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Studies of plant terpenoid biosynthesis using ^{13}C stable isotope labeling techniques

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vorgelegt von

Andrea Ghirardo

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Dekan:	Prof. Dr. Dr. h.c. Gero Becker
Referent:	Prof. Dr. Jörg-Peter Schnitzler
Koreferent:	Prof. Dr. Heinz Rennenberg
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SUMMARY

Studies of plant terpenoid biosynthesis using ^{13}C stable isotope labeling techniques

Terpenoids (or isoprenoids) are a large group of compounds that possess a wide spectrum of biological properties, which have made them widely used (e.g. as flavors, fragrances, pesticides, antineoplastic agents, antibacterial drugs, food additive, vitamins, etc.). Some volatile terpenoids like isoprene and monoterpenes are extensively studied for their physiological functions in plants, their ecosystem impacts, and their effects on the chemical and physical properties of the atmosphere. Isoprene and monoterpenes are largely produced and emitted by plants from the plastidic 2-C-methylerythritol-4-phosphate (MEP) pathway. Terpenoid biosynthesis is a function of genetic factors, environmental constraints, as well as biotic and abiotic stresses.

This thesis aims to deepen our understanding of plant terpenoid biosynthesis and the regulation of the MEP pathway with respect to isoprene and monoterpene biosynthesis. Stable isotope techniques offer a useful tool for labeling specific compounds such as organic matter and atmospheric gases. In contrast to radioactive isotopes, stable isotopes are harmless to the health. Labeled compounds (enriched in heavier isotopes) can be traced and separated from isotopes occurring at natural abundance. They can therefore be used to study terpenoid biosynthesis in plants, providing information on the carbon (C) sources for terpenoids biosynthesis and C fluxes within the MEP pathway at various spatial and temporal scales, as well as giving insight into the regulation of enzymes from the MEP pathway.

In this work, ^{13}C stable isotope labeling techniques have been used to answer three pertinent plant terpenoid issues, summarized as following:

The first question concerns the monoterpene emission from conifer trees that populate the boreal area. What fraction of total monoterpenes emitted by conifers originates directly from *de novo* synthesis in relation to monoterpenes emitted from storage pools? This is at present an important topic, since boreal forests are the most extensive forests on our planet and the fraction of monoterpenes originating from *de novo* synthesis is ignored in traditional atmospheric models. Because of extensive boreal forest coverage, and the impact of isoprene and monoterpene emissions on air quality and climate dynamics, reliable emission estimates are needed for present and predicted future climate assessments.

By applying $^{13}CO_2$ fumigation and analyzing the isotope label with proton transfer reaction mass spectrometry (PTR-MS) and classical gas chromatography mass spectrometry (GC-MS) the fractions of *de novo* synthesized monoterpenes were determined in the four most common European boreal/alpine trees species: *Pinus sylvestris* (58%), *Picea abies* (33.5%), *Larix decidua* (9.8%) and *Betula pendula* (98-100%). Application of the observed split between *de novo* and storage pool emissions from *P. sylvestris* in a hybrid emission algorithm resulted in a better description of ecosystem scale monoterpene emissions from a boreal Scots pine forest stand.

The second issue concerns the regulation of the plastidic terpenoid biosynthesis and the establishment of a new analytical method. 1-Deoxy-D-xylulose 5-phosphate synthase (DXS) catalyzes the first step of the MEP pathway and it is believed to play an important role in regulating the metabolic flux of this pathway. The enzymatic product, 1-deoxy-D-xylulose 5-phosphate (DXP) originates from an acyloin condensation of hydroxyethylthiamine, derived from

the decarboxylation of pyruvate (PYR), with the C_1-aldehyde group of D-glyceraldehyde 3-phosphate (GAP). Only recombinant expressed DXS proteins have been functionally characterized with the use of radioactive assays and therefore no data are available on DXS activity extracted from plants. At present, the lack of sensitive methods is the main obstacle to perform plant physiological studies and test the importance of DXS on the MEP pathway.

By using ^{13}C-labeling, a new sensitive assay was developed. The assay is based on the decarboxylation of labeled pyruvate (1-^{13}C)-PYR and detection of $^{13}CO_2$ by isotope ratio mass spectrometry (IRMS). This new method is suitable for the measurement of DXS activity directly from plant extracts, i.e. without over-expressing the enzyme in a host organism. It was successfully applied to characterize the apparent kinetic properties of the DXS activity in poplar leaves. Analysis of DXS activity along the plant axis showed that DXS undergoes developmental regulation and positively correlates with the activation of the gene expression of the isoprene synthase. The DXS activity was found to be lower in transgenic, non-isoprene-emitting poplars compared to the wild-type plants, suggesting that DXS may play an important role in the regulation of flux through the MEP pathway.

The third topic concerns the C source for isoprene biosynthesis. Approximately 75% of isoprene is synthesized directly from recently fixed carbon (photosynthetic C), whereas the remaining fraction originates from other 'alternative sources'. Part of this fraction is proposed to originate from the significant amount of carbon that is translocated within the plant. An aim of the present work was therefore to quantify the contribution of this translocated carbon as a C source for isoprene biosynthesis in young Grey poplar trees. Mature leaves were either fumigated with $^{13}CO_2$, or the plant xylem sap was fed with ^{13}C-glucose. Thus, the emission and ^{13}C incorporation into isoprene and respiratory CO_2 from different plant organs (i.e. the apical part of the plant, young leaves, mature leaves and root system) was measured with 'PTR-MS' and 'tunable diode laser absorption spectrometry' (TDLAS). The combination of TDLAS, PTR-MS and ^{13}C-labeling studies allowed non-invasive, high time resolution monitoring of C flux dynamics within poplar saplings, from CO_2 fixation in source leaves to C allocation in other plant parts, whereas the isotopic composition of bulk plant material allowed the final quantification of C allocation.

In poplar saplings, assimilated $^{13}CO_2$ became translocated via the phloem down to the roots within a few hours. ^{13}C-label was stored in the roots and partially reallocated to the plants' apical part the day after labeling, particularly in the absence of photosynthesis. Deprivation of the plants of their root system short-circuited C storage in the roots. The ^{13}C-labeled sugars entering the nutrient solution could immediately re-enter the xylem stream and be transported upward, mainly to the C sink tissue of the apex. The C loss as isoprene originated mainly (76-78%) from recently fixed CO_2, and to a minor extent from photosynthetic intermediates (8-11%) with slower turnover rates and xylem-transported sugars (7.4-10.8%). Overall, 93-99% of the C sources of isoprene formation could be explained.

ZUSAMMENFASSUNG

Studie über die Terpenoid-Biosynthese der Pflanzen unter Verwendung der Markierungsmethode mit stabilen ^{13}C-Isotopen

Terpenoide (oder Isoprenoide) stellen eine große Gruppe von Verbindungen dar, die ein weites Spektrum von biologischen Eigenschaften besitzen und vielfältige Verwendung beispielsweise als Geschmacksstoffe, Duftstoffe, Pestizide, antineoplastische Mittel, antibakterielle Pharmazeutika, Essenszusätze wie Vitamine usw. finden. Des Weiteren sind einige flüchtige Terpenoide wie Isopren und Monoterpene wegen ihrer physiologischen Funktionen in Pflanzen, ihrer Einflüsse auf das Ökosystem und ihrer Effekte auf die chemischen und physikalischen Eigenschaften der Atmosphäre, Gegenstand intensiver wissenschaftlichen Untersuchungen. Isopren und Monoterpene werden von Pflanzen in großen Mengen emittiert und über den plastidären 2-C-Methylerythritol-4-Phosphatweg (MEP) produziert. Genetische Faktoren wie auch biotische und abiotische Umweltfaktoren kontrollieren die Terpenoid-Biosynthese der Pflanze.

Diese vorliegende Dissertation trägt wesentlich zu einem vertieften Verständnis über die Terpenoid-Biosynthese der Pflanzen und die Regulierung des MEP-Stoffwechsels unter besonderer Beachtung der Isopren- und Monoterpenbiosynthese bei, ermöglicht durch moderne und neu etablierte Methoden, insbesondere der Verwendung von stabilen Isotopen. Stabile Isotope stellen ein nützliches Werkzeug dar, um spezifische Substanzen, wie organische Stoffe und atmosphärische Gase, zu markieren. Im Gegensatz zu radioaktiven Isotopen haben stabile Isotope den Vorteil, unschädlich zu sein. Die markierten Verbindungen (d.h. angereichert mit schwereren Isotopen) können zurückverfolgt und isoliert werden. Somit können stabile Isotope genutzt werden, um die Terpenoidbiosynthese in Pflanzen zu studieren, um Informationen zu Kohlenstoff-Quellen und -Flüssen innerhalb des MEP-Stoffwechsels in verschiedenem räumlichen und zeitlichen Zusammenhang zu untersuchen, sowie um Einblicke in die Regulierung der Enzyme des MEP-Stoffwechsels zu erhalten.

Für die vorliegende Doktorarbeit wurde die ^{13}C-Markierungmethode verwendet, um drei die pflanzlichen Terpenoide betreffenden Fragen zu beantworten:

Die erste Frage beschäftigte sich mit der Monoterpenemission von Koniferen, die in der borealen Zone beheimatet sind. Welcher Anteil der Gesamt-Monoterpenemission von Koniferen kann direkt auf die *de novo*-Synthese zurückgeführt werden im Vergleich zur Emission von Monoterpenen aus Speichervorräten? Diese Frage ist von großer Bedeutung: Boreale Wälder bedecken eine sehr große Fläche unseres Planeten, und die Fraktion der Monoterpene, welche *de novo* synthetisiert werden, wird in den traditionellen atmosphärischen Modellen ignoriert. Wegen der Größe der borealen Waldflächen und des Einflusses ihrer Isopren- und Monoterpenemissionen auf die Luftqualität und die Dynamik des Klimas werden verlässliche Schätzungen über diese Emissionen für das Vorhersagen des heutigen und des zukünftigen Klimas benötigt.

Unter Begasung mit $^{13}CO_2$ und der Analyse der Isotopen-Markierung mittels der Protonentransferreaktions-Massenspektrometrie (PTR-MS) und der klassischen Gaschromatographie-Massenspektrometrie (GC-MS) wurden die *de novo* synthetisierten

Monoterpene der vier am häufigsten vorkommenden europäischen borealen/alpinen Baumspezies bestimmt: *Pinus sylvestris* (58%), *Picea abies* (33.5%), *Larix decidua* (9.8%) und *Betula pendula* (98-100%). Die Verwendung der beobachteten Aufteilung zwischen *de novo*-Monoterpenbiosynthese und den Emissionen von Speichervorräten von *P. sylvestris* in einem Hybrid-Emissionsalgorithmus ergab eine bessere Beschreibung der Monoterpenemissionen eines borealen Schwarzkiefernbestandes.

Die zweite Frage befasste sich mit der Regulierung der Terpenoidbiosynthese und der Etablierung einer neuen Methode zu deren Analyse. 1-Deoxy-D-Xylulose 5-Phosphat-Synthase (DXS) katalysiert den ersten Schritt des MEP-Stoffwechsels, und es ist anzunehmen, dass dieser Schritt eine bedeutende Rolle in der Regulierung des Stoffwechselflusses spielt. Das enzymatische Produkt 1-Deoxy-D-Xylulose-5-Phosphat (DOXP) ist zurückzuführen auf eine Acyloin-Kondensation von Hydroxyethylthiamin, welches durch Decarboxylierung von Pyruvat (PYR) entsteht, mit der C_1-Aldehyd-Gruppe des D-Glyceraldehyd-3-Phosphat (GAP). Bis jetzt sind allein rekombinant hergestellte DXS-Proteine funktional charakterisiert worden, wobei deren Aktivität mittels radioaktiver Methoden bestimmt wurde und das Reaktionsprodukt entweder mit Dünnschichtchromatographie (TLC) oder Hochleistungsflüssigkeitschromatographie (HLPC) separiert werden musste.

Die ^{13}C-Markierung wurde dazu verwendet, um eine neue empfindliche Messung, basierend auf der Decarboxylierung von markiertem Pyruvat (1-^{13}C)-PYR und der Detektion von $^{13}CO_2$ durch Isotopenverhältnis-Massenspektrometrie (IRMS), zu entwickeln. Diese neue Methode eignet sich für die direkte Messung der DXS-Aktivität in Pflanzenextrakten, d.h. für die Charakterisierung der biochemischen Eigenschaften muss nicht mehr auf rekombinant hergestellte Enzyme zurückgegriffen werden. Diese Technik wurde erfolgreich bei der Charakterisierung der kinetischen Eigenschaften der DXS in Blättern von Pappeln angewandt. Die Analyse der DXS-Aktivität entlang der Pflanzenachse zeigte eine entwicklungsbedingte Regulierung und eine positive Korrelation mit der Aktivierung der Genexpression der Isoprensynthase. Schließlich wurde herausgefunden, dass die DXS-Aktivität in transgenen, nicht Isopren emittierenden Pappeln geringer ist verglichen mit Wildpflanzen. Daraus lässt sich schließen, dass die DXS eine wichtige Rolle in der Regulierung des C-Flusses im MEP-Stoffwechsel einnimmt.

Der dritte Fragenkomplex betrachtete die C-Quellen für die Isoprenbiosynthese. Ungefähr 75% des Isopren wird direkt aus kurz zuvor gebundenem Kohlenstoff (photosynthetischer Kohlenstoff) synthetisiert, während der restliche Anteil des Kohlenstoffs aus „alternativen“ C-Quellen stammt. Für einen Teil dieser Fraktion nahm man an, dass er von der beträchtlichen Menge an Kohlenstoff, die in der Pflanze verlagert wird, stammt. Das Ziel der vorliegenden Arbeit war es, den Beitrag dieses alternativen Kohlenstoffes als C-Quelle zur Isoprenbiosynthese in jungen Graupappeln zu quantifizieren. Hierfür wurden entweder ausgewachsene Blätter mit $^{13}CO_2$ begast oder der Xylem-Saft mit ^{13}C-Glucose angereichert. So konnte die Emission und die Einbindung von ^{13}C in Isopren mit PTR-MS und in Kohlendioxid (CO_2) aus der Atmung mit Absorptionsspektrometrie mit einem durchstimmbaren Diodenlaser (TDLAS) in den verschiedenen Organen der Pflanze (zum Beispiel im apikalen Teil der Pflanze, in jungen Blättern oder ausgewachsenen Blättern und im Wurzelsystem) gemessen werden. TDLAS und PTR-MS

wurden miteinander kombiniert, um parallel den Gasaustausch verschiedener Pflanzenteile zu erfassen. Die Kombination des TDLAS mit dem PTR-MS und der Untersuchung mit ^{13}C-Markierung ermöglichten es, nicht-invasiv und mit hoher zeitlicher Auflösung die Dynamik des C-Flusses in den Pappelsetzlingen von der CO_2-Bindung in „Quell"-Blättern bis hin zu der C-Anreicherung in anderen Pflanzenteilen zu erfassen. Zusätzlich wurde die Isotopen-Zusammensetzung von verschiedenen Pflanzengeweben mittels Elementaranalyse-(EA)-IRMS analysiert, um die endgültige ^{13}C-Anreicherung zu quantifizieren.

Bei intakten Pflanzen wurde ^{13}C-Markierung über das Phloem innerhalb weniger Stunden zu den Wurzeln verlagert. ^{13}C wurde einen Tag nach der Markierung in den Wurzeln sowie teilweise in apikalen Pflanzenteilen eingelagert, besonders während der Dunkelphase. Der Verlust der Wurzelsysteme führte zu einem Kurzschluss der C-Allokation innerhalb der Pflanze. Die mit ^{13}C markierten Zucker, welche über das Phloem in die Nährlösung gelangten, konnten sofort wieder über das Xylem aufgenommen und daraufhin aufwärts zu den „Senken"-Geweben transportiert werden.

Zusammenfassend ließ sich feststellen, dass der für die Isoprenbiosynthese verwendete Kohlenstoff überwiegend (76-78%) von kurz zuvor gebundenem CO_2 stammte und zu einem geringeren Ausmaß von photosynthetischen Zwischenprodukten (8-11%) sowie von über das Xylem transportierten Zuckern (7.4-10.8%). Insgesamt konnten 93-99% der C-Quellen für die Bildung von Isopren aufgeklärt werden.

CONTENTS

List of abbreviations

ADP	adenosine diphosphate
amu	atomic mass unit
ATP	adenosine triphosphate
BVOC	biogenic volatile organic compound(s)
C	carbon
cps	counts per second
DMADP	dimethylallyl diphosphate
CO_2	carbon dioxide
DOXP (or DXP)	1-deoxy-D-xylulose 5-phosphate
DXR	1-deoxy-D-xylulose 5-phosphate reductoisomerase
DXS	1-deoxy-D-xylulose 5-phosphate synthase
FDP	farnesyl diphosphate
Flash EA-IRMS	flash elemental analyzer-isotope ratio mass spectrometer
g	gram
GAP	glyceraldehyde 3-phosphate
GC	gas chromatography
GDP	geranyl diphosphate
GGDP	geranylgeranyl diphosphate
H	Henry's law constant
HDR	1-hydroxy-2-methylbutenyl 4-diphosphate reductase
HMG-CoA	3-hydroxy-3-methylglutaryl-Coenzyme A
HPLC	high performance liquid chromatography
h	hours
IDI	isopentenyl diphosphate isomerase
IRMS	isotope ratio mass spectrometer
IAEA	International Atomic Energy Agency
Km	Michaelis constant
L	liter
LC-IRMS	liquid chromatography isotope ratio mass spectrometry
LOD	limit of detection
m	milli (10^{-3})
min	minute
MEP	methylerythritol 4-phosphate
M	molarity
MS	mass spectrometry
MW	molecular weight
MVA	mevalonate
m/z	mass-to-charge ratio

μ	micro (10^{-6})
n	nano (10^{-9})
NADPH	nicotinamide adenine dinucleotide phosphate
NMHC	non-methane hydrocarbons
ORVOC	oxygenated reactive VOC
OVOC	oxygenated VOC
ppbv	parts per billion by volume
PPFD	photosynthetic photon flux density
ppmv	parts per million by volume
pptv	parts per trillion by volume
PEP	phosphoenolpyruvate
PYR	pyruvate
PPTtr	phosphoenolpyruvate translocator
PTR-MS	proton transfer reaction mass spectrometry
rTF	relative transmission factor
s	seconds
SD	standard deviation
SE	standard error
T3P	triose 3-phosphate
TDLAS	tunable diode laser absorption spectroscopy
TF	transmission factor
TLC	thin-layer chromatography
TPP	thiamine pyrophosphate
TR	transmission factor
V	volt
VOC	volatile organic compound(s)
v/v	volume per volume
w/v	weight per volume
WT	wild type
y	year

1. INTRODUCTION

1. Introduction

The object of this thesis was the biosynthesis of plant terpenoids, particularly of the volatile isoprene and monoterpenes.

After introducing the importance of terpenoids and biogenic volatile organic compounds in general and presenting the biosynthesis pathways of terpenoids, principles of the used stable isotope techniques and their application for the present thesis are given.

1.1. Volatile terpenoids as main biogenic volatile organic compounds

1.1.1. Definition and importance of terpenoids

Terpenoids (or isoprenoids) are a ubiquitous group of compounds occurring in all living organisms and the largest group of natural compounds existing in nature with more than 40,000 structures (Bohlmann & Keeling, 2008). Terpenoids possess many biological properties, which have made them widely used for traditional and modern purposes as pharmaceuticals, flavors, fragrances, pesticides, antineoplastic drugs, antibacterial agents, food additives (e.g. vitamins, etc.) (Mahmoud & Croteau, 2002; Kintzios, 2006; Bohlmann & Keeling, 2008). Terpenoids, up to now mostly synthesized from fossil oil, become very interesting for biotechnology since the production of terpenoids for industrial purposes can be largely increased by engineered microorganisms (Chang & Keasling, 2006). In plants, terpenoids are essential for metabolism, plant growth and development (Bohlmann & Keeling, 2008). Overall, their physiological roles are related to photosynthesis (e.g. chlorophyll, carotenoids) or the regulatory activities of plant hormones (phytohormones) such as abscisic acid, auxins, cytokinins and gibberellins (Lange & Ghassemian, 2003). Other terpenoids are known or assumed to have functions related to defense against or symbiosis with other organisms (Gershenzon & Dudareva, 2007). These terpenoids include volatile compounds with the function of attractants, repellents, toxins, and antibiotics (Pichersky & Gershenzon, 2002; Gershenzon & Dudareva, 2007).

Terpenoids are derived from the branched C_5 skeleton of isoprene. Head-to-tail condensations of isopentenyl diphosphate (IDP) with its allylic isomer dimethylallyl diphosphate (DMADP) starter unit generate the linear prenyl diphosphate precursors geranyl diphosphate (GDP) (Fig. 1).

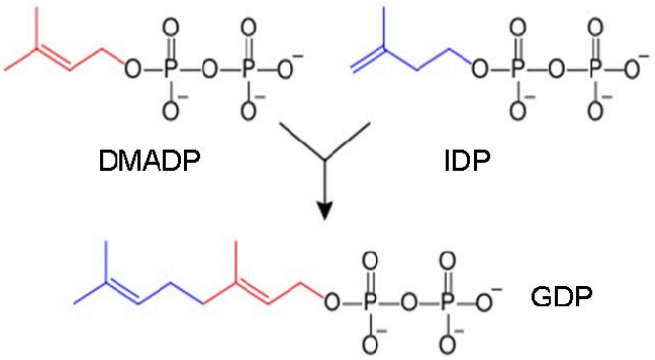

Figure 1. Schematic representation of the terpene skeleton and its formation from head-to-tail C_5 condensation. The C_5 units originating from DMADP and from IDP are indicated in red and in blue, respectively.

The classification of terpenoids is based on the number of isoprene (C_5) units (u.): hemi terpenoids (1 u.), monoterpenoids (2 u.) sesquiterpenoids (3 u.), diterpenoids, (4 u.), sesterterpenoids, (5 u.), triterpenoids (6 u.), tetraterpenoids, (8 u.) and polyterpenoids with a larger number of units (Table 1).

Table 1. Classification of terpenoids based on number of isoprene units. For each isoprenoid class, the isoprenoid precursors, examples and main functions are given.

Num. carbons	Isoprene units (u.)	Isoprenoid precursor	Isoprenoid class	Examples	Functions
C_5	u.	Isopentenyl diphosphate (IDP)	Hemiterpenes	Side chain of cytokinins	Phytohormones
		Dimethylallyl diphosphate (DMADP)		Isoprene	Thermotolerance/ oxidative stress /'safety valve' /interferes with the attraction of bodyguards
C_{10}	2u.	Geranyl diphosphate (GDP)	Monoterpenes	α-pinene Ocimene, Myrcene, Geraniol	Plant defence/ attractance
C_{15}	3u.	Farnesyl diphosphate (FDP)	Sesquiterpenes	Capsidal	Phytoalexin
C_{20}	4u.	Geranylgeranyl diphosphate (GGDP)	Diterpene	Giberellins	Phytohormone
C_{30}	6u.	2 x FDP	Triterpene	Cholesterin	Membrane stabilization
C_{40}	8u.	2 x GGDP	Tetraterpene	Carotenoids Xanthophylls	Accessories photosynthesis pigments Photochemical quenching
$C_{(n)}$	(n)u.	GGDP or FDP	Polyprenol	Prenylated proteins Prenylation of plastochinon	Regulation of cell growth Membrane solubility of photosynthetic apparate

The precursors of terpenoid biosynthesis are synthesized from two independent pathways localized in two separate cell compartments: (i) the cytosolic mevalonate (MVA) pathway and (ii) the plastidic 2-C-methylerythritol 4-phosphate (MEP) pathway.

The cytosolic mevalonate (MVA) or 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) pathway was discovered earlier (Agranoff et al., 1960) and is responsible for the synthesis of ubiquinone (Disch et al., 1998), cytokinins, sesquiterpenes (C_{15}), triterpenes (C_{30}), sterols and brassinosteroids (Suzuki et al., 2004). The MVA pathway is found in animals, fungi and phototrophic organisms (Chappell et al., 1995).

Biosynthesis of isoprene and monoterpenes in plants proceeds via the plastid MEP pathway, discovered in 1993 by Rohmer and co-workers (for review, Lichtenthaler, 1999). This pathway is also present in Eubacteria, green algae and *Plasmodium s*p. (Eoh et al., 2007; Cassera et al., 2004; Grauvogel & Petersen, 2007; Okada & Hase, 2005; Massé et al., 2004) and delivers the metabolic intermediates for the formation of higher isoprenoids such as carotenoids, phytol, tocopherols, phylloquinones (Lichtenthaler, 1999).

1.1.2. Importance of isoprene and monoterpenes as BVOC

1.1.2.1. Definition of BVOC

The biogenic volatile organic compounds (BVOC) embrace a large variety of chemicals, including alkanes, alkenes, alcohols, ketones, aldehydes, ethers, esters and carboxylic acid (Kesselmeier & Staudt, 1999). Some BVOC are volatile terpenoids (isoprene, monoterpenes, sesquiterpenes), other compounds are not (e.g. methanol, ethanol, acetaldehyde, formaldehyde etc.). Methane is the only biogenic compound emitted, which is not included in the BVOC group [BVOC is sometimes referred to as non-methane hydrocarbons (NMHC)]. Although BVOC are emitted by all biota, they are largely produced and emitted by plants. BVOC are important in the global C cycle. With an atmospheric carbon flux of 1200 Tg C yr^{-1}, BVOC represent circa 1% of the total carbon exchanged between biota and the atmosphere (Lal, 1999). The contribution of VOC from anthropogenic sources to the total VOC emission accounts for approximately 150 Tg C yr^{-1}, indicating that on the global scale biogenic emissions are dominant.

The most emitted BVOC are isoprene (2-methyl-1,3-butadiene) and monoterpenes (e.g. α-pinene, β-pinene, camphor, linalool, etc.). Still, there are large uncertainties associated with the global isoprene (250-450 Tg C yr^{-1}) and monoterpene (128-450 Tg C yr^{-1}) emission budgets, which are estimated to contribute approximately 40% and 30% to the total BVOC emission, respectively (Guenther et al., 1993). The flux of isoprene to the atmosphere is roughly similar to that of methane.

Table 2. Sources and amount of the most important biogenic volatile organic compounds (* oxygenated reactive VOC; ** oxygenated VOC).

Sources of the most important biogenic volatile organic compounds (BVOC)			
BVOCs	**Primary natural sources**	**Estimated global emissions y^{-1} [Mt C]**	**Mean atmos. Lifetime [days]**
Isoprene	Plants	250-450	0.2
Monoterpenes	Plants	128-450	0.1-0.2
Dimethylsulfide	Marine phytoplankton	15-30	<0.9
Ethene	Plants, Soils, Oceans	8-25	1.9
ORVOC* (e.g. aldehydes, MBO)	Plants	Ca. 260	<1
OVOC** (e.g. MeOH, EtOH, acetone, acids)	Plants, Soils	Ca. 260	>1

1.1.2.2. Roles in the atmosphere / physiological functions

BVOC are reactive molecules and play an important role in air quality and climate dynamics in the lower troposphere. In the presence of nitrogen oxides (NO_x), BVOC contribute to the formation of tropospheric ozone (Thompson, 1992). The dynamics of ozone, hydroxyl radical, carbon monoxide, methane formation/breakdown and secondary aerosol formation cannot be explained without taking into account the plant-produced reactive compounds (Trainer et al., 1987; Chameides et al., 1988; Jacob & Wofsy 1988; Novakov & Penner 1993; Biesenthal et al., 1997; Tunved et al., 2006; Kiendler-Scharr et al. 2009).

Besides their contribution to air chemistry, BVOC play a dominant role in the physiology and ecology of plants, as they are used for communication in plant-plant and plant-organism interactions (Kegge & Pierik, 2009; Dicke & Baldwin, 2010).

The physiological function(s) of isoprene is/are still a matter of debate. Different studies showed that isoprene can protect plant against transient heat (Sharkey & Singsaas, 1995; Behnke et al., 2007; 2010) and oxidative stress (Loreto et al., 2001; Loreto & Velikova, 2001). Isoprene functions as a metabolic 'safety valve' (Rosenstiel et al., 2004), can act as negative cue for herbivores (Laothawornkitkul et al., 2009) and interferes with the attraction of plant-protecting predators (Loivamäki et al., 2008). Although the physiological function of isoprene is still under debate, plants invest a significant amount of fixed C into isoprene, e.g. approximately 2% at 30°C relative to net CO_2 assimilation, and a dramatically increased amount under stress conditions when, for example, photosynthesis is reduced and isoprene is emitted at its highest rate (in poplar trees, at circa 36°C, the relative loss of carbon by emitting isoprene reaches 30% of the net CO_2

fixation rate) (Sharkey & Yeh, 2001). Moreover, isoprene emission costs the plants significant amounts of ATP and reducing (NADPH) equivalents (Niinemets et al., 2002).

Monoterpenes (C_{10}) can be acyclic (such as ocimene, myrcene, linalool) or contain rings (such as limonene, menthol, carene, sabinene, pinene, thujene) (Fig. 2). Monoterpenes are known as defense molecules against insects, bacteria, fungi, and herbivores. When these compounds are volatized, they can attract pollinators and act as signal for herbivores (Dicke & Baldwin, 2010). Also, the synthesis can be induced by herbivores and pathogenic attack (Dicke & Baldwin, 2010).

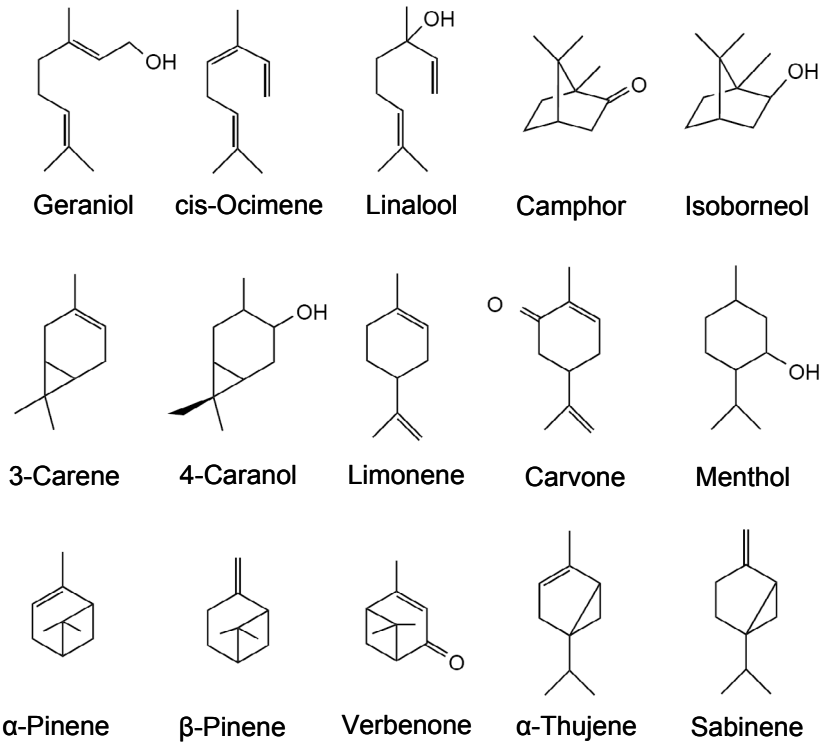

Figure 2. Diversity of monoterpenes (C_{10}) structures. Typical examples of acyclic or cyclic and oxygenated or non oxygenated forms.

1.1.2.3. Regulation and driving forces of BVOC emission

BVOC emissions from plants are controlled by different factors: (i) plant species (Kesselmeier & Staudt, 1999), environmental conditions such as (ii) temperature, (iii) light (Kesselmeier & Staudt, 1999), (iv) drought (Brilli et al., 2007), (v) ambient ozone (Loreto & Velikova, 2001), and (vi) CO_2 concentration (Rosenstiel et al., 2003). Plant BVOC emission are also largely induced by both abiotic and biotic stresses (for review see Loreto & Schnitzler, 2010; Niinemets, 2009; Holopainen & Gershenzon, 2010; Dicke & Baldwin, 2010). Low atmospheric CO_2 concentrations are associated with high isoprene emission rates (Sanadze, 1964), whereas the high atmospheric CO_2 concentrations expected in future suppress isoprene emission (Sharkey et al., 1991; Rosenstiel et al., 2003; Centritto et al., 2004; Pegoraro et al., 2004; Wilkinson et al., 2009). Drought reduces isoprene and monoterpene emissions only when the stress is severe and almost

completely inhibits photosynthesis (Sharkey & Loreto, 1993; Brüggemann & Schnitzler, 2002b; Fortunati et al., 2008). Temperature strongly influences the activity of the enzymes, which catalyze the synthesis of many BVOC (Monson et al., 1992), and increasing light directly influences the isoprene and monoterpene emission by enhancing photosynthesis (Sharkey et al., 2001). Plants produce a diversity of BVOC (e.g. monoterpenes and sesquiterpenes) in response to biotic stress, as for example, attack or egg deposition by herbivores for direct defense or to attract herbivore enemies (De Moraes et al., 2001; Kessler & Baldwin, 2001; Mumm et al., 2006; Fatouros et al., 2008; Dicke et al., 2009; Unsicker et al., 2009).

Many plants, especially conifers, mints and citrus, accumulate monoterpenes in specialized storage tissues, such as ducts, glands, and cavities, and the synthesis is located in cells lining these specialized structures (Schürmann et al., 1993). Conversely, some dicotyledon trees such as *Quercus ilex, Betula pendula* etc. do not possess such structures, and monoterpenes are immediately emitted after having been synthesized in the mesophyll cells (Loreto and Schnitzler, 2010). Isoprene is always emitted after synthesis due to its high volatility and absence of specialized storages tissues (Niinemets et al., 2004).

Isoprene and monoterpene emissions from many dicotyledon trees originate from recently fixed photosynthetic intermediates by *de novo* biosynthesis in a light- and temperature-dependent manner (Tingey et al., 1980; Loreto et al., 1996, 2000a) when photosynthesis is not impaired by environmental stresses (Kreuzwieser et al., 2002; Fortunati et al., 2008; Teuber et al., 2008). Monoterpene emissions from the reservoirs are only due to evaporation processes which are specific for each monoterpene (due to different volatility), thus, they are only temperature-dependent (Schürmann et al., 1993). Traditionally, monoterpenes emitted by coniferous trees have been assumed to originate only from these evaporation processes (Guenther et al., 1991; Grote & Niinemets, 2008). As a result, monoterpene emissions from coniferous trees are commonly calculated by temperature-dependent algorithms (Tingey et al., 1980; Guenther et al., 1991; Guenther et al., 1993). Even though specific storage structures are absent in most dicotyledon tree species, the temperature-dependent algorithm is often used also to describe the monoterpene emissions from these species as well (e.g. Tarvainen et al., 2007).

1.1.3. The MEP pathway: source of isoprene and monoterpenes; regulatory steps

In the plastidic MEP pathway, *D*-glyceraldehyde 3-phospate (GAP) and pyruvate are both precursors for DMADP (and its isomer IDP). These precursors are central metabolites involved in several pathways (glycolysis, gluconeogenesis, the tricarboxylic acid cycle and the pentose phosphate pathway) and thus subjected to many layers of regulation.

The MEP pathway starts with the formation of 1-deoxy-*D*-xylulose 5-phosphate (DXP) by an acyloin condensation of hydroxyethylthiamin derived from the decarboxylation of pyruvate (PYR) with the C_1-aldehyde group of GAP (Arigoni et al., 1997; Rohmer et al., 1993) (Fig.3). This first enzymatic step is catalyzed by 1-deoxy-*D*-xylulose 5-phosphate synthase (DXS, EC number

2.2.1.7), an enzyme that is assumed to be important in regulating the metabolic flux within the pathway (Estévez et al., 2001; Lois et al., 2000; Walter et al., 2000).

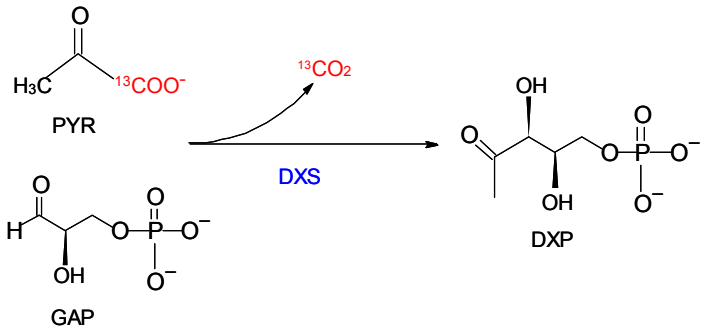

Figure 3. Schematic representation of the reaction catalyzed by 1-deoxy-*D*-xylulose 5-phosphate synthase (DXS, EC number 2.2.1.7): pyruvate (PYR) is decarboxylated and reacts with *D*-glyceraldehyde 3-phospate (GAP) to 1-deoxy-*D*-xylulose 5-phosphate (DXP).

DXS is a recently discovered thiamin-dependent transketolase-like enzyme. DXS was genetically characterized in *E. coli* (Lois et al., 1998; Sprenger et al., 1997) and several plant species (Arigoni et al., 1997; Lange et al., 1998). The DXS product 1-deoxy-*D*-xylulose 5-phosphate (DXP or DOXP) is converted by the enzyme 1-deoxy-*D*-xylulose 5-phosphate reductoisomerase (DXR) to form MEP (Fig. 4). DOXP is not only the first intermediate of the MEP pathway since it is also involved in the thiamine/shikimate pathway (Julliard and Douce, 1991; Belanger et al., 1995). Thus, it was supposed that DXR may play the regulatory role in the MEP pathway rather than DXS (Mayrhofer et al., 2005). Supporting this idea, Carretero-Paulet et al. (2002; 2006) showed that over-expression of the *DXR* gene in *Arabidopsis thaliana* increased the concentration of isoprenoid end products. By contrast, in poplar the *DXR* gene expression did not correlate with isoprene emission (Mayrhofer et al., 2005), and over-expression of *DXS* enhanced the production of essential oil in transgenic Spike Lavender (Munoz-Bertomeu et al., 2006). The regulatory roles of DXS and DXR in the MEP pathway therefore await further studies. The complete sequence of genes involved in the following five steps of the MEP pathway until the synthesis of the direct isoprenoid precursors IDP and DMADP were identified by Bacher and co-worker (for review see Eisenreich et al., 2001) (Fig. 4). The last step in the MEP pathway is catalyzed by the enzyme 1-hydroxy-2-methylbutenyl 4-diphosphate reductase (HDR) which converts 1-hydroxy-2-methyl-2-*(*E)-butenyl 4-diphosphate to DMADP, the immediate precursor of isoprene and of its isomeric form IDP, the precursor of higher isoprenoids. IDP can also be isomerized by isopentenyl diphosphate isomerase (IDI) to DMADP from which isoprene synthase (ISPS) synthesizes isoprene. In order to synthesize higher isoprenoids the two diphosphorylated C_5 units, DMADP and IDP, condense in a head-to-tail reaction to produce geranyl diphosphate (GDP; C_{10}). The

reaction can be repeated to further produce farnesyl diphosphate (FDP, C_{15}) or geranylgeranyl diphosphate (GGDP; C_{20}) (Ramos-Valdivia, 1998).

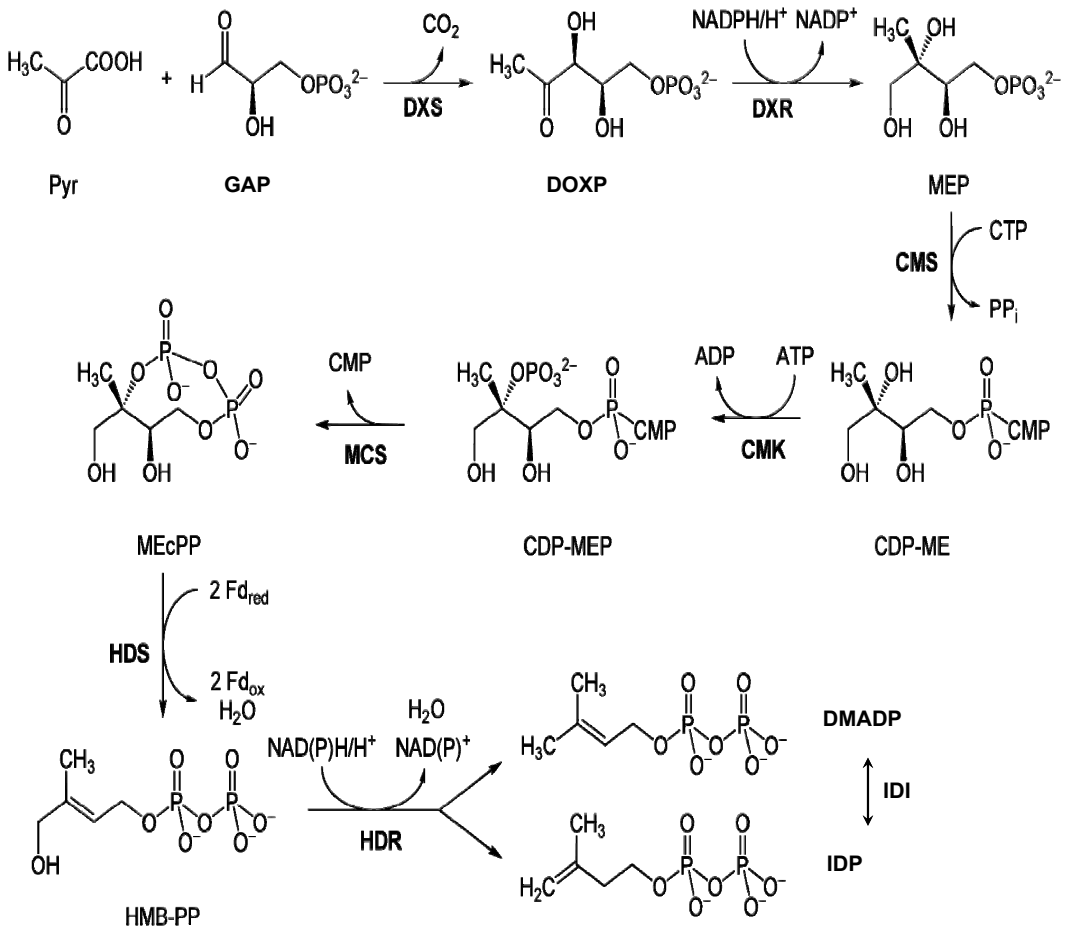

Figure 4. Scheme of the plastidic MEP pathway from the two precursor molecules pyruvate (**Pyr**) and D-glyceraldehyde 3-phosphate (**GAP**) to the formation of isopentenyl diphosphate (**IDP**) and dimethylallyl diphosphate (**DMADP**). [Acronyms: **DOXP** = 1-deoxy-*D*-xylulose 5-phosphate; **MEP** = 2-C-methyl-*D*-erythritol 4-phosphate; **CDP-ME** = 4-diphosphocytidyl-2C-methyl-*D*-erythritol; **CDP-MEP** = 4-diphosphocytidyl-2C-methyl-*D*-erythritol 2-phophate; **MEcPP** = 2C-methyl-*D*-erythritol 2,4-cyclodiphosphate; **HMB-PP** = 1-hydroxy-2-methyl-2-*(E)*-butenyl 4-diphosphate; **DXS** = DOXP synthase (EC 2.2.1.7); **DXR** = DOXP reductois**o**merase (EC 1.1.1.267); **CMS** = MEP cytidylyltransferase (EC 2.7.7.60); **CMK** = CPD-ME kinase (EC 2.7.1.148); **MCS** = MEcPP synthase (EC 4.6.1.12); **HDS** = HMB-PP synthase (EC 1.17.4.3); **HDR** = HMB-PP reductase (EC 1.17.1.2); **IDI** = DMADP/IDP isomerase (EC 5.3.3.2)].

In chloroplasts, pyruvate can be imported from the cytosol as phosphoenolpyruvate (PEP) and GAP as a triose 3-phosphate (T3P) (Fig. 5). Although DMADP is required in plastids, mitochondria and cytosol, only the plastidic DMADP is used for the biosynthesis of isoprene, while

cytosolic DMADP is used, for instance, to synthesize vitamins, sesquiterpenes and other compounds (Fig. 5).

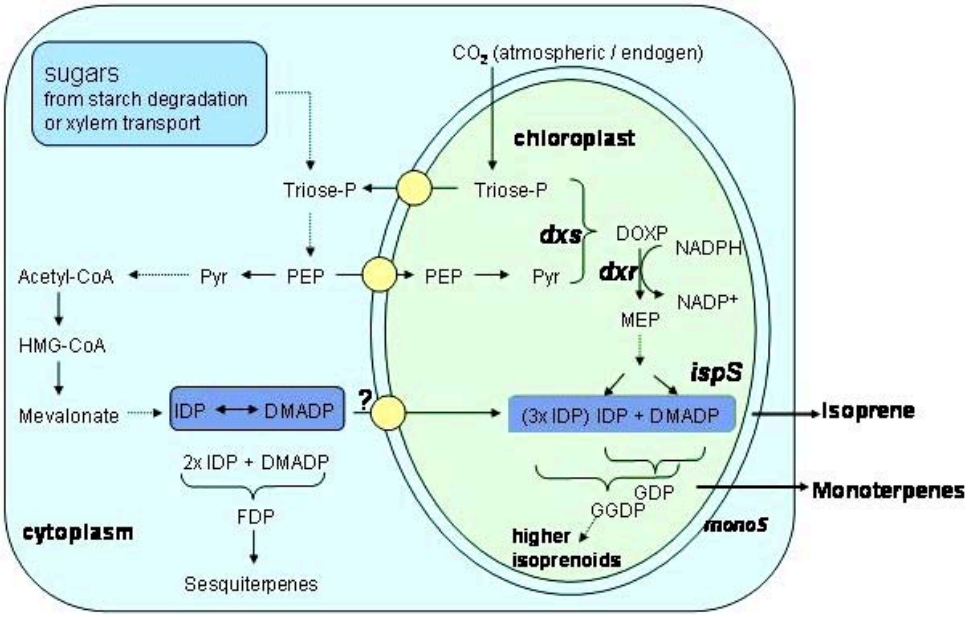

Figure 5. Schematic representation of main regulatory steps of the chloroplastic MEP pathway; the main C sources for terpenoid biosynthesis (atmospheric CO_2 and sugars); cross-talk between MEP and MVA pathways (the question mark indicates a hypothetical transfer of cytosolic DMADP to the chloroplast; Lichtenthaler, 1999).

The close relation between isoprene biosynthesis and photosynthetic activity was demonstrated by $^{13}CO_2$ labeling (Sanadze, 1991): ^{13}C was almost instantaneously (within a few minutes) incorporated into isoprene. However, isoprene was not fully ^{13}C-labeled (circa 80% in poplar) and this proportion of labeling decreases under stress conditions suggesting that 'alternative' C sources are used for isoprene biosynthesis. These C sources were proposed first by Karl et al. (2002a, 2002b) and Affek and Yakir (2003) as i) chloroplastic degradation of starch occurring simultaneously to its biosynthesis, ii) refixation of respiratory C, iii) influx of cytosolic precursors (pyruvate/PEP) into the chloroplast. It was demonstrated later by Kreuzwieser et al. (2002) in *Quercus robur* and Schnitzler et al. (2004b) in Grey poplar that xylem-transported sugars are additional C sources for isoprene formation. Brilli et al. (2007) showed in *Populus alba* a larger contribution of these alternative C sources to isoprene emission during dramatically limited photosynthesis activity caused by drought.

Volatile isoprenoids can be an important part of the carbon metabolism of a plant, and their biosynthesis is subject to various regulations not always understood. To further increase our knowledge on the C sources and the regulations of terpenoid biosynthesis, ^{13}C stable isotope labeling techniques were employed.

1.2. Stable isotopes: principles and instruments

1.2.1. ^{13}C stable isotope labeling techniques as a tool to study terpene biosynthesis

^{13}C stable isotope analysis is widely used to study physiological, ecological and biogeochemical processes related to ecosystems (Dawson et al., 2002).

The concept is based on the differences in stable C isotope composition in organic compounds well defined by physical and biological processes. The carbon in atmospheric CO_2 mainly consists of 98.892% ^{12}C and 1.108% ^{13}C (Dawson et al., 2002). Plants contain less ^{13}C relative to ^{12}C than atmospheric CO_2 in their tissues, i.e. plants are isotopically lighter (or depleted in ^{13}C) than atmospheric CO_2. This is due to the faster diffusion of $^{12}CO_2$ into the leaves and the faster reaction rate of Rubisco with ^{12}C during photosynthesis (Farquhar et al., 1982; Flanagan & Ehleringer, 1998). This effect on isotopes differs in various ecosystem processes and it is referred to as fractionation (Farquhar, 1989). Fractionation occurs because physical and chemical processes influence the partitioning of the isotopes in a particular way due to the difference of isotopes masses.

Isotopic signatures for ^{13}C are commonly noted with delta ($\delta^{13}C$) notation, which is defined by the equation:

$$\delta = \left(\frac{R_{sample}}{R_{VPDB}} - 1 \right) * 1000, \text{ ‰}$$

where R is the molar ratio of $^{13}C/^{12}C$ and VPDB is the Vienna Pee Dee Belemnite international standard (based on carbonate from the Pee Dee formation). Delta values are commonly given in "per mil" (‰) due to the small fractional differences in natural abundance (Dawson et al., 2002). Negative values stand for depletion versus the standard and positive values for enrichment in the heavy isotope. Isotopic discrimination against ^{13}C occurs during diffusion of CO_2 into leaves (4.4 ‰, Guy et al., 1993), CO_2 fixation by Rubisco (29 ‰; Guy et al., 1993), and during isoprene biosynthesis (3 ‰ with respect to photoassimilates, Sharkey et al., 1991). Further discriminations occur during biosynthesis of compounds like lipids (Melzer & Schmidt, 1987).

At natural abundance level stable isotopes are used mainly as natural tracers and integrators, allowing ecologists to evaluate the net result of processes, without disrupting the natural flow of the elements (Högberg, 1997). The isotopic composition of carbon can provide information at different temporal and spatial scales (Miller et al., 2003). For example, ecosystem photosynthesis and respiration can be separated, because these two processes have contrasting effects on the isotopic ratio of ^{13}C (Flanagan & Ehleringer, 1998). Thus, isotopic measurements can reveal climate change by variation of temperature, soil moisture and all other factors that influence photosynthesis and respiration (Flanagan et al., 1997).

Studying the isotopic composition of atmospheric trace gases is a useful tool to trace sinks and sources of these gases and underlying processes (Griffiths, 1998), and using plants as biomarkers for large-scale studies of photosynthesis has a great potential (Conte & Weber, 2002). The same potential may be associated with the isotopic composition of isoprene or other BVOC (Affek & Yakir, 2003).

In contrast to analysis at natural abundance level, isotope labeling methods involve applying specific amounts of an isotopically enriched substance (up to 99 atom% of the heavy isotope, e.g. ^{13}C) to plants, soil, animals etc. Thus, the flow and the fate of a specific (labeled) compound (e.g. gas, $^{13}CO_2$, sugars, ^{13}C-glucose) can be traced without altering its natural behavior (Schimel, 1993) and the addition of a labeled compound is a powerful tool for determining rates of biological processes within the system (Lajtha & Michener, 1994). For studies with enriched samples, the labeled substances usually have an isotopic composition that is largely greater in abundance of heavier isotopes than the substance occurring at natural abundance. It is common to use the expression 'atom%' (A_b) which is defined as:

$$A_b = \left(\frac{X_{heavy}}{X_{heavy} + X_{light}} \right) = 100 * \left(\frac{R_{sample}}{R_{sample} + 1} \right), \%$$

where $X_{heavy\ and}$ X_{light} are the number of heavy and light atoms present in the samples and $R_{samples}$ is the isotope ratio of the sample as for natural abundance (see above). Thus, a substance that exceeds 1 'atom %' compared to another substance, has a δ value of greater than 1000‰.

Labeling experiments represent a powerful tool since current technology allows labeling C with 99% of ^{13}C and (depending on the technique used) detecting variations in ^{13}C smaller than 0.0001 atom% ^{13}C. Thus, enriched compounds can be traced even after manifold dilutions with the non-enriched species. This represents a useful tool, for example, to study plant physiological processes. For instance, feeding plants with $^{13}CO_2$ allows following the fixation of ^{13}C by photosynthesis into its incorporation in biosynthesized plant compounds (e.g. Delwiche & Sharkey, 1993; Loreto et al., 1996; Shao et al., 2001). Labeling experiments with ^{13}C can also be used for studying the enzymatic properties of an enzyme (Everley et al., 2007). These approaches were followed in this thesis using different adapted instruments presented in the following sections.

1.2.2. Proton Transfer Reaction Mass Spectrometry (PTR-MS)

A proton transfer reaction mass spectrometer (PTR-MS) uses H_3O^+ ions to ionize volatile organic compounds present in the gas sample to be analyzed. The technique allows simultaneous online monitoring and has a detection limit of a few parts per trillion (pptv) (Hansel et al., 1995; Lindinger et al., 1998). The instrument is made of three main components: an ion source, a drift tube, and a mass analyzer (quadrupole mass spectrometer) in conjunction with an ion detector/amplifier (Fig.

6). The PTR-MS produces H_3O^+ ions at high concentration from pure water vapor by a hollow cathode discharge ion source. These ions are called primary ions and they pass into the drift tube where they mostly undergo non-dissociative proton transfer to the VOC analyzed. The air samples to be analyzed enter the PTR-MS from the inlet into the drift tube (Fig.6). Finally, the ions enter the quadrupole (QMS) where they are deflected according to their masses, and the ions are detected successively in the secondary electron multiplier (SEM). The instruments' response time is about 100 ms.

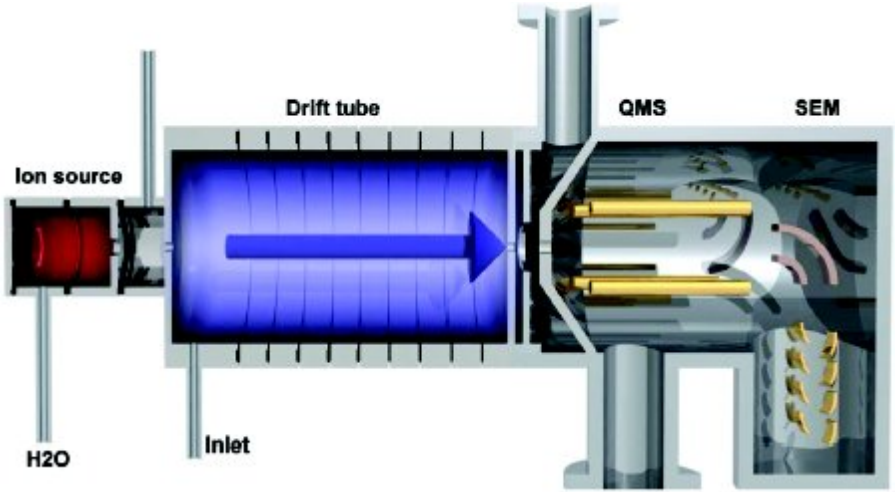

Figure 6. Schematic overview of PTR-MS main components (source: Ionicon Analytik Gesellschaft m.b.H., Innsbruck, Austria). The ion source produces the H_3O^+ ions; in the drift tube the protons are transferred from the H_3O^+ to the sample (e.g. isoprene); the quadrupole (QMS) separates the ions, which are detected with the secondary electron multiplier (SEM).

The big advantage of a PTR-MS for stable isotopic studies is that it allows measuring online isotopologues such as isoprene containing one or more ^{13}C atoms (Schnitzler et al., 2004b; Brilli et al., 2007). The mass range of a sensitive PTR-MS is 1-512 atomic mass units (amu) with a resolution of < 1 amu. Thus, compounds containing heavier isotopes can be distinguished from the lighter ones. For instance, a protonated isoprene molecule ($C_5H_9^+$) containing only five ^{12}C atoms has a molecular mass of 69 amu. The enrichment of isoprene with ^{13}C leads to an incorporation of 1 to 5 ^{13}C atoms, with molecular masses ranging from 70 to 74 amu for the protonated forms, respectively. Another advantage of PTR-MS is that the VOC samples do not need to be prepared before the measurement, thus, in plant cuvette measurements the air samples can be directly introduced into the inlet of the PTR-MS. Because the proton affinity (PA) of H_3O^+ (7.2 eV, 166.5 kcal mol^{-1}) is between the proton affinities of the major components present in air (i.e. N_2, O_2 , Ar, CO_2, CH_4, N_2O, CO, He, Ne; 1.8 eV < PA < 6.2 eV, or 42 kcal/mol < PA <142 kcal mol^{-1}) and the BVOC (PA > 7.22 eV; PA > approx. 168 kcal mol^{-1}), most of the relevant BVOC will undergo a proton transfer reaction with H_3O^+ ions. Also, the low PA difference results in an only slightly exergonic reaction, with the advantage that in most cases the BVOC remain undissociated.

1.2.3. Measurements of $^{13}C/^{12}C$ using LC-IRMS, EA-IRMS and GC-IRMS

The isotopic composition of elements in a sample can be determined by using isotope ratio mass spectrometry (IRMS) (Dawson et al., 2002). This requires a very sensitive instrument, due to the fact that the differences in isotope abundances of an element to be detected are usually very small. The IRMS uses a homogeneous magnetic field to deflect a beam of ionized particles according to their molecular masses towards a series of Faraday cups which convert particle impacts to electric current.

The Liquid Chromatography (LC)-IRMS system is an online coupling of a high performance liquid chromatography (HPLC) system and an isotope ratio mass spectrometer (Krummen et al., 2004; Godin, Fay & Hopfgartner, 2007). The instrument allows accurately determining the $^{13}C/^{12}C$ isotope ratios of all organic compounds eluted from an HPLC column, while maintaining the chromatographic resolution. The sample detection limit is in the low nanomole range.

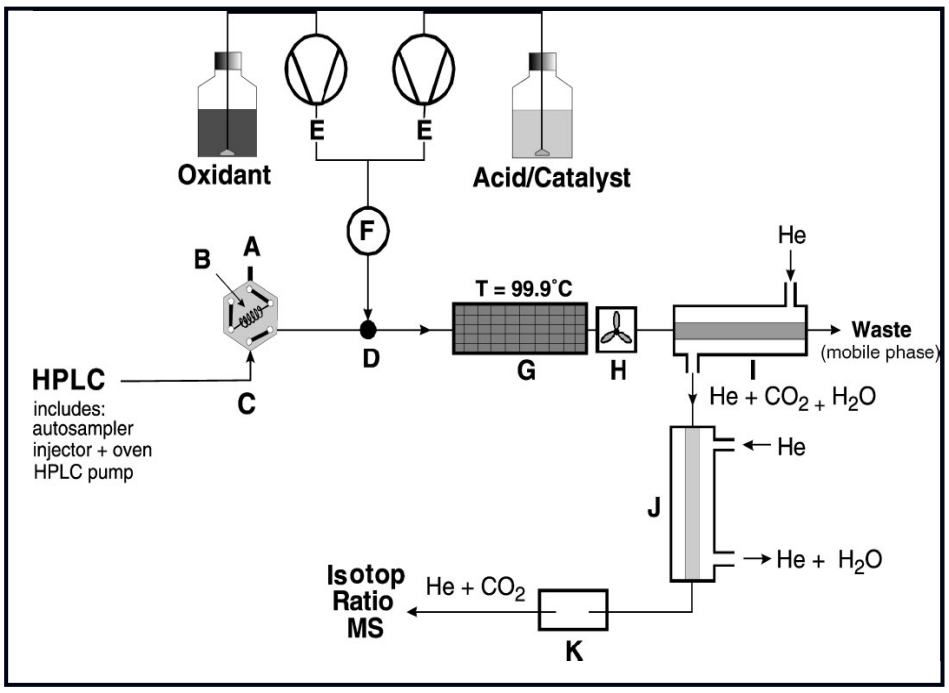

Figure 7. Scheme of a LC-IRMS system. A: Needle port; B: sample loop; C: 6-port valve; D: T-piece; E: two-head-pump; F: pulse damper; G: oxidation reactor; H: cooler; I: CO_2 separation unit; J: gas dryer; K: open split (Krummen et al., 2004).

In the reactor chamber all organic samples eluted from HPLC become oxidized to CO_2 while the samples are still in the aqueous solution. The column eluent is mixed with acid and oxidant (Fig. 7 D) and completely oxidized when passing through the heated reactor (T = 99.9 °C) (Fig.7 G). The reaction mixture is cooled (H) and then the CO_2 is removed from the aqueous solution in a downstream degassing unit (Fig. 7 I) due to the acidity of the solution. It enters a He stream and passes a drying unit (Nafion) (Fig. 7 J), before it reaches the inlet of the isotope mass

spectrometer via an open split interface for the quantification of amount and $^{13}C/^{12}C$ ratios of CO_2. The liquid phase containing the solute and the oxidant/acid leaves the instrument into a waste container. The whole process is quantitative and fractionation does not occur. Assessment of the authenticity of products and the determination of their origin is a typical example of LC-IRMS application (Krummen et al., 2004). For instance, the adulteration of honey by addition of sugar can be assessed by its isotopic signature (Krummen et al., 2004).

An Elemental Analyzer (EA) coupled to an IRMS analyzes the elemental (e.g. C, N) and the isotopic composition of a solid sample (Holt & Hughes, 1955; Brenna et al., 1997). The results of this analysis are both qualitative and quantitative. The instrument is based on quantitative high temperature oxidation of bulk material at high temperatures (approximately 1000 °C) to CO_2 and N_2. Nitrogen oxides which may be formed during combustion are reduced by Cu to N_2. After removal of H_2O, the CO_2 and N_2 are separated isothermally on a GC column prior to transfer to the ion source of the IRMS (Fig.8). Thus, the $^{13}C/^{12}C$ and $^{15}N/^{14}N$ isotope ratios can be measured sequentially.

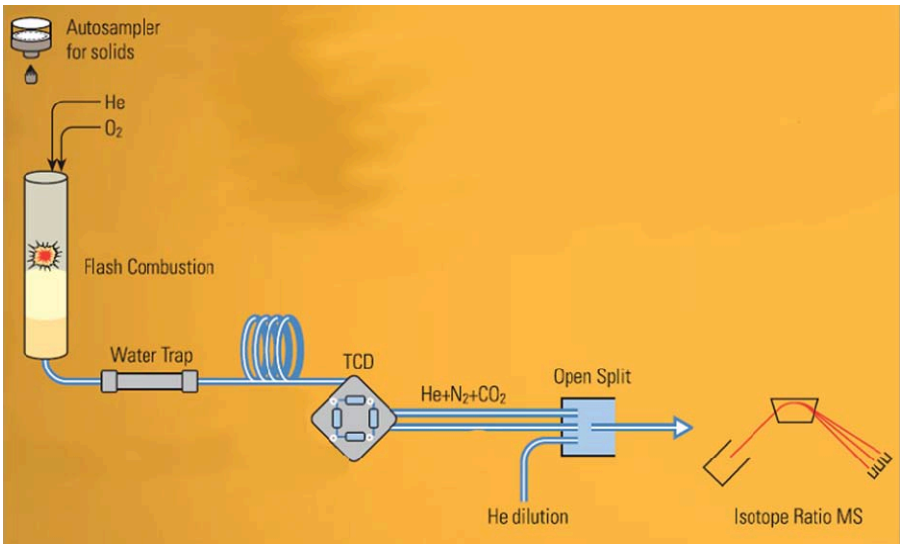

Figure 8. The Elemental Analyzer consists of an autosampler, where bulk organic material is loaded. The organic sample is rapidly combusted at high temperature (Flash combustion) in presence of the oxidant O_2 to CO_2 and nitrogen oxides, which are reduced to N_2 by copper in the following reduction oven. Then, the sample is moved by the He carrier to the water removal unit and the CO_2 and N_2 are separated isothermally on a GC column. The samples are transferred via an open split into the IRMS for the determination of the isotope ratios of the elements. (Source: http://www.thermoscientific.com).

The gas chromatography (GC) system, coupled to an IRMS, is used for the isotopic characterization of gaseous species from air samples. The samples can be as small as 200 nmol to 20 µmol. The GC system is composed of an autosampler (Fig. 9, n.1), where the air sample is placed into glass vials. A two-hole needle of the gas sampling system (Fig. 9, n.2) transfers the sample, which passes through a maintenance-free water removal system (Fig. 9, n.3) for the complete removal of H_2O, via a He carrier stream to the injection system. A loop injector (Fig. 9, n.4) injects aliquots of the sample gas into the isothermal gas chromatography column (Fig. 9, n.5) where the different gases (e.g. CO_2, N_2O) are separated. The samples enter the IRMS via an open split interface (Fig. 9, n.6).

Recently, GC-IRMS and EA-IRMS were used to link the ecosystem respiration to C assimilation in a deciduous forest (Knohl et al., 2005). Also, Ruehr and co-workers (2009) investigated the effect of environment changes on recently assimilated C to the soil from *Fagus sylvatica* and demonstrated that drought significantly affects (reduces) the soil CO_2 efflux. Gessler and co-workers (2009) provided the physiological explanation of the short-term variation of $\delta^{13}C$, showing the metabolic origin of CO_2 respired from leaves, roots and stems of *Ricinus communis.*

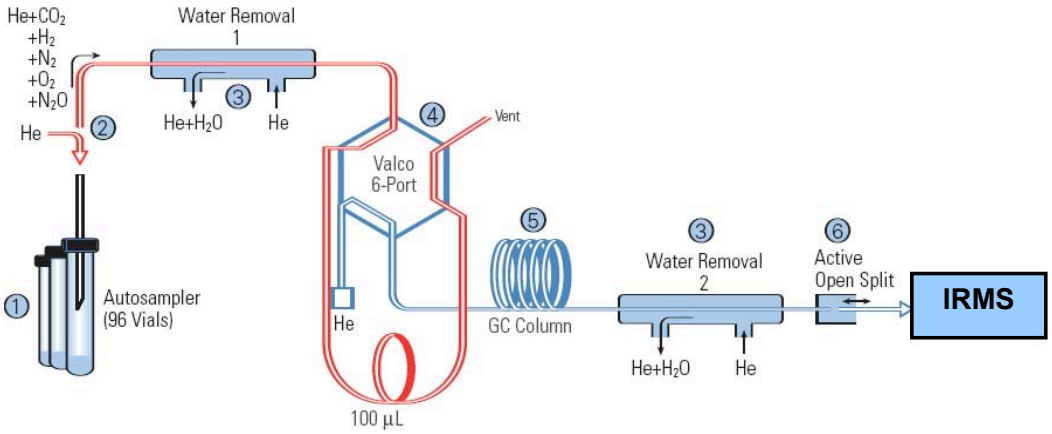

Figure 9. The GC system consists of an autosampler (1), a gas sampling system (2), a maintenance-free water removal system (3), a loop injection system (4), an isothermal gas chromatograph (5), an open split interface (6). (Source: http://www.thermoscientific.com).

1.2.4. Tunable Diode Laser Absorption Spectroscopy (TDLAS)

Tunable diode laser absorption spectroscopy (TDLAS) is a technique that allows measuring the concentration of certain gases such as CO_2 and water vapor (Cooper & Martinelli, 1992; Bomse, 1995; Bahn et al., 2009). The technique is based on light absorption of molecules in the infrared. The absorbed wavelengths are characteristic of each molecule. The amount of the light absorbed depends on two factors: i) the number of molecules in the light beam, and ii) the characteristics of the molecule.

The TDLAS is able to detect very low gas concentrations in the samples (in the lower ppbv range), with high speed and selectivity. Using TDLAS, the concentration and the isotopic composition of CO_2 can be quantified. Measurement of changes in the light intensity, as it passes through the absorption cell, and the use of calibration and reference gases, enables the determination of the concentration of the sample gas. The TDLAS uses a diode laser as light source for the absorption measurements. The emission wavelength of the laser is tuned over characteristic absorption lines of the gas to be measured, e.g. CO_2, in the path of the laser beam. This causes a reduction of the measured laser signal intensity, which can be detected by a photodiode, and then used to determine the gas concentration (Schiff, 1996) (Fig. 10).

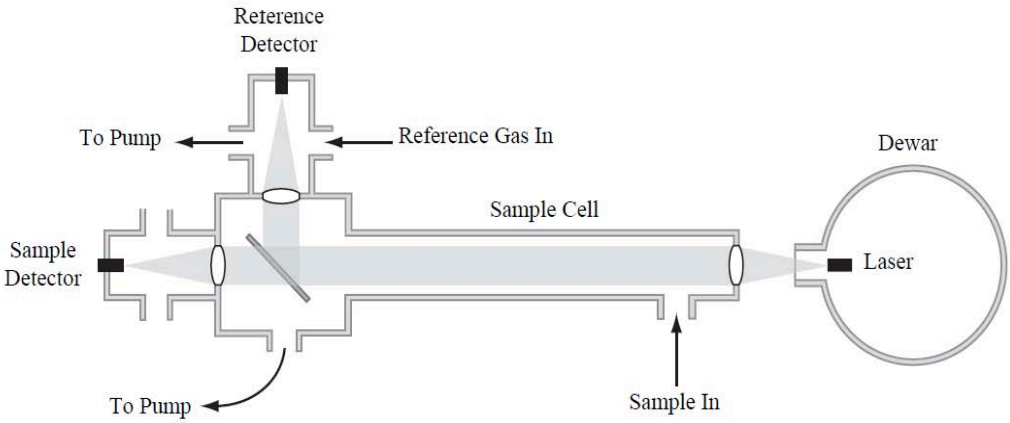

Figure 10. Scheme of the main components of a TDLAS instrument (source http://www.campbellsci.com). The instrument consists of a tunable diode laser light source, transmitting optics, optically accessible absorbing medium, receiving optics and detector/s.

TDLAS has been used largely in the measurement of trace gases such as N_2O, CO_2, CF_4, C_2F_6, CO (e.g., Schiff, 1996). Its ability to discriminate isotopically different CO_2 makes the TDLAS a powerful tool in ecology and plant ecosystem studies. For instance, Bahn and co-workers (2009) demonstrated the tight coupling of the plant-soil system and the importance of plant photosynthesis for soil CO_2 efflux in an alpine grassland ecosystem.

2. AIMS OF THE THESIS

This thesis aims at widening our understanding of plant terpenoid biosynthesis and regulation using ^{13}C-labeling techniques. The specific aims of the thesis are:

- to determine the fraction of monoterpene emissions originating from *de novo* biosynthesis and to separate this from the total emissions originating from the storage pool of four common boreal/alpine forest tree species (*Pinus sylvestris, Picea abies*, *Larix decidua* and *Betula pendula*);

- to develop a new, highly sensitive, biochemical assay for measuring *in vitro* DXS activity from plant extracts in order to characterize the apparent kinetic properties of DXS in Grey poplar leaves; to assay DXS activity in developing poplar leaves and to compare DXS activity in transgenic, non-isoprene emitting and wild-type poplar plants;

- to study the source-to-sink C translocation in young Grey poplar trees and to clarify the contribution of this transported C as a C source for isoprene formation.

This work includes a cumulative dissertation of previously published articles or data submitted for publication, which are enclosed in this doctoral thesis. The following pages give an overview of the scientific issues, the applied methods and conducted experiments, and finally the conclusion and outlook for future research.

3. MATERIALS AND METHODS

3.1. Plant material and growth conditions

Plants used during the present work are grouped as dicotyledons (broad leaf plants) and conifers (needle plants):

Dicotyledons:

- Hybrid Grey poplar (*Populus* x *canescens, syn. Populus tremula* x *P. alba*)
  - Wild-type
  - Transgenic non-isoprene emitting (Behnke et al., 2007)
  - Transgenic, carrying the reporter genes GUS/GFP under the control of the poplar *ISPS* promoter region (Cinege et al., 2009)
- Silver birch (*Betula pendula* L.)
- Holm oak trees (*Quercus ilex* L.)

Conifers:

- European larch (*Larix decidua* L.)
- Norway spruce (*Picea abies* (L.) Karst.)
- Scots pine (*Pinus sylvestris* L.)

The poplar plants were 1 to 3 years old. Silver birches were two years old and they were collected locally. Holm oaks were 5 years old and they originated from an Italian plant nursery (Balducci, Pistoia, Italy). Conifer trees were 2 years old and they were obtained from a pre-alpine plant nursery (Pflanzgarten, Laufen, Germany).

Poplar seedlings were amplified, micro-propagated and cultivated under controlled conditions inside a phytochamber (publications **I**, **II**, **III**). At the beginning of 2007 the plants were transferred into pots (25 cm diameter, 20 cm high) containing 50% (v/v) perlite (Agriperl Dämmstoff GmbH, Dortmund, Germany), 25% (v/v) silica sand (particle size 1-3 mm), 25% (v/v) potting soil (Fruhstorfer Einheitserde, Bayerische Gärtnereigenossenschaft, Aschheim, Germany) and 10 g of Osmocote and Triabon fertilizer (Scotts International GmbH, Nordhorn, Germany) and watered weekly. All the other plant species (publication **I**) were potted in the same way. Dicotyledon plants were exposed to temperatures ranging from 10 to 32°C and 16 h light conditions of 600 - 1600 PPFD (µmol photons m^{-2} s^{-1}), and relative humidity of 40 ± 20% in the greenhouse of the IMK-IFU, whereas the conifer plants were grown outside, in the garden of IMK-IFU at Garmisch-Partenkirchen throughout the growing season. The institute is located in the German Alps, at 730 m amsl, next to a forest dominated by Scots pine and Norway spruce trees. Summer temperatures ranged from 10 to 30°C, and photosynthetic photon flux density (PPFD) from 100 to 1500 µmol m^{-2} s^{-1}.

3.2. Methods

3.2.1. Dynamic cuvette system

Gas exchange and BVOC measurements were performed using four dynamic cuvette systems (450 cm^3 volume) able to host a poplar leaf, apical bud or conifer twigs (Fig.11a). They included Peltier elements to control the chamber temperature, measured with thermocouples (Fig. 11b), which could be adjusted dynamically (± 5 Cº in ~120 sec). The light was provided by 15 LEDs (DP3-W3-854, Osram, Germany) allowing the light intensity to be increased up to a photosynthetic photon flux density (PPFD) of 1300 µmol photons m^{-2} s^{-1} with low IR content (Fig. 11c). Lamps and Peltier-elements were cooled with fans. All the parameters were controlled by a personal computer and could be programmed for simulating changes in environmental conditions. In addition, a bigger perspex cuvette (19 cm inner diameter, 20 cm height; i.e. circa 5670 cm^3) was used to host the root system of poplar plants potted in a sterile hydroponic solution.

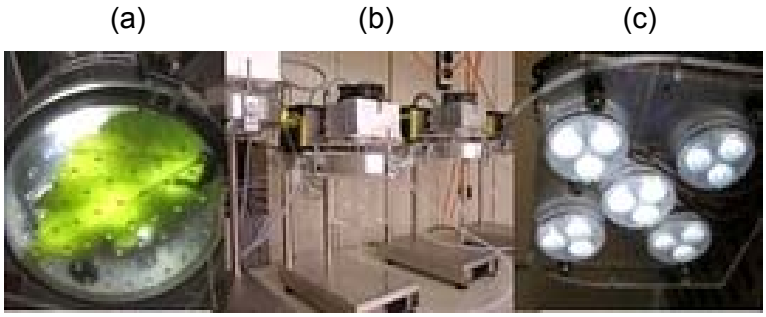

Figure 11. Views of the cuvette system. Cuvette body for hosting the leaf/twig (a); whole cuvette system with Peltier elements and cooling system on the top (b); the 15 LEDs light system (c).

3.2.2. Plant gas-exchange measurements

The plant gas-exchange measurements of H_2O and CO_2 were performed either with a portable gas exchange system (GFS-3000, Heinz Walz, Germany) or by TDLAS (TGA100A, Campbell Scientific, Inc., Logan, UT, U.S.A.). Net CO_2 assimilation rates were calculated according to von Caemmerer & Farquhar (1981).

3.2.3. PTR-MS analysis

Emission of isoprene and monoterpenes was measured online using PTR-MS (paragraph 1.2.2.) and the dynamic cuvette systems (paragraph 3.2.1.). Also, the quantification of the isoprenoid precursors DMADP and GDP and of endogenous monoterpenes was achieved by coupling the PTR-MS with a head-space analysis system (Behnke et al., 2007). Inside 2 ml glass vials, 5 mg of

leaf material containing the precursors DMADP and GDP were converted to isoprene and linalool by the catalyzed acid reaction according to Nogues et al. (2006), using a modified extraction protocol of Brüggemann & Schnitzler (2002a) (publication **I**).

All the compounds measured were analyzed for their $^{13/12}C$ isotope ratios. In order to quantify the incorporation of ^{13}C into the terpenoids, the PTR-MS was used to detect the protonated isotopologue masses of 69 ($^{12}C_5H_9^+$), 70 ($^{13}C^{12}C_4H_9^+$), 71 ($^{13}C_2{}^{12}C_3H_9^+$), 72 ($^{13}C_3{}^{12}C_2H_9^+$), 73 ($^{13}C_4{}^{12}C_1H_9^+$), and 74 ($^{13}C_5H_9^+$) for isoprene and similarly of 137 ($^{12}C_{10}H_{16}^+$) to 147 ($^{13}C_{10}H_{16}^+$) for monoterpenes. Then, the ^{13}C and ^{12}C were counted and related to the total C-monoterpene emitted (publication **I**).

The detection efficiency of the PTR-MS changes according to the molecular mass of the compounds (Steinbacher et al., 2004) following a typical transmission factor (TF) curve (Fig. 12). For the quantification of the monoterpene isotopologues (i.e. 137-147 amu), which lie in the descending part of the TF curve (e.g. Taipale et al*.,* 2008), each isotopologue was quantified using a relative transmission factor (rTF) based on the fractionation rate of a monoterpene standard and the mass discrimination of the instrument (i.e. TF).

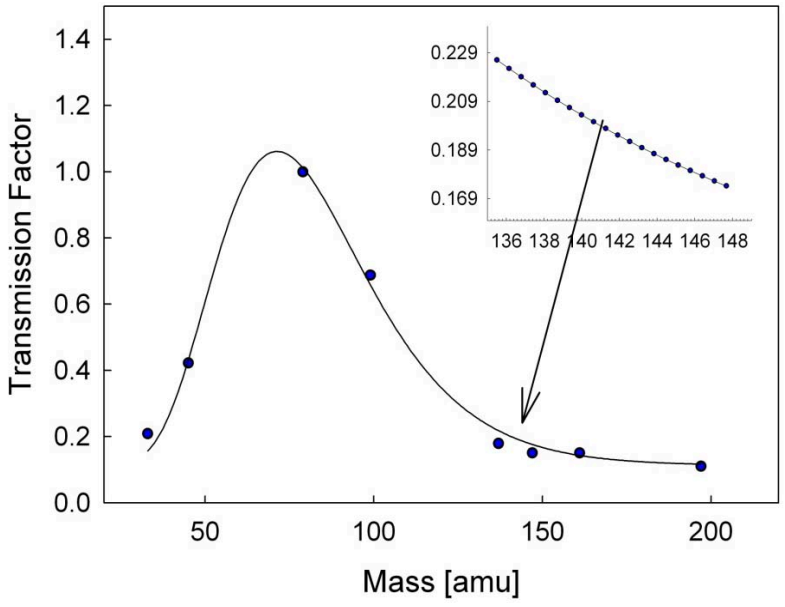

Figure12. Plot of a typical transmission factor (TF) curve of the PTR-MS for the quantification of substances without use of a standard (Hansel et al., 1995). Insert: descending part of the TF curve which reflects the efficiency of the instruments to detect the monoterpene isotopes.

The calibration of the instrument was performed using a standard mixture of 16 VOC in N_2 purchased from Apel-Riemer Environmental (Denver, CO, U.S.A.) with an uncertainty of $\leq$ 5%. This mixture contained both isoprene and monoterpene standards for the direct quantification of the samples (Hansel et al., 1995), and also compounds with mass range of 33-197 amu in order to calculate the TF of the PTR-MS for the quantification of samples not present in the standard

(Hansel et al., 1995). In order to assess the significance of compounds emitted at low quantities, the limit of detection (LOD) was calculated for each measurement. The LOD was set to twice the standard deviation in counts per second (cps) of the background signal divided by the sensitivity (cps $ppbv^{-1}$).

Further technical details can be found in publications **I** and **III**.

3.2.4. Determination of leaf and needle area

Leaves of branches and plants were counted and numbered from the top to bottom of each plant. Because the leaf/needle must be frozen in liquid N_2 within a few seconds after sampling, the determination of the leaf area was performed before enclosure in the cuvettes.
For dicotyledon plant species, leaves were traced on blank paper. The leaf area was then measured from paper traces using a leaf area scanner (Li-3100, Li-Cor Biosciences, Lincoln, NE, U.S.A.). Three replicate measurements were conducted to obtain mean and standard deviation of leaf area.

For coniferous species, the total needle area was calculated using the projected area and the cross-sectional shape for each needle-species as described elsewhere (Chen et al., 1997). Total needle area was calculated counting the number and measuring the length of the needles before the experiment, while the projected area was measured with a leaf area scanner from similar needles of other plants.

3.2.5. Biochemical analysis

All biochemical analyses were performed using frozen leaves/needles and twigs. Plant material was ground in liquid N_2 with a mortar and pestle. For leaves, the central vein and the petiole were removed. Protein extraction and protein determination followed established protocols (Mayrhofer et al., 2005). Endogenous monoterpene contents and pattern were measured with gas chromatography flame ionization detection (GC-FID) (Fischbach et al., 2002), whereas the isotopic distribution of the sample molecules was determined by PTR-MS as described in publication **I**. The amount of the terpenoid precursors DMADP and GDP and the subcellular pool determination was determined with PTR-MS by means of ^{13}C-labeling as also described in publication **I**. The enzyme activities of DXS, ISPS and terpenoid synthase (TPS) were assayed by the respective protocols that can be found in the publications **I**, **II** and **III**.

3.2.6. Statistical analysis

All experiments were performed independently with 3 biological replicates. Each analysis was assayed with three technical replicates. Statistical analyses (t-test, ANOVA) were performed using the Software package Origin (version 7.0; OriginLab, Northampton, MA, U.S.A.).

3.3. Experimental design

Overall, the experimental work has been carried out with a similar approach for both the determination of *de novo* monoterpene emission in boreal trees and the study of C translocation in poplar. Both experiments required cuvette measurements for characterizing the gas exchange and the BVOC emission under controlled environmental conditions. Then, a series of labeling experiments was performed by feeding plants with a ^{13}C-source (e.g. $^{13}CO_2$, ^{13}C-glucose) and measuring online the incorporation of ^{13}C into the volatile terpenoids by PTR-MS and into respired CO_2 by TDLAS. Once the ^{13}C was incorporated steadily into the terpenoids, different plant parts were harvested by freezing them in liquid N_2 for further biochemical analysis. The incorporation of ^{13}C into the terpenoids and their intermediates was then determined by means of online analysis and biochemical analysis.

In order to develop a new method for measuring the DXS activity, a different design was needed. Assays were prepared *in vitro* with poplar plant extracts. DXS was fed with labeled substrate (^{13}PYR) and the formation of $^{13}CO_2$ was measured with GC-IRMS. Finally, the enzyme product $^{13}CO_2$ was used to calculate the enzyme activity.

3.3.1. Determination of *de novo* and pool emissions of terpenes

In order to separate monoterpenes formed from recently fixed photoassimilates from the monoterpene fractions which evaporate from storage pools, $^{13}CO_2$-labeling experiments were performed in combination with enclosure cuvette measurements. Plants were fed with $^{13}CO_2$ until a steady incorporation of ^{13}C into the terpenoids was reached. The fraction of ^{13}C incorporated into monoterpenes was used to assess the fraction of *de novo* biosynthesis. Because other C sources than recently fixed photoassimilates are also supplying the formation of terpenoids in the MEP pathway, and because isoprene is always emitted after being synthesized, the incorporation of ^{13}C into isoprene was used to determinate these 'alternative C sources' that also contribute to *de novo* biosynthesis of monoterpenes.

The three conifer species *Larix decidua, Picea abies, Pinus sylvestris* and the broad-leaf species *Betula pendula* were chosen as representative of coniferous and dicotyledon trees of the boreal/Alps area, respectively. In addition, the dicotyledon species *Quercus ilex* was also investigated and compared to other species, since its emission behavior is well know in literature (Loreto et al., 2000a; Fischbach et al., 2002).

Twigs/leaves of trees were enclosed in the dynamic cuvette systems (paragraph 3.2.1.). The gas exchange was measured with a portable gas exchange system (GFS-3000, Heinz Walz, Germany), whereas the online quantification of labeled and unlabeled BVOCs was achieved with a PTR-MS (paragraph 1.2.2.). Briefly, twigs/leaves were enclosed already the day before labeling to avoid overestimation of monoterpene emissions from the storage pool (due to mechanical

stress following insertion into the cuvette) and to let the plants acclimatize to their new environment with a temperature of 30°C and light flux density of 1000 µmol m^{-2} s^{-1} PPFD.

Cuvettes were continuously flushed with synthetic, VOC-free and constantly humidified synthetic air (21% v/v O_2, 79% v/v N_2), amended with 385 ppmv CO_2 with natural abundance of $^{13}CO_2$. From the outlet air, 70 mL min^{-1} was continuously taken by the PTR-MS for VOC online measurements, and 133 mL min^{-1} for 30 min (total of 4 L) was passed through a three-bed-adsorbent tube (90 mg Carbotrap C, 60 mg Carbotrap, 60 mg Carbopack X, Supelco, Bellafonte, PA, U.S.A.). Another 250 mL min^{-1} of the outlet air from the cuvettes passed the GFS-3000 for photosynthetic gas exchange measurements. Tubes were analyzed with gas chromatography mass spectrometry (GC-MS; Perkin Elmer, Weiterstadt, Germany) as described by Schnitzler et al. (2004a) for the identification of monoterpenes.

$^{13}CO_2$-labeling was performed in the presence of light (i.e. photosynthesis) until incorporation of ^{13}C into isoprene and monoterpenes reached a steady state (8 hours for conifers and 5 for dicotyledons). Before and during the labeling procedure it was ensured that CO_2 assimilation and BVOC emissions were stable.

In addition, the endogenous monoterpenes were extracted from coniferous plants by a pentane extraction procedure (publication **I**), and the incorporation of ^{13}C was measured with PTR-MS. By this means, it should be investigated whether the storage pools of monoterpenes were influenced by the *de novo* biosynthesis.

The monoterpene and isoprene precursors GDP and DMADP were measured for the determination of precursor pools, and the terpenoid synthase (TPS) activities were assayed to prove and quantify the *de novo* terpene biosynthesis.

Detailed information on the materials and methods can be found in publication **I**.

3.3.2. Determination of DXS activity

Measuring the DXS enzymatic activity and characterizing its kinetic properties required three steps: (i) extracting the enzyme from the plant material; (ii) feeding the enzyme with labeled substrate and incubation under different, specific conditions (e.g. with varying substrate amount, temperature, pH); (iii) detecting and analyzing the reaction byproduct $^{13}CO_2$, in relation to reaction time and amount of enzyme.

In order to test the new method, DXS activity was also measured in transgenic non-isoprene emitting lines with isoprene synthase (*ISPS*) knocked down by RNA interference (Behnke et al., 2007). Due to the knock-down of ISPS downstream the terpenoid pathway and the consequent accumulation of the isoprene precursor DMADP, the DXS activity was hypothesized to be down-regulated (Wolfertz et al., 2004). Moreover, we also worked on transgenic poplars carrying the reporter genes GUS/GFP under the control of the poplar *ISPS* promoter region (Cinege et al.,

2009) and studied leaves of different developmental stages (starting from the first leaf on the apical part of the plant down to leaf n°20) in order to analyze developmental changes of DXS activity in relation to the activation of isoprene emission potential (as followed by ISPS promoter activity).

To measure DXS activity in all these cases, the protein extracts (containing DXS) were incubated with a reaction mixture containing the two DXS substrates GAP and the labeled 1-^{13}C-PYR. Because DXS decarboxylates specifically the carboxylate anion (COO^-) of pyruvic acid (CH_3COCOO^-), the head-space of the vial contained also the $^{13}CO_2$ originating from the DXS activity. Assays were performed in parallel with the specific DXS inhibitor *β*-fluoropyruvate (Eubanks and Poulter, 2003). The assay procedures and the compounds utilized in the reaction mixture are described in detail in publication **II**. The enzyme product $^{13}CO_2$ was analyzed with a Gas Bench II coupled to a DELTA plus XP IRMS (Thermo Fisher Scientific, Bremen, Germany). The amount of $^{13}CO_2$ produced by DXS activity was distinguished from the unspecific formation of $^{12}CO_2$ or $^{13}CO_2$ originating from other sources (e.g. oxidation of organic acid or decarboxylation of pyruvate) as described in publication **II**.

Detailed information about the materials and methods used to develop the DXS assay and to characterize the DXS activity in poplar leaves can be found in publication **II**.

3.3.3. Tracing the C fluxes within the plant

In order to study the C translocation in young wild-type Grey poplar saplings and its contribution as C source of isoprene formation, four cuvette systems (paragraph 3.2.1.) were used in parallel. One cuvette was fumigated with $^{13}CO_2$ (99 atom%, 385 ppmv) and the appearance of ^{13}C-label in isoprene and respiratory CO_2 in the other three cuvettes was monitored online with PTR-MS and TDLAS (TGA100A, Campbell Scientific, Inc., Logan, UT, U.S.A.), respectively. Two experiments were performed, one with the 4 cuvettes placed at different levels on the same intact plant, and one with two cut shoots in parallel, which were either fumigated with $^{13}CO_2$ via a mature leaf or fed with ^{13}C-glucose via the xylem sap. The setup of these two experiments is shown in Fig. 13. Experiments were conducted on either intact plants (Fig. 13, insert: 'intact plant') in hydroponic culture, or shoots (Fig. 13, insert: 'shoots') without root system. In order to measure root respiration without bacterial interference, poplar plant roots were cleaned of soil carefully 7-10 days prior to the start of the experiments, and plants were potted into 100% perlite substrate with sterilized Long Ashton nutrient solution (Ehlting et al., 2007). In experiments with intact plants, the four dynamic cuvettes were run in parallel on one plant to detect exchange of ^{13}C (source/sink) between mature leaves, apex and root system: one cuvette enclosed one fully expanded mature leaf, which was fumigated with $^{13}CO_2$. A second cuvette was attached to the apical bud plus the topmost 3-4 leaves.

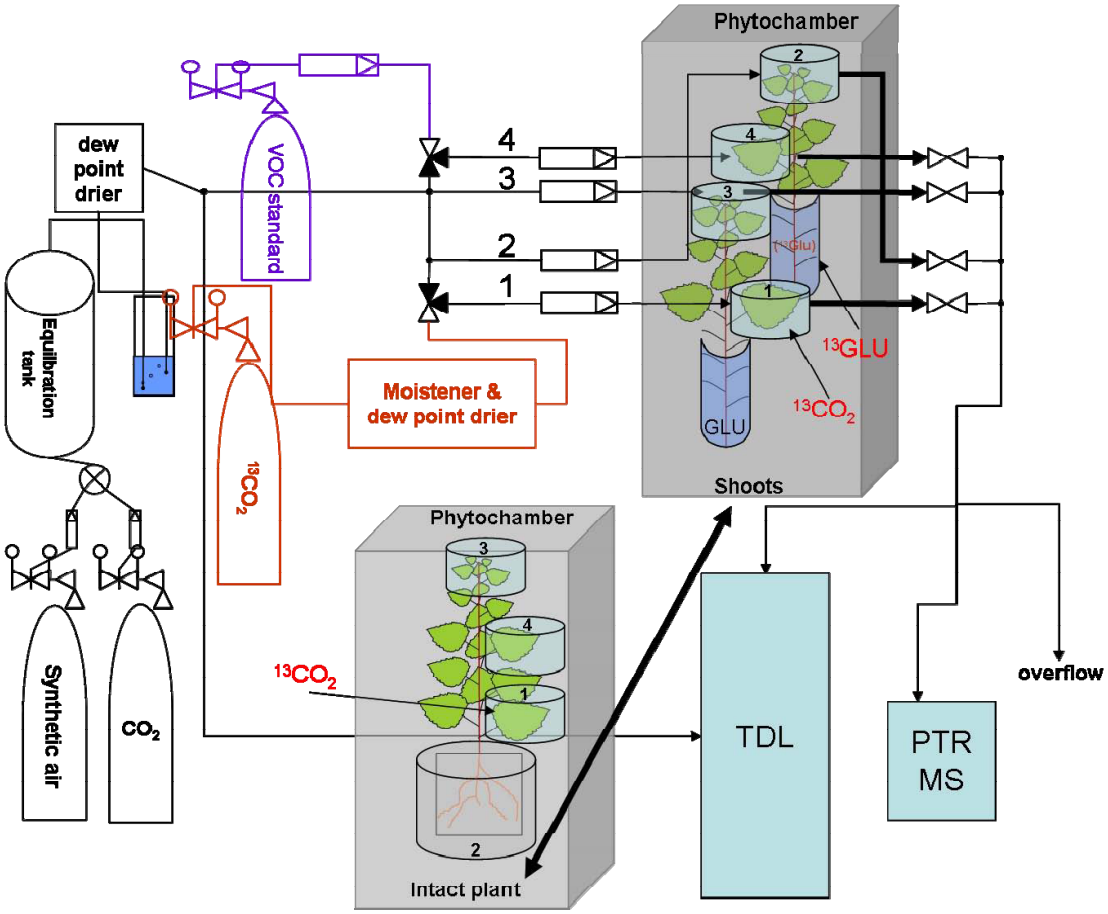

Figure 13. Scheme of the design of the C allocation experiments with Grey poplar. Ten liter per minute of synthetic VOC-free air (BASI Schöberl, Germany) were mixed with CO_2 (38.000 ppmv) to a final concentration of 380 ppmv, passing a 20 L equilibration tank before being completely humidified by bubbling the airstream through pure, distilled water. A dew point unit assured a stable humidity level before the airflow entered each of the four cuvettes through flow controllers, set at 2 L min^{-1}. One cuvette (no. 1) was connected via a 3-port valve to a separate $^{13}CO_2$ tank (in synthetic air) for ^{13}C-labeling. The gas was purchased already mixed at 385 ± 7.7 ppmv $^{13}CO_2$ (Air Liquide, Krefeld, Germany), and humidified separately using a Licor portable dew point generator (Li-610, Licor). Inlet and outlet of the cuvettes were directed sequentially via electronic, computer-controlled 3-port valves to the three gas analyzers [GFS-3000 (1), Heinz Walz, Germany; TDLAS (2); PTR-MS (3)], while the excess flow was directed to a vent which was used to monitor a surplus of air as indicator for the gas-tightness of the whole system. All lines were Teflon tubes sealed with stainless steel nuts and ferrules.

The third cuvette monitored a fully expanded leaf between the labeled leaf and the apex (i.e. younger than labeled leaf). The last cuvette was enclosing plant roots immersed in the hydroponic sterilized solution. For shoot experiments, the root system was cut off the same morning of the experiment and the shoots were put into 50-mL flasks containing autoclaved Long Ashton nutrient solution with 10 mM unlabeled glucose (^{12}C-glucose). Two shoots were analyzed in parallel, and each shoot was followed with two cuvettes: one cuvette enclosing a fully expanded mature leaf, the other one enclosing the apical bud plus 2-3 young developing leaves. For labeling, the ^{12}C-glucose solution of one shoot was exchanged with ^{13}C-glucose solution. The other shoot was labeled, like the intact plants, with $^{13}CO_2$ supplied with the air stream to the mature leaf. The solution of the latter shoot was continuously sampled for sugar analysis every 2 h during and after labeling. All experiments followed the same sequence with duration of 3 days. Briefly, the day before labeling (day 1), plants were adapted to the new environment; on day 2 the plants were labeled and on day 3 the plants were stressed by removing the CO_2 supply, while the effects of the treatments were observed. At the end, all leaves were immediately frozen in liquid N_2 and stored at -80°C for metabolic analysis. The bulk material of the whole plant was isotopically analyzed with EA-IRMS to elucidate where the recently fixed labeled C had been allocated. The terpenoid precursor DMADP was quantified according to the method described in paragraph 3.2.3., and the incorporation of ^{13}C into isoprene was analyzed by PTR-MS. Liquid samples of the nutrient solution were collected regularly during and after labeling the mature leaf with $^{13}CO_2$. The concentration of sugars in the solution samples was measured with a phenol-sulfuric acid assay (Buysse & Merckx, 1993). The isotopic composition of the nutrient solution of the shoot experiment was determined with LC-IRMS (Isolink DELTA V PLUS, Thermo Fisher Scientific, Bremen, Germany; Krummen et al., 2004). With this procedure, the source-to-sink ^{13}C-fluxes (as ^{13}C-enriched transported sugars) from a $^{13}CO_2$-labeled mature leaf into the nutrient solution containing the unlabeled ^{12}C-glucose (where the plant was placed in) were determined.

Detailed information about the materials and methods can be found in publication **III**.

4. RESULTS AND DISCUSSION

4. Results and discussion

The application of ^{13}C stable isotope labeling techniques with PTR-MS, IRMS and TDLAS allowed deepening our understanding on terpenoid biosynthesis and C-fluxes in several tree species. The present thesis demonstrated that these techniques were useful to (i) point out and quantify the *de novo* fraction of monoterpene biosynthesis (publication **I**); (ii) characterize the enzymatic properties of DXS (publication **II**); (iii) trace the C fluxes within poplar saplings and quantify the C-sources for terpene synthesis (publication **III**).

4.1. Determination of *de novo* and pool emissions of terpenes

4.1.1. $^{13}CO_2$-labeling experiments reveal light-dependent monoterpene biosynthesis in conifer trees: determination of *de novo* synthesis

In the present work, for the first time an accurate quantification of the fraction of monoterpene originating from *de novo* biosynthesis in respect to the overall monoterpene emission could be achieved for four of the most common European boreal/Alps trees species: *Pinus sylvestris* (58%), *Picea abies* (33.5%), *Larix decidua* (9.8%) and *Betula pendula* (98-100%) (publication **I**). ^{13}C-labeling was also used in earlier studies (e.g. Delwiche & Sharkey, 1993; Schürmann et al., 1993; Loreto et al., 1996; Shao et al., 2001). However, a proper quantitative estimation of *de novo* and pool emissions of terpenes was not achieved yet. Feeding plants (conifers and dicotyledons) with $^{13}CO_2$ leads to a ^{13}C incorporation into both monoterpene and isoprene (Fig. 14). As the origin of monoterpene emissions from conifer trees are both light-dependent (*de novo*) and temperature-dependent (Fig. 15) (Loreto & Schnitzler, 2010), at constant temperature the dark emissions are lower than light emissions (Staudt et al., 1997; Fig.14). However, the quantification of the *de novo* fraction by measuring light and temperature dependencies is difficult, due to the influence of the light on the different physicochemical characteristics (e.g. volatility, diffusion through phospholipidic membranes) of each monoterpene (Niinemets, et al., 2004). Henry's law constant (*H*) (equilibrium gas-aqueous phase partitioning coefficient) and octanol-to-water partition coefficient ($K_{o/w}$) (parameter to assess the hydrophobicity/ hydrophilicity of a substance) are strongly temperature-dependent: when temperature increases by 10 °C, *H* increases about 1.3- to 1.8-fold and $K_{o/w}$ decreases about of 1.15- to 1.32-fold (Copolovici & Niinemets, 2005). Illumination of leaves/twigs induces the emission of some monoterpenes due to the increase in temperature caused by irradiation. The infrared portion of the electromagnetic spectrum heats the water part of the plant cell. As a consequence, the changed physical conditions can enhance monoterpene emissions. The above-mentioned light effect is not linked to *de novo* terpene biosynthesis and therefore can be easily mistaken when light-to-dark experiments are conducted at constant temperature to determine the *de novo* emission.

Consequently, ^{13}C-labeling and PTR-MS represented a more feasible way to determine the *de novo* biosynthesis of monoterpenes. On the other hand, when using $^{13}CO_2$-labeling and PTR-MS other important aspects need to be taken into account. The first aspect is the 'alternative C source' for isoprenoid biosynthesis (Karl et al., 2002a, 2002b; Kreuzwieser et al., 2002; Affek & Yakir, 2003; Schnitzler et al., 2004b).

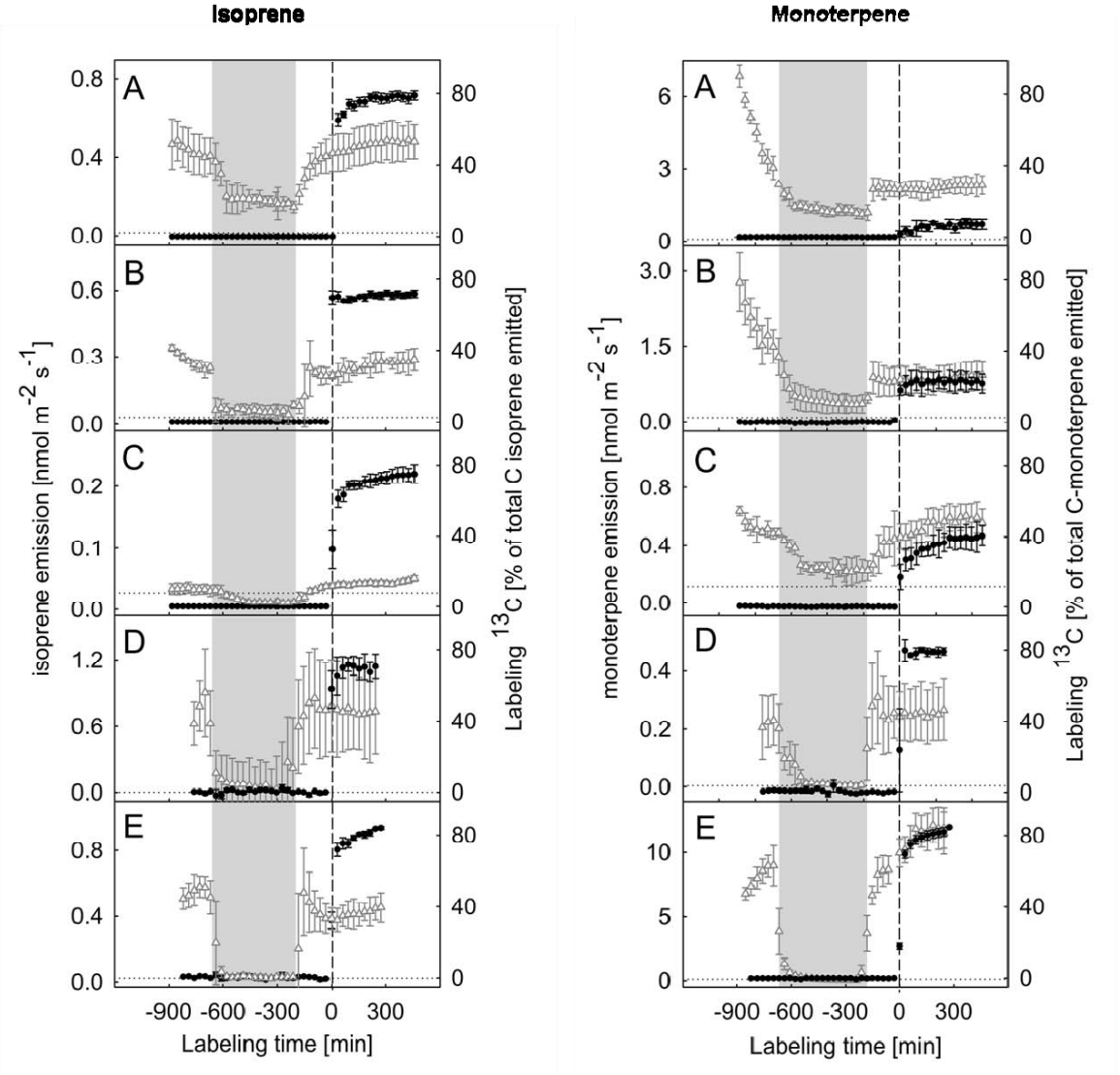

Figure 14. Isoprene and monoterpene emissions (Δ) and ^{13}C-labeling (●) from conifer trees (A) *Larix decidua*, (B) *Picea abies,* (C) *Pinus sylvestris* and from dicotyledon trees (D) *Betula pendula* and (E) *Quercus ilex*. The labeling $^{13}CO_2$ was applied at time 0 (vertical dash line). Isoprene shows similar incorporation of ^{13}C in both conifer and dicotyledon species, whereas monoterpenes show a different behavior between conifers (species specific) and dicotyledons (similar to isoprene). The horizontal dot line represents the limit of detection and the light grey square the night phase. Mean of 3 experiments ± S.E.

The incomplete labeling (e.g. at non-stressed condition, circa 75-80% of C are labeled, through photosynthesis) of isoprene and monoterpenes in dicotyledon species leaves a remaining unlabeled fraction (e.g. 20-25%) which also represents *de novo* biosynthesis and therefore must be accounted for (for details, see publication **I**). The second important aspect that needs consideration is the calibration of the instruments with independent monoterpene isotopologues. Heavier monoterpene isotopologues are less detectable than lighter isotopologues, due to the mass-specific detection efficiency of the instrument (Steinbacher et al., 2004). For this reason, a relative transmission factor was used in this study (paragraph 3.2.3.). The advantage of using the PTR-MS for the quantification of monoterpenes compared to traditional GC-MS is, however, that it measures the sum of monoterpene isomers. Thus PTR-MS detects the entire group of monoterpenes at once without omitting the less abundant monoterpenes as typically done, for technical limitations, in many studies with GC-MS (e.g. Steinbrecher 1989; Schürmann et al., 1993; Staudt et al., 1997; Tarvainen et al., 2007).

Up to now, neglecting the above discussed factors (incomplete ^{13}C-labeling of terpenes and declining instrument sensitivity) had hampered the correct determination of *de novo* emissions in monoterpene-storing plant species. Thus, previous quantifications reported significantly lower fractions of *de novo* biosynthesis within overall monoterpene emissions: for Scots pine, Shao et al. (2001) reported 20-30%, whereas Steinbrecher et al. (1999) found 25-37%, in contrast to the present experiment where the labeled fraction was 39±7%, but the calculated global *de novo* synthesis amounted to 58±4%. For Norway spruce, the data of Schürmann et al. (1993) showed a ^{13}C-incorporation of 20-30% against here reported 23±5%, with calculated *de novo* fraction of 34±8%. The observed split between *de novo* and pool emissions from *P. sylvestris* was applied in a hybrid emission algorithm that considers the two origins of monoterpene emissions (publication **I**). The hybrid model was, indeed, able to better describe (in particular the day-to-night variation of) ecosystem-scale monoterpene emission data measured at a Scots pine forest (publication **I**).

As a conclusion, stable isotope labeling techniques in combination with PTR-MS represent an appropriate tool to separate the monoterpene emission originating from *de novo* biosynthesis from emission due to evaporation processes of monoterpene storage pools in conifer trees.

4.1.2. Absence of ^{13}C-labeling of endogenous monoterpenes indicates complete split between *de novo* and storage pool emissions

Recently synthesized monoterpenes were not significantly stored in coniferous mature needles or bark (publication **I**), and the investigated endogenous monoterpenes were not significantly labeled after $^{13}CO_2$ fumigation, with the exception of those in Scots pine needles (1.2 ± 0.4%) (publication **I**).

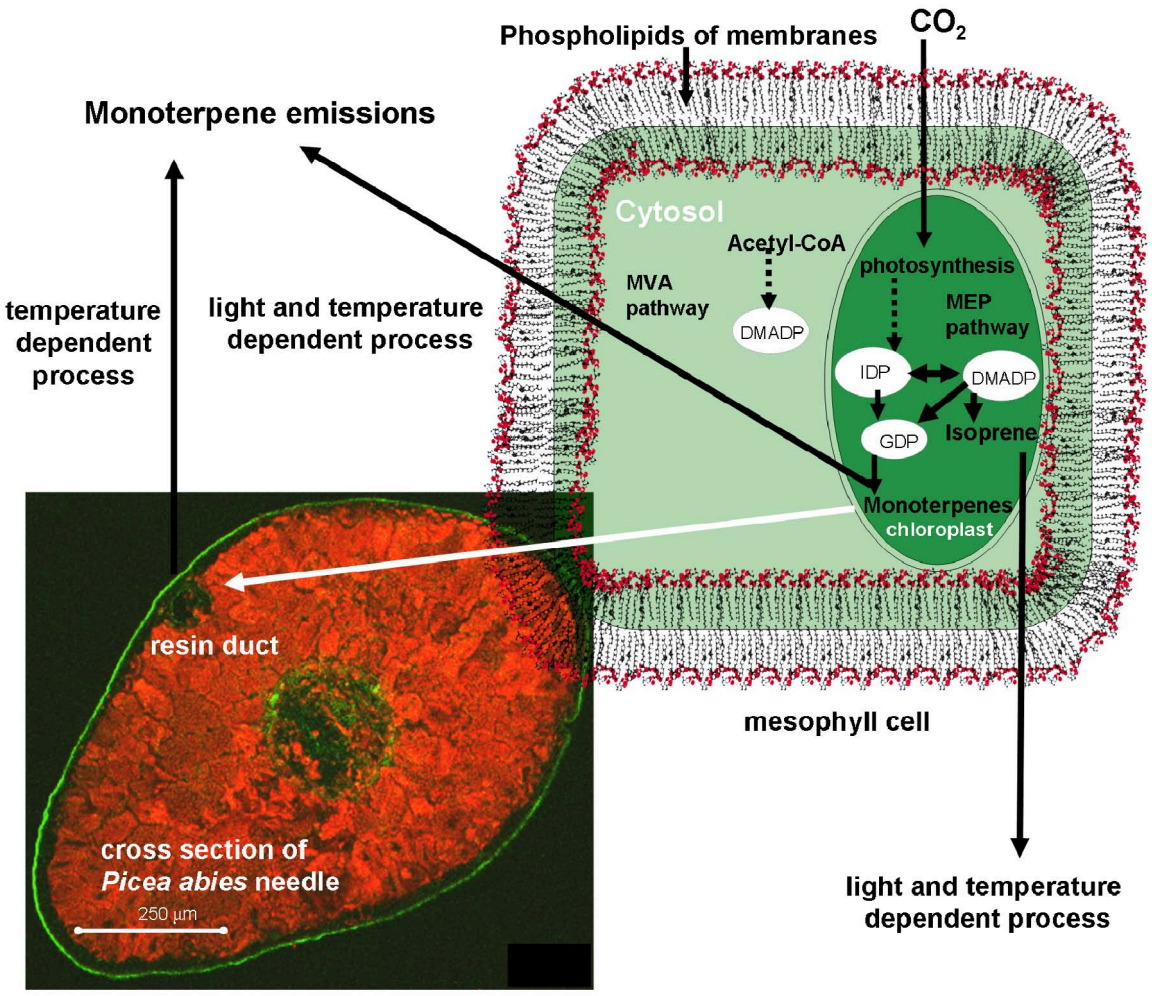

Figure 15. Scheme of cellular and tissue-specific biosynthesis of terpenes in conifers. Isoprene and monoterpenes are synthesized via the MEP pathway in the chloroplasts of mesophyll cells. Isoprene (C_5) is immediately emitted due to its high volatility and absence of storage compartments in a light- and temperature-dependent manner. The larger, more lipophilic monoterpene (C_{10}) molecules are also directly emitted after biosynthesis (*de novo*) or emitted as a consequence of a temperature-driven evaporation process especially from specific storage organs (e.g. resin ducts of conifer needles) or to a minor extent, from non-specific storage pools (e.g. membrane's phospholipids). The cross section of a Norway spruce needle shows the autofluorescence of chlorophyll (red) and of phenolic compounds (green) visualized by confocal laser scanning microscopy (picture: J.P.Schnitzler; for details see Hutzler et al., 1998).

De novo emission bypassed the storage structures and thus must have originated from the surrounding tissue of the resin ducts, i.e. the needle mesophyll. This is in agreement with the fact that in Norway spruce the secretory cells of the resin ducts die after the growing season of the first year (Schürmann et al., 1993) and therefore the pools of endogenous monoterpenes do not receive new monoterpenes. In Scots pine, the low amount of label found might be due to minor 'unspecific storage structures' (Niinemets et al., 2004), like lipophilic compounds of the needle, which generally account for 1-3% of total leaf dry mass (Niinemets et al., 2004). These 'unspecific storage structures' can trap a small amount of monoterpenes, which have lipophilic properties, and were also found in leaves of non-coniferous species, such as birch and oak (publication **I**). The 'unspecific storage structures' explain also very well why the emission of monoterpenes continues for longer periods (in general 10-15 min) than isoprene emission (which is sustained only for a few seconds by the pool of the precursor DMADP) when photosynthesis is rapidly ceasing after darkening (Fig.14).

The present thesis shows that ^{13}C-labeling experiments allow identifying the metabolic origins of monoterpene emission (see scheme Fig.15). Potentially, ^{13}C-labeling provides the information on the time courses of monoterpene emissions, as required in recently developed emission models (Noe et al., 2010).

4.1.3. Isotopic pattern of isoprene and DMADP reveals the subcellular DMADP pool size

^{13}C-labeling experiments allowed splitting the plastidic from the cytosolic DMADP pools, showing that approximatively 20-30% of total DMADP is localized in the chloroplasts during light conditions (publication **I**).

The principle is based on the ^{13}C-labeling pattern of isoprene which reflects the pattern of the plastidic DMADP, since (i) fully labeled DMADP is formed in the chloroplast shortly after $^{13}CO_2$ fumigation (Loreto et al*.,* 2004), (ii) the formation of DMADP is irreversible (Lichtenthaler, 1999), (iii) the cross-talk between chloroplast and cytosol of DMADP and IDP is minimal at short time scale (Lichtenthaler, 1999; Laule et al., 2003; Loreto et al., 2004; Wolfertz et al., 2004; Wu et al., 2006), and (iv) the labeling is kept at steady state conditions. Thus, chloroplastic DMADP molecules must have the same ^{13}C labeling pattern as isoprene (due to its high volatility), and the chloroplastic DMADP pool can therefore be calculated. The results of the cellular compartmentation of DMADP in *Picea abies*, *Pinus sylvestris*, *Betula pendula* and *Populus x canescens* were in good agreement with results obtained with other methods in *Quercus ilex* and *Populus tremuloides* (e.g. Loreto et al., 2004; Rasulov et al., 2009). Therefore, ^{13}C-labeling experiments as demonstrated herein allow determination of chloroplastic DMADP concentrations, which in combination with pH, temperature and bivalent cation concentrations in the chloroplast stroma are as assumed to be main controlling parameters of actual isoprene synthase activity (Zimmer et al., 2003; Magel et al., 2006; Monson et al., 2007).

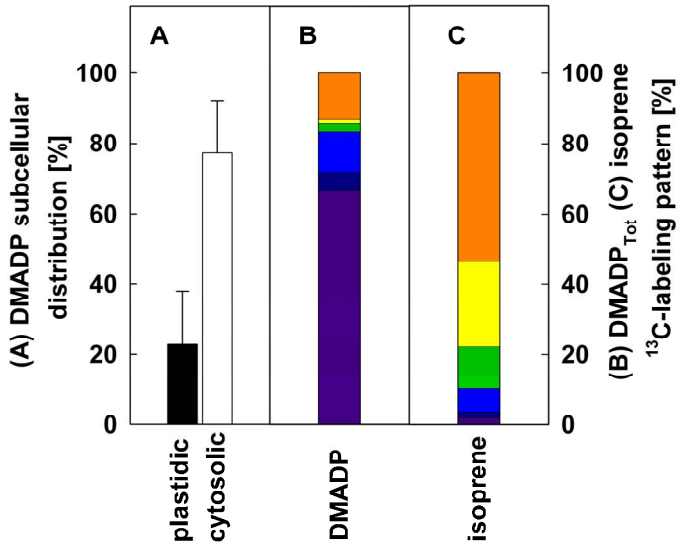

Figure 16. ^{13}C-labeling pattern (m69; m70; m71; m72; m73; m74) of isoprene and DMADP allows determination of cytosolic (___) and plastidic (■) DMADP pools. The DMADP pools (A) are calculated from a mass balance of ^{13}C:^{12}C in total DMADP (B) and in isoprene (C) during steady state $^{13}CO_2$-labeling. Because of marginal exchange of DMADP between cytoplasm and plastids, the pattern of ^{13}C incorporated into isoprene (C) reflects the pattern of DMADP in the plastids (B), and therefore the amount of plastidic DMADP can be calculated as a subtraction of the cytosolic DMADP from the total DMADP (for calculation see publication **I**) (n=3 ± SD).

4.2. Determination of DXS activity

During this PhD work, a new, highly sensitive biochemical assay for measuring DXS activity in plant extracts was developed (publication **II**). It was motivated by the absence of any technique to measure DXS activity in crude protein extracts from plant material. Information of apparent DXS activities under various environmental conditions is essential to analyze the assigned regulatory role of DXS in the MEP pathway. In most of the studies DXS activity is measured by radioactive assays where the reaction product is separated either by thin-layer chromatography (TLC) or HPLC (Sprenger et al., 1997; Lange et al., 1998; Lois et al., 1998; Estévez et al., 2000; Kuzuyama et al., 2000; Cane et al., 2001). Alternative methods include coupled spectrophotometric (Altincicek et al., 2000; Hahn et al., 2001) and fluorometric assays (Querol et al., 2001). However, in all of these studies heterologously expressed and purified DXS was used for biochemical analysis.

In this part (i) the main results of the development of this new assay, using stable isotope labeling with ^{13}C-enriched pyruvate (PYR), (ii) the apparent kinetic properties of DXS in Grey poplar, and (iii) the developmental changes of DXS activity and the regulatory role of DXS in the MEP pathway are presented and discussed.

Detailed information can be found in publication **II**.

4.2.1. Use of ^{13}C-labeling to determine the DXS activity

Using stable isotope labeling with ^{13}C-enriched PYR and IRMS analysis, the apparent activity of DXS could be determined in protein extracts from poplar leaves (publication **II**). The analytical principle of the enzyme assay is based on the PYR decarboxylation activity of the DXS. ^{13}C-labeled PYR with the specific label at the C_1 atom of PYR (1-^{13}C-PYR) forms labeled $^{13}CO_2$ and DOXP in presence of the second substrate glyceraldehyde 3-phosphate (GAP). The developed assay is highly sensitive, and well suitable for the analysis of DXS activity in crude plant extracts. The use of the isotopic signature of CO_2 was essential to separate the $^{13}CO_2$ produced by the enzymatic activity of DXS from the minor unspecific (not due to DXS activity) formation of CO_2 ($^{13}CO_2$ and/or $^{12}CO_2$) originating from other sources, such as (i) natural degradation of PYR, (ii) decarboxylation of PYR in presence of the cofactor thiamin pyrophosphate (TPP), and (iii) oxidation of organic acid of the crude extract.

Accurate quantification of specific DXS CO_2 formation was achieved by use of three groups of controls which consider the above mentioned (possible) sources of extra CO_2. The calculation was based on amount and isotopic signature of produced CO_2 as well as on the global carbon balance as described in detail in publication **II**.

4.2.2. Apparent kinetic properties of DXS and its regulation in the MEP pathway

Apparent kinetic properties of DXS extracted from Grey poplar leaves were determined (publication **II**). The temperature dependency of DXS followed a typical Arrhenius type curve with maximum activity at 45°C. This temperature optimum of DXS is in line with the temperature optima of isoprene emission and other enzymes of the chloroplastidic isoprenoid pathway [e.g. prenyltransferases (Tholl et al., 2001)]. Similar to other enzymes localized in the stroma of chloroplasts, the poplar DXS has a sharp pH activity optimum at 8.5 - 8.7. The apparent Michaelis constants (K_m values) of DXS (111 and 158 µM for GAP and PYR, respectively) were in the same concentration range as described for several other enzymes of the MEP pathway (e.g. Grote et al., 2006). All known isoprene synthases (ISPS) have 10- to 100-fold higher Michaelis constants for its substrate DMADP [e.g. 2.45 ± 0.1 mM in poplar (Schnitzler et al., 2005) and between 0.5 and 8 mM in oak (Lehning et al. 1999), velvet bean (Kuzma & Fall 1993), aspen (Silver & Fall, 1995), and willow (Wildermuth & Fall, 1996)] than monoterpene synthases for GDP [e.g. 10-100 µM in *Quercus ilex* (Fischbach et al., 2000), 2.6 µM in *Thymus vulgaris* (Alonso & Croteau, 1991)] and prenyltransferases for IPP and DMAPP [e.g. 9-18 µM in *Abies grandis* (Tholl et al., 2001)]. It is assumed that the low K_m of prenyltransferases may control the metabolic flux within the MEP pathway because downstream reactions leading to monoterpene and non-volatile terpene biosynthesis are favored over isoprene biosynthesis (Loreto & Schnitzler, 2010). Based on this finding, it was suggested that isoprene emission occurs only when the plants' need for essential, higher terpenoids [hormones (ABA, gibberellins), tocopherol, phytosterols, and photosynthetic pigments] are satisfied (Owen & Peñuelas, 2005). As a consequence for isoprene-emitting species, isoprene biosynthesis requires a much higher C flux through the MEP pathway than non-isoprene emitting plants where under non-stressed conditions only the essential non-volatile terpenoids have to be produced (Sharkey et al., 1991). From feeding experiments with dideuterated deoxy-xylulose (DOX-d_2), bypassing the intrinsic DXP biosynthesis, Wolfertz et al. (2004) proposed a strong *in vivo* feedback control on DXS mediated by DMADP and/or other MEP pathway intermediates.

In the present thesis the role of DXS in the regulation of chloroplastidic terpenoid biosynthesis in poplar could be investigated. DXS activity was found lower in poplar leaves of non-isoprene emitting plants (Behnke et al., 2007) which accumulate dramatically high amounts of DMADP compared to isoprene-emitting wild-type plants. Wolfertz et al. (2004) suggested that accumulation of downstream MEP pathway intermediates might have a negative feedback control on DXS. This hypothesis could not be tested with the *in vitro* analysis of DXS activity since it excluded a direct allosteric inhibition by DMADP or other metabolites due to the removal of low molecular compounds during protein extract preparation. On the other hand, the present result opens new ideas on the regulation of DXS, which may be due to post-translational modifications or down-regulation of the translation process or protein turnover, since no differences in gene expression of *DXS* between isoprene-emitting and non-emitting poplar lines were detectable (publication **II**).

DXS activity in poplar displayed a distinct developmental pattern, which correlated with the activation of the *ISPS* promoter (publication **II**). The results of the thesis support the idea that DXS activity might be a flux-controlling step of the MEP pathway (Munoz-Bertomeu et al., 2006; Lois et al., 2000a; Estévez et al., 2001). It gets support from the observation that DXS undergoes a similar leaf-age dependent regulation as isoprene emission (Cinege et al., 2009). Leaves with highest isoprene emission rates and hence highest requirement of MEP pathway activity displayed highest DXS activities. However, DXS activity is already present in very young developing leaves, which do not emit isoprene yet but the flux through the MEP pathway may be then directed to the biosynthesis of essential non-volatile terpenoids, i.e. photosynthetic pigments in developing and growing chloroplasts (Mayrhofer et al., 2005). This assumption agrees with the observation of high transcript levels of DXS (Lange et al., 1998) and other plastidic enzymes (DXR and phytoene synthase as shown by Mayrhofer et al., 2005) in young peppermint and poplar leaves. In summary, all these considerations support the 'opportunistic hypothesis' of Owen & Peñuelas (2005) that isoprene emission from leaves is limited by the MEP pathway capacity at early developmental stages (Zimmer et al., 2003; Magel et al., 2006; Monson et al., 2007) and hence become maximal when the plants' need for essential terpenoids is satisfied.

4.3. Use of ^{13}C-labeling for tracing the C fluxes within the plant

During the work for this thesis, ^{13}C-labeling experiments were carried out for tracing the major C fluxes within poplar saplings (publication **III**). PTR-MS and TDLAS were combined in order to detect the incorporation of ^{13}C into isoprene and respiratory CO_2 for studying (i) isoprene C sources and (ii) C translocation in plants.

4.3.1. Role of C translocation in the 'alternative C sources' for terpene biosynthesis

The C-sources for isoprene biosynthesis in poplar were identified to 93-99% using $^{13}CO_2$ and ^{13}C-glucose (publication **III**). Isoprene originated mainly (76-78%) from recently fixed CO_2, and to a minor extent from 'alternative C sources' of photosynthetic intermediates with slower turnover rates (8-11%) and xylem-transported sugars (7.4-10.8%) (publication **III**). The contribution of these 'alternative C sources' to isoprene formation increased during limited net CO_2 assimilation due to the experimental removal of CO_2, and is in good agreement with earlier studies of Schnitzler et al. (2004b) and Brilli et al. (2007), aiming to quantify the contribution of different carbon sources for isoprene emission in *Populus* x *canescens* and *Populus alba*, respectively. Stress conditions such as drought (Brüggemann & Schnitzler, 2002b; Fortunati et al., 2008) and salinity (Loreto & Delfine, 2000; Teuber et al., 2008) can reduce photosynthesis dramatically, whereas isoprene emission is less affected and can be sustained by 'alternative C sources' (Brilli et al., 2007). These abiotic stresses on volatile terpenoid synthesis have important implications for emission models that are based on the photosynthetic activity of the plant (e.g. Niinemets et al.,

1999; Zimmer et al., 2003, Grote et al., 2009), which would, for instance, predict a drastic reduction of terpene emissions at stress conditions.

Significant amounts of carbon are transported over long distances within the plant (publication **III**). The results from this study agree well with other studies of C supply in plants as from Heizmann et al. (2001) in pedunculate oak (*Quercus robur* L.) and Mayrhofer et al. (2004) in Grey poplar (*Populus* x *canescens*). Both studies showed that long-distance transport of carbon was an important C-source for plants when net CO_2 uptake by photosynthesis was reduced. However, in order to quantify the C translocation in both studies invasive methods, such as pressure vessel technique (Rennenberg et al., 1996) for obtaining xylem sap, were used.

Mature leaves of intact poplar saplings exported photoassimilates primarily downward to the root system via the phloem at a velocity of 9.4 ± 1.3 cm h^{-1} (publication **III**). The primary allocation of ^{13}C to the root system proves the function of roots as carbohydrate reserves (Loescher et al., 1990). This C is gradually reallocated to the plants' apical part via xylem transport, particularly under limited net assimilation (publication **III**). The remobilized C is continuously translocated mainly to the apical part of the plant to sustain metabolic processes and serves as an additional C source for isoprene biosynthesis (publication **III**). The results of the C allocation experiments agree with an old ^{14}C-study by Larson & Gordon (1969). The speed of relation between plant photosynthesis and root respiration agrees with field experiments in a coniferous forest ecosystem (Ekblad & Högberg, 2001) and in a deciduous forest (Knohl et al., 2005).

Performing ^{13}C-labeling experiments and using PTR-MS and TDLAS to detect labeled isoprene and respiratory CO_2, this thesis shows an alternative non-invasive way to study dynamically and with high time resolution C translocation within the plant (publication **III**). Moreover, online analysis allows to describe the dynamics of C translocation and to assess the phloem velocity, information, which is scarcely reported in the literature.

Using ^{13}C-labeling experiments together with PTR-MS and TDLAS, the dynamics of C allocation within tree saplings can be successfully studied (publication **III**).

5. CONCLUSION AND OUTLOOK

The large spectrum of plant terpenoids and their very different roles in plant metabolism, growth and development is the result of a very complex regulation of the metabolic fluxes through the MVA and MEP pathways providing the central intermediates for specific terpenoid biosynthesis. Both pathways are connected to a multitude of plant metabolic networks able to originate a large variety of compounds, including the volatile terpenoids. As demonstrated in the present thesis, fluxes of carbon, metabolic regulation and C sources for the biosynthesis of isoprene and monoterpenes can be investigated in detail using ^{13}C stable isotope labeling techniques.

The biosynthesis of isoprene and monoterpenes, the most commonly emitted volatile terpenoids, are closely connected to the photosynthetic net CO_2 uptake of mesophyll cells, and are readily visible by the rapid appearance of ^{13}C-labeled compounds after the fumigation of plants with $^{13}CO_2$ (publications **I** and **III**). However, the incorporation of ^{13}C in isoprene and monoterpenes is incomplete. Other 'alternative C sources' sustain the metabolic flux into terpenoid biosynthesis, particularly under stress conditions with limited net CO_2 assimilation (publication **III**). For plant species like conifers which possess specific structures for storing monoterpenes, the incomplete ^{13}C-labeling is also due to the emission of previously stored monoterpenes. The emission of monoterpenes from these large storage pools is driven solely by temperature-dependent evaporation processes. The fraction of monoterpene emissions originating from *de novo* biosynthesis is also light-dependent, a consequence of the light dependency of photosynthesis (publication **I**). Traditionally, the fraction of newly formed monoterpenes was thought to be small with respect to the total monoterpene emission of conifers. Therefore, models of monoterpene emissions of conifers were treated as a simple evaporation process from storage pools (Tingey et al., 1980; Guenther et al., 1991; Guenther et al., 1993; Arneth et al., 2008).

In the present thesis, the origin of monoterpene emissions from four common boreal/Alps forest tree species was investigated with ^{13}C stable isotope labeling techniques. By this, the fraction of monoterpene emissions originating from *de novo* biosynthesis was accurately determined for the first time (publication **I**). Using $^{13}CO_2$-labeling and considering the 'alternative C source' of terpenoid biosynthesis, it could be shown in the present work that a significant fraction of monoterpene emissions originate from *de novo* biosynthesis and that this fraction strongly depends on the plant species. Moreover, monoterpene emissions from conifer species are a mixture of both emission types, whereas terpenoid emissions from dicotyledonous trees originate from immediate biosynthetic activity. Nevertheless, the fraction of *de novo* monoterpene emissions differs between conifer species (in the present case: 58% in *Pinus sylvestris*, 33.5% in *Picea abies*, and 9.8% in *Larix decidua*).

It became evident that monoterpene emissions had to be handled differently in models by applying data from *P. sylvestris* in a hybrid emission algorithm. With different mathematical

treatments of *de novo* and pool monoterpene emissions, a more accurate description of monoterpene fluxes from a Scots pine forest stand became possible (publication **I**). Thus, the determination of *de novo* emission fractions has important consequences for developing new emission models that are able to assess more realistic monoterpene emissions from areas with extensive boreal forest coverage. Also, the monoterpene emission flux estimates (Guenther et al., 1997) for the boreal area or regions with high proportions of conifers (e.g. the Alps region in Central Europe) should be reevaluated.

However, further investigations are required to determine the fraction of *de novo* monoterpene emissions from conifers undergoing seasonal variations as a consequence of tree phenology. Future studies should also focus on to what extent the dynamics of abiotic and biotic stresses influence terpenoid emissions (Niinemets, 2009; Holopainen & Gershenzon, 2010; Loreto & Schnitzler, 2010). Consequently, the inclusion of specific characteristics of *de novo* monoterpene biosynthesis and terms describing the seasonality of *de novo* emissions of monoterpenes, as well as the influence of abiotic/biotic stresses in semi-empirical (e.g. hybrid method) or process-based models, are further steps towards a more realistic assessment of monoterpene emissions from conifer forests. Process-based models (Niinemets et al., 2002; Grote et al., 2009), in particular, are the ideal starting point for this effort. They are developed on the basis of photosynthetic carbon supply, enzyme activities, metabolic pools and phenological data that take into account the plant ontogenesis and the rapid acclimation to seasonal fluctuations of temperature and light (e.g. Grote et al., 2009). Nevertheless, current process-based models need more detailed information about different environmental constraints (e.g. light, temperature, CO_2 concentration). It is suggested here that the quantification of the plastidic DMADP pool by ^{13}C-labeling (instead of the total DMADP pool) could be included in models of isoprene biosynthesis (publication **I**).

Introducing the activities of those enzymes that are thought to play a key role in the regulation of MEP pathway fluxes (e.g. DXS; Lois et al., 2000; Walter et al., 2000; Estévez et al., 2001; Wolfertz et al., 2004) would be another important improvement in the models. At present, DXS activity at a range of environmental and developmental conditions is unknown due to a lack of suitable techniques for the measurement of DXS activity in plant crude extracts. Investigations into DXS activity in poplar trees here (publication **II**) is an important step towards understanding the regulatory role of DXS in poplar as well as in other plant species. The kinetic properties of poplar DXS were found to be similar to other enzymes of the MEP pathway. Poplar DXS activity undergoes developmental changes that correlate with leaf isoprene emission potential (publication **II**). In mature poplar leaves, isoprene emission is the main metabolic sink of plastidic terpenoid intermediates (Sharkey et al., 1991). The relatively large flux of carbon needed for isoprene synthesis is approximately 100 times the MEP pathway flux needed to synthesize the essential non volatile terpenoids in plastids (Sharkey et al., 1991). Thus, the observed lower DXS activities in non-isoprene emitting poplar mutants compared to wild type plants are an indication of a lower demand for metabolic fluxes within the MEP pathway of non–isoprene-emitting plants and confirm the regulatory role of DXS on the MEP pathway. Resolving the controlling step in the

MEP pathway is of key importance for enhancing the production of terpenoids for industrial purposes (Chang & Keasling, 2006) and for developing new models that are able to simulate the terpenoid emissions in plants under different environmental conditions (Monson et al., 2007; Grote & Niinemets, 2008).

In a mature poplar leaf, the 'alternative C sources' for chloroplastidic terpenoid biosynthesis can originate from C imported via xylem sap (publication **III**). Significant amounts of C are cycled within the plant and contribute to isoprene formation and the metabolism of green and non-green tissues (publication **III**). Thus, some recently fixed carbon intermediates might 'travel' around the plant and thereby have a relatively 'long life' before this C is either incorporated into terpenoids, or re-emitted as respiratory CO_2. Significant amounts of translocated C contribute to the plant metabolism (publication **III**; Heizmann et al., 2001; Mayrhofer et al., 2004) and biosynthesis of terpenoids (publication **III**). The C fluxes within the plant are so far not extensively studied due to the lack of appropriate methods. In the present thesis, the combination of PTR-MS and TDLAS allowed dynamic, high time-resolution tracing of C fluxes within poplar saplings. Application of ^{13}C-labeling and the availability of fast, online measurement systems (PTR-MS and TDLAS), as well as of off-line instruments for the quantification and isotopic discrimination of $^{12/13}C$ (EA-IRMS, LC-IRMS, GC-IRMS), permitted a comprehensive analysis of the origin and processing of carbon within plants from source to sink and with regard to isoprene biosynthesis and emission, C allocation, C translocation and the respiratory CO_2 emissions (publication **III**).

The combination of techniques shown here theoretically allows the quantification of the net C uptake of the plant, which is of increasing importance in the context of climate change. This is because plants are the main C sink for atmospheric CO_2 and their gross uptake is still 15-20 times larger than CO_2 emissions from anthropogenic sources (Forster et al., 2007). Any accurate estimation of a C budget should consider also the substantial part of fixed C which is either re-emitted as respired CO_2, BVOC (not only isoprene), or excreted into the soil as root exudates, and henceforth re-emitted into the atmosphere by microbial respiration. It has been shown that during severe drought conditions and/or salt stress, the C-uptake capacity of plants is reduced, whereas the emission of BVOC may be sustained (Loreto & Centritto, 2004; Rennenberg et al., 2006). Because half of the soil CO_2 efflux might originate from recent photoassimilates (Högberg & Read, 2006), the C loss of the plant as root exudates is another important aspect in constraining ecosystem C fluxes and C budgets. Thus, the use of techniques for quantification of the net (and not the gross) C uptake by plants, in a dynamic way and under changing environmental constraints, will improve our understanding of the global carbon cycle in a changing world.

A fascinating, yet widely unknown aspect of the terpenoid biosynthesis of plants is how they achieve a perfect regulation of C flux through the MEP pathway. Depending on their need, carotenoids, phytohormones, plant repellents of predators or attractants of pollinators are synthesized to varying degrees by plants (Gershenzon & Dudareva, 2007). The quantification of metabolic fluxes through the MEP pathway can be mathematically modeled by an experimental approach which includes ^{13}C-labeling methods (Rios-Estepa & Lange, 2007). A metabolic model incorporates knowledge of gene and protein expression, the kinetic properties of enzymes involved in a metabolic pathway and associated networks and transient fluxes of metabolites under non-steady-state conditions (Rios-Estepa & Lange, 2007). At least some of these aspects are already treated in the process-based emission model SIM-BIM (Zimmer et al., 2000; Grote et al., 2009). Metabolic control analysis (Rios-Estepa & Lange, 2007) could be implemented for understanding the regulatory step of the MEP pathway.

Finally, the construction of mathematical models will enable the simulation of terpenoid flux under different constraints (e.g. the environmental increase of CO_2 concentration or temperature) or the rational alteration of metabolic fluxes during the design of specific terpenoid production.

6. LITERATURE

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7. PUBLICATIONS

7.1. Publication I

Ghirardo A, Koch K, Taipale R, Zimmer I, Schnitzler JP, Rinne J (2010): Determination of *de novo* and pool emissions of terpenes in four common boreal/alpine trees by means of $^{13}CO_2$ labeling and PTR-MS analysis. Plant Cell and Environment 33: 781-792.

7.2. Publication II

Ghirardo A, Zimmer I, Brüggemann N, Schnitzler JP (2010): Analysis of 1-deoxy-D-xylulose 5-phosphate synthase activity in poplar leaves with isotope ratio mass spectrometry. Phytochemistry 71: 918-922.

7.3. Publication III

Ghirardo A, Gutknecht J, Zimmer I, Brüggemann N, Schnitzler J-P (2011): Biogenic Volatile Organic Compound and Respiratory CO_2 Emissions after ^{13}C-Labeling: Online Tracing of C Translocation Dynamics in Poplar Plants.
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