

# Protection by Isoprene against Singlet Oxygen in Leaves

Hagit P. Affek and Dan Yakir\*

Department of Environmental Sciences and Energy Research, Weizmann Institute of Science, Rehovot 76100, Israel

Isoprene (2-methyl-1,3-butadiene) protection against effects of singlet oxygen was investigated in *Myrtus communis* and *Rhamnus alaternus*. In *M. communis*, singlet oxygen produced in the leaves by Rose Bengal (RB) led to a 65% decrease in net assimilation rates within 3 h, whereas isoprene emission rates showed either a 30% decrease at ambient CO<sub>2</sub> concentrations or a 70% increase under high CO<sub>2</sub>. In both cases, these changes led to an increase in calculated internal isoprene concentrations. The isoprene protection effect was directly demonstrated by fumigation of young (non-emitting) leaves, treated with RB or bromoxynil (simulating photoinhibition). There was 42% and 29% reduction in the damage to net assimilation compared with non-fumigated leaves for RB or bromoxynil, respectively. In *R. alaternus*, similar effects of RB on net assimilation were observed, and additional fluorescence measurements showed a significantly smaller decrease in  $F_v/F_m$  in isoprene-fumigated young leaves treated with RB (from 0.78 to 0.52), compared with non-fumigated leaves (from 0.77 to 0.27). The internal isoprene concentrations used in this study and possible rate of <sup>1</sup>O<sub>2</sub> production in leaves indicate that the protective effects observed should be beneficial also under natural conditions.

Isoprene (2-methyl-1,3-butadiene) is emitted by a variety of plant species (Harley et al., 1999; Kesselmeier and Staudt, 1999). It influences the trace-gas composition of the troposphere by reacting with OH radicals and NO<sub>x</sub> to generate tropospheric ozone (Trainer et al., 1987; Chameides et al., 1988; Ryerson et al., 2001). Isoprene also affects the oxidizing capacity of the atmosphere by scavenging OH radicals (Jacob and Wofsy, 1988) and may indirectly influence atmospheric methane accumulation. Models predicting ozone concentrations such as that of Trainer et al. (1987) use emission rates and inventories of isoprene, as well as other volatile organic compounds, and must consider physiological and environmental influences on emission rates.

Isoprene production can consume a few percent of the carbon fixed in photosynthesis but, despite much research, the role of isoprene emission is not fully understood. It was suggested to be protection against sharp temperature increases by dissolving in the thylakoid membranes and stabilizing hydrophobic interactions (Sharkey and Singsaas, 1995; Singsaas et al., 1997; Sharkey et al., 2001). However, this effect was not observed in all studies (Logan and Monson, 1999). Isoprene was also suggested to potentially provide a more general protection against stress conditions (Sharkey and Loreto, 1993) and in particular against photooxidative stress (Zeidler et al., 1997) and was recently shown to protect leaves against ozone (Loreto et al., 2001; Loreto and Velikova, 2001). Other alkenes were also found to increase thermotolerance in leaves (Sharkey et al., 2001). The well-

known ability of alkenes, such as isoprene, to react with singlet oxygen (<sup>1</sup>O<sub>2</sub>), ozone and OH radicals led us to test the hypothesis that isoprene may help to protect leaves against oxidative stress by reacting with <sup>1</sup>O<sub>2</sub> and other radicals.

Singlet oxygen is produced in leaves by interaction of molecular oxygen with triplet state chlorophyll, which is formed under conditions of excessive excitation. This may occur under high-light intensities or because of environmental stress that limits the use of the absorbed sunlight (Demmig-Adams, 1990). Here, we used Rose Bengal (RB) as a photosensitizer that produces <sup>1</sup>O<sub>2</sub> upon absorbing green light and the herbicide bromoxynil (BX) that increases the sensitivity of PSII to light, thus, producing <sup>1</sup>O<sub>2</sub> through photoinhibition (Krieger-Liszka and Rutherford, 1998).

In this study, we used the Mediterranean shrubs *Myrtus communis* and *Rhamnus alaternus*. Both are evergreen shrubs that grow in northern Israel and throughout the Mediterranean region (Heler and Livneh, 1982). *M. communis* is a high isoprene emitter (Hansen et al., 1997) and emits only small amounts of monoterpenes (Owen et al., 1997). To our knowledge, there is no information of emission from *R. alaternus*, however, two other *Rhamnus* spp. are known to emit isoprene (Kesselmeier and Staudt, 1999).

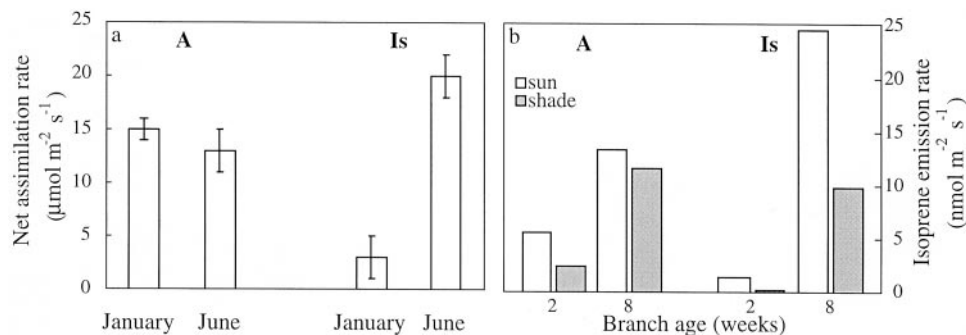
## RESULTS

### Isoprene Emission from *M. communis*

To characterize isoprene emission from *M. communis*, we examined rates of emission, seasonality, phenology, and CO<sub>2</sub> response. Large seasonal variations were observed in isoprene emission rates (Fig. 1a) with maximal rates during the summer and autumn

\* Corresponding author; e-mail dan.yakir@weizmann.ac.il; fax 972-8-9344124.

Article, publication date, and citation information can be found at [www.plantphysiol.org/cgi/doi/10.1104/pp.010909](http://www.plantphysiol.org/cgi/doi/10.1104/pp.010909).



**Figure 1.** Seasonal variations (a) and effects of branch age (b) on net assimilation (A) and isoprene emission rates (Is) from a shrub of *M. communis*. Measurements were performed at leaf temperature of 26°C and light intensity of 1,000  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . The seasonal variations were measured at intercellular  $\text{CO}_2$  concentration ( $c_i$ ) values between 450 and 500  $\mu\text{L L}^{-1}$ , and the effect of branch age was measured at ambient  $\text{CO}_2$  concentrations (approximately 350  $\mu\text{L L}^{-1}$ ).

(up to 30  $\text{nmol m}^{-2} \text{s}^{-1}$ ) and close to detection limit (approximately 0.2  $\text{nmol m}^{-2} \text{s}^{-1}$ ) in winter. Only small changes in net assimilation rates were observed over the same time (Fig. 1a).

Net assimilation and isoprene emission rates were measured in branches that developed from leaves marked at “age zero” (approximately 5  $\text{mm}^2$ ). Both net assimilation and isoprene emission rates increased with leaf age up to 2 months (Fig. 1b). Isoprene emission was first detected, however, only about 1 week later than net  $\text{CO}_2$  uptake in both sun and shade leaves. The lack of isoprene emission in young leaves was useful in fumigation experiments (see below). Throughout the experiment, both net assimilation and isoprene emission rates were higher in sun leaves than in shade leaves.

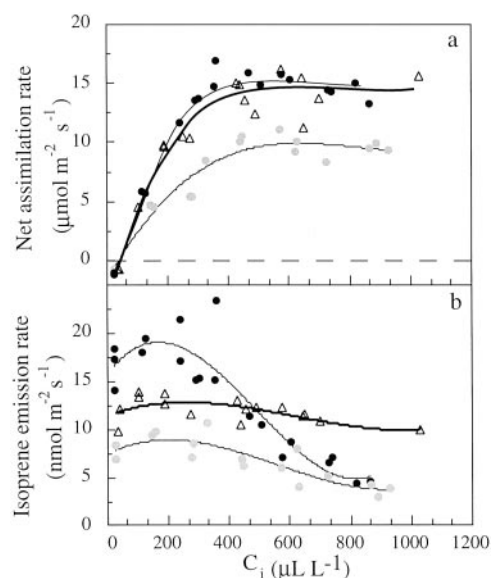
The short-term response of net  $\text{CO}_2$  assimilation and isoprene emission rates to changes in the intercellular  $\text{CO}_2$  concentration ( $c_i$ ) was measured at two leaf temperatures (26  $\pm$  0.5°C and 34  $\pm$  0.5°C). Emission rates were relatively constant at  $c_i$  values between 20 and 300  $\mu\text{L L}^{-1}$ , whereas net assimilation rates increased with  $c_i$  (Fig. 2). At  $c_i$  values of approximately 300 to 900  $\mu\text{L L}^{-1}$ , isoprene emission rates decreased by 55% (at 26°C), whereas net assimilation appeared to be  $\text{CO}_2$  saturated. The decrease of isoprene emission rates at high  $c_i$  values was much smaller at 34°C, and isoprene emission rates decreased only by approximately 25% between 300 and 1,000  $\mu\text{L L}^{-1}$  (Fig. 2b).

#### Effects of Singlet Oxygen in *M. communis*

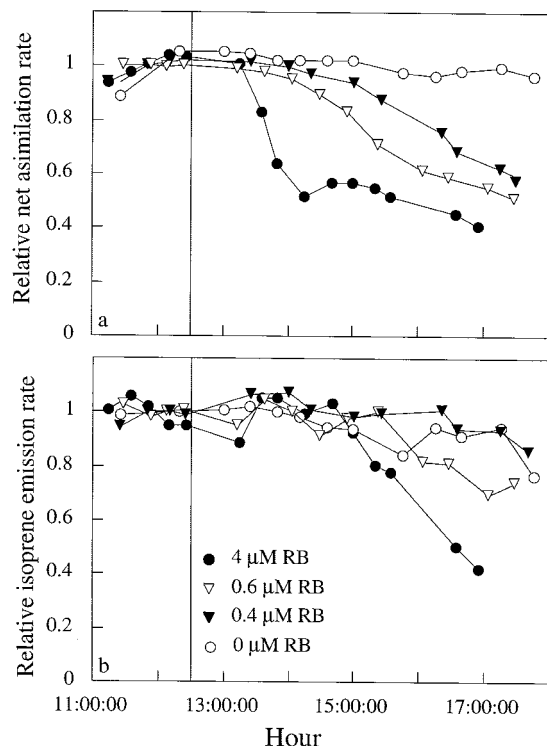
RB was used as a sensitizer to produce  $^1\text{O}_2$  under light, and its effect on net assimilation and isoprene emission rates was clearly concentration dependent (Fig. 3). Treatments with RB at ambient  $\text{CO}_2$  concentration resulted in a rather fast decrease in both net assimilation and isoprene emission rates (Figs. 3 and 4a), whereas lesions on the leaves were observed only after 2 d. In control plants (no RB), net assimilation and isoprene emission rates remained rela-

tively constant throughout the measurement day (Fig. 3).

An important check was made by repeating the treatment and control measurements using light filtered through purple zelofan but maintaining the same total photosynthetically active radiation at the leaf level (the zelofan filter had a broad absorption peak around  $\lambda_{\text{max}} = 567 \text{ nm}$ , as compared with  $\lambda_{\text{max}} = 547 \text{ nm}$  of RB). The filter prevented most of the photochemical formation of  $^1\text{O}_2$  by RB, and no effect of RB on net assimilation and isoprene emission rates was observed in this case. Net assimilation rates after 2.5 h of RB feeding were 36% of control with no filter (Fig. 4) but 70% of control with the filter. The decrease in net assimilation with filter was similar to that observed in untreated leaves. The



**Figure 2.** Effects of  $c_i$  on net assimilation (a) and isoprene emission rates (b) from branches of *M. communis* measured at 26°C (circles; two different branches) and 34°C (triangles) and light intensity of 1,000  $\mu\text{mol m}^{-2} \text{s}^{-1}$ .



**Figure 3.** Effects of different concentration of RB in the feeding solution on net assimilation (a) and isoprene emission rates (b) of *M. communis* at leaf temperature of 26°C, light intensity of 1,000  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , and  $c_i$  values of 200 to 250  $\mu\text{L L}^{-1}$ . The vertical line indicates the beginning of RB feeding. Values were normalized to the average before RB feeding.

results confirmed that the effect of RB was via the photochemical reaction that yields  $^1\text{O}_2$ , and not, for example, by a chemical poisoning.

Comparing the time response of isoprene emission and net assimilation rates showed that, at ambient  $\text{CO}_2$  concentrations, the decrease in isoprene emission rates was delayed by 1 to 2 h, and was smaller in magnitude, relative to that in net assimilation (Fig. 4a). As a result, the fraction of fixed C allocated to isoprene production increased from 0.5% to 1.1% within 3 h, as did estimated isoprene concentration in the leaf airspaces (Fig. 4c; calculated as by Singsaas et al. [1997]; note that consideration of stomatal patchiness may have small effect on our results). At high  $\text{CO}_2$  concentrations, net  $\text{CO}_2$  assimilation rates began to decrease approximately 1.5 h after the beginning of RB feeding, but, in contrast to ambient  $\text{CO}_2$ , isoprene emission rates either did not change or increased (Fig. 4b).

At high  $\text{CO}_2$  concentrations, the response of isoprene emission to RB treatments was well correlated with changes in  $c_i$ . In untreated leaves, isoprene emission normally decreased with increasing  $c_i$  (at 26°C; Fig. 2); whereas in RB-treated leaves, a decrease in  $c_i$  with time, because of RB effect, led to an increase in isoprene emission rates (comparison of absolute emission rates between Figs. 2 and 4 is difficult be-

cause of large differences among the different branches used in the different  $c_i$  experiments). This was most apparent over the seasonal cycle. During autumn and summer,  $c_i$  decreased and isoprene emission rates increased in response to RB treatments (Fig. 4, b and d). In contrast, during spring, both  $c_i$  and isoprene emission rates did not change (data not shown). Furthermore, in an RB treatment in autumn at 34°C and high  $\text{CO}_2$  concentration, a large decrease in net assimilation and in  $c_i$  but no change in isoprene emission rates were observed (data not shown). This is consistent with the notion of reduced effect of  $c_i$  on isoprene emission rate at elevated temperatures (Fig. 2b).

### Isoprene Fumigation in *M. communis*

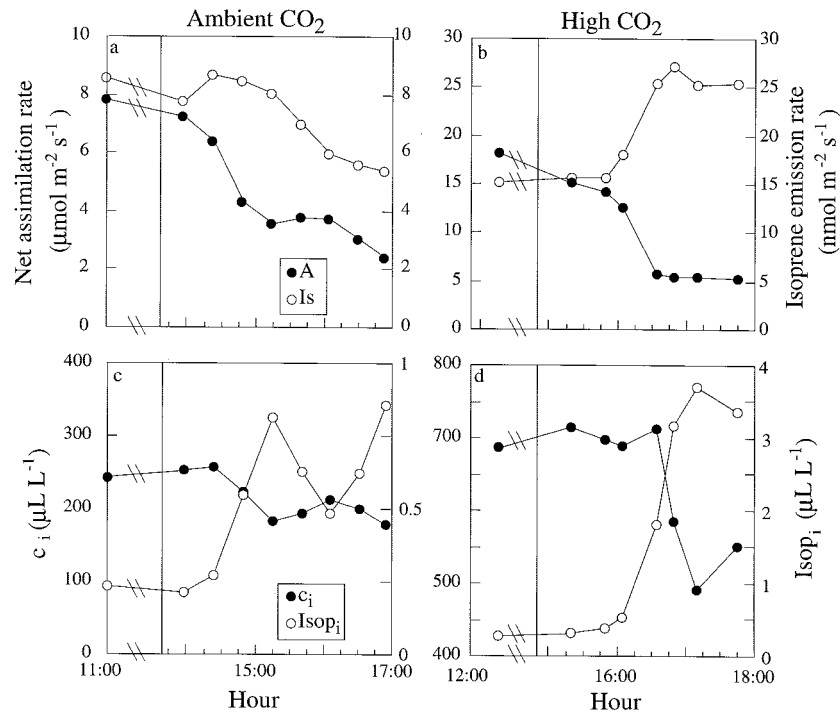
The possibility that isoprene may provide protection against  $^1\text{O}_2$  damage was further tested by isoprene fumigation of young, non-emitting branches of *M. communis* treated by RB or BX. In both isoprene-fumigated and non-fumigated young leaves, RB treatment (0.4  $\mu\text{M}$ ) led to a decrease in net assimilation rates. However, in isoprene-fumigated leaves (1–2  $\mu\text{L L}^{-1}$ ), the decrease in net assimilation was one-half that of non-fumigated leaves (Table I). Higher isoprene concentration (4–5  $\mu\text{L L}^{-1}$ ) did not increase the protection effects, and similar treatments in mature (isoprene-emitting) leaves showed no clear fumigation effects.

RB may produce  $^1\text{O}_2$  at concentrations and locations that do not occur naturally. A more natural cause for  $^1\text{O}_2$  production in the leaves is photoinhibition, during which the rate of absorption of photons is higher than the rate of utilization of the excitation energy. This leads to formation of triplet state chlorophyll that reacts readily with oxygen, yielding  $^1\text{O}_2$  (Demmig-Adams, 1990). To better simulate photoinhibition and  $^1\text{O}_2$  production in the vicinity of PSII, we repeated the isoprene fumigation experiments in leaves treated with the herbicide BX (50  $\mu\text{M}$ ). BX increases the sensitivity of PSII to light, leading to production of  $^1\text{O}_2$  near PSII. In non-fumigated leaves, the treatment resulted in approximately 50% decrease in net assimilation rates within 4 h (beginning 1.5–2 h after feeding). Isoprene-fumigated leaves (1–15  $\mu\text{L L}^{-1}$ ) showed approximately 30% smaller decrease in net assimilation due to BX (Table I).

### Isoprene Fumigation in *R. alaternus*

The isoprene  $^1\text{O}_2$  protection effect was further tested in a different isoprene-emitting species, *R. alaternus*. Similar fumigation experiments were carried out with young shade leaves treated with RB (0.1  $\mu\text{M}$ ). Young leaves of *R. alaternus* emitted small amounts of isoprene, and isoprene concentrations used in fumigation were up to 20  $\mu\text{L L}^{-1}$ . In non-

**Figure 4.** Effects of singlet oxygen in leaves of *M. communis* under ambient CO<sub>2</sub> concentration (approximately 350 μL L<sup>-1</sup>; a and b) and high CO<sub>2</sub> concentration (approximately 800 μL L<sup>-1</sup>; c and d) on net assimilation and isoprene emission rates (a and c) and on changes in the c<sub>i</sub> and isoprene (Isop<sub>i</sub>; b and d) at leaf temperature of 26°C and light intensity of 1,000 μmol m<sup>-2</sup> s<sup>-1</sup>. The vertical line indicates the beginning of RB feeding.



fumigated leaves, net assimilation decreased significantly and with increasing rates of decrease over time (i.e. increase in the slope of the time response curve) to  $0.53 \pm 0.02$  of control after 4 h ( $n = 4$ ). In isoprene-fumigated leaves, net assimilation rates decreased to  $0.66 \pm 0.05$  of control ( $n = 4$ ; Fig. 5; the difference between isoprene-fumigated and non-fumigated leaves was significant at the  $P < 0.04$  level). Interestingly, there was a consistent difference in response between isoprene-fumigated and non-fumigated leaves even before RB feeding, in which non-fumigated leaves of *R. alaternus* consistently showed a decrease in net assimilation rates from the onset of the experiment, whereas in isoprene-fumigated leaves, net assimilation rates began decreasing only after RB feeding (Fig. 5).

Isoprene protection in *R. alaternus* was also clearly observed in chlorophyll fluorescence measurements ( $F_v/F_m$ , an indicator of PSII efficiency, compared before and after each RB treatment experiment).  $F_v/F_m$

decreased significantly more in non-fumigated leaves than under isoprene fumigation (from  $0.765 \pm 0.010$  to  $0.274 \pm 0.027$ ,  $n = 5$ ; and from  $0.778 \pm 0.003$  to  $0.520 \pm 0.069$ ,  $n = 4$ , in non-fumigated and isoprene-fumigated leaves, respectively;  $P < 0.006$ ). Slightly better recovery of  $F_v/F_m$  after 24 h in low light was observed in isoprene-fumigated leaves ( $F_v/F_m$  after 24 h was  $0.700 \pm 0.020$  and  $0.639 \pm 0.016$  in isoprene-fumigated and non-fumigated leaves, respectively;  $P < 0.03$ ).

## DISCUSSION

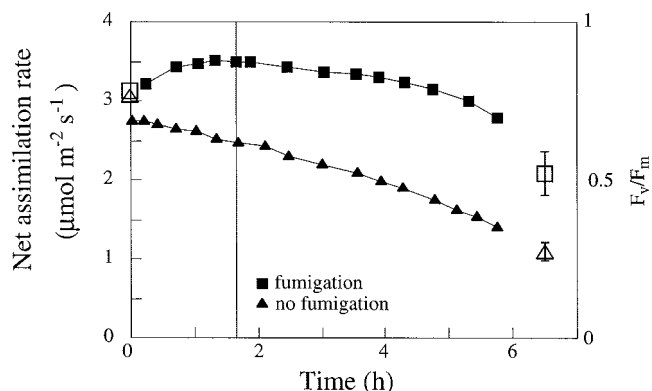
Characteristics of isoprene emission in *M. communis* reported here are consistent with reports on other plants (Sharkey et al., 1991; Sharkey and Loreto, 1993; Harley et al., 1994; Monson et al., 1994; Schnitzler et al., 1997). Such characteristics included pronounced seasonality, absence of isoprene emission in young leaves, delayed onset of emission with respect to

**Table 1.** Effect of isoprene fumigation on net assimilation rates (A) after 4 h of <sup>1</sup>O<sub>2</sub> treatment in young (non-emitting) leaves of *M. communis*

Data are presented as the percentage of A left after the treatment. Also presented is fumigation effect on percentage of A, as the ratio between consecutive fumigation and non-fumigation experiments. Light intensity was 1,000 μmol m<sup>-2</sup> s<sup>-1</sup> and leaf temperature was 26°C. Exogenous isoprene was added to give isoprene concentrations of 1 to 5 and 1 to 15 μL L<sup>-1</sup> in the leaf air spaces in the RB and BX experiments, respectively. The fumigation effect was significant at  $P < 0.005$  for RB and  $P < 0.025$  for BX (one tailed paired Student's *t* test). Data represent results of two shrubs each in the RB and BX experiments.

	Rose Bengal (0.4 μM)			Bromoxynil (50 μM)		
	Fumigation	No fumigation	Ratio	Fumigation	No fumigation	Ratio
	87	64	1.36	66	43	1.53
	79	49	1.61	62	49	1.27
	109	84	1.30	57	54	1.53
Average ± SE	92 ± 9	66 ± 10	1.42 ± 0.10	62 ± 3	49 ± 3	1.29 ± 0.14





**Figure 5.** Effect of RB ( $0.1 \mu\text{M}$ ) on net assimilation rates (full symbols) and chlorophyll fluorescence yield ( $F_v/F_m$ ; white symbols) in young shade leaves of *R. alaternus* at leaf temperature of  $26^\circ\text{C}$ , light intensity of  $1,000 \mu\text{mol m}^{-2} \text{s}^{-1}$ . The vertical line indicates the beginning of RB feeding. The fumigation effect was significant at  $P < 0.04$  and  $P < 0.006$  in net assimilation and fluorescence, respectively. The error bars of  $F_v/F_m$  before the RB treatment are smaller than the symbols.

photosynthetic activity, and higher activity of sun leaves. The effect of  $c_i$  on rates of net assimilation and isoprene emission at both high and moderate temperatures is similar to that observed in aspen and red oak (Monson and Fall, 1989; Loreto and Sharkey, 1990). This was related to ATP limitation at high  $c_i$  and moderate temperatures, which is reduced at high temperatures (Loreto and Sharkey, 1990). However, no decrease in isoprene emission rates was observed in *M. communis* at  $\text{CO}_2$  free air ( $c_i$  of approximately  $20 \mu\text{L L}^{-1}$ ) for extended time periods, similar to observations in aspen but not in red oak.

The results presented here show for two plant species and using two  $^1\text{O}_2$  production methods that isoprene can reduce oxidative damage to the photosynthetic apparatus. The beneficial effects are evident in the reduced effect of  $^1\text{O}_2$  on net assimilation rates and on PSII fluorescence yield ( $F_v/F_m$ ) in the presence of isoprene.

In discussing the isoprene protection effects observed in this study, it is important to consider the relevance of the experimental results to natural conditions. For this purpose, we consider the concentrations used, the production rates of isoprene as compared with possible production rates of  $^1\text{O}_2$  in leaves, and other sources for oxidative stress such as  $\text{O}_3$  and water stress.

A first indirect indication for the usefulness of isoprene in protection against  $^1\text{O}_2$  is an increase in internal concentration during  $^1\text{O}_2$  stress. Using RB as a photosensitizer, we observed a decrease in net assimilation and in stomatal conductance. Markedly smaller reduction in isoprene emission than in net assimilation rates (at ambient  $\text{CO}_2$ ) or even enhancement (at high  $\text{CO}_2$ ) was observed. As a consequence, the plant apparently invested more of the fixed carbon in isoprene formation, and the reduction in sto-

matal conductance led to an increase in isoprene concentrations in the leaf air spaces during stress. Such increases in intercellular isoprene concentrations may indicate enhancement of isoprene potential to scavenge  $^1\text{O}_2$ .

More directly, isoprene protection against  $^1\text{O}_2$  was observed in isoprene-fumigated young leaves (little or no endogenous isoprene emission; Table I; Fig. 5). Fumigation experiments are physiologically more relevant if the concentration of isoprene used for fumigation is consistent with concentrations that occur naturally within the leaf air spaces. The concentrations we used for fumigation ( $1\text{--}20 \mu\text{L L}^{-1}$ ) were higher than that occurring regularly in leaves of *M. communis* but were not unusual in mature stressed leaves (Fig. 4) and in leaves of other plants such as white oak and kudzu ( $>10 \mu\text{L L}^{-1}$ ; Singsaas et al., 1997). We, therefore, concluded that isoprene concentrations that are expected in the leaf air spaces of isoprene-emitting leaves, are effective in reacting with and scavenging  $^1\text{O}_2$ , as observed under the experimental conditions.

In addition to physiologically relevant concentrations, it also seems that potential rates of  $^1\text{O}_2$  scavenging by isoprene are physiologically relevant. Although detailed evidence is lacking, results from isolated PSII reaction centers show that approximately 30% of excitation yields  $^3\text{P680}$ , and most of it is reflected as  $^1\text{O}_2$  (Durrant et al., 1990; Telfer et al., 1994). In intact leaves, however, about 85% of the excitation of the reaction center leads to photochemistry (Papageorgiou, 1975), and in this case, one can assume that only a few percent of the excitation could yield  $^1\text{O}_2$ . Therefore, the naturally occurring production of  $^1\text{O}_2$  in the chloroplasts should, at least a priori, be comparable with the production of isoprene that can constitute up to a few percent of the carbon assimilation.

The fumigation experiments reported here seemed to be more efficient with RB than with BX. This, in fact, is consistent with the expected isoprene concentration gradients from a source in the atmosphere to the chloroplasts. RB-produced  $^1\text{O}_2$  is expected to spread across the leaf, and an external source of isoprene would be more efficient in reacting with it than with  $^1\text{O}_2$  produced specifically near PSII (i.e. by BX). Under natural conditions, however, a gradient in the opposite direction is likely to exist. In this case, isoprene production is in the chloroplasts, where protection against photoinhibition (simulated here by BX treatment) is expected to be more efficient.

Isoprene protection against  $^1\text{O}_2$  was not limited to *M. communis*, and similar effects were observed in young leaves of *R. alaternus*. Interestingly, non-fumigated leaves of this plant consistently showed a decrease in net assimilation rates even before RB treatment. This was probably due to exposure of shade-adapted leaves to high light intensity during measurements. Our interpretation is supported by

the results that fumigation always prevented this effect, apparently protecting the leaves against photoinhibition. We, therefore, speculate that the results show, in fact, two levels of protection before and after RB feeding.

Increased thermotolerance and ozone protection by isoprene were suggested to be achieved through strengthening of the thylakoid membranes (Loreto et al., 2001; Sharkey et al., 2001) and preventing peroxidation of membrane lipids (Loreto and Velikova, 2001). In *R. alaternus*, the fluorescence yield ( $F_v/F_m$ ) indicated significantly lower damage to the photosynthetic apparatus in isoprene-fumigated as compared with non-fumigated leaves. This also suggest that isoprene may protect the photosynthetic apparatus embedded in the thylakoid membranes, but, whereas the actual cause for the thermal or ozone damage is not clear, here, it was directly related to  $^1\text{O}_2$ .

Although not the only protection mechanism available, isoprene may have some specific advantages. For example, chloroplast membranes are highly sensitive to photooxidative damage that occurs because of excessive light intensity leading to  $^1\text{O}_2$  production. The extent of this damage/protection is related to the amount of  $\beta$ -carotene bound to the PSII reaction centers (Telfer et al., 1994). In that sense, isoprene may provide a more dynamic protection mechanism, considering the increase in isoprene emission rates with light intensities (Guenther et al., 1993). This enables the plant to rapidly raise the protection level when needed and before changes in carotenoid synthesis are effective.

The products of reactions between isoprene and  $^1\text{O}_2$  are likely to be toxic hydroperoxides similar to those produced in reactions with  $\text{O}_3$  (Mehlhorn and Wellburn, 1987; Hewitt et al., 1990; Salter and Hewitt, 1992). However, we show here that isoprene can protect leaves against  $^1\text{O}_2$ , and isoprene was shown lately to protect leaves against ozone by preventing the collapse of mesophyll cells and chloroplasts membranes (Loreto et al., 2001) and decreasing the amounts of  $\text{H}_2\text{O}_2$  and lipid peroxidation (Loreto and Velikova, 2001). The reaction products are apparently less toxic than  $^1\text{O}_2$  itself or are efficiently scavenged by other agents. Also, the possibility that isoprene could protect against  $^1\text{O}_2$  by strengthening the membranes, whereas the direct reaction with  $^1\text{O}_2$  is less significant, cannot be ruled out (isoprene protection against  $\text{O}_3$  was suggested to be achieved by both mechanisms; Loreto et al., 2001).

Photoinhibition and  $^1\text{O}_2$  stress are often associated with high light intensities together with low temperatures, as observed in clear winter days. It was consequently shown that in some plants, levels of the carotenoids of the xanthophyll cycle and de-epoxidation state are higher in winter than in summer (e.g. Adams and Demmig-Adams, 1994). However, isoprene emission, proposed here as a protection mechanism, was minimal in winter in *M.*

*communis*. But note that in Mediterranean plant species, the xanthophyll cycle de-epoxidation state was shown to be higher in summer, well correlated with low water potential (Kyparissis et al., 2000). In the eastern Mediterranean region, photoinhibition and oxidative stress are likely to be more pronounced in summer, when plants are exposed to high light and water stress, whereas winter conditions are optimal for growth. Low stomatal conductance during water stress periods would also enhance intercellular isoprene concentrations (Sharkey and Loreto, 1993; Fang et al., 1996) and the potential protective capacity.

We note in closing that the protective aspects of isoprene discussed above may also be consistent from an evolutionary perspective. Carotenoids of the xanthophyll cycle are known to scavenge both triplet chlorophyll and singlet oxygen (Demmig-Adams, 1990; Havaux and Niyogi, 1999). Zeaxanthin was suggested to decrease membrane fluidity and to increase thermotolerance (Tardy and Havaux, 1997; Havaux, 1998) as was suggested for isoprene (Sharkey et al., 2001; Sharkey and Singaas, 1995; Singaas et al., 1997). Isoprene shares a common biochemical production pathway with carotenoids, and it can be speculated that isoprene was a primitive protection mode against  $^1\text{O}_2$  that evolved into these more dedicated radical scavengers.

## MATERIALS AND METHODS

### Plant Material

Net assimilation and isoprene emission rates were measured for cut branches of *Myrtus communis* from plants growing on the campus of the Weizmann Institute of Science (Rehovot, Israel) and for attached branches from plants grown in pots under similar light and temperature conditions. Net  $\text{CO}_2$  assimilation ( $6.5 \pm 0.5$  and  $7.5 \pm 0.5 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) and isoprene emission rates ( $6.0 \pm 0.5$  and  $6.5 \pm 2.0 \text{ nmol m}^{-2} \text{s}^{-1}$ ) were similar in attached and cut branches, respectively. Hence, branches were cut under water and were kept with the stem immersed in deionized water for the experiments reported here. Cut branches containing young leaves from a shade growing shrub of *Rhamnus alaternus* in the Weizmann campus were used in isoprene fumigation experiments.

### Gas-Exchange Measurements

The sampling system was centered on a flow-through cuvette (volume of approximately 60 mL) in which the branch was sealed. The cuvette was equipped with a magnet-operated fan for mixing the air. Light ( $1,000 \mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetically active radiation) was supplied by a halogen lamp (250 W, Quartzline lamp, General Electric Co., Cleveland, OH); the infrared radiation was filtered out using a water bath (3 cm thick). The temperature in the cuvette was controlled by water circulation through a cooling bath (Haake D8-V, Karlsruhe, Germany) and through

the bottom of the cuvette and was set to give the desired leaf temperature (normally  $26 \pm 0.5^\circ\text{C}$ ). Air temperature was measured by a shaded thermocouple in the cuvette, and the leaf temperature was measured by a thermocouple touching the abaxial side of the leaf (type T thermocouple digital thermometer, HH82, Omega, Stamford, CT; precision of  $0.1^\circ\text{C}$ ). Dry air with various  $\text{CO}_2$  concentrations was supplied by mixing cylinder air with no  $\text{CO}_2$  and cylinder air having 1% or 2.5% (v/v)  $\text{CO}_2$  using two mass flow controllers (MKS1179A, Andover, MA;  $1,000 \text{ mL min}^{-1}$  and  $100 \text{ mL min}^{-1}$  full scale, respectively, used in a relative mode). Total flow rate through the cuvette was  $200 \text{ mL min}^{-1}$ . Both air flows passed through activated charcoal traps to remove hydrocarbons. The tubing (0.25- and 0.125-inch stainless steel) carrying the air exiting the cuvette was heated to  $80^\circ\text{C}$  to avoid condensation of water vapor.

$\text{CO}_2$  and  $\text{H}_2\text{O}$  concentrations in the air entering and leaving the leaf cuvette were measured by an infrared gas analyzer (LI-6262, LI-COR, Lincoln, NE), at a precision of  $\pm 1 \mu\text{mol mol}^{-1}$  for  $\text{CO}_2$  and  $\pm 0.1 \text{ mmol mol}^{-1}$  for water vapor. Net assimilation, stomatal conductance, and  $c_i$  were calculated according to von Caemmerer and Farquhar, (1981) assuming that stomatal patchiness has only small effects (van Kraalingen, 1990). Projected leaf area was estimated at the end of each experiment.

### Isoprene Measurements

For hydrocarbons measurements (Greenberg et al., 1994; R.K. Monson, personal communication) an aliquot of the air stream exiting the cuvette was pumped (bypassing the infrared gas analyzer) into a pre-evacuated glass bulb (2 L, 60 mtorr), through a trapping loop (0.125-inch stainless steel, 27 cm long in which the central approximately 9 cm was packed with 212- to  $300\text{-}\mu\text{m}$  glass beads [Sigma, St. Louis], with a  $2\text{-}\mu\text{m}$  filter [Valco, Houston, TX] placed at the outlet) connected to a six-port valve (Valco). The loop was cooled with either liquid nitrogen or a mixture of ethanol and dry ice ( $-75^\circ\text{C}$ ) for trapping the hydrocarbons in the air. The pressure (pressure transducer, Omega) at the entrance to the glass bulb was used to estimate the amount of air sampled (typical pressure used was 100 torr, namely the sample size was approximately 250 mL). After trapping, the valve was switched to a flow of helium ( $1.5 \text{ mL min}^{-1}$ ), the loop was rapidly heated (hot sand,  $200^\circ\text{C}$ ), and the trapped hydrocarbons passed directly to a gas chromatography (GC) column (HP 5890, Wilmington, DE).

The hydrocarbons were separated using a polar GC column (30 m long,  $0.25 \text{ mm i.d.}$ ,  $0.25 \mu\text{m}$  film; Supelcowax 10, Supelco, Bellefonte, PA; temperature program:  $35^\circ\text{C}$  for 1 min, temperature increase at  $10^\circ\text{C min}^{-1}$  to  $170^\circ\text{C}$  for 2 min). Isoprene was detected using a flame ionization detector kept at  $250^\circ\text{C}$ . The area of the peaks obtained was recorded and analyzed by a chromatography software (Borwin, version 1.21.60, JMBS developments, La Fontenil, France).

The GC peak area of isoprene was found to vary linearly with concentration, and to be constant over time. Isoprene gaseous standards were prepared and measured every few

months. The precision of isoprene concentration measurements was 4%. Standards were prepared by evaporating isoprene (99%; Aldrich, Milwaukee, WI) into a pre-evacuated 12-L bulb to a pressure of approximately 0.1 torr, on a vacuum line. Nitrogen was added to atmospheric pressure. The bulb was evacuated to approximately 1 torr and refilled with nitrogen to atmospheric pressure, to give 0.1 to  $0.2 \mu\text{L L}^{-1}$  isoprene.

### Singlet Oxygen Treatments

Singlet oxygen was produced in the leaves by RB (4,5,6,7-tetrachloro-2',4',5',7'-tetraiodofluorescein) or BX (3,5-dibromo-4-hydroxybenzonitrile; Agan chemical manufacturers, Ashdod, Israel) in the light. RB acts as a photosensitizer to singlet oxygen production upon absorbing green light,  $\lambda_{\text{max}} = 547 \text{ nm}$ . BX is a phenolic herbicide that increases the sensitivity of PSII to excess light through formation of P680 triplet (Krieger-Liszky and Rutherford, 1998). Both chemicals were fed as aqueous solutions through the petiole, and their effect on net assimilation and isoprene emission rates was examined at ambient and high  $\text{CO}_2$  concentrations,  $26^\circ\text{C}$ , and at light intensity of  $1,000 \mu\text{mol m}^{-2} \text{ s}^{-1}$ . To use physiologically relevant  $^1\text{O}_2$  concentrations, RB concentration used in the fumigation experiments ( $0.4 \mu\text{M}$  in *M. communis* and  $0.1 \mu\text{M}$  in *R. alaternus*) was the lowest to induce decrease in net assimilation rates (Fig. 3), and BX was used as a source for  $^1\text{O}_2$  near PSII, simulating photoinhibition more specifically than RB.

Isoprene fumigation was done together with the  $^1\text{O}_2$  treatment in young leaves, by passing the air entering the leaf cuvette through a piece of permeable tubing that was enclosed in an Erlenmeyer containing isoprene and kept in an ice bath. The fumigation dose ( $1\text{--}20 \mu\text{L L}^{-1}$ ) was determined by the permeability of the tubing and the isoprene concentration in the Erlenmeyer.

### Chlorophyll Fluorescence

Chlorophyll fluorescence yield ( $F_v/F_m$ ) was measured in dark-adapted leaves of *R. alaternus* before and after singlet oxygen experiments, using a portable fluorometer (PAM-2000, Walz, Germany).

### Statistical Analysis

The data analysis add-in from Microsoft Excel 1998 (Microsoft Corp., Redmond, WA) was used to calculate Student's *t* test for determining the significance of difference between fumigation and non-fumigation experiments.

### ACKNOWLEDGMENTS

We thank Y. Rudich, F. Loreto, R.K. Monson, and two anonymous reviewers for helpful comments and E. Negreanu for technical help. We thank Agan Chemical manufacturers for supplying BX.



Received October 5, 2001; returned for revision December 16, 2001; accepted February 4, 2002.

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