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Differentiation between de novo synthesized and constitutively released terpenoids from *Fagus sylvatica*

Johannes Kahl, Thorsten Hoffmann*, Dieter Klockow

Institut für Spektrochemie und Angwandte Spektroskopie (ISAS), Bunsen-Kirchhoff-Str. 11, D-44139 Dortmund, Germany

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Abstract

Plants can use different carbon sources for the biosynthesis of individual C_{10}-volatiles. Feeding $^{13}CO_2$ to *Fagus sylvatica* plants, we monitored the incorporation of ^{13}C into the emitted compounds by mass spectrometry. By analyzing the rate and the distribution of labelling in the different fragments of the released compounds, we distinguished the instant emission within minutes after CO_2 assimilation from the delayed emission from storage compartments. The experiments provide evidence that the carbon skeleton of the emitted monoterpenes derived from two different carbon sources and that the contribution of the sources can be completely different for individual monoterpenes. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: *Fagus sylvatica*; Volatile emission; Monoterpenes; Biosynthesis; Labelling; Mass-spectrometry

1. Introduction

Plants emit a wide range of volatile hydrocarbons into the atmosphere (Fehsenfeld et al., 1992). Numerous studies implicate that monoterpenes, next to isoprene, are the dominant class of released compounds (Guenther et al., 1995). Although the role of volatile terpenoids in secondary metabolism still represents a largely debated issue, models have been proposed describing for example the isoprene release as a means of controlling energy flow in plants (Sharkey, & Singsaas, 1995). The important role of volatile terpenoids in tritrophic plant–insect interactions is well established (Dicke, & Sabelis, 1988; Turlings, Tumlinson, & Lewis, 1990). Pare and Tumlinson showed that, in cotton, the volatiles induced by insect herbivore damage are synthesized de novo with little or no release from storage, an observation that was interpreted as an active channelling of energy towards the release of volatiles as a defensive response (Pare, & Tumlinson, 1997a). The induced terpenoids showed significant higher incorporation levels of ^{13}C than those which were released constitutively. Recently, Loreto et al. gave evidence for the photosynthetic origin of released monoterpenes from *Quercus ilex* by ^{13}C labelling (Loreto et al., 1996b). Terpenoids released from *Picea abies* plants were partly enriched with isotope label whereas analysis of endogenous terpenoids in the needles indicate that even after a 24 h exposure to $^{13}CO_2$ there was no incorporation of ^{13}C (Schürmann, Ziegler, Kotzias, Schönwitz, & Steinbrecher, 1993).

In this study we used ^{13}C labelling to distinguish whether the volatile terpenoids from *Fagus sylvatica* are emitted directly after CO_2 assimilation depending on photosynthetic activity (connected with a rapid turnover of carbon) or are emitted from preformed intermediates which are stored in different compartments of the plant (McGarvey, & Croteau, 1995). Recently, we have shown that the rate of emission of certain terpenes from *Fagus sylvatica* is closely coupled to the rate of biosynthesis (Schuh et al., 1997). Emissions of sabinene, the main compound emitted from *Fagus sylvatica*, was shown to be light-depended and decreased below the detection limit in darkness whereas emission

* Corresponding author.

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Table 1
Total monoterpene emission from *Fagus sylvatica* plants during labelling experiments (25°C, photon flux 300 μmol m^{-2} s^{-1})

Sabinene	490 pmol m^{-2} s^{-1}
α−pinene	70 pmol m^{-2} s^{-1}
Limonene	20 pmol m^{-2} s^{-1}

of another monoterpene, limonene, did not change in darkness (Table 1).

Here we present results, focussing on the monoterpenes α-pinene, sabinene and limonene, which give evidence that different carbon sources are used for the synthesis of the released terpenes and that the contribution of the sources vary depending on light condition and the particular terpenoid structure.

2. Results

After 66 min of exposure to $^{13}CO_2$ a fraction of the fragments in the mass spectrum of the emitted sabinene showed complete incorporation of ^{13}C into the carbon skeleton (Fig. 1). The high intensity of m/z 100 indicates that all carbons in the seven-carbon-fragment ($C_7H_9^+$) are replaced by ^{13}C. Similarly, m/z 130 as well as m/z 146 could only result if all carbons from the nine-carbon-fragment and the ten-carbon fragment were ^{13}C. The rapid shift of all fragments to higher masses shown in Fig. 1 proofs de novo synthesis of sabinene emitted from *Fagus sylvatica*. In contrast, the mass spectra of α-pinene showed that only a fraction the released volatile incorporated ^{13}C, whereas the majority of the α-pinene molecules were unlabelled. For limonene, we could not find any differences between the mass spectra of the hydrocarbon before and during the experiment.

To utilize the measured mass spectra as well as the observed time course of labelling in a more quantitative manner, we developed a model to predict the mass spectra of the volatiles after ^{13}C incorporation. The model is based on the statistical probability of ^{13}C-incorporation into a C_x-skeleton. We assumed either a random or preferential distribution for the incorporated carbon. The term ''random incorporation'' is used here in the sense that it defines an equal probability of all positions in the molecule to incorporate a labelled carbon, without subdividing the skeleton into possible precursor units (e.g. activated isoprene-units). For all calculations we corrected the gas phase-concentrations of both, labelled and unlabelled CO_2 as well as the emitted compounds for the residence time of a gas inside the plant chamber by treating the chamber as a continuously stirred tank reactor. Calculations of the temporal behavior of the incorporation from different carbon sources were carried out on the basis of the non-labelled mass-spectra. Modelled mass spectra were then fit to the measured spectra. We achieved best fit of calculated and measured mass spectra assuming:

1. that 100% of the emitted *sabinene* is formed de novo, whereby 90% of the carbon is supplied from photosynthetic intermediates (carbon source 1; rapid incorporation of ^{13}C) and 10% of the carbon in the sabinene molecules is from precursors that are not closely linked with photosynthesis intermediates (carbon source 2; constant incorporation of unlabelled carbon).
2. that 20% of the emitted *α-pinene* molecules are synthesized de novo, whereas 80% of α-pinene originated from a pool of the monoterpene inside the leaves.
3. that 100% of the released *limonene* originates from a monoterpene pool inside the leaves.
4. a lifetime for carbon (turnover) from source 1 in the intermediary pool leading to the monoterpenes (sabinene and α-pinene) of 5 min.
5. a random distribution for all incorporated carbons.

In Fig. 2 the temporal change of the calculated and measured fragments 93, 95 and 100 from sabinene is shown after fitting the model with the conditions described above. 5 min after starting the exposure the signal intensity of m/z 93 decreased exponentially with time. The slope indicated a rapid incorporation of ^{13}C in the sabinene skeleton at the beginning of the experiment. The m/z 95 increased during the first 20 min, according to its generation from 91, 92 and 93 and decreased when more than four ^{12}C are replaced by ^{13}C in the carbon-skeleton. The m/z 100 increased as soon as all ^{12}C of the seven carbon fragment are replaced by ^{13}C (93 + 7), which starts for the first molecules 20 min after beginning of the exposure. However, the signal intensity of m/z 100 is levelling off towards the end of the experiment and is not reaching the same intensity as the related m/z 93, strongly pointing on a permanent incorporation of ^{12}C from a different carbon source. This is also indicated in Fig. 3, which shows the best fit of calculated and measured mass-spectra of sabinene after ca. 1 h $^{13}CO_2$ exposure, assuming a second, unlabelled carbon source contribution of 10%.

3. Discussion

Two different carbon sources exist for the de novo biosynthesis of monoterpenes in the leaves of *Fagus sylvatica*. For sabinene the de novo synthesized intermediates supply all of the carbon inside the monoterpene-skeleton (whereby 90% originates from recently assimilated CO_2, 10% from preformed intermediates)

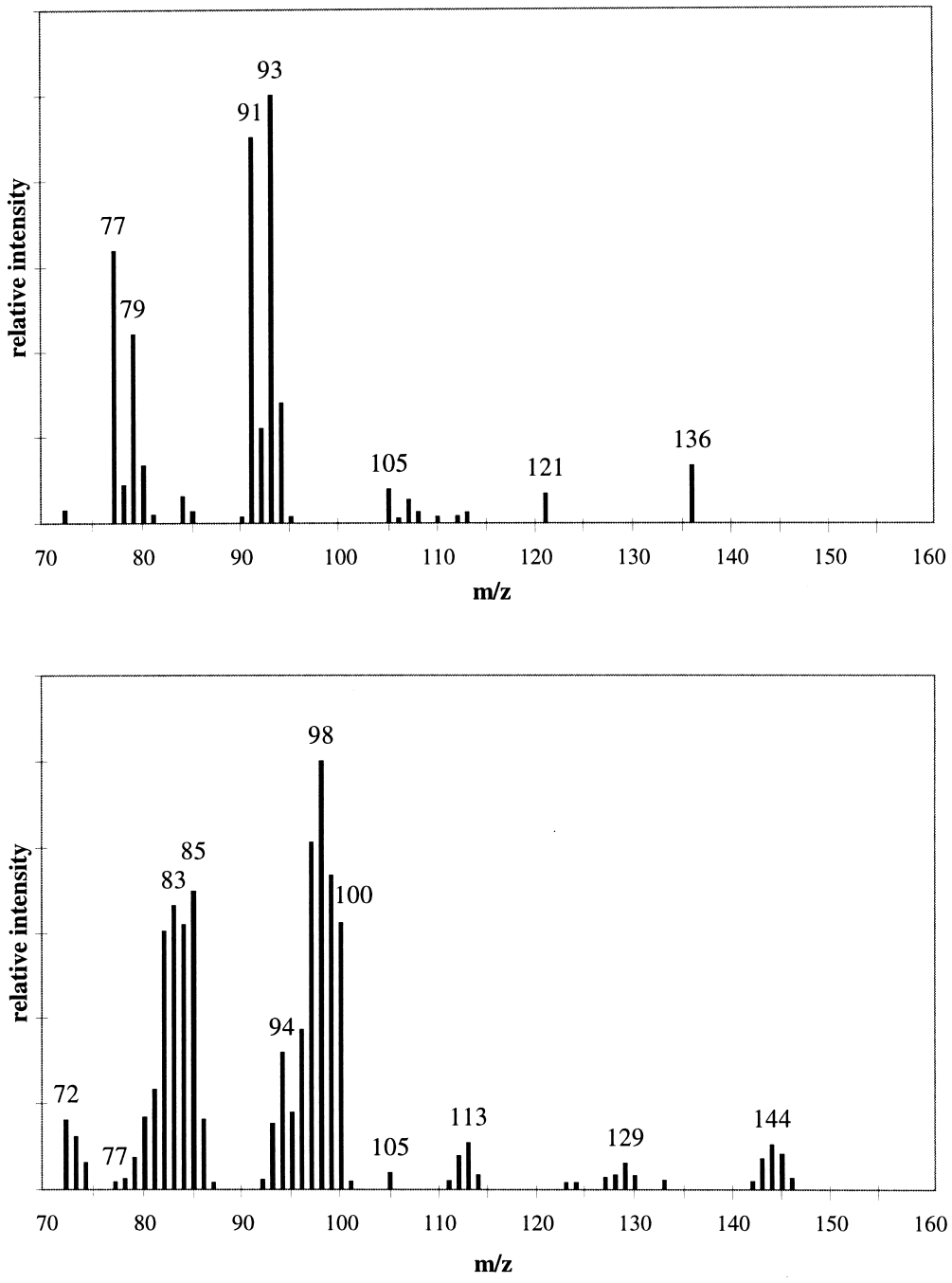

Fig. 1. Mass-spectra of sabinene emitted from *Fagus sylvatica* before and 66 min after starting the pulse label.

whereas 100% of the limonene molecules seem to come from a monoterpene pool. This is the first demonstration for intact plants, which gives a strong evidence that plant volatile terpenoid emission is based on both de novo biosynthesis from CO_2 *and* on emission from storage compartments.

Pare and Tumlinson measured the ^{13}C incorporation in different compounds emitted from *Gossypium hirsutum* after caterpillar feeding (Pare, & Tumlinson, 1997b). They found high incorporation of ^{13}C label into the induced compounds (ocimene/92%, (E,E)-α-farnesene/99%) whereas the constitutively emitted compounds incorporated very low levels of ^{13}C label (β-pinene/5%, α-humulene/5%) which suggest two different precursor pools. Although *Quercus ilex* has no specialized structures for terpene storage more than one pool of monoterpene precursors seem to be involved in volatile release, because different rates of labelling were determined for the investigated compounds (Loreto et al., 1996a). Whereas α-pinene was

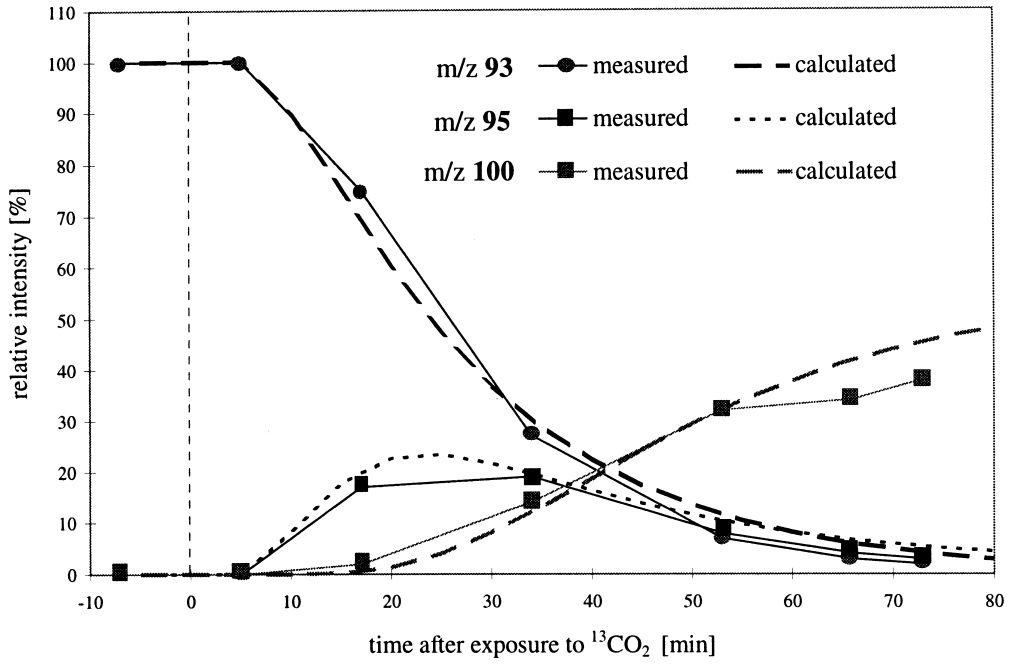

Fig. 2. Measured and calculated temporal changes of the fragment ions 93, 95 and 100 from the mass spectra of sabinene.

labelled quickly the emitted linalool showed a delay before label appeared. The authors suggest the existence of a reduced carbon pool possibly in non-photosynthetic plastids.

Previous studies (Delwiche, & Sharkey, 1993; Loreto et al., 1996a) had shown that 10% and 20% of the carbon leading to α-pinene and isoprene, respectively was not or slowly labelled during the $^{13}CO_2$ feeding experiments. The authors speculated that a second, endogenous source supplies part of the carbon for monoterpene formation which was labelled very slowly. However, they could not exclude errors in the experimental set-up. The measurements presented here support the concept that for one released substance

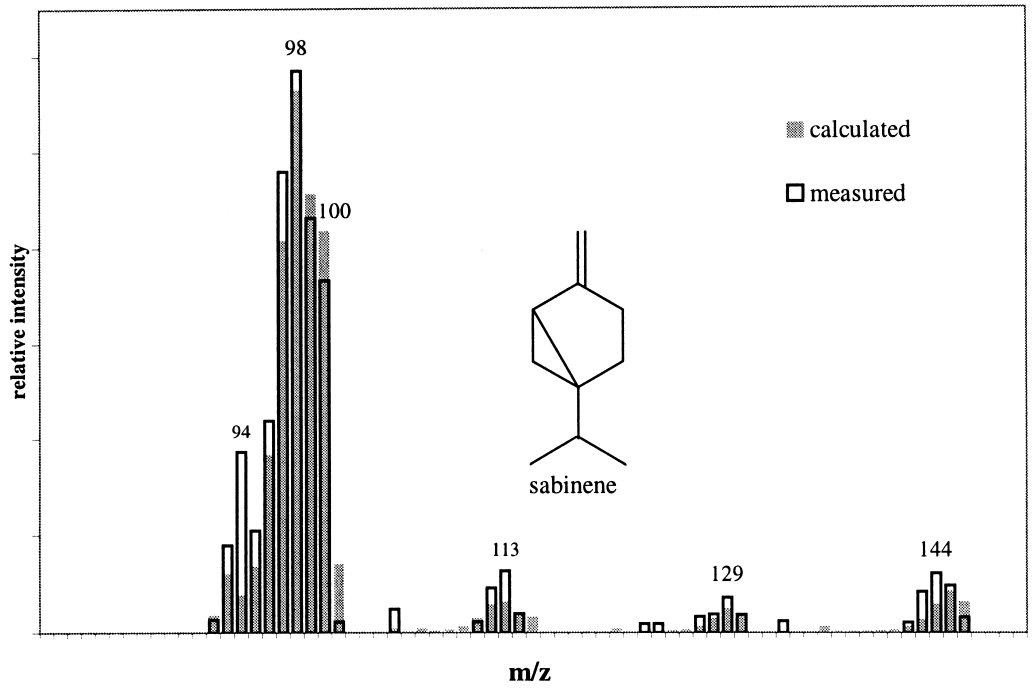

Fig. 3. Calculated and measured mass-spectra of sabinene (*m*/*z* 91–146) emitted from *Fagus sylvatica* 66 min after starting the pulse label.

two different carbon sources exists which supply the carbon for the terpene-skeleton. The 5 min turnover of the carbon of the de novo biosynthesized monoterpenes sabinene and α-pinene from *Fagus sylvatica* is similar to that reported from Loreto at al. for α-pinene from *Quercus ilex* leaves (Loreto et al., 1996b). In that study the rate of labelling found for the monoterpene was similar to that of isoprene as well as to that reported for phosphoglyceric acid, which indicates a close link between the carbon sources for isoprene and the photosynthetic carbon reduction pathway (Delwiche, & Sharkey, 1993). In agreement with the studies of Loreto et al., we achieved the best fit of the calculated and measured mass-spectra for sabinene and a pinene assuming a random distribution for the incorporated carbon from the two different sources into all fragments of the molecules.

The results presented here show that the contribution of the different sources of intermediates supplying the carbon for the terpene-skeleton varies not only between plant species for a selected molecule but also within one class of compounds emitted from one plant species. It might be speculated that the different sources found here are somehow connected with the two known major pathways for isoprenoid biosynthesis, the acetate/mevalonate pathway (Wright, 1961) and the glyceraldehyde phosphate/pyruvate pathway (Lichtenthaler, Schwender, Disch, & Rohmer, 1997; Zeidler, Lichtenthaler, May, & Lichtenthaler, 1997). However, this cannot be decided from our results and more work is needed in order to understand the regulation of terpenoid biosynthesis. Nevertheless, insight into both, the temporal and the spatial regulation of biosynthesis, might serve as a basis to comprehend the function of certain compounds in plant metabolism and/or catabolism as well as to develop biogenic hydrocarbon emission rate algorithms.

4. Experimental

4.1. Plant material

6 year old *Fagus sylvatica* plants were from commercial supplier. Plants were grown outside in 5 l pots in commercial soil under natural light conditions. Plants were watered the last time 12 h before the experiment.

4.2. Gas exchange and $^{13}CO_2$ administration

Three 6 year old *Fagus sylvatica* plants were placed 48 h before the experiment in a controlled plant enclosure chamber. The chamber allows the simulation of typical ambient conditions. The wall materials (PFA and a specific glass material (SANALUX©)) transmit the PAR and partly UV range of the solar radiation (details given in Kahl, Hoffmann, & Klockow (1996)). Air temperature and light intensity varied with sky conditions during the experiments between 21°C and 24°C and 200 μmol m^{-2} s^{-1} and 400 μmol m^{-2} s^{-1}, respectively. A ventilator inside the chamber minimized the boundary layer resistance on the surface of the leaves.

In order to perform the investigations under physiologically realistic conditions we used intact whole plants. Due to the high costs of the $^{13}CO_2$ needed for the whole plant investigations, the experiments were carried out only once.

The plants were exposed to a 17 l min^{-1} flow of synthetic air containing 350 ppm CO_2. The flow-rates were measured continuously with mass-flow-meters. Before CO_2 was added, the air stream was humidified by bubbling it into water ($r_H = 59 \pm 5\%$). The $^{13}CO_2$ was supplied by switching the CO_2-inlet to a 99% $^{13}CO_2$ tank (Promochem).

4.3. Volatile collection and analysis

Volatiles were collected at approximately 10 min intervals before and during the exposure. The sampling flow was 160 ml min^{-1}. Sampling air was taken from the outlet of the plant chamber and collected on Tenax TA©/Carbopack B© filled adsorbent traps (description is given in Hoffmann (1995)). The compounds were thermodesorbed and analyzed using a gas chromatograph/mass-spectrometer. Gas chromatographic operating conditions were as follows: 50 m × 0,2 mm, BPX5-columns (1 μm film thickness), oven temp 37°C for 2 min, then increased with a rate of 5°C min^{-1} to 80°C, followed by a second ramp of 20°C min^{-1} to 220°C. The transferline temperature was 220°C. Operation conditions of the mass spectrometer (Finnigan MAT-ITD 700): scan range 46–205 m/z, EI 70 eV. Compounds were identified using pure reference standards (Aldrich, Fluka, purity 93–96%).

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References

Delwiche, C. F., & Sharkey, T. D. (1993). *Plant Cell Environ.*, *16*, 587.

Dicke, M., & Sabelis, M. W. (1988). *Netherland J. of Zoology*, *38*, 148.

Fehsenfeld, F., Calvert, J., Fall, R., Goldan, P., Guenther, A. B., Hewitt, C. N., Lamb, B., Liu, S., Trainer, M., Westberg, H., & Zimmerman, P. (1992). *Global Biogeochemical Cycles*, *6*, 389.

Guenther, A., Hewitt, C. N., Erickson, D., Fall, R., Geron, C., Graedel, T., Harley, P., Klinger, L., Lerdau, M., McKay, W. A., Pierce, T., Scholes, B., Steinbrecher, R., Tallamraju, R., Taylor, J., & Zimmerman, P. (1995). *J. Geophys. Res.*, *100*, 8873.

Hoffmann, T. (1995). *Fres. J. Anal. Chem.*, *351*, 41.

Kahl, J., Hoffmann, T., & Klockow, D. (1996). *Proceedings of the EUROTRAC Symposium*, *96*, 197.

Lichtenthaler, H. K., Schwender, J., Disch, A., & Rohmer, M. (1997). *FEBS Letters*, *400*, 271.

Loreto, F., Ciccioli, P., Brancaleoni, E., Cecinato, A., Frattoni, M., & Sharkey, T. D. (1996a). *Proceedings of the National Academy of Science of the USA*, *93*, 9966.

Loreto, F., Ciccioli, P., Cecinato, A., Brancaleoni, E., Frattoni, M., Fabozzi, C., & Tricoli, D. (1996b). *J. Plant Physiol.*, *110*, 1317.

McGarvey, D. J., & Croteau, R. (1995). *Plant Cell*, *7*, 1015.

Pare, P. W., & Tumlinson, J. H. (1997a). *Nature*, *385*, 30.

Pare, P. W., & Tumlinson, J. H. (1997b). *J. Plant Physiol.*, *114*, 1161.

Schuh, G., Heiden, A. C., Hoffmann, T., Kahl, J., Rockel, P., Rudolph, J., & Wildt, J. (1997). *J. Atmos. Chem.*, *27*, 291.

Schürmann, W., Ziegler, H., Kotzias, D., Schönwitz, R., & Steinbrecher, R. (1993). *Naturwissenschaften*, *80*, 276.

Sharkey, T. D., & Singsaas, E. L. (1995). *Nature*, *374*, 769.

Turlings, T. C. J., Tumlinson, J. H., & Lewis, W. J. (1990). *Science*, *250*, 1251.

Wright, D. L. (1961). *Annu. Rev. Biochem.*, *30*, 525.

Zeidler, J. G., Lichtenthaler, H. K., May, H. U., & Lichtenthaler, F. W. (1997). *Z. Naturforsch.*, *52c*, 15.