

Thermotolerance of Leaf Discs from Four Isoprene-Emitting Species Is Not Enhanced by Exposure to Exogenous Isoprene¹

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The effects of exogenously supplied isoprene on chlorophyll fluorescence characteristics were examined in leaf discs of four isoprene-emitting plant species, kudzu (*Pueraria lobata* [Willd.] Ohwi.), velvet bean (*Mucuna* sp.), quaking aspen (*Populus tremuloides* Michx.), and pussy willow (*Salix discolor* Muhl). Isoprene, supplied to the leaves at either 18 $\mu\text{L L}^{-1}$ in compressed air or 21 $\mu\text{L L}^{-1}$ in N_2 , had no effect on the temperature at which minimal fluorescence exhibited an upward inflection during controlled increases in leaf-disc temperature. During exposure to 1008 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ in an N_2 atmosphere, 21 $\mu\text{L L}^{-1}$ isoprene had no effect on the thermally induced inflection of steady-state fluorescence. The maximum quantum efficiency of photosystem II photochemistry decreased sharply as leaf-disc temperature was increased; however, this decrease was unaffected by exposure of leaf discs to 21 $\mu\text{L L}^{-1}$ isoprene. Therefore, there were no discernible effects of isoprene on the occurrence of symptoms of high-temperature damage to thylakoid membranes. Our data do not support the hypothesis that isoprene enhances leaf thermotolerance.

More than 4 decades ago emission of isoprene (2-methyl-1,3-butadiene) from leaves of higher plants was first described (Sanadze, 1957). Since that time understanding of the biochemistry and environmental controls of isoprene emission has grown considerably, along with an appreciation for the role that phytochemical isoprene plays in critical oxidative atmospheric processes (for reviews, see Sharkey et al., 1991; Sharkey, 1996; Lerda et al., 1997). However, a function for isoprene in leaves remained elusive until Sharkey and Singsaas (1995) reported evidence from kudzu (*Pueraria lobata* [Willd.] Ohwi.) that isoprene protected thylakoid membranes against damage induced by high leaf temperatures. Under conditions that suppressed endogenous isoprene synthesis, isoprene supplied exogenously at physiologically realistic concentrations resulted in an increase in the temperature at which chlorophyll fluorescence emission exhibited a distinct upward inflection (Sharkey and Singsaas, 1995; Singsaas et al., 1997). Furthermore, it was reported that a linear relationship exists be-

tween the concentration of supplied isoprene and the extent of its effect on leaf thermotolerance (Singsaas et al., 1997). Subsequently, it was reported that certain monoterpenes, another class of phytochemical hydrocarbons, protect the photosynthetic apparatus of *Quercus ilex* L. from thermal damage (Loreto et al., 1998).

High temperature-induced inflection of chlorophyll fluorescence has been used widely as an indicator of thermal damage and correlates with the temperature at which leaves experience significant tissue necrosis (Bilger et al., 1984). Dislocation between the light-harvesting complexes and PSII reaction centers due to excessive membrane fluidity is thought to underlie this phenomenon (Armond et al., 1980), although Yamane et al. (1997) suggested that denaturation of PSII reaction center proteins may be involved as well.

Current understanding of leaf isoprene synthesis is largely consistent with the hypothesis that isoprene protects thylakoids from thermal damage. Isoprene is hydrophobic and presumably partitions into the interior of membrane bilayers. The final step in isoprene formation is catalyzed by isoprene synthase (Silver and Fall, 1995), an enzyme with stromal and thylakoid-bound isoforms (Wilderdmuth and Fall, 1998). This location for isoprene production would allow for its direct diffusion into thylakoids. The capacity to synthesize isoprene is found only in individuals acclimated to warm temperatures. In addition, isoprene emission rate exhibits a strong positive temperature response (Sanadze and Kursanov, 1966; Monson and Fall, 1989; Loreto and Sharkey, 1990). In fact, a Q_{10} for leaf isoprene emission as high as 8 has been reported (Sharkey and Loreto, 1993). High temperatures often occur simultaneously with other environmental factors, such as water stress, which lead to stomatal closure. Whereas the rate of isoprene emission is relatively unaffected by stomatal conductance (Monson and Fall, 1989; Fall and Monson, 1992), stomatal closure will increase isoprene concentrations inside the leaf at the time when enhanced thermotolerance is needed most. Isoprene concentrations inside leaves of a high isoprene-emitter such as kudzu can exceed 30 $\mu\text{L L}^{-1}$ (Singsaas et al., 1997).

Abbreviations: F_o , minimal chlorophyll fluorescence; F_v/F_m , ratio of variable to maximal fluorescence giving the maximum quantum efficiency of PSII photochemistry; Q_A , primary and secondary electron accepting plastoquinones of PSII; T_C , critical temperature for leaf damage.

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In the present study we surveyed the effect of isoprene on the thermotolerance of four isoprene-emitting plant species, kudzu, velvet bean (*Mucuna* sp.), quaking aspen (*Populus tremuloides* Michx.), and pussy willow (*Salix discolor* Muhl.). We used experimental methods analogous to those of Bilger et al. (1984) and Singaas et al. (1997) to determine the inflection temperature of chlorophyll fluorescence from leaf discs in the presence or absence of 18 to 21 $\mu\text{L L}^{-1}$ exogenously supplied isoprene. In addition, we measured the effect of exogenous isoprene on maximum quantum efficiency of PSII photochemistry (F_v/F_m) in kudzu during an increase in leaf-disc temperature.

MATERIALS AND METHODS

Kudzu (*Pueraria lobata* [Willd.] Ohwi.) and velvet bean (*Mucuna* sp.; J.L. Hudson, La Honda, CA) seeds were germinated on moist paper towels and transferred to 19-L pots filled with 3:1 Growing Mix no. 2 (Farfard, Agawam, MA): perlite. Plants were grown in a greenhouse exposed to direct full sunlight, watered daily, and fertilized with a complete nutrient medium three to four times weekly. Quaking aspen (*Populus tremuloides* Michx.; 1.5 m) and pussy willow (*Salix discolor* Muhl.; 2 m) trees were purchased at local nurseries. Trees were grown outdoors, exposed to direct full sunlight for a portion of the day, watered daily, and fertilized with Osmocote nutrient pellets (Scotts-Sierra, Marysville, OH) mixed into the soil medium. Leaf discs from mature, fully developed leaves were used for all of the experiments. During illumination in room air leaf discs of kudzu, velvet bean, and aspen emitted isoprene (data not shown; emission from leaf discs of pussy willow was not determined).

Determination of the T_C

The T_C measured as the temperature at which F_o exhibited an upward inflection, was determined using a modification of the method described by Bilger et al. (1984). Whole leaves were collected during midmorning and adapted to very low light ($2 \mu\text{mol m}^{-2} \text{s}^{-1}$) for between 1 and 6 h on moist paper towels in an unsealed plastic chamber. The duration of low-light adaptation had no effect on T_C (data not shown). Chlorophyll fluorescence emission was monitored from 2.54-cm² leaf discs in an LD-2 chamber (Hansatech, King's Lynn, Norfolk, UK) using a PAM 101 chlorophyll fluorometer (Walz, Effeltrich, Germany; settings: intensity = 8, gain = 7, damping = 7, and measuring beam frequency = 1.6 kHz). The fluorescence signal was recorded with a strip-chart recorder. A port on the leaf chamber was modified to bring the end of the fiber optics closer to the leaf disc. A nonfunctional platinum/silver electrode covered with Teflon tape was inserted in the chamber to maintain its seal. The foam spacer commonly located between metal screens in the chamber during measurements of O_2 evolution was replaced with wadded glass wool to minimize the reaction of isoprene with materials in the chamber. Leaf-disc temperature was controlled by a circulating water bath.

Prior to collecting fluorescence measurements we derived a reproducible linear relationship between water-bath and leaf-disc temperatures during increases from 32°C to 56°C. This relationship is described by the following equation: Leaf temperature = $(0.867 \times \text{water-bath temperature}) + 3.63$ ($r^2 = 0.999$; the relationship was derived from four independent temperature increases). During fluorescence measurements the rate of leaf-disc temperature increase was approximately $1.7^\circ\text{C min}^{-1}$. Leaf discs were exposed to very-low-intensity far-red illumination (less than $1 \mu\text{mol m}^{-2} \text{s}^{-1}$) from a Hansatech LS-2 light source with the appropriate filters during measurements to maintain PSII in the oxidized state (Bilger et al., 1984). Immediately prior to a measurement, a leaf disc was removed from a low-light-adapted leaf and placed in the chamber under measuring conditions (30°C and low-intensity far-red illumination under the appropriate gas) for 5 min before the temperature increase was initiated.

To determine the effect of exogenous isoprene on T_C , leaf discs collected from opposite sides of the leaf midvein were exposed to either a control gas (compressed air [Singaas et al., 1997] or N_2) or isoprene in compressed air (18 $\mu\text{L L}^{-1}$) or N_2 (21 $\mu\text{L L}^{-1}$) during measurement. Gases were humidified by passage through a ceramic diffuser in a flask of distilled water and flowed through the leaf chamber at a rate of $50 \text{ cm}^3 \text{ min}^{-1}$ (chamber volume was approximately 5.5 cm^3). All of the tubing used to direct gas from the cylinders to the measuring chamber was Teflon or glass, with the exception of two short connecting pieces. Potential effects of temporal artifacts on T_C were eliminated by conducting control and isoprene treatments immediately after one another and alternating (from leaf to leaf) between conducting the control or isoprene treatment first. The gas exiting the chamber was directed into a Fast Isoprene Sensor (Hills Scientific, Boulder, CO; described by Hills et al., 1991) to verify that the desired isoprene concentration had been achieved during the experimental treatment. The Fast Isoprene Sensor was also used to verify that control leaf discs exhibited no endogenous isoprene production. The "degree of thermoprotection" is defined

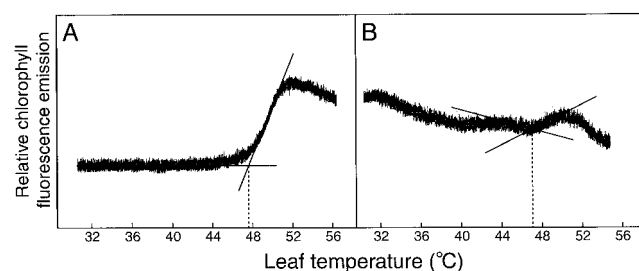


Figure 1. Representative traces of the response of chlorophyll fluorescence emission to increasing leaf temperature. A, The response of F_o during exposure to compressed air (F_o traces during exposure to N_2 as well as room air looked similar). B, The response of steady-state fluorescence during illumination with $1008 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and exposure to N_2 . The rate of leaf temperature increase was $1.7^\circ\text{C min}^{-1}$. Both traces are from kudzu; however, the responses of the other species were similar. Methods for determining T_C and the thermal breakpoint are exemplified.

Table I. T_c from leaf discs of four isoprene-emitting species exposed to compressed air or $18 \mu\text{L L}^{-1}$ isoprene in compressed air

T_c was measured as the temperature at which F_o exhibited an upward inflection (see Fig. 1). Leaf discs for each replicate measured in compressed air, with and without isoprene, were collected from opposite sides of the midvein of the same leaf. Paired Student's t tests examined if the degree of thermoprotection was different from zero. iso, Isoprene. sds are given.

Species	No. of Replicates	No. of Leaves Sampled	No. of Plants Sampled	Compressed Air (ca) T_c	$18 \mu\text{L L}^{-1}$ Isoprene in Compressed Air T_c	Degree of Thermoprotection $T_c(\text{iso in ca}) - T_c(\text{ca})$	Paired Student's t Test (P value)
Kudzu	10	10	3	49.3 ± 0.9	49.3 ± 0.8	0.0 ± 0.8	0.95
Velvet bean	8	8	5	47.5 ± 0.5	47.6 ± 0.4	0.1 ± 0.7	0.85
Quaking aspen	8	8	3	49.1 ± 0.8	48.9 ± 0.6	-0.2 ± 0.9	0.47
Pussy willow	3	3	2	47.4 ± 1.5	47.4 ± 1.0	0.0 ± 0.5	1.00

as: T_c (isoprene exposure) – T_c (control). Paired Student's t tests were used determine whether isoprene had a statistically significant effect on T_c .

Determination of the Thermal Breakpoint of Steady-State Fluorescence during Exposure to Actinic Light

The temperature at which steady-state fluorescence exhibited an inflection, defined as the "thermal breakpoint," was measured from leaf discs of kudzu and velvet bean. Measurements of the thermal breakpoint were made in N_2 , in the presence and absence of $21 \mu\text{L L}^{-1}$ isoprene, to suppress the endogenous production of isoprene (Singsaas et al., 1997). The absence of endogenous isoprene production from control leaf discs in the presence of pure N_2 was confirmed by directing gas from the chamber outlet into the Fast Isoprene Sensor. Measurements were made as described above, except that leaf discs received no period of adaptation to the measurement conditions, measurements were made in $1008 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, and the gas flow rate was reduced somewhat in the middle of the temperature induction.

Determination of F_v/F_m

Changes F_v/F_m during an increase in leaf-disc temperature, in the presence and absence of exogenous isoprene, were assessed in kudzu under the conditions described for the determination of T_c . To measure F_m , a flashlamp (model KL1500, Walz) was used to direct a saturating pulse of light (approximately $2500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) through the fiber optics to the leaf disc.

RESULTS

A representative trace of F_o during an increase in leaf-disc temperature from 32°C to 56°C is presented in Figure 1A. This trace was measured under conditions defined by Bilger et al. (1984), i.e. low-light adaptation prior to measurement and weak far-red illumination during measurement, and allowed for the unambiguous determination of the T_c (Fig. 1A). No leaf isoprene emission was detected under these conditions (data not shown). Exogenously supplied isoprene, either $18 \mu\text{L L}^{-1}$ in compressed air or $21 \mu\text{L L}^{-1}$ in N_2 , had no significant effect on the T_c of kudzu, velvet bean, quaking aspen, or pussy willow, all of which are isoprene emitters (Tables I and II). The degree of thermoprotection, T_c (isoprene) – T_c (control), did not differ significantly from zero in any of the four species (Tables I and II). Therefore, exogenously supplied isoprene had no measured effect on thermotolerance under these conditions.

In the studies by Sharkey and Singsaas (1995) and Singsaas et al. (1997), the most profound effect of exogenous isoprene on chlorophyll fluorescence was observed during exposure to saturating actinic light ($1000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$). Therefore, we measured the temperature response of steady-state fluorescence from leaf discs illuminated with $1008 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. These measurements were conducted during exposure to N_2 to suppress endogenous isoprene production (Monson and Fall, 1989; Sharkey and Singsaas, 1995; Singsaas et al., 1997). Under these conditions fluorescence inflections were less apparent and somewhat difficult to interpret (Fig. 1B). During exposure to approximately one-half full sunlight in N_2 , the effect of increasing temperature on chlorophyll fluorescence cannot be attributed to thylakoid membrane stability

Table II. T_c from leaf discs of four isoprene-emitting species exposed to N_2 , or $21 \mu\text{L L}^{-1}$ isoprene in N_2

T_c was measured as the temperature at which F_o exhibited an upward inflection (see Fig. 1). Leaf discs for each replicate measured in N_2 , with and without isoprene, were collected from opposite sides of the midvein of the same leaf. Paired Student's t tests examined if the degree of thermoprotection was different from zero. iso, Isoprene. sds are given.

Species	No. of Replicates	No. of Leaves Sampled	No. of Plants Sampled	N_2 T_c	$21 \mu\text{L L}^{-1}$ Isoprene in N_2 T_c	Degree of Thermoprotection $T_c(\text{iso in } \text{N}_2) - T_c(\text{N}_2)$	Paired Student's t Test (P value)
Kudzu	9	6	3	46.8 ± 1.3	46.7 ± 1.3	-0.1 ± 1.1	0.81
Velvet bean	7	6	2	46.3 ± 1.4	46.4 ± 1.4	0.1 ± 0.8	0.71
Quaking aspen	10	10	8	46.3 ± 2.0	46.6 ± 2.2	0.3 ± 1.1	0.49
Pussy willow	8	8	4	47.3 ± 1.1	47.5 ± 0.9	0.2 ± 0.9	0.54

Table III. The thermal breakpoint of leaf discs of kudzu and velvet bean illuminated with 1008 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and exposed to N_2 or $21 \mu\text{L L}^{-1}$ isoprene in N_2

Thermal breakpoint is defined as the temperature at which steady-state fluorescence exhibited an upward inflection (see Fig. 1). Leaf discs for each replicate, with and without isoprene, were collected from opposite sides of the midvein of the same leaf. Paired Student's *t* tests examined if the degree of thermoprotection, defined here as the (thermal breakpoint in $21 \mu\text{L L}^{-1}$ isoprene in N_2 -thermal breakpoint in N_2), was different from zero. SDs are given.

Species	No. of Replicates	Thermal Breakpoint		Degree of Thermoprotection	Paired Student's <i>t</i> Test (P value)
		N_2	$21 \mu\text{L L}^{-1}$ Isoprene in N_2		
Kudzu	4	46.1 ± 0.5	46.3 ± 1.0	0.2 ± 0.6	0.42
Velvet bean	4	46.2 ± 1.4	46.6 ± 1.7	0.4 ± 0.6	0.23

alone; temperature effects on photochemistry, xanthophyll cycle-dependent energy dissipation, and zeaxanthin-mediated changes in membrane stability (Havaux, 1998) could also influence the observed fluorescence traces. In addition, Q_A is likely to be highly reduced during exposure to these experimental conditions, and Bilger et al. (1984) noted the importance of maintaining Q_A in the oxidized state during determinations of T_C . Because of these concerns, we chose to define the temperature of fluorescence inflection measured under $1008 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ in an N_2 atmosphere as the "thermal breakpoint", as opposed to T_C , since the conditions for the determination of T_C were not satisfied. The thermal breakpoint and T_C of kudzu and velvet bean were similar (Tables II and III). Isoprene supplied exogenously at $21 \mu\text{L L}^{-1}$ had no effect on the thermal breakpoints of these two plant species and therefore offered no statistically significant enhancement of thermotolerance (Table III).

Decreases in the F_v/F_m of low-light-adapted leaves traditionally have been thought to reflect damage to the photosynthetic apparatus (Tyystjärvi et al., 1992; Aro et al., 1994; Osmond, 1994). However, it was recently suggested that persistent xanthophyll cycle-dependent energy dissipation may also be involved (Demmig-Adams et al., 1998). Increasing leaf-disc temperatures resulted in a profound decrease in F_v/F_m between 42°C and 50°C (Fig. 2). Exoge-

nous isoprene at $21 \mu\text{L L}^{-1}$ had no effect on this decrease in F_v/F_m in kudzu (Fig. 2).

DISCUSSION

Isoprene supplied exogenously at physiologically realistic concentrations had no effect on the temperature at which symptoms of thermal damage to thylakoids appeared (Tables I–III) or on decreases in F_v/F_m (Fig. 2) of leaf discs from four isoprene-emitting species subjected to controlled increases in leaf temperature. Therefore, our observations do not support the hypothesis that isoprene increases the thermotolerance of isoprene-emitting plant species.

Our findings differ from those reported by Sharkey and Singaas (1995) and Singaas et al. (1997). We have no certain explanation for this discrepancy, although there were several differences in the experimental approaches used that should be noted. Perhaps the most important difference is that we used leaf discs, whereas detached intact leaves were used primarily by Sharkey and Singaas (1995) and Singaas et al. (1997). It is possible that a difference in the physiological effect of a wound resulting from leaf-disc excision versus leaf detachment underlies the contrasting observations. Wounding of adjacent leaves has been shown to affect foliar isoprene emission rate, although that effect included both increases and decreases in isoprene emission rate, depending on conditions (Loreto and Sharkey, 1993). A second difference was that plants in our study were exposed to warmer growth temperatures than those of Singaas et al. (1997; growth temperatures were not reported by Sharkey and Singaas, 1995). Our plants were grown either inside or beside a greenhouse in Boulder, Colorado, during summer and regularly experienced midday temperatures above 35°C , whereas maximum air temperatures experienced by the plants of Singaas et al. (1997) did not exceed 26°C . Whereas this may have influenced our results, the need for enhanced thermotolerance would be greater in warm-grown plants and perhaps should have biased our results in favor of observing a thermotolerance effect of isoprene. However, it should be noted that acclimation to warmer growth temperatures has been shown to lead to lasting biochemical differences, such as increases in the degree membrane lipid saturation (Pearcy, 1978), which presumably also protect

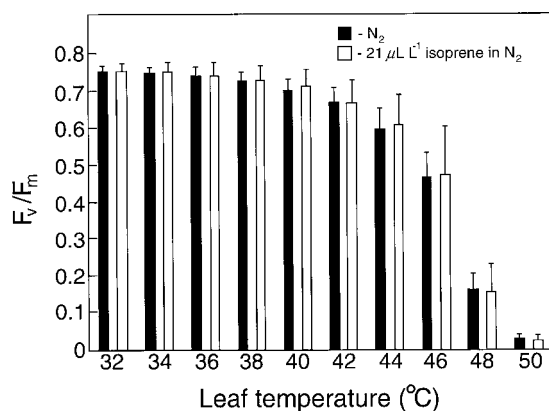


Figure 2. Changes in the F_v/F_m during an increase in the temperature of kudzu leaves exposed to N_2 (black bars) or $21 \mu\text{L L}^{-1}$ isoprene in N_2 (white bars). The rate of leaf temperature increase was $1.7^\circ\text{C min}^{-1}$. Error bars represent SD; $n = 3$.

membranes against high temperature damage. Such differences may explain why the fluorescence inflection temperatures we observed were slightly higher than those reported by Sharkey and Singaas (1995) and Singaas et al. (1997). Finally, we used a rate of leaf temperature increase of $1.7^{\circ}\text{C min}^{-1}$, whereas a rate of $1.0^{\circ}\text{C min}^{-1}$ was used by Singaas et al. (1997; rate of leaf temperature increase was not reported by Sharkey and Singaas, 1995). As was noted by Sharkey (1996), the volatile nature of isoprene makes it an ideal molecule to respond to the rapid, almost 10°C fluctuations in temperature experienced by canopy leaves under some conditions (Sharkey and Singaas, 1995). If isoprene emission evolved to aid plants coping with rapid temperature fluctuations, then our faster rate of leaf temperature increase better approximates relevant environmental conditions and should have also biased our results in favor of observing a thermotolerance effect of isoprene.

Although the function, if one exists, for isoprene in leaves remains an open question, we wish to emphasize that the data presented here do not disprove the thermotolerance hypothesis. They merely serve to weaken the current experimental evidence for thermotolerance. As was stated in the introduction, the thermotolerance hypothesis remains quite compelling because of its ability to explain the subcellular localization of isoprene synthesis, as well many of the short- and long-term responses of isoprene emission to the environment. It is possible that exogenously supplied isoprene, under the conditions imposed here, does not adequately approximate the effects of endogenous isoprene production and therefore does not allow for a clear demonstration of isoprene's role in leaves. Alternative means of investigating a function for isoprene production could shed light on this phenomenon.

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