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Evidence of the Photosynthetic Origin of Monoterpenes Emitted by *Quercus ilex* L. Leaves by ^{13}C Labeling[1]

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The carbon of the four main monoterpenes emitted by *Quercus ilex* L. leaves was completely labeled with ^{13}C after a 20-min feeding with 99% $^{13}CO_2$. This labeling time course is comparable with the labeling time course of isoprene, the terpenoid emitted by other *Quercus* species and synthesized in leaf chloroplasts. It is also comparable with that of phosphoglyceric acid. Our experiment therefore provides evidence that monoterpenes emitted by *Q. ilex* are formed from photosynthesis intermediates and may share the same synthetic pathway with isoprene. By analyzing the rate and the distribution of labeling in the different fragments, we looked for evidence of differential carbon labeling in the α-pinene emitted. However, the labeling pattern was quite uniform in the different fragments, suggesting that the carbon skeleton of the emitted monoterpenes comes from a unique carbon source.

Quercus ilex L. is the most common oak species in Mediterranean forests. *Q. ilex* leaves emit many monoterpenes, which may contribute to changes in the atmospheric composition (BEMA, 1994). Among the monoterpenes emitted, α-pinene, β-pinene, sabinene, and myrcene are the most abundant (BEMA, 1994; Kesselmeier et al., 1996). α-Pinene emission, in particular, represents about 40% of the total emission, and the emission rate ranges between 1 and 10 nmol m^{-2} s^{-1} in gas-exchange experiments performed under ambient air conditions. The emission is light dependent (Staudt and Seufert, 1995; Loreto et al., 1996) and CO_2 dependent (Loreto et al., 1996). The dependence on these environmental factors suggests that α-pinene biosynthesis may be related to photosynthesis and that the emission is controlled by the availability of photosynthesis intermediates (Loreto et al., 1996).

It is possible to monitor the appearance of ^{13}C in the emitted carbon by supplying $^{13}CO_2$ to the air flowing in a gas-exchange system. This technique was used to monitor the labeling rate of isoprene, a photosynthesis-dependent gas emitted by many oak species that is not emitted by *Q. ilex*. Sanadze et al. (1972) found that isoprene labeling was incomplete and that isoprene carbons were preferentially labeled, but these findings were contradicted by Delwiche and Sharkey (1993). In that experiment, there was no evidence of preferential labeling of isoprene carbon atoms. The time course of isoprene labeling was rapid (Delwiche and Sharkey, 1993) and similar to that of phosphoglyceric acid. Thus, that experiment supported previous indications that isoprene is formed in the chloroplasts (Mgaloblishvili et al., 1979) and that phosphoglyceric acid is the main carbon source for isoprene (Loreto and Sharkey, 1990).

$^{13}CO_2$ was also used to label monoterpenes emitted and stored by spruce (Schurmann et al., 1993). This led to the observation that the monoterpenes emitted were labeled faster than those stored in the specialized organs. It was proposed that a connection between photosynthesis and monoterpene synthesis and emission could exist. However, the time course of sampling (4 h after feeding) was too slow to determine whether the origin of the carbon used for monoterpene synthesis was photosynthetic.

In this study we used ^{13}C labeling to gain better insight into the biosynthetic pathway of the monoterpenes emitted by *Q. ilex* leaves. In particular, we investigated (a) whether the time course of labeling was comparable to that of photosynthesis intermediates and that of isoprene in isoprene-emitting species, to understand whether the synthetic pathway is similar for isoprene and monoterpenes, and (b) whether a preferential labeling in the monoterpene carbon was evident, to understand whether different carbon sources are necessary to form the monoterpene skeleton.

MATERIALS AND METHODS

Plant Material

Six 3-year-old seedlings of *Quercus ilex* were used for this experiment conducted in springtime. Each seedling was planted in a 50-L pot containing commercial soil and grown in a greenhouse. Air temperature and light intensity varied with sky conditions. On clear and sunny days, the light intensity at the canopy level was about 700 μmol photons m^{-2} s^{-1}, and the air temperature was between 25 and 30°C. During the night, the air temperature was between 15 and 20°C. Soil water and nutrient content were

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Abbreviation: ppm, parts per million.

maintained stable by irrigating pots every other day and occasionally adding soluble fertilizers.

Gas Exchange and $^{13}CO_2$ Feeding

Intact 1-year-old single leaves were clamped in a 0.5-L Plexiglas cuvette (Walz, Effeltrich, Germany). A film of Teflon was used to coat the cuvette interior to avoid the release of gas from the cuvette walls. The cuvette was thermostated with thermoelectric modules, and the temperature of the abaxial leaf side was sensed by a copper-constantan thermocouple (Walz). During the experiment, the leaf temperature was maintained at 30°C. The leaf was illuminated with an Osram (Munich, Germany) Power Star HQ-T 1000 bulb supplying 1000 μmol photons m^{-2} s^{-1} at the leaf level. This experiment was replicated three times in different leaves, but the results obtained were very similar. Therefore, only the result of one leaf feeding is shown.

At the beginning of the experiment, the leaf was exposed to a 2-L min^{-1} flow of air containing 350 ppm CO_2. Synthetic air was generated by mixing N_2, O_2, and 5% CO_2 in air having a 1.1% ^{13}C abundance with mass flow controllers. Before CO_2 was added, the air stream was humidified by bubbling it into water. The RH of air entering the cuvette was set to 50% by condensing the excess humidity in a water bath. When steady photosynthesis was measured, a Teflon valve was open to let 50 mL min^{-1} air coming out of the cuvette flow through a glass tube (15 × 0.3 cm i.d.) filled with Carbotrap C (0.034 g; Supelco, Bellefonte, PA) and Carbotrap (0.17 g). After 5 min, the Teflon valve was closed and all of the air was again directed to the IRGA for determination of CO_2 and H_2O exchange between leaf and air.

The CO_2 supplied was then substituted by switching to a 3-L tank containing 99% $^{13}CO_2$. To prepare this tank, we connected it to a vacuum line until a 10^{-2} torr vacuum was created. Then, the tank was placed in a liquid N_2 container, and 250 mL of 99% $^{13}CO_2$ (Sigma) were transferred into it. Finally, the tank was filled with pure N_2. The concentration of $^{13}CO_2$ in this mixture was 1500 ppm, but the concentration of CO_2 in the air entering the cuvette during the ^{13}C feeding was lowered to 350 ppm by mixing CO_2 with N_2 and O_2 as previously indicated. Since the IRGA sensitivity to $^{13}CO_2$ is much lower than that to $^{12}CO_2$, we analyzed the $^{13}CO_2$ concentrations in the tank and in the air entering the cuvette by MS. The signal was calibrated with the signal of a 354-ppm standard CO_2 tank (Caracciolo, Rome, Italy).

$^{13}CO_2$ feeding was carried out for 60 min, and four monoterpene traps were collected; the collection ended after 10, 20, 40, and 60 min. Each sample collection lasted 5 min. The $^{13}CO_2$ tank was again substituted with the tank having 1.1% $^{13}CO_2$, and four more monoterpene traps were collected after 10, 20, 40, and 60 min.

We measured the time required to exchange the volume of air into the cuvette. At the set flow rate, the residence time of the different CO_2 sources was 2 min. This time delay is considered in the presentation and discussion of data.

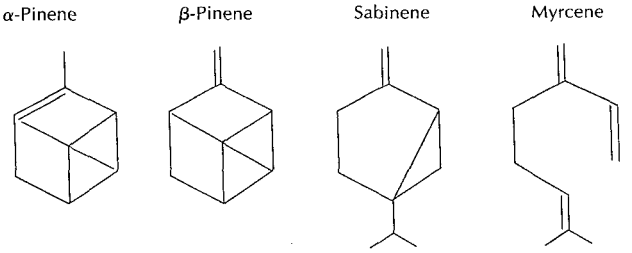
Each monoterpene trap was sealed and analyzed by GC-MS (HP 5890; Hewlett-Packard). Trapped monoterpenes were desorbed, cryofocused, and detected with a 60-m × 0.32-mm capillary column (J & W Fisons, Folsom, CA) coated with a 0.25-mm film of DB-1. Details of this procedure were given previously by Loreto et al. (1996). Monoterpene identification and ^{13}C-labeling pattern were carried out by selected-ion MS as reported by Ciccioli et al. (1992). Mass spectra were obtained by electron impact at 70 electron volts.

RESULTS

The ^{13}C-labeling pattern was similar in α-pinene, β-pinene, sabinene, and myrcene. The carbon of these monoterpenes was 90% ^{13}C after a 60-min feeding with $^{13}CO_2$ (Fig. 1). Since α-pinene is the most abundant among the monoterpenes emitted by *Q. ilex* leaves (Table I; Loreto et al., 1996), only the α-pinene data will be presented and discussed. Labeling of α-pinene following $^{13}CO_2$ feeding was fast. When examining the molecular ion (m/z = 136), we found that after a 10-min $^{13}CO_2$ feeding 60% of the α-pinene carbon was labeled (Fig. 2A). The α-pinene labeling increased to 90% after a 20-min feeding. We continued to feed $^{13}CO_2$ for 60 min, but ^{13}C incorporation in α-pinene carbon proceeded very slowly between 20 and 60 min. After a 60-min labeling, 7% of the

Table I. *Photosynthesis, monoterpene composition, and total emission of a Q. ilex leaf*

Monoterpenes were separated by GC and identified by selected-ion MS. Photosynthesis was measured before and after the $^{13}CO_2$ feeding; monoterpene composition and emission were measured also during the $^{13}CO_2$ feeding. No parameter varied significantly during the experiment. Measurements were done at a leaf temperature of 30°C, with an incident light intensity of 1000 μmol photons m^{-2} s^{-1} and at a CO_2 concentration in the air of 350 ppm.

Photosynthesis	Monoterpenes	α-Pinene	β-Pinene	Sabinene	Myrcene
					
$\mu mol\ m^{-2}\ s^{-1}$	$nmol\ m^{-2}\ s^{-1}$	% of monoterpenes			
5.25	9.55	34.3	24.9	17.5	9.2

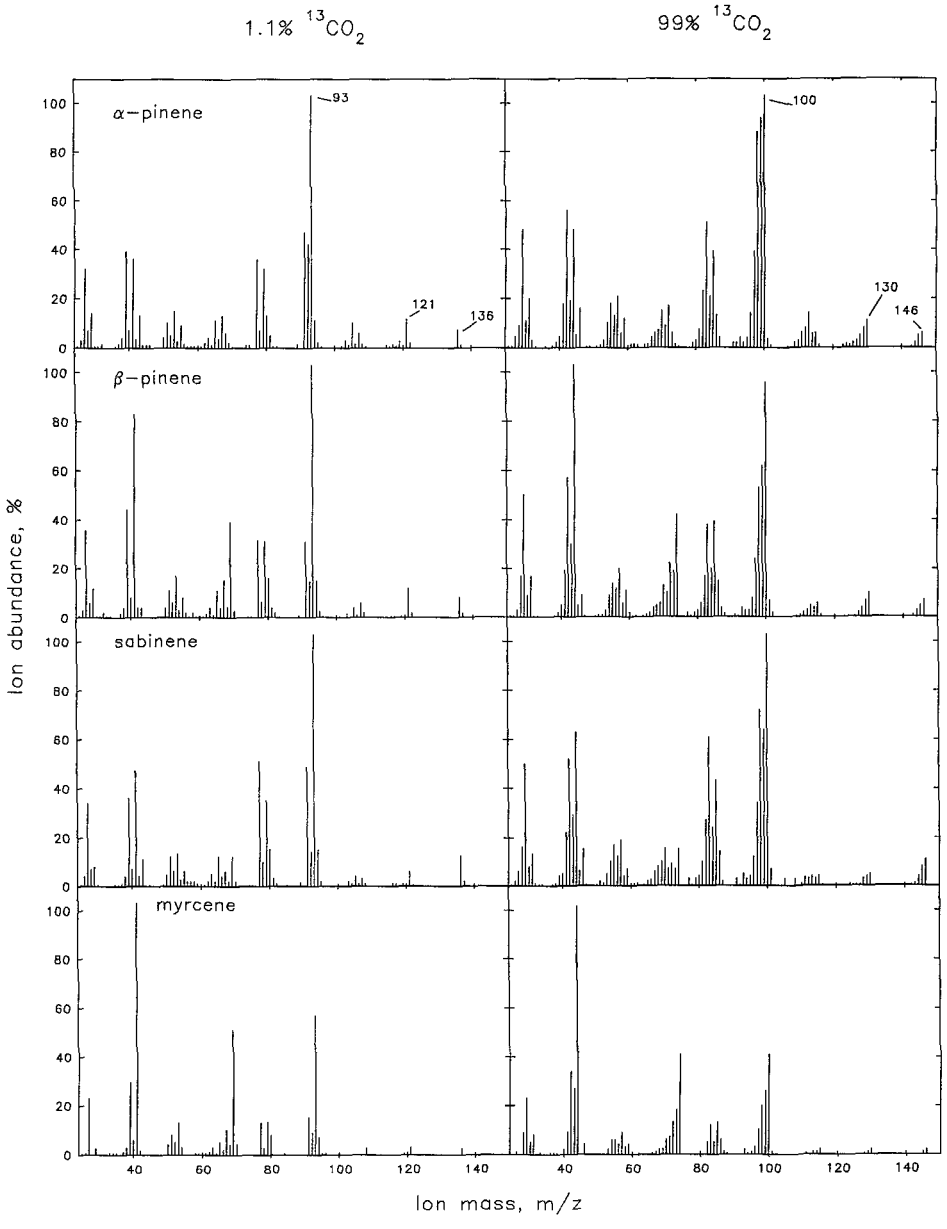


Figure 1. Electron impact (70 electron volts of energy) mass spectra of α-pinene, β-pinene, sabinene, and myrcene emitted by *Q. ilex* leaves before (left) and after (right) a 60-min feeding with 99% $^{13}CO_2$. The ion masses analyzed during this experiment are reported in the α-pinene panels.

α-pinene carbon was still unlabeled. When the natural abundance (1.1%) of ^{13}C was restored in the CO_2 entering the gas-exchange cuvette, the percentage of carbon emitted as α-pinene and labeled with ^{13}C changed again very rapidly. It decreased to 68% after 10 min and to 2.5% after 20 min and did not change significantly during the following 40 min (Fig. 2A).

The fragment with seven carbon atoms (m/z = 93) was the most abundant in the spectra of monoterpenes (Fig. 1). We compared the pattern of ^{13}C labeling of this fragment with that of a seven-carbon fragment of the molecular ion calculated on the basis of a random labeling of the α-pinene carbon atoms. The signal of the nine-carbon fragment was also stronger than that of the molecular ion in our spectra (Fig. 1). We also calculated the seven-carbon fragment resulting from the random labeling of the nine-carbon fragment. There was a general agreement between the measured and the calculated seven-carbon fragments (Fig. 3). Ten minutes after $^{13}CO_2$ feeding and 10 min after restoring the natural abundance of $^{13}CO_2$, some discrepancies were evident between fragments. However, after a 60-min feeding with $^{13}CO_2$ and 60 min after restoring the natural abundance of ^{13}C, the discrepancies between measured and calculated fragments were very small. The same pattern of labeling was found for each of the fragments visible in the spectrum of α-pinene (data not shown).

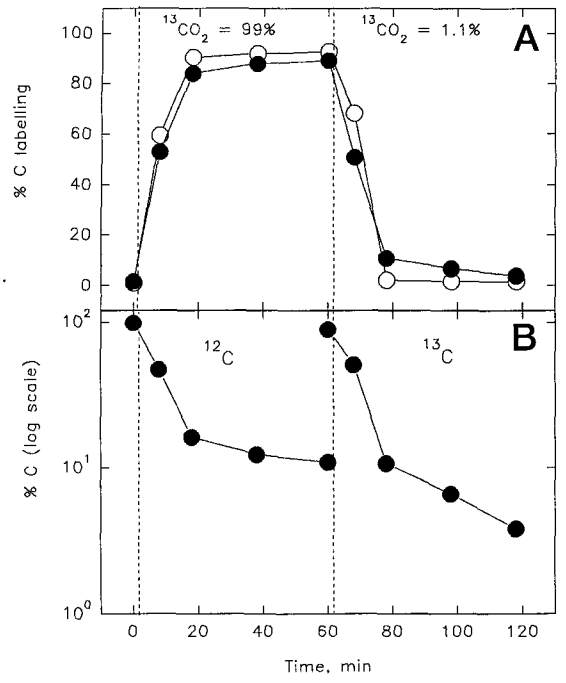


Figure 2. Time course of labeling and unlabeling with ^{13}C of the molecular ion (○) and of the seven-carbon fragment (m/z = 93, ●) carbon atoms in α-pinene emitted by *Q. ilex* leaves (A). The first vertical dashed line shows when CO_2 with a natural abundance of ^{13}C (1.1%) was substituted with 99% $^{13}CO_2$. At the second vertical dashed line the 1.1% $^{13}CO_2$ concentration was restored. In B, the time course of the disappearance of ^{12}C and ^{13}C in the α-pinene carbon after switching to different ^{13}C sources is plotted in a logarithmic scale. The symbol size is proportional to the sample collection time (5 min).

We compared the time course of labeling and unlabeling of the m/z = 93 fragment with that of the molecular ion and found no clear difference between them (Fig. 2A). The m/z = 93 fragment was also almost totally labeled with ^{13}C after 20 min of feeding. The same labeling time course was also found for the other α-pinene fragments (not shown).

By using the m/z = 93 fragment, we replotted in a logarithmic scale the time course of the disappearance of the ^{12}C ion during $^{13}CO_2$ feeding and compared it with the time course of the disappearance of the ^{13}C ion after restoring the natural abundance of ^{13}C in the CO_2 (Fig. 2B). The disappearance of the two ions was very fast during the first 20 min, as indicated by the steep slope, but was much slower between 20 and 60 min. The disappearance of the ^{13}C ion after restoring the 1.1% ^{13}C concentration was more complete than that of the ^{12}C ion after feeding ^{13}C.

DISCUSSION

The carbon of the four monoterpenes representing more than 80% of the total monoterpene emission by *Q. ilex* leaves was rapidly labeled with ^{13}C. Twenty minutes after feeding $^{13}CO_2$ (18 min when considering the residence time of the gas in the cuvette), about 90% of the α-pinene emitted was labeled. The time course and the extent of labeling was similar to that reported for isoprene emitted by *Quercus rubra* leaves (Delwiche and Sharkey, 1993). In that experiment, isoprene labeling was faster during the first 9 min and then proceeded at a slower rate. This suggested that isoprene carbon comes predominantly from a chloroplastic source and in part (about 20%) from a slower cytosolic pool. In our experiment, when the rate of α-pinene labeling and unlabeling was plotted in a logarithmic scale, the slope steepness did not clearly change during the first 20 min (Fig. 2B). However, gas collection for measurement of monoterpenes lasted 5 min; therefore, faster changes of monoterpene labeling could not be resolved. Overall, the rate of labeling of α-pinene was similar to that observed for phosphoglyceric acid (Canvin, 1979) and reported by Delwiche and Sharkey (1993). This supports the idea that monoterpenes emitted by *Q. ilex* leaves have the same carbon precursor and share the same metabolic pathway with isoprene emitted by other oak species (Loreto et al., 1996). Both isoprene and monoterpenes seem to be synthesized in the chloroplasts from photosynthetic carbon. This is at odds with previous observations from studies in which monoterpene emission was considered independent of photosynthesis (Lerdau et al., 1991; Tingey et al., 1991). On the other hand, plastids have already been determined to be the organelles where monoterpenes are synthesized (Kleinig, 1989; Soler et al., 1992).

If the monoterpenes were stored in specialized organs, then the tight relationship between recently fixed photosynthate and monoterpene synthesis would be difficult to demonstrate. Schurmann et al. (1993) found in spruce that the emitted monoterpenes are labeled when the stored monoterpenes are still unlabeled with $^{13}CO_2$. These authors concluded that monoterpenes may be synthesized from carbon recently fixed and then stored in the resin ducts. The emission of totally labeled monoterpenes in a plant species like *Q. ilex*, which does not store monoterpenes in large pools, may indicate that the interpretation of Schurmann et al. (1993) is correct. It has been suggested that plants emitting or storing a high amount of monoterpenes develop specialized organs like resin ducts and glands, whereas when the monoterpene emission is low the synthesis is restricted to the place of emission (McGarvey and Croteau, 1995). The second category includes plants that synthesize monoterpene in chromoplasts and leukoplasts (McGarvey and Croteau, 1995). The emission of chloroplast-synthesized monoterpene from *Q. ilex* leaves may also be included in this category. However, the amount of carbon emitted as monoterpene by *Q. ilex* leaves is not low. In fact, it is comparable to the amount of carbon emitted as isoprene by other oak species (Loreto and Sharkey, 1990) and even larger than the carbon emitted as monoterpenes from conifer stands (BEMA, 1994). The pool of monoterpenes present in *Q. ilex* leaves must be small, since (a) it is rapidly labeled and (b) it is rapidly depleted upon exposure to darkness (Loreto et al., 1996). Therefore, the amount of monoterpene emitted by *Q. ilex* leaves is representative of their formation. If the synthesis of monoterpenes occurs similarly in all plants, irrespective of the presence of storage organs, then *Q. ilex* can be used as a case study to

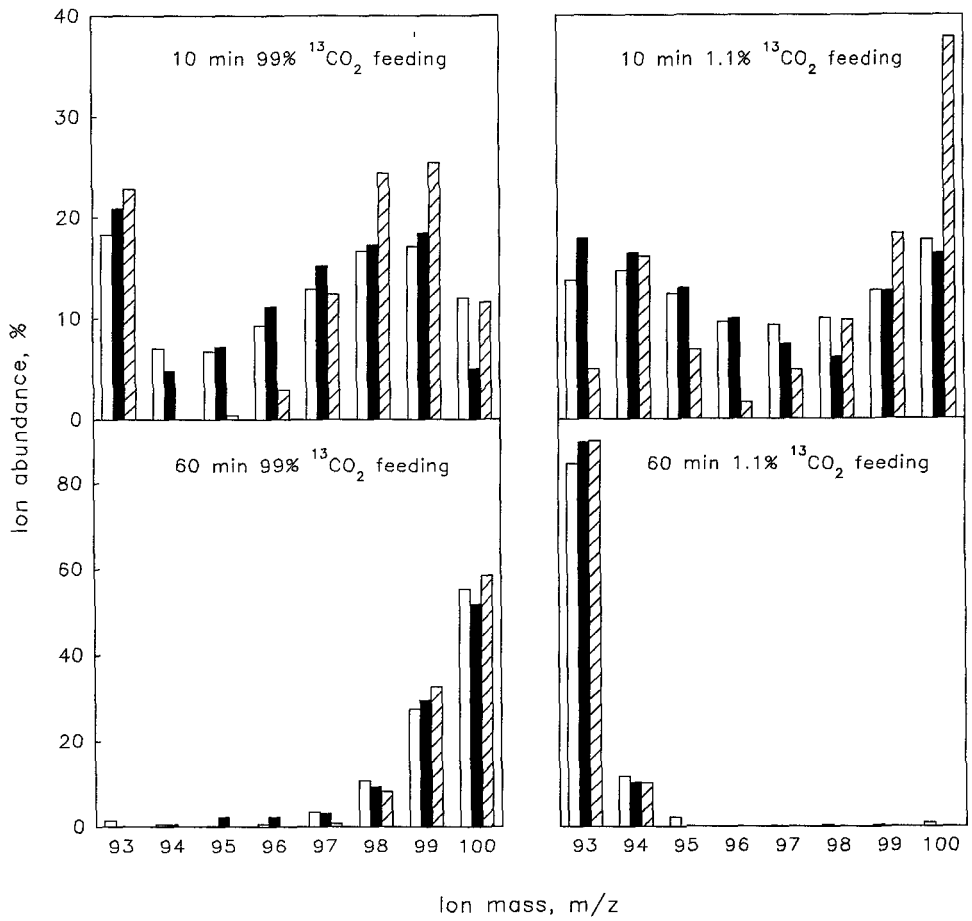


Figure 3. Distribution of carbon between different ions of α-pinene after feeding 99% $^{13}CO_2$ (left panels) and after restoring the 1.1% concentration of $^{13}CO_2$ in the air (right panels). The distribution observed in the seven-carbon fragment (open bars) was compared with the distribution calculated from the nine-carbon fragment (solid bars) and from the molecular ion (striped bars). These seven-carbon fragments were calculated assuming a random distribution of ^{13}C labeling between all of the carbons, both in the nine-carbon fragment and in the molecular ion. The relative abundance of each ion mass is shown 10 min (top panels) and 60 min (bottom panels) after switching to different $^{13}CO_2$ sources.

investigate monoterpene formation through gas-exchange experiments.

We continued to feed $^{13}CO_2$ to *Q. ilex* leaves for 60 min to determine whether total carbon labeling of monoterpene could be reached. However, only a small increase of the percentage of labeled carbon was observed (Fig. 2). The slope of the rate of unlabeling after restoring the 1.1% $^{13}CO_2$ composition was steeper than that observed during the labeling with 99% $^{13}CO_2$. This may indicate that the percentage of $^{13}CO_2$ in the cuvette was lower than 99% because of back diffusion of external, unlabeled CO_2 in the cuvette. If this interpretation is correct, then virtually all of the monoterpene carbon was labeled during the first 20 min. An alternative hypothesis is that a second, endogenous source supplies about 10% of the carbon for monoterpene formation and is labeled very slowly. This second interpretation is similar to that invoked by Delwiche and Sharkey (1993) to explain the slow labeling of about 20% of the carbon emitted as isoprene in *Q. rubra* leaves. An isoprene slow-labeling pool was not saturated after a 18-min $^{13}CO_2$ feeding, and this caused a more rapid unlabeling of isoprene. If this second carbon source is similar for isoprene and monoterpenes, our results indicate that even 60 min are not enough to saturate the pool, slowly labeling and contributing to both isoprene and monoterpene formation.

The signal given by the molecular ion was small in all of the monoterpenes examined. On the contrary, a strong signal came from the seven-carbon fragment ($m/z = 93$), which was therefore used in data analysis. We wanted to know whether carbon atoms of the monoterpene skeleton were differentially labeled and unlabeled. To do so, we calculated seven-carbon fragments from the molecular ion and from a nine-carbon fragment on the basis of random labeling of the carbon atoms and compared them with the measured seven-carbon fragment. After 60 min of labeling and unlabeling, the three fragments looked very similar. However, when the labeling pattern was examined 10 min after feeding 99% $^{13}CO_2$ and 10 min after restoring the 1.1% $^{13}CO_2$, the fragment calculated from the molecular ion showed a slower rate of labeling and unlabeling with respect to the measured

seven-carbon fragment. This difference was not found between the measured seven-carbon fragment and that calculated from the nine-carbon fragment. Therefore, although a preferential labeling of the last carbon is possible, we think that all carbon atoms were randomly labeled and that the observed discrepancy was caused by errors in the estimation of the small signal of the molecular ion. This error was more evident during the transients when several ion masses were present and the signal was particularly small.

This experiment supplies strong evidence for the photosynthesis origin of all of the carbon used to synthesize monoterpenes in *Q. ilex* and suggests that a photosynthesis intermediate may be a common precursor for both isoprene and monoterpene synthesis in plants. The occurrence of preferential carbon labeling in the monoterpene skeleton cannot be definitely discarded but seems rather unlikely.

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