Natural Abundance Carbon Isotope Composition of Isoprene Reflects Incomplete Coupling between Isoprene Synthesis and Photosynthetic Carbon Flow

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Isoprene emission from leaves is dynamically coupled to photosynthesis through the use of primary and recent photosynthate in the chloroplast. However, natural abundance carbon isotope composition ($\delta^{13}C$) measurements in myrtle (*Myrtus communis*), buckthorn (*Rhamnus alaternus*), and velvet bean (*Mucuna pruriens*) showed that only 72% to 91% of the variations in the $\delta^{13}C$ values of fixed carbon were reflected in the $\delta^{13}C$ values of concurrently emitted isoprene. The results indicated that 9% to 28% carbon was contributed from alternative, slow turnover, carbon source(s). This contribution increased when photosynthesis was inhibited by CO_2-free air. The observed variations in the $\delta^{13}C$ of isoprene under ambient and CO_2-free air were consistent with contributions to isoprene synthesis in the chloroplast from pyruvate associated with cytosolic Glc metabolism. Irrespective of alternative carbon source(s), isoprene was depleted in ^{13}C relative to mean photosynthetically fixed carbon by 4‰ to 11‰. Variable ^{13}C discrimination, its increase by partially inhibiting isoprene synthesis with fosmidomicin, and the associated accumulation of pyruvate suggested that the main isotopic discrimination step was the deoxyxylulose-5-phosphate synthase reaction.

2-Methyl-1,3-butadiene (isoprene) is emitted from leaves of various plant species (Kesselmeier and Staudt, 1999) and influences the trace-gas composition of the troposphere by reacting with OH radicals and NO_x to generate tropospheric ozone (Trainer et al., 1987; Chameides et al., 1988).

It has been demonstrated that isoprene is produced in the chloroplasts from primary photosynthetic products via the 2-methylerythritol-4-phosphate (MEP) pathway (Zeidler et al., 1997; Lichtenthaler, 1999). Fast labeling by 99% (v/v) $^{13}CO_2$ indicated direct coupling between isoprene and photosynthesis (Sharkey et al., 1991a), although uncertainty remains concerning the potential contribution of cytosolic substrates to chloroplastic isoprene production, such as isopentenyl pyrophosphate (IPP) produced via the mevalonic acid pathway (Lichtenthaler et al., 1997a). Process-based isoprene emission models use photosynthesis as a starting point (Niinemets et al., 1999; Zimmer et al., 2000) assuming direct coupling between the two processes. However, there are indications for incomplete coupling in some cases, such as emission of isoprene in the absence of net assimilation in the dark (Shao et al., 2001) or under CO_2-free air (Monson and Fall, 1989; Loreto and Delfine, 2000; Affek and Yakir, 2002), that requires isoprene synthesis using alternative carbon source(s). Isoprene protection, such as against oxidative damage (Affek and Yakir, 2002), may depend on such decoupling to maintain isoprene production when photosynthesis is at least partially inhibited.

Isotopic discrimination against ^{13}C (Δ, where $\Delta = R_{substrat}/R_{product} - 1$, and $R = {}^{13}C/{}^{12}C$) occurs during diffusion of CO_2 into leaves (4.4‰) and during CO_2 fixation by Rubisco (29‰; Roeske and O'Leary, 1984; Guy et al., 1993). The combined, scaled effects of diffusion and carboxylation lead to photosynthetically fixed carbon and organic matter depleted in ^{13}C with respect to atmospheric CO_2. Further carbon isotope discrimination occurs during lipid synthesis due to an isotopic effect that may be associated with decarboxylation in the production of acetyl CoA from pyruvate (DeNiro and Epstein, 1977; Melzer and Schmidt, 1987) and/or due to site-specific isotopic heterogeneity in pyruvate in which the carboxyl carbon is relatively enriched (Rinaldi et al., 1974; Gleixner and Schmidt, 1997).

Discrimination against ^{13}C was also observed in isoprene production. Isoprene emitted from red oak was depleted in ^{13}C by 3‰ with respect to photosynthetically fixed carbon (Sharkey et al., 1991b). This was suggested to be associated with isotopic discrimination by the pyruvate dehydrogenase complex as part of the mevalonate pathway. However, this interpretation should be revised to fit the currently accepted MEP pathway (Lichtenthaler, 1999), for which the possible discrimination steps have not yet been explicitly identified.

In the present work, we use the carbon isotopic compositions of isoprene and of newly fixed carbon to examine the coupling between isoprene produc-

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tion and photosynthetic carbon flow and to extend the knowledge of ^{13}C discrimination in isoprene synthesis in leaves of myrtle (*Myrtus communis*), reed (*Phragmites australis*), buckthorn (*Rhamnus alaternus*), and velvet bean (*Mucuna pruriens*).

RESULTS

Source Effect

A change in the isotopic composition of the CO_2 supplied to a leaf enclosed in a gas-exchange cuvette was immediately reflected in the ^{13}C content of the fixed carbon, δ_{fixed} (where $\delta = [R_{sample}/R_{standard} - 1] \times 10^3$‰, $R = {}^{13}C/{}^{12}C$ and the standard is Vienna Pee Dee Belemnite). It was partially reflected within 5 min in the ^{13}C content of isoprene, δ_{isop}, emitted from myrtle leaves and reached a constant value within about 30 min (Fig. 1). The relatively rapid response of δ_{isop} to labeling implied small isoprene pool size in the leaves, which was confirmed also by direct measurements. Only approximately 100 nmol m^{-2} isoprene was obtained by extractions from leaves of myrtle-1.

As expected, δ_{isop} values were significantly lower than the ^{13}C values of concurrently fixed carbon, δ_{fixed} (Fig. 1). In contrast to expectations, however, the apparent discrimination against ^{13}C from carbon fixed in photosynthesis to concurrently emitted isoprene, $\Delta_{C\text{-}isop}$ (where $\Delta_{C\text{-}isop} = [\delta_{fixed} - \delta_{isop}]/[\delta_{isop}/1{,}000+1]$), was sensitive to the $\delta^{13}C$ of the CO_2 supplied (Fig. 1). Similar results were obtained using leaves of myrtle, velvet bean, and buckthorn, as summarized in Figure 2. The variations in the apparent discrimination in response to changes in the $\delta^{13}C$ of the CO_2 supply are reflected in the slopes of δ_{isop} versus δ_{fixed}, which were smaller than 1 and varied between 0.72 ± 0.02 and 0.91 ± 0.03 (Fig. 2, a–e). In reed, on the other hand, the apparent discrimination did not vary significantly with changes in the $\delta^{13}C$ of the source CO_2, resulting in a slope of 1 (Fig. 2f).

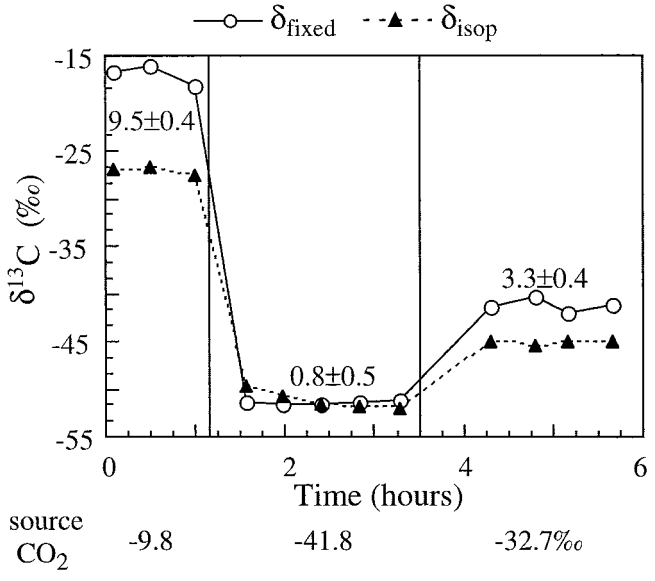

Figure 1. Changes in the isotopic composition of photosynthetically fixed carbon (from on-line gas exchange and isotopic measurements) and of isoprene emitted from a branch of myrtle-3, in response to a rapid change in the isotopic composition of the source CO_2 (at a time marked by the vertical lines). The numbers denote the discrimination in isoprene production relative to fixed carbon ($\Delta_{c\text{-}isop}$ in per mil). Photosynthesis was brought to steady state before the onset of the experiment and all conditions other than the $\delta^{13}C$ of the source CO_2 were kept constant throughout the experiments.

In myrtle, we also modified δ_{fixed} without changing the CO_2 supply, by changing c_i/c_a through stomatal closure induced, in turn, by abscisic acid (ABA) treatments (30–100 μM). Stomatal conductance decreased from 0.24 ± 0.09 to 0.02 ± 0.01 mol m^{-2} s^{-1} (average ± SE, $n = 3$) and net assimilation decreased from 7 to 1 μmol m^{-2} s^{-1}. This led to a decrease in c_i/c_a from 0.84 ± 0.002 to 0.61 ± 0.004 and in photosynthetic discrimination, yielding more positive δ_{fixed} (compare with Farquhar et al., 1982) and δ_{isop} values (Fig. 2a). The relationships of δ_{isop} versus δ_{fixed} showed an even lower slope than that observed in the CO_2-labeling experiments (above). Additional ABA treatments on other myrtle branches showed similar results with relatively large variations in slopes among branches (data not shown).

The variations in apparent discrimination indicated that some of the isoprene was not labeled by recently fixed carbon and that carbon from a source independent of current assimilation (termed below alternative carbon source) was incorporated into isoprene (see "Discussion"). The measurements described below were performed to characterize this unlabeled isoprene and to recognize its carbon source.

CO_2-Free Air

Measurements were carried out under CO_2-free air (or in the dark, see below) when there is no photosynthesis, to obtain isoprene that cannot be labeled by concurrent photosynthesis. After switching to CO_2-free air, isoprene emission was sustained for several hours, without significant change in emission rates (Affek and Yakir, 2002). δ_{isop} values under CO_2-free air were constant over approximately 2 h of treatment with a mean value of −41.7‰ ± 1.4‰ (average ± SE, $n = 6$, in myrtle-1 and -2 plants). This value was independent of δ_{isop} values during the several hours preceding the CO_2-free air treatment that ranged between −35‰ and −50‰, for different pretreatment in which $\delta^{13}C$ of the CO_2 supply ranged between −8‰ and −31‰. These results indicated that isoprene was produced under CO_2-free conditions from an unlabeled carbon source. Only when δ_{isop} preceding the treatment was as low as approximately −60‰ was some depletion in δ_{isop} observed, i.e. from −41.7‰ to −47.9‰ ± 1.4‰ ($n = 7$). Iso-

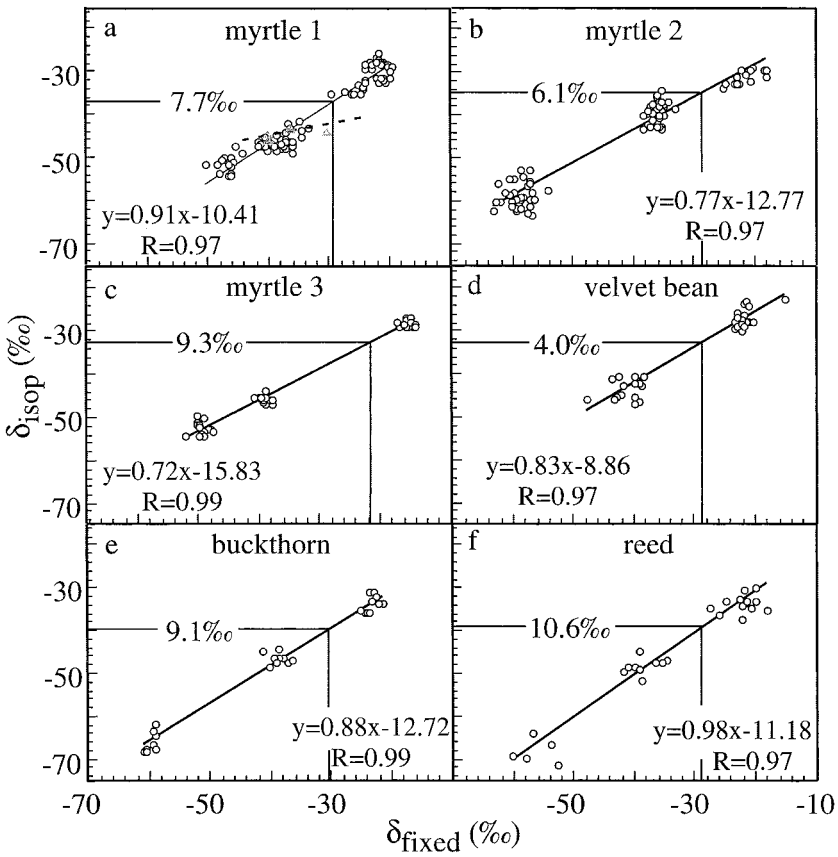

Figure 2. Relationships between the isotopic composition of the photosynthetically fixed carbon (δ_{fixed};3b from on-line gas exchange and isotopic measurements) and that of isoprene (δ_{isop}) emitted by leaves of myrtle-1 through -3 (a–c, ○), velvet bean (d), buckthorn (e), and reed (f). The vertical lines indicate the isotopic composition of leaf organic matter (δ_{leaf}; *n* between 4 and 10; SE between 0.2 and 0.6). The horizontal lines denote the apparent discrimination ($\Delta_{c\text{-}isop}$, calculated from the plot assuming that δ_{leaf} represents the mean δ_{fixed} during the growth period of the leaf). The slopes of myrtle, buckthorn, and velvet bean were significantly lower than 1 based on *t* test for ([slope − 1]/slope SE), with $n - 2$ degrees of freedom. Slope SE was 0.03 ($n = 106$), 0.02 ($n = 83$), 0.02 ($n = 42$), 0.04 ($n = 33$), 0.02 ($n = 28$), and 0.04 ($n = 30$) for plots a to f, respectively. The gray triangles and dashed line in plot a denote the influence of ABA on δ_{fixed} and δ_{isop} in a branch of myrtle-1.

prene emission under CO_2-free air was observed also in reed and buckthorn leaves with δ_{isop} values of $-44.4‰ \pm 2.2‰$ ($n = 2$) and $-45.8‰ \pm 0.6‰$ ($n = 7$), respectively, irrespective of the δ_{isop} values preceding the CO_2-free air treatment that ranged between $-35‰$ and $-50‰$.

Emission in the Dark

In the dark, isoprene emission from myrtle decreased rapidly to detection limit levels not sufficient for isotopic analysis. Consequently, δ_{isop} was measured in the light until a steady-state value was observed and then during the first few minutes of darkness. δ_{isop} values in the dark followed changes in δ_{isop} values in the preceding light period, such as due to changes in source CO_2. $\delta_{isop\text{-}dark}$ was slightly more depleted than $\delta_{isop\text{-}light}$ (by $2.7‰ \pm 0.7‰$; $n = 4$) for CO_2 source during the preceding light period with $\delta^{13}C$ values of either $-8‰$ or $-31‰$.

Glc Labeling

To examine incorporation of glycolytic carbon into isoprene (Fig. 3), we fed both myrtle and buckthorn leaves with ^{13}C-enriched Glc ($\delta^{13}C_{Glc}$ was $-10‰$ as compared with $\delta^{13}C$ of leaf organic matter of $-29‰$ or $-30‰$). No change in isoprene emission rates was observed during the feeding treatments. δ_{isop} and δ_{fixed} or $\delta_{respired}$ were measured at ambient or zero CO_2 concentrations, respectively, with and without Glc feeding. Under CO_2-free air, both respired CO_2 and isoprene were clearly labeled by the enriched Glc and both to a similar extent (Table I). Under ambient CO_2 concentration, Glc feeding led to a slight decrease in net assimilation rates, but neither δ_{isop} nor δ_{fixed} changed significantly.

Cytosolic IPP

To examine the potential effect of cytosolic IPP and the possibility that it contributes unlabeled carbon to isoprene, we reduced chloroplastic IPP formation using fosmidomycin. In myrtle, buckthorn, and reed, fosmidomycin led to inhibition of isoprene emission, although about 10% of the emission rates persisted even after treatment (Fig. 4; compare with Loreto and Velikova, 2001). Partial inhibition resulted in ^{13}C depletion of δ_{isop} and an increase of apparent discrimination, $\Delta_{C\text{-}isop}$, from an average of $9.4‰ \pm 0.5‰$ before treatment to $11.4‰ \pm 0.5‰$ during inhibition (average ± SE, $n = 8$, $P < 0.001$; Table II). This increase in $\Delta_{C\text{-}isop}$ was observed even when δ_{fixed} and δ_{isop} before the fosmidomycin treatment were highly depleted. Notably, inhibition with fosmidmycin resulted also in a 43% increase in leaf pyruvate content from 23 ± 3 μmol m^{-2} in control leaves to 33 ± 2

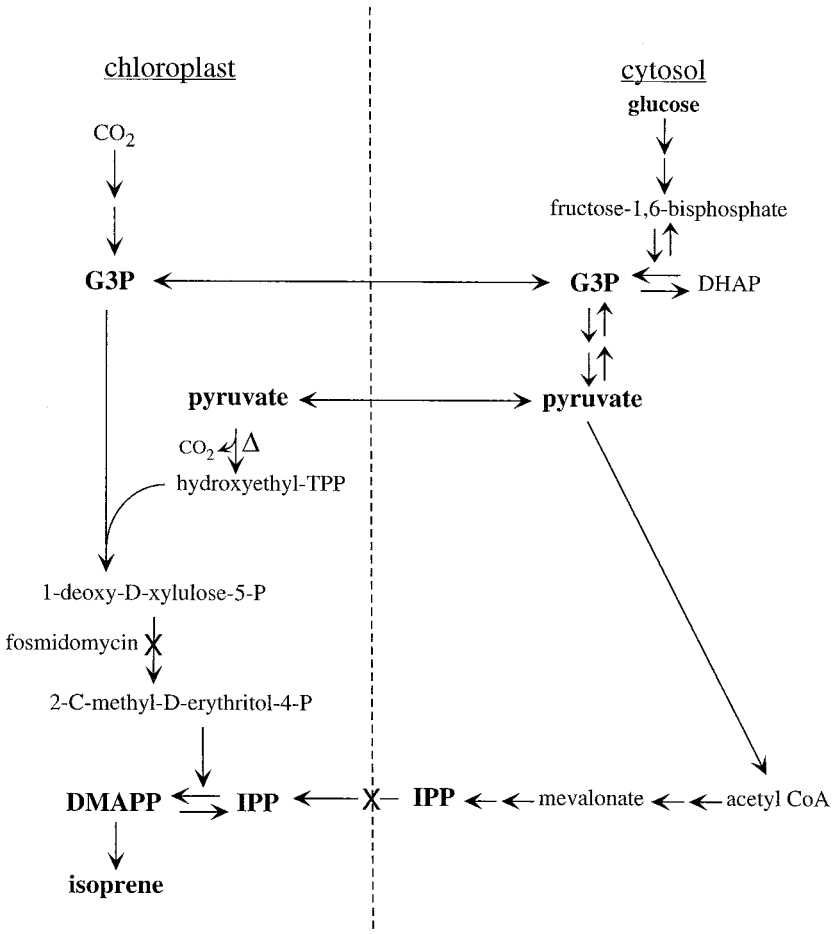

Figure 3. Schematic representation of isoprene biosynthesis pathway and possible coupling to cytosolic Glc metabolism and IPP. Also noted are the sites of action of the inhibitor fosmidomycin (Fellermeier et al., 1999) and of the isotopic discrimination by DXS. DHAP, dihydroxyacetone phosphate; DMAPP, dimethylallyl pyrophosphate; TPP, thiamine pyrophosphate.

μmol m^{-2} after 4 h of inhibitor treatment (3 μM; $P < 0.02$, $n = 4$).

Isotopic Composition of Isoprene Emitted to the Atmosphere

Short-term $\Delta_{C\text{-}isop}$ in myrtle-1 leaves was measured under ambient air and high flow rate, to obtain typical physiological c_i and δ_{fixed} values. This yielded $\Delta_{C\text{-}isop}$ of 7.7‰ ± 0.7‰ (δ_{isop} = −29.2‰ ± 0.6‰, δ_{fixed} = −21.7‰ ± 0.3‰, $n = 8$). However, measurements as in Figure 1 cannot be used to estimate the intrinsic isotopic discrimination in isoprene production, as indicated by the results presented in Figure 2, because of the deviations from the 1:1 in the δ_{fixed} versus δ_{isop} relationships. Alternatively, an estimate for long-term mean $\Delta_{C\text{-}isop}$ under natural conditions can be obtained by substituting $\delta^{13}C$ of total leaf organic matter (δ_{leaf}) for δ_{fixed}. For myrtle-1 this yielded mean $\Delta_{C\text{-}isop}$ value of 7.7‰ (Fig. 2a), consistent with the values obtained in the short-term measurement. Using the same approach, mean $\Delta_{C\text{-}isop}$ values in velvet bean, reed, and buckthorn were estimated to be 4.2‰, 11.0‰, and 9.6‰, respectively (Fig. 2).

Table I. *Effects of feeding leaves with ^{13}C-enriched Glc ($\delta^{13}C$ = −10‰) on the isotopic composition of isoprene and respired CO_2, under CO_2-free air conditions*

Each pair of lines denotes one branch. Average and SE refer to three or four measurements for either control of treatment.

Species	Glc Feeding	$\delta_{respired}$	δ_{isop}
		‰	
Myrtle	−	−22.8	−47.3 ± 0.3
	+	−16.4 ± 0.7	−41.7 ± 1.0
Myrtle	−	−24.3 ± 0.2	−54.4 ± 1.2
	+	−18.9 ± 2.2	−44.6 ± 1.1
Buckthorn	−	−19.6 ± 0.2	−46.1 ± 0.2
	+	−13.2 ± 0.3	−38.9 ± 0.7

DISCUSSION

Incomplete Coupling between Isoprene and Assimilation

The time course of changes in δ_{isop} values after a change in the isotopic composition of source CO_2 (Fig. 1) confirmed the dynamic connection between CO_2 assimilation and isoprene productions (Sharkey et al., 1991a). Quantitatively, however, the coupling

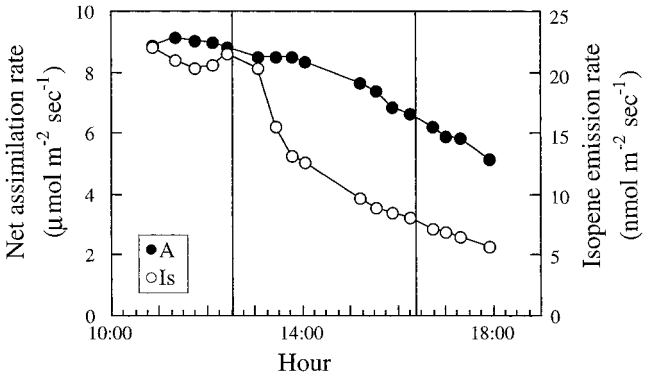

Figure 4. Effects of fosmidomycin inhibition on net assimilation (●) and isoprene emission rates (○) in a branch of myrtle-1. The vertical lines indicate the beginning of fosmidomycin feeding (5 μM at 12:30 and 10 μM at 16:20 PM). Leaf temperature was 26°C, light intensity was 250 μmol m^{-2} s^{-1}, and c_i varied between 220 and 250 μL L^{-1}. Due to the decrease in net assimilation observed at the high fosmidomycin used here, we used only up to 5 μM fosmidomycin in the isotopic analysis experiments.

between isoprene production and recently fixed carbon was clearly incomplete. If concurrent photosynthetically fixed carbon was the only carbon source for isoprene, it should be reflected in δ_{isop} versus δ_{fixed} relationships of 1:1. But the observed slopes of these relationships were considerably smaller than 1 (Fig. 2). Such behavior indicates a significant contribution from alternative carbon source(s), with ^{13}C content independent of the ^{13}C of the CO_2 source. Contribution from such alternative carbon source(s) with constant ^{13}C content would be expected to enhance $\Delta_{C\text{-}isop}$ when its $\delta^{13}C$ value is more negative than current δ_{fixed}, and vice-versa when it is more positive than current δ_{fixed}. Such response was clearly observed in the results reported in Figure 1.

As expected, possible contribution from stationary isoprene pool in the leaves could be ruled out based on the direct measurement of only approximately 100 nmol m^{-2} isoprene extractable from myrtle leaves. Such stationary pool would support about 100 s of emission (i.e. based on typical emission rate of 10 nmol m^{-2} s^{-1} and 10% contribution from alternative sources), whereas emission from an unlabeled carbon source was sustained for several hours.

Interestingly, in previously reported labeling experiments, partial ^{13}C labeling of isoprene has also been observed, even though the question of alternative carbon sources was not directly addressed. In the study of Sharkey et al. (1991b) with red oak, $\Delta_{C\text{-}isop}$ was slightly smaller when ^{13}C-depleted CO_2 was used (slope of 0.96 ± 0.04, $n = 6$). In another labeling study (Delwiche and Sharkey, 1993), most of the isoprene emitted from red oak was rapidly labeled by $^{13}CO_2$. But after 20 min, only 80% of the isoprene was labeled, which was similar in magnitude and time scale to the labeling pattern of phosphoglyceric acid (Canvin, 1979; Delwiche and Sharkey, 1993). Such results also suggest a contribution from an alternative, slow turnover, carbon source for isoprene or for phosphoglyceric acid and subsequently for isoprene (Delwiche and Sharkey, 1993). Such incomplete coupling between isoprene and concurrently assimilated carbon may be important in models using net assimilation to predict isoprene emission. Low isoprene emission was observed in Scots pine (*Pinus sylvestris*) with only approximately 90% labeling of the isoprene by $^{13}CO_2$ (Shao et al., 2001). This was explained by the possible existence of two carbon sources, whose turnover rates are different. Similarly, only approximately 90% labeling by newly fixed $^{13}CO_2$ was observed in non-stored sabinene in *Fagus sylvatica* (Kahl et al., 1999) and non-stored α-pinene and 3-methyl-3-buten-1-ol in *Quercus ilex* (Loreto et al., 1996a, 1996b). The question of alternative carbon source(s) for isoprene recently received renewed interest, and during the revisions of this paper, two other labeling studies provided evidence for contributions of extra-

Table II. *Effects of fosmidomycin on the isotopic composition of photosynthetically fixed CO_2 and of isoprene emitted from leaves of myrtle-1 and buckthorn and on pyruvate content in buckthorn leaves*

Each line denotes four control and four treatment measurements from one branch. For pyruvate content, leaves of four branches were extracted and measured for either control or treatment.

Species	$\delta^{13}C$ Source CO_2 (‰)	Untreated Control			Fosmidomycin Treated		
		δ_{fixed}	δ_{isop}	$\Delta_{C\text{-}isop}$	δ_{fixed}	δ_{isop}	$\Delta_{C\text{-}isop}$
		‰					
Myrtle	−13	−24.4 ± 0.3	−34.0 ± 0.5	9.9 ± 0.7	−27.3 ± 0.6	−39.6 ± 0.4	12.8 ± 0.9
	−13	−22.0 ± 0.7	−32.2 ± 0.2	10.5 ± 0.8	−27.8 ± 0.6	−39.0 ± 0.5	12.3 ± 0.8
	−49	−59.1 ± 0.4	−68.5 ± 0.6	10.1 ± 0.6	−61.8 ± 0.9	−72.7 ± 0.4	11.8 ± 0.9
	−49	−60.9 ± 0.3	−67.2 ± 0.2	6.8 ± 0.4	−62.9 ± 0.4	−71.4 ± 0.3	9.1 ± 0.7
	−27	−38.6 ± 0.3	−45.8 ± 0.1	7.5 ± 0.3	−39.7 ± 0.4	−50.1 ± 0.3	10.9 ± 0.3
	−27	−37.8 ± 0.2	−47.5 ± 0.2	10.2 ± 0.3	−40.4 ± 0.3	−51.6 ± 0.2	11.8 ± 0.2
Buckthorn	−27	−36.7 ± 0.2	−46.7 ± 0.3	10.5 ± 0.4	−38.1 ± 0.2	−49.1 ± 0.2	11.5 ± 0.4
	−13	−22.6 ± 0.2	−32.2 ± 0.1	9.9 ± 0.2	−24.3 ± 0.3	−34.5 ± 0.4	10.5 ± 0.4
				$\mu mol\ m^{-2}$			
Pyruvate		23 ± 3			33 ± 2		

chloroplastic carbon source (Karl et al., 2002b) or xylem-transported Glc (Kreuzwieser et al., 2002).

The results of our labeling experiments were supported by those for the ABA treatments. Modification of δ_{fixed} via changes in stomatal conductance and c_i/c_a, rather than CO_2 labeling, resulted in the expected enrichment in δ_{fixed} and δ_{isop}. But here too, coupling between δ_{fixed} and δ_{isop} was incomplete (Fig. 2a). The lower slope in the ABA treatments, as compared with the labeling experiments, was possibly due to decreased net assimilation rates with stomatal closure that would increase the relative contribution of the alternative source(s). Sharkey et al. (1991b) observed a more pronounced enrichment in δ_{isop} in red oak, under conditions of very low c_i, when δ_{fixed} is expected to become more enriched.

Large variations in the slopes of the δ_{isop} versus δ_{fixed} relationships from 1% to approximately 0.7% was observed (Fig. 2). This reflected species (genetic) effects, but likely also the effects of environmental factors on individual plants. The dynamic nature of the variable coupling between isoprene emission and concurrent photosynthesis was particularly evident in reed. In this case, a slope of 1 (Fig. 2) indicated full coupling to photosynthesis, but the emission under CO_2-free air suggested engagement of an alternative carbon source. Such dynamic response is significant because it may help explain the reduced sensitivity of isoprene emission to stress effect, as compared with photosynthesis (Sharkey and Loreto, 1993; Loreto and Delfine, 2000; Affek and Yakir, 2002). Further, such effect would enhance the potential protection effects by isoprene against, for example, oxidative stress when photosynthesis is partly inhibited (Affek and Yakir, 2002).

Alternative Carbon Source(s) for Isoprene

Characteristics

To characterize the isotopic composition of the alternative carbon source(s) for isoprene, we examined δ_{isop} when there was no net assimilation, such as under CO_2-free air (Monson and Fall, 1989; Loreto and Delfine, 2000; Affek and Yakir, 2002). Under CO_2-free air, we observed relatively constant δ_{isop} values, independent of $\delta^{13}C$ of source CO_2 or δ_{isop} during pretreatments. The results under CO_2-free air indicated also that the alternative carbon source(s) always produced isoprene with a $\delta^{13}C$ value in the range of −35‰ to −50‰ (approximately −42‰ on average). Labeling measurements in this $\delta^{13}C$ range were therefore insufficiently sensitive to clearly identify the influence of different carbon sources. Labeling with more depleted source CO_2 (leading to pretreatment δ_{isop} of −60‰) provided a clearer labeling effect. In this case, it could be estimated that approximately 30% of the alternative carbon was labeled by recent photosynthesis within 3 h. That is, labeling that produced 18‰ effect in δ_{isop} during 3 h of pretreatment, resulted in approximately 6‰ effect in δ_{isop} under CO_2-free air. Such results provide a first approximation for the turnover rate of the alternative carbon source(s), i.e. on the order of 10 h. The partial labeling of isoprene emitted under CO_2-free air is consistent with partial labeling of CO_2 respired into CO_2-free air (Ludwig and Canvin, 1971). Although in principle, the unlabeled carbon source could result from refixation of the respired CO_2, such refixation under CO_2-free air is very small (Loreto et al., 1999).

Photorespiration could also be involved in carbon supply to isoprene, and isoprene emission is inhibited when O_2 is lowered under CO_2-free air (Monson and Fall, 1989; Loreto and Sharkey, 1990). But Hewitt et al. (1990) showed that isoprene is not produced predominantly via photorespiration. It was previously suggested also that photorespiratory intermediates originate from short-term carbon storage, rapidly labeled by recent assimilation (Ludwig and Canvin, 1971; Loreto et al., 1999; Haupt-Herting et al., 2001). Such rapid labeling is inconsistent with the possibility that photorespiration is the source for unlabeled carbon in isoprene as observed here.

Isoprene emission in the dark may also indicate assimilation independent carbon source(s). Emission of small amounts of α-pinene in the dark was observed in *Q. ilex* (Loreto et al., 2000) and was mostly unlabeled by $^{13}CO_2$, indicating production de novo in the dark. In Scots pine, some emission of isoprene was observed during dark hours with approximately 90% labeling (Shao et al., 2001). In the present study, however, isoprene emission from myrtle leaves in the dark decreased rapidly while reflecting labeling of the source CO_2 previously assimilated. Unlike the sustained emission under CO_2-free air (several hours, under light), the small amounts of isoprene detected in the initial dark period probably reflected residuals and not de novo production. It seems that in the dark, the alternative carbon source(s) were not engaged, possibly due to light dependency of isoprene synthase (Wildermuth and Fall, 1996), and/or shortage in ATP, necessary for isoprene synthesis.

Glycolytic Sources

Isoprene is produced from pyruvate and glyceraldehyde-3-phosphate (G3P) in the chloroplasts, but these precursors can be derived either directly from concurrent Calvin cycle intermediates or from other metabolites such as those involved in glycolysis or from starch reserves (Fig. 3). Pyruvate may incorporate non-photosynthetic carbon in the cytosol before its import to the chloroplast (Givan, 1999). Incorporation of approximately 50% glycolitic carbon, such as was observed by Karl et al. (2002a), is consistent with the observed approximately 20% contribution to isoprene.

Carbon from Glc was incorporated into isoprenoids (Schwender et al., 1996; Lichtenthaler et al.,

1997b; Kreuzwieser et al., 2002). Feeding leaves with ^{13}C-enriched Glc under ambient CO_2 concentration did not produce a detectable signal because it was masked by much larger fluxes of CO_2 in the airflow through the leaf cuvette and isoprene production from concurrently fixed carbon. But under CO_2-free air, ^{13}C-enriched Glc clearly labeled both respired CO_2 and isoprene (Table I). The effect on respired CO_2 confirmed that labeled Glc was incorporated into leaf metabolism. Further, the labeling of isoprene clearly indicated that isoprene incorporated carbon via the glycolytic pathway. Glc metabolism is consistent with the characteristics of the alternative carbon source(s) for isoprene, as reflected under CO_2-free air (see above). The isotopic composition of leaf Glc is similar to δ_{fixed} under natural atmospheric conditions, and the products should undergo the same discrimination step as photosynthetically coupled isoprene. Glc, as was observed for Suc in wheat (*Triticum aestivum*; Gebbing and Schnyder, 2001), may also correspond well with a carbon source that is labeled by newly fixed carbon within several hours.

Cytosolic IPP

Among the possible carbon sources for the unlabeled isoprene could also be cytosolic IPP produced from pyruvate (itself containing approximately 50% glycolitic carbon; Karl et al., 2002a), through the mevalonic acid pathway (Fig. 3). The chloroplast envelope membrane is permeable to IPP (Kreuz and Kleinig, 1984; Heintze et al., 1990), and import of IPP is, in principle, possible (Lichtenthaler et al., 1997a).

This possibility, however, was not supported by our results for fosmidomycin treatments. Partial inhibition of the MEP pathway should enhance incorporation of cytosolic IPP, if this were an alternative carbon source. In this case, as was observed in Figure 1, the increased relative contribution of extrachloroplastic IPP should be accompanied by a shift in δ_{isop} toward that of the constant alternative $\delta^{13}C$ value (about −42‰, see above). Or in other words, increased contribution of cytosolic IPP would have depleted δ_{isop} in leaves where δ_{isop} before fosmidomycin treatment was approximately −30‰, would have enriched δ_{isop} of approximately −70‰, and would have had little effect on δ_{isop} of approximately −45‰. This was clearly not the case (Table II). The inhibitor treatments invariably resulted in more depleted isoprene, even when the pretreatment δ_{isop} was as low as −70‰. Such depletion could be the result of greater discrimination in the mevalonic acid pathway (Jux et al., 2001) only if cytosolic pyruvate is fully labeled by concurrently fixed C, in contrast to observations (Karl et al., 2002a). It is unlikely that any unlabeled intermediate in leaves is depleted enough to produce $\delta_{isop} < -70$‰. We therefore concluded that cytosolic IPP did not contribute to production of unlabeled isoprene, and we offer below an alternative explanation to the observed fosmydomicin-induced depletion in ^{13}C content of isoprene.

Isotopic Discrimination

Isotopic Discrimination in the MEP Pathway

Invoking the mevalonic acid pathway, Sharkey et al. (1991b) argued for discrimination by pyruvate dehydrogenase leads to ^{13}C-depleted isoprenoids. Recent works indicate, however, a non-mevalonate, MEP pathway (Zeidler et al., 1997; Lichtenthaler, 1999). Discrimination steps in the MEP pathway are not explicitly known, but the step highly prone to isotopic discrimination is the decarboxylation of pyruvate through deoxyxylulose-5-phosphate synthase (DXS). Pyruvate decarboxylation and reaction with G3P is achieved through thiamine pyrophosphate (Rohmer et al., 1996), as in the decarboxylation step of acetyl CoA production by mitochondrial pyruvate dehydrogenase complex, and is likely to have similar discrimination.

As mentioned above, whereas isoprene was always depleted in ^{13}C relative to photosynthetic intermediates, an increase in this depletion (i.e. increase in $\Delta_{C\text{-}isop}$) was observed in isoprene emitted from fosmidomycin-treated leaves. This is consistent with discrimination against ^{13}C occurring upstream from the inhibited step, namely the steps catalyzed by either DXS or deoxyxylulose-5-phosphate reductoisomerase (DXR). Kinetic isotopic discrimination, which is always expressed in the rate-limiting step (O'Leary, 1981; Cleland, 1982), downstream of DXR would be reduced or eliminated by the fosmidomycin inhibition, contrary to observations.

Furthermore, observations that deoxyxylulose 5-phosphate does not accumulate in the presence of fosmidomycin (Lange et al., 2001) argue against DXR as the discrimination step (but note that consumption of DOXP by other reactions cannot be ruled out at this stage). Our results of increased discrimination associated with pyruvate accumulation in conjunction with the currently held view of isoprene synthesis are therefore consistent with the DXS step as the rate-limiting and discriminating step (although this hypothesis will require further confirmation).

Isotopic Composition of Isoprene Emitted to the Atmosphere

The isotopic composition of atmospheric trace gases is a powerful tool to trace sinks and sources of these gases and underlying processes (Griffiths, 1998). Recently, the potential in using the isotopic composition of plant biomarkers in large-scale studies of terrestrial photosynthesis has been demonstrated (Conte and Weber, 2002). There is similar potential in using the isotopic composition of isoprene and other VOCs, that has not been realized. Very little information is available on the natural

abundance isotopic composition of isoprene, as well as on what influences it.

The isotopic composition of isoprene, δ_{isop}, must reflect the additive effect of Δ_A, the discrimination in photosynthetic carbon assimilation (Lloyd and Farquhar, 1994, and refs. therein), and that in the isoprene pathway, $\Delta_{C\text{-}isop}$, to produce the total discrimination $\Delta_{total} = \Delta_A + \Delta_{C\text{-}isop}$. Taking a typical mean Δ_A value of 17‰ (Bakwin et al., 1998), mean Δ_{total} values would be around 17‰ + 7‰ = 24‰.

It is now generally accepted that Δ_A can vary with time and plant species. In this study, we provide evidence that $\Delta_{C\text{-}isop}$ can vary at least between 4‰ and 11‰ (Fig. 2), a range that exceeds previously reported value for red oak (2.8‰ ± 0.4‰; Sharkey et al., 1991b). We further demonstrate that it is possible to separate estimates of $\Delta_{C\text{-}isop}$ from Δ_A by combining direct isotopic measurements of atmospheric CO_2 and isoprene and estimates of Δ_A based on gas-exchange approach of Evans et al. (1986; for the ecosystem scale, see Bowling et al., 2001) or by using $\Delta_{C\text{-}isop}$ in a monospecific canopy to estimate Δ_A. Better knowledge of Δ_{total} in different ecosystems will allow the use of atmospheric δ_{isop} measurements to trace sources of this compound. The ability to deconvolute the isotopic signal to Δ_A and $\Delta_{C\text{-}isop}$ can provide insights on processes associated with, for example, plants response to environmental stresses.

MATERIALS AND METHODS

Plant Material

Plants of velvet bean (*Mucuna pruriens*) were grown from seeds (Glendale Enterprises Inc., De Funiak Springs, FL) under ambient light and temperatures. Measurements were conducted on fully expanded, attached leaves. Mature branches of myrtle-1 and -2 (*Myrtus communis*) and buckthorn (*Rhamnus alaternus*) and leaves of reed (*Phragmites australis*) were cut under water from plants grown on the campus of the Weizmann Institute of Science (Rehovot, Israel) and were kept with the stem immersed in deionized water. Some measurements were done using attached myrtle leaves from plants grown in pots in a greenhouse. Potted plants were transferred to the campus at least a week before measurements (myrtle-3). Typical isoprene emission rates were approximately 10 nmol m^{-2} s^{-1} in all plants used.

Gas Exchange

Net assimilation rates, isoprene emission rates, and the isotopic composition of isoprene were measured with a leaf gas exchange system centered on a flow-through leaf cuvette in which the leaves were sealed. Net assimilation rates were measured using an infrared gas analyzer (Li-6262, LI-COR, Lincoln, NE) under light intensity of 1,000 μmol m^{-2} s^{-1} photosynthetically active radiation and leaf temperature of 26°C ± 0.5°C. An aliquot of the air in the leaf cuvette was pumped through a loop on a six-port valve (Valco Instruments Co, Houston, TX), which was cooled by a mixture of ethanol and dry ice (−75°C) for trapping the hydrocarbons. After trapping, the valve was switched to a flow of He, the loop was rapidly heated (200°C), and the trapped hydrocarbons passed to a gas chromatography (GC) column (GC-HP 5890, Wilmington, DE). The details of the gas exchange and isoprene sampling system are given in Affek and Yakir (2002).

Isotopic Analysis of Isoprene and Leaf Organic Matter

The isotopic composition of isoprene (δ_{isop}) was determined after GC separation using a Q-plot GC column (Supelco, Bellefonte, PA; 30 m long, 0.32 mm inner diameter; temperature program, 50°C for 1 min − 10°C min^{-1} − 170°C for 2 min) and combustion to CO_2. This GC column was selected because it provided good separation of isoprene and the large CO_2 peak (that interfered with isoprene in other columns). Retention times of CO_2 and isoprene were 300s and 700s, respectively, providing complete separation. A selection valve (see below) enabled us to direct the CO_2 peak to the flame ionization detector (FID), whereas only the isoprene peak was directed to the mass spectrometer. Testing on other columns (with myrtle and buckthorn) confirmed that they emit only isoprene (Affek and Yakir, 2002), and velvet bean and reed are commonly known as such. Furthermore, we tested separation of isoprene from several monoterpenes and 2-methyl-3-buten-2-ol and obtained good results with the Q-plot column.

A selection valve (MOVPT-1/100 pneumatic valve, SGE, Melbourne, Australia) was used to direct the flow eluting the GC column to either a FID or a combustion oven (CuO, 850°C) in which the desired GC peaks were each combusted quantitatively to CO_2. FID was used for isoprene concentration measurements. The CO_2 resulting from the combustion oven was used for isotopic composition measurements. The CO_2 from combustion was dried in a cold trap and fed in a flow of He, through an open split, to an isotope ratio mass spectrometer (IRMS; Optima, Micromass, Manchester, UK), where masses 44, 45, and 46 were measured. The ratio 45 to 44 was normalized to a pulse of CO_2 reference gas injected before each sample. The isotopic results are expressed in the δ (per mil) notation versus Vienna Pee Dee Belemnite standard, where $\delta = (R/R_{std} - 1) \times 1{,}000$ and R, R_{std} are the isotopic ratios $^{13}C/^{12}C$ of the sample and the standard, respectively.

For isotopic calibration, liquid isoprene was injected into a pre-evacuated glass bulb (10 L, 60 mtorr), and air or N_2 was added to atmospheric pressure. Aliquots were sampled and measured at the end of each experiment in a similar manner to the air in the leaf cuvette. Typical precision for isoprene standard measurements was ±0.3‰, and the $\delta^{13}C$ of isoprene standard in either air or in N_2 was the same, indicating no influence of CO_2 on δ_{isop}. The $\delta^{13}C$ of the liquid isoprene was predetermined by comparison with international and laboratory working standards, which were measured by conventional on-line combustion elemental analyzer (EA1109 CHN-O, Carlo Erba Instruments, Milan) connected to IRMS (Optima, Micromass). Ground whole dry leaves were measured by combustion in the same elemental analyzer to obtain $\delta^{13}C$ values of total leaf organic matter (δ_{leaf}).

Isotopic Analysis of CO_2

δ_{isop} was compared with the isotopic composition estimated for newly fixed carbon (δ_{fixed}), based on isotopic measurements of the CO_2 in the leaf cuvette. Samples of the air entering and leaving the leaf cuvette were dried and collected in glass flasks (100 mL) and $\delta^{13}C$ of the CO_2 was measured as described by Gillon and Yakir (2000). The CO_2 from each flask was trapped in liquid N_2 in a sample loop (1/16 inch outer diameter, 350 μL), which was then heated; and the sample was carried in a flow of He (80 mL min^{-1}), separated on a Porapak QS packed column (Supelco; 2 m, 50–80 mesh, 50°C) or Haysep D (Supelco; 3 m, 80°C), and analyzed for isotopic composition by IRMS (either Optima or a 20–20 [PDZ Europa, Crewe, Cheshire, UK]). δ_{fixed} was estimated by on-line discrimination calculations (Evans et al., 1986) using the CO_2 concentrations and $\delta^{13}C$ in the air entering and leaving the leaf cuvette.

CO_2 was calibrated by measuring air from cylinders of 400 or 1,000 μL L^{-1} CO_2 having different $\delta^{13}C$ values that were, in turn, calibrated by comparing with a cylinder of 400 μL L^{-1} CO_2 of known $\delta^{13}C$ value. Typical precision of ^{13}C analysis in CO_2 was ±0.15‰.

Air containing CO_2 of various isotopic compositions was supplied to leaves during experiments. Ambient air ($\delta^{13}C$ of approximately −8‰) was pumped through a 50-L external buffering volume and dried using drierite (8 mesh; W.A. Hammond Drierite, Xenia, OH). Cylinder air containing no CO_2 was mixed via mass flow controllers (MKS1179A, MKS instruments, Andover, MA) with cylinder air (Gordon Gas and Chemicals, Tel Aviv) containing 1% or 2.5% (v/v) CO_2 whose $\delta^{13}C$ value was −48‰, −33‰, or −27‰. Cylinder air mixture containing 383 or 366 μL L^{-1} CO_2 whose $\delta^{13}C$ value was −13‰ or −42‰, respectively, were also used.

CO_2-Labeling Experiments

Experiments testing the effect of the δ_{fixed} on δ_{isop} were performed by measuring both parameters when the leaf cuvette was supplied with air of

ambient CO_2 concentration and of a certain $\delta^{13}C$ value. After approximately 2 h of constant δ_{fixed} and δ_{isop}, the source CO_2 was rapidly switched, changing the $\delta^{13}C$ but not any of the gas-exchange parameters. δ_{fixed} and δ_{isop} were measured for an additional 2 h under the new source CO_2.

Few experiments were done in the dark or under CO_2-free air. Gas-exchange parameters and the isotopic composition of isoprene and CO_2 were measured in the light and under ambient CO_2 concentration during few hours to obtain control values. For CO_2-free air measurements, the air supply to the leaf cuvette was switched rapidly to CO_2-free air. CO_2 produced by respiration resulted in 10 to 20 μL L^{-1} CO_2 in the cuvette. Gas-exchange parameters and the isotopic composition of isoprene and CO_2 were measured under these conditions during 2 to 4 h. For measurements in the dark, the leaf cuvette was covered by a dark cloth. The isotopic composition of isoprene was measured within 10 min (after longer times, isoprene emission was too low for isotopic measurements).

Isoprene Pool in Leaves

The amount of isoprene stored in 10 myrtle leaves was measured by freezing leaves in liquid N_2 immediately after cutting and extracting the volatile fraction by heating under a flow of He (Loreto et al., 1998). The sample was dried by magnesium perchlorate (Aldrich Chemical Co., Milwaukee), trapped in a loop cooled by a mixture of ethanol and dry ice, heated, and measured by GC, as described above. Magnesium perchlorate was examined separately and was found to influence neither the concentration nor the isotopic composition of an isoprene standard.

Labeled Glc and Inhibitor Feeding

All chemicals used in our experiments were fed to the leaves as aqueous solutions through the petiole. Fosmidomycin (Molecular Probes, Eugene, OR) was fed at concentrations of 5 to 20 μM for emission rates measurements and 2 to 5 μM for isotopic analysis experiments. Measurements were performed during approximately 2 h before feeding and then during 2 h, beginning approximately 1 h after onset of fosmidomycin feeding. D-Glc (BDH Chemicals, Poole, Dorset, UK; $\delta^{13}C = -10‰$) was fed at concentration of 15 mM. Measurements were done during few hours before feeding at both ambient CO_2 concentrations and CO_2-free air. Then, feeding of Glc were performed during approximately 2 h under ambient CO_2 concentration and continued for an additional 2 h under CO_2-free air.

Pyruvate Content

Water soluble fraction of leaves was extracted from leaf-discs (total area of 15 cm^2) as described by Duranceau et al. (1999). Pyruvate was separated by ProStar HPLC (Varian, Palo Alto, CA) with an AS11 column (Dionex, Sunnyvale, CA) at 24°C, with NaOH concentration gradient of 0.4 to 22.5 mM as eluent, at 2 mL min^{-1}, and measured by a Dionex ED50 electrochemical detector.

Statistical Analysis

Statistical analysis was done using the *t* test and regression functions in the data analysis add-in from Microsoft Excel 2001 for Macintosh (Microsoft Corp., Redmond, WA).

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