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Effect of Methionine Sulfoximine on the Accumulation of Ammonia in C_3 and C_4 Leaves[1]

THE RELATIONSHIP BETWEEN NH_3 ACCUMULATION AND PHOTORESPIRATORY ACTIVITY

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ABSTRACT

Additions of methionine sulfoximine (MSX), an inhibitor of glutamine synthetase (GS), result in an increase in NH_3 in seedling leaves of C_3 (wheat [*Triticum aestivum* cv. *Kolibri*] and barley [*Hordeum vulgare* var Perth]) and C_4 (corn [*Zea mays* W6A × W182E] and sorghum [*Sorghum Vulgare* var MK300]) plants. NH_3 accumulation is higher in C_3 (about 17.8 micromoles per gram fresh weight per hour) than in C_4 (about 4.7 micromoles) leaves. Under ideal conditions, when photosynthesis is not yet inhibited by the accumulation of NH_3, the rate of NH_3 accumulation is about 16% of the apparent rate of photosynthesis. A maximum accumulation of NH_3 was elicited by 2.5 millimolar MSX and was essentially independent of the addition of NO_3^- during either the growth or experimental period. When O_2 levels in the air were reduced to 2%, MSX resulted in some accumulation of NH_3 (6.0 micromoles per gram fresh weight per hour). At these levels of NH_3, there was no significant inhibition of rates of CO_2 fixation. There was also a minor, but significant, accumulation of NH_3 in corn roots treated with MSX. Inhibitors of photorespiration (isonicotinic hydrazide, 70 millimolar; 2-pyridylhydroxymethanesulfonic acid, 20 millimolar) or transaminase reactions (aminooxyacetate, 1 millimolar) inhibited the accumulation of NH_3 in both C_3 and C_4 leaves. These results support the hypothesis that GS is important in the assimilation of NH_3 in leaves and that the glycine-serine conversion is a major source of that NH_3.

In 1974, Mahon *et al.* (9) suggested that the major reaction leading to the release of CO_2 in photorespiration was the conversion of glycine to serine. If this were true, the release of NH_3 by photorespiration would be as significant as the release of CO_2. In C_3 plants, the rates of photorespiration can be considerable, up to 25% the rate of photosynthesis (9). Thus, in a system where NH_3 accumulation can be measured, NH_3 derived from photorespiration could be far in excess of the NH_3 produced by the reduction of NO_3^-. Under normal conditions, however, little NH_3 is recovered from leaf tissue (1, 21). Miflin and Lea (11) suggested that an active GS[3] could be responsible for the efficient assimilation of NH_3, and subsequently additions of MSX, an inhibitor of GS, were shown to result in accumulations of NH_3 (4, 5, 10, 12, 18) and in the inhibition of photosynthesis (13, 14). In 1978, Keys *et al.* (7) proposed a photorespiratory nitrogen cycle whereby NH_3 released from glycine would be efficiently reassimilated by GS in the cytosol. The resultant glutamine (20) would be transferred to the chloroplast where it would serve as a substrate for GOGAT, and the resultant glutamate would serve as the *N*-donor in the transaminase reaction leading to glycine formation in the peroxisome. The overall reactions and potential sites of inhibition are illustrated in Figure 1. Sommerville and Ogren (16) showed that *Arabidopsis* mutants lacking GOGAT accumulated NH_3 under conditions which permitted photorespiration. In mutants lacking serinetranshydroxymethylase activity, NH_3 was necessary for the continued synthesis of glycine, again under conditions which permitted photorespiration (17). Thus, their results support the hypothesis of Keys *et al.* (7).

Recently, Platt and co-workers (13, 14, and personal communication) observed an accumulation of NH_3 in spinach leaf discs and an inhibition of photosynthetic activity in the presence of MSX. Under conditions where photosynthesis was inhibited, they still saw an accumulation of NH_3 in experiments where the O_2 content was reduced to 2%. These conditions should have inhibited photorespiration (15, 19) and hence NH_3 accumulation. These observations agree with neither the *Arabidopsis*-mutant studies (16, 17) nor with the original hypothesis of Keys *et al.* (7, 20) as it suggests alternate and significant sources of NH_3 in leaf tissue. Explanations for this apparent contradiction could be the excessively high levels of MSX (8 mM) used in Platt's experiments and to the use of leaf discs rather than whole leaves.

In view of the fact that corn leaf pieces showed an accumulation of NH_3 in the presence of MSX (12) and that high levels of MSX (2.5 mM) caused a general inhibition of root metabolism (A. Oaks, unpublished), it seemed to us that the role of MSX in leaves needed to be reexamined. In this paper, we show that a maximum level of NH_3 is released by relatively low concentrations of MSX (0.60–2.5 mM) and that this release is inhibited by low concentrations of O_2, by standard inhibitors of photorespiration (INH, HPMS) and by a transaminase inhibitor (AOA) in leaves of both C_3 and C_4 plants. Under ideal conditions, when photosynthesis is not inhibited, the levels of NH_3 accumulated in the presence of MSX are 3 to 4 times higher in C_3 than in C_4 leaves.

MATERIALS AND METHODS

Plant Material. Corn caryopsis (*Zea mays* W6A × W182E, supplied by the Wisconsin Seed Foundation, Madison, WI), wheat (*Triticum aestivum* cv Kolibri, supplied through the OECD program), barley (*Hordeum vulgare* var Perth, supplied by the Crop

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[3] Abbreviations: GS, glutamine synthetase; MSX, methionine sulfoximine; GOGAT, glutamate synthase; INH, isonicotinic hydrazide; α-HPMS, 2-pyridylhydroxymethanesulfonic acid; AOA, aminooxyacetate; GDH, glutamate dehydrogenase.

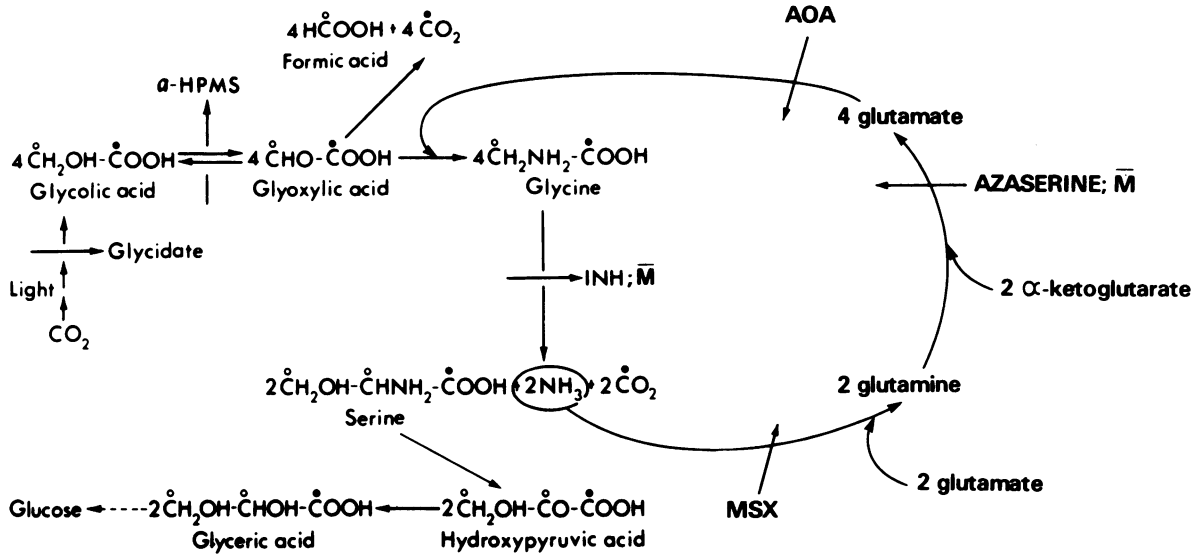

FIG. 1. Scheme describing the photorespiratory carbon and nitrogen cycles (adapted from Zelitch [22] and Keys *et al.* [7]. In the nitrogen cycle, four glycine molecules give rise to two serine, 2 CO_2, and 2 NH_3 molecules. The reaction is inhibited by INH (22) and there is an *Arabidopsis* mutant ($\bar{M}$) which lacks the required enzyme (17). The NH_3 reacts with glutamate to yield two molecules of glutamine. This reaction (GS) is inhibited by MSX. The glutamine then reacts with α-ketoglutarate to yield four molecules of glutamate; GOGAT is inhibited by azaserine (10, 11) and there are *Arabidopsis* mutants ($\bar{M}$) which lack this enzyme (16). The four glutamates could then serve as the nitrogen donor for the transaminase reaction leading to four molecules of glycine. This reaction should be inhibited by AOA. α-HPMS blocks the conversion of glycolate to glyoxylate an early step in the photorespiratory carbon cycle. In our experiments, we have used MSX which leads to an accumulation of NH_3 together with α-HPMS, INH, or AOA which should block the photorespiratory carbon cycle. If photorespiration is the major source of NH_3 resulting from the addition of MSX, then the addition of any of these inhibitors should lead to a reduction in the accumulation of NH_3.

Science Department, University of Guelph) and sorghum (*Sorghum vulgare* var MK300, supplied by the Crop Science Department, University of Saskatchewan) were grown in a mixture of sand and vermiculite (1:1) in a Conviron growth chamber (model E-7).

For corn and sorghum, the day length was 16 h, the day temperature was 28°C, and the night temperature was 26°C. For wheat and barley, the day (16 h) and night temperatures were maintained at 20°C. Radiant flux densities of approximately 150 $\mu E \cdot m^{-2} \cdot s^{-1}$ were supplied by cool white and incandescent light. The plants were watered each morning with 0.1 strength Hoagland solution which was modified to contain either 10 mM NO_3^- as the N source or no NO_3^- as required by the experimental protocol.

Incubation of Leaves.

Method 1. For most experiments, carefully matched primary leaves were detached and placed vertically in a small beaker containing 10 ml of water and MSX (Sigma), INH (Sigma), α-HPMS (Aldrich), or AOA (Sigma) as required. There was no external N during the actual experiment. The leaves were then illuminated for 90 min at 28°C. At the end of the experiment, the leaves were weighed (about 0.5 g/sample) and then placed in liquid N_2 and stored at −20°C. Whole roots from 7-d-old plants were also incubated in water or water plus MSX. In each experiment, duplicate tests were performed and each experiment was repeated at least twice.

Method 2. For these experiments, five carefully matched leaves were placed in water or water plus MSX (2.5 mM) in a flow through chamber (about 100 ml volume). Thus, both rates of photosynthesis and rates of NH_3 accumulation could be measured. Air samples were taken with a syringe from the air stream before and after passage through the cuvette. Radiant flux density at the surface of the cuvette was 150 $\mu E \cdot m^{-2} \cdot s^{-1}$ (400–700 nm; Lambda Instruments). The flow rate, for air or for an air mixture which contained only 2% O_2, was set so that CO_2 was not limiting the rate of photosynthesis (in excess of 200 μl/l at the gas exit). CO_2 concentrations were measured with a Beckman 865 IRGA (Beckman Instruments). Using this method, rates of NH_3 accumulation were faster than rates observed using method 1.

Ammonia and Nitrate Analysis. The leaves or roots were ground in a mortar with a methanol:chloroform:water (M:C:W ÷ 12:5:3) medium. The extract was then centrifuged in a Damon IEC-HW-5 centrifuge (Fisher Scientific) at full speed. The supernatant solutions from five successive washes in the MCW medium were pooled and concentrated to dryness with a rotovaporator (Buchi) set at 40°C. The residues were dissolved in 2 ml of 0.05 N HCl. For NH_3 determinations, it was also satisfactory to extract with phosphate buffer (200 mM; pH 7.5). No evaporation was required in this case. Ammonia was diffused from the extract after liberation by sodium tetraborate (pH 10) into traps of 0.1 N HCl in Conway dishes. The NH_3 was determined using a modified method of Kaplan (6). The reaction was initiated by adding successively 200 μl of sample, 1 ml of 0.17 mM Na nitroprusside in 1% (w/v) phenol, 1.0 ml of a solution containing 0.125 N NaOH, 0.25 M Na_2HPO_4 in 0.03% (w/v) NaOCl. The reaction was mixed vigorously on a Vortex mixer and incubated in a waterbath at 37°C for 30 min. The absorbance was read at 625 nm. Blank and standard assays with known concentrations of NH_4Cl were made up with 0.1 N HCl and were measured with each set of unknowns. Nitrate was analyzed by the method of Cataldo *et al.* (2).

RESULTS AND DISCUSSION

Preliminary experiments (Fig. 2) showed that NH_3 accumulated in a linear fashion during the initial 130 min after transferring wheat leaves to a MSX solution (2.5 mM). However, in experiments where photosynthesis was measured, the rate of NH_3 accumulation was faster and was linear for a much shorter period of time. In Table II, for example, photosynthesis was not inhibited during the initial 30-min exposure to MSX, but by 90 min there was a 64% inhibition. NH_3 production measured as a rate (μmol · g fresh weight^{-1} · h^{-1}) was also lower at 90 min when the total accumulation was used to calculate the rate than at 30 min. It is known that high levels of NH_3 inhibit the electron transport system in

Table I. *NO_3-N and the Photorespiratory Nitrogen Cycle as a Source of NH_3 Accumulation in the Presence of MSX*

The tissues were incubated in H_2O or H_2O + MSX (2.5 mM) for 90 min. See Figure 2 for protocol. Experimental temperature was 28°C (method 1).

Plant Type	Tissue	Experimental Treatment	Growing Conditions: No NO_3^-		Growing Conditions: 10 mM KNO_3	
			NO_3^-[a]	NH_3[b]	NO_3^-[a]	NH_3[b]
			μmol g fresh wt^{-1}	*Δμmol g fresh wt^{-1}*	*μmol g fresh wt^{-1}*	*Δμmol g fresh wt^{-1}*
C_3						
Wheat	Primary leaf	Control	4.2	0.5 (1.6)	90.5	0.05 (0.95)
		+ MSX		6.8		6.9
Barley	Primary leaf	Control	1.9	0.5 (0.9)	107.4	0.72 (0.97)
		+ MSX		8.1		7.53
C_4						
Corn	Primary leaf	Control	0.5	0.33 (1.45)	28.2	0.17 (1.62)
		+ MSX		1.42		2.95
Corn	Fifth leaf	Control			26.5	0.05 (0.52)
		+ MSX				3.17
Corn	Root	Control				0.51 (.50)
		+ MSX				0.95
Sorghum	Primary leaf	Control		0.1 (4.4)	28.5	0 (1.3)
		+ MSX		3.2		2.06

[a] NO_3^- values were obtained on the initial material.

[b] NH_3 values represent the increase over a 90-min period. Values in parentheses are the initial values (μmol per g fresh weight.

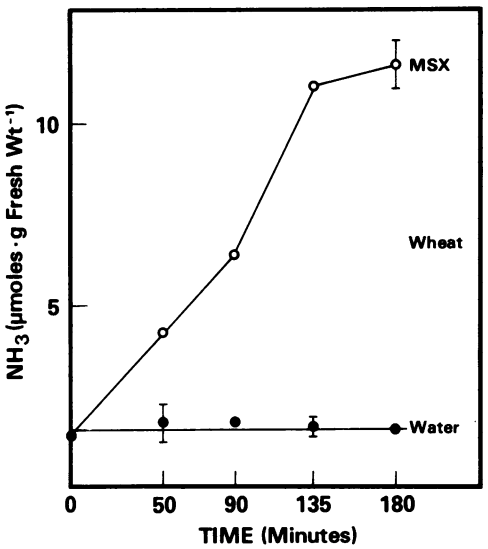

FIG. 2. Effect of time on the MSX-induced accumulation of NH_3 in wheat leaves. The leaves (five/sample) were incubated in open vials with H_2O or H_2O + MSX (2.5 mM) for the required time in a Conviron growth chamber at 20°C (method 1).

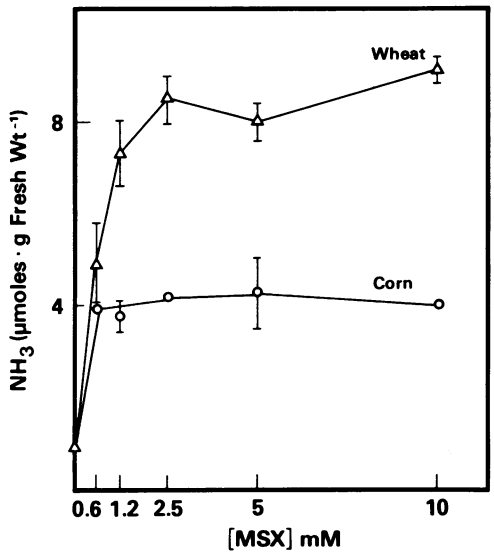

FIG. 3. Effects of MSX concentration on the accumulation of NH_3 in wheat (C_3) and corn (C_4) leaves. Experimental design as in Figure 2. Experimental temperature was 28°C for corn and 20°C for wheat. Experimental time was 90 min (method 1).

chloroplasts (see 7) and hence, indirectly CO_2 fixation as well. Under these conditions, one would expect an inhibition in photorespiration and of NH_3 production if that NH_3 was derived from photorespiration. When levels of NH_3 exceeded 8 to 10 μmol · g fresh weight^{-1} in wheat leaves, there was subsequently both an inhibition of photosynthesis and a reduction in the rate of NH_3 accumulation. Care was, therefore, taken to use time intervals when NH_3 accumulation was still in the initial linear phase. Increasing concentrations of MSX resulted in increasing accumulations of NH_3 up to a characteristic threshold concentration (Fig. 3). With corn, 0.6 mM MSX gave a maximum response; with wheat, 2.5 mM. In subsequent experiments, we used 2.5 mM MSX, the lowest concentration which gave a maximum accumulation of NH_3. In a preliminary survey of a number of C_3 and C_4 cereals, we found levels of accumulation of NH_3 of about 6 to 8 μmol per g fresh weight over a 90-min period for a number of wheat and barley cultivars and of 3 to 4 μmol for a number of corn and sorghum cultivars.

NH_3 could result from the reduction of NO_3^-, from photorespiration, or from some other unidentified source (3, 13, 14). The

Table II. *Effect of MSX on the Rates of Photosynthesis and NH_3 Accumulation*

The primary leaves were incubated in H_2O + MSX (2.5 mM) for 30 or 90 min. CO_2 uptake was measured at intervals throughout the incubation period. However, the 90-min values were used in most cases above. With wheat leaves, it took about 1 h before there was a high steady rate of CO_2 uptake. Values for CO_2 consumption or NH_3 production are calculated as rates per h. All treatments were run in an open cuvette system with a stream of air flowing past the leaves. Rates of NH_3 accumulation are faster with this treatment than with our growth chamber experiments. Experimental temperature was 25°C (method 2).

Plant	Incubation Time	Flow Rate	Photosynthesis		NH_3 Accumulation[a]	
			Control	+ MSX	Control	+ MSX
	min	*ml/min*	*μmol CO_2·g fresh wt^{-1}·h^{-1}*		*μmol·g fresh wt^{-1}·h^{-1}*	
C_3						
Wheat (air)	30	60	72	70 (97)	1.33	17.8
	90	60	88	31 (36)	0.93	8.4
Wheat (2% O_2 in air)	90	135	109	119 (110)	1.2	6.0
C_4						
Corn (air)	90	100	116	113 (97)	1.4	4.7

[a] Values of NH_3 accumulation become nonlinear when levels are in excess of 8 μmol/g fresh wt. Thus, for wheat leaves the 30-min value was used in calculating the rate of NH_3 production per h. With reduced O_2 concentrations, that rate was linear over a 90-min period; hence, any time interval could be used for calculating the rates of NH_3 accumulation.

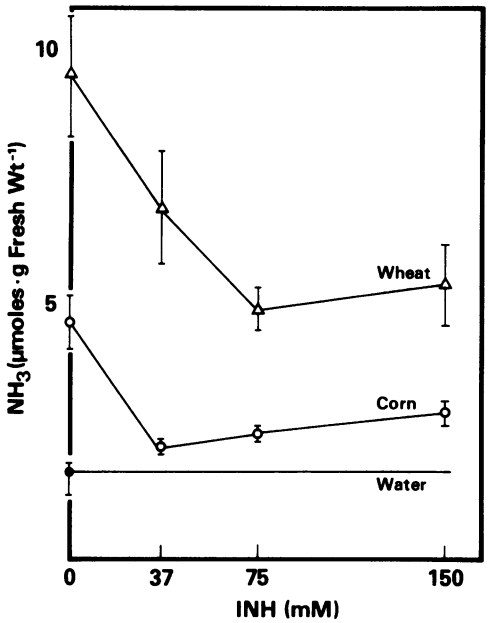

FIG. 4. Effect of INH on the MSX-induced accumulation of NH_3 in wheat (C_3) and corn (C_4) leaves. Experimental design as in Figure 3. (method 1).

results in Table I summarize the effect of NO_3^- during growth on the MSX-induced accumulation of NH_3 in C_3 and C_4 leaves. Plants grown with 10 mM KNO_3 had high endogenous levels of NO_3^- initially (about 100 μmol per g fresh weight for C_3 plants and about 30 μmol for C_4 plants), whereas those grown in the absence of NO_3 had low endogenous levels of NO_3^-. The recovery of NH_3 in MSX-treated leaves was not influenced by the presence of endogenous NO_3^- within the limits of our experiments. We take this as evidence that most of the NH_3 production over the 90-min experimental period originated from reactions other than the reduction of NO_3^-. In corn leaves (12) as well as in spinach (4, 13), the accumulation of NH_3 in the presence of MSX is light dependent and hence could originate from photorespiration. Results in Table I also show that the accumulation of NH_3 is similar in the primary and fifth leaves of corn and that the accumulation of NH_3 in the root is minor relative to accumulations seen in leaves.

Table III. *Effects of Potential Inhibitions of the Photorespiratory Nitrogen Cycle on the MSX-Induced Increase in NH_3*

The tissues were incubated in H_2O or H_2O plus the appropriate inhibitor(s) for 90 min. Values represent the total NH_3 after 90 min. Experimental temperatures were 28°C for corn and for wheat in air (method 1).

Treatment	Corn Leaf		Wheat Leaf (Primary)
	Primary	Fifth	
	NH_3 (μmol·g fresh wt^{-1})		
Control	1.78	0.57	1.1
MSX (2.5 mM)	2.90 (100)[a]	3.17 (100)	8.4 (100)
MSX + INH (70 mM)	1.85 (64)	1.89 (60)	4.9 (58)
MSX + HPMS (20 mM)	1.09 (37)		4.8 (57)
MSX + AOA (1 mM)	0.97 (33)		3.6 (42)

[a] Values in parentheses represent accumulations of NH_3 relative to accumulations with MSX alone.

If NH_3 accumulation originates primarily from photorespiration, then treatments designed to inhibit photorespiration should inhibit the accumulation of NH_3. We manipulated the rates of photorespiration in two ways: (a) by reducing the O_2 concentration from 20 to 2% (Table II); and (b) by adding presumed inhibitors of photorespiration to MSX-treated leaves (Table III; Fig. 4). Reducing the O_2 concentration resulted in higher apparent rates of photosynthesis and to reduced rates of NH_3 accumulation. Because the kinetics of NH_3 accumulation were different in the closed system required to control the O_2 concentration, we also tested the effect of MSX on corn leaves. Photosynthesis was not inhibited over a 90-min period, and the accumulation of NH_3 was lower than that seen in the wheat leaves. Our results are clearly at variance with those reported by Platt and Anthon (13). Inasmuch as we have deliberately chosen low concentrations of MSX, concentrations which do not inhibit photosynthesis but which do cause moderate accumulations of NH_3, we think we are looking at the effect of MSX on the GS reaction and that we have minimized other nonspecific effects of MSX that could result in accumulations of NH_3. Our results support the presence of a photorespiratory N cycle as proposed by Keys *et al.* (7). Results in Table III show that presumed inhibitors of photorespiration (see 8, 21; Fig. 1) also inhibit the MSX-induced accumulation of NH_3

in both wheat and corn. Because of the high levels of inhibitor required to see the effect (Fig. 4), there is some question concerning the specificity. However, they do lend support to the results obtained with low levels of O_2 (Table II).

The inhibition caused by the INH (Table III; Fig. 4) suggests that the glycine-serine conversion is a major source of NH_3 accumulation in MSX-treated leaves. The inhibition caused by MSX suggests that GS is important in the assimilation of that NH_3 in leaves. The minor effect of MSX on the accumulation of NH_3 in roots suggests that alternate mechanisms, perhaps the GDH reaction, are important in the assimilation of NH_3. The results with leaves agree with recent results of Lawyer *et al.* who examined the fate of exogenous NH_3 (8). In agreement with other methods of measuring photorespiration (15, 19), our results also indicate that the rate of photorespiration is much higher in C_3 than in C_4 leaves. If we look at rates of photosynthesis (about 114 μmol CO_2 · g fresh weight^{-1} · h^{-1} in 2% O_2) and of the accumulation of NH_3 (about 18 μmol · g fresh weight^{-1} · h^{-1} at a time before photosynthesis is inhibited) we see that photorespiration as measured by the accumulation of NH_3 is 16% of the apparent rate of photosynthesis. This is well within the range for previously calculated rates of photorespiration (1, 9, 15, 19, 22). Thus, if appropriate precautions are taken, NH_3 accumulations in the presence of MSX do give a reliable estimation of the relative rates of photorespiration in C_3 and C_4 leaves.

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