark (Figs. 4B and 5B). At the same time, NR activity underwent further reduction, both in control plants and those treated with simazine; in the case of cucumbers, it was totally inhibited (Fig. 3B). In the light, however, distinct decreases in the sugar content and NR activity were observed only in the presence of simazine. 3) Introduction of glucose into the medium stimulated the induction of NR (Fig. 1) in wheat and cucumber roots growing in the presence of simazine and clearly elevated the level of NR activity in cucumber roots growing in the presence of simazine (Fig. 3).

Reports have been published indicating that assimilation of NO_3^- requires metabolic energy and carbon for the carbon skeletons of amino acids, whose source are the carbohydrates arising during photosynthesis (Breteler and Hänisch ten Cate 1980). The indispensability of a steady reserve of assimilates for keeping up NO_3^- reduction in roots was demonstrated by Radin et al. (1978) and Aslam and Huffaker (1982) who measured the reduction of NO_3^- in the roots of plants growing in the light and in darkness. In turn, the experiments by Glabiszewski et al. (1966), Świętochowski et al. (1966) and Płoszyński and Żurawski (1967) showed that the supply of soluble carbohydrates in plants decreased under the influence of triazines. In light of the results of our study as well as on the basis of the cited papers, it seems that the reduction by simazine of the level of soluble sugars in roots is one of the causes of the decrease, and in the case of cucumbers, complete inhibition, of NR activity in roots. Our experiments with the introduction of glucose into the medium support this hypothesis.

However, our results indicate that the complete inhibition of NR activity in cucumber roots is not connected with the completed depletion of soluble sugars, understood as reducing and non-reducing ones. It is not excluded that other processes requiring carbohydrates, growth, for instance, compete with NO_3^- reduction, which has been observed by Radin et al. (1978). On the other hand, our studies have shown a high similarity between the changes in the level of reducing sugars in the roots of cucumbers growing in the dark or in the light in the presence of simazine and significant differences in the rate of disappearance. This fact may suggest a different mechanism of simazine action on the level of NR activity.

Our study has shown that the presence of simazine in the medium decreased the level of malate and glucose-6-phosphate dehydrogenase activities, which are a potential source of reduced nucleotides for NO_3^- reduction (Mann et al. 1978, Deane-Drummond et al. 1980, House and Anderson 1980, Kłobus 1984a and b). This fact may shed some light on a specific mechanism by which simazine inhibits root NR activity, through inhibition of redox processes supplying reducing equivalents for NR. It is not impossible that this herbicide may act directly on the dehydrogenases, which may be suggested by in vitro studies with simazine and these enzymes. On the other hand, simazine did not inhibit NR in vitro.

In would seem then, that the effect of simazine on the inhibition of NR in plant roots is rather complex. This pertains to the effect of this herbicide on lowering the carbohydrate level in roots, which indirectly influence the lowering of NR synthesis or induction, especially in darkness (Travis and Kay 1971, Ashton and Crafts 1973). On the other hand, simazine lowers the activity of dehydrogenases supplying reduced nucleotides, indispensable for nitrate reduction.

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Zależność aktywności reduktazy azotanowej od poziomu cukrów rozpuszczalnych w korzeniach pszenicy i ogórka rosnących w obecności symazyny w warunkach światła lub ciemności

Streszczenie

Symazyna w stężeniu 25 μm i 5 μm hamowała aktywność reduktazy azotanowej, odpowiednio, w korzeniach pszenicy i ogórka, zmniejszała zawartość cukrów rozpuszczalnych oraz obniżała poziom aktywności dehydrogenezy jabłczanowej zależnej od NADH i dehydrogenazy glukozo-6-fosforanowej zależnej od $NADP^+$. Wprowadzenie do pożywki 50 mM glukozy znosiło, częściowo, hamujący wpływ symazyny na aktywność reduktazy azotanowej w korzeniach ogórków oraz nieznacznie podwyższało aktywność enzymu w korzeniach pszenicy. Wyniki sugerują złożony wpływ herbicydu na aktywność reduktazy azotanowej: symazyna zmniejsza poziom cukrów rozpuszczalnych w korzeniach oraz obniża aktywność dehydrogenaz dostarczających zredukowane nukleotydy niezbędne do redukcji azotanów.