Inhibition of Nitrite Reduction by Photosynthetic Inhibitors[1]

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Abstract. Using a modified in vivo nitrate reductase assay, all photosynthetic inhibitors tested were shown to effectively block light-dependent nitrite reduction in green leaf tissue. Nitrate reduction continued so that nitrite accumulated. Nitrite, vacuum infiltrated into leaf tissue, disappeared linearly with time when incubated in the light. Nitrite, vacuum infiltrated with a photosynthetic inhibitor, did not disappear but accumulated to even higher levels. The degree of inhibition of nitrite reduction was a function of herbicide concentration and time of incubation.

INTRODUCTION

It has been shown recently by a modified in vivo nitrate reductase assay (2) that photosynthetic inhibitor herbicides interfere in the reduction of nitrite by green leaf tissue. Photosynthetic inhibitor herbicides were known to block electron flow within the chloroplast (4, 5), but interference with the normal process of nitrite reduction had not been reported. Nitrite reductase, located within the chloroplast (8), is directly dependent upon reduced ferredoxin as its electron donor (1). Hence, blockage of electron flow to ferredoxin within the chloroplast prevents nitrite reduction. Nitrate reductase, located outside the chloroplast (8), is not directly dependent upon photosynthetic energy (3). Nitrate reduction can continue after nitrite reduction is inhibited which results in nitrite accumulation (2). It has been postulated that nitrite is a secondary phytotoxic agent responsible for initial injury and final death of the plant after herbicide treatment (2).

The objectives of this study were to further verify and improve the basic technique as first reported (2) for adaptation as a rapid screening assay for photosynthetic inhibitor herbicides.

MATERIALS and METHODS

Reaction solution. The aqueous reaction solution contained 0.05 M KNO_3, 0.05 M KH_2PO_4, 0.3% Tergitol 15–S-7 surfactant (a non-ionic linear alcohol ethoxylate[3]) and 4% ethanol (v/v). Final pH was adjusted to 7.5 with 3 N KOH. When herbicides were included in the reaction solution, they were first dissolved in absolute ethanol and brought to proper volume with the reaction solution minus ethanol. Final concentration of ethanol in all solutions was 4%.

Plant tissue and culture. Green leaf tissue of winter wheat (*Triticum aestivum* L., 'Centurk') was used in all experiments. Plants were grown in growth chambers (16 hr at 25 klux, 25 C day, 18 C night) in vermiculite and watered daily with a modified Hoagland's nutrient solution (3). Leaf tissue was harvested 7 to 10 days after planting.

Basic technique. Leaf samples were cut (1-cm sections), weighed (0.25 g), and placed in 50-ml beakers containing 5 ml of the reaction solution. Vacuum infiltration of the tissue was done in the beakers within a vacuum desiccator using a rotary vacuum oil pump. A vacuum was applied (72 cm Hg) and released twice. The beakers and their contents were incubated in shaking water baths at 33 C in dark or in light (15 klux). Aliquots (normally 0.2 ml) of the reaction solutions were taken at timed intervals and analyzed for nitrite as previously described (2).

Herbicides used. The following technical grade herbicides were used during the course of this study: ametryn [2-(ethylamino)-4-(isopropylamino)-6-(methylthio)-*s*-triazine]; atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine]; bentazon [3-isopropyl-1*H*-2,1,3-benzothiadiazin-(4)3*H*-one 2,2-dioxide]; bromacil (5-bromo-3-*sec*-butyl-6-methyluracil); bromoxynil (3,5-dibromo-4-hydroxybenzonitrile); dicryl (3′,4′-dichloro-2-methylacrylanilide); metribuzin [4-amino-6-*tert*-butyl-3-(methylthio)-*as*-triazin-5(4*H*)one]; monuron [3-(*p*-chlorophenyl)-1,1-dimethylurea]; norea [3-(hexahydro-4,7-methanoindan-5-yl)-1,1-dimethylurea]; prometon [2,4-bis(isopropylamino)-6-methoxy-*s*-triazine]; prometryn [2,4-bis(isopropylamino)-6-(methylthio)-*s*-triazine]; propazine [2-chloro-4,6-bis(isopropylamino)-*s*-triazine]; simazine [2-chloro-4,6-bis(ethylamino)-*s*-triazine]; solan (3′-chloro-2-methyl-*p*-valerotoluidide). Fourteen photosynthetic inhibitor herbicides, representing six distinct chemical classes were tested.

Values presented for all experimental data represent the means of duplicate analyses and all experiments have been repeated at least twice. The coefficient of variation for this in vivo assay with wheat leaf tissue commonly varies between 8 and 12.

RESULTS and DISCUSSION

Inhibition of nitrite reduction. All herbicides tested inhibited light-dependent nitrite reduction in green, wheat leaf tissue within an hour (Table 1). Percent inhibition was calculated as the amount of nitrite accumulated with light incubation with a herbicide as compared to that accumulated in the normal in vivo nitrate reductase assay (dark incubation without a herbicide). All reaction solutions contained herbicide at the concentration of 10^{-4} M and did not decrease rates of nitrate reduction (data not shown).

The light control (without herbicide) did not accumulate

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[3] Manufactured by Union Carbide Corp.

nitrite since nitrite is normally further reduced in the light (1, 9). The dark control, with photosynthetic electron flow prevented by the darkness, permitted nitrate reduction to continue thereby allowing nitrite to accumulate. Bromoxynil was the least effective treatment. Other herbicides, such as bromacil and dicryl, appeared to inhibit nitrite reduction by more than 100%. It is not known whether these increased accumulations of nitrite were due to increased nitrate reduction or due to a more effective blockage of nitrite reduction than that of dark incubation.

The technique used to obtain the data as shown in Table 1 appears to be sufficiently effective to screen rapidly and accurately for inhibitors of photosynthetic electron flow. Similar results were obtained using the same technique but without solubilization of the herbicide in ethanol and with a different surfactant in the reaction solution (2). However, results were more variable and higher herbicide concentrations were required for approximately the same amount of inhibition. Pyne et al. (7) have successfully used *n*-propanol for solubilization of the herbicide to be tested. This portion of the technique does not appear to offer any measure of herbicide specificity but will permit identification of herbicidal activity.

Table 1. Inhibition of nitrite reduction in wheat leaves within 1 hr by photosynthetic inhibitor herbicides. (In vivo assay)

Treatment[a]		Nitrite accumulated (μmoles NO_2^-/g fresh wt/hr)	Inhibition (%)
Control	Light	0	0
Ametryn	Light	3.9	100
Atrazine	Light	3.9	100
Bentazon	Light	3.1	79
Bromacil	Light	4.5	115
Bromoxynil	Light	1.4	36
Dicryl	Light	4.6	118
Metribuzin	Light	4.2	108
Monuron	Light	3.9	100
Norea	Light	4.1	105
Prometon	Light	3.3	85
Prometryn	Light	3.3	85
Propazine	Light	2.4	62
Simazine	Light	2.6	67
Solan	Light	4.1	105
Control	Dark	3.9	100
LSD (0.05)[b]			30

[a]10^{-4} M concentration of all herbicides were used.
[b]Calculated using only treatment values. Neither control was included in the statistical analysis.

Table 2. Entry of herbicides and partial inhibition of nitrite reduction by floating wheat leaf tissue.[a]

Treatment		% Inhibition[b]	
		Wo/ETOH	W/ETOH
Control	Light	0	0
Ametryn	Light	14	24
Bentazon	Light	0	5
Bromacil	Light	7	18
Bromoxynil	Light	0	0
Metribuzin	Light	7	18
Norea	Light	0	8
Solan	Light	0	9

[a]10^{-4} M concentration of herbicides were used. Tests were conducted for 1 hr.
[b]Wo = without; W = with.

Floating leaf experiments. Wheat leaf sections were floated for 1 hr (no vacuum infiltration) on solutions of selected herbicides (10^{-4} M) to compare the inhibition of nitrite reduction with that obtained by vacuum infiltration. Also, the effect of solubilization of the herbicide with ethanol was tested (Table 2). Percent inhibition was calculated in the same manner as previously explained.

When the herbicides were first dissolved in ethanol, all herbicides except bromoxynil, penetrated the tissue in sufficient quantities to partially block nitrite reduction. Without ethanol, only ametryn, bromacil, and metribuzin inhibited nitrite reduction.

Although this floating technique is not suitable as a method for detection of herbicidal activity, it can possibly provide information on the ability of a herbicide to penetrate the foliage of a given plant species. Improper techniques could result in failure to identify a chemical as a potential herbicide. Examples of faulty techniques would be the elimination of vacuum infiltration, the use of ineffective surfactants (2), use of plant tissue low in nitrate reductase activity and poor solubility of the herbicide in the reaction solution. Pyne et al. (7) demonstrated a good correlation between nitrite accumulation and inhibition of the Hill reaction and pointed out the advantage of time saved using this assay with tissue sections rather than the necessary isolation of chloroplasts for the Hill reaction assay.

Vacuum infiltration of nitrite. Several investigators (3, 9) have shown that nitrite, vacuum infiltrated into green leaf tissue and incubated in the light is further reduced and disappears rapidly. This experiment was designed to determine whether this disappearance of nitrite would occur if a photosynthetic inhibitor was present (Figure 1). Nitrate was eliminated from all reaction solutions so that only endogenous nitrate would be available for reduction. Wheat leaf sections were vacuum infiltrated with various reaction solutions and all incubations were in the light.

The control (no atrazine) accumulated no nitrite. When only atrazine was added, nitrite accumulated. Nitrite alone decreased linearly throughout the incubation period. However, when nitrite and atrazine were in the reaction solution, nitrite not only did not disappear but increased to even higher levels. This provides further evidence that in the presence of a photosynthetic inhibitor, nitrate reduction can continue while further reduction of nitrite is blocked. This blockage of light-dependent nitrite reduction by diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] has also been demonstrated in vitro with isolated chloroplasts (6).

Concentration of herbicide and time of incubation. According to an older technique (2), vacuum infiltration of photosyn-

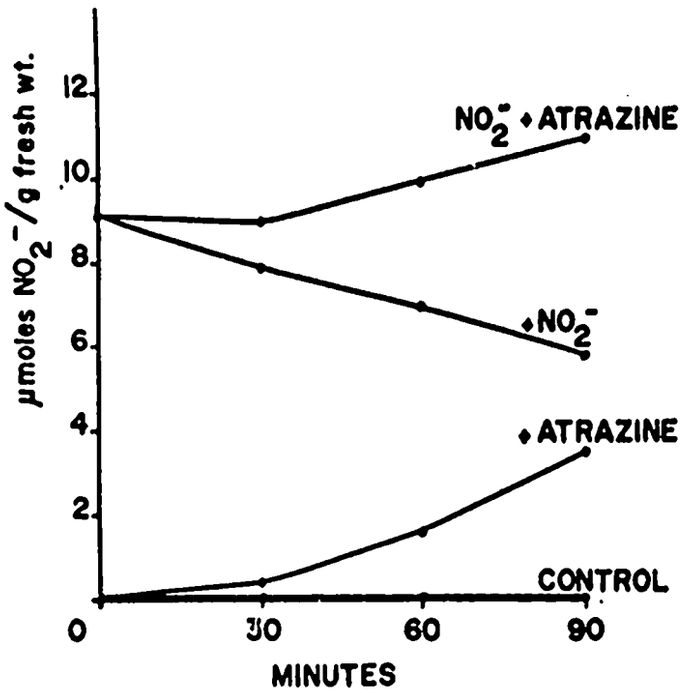

Figure 1. The accumulation or disappearance of nitrite dependent upon the presence of atrazine. All incubations were in the light and 10^{-6} M atrazine was vacuum infiltrated into the green leaf tissue

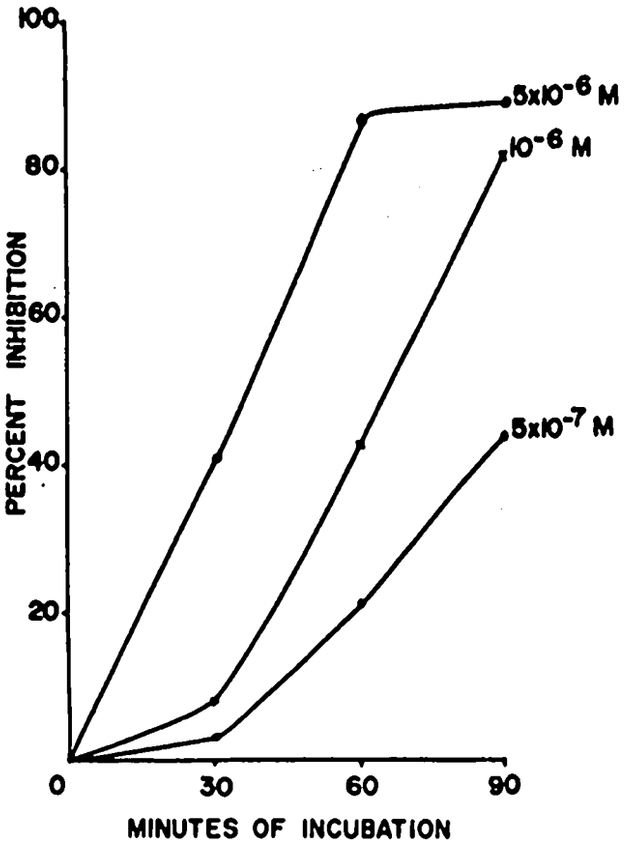

Figure 2. The inhibition of nitrite reduction dependent upon the time of incubation and ametryn concentration.

thetic inhibitors into plant tissues caused instant inhibition of nitrite reduction. This is true when high concentrations of herbicides are present in the reaction solutions. However, with more dilute concentrations (1×10^{-5} to 1×10^{-7} M), the amount of inhibition was increased with increased incubation times (Figure 2).

Three concentrations of ametryn were used and percent inhibition determined at 30, 60, and 90 min. At 30 min, inhibition by 5×10^{-7} M ametryn was barely detectable. At 60 min, the same concentration gave 20% inhibition and more than 40% inhibition after 90 min of incubation. The 10^{-6} M concentration gave approximately 10%, 40%, and 80% inhibitions during the same respective times of incubation.

From this experiment it is concluded that vacuum infiltration carries the herbicide rapidly into the leaf sections. However, with dilute concentrations of herbicides, more time is apparently required for further penetration by the herbicide into the chloroplast and for binding at the proper site.

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