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Sources of Ammonium in Oat Leaves Treated with Tabtoxin or Methionine Sulfoximine[1]

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ABSTRACT

Excised 7-day-old oat (*Avena sativa* L. cv. Jaycee) leaves were incubated in media containing 7.1 millimolar KNO_3 and 0.15 millimolar tabtoxin or 1 millimolar methionine sulfoximine (MSO) to investigate the sources of the observed ammonium accumulated. Tabtoxin and MSO are known inhibitors of glutamine synthetase, the first enzyme in the primary pathway of ammonium assimilation. During a 4- to 6-hour incubation, there was little net change in protein or total amino acid concentration. Alanine, aspartate/asparagine, and glutamate/glutamine decreased markedly under these treatments, whereas several other amino acids increased. Exogenous ^{15}N from $K^{15}NO_3$ was taken up and incorporated into the nitrate and ammonium fractions of leaves treated with tabtoxin or MSO. This result and the high *in vitro* activities of nitrate reductase indicated that reduction of nitrate was one source of the accumulated ammonium. Leaves incubated under 2% O_2 to reduce photorespiration accumulated only about 13% as much ammonium as did those under normal atmospheres. We conclude that most of the tabtoxin- or MSO-induced ammonium came from photorespiration, and the remainder was from nitrate reduction.

Tabtoxin, produced by certain phytopathogenic pathovars of *Pseudomonas syringae*, leads to the chlorotic halo that characteristically surrounds the infection site. In the infected plant, tabtoxin is hydrolyzed to yield its biologically active form, tabtoxinine-β-lactam (12) [2-amino-4-(3-hydroxy-2-oxo-azacyclobutan-3-yl)-butanoic acid]; this compound is an inhibitor of GS[3]. It has been postulated that GS inhibition leads to the accumulation of ammonium and that it is the ammonium that causes the chlorosis (25). Several findings support this proposal. First, ammonium *per se* is known to uncouple photophosphorylation (17), disrupt chloroplast ultrastructure, and cause chlorosis. Second, ammonium accumulation and chlorosis are both light-dependent reactions (11) and can be reversed by the application of DCMU. Third, MSO, a known inhibitor of GS, acts similarly to tabtoxinine-β-lactam (34).

There are three major potential sources for the ammonium: nitrogen assimilation; photorespiration; and protein hydrolysis followed by degradation of amino acids. Both of the first two sources involve GS; this enzyme is the first of two enzymes in the so-called GS-GOGAT cycle, which is considered to be the primary pathway for nitrogen assimilation in higher plants (8, 13, 18, 32). It also is reported (15, 16, 33) to play a role in photorespiration by recycling the ammonium released during the conversion of glycine to serine. At the flux rates calculated for this cycle in C_3 plants, considerable ammonium could be accumulated if it were not being reassimilated. Other nitrogen-containing compounds are probably not important sources, considering the rapidity and amounts of ammonium generated.

Knowing the source of the accumulated ammonium could contribute to our understanding of both the mechanism of action of tabtoxin and the pathways of nitrogen metabolism in plants. Accordingly, we have studied the contributions that these three sources make to the ammonium pool in oat leaves treated with either tabtoxin or MSO. We also examined the effects of these compounds on alternate pathways of ammonium utilization, particularly GDH because of the long-standing idea that it may be important in assimilation, especially at high levels of ammonium (1). Primary events were emphasized by limiting the experiments to short time periods.

MATERIALS AND METHODS

Plant Material. Oats (*Avena sativa* L. cv. Jaycee), susceptible to *Pseudomonas syringae* pv. *coronafaciens*, incitant of bacterial halo blight, were grown for 7 d in vermiculite in a controlled environment chamber with a 16-h photoperiod (500 μE m^{-2} s^{-1}; 400 to 700 nm) and 21°C/17°C day/night temperature. Plants were irrigated every 8 h with one-fourth strength Peterson and Schrader's (23) modified Hoagland solution.

Experimental Protocol. First leaves of 7-d-old seedlings were excised under water, and the cut ends were immersed in 7 ml aqueous media contained in a small circular well within a Petri dish. Covers with 24 4-mm diameter holes around the perimeter held the excised leaves at an angle of 15° from the incident light. The excised leaves were incubated in the same environmental chamber in which the seedlings were grown, except for the controlled atmosphere experiments.

The concentration of nitrate in the media used by other investigators has varied from 3 mM to 21 mM. We found that ammonium accumulation at 4 h increased slighty with increasing medium nitrate concentration. Although 34 μmol ammonium/g fresh weight accumulated using 21 mM medium nitrate compared to 28 μmol ammonium/g fresh weight using 7.1 mM, we used the latter concentration, inasmuch as it was nearer that used to grow the

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[3] Abbreviations: GS, glutamine synthetase; MSO, methionine sulfoximine; GOGAT, glutamate synthase; GDH, glutamate dehydrogenase; Asx, aspartate/asparagine; Glx, glutamate/glutamine; GOT, glutamate oxaloacetate transaminase; RuBPCase, ribulose 1,5-biphosphate carboxylase.

plants. Also, this value has been frequently used by others. Tabtoxin and MSO were added at 0.15 mM and 1 mM, respectively.

Transpiration and nitrate uptake occurred in our system, as indicated by a 4-ml decrease in medium volume and a 25-μmol decrease in medium nitrate after 4-h incubation. The decreases were the same for controls and treated leaves. A recent report (24) also presented evidence that MSO treatment of excised leaves did not affect stomatal resistance. It is possible that excision has reduced stomatal aperture (and consequently transpiration and photosynthetic rate); however, it did not close them completely, as shown by the present work and work by others (24).

Extraction and Assays. For determination of ammonium, nitrate, and amino acids, 0.15 to 2.5 g of leaves were minced and homogenized in 10 ml methanol:chloroform:water (12:5:3, v/v/v) (2). Three distinct, nonturbid layers were produced by the addition of 3 ml H_2O per 10 ml homogenate followed by centrifugation (3,000*g* for 10 min). The layers—upper layer of methanol-water containing the polar solutes; middle layer containing protein and cellular debris; and bottom chloroform layer containing Chl—were separated by aspiration. This separation resulted in a complete removal of Chl from the ammonium, nitrate, and amino acids, and they were not detected in the chloroform layer. Aliquots of the methanol-water phase were taken for assays. Ammonium was determined by the colorimetric method of Cataldo *et al.* (5), modified to increase the sensitivity of the assay. It was determined that 0.15 ml 1 N NaOH was consistently required for pH adjustment, so indicator dye was not used. Also, the reaction mixtures were not diluted after adding reagents C and D (5). A standard curve was linear between 0 and 0.15 mM ammonium. Nitrate was assayed by the Cataldo *et al.* method (4). Total amino acid concentration was measured by the method of Moore (20).

Protein was extracted with 0.1 N NaOH from the middle layer for ^{15}N analysis and protein quantitation. Whole-leaf tissue, when extracted with 0.1 N NaOH for protein quantitation, gave values equal to those found in the middle layer. Thus, results of protein values from both extraction methods were pooled. Protein concentration of these extracts was determined by the Lowry *et al.* procedure (19), with BSA as a standard.

Samples were extracted for nitrate reductase activity by the method of Schrader *et al.* (29) and assayed as described by Scholl *et al.* (28). GS, GDH, and GOT were extracted and assayed as described by Duke *et al.* (9).

^{15}N Analysis. Following incubation with $K^{15}NO_3$ (95 atom % excess), leaves were extracted as described above (2). The ammonium fraction was obtained by steam distillation of the upper layer (3). Nitrate was reduced to ammonium with Devarda's alloy (3), and this was distilled off as a second fraction. The solution remaining after distillation was centrifuged, and the supernatant was reduced in volume on a rotary evaporator. An equal volume of 20% (w/v) TCA was added to precipitate any residual protein. The amino acids in the supernatant were digested with H_2SO_4, and the ammonium generated was distilled off as a third fraction. Protein, extracted from the middle layer as described above, was precipitated with an equal volume of 20% (w/v) TCA, digested, and steam-distilled as a fourth fraction. The ammonium from these four fractions was converted to N_2 by the hypobromite method (27) and analyzed for ^{15}N on a mass spectrometer.

Controlled Atmosphere. Leaves were incubated in controlled atmosphere to compare ammonium accumulation with and without photorespiration. Petri dishes were placed in a 6.7-L acrylic plastic chamber containing inlet and outlet ports and a recirculation system. Gas supplied from cylinders of CO_2-free air, 5% CO_2 in air, O_2, and N_2 was metered through microneedle valves to achieve the desired composition. The mixed gases, humidified by bubbling through H_2O, flowed at 2 l/min. O_2 and CO_2 concentrations were continuously monitored at the inlet and outlet ports with an O_2 analyzer and an IR gas analyzer. CO_2 concentration was maintained at 300 μl/l (inlet), and O_2 concentration was either 21% or 2% (v/v). The chamber was maintained at 24°C with a copper cooling coil around the inside perimeter. Light, supplied by a 400-w Lucalox high-pressure sodium lamp (General Electric)[4], was filtered through 4 cm H_2O; it provided 500 μE m^{-2} s^{-1} (400 to 700 nm) at the base of the chamber.

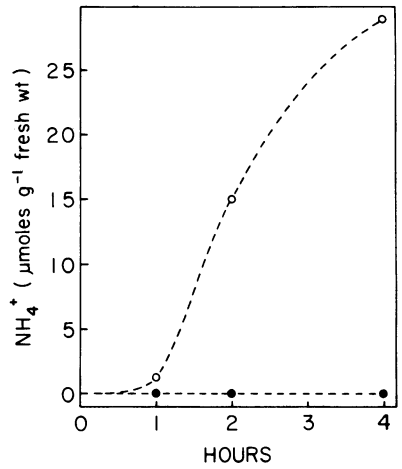

FIG. 1. Ammonium accumulation with time in oat leaves incubated in 7.1 mM KNO_3 with (○) or without (●) 1 mM MSO.

Table I. *Enzyme Activities of Oat Leaves Incubated with Tabtoxin*

Oat leaves were incubated with 7.1 mM KNO_3 ± 0.15 mM tabtoxin. Data represent one experiment consisting of one control Petri dish and two Petri dishes containing tabtoxin. Duplicate samples of six leaves each were taken from each Petri dish at sampling times. Thus, $n = 2$ for the controls and $n = 4$ for the tabtoxin treatments.

Treatment	Time	GS	GDH	GOT
	h	*μmol γ-glutamylhydroxamate min^{-1} g^{-1} fresh wt*	*μmol NADH oxidized min^{-1} g^{-1} fresh wt*	*μmol NADH oxidized min^{-1} fresh wt*
Control	0	0.95 ± 0.06	17.6 ± 8.9	3.07 ± 0.07
Control	4	0.89 ± 0.00	5.0 ± 3.8	2.93 ± 0.08
Tabtoxin	4	0.00	7.3 ± 3.7	2.67 ± 0.12
Control	19	1.06 ± 0.06	27.8 ± 1.2	3.09 ± 0.21
Tabtoxin	19	0.00	71.7 ± 14.9	2.68 ± 0.43

Tabtoxin Preparation. Tabtoxin was prepared from pv. *coronafaciens* (Pc-27) by the method of Ribeiro *et al.* (26). The bacteria were grown with agitation in Woolley's medium (35) at 22°C for 3 to 4 d until the medium pH rose to 7.4. Cells were removed by centrifugation, and the tabtoxin was purified from the supernatant fraction by ion-exchange chromatography on an Amberlite CG-120 column followed by gel filtration on a Sephadex LH-20 column. Fractions (10 ml) of the LH-20 eluate were bioassayed on tobacco leaves (31). Active fractions were pooled, analyzed for purity on an amino acid analyzer, and quantified relative to glycine.

RESULTS AND DISCUSSION

Excised oat leaves incubated with KNO_3 and MSO accumulated ammonium, whereas leaves in only KNO_3 did not (Fig. 1). Tabtoxin effects were similar (13.1 μmol/g fresh weight at 2 h and

[4] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

21.8 μmol/g fresh weight at 4 h) and were consistent with previous reports of ammonium accumulation after toxin treatment (21, 30).

GS activity was absent in extracts of leaves incubated for 4 h with tabtoxin (Table I). The known inhibition of GS by tabtoxin *in vitro* (30) and the tabtoxin-induced ammonium accumulation suggest *in vivo* inhibition of GS. GDH activity in leaf extracts from control plants increased slightly with time (Table I); the greater increase observed in tabtoxin-treated leaves may be an ammonium detoxification response (1, 14). However, these increases were not sufficient to assimilate the quantities of ammonium generated (*e.g.* at 4 h, 0.5 μmol NH_4^+/h could potentially be assimilated by GDH compared to the 6 μmol NH_4^+/h being generated).

The activity of GOT (Table I), an enzyme representative of constitutive enzymes and involved in the photorespiratory cycle, and that of NR (data shown later), an inducible enzyme, were unaffected by tabtoxin. Durbin (10) has reported that the activities of other major enzymes also were unaffected by tabtoxin *in vitro*. These data support the suggestion that tabtoxin specifically affects GS.

The concentration of total amino acids at 0 h, 4 μmol/g fresh weight, did not change significantly during th 4-h treatment with tabtoxin or MSO. Protein concentration remained constant at about 9 mg/g fresh weight. Inasmuch as ammonium accumulation occurred without a quantitative change in amino acids or protein, we conclude that these cannot be significant sources of ammonium

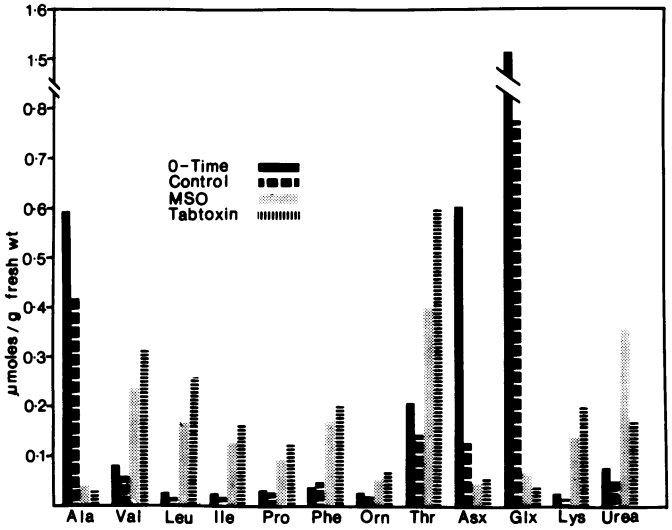

FIG. 2. Amino acid concentrations in leaves incubated for 6 h in 7.1 mM KNO_3 (control) and with either 1 mM MSO or 0.15 mM tabtoxin, as compared to 0-time concentrations. Amino acids not shown did not change significantly.

Table II. *Distribution of ^{15}N in Oat Leaves Incubated with $K^{15}NO_3$ and Tabtoxin or MSO for 6 Hours*

Leaves were incubated for 6 h in 95 atom % excess $K^{15}NO_3$ with 0.15 mM tabtoxin or 1 mM MSO, then homogenized in methanol-chloroform-water (2) (6 leaves/sample). The N-containing fractions were separated, as described in the text, converted to N_2 by the hypobromite method (27), and analyzed for atom % excess ^{15}N by mass spectrometry.

Treatment	NO_3^-	NH_4^+	Amino Acids	Protein
	atom % excess			
Tabtoxin	3.12 ± 0.35[a]	6.08 ± 0.42[a]	0.22 ± 0.03[b]	0.02 ± 0.003[b]
MSO	4.30 ± 0.17[b]	7.69 ± 0.98[b]		

[a] SE, $n = 8$.
[b] SE, $n = 3$.

Table III. *Effect of O_2 on Ammonium Accumulation and NRA in Oat Leaves Incubated with Tabtoxin or MSO*

Leaves were incubated in an acrylic plastic chamber where CO_2 was maintained at 300 μl/l and O_2 was either 2% or 21%. Each experiment at a given O_2 concentration consisted of one Petri dish each for a control, 1 mM MSO, and 0.15 mM tabtoxin treatment. Three experiments were done for each O_2 concentration.

Treatment	Time	NH_4^+		NRA	
		2% O_2	21% O_2	2% O_2	21% O_2
	h	*μmol g^{-1} fresh wt*		*μmol NO_2 g^{-1} fresh wt h^{-1}*	
Control	0	ND[a]	ND	13.8 ± 1.8	12.5 ± 1.3
Control	2	ND	ND	11.6 ± 1.3	11.4 ± 1.5
Control	4	ND	ND	11.0 ± 1.1	10.7 ± 1.3
Tabtoxin	2	1.7 ± 0.6[b]	12.6 ± 1.4	13.7 ± 1.7	11.7 ± 2.1
Tabtoxin	4	2.2 ± 0.2	20.7 ± 0.6	11.4 ± 1.2	10.9 ± 1.6
MSO	2	0.3 ± 0.2	4.9 ± 0.3	16.9 ± 3.4	12.6 ± 1.8
MSO	4	2.9 ± 0.4	12.7 ± 0.4	10.4 ± 0.6	10.5 ± 1.4

[a] None detectable.
[b] SE, $n = 3$.

at this early stage. Decreases in protein concentration and corresponding increases in amino acid concentration in lesions on tobacco leaves, reported earlier (10, 22), were measured 24 to 48 h after tabtoxin application, whereas our results reflect the metabolic changes which occur early after tabtoxin application.

The concentrations of individual amino acids and urea varied significantly, despite a lack of change in total amino acid concentration. Large decreases were observed for Ala, Asx, and Glx, whereas increases were noted for Val, Leu, Ile, Pro, Phe, Orn, Thr, Lys, and urea (Fig. 2). The Glx decrease of 94 and 98% in 6 h from the 0-h value for MSO and tabtoxin treatments, respectively, would be expected to result from GS inhibition. A 50% decrease, however was also noted in controls. The increases in ornithine and urea, coupled with the decrease in Asx, also suggest that increased activity of the ornithine cycle may be induced as a possible ammonium detoxifying mechanism (14). The changes observed in amino acid patterns were generally similar to the changes noted in chlorotic tissue of infected oat leaves (22).

When $K^{15}NO_3$ was used in 6-h incubations, nitrate pools of leaf extracts were enriched to 3.12 and 4.30 atom % excess ^{15}N for tabtoxin and MSO treatments, respectively (Table II). Inasmuch as the medium was 95 atom % excess in ^{15}N, these data indicate a 22- to 30-fold dilution by endogenous nitrate pools. The ammonium fraction was enriched about twice as much as was the nitrate fraction, indicating that exogenous nitrate was being preferentially reduced. Neither tabtoxin nor MSO inhibited nitrate reductase (Table III). Since NRA was not inhibited and enrichment of the ammonium fraction was observed, these experiments conclusively demonstrate that reduced nitrate contributes to the accumulated ammonium. However, the fact that enrichment of the ammonium fraction was considerably less than the 95 atom % excess of the medium ^{15}N can be interpreted in several ways. First, nitrate reductase acts on both exogenous nitrate (95 atom % excess) and endogenous nitrate (0 atom % excess) in a proportion that yields 6 to 8 atom % excess ammonium, and this is the sole source of the accumulated ammonium. Second, nitrate reductase acts only on exogenous nitrate, but ammonium also accumulated from internal sources, which results in a combined ammonium pool of 6 to 8 atom % excess. Third, both of the above occur. These possibilities will be discussed following evidence that photorespiration also contributes to the ammonium pool. The very low level enrichment of the amino acids and protein fractions (Table II) reflects the blocked assimilation of ammonium by GS inhibition. The organic N fraction may have been slightly enriched

by GDH or perhaps by GS before it was completely inhibited.

The ammonium generated by the glycine-to-serine conversion of photorespiration is believed to be assimilated by the GS-GOGAT cycle (15, 33). This was confirmed by Somerville and Ogren (32), using *Arabidopsis* mutants lacking GOGAT. We compared the ammonium accumulation in leaves incubated in an atmosphere of 2% O_2, which inhibits photorespiration (6), and in normal (21% O_2) atmosphere (Table III). Under low O_2, ammonium accumulation induced by tabtoxin or MSO averaged 13.4% that found in leaves under normal O_2 (mean of 2- and 4-h samples for both inhibitors). These values agree with values calculated by others, indicating that photorespiration at normal rates could potentially generate 10 times more ammonium than could nitrate reduction (16). If we assume that the ammonium accumulating under 2% O_2 comes from nitrate reduction, our data are consistent with these calculated estimates.

These data help explain the results of the $^{15}NO_3^-$ experiment. If 13.4% of the accumulated ammonium comes from reduction of nitrate, then the ^{15}N atom % excess of the ammonium pool would be 12.7 (0.134 × 95 atom % excess) if only exogenous nitrate were reduced. The observed value of only 7 atom % excess can be explained by reduction of endogenous, unlabeled nitrate, in addition to the exogenous ^{15}N nitrate.

It is important to note that 2% O_2 was not inhibitory to NRA (Table III). Although there was some reduction in activity with time in MSO or tabtoxin, activities in leaves in 2% O_2 were as high or higher than they were in those in 21% O_2.

Tabtoxin and MSO have been shown to affect photosynthesis. Platt and Anthon (24) reported a significant decrease in CO_2 uptake by spinach leaves within 1 h of incubation in 5 to 10 mM MSO. However, at 1 mM, MSO had only a slight inhibitory effect after 3 h of incubation. We used 1 mM MSO, a level which presumably had minimal effects on photosynthesis while still inhibiting GS activity. Tabtoxin was shown to inhibit RuBPCase *in vitro* (7). Surprisingly, the inhibition was inversely related to tabtoxin concentration. *In vivo* effects of tabtoxin on photosynthesis have not been reported.

In view of our present knowledge of photosynthesis and photorespiration in C_3 plants, our data suggest that tabtoxin and MSO inhibit GS with only slight disturbance of RuBPCase and photorespiration. Significant amounts of ammonium were accumulated, because further assimilation by the GS-GOGAT cycle was blocked. GDH, the other possible assimilatory enzyme for ammonium, was not being elevated to levels capable of assimilating this ammonium. These experiments confirm the primary role of GS-GOGAT in nitrogen assimilation. Photorespiration accounted for about 83% of the ammonium accumulated, and nitrate reduction apparently accounted for the remainder. Thus, our data lend support to the suggestion that the photorespiratory nitrogen cycle generates one order of magnitude more ammonium than does primary nitrate assimilation.

Our results are consistent with previous work and represent what we believe are the early effects of tabtoxin and MSO on metabolism in C_3 grasses. In corn, a C_4 species, a bacterium similar to pv. *coronafaciens* causing chocolate spot, has been shown to synthesize tabtoxin and produce a light-dependent chlorosis (26). Also, ammonium accumulates in corn leaves treated with tabtoxin or MSO (R. D. Durbin, unpublished data). How such observations in a C_4 plant correlate with the conclusions of the present work remains to be investigated.

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