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Methanol Emission from Leaves[1]

Enzymatic Detection of Gas-Phase Methanol and Relation of Methanol Fluxes to Stomatal Conductance and Leaf Development

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We recently reported the detection of methanol emissions from leaves (R. MacDonald, R. Fall [1993] Atmos Environ 27A: 1709–1713). This could represent a substantial flux of methanol to the atmosphere. Leaf methanol production and emission have not been investigated in detail, in part because of difficulties in sampling and analyzing methanol. In this study we used an enzymatic method to convert methanol to a fluorescent product and verified that leaves from several species emit methanol. Methanol was emitted almost exclusively from the abaxial surfaces of hypostomatous leaves but from both surfaces of amphistomatous leaves, suggesting that methanol exits leaves via stomates. The role of stomatal conductance was verified in experiments in which stomates were induced to close, resulting in reduced methanol. Free methanol was detected in bean leaf extracts, ranging from 26.8 μg g^{-1} fresh weight in young leaves to 10.0 μg g^{-1} fresh weight in older leaves. Methanol emission was related to leaf development, generally declining with increasing leaf age after leaf expansion; this is consistent with volatilization from a cellular pool that declines in older leaves. It is possible that leaf emission could be a major source of methanol found in the atmosphere of forests.

Plants are known to produce a large variety of VOCs (Isidorov et al., 1985; Winer et al., 1992; Teranishi et al., 1993; Guenther et al., 1994, 1995). These VOCs play a number of roles in plant physiology and signaling, in plant-herbivore relationships, and in defense against microorganisms (reviewed by Roshchina and Roshchina, 1993). Some of these VOCs are emitted in large enough amounts to result in significant impacts on chemical reactions in the atmosphere (Trainer et al., 1987; Chameides et al., 1988). For example, emission of isoprenoids such as isoprene and monoterpenes lead to ozone production in rural forest ecosystems (reviewed by Fehsenfeld et al., 1992). The emission of some of these compounds may represent a significant loss of carbon from the plant. Global emissions of isoprene and monoterpenes from plants are estimated to be very large, 5.0×10^{14} and 1.3×10^{14} g C year^{-1}, respectively. Recent evidence also suggests that, in addition to isoprene and monoterpenes, plants emit on the order of 5.2×10^{14} g C year^{-1} of other VOCs (Guenther et al., 1995). There is current interest in identifying plant sources for these other VOCs and understanding their impacts on atmospheric processes.

We recently reported substantial emissions of the VOC methanol from plants to the atmosphere (MacDonald and Fall, 1993). Methanol emission rates, which were determined by a GC method from 11 different plant species, ranged in magnitude from 0.6 to 17 μg C h^{-1} g^{-1} dry weight. By means of comparison, a typical leaf monoterpene emission rate is 1.6 μg C h^{-1} g^{-1} dry weight and a typical rate of isoprene emission is 35 μg C h^{-1} g^{-1} dry weight (Guenther et al., 1994). It is noteworthy that, although only a small fraction of U.S. woodland tree species and crops are isoprene and/or monoterpene emitters (Guenther et al., 1994), all of the tree and crop species we have tested so far emit methanol (MacDonald and Fall, 1993; Fall, 1994). Although the impact of plant methanol emission on atmospheric photochemistry is uncertain, it is interesting that methanol was the major VOC detected in the air, both day and night, during the summer in a southeastern U.S. pine forest (Fehsenfeld et al., 1992). We have been working to establish whether forest vegetation is a major source of atmospheric methanol.

The analysis of methanol in leaf emissions presents certain analytical challenges. Because of its complete miscibility with water, such a polar solute is easily lost in sampling lines if condensation occurs and in traps designed to remove water that interferes with GC. Because it contains only a single, partially oxidized carbon atom, methanol produces a weaker signal than hydrocarbons in a standard flame ionization detector. Analysis of methanol (mass 32) by GC-MS techniques is complicated by the fact that O_2

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Abbreviations: DDDP, 3,5-diacetyl-1,4-dihydro-2,6-dimethylpyridine; Fluoral-P, 4-amino-3-penten-2-one; LPI, leaf plastochron index; VOC, volatile organic compound.

gas, which is present in traces in reagent gases, has the same molecular ion mass. Here, we have used an enzymatic method with similar sensitivity to GC analysis that avoids many of these problems.

In this paper we have addressed some of the factors that may influence methanol emission in an attempt to understand how this flux is regulated and to try to provide some clues as to its biochemical origin in the leaf. Specifically, we have used detailed experiments to examine the relationship between methanol emission and stomatal conductance and leaf development. This work is a logical step in approaching the biochemical level of understanding of methanol dynamics in leaves.

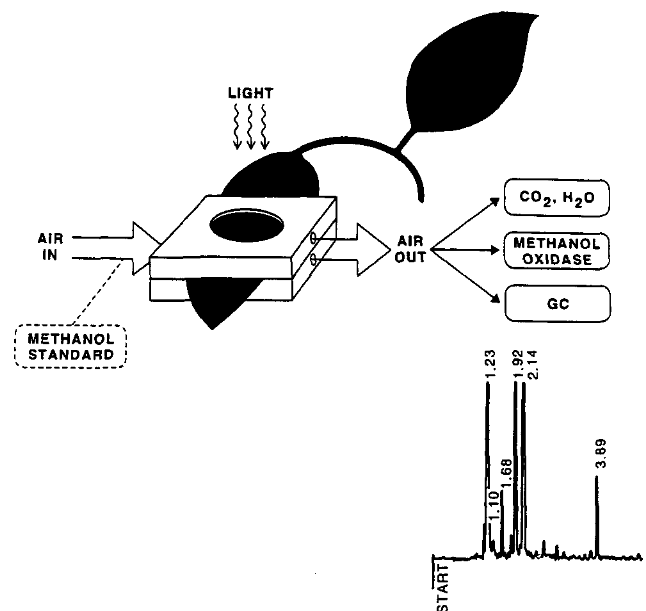

Figure 1. The experimental design for the measurement of methanol fluxes from leaves by GC or an enzymatic method (methanol oxidase). A clamp-on leaf cuvette (Fall and Monson, 1992) was used that allowed independent monitoring of gas exchange on the adaxial or abaxial leaf surfaces at constant light intensity (PAR) and temperature. Purified, methanol-free air that passed over the leaf could be analyzed in the exit stream for photosynthetic CO_2 assimilation and transpiration and for methanol by either a methanol oxidase procedure (see text and Fig. 2) or a GC procedure that involved cryogenic preconcentration of the gas stream and analysis by DB-WAX capillary chromatography and flame ionization detection (MacDonald and Fall, 1993). A typical GC chromatogram for cottonwood leaf VOCs is shown with methanol eluting at 1.92 min.

MATERIALS AND METHODS

Plant Material

Individual cottonwood (*Populus deltoides* var *occidentalis*) specimens were propagated from branch cuttings taken from a local tree. Seedlings of other tree species were obtained from local nurseries. In some experiments, branches of Russian olive (*Elaeagnus angustifolia*) from trees on the University of Colorado campus were cut under water and immediately transported to the laboratory with cut stems in water. All other plant material was grown from seed: Kentucky Wonder 125 bush bean (*Phaseolus vulgaris*; Lake Valley Seed, Boulder, CO), McCall soybean (*Glycine max*; Northern Soybean Germplasm Collection, Urbana, IL), and velvet bean (*Mucuna* sp.; Glendale Enterprises, DeFuniak Springs, FL). Plants were grown in pots in Agro-Mix No. 2 (Conrad Fafard Co., Springfield, MA), watered daily, and fertilized weekly with Peters Professional Soluble Plant Food (Peters Fertilizer Products, Fogelsville, PA). All plants were grown in a greenhouse at 27°C day and 21°C night temperatures with supplemental lighting (except in summer) from low-pressure sodium vapor lamps to provide a 16-h photoperiod.

Measurement of Methanol Emission from Separate Sides of the Leaf

Methanol emission was measured separately from the abaxial and the adaxial surfaces of the same leaf using a water-jacketed, clamp-on cuvette that allowed the leaf to act as a membrane separating the upper and the lower chambers of the cuvette (Fall and Monson, 1992). The experimental design is shown in Figure 1. Purified, methanol-free air was passed through each cuvette chamber, and methanol emission from each leaf surface was quantified using the GC method or methanol oxidase method described below. Light was supplied to the leaf by an incandescent bulb or a low-pressure sodium vapor lamp (PAR modulated with a water bath and screens), and PAR at the leaf surface was measured with a photon flux sensor (Li-Cor, Lincoln, NE). The temperature in the cuvette was maintained at 30°C with a circulating water bath.

Methanol Emission during Changing CO_2 Assimilation and Stomatal Conductance

For preliminary experiments of the relation between methanol emission and leaf gas exchange, leaves of *P. vulgaris* were placed in a 0.5-L nickel-plated gas-exchange cuvette described in detail elsewhere (Monson and Fall, 1989). Temperature inside the cuvette was maintained by Peltier cooling, and air in the cuvette was continuously stirred by two small fans. Air supplied to the cuvette was regulated with respect to CO_2, O_2, humidity, and total gas flow. Light was supplied from a low-pressure sodium vapor lamp, and intensity was modulated by placing various layers of shade cloth between the lamp and the cuvette. Different light treatments were applied in random order. Controls were the means of measurements made before and after each light treatment at a light intensity of 375 μmol m^{-2} s^{-1} (leaves at steady state). Stomatal conductance was calculated from measurements of RH made by dew-point mirrors (General Eastern Corp., Watertown, MA), and CO_2 assimilation was calculated from measurements made by an ADC 225-MK3 IRGA (Analytical Development Co., Ltd., Hoddesdon, UK). The outputs from all instruments used to measure gas exchange (not methanol emission) were scanned and analyzed every 10 s using a

microcomputer. Measurements of methanol emissions were accomplished by interfacing the GC system, described below, to the output from the leaf cuvette using a heated Teflon sample line.

Most of the measurements of stomatal conductance and methanol emission rate used a Li-Cor 6200 photosynthesis system (Li-Cor) modified to allow samples of gas exiting the cuvette to be sampled (Monson et al., 1994). The system was operated in the closed flow mode (for conductance measurements) and then switched to open flow mode (for methanol flux measurements). Flow rate in the open mode was measured with a rotameter, and at the time of methanol sampling the flow rate, leaf temperature, and incident photon flux density were noted. Methanol recovery in the system was evaluated with the methanol gas standard described below.

To observe stomatal closing induced by ABA the following procedures were used. Stems containing several leaves were cut from *P. vulgaris* plants that had been maintained in the dark overnight and then placed in water. A leaf on the stem was placed in the Li-Cor leaf cuvette under a light source (PAR, 450 μmol m^{-2} s^{-1}), and leaf conductance and methanol emission measurements were carried out until steady-state conditions were attained. At that time the cut stem was placed in a solution of 20 μM ($\pm$)-*cis-trans*-ABA (prepared from a 20 mM ABA stock solution in DMSO), and measurements were continued until stomatal conductance was essentially zero.

GC Method of Methanol Quantitation

Air exiting the leaf cuvette was passed through a sample loop submerged in liquid nitrogen to concentrate VOCs in the samples. The sample loop was flash heated to 100°C, and the gas sample was swept onto a GC column by H_2 carrier gas, as fully described by Monson and Fall (1989). Prior to cryotrapping, water was removed from the air stream by passage through a cold finger at 50°C (80% [w/v] ethylene glycol and dry ice). This step improved the chromatography with little loss of methanol through the system. GC was performed with a Hewlett-Packard 5790A GC with a DB-WAX column (0.32 mm × 30 m; J & W Scientific, Folsom, CA) and a H_2 carrier flow of 42 mL s^{-1} at 50°C. The detector temperature was 200°C. The system was calibrated by injection of an authentic methanol standard.

Methanol Oxidase Method for Methanol Quantitation

The assay of gas-phase methanol was based on a spectrophotometric determination of liquid-phase methanol (Klavons and Bennett, 1986; Hamano et al., 1990). Gas emerging from the leaf cuvette was bubbled via Teflon tubing through 2 mL of solution containing methanol oxidase (5 units, *Pichia* alcohol oxidase; Sigma), Fluoral-P (2 mg; Wako Pure Chemicals, Richmond, VA), and potassium phosphate buffer (0.2 M, pH 6.0). The Teflon tubing and reaction mixture (13 × 100-mm test tube) were wrapped in heat tape (32°C) to prevent condensation. Sampling was conducted at room temperature for 5 to 30 min, and then the enzymatic reaction was allowed to proceed after sampling for an additional 30 min. The fluorescence of the solution was measured with a fluorometer (excitation at 405 nm; emission at >500 nm). Control tubes included (a) reagent blanks (reaction mixture but not bubbled) and (b) reaction mixture minus methanol oxidase; the fluorescence signal of the latter was a measure of gas-phase formaldehyde, which was negligible in all of the experiments described here. Under these conditions a linear methanol response was obtained from 0.02 to 0.73 μg/mL. The methanol signal was constant over the flow range of 30 to 65 mL min^{-1} (at higher gas flows loss of gas-phase methanol was noted). At the flow typically used (40 mL min^{-1}) the cuvette was flushed approximately four times per min. Optimal conditions of the methanol oxidase/Fluoral-P reaction were checked and were identical with those described by Hamano et al. (1990).

HPLC analysis of reaction mixtures was used to identify the fluorescent product, DDDP, of the enzymatic procedure. Authentic DDDP was obtained from Aldrich. The solvent system used ion pairing to resolve the secondary amine DDDP from other components in leaf extracts; it was identical with that described by Krstulovic (1982) except that methanol was replaced by acetonitrile (0.0347 M KH_2PO_4, 3.0 mM sodium octyl sulfate, 0.03 M citric acid, 14% acetonitrile [v/v], pH 4.85). Chromatography was carried out on a C_{18} reversed-phase column (3.9 × 150 mm, 4-μm beads; Millipore); the flow rate was 1.2 mL min^{-1}, and detection at 412 nm (wavelength of maximum absorption for DDDP) was used.

Recovery of Methanol from Leaf Cuvettes

To measure recovery of methanol in the leaf cuvettes and sampling lines, a gas-phase methanol source was used. A methanol permeation tube, obtained from VICI Metronics (Santa Clara, CA), was housed in a Teflon container that was maintained in a constant temperature oven (58 ± 2°C) with a constant stream of methanol-free, compressed air (about 40 mL min^{-1}) passing over the permeation tube. The loss of weight of the permeation tube was determined during a period of several weeks to construct a methanol flux calibration plot. The temperature of the oven was chosen so that the emission rate, approximately 0.28 μg methanol min^{-1}, was similar in magnitude to many of the leaf methanol flux rates measured here. When this source was used in a timed methanol oxidase reaction assay (see above) and compared to assays with liquid methanol standards, the assayed methanol values agreed within 5%. To determine recovery of methanol from leaf cuvettes, the source methanol gas stream was allowed to mix with the air entering the cuvette and methanol emerging from the sample line was assayed until a steady-state value was obtained. Recovery of methanol was dependent on the type and stomatal conductance of the leaf present in the cuvette, ranging from 65 to 85% with the clamp-on leaf cuvette; during a period of several months, methanol recovery in the absence of a leaf ranged from 85 to 95%. Methanol recovery in the Li-Cor cuvette was much lower, only 25%, probably because of losses in the tubing and pumps of the instrument.

Methanol Content of Leaves

The methanol content of leaves was measured with the following procedure. Bush bean leaves from 6-week greenhouse-grown plants were excised, and leaf discs (2.6 cm diameter) or segments from each of three leaves of individual trifoliates were rapidly cut from each leaf, pooled, frozen in liquid nitrogen, and then ground to a powder with a cold mortar and pestle. Neutralized, perchloric acid extracts were prepared as described by Leegood (1993). These deproteinized extracts were analyzed for methanol by direct injection of 20-μL aliquots onto a Poropak Q column (80/100 mesh; 1/8 inch × 6 feet; Alltech, Deerfield, IL), housed in a Hewlett-Packard model 5790 gas chromatograph. The column was operated isothermally at 120°C with hydrogen as the carrier gas, and between injections water was removed from the column by ramping to 180°C (35°C min^{-1}) and holding at this temperature for 6 min. Methanol was quantitated by comparison to a standard curve.

Determination of the Effects of Leaf Ontogeny on Methanol Emission

Leaves of *G. max* and *P. vulgaris*, attached to the plants, were placed in the clamp-on cuvette described above but with the sample streams exiting abaxial and adaxial chambers combined prior to methanol analysis by GC or the enzymatic procedure. Leaves of *P. deltoides* were sampled under a low-pressure sodium vapor lamp (PAR, 500 μmol m^{-2} s^{-1}) in a greenhouse using the portable leaf cuvette system described by MacDonald and Fall (1993), which allows for determination of stomatal conductance and cryogenic collection of a methanol sample for later analysis by GC. The leaves of *G. max* and *P. vulgaris* were numbered starting with the first unfolding leaf. The LPI developed by Larson and Isebrands (1971) was used to number the leaves of *P. deltoides*. Leaves of different ages from each species were usually sampled in random order.

RESULTS

Use of Methanol Oxidase to Verify Methanol Emission from Leaves

We adapted an enzymatic procedure, originally developed to measure methanol in aqueous samples (Klavons and Bennett, 1986; Hamano et al., 1990), to the measurement of methanol in gases from leaf cuvettes. With this method, methanol partitions from the gas to liquid phase, where it is oxidized to formaldehyde by methanol oxidase, and the formaldehyde produced is continuously trapped by the reagent Fluoral-P to generate a stable, fluorescent product (Fig. 2). This procedure avoids complex sampling lines and traps. As shown in Figure 2, the analysis of methanol in *P. vulgaris* leaf gases by the methanol oxidase/Fluoral-P procedure yields a linear response with time. In the absence of methanol oxidase there was no increase in fluorescence due to formaldehyde emission from leaves. In the absence of Fluoral-P, essentially no fluorescence was seen. In the plants surveyed here, control reactions without

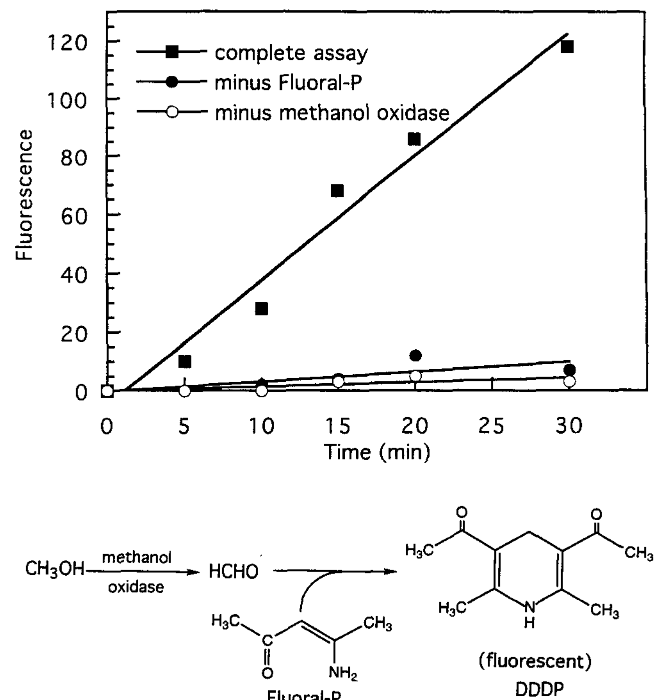

Figure 2. The production of fluorescent DDDP from leaf gases is dependent on the presence of methanol oxidase and Fluoral-P. In this experiment, which was replicated three times, a fully expanded bean leaf (*P. vulgaris*) near the top of the plant canopy was placed in the clamp-on leaf cuvette (Fig. 1; 30°C, 500 μmol $m^{-2}s^{-1}$), and after steady-state methanol flux was obtained, leaf gases were trapped in aqueous solutions containing (a) methanol oxidase and Fluoral-P, (b) methanol oxidase and no Fluoral-P, or (c) Fluoral-P and no methanol oxidase, as described in "Materials and Methods." The methanol oxidase/Fluoral-P reaction scheme is shown below the figure. The small fluorescence signal obtained for reaction mixtures lacking methanol oxidase is due to traces of formaldehyde.

methanol oxidase were routinely analyzed; no appreciable formaldehyde fluxes from leaves were seen; therefore, if formaldehyde was emitted from these leaves, it was below the detection limit (0.02 μg formaldehyde g^{-1} dry weight h^{-1}).

The fluorescence emission spectrum of the product of the methanol oxidase/Fluoral-P reaction with authentic methanol and leaf gas samples was identical with that for the product DDDP. With excitation at 412 nm, a broad emission maximum was seen centered at 505 nm (data not shown). Further verification of the products of leaf gases with methanol oxidase/Fluoral-P was undertaken by HPLC analysis. Resolution of reaction mixtures by reversed-phase HPLC demonstrated that the major product was DDDP. Figure 3A shows the HPLC profiles of bean leaf volatiles after reaction for 30 min with Fluoral-P; only a trace of DDDP is seen. HPLC analysis of bean leaf volatiles after reaction with methanol oxidase and Fluoral-P shows a major peak (Fig. 3B), which has a retention time identical with that for authentic DDDP (Fig. 3C). This analysis also shows that bean leaf gases contain methanol, and little formaldehyde. HPLC analyses of the reaction products from the enzymatic trapping of leaf gases were

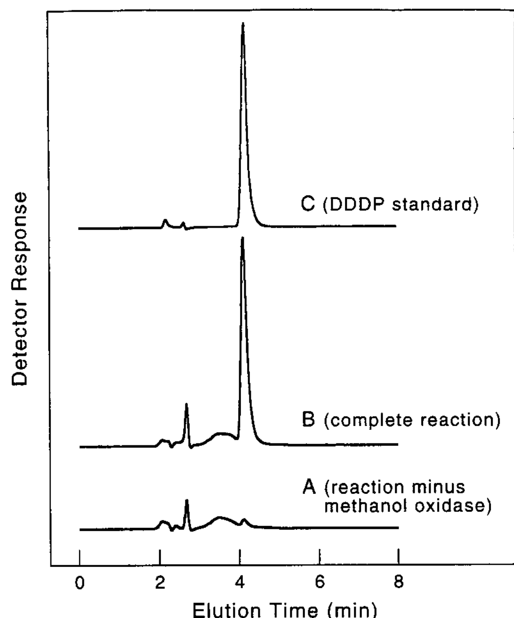

Figure 3. HPLC analysis of the products of the methanol oxidase/Fluoral-P reaction. A, Bean leaf VOCs trapped in a reaction mixture lacking methanol oxidase; B, bean leaf VOCs trapped in a complete reaction mixture; and C, authentic DDDP. HPLC conditions are described in "Materials and Methods." For samples in A and B, bean leaf VOCs were collected as in Figure 2, and 100-μL samples from 2-mL reaction mixtures were injected. For the sample in C, 100 μL of a standard solution of DDDP (0.625 mg ml^{-1}) were injected.

performed for the following plants: aspen, cottonwood, sweetgum, soybean, and velvet bean. Each showed results virtually identical with those for bush bean leaves.

The possibility of false positive signals in the enzymatic procedure for methanol by other potential leaf volatiles, such as ethanol and acetaldehyde, was also investigated. The methanol oxidase used here from the yeast *Pichia pastoris* is relatively specific for methanol but will catalyze oxidation of other short-chain alcohols (Hopkins and Muller, 1987). However, it is also known that products of the Fluoral-P reaction with aldehydes other than formaldehyde are essentially nonfluorescent (Dong and Dasgupta, 1987). We observed no significant fluorescence interference with ethanol or acetaldehyde or production of a peak chromatographing with DDDP when these samples were analyzed by HPLC.

Relationship between Methanol Emission and Stomatal Distribution

It was possible to simultaneously measure leaf gas exchange from the abaxial and adaxial surfaces of leaves using a clamp-on leaf cuvette. Virtually all methanol emission occurred from the surfaces containing stomates when these leaf gases were analyzed for methanol, using both GC and methanol oxidase procedures (Fig. 4). For four hypostomatous plant species, including aspen, sassafras, sweetgum, and velvet bean, the methanol flux from the abaxial surface ranged from 94 to 97% of the total leaf flux with only a fraction of the methanol, 3 to 6%, from the adaxial surface. For three amphistomatous plants, including bush bean, soybean, and cottonwood, methanol was emitted from both leaf surfaces, as shown in Figure 4. These results are consistent with the idea that methanol is emitted primarily from stomates and not from cuticular surfaces.

Variability of Methanol Emission Rate

When attempting to measure steady-state leaf methanol fluxes, we noted large transient variations in methanol emissions, especially with leaves tested in the morning. Figure 5 shows a typical experiment of this type, in which bean leaf methanol emission was tested in the morning after removing plants from a darkened cabinet. Initially, a large transient emission of methanol was seen, and then the methanol emission rate decreased with time, finally reaching a lower steady state. A similar phenomenon was observed with a variety of plants in these experiments and also in field studies we reported with sweetgum leaves (MacDonald and Fall, 1993). These transient peaks could be due to volatilization of methanol condensed on leaf surfaces or leaf damage or disturbance by the cuvette. We have seen the latter phenomenon in the case of monoterpene emission from eucalyptus leaves (Guenther et al., 1993). To resolve these possibilities, methanol emissions from leaves were analyzed (a) early in the morning or (b) after several hours in the light. When this was done, the leaf methanol emission rate was lower and at apparent steady state in leaves illuminated for 1 to 2 h.

Separate experiments were conducted with Russian olive leaves (*E. angustifolia*), which have elongated leaves that could be placed in the Li-Cor leaf cuvette either (a) longi-

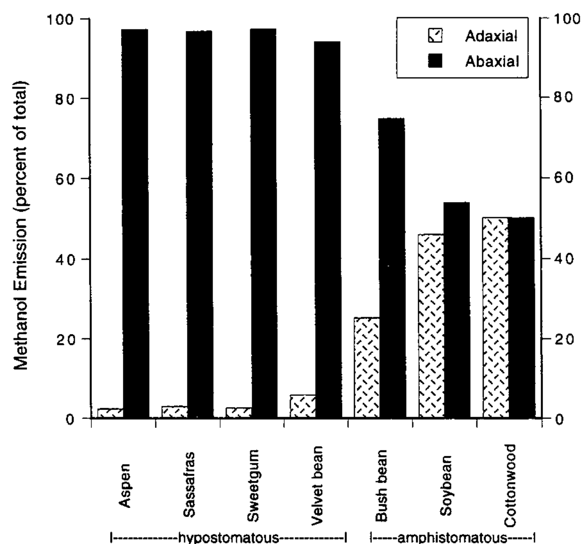

Figure 4. Methanol emission from adaxial and abaxial leaf surfaces of hypostomatous and amphistomatous plants. Using the clamp-on leaf cuvette (Fig. 1), methanol fluxes from different plant species were measured under similar conditions (375–500 μmol m^{-2} s^{-1}; 28–30°C). The data presented are the averages of duplicate determinations for leaves under steady-state conditions. Methanol was analyzed by either the GC method (aspen, bush bean, sweetgum, velvet bean) or the methanol oxidase procedure (cottonwood, soybean).

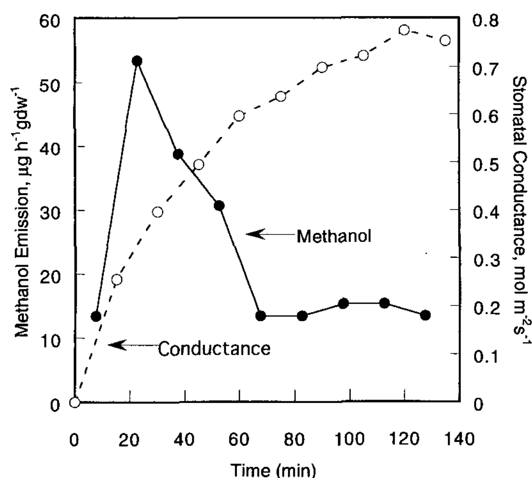

Figure 5. Transient methanol fluxes from leaves are seen in the morning. In this experiment an older *P. vulgaris* stem (node 4) bearing several trifoliates was cut under water from a plant kept in the dark overnight and until the start of the experiment. One of the leaves was placed in the leaf cuvette in the light (30°C, 500 μmol m^{-2} s^{-1}), and sampling was initiated immediately and continued until steady-state methanol emission and leaf conductance were obtained. Methanol was assayed by the methanol oxidase/Fluoral-P procedure. These results were replicated several times. gdw, g dry weight.

tudinally, so that only the petiole was in contact with cuvette seal, or (b) in the normal transverse orientation, so that two leaf regions were in contact with the cuvette seal. The resulting methanol fluxes from leaves of similar age (in the light) were virtually identical, 29.8 versus 34.0 μg g^{-1} dry weight h^{-1}, regardless of leaf orientation; this result was replicated in a second experiment. These results suggest that for this species physical damage to the leaf was not an important factor in methanol emission rate. As with bean leaves, Russian olive and aspen leaves sampled in the early morning showed a similar pattern of higher methanol fluxes, followed by lower steady-state fluxes after photosynthesis and stomatal conductance had maximized.

Leaf Methanol Emission and Stomatal Conductance

Using a highly controlled leaf cuvette that we have used extensively in isoprene emission measurements (Monson and Fall, 1989; Monson et al., 1994), in preliminary experiments we examined the effect of light on current photosynthesis and methanol emission. Leaves of *P. vulgaris* were exposed to varying light intensities in conditions of constant temperature and humidity. Methanol emission, stomatal conductance, and CO_2 assimilation were measured under each light treatment. Measurements were made during steady-state conditions as well as when the leaves were in the process of responding to altered light levels. This methodology allowed some separation of the effects of light on photosynthesis and stomatal conductance. Methanol emission from leaves was positively correlated with light intensity (r = 0.64) and with CO_2 assimilation (r = 0.66), although methanol emission was not eliminated after 4 h in the dark or when CO_2-free air was used (data not shown). There was a stronger relationship between stomatal conductance and methanol emission, with methanol emission increasing with increasing stomatal conductance (r = 0.83). As will be shown below, this relationship holds for leaves of the same developmental age but not for leaves of different ages.

To directly demonstrate the linkage between methanol fluxes and stomatal conductance, we measured leaf methanol emission during the onset of leaf photosynthesis and then after closing stomates by (a) cutting the petiole or (b) adding ABA to the transpiration stream. These results are shown in Figure 6. In each case, bean leaf methanol emission rate decreased with decreasing stomatal conductance but did not exactly parallel stomatal conductance. When stomates were induced to close, methanol emission rate decreased 64 to 100%, although in most experiments the decline in methanol emission rate lagged behind stomatal closure (Fig. 6). This lag may be due to a slower volatilization of some methanol deposited or condensed on the leaf surface. Nevertheless, the decline of methanol emission, in some cases to below the detection limit, after stomates were closed clearly suggests that the alcohol was derived from within bean leaves. Similar results were obtained with aspen leaves that were treated with ABA. In two different experiments, ABA inhibition of stomatal conductance (60–94%) produced a 83 to 84% decrease in methanol flux from the control values of 7.4 to 10.9 μg g^{-1} dry weight h^{-1}.

Detection of Free Methanol in Leaves

We attempted to use the methanol oxidase/Fluoral-P procedure to determine whether a free methanol pool exists in leaves of the plants studied here, but the procedure was complicated by interferences due to (a) fluorescent materials in leaf extracts, (b) fluorescent products from reaction of Fluoral-P with cell components other than methanol, and (c) variable inhibition of the methanol oxidase reaction by unknown leaf components. Alternatively, we froze and ground leaves in liquid nitrogen, prepared neutralized, perchloric acid extracts, and analyzed these extracts by direct injection onto a GC column. This procedure is similar to that developed by Obendorf et al. (1990) but includes a perchloric acid extraction step to inactivate pectin methylesterase activity and deproteinize the leaf extracts. For bean leaves from greenhouse-grown plants we could clearly demonstrate the presence of free methanol: young, fully expanded leaves contained 26.8 ± 14.6 μg methanol g^{-1} fresh weight (n = 8; range 10.4–58.8 μg methanol g^{-1} fresh weight), whereas the oldest true leaves on the same plants contained 10.0 ± 3.8 μg methanol g^{-1} fresh weight (n = 8; range 6.9–18.7 μg methanol g^{-1} fresh weight).

Leaf Development and Methanol Emission Rate

The effect of leaf development on methanol emission was examined in several experiments with greenhouse-grown plants, including bush bean, soybean, and cottonwood. For bush beans, methanol emission was highest in the youngest leaves measured and decreased linearly with increasing

leaf age (Fig. 7A). However, in another leguminous plant species, soybean, we found that methanol emission increased with leaf age up to leaf node 4 before declining in older leaves (Fig. 7B). In the experiment shown in Figure 7B, leaf 6 was observed to be the first fully expanded soybean leaf.

We repeated this experiment using cottonwood leaves. This species has the experimental advantage that an LPI has been established, which allows the comparison of a number processes throughout the course of leaf develop-

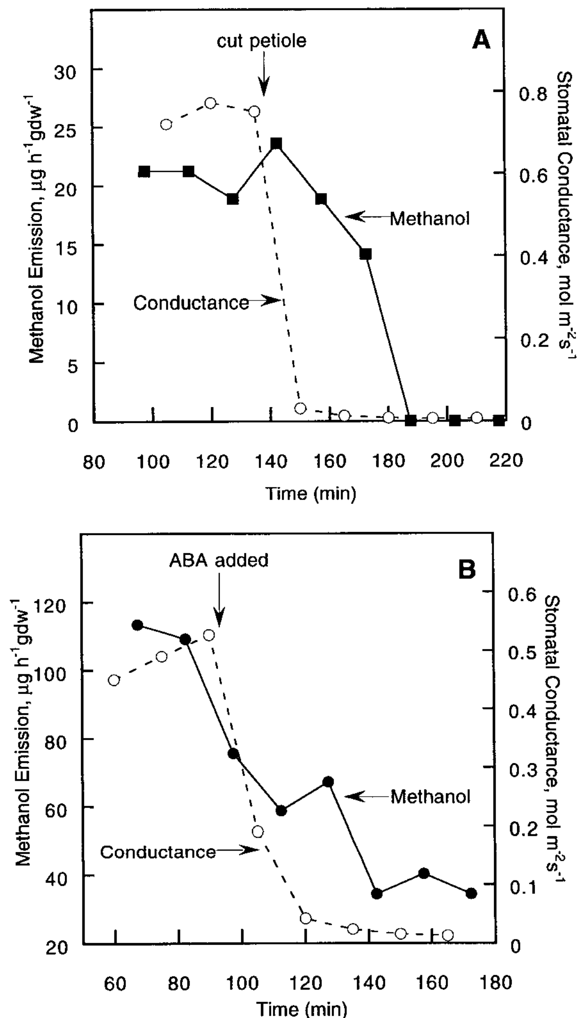

Figure 6. Relationship between methanol emission and stomatal conductance of leaves of *P. vulgaris*. A, The effect of excision of the petiole on methanol emission and stomatal conductance. In this experiment, an older stem (node 4) was treated as described in Figure 5; the petiole of the leaf sampled was cut at the time indicated on the figure after steady-state methanol emissions and leaf conductance were attained. This experiment was replicated two other times. B, The effect of ABA treatment on methanol emission and stomatal conductance. In this experiment, a young stem (node 2) was cut as above and was incubated in the light for approximately 45 min before sampling began; 20 μM (±)-*cis-trans*-ABA was added to the solution bathing the cut stem at the time shown. For both experiments, methanol was assayed by the methanol oxidase/Fluoral-P procedure. gdw, g dry weight.

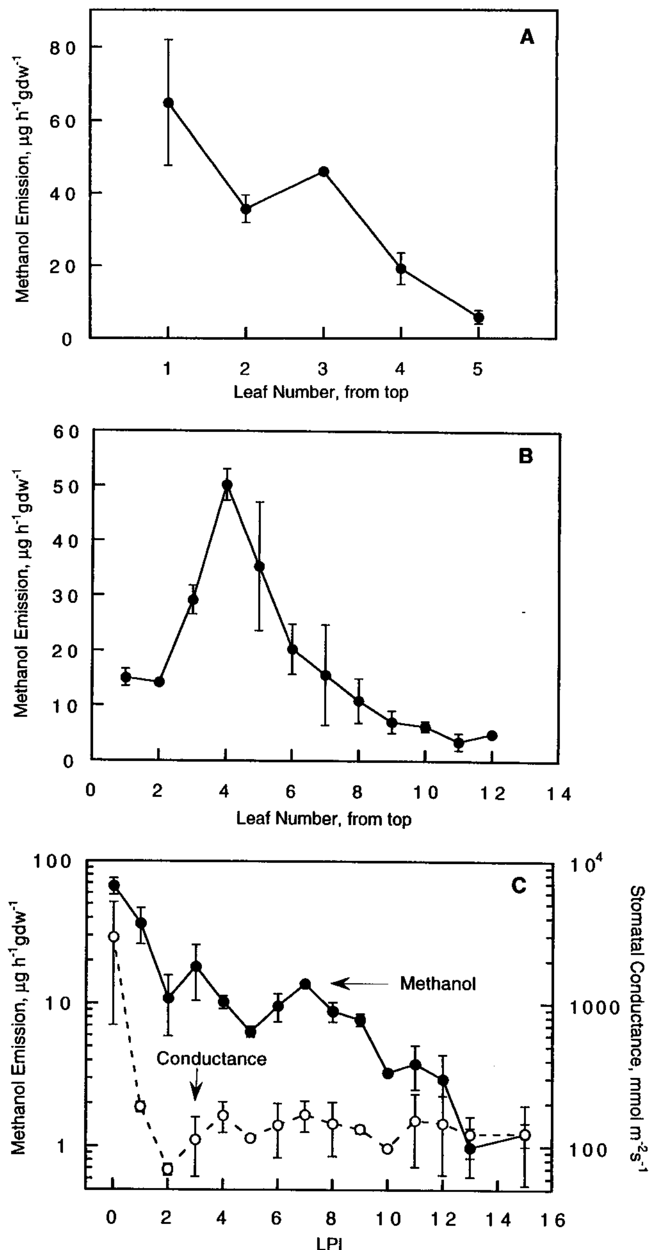

Figure 7. Relationship between methanol emission and leaf development in *P. vulgaris* (A), *G. max* (B), and *P. deltoides* (C). Data are the means ± SE of two replicate plants. For bean and soybean leaves, leaf age is expressed as node number from the top of the plant. For soybean, leaf 6 was observed to be the first fully expanded leaf. For cottonwood, the LPI is that described by Larson and Isebrands (1971) as detailed in the text; LPI 6 is the first fully expanded leaf in this system. Stomatal conductance is also shown for cottonwood leaves in C. Methanol was assayed by the GC procedure. gdw, g dry weight.

ment in greenhouse-grown plants. In this system, the first unfolding leaf of 2 cm in length is designated LPI 0, and LPI 6 is the first fully expanded leaf and the first leaf to become a net source of carbon to the plant (Larson and Isebrands, 1971; Larson and Dickson, 1973). Methanol emission declined in a log-linear fashion with increasing leaf age in *P. deltoides* (Fig. 7C). This decrease was not

paralleled by changes in stomatal conductance; for example, from leaf LPI 3 to 13, methanol emission rate declined about 20-fold, whereas stomatal conductance was relatively constant, approximately 120 mmol m^{-2} s^{-1} (Fig. 7C).

Even the oldest leaves of greenhouse-grown plants, and the cotyledons in the case of soybeans and bush beans, continued to emit significant amounts of methanol. Similarly, cut branches of field-grown aspen continued to emit methanol even when leaves began to senesce and turn yellow; analysis of such yellow leaves gave methanol flux rates of 6.8 ± 3.2 μg methanol g^{-1} dry weight h^{-1} at 30°C (n = 9). These flux rates are about 30% of that of typical young, fully expanded aspen leaves.

DISCUSSION

We have described a new method for assaying gas-phase methanol fluxes from leaves. Leaf gases are passed through a solution containing methanol oxidase and the formaldehyde-trapping reagent, Fluoral-P. Because of its miscibility with water, methanol is effectively stripped from the gas stream and is converted to a stable, fluorescent product under very mild reaction conditions. In addition to providing a simple, high-yield method for methanol detection, this procedure also provides a specific method for validating the presence of methanol; with the exception of formaldehyde there is virtually no interference from other volatile leaf alcohols or aldehydes, such as ethanol, acetaldehyde, and hexanal (Compton and Purdy, 1980; Klavons and Bennett, 1986; Dong and Dasgupta, 1987). Confirmation that the fluorescent product resulting from reaction of leaf gases with methanol oxidase and Fluoral-P is the expected dihydropyridine product, DDDP, can easily be carried out by HPLC analysis (Fig. 3). In the leaves analyzed here, only traces of fluorescent product (i.e. formaldehyde) were detected in the absence of methanol oxidase in the reaction mixture. This suggests that little free formaldehyde was present in leaf gases sampled here. Growing leaves are more likely to be a sink than a source for formaldehyde, since some plants contain formaldehyde dehydrogenase activity and metabolize this aldehyde (Giese et al., 1994).

Methanol fluxes from leaf surfaces were correlated with stomatal distribution and conductance. Although methanol emission from leaves increased with increasing light intensities, it is apparent that this result is due to the effects of light intensity on stomatal conductance rather than any direct effect of light itself or the effect of light through photosynthesis. Also, methanol emission was observed in the dark when stomates opened spontaneously (MacDonald and Fall, 1993) and in CO_2-free conditions (data not shown), which would rule out any close linkage to photosynthesis. In addition, we observed that (a) the majority of methanol emitted from hypostomatous leaves occurred from the abaxial surfaces and (b) methanol was emitted at similar rates from both abaxial and adaxial surfaces of amphistomatous leaves (Fig. 4), consistent with the linkage of methanol fluxes to stomatal conductance. The direct laboratory demonstration of decreased methanol emission rates with decreased stomatal conductance was seen when stomates were induced to close by excision of leaf petioles or administration of ABA (Fig. 6). In these experiments, decreases in methanol fluxes lagged behind decreases in stomatal conductance, perhaps because some of the methanol flux is from a film of the alcohol that has condensed on external leaf surfaces. The existence of such a surface film would explain large transient fluxes of methanol seen with leaves tested in the early morning (Fig. 5; also fig. 2 of MacDonald and Fall, 1993).

Although stomatal conductance apparently has substantial control over the short-term rate of methanol emission, it is likely that factors that affect the rate of methanol synthesis, such as temperature and leaf age, would have long-term effects on methanol emission, as equilibria are established between changes in internal methanol concentrations and stomatal opening. Here we observed that the stage of leaf development has a profound effect on methanol emission rate and that different plants display different developmental patterns of methanol emission. These different patterns are not readily explained, in part because the biochemical source of methanol in leaves is not known with certainty. Obendorf et al. (1990), who measured the accumulation of free methanol in maturing soybean seeds, reviewed earlier observations of methanol production during seed growth and maturation and suggested that pectin methylesters in cell walls are a likely source of methanol. Methanol is known to be a product of pectin demethylation in the cell walls of roots, stems, leaves, and fruits by pectin methylesterase (reviewed by Gaffe et al., 1994). Pectin demethylation occurs during growth and development as well as during aging and senescence of plant tissues. Other possible methanol sources could be C_1 intermediates of the tetrahydrofolate pathway (Cossins, 1987), protein methyltransferase and protein repair reactions that occur in all plant tissues (Mudgett and Clarke, 1993), and fungal degradation of lignin in plant secondary cell walls (Ander et al., 1985).

Methanol formation in leaves may be associated with cell-wall loosening during cell expansion and with the formation of intercellular air spaces (McCann and Roberts, 1991; Levy and Staehelin, 1992). It has been hypothesized that pectin demethylation occurs in the formation of intercellular air spaces such as would occur during leaf expansion (Knox et al., 1990; McCann and Roberts, 1991). If methanol emission is exclusively the result of this demethylation, it should be highest in younger, rapidly expanding leaves. The developmental pattern displayed by *G. max* fits this hypothesis well. Leaves undergoing the most growth by cell expansion are those intermediate between the youngest and the first fully expanded leaves (Sunderland, 1960). In our experiments with *G. max*, these immature, presumably rapidly expanding leaves emitted methanol at the greatest rates (Fig. 7B). However, in *P. vulgaris* and *P. deltoides*, methanol emission declined continually from the very youngest leaves (Fig. 7, A and C). It is doubtful that the greatest rates of formation of intercellular air spaces occurs in the very youngest leaves of these species.

Other factors that may influence methanol emission rates during leaf development include (a) methanol pool size, which may vary with changing metabolism, and (b) leaf methylotrophic bacterial populations. Since plants are able to metabolize methanol (Cossins, 1964), the amount of methanol emitted from leaves may represent only a fraction of that produced within the leaves. Methanol metabolism in plant cells might change during the course of leaf development. The leaves of most or all plants harbor methylotrophic bacteria that can utilize methanol as their sole carbon source (Corpe and Rheem, 1989); sometimes, as in the cases of bush beans and soybeans, these bacteria are the dominant viable bacterial species that can be isolated from leaves (Hirano and Upper, 1991; Holland and Polacco, 1994). Such methylotrophs are seed borne in the case of soybean (Holland and Polacco, 1994). It is probable, but not proven, that methylotrophs consume leaf methanol; as a result, methanol fluxes from leaves might be mediated by the population size and metabolic activity of these bacteria. We are currently working to clarify the significance of methylotrophic bacteria to methanol emission fluxes.

Given the magnitude of the methanol fluxes we measured, it seems probable that leaves contain a pool of free methanol. Although there are numerous reports of free methanol in developing plant tissues and in the volatiles released from maturing and germinating seeds (reviewed by Obendorf et al., 1990), there are few reports of the methanol contents of leaves. Corpe and Rheem (1989) found 1 to 2 mg methanol g^{-1} fresh weight in clover leaves, with higher levels of alcohol released when leaves were treated with detergent. They suggested that the waxy cutin layer of the leaves deters the rapid release of methanol to the atmosphere, and as a result methanol may be concentrated in the cutin layer, where it could then be available for metabolism by leaf surface methylotrophic bacteria. Since these workers used a microdiffusion method (Feldstein and Klendshoj, 1954) for assaying methanol, it is possible that some of the methanol detected resulted from breakdown of potential precursors, such as pectin methylesters (discussed above). We measured leaf methanol by extracting leaf metabolites in cold perchloric acid, a method that inactivates most cellular enzymes (Leegood, 1993). Analysis of methanol in such leaf extracts by GC demonstrated that free methanol is present in bean leaves, at levels ranging from about 10 to 27 $\mu g\ g^{-1}$ fresh weight; these values are much lower than those reported by Corpe and Rheem (1989) for clover leaves. It is currently difficult to reconcile these low methanol levels with the relatively high fluxes of methanol from the same leaves. For example, if young bush bean leaves contain 27 μg methanol g^{-1} fresh weight, or about 190 μg methanol g^{-1} dry weight, and emit on the order of 60 μg methanol g^{-1} dry weight (Fig. 7A), this would suggest that a large fraction of leaf methanol is constantly emitted when stomates are open. On the other hand, since methanol has a large water:air partition coefficient (i.e. about 3700 at 34°C; Jones et al., 1990), it seems likely that only a small fraction of a leaf aqueous methanol pool would partition into the leaf air space and be emitted. It is possible that some leaf methanol is highly concentrated in cell walls at the site of action of pectin methylesterase (Knox et al., 1990; Levy and Staehelin, 1992), where there is a lower effective water content and relatively high surface contact with the intercellular air space. We are currently working to validate our methanol measurements and better correlate leaf methanol pool size with methanol emission rate.

A plausible model to explain the range of methanol fluxes from fully expanded leaves of different ages, at a given leaf temperature, is that the flux is primarily a function of leaf methanol pool size and stomatal conductance. Thus, as leaves age, the pool of free methanol might decrease, leading to declining methanol fluxes. For example, the older cottonwood leaves shown in Figure 7C have relatively constant stomatal conductance but show a decline in methanol fluxes with increasing leaf age. When a reliable method for measuring leaf methanol pools is available, it should be possible to test this idea.

It seems likely that the leaf methanol fluxes described in this paper and their linkage to stomatal conductance could explain the observed daily increase in methanol in forest air (P.D. Goldan, W.C. Kuster, F.C Fehsenfeld, S.A. Montzka, unpublished data). Since leaves of all the C_3 tree and crop species we have tested emit methanol at significant rates, it is possible that a large fraction of atmospheric methanol is a product of plant metabolism. The global magnitude of leaf methanol fluxes to the atmosphere will require extensive field measurements. The method for methanol flux analysis described here may be useful for such measurements.

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