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**ISOPRENE EMISSION FROM PLANTS:
PHYSIOLOGICAL ROLE AND ISOTOPIC COMPOSITION**

פליטת איזופרן מצמחים: תפקיד פיזיולוגי והרכב איזוטופי

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Thesis for the degree of Ph.D.

presented to the Scientific Council of the Weizmann Institute of Science

April 2003

UMI Number: DP16824

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*This work is dedicated to my grandmother
whose encouragement accompanied me throughout this project.*

ACKNOWLEDGMENTS

Naturally, many helped me to complete this research, and I feel grateful to all of them. It is my pleasure to thank them here:

First of all is Prof. Dan Yakir, who guided me along this research during which, for five years, I have also changed from a young to mature person. Second, is my family who performed two roles: they strengthened me in difficult times and in many discussions gave me the so important outsider's point of view.

The advisory committee, Profs. Avigdor Schertz and Mordechai Sheves, gave me useful ideas, which improved this work; Prof. Yinon Rudich had useful ideas; Dr. Arye Tishbee guided me in my preliminary steps in chromatography and in tackling instrumentation problems; Drs. Amnon Schwartz and Victor Raskin, from the faculty of Agriculture of the Hebrew university, let me use their ozone fumigation equipment and shared with me their knowledge in related subjects; Dr. Francesco Loreto and Paola Pinelli from CNR-IBEV, Italy, introduced me to the area of field measurements and gave me their great hospitality, during my visit in their laboratory.

I'm happy to thank many others: Andy from Agan chemical manufacturers who made great efforts to supply me with bromoxynil; to Alla and Olga helped in pyruvate content measurements; Hagay, Avram, and Yankaleh assisted me in building and fixing my sampling system; Neomi, Dalia and Shoshana relieved me from coping with bureaucracy; Roberto, spent many hours trying to make my computer work. Michal Lavy and Burkhard Wilske discussed with me issues of terpenes and VOCs and Keren and Tamar - of atmospheric chemistry.

Kadmiel not only taught me how to measure chlorophyll fluorescence but also was a good friend and dragged me to many talks away from work and into the real world. Others in Dan's group and in the department helped me with their friendly relations and occasional advice. Ruth Yam, with whom I had many discussions on isotopes and biochemistry, was very patient even when I raised "crazy" questions.

Finally, I'm grateful to Emanuela Negreanu, with whom I spent many hours in the laboratory, gave me advice on mass spectrometry and graphical presentation of data, and without whom I could never cope with the amount of CO₂ isotopic analyses.

ABSTRACT

Isoprene is a major hydrocarbon emitted from plants. It influences the oxidative capacity of the troposphere and leads, in the presence of NO_x , to formation of tropospheric ozone. Isoprene production is strongly associated with photosynthetic activities and it utilizes up to a few percent of the carbon fixed in photosynthesis, but its physiological role is not fully known. The objectives of this study were first to characterize isoprene emission in a typical isoprene emitting Mediterranean plant (*Myrtus communis*). Second, to test the hypothesis that isoprene provides a protection mechanism against oxidative stress in leaves. Third, to examine, using natural abundance ^{13}C analysis, the quantitative coupling between isoprene production and concurrent photosynthetic carbon assimilation. Fourth, using the results to provide first estimates of the potential variations in the ^{13}C content of isoprene in nature.

M. communis showed large seasonal variations in isoprene emission rates, J_{isop} , from maximum rates of $30 \text{ nmol m}^{-2} \text{ s}^{-1}$ in summer to close to zero in winter. Rates of photosynthetic assimilation, A , showed relatively small changes over the same time (from $17 \mu\text{mol m}^{-2} \text{ s}^{-1}$ in winter to $13 \mu\text{mol m}^{-2} \text{ s}^{-1}$ in summer). Both J_{isop} and A increased with leaf age. In developing leaves, isoprene emission appeared a week later than the appearance of net assimilation. CO_2 concentrations greatly influenced J_{isop} , with a 2 to 4 fold decrease at high CO_2 concentrations (c_i of $900 \mu\text{L L}^{-1}$), as compared to ambient concentration (c_i up to $300 \mu\text{L L}^{-1}$). Isoprene emission was sustained at CO_2 -free air for several hours.

Potential protection of isoprene against oxidative stress focused on the effects of singlet oxygen. Singlet oxygen produced in the leaves of *M. communis* by Rose Bengal (RB) led to a 65% decrease in net assimilation rates within 3h, whereas isoprene emission rates showed either a 30% decrease and ambient CO_2 concentrations or even an increase (70%) under high CO_2 . In both cases, the treatments led to increase in calculated internal isoprene concentrations, suggesting a potential protection effect. Protection effects were assessed directly by fumigating young, non-emitting, leaves with exogenous isoprene, together with treatment with RB or bromoxynil (BX, simulating photoinhibition). The fumigation alleviated oxidative damage to the photosynthetic apparatus. In fumigated *M. communis* leaves there was 42% and 29% reduction in the damage to

net assimilation compared to non-fumigated leaves, for RB or BX treatments, respectively. In RB treated *Rhamnus alaternus* leaves fumigation resulted in 24% reduction in singlet oxygen damage. The isoprene protection against singlet oxygen was also evident in chlorophyll fluorescence yield (F_v/F_m) that showed a significantly smaller decrease in fumigated *R. alaternus* leaves (from 0.78 to 0.52) as compared to non-fumigated leaves (from 0.77 to 0.27).

The ^{13}C content of isoprene reflected within a few minutes the changes in the isotopic composition of ambient, atmospheric, CO_2 . Quantitatively, however, the labeling was incomplete even after several hours in leaves of *M. communis*, *R. alaternus* and *Mucuna pruriens* (only 72% to 91% of the labeling in the photosynthetically fixed C was reflected in the ^{13}C content of isoprene). These results indicated that some of the carbon in isoprene was not derived from concurrently assimilated CO_2 . The ^{13}C content of the alternative, slow turnover, C source was estimated from measurements in CO_2 -free air to be $\sim -42\text{‰}$. Using this estimate during labeling experiments indicated that the alternative C source had a turnover rate of ~ 10 h. This was consistent with labeled glucose feeding experiments, and contribution of glycolytic intermediates to isoprene production. The contribution of extra-chloroplastic C source to isoprene production is likely to be significant in enabling isoprene production, and its associated protection, under oxidative stress conditions when photosynthesis is limited.

The inhibitor of chloroplastic isoprene production through the MEP pathway, fosmidomycin, led to a decrease in isoprene emission rates accompanied by a depletion of ^{13}C in the remaining isoprene flux. This reflected an increase in the apparent discrimination against ^{13}C in isoprene production from $9.4 \pm 0.5\text{‰}$ to $11.4 \pm 0.5\text{‰}$, and was associated with 43% increase in the leaf pyruvate accumulation. Based on these results we hypothesize that the step catalysed by deoxyxylulose-5-phosphate synthase (DXS), is the rate-determining step and the main discriminating step in isoprene production. Variations in the relative rate of DXS among species may therefore explain the observed variations in the apparent discrimination (between 4 and 11‰) among species or individual plants under different conditions.

איזופרן הינו הפחמימן העיקרי הנפלט מצמחים. הוא משפיע על הכושר החימצוני של הטרופוספירה וגורם, בנוכחות תחמוצות חנקן (NO_x), ליצירת אוזון טרופוספירי. ייצור איזופרן קשור בקשר הדוק לפעילות פוטוסינתטית והוא צורך מספר אחוזים מהפחמן המקובע בפוטוסינתזה, אך עם זאת תפקידו הפיזיולוגי אינו ברור לחלוטין. מטרת המחקר היו ראשית, לאפיין פליטת איזופרן מצמח ים-תיכוני פולט איזופרן אופייני (הדס מצוי). שנית, לבדוק את ההשערה לפיה איזופרן מספק לעלים הגנה מפני עקה חימצונית. שלישית, לבחון, תוך שימוש בניתוח ^{13}C ברמת שכיחות טבעית, את הצימוד הכמותי בין ייצור איזופרן וקיבוע פחמן בו זמנית על-ידי פוטוסינתזה. ולבסוף, שימוש בתוצאות לקבלת הערכה ראשונית של השונות האפשרית בתכולת ^{13}C באיזופרן בטבע.

השתנות עונתית רבה נצפתה בקצב פליטת איזופרן, J_{isop} , בהדס, מ- $30 \text{ nmol m}^{-2} \text{ s}^{-1}$ בקיץ עד קרוב לאפס בחורף. קצב ההטמעה הפוטוסינתטית, A , השתנה יחסית מעט במהלך אותו זמן (מ- $17 \mu\text{mol m}^{-2} \text{ s}^{-1}$ בחורף ל- $13 \text{ mmol m}^{-2} \text{ s}^{-1}$ בקיץ). הן J_{isop} והן A גדלו עם גיל העלה. בעלים מתפתחים פליטת איזופרן הופיעה כשבוע לאחר הופעת קיבוע נטו של CO_2 . ריכוזי CO_2 השפיעו במידה ניכרת על J_{isop} , עם ירידה של פי 2 עד 4 בריכוזי CO_2 גבוהים ($c_i=900 \mu\text{L L}^{-1}$), בהשוואה לריכוזי אטמוספירי (c_i עד $300 \mu\text{L L}^{-1}$). פליטת איזופרן נשמרה באוויר נטול CO_2 במשך מספר שעות.

בחינת פוטנציאל ההגנה על-ידי איזופרן מפני עקה חימצונית התמקדה בהשפעה של חמצן סינגלטי. חמצן סינגלטי, שיוצר בעלי הדס על-ידי רוח בנגל (RB), גרם לירידה של 65% בקצב ההטמעה נטו בתוך 3 שעות, בעוד שקצב פליטת איזופרן ירד ב-30% בתנאים של ריכוז CO_2 אטמוספירי או אף עלה (70%) בתנאים של ריכוז CO_2 גבוה. בשני המקרים הטיפול גרם לעלייה בריכוז המחושב של איזופרן בתוך העלה, דבר המרמז על השפעה הגנתית אפשרית. ההשפעה ההגנתית הוערכה ישירות על-ידי טיפול של איזופרן חיצוני (פומיגציה) בעלים צעירים, שאינם פולטים איזופרן, תוך כדי טיפול ב-RB או בברומוקסיניל (BX, המדמה פוטואינהיביציה). האיזופרן החיצוני הקל על הנזק החימצוני למערכת הפוטוסינתטית. בעלי הדס שטופלו באיזופרן חיצוני הייתה הפחתה של 42%-ו-29% בנזק לקיבוע נטו של CO_2 , עבור טיפולי RB ו-BX בהתאמה, בהשוואה לעלים שלא טופלו באיזופרן. בעלים של אשחר רחב-עלים שטופלו ב-RB, הוספת איזופרן חיצוני גרמה להפחתה של 24% בנזק מחמצן סינגלטי. הגנה על-ידי איזופרן כנגד חמצן סינגלטי נצפתה גם בניצולת הפלורסנציה של כלורופיל (F_v/F_m), שבה הייתה ירידה קטנה יותר באופן משמעותי בעלי אשחר שטופלו באיזופרן חיצוני (מ-0.78 ל-0.52) בהשוואה לעלים שלא טופלו (מ-0.77 ל-0.27).

תכולת ה- ^{13}C באיזופרן שיקפה בתוך מספר דקות את השינויים בהרכב האיזוטופי של ה- CO_2 בסביבה, או באטמוספירה. לעומת זאת, באופן כמותי, הסימון לא היה מלא אפילו לאחר מספר שעות בעלים של הדס, אשחר ו-*Mucuna pruriens* (רק 72% עד 91% מהסימון של הפחמן המקובע בפוטוסינתזה השתקף בתכולת ה- ^{13}C של האיזופרן). התוצאות הצביעו על כך שמקורו של חלק מהפחמן באיזופרן לא היה פחמן שקובע באותו הזמן.

תכולת ה- ^{13}C במקור הפחמן החלופי, שהינו בעל קצב תחלופה איטי (slow turnover rate), הוערכה על-פי מדידות באוויר נטול CO_2 כ-42%. שימוש בהערכה זו יחד עם ניסויי סימון הצביע על כך שמקור הפחמן החלופי הוא בעל זמן תחלופה של כ-10 שעות. ממצא זה התאים לניסיונות עם גלוקוזה מסומנת ולתרומה של תוצרי ביניים גליקוליטיים לייצור איזופרן. תרומה של מקור פחמן חוץ-כלורופלסטי לייצור איזופרן צפויה להיות חשובה בתנאי עקה כימיונית, באפשרה ייצור איזופרן עם ההגנה הקשורה בו, כאשר הפוטוסינתזה מוגבלת.

המעכב של ייצור איזופרן כלורופלסטי במסלול ה-MEP, פוסמידומיצין, הביא לירידה בקצב פליטת איזופרן שהייתה מלווה בירידה בתכולת ה- ^{13}C בשטף האיזופרן הנותר. ירידה זו שיקפה עלייה בדיסקרימינציה כנגד ^{13}C בייצור איזופרן מ- $9.4 \pm 0.5\%$ ל- $11.4 \pm 0.5\%$, ונקשרה לעלייה של 43% בתכולת הפירובאט בעלה. מתוך תוצאות אלה אנו משערים כי השלב המזורז על-ידי DXS (דאוקסי קסילולוז-5-פוספט סינטאז) הוא השלב קובע המהירות ולכן השלב שבו מתרחשת רב הדיסקרימינציה בייצור איזופרן. שינויים בקצב היחסי של DXS בין זנים שונים יכולים, אם כן, להסביר את השינויים שנצפו בדיסקרימינציה (בין 4% ל-11%) בין זנים או צמחים שונים ובתנאים שונים.

TABLE OF CONTENTS

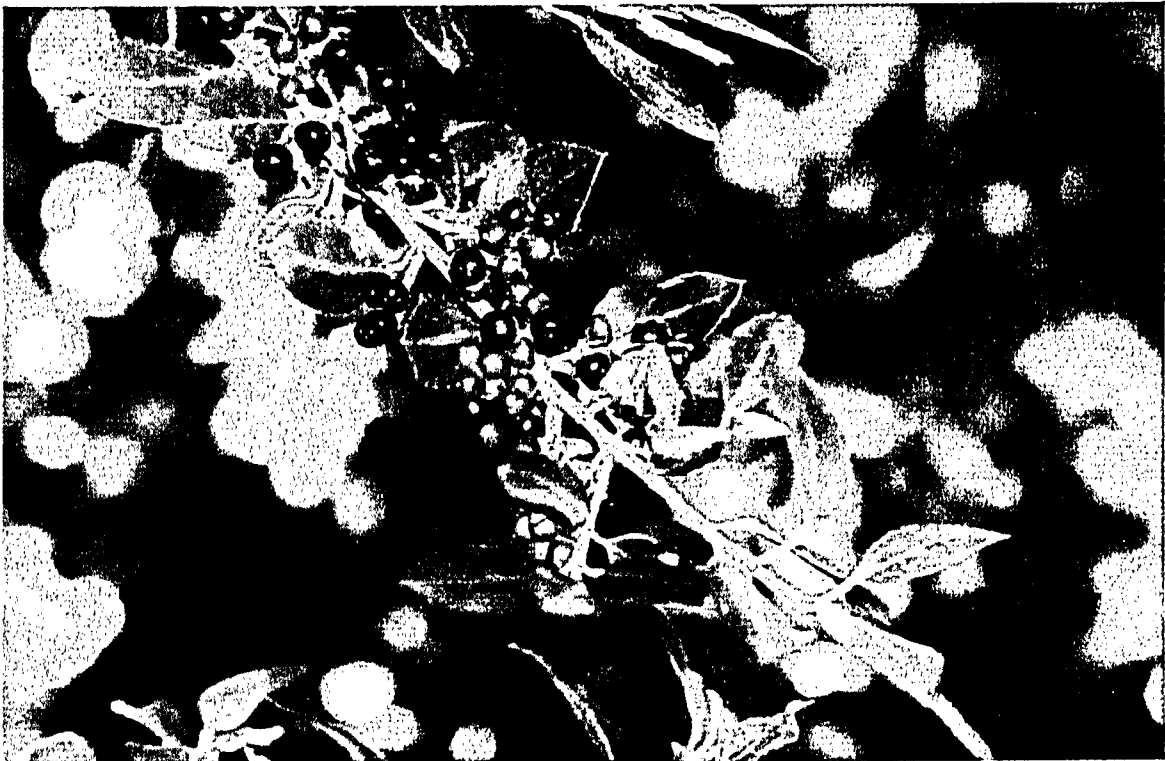
Abstract	4
List of abbreviations	10
1. Introduction	12-23
1.1. Motivation of the research	12
1.2. Background	12
1.2.1. Hydrocarbons and tropospheric ozone formation	12
1.2.2. Emission of hydrocarbons from leaves	14
1.2.3. Biochemical pathways of isoprenoids production	15
1.2.4. Influence of environmental conditions on isoprenoid emission	16
1.2.5. The physiological role of isoprene and terpenes	18
1.2.6. The isotopic composition of isoprene	19
1.3. Main goals of the research	20
1.4. Main findings and conclusions	20
1.4.1. Characterization of isoprene emission from myrtle leaves	20
1.4.2. Protection by isoprene against singlet oxygen	20
1.4.3. Isotopic composition and metabolic sources of isoprene	20
2. Methodology	24-33
2.1. Experimental approach	24
2.2. Plant material	24
2.3. Gas exchange measurements	25
2.4. Isoprene concentration measurements	27
2.5. Isotopic analysis of isoprene	28
2.6. Isotopic analysis of CO ₂	29
2.7. Singlet oxygen treatments	30
2.8. Chlorophyll fluorescence	31
2.9. Isoprene pool in leaves	32
2.10. CO ₂ labeling experiments	32
2.11. Labeled metabolites and inhibitor feeding	32
2.12. Pyruvate content	33
2.13. Statistical analysis	33

3. Isoprene emission from <i>Myrtus communis</i>	34-39
3.1. Seasonality	34
3.2. Leaf age and growth light	35
3.3. CO ₂ concentration	37
4. Protection by isoprene against singlet oxygen in leaves	40-51
4.1. Effect of singlet oxygen in myrtle	40
4.2. Isoprene fumigation in myrtle	45
4.3. Isoprene fumigation in buckthorn	47
4.4. Discussion	48
5. Isotopic composition and metabolic sources for isoprene	52-67
5.1. Effect of CO ₂ source	52
5.2. CO ₂ -free air	54
5.3. Emission in the dark	55
5.4. Glucose labeling	56
5.5. Cytosolic IPP	57
5.6. Isotopic composition of isoprene emitted to the atmosphere	59
5.7. Discussion	59
5.7.1. Incomplete coupling between isoprene and assimilation	59
5.7.2. Alternative C sources for isoprene	61
5.7.3. Isotopic discrimination	64
6. Summary and conclusions	68-71
7. Appendix 1: Dependence of the isotopic discrimination on reaction rates	72-75
8. Appendix 2: Dependence of the isotopic discrimination on reaction rates	76-77
9. Literature cited	78-91

LIST OF ABBREVIATIONS

A	net assimilation rate
ABA	2,cis-4,trans-abscisic acid
BX	bromoxynil, 3,5,-dibromo-4-hydroxybenzotrile
c_i	intercellular CO ₂ concentration
CoA	coenzyme A
DHAP	dihydroxyacetone phosphate
DMAPP	dimethylallyl pyrophosphate
DOXP	1-deoxy-D-xylulose-5-phosphate
DXR	1-deoxy-D-xylulose-5-phosphate reductoisomerase
DXS	1-deoxy-D-xylulose-5-phosphate synthase
F_v/F_m	chlorophyll fluorescence yield
GC-FID	gas chromatograph-flame ionization detector
GC-C-IRMS	gas chromatograph -combustion-isotope ratio mass spectrometer
G3P	glyceraldehyde-3-phosphate
HMG	hydroxymethylglutaryl
IPP	isopentenyl pyrophosphate
IRGA	infra red gas analyzer
Isop _i	intercellular isoprene concentration
MEP	2-methylerythritol-4-phosphate
NO _x	NO+NO ₂
¹ O ₂	singlet oxygen
PAR	photosynthetic active radiation (400-700nm)
3-PGA	3-phosphoglycerate
PSII	photosystem II
RB	Rose Bengal, 4,5,6,7-tetrachloro-2',4',5',7'-tetraiodofluorescein
RubP	ribulose-1,5-bisphosphate
VOC	volatile organic carbon

Chapter 1
INTRODUCTION



Leaves and fruits of *Rhamnus alaternus*

MOTIVATION OF THE RESEARCH

Plants play an important role in determining the chemical composition of the atmosphere. Oxygen, water vapor, and carbon dioxide are major constituents, whose atmospheric concentration is controlled by photosynthesis and respiration. Plants also influence the trace gas composition of the atmosphere and its oxidizing capacity, through the emission of reactive hydrocarbons. Emission of biogenic hydrocarbons, of which isoprene is a major player, is significant and led the American president Ronald Reagan, in 1980, to blame vegetation as being a major source for air pollution.

Isoprene (2-methyl-1,3-butadiene) is emitted by a variety of plant species (Kesselmeier and Staudt, 1999) and is a major hydrocarbon emitted from vegetation. It influences the trace-gas composition of the troposphere by reacting with OH radicals and NO_x (whose sources are mainly anthropogenic) 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 which are in turn, based on physiological emission studies and must consider biochemical and environmental influences on emission rates.

In order to improve emission models and to better predict the variations in isoprene emission rates, a better understanding of its physiological role as well as of the processes leading to its production should be obtained. This study tested the hypothesis that isoprene can protect plants from oxidative damage inflicted by singlet oxygen and developed the use of the carbon isotopic composition of isoprene to trace its metabolic sources.

Following is an overview of background information, regarding the atmospheric significance and biochemistry of isoprene emission. The goals and main findings of this work are presented. Then, the experimental approach and methodology to achieve the goals of the study are described and the main findings are presented in details.

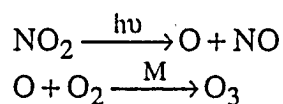
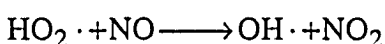
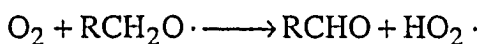
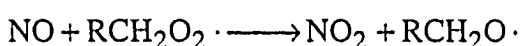
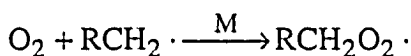
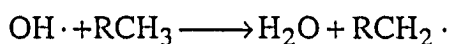
BACKGROUND

Hydrocarbons and tropospheric ozone formation

Tropospheric ozone, unlike its stratospheric counterpart, is harmful. It is considered "air pollution" on local scale and is a greenhouse gas on the global scale. Ozone damage to plants has

global implications such as affecting crop yield (Chameides et al., 1994) and leading to forest decline (e.g. in Israel: Naveh et al., 1980). In men and animals it can cause respiratory problems (Lippmann, 1991).

Unlike most pollutants, which are directly emitted to the atmosphere, tropospheric ozone is a secondary pollutant. Ozone is generated *in situ* from oxidation of hydrocarbons (VOCs, volatile organic carbon) in the presence of NO_x ($\text{NO} + \text{NO}_2$) and sunlight (Thompson, 1992). NO_2 is formed in oxidation of hydrocarbons by OH radicals. Photolysis of NO_2 yields ground state oxygen atoms (^3P), which recombine with O_2 to form ozone. This cycle is catalytic in OH and NO:



Tropospheric ozone is lost by photochemical dissociation ($\lambda < 310$ nm) forming $\text{O}(^1\text{D})$ that reacts with water to yield OH radicals (Thompson, 1992). Unlike the above production mechanism, in a clean atmosphere, when NO_x concentrations are low, ozone is destroyed by reacting with CO and hydrocarbons. Also, ozone is lost by deposition to the surface.

The major source for NO_x is anthropogenic emissions, primarily from transportation but also from electric utility power plants (Ryerson et al., 2001). VOCs, on the other hand, are emitted from both anthropogenic and natural biogenic sources. Lamb et al. (1987) estimated the total biogenic emission of VOCs in the USA to be 31 TgC/y ($31 \cdot 10^{12}$ gC per year), compared to 18 TgC/y anthropogenic emissions in 1983. Early ozone abatement attempts concentrated on reducing anthropogenic VOC emissions, mainly from automobile exhaust. This was successful in

areas where ozone formation is limited by VOC concentration (Fiore et al., 1998). But this strategy failed in vegetated cities and rural areas in which large VOC emission from vegetation led to ozone formation (Trainer et al., 1987; Chameides et al., 1988; Ryerson et al., 2001). In such places, ozone mitigation strategies should be based on reduction of anthropogenic NO_x emissions (Trainer et al., 1987; Ryerson et al., 2001).

Emission of hydrocarbons from leaves

Large amount of organic carbon is emitted from vegetation and is available for tropospheric ozone formation. Global biogenic VOCs emissions were estimated at 1150 TgC/y for the year 1990. This includes 503 TgC/y of isoprene and 127 TgC/y of monoterpenes (Guenther et al., 1995). Within the biogenic emissions isoprene (2-methyl-1,3-butadiene) is the most important component. The isoprene flux is comparable in magnitude to the total source of methane emission to the atmosphere (approximately 500 TgC/y; Crutzen, 1991). Isoprene has, therefore, an atmospheric significance besides its role in ozone production, as a scavenger of OH radicals (Jacob and Wofsy, 1988; Carslaw et al., 2001). Non-methane VOCs in general may decrease atmospheric OH concentrations by ~20% globally (Wang et al., 1998). As such they compete with methane over this major sink, and may contribute to atmospheric methane accumulation.

Isoprene is emitted by many, though not all, plant species (Harley et al., 1999; Kesselmeier and Staudt, 1999). It is mostly emitted from deciduous broad-leaved species and is well known to be dominant in North American oak forests. Evergreen trees, such as conifers, are characterized normally by monoterpenes emission. Typical atmospheric concentrations in a mixed forest are 1-10 nL L⁻¹ and 0.1-1 nL L⁻¹ for isoprene and monoterpenes (represented by α -pinene), respectively (Goldan et al., 1995). Recently, 2-methyl-3-buten-2-ol was found to be emitted as well from several pine species with concentrations, emission patterns and biochemistry similar to those of isoprene (Harley et al., 1998; Schade et al., 2000; Zeidler and Lichtenthaler, 2001). Grasses and agricultural crops were considered to emit only small amount of VOCs (Lamb et al., 1987; Hewitt and Street, 1992) but recently were found to emit a variety of oxygenated VOCs (Kristine et al., 1998).

In the Mediterranean forest, trees emit mostly monoterpenes whereas isoprene is generally emitted from shrubs (Hansen et al., 1997; Owen et al., 1997; Owen et al., 1998). Emission in the Mediterranean pseudosteppe habitat was found to be dominated by isoprene (emitted primarily

from *Asphodelus microcarpus*) (Ciccioli et al., 1997). Unlike the highly dominant isoprene emission from North American oak species, Mediterranean oaks were found to emit either isoprene or monoterpenes and few species do not emit isoprenoids at all (Steinbrecher et al., 1997; Loreto et al., 1998a; Loreto et al., 1998b; Csiky and Seufert, 1999).

In most monoterpene emitting species the monoterpenes are stored in the leaves in specialized oil glands or resin canals and are emitted from these reservoirs (Seufert et al., 1995). This is in contrast to isoprene, which is considered to be emitted as produced, without storage in the leaf (Sharkey et al., 1991a). However, some Mediterranean species, primarily oaks (though not only; Llusia and Peñuelas, 2000), were found not to store monoterpenes but to emit them as produced, as is the case with isoprene (Staudt and Seufert, 1995; Loreto et al., 1996c; Llusia and Peñuelas, 2000).

Biochemical pathways of isoprenoids production

Isoprene is produced in the leaf chloroplasts from isopentenyl pyrophosphate (IPP) through isomerization to dimethylallyl pyrophosphate (DMAPP) and subsequent elimination of the pyrophosphate. This stage is catalyzed by the enzyme isoprene synthase. This enzyme has both soluble (stromal) (Silver and Fall, 1991; Silver and Fall, 1995) and thylakoid-bound forms (Wildermuth and Fall, 1996; Wildermuth and Fall, 1998). Soluble isoprene synthase was found to be composed of two subunits, having molecular weights of 58 and 62 kDa (Silver and Fall, 1995). The enzyme requires Mg^{+2} or Mn^{+2} for activity as well as free thiols (Silver and Fall, 1995). Maximal enzymatic activity was obtained at pH=7 – 9 in the soluble form and around pH=10 for the thylakoid-bound enzyme (Silver and Fall, 1991; Wildermuth and Fall, 1996), whereas non-enzymatic conversion of DMAPP to isoprene is significant only at pH<3 (Silver and Fall, 1991). The temperature response pattern of isoprene synthase activity correlates well with that of isoprene emission (Monson et al., 1992). Isoprene synthase was suggested to be light regulated, explaining the light response of isoprene emission rates (Wildermuth and Fall, 1996).

In the past isoprene was considered to be produced by the plant as a part of the mevalonic acid pathway. In this pathway, three acetyl CoA molecules produce hydroxymethylglutaryl CoA (HMG CoA), whose reduction yields mevalonic acid, which is then converted, to IPP. However, in recent years it became increasingly accepted that this pathway exists in the cytosol but not in the chloroplast and as such it is used for sterol production but not for the synthesis of isoprene and

monoterpenes. The alternative pathway suggested for the production of IPP (MEP pathway) begins with addition of glyceraldehydes-3-phosphate (G3P) to decarboxylated pyruvate, yielding 1-deoxy-D-xylulose-5-phosphate (DOXP), which is rearranged and converted to IPP (Zeidler et al., 1997; Lichtenthaler, 1999). Uncertainty remains concerning possible influence of the cytosolic pathway on isoprene production through leakage of cytosolic IPP into the chloroplast (Lichtenthaler et al., 1997a).

Influence of environmental conditions on isoprenoid emission

Emission rates of monoterpenes in species that have storage oil glands depend on the pool size and the vapor pressure of each monoterpene. Hence, emission rates vary exponentially with temperature, although the temperature dependence is larger than that predicted by vapor pressure only (Tingey et al., 1980; Tingey et al., 1991). Monoterpenes production is light dependent but emission rates do not depend on light intensity and current photosynthetic rates (Tingey et al., 1991).

Emission rates of isoprene and non-stored monoterpenes depend on both temperature and light (Loreto et al., 1996c; Llusia and Peñuelas, 2000). Isoprene emission increases with increasing temperature (Sharkey and Loreto, 1993; Sharkey et al., 1996), to a maximal emission at temperatures around 40°C (Monson et al., 1992; Singsaas and Sharkey, 2000), whereas the optimal temperatures for photosynthesis are lower by 5 to 10°C (Monson et al., 1992). Fast response of isoprene emission was observed following fast changes in leaf temperature (Singsaas and Sharkey, 1998).

Plant growth at high temperatures leads to higher isoprene emission, at high temperatures, relative to plants grown at lower temperature (Monson et al., 1992). Also, isoprene emission was found to begin, in trees grown under different conditions, after a similar cumulative temperature (Monson et al., 1994). This leads to seasonal variations in isoprene emission rates with maximal isoprene emission rates in the summer and minimal rates in spring and autumn (in deciduous trees), well correlated with variations in the activity of isoprene synthase (Monson et al., 1994; Schnitzler et al., 1997).

Isoprene emission is light mediated and normally no emission is observed in the dark. In some cases the light response of isoprene emission is similar to the light response of photosynthesis (Monson and Fall, 1989) but in others, isoprene emission continues to increase also at light

intensities in which photosynthesis is saturated (Sharkey and Loreto, 1993). Isoprene synthase is bound to the thylakoids, facing the stroma, where it can be light regulated (Wildermuth and Fall, 1996). The light response of isoprene emission can be, therefore, related to light activation of the enzyme or light influence on the supply of the substrate DMAPP.

As in isoprene emission, the emission of non-stored α -pinene from *Quercus ilex* increases with light intensity. But unlike photosynthesis and isoprene, α -pinene emission in the dark decreased fast to 20% of the emission in the light and the residual emission decreased much slower (Loreto et al., 1996c).

Growth light intensity may also influence the emission rates of isoprene, with higher rates of net assimilation and isoprene emission in sun vs. shade leaves (Sharkey et al., 1991b; Litvak et al., 1996; Harley et al., 1994). This effect may be merely due to larger biomass per leaf area in sun grown leaves (Harley et al., 1994) but in other cases more isoprene is emitted from leaves grown at high light also on a dry mass basis (Litvak et al., 1996). The difference in isoprene emission between sun and shade leaves was shown to be due to development of lower activity of the enzyme isoprene synthase in leaves growing in the shade (Lehning et al., 1999).

The influence of temperature and light intensity lead to diurnal and seasonal variations in the emission of isoprenoids. Isoprene emission varies during the day together with light intensity, with maximal emission around noon and no emission at night. The emission of stored monoterpenes is also maximal around midday and night-time emission is low, but not negligible (e.g. Hansen et al., 1997).

Isoprene is not produced in very young leaves, lagging behind photosynthesis and stomatal conductance (Grinspoon et al., 1991; Sharkey and Loreto, 1993; Harley et al., 1994). A similar pattern was observed for the activity of the enzyme isoprene synthase suggesting that the level of the enzyme regulates the amount of isoprene emitted (Kuzma and Fall, 1993).

A pronounced diversion from the coupling of isoprene emission and assimilation rates is observed in the influence of intercellular CO_2 concentration (c_i) on isoprene emission. At low and ambient CO_2 concentrations, in which photosynthesis is CO_2 limited and assimilation rates increase with CO_2 concentration, isoprene emission rates are constant. Under high CO_2 concentrations, when photosynthesis is saturated, isoprene emission rates decrease with the increase in CO_2 (Monson and Fall, 1989; Loreto and Sharkey, 1990). Isoprene inhibition at high c_i

was explained as resulting from limitation of ATP, needed for isoprene production, due to feedback conditions (Loreto and Sharkey, 1990; Sharkey et al., 1991b).

Under CO₂ free air isoprene emission from red oak decreased markedly (Loreto and Sharkey, 1990) but no such decrease was observed in aspen leaves (Monson and Fall, 1989). In both species, however, very low isoprene emission was observed when both CO₂ and O₂ were removed from the air. Emission of non-stored α -pinene from *Q. ilex* decreased gradually after removal of CO₂, until it reached a steady state of 25% of the emission under ambient CO₂ concentration. When CO₂ was removed under low O₂ concentration, α -pinene emission decreased fast to a steady state of 20% of the emission under ambient air (Loreto et al., 1996c). The difference in response to CO₂-free air at low and ambient O₂ concentrations was attributed to the availability of 3-PGA, in spite of the inhibition of photosynthesis, by production in photorespiration, under ambient O₂ (Loreto et al., 1996c).

Isoprene is emitted through the stomata (Tingey et al., 1981; Fall and Monson, 1992), but the flux does not change significantly when stomatal conductance is reduced by abscisic acid (Monson and Fall, 1989; Fall and Monson, 1992). Isoprene emission was found to be much less sensitive to drought comparing to assimilation rates (Fang et al., 1996). The lack of stomatal control on isoprene emission was explained by accumulation of isoprene, which continues to be produced, in the leaf air spaces leading to an increase in its concentration gradient to the point where it balances the decrease in conductivity (Fall and Monson, 1992).

The physiological role of isoprene and terpenes

Isoprene and monoterpenes production consume a considerable amount of the carbon fixed in photosynthesis (up to a few percent in isoprene production, (Monson and Fall, 1989) as well as additional energy as ATP and NADPH consumed in the synthesis). But in spite of much research, the role of isoprene emission is not fully understood. Plants use mono and sesquiterpenes as defense against herbivores, and their concentration is a tradeoff in the carbon balance between protection and growth. It seems that the terpenes protect the plant both by deterring the herbivores and by attracting their natural enemies (e.g. Turlings et al., 1990; Paré and Tumlinson, 1997).

Isoprene was suggested to protect against sharp temperature increase, by dissolving in the thylakoid membranes and stabilizing the hydrophobic interactions (Sharkey and Singaas, 1995; Singaas et al., 1997; Sharkey et al., 2001). Other alkenes (Sharkey et al., 2001) and to some

extent also monoterpenes (Loreto et al., 1998c; Delfine et al., 2000) were found to increase thermotolerance in leaves as well. However, protection by isoprene was not observed in all studies (Logan et al., 1999; 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).

The well known ability of alkenes, such as isoprene, to react with singlet oxygen ($^1\text{O}_2$), ozone, and OH radicals led us, in this work, to test the hypothesis that isoprene may help to protect leaves against oxidative stress by scavenging $^1\text{O}_2$ 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 due to environmental stress that limits the use of the absorbed sunlight (Demmig-Adams, 1990). Here we used Rose Bengal (RB) as a photosensitizer that produces $^1\text{O}_2$ upon absorbing green light, and the herbicide bromoxynil (BX) that increases the sensitivity of PSII to light, thus producing $^1\text{O}_2$ through photoinhibition (Krieger-Liszkay and Rutherford, 1998).

The isotopic composition of isoprene

Further insight to understanding of processes and conditions underlying the formation of isoprene may be gained by the use of the carbon stable isotopic composition of isoprene. Isotopic discrimination against ^{13}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 that are ^{13}C depleted with respect to atmospheric CO_2 . Further, carbon isotope discrimination occurs in lipid synthesis due to isotopic effect associated with decarboxylation in the production of acetyl CoA from pyruvate (DeNiro and Epstein, 1977; Monson and Hayes, 1982; Melzer and Schmidt, 1987).

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., 1991c). 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 MEP biochemical pathway, for which the discrimination steps have not yet been explicitly identified.

The use of photosynthetic intermediates in isoprene formation as well as the similar response to environmental conditions led to a general assumption of tight coupling between isoprene production and photosynthesis. This assumption is reflected in the use of photosynthesis as the starting point in process based models for isoprene emission (Niinemets et al., 1999; Zimmer et al., 2000). However, some deviations from this coupling were observed (see in the section of environmental conditions, above). In this work we further study the carbon isotopic composition of isoprene and use it to examine the extent of coupling between isoprene production and photosynthesis.

MAIN GOALS OF THE RESEARCH

In this thesis experimental work was performed in order to:

- Build an experimental system for sampling and measuring isoprene emission rates from leaves, together with net assimilation and other leaf-gas exchange parameters, and to adapt this system for measuring carbon isotopic composition of isoprene.
- Characterize isoprene emission from the model plant species (*Myrtus communis*, myrtle).
- Characterize the effect of singlet oxygen on net assimilation and isoprene emission rates.
- Test the ability of isoprene to alleviate the damage inflicted by singlet oxygen by examining the influence of isoprene fumigation on the decrease in net assimilation rates and PSII fluorescence yield (F_v/F_m).
- Examine the isotopic discrimination in isoprene production in order to characterize the isotopic composition of the isoprene emitted to the atmosphere.
- Use the isotopic composition of isoprene, in comparison to that of the fixed C, to trace the metabolic sources of isoprene.

MAIN FINDINGS AND CONCLUSIONS

Characterization of isoprene emission from myrtle leaves

- Large seasonal variations were observed in isoprene emission rates from myrtle leaves (*Myrtus communis*) with maximal rates during the summer and autumn and close to detection limit in winter, accompanied by only small variations in net assimilation rates.

- Young leaves emitted very little isoprene. Both net assimilation and isoprene emission rates increased with leaf age up to two months. Isoprene emission was first detected about one week later than net CO₂ uptake. Both net assimilation and isoprene emission rates were higher in sun leaves than in shade leaves.
- Short term increase in the intercellular CO₂ concentration (c_i), at moderate temperatures, led to a sharp decrease in isoprene emission rates, whereas a much lower decrease was observed at high temperature. Isoprene was emitted also under CO₂ free-air at emission rates similar to those observed under ambient CO₂ concentrations.

Protection by isoprene against singlet oxygen

- Singlet oxygen (¹O₂) led to a decrease in net assimilation rates but isoprene emission was less sensitive and intercellular isoprene concentration increased under ¹O₂ treatment, making it a potential protection agent.
- In young leaves of myrtle and buckthorn (*Rhamnus alaternus*) isoprene fumigation led to reduced singlet oxygen damage to both net assimilation rates and F_v/F_m , indicating protection by isoprene.

Isotopic composition and metabolic sources of isoprene

- Isoprene was found to be ¹³C depleted by 4 and 11‰ (in different species) as compared to the concurrently fixed C, indicating isotopic discrimination in its production, on top of the isotopic discrimination in photosynthetic CO₂ fixation.
- ~20% of the isoprene was not labeled by concurrently fixed C but was produced from a long-term C source.
- No isoprene was found to be stored in myrtle leaves, indicating that a pool of isoprene cannot explain unlabeled isoprene and that there must be a long-term source for isoprene precursors.
- Labeled glucose was incorporated into isoprene, suggesting that glyceraldehyde-3-P (G3P) and pyruvate, produced in glycolysis may serve as a long-term C source for isoprene.
- Fosmidomycin led to a decrease in isoprene emission rates and to a ¹³C depletion of the remaining isoprene emission, at various pretreatment $\delta^{13}\text{C}$ values of isoprene, resulting in increased apparent discrimination. This rejects the hypothesis that cytosolic IPP is the source for unlabeled isoprene.

- Fosmidomycin induced depletion in isoprene suggested increased manifestation of the discrimination due to slowing down of the step in which the major discrimination occurs (rate determining step). Fosmidomycin induced accumulation of pyruvate (observed in this work) and the lack of accumulation of deoxyxylulose-5-phosphate (observed by Lange et al., 2001) were consistent with the reaction catalyzed by deoxyxylulose-5-phosphate synthase as the major discriminating step.
- A long-term isoprene source may be significant for protection against reactive oxygen damage by reducing the direct dependence of isoprene production on current photosynthesis, thus enabling isoprene production also under low net assimilation, during stress.

Chapter 2
METHODOLOGY



Leaves of Phragmites australis

EXPERIMENTAL APPROACH

The study described in this thesis was performed in two directions, looking at the ability of isoprene to protect the leaves against singlet oxygen and using the isotopic composition of isoprene to study the metabolic sources of isoprene, bearing in mind that in order to protect against stress, isoprene production cannot be fully coupled to photosynthesis, as is the current dogma.

The major model plant was *Myrtus communis* (myrtle), a common Mediterranean evergreen shrub. Other plant species were used for comparison in order to confirm the trends observed in myrtle. The first step of the work was characterization of isoprene emission from myrtle, including the influence of seasonality, growth light conditions, leaf age, and CO₂ concentration on net assimilation and isoprene emission rates. For consistency all characterization was done using branches of the same shrub (myrtle-1).

To examine the effect of singlet oxygen, both net assimilation and isoprene emission rates were measured until they were constant before the singlet oxygen treatment was given (this part of the experiment was used as a control). The controls measured throughout the year were used to characterize the seasonal trend. To directly examine protection by isoprene we fumigated the leaves with isoprene, using the same approach taken in thermotolerance (e.g. Singaas et al., 1997) and ozone protection studies (Loreto et al., 2001). Unlike in the thermotolerance studies (Singaas et al., 1997) we could not suppress endogenous isoprene production by performing the experiments in the dark or under nitrogen, since singlet oxygen formation requires both oxygen and light. We therefore used young leaves that emitted only very small amounts of isoprene.

The isotopic measurements were based on comparison between the isotopic composition of the emitted isoprene and that of the concurrently fixed C. Measurements were performed using several isotopically distinct CO₂ sources. Gas exchange parameters and isotopic composition of the isoprene were measured until they were constant (2 to 3h), followed by a rapid switch in the isotopic composition of the source CO₂.

PLANT MATERIAL

Plants of *Mucuna pruriens* (velvet bean) were grown from seeds (Glendale Enterprises Inc., De Funiak Springs, FL) under ambient light and temperatures. Measurements were conducted on

fully expanded, attached leaves. Branches of *Myrtus communis* (myrtle-1, 2, 4) and *Rhamnus alaternus* (buckthorn) and leaves of *Phragmites australis* (reed) were cut under water from several plants grown on the campus of the Weizmann institute of Science (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).

Net assimilation and isoprene emission rates were measured for cut as well as attached myrtle branches. Net CO₂ assimilation (6.5 ± 0.5 and $7.5 \pm 0.5 \mu\text{mol m}^{-2} \text{s}^{-1}$) and isoprene emission rates (6.0 ± 0.5 and $6.5 \pm 2.0 \text{ nmol m}^{-2} \text{s}^{-1}$) were similar in attached and cut branches, respectively, enabling the use of cut branches for the experiments reported here.

GAS EXCHANGE MEASUREMENTS

The sampling system (Fig. 1) was centered on a flow-through cuvette (volume of ~60 ml) in which the branch was sealed. The cuvette was equipped with a magnet-operated fan for mixing the air. Light ($1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR) was supplied by a halogen lamp (250W, Quartzline® lamp, General Electric Co., Cleveland, O., USA); 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, Omega digital thermometer, HH82, Stamford, Conn., USA; precision of 0.1°C). Dry air with various CO₂ concentrations was supplied by mixing cylinder air with no CO₂ and cylinder air having 1% or 2.5% CO₂ using two mass flow controllers (MKS1179A, Andover, Mass., USA; 1000 ml min^{-1} and 100 ml min^{-1} full scale, respectively, used in a relative mode). Total flow rate through the cuvette was normally 200 ml min^{-1} . Both air flows passed through activated charcoal traps to remove hydrocarbons. The tubing ($1/4$ " and $1/8$ " stainless steel) carrying the air exiting the cuvette, were heated to 80°C to avoid condensation of water vapor.

CO₂ and H₂O concentrations in the air entering and leaving the leaf cuvette were measured by an Infra Red Gas Analyzer (IRGA; Li-6262, LiCor, Lincoln, Neb., USA), at a precision of $\pm 1 \mu\text{mol mol}^{-1}$ for CO₂ and $\pm 0.1 \text{ mmol mol}^{-1}$ for water vapor. Net assimilation, stomatal conductance

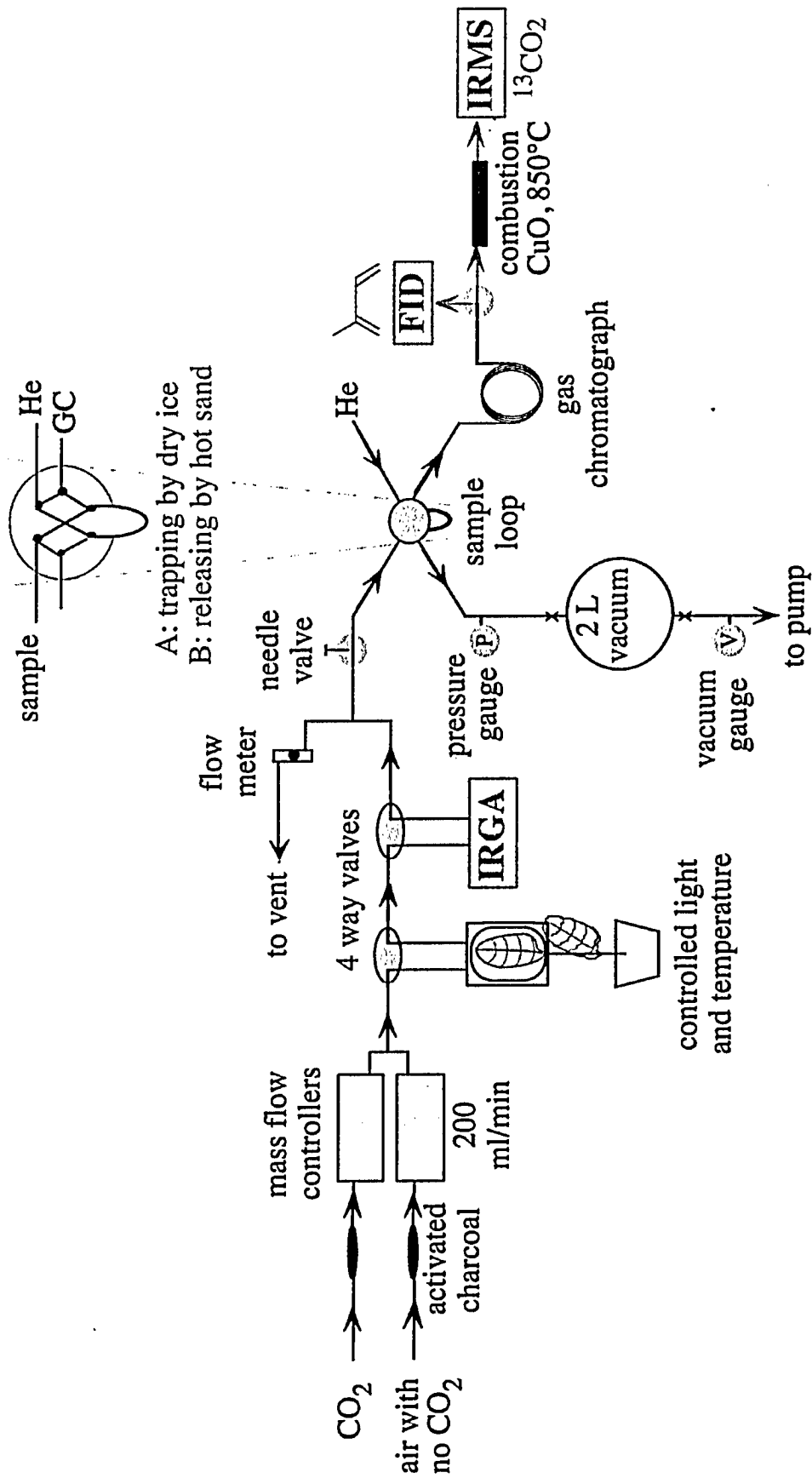


Figure 1: The experimental setup for measuring leaf gas exchange and for sampling VOCs in the air surrounding a leaf. Net assimilation rates were measured using the IRGA. Isoprene was sampled by pre-concentration in the sample loop and measured by GC-FID for emission rates and by GC-C-IRMS for isotopic composition.

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 CONCENTRATION MEASUREMENTS

For hydrocarbons measurements (Greenberg et al., 1994; Monson, R.K., personal communication) an aliquot of the air stream exiting the cuvette was pumped (bypassing the IRGA) into a pre-evacuated glass bulb (2 L, 60 mtorr), through a trapping loop (1/8" stainless steel, 27 cm long in which the central ~9 cm were packed with 212 to 300 μm glass beads (Sigma, St. Louis, Mo., USA), with a 2 μm filter (Valco, Houston, Tex., USA) placed at the outlet) connected to a six-port valve (Valco, Houston, Tex., USA). The loop was cooled with either liquid nitrogen or a mixture of ethanol and dry ice (-70°C) for trapping the hydrocarbons in the air. The pressure (pressure transducer, Omega, Stamford, Conn., USA) 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 ~250 ml). After trapping, the valve was switched to a flow of helium (1.5 ml min^{-1}), the loop was rapidly heated (hot sand, 200°C) and the trapped hydrocarbons passed directly to a GC column (HP 5890, Wilmington, Del., USA).

The hydrocarbons were separated using a polar GC column or a column dedicated for small molecules (Supelco, Bellefonte, Penn., USA: SupelcowaxTM 10, 30 m long, 0.25 mm ID, 0.25 μm film, temperature program: 35°C for 1 min, temperature increase at $10^\circ\text{C min}^{-1}$ to 170°C for 2 min; or Supel-QTMplot, 30m long, 0.32mm ID, temperature program: 50°C for 1 min, temperature increase at $10^\circ\text{C min}^{-1}$ to 170°C for 5 min). Isoprene was detected using a flame ionization detector (FID) kept at 250°C . The area of the peaks obtained was recorded and analyzed by a chromatography software (BorwinTM version 1.21.60, JMBS developments, La Fontenil, France). Typical chromatograms are depicted in Fig. 2.

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, Wis., USA) into a pre-evacuated 12 L glass bulb to a pressure of ~0.1 torr, on a vacuum line. Nitrogen was added to atmospheric pressure. The

bulb was evacuated to ~1 torr and refilled with nitrogen to atmospheric pressure, to give 0.1 to 0.2 $\mu\text{L L}^{-1}$ isoprene.

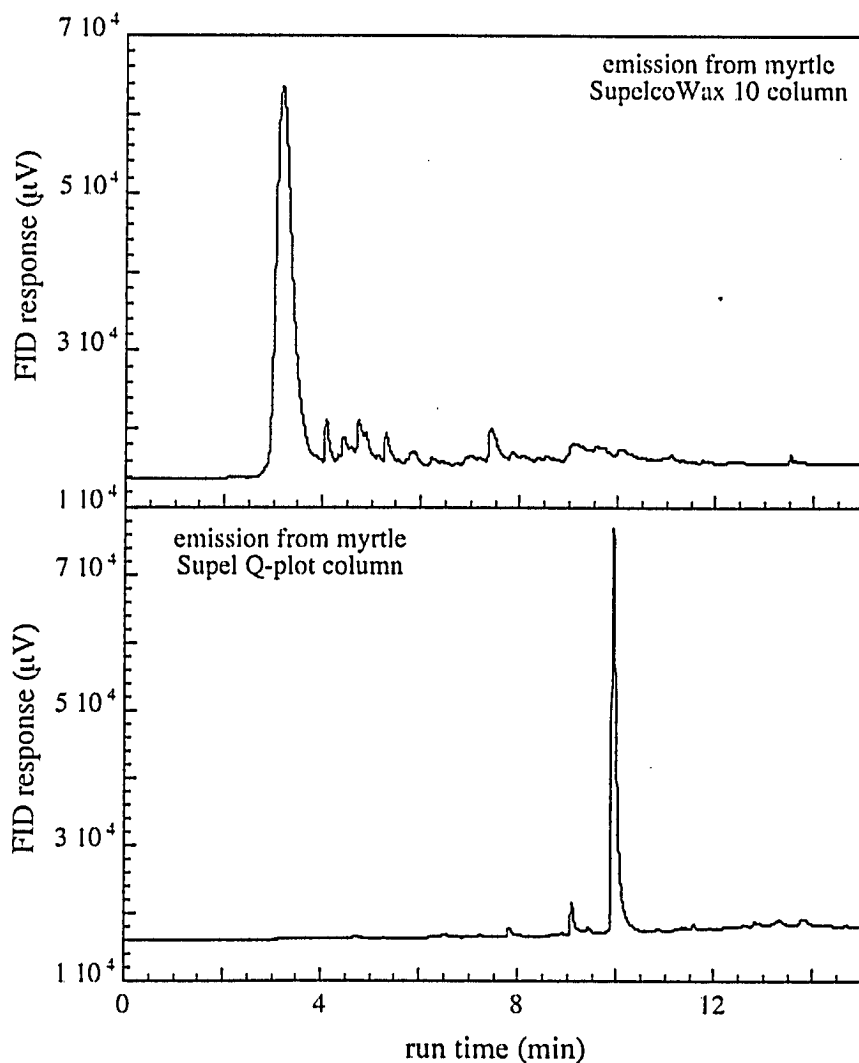


Figure 2: Typical chromatograms of emission from myrtle, separated by either a Supelcowax10 or a Supel-Qplot GC column. In both chromatograms isoprene is the major peak.

Isoprene emission rates (J_{isop}) were calculated from the concentration of isoprene in the air exiting the leaf cuvette (C_{isop}), the molar flow rate of air through the cuvette (U) and the projected leaf area (L):

$$J_{\text{isop}} = \frac{U}{L} C_{\text{isop}} \quad (1)$$

ISOTOPIC ANALYSIS OF ISOPRENE

The carbon isotopic composition of isoprene (δ_{isop}) was determined after GC separation and combustion to CO_2 . Separation between the peaks of isoprene and CO_2 in the air sample before combustion was achieved with the SupelQ-plot GC column. A selection valve (MOVPT-1/100 pneumatic valve, SGE, Australia) was used to direct the flow eluting the GC column to either a flame ionization detector (FID) or a combustion oven (CuO , 850°C) in which the desired GC peaks were each combusted quantitatively to CO_2 . This CO_2 was used for isotopic composition measurements. The CO_2 from combustion was dried in a cold trap and was fed in a flow of He , through an open-split, to an isotope ratio mass spectrometer (IRMS; Optima, Micromass, UK), where masses 44, 45, and 46 were measured. The ratio 45/44 was normalized to a pulse of CO_2 reference gas injected prior to each sample. The isotopic results are expressed in the $\delta(\text{‰})$ notation versus the V-PDB standard, where $\delta = (R/R_{\text{std}} - 1) \cdot 1000$ and R , R_{std} are the isotopic ratios $^{13}\text{C}/^{12}\text{C}$ of the sample and the standard, respectively.

For isotopic calibration, liquid isoprene was injected into a pre-evacuated glass bulb (12 L, 60 mtorr) and air or N_2 was added to atmospheric pressure. Aliquots were sampled and measured in a similar manner to the air in the leaf cuvette, at the end of each experiment. Typical precision for isoprene standard measurements was $\pm 0.3\text{‰}$. The $\delta^{13}\text{C}$ of the liquid isoprene was predetermined by comparison to international and laboratory working standards, which were measured by conventional on-line combustion elemental analyzer (EA1109 CHN-O; Carlo Erba Instruments, Italy). The same elemental analyzer was used also for measuring the $\delta^{13}\text{C}$ of total leaf organic matter.

ISOTOPIC ANALYSIS OF CO_2

δ_{isop} was compared with that estimated for the concurrently fixed C (δ_{fixed}), based on the 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 the $\delta^{13}\text{C}$ 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" O.D, 350 μl), which was then heated and the sample was carried in a flow of helium (80 ml min^{-1}), separated on a Porapak QS (Supelco, Bellefonte, Penn., USA; 2m, 50-80 mesh, 50°C) or Haysep D (Supelco, Bellefonte, Penn., USA; 3m, 80°C) packed columns, and analyzed for isotopic composition by the IRMS (either Optima, or a 20-20, PDZ Europa, UK).

CO₂ was calibrated by measuring air from cylinders of 400 or 1000 μL L⁻¹ CO₂ having different δ¹³C values that were, in turn, calibrated by comparing to a cylinder of 400 μL L⁻¹ CO₂ of known δ¹³C value. Typical precision of ¹³C analysis in CO₂ was ±0.15‰.

The δ¹³C of the CO₂ fixed by the plant was estimated by on-line discrimination calculations (Evans et al., 1986) using the CO₂ concentrations and δ¹³C in the air entering and leaving the leaf cuvette:

$$\delta_{\text{fixed}} = \delta_{\text{out}} - \Delta \quad \text{where} \quad \Delta = \frac{\xi(\delta_{\text{out}} - \delta_{\text{in}})}{1000 + \delta_{\text{out}} - \xi(\delta_{\text{out}} - \delta_{\text{in}})} \quad \text{and} \quad \xi = \frac{C_{\text{out}}}{C_{\text{out}} - C_{\text{in}}} \quad (2)$$

where δ_{fixed} is the estimated δ¹³C value of the C fixed in photosynthesis, δ_{out} and δ_{in} are the measured δ¹³C values of the CO₂ in the air leaving and entering the leaf cuvette, C_{out} and C_{in} are the respective CO₂ concentrations.

Air containing CO₂ of various isotopic compositions was supplied to leaves during experiments in order to obtain variations in the isotopic composition of the fixed C. Ambient air (δ¹³C ≈ -8‰) was pumped through a 50 L external buffering volume and dried using drierite (8 mesh; W.A. Hammond Drierite company, Xentia, O., USA). Cylinder air containing no CO₂ was mixed via mass flow controllers (MKS1179A, MKS instruments, Andover, Mass., USA) with cylinder air (Gordon gas and chemicals, Israel) containing 1% or 2.5% CO₂ whose δ¹³C value were -48‰, -33‰, or -27‰. Cylinder air mixture containing 383 or 366 μL L⁻¹ CO₂ whose δ¹³C value was -13‰ or -42‰, respectively, were also used. Variation in the isotopic composition of the fixed C was obtained also by inducing stomatal closure using 2-cis,4-trans-abscisic acid (ABA, 30 to 100 μM, Aldrich, Milwaukee, Wis., USA).

SINGLET OXYGEN TREATMENTS

Singlet oxygen was produced in the leaves (myrtle-1, 4, and buckthorn) by Rose Bengal (RB: 4,5,6,7-tetrachloro-2',4',5',7'-tetraiodofluorescein) or bromoxynil (BX: 3,5-dibromo-4-hydroxybenzotrile; Agan chemical manufacturers, Ashdod, Israel) in the light. RB acts as a photosensitizer to produce singlet oxygen upon absorbing green light (λ_{max}=547 nm, Fig. 3). Bromoxynil is a phenolic herbicide that increases the sensitivity of PSII to excess light through formation of P680 triplet (Krieger-Liszkay and Rutherford, 1998) and was used as a source for ¹O₂ near PSII, simulating photoinhibition more specifically than RB. 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 CO₂ concentrations, leaf temperature of 26°C and at light intensity of 1000 μmol m⁻² s⁻¹.

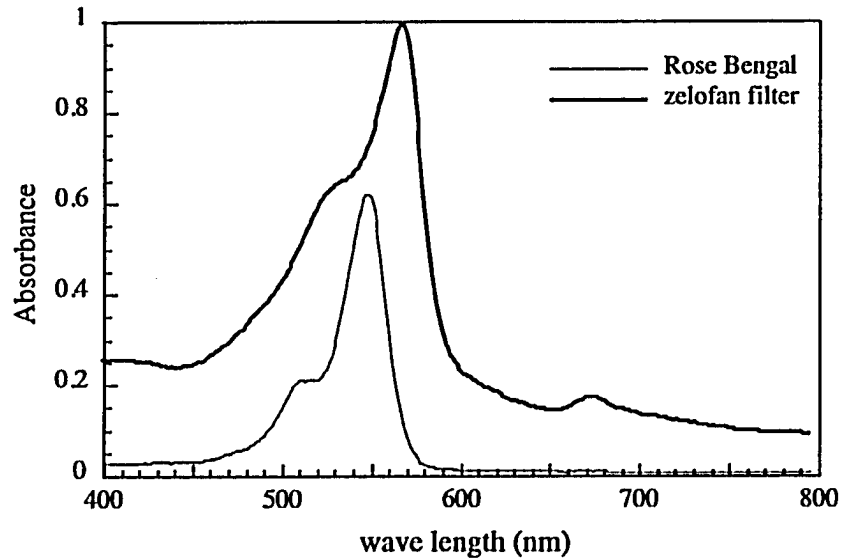


Figure 3: Spectra of the absorbance of 6 μM Rose Bengal (red) and the purple zelofan filter (blue) used to prevent the excitation of Rose Bengal, measured by Cary 118 spectrophotometer.

Isoprene fumigation was done together with the ¹O₂ treatment in young leaves, by passing the air entering the leaf cuvette through a piece of permeable tubing that was enclosed in an Erlenmeyer bottle containing isoprene and kept in an ice bath. The fumigation dose (1 to 20 μL L⁻¹) was determined by the permeability of the tubing and the isoprene concentration in the Erlenmeyer bottle.

In experiments used for either characterization of the response to ¹O₂ or fumigation, the control values of both net assimilation and isoprene emission rates were measured during 2 to 3h, followed by ¹O₂ treatment (feeding of either RB or BX).

CHLOROPHYLL FLUORESCENCE

Chlorophyll fluorescence yield (F_v/F_m) was used as a measure for oxidative damage, together with following the change in net assimilation rates, in the series of isoprene fumigation experiments in buckthorn leaves. F_v/F_m was measured in dark-adapted leaves before and after singlet oxygen experiments, using a portable fluorometer (PAM-2000, Walz, Germany).

ISOPRENE POOL IN LEAVES

The amount of isoprene stored in 10 myrtle-1 leaves was measured by freezing the leaves in liquid N₂, immediately after cutting, and extracting the volatile fraction by heating under a flow of helium (Loreto et al., 1998a). The sample was dried by magnesium perchlorate (Aldrich chemical Co., Wis., USA), trapped in a loop cooled by a mixture of ethanol and dry ice, heated and measured by the GC, as described above. Magnesium perchlorate was examined separately and was found not to influence either concentration or isotopic composition of an isoprene standard.

CO₂ LABELING EXPERIMENTS

Experiments testing the effect of δ_{fixed} on δ_{isop} were performed by measuring both parameters when the leaf cuvette was supplied with air of ambient CO₂ concentration and of a certain $\delta^{13}\text{C}$ value. After ~2h of constant δ_{fixed} and δ_{isop} , the source CO₂ was rapidly switched, changing the $\delta^{13}\text{C}$ but not any of the gas-exchange parameters. δ_{fixed} and δ_{isop} were measured for additional 2h under the new source CO₂.

Few experiments were done in the dark or under CO₂-free air. Gas-exchange parameters and the isotopic composition of isoprene and CO₂ were measured in the light and under ambient CO₂ concentration during few hours, to obtain control values. For CO₂-free air measurements, the air supply to the leaf cuvette was switched rapidly to CO₂-free air. CO₂ produced by respiration resulted in 10 to 20 $\mu\text{L L}^{-1}$ CO₂ in the cuvette. Gas-exchange parameters and the isotopic composition of isoprene and CO₂ were measured under these conditions during 2 to 4 hours. 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).

LABELLED METABOLITES AND INHIBITOR FEEDING

As part of the isotopic study attempts were made to identify a long-term carbon source for isoprene production by examining the influence of labeled glucose and acetate on the isotopic signature of isoprene. Also tested was the influence of fosmidomycin, an inhibitor of the MEP pathway for isoprene synthesis. All chemicals were fed to the leaves as aqueous solutions through

the petiole. Fosmidomycin (Molecular probes Inc.) was fed at concentrations of 5 to 20 μM for emission rates measurements and 2 to 5 μM for isotopic analysis experiments. D-glucose (BDH chemicals LTD, England; $\delta^{13}\text{C}=-10\text{‰}$) was fed at concentration of 15 mM. Sodium acetate solutions of varying isotopic composition were prepared by dissolving sodium [^{13}C]acetate (99% ^{13}C ; Fluka chemie AG, Switzerland) and unlabeled NaAc ($\delta^{13}\text{C}$ of -20‰ ; Merck, Germany). Concentrations of 30 to 120 mM and $\delta^{13}\text{C}$ of 0, +25, or +150‰ were fed to the leaves. To verify that the fed acetate indeed labeled the acetate in the leaves, two buckthorn branches were fed with 60 mM NaAc with $\delta^{13}\text{C}$ values of -20 and $+150\text{‰}$, respectively. The water soluble fraction was extracted (3 extractions per branch) as described below for pyruvate content, freeze dried, combusted (EA1109 CHN-O; Carlo Erba Instruments, Italy) and measured for $\delta^{13}\text{C}$ by IRMS (Optima, Micromass, UK).

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). The leaf-discs were crushed under liquid N_2 and extracted in water, on ice. The supernatant was separated by centrifuge, boiled and recentrifuged. The solution was diluted 1:10 and measured using the standard addition method. Pyruvate was separated by Varian ProStar HPLC (Varian, USA) with Dionex AS11 column (Dionex, USA) at 24°C , with NaOH concentration gradient of 0.4 to 22.5 mM as eluent, at 2 ml min^{-1} , and measured by Dionex ED50 (Dionex, USA) electrochemical detector.

STATISTICAL ANALYSIS

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

Chapter 3

ISOPRENE EMISSION FROM *MYRTUS COMMUNIS*



Leaves and flowers of *Myrtus communis*

In the experiments described in chapters 4 and 5, treatment was given to affect either net assimilation or isoprene emission rates, and features of the isoprene emission were exploited. To enable that, net assimilation and isoprene emission rates from myrtle, the major model plant used in this study, had to be characterized, as described in this chapter. Measurements were performed on cut myrtle branches, from a shrub growing on campus (myrtle-1) and focused on the seasonal trend, the influence of leaf age, and the effect of CO₂ concentration.

SEASONALITY

Net assimilation and isoprene emission rates were measured (several measurements per month throughout the year) at leaf temperature of $26 \pm 0.5^\circ\text{C}$ and different c_i values. Within each c_i group, net assimilation and isoprene emission rates were averaged monthly. Myrtle, a high isoprene emitter, showed during the summer net assimilation and isoprene emission rates comparable to those observed in a field study in Italy (Hansen et al., 1997) and in mature leaves of velvet beans (Harley et al., 1994) and of kudzu (Sharkey and Loreto, 1993).

As expected, large seasonal variations were observed in isoprene emission rates, accompanied by relatively small changes in net assimilation rates (Fig. 4). Net assimilation decreased from $17 \mu\text{mol m}^{-2} \text{s}^{-1}$ in December 1998 to $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ in April 1999, and increased to a maximum of $25 \mu\text{mol m}^{-2} \text{s}^{-1}$ in September 1999 (at $c_i=550$ to $650 \mu\text{L L}^{-1}$). Isoprene emission rates were maximal during the summer and autumn (24 to $28 \text{ nmol m}^{-2} \text{s}^{-1}$, at $c_i < 400 \mu\text{L L}^{-1}$, but lower in August, for unknown reasons) and close to detection limit ($\sim 0.2 \text{ nmol m}^{-2} \text{s}^{-1}$) in winter. A similar trend was observed in 2000. Under field conditions, where summer temperatures are higher and winter temperatures are lower than the measurement temperature used (26°C), variations in isoprene emission are likely to be even more pronounced.

The observed seasonal variations in isoprene emission are consistent with reports on aspen (Monson et al., 1994), with maximal isoprene emission rates in July and minimal rates in May and October, and on the activity of isoprene synthase in oak (Schnitzler et al., 1997). Note that myrtle is evergreen and emission could be measured throughout the year mostly on mature leaves (to reduced the phenological influence that partly overlaps seasonality in these other studies).

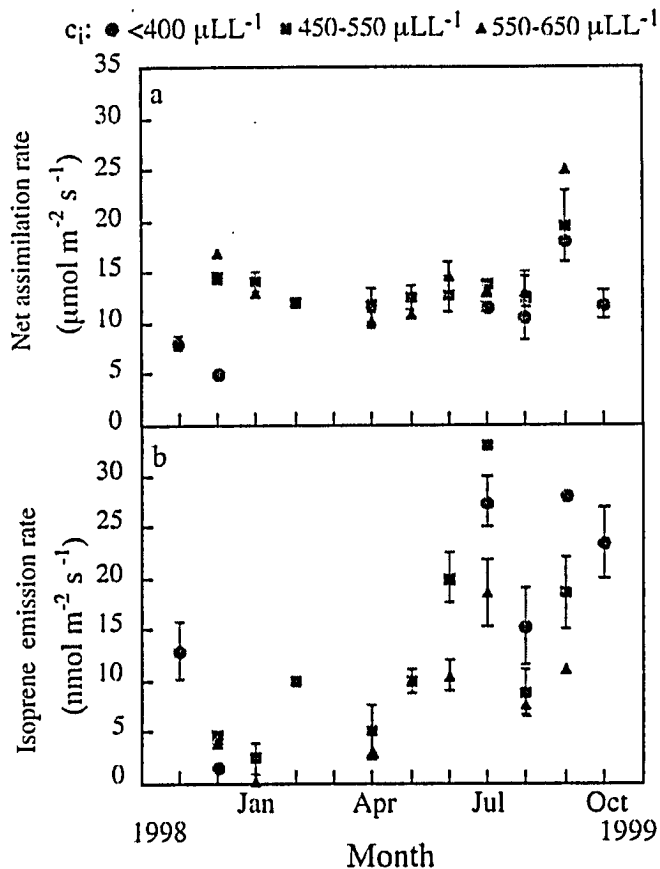


Figure 4: Seasonal variations in net assimilation and isoprene emission rates from myrtle-1. The data presented are averaged values (\pm SE) for several measurements taken each month. The different symbols refer to measurements taken under different intercellular CO_2 concentrations (c_i). Leaf temperature was 26°C and light intensity was $1000 \mu\text{mol m}^{-2} \text{sec}^{-1}$.

Although isoprene emission in myrtle was maximal during summer, autumn emission rates were also high (Fig. 4) due probably to high average temperature (daily max/min $32/22^\circ\text{C}$ in July to September and $29/17^\circ\text{C}$ in October, 1999 measured in the nearest meteorological station). Leaves were photosynthetically active throughout the winter, but isoprene emission in most branches was below the detection limit. Lack of correlation between variations in the rates of isoprene emission and net CO_2 assimilation over the annual cycle is similar to that in the Mediterranean oak *Quercus ilex* that emits non-stored monoterpenes (Peñuelas and Llusà, 1999).

LEAF AGE AND GROWTH LIGHT

Very young leaves ($\sim 5 \text{ mm}^2$), defined as age zero, were marked on both the sunny and shaded sides of myrtle-1. Branches that developed from the marked leaves were measured at different ages (each branch integrated leaves aging from zero to the time of measurement). Net assimilation

and isoprene emission rates were measured both at $26\pm 0.5^\circ\text{C}$ and $34\pm 0.5^\circ\text{C}$ during September-October 1999.

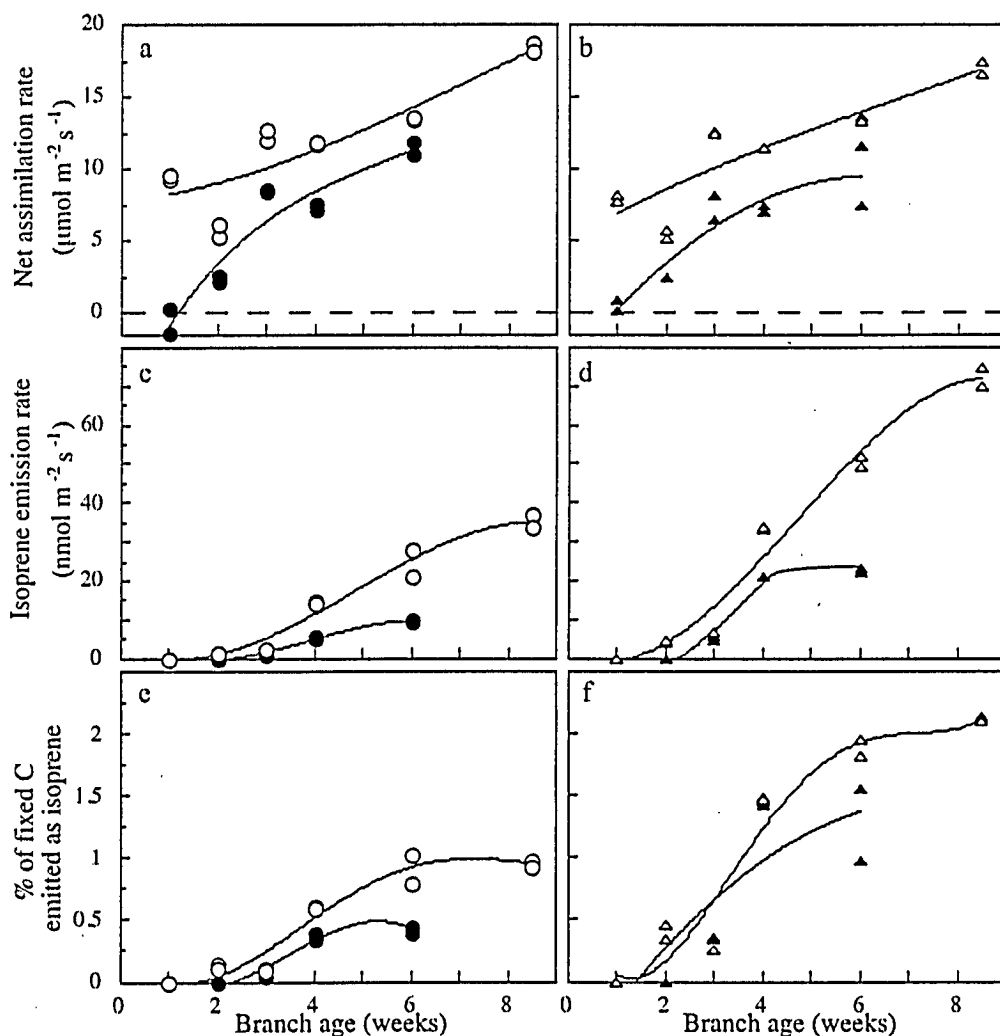


Figure 5: The effect of leaf age on photosynthesis and isoprene emission rates from myrtle-1 measured at 26°C (circles) and 34°C (triangles). The bold symbols denote shade leaves and the empty symbols denote sun leaves. In each age interval the branches measured included leaves aging from zero to the age interval. The data is presented on projected leaf area basis, and leaf dry weight basis gave similar trends.

Both net assimilation and isoprene emission rates increased with leaf age up to two months, during the study period (Fig. 5). Isoprene emission rates more than doubled with increasing leaf temperature during measurement from 26°C to 34°C , whereas net assimilation rates were not significantly affected (Fig. 5a,c vs. 5b,d). A clear difference was observed between sunny and shaded leaves. On the sunny side of the shrub, a one-week-old branch was already photosynthetically active, while net assimilation was first detected only in a two weeks old branch

in the shaded side. Isoprene emission was first detected only about a week after net CO₂ uptake was observed in both sun and shade leaves. Both net assimilation and isoprene emission rates were higher in sun leaves, than in shade leaves, at any given age. But the higher fraction of fixed C that was emitted as isoprene in the sun leaves (Fig. 5e,f) indicated greater sensitivity of isoprene emission than CO₂ assimilation to growth light conditions, and any associated temperature effect.

The absence of isoprene emission in young leaves, and the delay in the onset of emission with respect to photosynthetic activity is consistent with reports on other plants. However, in kudzu and velvet beans isoprene emission began in a few days old leaves and reached a maximal value after two weeks (Sharkey and Loreto, 1993; Harley et al., 1994). In myrtle, isoprene emission was first detected only after one to two weeks and did not reach a maximum even after eight weeks of the study period. Both kudzu and velvet beans are fast growing plants while myrtle is a slow growing perennial shrub. The phenological response of isoprene emission from myrtle may be comparable to that observed in aspen leaves, in which isoprene emission from leaves growing during the summer began at 6 days old leaves and increased to a maximum in 30 days old leaves (Monson et al., 1994).

The higher rates of net assimilation and isoprene emission in sun vs. shade leaves were observed also in other plant species (Sharkey et al., 1991b; Harley et al., 1994; Litvak et al., 1996). In velvet beans, white oak, and aspen the higher isoprene emission from sun leaves could be explained fully or partially by the higher leaf mass per unit area in sun leaves (Harley et al., 1994; Litvak et al., 1996). In myrtle, however, there was no significant effect on the sun/shade differences when results are expressed on leaf area or leaf dry weight basis. It was recently shown that the difference in isoprene emission between sun and shade leaves may be due to development of lower activity of the enzyme isoprene synthase in leaves growing in the shade (Lehning et al., 1999).

CO₂ CONCENTRATION

The short-term response of net CO₂ assimilation and isoprene emission rates to variations in intercellular CO₂ concentration (c_i) was measured at leaf temperatures of $26 \pm 0.5^\circ\text{C}$ and $34 \pm 0.5^\circ\text{C}$ (Fig. 6). Emission rates were relatively constant at c_i values between 20 and 300 $\mu\text{L L}^{-1}$ (9 and 17 $\text{nmol m}^{-2} \text{s}^{-1}$ at 26°C , in two different branches). Rates of net assimilation increased with c_i under the same conditions from -1 to +10 or +15 $\mu\text{mol m}^{-2} \text{s}^{-1}$. At c_i values of ~ 300 to 900 $\mu\text{L L}^{-1}$ (at

26°C), isoprene emission rates decreased by 55 to 75%, to 4 nmol m⁻² s⁻¹, while net assimilation appeared to be CO₂ saturated.

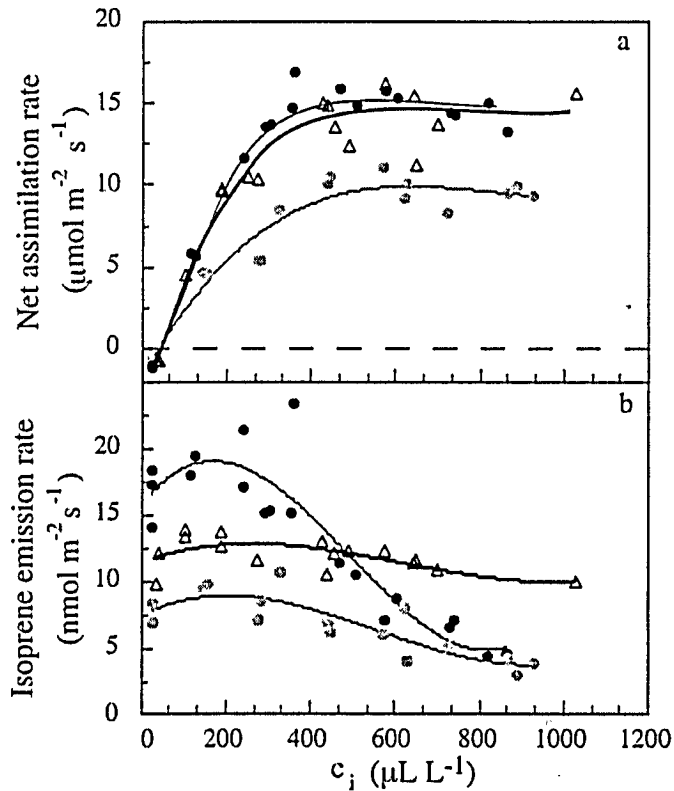


Figure 6: Effects of intercellular CO₂ concentration (c_i) on net assimilation (a) and isoprene emission rates (b) from myrtle leaves at 26°C (circles; two different branches) and 34°C (triangles) and light intensity of 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

The effect of c_i on isoprene emission rates at 34°C was much smaller than that observed at 26°C (Fig. 6). Isoprene emission rates decreased by only ~25% from a mean value of 13 $\text{nmol m}^{-2} \text{s}^{-1}$, at c_i values between 20 and 300 $\mu\text{L L}^{-1}$, to 10 $\text{nmol m}^{-2} \text{s}^{-1}$ when c_i was 1000 $\mu\text{L L}^{-1}$. Net assimilation rates increased from -1 to +11 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with increasing c_i values from 20 to 300 $\mu\text{L L}^{-1}$ and was on average ~15 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at c_i above 300 and up to 1000 $\mu\text{L L}^{-1}$.

The effect of c_i on net assimilation and isoprene emission rates in myrtle was similar to that observed in aspen and red oak (Monson and Fall, 1989; Loreto and Sharkey, 1990). Notably, however, unlike in red oak but similar to aspen, no decrease in isoprene emission rates was observed in myrtle for several hours under CO₂-free air ($c_i \approx 20 \mu\text{L L}^{-1}$).

At high leaf temperature (35°C), similar emission rates were observed in red oak at 390 and 2200 $\mu\text{L L}^{-1} \text{CO}_2$, as opposed to the decrease in isoprene emission rates at high c_i at 25°C (Loreto and Sharkey, 1990). Similarly, in myrtle the effect of high c_i values on isoprene emission was much smaller at 34°C as compared to 26°C (Fig. 6). The decrease in isoprene emission rates at high c_i values and moderate temperatures was suggested to be due to ATP limitation, when photosynthesis is feedback limited, which is less likely to occur at high temperatures (Loreto and Sharkey, 1990).

Chapter 4

**PROTECTION BY ISOPRENE AGAINST SINGLET
OXYGEN IN LEAVES**



Leaves and flowers of *Rhamnus alaternus*

This chapter describes experiments that were performed to test the hypothesis that isoprene can act as a protection mechanism against oxidative stress, focusing on singlet oxygen ($^1\text{O}_2$). We examined the influence of Rose Bengal (RB) and bromoxynil (BX), used as sources for singlet oxygen, on net assimilation and isoprene emission rates, and the effect of exogenous isoprene on the damage to photosynthesis.

EFFECT OF SINGLET OXYGEN IN MYRTLE

RB was used as a photosensitizer to produce $^1\text{O}_2$ under light. Treatments with RB at ambient CO_2 concentration resulted in a rather fast decrease in both net assimilation and isoprene emission rates (Fig. 8, 9a), whereas lesions on the leaves were observed only after two days (Fig. 7). The effect on net assimilation and isoprene emission rates was clearly concentration dependent (Fig. 8). In control plants (no RB) net assimilation and isoprene emission rates remained relatively constant throughout the measurement day (Fig. 8).

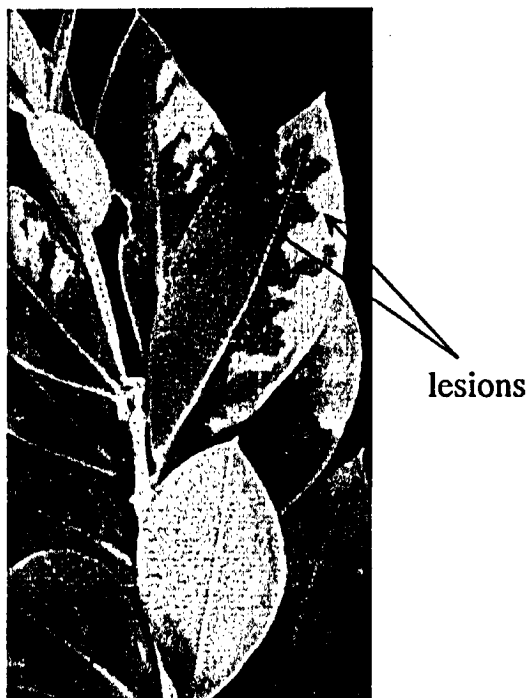


Figure 7: Lesions on myrtle leaves, 3 days after a RB treatment ($0.4\mu\text{M}$).

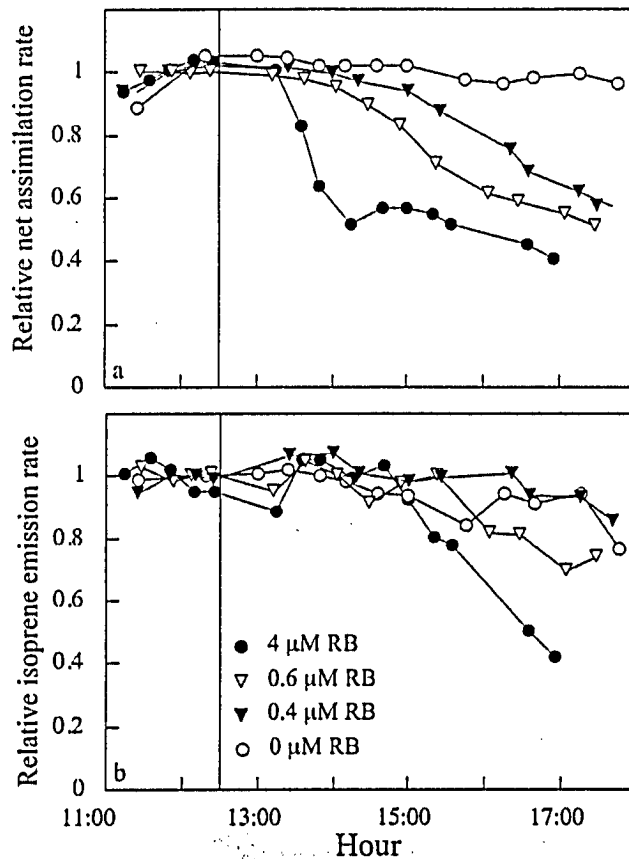


Figure 8: Effects of different concentrations of RB in the feeding solution on net assimilation (a) and isoprene emission rates (b) of myrtle leaves at light intensity of $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$, leaf temperature of 26°C , and c_i values of 200 to $250 \mu\text{L L}^{-1}$. The vertical line indicates the beginning of RB feeding.

An important check was made by repeating the treatment and control measurements using light filtered through purple zelofan but maintaining the same total PAR at the leaf level (the zelofan filter had a broad absorption peak around $\lambda_{\text{max}}=567\text{nm}$, as compared to $\lambda_{\text{max}}=547\text{nm}$ of RB, Fig. 3). 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 2.5h of RB feeding were 36% of control with no filter (Fig. 9b) but 70% of control with the filter (Fig. 10). The decrease in net assimilation with filter and RB was similar to that observed in untreated leaves (Fig. 10). The 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.

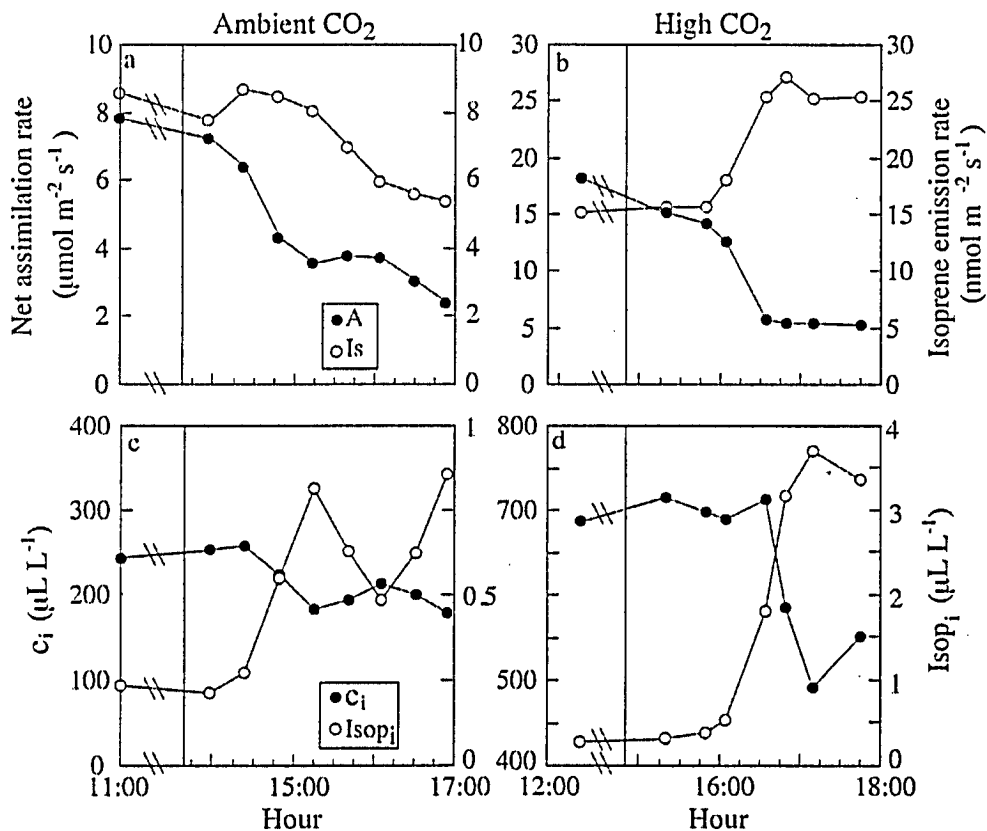


Figure 9: Effects of singlet oxygen in myrtle leaves under ambient CO₂ concentration (~350 μL L⁻¹; a,c) in autumn and high CO₂ concentration (~800 μL L⁻¹; b,d) in summer on net assimilation and isoprene emission rates (a,b) and on changes in the intercellular concentration of CO₂ (c) and isoprene (Isop_i; c,d) at leaf temperature of 26°C, light intensity of 1000 μmol m⁻² s⁻¹. The vertical line indicates the beginning of RB feeding. (The figure depicts one typical experiment for each case. The average of other experiments is given in appendix 1).

In order to provide protection isoprene should be able to either strengthen the membranes as suggested for thermotolerance or to scavenge ¹O₂ by reacting with it. ¹O₂ is known to react with alkenes. We tested the reaction of ¹O₂ produced by RB with isoprene by following the concentration of isoprene (initial concentration of 6 mM in CCl₄) in contact with an aqueous solution of RB (7 mM) at light intensity of 1000 μmol m⁻² s⁻¹. This was compared to similar reaction flasks containing deionized water instead of the RB solution or kept in the dark. Illuminated RB led to a 60% decrease in isoprene after 5 hours and 100% decrease after 20 hours (Fig. 11). Only 25% decrease in isoprene was observed after 20 hours of illumination with no RB and no decrease in the dark. This indicated reaction of isoprene with the ¹O₂ produced by illumination of RB, making isoprene a potential scavenger.

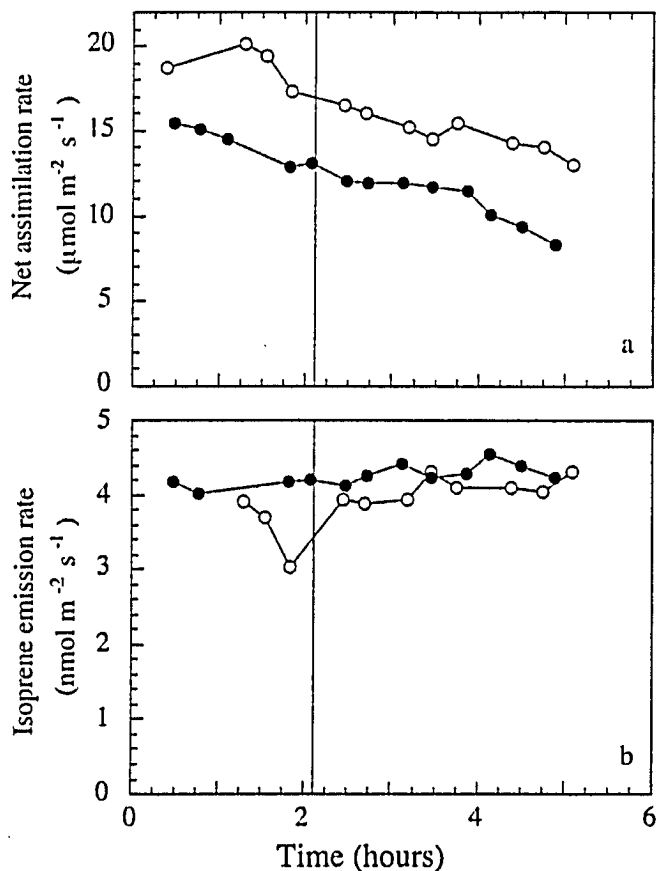


Figure 10: Control experiments for singlet oxygen treatments, in myrtle leaves under high CO_2 concentration: net assimilation rates (a) and isoprene emission rates (b). In both experiments light was filtered through purple zelofan ($\lambda_{\text{max}}=567 \text{ nm}$) to prevent formation of singlet oxygen by RB ($\lambda_{\text{max}}=547 \text{ nm}$). The bold symbols indicate RB treatment. The vertical line indicated the beginning of RB feeding. The empty symbols indicate a control experiment in which no RB was added.

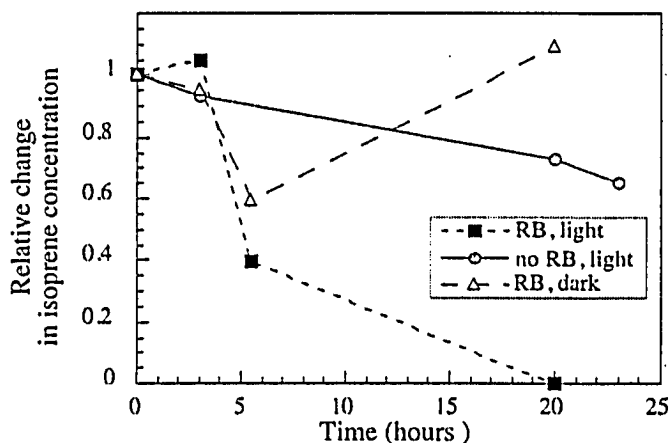


Figure 11: Consumption of isoprene (6 mM in CCl_4) by reaction with singlet oxygen produced by RB (7mM in water) under light intensity of $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ (squares). No reaction was observed in controls containing water instead of RB solution (circles) or not exposed to light (triangles). Isoprene concentration is presented relative to the initial concentration.

Comparing the time response of isoprene emission and net assimilation rates showed that at ambient CO₂ concentrations the decrease in isoprene emission rates was delayed by 1 to 2 hours, and was smaller in magnitude, relative to that in net assimilation (Fig. 9a). 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. 9c; calculated as in Singaas et al., 1997). At high CO₂ concentrations, net CO₂ assimilation rates began to decrease ~1.5 hours after the beginning of RB feeding but in contrast to ambient CO₂, isoprene emission rates did not change, or even increased (Fig. 9b). The lower sensitivity to ¹O₂ of isoprene production, with respect to net assimilation, and the increase in leaf internal isoprene concentration indicates a potential for isoprene to act as a protection mechanism.

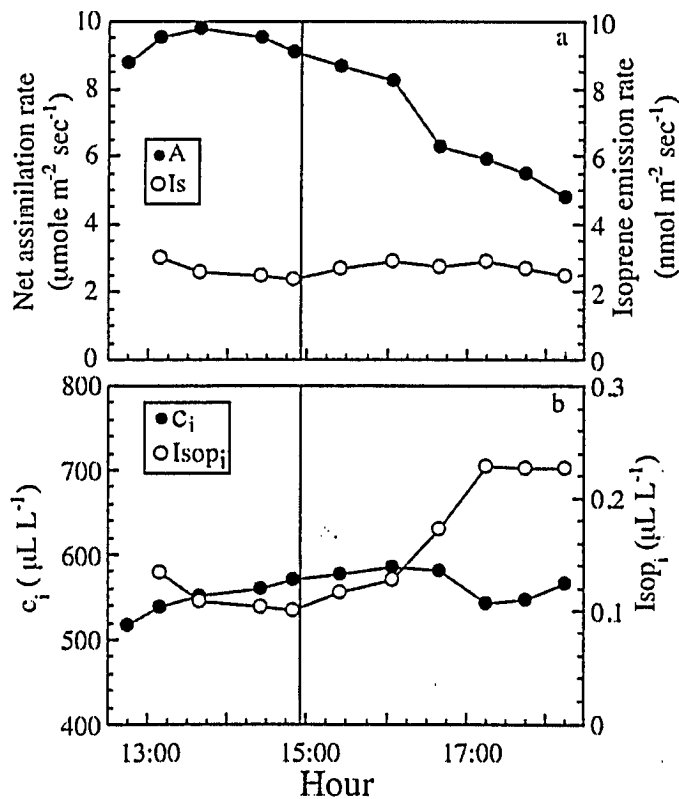


Figure 12: Effects of singlet oxygen in myrtle leaves at high CO₂ concentration (~550 μL L⁻¹) in spring on net assimilation and isoprene emission rates (a) and on changes in the intercellular concentration of CO₂ (c_i; b) and isoprene (Isop_i; b) at leaf temperature of 26°C, light intensity of 1000 μmol m⁻² s⁻¹. The vertical line indicates the beginning of RB feeding. (The figure depicts one typical experiment for each case. The average of other experiments is given in appendix 1).

At high CO_2 concentrations, the response of isoprene emission to RB treatments was well correlated with variations in c_i . In untreated leaves isoprene emission normally decreased with increasing c_i (at 26°C ; Fig. 6), while in RB treated leaves a decrease in c_i with time, due to RB effect, led to an increase in isoprene emission rates (comparison of absolute emission rates between Figures 6 and 9 is difficult due to large differences between 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. 9b, d). In contrast, during spring both c_i and isoprene emission rates did not change (Fig. 12). Furthermore, in a RB treatment in autumn at 34°C and high CO_2 concentration a large decrease in net assimilation and in c_i but no change in isoprene emission rates were observed (Fig. 13). This is consistent with the notion of reduced effect of c_i on isoprene emission rates at elevated temperatures (Fig. 6).

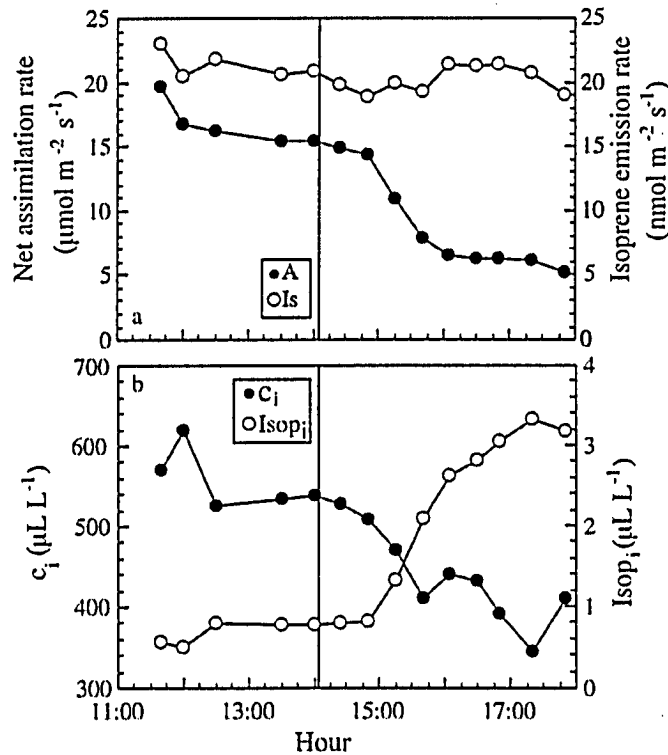


Figure 13: Effects of singlet oxygen in myrtle leaves at high CO_2 concentration ($\sim 500 \mu\text{L L}^{-1}$) in summer on net assimilation and isoprene emission rates (a) and on changes in the intercellular concentration of CO_2 (c_i ; b) and isoprene (Isop_i ; b) at leaf temperature of 34°C , light intensity of $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$. The vertical line indicates the beginning of RB feeding.

ISOPRENE FUMIGATION IN MYRTLE

The possibility that isoprene may provide protection against $^1\text{O}_2$ damage was further tested by isoprene fumigation of young, non-emitting myrtle branches treated by RB or BX, under ambient CO_2 concentration. 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 to $2 \mu\text{L L}^{-1}$) the decrease in net assimilation was half that of non-fumigated leaves (Table I). Higher isoprene concentration (4 to $5 \mu\text{L L}^{-1}$) 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 (Krieger-Liszky and Rutherford, 1998). In non-fumigated leaves, the treatment resulted in a $\sim 50\%$ decrease in net assimilation rates within 4 hours (beginning 1.5 to 2 hours after feeding). Isoprene-fumigated leaves (1 to $15 \mu\text{L L}^{-1}$) showed $\sim 30\%$ smaller decrease in net assimilation due to BX (Table I).

Table I: Effect of isoprene fumigation on net assimilation rates (A) after 4 hours of $^1\text{O}_2$ treatment in young (non-emitting) myrtle leaves (myrtle-1, 4).

Data are presented as the percentage of A left after the treatment. Also presented is fumigation effect on %A, as the ratio between consecutive fumigation and no-fumigation experiments. Light intensity was $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ and leaf temperature was 26°C . Exogenous isoprene was added to give isoprene concentrations of $1-5 \mu\text{L L}^{-1}$ and $1-15 \mu\text{L L}^{-1}$ 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).

	Rose Bengal ($0.4 \mu\text{M}$)			Bromoxynil ($50 \mu\text{M}$)		
	Fumigation	No fumig.	Ratio	Fumigation	No fumig.	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
AVE \pm SE	92 \pm 9	66 \pm 10	1.42 \pm 0.10	62 \pm 3	49 \pm 3	1.29 \pm 0.14

ISOPRENE FUMIGATION IN BUCKTHORN

To verify the $^1\text{O}_2$ protection effect of isoprene, it was tested in another isoprene emitting species – buckthorn (*Rhamnus alaternus*). Similar fumigation experiments were carried out with young shade leaves treated with RB ($0.1 \mu\text{M}$). Young buckthorn leaves emitted small amounts of isoprene and isoprene concentrations used in fumigation were up to $20 \mu\text{L L}^{-1}$. In non-fumigated leaves net assimilation decreased significantly and with an increasing rate of decrease over time (i.e. increase in the slope of the time response curve) to 0.53 ± 0.02 of control after 4h ($n=4$). In isoprene-fumigated leaves net assimilation rates decreased to 0.66 ± 0.05 of control ($n=4$; Fig. 14; 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 buckthorn leaves consistently showed a decrease in net assimilation rates from the onset of the experiment while in isoprene-fumigated leaves net assimilation rates began decreasing only after RB feeding (Fig. 14). 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.

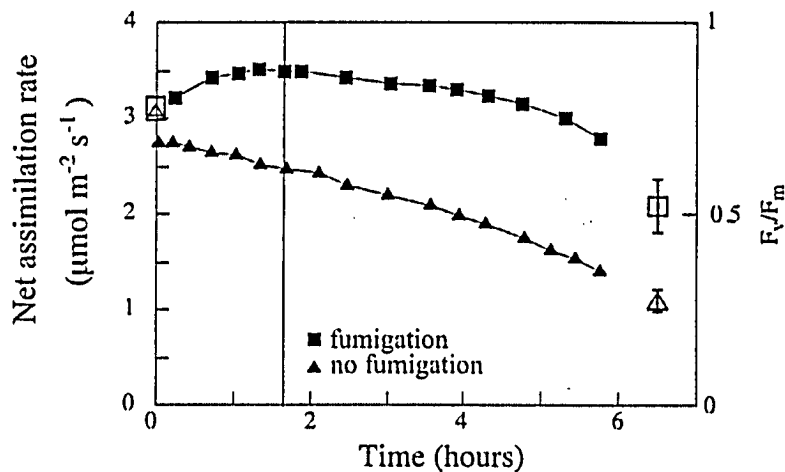


Figure 14: Effect of RB ($0.1 \mu\text{M}$) on net assimilation rates (bold symbols) and chlorophyll fluorescence yield (F_v/F_m ; empty symbols) in young shade buckthorn leaves at leaf temperature of 26°C and light intensity of $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$. The vertical line indicates the beginning of RB feeding. The error bars of F_v/F_m before the RB treatment are smaller than the symbols.

Isoprene protection in buckthorn 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 ± 0.010 to 0.274 ± 0.027 , $n=5$, and from 0.778 ± 0.003 to 0.520 ± 0.069 , $n=4$, in non-fumigated and isoprene-fumigated leaves, respectively; $P < 0.006$). Slightly better recovery of F_v/F_m after 24h in low light was observed in isoprene-fumigated leaves (F_v/F_m after 24h was 0.700 ± 0.020 and 0.639 ± 0.016 , in isoprene-fumigated and non-fumigated leaves, respectively; $P < 0.03$).

DISCUSSION

Thus, the results described above show for two plant species, using two 1O_2 production methods, that isoprene can reduce oxidative damage to the photosynthetic apparatus. The beneficial effects are evident in the reduced effect of 1O_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 1O_2 in leaves, and consider other sources for oxidative stress such as O_3 and water stress.

A first indirect indication for the usefulness of isoprene in protection against 1O_2 is an increase in internal concentration during 1O_2 stress. Using RB as a photosensitizer, we observed a decrease in net assimilation and in stomatal conductance. Either markedly smaller reduction in isoprene emission than in net assimilation rates (at ambient CO_2), or even enhancement (at high CO_2) was observed. Consequently, the plant apparently invested more of the fixed carbon in isoprene formation, and the reduction in stomatal conductance led to an increase in isoprene concentrations in the leaf air spaces during stress. Such increase in intercellular isoprene concentrations may indicate enhancement of isoprene potential to scavenge 1O_2 .

More directly, isoprene protection against 1O_2 was observed in isoprene fumigated young leaves (little or no endogenous isoprene emission; Table I, Fig. 14). 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 to $20 \mu L L^{-1}$) were higher than that occurring regularly in myrtle leaves, but not

unusual in mature stressed leaves (Fig. 9), and in leaves of other plants such as white oak and kudzu ($>10 \mu\text{L L}^{-1}$; Singaas 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 $\sim 30\%$ of excitation yields $^3\text{P680}$ and most of it is reflected in $^1\text{O}_2$ (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 percents of the excitation could yield $^1\text{O}_2$. The naturally occurring production of $^1\text{O}_2$ in the chloroplasts should therefore, 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.

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). The fluorescence yield (F_v/F_m) in buckthorn indicated significantly lower damage to the photosynthetic apparatus in isoprene-fumigated as compared to non-fumigated leaves. This suggests as well 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 clearly not the only protection mechanism available, isoprene may have some specific advantages. For example, chloroplast membranes are highly sensitive to photooxidative damage that occurs due to excessive light intensity leading to $^1\text{O}_2$ production. The extent of this damage/protection is related to the amount of β -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 O_3 (Mehlhorn and Wellburn, 1987; Hewitt et al., 1990a; 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 H_2O_2 and lipid peroxidation (Loreto and Velikova, 2001). Apparently, the reaction products are 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, while the direct reaction with $^1\text{O}_2$ is less significant, cannot be ruled out (isoprene protection against O_3 was suggested to be achieved by both mechanisms; Loreto et al., 2001).

Photoinhibition and $^1\text{O}_2$ 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 as well as 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 myrtle (Fig. 4). 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.

To conclude, isoprene was shown to protect the photosynthetic apparatus against stress inflicted by singlet oxygen. This may be particularly important in dry summer days in plants exposed to water stress. Isoprene is probably less efficient scavenger as compared to carotenoids, but it may have an advantage in fast response to increased light intensity.

Chapter 5
**ISOTOPIC COMPOSITION AND METABOLIC
SOURCES FOR ISOPRENE**



Leaves of Mucuna pruriens

Recent isoprene emission models use photosynthesis as a starting point, assuming direct coupling between isoprene production and photosynthesis and instantaneous emission without storage (Niinemets et al., 1999; Zimmer et al., 2000). However, protection by isoprene against oxidative stress, as shown in the previous chapter, requires that isoprene is produced under stress conditions when photosynthesis is limited. Incomplete coupling between isoprene emission and photosynthesis is indicated, for example, by emission of isoprene in the absence of net assimilation under CO₂-free air (see chapter 3 and Monson and Fall, 1989). In this chapter the difference between the carbon isotopic compositions of isoprene (δ_{isop}) and of newly fixed carbon (δ_{fixed}) are used as an indicator for the extent of such metabolic de-coupling between isoprene production and photosynthesis.

The concept apparent discrimination ($\Delta_{\text{C-isop}}$) is used, denoting the difference between the isotopic composition of isoprene and that of the concurrently fixed C and is calculated as:

$$\Delta_{\text{C-isop}} = \frac{\delta_{\text{fixed}} - \delta_{\text{isop}}}{\delta_{\text{isop}} + 1000} \cdot 1000 \quad (3).$$

In order to study variations in $\Delta_{\text{C-isop}}$, δ_{fixed} and δ_{isop} were measured using varying isotopic composition of the C source, resulting in large variations in δ_{fixed} and δ_{isop} .

EFFECT OF CO₂ SOURCE

Values of δ_{fixed} and δ_{isop} were measured during steady state leaf gas exchange before and after a rapid change between isotopically distinct sources of CO₂, with no change in the gas-exchange conditions. The change in CO₂ supplied to the leaf was reflected in δ_{fixed} but only partly reflected in δ_{isop} within 5 min. But within about 30 min of source change, newly established δ_{isop} values became relatively constant (Fig. 15). 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 ~100 nmol m⁻² isoprene were obtained by extractions from leaves of myrtle-1.

As expected, δ_{isop} values were significantly lower than the ¹³C values of recently fixed carbon, δ_{fixed} (Fig. 15). In contrast to expectations, however, the apparent discrimination against ¹³C in going from photosynthetically fixed carbon to isoprene was sensitive to the $\delta^{13}\text{C}$ of the source CO₂ (Fig. 15).

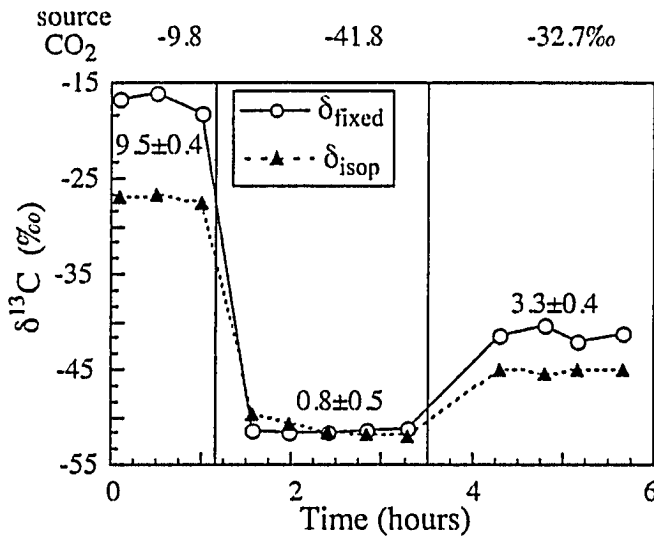


Figure 15: Changes in the isotopic composition of photosynthetically fixed carbon (δ_{fixed}) and of isoprene emitted (δ_{isop}) from a branch of myrtle-3, in response to rapid changes in the isotopic composition of the source CO_2 (at a time marked by the vertical lines). The numbers denote the apparent discrimination in isoprene production from fixed C ($\Delta_{\text{c-isop}}$ in ‰). Photosynthesis was at steady state before the onset of the experiment and all conditions, other than the CO_2 source, were kept constant throughout the experiments.

Similar results were obtained using leaves of myrtle, velvet bean and buckthorn, as summarized in Fig. 16 and Table II. The variations in the apparent discrimination in response to changes in the $\delta^{13}\text{C}$ of the CO_2 supply are reflected in the slopes of δ_{isop} vs. δ_{fixed} , which were smaller than 1 and varied between 0.72 ± 0.02 and 0.91 ± 0.03 . In reed, on the other hand, the apparent discrimination did not vary significantly with changes in the $\delta^{13}\text{C}$ of the source CO_2 , resulting in a slope of 1 (Fig. 16f).

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 treatments (ABA; 30 to 100 μM). Stomatal conductance decreased from 0.24 ± 0.09 to 0.02 ± 0.01 $\text{mol m}^{-2} \text{s}^{-1}$ (average \pm SE, $n=3$) and net assimilation decreased from 7 to 1 $\mu\text{mol m}^{-2} \text{s}^{-1}$. This led, in turn, to a decrease in c_i/c_a from 0.84 ± 0.002 to 0.61 ± 0.004 and hence in photosynthetic discrimination, yielding more positive δ_{fixed} (cf. Farquhar et al., 1982) and δ_{isop} values (Fig. 16a). The relationships of δ_{isop} vs. δ_{fixed} showed an even lower slope than that observed in the CO_2 labeling experiments (above). Similar ABA treatments on other myrtle branches showed variations in slopes among branches.

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 in order to characterize this unlabeled isoprene and to recognize its carbon source.

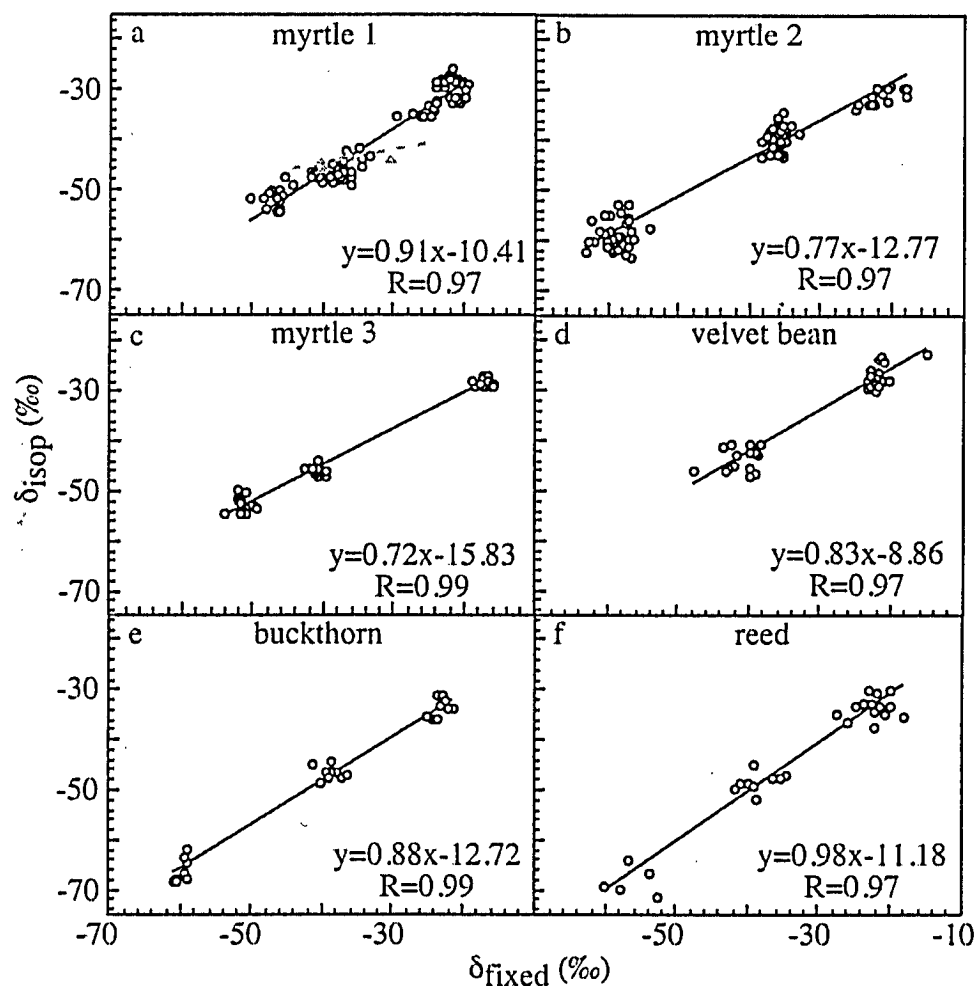


Figure 16: Relationships between the isotopic composition of the photosynthetically fixed carbon (δ_{fixed}) and that of isoprene emitted (δ_{isop}) by leaves of myrtle-1-3 (a-c, open circles), velvet bean (d), buckthorn (e), and reed (f). (Statistical analysis and additional information are given in Table II). The gray triangles and dashed line in (a) denote the influence of abscisic acid on δ_{fixed} and δ_{isop} in a branch of myrtle-1.

CO₂-FREE AIR

Measurements were carried out under CO₂-free air (or in the dark, see below) when there is no photosynthesis, in order to obtain isoprene that cannot be labeled by concurrent photosynthesis. After switching to CO₂-free air, isoprene emission was sustained for several hours, without significant change in emission rates (Fig. 6). δ_{isop} values under CO₂-free air were constant over ~2h of treatment with a mean value of $-41.7 \pm 1.4\%$ (average \pm SE, $n=6$, in myrtle-1 and 2 plants).

This value was independent of δ_{isop} values during the several hours preceding the CO₂-free air treatment that ranged between -35 and -50‰, for different pretreatment in which $\delta^{13}\text{C}$ of the CO₂ supply ranged between -8 and -31‰. These results indicated that isoprene was produced under CO₂-free conditions from an unlabeled carbon source. Only when δ_{isop} preceding the treatment was as low as \sim -60‰, some depletion in δ_{isop} , i.e. from -41.7 to $-47.9 \pm 1.4\%$ (n=7), was observed. Isoprene emission under CO₂-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 ± 0.6 (n=7), respectively, irrespective of the δ_{isop} values of preceding the CO₂-free air treatment that ranged between -35 and -50‰.

Table II: Slopes and regression coefficients (R^2) of the relationship between observed isotopic composition of fixed C (δ_{fixed}) and isoprene (δ_{isop}), and the apparent discrimination factor in isoprene synthesis from fixed C ($\Delta_{\text{C-isop}}$). Estimates of $\Delta_{\text{C-isop}}$ are based on the assumption that the isotopic composition of leaf organic matter (δ_{leaf}) represents mean δ_{fixed} during the growth period of the leaf. Slopes in myrtle, buckthorn, and velvet bean are significantly lower from 1, based on t-test on $((\text{slope}-1)/\text{slope SE})$.

Species	Slope	R^2	$\delta_{\text{leaf}}(\text{‰})$	$\Delta_{\text{C-isop}}(\text{‰})$
Myrtle 1	0.91 ± 0.03	0.932	-29.0 ± 0.2	7.7
Myrtle 2	0.77 ± 0.02	0.944	-28.4 ± 0.2	6.3
Myrtle 3	0.72 ± 0.02	0.981	-23.4 ± 0.3	9.6
Velvet bean	0.83 ± 0.04	0.936	-28.5 ± 0.6	4.2
Reed	0.98 ± 0.04	0.946	-28.8 ± 0.3	11.0
Buckthorn	0.88 ± 0.02	0.981	-30.2 ± 0.4	9.5
Red Oak: (Sharkey et al. 1991c)	0.96 ± 0.04	0.992	-25.8 to -26.8	2.8

EMISSION IN THE DARK

In the dark, isoprene emission from myrtle decreased rapidly to detection limit levels (Fig. 17), 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 . $\delta_{\text{isop-dark}}$ was slightly more depleted than $\delta_{\text{isop-light}}$ (by $2.7 \pm 0.7\%$; $n=4$), for CO_2 source during the preceding light period with $\delta^{13}\text{C}$ values of either -8% or -31% .

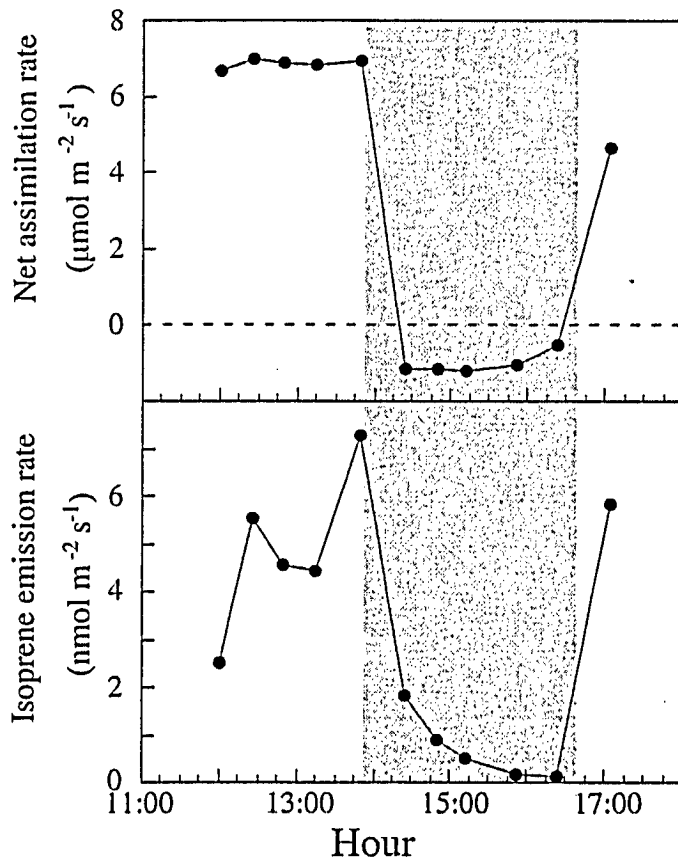


Figure 17: Net assimilation and isoprene emission rates in light and dark in myrtle-1. The shaded area denotes the dark time period. The white area is time with light intensity of $1000 \mu\text{mol m}^{-2} \text{sec}^{-1}$. Leaf temperature was 26°C throughout.

GLUCOSE LABELING

To examine incorporation of glycolytic carbon into isoprene (Fig. 19) we fed both myrtle and buckthorn leaves with ^{13}C -enriched glucose ($\delta^{13}\text{C}_{\text{glucose}}$ was -10% as compares to $\delta^{13}\text{C}$ 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 δ_{respired} were measured at ambient or zero CO_2 concentrations, respectively, with and without glucose feeding. Under CO_2 -free air, both respired CO_2 and isoprene were clearly labeled by the enriched glucose, and to a similar extent (Table III). Under

ambient CO₂ concentration, glucose feeding led to a slight decrease in net assimilation rates, but both δ_{isop} and δ_{fixed} did not change significantly.

Table III: Effects of feeding leaves with ¹³C enriched glucose on the isotopic composition of isoprene and respired CO₂, under CO₂-free air conditions. Each pair of lines denotes one branch. Average and SE refer to 3 or 4 measurements for either control of treatment.

Species	Glucose feeding	$\delta_{\text{respired}}(\text{‰})$	$\delta_{\text{isop}}(\text{‰})$
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

CYTOSOLIC IPP

In order 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 emission rates persisted even after treatment (Fig. 18 cf. Loreto and Velikova, 2001). Partial inhibition resulted in ¹³C depletion of δ_{isop} and an increase of apparent discrimination, $\Delta_{\text{C-isop}}$, from an average of 9.4±0.5‰ before treatment to 11.4±0.5‰ during inhibition (average±SE, n=8, P<0.001; Table IV). This increase in $\Delta_{\text{C-isop}}$ was observed even when δ_{fixed} and δ_{isop} before the fosmidomycin treatment was highly depleted. Notably inhibition with fosmidomycin resulted also in a 43% increase in leaf pyruvate content by, from 23±3 $\mu\text{mol m}^{-2}$ in control leaves to 33±2 $\mu\text{mol m}^{-2}$ after 4h of inhibitor treatment (3 μM ; P<0.02, n=4).

Assuming that cytosolic IPP, should be produced from acetate through acetyl CoA, leaves were fed also with labeled acetate. Labeled acetate (30 to 120 mM, $\delta^{13}\text{C}=+150\text{‰}$) led to a 2±0.1‰ increase in the $\delta^{13}\text{C}$ of the water-soluble fraction of leaf extracts, indicating that the leaves took up the labeled acetate. However, no effect was observed on the isotopic composition of either respired CO₂ or isoprene under either normal or zero CO₂ concentrations.

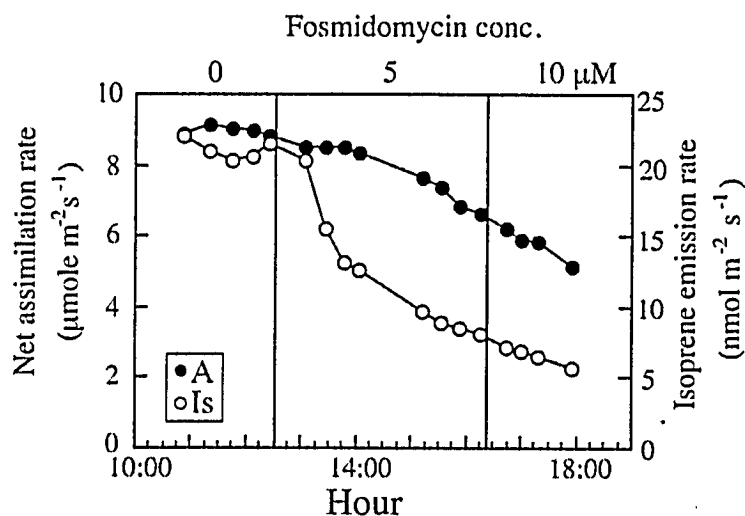


Figure 18: Effects of fosmidomycin inhibition on net assimilation (bold circles) and isoprene emission rates (empty circles) in a branch of myrtle-1. The vertical lines indicate the beginning of fosmidomycin feeding (5 μM at 12:30 and 10 μM at 16:20). Light intensity was 250 $\mu\text{mol m}^{-2} \text{sec}^{-1}$, leaf temperature was 26°C and c_i varied between 220 and 250 $\mu\text{L L}^{-1}$. 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.

Table IV: Effects of fosmidomycin on the isotopic composition of photosynthetically fixed CO_2 and of isoprene emitted from leaves of myrtle and buckthorn and on pyruvate content in buckthorn leaves. Each line denotes 4 control and 4 treatment measurements from one branch. For pyruvate content leaves of 4 branches were extracted and measured for either control or treatment.

Species	$\delta^{13}\text{C}$ source	Untreated control			Fosmidomycin treated		
		$\delta_{\text{fixed}}(\text{‰})$	$\delta_{\text{isop}}(\text{‰})$	$\Delta_{\text{C-isop}}(\text{‰})$	$\delta_{\text{fixed}}(\text{‰})$	$\delta_{\text{isop}}(\text{‰})$	$\Delta_{\text{C-isop}}(\text{‰})$
Myrtle	$\text{CO}_2(\text{‰})$						
	-13	-24.4±0.7	-34.0±0.5	9.9±0.7	-27.3±0.6	-39.6±0.4	12.8±0.9
	-13	-22.3±0.5	-32.2±0.2	10.2±0.4	-25.6±0.2	-39.0±0.5	14.1±0.8
	-49	-58.6±0.4	-68.5±0.6	10.7±0.6	-62.2±1.1	-72.7±0.4	11.4±1.0
	-49	-60.7±0.3	-67.2±0.2	6.9±0.4	-62.8±0.4	-71.4±0.3	9.3±0.7
	-27	-37.7±0.2	-45.8±0.1	8.4±0.1	-39.8±0.3	-50.1±0.3	10.9±0.2
Buckthorn	-27	-37.5±0.2	-47.5±0.2	10.4±0.3	-39.7±0.4	-51.6±0.2	12.5±0.5
	-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
Pyruvate ($\mu\text{mol m}^{-2}$)		23±3			33±2		

ISOTOPIC COMPOSITION OF ISOPRENE EMITTED TO THE ATMOSPHERE

Short-term Δ_{C-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 Δ_{C-isop} of $7.7 \pm 0.7\text{‰}$ ($\delta_{isop} = 29.2 \pm 0.6\text{‰}$, $\delta_{fixed} = -21.7 \pm 0.3\text{‰}$, $n=8$). However, measurements as in Fig. 15 cannot be used to estimate the intrinsic isotopic discrimination in isoprene production, as indicated by the results presented in Fig. 16, because of the deviations from the 1:1 in the δ_{fixed} vs. δ_{isop} relationships. Alternatively, an estimate for long-term mean Δ_{C-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 Δ_{C-isop} value of 7.7‰ (Table II), consistent with the values obtained in the short-term measurement. Using the same approach, mean Δ_{C-isop} values in velvet bean, reed and buckthorn were estimated to be 4.2‰ , 11.0‰ , and 9.5‰ , respectively (Table II).

DISCUSSION

Incomplete coupling between isoprene and assimilation

The time course of changes in δ_{isop} values following a change in the isotopic composition of source CO_2 (Fig. 15) confirmed the dynamic connection between CO_2 assimilation and isoprene productions (Sharkey et al., 1991a). Quantitatively, however, the coupling 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} vs. δ_{fixed} relationships of 1:1. But the observed slopes of these relationships were considerably smaller than 1 (Fig. 16). 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 Δ_{C-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 Fig. 15.

As expected, possible contribution from stationary isoprene pool in the leaves could be ruled out based on the direct measurement of only $\sim 100 \text{ nmol m}^{-2}$ isoprene extractable from myrtle leaves. Such stationary pool would support about 100 sec of emission (i.e. based on typical

emission rate of $10 \text{ nmol m}^{-2} \text{ s}^{-1}$ and 10% contribution (Fig. 15a) from alternative source(s)), 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. (1991c) with red oak, $\Delta_{\text{C-isop}}$ 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 $^{13}\text{CO}_2$. 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 (PGA, Calvin, 1979; Delwiche and Sharkey, 1993). Such results also suggest contribution from an alternative, slow turnover, carbon source for isoprene, or for PGA 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 with only ~90% labeling of the isoprene by $^{13}\text{CO}_2$ (Shao et al., 2001). This was explained by the possible existence of two carbon sources, whose turnover rates are different. Similarly, only ~90% labeling by newly fixed $^{13}\text{CO}_2$ 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; Loreto et al., 1996b). The question of alternative carbon source(s) for isoprene recently received renewed interest, and during the writing of this thesis two other labeling studies provided evidence for contributions of extra-chloroplastic carbon source (Karl et al., 2002b) or xylem-transported glucose (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. 16a). The lower slope in the ABA treatments, as compared to 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. (1991c) 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} vs. δ_{fixed} relationships from zero to ~30% was observed (Fig. 16). 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. 16) indicated full coupling to photosynthesis, but the emission under CO₂-free air suggested engagement of an alternative carbon source. Such dynamic response is significant since it may help explain the reduced sensitivity of isoprene emission to stress effect, as compared to photosynthesis (Sharkey and Loreto, 1993; Loreto and Delfine, 2000; and see chapter 4). Further, such effect would enhance the potential protection effects by isoprene against, for example, oxidative stress when photosynthesis is partly inhibited (see chapter 4).

Alternative C sources 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 CO₂-free air (Monson and Fall, 1989; Loreto and Delfine, 2000; and see Fig. 6). We observed under CO₂-free air relatively constant δ_{isop} values, independent of $\delta^{13}\text{C}$ of source CO₂ or δ_{isop} during pretreatments. The results under CO₂-free air indicated also that the alternative carbon source(s) always produced isoprene with a $\delta^{13}\text{C}$ value in the range of -35 to -50‰ (~-42‰ on average). Labeling measurements in this $\delta^{13}\text{C}$ range were, therefore, insufficiently sensitive to clearly identify the influence of different carbon sources. Indeed, labeling with more depleted source CO₂ (leading to pre-treatment 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 3h. That is, labeling that produced 18‰ effect in δ_{isop} during 3 h of pretreatment, resulted in ~6‰ effect in δ_{isop} under CO₂-free air. Such results provide a first approximation for the turnover rate of the alternative carbon source(s), i.e. in the order of 10h. The partial labeling of isoprene emitted under CO₂-free air is consistent with partial labeling of CO₂ respired into CO₂-free air (Ludwig and Canvin, 1971). While in principle, the unlabeled carbon source could result from refixation of the respired CO₂, such refixation under CO₂-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 O₂ is lowered under CO₂-free air (Monson and Fall, 1989; Loreto and Sharkey, 1990). But Hewitt et al. (1990b) showed that isoprene is not produced predominantly via photorespiration. It was previously suggested also that photorespiratory intermediates originate from short-term carbon storage, that was 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 $^{13}\text{CO}_2$, indicating production de-novo in the dark. In Scots pine some emission of isoprene was observed during dark hours with ~90% labeling (Shao et al., 2001). In the present study, however, isoprene emission from myrtle leaves in the dark decreased rapidly (Fig. 17) 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 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. 19). Pyruvate may incorporate non-photosynthetic carbon in the cytosol prior to its import to the chloroplast (Givan, 1999). Incorporation of ~50% glycolytic carbon, such as observed by Karl et al., (2002a) is consistent with the observed ~20% contribution to isoprene.

Carbon from glucose incorporated into isoprenoids (Schwender et al., 1996; Lichtenthaler et al., 1997b; Kreuzwieser et al., 2002). Feeding leaves with ^{13}C labeled glucose 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 glucose clearly labeled both respired CO_2 and isoprene (Table III). The effect on respired CO_2 confirmed that labeled glucose was incorporated into leaf metabolism. Further, the labeling of isoprene clearly indicated that isoprene incorporated carbon via the glycolytic pathway. Glucose 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 glucose is similar to δ_{fixed} under natural atmospheric conditions, and the products should undergo the same discrimination step as photosynthetically coupled isoprene.

Glucose, as was observed for sucrose in wheat (Gebbing and Schnyder, 2001), may also correspond well with a carbon source that is labeled by newly fixed carbon within several hours.

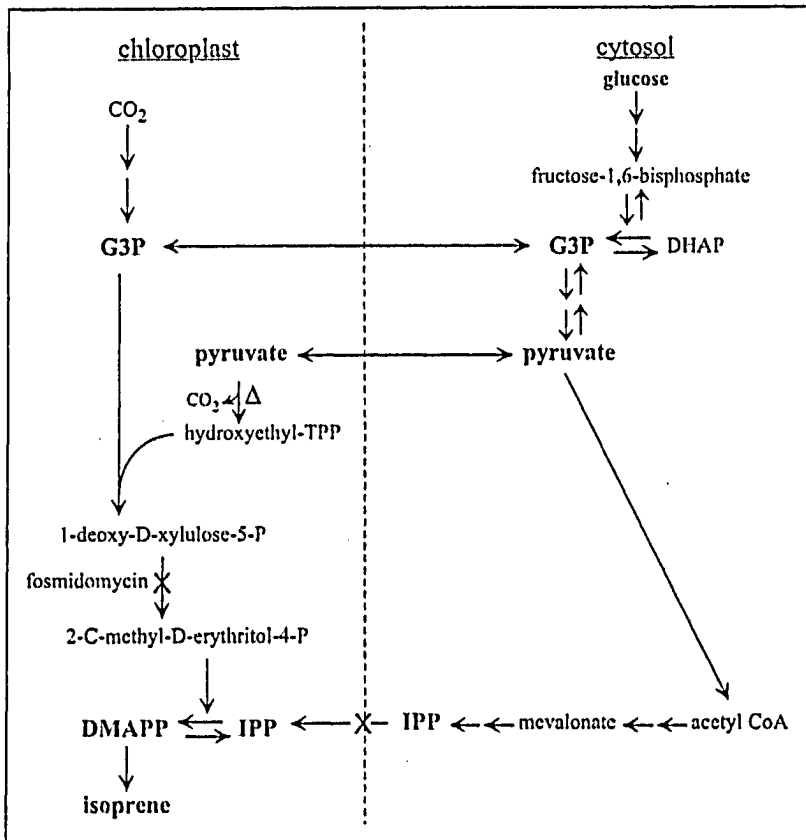


Figure 19: Schematic representation of the MEP biosynthesis pathway for isoprene, and possible coupling to cytosolic glucose metabolism and IPP. Also noted are the sites of action of the inhibitor fosmidomycin (Fellermeier et al., 1999) and of the isotopic discrimination by deoxyxylulose-5-phosphate synthase. Abbreviations: DHAP dihydroxyacetone phosphate, DMAPP dimethylallyl pyrophosphate, G3P glyceraldehyde-3-phosphate, IPP isopentenyl pyrophosphate, TPP thiamine pyrophosphate.

Cytosolic IPP: Among the possible carbon sources for the unlabeled isoprene could also be cytosolic IPP produced from pyruvate (itself containing ~50% glycolytic carbon Karl et al., 2002a), through the mevalonic acid pathway (Fig. 19). 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 indeed this was an alternative carbon source. In this case, as was observed in Fig. 15, the increased relative contribution of extra-chloroplastic IPP should be accompanied by a shift in δ_{isop} toward

that of the constant alternative $\delta^{13}\text{C}$ 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 $\sim -30\text{‰}$, enriched δ_{isop} of $\sim -70\text{‰}$ and would have little effect on δ_{isop} of $\sim -45\text{‰}$. 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 carbon, in contrast to observations (Karl et al., 2002a). It is unlikely that any unlabeled intermediate in leaves is depleted enough to produce $\delta_{\text{isop}} < -70\text{‰}$. We therefore concluded that cytosolic IPP did not contribute to production of unlabeled isoprene, and offer below an alternative explanation to the observed fosmidomycin-induced depletion in isoprene ^{13}C content.

Isotopic Discrimination

Isotopic discrimination in the MEP pathway: Invoking the mevalonic acid pathway, Sharkey et al. (1991c), argued for discrimination by pyruvate dehydrogenase leading 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 DXS (deoxyxylulose-5-phosphate synthase). 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 $\Delta_{\text{C-isop}}$) 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 DXR (deoxyxylulose-5-phosphate reductoisomerase). Kinetic isotopic discrimination, which is only 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.

Further, 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). The results presented here, 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 (see Appendix 2).

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.

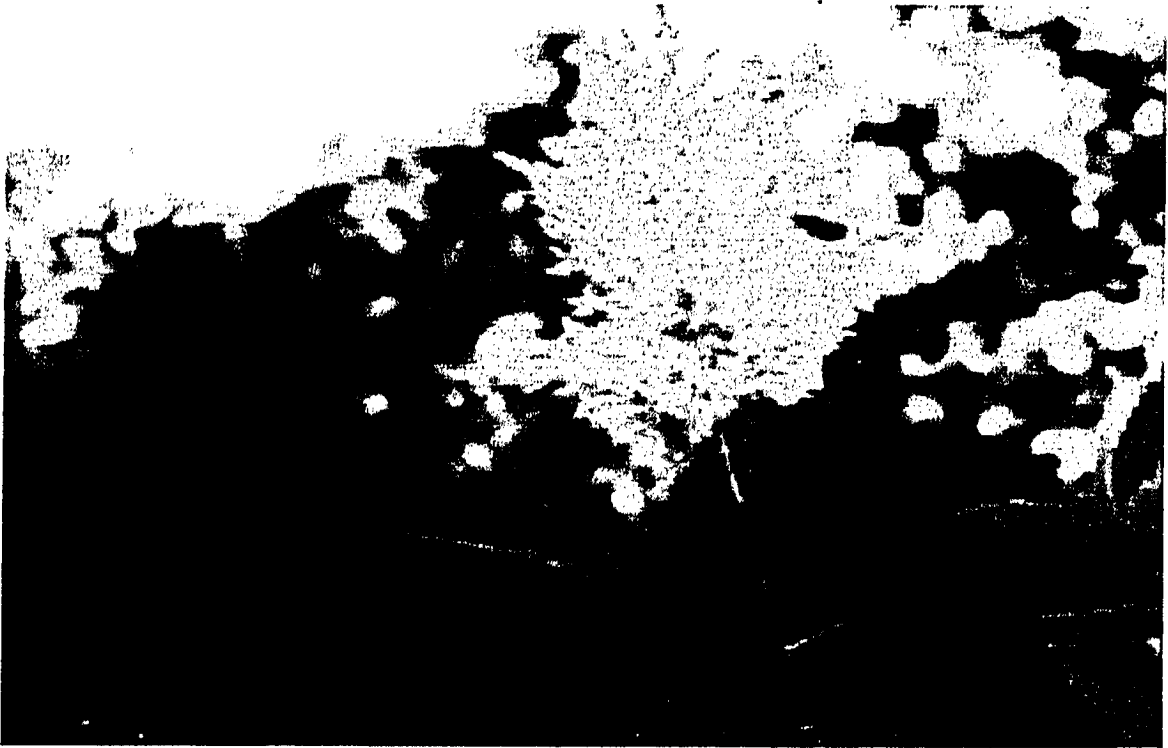
The isotopic composition of isoprene, δ_{isop} , must reflect the additive effect of Δ_A , the discrimination in photosynthetic carbon assimilation (Lloyd and Farquhar, 1994, and references therein), and that in the isoprene pathway, $\Delta_{\text{C-isop}}$, to produce the total discrimination $\Delta_{\text{total}}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

It is now generally accepted that Δ_A can vary with time and plant species. In this study we provide evidence that $\Delta_{\text{C-isop}}$ can vary at least between 4 and 11‰ (Table II), a range that includes previously reported value for red oak ($2.8 \pm 0.4\text{‰}$, Sharkey et al., 1991c). We further demonstrate that it is possible to separate estimates of $\Delta_{\text{C-isop}}$ 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; and see Bowling et al., 2001 for the ecosystem scale) or by using $\Delta_{\text{C-isop}}$ in a mono-specific 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 and fate of this compound. The ability to de-convolute the isotopic signal to Δ_A and $\Delta_{\text{C-isop}}$ can provide insights on processes associated with, for example, plants response to environmental stresses.

In conclusion, this chapter shows that although isoprene is produced mostly from newly fixed C through the MEP pathway and is not stored in the leaves, a significant fraction incorporates carbon from a slow-turnover source. The importance of this long-term source increases when assimilation diminishes. A few alternatives for this long-term carbon source were tested (Fig. 19) suggesting that cytosolic IPP does not significantly contribute to isoprene production, but

glycolytic precursors that lead to isoprene via pyruvate and G3P are likely to be involved. Also suggested is that the major discrimination occurs in the MEP pathway in the step catalyzed by DXS and the extent to which it is rate-limiting can modify the $\delta^{13}\text{C}$ values of isoprene, leading to variations in apparent discrimination, $\Delta_{\text{C-isop}}$, in a range of between 3 and 11‰ among plant species.

Chapter 6
SUMMARY AND CONCLUSIONS



A flower of *Phragmites australis*

Isoprene is a major hydrocarbon emitted from vegetation to the atmosphere and as such it influences the oxidative capacity of the troposphere and leads, in the presence of NO_x , to formation of tropospheric ozone. Isoprene production is strongly associated with photosynthetic activity and it utilizes up to a few percent of the carbon fixed in photosynthesis, but its physiological role is not fully known.

The study presented in this thesis characterized isoprene emission in a typical isoprene emitting Mediterranean plant (*Myrtus communis*), examined the ability of isoprene to act as a protection mechanism against stress in leaves, examined, using natural abundance ^{13}C analysis, the quantitative coupling between isoprene production and concurrent photosynthetic carbon assimilation, and provided first estimates of the potential variations in the ^{13}C content of isoprene in nature.

M. communis showed large seasonal variations in isoprene emission rates with maximum rates in summer and to close to zero in winter. Both isoprene emission and net assimilation rates increased with leaf age so that young leaves emitted only small amount of isoprene. CO_2 concentrations greatly influenced isoprene emission rates with a sharp decrease at high CO_2 concentrations, as compared to ambient concentration. Isoprene emission was sustained at CO_2 -free air for several hours.

The study of the protection capability of isoprene was focused on oxidative stress due to singlet oxygen. Singlet oxygen is normally produced in the leaves as a result of excessive light, with chlorophyll acting as a photosensitizer, transferring the light energy to molecular oxygen, in a process termed photoinhibition. In this study an external photosensitizer was used (Rose Bengal, RB) as well as an herbicide (bromoxynil, BX) that led to an enhanced sensitivity to light, thus simulating photoinhibition.

The influence of singlet oxygen on net assimilation and isoprene emission rates was tested, indicating damage to both. But isoprene emission was less sensitive and showed either a smaller decrease (at ambient CO_2 concentrations) or even an increase (under high CO_2). In both cases, the treatments led to increase in calculated internal isoprene concentrations, suggesting a potential protection effect. Protection effects were assessed directly by fumigating young, non-emitting, leaves with exogenous isoprene, during treatment with RB or BX. The fumigation alleviated oxidative damage to the photosynthetic apparatus. In fumigated leaves of *M. communis* and *Rhamnus alaternus* there was reduction in the damage to net assimilation as compared to non-

fumigated leaves. The isoprene protection against singlet oxygen was also evident in chlorophyll fluorescence yield (F_v/F_m) that showed a significantly smaller decrease in fumigated *R. alaternus* leaves as compared to non-fumigated leaves. This indicates the ability of isoprene to act as protection mechanism against singlet oxygen in leaves.

Some indication was observed for protection by isoprene against high light intensity even without chemical enhancement of singlet oxygen. This may also be the reason for the observed decrease in net assimilation, as a result of fosmidomycin inhibition of isoprene formation. Eliminating isoprene (using fosmidomycin with and without isoprene fumigation) may be used in a further study to examine more specifically protection by isoprene against photoinhibition under excess light.

Studying the degree of coupling between isoprene synthesis and photosynthetic carbon fixation was performed using the carbon isotopic composition of isoprene and fixed C. The ^{13}C content of isoprene reflected within a few minutes the changes in the isotopic composition of ambient CO_2 . Quantitatively, however, the labeling was incomplete even after several hours in leaves of *M. communis*, *R. alaternus* and *Mucuna pruriens* and ~20% of the carbon in isoprene was not derived from concurrently assimilated CO_2 . The relative contribution of this slow turnover source increased when photosynthesis was inhibited by lack of CO_2 . This allowed estimation of the ^{13}C content of the alternative source as ~-42‰ and labeling under CO_2 -free air indicated its turnover rate to be ~10h. This was consistent with labeled glucose feeding experiments, and contribution of glycolytic intermediates to isoprene production.

Inhibition of isoprene production through the MEP pathway led to a decrease in isoprene emission rates accompanied by depletion of ^{13}C in the remaining isoprene flux. This reflected an increase in the apparent discrimination against ^{13}C in isoprene production and was associated with accumulation of leaf pyruvate. Based on these results we hypothesize that the step catalysed by deoxyxylulose-5-phosphate synthase (DXS), is the rate-determining step and therefore the main discriminating step in isoprene production. Variations in the relative rate of DXS among species may therefore explain the observed variations in the apparent discrimination (between 4 and 11‰) among species or individual plants under different conditions.

The results of this study improved our understanding of the possible physiological function of isoprene, its metabolic pathway, and its isotopic composition. Combined, the results provide also

some insights to observations of isoprene emission in nature. For example, isoprene production is maximal in summer and very low in winter making it an inefficient protection against photoinhibition in clear winter days. In the Mediterranean, however, it may be useful in plants growing in dry conditions as protection against photoinhibition due to water stress, which is more pronounced in summer. Further, protection requires isoprene synthesis under stress conditions when photosynthesis is limited. The observed contribution of extra-chloroplastic C sources to isoprene is likely a key element in this respect. Confirming this point will require further study to examine the effect of stress on the level of the isoprene/concurrent photosynthesis coupling. The isotopic composition (^{13}C) of isoprene reflects the combined discrimination effects during both photosynthesis, Δ_A , and isoprene production, $\Delta_{c\text{-isop}}$. Under natural conditions the involvement of extra-chloroplastic carbon will have relatively little effect, as the ^{13}C of the source CO_2 is relatively constant. Our results should, therefore, facilitate the use of ^{13}C in isoprene as an indicator of $\Delta_{c\text{-isop}}$ and/or Δ_A . When estimates of Δ_A are well constrained, variations in the isotopic composition of isoprene may be used as indicator of changes in DXS activity. This, in turn, could help future studies to better understand the physiological role of isoprene emission in plants and its response to environmental change. Alternatively, when $\Delta_{c\text{-isop}}$ can be assumed to be relatively constant, atmospheric measurements of ^{13}C in isoprene can provide an integrated indicator of Δ_A . This will provide a unique contribution to ecosystem-scale studies of carbon exchange in the terrestrial biosphere.

Appendix 1

**EFFECT OF SINGLET OXYGEN IN MYRTLE,
AVERAGED DATA OF ALL EXPERIMENTS**



Leaves and flowers of *Myrtus communis*

Time response of isoprene emission and net assimilation rates to singlet oxygen treatment by RB is shown for representative experiments in figures 9 and 12 above. These measurements were repeated on several branches ($n=20$) that showed significant variations in rates under control conditions. Here, the results for all branches are normalized and the relative changes and their variations (SE) are reported and are consistent with the results discussed in chapter 4. The pooled results show that at ambient CO_2 concentrations the decrease in isoprene emission rates was delayed and was smaller in magnitude, relative to that in net assimilation (Fig. 20). As a result the estimated isoprene concentration in the leaf airspaces increased.

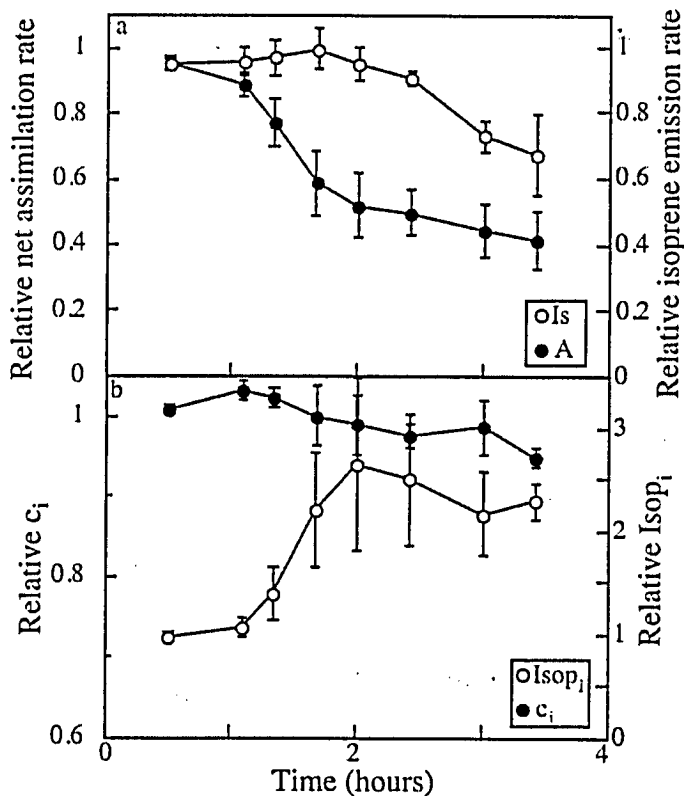


Figure 20: Average effects of singlet oxygen in myrtle leaves at ambient CO_2 concentration on net assimilation and isoprene emission rates (a) and on changes in the intercellular concentration of CO_2 (c_i ; b) and isoprene (Isop_i; b) at leaf temperature of 26°C , light intensity of $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$. Time zero indicates the beginning of RB feeding. The values in each experiment were normalized to the average value before RB feeding, in the same experiment. Averages and SE (depicted by the error bars) of 4 experiments are presented. A representative experiment is given in figure 9 a, c above.

At high CO_2 concentrations isoprene emission rates did not change in spring and winter (note that the emission rates in winter were very low; Fig. 21), or even increased in summer and autumn (Fig. 22). This response was well correlated with variations in c_i . During autumn and summer, c_i

decreased and isoprene emission rates increased (Fig. 22) in response to RB treatments whereas during winter and spring both c_i and isoprene emission rates did not change (Fig. 21).

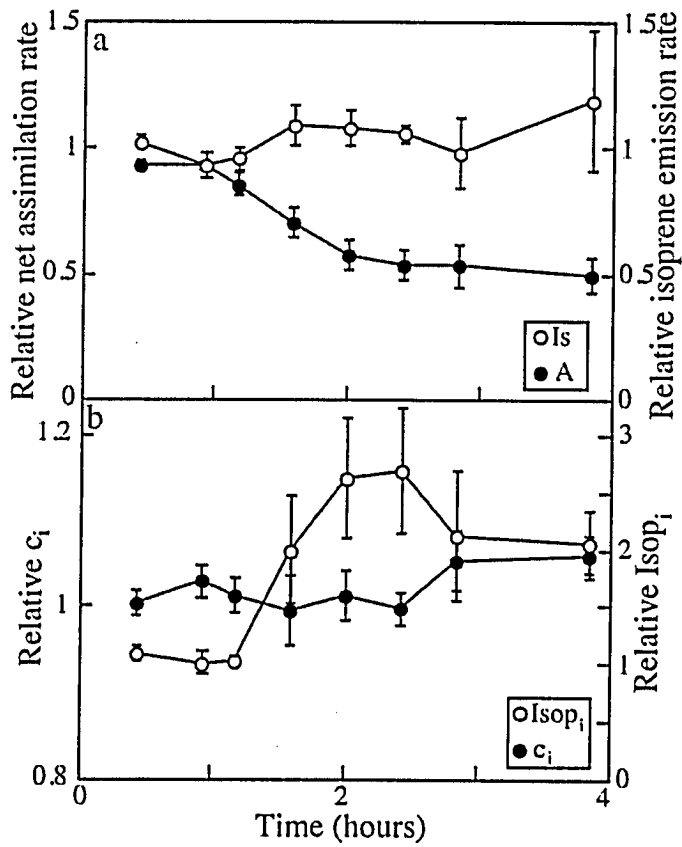


Figure 21: Average effects of singlet oxygen in myrtle leaves at high CO₂ concentration in spring and winter on net assimilation and isoprene emission rates (a) and on changes in the intercellular concentration of CO₂ (c_i ; b) and isoprene (Isop_i; b) at leaf temperature of 26°C, light intensity of 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Time zero indicates the beginning of RB feeding. The values in each experiment were normalized to the average value before RB feeding, in the same experiment. Averages and SE (depicted by the error bars) of 8 experiments are presented. A representative experiment is given in figure 12 above.

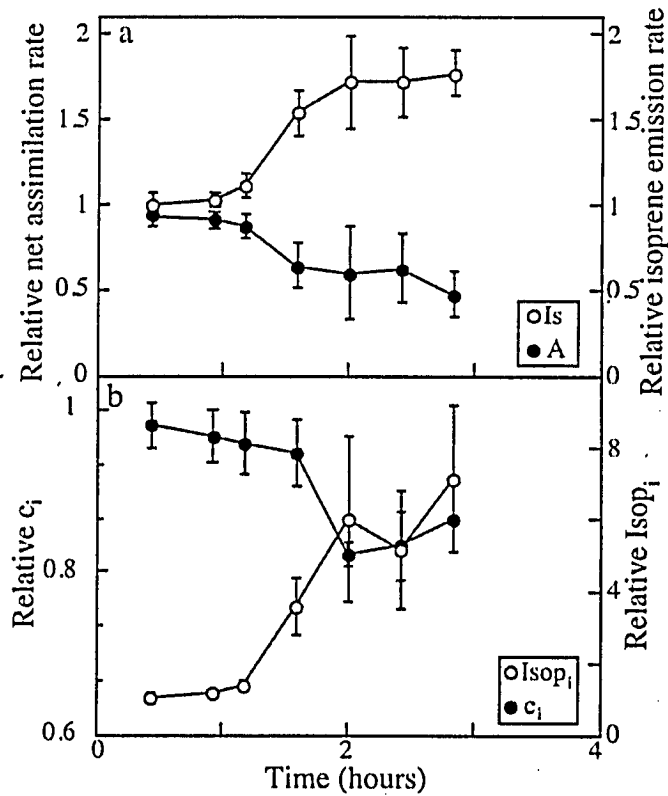


Figure 22: Average effects of singlet oxygen in myrtle leaves at high CO₂ concentration in summer and autumn on net assimilation and isoprene emission rates (a) and on changes in the intercellular concentration of CO₂ (c_i ; b) and isoprene (Isop_i; b) at leaf temperature of 26°C, light intensity of 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Time zero indicates the beginning of RB feeding. The values in each experiment were normalized to the average value before RB feeding, in the same experiment. Averages and SE (depicted by the error bars) of 8 experiments are presented. A representative experiment is given in figure 9 b, d.

Appendix 2

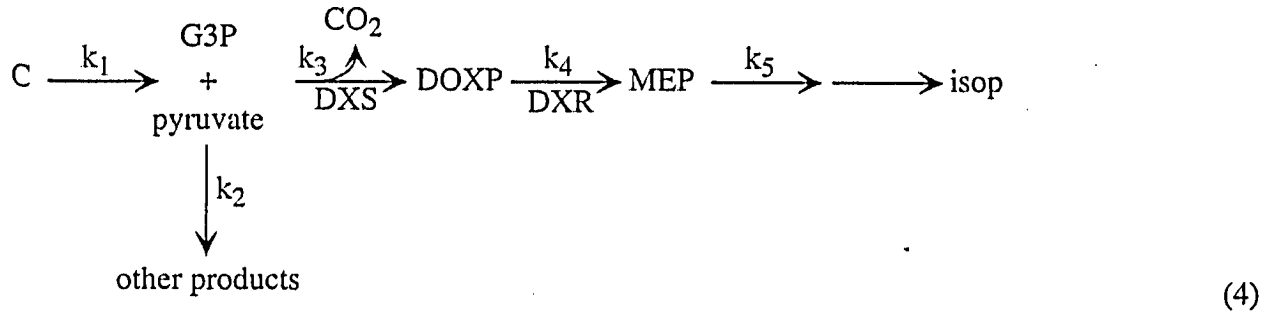
**DEPENDENCE OF THE ISOTOPIC
DISCRIMINATION ON REACTION RATES**



Leaves and fruits of *Rhamnus alaternus*

A model is developed to describe the effect of changing reaction rates on the isotopic discrimination in isoprene production, based on the kinetic isotope effect (KIE) derived by O'Leary, (1981).

Isoprene synthesis can be described schematically as,



Apparent discrimination against ^{13}C in isoprene production, $\Delta_{\text{c-isop}}$, is defined as,

$$\Delta_{\text{c-isop}} = (R_{\text{substrate}}/R_{\text{product}} - 1) \cdot 1000 = (\text{KIE} - 1) \cdot 1000 \quad \text{where } R = ^{13}\text{C}/^{12}\text{C}.
 \tag{5}$$

The rate of isoprene synthesis is:

$$\frac{\partial[\text{isop}]}{\partial t} = k_5[\text{MEP}]
 \tag{6a}$$

The rate of change in concentration of the intermediates in the reaction is:

$$\frac{\partial[\text{MEP}]}{\partial t} = k_4[\text{DOXP}] - k_5[\text{MEP}]
 \tag{6b}$$

$$\frac{\partial[\text{DOXP}]}{\partial t} = k_3[\text{pyruvate}][\text{G3P}] - k_4[\text{DOXP}] = k_G[\text{pyruvate}] - k_4[\text{DOXP}]
 \tag{6c}$$

Since G3P is produced in the chloroplast and pyruvate has to be (at least to some extent) imported from the cytosol, it can be assumed that the concentration of G3P is relatively constant. Therefore, it is included in the rate coefficient as $k_G = k_3[\text{G3P}]$.

$$\frac{\partial[\text{pyruvate}]}{\partial t} = k_1[\text{C}] - (k_G + k_2)[\text{pyruvate}]
 \tag{6d}$$

At a steady state the concentration of the intermediates is constant and:

$$[\text{pyruvate}] = \frac{k_1[\text{C}]}{k_2 + k_G}
 \tag{7a}$$

$$[\text{DOXP}] = \frac{k_G[\text{pyruvate}]}{k_4} = \frac{k_1 k_G [\text{C}]}{k_4(k_2 + k_G)}
 \tag{7b}$$

$$[\text{MEP}] = \frac{k_4[\text{DOXP}]}{k_5} = \frac{k_1 k_G [\text{C}]}{k_5(k_2 + k_G)}
 \tag{7c}$$

Then, the rate of isoprene production is:

$$\frac{\partial[\text{isop}]}{\partial t} = k_5[\text{MEP}] = \frac{k_1 k_G [C]}{k_2 + k_G} \quad (8)$$

Considering the isotopic species (^{13}C) and its reaction rate coefficient for the respective step:

$$E = k/k' \quad (9)$$

where k is the rate coefficient of the step in question and k' is the corresponding rate coefficient for a ^{13}C form of the substrate.

Then, KIE, the isotope effect for the overall reaction:

$$\text{KIE} = \frac{k_1 k_G}{k_2 + k_G} \cdot \frac{k'_2 + k'_G}{k'_1 k'_G} = E_1 E_G \frac{k_2/k_2 + k'_G/k_2}{1 + k_G/k_2} = E_1 \frac{E_G/E_2 + k_G/k_2}{1 + k_G/k_2} \quad (10)$$

In that case, a decrease in k_G would lead to an increase in KIE and hence in $\Delta_{\text{C-isop}}$ (see Eq. 1) if the isotope effect in the decarboxylation of pyruvate (E_G) is larger than in other reactions of pyruvate (E_2). This may explain the increased apparent discrimination observed in fosmidomycin treatments.

Using similar approach, KIE can be derived for the case in which the reaction catalyzed by DXS is reversible, with k_3 as the rate coefficient for the backward reaction, (O'Leary, 1981):

$$\text{KIE} = E_1 \frac{E_G E_4 / E_2 E_{-3} + (E_G / E_2)(k_4 / k_{-3}) + (k_G / k_2)(k_4 / k_{-3})}{1 + (k_4 / k_{-3}) + (k_G / k_2)(k_4 / k_{-3})} \quad (11)$$

If k_4 is large, namely the step catalyzed by DXR is fast, then Eq. 11 is reduced to Eq. 10. If, on the other hand, the DXR step is rate limiting and the DXS step is fast (large k_3) then KIE is:

$$\text{KIE} = \frac{E_1 E_G}{E_2} \cdot \frac{E_4 / E_{-3} + (k_4 / k_{-3})}{1 + (k_4 / k_{-3})} \quad (12)$$

However, if the DXR step was rate limiting there should have been accumulation of DOXP due to its inhibition with fosmidomycin, which was not observed (Lange et al., 2001), whereas the observed accumulation of pyruvate (Table IV) supports DXS as rate limiting and Eq. 10 for KIE.

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PUBLICATIONS RESULTING FROM THIS STUDY

Affek HP, Yakir D (2002), Protection by isoprene against singlet oxygen in leaves. *Plant Physiology* 129: 269-277

Affek, HP and Yakir D (2003), Natural abundance carbon isotope composition of isoprene reflects incomplete coupling between isoprene synthesis and photosynthetic carbon flow. *Plant Physiology*, 131: 1727-1736.

I hereby declare that this thesis summarizes my independent efforts, conducted under the guidance of Prof. Dan Yakir.