Plant Physiol. (1979)64, 273–275
0032-0889/79/64/0273/03/$00.50/0

Effects of Certain Herbicides and Their Combinations on Nitrate and Nitrite Reduction[1]

Received for publication October 20, 1978 and in revised form March 6, 1979

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ABSTRACT

A study was made concerning the effect of various herbicides, when used alone or in combination, on nitrite accumulation in excised leaves of wheat (*Triticum aestivum* L., var. 'Centurk'). Treatment of leaves with photosynthetic inhibitor herbicides, known to interfere with the transfer of light energy, caused accumulation of nitrite under illuminated, aerobic conditions. When certain other herbicides, which do not interfere with the photosynthetic process, were applied to leaves and incubated under dark, aerobic conditions, nitrite accumulations were enhanced over those treated with photosynthetic inhibitors or the controls. The combination of photosynthetic inhibitor herbicides and certain other "nonphotosynthetic inhibitor" herbicides caused relatively large amounts of nitrite to accumulate in light or in darkness. Nitrite accumulation occurs when nitrate and nitrite reduction are not in balance. The proposed actions of the herbicides used in this study are discussed. This discussion provides a rationale for the accumulation of nitrite by the herbicide-treated leaves.

Two important enzymes involved in nitrate assimilation are nitrate reductase and nitrite reductase. In leaves, these enzymes differ in cellular location, electron donor, and energy generation system. Nitrate reductase is located in the cytoplasm (16), uses NADH as its electron donor (2), and derives its source of NADH primarily from the oxidation of 3-P-glyceraldehyde (11) or malate (15). Nitrite reductase is located in the chloroplast (16), utilizes reduced ferredoxin as its electron donor (14), and derives its reductive energy from photosynthetic electron flow (14).

Nitrite reduction is inhibited by photosynthetic inhibitor herbicides (6, 8, 9, 14) with little or no immediate effect upon nitrate reduction. In contrast, certain "nonphotosynthetic inhibitor" herbicides stimulate nitrate reduction and permit it to occur in darkness with little or no immediate effect upon photosynthetic nitrite reduction (10). Thus, several chemical classes of herbicides have been shown to cause free nitrite to accumulate within treated plant tissues: the photosynthetic inhibitors by inhibiting nitrite reduction in light while nitrate reduction continues; certain other herbicides by permitting nitrate reduction to occur in the absence of photosynthetic nitrite reduction (aerobically in darkness).

I investigated the effects that combinations of herbicides exerted on nitrate and nitrite reduction, and determined whether a combination would cause nitrite accumulation, both in light and in darkness.

MATERIALS AND METHODS

Plant Culture. Leaves of wheat (7- to 10-day-old) seedlings were used as the test material. Plants were grown in growth chambers (16 h at 900 μE s^{-1} m^{-2}, 25 C day, 18 C night) in a Vermiculite base and watered daily with a nutrient solution (12).

Treatment Solutions. The aqueous treatment solutions contained 2% ethanol, 0.3% Tergitol 15-S-7 (a non-ionic linear alcohol ethoxylate surfactant), and herbicides as noted in legends of the tables and figures. Control solutions contained all ingredients except herbicides. The herbicides were first dissolved in ethanol, surfactant added, and the solution brought to proper volume with distilled, deionized H_2O.

Techniques. Leaves (distal 6–8 cm) were cut from seedlings and immediately floated on the treatment solutions (100 ml). Treatment solutions were contained in Pyrex pie plates (24-cm diameter). The leaves were floated on the solution in light (300 μE s^{-1} m^{-2}) for 20 min. They were then removed, gently blotted dry with paper toweling, counted, and weighed. Groups of five leaves (0.40–0.50 g fresh weight) were placed into separate Petri dishes (9-cm diameter). A moist, sterile cotton ball was also placed in the Petri dish to maintain humidity. Treated and control leaves were incubated in light or in darkness for periods up to 2 h. During this incubation, the Petri dish lids were lifted every 20 min to maintain aerobic conditions. At the end of incubation, the leaves were tested for nitrite content.

Assays. For nitrite analyses, extracts of the leaf tissues were prepared by using a Polytron homogenizer and 10 ml of a 4:1 ratio of water-methylene chloride. After centrifugation (10,000*g*), an aliquot of the clear, aqueous portion of the extract was analyzed for nitrite as previously described (8).

Herbicides and Chemicals Used. The following herbicides and chemicals were used: ametryn, 2-(ethylamino)-4-(isopropylamino)-6-(methylthio)-*S*-triazine; atrazine, 2-chloro-4-(ethylamino)-6-(isopropylamino)-*S*-triazine; dicamba, 3,6-dichloro-*O*-anisic acid; diuron, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DNP[2]; metribuzin, 4-amino-6-*tert*butyl-3-(methylthio)-*as*-triazin-5(4H)-one; 2,4-D. Concentrations of chemicals used in each experiment are denoted in each table and figure.

Values presented for all experimental data are the means of quadruplicate analyses. Tables contain the means ± SD. All experiments have been repeated at least three times. Results from single, typical experiments were selected for presentation.

RESULTS AND DISCUSSION

Atrazine and DNP were used alone and in combination, in light and in darkness (Table I). Control leaves, in light or dark, did not accumulate significant amounts of nitrite. Controls were included in all tests to ensure that the surfactant used would not cause nitrite accumulation.

Atrazine, a photosynthetic inhibitor herbicide, caused the accumulation of more than 100 nmol nitrite g fresh weight^{-1} in light and 67 in darkness. In all experiments, nitrite accumulations were not as high as some previously reported (6, 8, 9) because exogenous

[1] Cooperative investigations of the Nebraska Agricultural Experiment Station under Contract AID/csd-1208 and AID/cm/ta-C-73-71 with the Agency for International Development, Department of State. Published as Paper 5656, Journal Series, Nebraska Agricultural Experiment Station.

[2] Abbreviation: DNP: 2,4-dinitrophenol.

Table I. *Nitrite Accumulation in Light and Darkness by Treatments of Atrazine, DNP, and Their Combination*

Treatment[1]	Light	Dark
	nmol NO_2^- g fresh weight^{-1} (± SD)	
Control	8 ± 2	10 ± 4
Atrazine	101 ± 15	67 ± 22
DNP	11 ± 6	742 ± 52
DNP + atrazine	683 ± 86	876 ± 69

[1] Wheat leaves were treated with solutions of 9.3×10^{-4} M atrazine, 5.4×10^{-4} M DNP. The combination solution contained both. Nitrite was determined after a 2-h incubation period.

nitrate was not supplied. Only endogenous nitrate was available for reduction to nitrite. In these experiments there was no aqueous medium during incubation. Consequently, there was no efflux of nitrite from the tissue. All nitrite was retained in the leaves.

Treatment with 0.5 mM DNP alone caused large amounts of nitrite to accumulate in darkness but not when the leaves were illuminated. This result is consistent with previous research (10) which showed that DNP did not interfere with light-dependent nitrite reduction but allowed leaf tissue to reduce nitrate in darkness.

When DNP and atrazine were used in combination, nitrite accumulated to high levels in light and in darkness. In light, an apparent synergistic effect was observed inasmuch as leaves treated with the combination produced a higher level of nitrite than the sum of separate treatments. In darkness, there was little difference between DNP alone and DNP plus atrazine treatment.

The DNP-dark treatment appears to have stimulated nitrate reduction to occur at a faster rate than the control rate. It is assumed that this increased rate of nitrate reduction occurred also in the DNP-light treatment. However, nitrite did not accumulate because nitrite reduction was not inhibited in the light.

Ametryn, another photosynthetic inhibitor herbicide, was used alone and in combination with 2,4-D (Table II). 2,4-D has been shown to permit nitrate reduction in darkness (10). Data similar to that presented in Table I were obtained. Ametryn alone was more effective in light than in darkness. Analyses revealed that a small amount of nitrite present in ametryn-treated leaves (Table II) and atrazine-treated leaves (Table I) was accumulated during the 20-min herbicide pretreatment or during preparation prior to the incubation (data not shown).

Leaves treated with 2,4-D only accumulated 65 nmol NO_2^- g fresh weight^{-1} under light. In darkness, 2,4-D permitted nitrite to accumulate to a degree similar to that of DNP (Table I). The combination of 2,4-D and ametryn allowed nitrite accumulation in light and in darkness. It required approximately twice the concentration of 2,4-D to that of DNP to permit nearly equivalent production of nitrite.

Several researchers have reported synergistic responses of herbicidal action when these same or similar herbicides (Tables I and II) were used in combination. Colby *et al.* (4) reported increased herbicide toxicity with combinations of DNBP (2-*sec*-butyl-4,6-dinitrophenol) or DNP with simetone (2,4-*bis* (ethylamino)-6-(methoxy-*S*-triazine). They concluded that the nitrophenols interfered with respiratory phosphorylation and that simetone (a photosynthetic inhibitor herbicide) interfered with photosynthetic phosphorylation. Diem and Davis (5) found that low levels of 2,4-D increased the toxicity of ametryn. Data from Tables I and II could help explain the increased toxicity noted by these researchers because these combinations of herbicides caused excessive nitrite accumulations in light and in darkness.

Wheat leaves treated with diuron and 2,4-D and their combination were incubated under light to determine the effects of these treatments on nitrite accumulation (Fig. 1). Like the triazines, atrazine and ametryn, the substituted urea, diuron, is a potent photosynthetic inhibitor herbicide. The control and 2,4-D-alone-treated leaves accumulated only traces of nitrite. After an initial lag phase the leaves treated with diuron alone accumulated nitrite at a nearly constant rate. After 120 min, more than 400 nmol g fresh weight^{-1} had accumulated. The combination of 2,4-D plus diuron evoked a synergistic response in respect to nitrite accumulation. Leaves treated with both herbicides accumulated 918 nmol nitrite g fresh weight^{-1} during 2 h of incubation. Again, 2,4-D was apparently stimulating nitrate reduction but the effect was not noted unless a photosynthetic inhibitor was present to inhibit photosynthetic electron flow necessary for nitrite reduction.

An observation noted in several experiments using photosynthetic inhibitors alone is illustrated in Figure 2. Leaves treated with metribuzin and dicamba alone and in combination were incubated under light. The photosynthetic inhibitor (in this case, metribuzin) did not cause nitrite accumulation when used alone as was demonstrated by the photosynthetic inhibitors in Figure 1 and Tables I and II. Although metribuzin and many other photosynthetic inhibitors are capable of inhibiting the reduction of nitrite, nitrite cannot be accumulated unless the leaf tissue is

Table II. *Nitrite Accumulation in Light and Darkness by Treatments of Ametryn, 2,4-D, and Their Combination*

Treatment[1]	Light	Dark
	nmol NO_2^- g fresh weight^{-1} (± SD)	
Control	8 ± 3	12 ± 1
Ametryn	276 ± 27	152 ± 46
2,4-D	65 ± 10	688 ± 104
2,4-D + ametryn	726 ± 59	849 ± 58

[1] Wheat leaves were treated with solutions of 8.8×10^{-4} M ametryn and 9×10^{-4} M 2,4-D. The combination solution contained both. Nitrite was determined after a 2-h incubation period.

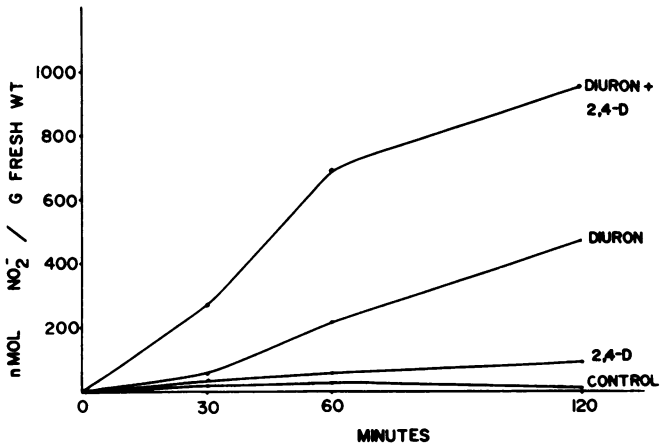

FIG. 1. Accumulation of nitrite in wheat leaves in light as influenced by treatment with 2,4-D, diuron, or both combined. Treatment solutions contained 9×10^{-4} M 2,4-D and 8.6×10^{-4} M diuron.

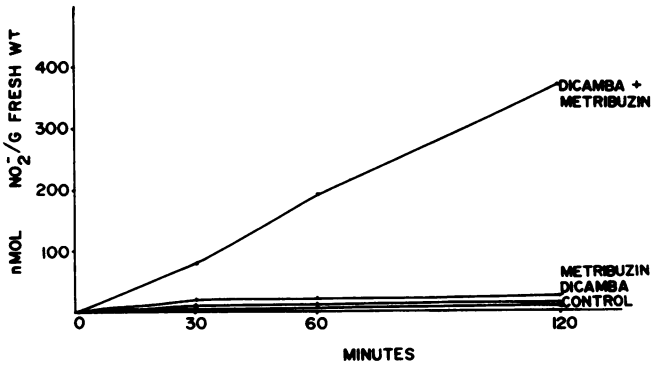

FIG. 2. Accumulation of nitrite in illuminated wheat leaves as influenced by treatment with dicamba and metribuzin or both combined. Treatment solutions contained 9×10^{-4} M dicamba and 9.3×10^{-4} M metribuzin.

reducing nitrate. It is postulated that in this experiment nitrite accumulation did not follow treatment with metribuzin alone because: (*a*) the leaf tissue did not reduce nitrate during the 2-h incubation period; or (*b*) the leaf tissue slowly reduced nitrate but metribuzin had not inhibited all sites of reduction within the chloroplast. Because nitrite reductase is normally much higher in activity than nitrate reductase (2), a partially inhibited system of nitrite reduction could keep pace with low rates of nitrate reduction.

Dicamba alone, which acts similarly to 2,4-D and DNP, permitted nitrate reduction to occur (Fig. 2) but nitrite accumulation was not observed under light due to the occurrence of photosynthetic nitrite reduction. Leaves treated with the combination of dicamba and metribuzin accumulated more than 350 nmol NO_2^- g fresh weight^{-1} during the 120-min incubation. Dicamba caused nitrate reduction to occur and metribuzin blocked further reduction of nitrite.

In wheat leaves, nitrate reduction is accompanied by NADH oxidation. The NADH is provided by carbohydrate oxidation (11). Nitrite reduction is accompanied by ferredoxin oxidation. Light via the photosynthetic electron pathway provides energy for ferredoxin reduction. The level of nitrite present in plants is dependent upon the balance between rates of nitrate reduction and nitrite reduction. Since nitrite reductase activity is normally much higher than nitrate reductase activity, nitrite should not accumulate in light. Leaves, under dark, aerobic conditions do not reduce nitrate (3). Thus, nitrite does not accumulate in normal, healthy plant tissues in light or in darkness (7, 8).

Photosynthetic inhibitor herbicides block the transfer of energy from light to nitrite reductase without immediately affecting nitrate reduction. Thus, nitrite accumulated if nitrate reduction occurred or continued. Certain other chemicals and herbicides such as 2,4-D, DNP, dicamba, and others (10) do not block light-dependent nitrite reduction. They appear to stimulate nitrate reduction. This stimulation was not detected unless it occurred in the absence of nitrite reduction (illumination but with the presence of a photosynthetic inhibitor or in darkness). The combination of herbicides (a stimulator plus a photosynthetic inhibitor) produced nitrite accumulation in light and in darkness.

This stimulation of nitrate reduction is not well understood. It is thought that nitrate reductase *per se* is not affected. Rather, the herbicides exert an effect on other metabolism which indirectly affects the process of nitrate reduction. As previously reported (10), to obtain this type of stimulation, aerobic incubation must be maintained. The stimulation of nitrate reduction by anaerobioses (such as the *in vivo* assay) tends to mask the herbicide stimulation. It is tempting to theorize that these herbicides are interfering with normal aerobic oxidation of NADH by the Cyt system or are causing an acceleration of glycolysis. It was recently reported that 2,4-D specifically inhibited NADH dehydrogenase on the outer membrane of plant mitochondria (13). Such an inhibition could furnish additional NADH for nitrate reduction. Respiration and oxidative phosphorylation studies with several of these herbicides have yielded mixed results (1) so that no clear answer is presently available.

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