*THE INCREASE IN NITRATE REDUCTASE ACTIVITY AND PROTEIN CONTENT OF PLANTS TREATED WITH SIMAZINE**

By S. K. Ries, H. Chmiel, D. R. Dilley, and P. Filner

DEPARTMENT OF HORTICULTURE AND MSU/AEC PLANT RESEARCH LABORATORY, MICHIGAN STATE UNIVERSITY, EAST LANSING

Communicated by Anton Lang, June 12, 1967

Simazine (2-chloro-4,6-bis ethylamino-s-triazine) is an herbicide with the unusual property that at subtoxic levels it increases the growth and nitrogen content of certain plant species.[1-5] In experiments with corn (*Zea mays* L.), these responses to simazine occur in plants grown with nitrate, but not in plants grown with ammonia as the source of nitrogen, and are greatest when nitrate and temperature are at suboptimal levels. Nitrate reductase activity in corn growing on suboptimal levels of nitrate increases with simazine concentration.[6]

The previous research suggested the hypothesis that simazine enhanced nitrate utilization. If this interpretation is correct, an increase in total protein per plant might be expected. The research reported in this paper confirms this expectation. There are many reports in the literature on the chemical alteration of nitrogen metabolism and percentage increases in protein;[7,8] however, there is no evidence of a chemical's consistently and appreciably increasing the amount of protein per plant in shoot and seed.

Materials and Methods.—Rye (*Secale cereale* L. cv. MSU Exp. 1) and peas (*Pisum sativum* L. cv. Little Marvel) were seeded in 11- × 7-cm plastic cups containing vermiculite. Nutrient solutions were prepared according to Hoagland and Arnon.[9] Nitrogen was supplied as KNO_3 for the nitrate studies, and $(NH_4)_2SO_4$ for the ammonium studies. The pH of the NH_4^+ nutrient solution was adjusted with KOH to that of the NO_3^- nutrient solution (pH 6.5). There was no appreciable change in the pH of the growing media during the course of the experiments.

In tests where NH_4^+ was compared with NO_3^- as a source of nitrogen, 2-chloro-6-trichloromethyl pyridine was used to prevent oxidation of NH_4^+ by *Nitrosomonas* sp.[10] This compound was applied in all treatments at the rate of 10 per cent of the respective N level.

Plants were grown at 22° for 16 hours with 2,000 ft-c of light and at 17° for an 8-hour night. Low-temperature studies were conducted at a 17° day and a 12° night temperature. Simazine treatments were made by adding aliquots from a 25.0-μM stock solution to the nutrient solution and applying it at regular intervals commencing approximately one week after seeding. Approximately 750 and 600 ml of treating solution were applied at the high and low temperatures, respectively, over the duration of the experiments.

Plants were harvested, the aerial portion cut into approximately 5-mm segments, weighed, and 1-gm samples held in ice for subsequent assay of nitrate reductase and protein content. Another 1-gm sample was used for determining dry weight and total Kjeldahl N. A crude enzyme extract was prepared and assayed for nitrate reductase by the method of Sanderson and Cocking.[11]

Protein determinations were made by the method of Lowry *et al.*[12] and by a micro-Kjeldahl procedure. The protein profile of the crude enxyme extracts was obtained by acrylamide gel electrophoresis.[13]

TABLE 1

EFFECT OF SIMAZINE CONCENTRATION ON GROWTH AND PROTEIN CONTENT OF 26-DAY-OLD RYE RECEIVING 6 mM NO_3^- N IN THE NUTRIENT SOLUTION

Simazine conc. (μM)	Amount per Plant: Fresh wt (mg)	Dry wt (mg)	Dry wt (%)	Protein (mg)	Protein increase†: Per plant (%)	Per dry wt (%)
0.00	361	77	21.2	2.9*		
0.05	367	77	21.0	3.2	10	10
0.10	373	71	19.1	4.2	45	57
0.20	350	57	16.3	3.6	24	68
0.40	374	59	15.7	3.8	31	71
0.80	344	42	12.3	3.8	31	140

* Control significantly lower than simazine treatments at 5% level.
† Expressed as percentage increase over the control.

Respiration rate of intact plants was determined on two successive nights by measuring O_2 uptake and CO_2 evolution in an automatic gas analyzing system.[14]

All treatments were replicated two or three times and arranged in a completely randomized design within the growth chambers. The data were subjected to analysis of variance and the means compared by the F test or Duncan's multiple range procedure. All data with the exception of the respiration measurements are expressed as amounts per plant. This expression best illustrates the changes in absolute amounts of protein.

Results.—Simazine concentration: Rye plants receiving 0.05–0.8 μM simazine contained up to 45 per cent more water-extractable protein per plant than the controls (Table 1). The dependence on simazine concentration was between 0 and 0.1 μM, but not above 0.1 μM. The fresh weight per plant was not changed, but there was a progressive decrease in per cent dry weight with increasing simazine concentration. Simazine increased the respiration rate more than 10 per cent without affecting the respiratory quotient (Table 2). This may account for the decrease in dry weight.

Nitrate level and temperature: Protein content and nitrate reductase activity per plant increased with increasing levels of NO_3^- nitrogen in the nutrient solution (Table 3). Regardless of nitrogen level, there was a concomitant increase in both nitrate reductase activity and water-extractable protein per plant from simazine applications. Enzyme activity increased substantially more than protein content from simazine applications at all NO_3^- levels. However, the relative increase from simazine applications decreased with increasing NO_3^- levels.

Simazine applications to rye plants grown under two different temperature regimes increased protein content and nitrate reductase activity at both temperatures (Table 4). The enzyme activity more than doubled with a 5° increase in temperature, with or without simazine. The increase in protein content caused by 0.4 μM simazine was 79 and 59 per cent at the high and low temperatures, respectively.

TABLE 2

EFFECT OF SIMAZINE ON THE RESPIRATION RATE OF 21-DAY-OLD RYE PLANTS GROWN ON NO_3^- NITROGEN

Simazine conc. (μM)	Average Cumulative Respiration for Two 12-hr Periods: Ml/100 gm Fresh Wt (CO_2)	Ml/100 gm Fresh Wt (O_2)	Ml/10 gm Dry Wt (CO_2)	Ml/10 gm Dry Wt (O_2)	Respiratory quotient
0.0	332	356*	316*	339*	0.93
0.2	364	384	372	392	0.94
0.4	363	386	361	384	0.94
0.8	342	362	386	410	0.94

* Control is significantly lower than treatments at 5% level.

TABLE 3

RESPONSE OF 25-DAY-OLD RYE TO SIMAZINE TREATMENT AT VARIOUS LEVELS OF NO^{3-} NITROGEN

Treatment		Amount per Plant				Protein Increase†	
N level (mM)	Simazine conc. (μM)	Fresh wt (mg)	Dry wt (mg)	Nitrate reductase* (mμmoles NO_2/hr)	Protein* (mg)	Per plant (%)	Per dry wt (%)
3	0.0	302	43	66	4.26		
3	0.4	344	43	408	5.94	39	39
6	0.0	398	54	386	7.23		
6	0.4	387	46	840	8.83	22	43
12	0.0	525	68	1,305	11.17		
12	0.4	553	65	1,876	13.65	22	28

* F value for comparison of control versus treatment, within nitrogen levels, significantly different at 1% level.
† Expressed as percentage increase over the control.

TABLE 4

RESPONSE TO SIMAZINE OF RYE PLANTS GROWN FOR 33 DAYS AT DIFFERENT TEMPERATURES

Treatment		Amount per Plant*				Protein Increase†	
Temp. (°C)	Simazine conc. (μM)	Fresh wt (mg)	Dry wt (mg)	Nitrate reductase (mμmoles KNO_2/hr)	Protein (mg)	Per plant (%)	Per dry wt (%)
17–12	0.0	325 a	76 a	31 a	3.18 a		
17–12	0.2	319 a	60 a	99 b	4.59 b	44	83
17–12	0.4	328 a	64 a	192 c	5.05 c	59	89
22–17	0.0	380 a	59 a	73 a	4.36 a		
22–17	0.2	456 b	67 a	266 b	5.98 ab	37	21
22–17	0.4	463 b	68 a	603 c	7.82 c	79	56

* Means followed by unlike letters are significantly different at the 5% level within a temperature.
† Expressed as percentage increase over the control.

TABLE 5

INCREASE IN NITRATE REDUCTASE ACTIVITY AND PROTEIN CONTENT OF SIMAZINE-TREATED RYE WITH NO_3^- COMPARED TO NH_4^+ AS A SOURCE OF NITROGEN

Treatment		Amount per Plant*				
N source (6 mM)	Simazine conc. (μM)	Fresh wt (mg)	Dry wt (mg)	Dry wt (%)	Nitrate reductase (mμmoles KNO_2/hr)	Protein (mg)
NO_3^-	0.0	363 a	82 a	22	54 a	2.24 a
NO_3^-	0.4	331 a	56 b	16	400 b	2.88 b
NH_4^+	0.0	317 a	76 a	23	73 a	2.76 b
NH_4^+	0.4	321 a	77 a	23	54 a	2.60 b

* Means followed by unlike letters are significantly different at 5% level.

Dependence on NO_3^- as nitrogen source: Rye plants treated with simazine contained more protein and had a higher level of nitrate reductase activity when grown with NO_3^- but not when grown with NH_4^+ as the nitrogen source(Table 5). Although the fresh weight did not vary with treatments, the dry weight of simazine-treated plants grown on NO_3^- was lower, as was observed in the earlier experiments.

Light intensity: Rye plants grown under three different light intensities responded to simazine applications at both 1,200 and 2,000 ft-c, but not at 150 ft-c (Table 6). Nitrate reductase activity was highest in the etiolated plants grown at 150 ft-c, but there was no response to simazine.

Seed protein: Pea plants treated with simazine and grown to maturity contained more seed protein (Table 7). Simazine levels as low as 0.05 μM resulted in more than a 40 per cent increase in water-extractable protein and total N per plant. At

TABLE 6

EFFECT OF LIGHT INTENSITY ON THE NITRATE REDUCTASE ACTIVITY AND PROTEIN CONTENT OF 34-DAY-OLD RYE PLANTS RECEIVING SIMAZINE AT LOW TEMPERATURES (17–12°) AND 3 mM NO_3^-

Treatment: Light intensity (ft-c)	Treatment: Simazine conc. (μM)	Amount per Plant: Fresh wt (mg)	Dry wt (mg)	Dry wt (%)	Nitrate reductase (mμmoles KNO_2/hr)	Protein* (mg)	Protein Increase†: Per plant (%)	Per dry wt (%)
2,000	0.0	570	136	23.9	12	4.1		
2,000	0.2	610	124	20.3	36	5.2	27	40
2,000	0.4	506	85	16.8	60	4.6	12	80
1,200	0.0	654	134	20.5	18	4.7		
1,200	0.2	688	130	18.9	41	5.5	17	20
1,200	0.4	518	82	15.8	100	4.8	2	69
150	0.0	487	49	10.1	472	3.8		
150	0.2	472	46	9.7	474	3.7	0	3
150	0.4	416	40	9.6	443	3.3	0	5

* F-value for comparison of average protein content of 0.2 μM simazine at 2,000 and 1,200 ft-c with control and 0.4 μM simazine significant at 5% level.
† Expressed as percentage increase over the control.

TABLE 7

INFLUENCE OF SIMAZINE ON GROWTH AND PROTEIN CONTENT OF MATURE PEA SEEDS*

	Dry Wt		Seed Protein† — Water-Extractable — Lowry			Kjeldahl			Total		
Simazine conc. (μM)	Per plant (gm)	Seeds per plant (gm)	Protein per plant (mg)	Protein Increase: Per plant (%)	Per dry wt. (%)	Protein per plant (mg)	Protein Increase: Per plant (%)	Per dry wt. (%)	Protein per plant (mg)	Protein Increase: Per plant (%)	Per dry wt. (%)
0.00	1.36 a	0.88 a	83 a			94 c			156 b		
0.05	1.26 a	1.03 a	117 b	41	21	147 a	56	34	219 a	40	20
0.15	1.21 a	0.82 a	103 ab	24	34	122 b	30	39	200 a	28	38
0.30	0.95 b	0.58 b	89 a	7	63	107 bc	14	72	150 b	0	46

* Means followed by unlike letters are significantly different at the 5% level.
† Mature seeds were extracted as stated in *Materials and Methods*. Proteins were precipitated with TCA and aliquots taken for Lowry and Kjeldahl determinations. Total N in seed determined by Kjeldahl and converted to protein by factor 6.25.

higher levels (0.30 μM of simazine) plant growth was inhibited and although there was 63 per cent more protein per gram dry weight, there was less total protein per plant.

These data indicate that the increased protein synthesis from simazine applications observed in young plants may also be manifested in the maturing seed, the major storage organ for protein in many plant species.

Protein profile: Acrylamide gel electrophoresis of proteins extracted from rye plants and pea seeds indicated no obvious alteration in the protein pattern by simazine application. Instead, more of the same types of proteins were synthesized (Figs. 1 and 2).

Discussion.—Clearly, simazine accelerates the rate of protein accumulation in rye and peas. It remains to be determined whether this increase in protein is due to more rapid synthesis or less rapid degradation. The increased rate of respiration combined with the lower rate of carbohydrate accumulation suggest that there is a greater energy requirement in simazine-treated plants. This favors the idea that the increase in protein is due to more rapid synthesis.

The effect of simazine on protein accumulation is observed in plants grown on nitrate, but not in plants grown on ammonia as a nitrogen source. This implies that the action of simazine involves some step in the reduction of nitrate to am-

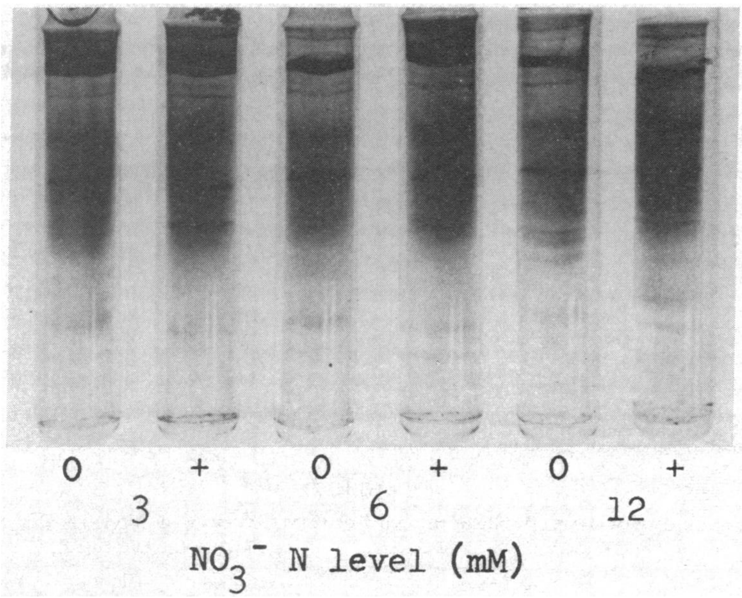
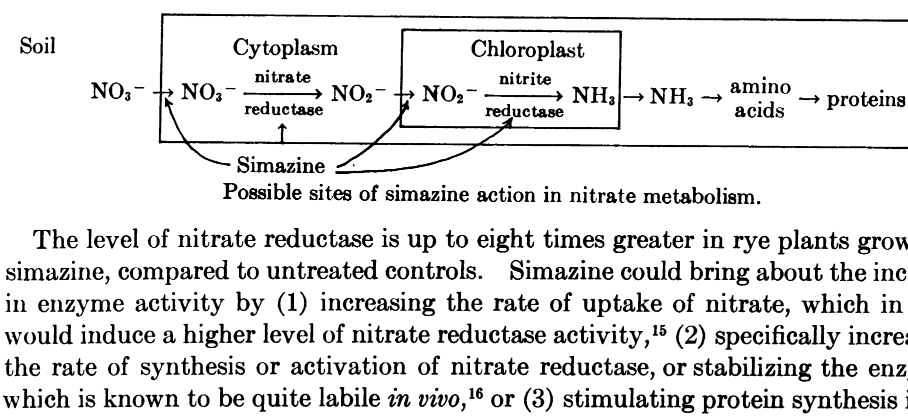


FIG. 1.—Acrylamide gel electrophoresis of protein in a crude extract of rye plants grown on various NO_3^- nitrogen levels comparing no simazine (0) with 0.2 μM simazine (+) level.

monia, or perhaps earlier in the uptake of nitrate, but no step in the further conversion of ammonia (see diagram below). Furthermore, the effect of simazine decreases as the nitrate level approaches the optimum.



Possible sites of simazine action in nitrate metabolism.

The level of nitrate reductase is up to eight times greater in rye plants grown on simazine, compared to untreated controls. Simazine could bring about the increase in enzyme activity by (1) increasing the rate of uptake of nitrate, which in turn would induce a higher level of nitrate reductase activity,[15] (2) specifically increasing the rate of synthesis or activation of nitrate reductase, or stabilizing the enzyme, which is known to be quite labile *in vivo*,[16] or (3) stimulating protein synthesis in an unspecified way, with the secondary and nonspecific consequence that the enzyme activity rises. If either (1) or (2) is correct, the nitrate reductase reaction would be the rate-limiting step for protein synthesis in plants growing on suboptimal levels of nitrate.

Some support for the idea that nitrate reduction is in fact the rate-limiting step can be found in the literature. Experiments with cultured tobacco cells have provided evidence that the level of nitrate reductase can limit the rate of proliferation of plant cells.[17] Nitrate reductase is well known to be a substrate-inducible enzyme

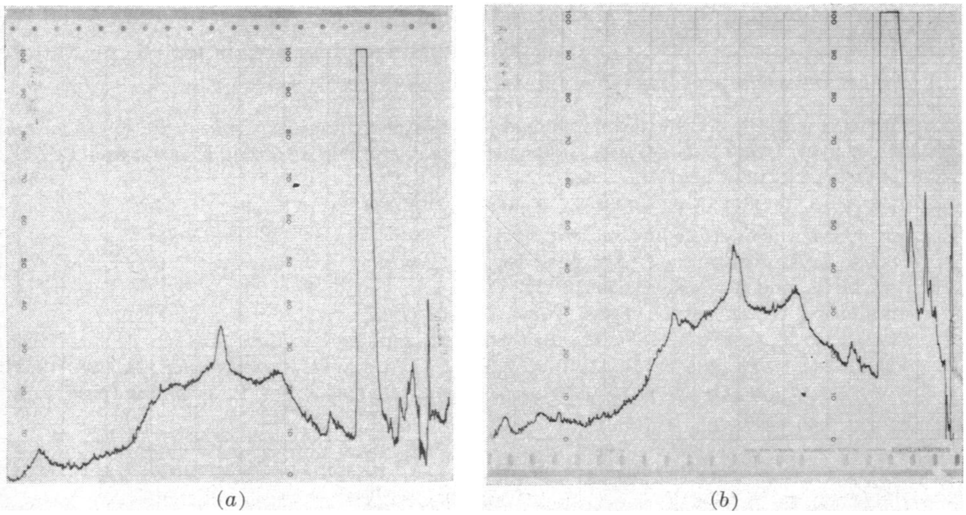


FIG. 2.—Densitometer traces of acrylamide gel columns depicting protein profile of rye plants grown with 84 ppm NO_3^- nitrogen in the absence (*a*) or presence (*b*) of 0.2 μM simazine.

activity in higher plants, and its level increases with nitrate level.[15] The enhancement of the response to simazine with higher temperature and higher light intensity is consistent with the reported effects of temperature and light on the formation of nitrate reductase.[15] However, one noteworthy contradiction to the literature was found in experiments on the effect of light on the response of rye to simazine. Plants grown in low light (150 ft-c) had higher enzyme activity per plant than plants grown at 2,000 ft-c, although the effect of simazine was most pronounced at the higher light intensity.

The accumulation of protein in response to simazine may or may not be related to its herbicidal action. Simazine and related triazines are inhibitors of the Hill reaction.[18, 19] Previously it had been assumed that this effect was the basis of herbicidal activity of these compounds. However, the effect of simazine on nitrogen metabolism raises the possibility that simazine toxicity is the result of excess production of nitrite or ammonia, both of which are known for their toxic effects on plants.

The experiments with peas demonstrate that a higher-protein food may be obtained from plants grown on extremely low levels of simazine. In developing countries, the use of simazine[20] may significantly improve food and forage crops as sources of protein.

Summary.—Simazine (2-chloro-4,6-bis ethylamino-s-triazine) applied at optimum levels causes markedly increased accumulation of protein in rye plants. The maximal increase observed was 79 per cent. Relative to untreated plants, those grown in the presence of simazine show a slight increase in respiration with the respiration quotient unchanged, and a somewhat lower dry weight, but similar fresh weight. The level of nitrate reductase activity is greatly increased. These phenomena occur only in plants grown on nitrate, not in plants grown on ammonia. The magnitude of the response decreases as the nitrate concentration approaches the optimal nutritional level. Pea plants grown to maturity in the presence of 0.05 μM sima-

zine yield the same weight of seed as untreated plants, but the seed contains 40 per cent more protein. Acrylamide gel electrophoresis has not indicated qualitative differences between proteins from treated versus untreated plants.

* Published with the approval of the Director of the Agricultural Experiment Station as journal article no. 4090. This research was supported in part by USPHS grant ES0043, and USAEC contract AT(11-1)-1338.

[1] Ries, S. K., R. P. Larsen, and A. L. Kenworthy, *Weeds*, **11**, 270 (1963).

[2] Karnatz, H., *Mitt. Obstbauversuchsringes Alten Landes*, **19**, 109 (1964).

[3] Freney, J. R., *Australian J. Agr. Res.*, **16**, 257 (1965).

[4] Ries, S. K., and A. Gast, *Weeds*, **13**, 272 (1965).

[5] Gramlich, J. V., and D. E. Davis, *Weeds*, **15**, 157 (1967).

[6] Tweedy, J. A., and S. K. Ries, *Plant Physiol.*, **42**, 280 (1967).

[7] Sell, H. M., R. W. Luecke, B. M. Taylor, and C. L. Hamner, *Plant Physiol.*, **24**, 295 (1949).

[8] Wort, D. J. in *The Physiology and Biochemistry of Herbicides*, ed. L. J. Audus (New York: Academic Press, 1964), p. 313.

[9] Hoagland, D. R., and D. I. Arnon, *Univ. Calif. Agr. Expt. Sta. Circ.* 347 (1938).

[10] Goring, C. A. I., *Soil Sci.*, **94**, 211 (1962).

[11] Sanderson, G. W., and E. C. Cocking, *Plant Physiol.*, **39**, 416 (1964).

[12] Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

[13] Davis, B. J., *Ann. N. Y. Acad. Sci.*, **121**, 404 (1964).

[14] Halevy, A. H., D. R. Dilley, and S. H. Wittwer, *Plant Physiol.*, **41**, 1085 (1966)

[15] Beevers, L., L. E. Schrader, D. Flescher, and R. H. Hageman, *Plant Physiol.*, **460**, 691 (1965).

[16] Hageman, R. H., and D. Flescher, *Plant Physiol.*, **35**, 700 (1960).

[17] Filner, P., *Biochim. et Biophys. Acta*, **118**, 299 (1966).

[18] Moreland, D. E., W. A. Genter, J. L. Hilton, and K. L. Hill, *Plant Physiol.*, **34**, 432 (1959).

[19] Good, N. E., *Plant Physiol.*, **36**, 788 (1961).

[20] Simazine has an extremely low mammalian toxicity of 5000 mg/kg in rats.