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Understanding the link between photosynthesis, growth and emissions of biogenic volatile organic compounds (BVOCs) in beech, oak and ash

Thesis submitted in fulfilment of the requirements for the degree of

Doctor (PhD) in Applied Biological Sciences

Dutch translation of the title:

Het begrijpen van de link tussen fotosynthese, groei en emissies van biogene vluchtige organische stoffen (BVOCs) van beuk, eik en es

Cover illustration:

The leaves of beech, oak and ash indicating chemical structure of major BVOCs always present throughout the growing season for each tree species (green: sabinene from beech; orange: isoprene from oak; red: ocimene from ash)

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The author,	The promoters,	Ghent, 2011
Maja Šimpraga	Prof. dr. ir. Kathy Steppe	
	dr. ir. Hans Verbeeck	

To Amon, Ine & Tom, for your patience

A manuscript is like red wine, it needs maturation

Manuskript je kao crveno vino, zahtijeva sazrijevanje

M. C.


INTERNATIONAL YEAR
OF FORESTS • 2011


2011
C
CHEMISTRY
International Year of
CHEMISTRY
2011

Dankwoord

Gaining knowledge emerged from a thesis on cherry trees and infestation, followed by a thesis on wetland chemistry, this PhD became a joint project on trees, chemistry, and infestations.

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Het boek is geschreven. Knjiga je napisana.

Gent, mei 2012

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http://www.impecvoc.ugent.be/

http://www.aelmoeseneiebos.ugent.be/

List of abbreviations

ACCENT	Atmospheric composition change – the European Network
AVOC	Anthropogenic volatile organic compound
BELSPO	Belgian Federal Science Policy Office (Belgisch Federaal Wetenschapsbeleid)
BEMA	Biogenic Emissions in the Mediterranean Area
BEWA	Process studies, modelling and validation experiments
BIATEX	BIosphere ATmosphere EXchange of Trace Gases and Aerosols
BIPHOREP	BVOC emissions and photochemistry in the boreal regions of Europe
BIRA	Belgisch Instituut voor Ruimte-Aëronomie (BIRA) / Belgian Institute for Space Aeronomy (BISA)/ Institut d'Aéronomie Spatiale de Belgique (IASB)
BVOC	Biogenic volatile organic compound
C	Carbon
CCI	Chlorophyll Content Index
$[CO_2]$	Atmospheric CO_2 concentration
Chl	Chlorophyll
DAQ	Data acquisition unit
DELLA	Protein class containing a DELLA amino acid sequence, necessary for degradation
EnVOC-UG	Research Group Environmental Organic Chemistry and Technology, Ghent University
EQ	Equitensiometer
ESF	European Science Foundation
EuroVOC	European VOC project
DMAPP	Dimethylallyl pyrophosphate
DMNT	4,8-dimethyl-1,3,7-nonatriene
$FADH_2$	Flavin adenine dinucleotide
FPP	Farnesyl diphosphate
FID	Flame ionization detection
GAP	Glyceraldehyde-3-phosphate
GC/MS	Gas chromatography / mass spectrometry
GGPP	Geranylgeranyl diphosphate
GLV	Green leaf volatile
gs	Stomatal conductance
HIPV	Herbivore-induced plant volatiles
IMPECVOC	Impact of Phenology and Environmental Conditions on BVOC Emissions from Forest Ecosystems
IR	Infrared radiation
IRGA	Infrared gas analyzer
IRTC	Infrared thermocouple
IPP	Isopentenyl diphosphate; “active isoprene”
JA	Jasmonic acid

LA	Leaf area
LAI	Leaf area index
LRTAP	Long-range Transboundary Air Pollution
LVDT	Linear variable displacement transducer
MACR	Methacrolein
MBO	2-methylen-3-buten-2-ol
m/z	mass to charge ratio
MeJA	Methyl jasmonate
MeSA	Methyl salicylate
MPAN	Methyl peroxy acyl nitrate
MT	Monoterpenoid
MVK	Methyl vinyl ketone
Non-VIP	Non volatile plant isoprenoid
NOx	Nitrogen oxides
$OH^{\bullet}$	Hydroxyl radical; atmosphere's detergent; very reactive
OVOC	Oxygenated volatile organic compound
ORVOC	Other reactive VOC
Pn or A	Net photosynthesis or net CO_2 assimilation rate (μmol-CO_2 m^{-2} s^{-1})
PAR	Photosynthetically active radiation (μmol-photons m^{-2} s^{-1})
PAN	Peroxy acyl nitrate
PE-UG	Laboratory of Plant Ecology, Ghent University
Pg	Petagram
PPFD	Photosynthetic photon flux density (μmol-photons m^{-2} s^{-1})
ppbv	parts per billion (by volume)
ppm	parts per million
ppmv	parts per million (by volume)
pptv	parts per trillion (by volume)
PTR-MS	Proton transfer reaction - mass spectrometry
PV	Plant Volatile
PVOC	Plant VOC
QUPIFE	*Quercus, Pinus, Fraxinus* FWO project
RH	Relative humidity
Rubisco	Ribulose-1,5-bisphosphate carboxylase oxygenase
SQT	Sesquiterpenoid
SWC	Soil water content
TDP	Thermal dissipation probe
TMTT	4,8,12-trimethyltrideca-1,3,7,11-tetraene
UNECE	United Nation Economic Commission for Europe
VIP	Volatile isoprenoid
VOC	Volatile organic compound
VR-BVOC	Very reactive BVOC

Preface

All terrestrial vegetation takes up carbon dioxide (CO_2) and the majority emits biogenic volatile organic compounds (BVOCs). Air quality and climate issues are linked with BVOC emissions. These BVOC emissions are recognized as a source of aerosols, an important tropospheric ozone (O_3) precursor and play a role in global warming.

Already from 1979, the convention on Long-range Transboundary Air Pollution (LRTAP) of the United Nation Economic Commission for Europe (UNECE) has addressed some of these major environmental problems. The LRTAP convention aims to gradually reduce and mitigate air pollution. Focusing initially on sulphur (S) emissions, the LRTAP convention has been extended by eight protocols. Amongst others, in 1991, a protocol on the control of emissions of volatile organic compounds or their transboundary fluxes was adopted.

Being not yet implemented in policy making, the chemistry of trees and the chemistry of the atmosphere are interlinked in ways that scientists are only just beginning to understand. The year 2011, when this dissertation was written, is an international year of chemistry and an international year of forests representing an important step towards a deeper understanding of the volatile language of plants linking trees to atmospheric chemistry. Up till now many scientific breakthroughs have been achieved including knowledge about the importance of BVOC emissions in aerosol formation, photochemical smog, global warming, and the impact on greenhouse gasses such as methane. BVOC emissions are important in air quality and climate issues influencing the health of living beings. Indirect effects on respiratory organs led to the conclusion that in order to mitigate air pollution, certain tree species should be planted in certain areas, depending on urban or rural nature.

With this dissertation I hope to provide a stimulating interdisciplinary approach to the chemistry and the biology of BVOC emissions. However, it is clear that many more interactions will be detected in the future. Will we end up in an increasingly scented world?

Introduction and outline of the dissertation

Plants are chemical factories. They perfume the air around them. By releasing various biogenic volatile organic compounds (BVOCs), trees communicate, signal and defend themselves in order to overcome stationary life constraints (Piel et al., 1998). BVOC emissions are present in our everyday life. We smell them and we even drink them. The typical pine smell (pinene), lemon scent (limonene), black pepper spiciness (sabinene) and eucalyptus smell (eucalyptol/cineole) are all familiar examples of BVOC emissions from plants belonging to the group of monoterpenoids (MTs). BVOC emissions have multiple functions, and have long been studied by chemists and biologists.

There are many BVOC projects running in the tropics, revealing their functioning, beneficial, and adverse effects. There is also growing interest in BVOC emissions from temperate regions where air quality is severely affected as they contribute to tropospheric ozone formation, secondary aerosols and the depletion of hydroxyl ($OH^{\bullet}$) radicals in the troposphere having an important impact on the cleansing capacity of the lower atmosphere (Roelofs and Lelieveld, 2000; Müller and Brasseur, 1995) and climate forcing (Andreae and Crutzen, 1997).

Here we focus on Belgium, Flanders region. In the centre of Flanders, long-term (1981-2010) mean annual precipitation is 852 mm and mean annual temperature is 10.5 °C (Royal Meteorological Institute of Belgium). Belgian forests cover an area of 693 kha (2000), of which 146 kha belongs to Flanders and 544 kha to Wallonia (Vande Walle et al., 2005). In Belgium, where air pollution is relatively high, including high photochemical smog episodes, BVOC emission air chemistry is important and highly active. In these regions BVOC emissions from trees are quite important due to climate change and the widespread emission of the precursors of photochemical oxidants (e.g. NO_x, VOCs). This suggests that specific pollutants can become additional stress factors for plants, thus making European forests potentially sensitive to climatic fluctuations (Bussotti and Ferretti, 1998). In order to reveal the physiological conditions of trees and to help us understanding how primary (Pn) and secondary (BVOC) plant metabolism is linked, net photosynthesis (Pn) and BVOC emissions are studied on trees in three different environments.

Therefore, the main objective of this dissertation was to study, understand and characterize the relationship between Pn and BVOC emissions under controlled and natural conditions. The following specific research questions are addressed:

- What do we currently know about Pn and BVOC emissions and what are the concerns about measuring BVOC emissions?
- Which variables are needed to gain insight into the dynamics of Pn and BVOC emissions?
- How do Pn and BVOC emissions relate to temperature in indoor and outdoor conditions and to which extent are these processes related?
- How do Pn and BVOC emissions change under drought stress and what are the effects of drought stress?
- May seasonal variability in Pn contribute to large variations BVOC emission or are other factors influencing the emissions?
- Is there a vertical gradient in Pn and BVOC emissions along tree canopies?

This doctoral study is embedded into two research projects (December 2006-June 2011): (1) the Belgian Federal project IMPECVOC (***Im**pact of **P**henology and **E**nvironmental **C**onditions on B**VOC** Emissions from Forest Ecosystems*, funded by BELSPO), where the link between BVOC emissions and tree physiology is investigated for various tree species and (2) QUPIFE (***Q**uercus **r**obur, **Pi**nus nigra* and ***F**raxinus **e**xcelsior*) of the Research Foundation - Flanders (FWO-Vlaanderen).

Many studies have already been performed concerning BVOC emissions released by plants, but the analytical aspects are often not evaluated properly. Reliable analytical techniques are necessary for an accurate determination of volatiles (Dewulf et al., 2002). Therefore, this work was performed in collaboration with two other departments, namely, the Belgian Institute for Space Aeronomy (BIRA) and the UGent research group Environmental Organic Chemistry and Technology (EnVOC). Other PhD manuscripts within the IMPECVOC and FWO project have focussed on the BVOC emissions modelling aspect (Demarcke, 2011), BVOC emissions and stressors (Joó, 2011) and analytical aspects of BVOC emissions of deciduous and coniferous tree species (Pokorska, 2012). Several M. Sc. theses (Neina, 2008; Schieste, 2009; Bloemen, 2010; Minnaert, 2011; Vandenbussche, 2011) have emerged as well. Past or running BVOC emission projects include “BVOC emissions and photochemistry in the boreal regions of Europe” (BIPHOREP, 1999), “Regional biogenic emissions of reactive BVOCs from forests: BIosphere ATmosphere EXchange of Trace Gases and Aerosols” (BIATEX, 1991), “Process studies, modelling and validation experiments” (BEWA, 2000), “Biogenic Emissions in the Mediterranean Area” (BEMA, 1994), “Volatile Organic Compounds in the Biosphere-Atmosphere System” (VOCBAS, 2004-2009) and “Biogenic Volatile Organic Compounds in the Carbon–Chemistry–Climate System:

present, past, and future (EuroVOC, 2008-now), “Atmospheric composition change – the European Network” (ACCENT, 2004-2009).

In the experiments for this dissertation, three deciduous tree species were used, i.e. European beech (*Fagus sylvatica* L.), common ash (*Fraxinus excelsior* L.) and pedunculate oak (*Quercus robur* L.). European beech is the most important deciduous tree species in the cool-temperate deciduous forests of central and northern Europe (Dindorf et al., 2006).

Experiments were conducted at three sampling locations: facilities of the Faculty of Bioscience Engineering (51°03'N, 3°42'E) (1) indoor (controlled) experiments on potted trees in the growth rooms, (2) outdoor experiments on potted trees on the campus, (3) outdoor experiments conducted in a temperate broadleaf deciduous forest (Aelmoeseneie forest, Gontrode, (50°58'N, 3°47'E) on the canopy of a mature European beech (*Fagus sylvatica* L.), where an experimental tower is available.

The dissertation has the following structure (Fig. 1):

Chapter 1 provides an introduction and outline of the dissertation. First, it gives an overview of Pn and carbon allocation to growth, followed by BVOC emissions and carbon allocation to defense. Biosynthesis, influencing factors and used measuring techniques for these processes are discussed. It reveals up-to-date knowledge on Pn and BVOC emissions, especially MT emissions.

Chapter 2 deals with a comparison of European beech MT emissions in controlled and natural environmental conditions, focusing on the temperature variation. It describes the case of temperature-dependent MT emissions observed in young beech saplings and an adult beech. The principal aim of the investigation was to determine the effects of temperature fluctuations on Pn and MT emissions requiring the integration of these plant processes. The focus is on beech due to the availability of a beech tree accessible from the measuring tower in the Aelmoeseneie forest, Gontrode (Belgium).

Chapter 3 deals with indoor experiments in the growth chamber in order to study the influence of drought on Pn, MT emissions, and radial stem growth. The long-term effects of drought stress as well as their diurnal patterns are studied. Effects of drought are discussed from an ecophysiological point of view. This gave rise to a new view of BVOC emissions control.

Chapter 4 compares seasonal differences in Pn and total BVOC emissions between anatomically different species in outdoor conditions (potted oak, ash, and beech). The experiments were conducted outdoors on potted trees. Effects of seasonality are discussed incorporating a basic temperature model used for prediction of point-

sampled BVOC emissions throughout the season. Infestations showed to play a possible role influencing total BVOC emissions requiring further research.

Chapter 5 deals with a vertical canopy gradient of Pn and MT emissions in a beech canopy distinguishing between sun, semi shade, and shade leaves on the selected sunny and cloudy days throughout the growing season. It discusses diurnal patterns of Pn and MT emissions along vertical gradient in outdoor conditions on an adult beech tree. Additionally, it shows physiological traits reflecting the significance of sun/semi-shade/shade leaves and their canopy positioning.

Finally, we conclude the thesis by **Chapter 6** with a general discussion in which the main findings are summarized and prospective ecophysiological research areas are suggested. Further discussion is based on questions that remained unanswered and where promising areas of future research lie.

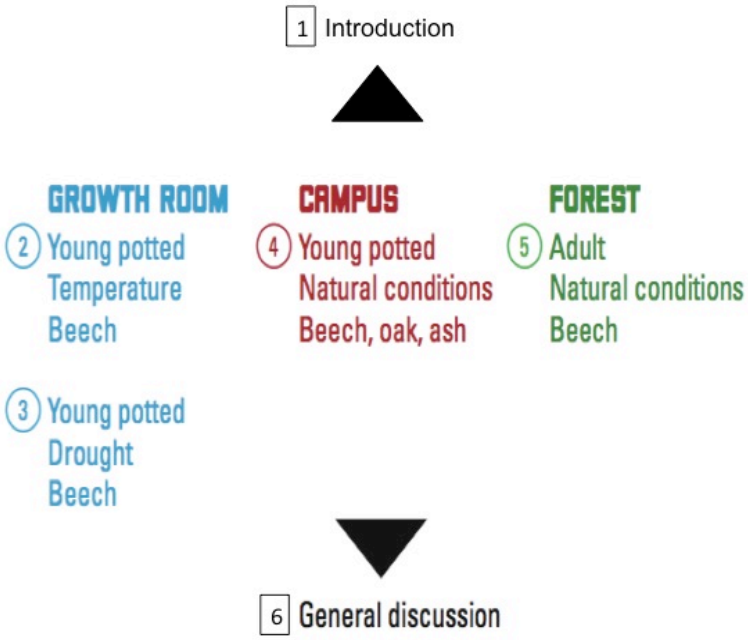

Figure 1: Thesis outline indicating species used and performed experiments: indoor (growth room), outdoor (campus), and outdoor (forest). The chapters are indicated by numbers 1-6.

Chapter 1

Photosynthesis and BVOC emissions

1.1 Plant processes: photosynthesis and BVOC emissions

1.1.1 Photosynthesis

Photosynthesis acts as a pump bringing carbon from the atmosphere into terrestrial ecosystems. It is a key component of the global carbon cycle (Tissue et al., 2002). Photosynthesis affects the carbon cycle at scales ranging from the leaf to the globe (Reich et al., 1998; Cavaleri et al., 2008; Imada et al., 2010; Chu et al., 2011). Terrestrial photosynthesis is the basis of life. As it is the process required for plant growth, a complex set of reactions must occur in a coordinated manner for the carbohydrates synthesis (Baker, 2004) (Fig.1.1).

Chloroplasts are the organs where photosynthesis occurs and they provide all of the reduced carbon in higher plants: from photosynthesis during the day and from starch degradation at night. During the day, reduced carbon is exported as triose phosphate, especially dihydroxyacetone phosphate (Walker and Herold, 1977), used to make sucrose and similar transport sugars (see 1.1.1.1).

Besides photosynthesis, the growth of plants depends upon translocation of photosynthates (products of photosynthesis) and its utilization (Guimaraes et al., 2009). As photosynthesis is linked to photosynthate allocation, partitioning, translocation, growth, and defense, these terms will be described briefly hereafter. Carbon allocation is the regulation of the distribution of fixed carbon into various metabolic pathways (Taiz and Zeiger, 2010). Optimal allocation reduces the risk of mortality particularly in resource-limited environments (Imaji and Seiwa, 2010). Photosynthate partitioning is the differential distribution of photosynthates within the plant (Taiz and Zeiger, 2010; Pien, 2008). Translocation is the process of translocating photosynthates from mature leaves to areas of growth and storage. Additionally, it should be noted that different terminology exists in the literature to indicate the difference between gross photosynthesis and respiration: net photosynthesis (Pn) (Loreto et al., 2004), carbon dioxide (CO_2) assimilation rate (An) (Smith and Stitt, 2007; Neubauer et al., 2011), net photosynthetic rate (NPR) (Qingcheng et al., 1997) or net assimilation rate (NAR) (Lambers et al., 1998). Throughout this thesis, we will refer to net photosynthesis (Pn) unless stated otherwise.

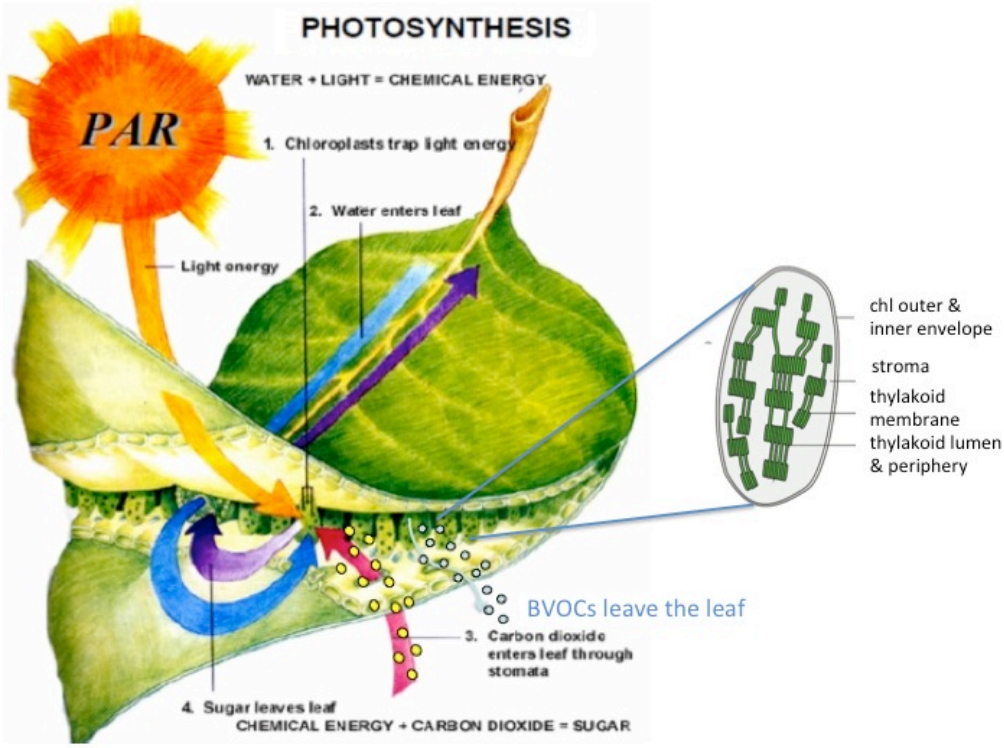

Figure 1.1: Simplified scheme of photosynthesis indicating a leaf and positioning of chloroplasts. Arrows indicate the sugar transport direction (purple), water transport (blue) and CO_2 entry (red). PAR represents photosynthetically active radiation. Full yellow and full blue circles represent the entry of CO_2 and the emission of biogenic volatile organic compounds (BVOCs), respectively (adopted from Dye, 2007; Armbruster et al., 2011).

A plant process closely linked with photosynthesis is photorespiration. This process depends on light and occurs when ribulose-1,5-biphosphate (RuBP) carboxylase becomes oxygenase, consuming oxygen (O_2) instead of carbon dioxide (CO_2). It occurs when the concentration of O_2 in mesophyll cells is high relative to CO_2. Mostly, photorespiration increases with temperature. This process reduces the photosynthetic efficiency and produces no adenosine-5-triphosphate (ATP) resulting in a net carbon and nitrogen (N) loss. Consequently, plant growth is slowed down.

In contrast, the nighttime counterpart, dark respiration (Rd) represents the usage of CO_2 for maintenance of existing biomass and growth of new tissues (Kozlowski, 1992; Taiz and Zeiger, 2003). This process is needed to produce the energy and carbon skeletons to sustain plant growth (Lambers et al., 1998).

The magnitude of carbon fluxes between the atmosphere and the terrestrial ecosystems is large: photosynthesis assimilates about 120 Pg C $year^{-1}$, whereas plant respiration and soil respiration each release about 60 Pg C $year^{-1}$ into the atmosphere (Amthor, 1991; Tissue et al., 2002).

1.1.1.1 Photosynthesis process

Photosynthesis is a two-stage process consisting of light reactions (electron and proton transfer reactions) and carbon (previously known as dark) reactions (biosynthesis of carbohydrates from CO_2) (Fig. 1.2).

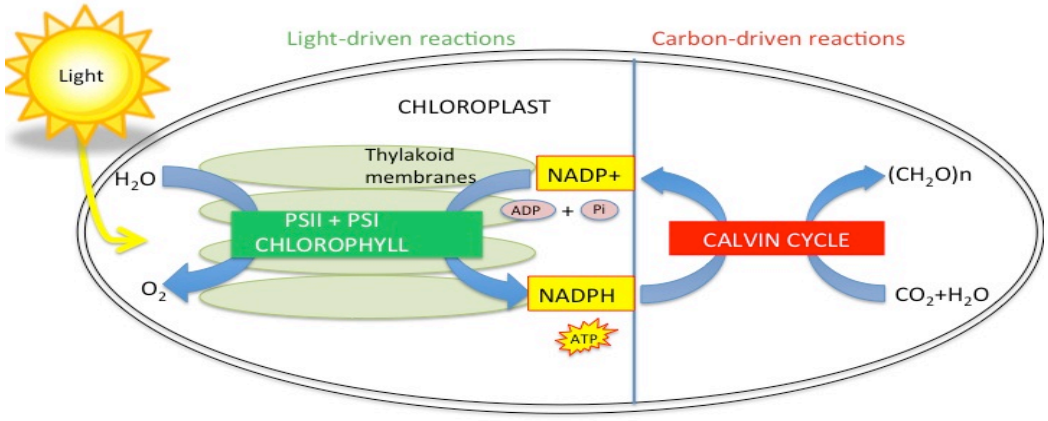

Figure 1.2: Light and carbon-driven reactions of photosynthesis in chloroplasts. Light excitation of chlorophyll in the photosynthetic electron transport system (PSII and PSI) elicits the formation of of ATP and NADPH in thylakoid membranes. Afterwards, both ATP and NADPH are consumed by the Calvin cycle in stroma, reducing CO_2 to carbohydrates (triose-phosphates) (after Taiz and Zeiger, 2010).

The light reactions occur in a complex membrane system that is made up of protein complexes, electron carriers, antenna pigments (chlorophyll), and lipid molecules. In the light reactions, two different reaction centra, known as photosystem I and II (PSI and PSII), work concurrently. As a result, electrons are transferred from a water molecule to nicotineamide adenine dinucleotide monophosphate ($NADP^+$), producing the reduced form, i.e. nicotineamide adenine dinucleotide phosphate (NADPH). NADPH together with ATP formed by the light reactions provide the energy for the dark reactions of photosynthesis, known as the Calvin-Benson cycle or photosynthetic carbon reduction cycle (Baker, 2004; Taiz and Zeiger, 2010). As CO_2 is fixed via the Calvin-Benson cycle through the use of ATP and NADPH, CO_2 and water are enzymatically combined with 5-C acceptor molecules (ribulose 1,5-biphosphate) to generate two molecules of a 3-C intermediate. This intermediate is reduced to carbohydrate by enzymatic reactions driven by ATP and NADPH. The cycle is completed by regeneration of the ribulose 1,5-biphosphate. Overall, the reactions of the Calvin-Benson cycle reduce atmospheric carbon for its incorporation into organic compounds used by the cell (Fig. 1.3).

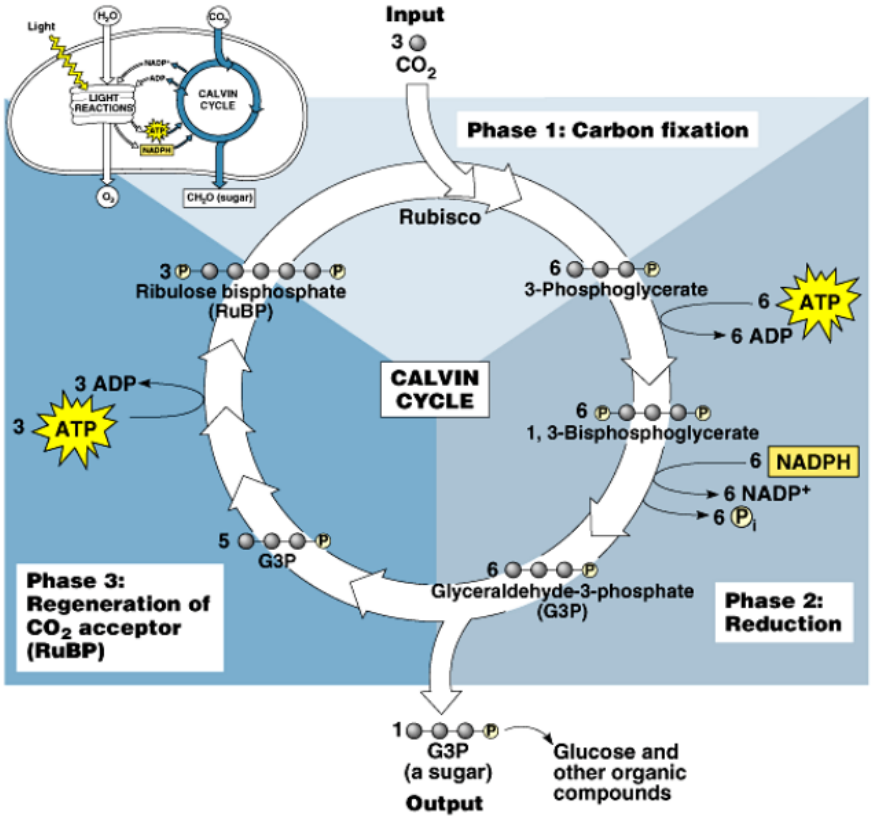

Figure 1.3: Net photosynthesis in a C3-plant. The Calvin-cycle consists of three phases: carbon fixation, reduction and regeneration of the CO_2 acceptor (after Taiz and Zeiger, 2003; Heldt, 2005).

1.1.1.2 Carbon allocation to growth

Plants allocate their photosynthates to growth, defense and storage (Chapin et al., 1990; Imaji and Seiwa, 2010). In most tree species, sucrose (a disaccharide) is the main transport sugar accumulating in the cell cytosol, while starch (a complex polysaccharide) is the main storage compound, accumulating in the chloroplast. Consequently, as starch storage occurs in the chloroplast, its synthesis and breakdown are tightly coupled to photosynthesis (Chapin et al., 1990). Moreover, starch is considered as an integrator of plant metabolism and growth in response to environmental changes (Pinheiro and Chavez, 2011). The concentration of sucrose in the leaf cytosol largely depends on (1) photosynthesis (because triose phosphates are exported from chloroplasts into the leaf cytosol) and (2) export of carbon from leaves (because sucrose fulfils the energy demands of other tissues) (Taiz and Zeiger, 2010). The transport between tissues or from cell to cell is essential for a normal growth rate and development (Pien, 2008). As phloem translocates photosynthates from sources (the area of production, meristem and developing organs as leaves, fruits, and flowers) to sink organs (consumption/storage) (Wareing

and Partick, 1975; Pien, 2008), the glycolysis (degradation of sugars) pathway plays a central role in the utilization of carbohydrates. This pathway provides precursors for a multitude of cell components such as biogenic volatile organic compounds (BVOCs) originating from different biochemical pathways (see 1.1.2.1). When using newly fixed carbon in leaves, the partitioning between starch and sucrose becomes important. These pathways compete for the same pool of triose phosphates, generated by the Calvin-Benson cycle in the chloroplasts (Pien, 2008).

Zweifel et al. (2006) indicated a balance between growth and carbon reserves in trees: the more a tree part grows, the more carbon it stores and utilizes. During tree growth there is a continued translocation of photosynthates within the plant. Once the transport carbohydrates have been unloaded and entered the sink cells, they can remain as such or be transformed into various compounds. In storage sinks, fixed carbon can be accumulated as sucrose or hexose in vacuoles or as starch in amyloplasts. However, insufficient sink strength can cause an accumulation of carbohydrates in the sources. In growing sinks, carbohydrates can be utilized for respiration and for synthesis of other molecules required for growth. In contrast, when carbohydrates accumulate in leaves, photosynthesis is suppressed. However, it is the integrated response at the whole plant level, including photosynthesis and the allocation of photosynthates to different plant parts, which finally dictates the survival and persistence of plants under environmental stresses (Chavez et al., 2003). This makes carbohydrate-allocation patterns increasingly complex. Understanding the photosynthesis and growth mechanisms is therefore critical in order to interpret photosynthesis measurements in growth rooms as well as in field conditions.

1.1.1.3 Importance and function of photosynthesis

As an ancient process, photosynthesis has adapted in the past to different life conditions and environmental changes. On the other hand, photosynthesis is very sensitive to the environment, immediately sensing minimal environmental changes and triggering a series of adjustments eventually leading to adaptation through changes in primary production (Centritto et al., 2004). Photosynthesis provides multiple functions ranging from system functioning to being the only natural process sequestering a massive amount of CO_2 from the atmosphere (Loreto and Centritto, 2004). This sink effect is of extraordinary importance, because it could partly counteract the present trend toward an atmospheric CO_2 concentration rise. In return, trees are the Earth's main source of O_2. Additionally, trees contribute to urban air quality by trapping particulate pollutants (dust, ash, pollen, and smoke). Carbon is taken up in gaseous form and a large amount of energy is needed for its reduction (see above). Plants maintain a balance between photosynthesis, storage and growth in response to developmental and environmental signals (Smith and Stitt, 2007; Neubauer et al., 2011). Moreover, plants are subjected to many signals to be

recognized and translated into cellular responses. Because of their sessile nature, plants must adapt to changing environmental conditions (Piel et al., 1998). Many internal and external (abiotic and biotic) factors influence photosynthesis, growth, and emissions of biogenic volatile organic compound (BVOCs or BVOC emissions) (see 1.2). However, whether these factors will positively or negatively affect photosynthesis and will trigger the onset of adaptive responses on photosynthesis (Long et al., 2004), remains to be determined.

1.1.2 Biogenic volatile organic compounds (BVOCs)

Besides carbon dioxide (CO_2), there are other gases exchanged between vegetation and the atmosphere. BVOC emissions are such gasses released by vegetation. During photosynthesis (Pn) and respiration (Rd), where plants exchange non-organic volatiles such as CO_2 and oxygen (O_2), most of them also contain many more secondary metabolism compounds. Amino acids, nucleotides or simple carbohydrates are some of the primary plant metabolites, and not the focus of this study. Secondary metabolites are generally defined as plant chemicals with no role yet found in growth, photosynthesis, reproduction or other primary functions (Iriti and Faoro, 2009). Recent research, however, is identifying more and more primary roles for these chemicals as signals or antioxidants, and being involved in other functions (see 1.1.2.3). Therefore, the term secondary may require a change or adaptation in the future. Primary metabolic processes are relatively well characterized in contrast to the fate of secondary metabolites. Besides being present in plants and animals, BVOCs also are emitted by fungi (Tertuliano et al., 2005; Jurjević et al., 2008) and viruses (Mauck et al., 2010). As BVOCs originate from living beings the prefix ‘biogenic’ is justified. These semiochemicals (Greek “semeion,” a mark or signal) represent the volatile language of plants, functioning in communication between and among species, as well as serving as messengers between members of the same species (Paré and Tumlinson, 1996) (see section 1.1.2.3).

There is no agreement about the definition of volatile organic compounds (VOCs). However, frequently used are definitions based on American and European legislation. Per definition and in general, in the USA, VOCs denote “any compound that participates in atmospheric photochemical reactions except those designated by United States Environmental protection Agency (US EPA) as having neglibile photochemical reactivity” (USEPA; Joó, 2011). In the European Union (EU), a common definition is that VOCs are “any organic compound having an initial boiling point less than or equal to 250 °C measured at standard pressure of 101.3 kPa” (EU Directive 2004/42/CE, 2008; Joó, 2011). VOCs include hydrocarbons, oxygenated hydrocarbons and organic compounds containing N or sulphur (S) (Maes, 2001). VOCs classes have a large variety of acronyms. Each class defines a special class of VOCs such as BOVOCs (biogenic oxygenated VOCs), ORVOCs (other reactive VOCs), BVOCs, AVOCs (anthropogenic volatile organic compounds) and OVOCs

(other VOCs) (Kesselmeier and Staudt, 1999). Other authors use still VR-BVOCs (very reactive), WI-VOCs (wound-induced), HIPVs (herbivore-induced plant volatiles), GLVs (green leaf volatiles also called green-odor compounds (GOCs), and PVOCs (phytogenic VOCs) (Peňuelas and Llusia, 2004; Koppmann, 2007; Davidson et al., 2008).

Annual worldwide emissions of BVOCs are estimated to be almost ten times greater than those of all combined AVOCs (Atkinson and Arey, 2003; Amelynck et al., 2005; Fig. 1.4). On a global scale, vegetation, and forests in particular, acts as a major source of BVOC release into the atmosphere. Nevertheless, the uncertainty in the global emission estimates is thought to be a factor of three (Sharkey et al., 1991). Interestingly, the global annual isoprene flux from vegetation is of a magnitude similar to that of methane (Guenther et al., 1995). Globally, this makes fast-reacting BVOC emissions more important than AVOCs. In order to evaluate the potential impacts of BVOCs, it is essential to understand the processes that determine their emission, understand abiotic and biotic effects, as well as their atmospheric fate. Within this context, and due to the development of ultrasensitive analytical methods, advanced measurements of BVOC emissions became possible (see 1.3.2).

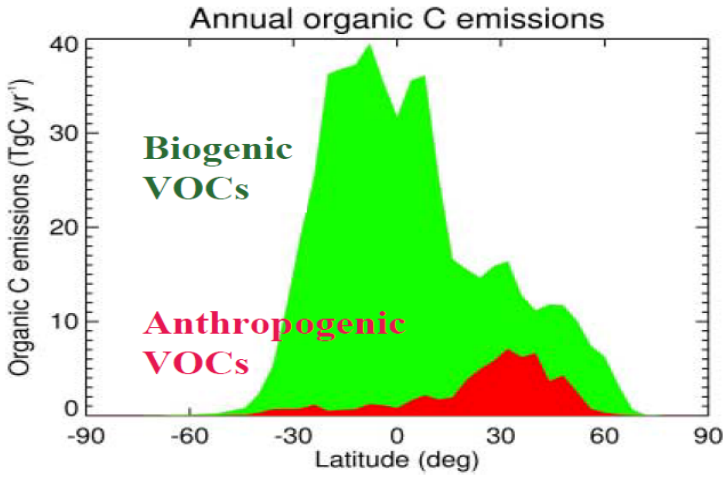

Figure 1.4. Forests are a major source of reactive trace gasses, especially volatile organic compounds. Volatile organic compound (VOC) emissions are separated in two different sources: biogenic (BVOC) and anthropogenic (AVOC) indicating their emission magnitude depending on latitude (Courtesy Nick Hewitt, personal communication).

BVOC emissions, also refered to as non-methane hydrocarbons (NMHCs), include a complex mixture of chemical species. They include the isoprenoids as well as alkanes, alkenes, carbonyls, alcohols, esters, ethers, and acids. Additionally, recent literature indicates isoprenoids (terpenoids) represent the largest part of BVOC emissions (Kesselmeier et al., 2002). Due to the inconsistency of the isoprenoid nomenclature, we explain further the commonly used terminology. There

is a tendency to use the more general name terpenoids or isoprenoids rather than terpenes. Because the suffix 'ene' signifies unsaturated hydrocarbons, the name terpene is inappropriate to include compounds such as alcohols, aldehydes, ketones, etc. The term terpene is restricted to the hydrocarbons $C_{10}H_{16}$. Thus, all terpenes (monoterpenes, diterpenes, etc.) essentially have a chemical formula being C_5H_8 or a multiple thereof (e.g. $C_{10}H_{16}$ for monoterpenes consisting of two isoprene units, MTs, C_{10} hydrocarbons). On the other hand, terpenoids (isoprenoids) (Table 1.1.) are all kinds of derivatives thereof; with substituents of diverse chemical nature. Hence, essentially terpenoids and terpenes are not true synonyms although they are often being used as such. Isoprenoids (terpenoids) cover predominantly the subgroup hemiterpenoids (HTs, C_5 hydrocarbons) such as isoprene, 2-methylen-3-buten-2-ol (MBO), 4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT), 4,8-dimethylnona-1,3,7-triene (DMNT) and the subgroup monoterpenoids (MTs, C_{10} hydrocarbons) (Kesselmeier and Staudt, 1999; Eisenreich et al., 2004; Owen and Peñuelas, 2005). MTs are the most representative BVOCs emitted by plants in the atmosphere (Vitale et al., 2008) and the most studied group of the isoprenoid compounds (Koppmann, 2007). The history of isoprenoids is acknowledged to Otto Wallach (1847-1931), who received a Nobel Price (1910) and clarified in 1891 the relations between twelve different MTs related to pinene (Singh, 2007). Another significant breakthrough was that Lavoslav Ružička (1887-1976) discovered in 1953 the isoprene rule, signifying that isoprenoids are built of isoprene units representing a shared biochemical pathway (see 1.1.2.1). Due to their importance and large emissions, we focus in this thesis on the MTs emitted from leaves. In addition, because of the inconsistency of nomenclature, we will use the term isoprenoids and more particularly MTs throughout this thesis, unless stated otherwise. Consequently, each chapter explains whether we deal with monoterpenes, monoterpenoids (MTs including oxygenated ones) or total BVOCs. Beech, oak and ash are shown to be isoprenoid emitters (Dindorf et al., 2006; Tani and Kawawata, 2008; Pokorska et al., 2011). However, identification (fingerprint) of the emitted BVOCs is not the scope of the current work. We rather focus on plant physiology, total BVOCs and MTs throughout this thesis, depending on the technique used.

Most of the BVOCs are emitted from vegetative parts (leaves and flowers) and some even from roots (Steeghs et al., 2004; Dudareva et al., 2004). We focus on BVOC emissions from foliage. Hundreds of MT compounds have been identified in plants with up to 15 or more foliar MTs found in an individual tree species (Sharkey et al., 1991).

Table 1.1 Isoprenoids (terpenoids) classified according to the number of isoprene units used (after Singh, 2007).

Types of terpenoids	Isoprene C_5 unit	Number of C atoms
Monoterpenoids ($C_{10}H_{16}$)	2	10
Sesquiterpenoids ($C_{15}H_{24}$)	3	15
Diterpenoids ($C_{20}H_{32}$)	4	20
Sesterterpenoids ($C_{25}H_{40}$)	5	25
Triterpenoids ($C_{30}H_{48}$)	6	30
Tetraterpenoids (carotenoids) ($C_{40}H_{64}$)	8	40
Polyterpenoids (C_5H_8)	> above	> 40

Emitted BVOCs, as secondary metabolites, can be classified on the basis of chemical structure (e.g. having rings, containing a sugar), composition (containing N or not), solubility in various solvents, or biosynthesis pathway (e.g., phenylpropanoid, which produces tannins) (Dudareva et al., 2006). A simple BVOC classification includes three groups: (1) terpenoids, which are made from mevalonic acid, and are almost entirely composed of carbon and hydrogen (H), (2) phenolics, made from simple carbohydrates, and containing benzene rings, hydrogen, and oxygen (O) and (3) N-containing compounds (extremely diverse, may also contain S) (Taiz and Zeiger, 2010). On the other hand, Dudareva et al. (2006) classified them as (1) terpenoids, (2) phenylpropanoids and benzenoids, (3) derivatives of amino acids, and (4) fatty acid derivatives, including lipoxygenase pathway products (Fig. 1.5).

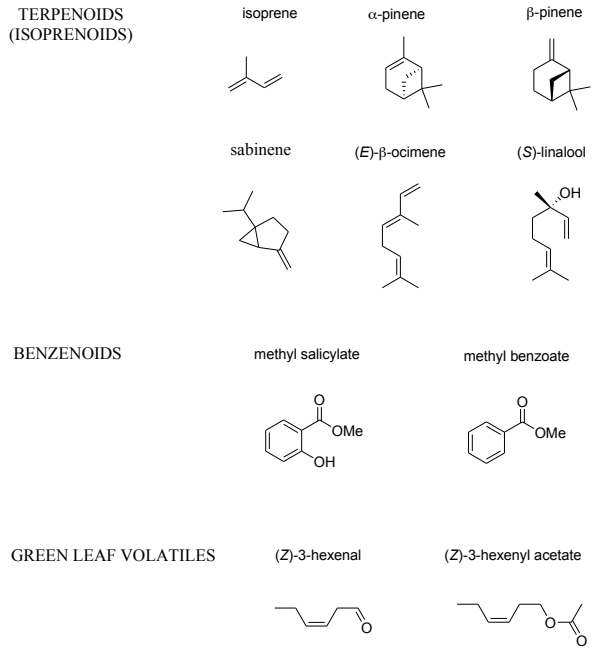

Figure 1.5: Biochemical structures of secondary metabolites indicating certain classification groups according to Dudareva et al. (2006). Structures are drawn in ChemOffice Ultra chemdraw 7.0 software.

From a synthesis point of view, isoprenoid division can be made into *de novo* and storage pool emission. *De novo* synthetized BVOC emissions originate from recently fixed carbon (Ghirardo et al., 2010). Isoprenoid storage pool emission distinguishes nonstoring (e.g. *Arbutus unedo, Erica* sp.) and storing *(*e.g. *Bupleurum fruticosum*, *Cistus albidus* and *Pinus halepensis*) plant species*.* In storing pool emission species, a temporary pool (max 4 h) and a permanent pool (e.g. in resin ducts in conifers, few days) can be distinguished. *De novo* biosynthesis and emission of VOCs include products of the lipoxygenase (LOX) pathway, such as oxylipins, GLVs, as well as many terpenoids, including isoprene, some carotenoid derivatives, indoles and phenolics, including MeSA and aromatic VOCs (Tholl et al., 2006; Maffei, 2010). Additionally, not all isoprenoids are volatile; hence, a classification is made into volatile isoprenoids (VIPs) and non-volatile (non-VIPs) isoprenoids (Vickers et al., 2009). Finally, BVOCs can be classified into essential (carotenoids and gibberellins) and non-essential (isoprene and MTs) (Owen and Peňuelas, 2005). Depending on classification, storage possibility, volatility and function, BVOC emissions act differently (see 1.2).

1.1.2.1 Biosynthesis of BVOCs

Earlier studies indicate only one biochemical pathway for isoprenoids in higher plants, i.e. the acetyl-CoA – mevalonate pathway (MVA) in the cytosol. However, since 1990 two biochemical pathways have been extensively reviewed (Croteau, 1987; Eisenreich et al., 2004; Ershov, 2007; Chang and Keasling, 2006; Lichtenthaler, 2007; Maffei, 2010). Fig. 1.6 illustrates the distinction between the primary and secondary metabolism.

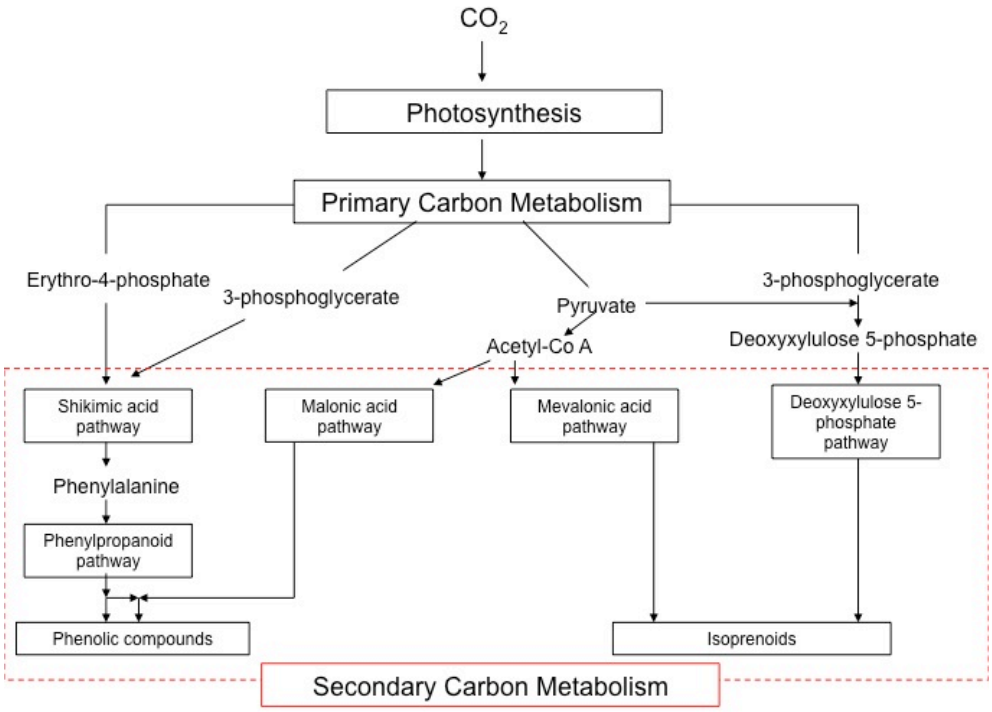

Figure 1.6: Generalized outline of terpene biosynthesis indicating the interconnection with relevant primary compounds and processes (after Langenheim, 2003).

Isoprenoid biosynthesis central intermediates are isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Their formation is divided into two cell compartments: (1) cytosolic MVA-dependent pathway and (2) plastidial non-mevalonate pathway (MVA-independent pathway or non-MVA or DOXP/MEP) (Laule et al., 2003). The MVA-dependent pathway is responsible for the synthesis of sterols, certain sesquiterpenes (SQTs) and the side chain of ubiquinone. In contrast, the recently discovered MVA-independent pathway is involved in providing the precursors for MTs, certain SQTs, diterpenes, carotenoids, and the side chains of chlorophylls and plastoquinone (Laule et al., 2003). However, most recent studies indicate localization of IPP formation in peroxisomes as well (Croteau et al., 2000). Finally, the compartmental separation of these isoprenoid pathways is not absolute (Maes, 2001; Eisenreich et al., 2004).

Biochemically acetyl-CoA is the precursor for the synthesis of isoprenoids in the cytosol, while pyruvate and D-glyceraldehyde-3-phosphate are the precursors in the plastids. Although this subcellular compartmentation allows both pathways to operate independently in plants, there is evidence that they cooperate in the biosynthesis of certain metabolites (Laule et al., 2003). For example, chamomile SQTs are composed of two C_5 isoprenoid units formed via the MVA-independent pathway, with a third unit being derived from the MVA-dependent pathway (Laule et

al., 2003). The cited study indicates an interaction of both pathways for MT and SQT volatiles. Finally, isoprene and MTs are formed in plastids, while SQTs are formed in the cytosol (Fig. 1.7). Likewise, a possible link with the synthesis of starch and sucrose is hypothesized where starch is mainly accumulated in plastids (chloroplasts) and sucrose is accumulated in the cytosol requiring further research.

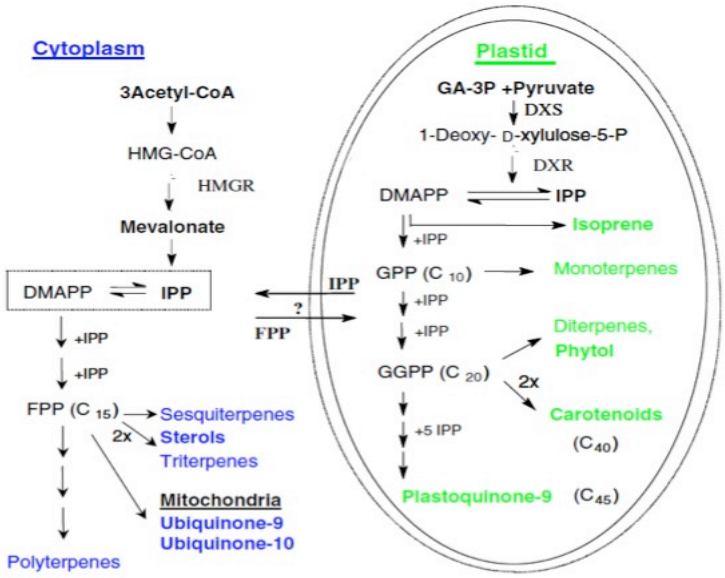

Figure 1.7: Overview of isoprenoid biosynthesis in plants according to plant cell compartment division. (1) the plastidic DOXP/MEP pathway (also termed: MEP pathway, non-mevalonate or non-MVA pathway) for the biosynthesis of the active C_5 unit (IPP) for chlorophylls (phytyl side-chain), carotenoids and prenylquinones (isoprenoid side-chains) and (2) the cytosolic acetate/mevalonate pathway of IPP biosynthesis for the formation of sterols and the prenyl side-chain of the mitochondrial ubiquinones. IPP-isopentenyl diphosphate, FPP-farnesyl diphosphate; GPP-geranyl diphosphate; GGPP-geranylgeranyl diphosphate; HMGR-hydroxymethylglutaryl-CoA reductase (adopted from Lichtenthaler, 2007).

1.1.2.2 Carbon allocation to defense

Plant defense systems depend on energy in the form of carbohydrates (Christiansen et al., 1987). When there is a reduction in some physiological rate (water or nutrient absorption, photosynthesis, respiration, growth, development, reproduction or others) below the maximum possible rate expressed under optimal conditions, a plant is considered as stressed (Reigosa et al., 2006). In order to protect themselves, plants developed defense systems. Many plant studies up till now verified the mechanisms of a self-defense system, including allelopathy, particularly isoprenoid metabolism. The increase of isoprenoid compounds under environmental stresses has been well documented. As photosynthesis under stress conditions could decrease, consequently altering carbon allocation, a difference should be made between normal and stress metabolism (as a shift occurs when stressors are present).

Moreover, stress elicits changes and responses on all functional levels of the organism depending on the degree of severity, time of the year, phenophase, and change in enzyme activity (Larcher, 2003).

Carbon allocation patterns are most vunerable to stress during early life stages, when seedlings are highly vulnerable to herbivores, pathogens, and physical disturbance (Moles and Westoby, 2004; Imaji and Seiwa, 2010). Carbohydrate allocation varies seasonally (and between deciduous and evergreen species). Woody plants store starch and simple carbohydrates (glucose, fructose) (Barbaroux and Breda, 2002; Hoch et al., 2003) as energy reserves supporting recovery from stress and damage (Imaji and Seiwa, 2010), commonly called nonstructural carbohydrates (NSC). Defense is related to a tree's carbon balance in appearance of abiotic and biotic stressors and affected by many internal and external factors, e.g. fluctuating temperatures and drought (Christiansen et al., 1987) (see 1.2).

In woody plants, respiration depletes a significant amount of the daily photosynthate production. Moreover, Rd is a major drain on the carbohydrate resources of infected plants, while reserve carbohydrates are important in preventing infestation: e.g. reduced capacity of stressed trees to withstand bark beetle attack is correlated with low carbohydrate reserves (Christiansen et al., 1987; Kozlowski, 1992). Defense compounds include large amounts and many species of emissions of BVOC. Photosynthesis decreases as a consequence of CO_2 diffusion limitations, or changes in leaf biochemistry resulting in the down-regulation of photosynthesis (Chaves et al., 2003). The concentration of secondary plant compounds depends on abiotic and biotic as well as external and internal factors (e.g., light intensity, drought, waterlogging and flooding, frost, pollution, nutrient supply). Finally, the total tree defensive capacity depends on the tree's capability to mobilize defense chemicals in the distinctive reaction zones surrounding the points of attack (Christiansen et al., 1987). Understanding the carbon allocation to defense is therefore critical in order to interprete Pn-BVOC measurements under stressed conditions. For further reading on stress signalling we refer to box 1.1.

Box 1.1

Plant stress signalling beyond physiological level

Gaseous compounds act as airborne signals mediating inter-plant communication thus affecting not only the challenged plant but also its neighbors (Cheong and Choi, 2003). These airborne BVOC emission signals enter the plant triggering a cascade of cellular signalling events. This results in the defensive chemical(s) production. Thus, this increases plant survival chances (Farmer, 2001). Plant stress signalling is complex and involves gene activation at the nuclear level. Here, focus on cellular level is briefly given. At the cellular level, plants must first perceive stress and then trigger the necessary adaptations. As well, these signalling events may be initiated by small changes in the carbon status (Pinheiro and Chavez, 2011).

Stress signalling is often mediated by reactive oxygen species (ROS), reactive nitrogen species (RNS) and stress hormones such as ethylene (ET), salicylate (SA), jasmonates (JAs), and abscissic acid (ABA). ROS are molecules acting in response to biotic and abiotic stress serving as chemical weapons against attack (Foyer and Noctor, 2005). ROS production is an important inducer of stomatal closure, acting in the pathway for abscisic acid response. Beside ROS, NO radicals are formed serving as hormonal and defense messenger (Wendehenne and Hancock, 2011). This ROS and RNS rise cumulates into a situation termed oxidative stress. Furthermore, all abiotic and biotic stresses impairing net photosynthesis (Pn) and dark respiration (Rd) increase production of ROS (Kranner et al., 2010) and RNS. Together with hormones and antioxidants (Box 1.2) they are components of signalling networks.

As defense reactions are triggered by the interplay of several signal substances in a network they often include other messengers. Therefore, plants produce natural stress hormones for the purpose of signalling adverse conditions. Regarding hormones, the family of negative regulators of gibberellin response, called the DELLA proteins, have an important role in balancing the growth response in both biotic (Navarro et al, 2008) and abiotic stress (Achard et al., 2006). DELLAs are a family of nuclear proteins, mainly studied in *Arabidopsis sp.* with five members acting as growth repressors (Navarro et al., 2008). Additionally, two classes of stress-activated protein kinases (enzymes responding to extracellular stimuli) exist in plants: mitogen-activated protein kinase (MAPKs) and Ca^{2+} dependent protein kinases (CDPKs) (Ludwig et al., 2005). As a response to biotic or abiotic stress, signal cascades are initiated which lead to an increase in Ca^{2+} concentration in the cytosol, by which CDPKs are activated (Ludwig et al., 2005). Stress hormones activate plant defense mechanisms such as jasmonates for example (Reymond and Farmer, 1998). They act as signal transduction intermediates when plants are subjected to environmental stresses such as UV radiation, osmotic shock, heat (Cohen and Flescher, 2009), insects/pathogens, and drought (Cheong and Choi, 2003). Further efforts in oxidative stress characterization and quantification are necessary. The reader is suggested to consult Knight and Knight (2001) and/or Kranner et al. (2010).

1.1.2.3 Importance and function of BVOCs

BVOC emission chemodiversity is just as much a characteristic of life on Earth as biodiversity (Gershenzon and Dudareva, 2007). Many of the BVOC emissions have no apparent function in the basic processes of growth and development, and have been historically referred to as natural products or secondary metabolites (Gershenzon, 2007). However, BVOC emissions have multiple beneficial and adverse functions, and have long been studied for their roles as perfumes, flavorings, pharmaceuticals, pest protection agents (Tillman et al., 1999), importance in insect ecology and neurobiology (pherobase development and odor learning ability of insects (Tertuliano et al., 2005), plant enzymology, pollination ecology, phytochemistry, and plant engineering (Drewnowski and Gomez-Carneros, 2000). Additionally, BVOC emissions are used in human medicines with beneficial (Salminen et al., 2008) and adverse effects (Paulsen et al., 2002). The multiple functions of BVOC emissions have been classified into three groups (Table 1.2).

BVOC ecological functions include antiherbivory and antimicrobial defense, pollinators attraction, allelopathic roles (Owen and Peñuelas, 2005), protection against high temperature episodes, i.e. thermotolerance (Sharkey et al., 1991), communication with other plants or organisms, i.e. plant-insect interactions (Peñuelas and Llusia, 2003; Staudt and Lhoutellier, 2007), and insect pheromone precursors (Tillman et al., 1999). Through their diversity of functions they can be involved in the non-enzymatic plant defense strategy (Edreva et al., 2008) (Box 1.2).

BVOC physiological functions include their role as antioxidants in leaves safety valves, releasing excess carbon and energy (Sharkey et al., 1991), plant ammunition to cope with environmental constraints, deterrents for pathogens and herbivores, contribution to wound sealing (antimicrobial), attraction of pollinators, a possible tool for organic pollutant remediation (enhancing the process of biodegradation), contribution to activity of microbes, and nutrient availability, e.g., MT in the rhizosphere (Lin et al., 2006). The isoprenoids, which constitute the most diverse group of natural products, serve even more numerous biochemical functions in plants. They play important roles as quinones in electron transport chains, as components of membranes (sterols), in subcellular targeting and regulation (prenylation of proteins), as photosynthetic pigments (carotenoids, side chain of chlorophyll), as hormones (gibberellins, brassinosteroids, abscisic acid, and cytokinins), and as plant defense compounds as well as attractants for pollinators (MTs, SQTs, and diterpenes). Secondary metabolites, through their diversity of functions, can be involved in the non-enzymatic plant defense strategy (Edreva et al., 2008) whereas some defensive chemicals do not have essential roles, others do. A variety of secondary compounds, such as BVOCs, are primarily produced for the purpose of protection from biotic and abiotic stress. By emitting various BVOCs, trees communicate, signal and defend themselves in order to overcome stationary life constraints (Piel et al., 1998).

Table 1.2 Overview of some currently known BVOC functions divided into ecological, physiological and atmospheric functions.

Ecological	Physiological	Atmospheric
Plant-plant interactions	Pathogen defense	Oxidative capacity
Plant-insect interactions	Thermo and – photo protection	Photochemical smog
	Oxidative stress tolerance	Maintain redox potential of atmosphere
Herbivory protection	ROS detoxification	Influencing methane lifetime
Attractants		Air quality
Repellents		
Toxins		

Besides the importance of their ecological and physiological functions, VOCs and BVOCs (as well as AVOCs) play an important role in the composition and chemistry of the atmosphere. This has been well accepted since the pioneering work of Frits Went (1960), and AVOCs have been measured for about the last fifty years. As a consequence, VOC emission control strategies have been implemented. In 1952, the biochemist Arie J. Haagen-Smit discovered that the primary constituent in Los Angeles' smog was tropospheric ozone (O_3), an air pollutant. Ozone is an irritant of human airways, making it a serious health threat (Lippman, 1991; Gurjar et al., 2010). In contrast, BVOCs do not have a long history of measurements. However, nowadays, it is known that they indirectly contribute to the formation of harmful components such as photochemical smog, tropospheric O_3, secondary organic aerosol (SOA) (Claeys, 2004) and (methyl) peroxy acyl nitrate (PAN and MPAN) formation (Koppmann, 2007). Aerosols have an effect on the solar radiation balance and global radiative forcing. Some aerosols cause a positive forcing (warming) but others, such as biogenic secondary organic aerosol cause a negative forcing (cooling). Aerosols such as biogenic secondary organic aerosol also have an indirect effect on climate through their effects on cloud properties.

Box 1.2

BVOC and oxidative stress

Paracelsus "it is the dose that makes the poison" (1538)

Kranner "free radicals are not all bad, nor antioxidants all good" (2010)

Plants have developed antioxidant strategies to scavenge toxic compounds. All stressors ultimately lead to oxidative stress. Oxidative stress is characterized by formation of ROS such as superoxide radicals ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2) and $OH^{\bullet}$. The antioxidant system is composed of enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants (ROS scavengers) include enzymes such as catalase, superoxide dismutase (SOD), ascorbate peroxidase (APX), and glutathione reductase (GHS). Non-enzymatic molecules include ascorbate, glutathione, carotenoids, and anthocyanins (Wang et al., 2003). The reader is further guided to read Foyer and Noctor (2005) and Gan (2007).

1.1.3 Link between photosynthesis and BVOC emissions

Photosynthesis belongs to the primary metabolism, while BVOC emissions relate to the secondary metabolism. However, possible crosstalks between both processes exist. Photosynthesis is related to VOC production as trees emit a fraction of their carbon fixed by photosynthesis into the atmosphere as BVOC emissions (Yuan et al., 2009). Many studies have demonstrated a link between photosynthesis and the instantaneous BVOC emissions (Sanadze, 1969; Rasmussen, 1972). Isoprene emission occurs without a leaf reservoir and is linked to the photosynthetic metabolism (Monson et al., 1995; Koppmann, 2007). However, other carbon reserves can be tapped to produce isoprene even when the photosynthetic cycle is operating under drought or temperature stress (Koppmann, 2007). In contrast, MT emission occurs from a large leaf reservoir and is likely linked to whole-plant carbon allocation patterns. A basic tenet of applying ecological resource models to basal MT emission is that MT production is controlled by the plant carbon balance, i.e. the balance between photosynthetic carbon assimilation and its utilization in the production of new growth (Monson et al., 1995). The major biological role of MTs is herbivore defense. Deterrence of herbivores is most likely due to toxic effects of MT ingestion on animal mitochondrial function. Species-specific isoprenoids carbon loss through BVOC emission, and thus the plant's carbon budget, might change with global warming due to rising air temperatures (Box 1.3). When released into the atmosphere via stomata (Rennenberg et al., 2006; Tani and Kawawata, 2008), BVOC emissions represent a large carbon loss, which can be up to ~10% of that fixed by Pn (Sharkey and Singsaas, 1995; Peňuelas and Llusia, 2003; Firn and Jones, 2006) and even higher in stress conditions (25-36%) (Kesselmeier et al., 2002; Brilli et al., 2009). The main conclusion is that indeed a strong relationship between photosynthesis and BVOC emissions may exist (Fig. 1.8). Unravelling the right mix of chemical processes responsible for this effect remains a challenge (Bruce and Pickett, 2011).

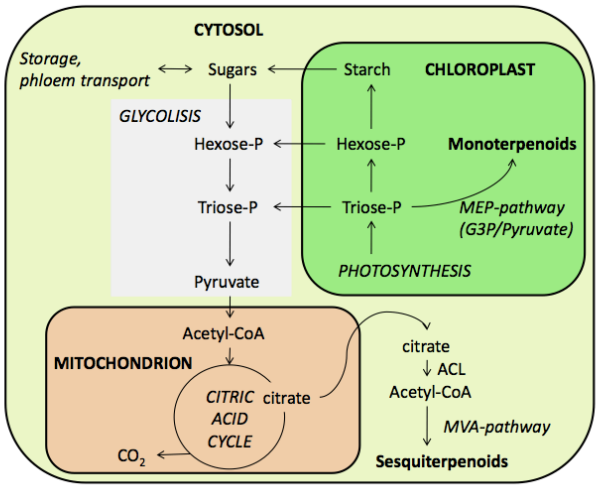

Figure 1.8: Schematic overview of photosynthesis and BVOC biosynthesis in the plant cell. Photosynthesis, starch synthesis and monoterpenoids (MT) synthesis occur in the chloroplasts. Glycolysis in the cytosol leads to the formation of pyruvate, ATP, and NADPH. The formed pyruvate drives the Krebs cycle (lemon acid cycle) in mitochondria with formation of CO_2, ATP, and $FADH_2$. A part of this formed citric acid goes to the cytosol for the production of acetyl-CoA under ATP-citrate lyase (ACL). This source of acetyl-CoA transforms to the mevalonate (MVA) pathway with formation of sesquiterpenoids (adapted from Fatland et al., 2005; Taiz and Zeiger, 2010).

Box 1.3.

Photosynthesis and BVOC emissions under climate change

The short-term responses of Pn to increased atmospheric CO_2 concentrations are well understood: the rate of Pn increases (in the order of 40-80%) due to an increased amount of substrate (Medlyn et al., 2002; Hyvonen et al., 2006). Large-scale free air CO_2 enrichment (FACE) experiments in the USA have also shown increased Pn upon enhanced CO_2 treatment on a short-term (few weeks). In contrast, long-term (few months) responses are however less understood. Effects of enhanced CO_2 on Pn and BVOC emissions yield conflicting results, and further research is still needed. Laboratory measurements showed reduced emissions or no effect on a short term (Laothawornkitkul et al., 2009). In contrast, long-term results indicated consistent, but apparently species-specific results. Elevated CO_2 reduced isoprene emissions from aspen while in contrast emissions increased in oak. Reductions in MT emissions have been explained by a decreased enzyme activity (Loreto and Velikova, 2001), while increases in emissions are explained by protection against oxidative stress (Yuan et al., 2009).

Besides increasing atmospheric CO_2 concentrations, tropospheric O_3 has also been increasing for several decades (Brasseur et al., 1999). For clarification, there exist two kinds of O_3, beneficial (stratospheric) and detrimental (tropospheric). Here, focus on tropospheric O_3 is given. In general, it may be considered that the primary effect of O_3 is disrupting the carbon assimilation in leaves and decreases the supply of carbon to roots (Monson, 2003). More specifically, O_3 affects Pn by altering stomatal functioning, decreasing the activity and concentration of Rubisco, decreasing protein synthesis associated with photosynthetic enzymes (Dann and Pell, 1989). Additionally, it reduces the

amount of light harvesting complexes (LHC), negatively affects carbon translocation to the roots, reduces leaf longevity, increases leaf senescence and reduces the overall carbon gain (Dan and Pell, 1989; Zheng et al., 2002). Increased BVOC emissions under increased O_3 exposure have been observed for isoprene. Recently, endogenous isoprene was identified as an antioxidant, protecting against O_3 (Loreto and Velikova, 2001). Ozone stimulates a shift of the available resources in favor of the synthesis of secondary products (Iriti and Faoro, 2009) causing oxidative stress in plants (Yuan et al., 2009). Once O_3 enters the cell it is converted to reactive oxygen species (ROS) and additionally triggers oxidative burst, developing hypersensitive response (HR) and protecting plants from oxidative stress (Yuan et al., 2009).

Climate change influences Pn and BVOC emissions by prolonging the growing season (Peñuelas and Staudt, 2010). Additionally, climate change influences plant physiology and stomatal functioning, leading to changes in BVOC biosynthesis pathways (Yuan et al., 2009). For example, SQTs react with O_3, having a high potential to form secondary (formed in the atmosphere from gaseous precursors) organic aerosols (Tarvainen et al., 2005). This threat indicates that more research is needed.

Both trace gasses, CO_2 and O_3, and climate change affect trees by impairing Pn and BVOC emissions, making them more susceptible to other physiological stressors such as pests and herbivore attacks. Exposure to O_3 can also modify the pattern of BVOC emissions in response to herbivore feeding, altering interactions among plant, phytofagi and natural enemies (Iriti and Faoro, 2009). Changes in CO_2 and O_3 concentrations in future atmospheres could also have significant effects on the patterns, magnitude and stability of BVOCs that affect atmospheric reactions contributing to SOA formation, as well as on those emissions released by the actions of predator and parasitoid insects during multitrophic signaling (Yuan et al., 2009).

Increased CO_2, O_3 and H_2O could have significant effects on Pn/growth and BVOC emissions/defense. Much less is however known about feedback effects on Pn and BVOC determined by multiple influencing factors.

1.1.4 Photosynthesis and BVOC emissions under normal and stressed conditions

Environmental stresses and air pollution cause serious problems to photosynthesis and BVOC emissions. Due to the uncertainty in this field, intensive research should contribute to a deeper understanding of the mechanisms by which plants adapt to and cope with adverse environments and survive (Centritto et al., 2004). In nature, trees are exposed naturally to beneficial and harmful pests, possibly altering the leaf carbon budget. Under stress, leaves could release more carbon as isoprenoids (and Rd) than gained thorough Pn (Fowler et al., 2009). Stressed trees were reported to mobilize a large part of stored carbohydrates into sugars and to convert proteins and other nitrogenous compounds to more soluble forms of amino acids (Kozlowski, 1992). A portion of the carbohydrate pool is diverted for production of chemicals involved in defense against fungi, herbivores, and competing plants (Kozlowski, 1992) (see 1.1.2.2). Stored carbohydrates play an important role in metabolism, growth, defense, cold hardiness, and delay or prevention of plant mortality

(Kozlowski, 1992; Dickson and Tromlinson, 1996). All abiotic and biotic stresses impairing Pn and Rd electron transport increase the production of reactive oxygen species (ROS) (Kranner et al., 2010).

However, an undamaged plant maintains a baseline level of BVOC emissions released from the leaf surface and/or from leaf accumulated storage sites. These constitutive chemical reserves, which often include MTs, accumulate to high levels in specialized glands or trichomes (Paré and Tumlinson, 1997a; Maes, 2001).

Obvious differences in BVOC emissions exist between non-stressed and stressed plants. Under stressed conditions, emitted BVOCs include increased levels of MTs and/or SQTs. Under acute stress conditions (e.g. insect attacks, O_3, heat), other BVOCs, such as green leaf volatiles (GLVs), have been reported (Hatanaka, 1993; Fowler et al., 2009) produced by another biosynthetic pathway (i.e. octadecanoid pathway). A variety of pathogens and plant-produced molecules, collectively known as elicitors, will induce BVOC emissions and other defense responses (see 1.2.5).

1.2 Factors affecting photosynthesis and BVOC emissions

Environmental influencing factors of photosynthesis and BVOC emissions can be classified into two major categories: (1) internal factors (genetical and biochemical) and (2) external factors, subdivided into biotic and abiotic factors (Marin-Loaiza and Cespedes, 2007). Here, we focus on light, temperature, drought, seasonality, and infestations only.

1.2.1 Light

Light is electromagnetic radiation and is characterized by its wavelength where radiation with wavelengths 400-700 nm is regarded as photosynthetically active. Its energy can be absorbed by plant pigments and transduced to chemical energy through Pn (Porcar-Castell, 2008). Light is vital for plant growth and development and plants utilize it in two distinctive ways: as a source of energy and as a source of spatial and temporal information (Heldt, 2005). As it is a driving factor for Pn, a typical result of Pn as a function of light is a light response curve, saturating at a higher light intensity. Similarly, in response to radiation, the BVOC emission curve shows a saturating behavior (Kesselmeier and Staudt, 1999).

Leaf position in the canopy determines the light intensity and important leaf physiological, biochemical, and anatomical differences (Johnson et al., 1997). Hereafter, sun and shade leaves pose important differences. Consequently, these sun and shade differences influence photosynthesis and BVOC emissions (Fig. 1.9).

Moreover, besides differences in leaf anatomy also differences in sun and shade chloroplast types exist (Lichtenthaler, 2007; Sarijeva et al., 2007).

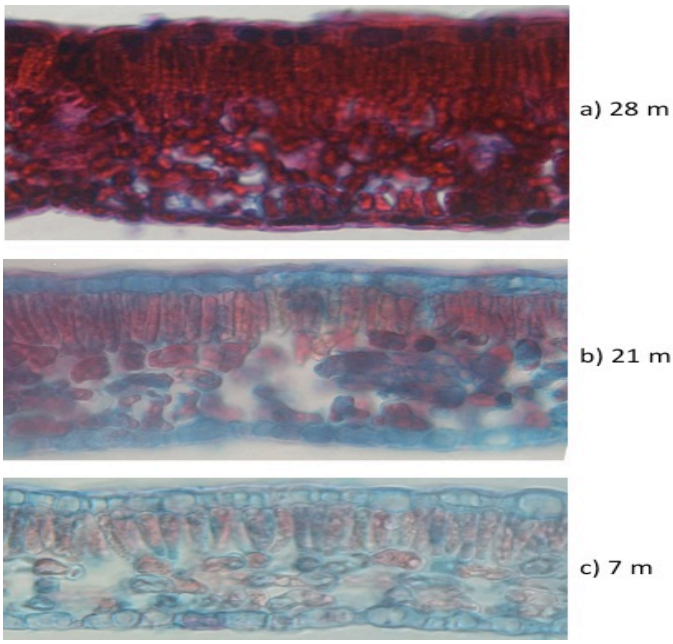

Figure 1.9: Anatomical differences between sun (a, 28m), semi-shade (b, 21m) and shade (c, 7m) leaves of an adult beech tree showing difference in amount of palisade parenchyma and amount of chloroplasts performed in Aelmoeseneie experimental forest on beech leaves in the 2008 growing season (adopted from Van Wittenberghe et al., 2012).

The light capture and energy utilization are regulated in a coordinated manner to prevent oxidative damage to the photosynthetic apparatus (Busch et al., 2008). In general, the higher the light intensity, the greater Pn (Lambers et al., 1998; Casella and Ceulemans, 2002; Le Goff et al., 2004; Lombardini et al., 2009), resulting in clear differences in Pn rate between sun and shade leaves (Larcher, 2003). Additionally, sun/shade leaves differ in antioxidant (carotenoid, α-tocopherol and plastoquinone) content, with sun leaves containing more antioxidants than their shaded counterparts.

In many plant species, the rates of MT synthesis depend on light, similarly to Pn rates (Schuh et al., 1997; Tarvainen et al., 2005), and there is conclusive evidence that part of the emitted compounds originates from a small pool of immediately assimilated carbon (Niinemets et al., 2002). Even species with extensive MT storage pools in resin ducts, such as conifers, often show light-dependent MT emission patterns (Staudt et al., 1997; Kesselmeier and Staudt, 1999; Noe et al., 2006). While many studies have shown MT emissions are dominantly a function of temperature and are not impacted by light (Dement et al., 1975; Guenther et al., 1993; Koppmann, 2007), others have reported MT emissions are a function of both light and temperature (Koppmann, 2007). The mechanisms and functions of these two categories of isoprenoid-emitting plants, i.e. (1) temperature-dependent only and

(2) temperature- and light-dependent emitters, are quite different (Koppmann, 2007). Isoprene emission is strongly light dependent. Even long term light effects have been reported for isoprene, with higher emission capacities for sun rather than shaded leaves (Harley et al., 1998; Lerdau and Throop, 1999; Litvak et al., 1999; Koppmann, 2007). Light indirectly influences the MT pool by (1) providing biosynthetic energy, (2) controlling leaf anatomical changes, and (3) photoregulating MT synthesis (Koppmann, 2007). Interestingly, in light to dark transition experiments, Pn stopped after one minute, while BVOC emissions stopped after fifteen minutes indicating a lack of reducing power (Sharkey et al., 1991).

1.2.2 Temperature

Temperature affects all biochemical reactions, including those involved in the photosynthesis process, so it is not surprising that the responses to temperature are complex. Changes in temperature trigger physiological and seasonal developmental processes. Temperature affects metabolic processes influencing the reaction kinetics of chemical events and the activities of the various enzymes involved (Larcher, 2003). Photosynthesis responds to temperature changes in the form of an optimum curve. Suboptimal temperatures cause slow carbon fixation (Fig 1.10). The rates at which carbon is fixed increase as temperature rises until an optimal value. Above optimal values the CO_2/O_2 ratio decreases, resulting in a decrease in carboxylation efficiency of the Rubisco (RuBP carboxylase/oxygenase) (Larcher, 2003). However, the range of the temperature optimum is plant-specific. The rates of Pn (and Rd) adapt to the temperature prevailing at a given time. When acclimating to the thermal conditions of a habitat, the temperature optimum and limit of Pn undergo modulative shifts (Larcher, 2003).

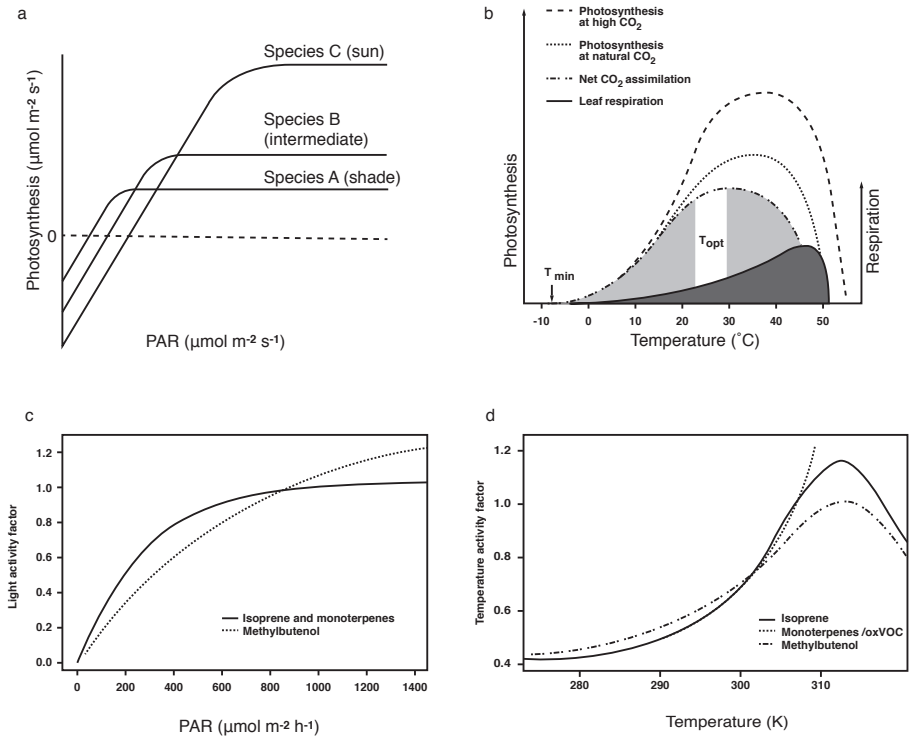

Figure 1.10: Light and temperature impact on net photosynthesis (Pn) and BVOC emissions. a) light response curves of Pn in plants acclimated to low, intermediate, and intense light. b) temperature response curves of Pn in plants at high and natural CO_2 concentration, c) light response curves for BVOC emissions, and d) temperature response curves of BVOC emissions (after Koppmann, 2007). PAR indicates photosynthetically active radiation.

Already in 1975, Dement et al. (1975) noted a temperature dependence of BVOC emissions. Afterwards, many authors observed a similar behavior (Tingey et al., 1980; Guenther et al., 1993; Harley et al., 1998) isoprene emissions increasing with temperature up to an optimum at 35-40 °C afterwards emissions decrease. This implies enzymatic dependence in emissions as well as in Pn. Recently, Singsaas and Sharkey (2000) explained this phenomenon by a regulatory mechanism rather than isoprene synthase destruction (Koppmann, 2007). As isoprene and light-dependent MT emitters have a different storage and release mechanism than non-light dependent MT emitters, their temperature dependences differ as well (Koppmann, 2007). Emission rates of temperature-dependent MT emitters increase exponentially with increasing temperature. This can be explained by the MT storage pool (Fig. 1.11) linking the emissions to MT volatility and Henry's law constant. Temperature increases the emission rate of most BVOCs exponentially by enhancing the enzymatic activities of synthesis, by raising the BVOC vapor pressure and by decreasing the resistance of the diffusion pathway (Peňuelas and Llusia, 2003).

Finally, various authors also observed longer-term effects of temperature, with different past number of days (Sharkey and Lerdau, 1999; Demarcke et al., 2010).

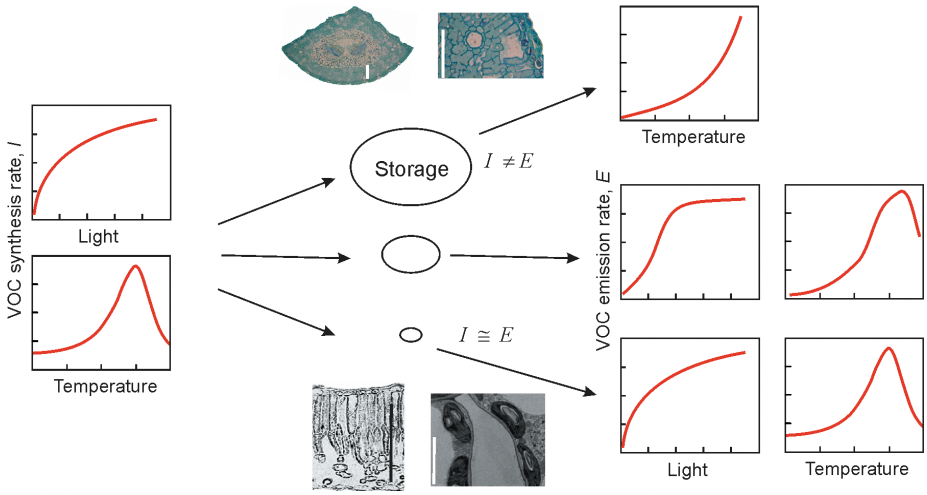

Figure 1.11: Light and temperature effects on BVOC emissions for storing and non-storing plant species (Grote and Niinemets, 2008; Laothawornkitkul et al., 2009).

1.2.3 Drought

Photosynthesis and BVOC emissions are both affected by drought (Delfine et al., 2005; Hummel et al., 2010; Pinheiro and Chavez, 2011). Drought reduces Pn by stomatal closure, leading to large morphological and physiological changes (Hummel et al., 2010), increasing leaf temperature and metabolic impairment (Escalona et al., 2002). Growth and carbon fluxes may severely be altered during drought (Hummel et al., 2010) typically allocating more carbon to non-photosynthetic organs (e.g. roots, change in root/shoot ratio) (Vivin et al., 1996) or supporting structures (Hummel et al., 2010). Besides Pn and growth reduction, drought affects many aspects of plant physiology: transpiration, sap flow, leaf water potential, C/N ratio, specific leaf area (SLA), ability to translocate photosynthates, accumulation of osmotics, sink-source relationship, and ratio between respiratory needs for growth and maintainance (Pinheiro and Chavez, 2011). Thus, osmotic adjustment on the one hand and growth and metabolic processes on the other hand may compete for the limited carbon supply under drought (Vivin et al., 1996). Drought-stressed plants show decreased carbohydrate export rates, signifying the translocation of photosynthates is affected. When Pn is affected by stress, the consumption of photosynthates decreases.

All these altered physiological processes could lead to altered BVOC emissions and various effects depending on stress severity. Pn decrease leads to a change in carbon supply for the BVOC biosynthesis pathways. During moderate drought stress, emissions could be explained by usage of alternative carbon sources

at the moment when Pn is drastically limited (Brilli et al., 2007; Loreto and Schnitzler, 2010). Under these drought conditions, Pn is inhibited, but not the electron transport for synthesizing ATP and NADPH. These energy sources may, together with alternative carbon sources (sugars and starch), maintain isoprene synthesis (Niinemets, 2010). In contrast, in young plants, Loreto and Schnitzler (2010) observed moderate drought stress had no effect on BVOC emissions. Others observed a BVOC increase during moderate stress (Bertin and Staudt, 1996; Pegoraro et al., 2004). In the same study, acute drought stress reduced emissions, explained by exhausted carbon reserve sources (Pegoraro et al., 2004; Loreto and Schnitzler, 2010). These observations might be partly explained by changes in DMAPP allocation in the isoprenoid biochemical pathway to respond chemically to the needs of the stressed plant (Owen and Peñuelas, 2005). Drought stress in *Pinus taeda* caused an increase in MT emissions by the needles (Sharkey et al., 1991). Changes in leaf water potential (from -0.6 to -0.9 MPa) corresponded with significant increases in MT emissions in *Satureja douglassi* (Gershenzon et al., 1978). Finally, after acute stress and re-watering BVOC emissions could again appear and/or even increase (Kesselmeier and Staudt, 1999).

1.2.4 Seasonal variation (phenology)

Phenological changes in leaves include physical, biological, and chemical changes. Tree phenology depends on the anatomical properties of a tree species, with ring-porous species starting wood growth before leaf unfolding. Diffuse-porous trees start growth after leaf unfolding. Therefore, the seasonal Pn variation is related to the type of wood formation (Prislan et al., 2009). Seasonal Pn as well as isoprene synthase activity increases gradually until full leaf maturation and decreases thereafter with the onset of leaf senescence (Schnitzler et al., 1997; Niinemets et al., 2010). An increase in Pn is explained by accumulation of Rubisco in the leaves (Eichelmann et al., 2004), changes in total non-structural carbohydrate (starch and sugars, NSC) concentration (Barbaroux and Breda, 2002) and an increase in chlorophyll (Richardson et al., 2011) and leaf N (Montpied et al., 2009; Varinderpal-Singh et al., 2010) content. Additionally, Pn changes as foliage ages and senesces (Medlyn et al., 2002). In late August for temperate forests, this is typically explained by decreasing tree metabolism and reallocation of nutrients (Keskitalo et al., 2005). This could be interpreted as the anticipated leaf senescence symptom (Grassi and Magnani, 2005).

Seasonal factors such as budburst, growth, aging, and senescence, all influence BVOC emissions. Emissions are known to begin 2-4 weeks after budburst (Owen and Peňuelas, 2005). Another important factor regarding phenology is leaf age and development. For example, MBO emissions were found to decrease with increasing leaf age (Harley et al., 1998) and leaf MT concentrations changed greatly during the first six months of growth and then gradually stabilized (Sharkey et al., 1991). Earlier studies indicated beech as a non-MT emitter, but recent studies show

it can emit MT, mostly sabinene and α-pinene (Schuh et al., 1997; Holzke et al., 2006; Joó et al., 2010). At the stand level, Gallagher et al. (2000) found Δ³-carene as the most abundant emitted MT by a beech forest. Extensive literature indicates that oak emissions are dichotomized: MT emitters and isoprene emitters exist depending on the species. Literature about ash is more limited. Phenological periods during which Pn is high, but growth is limited such as under stress might correspond to a high MT production. After bud break, when both Pn and growth are high, plants are expected to allocate less to MTs in non-stressed conditions. Concurrently, isoprenoid production declines later in the season when growth and Pn decline. Many plants also show a general pattern of decreasing their allocation to mobile defense compounds across the growing season (Sharkey et al., 1991).

1.2.5 Infestation

It is well known that infestations have an impact on both Pn and BVOC emissions. Many authors have demonstrated infestation influences on Pn, where clear decreasing trends in Pn have been observed due to trips, stink bugs, and gypsy moths (Ellsworth and Reich, 1993; Staudt and Lhoureillier, 2007; Velikova et al., 2010). Additionally, other processes are affected by infestation. Flinn et al. (1990) reported that infestation by the potato leafhopper (*Empoasca fabae*) increased leaf total non-structural carbohydrates compared to non-infested alfalfa plants (Watanabe and Kitagawa, 2000). The same authors reported a disruption of translocation by *Empoasca fabae* feeding by using a ^{14}C fixation method. Infestation with the brown planthopper (*Nilaparvata lugens)* accelerated the decomposition of Rubisco to amino acids and Pn reduction started before the reduction of total N in the leaf (Watanabe and Kitagawa, 2000). Nabity et al. (2009) proved an indirect suppression of Pn on individual leaves by arthropod herbivory. These studies show that infestations cause physiological as well as metabolic changes. Besides insects, also pathogens (fungi, bacteria, viruses) increase the activity of several enzymes, particularly those associated with the generation of energy (Rd) or with production/oxidation of various defense-related compounds (Agrios, 2005).

Plant defenses can be induced by pathogens and herbivores (Walling, 2000). The mechanisms involved in these defenses are being elucidated rapidly (Kessler and Baldwin, 2002; De Vos et al., 2005; Kant and Baldwin, 2007; Pieterse and Dicke, 2007). Leaves normally release small quantities of BVOCs, but when herbivores damage the plant, more volatiles are being released (Paré and Tumlinson, 1997b). These are commonly called herbivore-induced plant volatiles (HIPVs). *Nicotiana tabacum* for example releases several HIPVs exclusively at night. These nocturnally emitted compounds repel female moths (*Heliothis virescens*) searching for oviposition sites (Dudareva et al., 2006).

A distinction can be made between constitutive and induced emissions (Fontana et al., 2011). Constitutive emissions are already present in plants, while

induced emissions are synthesized after infection. These inducible defense substances are called phytoallexins, comprising isoprenoids. Their synthesis is induced by so-called elicitors-pathogen excreted proteins (Heldt, 2005). They signal other parts of the same plant as well as uninfected plants to induce defense mechanisms. Within induced emissions there are two classes: (1) green leaf volatiles (GLVs), emitted immediately after wounding (minutes) and not specifically linked to infestations (Davidson et al., 2008; Brilli et al., 2009; Arneth and Niinemets, 2010; Niinemets, 2010); and (2) compounds that are emitted hours-days after infection, indicating induction of specific genes (Brilli et al., 2009). They consist of, for example, MTs, SQTs, MeSA and methyl jasmonate (MeJA). Interestingly, Kant et al. (2008) showed the capability of the two-spotted spider mite (*Tetranychus urticae)* to suppress the induced defenses of tomato plants (Kant et al., 2008; Sarmento et al., 2011). Finally, Holopainen (2004) indicated multiple functions of inducible BVOCs (1.1.2.3).

1.2.6 Other factors

Besides the above mentioned common factors, other factors might influence Pn and BVOC emissions, such as an increased atmospheric CO_2 concentration, tropospheric O_3 (Box 1.4), flooding (Copolovici and Niinemets, 2010), relative humidity, leaf wetness (Kim, 2001), nutrient status (Sharkey et al., 1991), circardian rhythms (Yuan et al., 2009), mesophyll CO_2 concentration (Ruuskanen et al., 2005), leaf oil content (Lerdau et al., 1994), genetic variability, and cultivar type. Even within tree species, diversity exists in BVOC emissions between different cultivars and ecotypes (Yuan et al., 2009).

Box 1.4

Tropospheric chemistry – BVOC perspective

Climatic and anthropogenic conditions facilitate the formation of photochemical oxidants (Manes et al., 1999). The main drivers of the tropospheric chemistry are the nitrogen oxides (NOx) family, grouping nitrogen oxide (NO) and nitrogen dioxide (NO_2) (Capouet, 2005). When VOCs are degraded in polluted air, NO is oxygenated to NO_2 that photolyzes and contributes to the formation of tropospheric O_3, which is 'the bad O_3'. In the case of high NOx levels, isoprene breakdown can lead to the formation of peroxyacetyl nitrates (PAN) and methyl PAN (MPAN). In general, the larger the carbon component, the more toxic the compounds are. The concentration of isoprene in the atmosphere is generally very low (< 10 ppb). Certain hydrocarbons, such as ethylene (CH_2CH_2), are plant growth regulators, while others are harmful to humans. Unsaturated hydrocarbons have the ability to promote the formation of photochemical smog in the presence of NOx, strong sunlight and stable meteorological conditions. Photochemical smog is the product of chemical reactions driven by sunlight, involving NOx of urban and industrial origin and VOCs from either

vegetation (biogenic, BVOC) or human activities (anthropogenic, AVOC). Finally, the detrimental O_3 and PANs produced are injurious to many life forms including trees (Dittmar et al., 2005). The principal global sink for O_3 is photolysis in the presence of water vapor (Jacob and Winner, 2009). However, higher water vapor in the future climate is expected to decrease the O_3 background so that pollution and background O_3 have opposite sensitivities to climate change.

1.3 Measurement techniques

Over the past years, Pn measurement techniques have not changed much, while, on the contrary, important scientific breakthroughs have been achieved in BVOC measurement techniques. In order to provide more representative BVOC profiles, traditional methods (e.g. solvent extraction or steam distillation) have been replaced by headspace (gas-phase) analysis techniques (Dewulf et al., 2002; Tholl et al., 2006). Besides manually operated BVOC sampling methods, automated BVOC analysis systems are now also available (Tholl et al., 2006).

The principal methods and latest technologies used in the analysis of BVOC emissions are discussed in detail in Tholl et al. (2006). Dynamic headspace adsorption, static headspace adsorption (e.g. Solid-Phase Microextraction (SPME)), gas chromatography (GC) coupled to different detectors, flame ionization detection (FID), mass spectrometry (MS) and electroantennogram detection (EAD) are important examples of commonly used techniques for measuring plant volatiles. Recently, real-time monitoring with proton transfer reaction mass spectrometry (PTR-MS) and selected ion-flow-tube mass spectrometry (SIFT-MS) have been developed (Biasioli et al., 2011). In this PhD work, both manual and automated measurement techniques for Pn and BVOC emissions were used. A brief overview is presented here. The calibration is a critical step in experimental techniques and therefore will be described briefly for each instrument.

1.3.1 Measurement of photosynthesis

For the measurement of Pn, different CO_2 gas analyzers exist, differing in the detection of infrared energy. Here, only the open differential infrared gas analyzer (IRGA) will be discussed (Fig. 1.12a). Two air streams are used in this system. One is the reference air stream that always flows through the reference tube towards the reference detector of the analyzer. The second one is an air stream passed over the leaf and flows through the sample (measuring) tube towards the sample detector of the analyzer. The difference in CO_2 level is detected and is determined from the instrument calibration (Sharkey et al., 1991). Calibration CO_2 bottles were used to calibrate the differential IRGA. The calibration gas mixtures consisted of N_2 and CO_2 in different

known concentrations. Hereafter, zeroing and spanning were performed. Zeroing sets the actual measured gas concentration to zero. Afterwards, spanning was performed to set actually measured gas concentration to span gas setpoint. Difference in CO_2 concentration registered with IRGA was used for determination of Pn rate in the cuvettes according to the formula:

$$Pn = \frac{\left[\left[(\mathrm{CO}_2)out - (\mathrm{CO}_2)in\right] \times F\right]}{LA} \qquad (1)$$

where Pn is net photosynthesis; CO_2out is CO_2 concentration outside cuvette; CO_2in is CO_2 concentration inside cuvette; F is the flow entering the cuvette in L min^{-1} and LA is the leaf area enclosed in the cuvette in m^2.

Measurements of Pn and Rd are usually expressed as rates of CO_2 exchange per unit time per unit leaf area (μmol-CO_2 m^{-2} s^{-1}). Pn is measured as a decrease in CO_2 concentration in the sample air when light is provided to the leaves. In contrast, Rd is measured as an increase in CO_2 concentration in the sample air when the leaves are kept in darkness.

Three types of IRGAs were used in this PhD work: BINOS 100 4P (Effeltrich, D), ADC 2250 (IRGA, Bioscientific, UK) and LI-COR 6400xt (IRGA, LI-COR, Lincoln, NE, USA) (Fig. 1.12b-d).

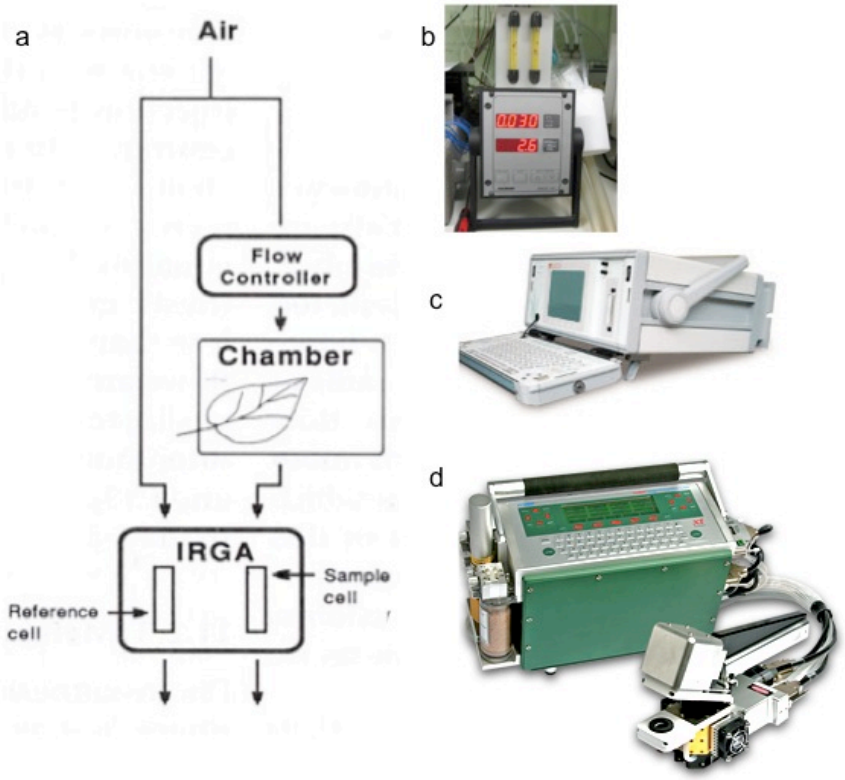

Figure 1.12: (a) Simplified scheme of an open differential infrared gas analyzer (IRGA) system, (b) stationary differential BINOS, (c) stationary ADC and (d) portable LICOR 6400xt used for photosynthesis (Pn) measurements.

1.3.2 Measurement of BVOC emissions

There is no single method which meets the requirements for determining the whole set of BVOCs emitted by plants. In this PhD thesis, two complementary analytical techniques, namely, off-line thermal desorption gas chromatography mass spectrometry (TD-GC/MS) and on-line proton transfer reaction mass spectrometry (PTR-MS), were used. Each technique is presented in detail in other IMPECVOC publications (Demarcke et al., 2010; Joó et al., 2010a,b; 2011; Pokorska et al., 2011; Pokorska, 2012) and is briefly described below.

PTR-MS is ideally suited for long-term, fast, on-line monitoring of BVOCs, but lacks BVOC identification possibilities. In contrast, TD-GC/MS is labour-intensive, limiting the measurement frequency, but it is an excellent technique for identification and quantification of individual BVOC species. The use of Tenax/Carbotrap sorption media, however, does not allow the collection of lower molecular weight ($<C_5$) VOCs quantitatively when used at ambient temperatures (Helas et al., 1997). PTR-MS, on the other hand, is well suited to measure low molecular weight oxygenated compounds (e.g. methanol, acetone, acetaldehyde).

1.3.2.1 BVOC analysis using PTR-MS

For automatic BVOC quantification, proton transfer reaction mass spectrometry (PTR-MS) was used (Fig. 1.13a). It is a fast, sensitive and continuous technique applicable to any compound that has a higher proton affinity than H_2O, as is the case for most VOCs (Koppmann, 2007). The instrument itself consists of (1) an ion source, (2) a drift tube reactor (where the gas-phase analytes are ionized), (3) a mass spectrometer, and (4) an ion detection system. In contrast to TD-GC/MS, PTR-MS is an on-line technique and does not require sample preconcentration. This results in a high measurement frequency (typically 0.1-10 Hz, depending on the number of compounds being measured) and reduces the risk of sampling artefacts. The fundamental difference from conventional MS is that PTR-MS uses soft chemical ionization of VOC molecules by reaction with hydronium ions (H_3O^+), which are produced in a hollow cathode discharge in pure H_2O vapor. Primary H_3O^+ ions enter the drift tube that is flushed continuously with ambient air and undergo non-reactive collisions with any of the main air components (N_2, O_2, Ar, CO_2). Typically 1% of the primary H_3O^+ ions transfer a proton to VOCs which are present as trace gases in air and which have proton affinities higher than that of H_2O (Tani et al., 2003; Tholl et al., 2006). The resulting protonated analyte molecules and possible fragment ions, which are considered as estimator ions of the VOCs of interest, are sampled downstream the drift tube and analyzed in the mass spectrometer.

The PTR-MS ion signal at *m/z* 137 (monoterpenoids) was used to monitor the sum of monoterpenoids. Ion signals at *m/z* 33, *m/z* 59, *m/z* 69 and *m/z* 81 were also continuously recorded as signatures for methanol, acetone, isoprene and monoterpenes, respectively. These ion signals however were not the focus of this PhD dissertation with the exception of *m/z* 81 as additional signal to *m/z* 137 in Chapter 3 only. For the PTR-MS analysis, the mixing ratio X_{voc} (ppbv) of the reactant VOC in ambient air is calculated from:

$$x_{VOC} = \frac{P_m \times 10^6}{S_{VOC,m} \times R_{21} \times 500} \qquad (2)$$

where P_m the VOC product ion signal at mass m expressed in normalized counts per second (ncps) where normalization refers to the product ion count rate that would be obtained at a reactant H_3O^+ count rate of 10^6 counts per second; R_{21} the count rate of the isotope of the reactant H_3O^+ ion at *m/z* 21, $S_{voc,m}$ (ncps $ppbv^{-1}$) the sensitivity of the instrument for detection of the VOC based on the H_3O^+/VOC reaction product at mass m. The sensitivity factor $S_{voc,m}$ is obtained by calibrating the PTR-MS using a gravimetrically prepared mixture of methanol (1.01 ppmv), acetone (1.01 ppmv), isoprene (0.52 ppmv), α-pinene (0.47 ppmv) and sabinene (0.41 ppmv), in nitrogen

(Apel-Riemer Inc., Denver, CO, USA), with a certified accuracy of 5%. Based on the mixing ratios X_{voc} obtained from equation (2), emission fluxes Φ_{voc} (µg m^{-2} h^{-1}), were calculated according to the following formula:

$$\Phi_{voc} = 10^6 \times 3600 \times X_{voc} \times Q_0 \times \frac{M_{VOC}}{R \times T_0} \times \frac{1}{LA} \qquad (3)$$

Q_0 [Pa m^3 s^{-1}] is the flow through the cuvette at standard conditions (101325 Pa and 273.15 K); M_{voc} the molar mass of the VOC (g/mol); R the universal gas constant, T_0 the standard temperature (273 K) and LA is the leaf area enclosed in the cuvette in m^2. Finally, BVOC fluxes based on the ion signals monitored from branch enclosures were averaged over the sampling interval and corrected for the averaged background fluxes registered in the empty reference cuvettes (Demarcke, 2011).

Although PTR-MS has unique features that are very useful in many applications of VOC analysis, complementary TD-GC/MS measurements are necessary because identification in PTR-MS is based on the mass to charge (*m/z*) ratio of the protonated compounds or some of their important fragments. This identification may be hampered by the presence of spectral interferences and the difficulties in PTR-MS to distinguish isobaric and isomeric compounds. For details of PTR-MS analytical technique and laboratory PTR-MS experiments, the reader is referred to Lindinger et al. (1998), de Gouw and Warneke (2007), or Demarcke et al. (2010).

1.3.2.2 BVOC analysis using TD-GC/MS

Manual BVOC emission analysis consisted of quantification and identification by using Thermal Desorption - Gas Chromatography/Mass Spectrometry (TD-GC/MS). As TD-GC/MS BVOC species identification was not within the scope of this PhD thesis, we focused on total BVOC and/or MT emissions, unless specified otherwise. Other IMPECVOC PhD dissertations are dealing more in detail with TD-GC/MS measurements (Joó, 2011; Pokorska, 2012).

A multistep procedure is used. Firstly, this included preconditioning of adsorbent sampling tubes (Fig. 1.13d) containing Tenax TA (Markes International, Pontyclum, UK) and Carbotrap (Markes International, Pontyclum, UK). Preconditioning consisted of passing a helium gas (300° C and 46 ml min^{-1}) through the tubes stationed in the oven (Carlo Erba instruments) for a duration of 1 h. Secondly, for BVOC quantification, adsorbent tubes were loaded with toluene-D_8 (Acros Organics, Geel, Belgium), used as an internal standard (Joó, 2010). After preconditioning, loading and sampling, the next step consisted of thermal desorption. Trapped compounds were thermally desorbed from the adsorbent tubes, separated

by gas chromatography (GC Trace 2000, ThermoFinnigan, Italy) (Fig. 1.13c) and identified by mass spectrometry (MS TracegDSQ WE-250, ThermoFinnigan, USA). The whole system was controlled from a personal computer with Unity 1.2.0 (Markes International, Pontyclum, UK) and XCalibur 1.3 (ThermoFinnigan, Austin, TX, USA) software. TD-GC/MS calibration was performed monthly using a standard mixture (Luxfer, Inc., Riverside, CA, N150, 1800 psig) containing isoprene (0.515 ppmv), α-pinene (0.496 ppmv), β-pinene (0.501 ppmv), sabinene (0.492 ppmv), limonene (0.486 ppmv), linalool (0.473 ppmv) and (Z)-3-hexenyl-acetate (0.499 ppmv). For the quantification of BVOCs not available in the gas standard, response factors of sabinene were used (Joó et al., 2010). Additionally, pure standards of (Z)-β–ocimene (Fluka≥90%), MeSA (TCI Europe>99%), β-caryophyllene (Fluka>90%), and α-farnesene (extraction form *Chaenomeles superba*, Poland) and methyl dihydrojasmonate (Sigma Aldrich 96%) were used. For details of this analytical technique, the reader is referred to Joó et al. (2010, 2011), Joó, 2011, and/or Pokorska et al. (2011).

TD-GC/MS emissions were calculated according to the following formula:

$$E_x = \frac{AR_x \times F \times 600}{S_x \times V \times LA} \qquad (4)$$

where E_x is the emission of compound x in μg m^{-2} h^{-1}; AR_x is the area ratio in the samples, calculated from the integrated area of compound x divided by the area of the internal standard toluene-D_8 (ISTD); F is the flow entering the cuvette in L min^{-1}; S_x (ng^{-1}) is the response facor obtained from the calibration; V (l) is the sampling volume on the tube; LA is the leaf area enclosed in the cuvette in cm^2 (Joó, 2011). In cases where leaf dry weight (LDW) was used, the formula was as follows:

$$E_x = \frac{AR_x \times F \times 600}{S_x \times V \times LDW} \qquad (5)$$

where all parameters are the same as above.

Finally, significant breakthroughs have been achieved in relation to GC/MS principles by the recent development of a miniaturized GC instrument (e.g. the so-called zNose; Electronic Sensor Technology, Newbury Park, CA, USA), combining fast VOC trapping with automatic GC analysis (Tholl et al., 2006).

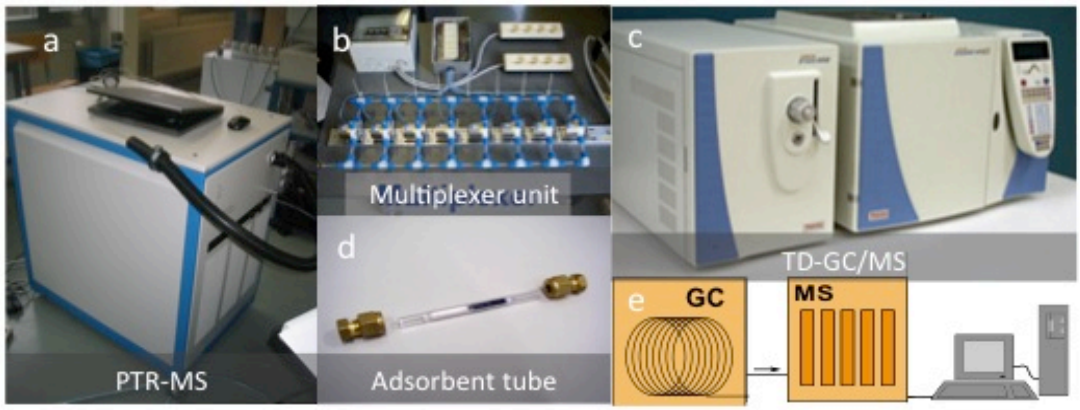

Figure 1.13: From left to right: (a) PRT-MS instrument, (b) PTR-MS multiplexer, c) TD-GC/MS oven and column d) adsorbent sampling tubes containing Tenax (white) and Carbotrap (black), and e) GC/MS work principle scheme.

In this study, a whole sampling unit for simultaneous analysis of Pn and BVOC emissions was developed. The sampling unit consisted of a coupling between an IRGA, a PTR-MS and a TD-GC/MS instrument (Fig. 1.14a-c). For TD-GC/MS sampling, an additional inlet, situated close to the IRGA/PTR-MS inlet line location, was established. A custom-made multiplexer system (Fig. 1.13b) was developed for sequential measurement of different cuvettes. Finally, data acquisition units of the IRGA and the PTR-MS instruments were controlled via separate computers using custom-made LabVIEW 7 software. All data were stored into two different MySQL databases, one for Pn and one for BVOC emissions.

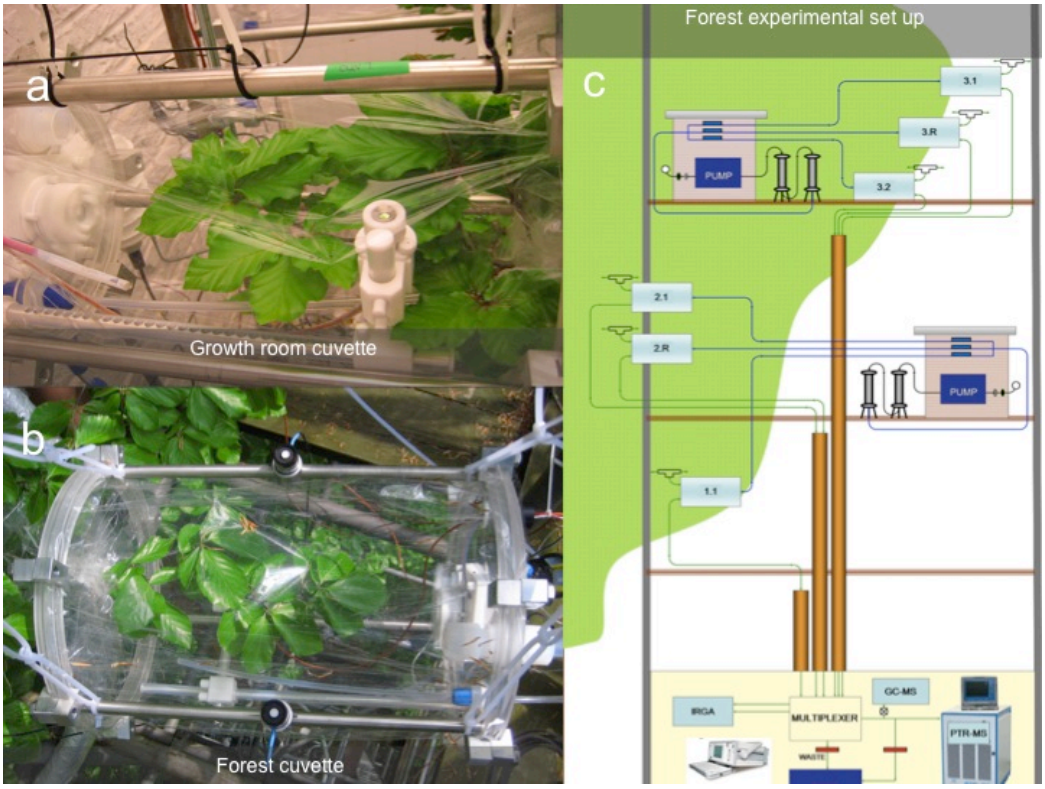

Figure 1.14: From left to right: a) Growth room 7-L cuvette, b) Forest and campus 31-L cuvette containing enclosed branches, and c) Aelmoeseneie forest schematic representation of set up with a IRGA-PTR-MS-GC/MS coupling (tower scheme courtesy Demarcke, 2011).

Chapter 2

Comparing monoterpenoid emissions and net photosynthesis of beech (Fagus sylvatica L.*) in controlled and natural conditions*

Adapted from: Šimpraga M, Verbeeck H, Demarcke M, Joó E, Amelynck C, Schoon N, Dewulf J, Van Langenhove H, Heinesch B, Aubinet M, Müller J-F, Steppe K. (2011). Comparing monoterpenoid emissions and net photosynthesis of beech (*Fagus sylvatica* L.) in controlled and natural conditions. Atmospheric Environment 45, 2922-2928.

Abstract

Although biogenic volatile organic compounds (BVOCs) only represent a very limited fraction of the plant's carbon budget, they play an important role in atmospheric chemistry, for example, as a precursor of tropospheric ozone. We performed a study comparing BVOC emissions of European beech (*Fagus sylvatica* L.) in controlled and natural environmental conditions. Young and adult beech trees were exposed to short-term temperature variations in growth room conditions and in an experimental forest, respectively. This study attempts to clarify how short-term temperature variations between days influenced the ratio between monoterpenoid (MT) emissions and net photosynthesis (Pn). Within a temperature range of 17-27 °C and 13-23 °C, the MT/Pn carbon ratio increased 10-30 fold for the growth room and forest, respectively. An exponential increasing trend between MT/Pn carbon ratio and air temperature was observed in both conditions. Beech trees re-emitted a low fraction of the assimilated carbon back into the atmosphere as MT: 0.01-0.12% and 0.01-0.30% with a temperature rise from 17-27 °C and 13-23 °C in growth room and forest conditions, respectively. However, the data showed that the MT/Pn carbon ratio of young and adult beech trees responded significantly to changes in temperature.

2.1 Introduction

Not only anthropogenic sources disturb air quality, but BVOCs (biogenic volatile organic compounds) alter it as well. Already in 1960, Went (1960) linked the formation of “blue haze” over coniferous forests to the biogenic emission of monoterpenes. Due to the magnitude of their emissions as well as their photochemical reactivity (e.g. Guenther et al., 2000; Scott and Benjamin, 2003), BVOCs could significantly impact regional air quality and human health. Their very short lifetimes represent a challenge to quantify them (Kesselmeier and Staudt, 1999). Isoprene (C_5 hydrocarbon), monoterpenes (C_{10} hydrocarbons), and derivatives thereof (the so-called isoprenoids or terpenoids) are the most representative BVOCs emitted by plants in the atmosphere (Vitale et al., 2008). Moreover, these volatile compounds alter climate by influencing the residence time of greenhouse gases, such as methane, and by the formation of aerosols and cloud condensation nuclei, a major concern of current climate research (e.g. Kesselmeier et al., 2002; Peñuelas and Llusia, 2003; Copolovici et al., 2005). Once emitted into the atmosphere, BVOCs undergo transformations including reactions with hydroxyl radicals ($OH^{\bullet}$), nitrate radicals ($NO_3^{\bullet}$) and ozone (O_3) (Räisänen, 2008). In the presence of sunlight, BVOCs and oxides of nitrogen (NO_x) produce tropospheric O_3, an important air pollutant. These reactions are particularly important in urban, polluted areas where the air quality standards are often violated (Räisänen, 2008). BVOCs decrease the level of $OH^{\bullet}$ radicals, normally contributing to the breakdown of greenhouse gases. As such, BVOCs indirectly contribute to global warming (Graedel and Crutzen, 1993, Grote et al., 2006).

Some authors state that BVOCs have no apparent function in the basic processes of plant growth and development, and have been historically referred to as natural products or secondary metabolites (Gershenzon and Dudareva, 2007). However, according to other authors, BVOCs have various ecological and physiological roles in plants: antiherbivory and antimicrobial defense, pollinator attraction, allelopathic role (Owen and Peñuelas, 2005), protection against high temperature episodes (i.e. thermo tolerance; Sharkey and Yeh (2001)), communication with other plants or organisms (i.e. plant-plant and plant-insect interactions; Peñuelas and Llusia (2003); Staudt and Lhoutellier (2007)).

Net photosynthesis (Pn) also provides an important contribution to exchange processes between atmosphere and vegetation (Cieslik et al., 2009). Pn and MT emission change significantly during the year (Grote and Niinemets, 2008). Aging causes anatomical and physiological changes in shade and sun adapted leaves. Consequently, MT emission and Pn potentially decrease gradually with increasing age (Steppe et al., 2011). Beech is a species with high shade and high irradiation adaptation (Druebmer et al., 2009); therefore, sun and shade leaves have considerably different Pn rates, photosynthetic pigments

composition, electron carriers, chloroplast ultrastructure (Sarijeva et al., 2007) and display considerable phenotypic and ecophysiological plasticity (Druebmer et al., 2009).

Relations between MT emissions and Pn are still poorly understood and besides their strong dependence on light and temperature (Schuh et al., 1997), a close relationship is suggested by their common synthesis place, the use of primary products of Pn and the high requirements for energy (Grote and Niinemets, 2008). There is evidence that a part of the emitted compounds originates from a small pool of immediately assimilated carbon (Niinemets et al., 2002). It has been reported that plants might allocate up to 2% of their assimilated carbon to the production of volatile secondary metabolites (Sharkey and Singsaas, 1995; Firn and Jones, 2006; Tani and Kawawata, 2008) under normal conditions. Nevertheless, in their natural habitat, trees encounter biotic and abiotic stresses possibly altering carbon allocation simultaneously. Consequently, these carbon ratios may increase up to 10% (Peňuelas and Llusia, 2003) and even up to 67% according to other authors (Sharkey and Loreto, 1993, Kesselmeier et al., 2002) under stress conditions.

The aim of this study is to investigate in which way BVOCs and Pn behave when exposed to short-term temperature variation under (1) controlled and (2) natural environmental conditions in young and adult European beech (*Fagus sylvatica* L.). To our knowledge, when relating BVOC emissions and Pn, we are the first to perform such a detailed comparison between these two environments. Focusing on the leaf carbon balance and BVOC emissions, we investigated the drivers of the ratio between the carbon release through BVOC emissions (in particular monoterpenoids) and the net carbon uptake through Pn.

2.2 Material and methods

2.2.1. Plant material and experimental conditions

For the growth room experiment, potted beech trees of approximately 1.25 m high (3-4 years old), were grown in 30-L containers during the vegetation year 2007. The soil consisted of a commercial soil mixture (Agrofino, Arendonk, Belgium) and was fertilized with ozmocote (Ingelheim, Germany). The trees were placed in a walk-in growth room (2 x 1.5 x 2 m; height x width x length) during a 14 day period, under a constant day/night air temperature of 21 °C. In the growth room, hourly stepwise varying light intensities of photosynthetic photon flux density (PPFD) were used to simulate the natural daylight pattern (08-22h) by means of a set of 40 fluorescent lamps (type PHILIPS Master TL-D fluorescent lamps 36W/830 warm white, super 80). Growth room PPFD-values at the top of the trees were 155±4 µmol m^{-2} s^{-1} (Fig. 2.1a). These low light intensities corresponded to typical shady conditions in which young trees at the forest floor in Belgium grow (Steppe and Lemeur, 2004). For the growth room, the horizontal

fixation of the leaves in a frame served for equal light interception on every single leaf to ensure a homogeneous light distribution.

The forest measurements (vegetation year 2008) were performed on a 30 m-high 85-year old beech tree (LAI of 3.5 m² leaf/m² soil) in the experimental forest Aelmoeseneie, which is under management of Ghent University and INBO (Instituut voor Natuur en Bosonderzoek), Belgium. This forest is located about 15 km from Ghent, Belgium (50°58' N, 3°48' E, 21 m altitude). The mixed temperate forest covers an area of 28 ha having temperate maritime climate. Further details of the site description can be found in Saveyn et al. (2008). Forest PPFD was measured with two quantum flux PPFD sensors attached in a horizontal arrangement at each side of a branch bag (i.e. cuvette). The mean value was used in the analysis. Forest branch maximum PPFD reached 321±208 μmol m^{-2} s^{-1} (Fig. 2.1b) for the selected days. The measured branch was selected at 24 m height facing west. An enclosed branch bag system was used for the simultaneous flux measurements of net photosynthesis (Pn) and monoterpenoids (MT). Both the growth room and the forest measurement systems consisted of a reference cuvette (without leaves), and of two branch cuvettes (with leaves). In the growth room two different trees were used.

Only daytime values of Pn and MT emissions were used, because night values of CO_2 assimilation represent the loss of carbon by respiration. Consequently, we included day values from 08-22 h (growth room) and 06-21h (forest). Both the growth room and the forest were equipped with sensors for measurements of the air temperature (type Pt 100; Rotronic, Bassersdorf, Switzerland), the relative humidity (type RHa; Rotronic, Bassersdorf, Switzerland); and photosynthetically active radiation (type Li-190S; Li-COR, NE, USA, PPFD). All sensors were placed at cuvette level, being 1.2 m and 24.0 m high in growth room and forest, respectively. Calibration of all sensors was carried out prior to the start and at the end of the experiment. The data were measured every 4 s and the 1 min averages were logged.

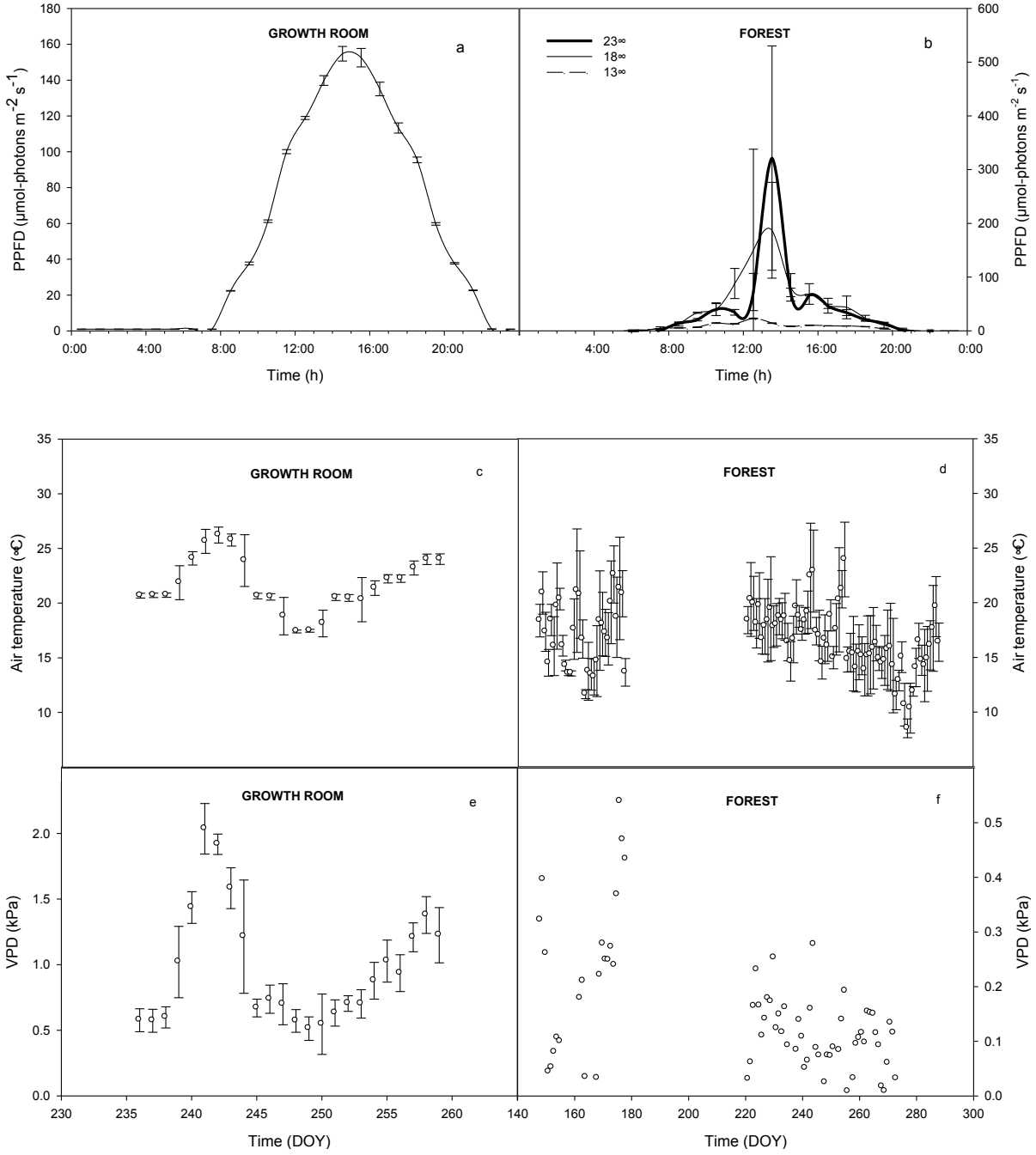

Figure 2.1: (a) Mean stepwise variation in photosynthetic photon flux density (PPFD) in the growth room simulating the natural forest understory daylight pattern. The values are averages of all days during the experiment (mean SD=0.73). (b) PPFD values in forest conditions (c) Cuvette air temperature in the growth room and (d) in the forest. (e) Cuvette vapour pressure deficit (VPD) in growth room, and (f) in the forest, respectively. The error bars represent the day mean standard deviation (SD).

2.2.3. Temperature conditions

Growth room air temperature was kept constant during the day and varied from 17-27 °C between days (Fig. 2.1c). The temperature variation was randomly applied, with 21 °C taken as reference temperature. The transitions days (when temperature was changed) or days with data failure were not taken into account, unless specified. In the forest experiment, the air temperature followed a natural pattern and varied from 9 to 23 °C (Fig. 2.1d). Both temperature ranges are typical for Belgian understory forest conditions.

2.2.4. Leaf-level gas exchange measurements

Teflon® was used for the cuvette construction as it minimizes wall-losses of the emitted substances and transmits a wide spectral range of short-wave radiation, especially the photosynthetically active range (Schuh et al., 1997). A Teflon®-coated ventilator mixed the air homogeneously inside the cuvette and reduced the leaf boundary layer resistance. The branch cuvette was equipped with sensors for relative humidity (RH; type HIH-3610, Honeywell, NJ, USA) and air temperature (Tair; type thermistor 10k-NTC, TH-44031-36-T, Omega, USA).

Pn was measured with an infrared gas analyzer (IRGA CO_2 model BINOS 4P, Effeltrich, Germany and IRGA CO_2 model ADC 2250, ADC BioScientific, UK, in the growth room and forest, respectively) based on the difference between air entering the cuvette and air leaving the cuvette. The leaves take up CO_2 by Pn or produce CO_2 by dark respiration (Rd).

MT emissions were measured using Proton-Transfer-Reaction-Mass-Spectrometry (PTR-MS, IONICON Analytik GmbH, Innsbruck, Austria). This fast and sensitive on-line VOC analyser relies upon ionization of target compounds by exothermic reactions with H_3O^+ ions in a medium-pressure flow-drift tube reactor, which is coupled to a quadrupole mass spectrometer. Measurements were performed at a drift tube pressure and temperature of 2.35 hPa and 333K, respectively, and at an *E/N* value of 128 Td (*E*: electric field; *N*: buffer gas number density, 1 Td = 10^{-17} V cm^2). Detailed information on the technique can be found in a number of extensive review articles (e.g. de Gouw and Warneke, 2007). Regular growth room sampling of cuvette air followed by Thermal Desorption Gas Chromatography – Mass Spectrometry (TD-GC/MS) analysis revealed that monoterpenoid emissions from the beech trees were mainly composed of sabinene ($C_{10}H_{16}$) and linalool ($C_{10}H_{18}O$), an oxygenated monoterpene (Joó et al., 2010a, Demarcke et al., 2010). Both species have a PTR-MS estimator product ion at the same mass-to-charge ratio (137), but with different detection sensitivity. Careful intercomparison of TD-GC/MS and PTR-MS techniques, however, has shown that by using a weighted PTR-MS sensitivity factor, accurate emissions of the sum of monoterpenes and linalool could be

obtained. This weighted sensitivity factor takes into account the distribution of monoterpenes and linalool obtained with TD-GC/MS. Details are described in a separate paper on the TD-GC/MS/PTR-MS intercomparison (Joó et al., 2010b). Both the Pn and the MT emissions were normalized for leaf area, measured throughout the growing seasons with a leaf area meter (type LI-3000, Lincoln, NE, USA) coupled with a LI-3050A transparent conveyer belt.

2.2.5. Phenological indicator

Chlorophyll content index (CCI), a phenological indicator, was measured using a portable chlorophyll content reflectometer measuring the reflection coefficient of the green wavelengths (type SPAD-500 Minolta). CCI expresses the relative amount of chlorophyll pigments in leaf tissue and is related to the green coloration. On each tree 15 carefully selected leaves (representing the branch inside cuvette) were measured on the right and left side from the leaf main vein. Means thereof were used in further analysis.

2.2.6. Statistical analysis

All statistical analysis was performed with R version 2.11.1 statistical software (R Development Core Team 2010). A linear regression was used to test the significance for the MT/Pn ratio trend. Additionally a repeated measures analysis of variance (ANOVA) was performed on the Pn and MT emission rates with temperature (n=5) and time of the day (n=24) as fixed factors.

2.1 Results

2.3.1. Chlorophyll level

The qualitative non-destructive CCI-measurements revealed a strong variation in leaf chlorophyll content in growth room and forest conditions (Fig 2.2a,b). During leaf development, increasing CCI-values were obtained indicating rising plant metabolic activity. Leaf senescence caused a decline in CCI by 50% across a two month period resulting in lower Pn values.

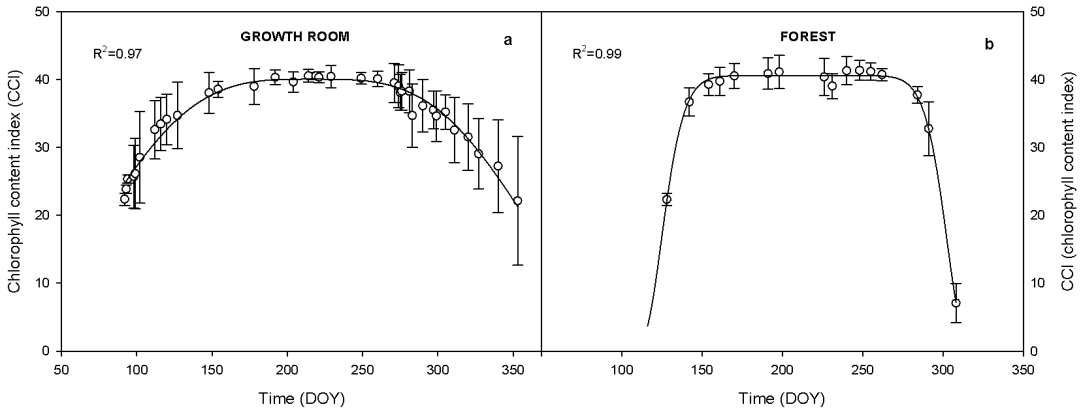

Figure 2.2: Beech chlorophyll content index (CCI) in (a) the growth room and (b) the forest. Standard deviations are given as ±SD error bars. Modified Gaussian equation was fitted of the form: $y=a \times \exp(-bx((x-c)/d)^2)$

2.3.2. Microclimate effects and diurnal dynamics of Pn and MT emissions

We characterized the microclimate by measurements of PPFD, air temperature and VPD_{air} (air vapour pressure deficit). PPFD results showed important differences between growth room and forest conditions. This indicated that growth room trees were shade adapted as they received low PPFD levels. Forest leaves were also shade adapted, but with occasional sunflecks exposures. MT emissions and Pn exhibited pronounced diurnal dynamics as light/sun gradually appeared/disappeared. A clear effect of temperature variation on MT emissions was observed (Fig. 2.3c,d). Interestingly, when exposed to higher temperatures (up to 27 °C or 23 °C in growth room and forest, respectively), trees showed pronounced higher MT emissions. The repeated measures ANOVA applied on the growth room data showed that temperature was no significant factor for Pn. The MT emissions showed to be significantly higher ($p<0.05$) only for the 27 °C situation during the entire daytime (8-21 h). At 27 °C and 23 °C, emissions reached a peak value of 334 and 567 µg m^{-2} h^{-1}, in the growth room and forest, respectively. MT emissions were low when exposed to low temperatures. At 17 °C, peak emissions were only 34 µg m^{-2} h^{-1} and 28 µg m^{-2} h^{-1}, respectively. In contrast, except for 13 °C in the forest, Pn appeared to be less affected by the temperature range, indicating optimal growth conditions for beech (Fig. 2.3a,b). Within the small temperature range, MT indicated high temperature influence while Pn showed only minor changes. The optimum temperature for Pn was found at 21 °C in the growth room, while in the forest Pn was highest at 23 °C for the investigated days.

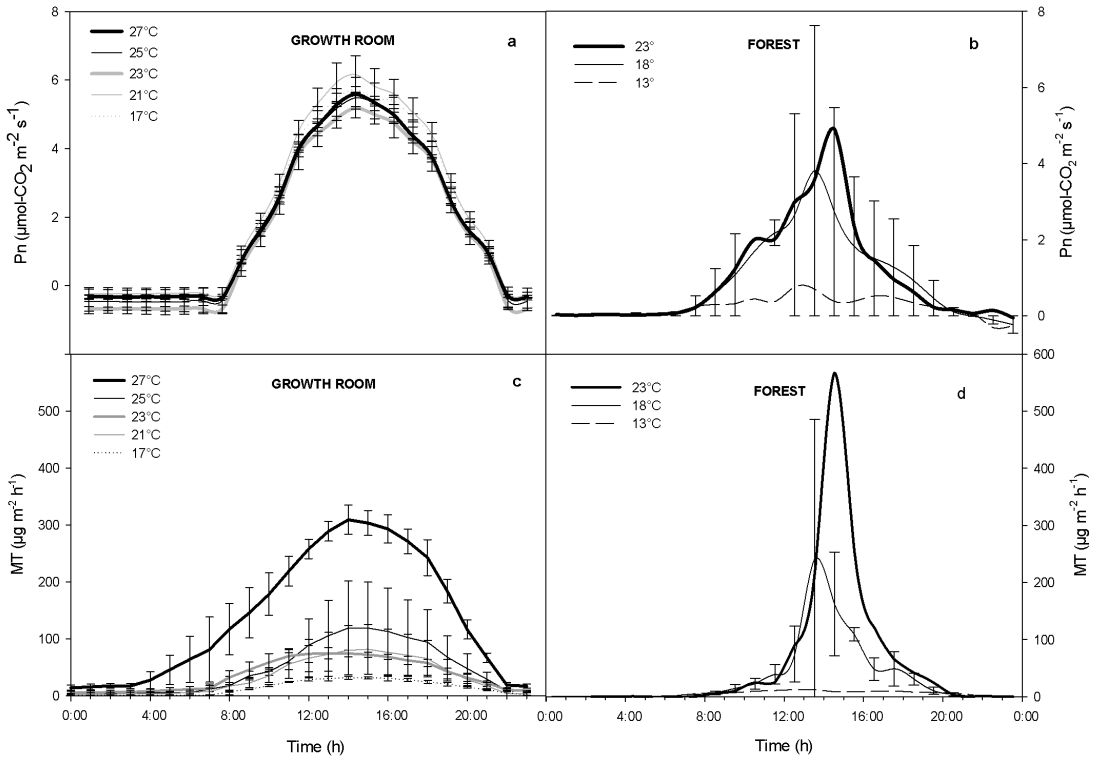

Figure 2.3: Mean diurnal patterns of net photosynthesis (Pn) of (a) a young beech in the growth room and (b) adult beech in the forest and total monoterpenoid emission (MT) (c) young beech in the growth room, and (d) adult beech in the forest for each temperature.

3.3.3. Diurnal pattern of the MT/Pn carbon ratio

The fraction of assimilated carbon re-emitted back into the atmosphere through MT emission was represented by the carbon ratio between diurnal total emitted MT and Pn. The mean diurnal pattern of the MT/Pn carbon ratio measured at days with a temperature of 21 °C in the growth room showed that the ratio remained quite constant during the day, with an increase only in the late afternoon (Fig. 2.4a). The increase in the course of the day was not significant (y=0,015+0,00044x; p=0,30). Hence, at the low light intensities prevailing in the growth room no effect of diurnal light patterns could be observed. The reason for the slight increase in the ratio as light diminished at the end of the day is that Pn decreased more strongly compared to MT emissions (Fig. 2.3a,c). In the forest, the MT/Pn carbon ratio showed an increasing trend (Fig. 2.4b) that could be linked to temperature and/or radiation (Fig. 2.3b,d). This trend was significant (y=-0,052+0,0082x; p<0,001).

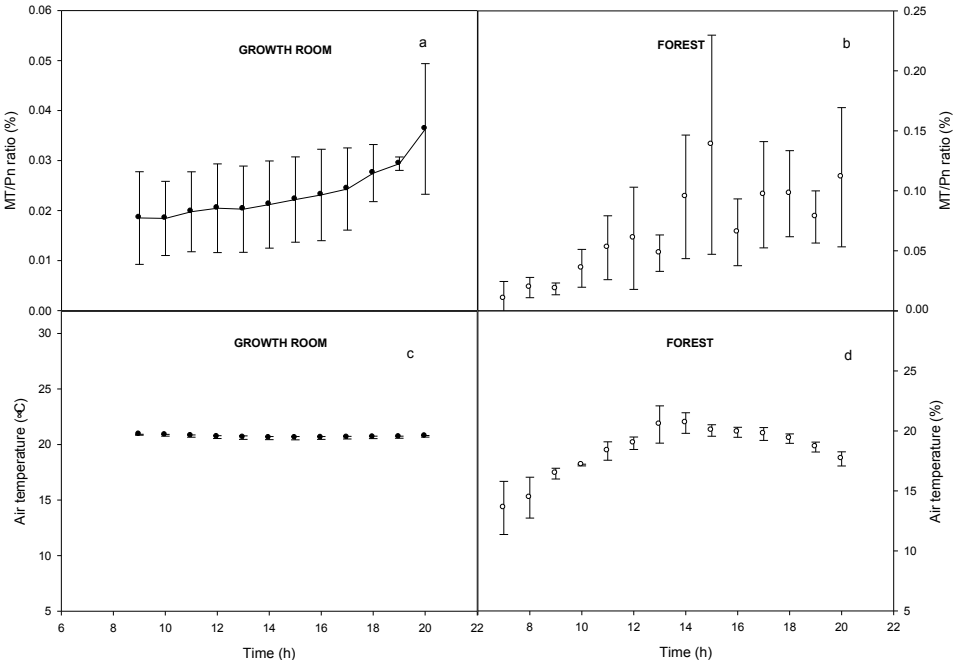

Figure 2.4: Diurnal trend in total monoterpenoid/net photosynthesis (MT/Pn) carbon ratio of (a) a young beech in the growth room and (b) an adult beech in the forest, respectively. The growth room curve represents the mean of 6 different days (± SD) with a temperature of 21 °C. The forest curve represents a mean of three days with temperature of 18 °C. Air temperature mean diurnal patterns of temperatures for the different days (c) in the growth room and (d) in the forest.

2.3.4. Temperature response

Temperature variation affected growth room and forest trees. These responses indicate that enzymatic processes are at work. The MT/Pn carbon ratio showed an exponential rising trend with temperature, both in the growth room and forest (Fig. 2.5). The results revealed that the potted beech under well-watered conditions re-emitted a rather low fraction, while the adult beech tree indicated a slightly higher fraction of the assimilated carbon re-emitted back into the atmosphere as MTs. This fraction ranged from 0.01-0.12% and 0.01-0.30% with a temperature rise from 17-27 °C and 13-23 °C in growth room and forest, respectively (Fig. 2.5a,b). The coefficient of determination (R^2) for this relationship with temperature is higher in case of growth room (0.93) compared to the forest (0.52).

When we consider the temperature transition day from a higher temperature of 27 °C to a lower temperature of 21 °C (Fig. 2.5a, symbol ∆), the beech needed the two-day adaptation time and showed a higher calculated MT/Pn carbon ratio.

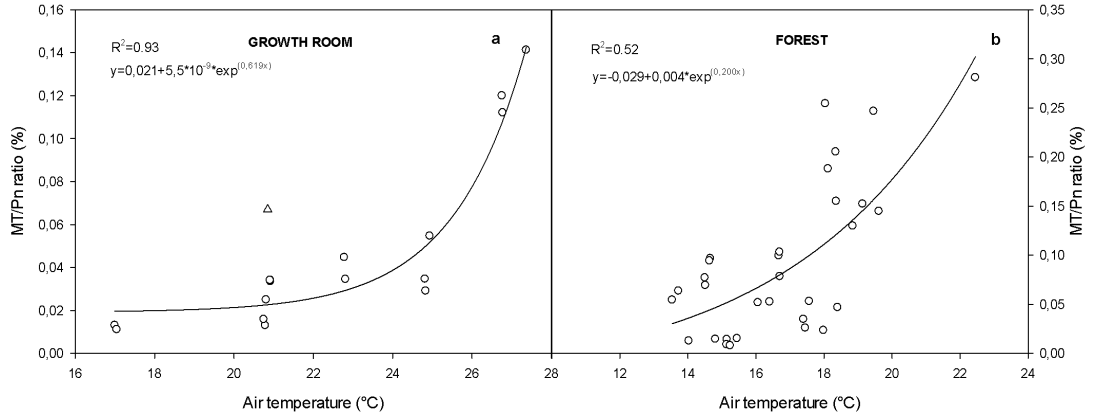

Figure 2.5: (a) Exponential relationship of the total monoterpenoid/net photosynthesis (MT/Pn) carbon ratio in function of temperature in the growth room under controlled conditions. The triangle (△) represents the MT/Pn carbon ratio measured the day after transition from 27 to 21 °C. This day demonstrates the adaptation of the beech tree to larger temperature steps, and is not included in the calculation of the mean value for 21 °C; (b) An exponential trend of the total MT/Pn carbon ratio as a function of temperature in the forest under natural conditions.

2.4 Discussion

2.4.1. Carbon loss through MT emissions

This study revealed that young potted beech trees growing under well-watered controlled conditions and the adult beech tree in natural conditions showed pronounced diurnal dynamics in Pn and MT emissions. In contrast with the MT emissions, growth room Pn patterns showed only minor changes within the imposed air temperature range: nor the highest temperature (27 °C) neither the lowest temperature (17 °C) inhibited Pn (Fig. 2.3a). Forest Pn was clearly affected at 13 °C (Fig. 2.3b) indicating a decreased carboxylation efficiency of RuBisCO (ribulose-1,5 bisphosphate carboxylase/oxygenase) (Medlyn et al., 2002; Larcher, 2003). This different dependence of MT and Pn on temperature caused an important change in the fraction of assimilated carbon re-emitted back into the atmosphere as MT. The observed ratios (0.01- 0.12% in the growth room and 0.01-0.30% in the forest) correspond to the ratios found in literature for other deciduous (oak, poplar) and even some coniferous (spruce) tree species, ranging between 0.01-13% (Tingey et al., 1980; Street et al., 1996; Staudt and Bertin, 1998; Kesselmeier et al., 2002; Grabmer et al., 2006) under normal conditions. The temperature dependence of assimilated carbon loss by MT emissions was observed by Staudt and Bertin (1998), who found an increase from 0.5 % at 30 °C up to 2-6% between 40 and 45 °C for a 10-year-old potted oak tree (*Quercus*

ilex). Under natural conditions, Dindorf et al. (2005) found MT/Pn carbon ratios between 0.5 and 1.7% for an adult beech tree, while under stress conditions other studies mention a large re-emitted carbon fraction of 20% as MTs (Staudt and Bertin, 1998) and up to 67% as isoprene (Sharkey and Loreto, 1993). Similar to our results Tani and Kawawata (2008) indicated a carbon ratio lower then 1% in different *Quercus* species for temperatures between 20-30 °C. Carbon losses under the form of MTs are thus small, but may become a significant component of the carbon budget in specific tree species under stress conditions.

2.4.2. MT and Pn light and temperature dependency

While some studies indicate that MT emissions are dominantly a function of temperature and are not impacted by PPFD (Dement et al., 1975; Tingey et al., 1980; Koppmann, 2007), other studies show that both Pn and the biosynthesis of some MT are strongly influenced by PPFD (Loreto et al., 2001). Our results showed that Pn and MT followed a diurnal variation in PPFD under constant temperature, supporting the light dependency hypothesis. MT emissions and Pn were clearly dependent on changing light intensities (Fig. 2.1a,b), but we found that the MT/Pn carbon ratio was not affected by the stepwise changes in PPFD (Fig. 2.4a,b), when the temperature was kept constant. Similar light-independency was reported for *Quercus sp*. (Tani and Kawawata, 2008). The light dependency is probably linked to the BVOC-specific synthase activity and the available amount of substrate (DMAPP, 1-deoxy-D-xylulose-phosphate) (Sharkey and Yeh, 2001; Tani and Kawawata, 2008). An increasing trend of MT/Pn carbon ratio with temperature was shown for all trees. This exponential relationship between emissions and air or leaf temperature has been reported earlier for different plant species (Tingey et al., 1980; Lerdau et al., 1997; Schuh et al., 1997; Harley et al., 1998) and at stand level for beech (Gallagher et al., 2000). To our knowledge, we report for the first time an exponential relationship between the MT/Pn carbon ratio and rising temperatures. Our results are consistent with other studies suggesting that plants adapt to fluctuating temperatures (Sharkey and Singsaas, 1995; Street et al., 1996) by balancing their investments in BVOCs versus other secondary metabolites (e.g. carotenoids).

2.5 Conclusions

Simultaneous measurements of MTs and Pn were compared for young beech trees growing in a growth room and an adult forest beech tree growing in an experimental forest. While the daily dynamics of MTs and Pn were different under growth room and natural conditions, the MT/Pn carbon ratio showed in both

cases an increasing exponential trend with increasing air temperatures. This demonstrates that the MT/Pn carbon ratio of the studied young and adult beech trees responded in a similar way to short term changes in air temperature.

Chapter 3

Clear link between drought stress, photosynthesis and biogenic volatile organic compounds in beech (Fagus sylvatica L.*)*

Adapted from: Šimpraga M, Verbeeck H, Demarcke M, Joó E, Pokorska, O, Amelynck C, Schoon N, Dewulf J, Van Langenhove H, Heinesch B, Aubinet M, Laffineur, Q, Müller J-F, Steppe K. (2011). Clear link between drought stress, photosynthesis and biogenic volatile organic compounds in *Fagus sylvatica* L. Atmospheric Environment 45, 5254-5359.

Abstract

Direct plant stress sensing is the key for a quantitative understanding of drought stress effects on biogenic volatile organic compound (BVOC) emissions. A given level of drought stress might have a fundamentally different effect on the BVOC emissions of different plants. For the first time, we continuously quantified the level of drought stress in a young potted beech (*Fagus sylvatica* L.) with a linear variable displacement transducer (LVDT) installed at stem level in combination with simultaneous measurements of BVOC emissions and photosynthesis rates (Pn) at leaf level. This continuous set of measurements allowed us to examine how beech alters its pattern of photosynthesis and carbon allocation to BVOC emissions (mainly monoterpenes, MTs) and radial stem growth during the development of drought stress. We observed an increasing-decreasing trend in the MT emissions as well as in the fraction of assimilated carbon re-emitted back into the atmosphere (ranging between 0.14 and 0.01%). We were able to link these dynamics to pronounced changes in radial stem growth, which served as a direct plant stress indicator. Interestingly, we detected a sudden burst in emission of a non-identified, non-MT BVOC species when drought stress was acute (i.e. pronounced negative stem growth). This burst might have been caused by a certain stress-related green leaf volatile, which disappeared immediately upon re-watering and thus the alleviation of drought stress. These results highlight that

direct plant stress sensing creates opportunities to understand the overall complexity of stress-related BVOC emissions.

3.1 Introduction

The atmosphere receives a chemodiversity of reactive biogenic volatile organic compounds (BVOCs), predominantly emitted by plants. BVOCs cover a wide range of organic species, including hemiterpenes, monoterpenes and sesquiterpenes. The importance to study BVOCs lays in their high reactivity with oxidants in the air (such as ozone (O_3) as well as hydroxyl ($OH^{\bullet}$) and nitrate ($NO_3^{\bullet}$) radicals) leading to the formation of secondary organic aerosol (SOA) (Cahill et al., 2006; Rennenberg et al., 2006). On the other hand, SOA plays an important role in the chemistry of the atmosphere and contributes to climate change by scattering the incoming solar radiation back to space. Some aerosols cause a positive radiative forcing (warming), while others cause a negative one (cooling); however, their net effect is cooling (IPCC, 2007). Moreover, in the presence of anthropogenic pollutants, BVOCs are main precursors of the photochemical tropospheric O_3 production (Cahill et al., 2006). At current concentrations, the O_3 radiative forcing potential is of near-equal magnitude to that of methane, making it the third largest contributor to anthropogenic warming (Tsigaridis and Kanakidou, 2007). In addition, it is known that BVOCs have an important role in the plant carbon balance representing a loss of carbon (Sharkey and Loreto, 1993). Under normal conditions (no stress) and during application of certain stressors (e.g. heat), it has been shown that plants allocate up to 2% of their assimilated carbon to the production of volatile secondary metabolites (Sharkey and Singsaas, 1995; Firn and Jones, 2006; Tani and Kawawata, 2008; Šimpraga et al., 2011a). When dealing with multiple stressors (heat, high radiation and/or drought) carbon ratios increase up to 10% (Peňuelas and Llusia, 2003) and even up to 67% (Sharkey and Loreto, 1993).

However, regarding drought stress effects on BVOC emissions the literature is conflicting. Upon imposing a drought stress, BVOC emissions are reported to be enhanced (Delfine et al., 2005; Blanch et al., 2009), reduced (Brilli et al., 2007; Fortunati et al., 2008; Lavoir et al., 2009; Peňuelas et al., 2009), or increased followed by a decrease (Sharkey and Loreto, 1993; Bertin and Staudt, 1996; Ormeňo et al., 2007). Moreover, drought stress can alter BVOC species composition and/or induce new emissions depending on the level of stress (Niinemets, 2009). Besides BVOC emissions, drought stress also affects net photosynthesis and radial stem growth (Saveyn et al., 2007; De Swaef and Steppe, 2010). Because photosynthesis represents the main carbon source for certain BVOCs (Schnitzler et al., 2004), BVOC emissions are indirectly influenced by drought stress when photosynthesis is affected. When photosynthesis is not impaired by stress, carbon for BVOC in trees comes from recently fixed

photosynthetic intermediates (i.e *de novo* synthesis), being plant species rather than compound dependent (Ghirardo et al., 2010).

Drought stress in the above mentioned BVOC-related studies is typically quantified using measurements of leaf water potential (Delfine et al., 2005; Lavoir et al., 2009), photosynthesis (Sharkey and Loreto, 1993; Fortunatti et al., 2008), soil water potential (Bertin and Staudt, 1996), leaf relative water content (Blanch et al., 2009) and substrate water content (Ormeňo et al., 2007). These measurements are however either labour-intensive or soil-water based approaches. In contrast, the use of linear variable displacement transducers (LVDTs), positioned perpendicular to a tree stem and allowing continuous measurements of radial stem growth, enables direct plant stress sensing and is therefore very valuable to monitor the plant's response to drought stress (Steppe et al., 2006; Saveyn et al., 2007; De Pauw et al., 2008; De Swaef et al., 2009; Villez et al., 2009). Under normal conditions LVDTs register reversible morning shrinkage and evening swelling, in addition to radial stem growth. In contrast, under drought stress conditions, internal stem water reserves are depleted more, resulting in more stem shrinkage, a decrease in stem turgor and a change in radial stem growth.

Recently, Niinemets (2009) highlighted the lack of data on quantitative plant stress detection versus BVOC emission responses, despite its importance for current emission models. Thus, we conducted measurements to assess potential links between net photosynthesis and BVOC emissions occurring in young potted beech trees (*Fagus sylvatica* L.) during drought stress. For the first time, we quantified continuously the tree's stress level using LVDTs and measured net photosynthesis and BVOC emissions, simultaneously. In this context, two research questions were put forward: (i) does beech alter its patterns in photosynthesis, carbon allocation to BVOC emissions and radial stem growth on a day-to-day basis during drought stress development; and (ii) how do different PTR-MS signals behave during drought stress development and what is their significance.

3.2 Materials and methods

3.2.1. Plant material and drought stress treatment

The experiment was performed in the growth room (2 x 1.5 x 2 m; height x width x length) of the Laboratory of Plant Ecology at Ghent University, in which radiation and air temperature were controlled. The experiment was conducted on a potted four-year-old European beech tree (*Fagus sylvatica* L.) grown in a 40-L container during the growing season of 2008. Soil consisted of a commercial soil mixture (Agrofino, Arendonk, Belgium) homogenously mixed with fertilizer basacote (Basacote Plus® 12M, COMPO Benelux, Belgium). The experiment

was performed when the leaves were fully developed, because developing leaves might produce different BVOC compounds during the expansion phase (Vitale et al., 2008). Drought stress was applied from Day Of the Year (DOY) 186 to DOY 204 by withholding water supply. The measurement campaign hence lasted 18 days during drought stress development. Last watering and subsequent re-watering occurred on DOY 186 and DOY 204, respectively.

3.2.2. Microclimatic measurements

The growth room was equipped with sensors for measurements of air temperature (type Pt 100; Rotronic, CH), relative humidity (type RHa; Rotronic, CH) and photosynthetically active radiation (PAR; type Li-190S; Li-COR, NE, USA). Light was supplied by lamps (type PHILIPS Master TL-D fluorescent lamps 36W/830 warm white, super 80, Eindhoven, The Netherlands), providing a mean PAR level of 225 µmol m^{-2} s^{-1} at a height of 1.5 m during the daytime period (from 08h till 20h). The air temperature was held constant at (21 ± 0.95) °C. Data were collected with a data acquisition system (type 34970a Agilent Technologies, CA, USA), storing 20 s data. Five min mean values were used in the analysis.

3.2.3. Experimental setup

A dynamic branch enclosure system consisting of a branch cuvette (with leaves) and a reference cuvette (without leaves) was used for leaf-level carbon dioxide (CO_2) and BVOC emission measurements. The grid-hold horizontal placement of the leaves served for equal light interception on every leaf avoiding mutual shading (Demarcke et al., 2010). Teflon-PFA (perfluoroalkoxy polymer foil) was used for the cuvette construction as it minimizes wall-losses of the emitted substances and transmits a wide spectral range of shortwave radiation, especially the photosynthetically active range (Schuh et al., 1997; Šimpraga et al., 2011a). Teflon-PFA-coated ventilator mixed the air homogeneously inside the cuvette and reduced the leaf boundary layer resistance. The branch cuvette was equipped with sensors for relative humidity (RH; type HIH-3610, Honeywell, NJ, USA) and air temperature (Tair; type thermistor 10k, NTC, Omega, NL).

3.2.4. Leaf-level gas exchange measurements

Net photosynthesis (Pn) and dark respiration (Rd) were measured continuously with an infrared gas analyzer (CO_2 IRGA BINOS 4P, Effeltrich, Germany) based on the difference between air entering the cuvette and air leaving the cuvette. The leaves take up CO_2 by Pn during the daytime or produce CO_2 by Rd at night.

BVOC emissions were measured continuously using Proton-Transfer-Reaction-Mass-Spectrometry (PTR-MS, IONICON Analytik GmbH, Innsbruck, Austria). Measurements were performed at a drift tube pressure and temperature

of 2.35 hPa and 333 K, respectively, and at an *E/N* value of 128 Td (*E*: electric field; *N*: buffer gas number density, 1 Td = 10^{-17} V cm^2). Detailed information on the technique can be found in review articles (de Gouw and Warneke, 2007, Blake et al., 2009). For validation of the PTR-MS analysis, measurements were extended with Thermal Desorption Gas Chromatography/Mass Spectrometry (TD-GC/MS) analysis. This was done in order to determine which VOC species contributed to the PTR-MS signal at a certain *m/z* value (Joó et al., 2010a). Sampling was performed using Tenax/Carbotrap multi-adsorption tubes. Details of the applied PTR-MS and TD-GC/MS techniques are explained in section 1.3.2.1. and 1.3.2.2 and have been published previously (Demarcke et al., 2010; Joó et al., 2010b; Pokorska et al., 2011). Regular sampling of cuvette air followed by TD-GC/MS analysis revealed that isoprenoid emissions from beech during the measurement campaign consisted of monoterpenes (MTs). These species have a PTR-MS estimator protonated molecule at *m/z* 137 ($C_{10}H_{17}^+$). In addition to *m/z* 137, the fragment ion *m/z* 81 was also measured. Henceforth, we considered the *m/z* 137 signal as MT emission.

The fraction of assimilated carbon re-emitted back into the atmosphere through MT emission was represented by the carbon ratio between daily total emitted MT and Pn. The 81/Pn carbon ratio could however not be calculated due to the contribution of different unidentified molecules with the same molecular mass. Both Pn and MT emission were normalized for leaf area, measured with a leaf area meter (type LI-3000, Lincoln, NE, USA) coupled with a LI-3050A transparent conveyer belt.

3.2.5. Radial stem growth and water potential

Linear variable displacement transducers (LVDTs) (type LVDT DF5.0, Solartron Metrology, Leicester, UK) were used to measure stem diameter variations (i.e., stem shrinkage/swelling and radial stem growth). These measurements served for direct plant stress sensing. The sensors were installed at stem level about 20 cm above the soil surface. The LVDTs were stem-attached by custom-made stainless steel holders (Steppe and Lemeur, 2004) (Fig. 3.1).

Stem diameter measurements were complemented with leaf (Ψ_{leaf}) and soil (Ψ_{soil}) water potential measurements. Ψ_{leaf} was measured with a standard Scholander pressure bomb (PMS Instruments, Corvallis, OR, USA). Measurements were made on ten different days, on three individual leaves selected at three different heights. Averages were used in further data analysis. Ψ_{soil} was measured with equitensiometers (type EQ2, Delta-T devices, Cambridge, UK) at a depth of 20 cm (Fig.3.2a and b).

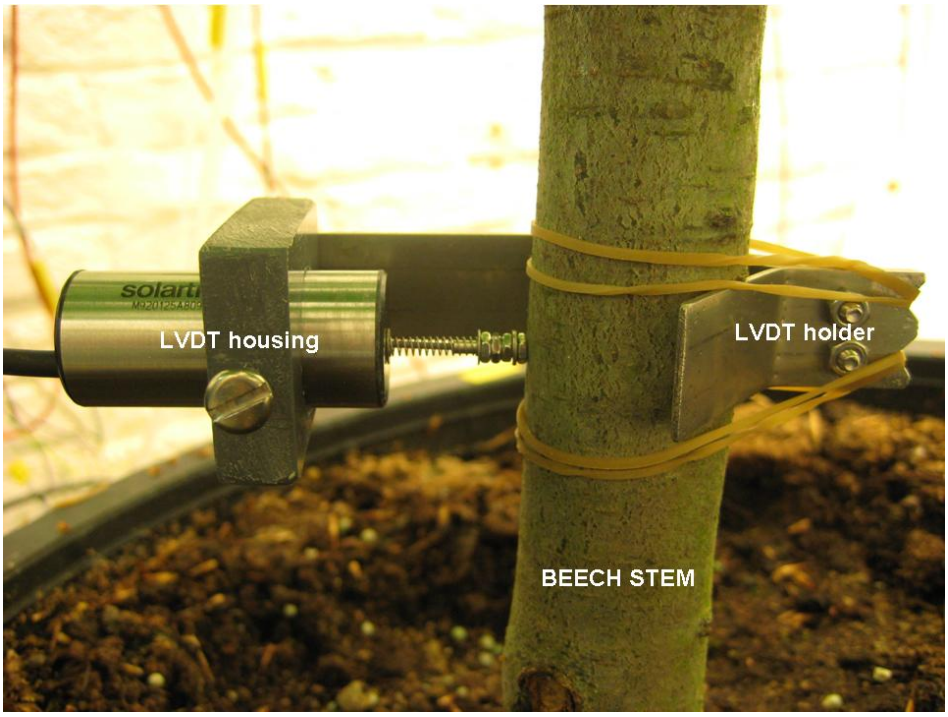

Figure 3.1: Used plant sensors: LVDT (linear variable displacement transducer) sensor for measurement of stem diameter variations as growth component including installed parts on beech stem.

3.3 Results

3.3.1. Drought stress effect on time series of Pn and MT emission

Both Pn and MT emission showed daily dynamics that were affected by the imposed drought stress (Fig. 3.3a and b). Pn started to decrease ten days after withholding the water supply, followed by a decrease in MT emission. Pn decreased from daily maxima of 8.4 µmol-CO_2 m^{-2} s^{-1} (DOY 186-195) to daily maxima of only 2.03 µmol-CO_2 m^{-2} s^{-1} towards the end of the drought stress period (DOY 204) (Fig. 3.3a), representing a reduction of 76%. In contrast to Pn, MT emissions first increased when drought stress was imposed, reaching daily maxima of 470 µg m^{-2} h^{-1} (DOY 195). Then, MT emission started to decrease (to 95 µg m^{-2} h^{-1} at DOY 204). Emission linked to the *m/z* 81 signal showed a similar trend as the MT emission during most of the time, but interestingly a clear increase was observed at the end of the drought stress period, while the MT emission kept decreasing. The different behaviour of MT emission and the *m/z* 81 signal became clear from DOY 203 onwards (Fig. 3.3b). During the imposed drought stress period, Rd slightly decreased (less negative) (range: -0.47 µmol-CO_2 m^{-2} s^{-1} at the beginning of experiment to -0.32 µmol-CO_2 m^{-2} s^{-1}, DOY 204 at the end of experiment). Immediately after re-watering (DOY 204), Pn stabilized

and did not show a further decrease. In contrast, MT emission decreased further reaching its lowest values. Upon re-watering, the *m/z* 81 signal showed an immediate response to the new soil water availability and showed a sudden decrease, remaining low afterwards.

Simultaneous measurements made by TD-GC/MS confirmed the *m/z* 137 signal measured with PTR-MS is characteristic for MT emissions (i.e. sabinene, limonene, α-pinene, β-pinene, α-terpinene, 3-thujene and γ-terpinene). No changes in compound composition of the emissions due to drought stress were detected (data not shown). This contrasts with the findings of Ormeňo et al. (2007) who observed changes in emission profile on Mediterranean species (oak, pine, rosemary, and spring rock-rose). Also the TD-GC/MS measurements showed a progressive increase in total MT emission at the beginning of the drought stress, followed by a decrease (Fig. 3.4).

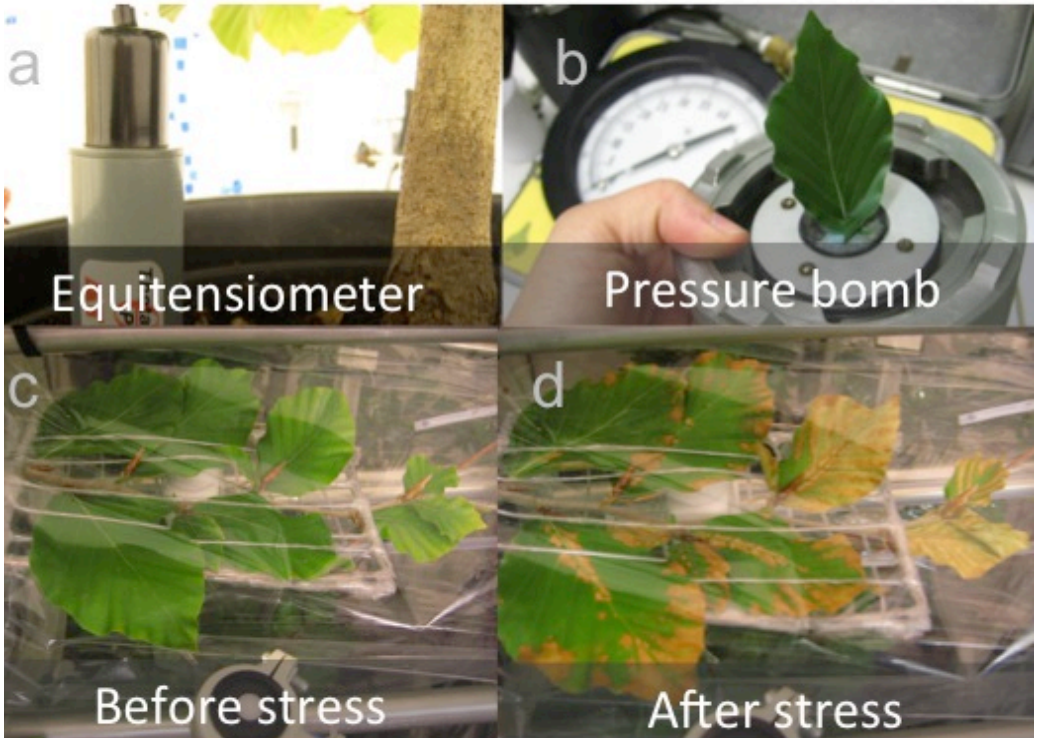

Figure 3.2: a) Equitensiometer for measurement of soil water potential, b) Scholander pressure bomb for measurement of leaf water potential, c-d) visual observation of leaves inside the cuvette before and after drought stress.

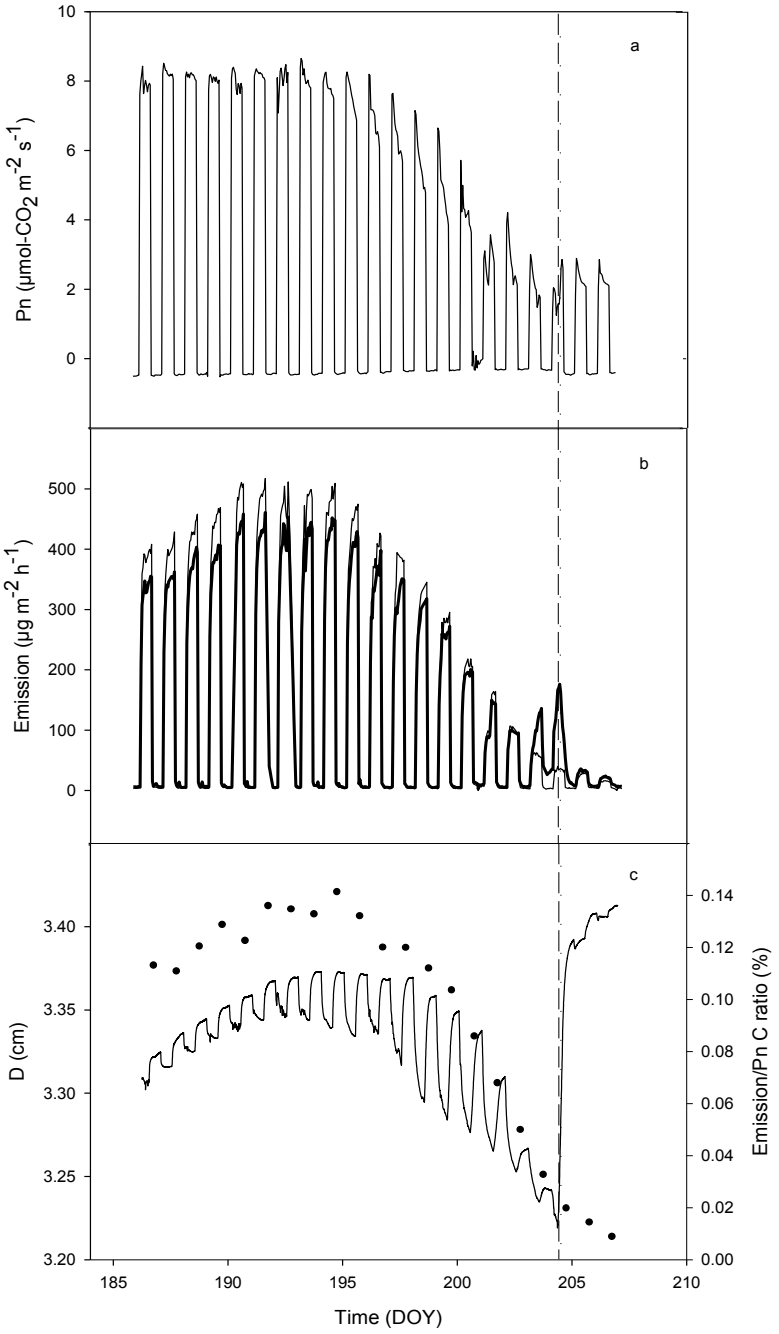

Figure 3.3: From up to down: a) Net photosynthesis (Pn) and dark respiration (Rd), b) monoterpene (MT) emission (m/z 137 signal, black fine) and m/z 81 signal (black bold), c) stem diameter (-, D) variations and daytime carbon (C) ratio for monoterpenes/net photosynthesis (●, emission/Pn carbon ratio). The vertical dashed lines indicate the moment of re-watering (DOY 204 at 15:15h).

3.3.2. Link between Pn, MT emission, and radial stem growth

Observed stem diameter variations showed the strong effect of limiting soil water availability on radial stem growth. At the beginning of the experiment, no drought stress was detected and a normal shrinkage/swelling pattern was observed (Fig. 3.13c) similar to reports of other authors (Steppe et al., 2006; 2007; Saveyn et al., 2007; De Swaef et al., 2009, De Swaef and Steppe, 2010; De Schepper and Steppe, 2010). These regular variations showed a sharp decline in stem diameter in the morning, followed by constant stem diameter representing steady-state conditions. In the evening, an opposite trend was observed, where the stem started swelling due to refilling of internal water reserves (stem diameter increased). From DOY 193 onwards, stem diameter variations became deeper and steeper, indicating the tree's need to rely more and more on its internal water reserves, leading to net growth stagnation. With drought stress continuing, daily shrinkages increased and due to the lack of night-time recovery there was a net daily decrease in stem diameter (from DOY 198 onwards). As tree growth was impaired, the beech's normal ability to photosynthesize (Fig. 3.2c and d) and emit MTs was disturbed, probably due to disturbances in biochemical processes (Grote and Niinemets, 2008). Additionally, by imposing drought stress, Ψ_{leaf} and Ψ_{soil} decreased and corresponded with a decrease in Pn and MT emission (Fig. 3.5a, b). Before the acute drought stress, a linear relationship was found between Ψ_{leaf} and Pn, MT emission and *m/z* 81 signal (R^2=0.94, 0.95 and 0.93, respectively), while an exponential relationship was found between Ψ_{soil} and Pn, MT emission and *m/z* 81 signal (R^2=0.97, 0.92 and 0.87, respectively) (Fig. 3.5a, b).

Differentiation between MT emission and the *m/z* 81 signal became most clear when the plant really started suffering from the imposed drought stress (i.e. negative stem growth). During the days just before re-watering (DOY 204), the *m/z* 81 signal increased and deviated clearly from MT emission. Immediately after re-watering the *m/z* 81 signal started to decline. Upon re-watering, also stem diameter responded immediately to the water supply, resulting in a recovery in radial stem growth. At that same time instance, the fraction of assimilated carbon re-emitted back into the atmosphere through the *m/z* 81 signal became low again. Noteworthy is that, in contrast to MT emission, the *m/z* 81 signal remained substantial during the night (DOY 204 and 205, nighttime mean value of 52.38 and 18.35 µg m^{-2} h^{-1}, respectively, compared to 8.38 and 8.13 µg m^{-2} h^{-1} on DOY 203 and 206, respectively).

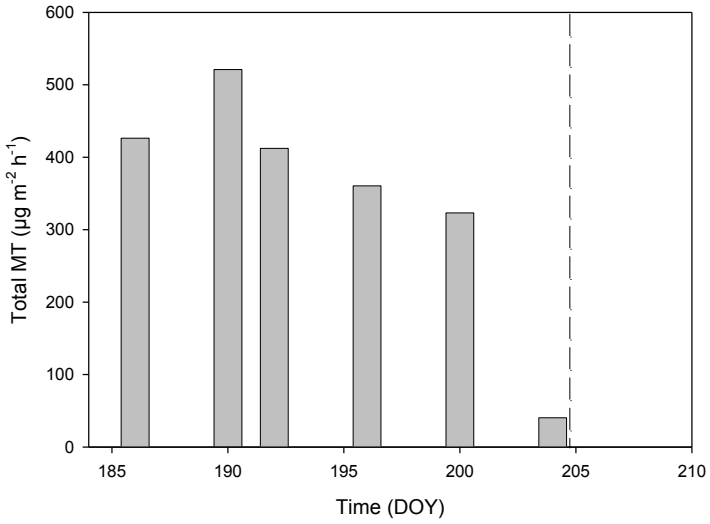

Figure 3.4: Dynamics of during the development of drought stress measured by the TD-GC/MS technique. The line indicates the re-watering day.

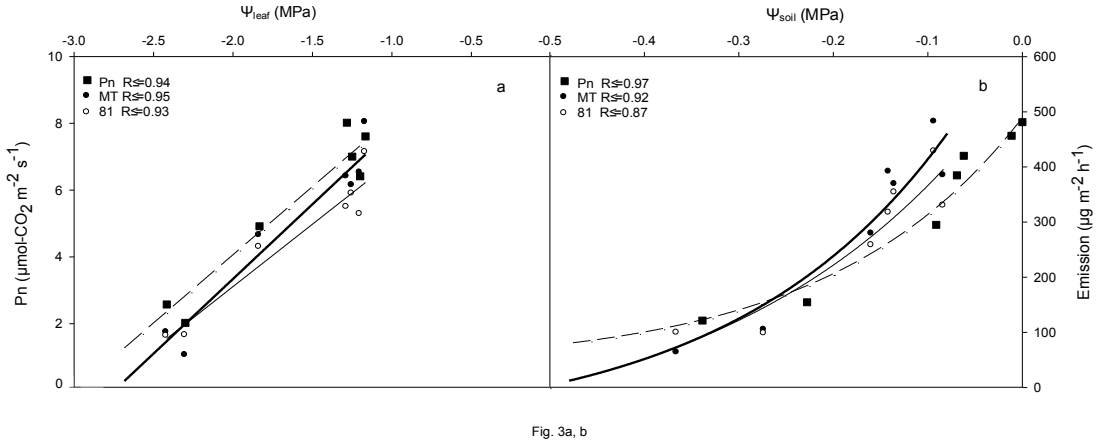

Figure 3.5: Net photosynthesis (Pn, ■), monoterpenes as MT (emission, ●) and m/z 81 signal (emission, ○) as function of a) leaf water potential Ψ_{leaf} (MPa) (n=3) and b) soil water potential Ψ_{soil} (MPa) before acute drought stress.

3.4 Discussion

3.4.1. Link between radial stem growth and changes in Pn, MT, and carbon ratio

During the first days following the application of drought stress, the stem diameter measurements still indicated radial stem growth and no stress effects on stem diameter were observed (Fig. 3.3c). Daily dynamics showed a typical decline in stem diameter (stem shrinkage) during the day due to leaf transpiration exceeding root water uptake, while the stem diameter increased (stem swelling)

during the night due to refilling of internal water reserves. During this period of positive radial stem growth, Pn was not impaired and could be explained by an increased carbon allocation to growth. Also Ψ_{leaf} and Ψ_{soil} were not affected as long as the beech tree kept growing (Fig. 3.3a, b). When drought stress continued, it however triggered responses in both tree water relations (stem diameter, Ψ_{leaf}, Ψ_{soil}) and carbon metabolism (stem diameter, Pn, MT emission and their carbon ratio) (Fig. 3.3 and 3.5). Interestingly, the increase in stem diameter (radial stem growth) ceased at the same moment Pn started to decrease. Pn inhibition could be the result of leaf CO_2 diffusion limitations, decreasing leaf internal CO_2 concentration, leaf biochemical changes, such as a decline in Rubisco abundance and/or activity (Rennenberg et al., 2006) or a carbon allocation divergence to non-photosynthetic organs and/or defense molecules (Chavez et al., 2003, Llusia et al., 2006; Pinheiro and Chavez, 2011). As a consequence of the reduction in Pn, Rd gradually decreased (less negative), possibly meaning less investment into maintenance (R_m) and/or growth (R_g) respiration (Larcher, 2003). A decline in R_m typically shows the overall slowing of the metabolic activity during drought (Gratani et al., 2008). In our study Rd (-0.47 μmol-CO_2 m^{-2} s^{-1} to -0.32 μmol-CO_2 m^{-2} s^{-1}) was similar to Rd for other drought stressed tree species ranging between -2.11 and -0.20 μmol-CO_2 m^{-2} s^{-1} (Morecroft and Roberts, 1999, Yin et al., 2006, Lombardini et al., 2009).
Stem growth cessation could be explained by a lower turgor pressure, limited carbohydrate supply or both (Christiansen et al., 1987; Daudet et al., 2005; Saveyn et al., 2007; Steppe et al., 2008; De Swaef et al., 2009; Lavoir et al., 2009; De Schepper and Steppe, 2010). Re-watering (DOY 204) caused an immediate increase in stem diameter, indicating tree storage compartment re-filling and stem growth continuation. After re-watering, only a partial recovery in Pn was observed, meaning possible irreversible tree damage, and also MT emission and MT/Pn carbon ratio remained low.

In accordance with other authors (Sharkey and Loreto, 1993; Bertin and Staudt, 1996; Ormeňo et al., 2007), an increasing-decreasing trend in MT emission was observed. In these earlier studies, stress was monitored using measurements of Pn (Sharkey and Loreto, 1993), Ψ_{soil} (Bertin and Staudt, 1996) or Ψ_{leaf} (Ormeňo et al., 2007), while we continuously sensed plant stress with LVDTs. The different, sometimes conflicting, BVOC-related drought stress responses reported in the literature depends on the drought stress level (Ormeňo et al., 2007; Niinemets, 2009), the level of proteins and/or the substrate supply (Fortunatti et al., 2008). In our study, the MT emission (as reflected by both the MT and the *m/z* 81 signal) and the MT/Pn carbon ratio increased as long as the beech stem was growing (Fig. 3.3a, b, c). This increase in MT emission and in the MT/Pn carbon ratio could be explained by a protection function used by the tree (Delfine et al., 2005). Indeed, a larger fraction of carbon might be allocated to MT formation to mitigate chloroplast oxidative damage, as trees might

accumulate harmful free radicals during drought stress (Apel and Hirt, 2004; Delfine et al., 2005; Galle and Feller, 2007). Not only Pn and MT emission, but also the MT/Pn carbon ratio started to decrease from the moment the beech stem stopped growing (Fig. 3.3c). This implies that the MT emission was more affected than Pn during drought stress. The decreases became most pronounced when negative radial stem growth was detected. Beech was no longer able to refill its internal water reserves overnight and, hence, keep sufficient turgor, affecting its carbon metabolism (Saveyn et al., 2007).

3.4.2. Drought stress induced differentiation in m/z 137 and m/z 81 signals

In contrast to the MT emission, the *m/z* 81 signal showed a deviating but clear increase when pronounced negative radial stem growth occurred (Fig. 3.3b, c). At this level of drought stress, beech seemed to allocate a small portion of carbon to a certain non-identified *m/z* 81 compound. Possibly, a biosynthetic shift happened in the beech under this level of drought stress, leading to an emission burst of a BVOC that is different from a MT. However, individual MT speciation is beyond the scope and is covered in depth elsewhere (Joó, 2011; Pokorska, 2012) and will not be discussed in detail. Interestingly, in contrast to the MT emissions, at night before and after re-watering (DOY 204 and 205), the *m/z* 81 signal showed a substantial night-time emission likely indicating beech's non-specific storage pool(s) usage (Demarcke et al., 2010) from specialized organs in leaves and/or stem (Peňuelas and Llusia, 2003). Upon re-watering, this burst in *m/z* 81 signal disappeared immediately. Earlier, Davison et al. (2008) and Niinemets (2009) reported about the existence of green leaf volatiles (GLVs) or lipoxygenase (LOX) pathway volatiles. GLVs include 6 carbon-containing (C_6) aldehydes, alcohols, and esters (Holopainen and Gershenzon, 2010). They function as a fast and efficient airborne signaling way to pass stress information onto neighboring trees (Gershenzon, 2007; Niinemets, 2009). A study performed on agricultural grasslands demonstrated that the *m/z* 81 signal is actually stress-related and could be attributed to *Z*-3-hexenal, a GLV (Davison et al., 2008; Ruuskanen et al., 2011). Additionally, it has been documented that plants show higher emissions of *Z*-3-hexenal following biotic stress (Kant et al., 2009; Laothawornkitkul et al., 2009; Holopainen and Gershenzon, 2010). We therefore believe that the measured response in the *m/z* 81 signal might be attributed to such a GLV, but additional work is warranted to chemically characterize this compound in beech using a GC-based approach.

3.5 Conclusions

Responses in Pn, BVOC emissions and their carbon ratio varied strongly depending on the physiological status of beech under drought stress. This illustrates that direct plant stress sensing and drought detection is needed for a quantitative assessment of BVOC emissions from vegetation. The use of LVDTs enabled us to continuously monitor the drought stress level. It revealed that the burst of a certain stress-related green leaf volatile (linked to the *m/z* 81 signal) occurred when beech suffered from acute drought stress, indicated by pronounced negative stem growth. Being valid for young potted beech, our approach however should be tested on adult field-grown trees. Future research should elaborate to a mechanistic translation of short and long-term stress effects into BVOC emission models.

Chapter 4

Seasonality of photosynthesis and BVOC emissions for anatomically different tree species under natural biotic stress

Abstract

Increasing levels of air pollution, atmospheric CO_2 concentrations, changing climate and infestations strongly influence light-saturated net photosynthesis (Pn_{max}), dark respiration (Rd) and emissions of biogenic volatile organic compounds (BVOCs) in trees. We studied the seasonality of Pn_{max}, Rd and BVOC emissions (with focus on specific isoprenoids) in young trees of oak (*Quercus robur* L.), ash (*Fraxinus excelsior* L.) and beech (*Fagus sylvatica* L.) in outdoor conditions. Seasonal BVOC emission patterns were compared with seasonal variations in microclimatological and physiological variables (Pn_{max}, Rd, specific leaf area: SLA, chlorophyll content index: CCI). Strong but different seasonal patterns in Pn_{max} were found for all tree species. In contrast, Rd did not show any straightforward seasonal trend. Overall, BVOC emissions showed a seasonal trend, mainly driven by temperature. However, sudden non-temperature related peaks appeared with a species-specific timing probably related to infestations.

4.1 Introduction

Vegetation emits biogenic volatile organic compounds (BVOCs) into the atmosphere (Kesselmeier and Staudt, 1999), of which isoprenoids comprise the largest class (Gershenzon and Dudareva, 2007). The isoprenoids encountered in nature are biosynthesized by two different biochemical pathways: the mevalonic acid pathway (known as the MVA pathway) (Goodwin, 1971) in the cytosol and the deoxyxylulose-5-phosphate pathway (known as the MEP pathway) in the chloroplasts (McGarvey and Croteau, 1995; Lichtenthaler et al., 1997; Loreto and Schnitzler, 2010; Holopainen, 2011) without a strict separation of these pathways. Isoprenoids have multiple functions mediating antagonistic and

beneficial interactions among different organisms (Gershenzon and Dudareva, 2007). On the one hand, isoprenoids have multiple beneficial functions: physiological functions (like temperature (Singaas et al., 1997) and ozone protection (Loreto and Velikova, 2001)) and ecological functions (like plant-insect and plant-plant communication or herbivore repellent). On the other hand, isoprenoid emissions enhance biogenic secondary organic aerosol formation, modification of the ground-level tropospheric ozone budget and provoke indirect radiative forcing enhancement (Cahill et al., 2006), all associated directly or indirectly with human health impacts.

Seasonality in the physiology of deciduous trees is usually described by major visible phenological events like budburst and leaf unfolding followed by leaf development (spring), leaf maturation (summer), general leaf coloring and leaf senescence (autumn) (Kodani et al., 2002; Wang et al., 2005). These events critically influence light-saturated net photosynthesis (Pn_{max}) (Morecroft and Roberts, 1999; Seiwa, 1999) and isoprenoid emissions (Schnitzler et al., 1997; Owen and Peñuelas, 2005; Laffineur et al., 2011). In contrast to Pn_{max}, there is very little information available about changes in dark respiration (Rd) and isoprenoid emissions across the season and with tree age (Steppe et al., 2011). Leaf Rd is however an important component in the tree's carbon balance, expected to vary diurnally, seasonally and with tree age (Gratani et al., 2008). Additionally, the emissions depend on tissue type, tree age, time of the day, growth conditions (Kant et al., 2009), leaf tissue quality (Bignucolo and Körner, 2010), leaf positioning (sun/shade) (Niinemets and Tenhunen, 1997) and carbon allocation patterns which are different in shade tolerant or intolerant species (Imaji and Seiwa, 2010).

Plants allocate their net photosynthetic production to growth, defense and storage (Chapin et al., 1990). Natural stress occurrences (Niinemets, 2010) can induce or inhibit isoprenoid emissions (Loreto and Schnitzler, 2010) and impact plant carbon allocation patterns and tree physiology (Šimpraga et al., 2011a, b). Seasonal abiotic (e.g. temperature) and biotic (e.g. infestations) factors significantly affect growth, photosynthetic responses (Myers et al., 1999) and/or isoprenoid emissions (Niinemets, 2010). Besides light, temperature is the major determinant of photosynthetic and respiratory activity (Armstrong et al., 2006; Gratani et al., 2008; Richardson et al., 2011) and one of the main driving variables of isoprenoid emissions (Kesselmeier et al., 2002; Laffineur et al., 2011).

Amongst deciduous tree species, a distinction can be made between ring-porous and diffuse-porous wood anatomy. Oak and ash (*Querus robur* L. and *Fraxinus excelsior* L., respectively), both ring-porous species, have larger early wood vessels arranged in a ring and a large part of their early wood is formed before leaf expansion in spring. In contrast, beech (*Fagus sylvatica* L.), a diffuse-porous species, contains vessels of more unique dimensions which are more

evenly distributed over the growth ring and its leaf expansion starts before stem growth (Barbaroux and Breda, 2002) or coincides with the onset of wood formation (Čufar et al., 2008). Contrasting phenology and wood anatomy accompanied by differences in stored carbohydrate dynamics (Barbaroux and Breda, 2002), seasonal dynamics in wood formation (Čufar et al., 2008) and different naturally present stressors (Niinemets, 2009) could possibly explain specific seasonal patterns observed in Pn_{max} and emissions.

We compared the seasonal variation in BVOC emissions, with focus on monoterpenoids (MTs) and light-saturated net photosynthesis from two ring-porous (*Querus robur* L. and *Fraxinus excelsior* L.) and one diffuse-porous (*Fagus sylvatica* L.) tree species with the main objective to quantify and compare seasonal Pn_{max} and Rd with BVOC emissions.

4.2 Materials and methods

4.2.1 Plant material and experimental set up

The experiments were conducted during the growing season 2009 on three to four-year-old trees. In total, three potted beech (*Fagus sylvatica* L.), three potted ash (*Fraxinus excelsior* L.) and four potted oak (*Quercus robur* L.) trees were used. The young trees were grown in 40-L containers filled with a commercial soil mixture (Agrofino, Arendonk, Belgium) fertilized with basacote (Basacote Plus® 12M, COMPO Benelux, Belgium). Water was supplied regularly to keep the soil well-watered. All trees were on average 1.80 m high at the beginning of the experiment and their stem diameter ranged from 21 to 34 mm. The measurements were conducted outdoors at the campus of the Faculty of Bioscience Engineering, Ghent University (Belgium, 51°3'N, 3°43'E).

A dynamic branch enclosure system was used for isoprenoid emission measurements with the focus on specific monoterpenoids (MTs) and was operated according to Joó et al. (2010a) and Pokorska et al. (2011). In total, three cuvettes were used sequentially, measuring selected branches (at 1.20 m height) of all tree individuals throughout the growing season. The branch enclosure system was equipped with air temperature (type thermistor 10k, NTC, Omega, The Netherlands) and photosynthetically active radiation (PAR; type Li-190S; Li-COR, Nebraska, USA) sensors (Fig. 4.1). Scanning frequency of all sensors was 30 s and data were saved onto a datalogger extended with a multiplexer (type Cambell Scientific CR1000 and Cambell Scientific AM16-32, Loughborough, United Kingdom).

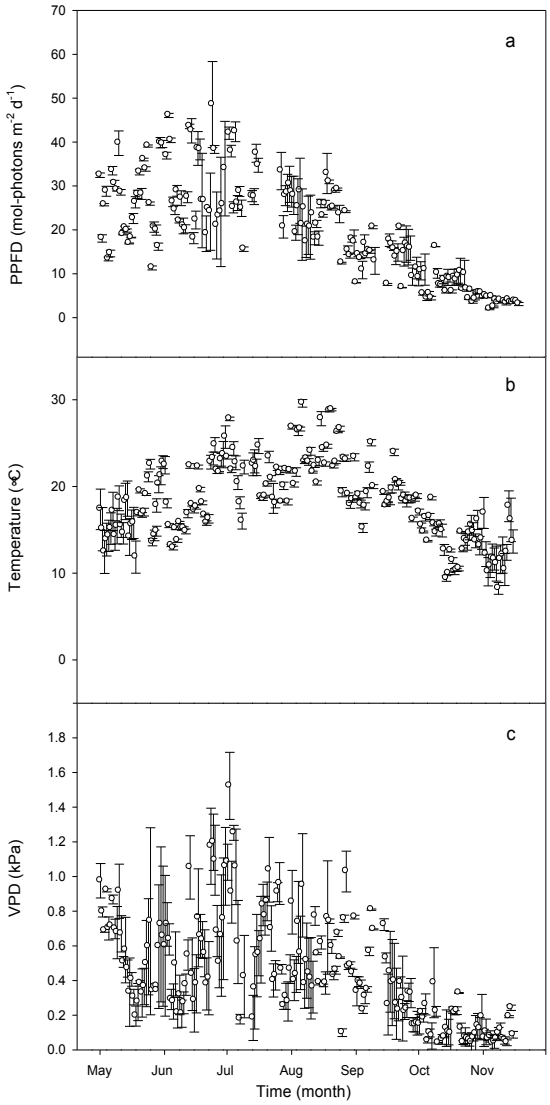

Figure 4.1: Microclimatic conditions characterized by (a) photosynthetic photon flux density (PPFD) (b) air temperature, and (c) air vapor pressure deficit (VPD). Day-time values are represented indicating standard variation (±SD).

4.2.2 Chlorophyll content index, specific leaf area and leaf area

Leaf phenology of all trees was monitored by recording the budburst and the start of leaf fall. Leaf budburst of the investigated trees was defined when approximately 20% of the leaf buds were open and leaf fall when 20% of leaves fell off (Hideyuki and Izumi, 2007). Chlorophyll content index (CCI) was used as an additional phenological indicator and was measured using a portable chlorophyll content reflectometer (type SPAD-500, Konica Minolta, Osaka, Japan), which detects the reflection coefficient of green wavelengths. CCI expresses the relative amount of chlorophyll pigments in the leaf tissue and is related to its green coloration. On

each tree, two selected leaves were measured. The average was used in further analysis. Sampling included in total 14 dates from April to November 2009.

Additionally, at all followed phenological stages (leaf development, leaf maturity and leaf fall) the specific leaf area (SLA, ratio of leaf surface to leaf dry mass, $m^2\ kg^{-1}$) was determined. In total, eight leaves were used per phenological stage and per tree. Leaf fresh weight and leaf area were determined by using a scale and a leaf area meter (type LI-3000, Lincoln, NE, USA) coupled with a LI-3050A transparent belt conveyer, respectively. Afterwards, the leaves were dried in an oven at 60 °C to constant weight for determination of leaf dry weight. At the end of the experiment dry weight of the leaves in the cuvette was determined and used for BVOC emissions normalization.

4.2.3 Light-saturated net photosynthesis and dark respiration

An open differential infra red gas analyzer (type IRGA LI-6400, Li-cor, Lincoln, Nebraska, USA) was used for measurements of light-saturated net photosynthesis (Pn_{max}) and dark respiration (Rd) (Fig. 4.4). The LI-6400 consisted of a 2x3 cm leaf chamber, containing a blue and red light emitting diodes (LED) light source. Pn_{max} was measured at an applied light intensity of 1800 µmol-photons $m^{-2}\ s^{-1}$, while Rd was determined at 0 µmol-photons $m^{-2}\ s^{-1}$. The IRGA leaf chamber conditions were set on temperature of 25 °C and 400 ppm CO_2 concentration. Measurements were performed between 11-13 h (n=1-4 leaves of each tree per sampling date). Averages were used in further analysis.

4.2.3 Gas chromatographic BVOC analysis

Measurements of BVOC emissions were performed at the same sampling moment as the Pn_{max} and Rd measurements. Teflon-perfluoroalkoxy foil (Teflon-PFA) (type WF Norton, Saint Gobain Performance Plastics, Kontich, Belgium) was used for the cuvette construction, minimizing wall-losses of the emitted substances and transmitting a wide spectral range of shortwave radiation, especially the photosynthetically active range (Schuh et al., 1997). A Teflon-PFA-coated ventilator mixed the air homogeneously inside the cuvette and reduced the leaf boundary layer resistance. Dust filters (type Zefluor TM PTFE Membrane filter, Pall, MI, USA) as well as a manganese dioxide (MnO_2) ozone filter (type ETO341FC003, Ansyco, Karlsruhe, Germany) were added to provide respectively dust and ozone-free air. The air passed through active coal filters removing VOCs from the outside air entering the branch enclosure system. Sampling was performed using Tenax/Carbotrap multi-adsorption tubes and TD-GC/MS technique was used for analysis. Adsorbent tubes were first conditioned at 300 °C under a helium (He) flow for 1 h, then covered with aluminium foil and stored in a drier for maximum seven days. Toluene D-8 was used as an internal

standard. Desorption of tubes was performed with a thermal desorber and autosampler (type ULTRA 50-50 UNITY Markes International, Llantrisant, UK) while separation was done using a GC Trace 2000 gas chromatograph (type ThermoFinnigan, Milan, Italy). The mass analyzer used for compound detection was a Trace DSQ Quadrupole mass spectrometer (type Thermo Finnigan, Houston, TX, USA). Due to switching of the cuvettes, BVOC emissions were sampled as point measurements at irregular intervals. TD-GC/MS calibration was performed monthly using a standard mixture (Luxfer, Inc., Riverside, CA, N150, 1800 psig) containing isoprene (0.515 ppmv), α-pinene (0.496 ppmv), β-pinene (0.501 ppmv), sabinene (0.492 ppmv), limonene (0.486 ppmv), linalool (0.473 ppmv) and (Z)-3-hexenyl-acetate (0.499 ppmv). For the quantification of BVOCs not present in the gas standard, response factors of sabinene were used (Joó et al., 2010). Pure standards of (Z)-β–ocimene (Fluka≥90%), MeSA (TCI Europe>99%), β-caryophyllene (Fluka>90%), and α-farnesene (extraction form *Chaenomeles superba*, Poland) and methyl dihydrojasmonate (Sigma Aldrich 96%). Further details of the used TD-GC/MS technique are explained in Joó et al. (2010) and Pokorska et al. (2011). We emphasize that BVOC speciation is beyond the scope of the present work and is covered in depth elsewhere (Joó, 2011; Pokorska, 2012). For this reason, it will not be discussed here in detail. The focus of this work is rather on the related tree physiology.

4.2.4 Entomological sampling and identification

In this study, Pn measurements and BVOC emissions were accompanied by entomological identification. Seasonal entomological observations were followed on all trees by using a loupe and microscopes (type SZX12-ILLK200 and type BX51TF, Olympus corporation, Tokyo, Japan) with Color View III camera imaging system and the software package CELL-F (Olympus Soft Imaging Solutions).

4.2.5 Statistical analysis

All statistical analysis was performed with R version 2.11.1 statistical software (R Development Core Team 2010). The influence of tree species and time on Pn_{max} and Rd was assessed by applying a linear mixed model (Pinheiro et al., 2009) with tree species and time as fixed effects and leaf within an individual tree as random effect to account for the repeated observations on the same leaf. In case of a significant effect between tree species and time, data were split up per date and the effect of tree species was tested on each date by means of Tukey's honestly significant difference test (Hothorn et al., 2008) (Table 4.1). The threshold for statistical significance was $P<0.05$. An exponential model in function

of temperature was fitted to the BVOCs data. The residuals between the data and temperature-based estimates were tested for their correlation with light.

4.3 Results

4.3.1 Phenology, specific leaf area and chlorophyll content index

As expected, the timing of budburst of the three tree species differed. The budburst of ash was initiated first (DOY 105), followed by oak (DOY 113) and beech (DOY 116). Not all buds expanded into shoots, because some remained dormant or died.

Except for ash, all trees showed a clear seasonal CCI trend linked to leaf phenology. In the beginning of the growing season, a CCI increase was observed for all tree species (Fig. 4.2) and a maximum was reached approximately 60 days after budburst. In the middle of July, the CCI of oak and ash ranged between 30 and 45, while for beech it ranged between 25 and 35. During leaf maturation, high and constant CCIs were obtained. At the end of the growing season, when leaves started to senescence, the CCI of oak and beech declined by 50% over a two month period, while the CCI of ash remained high.

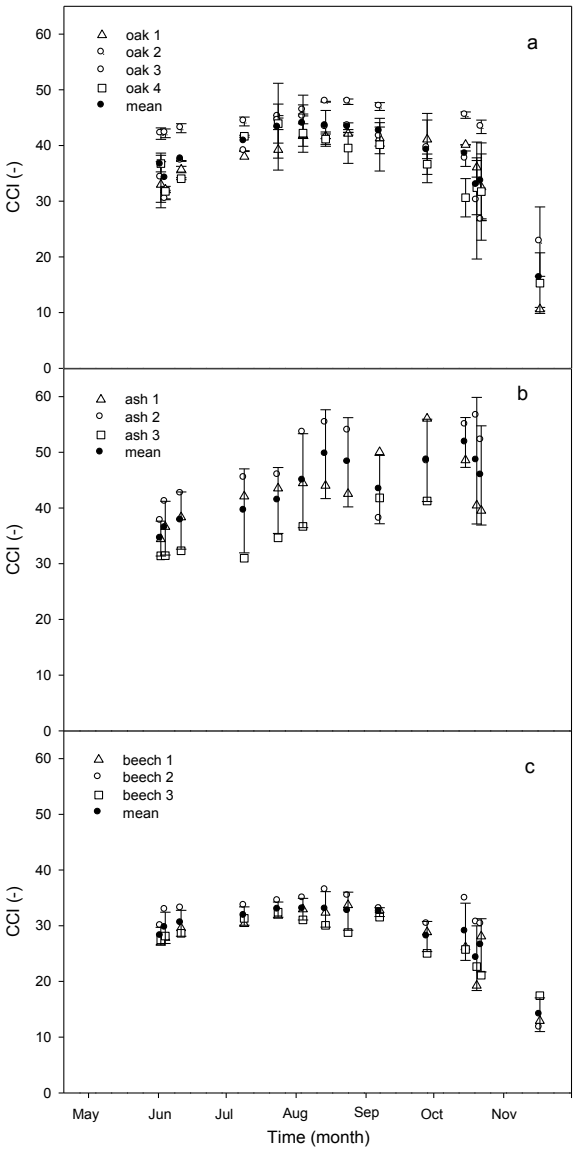

Figure 4.2: Seasonal dynamics of chlorophyll content index (CCI) of oak (a), ash (b), and beech (c). The error bars indicate the standard deviation (±SD).

In contrast to CCI, SLA showed no strong seasonal patterns (Fig. 4.3). A decreasing trend was observed for SLA in oak (from 25 to 11 $m^2 kg^{-1}$) and ash (from 24 till 10 $m^2 kg^{-1}$), while beech showed a slight increase at the end of the growing season.

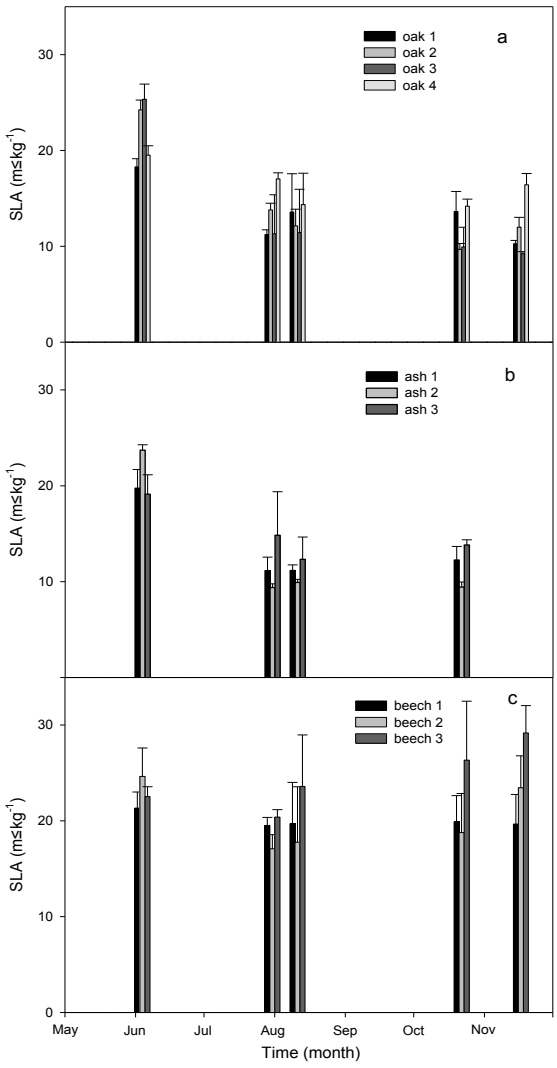

Figure 4.3: Seasonal dynamics of specific leaf area (SLA) of oak (a), ash (b), and beech (c). The error bars indicate the standard deviation (±SD).

Ash (DOY 295) lost its leaves before oak (DOY 321) and beech. The young potted beech trees kept their senescent leaves during winter until the next growing season (DOY 114). As expected, time of leaf shedding and duration of leafless periods varied strongly amongst the investigated tree species.

4.3.2 Seasonal trends in Pn_{max}, Rd and BVOC emissions

Pn_{max} was significantly affected by time and by the interaction between tree species and time (Table 4.1). The oak, ash and beech seasonal variation in Pn_{max} ranged between 2 to 15 µmol-CO_2 m^{-2} s^{-1}, 1.8 to 18 µmol-CO_2 m^{-2} s^{-1} and 3.5 to 13 µmol-CO_2 m^{-2} s^{-1}, respectively (Fig. 4.4a, c, e).

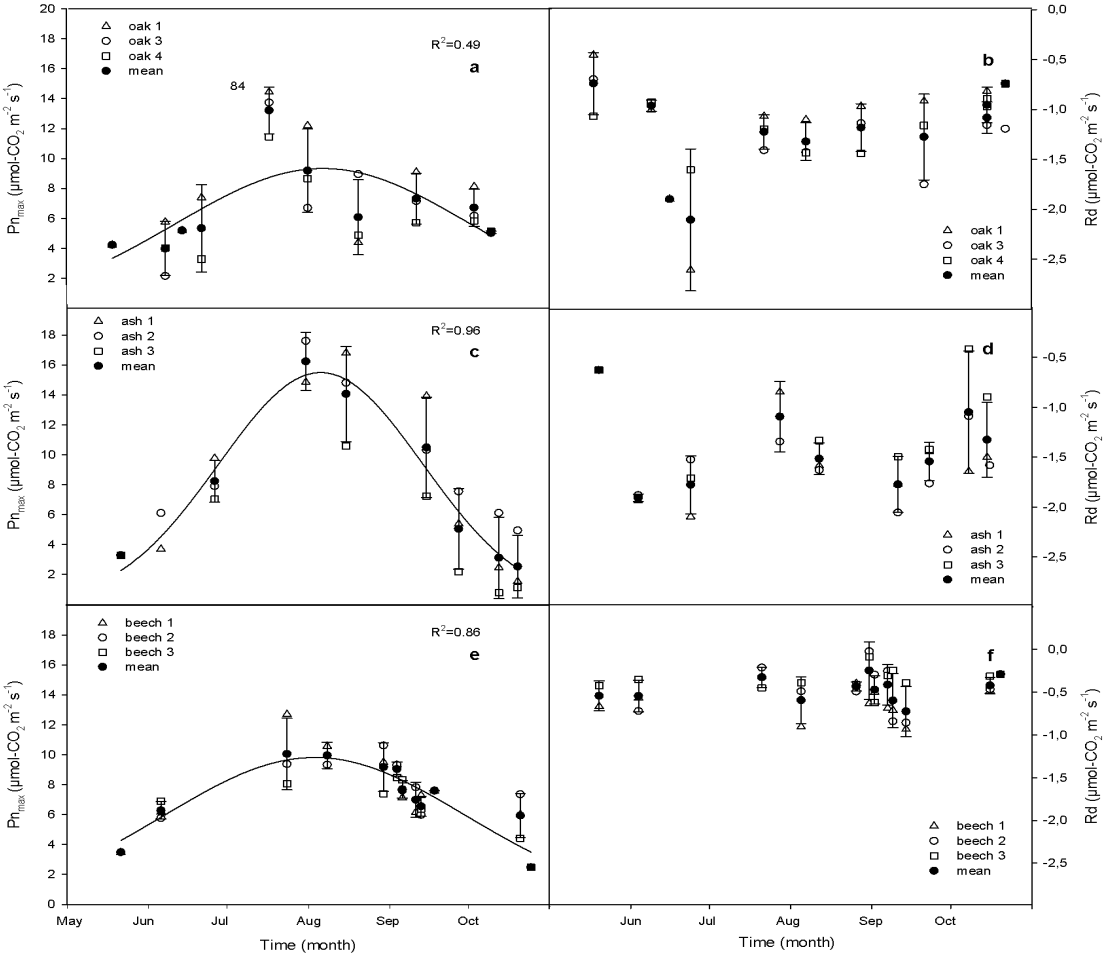

Figure 4.4: Seasonal dynamics of light-saturated net photosynthesis (Pn_{max}) and dark respiration (Rd) of oak (a, d), ash (b, e), and beech (c, f). The error bars indicate the standard deviation (±SD). The light-saturated net photosynthesis (Pn_{max}) trends are indicated by a black solid line. The fitted curves are of the form: $y=a \times exp(-bx((x-c)/d)^2)$.

Leaves of all tree species reached full Pn_{max} around mid-July. Ash showed no significant differences in the beginning and at the end of the growing season compared to oak and beech, while in June, July and August significantly higher (P<0.05) Pn_{max} values were found (18 µmol-CO_2 m^{-2} s^{-1}) compared to oak and beech. Oak and beech showed a seasonal trend, but no significant differences could be detected between both species. In all species, higher Pn_{max} values were associated with higher stomatal conductance (data not shown), indicating stomatal control over the photosynthetic carbon assimilation.

In contrast to Pn_{max}, in all tree species, no clear seasonal trends in leaf Rd were observed, similar to what has been found for other tree species (Lombardini et al., 2009; Chu et al., 2011). However, the absolute values of Rd differed between the investigated species. Rd in oak and ash ranged from -2.7 to -0.4 μmol-CO_2 m^{-2} s^{-1} and from -2.1 to -0.4 μmol-CO_2 m^{-2} s^{-1}, respectively. Beech had significantly smaller (P<0.001) Rd (-0.1 to -1.0 μmol-CO_2 m^{-2} s^{-1}) than oak and ash (Fig. 4.4b, d, f).

Measured seasonal variation in BVOC emissions for oak and beech ranged from 0.50 to 110.10 μg g_{dw}^{-1} h^{-1} and from 0.08 to 18.42 μg g_{dw}^{-1} h^{-1}, respectively. Ash indicated variations between 0.01 and 0.74 μg g_{dw}^{-1} h^{-1}. We established temperature relationships based on simple exponential regression (Fig. 4.5b, d, f). This was used for quantifying temporal trends in BVOC emissions. Moreover, the residuals were not significantly correlated with light (a very low R^2), indicating the dominance of temperature in the pattern (data not shown).

In general, we observed the highest BVOC emissions in oak, followed by beech and ash, the latter two orders of magnitude lower compared to oak. Temperature-based estimations in BVOC emissions indicated distinct peaks for each tree species, but the actual measurements showed sometimes different peaks with different timing. The residuals between the measurements and the estimated emissions based on temperature showed only a small correlation with light for beech (R^2=0.21) and no correlation for oak (R^2=0.03) and ash (R^2<0.01) (data not shown). Depending on tree species, different insects or fungi were identified (Table 4.2).

4.3.3 TD-GC/MS identification

The detected emissions indicated a mixture of various identified compounds classified as hemiterpenes, MTs, SQTs and benzenoids. In total a mixture of 7, 12 and 17 compounds in oak, ash and beech, respectively, were identified. Major MTs and some other non-MT compounds with minor emission rates at the lower limit of detection were detected. The following species were identified: sabinene, limonene, α-pinene, β-pinene, α-terpinene, 3-thujene, g-terpinene, (Z)-β-ocimene, (E)-β-ocimene, linalool, methyl salicylate (MeSA), 4,8-dimethyl-1,3,7-nonatriene (DMNT), isoprene, β-caryophyllene, α-farnesene, methyl dihydrojasmonate, 6-methyl-5-heptene-2-one, 3-thujene, α-phellandrene, α-terpinene, γ-terpinene, terpinolene, α-humulene depending on species. Details are available in Pokorska et al. (2011). Some of these identified BVOC species are discussed below as an indication for the contribution of infection-based emissions.

Table 4.1 Statistical analysis of light-saturated net photosynthesis (Pn_{max}) and dark respiration (Rd). P-values for the significance of species, time and their interactions are shown. Star (*) indicates significant difference, while non-significant difference is indicated with ns.

Variable	Species	Time	Species x time
Pn_{max}	0.1857^{ns}	$<0.001^{*}$	$<0.001^{*}$
Rd	$<0.001^{*}$	$<0.001^{*}$	0.2055^{ns}

Table 4.2 Identified pests (insects/fungi) on the studied oak, ash and beech trees throughout the 2009 growing season.

Oak	Species latin name	Species english name	Family/Order
June	*Tuberculoides annulatus*	oak leaf aphid	family *Aphididae*, insect order *Homoptera*
End of June-September	*Microsphaera alphitoides*	powdery mildew	*family Erysiphaceae, fungi order Erysiphales*
Ash			
May	unidentified *Heteroptera*	true bugs	insect order *Heteroptera*, insect order Colleoptera
June	*Psyllopsis fraxini*	ash plant louse	family *Psyllidae*, insect order Hemiptera
June	unidentified species of family *Cecidomyiidae*	*gall midges*	*family Cecidomyidae,* insect order *Diptera*
July-September	*Phyllactinia fraxini*	powdery mildew	*family Erysiphaceae,* fungi order *Erysiphales*
Beech			
May-September	*Phyllaphis fagi*	Beech woolly aphid	family *Aphididae*, insect order *Homoptera*

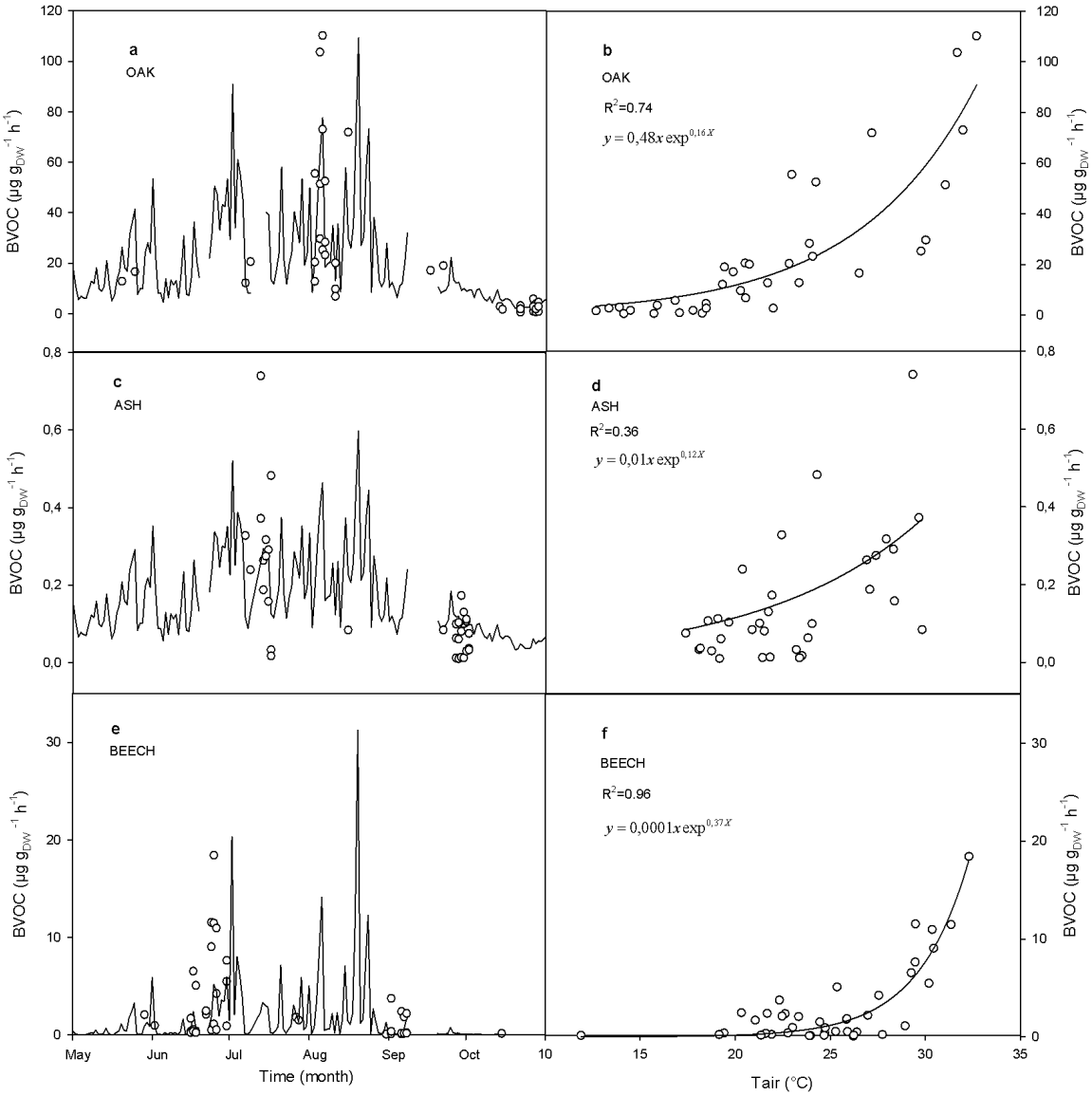

Figure 4.5: Seasonal dynamics of total measured (points) and modelled (line) BVOC emissions of oak (a-b), ash (c-d), and beech (e-f). The exponential function was fitted as indicated in b, d and f.

4.4 Discussion

Seasonal trends in Pn_{max}

Clear seasonal trends were observed in Pn_{max} for all investigated tree species. This is in accordance with previous studies (Morecroft and Roberts, 1999; Staudt et al., 2002; Grassi and Magnani, 2005; Manzanera and Martínez-Chacón, 2007; Gratani et al., 2008; Tani and Kawawata, 2008; Porcar-Castell et al., 2009). In contrast, no clear seasonal trends were observed in Rd for all species.

The Pn_{max} seasonality was however different for each tree species. Compared to oak and beech, ash reached significantly higher Pn_{max} values in July, possibly associated with higher carboxylation rates and Rubisco efficiency (Crafts-Brander and Salvucci, 2000), resulting in higher growth rates (Dobrovolska et al., 2011). These features are reflected in the growth form of ash with long upwards oriented leaves (Saxe and Kerstiens, 2005) allowing for higher light capture and maximizing net carbon gain (Fig. 4.4c). In contrast, beech, classified as a shade-tolerant tree species with mosaic-like leaf positioning and dense canopy (Saxe and Kerstiens, 2005) had a lower but overall not significantly different Pn_{max} (Kozlowski, 1992; Sarijeva et al., 2007) compared to oak. Throughout the growing season, as expected (Zaragoza-Castells et al., 2007; Rodriguez-Calccerada et al., 2009), the shade-tolerant beech showed the lowest leaf Rd rates. This could be explained by shade-induced reduction in the respiratory capacity (i.e. lower amount of mitochondria and amount/activity of mitochondrial enzymes) and/or the lower availability of carbohydrates (Rodriguez-Calccerada et al., 2009). In literature, no values of ash Rd were found to compare with. In our study, oak leaf Rd was similar to leaf Rd of other field-grown oak trees (Bolstad et al., 2003).

BVOC emissions variability between species

The presence of BVOC emissions in oak, ash and beech has been reported earlier (Staudt et al., 2002; Tani and Kawawata, 2008; Joó et al., 2010a; Pokorska et al., 2011). In absolute values, the range of oak, ash and beech is in accordance with observations of others (Staudt et al., 2002; Joó et al., 2010a; Pokorska et al., 2011). The magnitude of the BVOC emissions varied significantly among the tree species. Ash had significantly lower BVOC emission rates compared to the other two species (Fig. 4.5c) while it had higher Pn_{max} rates. Ash clearly invested relatively more carbon in growth than defense (Imaji and Seiwa, 2010). It is known that oak has a high carbon storage capacity (carbohydrate reserves) (Barbaroux and Breda, 2002) possibly responsible for the three-fold higher BVOC emissions compared to beech, despite its similar Pn_{max}. Beech is known to have relatively high SLA and low chlorophyll values (Vandenbussche et al., 2005), which is confirmed in our study. Therefore, beech is possibly allocating more carbon to defense (BVOC) rather than storage (Imaji and Seiwa, 2010).

Temperature dominated the seasonal BVOC pattern

The seasonal trend in BVOC emission was less straightforward compared to the trends in Pn_{max}, and the limited number of sampling points did not allow us to draw strong conclusions on the overall seasonal pattern of the emissions. Nevertheless, some patterns and some remarkable differences between the

species were observed. These differences are driven by differences in allocation patterns, phenology and infestations.

Most of the day-to-day variations in BVOC emissions could clearly be explained by temperature. For all tree species, a species-specific exponential response of BVOC emissions to air temperatures (Fig. 4.5) was observed which corresponded well with previous studies (Staudt et al., 2002; Räisanen et al., 2009; Laffineur et al., 2011; Šimpraga et al., 2011a). This temperature dependency indicated possible enzymatic control (Lerdau et al., 1997) and/or volatility (Niinemets et al., 2002), which is compound dependent (Peňuelas and Llusia, 1999). The residuals between the measurements and the emission estimates based on temperature showed to be independent from light. Therefore, we suggest that other factors such as phenology and infestation may have influenced the emissions.

Carbon allocation and phenology

Oak's BVOC emission peak could be possibly explained by fructification, when acorns become a CO_2 source and not longer a CO_2 sink. Fruits are known to emit other BVOC species (Laothawornkitkul et al., 2009). Our investigated oak species (*Quercus robur*) is known to be isoprene-dominated (Schnitzler et al., 1997; Pokorska et al., 2011) in contrast to some other oak species that are MT emitters only. For *Quercus ilex* and *Quercus suber* bell-shaped MT seasonal trends were found (Schnitzler et al., 1997; Staudt et al., 2002; Lavoir et al., 2009) and were related to activities of terpene synthases (Loreto and Schnitzler, 2010). The observed trends in BVOC emissions were however too fragmented to be linked with the seasonal pattern of terpene synthase activity, which is known to increase until full leaf maturation and decrease with the onset of leaf senescence (Schnitzler et al., 1997; Eichelmann et al., 2004; Niinemets et al., 2010). Additionally, leaf starch levels might control BVOC emissions by increasing substrate availability or enzyme activity (Lerdau et al., 1997), but this unfortunately was not quantified in our study.

Influence of infestations

There are several indications that infestations have strongly influenced the observed emission peaks. For example, a possible explanation for the pronounced peak observed in mid July for ash could be the presence of an infestation with powdery mildew (caused by *Phyllactinia fraxini* L.). Additionally, likely oak's double infestation with powdery mildew (caused by *Microsphaera alphitoides*) from July to October and an oak leaf aphid (*Tuberculoides annulatus*) in July and August (Fig. 4.5; Table 4.2) are responsible for an additive BVOC

emission effect (Holopainen, 2011). In the case of beech, the peak at the end of June was possibly caused by the presence of woolly beech aphid (*Phyllaphis fagi* L.). The release of (Z)- and (E)-β-ocimene, a stress compound, throughout the season is probably part of the tree's chemical defense system against *Phyllaphis fagi* L. (Joó et al., 2010a). Chemical speciation of identified BVOCs indicated large variability in emissions throughout the season. More details on the BVOC fingerprints are available in Pokorska et al. (2011). Finally, in our experiments the following biotic stress-related compounds were detected: DMNT (a hemiterpene) (Z)- and (E)-β-ocimene (MTs), α-farnesene and β-caryophyllene (SQTs) and MeSA (a benzenoid), which all support infestation-induced emissions (Holopainen, 2011; Mann et al., 2012). These stress compounds are main internal signals for activation of plant anti-herbivory defenses and/or attraction of herbivore enemies involving biochemical pathways other than MVA and MEP pathways and require further research.

4.5 Conclusions

This study describes simultaneously seasonal dynamics in Pn_{max} and BVOC emissions in oak, ash and beech. Pn_{max} showed seasonality in oak, ash and beech. In contrast, Rd indicated no clear seasonal trend. BVOC emissions showed a less clear seasonal trend, and most of the variability could be explained by temperature. Other influences were acting on the BVOC emissions possibly related to infestation as trees were heavily infested throughout the entire measurement period. It should also be taken into account that our observations are valid for young potted trees and that the seasonal behavior might deviate and should be tested for adult trees. The need for identification of BVOC fingerprints using TD-GC/MS in future research is emphasized. We can conclude that long-term studies in field and controlled conditions are important to further unravel the effects of multiple stressors on BVOC emissions.

Chapter 5

Vertical gradient in photosynthesis and monoterpenoid emissions in a beech (Fagus sylvatica L.*) canopy under varying sky conditions*

Abstract

It is well known that vertical canopy gradients and varying sky conditions influence photosynthesis (Pn), specific leaf area (SLA), leaf thickness (LT) and leaf pigments (lutein, β-carotene and chlorophyll). In contrast, little is known about these effects on monoterpenoid (MT) emissions. Our study examines simultaneously measured Pn, MT emissions and the MT/Pn ratio along the canopy of an adult European beech tree (*Fagus sylvatica* L.) in natural forest conditions. Dynamic branch enclosure systems were used at four heights in the canopy (7, 14, 21 and 25 m) in order to establish relationships and better understand the interaction between Pn and MT emissions under both sunny and cloudy sky conditions. Clear differences in Pn, MT emissions and the MT/Pn ratio were detected within the canopy. The highest Pn rates were observed in the sun leaves at 25 m due to the higher intercepted light levels, whereas MT emissions (and the MT/Pn ratio) were unexpectedly highest in the semi-shaded leaves at 21 m. The higher Pn rates and, apparently contradictory, lower MT emissions in the sun leaves may be explained by the hypothesis of Owen and Penuelas (2005), stating that the synthesis of more photo-protective carotenoids might decrease the emissions of volatile isoprenoids (including MTs) because they both share the same biochemical precursors. In addition, leaf traits like SLA, LT and leaf pigments clearly differed with height in the canopy, suggesting that the leaf's physiological status cannot be neglected in future research on biogenic volatile organic compounds (BVOCs) when aiming at developing new and/or improved emission algorithms.

5.1 Introduction

Plants take up carbon dioxide (CO_2) from the atmosphere (Taiz and Zeiger, 2010) and re-emit carbon back into the atmosphere via monoterpenoids. Monoterpenoids (MTs) are a subgroup of the biogenic volatile organic compounds (BVOCs) (Tillman et al., 1999; Kesselmeier and Staudt, 1999; Dudareva et al., 2006). In changing environmental conditions, persisting within a single canopy, plants must continuously optimize Pn (Franklin, 2008). In addition, they need to invest in defense by emitting MTs. Variation in Pn with height in the canopy has been characterized in many studies (e.g. Ellsworth and Reich, 1993; Frak et al., 2002; Petritan et al., 2010). In contrast, to the best of our knowledge, such information is lacking for MT emissions, as well as the link with some structural leaf traits, like leaf pigments, specific leaf area (SLA) and leaf thickness (LT), which might substantially vary with height (Terashima et al., 2006; Montpied et al., 2009).

Some investigations showed an optimized distribution of Pn in relation to light quantity (Griffin et al., 2001), while others have related it to the spectral quality of light (Franklin, 2008). In any case, it is known that light is the most powerful factor determining morphological, physiological and anatomical variations in leaves (Kim et al., 2005) resulting in the specific features of sun and shade leaves (Boardman, 1977; Lichtenthaler et al., 1997, Morecroft and Roberts, 1999; Kim et al., 2005; Terashima et al., 2006; Taiz and Zeiger, 2010). Leaves at the outside of the tree canopy are typically adapted to high light conditions (hereafter sun leaves), whereas leaves growing in the shaded canopy generally exhibit adaptations to low light conditions (hereafter shade leaves). Semi-shaded leaves that operate at the transient of sun and shade are often left out in scientific studies. The contrasting leaf morphology and anatomy of sun and shade leaves is required for Pn to acclimate to high or low light levels (Marshall and Monserud, 2003; Lombardini et al., 2006; Taiz and Zeiger, 2010). Sun leaves typically have less chlorophyll per reaction centre, are usually thicker, have more Rubisco (and therefore a higher potential photosynthesis level) and a larger pool of carotenoids ($C_{40}H_{56}$) than their shady counterparts. This higher carotenoid pool in sun leaves growing under high light intensities is known to have an important photoprotective role (Lambers et al., 1998; Hansen et al., 2002; Taiz and Zeiger, 2006; Lichtenthaler, 2007; Sarijeva et al., 2007). Owen and Peňuelas (2005) hypothesized that conditions affecting the synthesis of these essential carotenoids might affect the production and emission of the so-called non-essential volatile isoprenoids, because they share the same biochemical precursors dimethylallyl diphosphate (DMAPP) and its isomer isopentenyl diphosphate (IPP) (Lichtenthaler et al., 1997; Eisenreich et al., 2004; Owen and Peñuelas, 2005). MT emissions, as part of the low molecular weight volatile isoprenoid group, are influenced by light and temperature, with higher emissions

expected to occur under high light intensities (e.g., Kesselmeier and Staudt, 1999; Dindorf et al., 2006). Little is however known about how MT emissions are influenced by vertical gradients in the microclimate along a canopy (Ghirardo, 2010; Holopainen and Gershenzon, 2010) and leaf traits prevailing in a canopy. Leaf traits may not directly affect MT emissions, but by influencing the leaf's photosynthetic performance, leaf traits may indirectly affect MT production (Brilli, personal communication).

The objectives of this paper were therefore to: (i) simultaneously investigate and quantify Pn and MT emission profiles along the canopy of an adult beech tree (*Fagus sylvatica* L.), (ii) quantify vertical variations in key leaf traits along the canopy and investigate their influence on Pn and MT emissions, and (iii) examine underlying mechanisms of the observed vertical canopy gradients.

5.2 Material and methods

5.2.1 Field study site and experimental set up

The semi-urban Aelmoeseneie research forest is located about 15 km southeast from Ghent, Belgium (50°58' N, 3°48' E, 21 m altitude). In 1920, after World War I, the site was re-planted with beech (*Fagus sylvatica* L.), oak (*Quercus robur* L.) and ash (*Fraxinus excelsior* L.), interspersed with a few individuals of other tree species. This mixed temperate forest covers an area of 39.5 ha of which 1.85 ha serves for research purposes. This experimental site is divided into a beech-oak plot and an ash plot. The climate is temperate maritime with mean annual high/low temperature of 13.9/6.7 °C, precipitation of 750 mm and 1504 h of annual sunshine. Measurements were performed during the growing season of 2008. A 36 m high experimental tower provided accessibility to a 90-year old beech tree (30 m high). The tower consists of five platforms (7, 14, 21, 28 and 35 m). In this study, we reached two heights (21 and 25 m) from the platform at 21 m. Hereafter, a distinction between sun (25 m), semi-shaded (21 m) and deep-shaded (14 and 7 m) leaves is made. Classification into sun, semi-shaded and deep-shaded leaves is based on leaf pigments, specific leaf area (SLA) and leaf thickness (LT). In total six branch enclosure systems were used throughout the beech canopy and equipped with sensors for relative humidity (type HIH-3610, Honeywell, NJ, USA) and air temperature (type thermistor 10k-NTC, TH-44031-36-T, Omega, USA): four of these cuvettes enclosed leaves on branches at the specified heights, while two cuvettes were used as control. Intercepted photosynthetic photon flux density (PPFD) at the height of each cuvette was measured with quantum sensors (type Li-190S; Li-COR, NE, USA). The sensors were attached in a horizontal arrangement at each side of the cuvette at 25 and 21 m and one sensor per cuvette was installed at 14 and 7 m height. Additionally, a quantum sensor was installed at the tower top level (36 m). Differences in sky

conditions (sunny *versus* cloudy) were defined based on the light intensity measured at 36 m. A daily average of 400 µmol-photons m^{-2} s^{-1} was used as threshold: days with an average value above this threshold were considered as sunny, while the others were classified as cloudy. In total 79 days were analyzed. All data were logged (logging frequency 4 s) with a data acquisition system.

5.2.2 Gas exchange measurements

A dynamic branch enclosure system was used for the simultaneous measurements of photosynthesis (Pn) and monoterpenoid (MT) emissions. Stationed in the ground-level tower cabin, the system consisted of an infrared gas analyzer (IRGA CO_2 model ADC 2250, ADC BioScientific, Great Amwell, United Kingdom) and Proton-Transfer-Reaction-Mass-Spectrometry (PTR-MS, IONICON Analytik GmbH, Innsbruck, Austria, Lindinger et al., 1998) measuring automatically Pn and MT emissions, respectively.

Additionally, a separate sampling line was also included for manual Thermal Desorption Gas Chromatography/Mass Spectrometry (TD-GC/MS) MT analysis. GC/MS periodic sampling was performed using multiple Tenax® TA and Carbotrap™-filled adsorbent tubes (Markes International, Llantrisant, United Kingdom) according to Joó et al. (2010a, b) and Joó (2011).

Cuvette sampling air passed through Teflon-PFA tubings towards the dust filters and manganese dioxide (MnO_2) as ozone (O_3) scrubber, removing O_3 from the sampled air. Air continued to two active coal filters (Airpel 10 and Organosorb 10-CO, Desotec N.V., Roeselare, Belgium) releasing VOC-free air. PTR-MS and IRGA analyzers received the cuvette outcoming air. Both Pn and MT emissions were expressed on a leaf area basis. The leaf area was followed on reference branches throughout the growing season by drawing leaf circumferences and subsequent cut paper measurements using a leaf area meter (type Li-3050A, Lambda instruments corporation, Blacksburg, VA, USA).

In addition, light response curves were measured at the different canopy heights. A portable IRGA (LI-6400, Li-cor, Lincoln, Nebraska, USA) was used for manual net photosynthesis (Pn) and dark respiration (Rd) measurements. Additionally, measurements of internal CO_2 concentration (Ci) and transpiration (Tr) were measured simultaneously. The LI-6400 consisted of a 2x3 cm leaf chamber, containing a blue and red light emitting diodes (LED) light source. The IRGA leaf chamber conditions included set temperature of 25 °C and 400 ppm CO_2 concentration.

5.2.3 Foliage phenological indicators

The chlorophyll content index (CCI) was measured *in vivo* by using a portable chlorophyll content reflectometer (type Minolta SPAD-500, Spectrum

Technologies, IL, USA). CCI expresses the relative amount of chlorophyll pigments related to the green coloration that is positively related to leaf nitrogen (N) content (Varinderpal-Singh et al., 2010; Boussadia et al., 2011). Triplicate readings were taken from the left and right midpoint of each leaf and averaged. Reference branches adjacent to each cuvette were selected. Fifteen labeled leaves per branch were followed. Chlorophyll a+b was quantified according to the model of Gielen et al. (2007):

$$chla + b = 4.181 + 1.956 x CCI \quad (5.1)$$

where chl a+b is chlorophyll a+b concentration (µg cm^{-2}). In total, 16 days per height were measured throughout the 2008 growing season.

Specific leaf area (SLA, $m^2\ kg^{-1}$) was measured according to Vande Walle, (2007). In total eight leaves were used per phenological stage from reference branches near each cuvette from June till November 2008.

Leaf thickness (LT) was determined according to Vile et al. (2005). Visual assessment of changes in leaf pigments was possible via a photographical (Canon IXUS 70) overview of the leaves inside the cuvettes.

Seasonal entomological identifications were run from May till October on all vertical levels by using a loupe and microscopes (type SZX12-ILLK200 and type BX51TF, Olympus corporation, Tokyo, Japan) with Color View III camera imaging system and the software package CELL-F (Olympus Soft Imaging Solutions). Infestation severity was based on the visual observations.

5.2.4 LC-MS/MS analysis of leaf pigments

Towards the end of the growing season (late September), leaf pigments were determined. All-*trans*-β-carotene, all-*trans*-lutein and chlorophyll *a* were obtained from Sigma-Aldrich (St. Louis, MO, USA) and used as authentic standards. β-apo-8′-carotenal, purchased from Sigma-Aldrich, served as an internal standard. The HPLC-grade solvents including ethanol, n-hexane acetonitrile, methanol and isopropanol were from Fisher Scientific (Tournai, Belgium). Carotenoids in beech leaves were extracted by using a protocol adapted from Lin and Chen (2003). Briefly, samples (*ca*. 0.5 g of leaf material) were homogenized under liquid nitrogen, mixed with 1 mL of an aqueous magnesium carbonate solution (25 g/L) and repeatedly extracted with 5 mL of a mixture of ethanol and hexane (4:3, v/v) while shaking for 10 min until almost no color remained in the leaf residue. Subsequently, samples were filtered over a Whatman No. 1 filter paper, the filtrates pooled and poured into the same flask. Distilled water (25 mL) and 25 mL 10% aqueous NaCl solution were added for partitioning, and the supernatant was collected. A fraction of 1 mL of the filtrate was evaporated to dryness under a

gentle nitrogen stream and redissolved in 1 mL of acetonitrile:methanol (1:1, v/v). Of the final solution, 10 μL was used for LC-MS/MS analysis.

The analysis of carotenoids in the beech leaf extracts was performed using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Chromatography was carried out on a Thermo Finnigan Surveyor LC system (San Jose, USA) comprising a quaternary pump and an autosampler, equipped with a 5 µm 2.1 x 150 mm Symmetry C_{18} column obtained from Waters (Milford, USA). Metabolites were eluted using a gradient of solvent A (water), solvent B (isopropanol), solvent C (methanol), solvent D (acetonitrile) with the following profile: 0-2 min, 10% B, 50% C and 40% D; 2-4 min, from 10% to 0% A and from 40% to 50% D; 4-11.5 min, keeping these conditions; 11.5-19 min, from 0% to 6% B and from 50% to 44% D; 19-19.1 min, from 6% to 30% B and from 44 to 20% D; 19.1-29 min, keeping these conditions; 29-29.1 min, back to the initial conditions and keeping these for 8 min. The flow rate was 300 µL/min and the column oven temperature 30 °C. Analytes were detected with an LTQ ion trap mass spectrometer (Thermo Finnigan, USA) in the MS/MS positive ion mode using an Atmospheric Pressure Chemical Ionization (APCI) interface. A capillary temperature of 250 °C, a vaporizing temperature of 275 °C, a sheath gas flow of 20 units and an auxiliary gas flow of 5 units were used. Alternating scans were used to isolate $[M+H]^+$ ions for the different compounds: *m/z* 551.5 for lutein, *m/z* 537.5 for β-carotene, *m/z* 893.5 for chlorophyll *a,* and *m/z* 417.5 for β-apo-8′-carotenal. The precursor isolation width was set to 2 Da, the activation Q at 0.25 and the collision energy ranged from 23 to 27%.

5.2.5 Statistical analysis

Comparison between different canopy heights was done using R version 2.11.1 statistical software (R Development Core Team 2010) to test significant differences in Pn, MT, MT/Pn ratio as well as Chl a+b, SLA and LT measured during the growing season. Differences in Chl a+b, SLA and LT related to canopy height were assessed using Analysis of Variance (ANOVA). The influence of canopy height as well as differences between sunny and cloudy days on Pn, MT and MT/Pn ratio was assessed by applying a linear mixed model (Pinheiro et al., 2009). In case of a significant effect between height and leaf positioning, data were split up per date and the effect of height was tested on each date by means of Tukey's honestly significant difference test (Hothorn et al., 2008). To test difference between leaf pigments SPSS v.19 was used. The threshold for statistical significance using both software packages was $P<0.05$.

5.2 Results

5.2.1 Diurnal patterns in Pn and MT emissions during sunny and cloudy days

Averaged diurnal patterns of PPFD and VPD on sunny and cloudy days illustrate the microclimate under varying sky conditions and along the canopy height (Fig. 5.1 and 5.2). Maximum light intensity under a cloudy sky at 25 m height (205 µmol-photons m^{-2} s^{-1}) is for example three times less than under sunny skies (628 µmol-photons m^{-2} s^{-1}) (Fig. 5.1a and Fig. 5.2a, respectively). Sun leaves at 25 m height received a significantly lower amount of light than that received above the canopy (36 m): maxima of 555 and 1049 µmol-photons m^{-2} s^{-1} on cloudy and sunny days, respectively. Sunny days typically have high VPD with maxima of 1,01 kPa (at 25 m) compared to only 0,30 kPa on cloudy days.
On sunny days, Pn resulted in a characteristic diurnal pattern with typical mid-day depressions for sun, semi-shaded and deep-shaded (25, 21 and 14 m) leaves (Fig 5.2). In contrast, on cloudy days, this phenomenon was only present in sun and semi-shade leaves (25 and 21 m) (Fig. 5.1). Pn of sun leaves (25 m) rarely exceeded daily maxima of 3,00 µmol-CO_2 m^{-2} s^{-1} for sunny days and 1,90 µmol-CO_2 m^{-2} s^{-1} for cloudy days. Obviously, lower Pn was found deeper (14 and 7 m) in the canopy.
The MT emission however, showed an unexpected pattern under sunny conditions. On average, sun (25 m) leaves emitted less MTs than semi-shade leaves (21 m) (Fig. 5.2e and 5.2f). The MT emissions on the highest level (daily maxima 76 µg m^{-2} h^{-1}) were two times lower than those at 21 m (144 µg m^{-2} h^{-1}) height. The emissions of both the sun and the semi-shaded leaves were still significantly higher than the emissions from deep-shaded leaves (7 and 14 m), where emissions were very low and sometimes even below the detection limit.

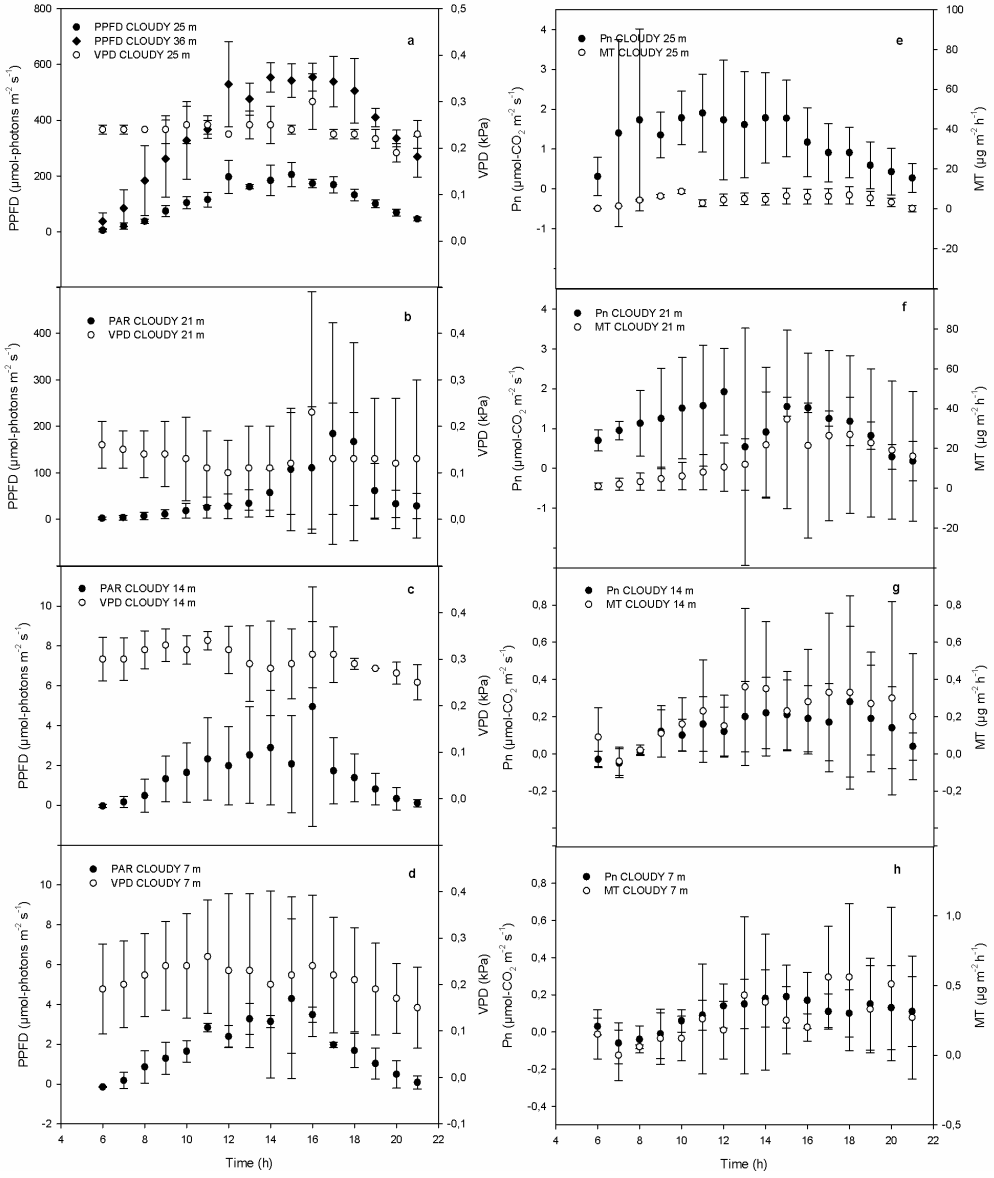

Figure 5.1: Averaged diurnal trends for photosynthetic photon flux density (PPFD), vapour pressure deficit (VPD), net photosynthesis (Pn), and monoterpenoids (MT) of (a, e) sun (25 m), (b, f) semi-shaded (21 m) (c, g) deep-shaded (14 m) and (d, h) deep-shaded (7 m) leaves for the cloudy days (n=13 up to 33 depending on the cuvette) in the Aelmoeseneie forest (Belgium) during the 2008 growing season. The error bars indicate standard deviation (±SD).

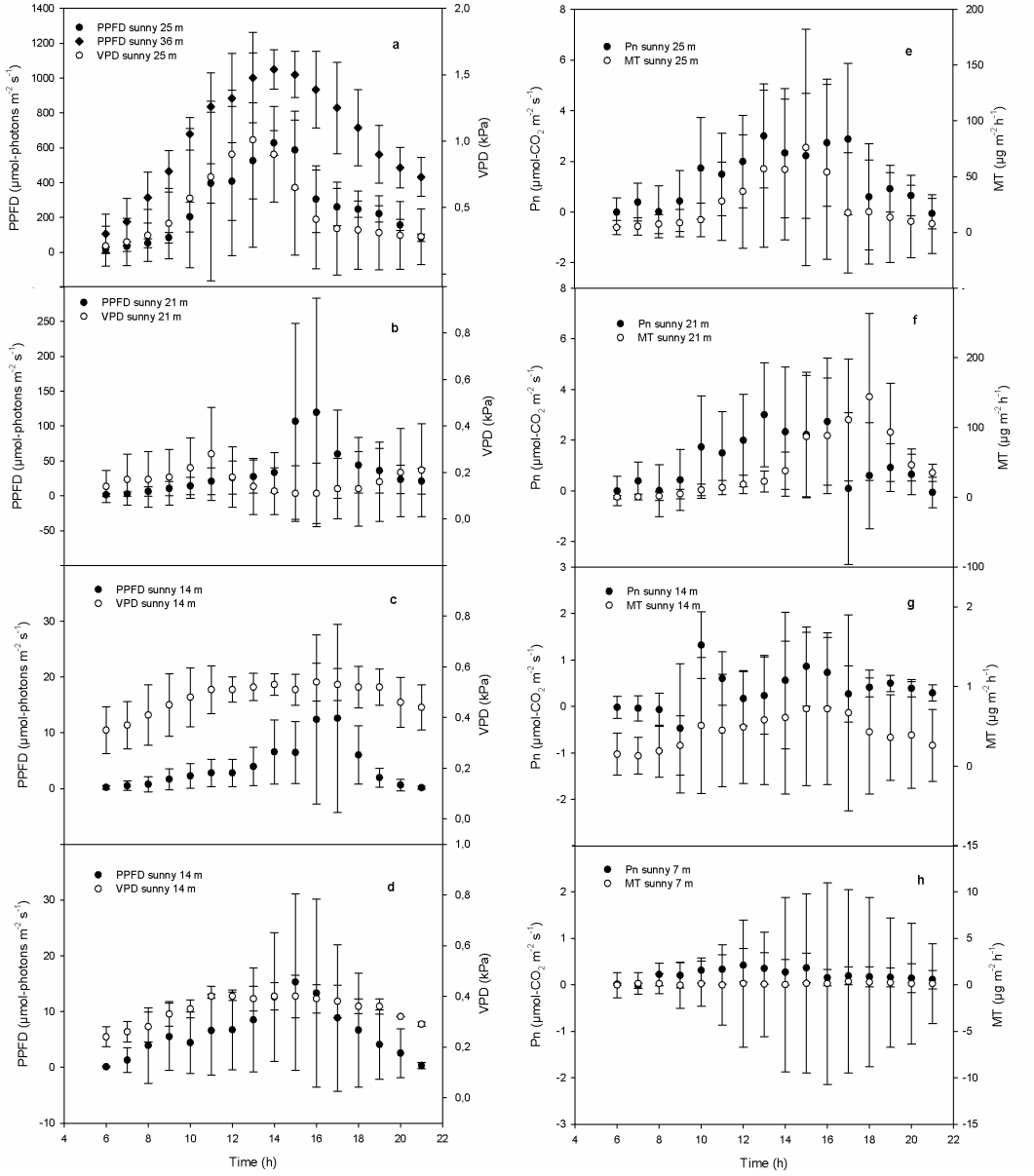

Figure 5.2: Averaged diurnal trends for photosynthetic photon flux density (PPFD), vapour pressure deficit (VPD), net photosynthesis (Pn) and monoterpenoids (MTs) of (a, e) sun (25 m), (b, f) semi-shaded (21 m) (c, g) deep-shaded (14 m) and (d, h) deep-shaded (7 m) leaves for sunny days (n=13 up to 33 depending on the cuvette) in the Aelmoeseneie forest (Belgium) during the 2008 growing season. The error bars indicate standard deviation (±SD).

5.2.2 Vertical canopy gradients in Pn and MT emissions

Averaged Pn values showed an increasing trend with canopy height both during sunny and cloudy days (Fig. 5.3a). In contrast, MT emissions showed very low values at the bottom of the canopy, a distinct peak at 21 m (semi-shaded leaves)

and clearly lower values at 25 m height (sun leaves) (Fig. 5.3b). The MT/Pn ratio followed this MT trend (Fig. 5.3c), with significantly higher values for this ratio on sunny days for the semi-shaded leaves. On sunny days, MT/Pn ratio ranged from 0,1 to 0,8%, while on cloudy days the ratio ranged from 0,02 to 0,10%.

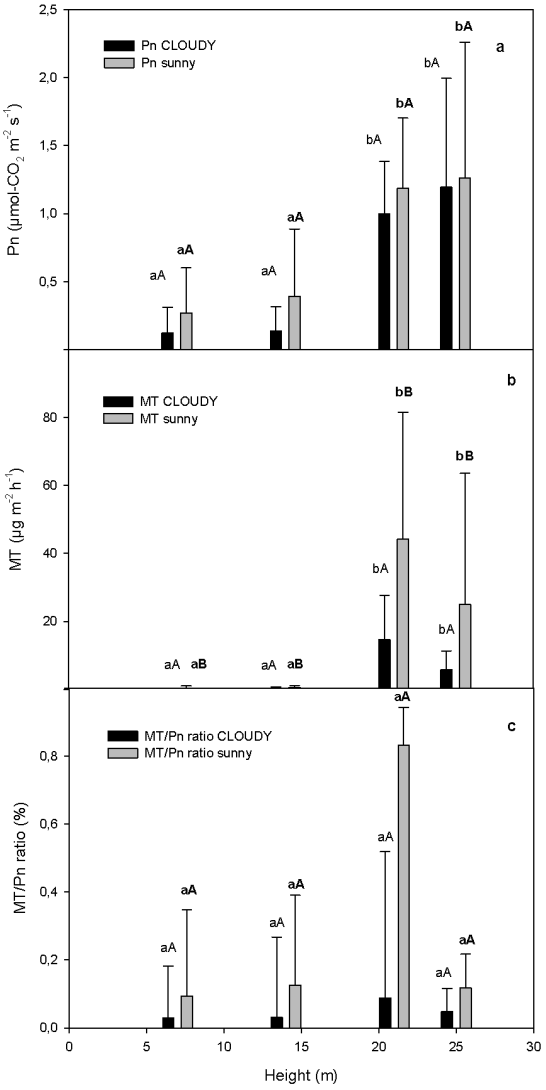

Figure 5.3: Vertical profiles of (a) net photosynthesis (Pn), (b) monoterpenoids (MTs), and (c) MT/Pn ratio averaged for sunny and cloudy days (n=13 up to 33 depending on the cuvette). The error bars indicate standard deviation (±SD). Lowercase: comparison between different heights. Uppercase: comparison between sunny (bold) and cloudy (not bold) days. Values followed by the same letter are not significantly different (P<0.05).

5.2.3 Light response curves along the canopy gradient

The observed light response curves at different heights in the canopy confirm the physiological adaptation of leaves to their light environment (Fig. 5.4). The curves at all canopy heights show a typical light limited behaviour at low light intensities. Above 200 µmol photons m^{-2} s^{-1}, Pn becomes carboxylation limited and significant differences in absolute values were observed between the different

heights. Sun adapted leaves typically showed higher maximum Pn rates at light saturation that was achieved at 1200 µmol photons m^{-2} s^{-1}. These differences in height were also reflected in the Ci and Tr values where Tr showed a response to light that was very similar to the Pn response.

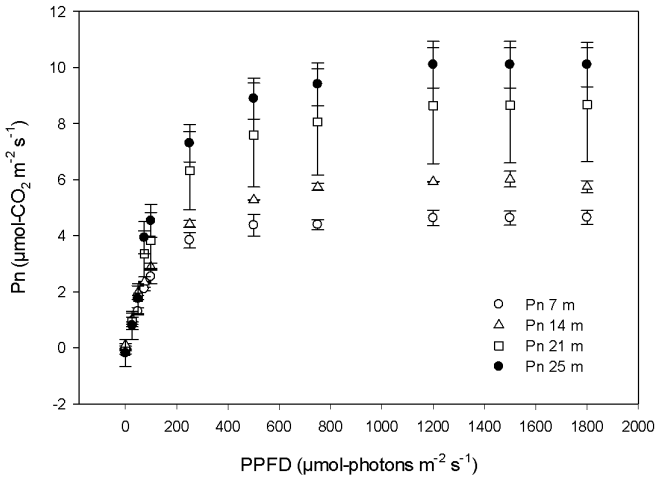

Figure 5.4: Single leaf level light response curves of net photosynthesis (Pn) (a) at four canopy heights (empty circle=7 m; triangle=14 m; square=21 m; full circle=25 m) measured in the middle of the growing season (July 9th, 2008). The error bars indicate standard deviation (±SD).

5.2.4 MT species quantification

The emitted MT compounds were quantified on a cloudy and a sunny day for both the sun (25 m) and the semi-shaded (21 m) leaves (Fig. 5.5). A clear difference in emitted compounds was observed between sun and semi-shaded leaves. Clearly more different MT compounds were emitted by semi-shade leaves (21 m) compared to sun leaves (25 m). On the sunny day, sun leaves emitted only sabinene, whereas an additional six compounds were identified in their semi-shaded counterparts. A similar pattern was observed on the cloudy day where additional new compounds were identified. For the sun leaves, sabinene dropped under 80%, to the same level as the semi-shaded leaves. On the selected cloudy day, both the sun and the semi-shaded leaves emitted methyl salicylate (MeSA), a non-MT anti-herbivore defense compound. The sun leaves had threefold-higher levels of MeSA compared to the semi-shaded ones (see also Joó et al., 2010).

It is important to mention that in June 2008 the semi shade leaves were infested with beech weevil (*Rhynchaenus fagi* L.) having a chewing feeding behavior, while the sun leaves were infested with beech wooly aphid (*Phyllaphis fagi* L.)

having a piercing-sucking feeding behavior. Additionally, beneficial insects (such as *Chrisopa* sp.*)* were identified and observed. From our study, it was not possible to determine whether MeSA was related to the infestations and further investigation is required to do so (Joó et al., 2010a).

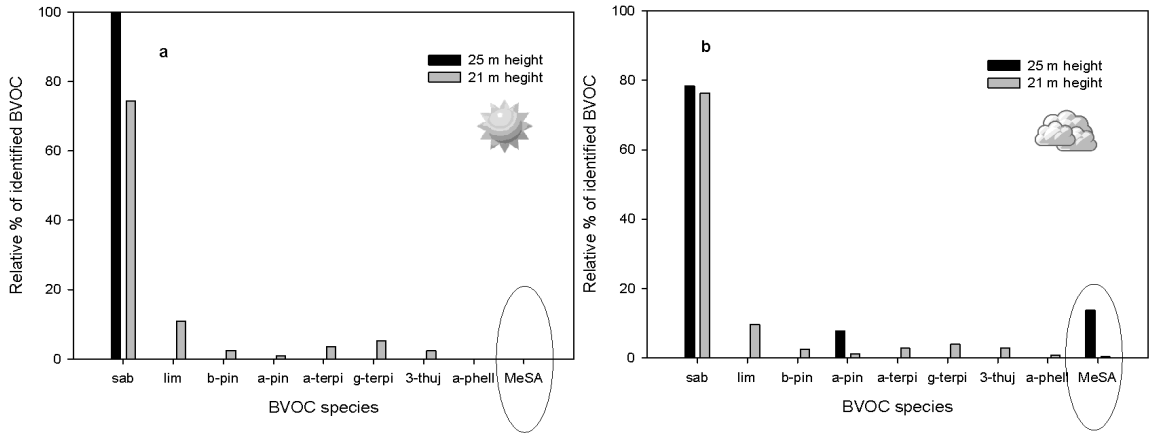

Figure 5.5: TD-GC/MS quantifications of monoterpenoid (MT) compounds for (a) a sunny (20/08/08) and (b) a cloudy day (03/09/08) in sun (25 m) and semi-shaded (21 m) leaves. Abbreviations: sabinene (sab), limonene (lim), α-pinene (a-pin), β-pinene (b-pin), α-terpinolene (a-terpi), g-terpinolene (g-terpi), 3-thujene (3-thuj), α-phellandene (a-phell), and the non-monoterpenoid methyl salicylate (MeSA).

5.2.5 Vertical leaf trait gradients

The seasonal averaged Chl a+b showed a clear but non-significant vertical linear decline (R^2=0.99) with canopy height. The sun leaves contained the lowest chlorophyll concentration per leaf mass, while semi-shaded and deep-shaded leaves contained higher concentrations (Fig 5.6a). SLA showed a significant linear decrease (R^2=0.99) with height in the canopy (Fig. 5.6b). Furthermore, sun leaves were thicker than semi-shaded and deep-shaded leaves (Fig. 5.6c). An exponential increase with height for LT was found (R^2=0.98). Leaf age and phenophase had minor effects on these relationships.

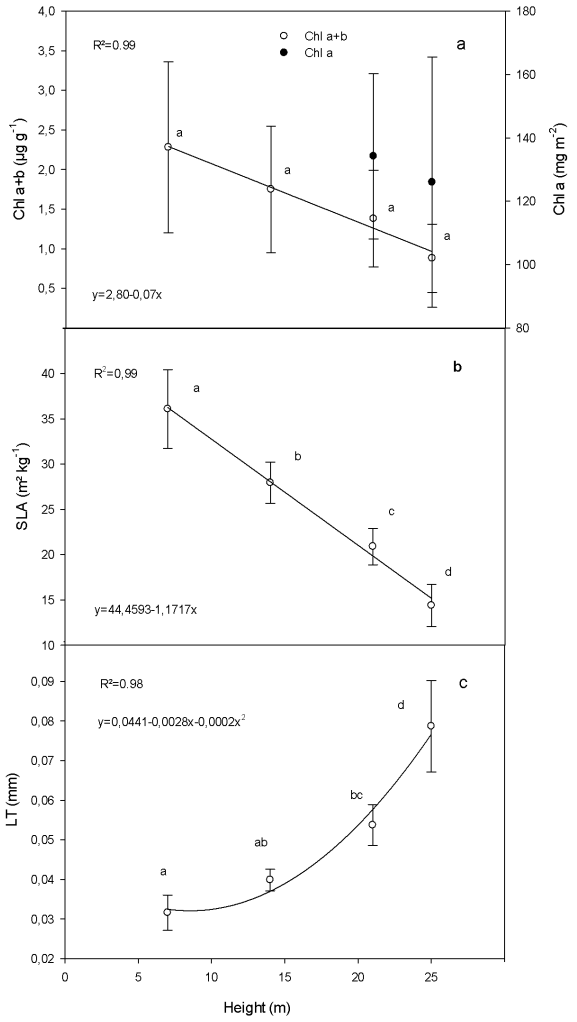

Figure 5.6: Vertical profiles of leaf physiological traits: (a) chlorophyll a+b (chl a+b) (n=15) and chl a (n=13), (b) specific leaf area (SLA) (n=8), and (c) leaf thickness (LT) (n=8). The error bars indicate standard deviation (±SD). (a) The two full points indicate LC/MS chlorophyll a measurements. Means with the same letter are not significantly different from each other (p<0,05).

The differences in leaf pigments (lutein, β-carotene and Chl a) were investigated in detail with LC-MS/MS analysis for the sun (25 m) and semi-shaded (21 m) leaves. Significant differences in content were observed between the two leaf types for lutein and β-carotene (Table 5.1).

Table 5.1 Concentration of lutein, β-carotene and chlorophyll a in sun and shade leaves of beech in mid-October. Mean ± SD of n=13. * Significant differences (P<0.01).

		Sun leaves	Shade leaves
Lutein	(mg m^{-2} leaf area)*	11.4 ± 2.1	8.9 ± 1.4
β-Carotene	(mg m^{-2} leaf area)*	44.9 ± 7.2	24.3 ± 3.8
Chlorophyll *a*	(mg m^{-2} leaf area)	126.0 ± 39.5	134.2 ± 26.1

Based on a visual assessment, a clear difference in phenological evolution between sun and semi-shaded leaves was observed towards the end of the growing season (Fig. 5.7).

Figure 5.7: Visual changes in leaf color intensity for the leaves in the sun (top) and semi-shade (bottom) cuvette in the growing season 2008: A = DOY (Day of Year) 262, B=DOY 291, C = DOY 305, D = DOY 308, E = DOY 312 and F = DOY 325.

First, a difference in timing of leaf senescence was detected. The greenish leaf color, an indicator for the presence of chlorophyll in the leaf tissue, started to disappear approximately two weeks earlier in sun leaves (17 October 2008 (DOY = 291)) compared to semi-shaded leaves (31 October 2008 (DOY = 305)). This

earlier green discoloration in the sun leaves was confirmed by CCI measurements on representative leaves selected in the close vicinity of the cuvettes (data not shown). Second, for the stage in which the yellow color of certain carotenoids was predominant, a time lag of approximately one week existed between sun and semi-shaded leaves (31 October (DOY = 305) versus 7 November 2008 (DOY = 312), respectively). Third, the final stage before leaf shed, which is typically marked by a deep reddish leaf color due to the presence of anthocyanins in the leaf tissues, could clearly be distinguished in sun leaves, but was visually absent in the semi-shaded leaves.

5.3 Discussion

Our measurements on beech confirmed the presence of gradients in leaf traits with height in the canopy. SLA values were generally in agreement with values obtained earlier for beech (Sarijeva et al., 2007; Vande Walle, 2007; Closa et al.; 2010). The shading of lower leaves caused increased SLA, resulting in thin shade adapted leaves at the bottom and thicker sun adapted leaves at the top of the canopy. Leaf thickness showed an exponentially increasing trend with height, which is in agreement with previous results (Lambers et al., 1998; Grossoni et al., 1998; Bussotti et al., 2000; Uemura et al., 2000; Larcher, 2003). On the same beech tree in the same growing season Van Wittenberghe et al. (2012) showed significantly thicker histological leaf layers for leaves in the upper canopy compared to the lower canopy.

The observed Chl a+b values agreed well with literature (Kozlowski and Pallardy, 1997; Morecroft and Roberts, 1999; Uemura et al., 2000; Hansen et al., 2002; Lenk and Buschmann, 2006; Closa et al., 2010) and indicated a decrease with increasing height, which was also confirmed by the independent LC-MS/MS data. However, the observed differences in Chl a+b between the different canopy heights were not significant. The higher SLA and higher Chl a+b concentration in the deep-shaded leaves is explained by light harvesting optimization (Boardmann, 1977; Lenk and Buschmann, 2006).

Additionally, some specific differences in leaf traits were found between sun and semi-shaded leaves. Visual assessment clearly showed the presence of anthocyanins (red) and carotenoids (yellow) (Fig. 5.7). When the green color disappeared and specific carotenoids predominated, sun leaves showed a more intense yellow coloring compared to the semi-shaded ones, suggesting the presence of higher carotenoid concentrations. Just before leaf shed, only sun leaves showed the presence of red anthocyanin pigments. These visual trends and differences between sun and semi-shaded leaves were confirmed by LC-MS/MS analysis of chlorophyll *a* and two important carotenoids for beech leaves (lutein and β-carotene) (Table 5.1). Significant ($p<0.01$) higher lutein and β-carotene concentrations (mg m^{-2} leaf area) were measured in sun leaves

compared to semi-shade leaves, while for Chl *a* no significant differences were noted.

Pn is strongly correlated with leaf traits such as SLA, LT and chlorophyll content (Terashima et al., 2006) and the observation of higher Pn rates for sun adapted leaves is well known in the literature (e.g. Lambers et al., 1998; Casella and Ceulemans, 2002; Le Goff et al., 2004; Lichtenthaler, 2007; Sarijeva et al., 2007; Lombardini et al., 2009; Steppe et al., 2011). We observed vertical gradients in Pn that differed on cloudy and sunny days. However, Pn differences between sun (25 m) and semi-shaded (21 m) leaves are not significant. The Pn decrease from the upper to the lower canopy could be explained by the vertical light gradient observed in our study and by many other authors (Le Goff et al., 2004; Čater and Simončič, 2009; Petritan et al., 2010). In addition, the vertical gradient in Pn capacity was also supported by a gradient in leaf nitrogen content (data not shown), reflecting a decrease in Rubisco and ATP synthase from upper to lower levels (Kozlowski and Pallardy, 1997; Medlyn et al., 2002; Valladares et al., 2002; Terashima et al., 2006; Closa et al., 2010; Adriaenssens et al., 2012).

Remarkably, the MT gradient contrasted the Pn and leaf traits trends. Highest MT emissions were observed in semi-shaded leaves (21 m). All currently available evidence demonstrates that higher PPFD levels and/or higher temperatures cause higher emissions of MT in beech (e.g. Schuh et al., 1997; Dindorf et al., 2006; Holzke et al., 2006). In our study, however, the opposite response occurred: higher MT emissions were found in semi-shaded leaves that were subjected to lower PPFD levels in comparison to sun adapted leaves that had to deal with on average a four times higher PPFD level (Fig 5.1, 5.2).

We hypothesize that the underlying reason for these unexpected higher MT emissions in semi-shaded leaves compared to sun leaves is likely the investment leaves make in the synthesis of either essential carotenoids or non-essential volatile compounds from the same biochemical precursors (DMAPP and IPP). Owen and Peñuelas (2005) hypothesized that any condition affecting the synthesis of these higher essential carotenoids might affect the production and emission of the volatile isoprenoids, but they lacked experimental data to underpin their hypothesis.

We give the first supportive evidence that this opportunistic hypothesis holds for European beech. The lower MT emissions in sun leaves under high PPFD levels compared to semi-shaded leaves can be explained by the synthesis of a higher amount of essential carotenoids from the precursors' pool in comparison to semi-shaded leaves, leaving less precursors available for the biosynthesis of MTs (Fig. 5.7 and Table 5.1). We argue that this difference is caused by a distinct difference in leaf physiology between both leaf types. Sun leaves indeed require more carotenoids as photo-protective agent during Pn than semi-shaded leaves due to the harsher light conditions to which they are exposed. Carotenoids help to destroy reactive oxygen species that might be created by interception of too much light energy and dissipate excessive energy,

preventing as such oxidative damage of photosynthetic structures in leaf cells (Lambers et al., 1998; Hansen et al., 2002). Therefore, in sun leaves larger amounts of essential isoprenoids were synthesized (carotenoids) rather than their volatile counterparts.

The presence of a larger carotenoid content in sun leaves compared to semi-shaded leaves in European beech was confirmed by the visual changes in leaf color towards the end of the growing season (Fig. 5.7) and by the substantially higher levels of both lutein and β-carotene (Table 5.1). The β-carotene levels measured in mid-October were very similar to those observed by Lichtenthaler (2007) in mid-July for beech, indicating that β-carotene levels remain rather constant during this part of the season. At the end of the growing season, the sun leaves no longer showed a higher Chl a content compared to semi-shaded leaves as was reported by Lichtenthaler (2007) for sun/shade leaves in mid-July. This resulted in the expected higher relative amounts of β-carotene per unit Chl a as is well known for sun leaves in beech (Hansen et al., 2002; Lichtenthaler, 2007). By linking these higher levels of carotenoid synthesis to the lower MT emissions in sun leaves, and vice versa in semi-shaded leaves, we were able to prove for beech the opportunistic hypothesis postulated earlier by Owen and Peñuelas (2005).

It is important to mention here that our observations might be affected by other mechanisms than the ones discussed above. First, the higher VPD levels at the top of the canopy might have induced some stress on the sun leaves, influencing the observed MT emissions. Second, the fact that emissions of MeSA (a non-MT) were observed (Fig. 5.5), is a strong indication that the observed emission patterns were at least partly induced by herbivore activity. MeSA is a physiologically active compound serving as a proof of herbivory (Shualev et al., 1997; Dudareva et al., 2006; Yuan et al., 2009; Shah, 2009; Joó et al., 2010a; Loreto and Schnitzler, 2010; Bruce and Pickett, 2011; Mann et al., 2012). On both the sun and semi-shaded leaves, infestations were observed during the growing season: beech weevil (*Rhynchaenus fagi* L.) and beech wooly aphid (*Phyllaphis fagi* L.). MeSA is a stress hormone with different functions, including recruiting beneficial insects (which were also observed and identified during our study). Different types of herbivore damage at different canopy heights might complicate the results. It is clear that such patterns can only be investigated by combining detailed herbivory quantification (not only identification) with detailed identification and quantification of key BVOC species, but which was beyond the scope of our present study.

5.5 Conclusions

It can be concluded that clear vertical canopy gradients were observed in leaf traits, Pn and MT emissions. Relationships between leaf traits and photosynthesis confirmed previous results, but leaf physiology affected the MT

emissions in European beech in an unexpected way. The apparent contradictory results could, however, be possibly explained by the opportunistic hypothesis of Owen and Peñuelas (2005). This suggests that the leaf's physiological status should be included in future research on BVOCs, even in global scale modelling and when developing new emission algorithms. Finally, caution for other influencing factors such as genetic control, growth conditions, seasonality, or other stress factors (including herbivory) as well as individual MT species may confound the interpretation of the MT variation requiring further research.

Chapter 6

General discussion and future perspectives

In the framework of this PhD, net photosynthesis (Pn) and biogenic volatile organic compound (BVOC) emissions in indoor (**Chapter 2** and **Chapter 3**) and outdoor (**Chapter 2** and **Chapters 4 to 5**) conditions were investigated for young and adult trees. Results of the investigations have been extensively discussed in previous chapters. The aim of this concluding chapter is to provide a more general discussion of the findings. Afterwards, the implications for future research are given.

6.1. General discussion

This PhD study integrates temperature, drought, seasonal and vertical canopy gradient effects and their impact on Pn and BVOC emissions with a focus on monoterpenoid (MT) emissions. Hypotheses on BVOC emissions that were confirmed included an exponential emission trend with increasing temperature, an increasing and subsequent decreasing trend during drought, seasonality effects as well as the occurrence of vertical canopy gradients. Enclosed branch systems were used in all experiments. The *in situ* measured Pn, BVOC emissions and most ecophysiological parameters under both normal and stressed conditions showed interesting interrelated trends. The interplay of these variables resulted in specific Pn and BVOC emission relationships, which are explained separately in the next sections.

Temperature effects

Temperature above the optimal growth range can affect many metabolic processes. In **Chapter 2** the effect of indoor and outdoor temperature variations on Pn, MT emission and the MT/Pn carbon ratio were compared for beech. Despite the distinct differences in experimental conditions, a similar behavior was observed in both the growth room and the forest: a strong MT exponential dependency on air temperature, with a larger fraction of carbon re-emitted back

into the atmosphere in the field than in the growth room. This implies a high sensitivity towards temperature and possible feedbacks to globally increasing air temperatures. The growth room-forest intercomparison study of the temperature effects on Pn, MT emissions and the MT/Pn carbon ratio indicated the importance of the supporting ecophysiological data.
In general, MT emission responses to the environment show a high natural variability, including inter (different species) and intra-species (same species) differences. As our results are based on experiments that were limited in time and number of replicates, we suggest that long-term measurements conducted on multiple replicates are needed to improve our understanding of the temporal and tree to tree variability of the BVOC emissions in response to temperature.

Drought effects

Besides temperature effects, Pn, MT emissions and the MT/Pn carbon ratio during drought stress (**Chapter 3)** were examined for beech. During drought, Pn, stem diameter (D) changes and MT emissions showed a different, but intertwined behavior. The MT/Pn carbon ratio followed the MT trend. A first notable finding of the study was that MT emissions were more sensitive to drought than Pn. A second interesting observation was the increasing-decreasing MT trend. At the beginning of the stress treatment, MT emissions showed an increasing trend. It is argued that such a trend could be due to the protective nature of MT emissions. Pn and stem D were not yet affected at this time. This indicated a high turgor in the living stem tissues, allowing radial stem growth. In contrast, mild drought stress influenced both Pn and stem D, possibly caused by a decline in the carbohydrate supply and a lower turgor pressure. Reaching acute drought stress, a Pn-MT switch was observed where a carbon allocation divergence towards non-photosynthetic organs and/or defense molecules was present (Christiansen et al., 1987). Stem growth did no longer occur. For the first time, detailed stem D measurements were combined with MT emission studies. D variations changed in correspondence to the modified supply of carbohydrates as well as the tree water status. Further research on this aspect is recommended.

Another notable finding of the study was that the signals of the MT emission (*m/z* 137) and the MT fragment (*m/z* 81) deviated from each other (MT decreased, *m/z* 81 increased) upon severe drought stress, signifying emission of BVOCs other than MTs (most likely green leaf volatiles (GLVs)).
Thus, it is highly desirable to evaluate individual MT compounds and other non-MTs such as GLVs in future experiments to identify which compounds are contributing to the *m/z* 81 signal. Overall, the strength of this study is the combination of MT emission measurements with a detailed monitoring of the plant physiological status through LVDTs. Unfortunately, due to the limited availability of the PTR-MS instrument, we were not able to monitor the BVOC emissions during the drought stress recovery. A drought experiment including an

intensive monitoring during the recovery phase is highly recommended to gain additional insight in the relation between BVOC emissions and plant physiology under drought stress and its relief.

Seasonal effects

Seasonal Pn and BVOC emissions in **Chapter 4** indicated differences between beech, ash and oak throughout the growing season. In this chapter, we further aimed at deepening our understanding on how seasonal Pn and BVOC emissions relate to each other under natural weather conditions in potted trees. In all trees, seasonality had an impact on both Pn and BVOC emissions. While Pn trends did not differ much among tree species, BVOC emissions showed clear differences with different peaks in different periods of the growing season. Linked with **Chapter 2**, **Chapter 4** investigated temperature (and light) effects on Pn and BVOC emissions. By using a simple temperature model, we simulated BVOC emission patterns throughout the season. The temperature based model was able to explain the majority of the variability in the observed data. Nevertheless, the temperature response was not able to explain several observed peaks in the BVOC emissions. These higher emissions with different timing in different tree species coincided with infestation appearance and emissions of stress compounds (identified by TD-GC/MS). Several stress related compounds (e.g. benzenoid MeSA, a SQT β-caryophyllene) proved the strong influence of infestation on the emissions. In addition, this observation showed that different herbivory pressures are present in certain plant phenophases. Herbivory should therefore not be neglected in future experiments, despite the complexity of its seasonal variation among tree species, genotypes, and locations.

Vertical canopy effects

To the best of our knowledge, this is the first study in which measurements have been performed using enclosure systems at different canopy heights and where results of leaf anatomy, physiology, Pn, MT and the MT/Pn carbon ratio have been combined. Our findings document interesting trends along the canopy of an adult beech tree, but also point to the need of further research on more replicates and other tree species. **Chapter 5** explored how Pn and MT emissions behave on a daily basis along a vertical canopy gradient, distinguishing sun, semi-shaded and shaded leaves involving almost the entire growing season in the canopy of an adult beech tree. Data are discussed based on the correlation with other leaf physiological parameters. It became clear MT emissions did not correlate directly with Pn, especially not in sun leaves. Therefore, it seems unlikely that beech MTs function solely in mitigating heat or oxidative stresses (as isoprene). Moreover, this is the first empirical evidence supporting the hypothesis of Owen and

Peñuelas (2005) where in sun leaves a higher amount of essential carotenoids are synthesized from the shared precursors' pool in comparison to shade leaves, leaving less precursors available for MT biosynthesis. The role for MT in direct defense against herbivores or indirect defense (attraction of herbivore enemies) is well-known in literature. This might have influenced the observed vertical distribution in the canopy. The observation of herbivory stress related compounds in our study indicate infestations played indeed a role. For example, on cloudy days, MeSA (an infestation related stress compound) was produced in elevated quantities especially on cloudy days at a canopy height of 25 m where aphids were present. The main finding was however the discovery of a very interesting pattern of MT emission related to leaf physiology (sun versus (semi) shaded leaves) within the vertical dimension of the canopy.

Infestation effects

BVOC emissions are released from vegetation under normal (constitutive emissions) and stress (induced emissions) conditions, depending on the occurrence, severity and duration of stressors (Loreto and Schnitzler, 2010; Niinemets, 2010). In natural conditions, stress occurrence is a part of plant life (Niinemets, 2010) inducing or quenching BVOC emissions (Loreto and Schnitzler, 2010) as well as impacting tree physiology. Biotic stressors (infestations) possibly significantly affected Pn, growth and/or BVOC emissions. These infestations demonstrated that identification of BVOCs on the group level (e.g. MTs) is not sufficient to explain certain trends, but that rather identification of individual MTs as well as non-MTs is required. In the context of different herbivore pressures, their occurrence and possible interaction should not be neglected in the future. Especially, the distinction of the right mixture of chemicals should be taken into account (Bruce and Pickett, 2011). Proposal of including multiple types of insect traps with different capture efficiencies that would allow various life stages and species of interest for monitoring, might be advised. Furthermore, all these data might help in updating and improving prediction algorithms of BVOC emissions in response to herbivory patterns. Recent literature shows insect activities such as oviposition and/or pheromone release may elicit changes related to defenses (Alba et al., 2011). Clearly, efforts to interpret BVOC signals in relation to prevailing environmental conditions without understanding the ecophysiology and influence of herbivory behind, are likely to fail.

6.2. Directions for future research

This study has led to new insights into the interrelated dependency of Pn, growth and BVOC emissions (especially MT emissions), and pointed towards new areas of research, which are summarized below. Additionally, these insights may have important implications for Pn as well as BVOC modeling, plant ecology, phytopathology and entomology. Finally, this dissertation has contributed to a significant improvement of our current understanding of the processes controlling Pn and BVOC emissions leaving still some explanations elusive.

BVOC emission mixtures rather than individual substances

Most BVOC emissions, as secondary metabolites (alkaloids, phenolics, terpenoids, …) invariably occur in mixtures rather than as individual substances (Gershenzon et al., 2012). Therefore, there is a need for techniques to measure simultaneously these various compounds originating from different biosynthetic pathways. In this study, the IRGA-PTR/MS-GC/MS measurements were supported by ecophysiological data sets. As other BVOC emission compounds can be emitted upon stress, there is a need for other techniques that would help identifying and quantifying stress in an early stage. Furthermore, emission of BVOCs other than isoprenoids needs to be elucidated. As individual MTs (α-pinene, β-pinene, sabinene,…) may play different roles in plant chemical signaling, the manual GC/MS technique was required for unravelling specific compound distributions in this study.

Our understanding of Pn and BVOC emissions and physiological control has greatly improved in the last years. However, the molecular information is much less clear. It is known that different BVOCs may result from different biosynthetic pathways. However, yet it is not clear how the controls of these pathways are co-ordinated emitting specific BVOC mixture(s) (Laothawornkitkul et al., 2009). Furthermore, molecular techniques are imperative to a better understanding of the plant BVOC emissions and Pn response to stressors. This molecular knowledge could pave the way for engineering stress-tolerant plants. Our results thus encourage further studies on these relationships in the field, with more species and biological replicates. However, this would increase the experiment cost.

Unravelling Pn-BVOC emission-carbohydrate(s) dynamics

Photosynthesis sequesters carbon from the atmosphere. The release of carbon via BVOC emissions is much smaller than Pn but significant for the local air quality. Belgium experiences a continuous rise in air temperature with irregularly scattered precipitation, increasingly dry soil in summer months, and rising air pollution, especially in Flanders. The integrated approach used in this study, including both ecophysiological and chemical research allowed us to gain insights into Pn-BVOC links and related processes. However, many unanswered questions remain. The high variability in BVOC emissions found among species creates difficulties in making predictions. Future investigations may therefore include carbohydrate analysis by using stable ^{13}C and/or radioactive ^{14}C isotopes. However, the carbohydrate distribution may not be sufficient to explain observed patterns in Pn and BVOC emissions and deviations from theoretical expectations might occur. This needs to be fulfilled with integration of other field experts such as entomologists and atmospheric chemists. Overall, this study underscores the importance of BVOC studies from an ecophysiological point of view (**Chapter 2-5**).

Increasing knowledge on plant-insect and plant-plant interactions

This PhD highlighted the importance of entomological identification and the need for entomological quantification in BVOC studies. Little is known about the molecular mode of action of most plant defenses at the individual level and this is even more true for BVOC species mixtures (Gershenzon et al., 2012), opening new research areas. Observation of changes in BVOC species mixtures in response to stress (such as herbivory) can lead to a better understanding of interactions between the plant and the stressor. Different phenological stages differ in their sensitivity to abiotic stress such as high temperature; however, this depends on species and genotype. The global change rapidity makes it difficult to predict future climate and how Pn and BVOC emissions will adapt to a changing environment (Loreto and Centritto, 2004; IPCC, 2007). Since global climate models predict a further 1.5-5.5 °C warming for this century as a result of increased atmospheric concentrations of CO_2 and other greenhouse gases (IPCC, 2007), MT emissions might rise, affecting plant-plant and plant-insect interactions. However, it is clear that an understanding of the interrelation between Pn, growth, BVOC emissions and defense in the atmosphere-biosphere exchange is important in a polluted atmosphere, under stress conditions and for predicting future global change. In **Chapter 1** the state-of-the-art BVOC

measurement techniques are mentioned that should be implemented for measurements of GLVs (**Chapter 3**). Beforehand, it should be first experimentally checked whether these observations are tree species-specific or of general nature. To our knowledge, this fifth Belgian BVOC-related PhD work (after Maes, 2001, Joó, 2011, Demarcke, 2011, and Pokorska, 2012) brought forward that BVOC leaf-level emissions are important in plant defense against abiotic and biotic stresses. However, this work is different from the above mentioned theses linking ecophysiology with BVOC emissions. Further research should focus on controlled experiments with artificial infestation and/or application of isoprenoid biosynthetic pathway inhibitors such as fosmidomycin (MVA-independent inhibitor), lovastatin (MVA-dependent inhibitor) or MeSA (**Chapter 5**; a stress hormone) treatments. Lastly, MTs emitted from trees not only attract herbivores, but also attract insect herbivore predators (parasitoid wasps and predatory mites) (Dicke and van Loon, 2000), which is another relevant new research area.

Additionally, it is emphasized that detailed entomological identification (as done in the present study) as well as quantification (as unfortunately was not done in this study) should be incorporated into future Pn-MT emission studies, divided per phenological phase. Finally, these experiments have underscored the need for long-term field experiments on more replicates and tree species to allow the evaluation of responses to complex multiple stressors. In the future, possible changes in MT composition along a vertical canopy gradient should be further investigated, in addition to the quantitative changes described here.

Developing an urban tree air quality score for Flanders (Belgium), updating inventories and improving predictions

In order to mitigate air pollution, certain tree species should be planted in certain areas. Since 1979, the Convention on Long-range Transboundary Air Pollution (LRTAP) aims to gradually reduce air pollution. As BVOC emissions are very reactive and alter the atmosphere's oxidation capacity on local scales (Holzinger et al., 2006), tree species planted in urban areas should be carefully selected to maximally include non-emitters. Special emphasis should be given to air quality mitigation and to possibly plant tree species with low BVOC emissions in urban areas. In contrast, in rural areas high BVOC emitters could be allowed. This research revealed differences between tree species (**Chapter 4**), which should be taken into account when choosing species for use in urban and/or rural areas. Donovan et al. (2005) established an urban tree air quality score (UTAQS) for the United Kingdom including best and worst tree species influencing air quality (Fig. 6.1). Trees not emitting the most reactive BVOCs and with a large leaf area index (LAI) have the best effects on air quality (Donovan et al., 2005). These observations may have important implications for BVOC emission assessments

with consequences for tropospheric chemistry on a local scale and show the need for developing an urban tree air quality scale for Flanders, requiring further research.

Fig. 6.1. Tree scheme from best to worst tree species regarding air quality improvement (Donovan et al., 2005; Nick Hewitt, personal communication).

However, to ensure an improvement of air quality, concerted efforts of plant physiologists, molecular biologists, air chemists and entomologists are an imperative. In order to combat this environmental adversary, the mentioned air quality scores should be implemented especially in industrial regions such as Flanders.

Research on plant-based BVOC emissions presents several challenging opportunities for plant ecophysiology, entomology/phytopathology as well as air chemistry. Through controlled laboratory and field experiments, BVOC emissions in relation to air quality warrants more research attention than has been given in the past.

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Summary

Gas exchange between vegetation and the atmosphere is very dynamic. In addition to gases such as carbon dioxide (CO_2), water vapor, oxygen, nitrogen oxides (NO_x), sulphur dioxide, ammonia and ozone (O_3), also biogenic volatile organic compounds (BVOCs) are exchanged between the vegetation and the atmosphere. This PhD focussed on the exchange of CO_2 and BVOCs, since net photosynthesis (Pn) and BVOC emission are two plant processes important in plant functioning. Vegetation, and forests in particular, acts as a major source of BVOCs. The importance of the study lays in understanding the link between Pn, BVOC emissions and tree growth. BVOC emissions indirectly affect climate change as BVOCs are in combination with atmospheric NO_x the main precursors of photochemical O_3 in the troposphere, where it acts as potential greenhouse gas, damaging vegetation and affecting human respiratory organs. BVOCs are therefore dominant reactive compounds in the troposphere and important in atmospheric chemistry and climatology. Understanding tree chemistry and ecophysiology is crucial to predict future changes in the Earth's carbon balance as well as to update BVOC inventories and improve predictions in tropospheric air chemistry. Accordingly, the main goals of the PhD were to identify and quantify the effects of temperature, drought, seasonality and vertical canopy gradients on Pn and BVOC emissions.

The general methodology consisted of developing and constructing enclosure systems for gas exchange measurements indoors and outdoors, where coupling of an infra-red gas analysis (IRGA), proton transfer reaction-mass spectrometry (PTR-MS) and thermal desorption gas chromatography/mass spectrometry (TD-GC/MS) represented a major challenge. With respect to tree species, the focus was on European beech (*Fagus sylvatica* L.), while additionally common ash (*Fraxinus excelsior* L.) and northern red oak (*Quercus rubra* L.) were examined in Chapter 4. The trees were examined in growth room conditions, at the campus and in the Aelmoeseneie experimental forest. The main variables measured were Pn and BVOC emissions, in particular of monoterpenoids (MTs). In addition, microclimatic variables (air temperature, photosynthetic photon flux density, soil water potential, and vapor pressure deficit) and leaf characteristics (specific leaf area, leaf temperature, leaf pigments, and leaf water potentials) were measured. In the growth room experiments, stem diameter variations and chlorophyll indices were measured to

explain the behavior of MT emissions by young beech trees. In the forest, the experimental tower showed to be an important facility for adequate local characterization of adult beech Pn and BVOC chemistry. Leaf level studies showed to be crucial for unraveling the mechanisms behind the emission of BVOCs.

The results indicated a large variability in BVOC emission patterns of different tree species. Temperature, drought, seasonality, vertical canopy gradients differently influenced Pn and BVOC emissions (and in particular MTs), as well as their ratio. Indoors and outdoors day-time Pn, MT emissions and MT/Pn carbon ratio varied in a systematic manner following light and temperature changes. The results indicated that not only light affected Pn, MT emissions and MT/Pn ratio, but also showed a pronounced temperature effect on MT emissions (and hence on the MT/Pn carbon ratio), with an increasing exponential trend with rising air temperatures. Furthermore, during drought stress MT emissions showed an increasing-decreasing trend depending on the drought severity. Linear variable displacement transducers (LVDTs) showed to be useful for stress quantification in BVOC studies. Another notable finding was that, under severe drought stress, two PTR-MS signals diverged from each other, indicating the possible presence of BVOC species other than MT such as green leaf volatiles (GLVs). Seasonal measurements on anatomically different trees indicated a strong temperature rather than light dependency when looking at total BVOC emission trends. Beside substantial quantities of MTs released from leaves into the atmosphere, driven by light and temperature, beside non-MTs, MTs also showed to play a role in plant-insect interactions. Detected stress compounds proved infestiation-based emissions. Consequently, plant-insect relationships require additional research, identifying individual MT species using the GC/MS speciation approach and looking at their relationships with ecophysiological parameters.

In conclusion, the performed indoor and outdoor studies demonstrated that Pn and BVOC emissions are strongly interrelated. Proposed hypotheses were tested and confirmed. However, many unanswered questions remain, e.g. how the distribution of individual BVOC compounds correlated with temperature and drought stress as well as along the vertical canopy gradient.

Samenvatting

Gasuitwisseling tussen vegetatie en de atmosfeer is zeer dynamisch. Naast gassen als koolstofdioxide (CO_2), waterdamp, zuurstof, stikstofoxides ($NO_{x)}$, zwaveldioxide, ammoniak en ozon ($O_{3)}$, worden ook biogene vluchtige organische stoffen (BVOCs) uitgewisseld tussen de vegetatie en de atmosfeer. Deze studie richtte zich op de uitwisseling van CO_2 en BVOCs, aangezien fotosynthese (Pn) en BVOC-emissie twee belangrijke processen zijn voor het functioneren van planten. Vegetatie, en bos in het bijzonder, treedt op als een belangrijke bron van BVOCs. Het belang van dit onderzoek ligt in het begrijpen van het verband tussen Pn, BVOC-emissie en boomgroei. BVOC-emissies hebben een indirect effect op de klimaatverandering, waarbij BVOCs in aanwezigheid van NO_x één van de belangrijkste precursoren vormen van fotochemisch O_3 in de troposfeer, waar het als een krachtig broeikasgas werkt en schade veroorzaakt aan vegetatie en de menselijke ademhalingsorganen. Inzicht in boomchemie en ecofysiologie is belangrijk om toekomstige veranderingen in de globale koolstofbalans te voorspellen, om BVOC-inventarissen te actualiseren en om voorspellingen in troposferische chemie te verbeteren. Daarom is het belangrijkste doel van dit doctoraat het identificeren en kwantificeren van effecten van temperatuur, droogte, seizoensgebondenheid en verticale kruingradiënten op Pn en emissies van BVOCs.

De algemene methodiek van dit werk bestond uit het ontwikkelen en bouwen van cuvette-systemen om de uitwisseling van gassen te meten, waarbij de koppeling van een infrarood gasanalyse (IRGA), protonentransfer reactie-massaspectrometrie (PTR-MS), en thermal disorptie-gaschromatografie/massaspectrometrie (TD-GC/MS) de belangrijkste uitdaging was. Qua boomsoorten lag de nadruk op beuk (*Fagus sylvatica* L.), al werden ook es (*Fraxinus excelsior* L.) en Amerikaanse eik (*Quercus rubra* L.) onderzocht in hoofdstuk 4. De bomen werden onderzocht in groeikamers, op de campus en in het proefbos Aelmoeseneie. De gemeten variabelen waren Pn en emissies van BVOCs, in het bijzonder van monoterpenoïden (MT). Daarnaast werd ook het microklimaat (luchttemperatuur, fotosynthesische fotonfluxdichtheid, bodemwaterpotentialen en dampdruk tekort) en bladkenmerken (specifieke bladoppervlakte, bladtemperatuur, bladpigmenten en bladwaterpotentialen) gemeten. In de groeikamer werden diameter-variaties en chlorofyl-indices gemeten om het MT-emissie gedrag te verklaren bij jonge beuken. In het proefbos bleek de experimentele toren een belangrijke faciliteit voor de lokale karakterisering van

Pn en BVOC emissies bij een volwassen beuk. Studies op bladniveau zijn immers cruciaal voor het ontrafelen van de mechanismen achter de emissies van BVOCs.

De resultaten toonden een grote variatie in de emissiepatronen van diverse boomsoorten. Pn en BVOC-emissies (en MT-emissies in het bijzonder) werden op een verschillende manier beïnvloed door temperatuur, droogte, seizoensgebondenheid en de verticale kruingradiënt. Zowel in binnen- als buitenomstandigheden varieerden Pn, MT-emissies en de MT/Pn-verhouding op systematische wijze met licht en temperatuur. De resultaten toonden aan dat niet enkel licht een effect heeft op de MT-emissies (en de MT/Pn-verhouding), maar ook temperatuur, met een stijgende exponentiële trend met stijgende temperatuuur. Bovendien werd tijdens een droogtestress-experiment een stijgende-dalende trend in BVOC-emissies aangetoond, afhankelijk van de stressintensiteit. Metingen van de variaties in stamdiameter (LVDTs) bleken een nuttig hulpmiddel voor de kwantificering van stress in BVOC-studies. Een andere opmerkelijke bevinding was dat tijdens ernstige droogte twee verschillende PTR-MS signalen werden onderscheiden. Dit is een indicatie voor de aanwezigheid van andere (niet-MT) BVOC-verbindingen, zoals ‘Green Leaf Volatiles’. Bij seizoensgebonden metingen op anatomisch verschillende bomen werd een sterke temperatuursafhankelijkheid waargenomen, eerder dan een lichtafhankelijkheid. Naast aanzienlijke MT-emissies, gedreven door licht en temperatuur, toonden deze experimenten ook de rol aan van plant-insect interacties. Extra onderzoek naar plant-insect relaties is daarom nodig, waarbij het identificeren van individuele MT-componenten (en niet-MT componenten) met behulp van GC/MS belangrijk is.

Samenvattend kan gesteld worden dat Pn en BVOC-emissies nauw met elkaar verbonden zijn, zowel in binnen- als buitenomstandigheden. De voorgestelde hypotheses werden getest en bevestigd, maar er blijven verschillende vragen onbeantwoord, bv. hoe individuele BVOC-verbindingen (en niet-MTs) gecorreleerd zijn met temperatuur, droogtestress en verticale kruingradiënten.

Curriculum vitae

Personalia

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Education

2003-2005	Master's in Environmental Sanitation, Faculty of Bioscience engineering, Ghent University, Ghent, Belgium
2004	Low country studies, Ghent University, Ghent, Belgium
1996-2003	Plant protection, Faculty of Agriculture, University of Zagreb, Zagreb, Croatia
2001-2002	Portuguese Language and Literature, Faculty of Philosophy, University of Zagreb, Zagreb, Croatia
1995-1996	Oroville High School, Oroville, California, USA
1992-1995	IIIrd Gymnasium, natural and mathematical sciences high school, Zagreb, Croatia

Awards

University of Zagreb Rector's award for best student research in biotechnical sciences:

Apr 2002 Šimpraga, M. 2002. 'An issue of pear rust (*Gymnosprangium fuscum* DC. (Hedw.*)*) at urban areas' – Zagreb University Rector's Award. Laboratory of Phytopathology, Faculty of Agriculture, University of Zagreb, Zagreb, Croatia. 26p.

Grants

Apr–May 2002 Research assistent, Royal Research Station of Gorsem (Koninklijk Opzoekingsstation van Gorsem), Sint-Truiden, Belgium

1995-1996 IEF exchange student - High school education - Oroville High School, Oroville, California, USA

Theses

1. **Šimpraga, M. 2005.** 'Metal accumulation in a surface flow wetland - Accumulatie van metalen in een vloeirietveld'. M.Sc. dissertation. Laboratory of Analytical Chemistry and Applied Ecochemistry, Faculty of Bioscience engineering, Ghent University, Ghent, Belgium. 82p.

2. **Šimpraga, M. 2003.** *'Rhagoletis cerasi* L. , cherry fruit fly – economically important pest of cherry and sour cherry'. Thesis. Department of Agricultural zoology, Faculty of Agriculture, University of Zagreb, Zagreb, Croatia. 35p.

Work experience

Aug 2011- now	Laboratory of Analytical Chemistry and Applied Ecochemistry, Faculty of Bioscience engineering, Ghent University, Ghent, Belgium, Erasmus Mundus scientific/technical IMETE (International Master in Environmental Technology and Engineering) coordinator.
Feb 2011- 2012	Laboratory of Plant Ecology, Faculty of Bioscience engineering, Ghent University, Ghent, Belgium; volontary work as researcher.
Mar 2007 -Jan 2011	Laboratory of Plant Ecology, Faculty of Bioscience engineering, Ghent University, Ghent, Belgium; research scientist.
Oct 2002 – Jun 2003	Central Agricultural Library (Središnja agronomska knjižnica), FAO depositary library for East Europe, Faculty of Agriculture, University of Zagreb, Zagreb, Croatia.
Nov 2002 – Jan 2003	Laboratory of Botany, University of Zagreb, Faculty of Agriculture, Zagreb, Croatia, botany practicum assisstant.

Professional experience

2004 - 2005	Practical experience in soil sanitation, heavy metal accumulation in wetlands, Laboratory of Analytical Chemistry and Applied Ecochemistry, Faculty of Bioscience engineering, Ghent University, Ghent.
2001 - 2003	Practical experience in monitoring and biological pest management of cherry fruit fly *Rhagoletis cerasi* L. Thesis research. Laboratory of Agricultural Zoology, Faculty of Agriculture, University of Zagreb, Zagreb, Croatia.
2000 - 2002	Practical experience in identification of *Gymnosprangium fuscum* DC. (Hedw.) and *Juniperus* sp. Department of Phytopathology, Faculty of Agriculture, University of Zagreb, Zagreb, Croatia.

May 2000	One week of practical experience in monitoring and indentification of beetle *Leptinotarsa decemlineata* (Say) on potatoes. Department of Agricultural Zoology, Faculty of Agriculture, University of Zagreb, Zagreb, Croatia.
2000	One week of practical experience in indentification of funghi *Botrytis cinerea* on grapes. Department of Phytopathology, Faculty of Agriculture, University of Zagreb, Zagreb, Croatia.
Oct 1999	Two weeks of practical experience in management and production of grapes (*Vitis vinifera* L.) and viticulture techniques. Scientific vinyard '*Jazbina',* Faculty of Agriculture, University of Zagreb, Zagreb, Croatia.

Educational activities

2007-2009	Guidance for practical classes of the courses “Ecology” and “Aquatic and terrestrial ecology” taught by the Department of Applied Ecology and Environmental Biology, Ghent University (Belgium). Teaching language: Dutch.
2008	3-day forest excursions.
2009-2010	Guidance of the master thesis of Jasper Bloemen under title Dynamische interactie tussen fotosynthese, transpiratie en BVOS emissies in bosecosystemen.

Publications

***Publications in international journals with peer review** (impact factor=IF 2010)*

- Joó É., Van Langenhove H., **Šimpraga M.,** Steppe K., Amelynck C., Schoon N., Müller J-F., Dewulf J., 2010. Variation in biogenic volatile organic compound emission pattern of *Fagus sylvatica* L. due to aphid infection. Atmospheric Environment 44, 227-234. IF=3.139
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- Laffineur Q., Heinesch B., Demarcke M., Schoon N., Amelynck C., Müller J.-F., Dewulf J., Van Langenhove H., Steppe K., **Šimpraga M.,** Aubinet M., (2011). Isoprene and monoterpene emissions from a mixed temperate forest. Atmospheric Environment 45, 3157-3168. IF=3.139
- Joó É., Dewulf J., Amelynck C., Schoon N., Pokorska O., **Šimpraga M.**, Steppe K., Aubinet M., Van Langenhove H., (2011). Constitutive versus heat and biotic stress induced BVOC emissions in *Pseudotsuga menziesii*. Atmospheric Environment 45, 3655-3662. IF=3.139
- Pokorska O., Dewulf J., Amelynck C., Schoon N., Joó É., **Šimpraga M.,** Bloemen J., Steppe K., Van Langenhove H., (2011). Emissions of biogenic volatile organic compounds from *Fraxinus excelsior* and *Quercus robur* under ambient conditions in Flanders (Belgium). International Journal of Environmental Analytical Chemistry 1-13.

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- **Šimpraga M.,** Verbeeck H., Demarcke M., Joó É., Pokorska, O., Amelynck C., Schoon N., Dewulf J., Van Langenhove H., Heinesch B., Aubinet M., Müller J-F., Steppe K., (2011). Clear link between drought stress, photosynthesis and biogenic volatile organic compounds in *Fagus sylvatica* L. Atmospheric Environment 45, 5254-5259. IF=3.139
- Pokorska O., Dewulf J., Amelynck C., Schoon N., Joó É., **Šimpraga M.**, Steppe K., Van Langenhove H., (2011). Isoprene and terpenoid emissions from *Abies alba*: identification and emission rates under ambient conditions. Accepted.

Publications in congress proceedings

- **Šimpraga M.**, Steppe K., Demarcke M., Amelynck C., Schoon N., Müller JF., Joó É., Dewulf J., Vanlangenhove H., Lemeur R., Steppe K., (2008). Preliminary observations of temperature effects on carbon losses through BVOC emissions in *Fagus sylvatica* L. EGU Conference Vienna, Austria.
- Demarcke M., Amelynck C., Schoon N., Müller JF., Joó É., Dewulf J., Vanlangenhove H., **Šimpraga M.,** Steppe K., Samson R., Lemeur R., (2008). Measurements of BVOC emissions from *Fagus sylvatica* L. in controlled environmental conditions: preliminary results. EGU Conference Vienna, Austria.
- **Šimpraga M.,** Steppe K., Demarcke M., Amelynck C., Schoon N., Samson R., Lemeur R., (2009). How does drought stress in a potted beech tree affects the relationship between photosynthesis and BVOC emissions? Submitted abstract for Starters in Bosonderzoek conference, Brussels, Belgium.
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- Demarcke M., Amelynck C., Schoon N., Müller J.-F., Joó É., Dewulf J., Van Langenhove H., **Šimpraga M.,** Steppe K., Laffineur Q., Heinesch B., Aubinet M., (2011). Effect of seasonality and short-term light and temperature history on monoterpene emissions from European beech (*Fagus sylvatica* L.). Submitted abstract for PTR-MS conference, Innsbruck, Austria. January.
- **Šimpraga M.,** Verbeeck H., Jonckheere I., Heinsch B., Laffineur Q., Aubinet M., Steppe K., (2011). Spatial and seasonal variation of LAI in the footprint of a flux measurement tower. Submitted abstract for Starters in Bosonderzoek conference (oral presentation). March.
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- Lemeur R., **Šimpraga M.**, Joó É., Dewulf J., Vanlangenhove H., Demarcke M., Amelynck C., Schoon N., Müller JF., Laffineur Q., Heinesch B., Aubinet M., (2008). **Im**pact of **P**henology and **E**nvironmental **C**onditions on B**VOC** Emissions from Forest Ecosystems. Science for Sustainable Development. Activity BELSPO report intended for the intermediary evaluation.
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Congresses, symposia and workshops with active participation

- 42nd World Congress, Sweden and Norway, 1999*; 'Food and Health' (poster)*
- 43rd World Congress, Mexico, 2000*; 'Water: A challenge for the next century' and 'Tropical and Organic agriculture' (poster)*
- 44th World Congress, Portugal, 2001*; 'Sustainable Development (poster)'*

- 45th World Congress, Indonesia, 2002*; 'Agribusiness: Now and Then' (poster)*
- Kick off meeting - presenting all selected BELSPO projects, Brussels, Belgium, 26th March, 2007 *(oral)*
- EGU (European Geosciences Union) General Assembly, Vienna, Austria, 13-18th April, 2008 *(poster)*
- Starters in Forest research, Brussels, Belgium, 19th March, 2009 *(poster)*
- Starters in Forest research, Brussels, Belgium, 17th March, 2011 *(oral)*
- Erasmus Mundus IMETE presentation, Zagreb, Croatia, 20th December, 2011 *(oral)*

Congresses, symposia and workshops with passive participation

- IAAS motivation week, Slovenia, 1999
- SRM (Subregional Meeting) en seminarie 'Save the Donkey', Croatia, 1999
- 35th Croatian Symposium on Agriculture, Croatia, 1999
- 'Olive Oil, Wine and Cheese – Typical Spanish Products', Spain, 2000
- Summer School 'Renewable Energy Sources', Republic of Serbia, 2000
- 'Exchange week Croatia – Macedonia', FYR of Macedonia, 2001
- 'VisApis2001 – Bee Selection', Croatia, 2001
- 'Exchange week Croatia – Belgium', Belgium, 2002
- Lessons from ten years of eddy covariance and carbon flux measurements, Gembloux, Belgium, 8th May, 2007
- Climate change: challenges, risks and impacts on cropping systems, Symposium of the Benelux Society of Horticultural Science (BNL-SHS), Gembloux, Belgium, 3rd April, 2009
- ACCENT EU Symposium, Air pollution and climate interactions – contribution to European Policy Development, Brussels, Belgium, 03-04th November 2009
- Sap flow index and its potential use in tree water relation studies (Nadezhdina Nadezhda) 28th February, 2009

- Fluorescence as a tool to communicate with plants (Roeland Valcke) 17th March, 2009
- The climate tug-of-war: greenhouse gases vs. aerosols in the climate of the 21 st century (Meinrat O. Andreae) 19th March, 2010
- MySQL database usage (Marie Demarcke) 24th April, 2010
- Tropospheric ozone and BVOC emissions (Silvano Fares) 14th October, 2011
- EM-IDEA Conference, 23rd November, 2011
- Trace gas fluxes from/to mountain grasslands (Georg Wohlfahrt) 2nd March, 2012

Varia

2001-2002 exchange coordinator for IAAS Croatia (International Association of Agricultural Students)

Member of:

◊ Vereniging voor bos in Vlaanderen (VBV)
◊ American Society of Plant Biologist (ASPB)
◊ International Society of Chemical Ecology (ISCE)
◊ Hrvatsko društvo za biljnu biologiju (Croatian Society of Plant Biology) (HDBB)
◊ IAAS alumni