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Influence of simazine on nitrate uptake and nitrate reductase activity in *Triticum aestivum* (L.) and *Cucumis sativus* (L.) seedlings

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Abstract

The influence of simazine (2-chloro-4,6-bis(ethylamine)-1,3,5-triazine) on NO_3^- uptake and nitrate reductase (NR) activity in wheat *(Triticum aestivum)* and cucumber *(Cucumis sativus)* seedlings was investigated. It was found that at first (up to 12 h after herbicide treatment) simazine enhanced and then inhibited NR activity in the leaves and roots of the tested plants. After seven days of growth in nutrient medium containing 2.5 and 25 μM simazine the herbicide inhibited by 25 and 70 per cent, respectively, NR activity and reduced by 30 per cent in wheat and 50 per cent in cucumber seedlings nitrate uptake. The probable mechanism of NR inhibition by simazin is discussed and it is suggested that the influence of the herbicide on NR activity may not be exclusively connected with photosynthesis inhibition.

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

INTRODUCTION

The inductive enzyme, nitrate reductase, utilises NADH as donor of electrons indispensable for nitrate reduction (Beevers and Hageman 1969). The source of NADH is the process of 3-phosphoglyceric aldehyde oxidation (Klepper at al. 1971) or malate (Neyra and Hageman 1976, Deane-Drummond et al. 1979). NR activity is, thus, indirectly dependent on light, in view of the indispensability of carbohydrates (Aslam et al. 1973) and the production in chloroplasts of reducing equivalents which may be utilised in the cytoplasm by NR owing to the shuttle systems malate-oxaloacetate (House and Anderson 1980).

Triazine herbicides are known inhibitors of electron transport in the photophosphorylation process (Moreland 1980, van Ransen

1982), therefore, their indirect influence on NO_3^- reduction is quite probable by way of inhibition of the photosynthesis process. It has been found, however, that triazine herbicides in sublethal concentration enhance the NR activity and NO_3^- uptake (Ries et al. 1967, Tweedy and Ries 1967, Wu et al. 1972, Mahanandas et al. 1978) and in short lasting experiments increase the extracting activity of NR and the NO_3^- content in leaves, although the NR activity *in vivo* was reduced (Aslam and Huffaker 1973). Decleire et al. (1976), on the other hand, demonstrated a diminished NR activity during longer exposure to triazines. In this connection investigations were performed on the effect of long and short exposure to the action of simazin on the NR activity in green and not green plant tissues in order to find an answer, whether the influence of triazines on NR activity is associated exclusively with photosynthesis inhibition.

MATERIAL AND METHODS

Wheet grains (*Triticum eastivum* L. var. Luna) after germination were placed in distilled water, exposed to light and after five days the selected seedlings were transferred to Hoagland's medium consisting of (in mM per dm^3): KNO_3 — 5, $Ca(NO_3)_2 \cdot 2H_2O$ — 5, KH_2PO_4 — 1, $MgSO_4 \cdot 7H_2O$ — 2. In some experiments the plants were transferred to medium deprived of nitrogen, while potassium and calcium were introduced in the form of sulphates instead of the nitrate salts. To all nutrient media microelements were added in the amount of 1 cm^3 per 1 dm^3 of medium from the basic solution containing the following components (in mg per 100 cm^3): iron citrate — 1973.0, $MnSO_4 \cdot 5H_2O$ — 241.0, H_3BO_3 — 30.0, $CuSO_4 \cdot 5H_2O$ — 25, $ZnSO_4 \cdot 7H_2O$ — 0.3, Na_2MoO_4 — 0.2. The media were adjusted to pH 6.0. Germinated cucumber seeds (*Cucumis sativus* L. var. Monastyrski) were placed for 3 days in threefold diluted Hoagland's medium not containing nitrogen, with microelements added and transferred to threefold diluted Hoagland's medium containing nitrates, with pH adjusted to 6.0. Simazine was introduced into the media after previous dissolution in 2 per cent (w/v) ethanol. To the control media equivalent quantities of ethanol were added. Water cultures of wheat and cucumber grew in a photothermostated room under conditions of 16 h light (14.7 $W \times m^{-2}$ and $\pm$ 25°C) and 8 h of darkness (19-20°C).

Nitrate reductase activity *in vivo* was determined after Jaworski (1971). To wheat leaf fragments (0.5 cm^2) and whole cucumber cotyledones (ca. 500 mg fresh wt.) 10 cm^3 of solution containing 0.1 M KNO_3 in 0.1 M K-phosphate buffer, pH 7.5 was infiltrated. Then they were placed in the same solution with 1 per cent propanol added. The tissues were incubated for 1 h in a water bath at 33°C in darkness. Then a suitable

amount of the solution was taken for nitrate determination. The latter were determined by adding to the sample 1 cm of 1 per cent (w/v) sulphanilamide in 1 N HCl and 1 cm^3 0.01 per cent (w/v) N-1-naphthyl/ethylenediamine dihydrochloride solution. The whole was diluted to 5 cm^3 with distilled water. After 30 min absorption was read at 540 nm. NR activity *in vivo* was expressed in nmoles of NO_2^- produced per 1 g of fresh weight per hour. The results in the tables are means from 5 replications for each combination. Each experiment was repeated 3-4 times. In some experiments NR activity *in vivo* was determined both in the presence of 0.1 M KNO_3 in the incubation solution (referred to as "potential" activity) and without any substrate (referred to as "actual" activity), according to the method described by Breteler et al. (1979). In the latter case KNO_3 in the incubation solution was substituted with K_2SO_4 so that the amount of potassium would remain the same. The "potential" activity was defined as activity induced by exogenous substrate (NO_3^-), whereas the activity of the enzyme the level of which was described by the current content of nitrates available for reduction (called the metabolic pool, Ferrari et al. 1973, Aslam et al. 1976) was considered as "actual".

Activity *in vitro* of nitrate reductase was determined in extracts (extraction NR) from the assayed tissues. Leaves, cotyledones or roots, after thorough washing were ground in a mortar in a 50 mM K-phosphate solution (pH 7.8), 0.1 mM cysteine and 3 mM EDTA in relation to 5 cm^3 solution per 1 g fresh tissue. The homogenate was centrifuged for 15 min at 20 000 × g and the supernatant was used for NR activity determination. All the above desribed manipulations were done at 0-4°C. Enzyme activity was determined according to Hageman and Flesher (1960) by measuring the NADH-dependent nitrite production. The enzyme activity was expressed in nmoles $NO_2^- \times g^{-1}$ fresh weight × × h^{-1}. The results are means from three replications for each combination.

The nitrates were colorimetrically determined by the method of Cataldo et al. (1975). The reaction samples were mixed thouroughly with 0.8 cm^3 of 5 per cent (w/v) salicylic acid in concentrated H_2SO_4. After 20 min at room temperature 19 cm^3 of 2 N NaOH was added, the whole was cooled under running water and absorption was read at 410 nm. The amount of NO_3^- taken up was calculated from the difference between its initial and end values in the medium. The nitrate content in the seedlings was determined in the dry matter after preparing water extracts. The ground material (100 mg d. wt.) was heated for 2 h with 10 cm^3 of water at 80-90°C and then for 10 min at 100°C. Nitrates were determined in the extract obtained. The amount of reduced NO_3^- was calculated from the difference between the amount of taken up and accumulated nitrates in the plant tissues. The results of measurement are

means from seven replications for each combination. The test for significance of differences between means consisted in calculating the standard error of the difference from the standard errors of two means (SE_1 and SE_2) by using the expression: SE of difference $= \sqrt{(SE_1)^2 + (SE_2)^2}$. Differences which exceeded twice the standard error were considered to be significant at the 5 per cent level.

Reagents used: Simazin (2-chloro-4,6,bis(ethylamino)-1,3,5-triazine) as pure substance (Institute of Organic Industrial Chemistry, Warsaw), NADH (Reanal, Hungary) the remaining reagents were from POCH Gliwice.

RESULTS

Infiltration of simazine into fragments of wheat leaves cut off from 7-day-old seedlings, which for 24 h before NR activity measurement were placed in nitrate nutrient medium, increased the enzyme activity (Table 1). This increase was the greater the higher was the concentration of simazine infiltered into the tissues. A similar stimulation of NR activity was noted in wheat leaf segments cut from plants which grew on medium with rising simazine concentrations added (Table 2). It was found that simazin enhanced both the "potential" and the "actual" activity. After 6 h ("potential" activity) and after 12 h ("actual" activity), however, the NR activity diminished down to its distinct inhibition, especially when higher herbicide concentration was used in the medium. The data in Table 2, indicate that the decrease in "potential" activity preceded in time the depression of "actual" activity, this possibly indicating certain disturbances in nitrate transport to the cells.

Calculation of the "actual" to "potential" activity ratio shown in Fig. 1 seems to confirm the above mentioned supposition. In control

Table 1

Influence of simazine *in vivo* on nitrate reductase activity (nmoles $NO_2^- \times g^{-1}$ fresh weight) in wheat leaf fragments

Simazine concentration, μM	Time of measurement, after hours			
	0.5	1.0	1.5	2.0
—	125±12	285±16	532±23	784±36
25	156±11	345±15	560±25	892±42
250	267±12	480±22	770±37	1092±46
2500	203±10	660±28	955±42	1338±48

Wheat seedlings 7-days old grew for 24 h in nitrate medium. The leaves were then detached, cut in to 0.5-cm^2 fragments infiltrated with incubation medium containing simazine and after the time intervals marked in the table 0.1 cm^3 samp e s were taken for NR aetivity determination.

Table 2

"Potential" and "actual" NR activity (nmoles $NO_2^- \times g^{-1}$ fresh weight $\times h^{-1}$) in fragments of 7-day wheat seedlings transferred to nitrate medium (at time 0) containing increasing simazine concentrations

	Simazine concentration, μM	Times of measurement, after hours					
		0	3	6	9	12	24
"Potential" activity	—	175	275± 9 a	425±23 c	775±33 d	625±24 f	525±23 g
	2.5		275± 12 a	425±19 c	550±27 e	450±25 c	150± 7 h
	25.0		325± 11 b	400±26 c	550±23 e	420±22 c	50± 1 i
	250.0		425± 15 c	325±15 b	480±22 e	400±21 c	25±0.8 j
"Actual" activity	—	0	25±0.7 a	175± 6 d	385±12 e	425±23 f	425±21 f
	2.5		25±0.9 a	175± 9 d	300±12 e	275±14 d	75± 4 h
	25.0		50±2.0 a	200±12 d	300± 9 e	170± 9 g	10±0.5 j
	250.0		70±2.0 c	200±12 d	300±11 e	85± 4 h	5±0.2 i

The plants grew for 7 days on nitrogen-free medium, then were transferred to nitrate medium (time 0) containing simazine and at indicated time intervals leaf samples were taken for NR activity determination. Mean values marked by the same letter do not differ for 5 per cent of error.

plants, namely, this ratio after 24 h approached unity, this seemingly suggesting a full activation of the inductive enzyme — nitrate reductase — and saturation of the tissues with nitrates. In plants growing in the presence of 2.5 μM simazine, however, a distinct depression of the above named ratio is observed after 12 h. Higher simazine concentrations caused

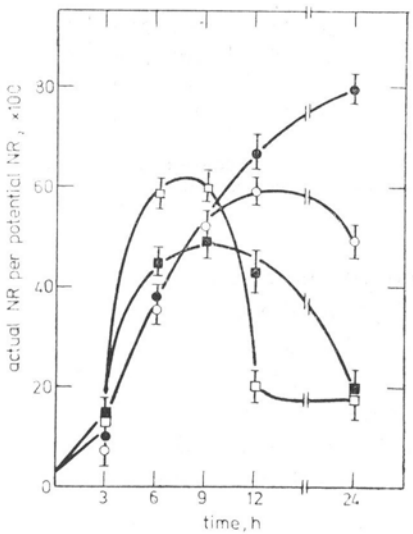

Fig. 1. Ratio of "actual to "potential' NR activity in wheat leaves, calculated from data in Table 2. ● — control, ○ — 2.5 μM simazine, ■ — 25 μM simazine, □ — 250 μM simazine

at first a drastic rise and subsequently a decrease of the value of the ratio of these two activities. It would seem, therefore, that simazine modifies nitrate transport to the cells.

NR activity measurements by the *in vitro* method performed on the cotyledones and roots of cucumber gave a similar picture of the influence of simazine on activity of the enzyme (Table 3). In cucumber roots (not green tissues) at first a rise, and then a decrease of NR activity was observed under the influence of simazine present in the medium, similarly as in the wheat leaves. In cucumber cotyledones, however, simazine did not influence the NR activity level in the initial period of its action, but after 24 h the enzyme activity decreased under the effect of 2.5 and 25 μM simazine.

Table 3

Effect of simazine on NR activity (nmoles $NO_2^- \times g^{-1}$ fresh weight $\times h^{-1}$) in cotyledones and roots of 5-day cucumber seedlings transferred at 0 time to nitrate medium containing increasing simazine concentrations

	Simazine concentration, μM	Time of measurement, after hours			
		3	6	12	24
Cotyledones	—	470±23 a	1060±56 b	2940±149 c	2920±137 c
	0.25	430±19 a	1200±59 b	3295±165 c	3212±152 c
	2.5	400±20 a	972±48 b	2322±104 d	2219±112 d
	25.0	420±21 a	922±46 b	1999± 92 e	1956± 89 c
Roots	—	250±14 a	600±23 c	1700± 87 e	1000± 51 d
	0.25	200±16 a	700±25 c	1690± 92 e	1210± 72 d
	2.5	400±20 b	690±27 c	1900±100 e	910± 29 d
	25.0	650±27 c	920±32 d	1580± 93 e	400± 19 b

Five-day plants transferred from medium free of nitrogen to nitrate medium with simazine. At intervals marked in table cotyledones and roots were cut off and NR activity was determined immediatelly by *in vitro* method. Further notation as in Table 2.

Increasing simazine concentration in the medium (beginning with 2.5 μM) diminished NO_3^- uptake by wheat and cucumber seedlings after seven days of growth of the plants. This reduction of nitrate uptake by wheat seedlings did not exceed 30 per cent, but in cucumber seedlings it reached about 40-50 per cent as compared with the control. Simazine was found to have no influence on the amount of accumulated NO_3^- in cucumber seedlings, whereas it markedly increased nitrate concentration in wheat (Table 4). The NO_3^- content in wheat seedlings growing in the presence of 2.5, 25 and 250 μM simazine exceeded 5-6 times the content in control plants. Notwithstanding the observed differences in the amount of accumulated nitrates in wheat and cucumber, the quantity of reduced

NO_3^- (calculated from the difference between the quantity of taken up and accumulated NO_3^-) in both plant species decreased after seven days of growth in the presence of increased simazine concentration. The data in Table 4 indicate that simazine in a 0.25 μM concentration decreased

Table 4

Effect of simazine on dry matter yield (mg per plant*), uptake, accumulation and reduction of nitrates (μmoles $NO_3^- \times g^{-1}$ dry weight**) by 10-day and 7-day wheat and cucumber seedlings respectively and NR activity (nmoles $NO_2^- \times h^{-1} \times g^{-1}$ fr. weight***) in leaves and cotyledons of the tested plants

Plant	Simazine concentration, μM	Dry matter*	Uptake**	Accumulation**	Reduction**	NR activity***
Wheat	—	43 a	2978 a	230 a	2748	5503 a
	0.025	36 a	2890 a	408 b	2482	4811 a
	0.25	28 b	2396 a	467 b	1920	4512 a
	2.5	14 c	2247 b	1059 c	1188	4127 b
	25.0	12 c	2076 c	1339 c	737	2100 c
	250.0	11 c	2037 c	1373 c	664	2050 c
Cucumber	—	54 a	3302 a	1295 a	2007	7900 a
	0.025	56 a	3403 a	1465 a	1938	8902 a
	0.25	44 a	2919 a	1508 a	1411	6320 b
	2.5	25 b	1984 b	1295 a	689	6100 b
	25.0	19 b	1742 b	1184 a	558	2408 c

The amount of reduced nitrates was calculated from the difference between the taken up amount and NO_3^- amount accumulated in the whole plants.

the amount of reduced NO_3^- by 30 per cent, whereas in a 25 μM concentration, by about 70 per cent. The NR level dynamics was similar in wheat leaves and cucumber cotyledons. NR activity, namely, determined by the *in vitro* method diminished by about 20 per cent in the leaves and cotyledones of plants growing in the presence of 0.25 μM simazine and by about 70 per cent at a 25 μM herbicide concentration. As expected, simazine also reduced the dry weight increment after 7 days of growth of wheat and cucumber seedlings.

DISCUSSION

Simazine introduced directly into plant tissues (infiltration) or into the nutrient medium at first enhanced (up to 12 h) nitrate reductase activity in wheat leaves and cucumber roots. The stimulation of NR activity observed in the present experiments has been also reported by Tweedy and Ries (1967) in maize leaves and by Aslam and Huffaker (1973) in barley leaves. On the other hand, Klepper

(1976, 1979) did not find a distinct influence of triazine herbicides on NR activity in wheat leaves.

Stimulation of NR activity by simazine is not clear if we take into account the fact of photophosphorylation inhibition by triazines (Ashton et al. 1960, Rensen 1982) and the indirect dependence of NR on photosynthesis (Klepper et al. 1971, Aslam et al. 1973). It seems, however, that the enhanced NR activity observed in the present experiments in the early period of herbicide action was the result of the relatively high "actual", NR activity. At the same time in the control plants the "actual" activity level was quite low. Since "actual" activity depends on the amount of endogenous nitrates (metabolic pool) available for reduction (Heimer and Filner 1971, Aslam and Oaks 1975, Aslam et al. 1976, Bar-Akiva et al. 1970, Breteler et al. 1979), one may suppose that simazine in the early period of its action causes a higher saturation of the tissues with nitrates, as has been noted by Aslam and Huffaker (1973). It would seem that the tenfold increase of NR activity in maize leaves observed by Tweedy and Ries (1967) confirms to some extent this supposition. These authors, namely, noted NR activity stimulation only in the presence of low nitrate concentrations in the medium, not exceeding 3 mM. At higher concentrations of NO_3^-, however, simazine had no effect on the NR activity level.

The fact that after about 12 h of plant growth on medium containing simazine, a decrease, and further inhibition of NR is observed, both in green and not green tissues, points to a distinct inhibition of nitrate assimilation, and particularly NR inhibition. On the basis of the available information it may be supposed that inhibition of NR activity by simazine is caused, at least in green plant tissues, by inhibition of electron transport in the process of photosynthesis (Rensen 1982) and CO_2 assimilation (Ashton et al. 1960). It would seem, therefore, that the inhibitory influence of simazine on NR activity in leaves is not direct (Klepper 1979), running *via* inhibition of carbohydrate production and reduction equivalents. Since NR activity depends on NADH formed in the process of carbohydrate oxidation in glycolysis (Klepper et al. 1971), indirect NR activity inhibition by simazine seems obvious. The fact, however, that this herbicide inhibited NR activity in roots as well suggests that the mechanism of NR inhibition should not be searched for exclusively in the indirect influence of simazine on the photophosphorylation process.

Another mechanism responsible for depression of the NR activity might involve the but little known influence of triazines on the permeability of cell membranes (Moreland 1980). This is connected with nitrate uptake indispensable for induction or activation and function of NR in plant tissues (Aslam and Oaks 1975). In the present experiments a distinct decrease of NO_3^- uptake by wheat seedlings (by 30%)

and by cucumber seedlings (by 50%) was observed. The fact that, independently of the degree of nitrate uptake decrease, simazine depressed by about 70 per cent nitrate reduction and NR activity in leaves or cotyledones, may suggest a mechanism of nitrate assimilation inhibition independent of NO_3^- uptake. It would seem, therefore, that simazine, although it inhibits the process of photosynthesis causing a diminution in dry matter increment of the plants, affects by some other pathway the process of NO_3^- reduction.

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Wpływ symazyny na pobieranie azotanów i aktywność reduktazy azotanowej w siewkach Triticum aestivum (L.) i Cucumis sativus (L.)

Streszczenie

Badano wpływ symazyny (2-chloro-4,6-bis(ethylamino)-1,3,5-triazine) na pobieranie NO_3^- oraz aktywność reduktazy azotanowej (NR) w siewkach pszenicy *(Triticum aestivum)* i ogórka *(Cucumis sativus)*. Stwierdzono, że symazyna początkowo (do około 12 godziny po podaniu) zwiększała a następnie hamowała aktywność NR w liściach i korzeniach badanych roślin. Po 7 dniach wzrostu roślin w pożywce zawierającej 2,5 i 25 μM symazyny, herbicyd hamował odpowiednio o 25 i 70 procent aktywność NR oraz zmniejszał o 30 procent w siewkach pszenicy i o 50 procent w siewkach ogórka pobieranie azotanów. Przedyskutowano prawdopodobny mechanizm inhibicji NR przez symazynę, sugerując, nie związany wyłącznie z inhibicją fotosyntezy, wpływ herbicydu na aktywność NR.