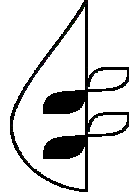


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***STUDY OF CONIFEROUS NEEDLES IN RELATION TO
ENVIRONMENTAL FACTORS USING APPROACHES OF
QUANTITATIVE ANATOMY AND LABORATORY SPECTROSCOPY.***



PhD Thesis

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Declaration:

I declare that I created this thesis on my own under supervision of Doc. RNDr. Jana Albrechtová PhD. and I have not submitted it to obtain any other academic degree.

Prague, November 2008

Abbreviations:

2D	Two-Dimensional
3D	Three-Dimensional
A	Leaf Area Surface
ABA	Abscisic Acid
A_{mes}	Area Of Mesophyll Cells Exposed To Intercellular Spaces
$ANMB_{650-725}$	Area Under Curve Normalized to Maximal Band Depth Between 650 and 725 nm
AR4	The Fourth Assessment Report "Climate Change 2007" by Intergovernmental Panel on Climate Change (http://ipcc-wg1.ucar.edu/wg1/wg1-report.html)
Chl NDI	Chlorophyll Normalized Difference Index
CHRIS	Compact High Resolution Imaging Spectrometer
CJRS	Canadian Journal of Remote Sensing
CKs	Cytokinins
$[CO_2]$	Carbon Dioxide Concentration
DOC	Dissolved Organic Carbon
DON	Dissolved Organic Nitrogen
ETM+	Enhanced Thematic Mapper Plus
FACE	Free Air Carbon dioxide Enrichment
GAs	Gibberelins
g_m	Mesophyll Conductance to CO_2
g_s	Stomatal Conductance
<i>HIC</i>	High Carbon Dioxide
IAA	Indole Acetic Acid
LAI	Leaf Area Index
LMA	($g \cdot m^{-2}$), Leaf Mass per Unit Area
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced)
OSAVI	Optimized Soil-Adjusted Vegetation Index
ppm	Parts Per Million
PROBA	Project for On Board Autonomy
PS I	Photosystem I
PS II	Photosystem II
Rubisco	Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase
TCARI	Transformed Chlorophyll Absorption in Reflectance Index
XET	Xyloglucan Endotransglycosylase

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GENERAL INTRODUCTION

The essentiality of Earth's vegetation as the most important primary producer of biomass, the base of the food chain and an important landscape-creating factor is generally acknowledged. In recent decades of constantly increasing CO₂ concentration in the atmosphere, a function of vegetation as a carbon sink is widely discussed. Particularly forests play an important role in the global carbon cycle by sequestering large amounts of atmospheric carbon, and are thought to offer a possible mitigation strategy to reduce global warming. Although in forest ecosystems the great amount of carbon is fixed in non-living biomass of wood, litter, or soil, the green photosynthesizing foliage represents the gate for the carbon, whatever metabolized by the plant itself or fixed into the biomass. As will be mentioned further in more detail, leaf structure is tightly connected with leaf function and vice versa. Not only from the point of view of the plant ecological physiology it is important to understand relationships between leaf structure, functions and environmental factors, to elucidate mechanisms that trigger and modify morphogenetic development and changes at an organ, cell or molecular levels. The knowledge of these mechanisms may help to understand the potential of an individual plant species or the whole ecosystems in carbon sequestration.

The present thesis is focused on conifer leaf anatomy in the context of leaf function under specific environmental conditions. As the main plant material, Norway spruce (*Picea abies* L. Karst), the most frequently planted tree species in the Czech Republic (53% of the forested area in the Czech Republic consisted of Norway spruce in 2006, <http://www.uhul.cz/zelenazprava/index.php>) and in other countries in the Central and Northern Europe, was selected. In addition, the study was performed on two very common North-American conifers – red spruce (*Picea rubens* Sarg.) and balsam fir (*Abies balsamea* L. Mill.) in collaboration with American colleagues from the University of New Hampshire, Durham, U.S.

Norway spruce is a very sensitive species to atmospheric pollution, particularly to deleterious impact of acid rain (e.g. Smith 1990). During the second half of the 20th century loads of SO₂, NO_x and ozone were extremely high in the Central Europe including the Czech Republic causing tremendous large-scale forest dieback, therefore the forest decline of Norway spruce was in the focus of extensive research in Europe (e.g. Smith 1990, Masuch 1992; Viskari *et al.* 2000; Soukupová *et al.* 2001). Alterations in needle anatomy caused by the effect of acid air pollution alone or in combination with harsh climatic conditions have been classified as so called „microscopical markers of forest damage“ and widely used by researchers including the team of Dr. Albrechtová from the Department of Plant Physiology, Faculty of Science, Charles University in Prague and their American collaborators from the University of New Hampshire (Albrechtová *et al.* 2001; Moss *et al.* 1998; Soukupová *et al.* 2001).

Despite that quantitative approach in description of inner leaf structure has quite a long tradition launched by Turrel in the 1930's (Turrell, 1936), the majority of studies concerning forest decline described the anatomical alterations of leaves only qualitatively, for example, by the loss of integrity of chloroplasts (Moss *et al.* 1998). However in several cases quantitative evaluation of anatomical changes appeared (e.g. proportion of intercellular spaces in mesophyll (Kukkola *et al.* 2005). Nowadays, modeling of gas transport (Aalto and Juurola 2002; Juurola *et al.* 2005), radiative transfer across the leaf (Ustin *et al.* 2001) in consequence with photosynthesis and carbon sequestration became the cardinal issue of plant ecophysiology and biophysics. Thus, there is a clear need for efficient and unbiased methods for quantitative description of important leaf anatomical characteristics, such as internal leaf surface area, mean mesophyll cell volume, and cell number in the leaf. Several methods for quantitative description of photosynthetic leaf tissue, the mesophyll, were established: e.g. model-based methods using correction shape factors (Oguchi *et al.*



2003) or image analysis approach (Niinemets *et al.* 2007). However, model-based methods for estimation of geometrical parameters of mesophyll were found to be biased in some cases (Slaton and Smith 2002) and image analysis cannot be always applied. The recently developed methods, based on confocal microscopy and stereology were tested on different kind of plant samples (Kubínová and Janáček 1998; Kubínová *et al.* 2001) and appeared to be suitable for use in ecophysiological studies. Feasibility of application of confocal microscopy and stereological methods in purpose to evaluate impact of environmental stress on leaf anatomy was tested on young Norway spruce potted trees treated with simulated acid rain; the first paper included in this thesis is based on the results of this pilot study (Albrechtová *et al.* 2007, Paper I.). The quantitative studies on leaf internal structure always used sections of fixed and embedded plant material (e.g. Turrell, 1936; Niinemets *et al.* 2007) but during fixation or embedding the tissue deformation and other artifacts may occur (Uwins *et al.* 1993; Dorph-Petersen *et al.* 2001), moreover sample preparation is time consuming and laborious. In Albrechtová *et al.* (2007) thick sections of fresh needles were used for confocal microscopy. However, fresh material limits the method application in ecophysiological studies, as only few fresh samples can be processed immediately if at all under condition that the experimental plots could not be located too far from the place of analysis. The aim of the second paper included in this thesis is addressed to test feasibility of using frozen stored Norway spruce needles for confocal microscopy and stereological processing and further to deal with another technical problem resulting in biased measurements, caused by the possible distortions of cutting surfaces of transversely cut needle sections (Lhotáková *et al.* 2008, Paper II.).

Sharp increase of carbon dioxide atmospheric concentration over the last 150 years by app. 1.3 times (AR4 2007), caused mainly by anthropogenic carbon emissions, induces many demands on physiological and developmental changes in plants, which may have strong impact on future crop and wildland productivity, biogeochemical cycling, water resources and other natural processes at global scale. The direct responses of plants to elevated carbon dioxide concentration have been in focus of extensive research at least for two decades and wide spectrum of changes on the physiological level was described (rev. by Urban, 2003). Mostly reported primary physiological responses on photosynthetic level to carbon dioxide enrichment are activation of photosynthetic enzyme Rubisco, decrease of its oxygenase activity followed by the subsequent decrease of photorespiration, stimulation of net photosynthetic rate (Maier *et al.* 2008) or decreased stomatal conductance (Zhang *et al.* 2008a). These primary responses lead to the secondary effects like increased content of sucrose and other non-structural carbohydrates (Cabálková *et al.* 2007), changes in their allocation within the plant or dilution of mineral nutrients, particularly nitrogen, what often leads to acclimation – down-regulation of photosynthesis (Zhang *et al.* 2008a). However, the plant responses are quite variable depending on a species, age of the plant, mineral nutrition as well as duration and method of CO₂ enrichment. The Paper III. included in this thesis focuses on evaluation of changes in photosynthesis (maximum assimilation rate and apparent quantum efficiency) and respiration together with structural acclimation to elevated CO₂ in sun and shade needles of Norway spruce, while stereological methods adjusted previously in Papers I. and II. are therein applied in the field study (Lhotáková *et al.*, submitted, Paper III.).

It is not only the elevated carbon dioxide concentration alone, which plants need to evolve acclimations to. Moreover, consequent forcing of atmospheric CO₂ enrichment as a temperature rise or changed precipitation patterns and water regimes may fundamentally change species geographical distribution, ecosystems stability or even reduce biodiversity (Kleidon and Mooney 2000). Despite plants are able to develop a broad spectrum of adaptive responses to several environmental factors, they have not experienced such high CO₂ concentrations as nowadays for over 20 million years (Pearson and Palmer 2000) and in consequence of rapid rise of CO₂



concentration there are uncertainties whether genetic variation is sufficient to manage this shift in the few generations before CO₂ concentration doubles or triples (Gutschick *et al.* 2003). In terrestrial ecosystems, photosynthesis plays a major role of coupling process between carbon and nitrogen cycles. So does soil organic matter, its production and decomposition by soil microbial and fungal societies, which interconnect demands for nutrition resources by vegetation to pools of those resources available for plants (McDowell, 2003b; Pritchard *et al.* 2008). Particularly in forests, nitrogen often stands as a limiting factor for tree productivity and thus influences the potential of carbon fixation. In several carbon dioxide enrichment experiments nitrogen availability modulated response of trees to elevated CO₂ (Eguchi *et al.* 2004; Eguchi *et al.* 2008; Maier *et al.* 2008), which only emphasizes the importance of elucidating plant–litter–soil interactions. Fluxes of carbon and nitrogen between soil and vegetation are often species-specific, even within one ecosystem or biome and may be influenced by human management (Ollinger *et al.* 2002). The knowledge of mechanisms, how vegetation and soil processes interconnect carbon and nitrogen cycles, is needed at least at regional scale, which could be further upscaled to continental or global scale. However, it would be extremely time and resource demanding to conduct such an ecosystem field study; therefore robust tools as remote sensing or ecosystem modeling seem to be more suitable.

Linkages among foliar chemistry, decomposition, nitrogen cycling, and productivity in forest ecosystems have been documented in literature frequently. Several specific indices and model-based methods derived from foliar reflectance airborne or spaceborn data have been developed and used for foliar chemistry (content of chlorophyll, lignin, nitrogen) estimation at regional scale (Ollinger *et al.* 2002; Serrano *et al.* 2002; Ollinger and Smith 2005; Zhang *et al.* 2008b). Leaf chlorophyll content is the main parameter determining leaf spectral variation in the visible regions, therefore spectral reflectance indices derived from chlorophyll absorbing wavelengths are considered as quite efficient, and sensitive non-destructive method of chlorophyll content assessment. However, those indices are usually developed for an individual species and in case of application on different species or a vegetation type, validation of such an index is needed. Recently, continuous reflectance data with high spectral resolution are used for vegetation monitoring. Similarly, spatial resolution of reflectance data is also very high (Malenovský *et al.* 2008) and in such a case one tree canopy is represented with several pixels in remote sensing data. The remote sensing approaches developed to estimate the leaf chlorophyll content had been based on the assumption that the leaf optical properties vary little with their location in the canopy. Considering possible physiological and structural variability in Norway spruce needles, a detailed study investigating spatial variability of the leaf optical properties within the tree crowns is required to confirm or reject this assumption. Paper IV. (Lhotáková *et al.* 2007, Paper IV.) is focused on assessment of variation in selected biochemical, structural and spectral parameters of Norway spruce needles within the upper sun-lit part of the canopy considering orientation of branches in different azimuth directions.

As mentioned above, foliar chemistry, which may be quite easily observable property across broad spatial scales by approach of high spectral resolution remote sensing, could serve as indicator of carbon and nitrogen cycling. The last paper included in this thesis is based on the study conducted on red spruce and balsam fir in New Hampshire focused on linking chlorophyll content in needles and amount of dissolved organic carbon and nitrogen in forest floor with spectral properties of foliage (Albrechtová *et al.* 2008, Paper V.).



OBJECTIVES AND HYPOTHESES:

- 1. To develop and adjust an efficient method based on confocal microscopy and stereology for detailed quantitative description of changes in geometrical parameters of coniferous mesophyll.**

Hypotheses:

- Subtle changes in needle structure, elicited by environmental factors such as simulated acid rain, can be quantitatively characterized using selected sensitive stereological methods: fakir and disector.
- Hand-cut thick sections of fresh and frozen stored needles are suitable for confocal microscopy and subsequent stereological analysis.
- Selected geometrical parameters of mesophyll: proportion of intercellular spaces and internal surface density do not suffer from deformation by freezing during needle storage.

- 2. To characterize changes in Norway spruce mesophyll anatomy under impact of elevated CO₂ concentration, using methods developed and adjusted in Aim 1.**

Hypotheses:

- Volume proportion of mesophyll in needles increases under impact of elevated CO₂ concentration.
- Internal needle surface increases under impact of elevated CO₂ concentration.
- Trees planted under impact of elevated CO₂ concentration will show less pronounced structural differences between sun and shade needle ecotype.

- 3. To characterize how needle position (azimuth orientation) influences selected biochemical, anatomical and spectral properties of needles.**

Hypothesis:

- Selected biochemical, anatomical and spectral properties of sun-exposed Norway spruce needles from the upper part of tree crown are different according to branch azimuth orientation.

- 4. To examine potential links among selected biochemical and spectral properties of needles and water extractable dissolved organic C and N in forest floor of mixed coniferous forest in New Hampshire.**

Hypotheses:

- There are links between foliage biochemistry and possibly chlorophyll content in Red spruce and Balsam fir needles and soil DOC and DON concentration.
- These links could be detected by chlorophyll spectral reflectance indices.



LIST OF INCLUDED PUBLICATIONS

Paper I.

Novel efficient methods for measuring mesophyll anatomical characteristics from fresh thick sections using stereology and confocal microscopy: application on acid rain treated Norway spruce needles. Albrechtová Jana, Janáček Jiří, **Lhotáková Zuzana**, Radochová Barbora, Kubínová Lucie. JOURNAL OF EXPERIMENTAL BOTANY 58 (6): 1451-1461, 2007

Paper II.

Advantages and pitfalls of using free-hand sections of frozen needles for three-dimensional analysis of mesophyll by stereology and confocal microscopy. Lhotáková Zuzana, Albrechtová Jana, Janáček Jiří, Kubínová Lucie. JOURNAL OF MICROSCOPY, 232: 56-63, 2008

Paper III.

The impact of long-term CO₂ enrichment on sun and shade needles of Norway spruce: photosynthetic performance, needle anatomy and phenolic compounds. Lhotáková Zuzana, Urban Otmar, Dubánková Marianna, Cvikrová Milena, Tomášková Ivana, Kubínová Lucie, Zvára Karel, Marek Michal V. and Albrechtová Jana, submitted

Paper IV.

Does the azimuth orientation of Norway spruce (*Picea abies* /L./ Karst.) branches within sunlit crown part influence the heterogeneity of biochemical, structural and spectral characteristics of needles? Lhotáková Zuzana, Albrechtová Jana, Malenovský Zbyněk, Rock Barrett N., Polák Tomáš, Cudlín Pavel. ENVIRONMENTAL AND EXPERIMENTAL BOTANY 59 (3): 283-292, 2007

Paper V.

Spectral analysis of coniferous foliage and possible links to soil chemistry: Are spectral chlorophyll indices related to forest floor dissolved organic C and N? Albrechtová Jana, Seidl Zdeněk, Aitkenhead-Peterson Jacqueline, Lhotáková Zuzana, Rock Barrett N., Alexander Jess E., Malenovský Zbyněk, McDowell William H. SCIENCE OF THE TOTAL ENVIRONMENT 404: 424–432, 2008



CRITICAL REVIEW

1 Why to study internal leaf structure in detail

Primarily, growth, development and final structure of mature plant organs, including leaves, are given by endogenous factors like genotype or plant age in case of trees and perennial herbs. On the other hand, structure, morphology and size of mature leaf are strongly influenced by the environmental conditions that prevail during the leaf morphogenesis. Once leaves are fully expanded, the potential for further structural changes is constrained. For evergreen species with long lasting leaves this could bring a disadvantage under changing microclimatic conditions in the canopy, for example, after thinning, natural gap formation or in reverse after shading caused by canopy closure. However, a plant could acclimate to changing environment at the subcellular level without structural alterations at the tissue level as documented by an increasing number or volume of chloroplasts and filling the vacant space along the cell walls of mesophyll to increase the photosynthetic capacity (Oguchi *et al.* 2005; Oguchi *et al.* 2008).

The knowledge of leaf structure development and how this development and final leaf structure itself is influenced by environment may help to understand the potential of individual plant species or the whole ecosystems in carbon sequestration. Wide range of studies focused on internal leaf structure and its interconnection with physiological processes were conducted on crops (Parker and Ford 1982; Masle, 2000; Barbour and Farquhar 2003), because of their importance in human nutrition. In general, broadleaved species are easier to study due to their leaf size and shape (James *et al.* 1999; Miyazawa and Terashima 2001), and only few studies focused on conifers (Warren *et al.* 2003). It is worth to extend research focus and achieve information about conifer leaves, because boreal forests, which occupy 25% of global forest area, consist mostly of cold-tolerant evergreen conifers with needle-like leaves, such as pine, fir, and spruce. Internal needle structure represented by 3D geometrical parameters of leaf mesophyll tissue may play an important role in physiological processes on the leaf level, such as gas transport and exchange as well as radiative transfer across the leaf. Consequently it influences the radiative use efficiency for photosynthesis (Vogelmann *et al.* 1996) and leaf optical properties, such as reflectance and transmittance (Slaton *et al.* 2001), which are exploited in ecosystem, landscape or global scale monitoring of forest health, chemical composition of foliage or its photosynthetic activity by remote sensing techniques (Myneni *et al.* 2001; Ollinger *et al.* 2002; Huber *et al.* 2008).



2 Endogenous factors determining leaf physiology and anatomy

The major endogenous factor that determines the structure of a plant organ is the genotype. The level of genotype ploidy appeared to influence strongly leaf anatomy in wheat, when mesophyll cell size, number of chloroplasts per cell or leaf thickness showed to be determined by nuclear genome size in nine wheat genotypes with different ploidy (Jellings and Leech 1984). Nassar *et al.* (2008) made similar observations in stems of Cassava (*Manihot esculenta* Crantz), when the stems of tetraploid plants exhibited several anatomical differences from diploid plants, such as wider secondary xylem with thinner cell walls and less starch amount. Sugiyama (2005) tried to elucidate the mechanism, which leads to a higher leaf size in polyploid genotypes in leaves of two grass, *Lolium perenne* L. and *Lolium multiflorum* (Lam.) and he examined parameters related to cell division and elongation on diploid and autotetraploid cultivars. Polyploidy lead to increase in leaf size mainly due to increasing the cell elongation rate, which also resulted in longer mature mesophyll and epidermal cells (Sugiyama, 2005). On the other hand cell division parameters, such as cell production rate or cell cycle duration did not contribute to final difference in leaf size between the cultivars. Several other studies were conducted preferentially on monocot grass species leaves, in order to elucidate, whether cell division rather than cell expansion or both play the crucial role in leaf development and leaf final size (Kinsman *et al.* 1997; Fiorani *et al.* 2000; Sugiyama, 2005), but precise mechanism still remains to be explored and elucidated. In perennial herbs or in trees, the age of a plant may also determine the leaf structure. Increase in leaf mass per unit area (LMA) in older trees comparing to younger ones was described for several coniferous species: including *Picea sitchensis* (Bong.) Carr (Steele *et al.* 1989), *Picea engelmanni* Parry × *Picea glauca* (Moench) Voss × *Picea sitchensis* (Richardson *et al.* 2000), and Douglas-fir (*Pseudotsuga menziesii* (Mirbel) Franco (Bond, 2000). With increase in tree age, higher LMA in red spruce needles (*Picea rubens* Sarg.) was accompanied with increase of mesophyll proportion on the cross section and decrease of proportion of intercellular air spaces, which were associated with a decrease in photosynthetic capacity (Greenwood *et al.* 2008).



3 Environmental factors determining leaf physiology and anatomy

Internal leaf structure, especially geometrical parameters of mesophyll and chloroplast arrangement, influences the interception of radiation and diffusion of carbon dioxide within the leaf, which are both essential for photosynthesis process (Pandey and Kushwaha 2005). Several environmental factors such as irradiance, surrounding carbon dioxide concentration, temperature, and water availability or air pollution are known to affect both, leaf structure and function. Recent studies show that relationships between leaf anatomical parameters and photosynthesis are important in leaf acclimation to high or low irradiances (Oguchi *et al.* 2005; Pandey and Kushwaha 2005) or elevated CO₂ concentrations (Eguchi *et al.* 2004). Selected examples of the focus of environmental factors on leaf development; structure and function are discussed in following subchapters with major stress given to carbon dioxide enrichment, because in Paper III. (Lhotáková *et al.* submitted) structure of Norway spruce needles under impact of elevated CO₂ was explored. However, it is important to keep in mind that environmental factors usually interact between each other and foliage is often exposed to simultaneous light, heat and water stresses (Niinemets, 2007).

3.1 Irradiance

As an acclimation to different irradiance environment, sun and shade leaf ecotypes develop frequently (Boardman, 1977). From the anatomical point of view, for Norway spruce generally thicker sun needles were described (Niinemets, 1997), needles of two pine species (*Pinus patula* Schiede ex Schtdl. & Cham. and *Pinus radiata* D. Don) exhibited an increased fraction of mesophyll cells with increased irradiance (Niinemets *et al.* 2007) and needles with higher thickness of mesophyll were observed for sun needles of grand fir (*Abies grandis* (Dougl. ex D. Don) Lindl.) and Engelman spruce (*Picea engelmannii* Parry ex Engelm.) (Youngblood and Ferguson 2003). In the latter case the increase in mesophyll thickness was probably caused by expansion of intercellular spaces, as a number of mesophyll layers and cell size remained unchanged. The shape of needle a cross-section also differs between sun and shade-ecotypes in such a manner that shaded needles are often flatter with a less circular cross section (Cescatti and Zorer 2003) (Figure 1). Flatter shape of needles results in a larger ratio of projected to total area, which increases the light-harvesting efficiency of shaded needles (Niinemets, 2007). In general, higher dry mass per unit of leaf area and a higher leaf thickness under stronger irradiation seem to be widespread anatomical traits for high light environment acclimation in several different plant species groups like herbs, deciduous broad-leaved



trees, evergreen warm-temperate and tropical broad-leaved trees, broad-leaved Mediterranean sclerophylls and evergreen conifers (Niinemets, 2007).

Not only anatomy of individual needles but the needle angular distribution on the shoot affects light-harvesting efficiency: in sun shoots of several spruce or fir species needles are symmetrically distributed around the twig with higher needle aggregation, in contrary to shade shoots, where needles are distributed nearly on the same horizontal plane (Stenberg, 1996; Cescatti and Zorer 2003).

Sun and shade adapted leaves usually differ with their photosynthetic capacity (light-saturated rate of photosynthesis on a leaf area basis), which is higher for sun needles. In general, leaves acclimated for lower irradiance levels contain more photosynthetic pigments (expressed for leaf mass basis) (Lewandowska and Jarvis 1978). Similar vertical profile of Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase, EC 4.1.1.39) enzyme activity and the vertical profile of irradiance was also observed in Norway spruce canopy (Hrstka *et al.* 2005).

Figure 1.

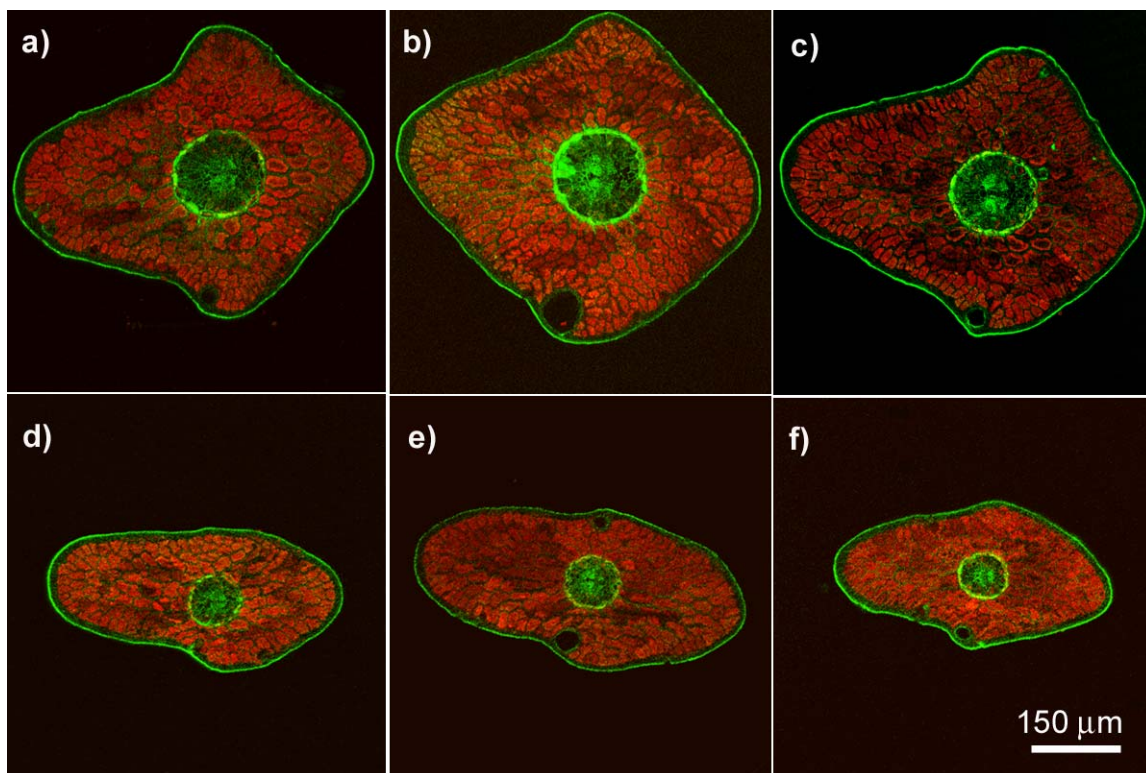


Figure 1: Transverse sections of Norway spruce needles from the middle part of a needle from trees grown for 8 years at the open air forest stand (a, d), glass dome with ambient ($365 \mu\text{mol CO}_2 \text{ mol}^{-1}$) CO_2 concentration (b, e) and glass dome with elevated ($700 \mu\text{mol CO}_2 \text{ mol}^{-1}$) CO_2 concentration (c, f). Top images display sections of sun needles; bottom images display sections of shade needles. Note that elevated CO_2 concentration did not influence the anatomical differences between sun and shade needles, which were proved also by quantitative measurements, see Lhotáková *et al.* submitted, Paper III. Fresh thick free-hand sections, confocal microscopy, autofluorescence. Bar corresponds to $150 \mu\text{m}$ in all sections.



3.2 Water availability, water stress

Although the irradiance is widely accepted as the main environmental factor driving physiological, anatomical and morphological acclimation of leaves, water availability or water stress gradient also play a role, especially in high forest trees. For example, in dry seasons the water potential in *Sequoia sempervirens* ((D. Don.) Endl.) leaves 100m above the ground could decrease to -1.23 and -1.71 MPa at predawn and midday during the dry season (Ishii *et al.* 2008). Low water potentials near to the tree top results in decline in turgor pressure and could negatively influence cell division and expansion. In this consequence, shorter shoots and needles were found in upper crown parts of 60-m-tall Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco. var. *menziesii*) trees (Woodruff *et al.* 2004). However, under long-lasting water stress, accumulation of soluble carbohydrates rather than growth may occur, in purpose to adjust water potential osmotically (Niinemets and Kull 1998).

3.3 Elevated carbon dioxide concentration

As carbon dioxide plays a role of a substrate for photosynthesis and the main source of carbon for building plant's body, concentration of CO₂ obviously influences leaf organogenesis, physiological processes and consequently the leaf structure.

3.3.1 Impact on cell division, expansion and leaf development

In controlled conditions, several studies showed stimulative effect of elevated CO₂ concentration on leaf expansion (Ferris and Taylor 1994) or whole-plant leaf area development (Pritchard *et al.* 1999). Both processes participating on leaf blade development, cell division in a meristem and cell expansion were reported to be sensitive to CO₂ supply. It is likely, that an enhancement of cell division caused by elevated CO₂ is mediated by changes in duration of cell cycle or its particular phases and then shortening of cell cycle and increased cell division may cause changes in leaf growth rate and final leaf structure and size (Masle, 2000). In shoot apical meristem of grass *Dactylis glomerata* (L.) exposed to elevated CO₂, proportion of actively dividing cells increased together with shortening of the cell cycle duration, particularly the G1 phase, which all resulted in faster leaf growth (Kinsman *et al.* 1997). Similarly in wheat leaves, CO₂ enrichment induced faster cycling cell divisions (Masle, 2000).

Elevated CO₂ levels increase the photosynthetic yield and soluble saccharides levels (DeLucia *et al.* 1985; Cure *et al.* 1987) and in most plants, sucrose functions not only as the major transported assimilate from source photosynthetic tissues to sinks, but plays a role of signaling molecule in many processes as well (rev. by Lipavská and Konrádová 2004). Carbon source availability appears to be a major factor determining cell



division and it has been confirmed that carbon availability in the form of sucrose is important during the G1 phase of the cell cycle by controlling the expression of CycD cyclins in *Arabidopsis thaliana* (L.) (Riou-Khamlichi *et al.* 2000). Clear impact of elevated CO₂ on cell expansion was observed in *Phaseolus vulgaris* L. (cv. Tender Green) by (Ranasinghe and Taylor 1996), triggered by changes in biophysical properties of the cell wall, namely increased wall extensibility, which was probably caused by higher activity of cell-wall loosening enzyme xyloglucan endotransglycosylase (XET).

Elevated CO₂ induces also changes at transcription level; the leaf transcriptome analysis of Soybeans (*Glycine max* L. cv 93B15; Pioneer Hi-Bred) growing under elevated CO₂ in a free-air CO₂ enrichment FACE (Free Air Carbon Dioxide Enrichment) facility revealed higher expression of transcripts involved in cell growth and proliferation; simultaneously it was shown that at the transcript level, CO₂ enrichment enhances respiratory breakdown of carbohydrates, which enables better energy and precursor supply for leaf expansion and enhanced growth in elevated CO₂ (Ainsworth *et al.* 2006).

Importance of cell wall extensibility and activity of XET for the rate of leaf expansion and increased final leaf size under elevated CO₂ was confirmed for three poplar species prior to canopy closure under FACE treatment (Ferris *et al.* 2001). However, mechanistic differences in leaf growth response to FACE treatment were also found between individual species: enhanced leaf expansion in *Populus. x euramericana* and *Populus. nigra* resulted from higher production of larger cells, in contrast to *P. alba*, where higher production of smaller cells was observed (Ferris *et al.* 2001). Authors pointed out that in highly controlled experimental environments, particularly if dealing with long-living plants like trees, several plant-soil feedbacks are missing and process of canopy closure is often neglected. They also discuss a question of long-term stimulation effects of FACE on leaf expansion: prior to canopy closure, rapidly expanding leaves act as strong sink for assimilated carbon; however, after reaching final canopy closure and higher leaf area index (LAI), shading and spatial constraints may change source-sink balance and diminish FACE effect. Taylor *et al.* (2003) confirmed stimulation of poplar leaf expansion in the above-cited FACE system even after canopy closure had been achieved, but main stem leaves were not shaded. Authors stressed out the importance of cell production over cell expansion for increased final leaf size and altered leaf shape in elevated [CO₂]. Third growing season after the full canopy closure and stronger tree competition, the initial stimulation of leaf area development was suppressed in *P. nigra* and *P. alba*, but in *P. euramericana* the increase in leaf area of sun leaves was still significant under elevated CO₂ (Tricker *et al.* 2004). In the latter case, the increased number of epidermal cells and acceleration of mesophyll expansion, particularly palisade parenchyma, during leaf growth seemed to be the basis for an adaptation in leaf



morphology over long term. It appears that effect of elevated CO₂ concentration on rate of cell division and expansion during leaf development is species-specific and further influenced by additional environmental factors (as shown for irradiance). According to my knowledge, no similar study focusing on needle cell production and expansion has been conducted on conifers. In so far available literature the mechanism how cell division and expansion interact in the control of needle growth remains speculative.

As shown, CO₂ enrichment stimulates plant growth and development by influencing cell division and elongation (Masle, 2000; Ferris *et al.* 2001; Luomala *et al.* 2005), which processes are known to be regulated by plant hormones, including auxins, gibberellins (GAs), cytokinins (CKs) and abscisic acid (ABA). Carbohydrates are the most readily available form of energy and carbon skeletons also for plant hormone metabolism and therefore a hypothesis was proposed that higher assimilate production may result in increase in phytohormone concentrations, which consequently enhance plant growth. In *Arabidopsis thaliana* L., the concentrations of carbohydrates and plant hormones (IAA, gibberellic acid GA₃, zeatin riboside, dihydrozeatin riboside and isopentenyladenosine) increased under elevated CO₂ and consequently growth rate of treated plants was enhanced (Teng *et al.* 2006). Although in many ways responses of plants to elevated CO₂ may be species specific, some basic universal principles of response may exist. It seems, that higher accumulation of assimilates and at least increased auxin levels may be an example of such a common principle, because similar impact of elevated CO₂ on growth and auxin levels as in *Arabidopsis* were shown also in pine species. CO₂ enrichment resulted in an increase of axial shoot growth and needle length in Chinese pine (*Pinus tabulaeformis*) and simultaneously significantly higher content of IAA in needles was detected (Li *et al.* 2007). Auxin plays a critical role in regulating many plant responses, including cell elongation, cell division and differentiation. Thus, increased levels of auxin (induced by higher availability of assimilates) may enhance plant growth and development by stimulating cell division and cell elongation. It is also known that plant hormones, auxins and cytokinins, are associated with progression through the G1–S and the G2–M control points within the cell cycle. Moreover, cytokinin has been shown to induce expression of Cyclin D (CycD3) in cultured cells and intact *A. thaliana* plants, functioning as a major target in the cytokinin control of cell division (Riou-Khamlichi *et al.* 1999).

3.3.2 Impact on stomatal conductance and density

Increasing concentration of CO₂ in the atmosphere sharpens concentration gradient of CO₂ between the outside environment and leaf intercellular spaces what facilitates the diffusion of CO₂ into the leaf. Plants usually respond with decreased stomatal conductance to increased carbon dioxide concentration within intercellular



spaces. A synthesis analysis of 13 long-term studies with woody forest species confirmed reduction of stomatal conductance (g_s) under elevated CO_2 with several differences in stomatal response between functional groups of tree species: coniferous trees reduced g_s less than broadleaved species (Medlyn *et al.* 2001).

Contrary to quite a consistent reducing effect of elevated CO_2 on stomatal conductance, considerable variation appears in results regarding responses in stomatal density ranging from most frequently shown decrease (Lin *et al.* 2001; Teng *et al.* 2006) through no change (Luomala *et al.* 2005), to increase in density (Ferris and Taylor 1994). Regarding a mode of needle development and subsequent stomatal patterning, stomatal frequency (number of stomata per millimetre of needle length) appears to be a suitable parameter for evaluating conifer responsiveness to changes in atmospheric CO_2 concentration. Despite controversial results in changes of stomatal frequency in response to $[CO_2]$ in several controlled experiments, Kouwenberg *et al.* (2003) proved the capability of selected conifers *Tsuga heterophylla* ((Raf.) Sarg.), *Picea glauca* ((Moench) Voss), *P. mariana* (Mill.), and *Larix laricina* (K. Koch) to adjust their stomatal frequency to changes in atmospheric $[CO_2]$ in long-time scale. The study was based on comparison of recently collected samples with herbarium sheets of needles and revealed that all explored species responded to a $[CO_2]$ rise of 80 ppm over the last century with a significant reduction in stomatal frequency (Kouwenberg *et al.* 2003). However, one must take into account that plant response to single-step atmospheric CO_2 enrichment may not be comparable to response to long-term incremental increases of 1 to 2 ppm CO_2 per growing season. However, stomatal density or stomatal index may be used reliably as indicators of paleoatmospheric CO_2 concentration (Royer, 2001). Conifer needles have high preservation capacity, moreover, conifers are dominant species in temperate and boreal forest ecosystems, thus fossil needles may bring great potential for paleo-atmospheric CO_2 concentration reconstructions. Considering the role of stomata in various physiological processes, such as transpiration or water use efficiency, the parallel changes in the stomatal density and stomatal conductance may have important impacts on water balance on the whole plant, canopy or even ecosystem levels (Hetherington and Woodward 2003). However, some authors propose that reduction in stomatal opening alone rather than stomatal density is the main factor improving leaf water use efficiency (Tricker *et al.* 2005).

Reduction of stomatal conductance as well as stomatal density and transpiration rate was described also in a model plant *A. thaliana* (Teng *et al.* 2006) and a gene, *HIC* (for high carbon dioxide), which is involved in the signal transduction pathway responsible for controlling stomatal numbers at elevated CO_2 was identified (Gray *et al.* 2000). It is proposed that CO_2 concentration or its changes are detected by mature leaves, a



systemic signal is generated there and transported to developing leaves, which are optimized for performance under the prevailing environmental conditions (Coupe *et al.* 2006). Based on gene expression patterns and transcriptomics analysis, authors suggested that both sugars and hormones may play a part in systemic signaling to changes in stomatal development under particular atmospheric CO₂ concentration.

3.3.3 Impact on photosynthetic parameters, dark respiration and final leaf structure

Generally, ambient atmospheric carbon dioxide concentration (383 $\mu\text{mol mol}^{-1}$, 2007; <http://www.esrl.noaa.gov/gmd/ccgg/trends/>) is not saturating for photosynthesis of C3 plants. Stimulation effect of elevated [CO₂] on light-saturated net photosynthesis was observed for a wide range of plant species including conifers (rev. by Körner, 2006). Rubisco is one of the most abundant enzymes on the Earth and at the same time it is the first target of elevated [CO₂] effect in C3 plants. Increased carboxylation and simultaneously decreased competitive inhibition of oxygenase reaction of Rubisco, which reduces carbon losses due to photorespiration, results in increased net CO₂ assimilation rates (Von Caemmerer *et al.* 1981). Subsequent increase in carbohydrate production, particularly sucrose, other non-structural carbohydrates and starch usually occur (DeLucia *et al.* 1985).

However, after initial stimulation, an acclimation depression of photosynthesis may occur under prolonged exposure to elevated [CO₂] as reported for many forest trees including Scots pine (Luomala *et al.* 2003) or Norway spruce (Špunda *et al.* 2005). Photosynthetic adjustments are often accompanied with changes at biochemical level such as decrease of Rubisco amount or activity (Sage, 1994; Eguchi *et al.* 2008; Zhang *et al.* 2008a), dilution and redistribution of nitrogen (Eguchi *et al.* 2008), changes in light harvesting complexes or increased levels of soluble carbohydrates, which have been shown to down-regulate photosynthesis gene transcription (Smith and Stitt 2007). Furthermore, alterations at the whole plant sink-source relationships may occur (rev. by Urban, 2003). The extent of down-regulation response to elevated [CO₂] may be modulated by additional factors, such as leaf position within the canopy or leaf age (Maier *et al.* 2008) in evergreen species. Stronger photosynthetic down-regulation was shown in sun needles of Loblolly pine (*Pinus taeda* L.) (Crous *et al.* 2008) and Norway spruce (Špunda *et al.* 2005). In spite of photosynthetic down-regulation, we recorded the positive effect of elevated CO₂ concentration on net CO₂ assimilation rates for Norway spruce even after 8 years of CO₂ enrichment (Lhotáková *et al.*, submitted, Paper III.). The similar phenomenon was confirmed also in the long-term FACE studies with Loblolly pine (Maier *et al.* 2008; Crous *et al.* 2008). However, observed long-term increases in net



photosynthesis are usually lower than the short-term responses and could slightly vary with leaf position within the crown and age (Crous and Ellsworth 2004).

Primary assimilates such as trioses or hexoses produced through photosynthesis serve as precursors for synthesis of structural compounds of plant body, therefore it is likely that leaf structure would be altered under elevated $[CO_2]$. Moreover, saccharides do not play only the role of building stones and source of energy for plant growth. They serve also as important signaling molecules, which are supposed to be involved in regulatory pathways of genes encoding enzymes of photosynthesis, sugar and starch metabolism (Smith *et al.* 2007).

Many studies evaluating the impact of elevated $[CO_2]$ on leaf structure on wide spectrum of plant species have been conducted (rev. by Pritchard *et al.* 1999). The results varied depending on plant species, age of plants, length of elevated $[CO_2]$ treatment, and other treatment conditions such as nutrients availability. Impact of elevated $[CO_2]$ on rate of cell division and expansion during leaf development has been already discussed; in this section the alteration of the final leaf structure is stressed out. At the subcellular level, changes in chloroplast structure, number or size appear to be obvious under elevated CO_2 , as chloroplasts are organelles, where photosynthetic reactions take place. Increased volume of chloroplasts on the cross section seem to be typical ultrastructural trait as reported for Longleaf pine (*Pinus palustris* Mill.) (Pritchard *et al.* 1997), aspen (*Populus tremuloides* Michx.) or birch (*Betula pendula* Roth) (Pritchard *et al.* 1997; Oksanen *et al.* 2005). In several cases, increased volume of chloroplasts resulted from accumulation of larger starch grains (Oksanen *et al.* 2001; Oksanen *et al.* 2005; Teng *et al.* 2006), which may result in distortion of thylakoid membranes and change of chloroplast shape to be more rounded (Kutík *et al.* 1995; Teng *et al.* 2006). Chloroplast distortion due to massive starch grain accumulation was suggested as a cause of photosynthetic acclimation (Pritchard *et al.* 1997; Teng *et al.* 2006). Increase in volume ratio of stromal to granal thylakoids were detected in the following tree species: *Acer rubrum* L., *Cercis canadensis* L., *Piper auritum* Kunth, and *Liquidambar styraciflua* L. (Griffin *et al.* 2001) as well as in herbaceous *A. thaliana* (Griffin *et al.* 2001; Teng *et al.* 2006). As generally accepted, photosystems I and II have preferentially different spatial location: PSI, where NADPH is produced, is more abundant at stromal thylakoids; whereas PSII containing an oxygen-evolving complex dominates at granal thylakoids. The shift in thylakoid arrangement in favour of stroma thylakoids could be explained by increasing demands for NADPH, which is needed for CO_2 reduction and its further incorporation into carbon intermediates of the Calvin cycle during enhanced photosynthesis under elevated $[CO_2]$. Decrease in volume proportion of granal thylakoids in sugar beet (*Beta vulgaris* L., cultivar Celt) chloroplasts was also described by Kutík *et al.* (1995) suggesting that elevated $[CO_2]$ could alter light



harvesting and energy transducing capacity of the leaf. Moreover, ultrastructural changes under CO₂ enrichment were influenced by nitrogen supply (Kutík et al. 1995).

Mitochondria are supposed to be after chloroplasts the most responsive organelles to changes in carbon dioxide concentration. The most frequently reported alteration under elevated [CO₂] appeared to be an increased number of mitochondria (Griffin *et al.* 2001; Oksanen *et al.* 2005), which was almost doubled in *B. pendula* leaves after three growing seasons under elevated [CO₂] (Oksanen *et al.* 2005). However, no change in size of the mitochondria was observed (Griffin *et al.* 2001). Enhanced photosynthesis and plant growth under elevated [CO₂] is supposed to result in higher respiration rates due to increased cellular energy demands. However, similarly like photosynthetic responses, short- and long-term responses of dark mitochondrial respiration to elevated [CO₂] may differ as shown for two species of pines (*Pinus koraiensis* Siebold & Zucc. and *Pinus sylvestrifomis*), when during the second growing season of elevated [CO₂] treatment needle dark respiration rate increased, however during the fourth growing season under treatment the dark respiration was inhibited (Zhou *et al.* 2007). Our observation on Norway spruce appears to be quite consistent with the latter study, as dark respiration in CO₂ enriched trees was suppressed in the 8th growing season under the treatment (Lhotáková et al. submitted, Paper III.) The direction and magnitude of changes in dark respiration under elevated [CO₂] largely depends on the hierarchical level (from enzymes to ecosystems) at which respiration is measured (rev. by Gonzalez-Meller *et al.* 2004).

At the level of leaf tissues, primarily structural changes in mesophyll, the photosynthetic tissue, caused by elevated [CO₂] will be now discussed. A tight connection between the leaf structure and photosynthetic performance is obvious: A positive correlation between the surface area of mesophyll cells exposed to intercellular air spaces and mesophyll CO₂ conductance (g_m) (Kogami *et al.* 2001) was reported; similarly the mesophyll surface area exposed to intercellular space per unit leaf area (A_{mes}/A) is considered to be an important anatomical parameter that is correlated with the photosynthetic rate (Slaton and Smith 2002). However, the character of changes in A_{mes}/A in Japanese larch (*Larix kaempferi* Carr.) needles under the impact of elevated [CO₂] have been observed to be strikingly different (opposite) depending on nitrogen supply (Eguchi *et al.* 2004). The authors attributed changes in A_{mes}/A to changes in mesophyll cell size and mesophyll cell number: The cell size increased but mesophyll cell number decreased in high nitrogen supply, while under low nitrogen supply a higher number of smaller cells was observed. These findings suggest that nitrogen supply modulates mesophyll cell response to elevated [CO₂] in the sense of different impact on cell division and cell expansion. In our study on Norway spruce, the trees were not suffering from nitrogen or any nutrient shortage; therefore we rather expected internal needle surface to increase.



Nevertheless, we found all selected structural parameters (needle volume, internal needle surface, internal needle surface density and volume ratios of individual tissues) unchanged under elevated CO₂ (Lhotáková *et al.* submitted, Paper III.).

A detailed study on conifer needle development under elevated [CO₂] similar to those conducted on poplar leaves (Ferris *et al.* 2001; Tricker *et al.* 2004) has not been performed yet. Proportion of intercellular air spaces in mesophyll, i.e. mesophyll porosity, has been also considered as an important anatomical parameter of leaf, which affects photosynthetic performance via influence on mesophyll conductance to CO₂ (Flexas *et al.* 2008). However, effects of elevated [CO₂] on mesophyll are not uniform, for example, while decrease of mesophyll porosity in birch leaves was reported (Oksanen *et al.* 2005), no effect on Scots pine (*Pinus sylvestris* L.) needles (Luomala *et al.* 2005) and increase in aspen leaves (Oksanen *et al.* 2001) was recorded.

The mesophyll thickness or generally leaf thickness is an anatomical parameter, which is much more easy to measure, and it was found to be positively correlated with the A_{mes}/A , and thus with photosynthetic rate (Hanba *et al.* 1999). Therefore, leaf thickness, mesophyll thickness or area of mesophyll on the cross section are often evaluated under the impact of elevated [CO₂]. An increased mesophyll thickness was shown in the leaves of aspen (*Populus tremuloides* Michx.) (Oksanen *et al.* 2001). Similarly, the increase in needle thickness due to a large increase in mesophyll thickness was reported for Scots pine seedlings after 4 years of exposure to elevated [CO₂] (Lin *et al.* 2001). However, even opposite or no effects of elevated [CO₂] were observed: decreased mesophyll thickness in Scots pine needles (Luomala *et al.* 2005) or European silver birch (*Betula pendula* Roth) (Oksanen *et al.* 2005). No effect of CO₂ enrichment on proportion of mesophyll per the needle cross-sectional area and the needle cross-section area alone was reported in Douglas fir needles (Olszyk *et al.* 2005).

Primary assimilates serve as precursors for synthesis of structural compounds of plant body such as cellulose and also secondary metabolites of structural character, such as lignin or other non-structural secondary metabolites, e.g. soluble phenolic compounds. Therefore, it is likely that not only leaf structure would be altered under elevated [CO₂] but also amount and/or composition of phenolic compounds and other secondary metabolites. In some cases, the impact of elevated CO₂ on mesophyll cell wall thickness was shown, as in birch the mesophyll cell wall thickened together with reduction of intercellular spaces volume, which led to greater limitations of CO₂ diffusion within the leaf (Oksanen 2005). For birch changes in cell wall composition were reported also, when concentration of hemicellulose increased, while the opposite was found for α -cellulose and acid-soluble lignin (Oksanen 2005). In our study on Norway spruce under the impact of elevated [CO₂], we did not find any changes in lignin and soluble phenolic compounds (Lhotáková *et al.*



submitted, Paper III.). We also investigated how the elevated [CO₂] influenced differentiation of sun and shade needle ecotypes regarding the mesophyll structure and accumulation of phenolic compounds. Since sun and shade needles exhibited the same pattern of differences in needle anatomy and content of phenolic compounds irrespective of the CO₂ enrichment, it appears that irradiance might be a higher priority environmental factor for leaf structure differentiation, morphogenesis and secondary metabolite synthesis and accumulation than enriched CO₂ concentration in the atmosphere.

The increase of specific leaf weight (leaf mass per area, LMA) as a result of stimulated biomass production may be supposed, however only slight effect was detected for Douglas fir needles (Apple *et al.* 2000; Olszyk *et al.* 2005) and in other cases elevated CO₂ had no effect on LMA of poplar leaves (Ferris *et al.* 2001) or Japanese larch (Eguchi *et al.* 2004) and Loblolly pine needles (Maier *et al.* 2008). Nevertheless, in the longest running forest free-air CO₂ enrichment (FACE) experiment to date (the Duke Forest FACE facility, <http://face.env.duke.edu/main.cfm>, in operation since June, 1994) conducted on coniferous species (loblolly pine – *Pinus taeda*), only small effects on needle morphology (needle length, needle diameter, LMA) were reported (Maier *et al.* 2008), although it has to be mentioned that a detailed survey of needle anatomical characteristics has not been performed yet.

3.4 Air pollution

During the second half of the 20th century, large-scale forest decline occurred in Europe and North America, which could not be attributed to a single factor, because of broad spectrum of affected forest tree species and enormous spatial extent. As defined by Innes (1987), individual stress factors, such as air pollution, drought periods, frost, poor soil conditions, root pathogens and pest insects act as a complex termed 'multiple impacts of stress factors'. However, air pollution including SO₂, being part of acid rain, and ozone, were considered to be the most important anthropogenic factors damaging European forests.

Coniferous tree species with long-lasting foliage are supposed to be more endangered by air pollution than deciduous ones. Norway spruce (*P. abies*) is one of the most common and economically most important tree species in Northern and Central Europe, very sensitive to acid pollution. Thus, mainly effects of acid rain or air pollution on structure of coniferous needles are discussed in this section. At the level of leaf structure, anatomical alterations caused by impact of acid rain were quite well described (Masuch *et al.* 1992; Moss *et al.* 1998; Viskari *et al.* 2000) and used in many studies as „microscopical markers of forest damage“, as was in studies of of our team (Albrechtová *et al.* 2001; Moss *et al.* 1998; Soukupová *et al.* 2001). In our study Albrechtová *et al.* (2007, Paper I.)



we evaluated anatomical changes in Norway spruce needles caused by simulated acid rain quantitatively, using stereological methods. Our results were in accordance with those observed in several field studies, such as increased volume of intercellular spaces in our simulation experiment and large cavities in red spruce mesophyll caused by the acid mist event in the field (Moss *et al.* 1998). Other observed change was a significant decrease of needle volume caused by a reduced area of the needle cross-sections, while needle length remained unchanged, similarly like observed by Masuch *et al.* (1992).

Loads of acidic air pollution, particularly of SO₂, decreased significantly during the 1990's and recently it is ozone, which appears to be the main pollution agents. Consequently, interactions of elevated [CO₂] and ozone on leaf structure, photosynthesis and respiration, which may have a significant influence on the leaf, tree, and ecosystem carbon balance under changing atmospheric conditions, were reported (Oksanen *et al.* 2005).

4 Quantitative Approaches in Plant Anatomy

The following approaches could be applied in plant anatomy analysis: descriptive, semi-quantitative and quantitative ones. The first two approaches, descriptive and semi-quantitative, are discussed briefly. The quantitative approach has been used in four of five papers included in the present thesis; therefore a special attention is devoted to application of quantitative methods in studies of leaf anatomy.

A descriptive approach to analysis of plant morphology has been used since the early history of plant anatomy. Descriptive analysis is still a very useful tool included in recent molecular biological or developmental studies dealing with mutants, when altered internal plant structure and tissue development may indicate particular gene expression or developmental program (DiLaurenzio *et al.* 1996). The descriptive approach also serves as a suitable tool in detection of localization of specific compounds in plant tissues (Soukupová *et al.* 2001; Soukupová and Albrechtová 2003) or at the cellular or subcellular level. In the semi-quantitative approach, the studied phenomenon, e.g. intensity of histochemical staining (Kutík and Beneš 1981) is classified into several discrete categories. A number of categories usually should not exceed four to enable unambiguous classification and avoid bias due to observer's subjectivity.

In quantitative approach characteristics of plant organs, tissues and cells are expressed numerically what offers great advantage when searching for empirical relations between structural and physiological or biochemical parameters of plants e.g. mesophyll geometrical parameters and mesophyll conductance to CO₂ (Miyazawa and Terashima 2001; Hanba *et al.* 2002).



The Department of Plant Physiology at the Charles University in Prague has a long tradition in plant anatomy research, which in its beginnings includes Julius von Sachs (1834-1897), who worked in Prague as Associated Professor with Jan Evangelista Purkyně. In the first half of the 20th century, Professor Bohumil Němec contributed to plant anatomy development remarkably and his most famous discovery is the function of statolith starch grains in the plant root gravity perception (Němec 1901). Further, the work of Professor Jaroslav Pazourek (1925-1999) a Němec's follower, should be mentioned. His credit lays in introduction of quantitative approach to plant anatomy in the Czech Republic (Pazourek, 1977). He defined quantitative plant anatomy as follows: „It is a field of science focusing on study of quantitative relationships of specific structures, tissues and tissue complexes in plant organs”. Professor Lubomír Nátr used for the first time computer-aided image analysis for measurements of the leaf area (Nátr 1968). The tradition of quantitative plant anatomy has continuation in the Department till nowadays as can be documented also by the present thesis.

4.1 Development of quantitative plant anatomy

Only selected milestones of the evolution of plant quantitative anatomy are discussed in this thesis, further details covering beginnings of the field could be found in a comprehensive review by Pazourek (1988), further field developments are discussed in Kubínová (1987, 1990) and Kubínová et al. (2005). The first quantitative studies on plants are known since the 18th century, when Humboldt (1786) published results of stomatal density on leaf of *Hyacinthus*. The work of Salisbury (1927) brought a breakthrough in quantitative plant anatomy in the sense of introduction of statistical evaluation of quantitative data. The majority of the early plant quantitative studies were dealing with leaf, particularly stomatal density, number or size and included planar measurements. Much less attention was devoted to mesophyll or internal structure in general, because of laboriousness of the sample preparation and the quantitative evaluation itself.

Turrell (1933) introduced measurements of internal mesophyll surface area, which corresponds to the area of mesophyll cell surface exposed to intercellular spaces, where the gas exchange takes place. This anatomical parameter became quite respected and have been included in several recent anatomical studies (Slaton and Smith 2002; Eguchi *et al.* 2004) or photosynthetic modeling (Nobel 1975). Until the end of the 20th century, the majority of quantitative anatomical parameters were achieved by model-based approach (4.2.1.), however in the 1980's, design-based methods such as stereology were introduced in plant anatomy by Parkhurst (1982), who emphasized an issue of cell shape irregularity and a need of methods independent on the shape of measured object. In fact, stereological unbiased methods had been used earlier, but rather occasionally (Pazourek



1988). Later on, a number of applications of design-based stereological methods was described for measurements of leaf anatomical characteristics by Kubínová (1991; 1993; 1994).

During the the 20th century, several revolutionary microscopic techniques and technologies have been developed such as electron, fluorescence or confocal microscopies, digital image acquisition, processing and analysis. The progress in computer technologies resulted in advancement in quantitative approaches, not only in plant anatomy, as the personal computers are today a common equipment of every researcher and a wide range of softwares suitable for image processing, image analysis either commercialized (e.g. Lucia G, LIM CZ, ACC SoFo Brno, CZ) or free-ware (e.g. Image J) up to digital applications of stereological methods (e.g. Ellipse, ViDiTo, SK) are available.

4.2 Two basic approaches in quantitative anatomy

This thesis focuses mainly on conifer needle anatomy evaluated by quantitative approaches and application of the quantitative data in ecophysiological research. Therefore, the two basic approaches in quantitative plant anatomy, i.e. model-based and design-based approaches, are discussed in the following section on the development of quantitative methods used for leaf studies, particularly for measurement of geometrical characteristics of mesophyll.

4.2.1 Model-based approach

In early studies of quantitative parameters of the leaf conducted by Turrell (1933, 1936), a model based-approach was used. The principle of model-based approach consists in a presumption that measured objects can be substituted by simple geometrical bodies, in case of mesophyll cells by spheres or cylinders. When measuring geometrical parameters of leaves with differentiated pallisade and spongy parenchyma, different correction factors and computing methods need to be used for different tissue types and as well for different leaf functional types (mesomorphic, succulet or xeromorphic leaf) (Turrell 1936). Even in quite recent studies mesophyll cells were classified into several shape types and approximated to the cylinders with hemispherical ends or spheres together with developing specific curvature correction factors for individual cell types (Barbour and Farquhar 2003; Burundukova *et al.* 2003; Khramtsova *et al.* 2003). Constraints of model-based approach to acquisition of quantitative parameters of mesophyll were pointed out by many authors (Parkhurst, 1982; Kubínová, 1993; 1994; Slaton and Smith 2002) stressing inaccuracy and bias, especially in surface area measurements. One of the most important pitfalls consists in the fact that the methods are dependent on cell shape or tissue architecture and thus not universal. Parkhurst (1982)



made a step forward to design-based approach, when he applied stereological method (counting intersections of random line testing system with cell surface on the sections with fixed orientation) on measurement of internal leaf surface area. However, he still used several model-based presumptions and shape factors for computing surface area of palisade and spongy parenchyma. For detail insight into progress from model-based to design-based approach to measurement of geometrical parameters of the leaf see Kubínová (1990).

4.2.2 Design-based approach

The main advantage of design-based methods for estimating geometrical characteristics of three-dimensional particles lays on their independence from measured object shape. Mesophyll cells are hardly ever of regular shape as discussed in (Albrechtová *et al.* 2007; Lhotáková *et al.* 2008, Papers I. and II.). Particularly Norway spruce mesophyll cells have an irregular lobed shape (Figure 2), and thus modelling them with cylinders or other geometrical bodies is not appropriate, and would yield biased results if possible at all. Therefore, only design-based methods appear to be suitable for unbiased estimation of mesophyll geometrical parameters of spruce needles. When discussing design-based approach, the main attention is going to be devoted to stereology, the principles of which were used for quantitative evaluation of mesophyll geometrical parameters in (Albrechtová *et al.* 2007, Lhotáková *et al.* 2008, Lhotáková *et al.* submitted, Papers I., II., and III.). Stereology as a scientific approach was established during the 1960's and was defined as methodology enabling three-dimensional (3D) characterization of structures on the base of their observation in two-dimensional (2D) sections of the structures. Today stereology gives description of structures on the base of stochastic geometry using unbiased estimators (for review see Weibel 1979, Howard and Reed 1998, Kubínová *et al.* 2005). Since the 1990's, stereological methods have been widely used for estimation of volume or volume density of subcellular structures such as starch grains (Redway, 1991) or organelles (Winter *et al.* 1993) and volume density of individual tissues in a leaf (Lukovic, 2006).

Recent development of non-invasive methods for image acquisition of serial sections of a single object, such as confocal microscopy, magnetic resonance imaging, computed tomography, etc. makes it possible to estimate geometric characteristics from perfectly registered serial sections. The idea of estimating stereological parameters from optical sections within a thick slice was first used for counting particles with the optical disector principle (Gundersen, 1986; Sterio, 1984) enabling to determine a number and volume of cells composing a tissue.

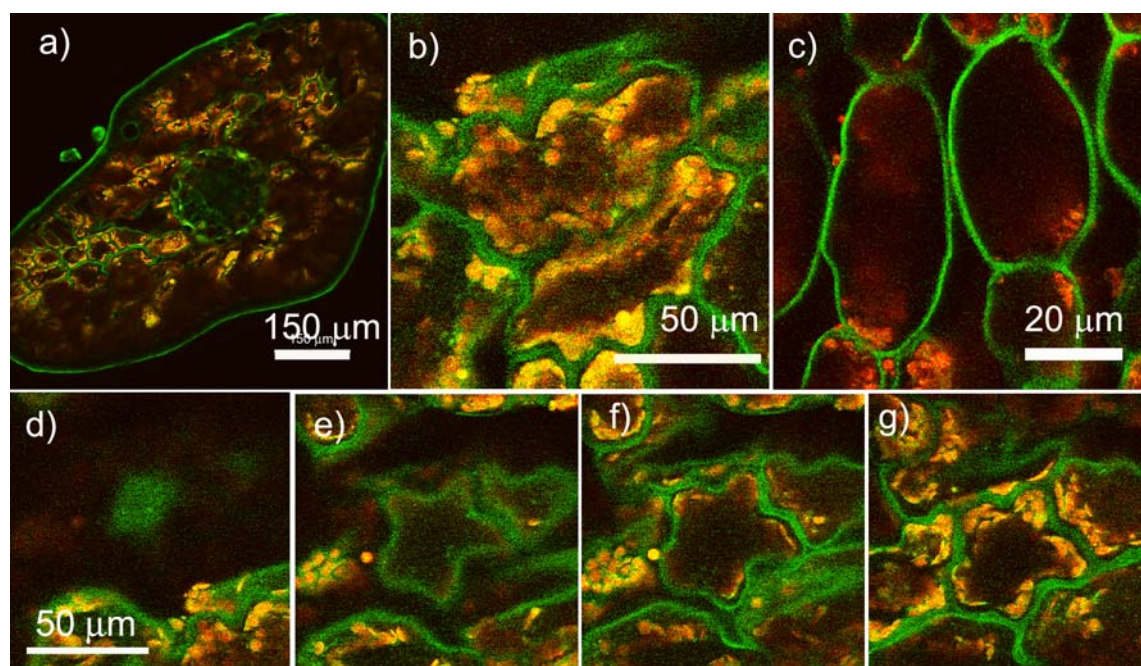
**Figure 2:**

Figure 2: Optical sections of Norway spruce needle acquired by confocal microscopy. Autofluorescence of phenolic acids bond in the cell walls in green, autofluorescence of chlorophyll in red. a) Transverse section of Norway spruce needle. b) Detail of mesophyll cells on the transverse needle section. c) Detail of mesophyll cells on the longitudinal needle section. d-g) Series of 4 optical sections of a mesophyll cell, note the irregularity of the cell shape. Bars correspond to 150 μm (a), 50 μm (b,d-g) and 20 μm (c).

Efficient design-based methods for surface area estimation based on evaluation of stacks of serial sections have emerged - spatial grid method (Sandau, 1987) or vertical spatial grid (Cruz-Orive and Howard 1995). For measuring surface area of barley mesophyll (Kubínová, 1991), a method of vertical sections developed by (Baddeley *et al.* 1986) was used and its modification – a method of local vertical windows proved to be suitable for estimation of thylakoid surface area estimation in maize leaves (Kubínová *et al.* 2007). All of these new methods together with previous methods listed by Parkhurst (1982) require certain randomization of the section direction what is not easily applicable in all cases like for elongated objects such as needles or grass leaves. In such cases, cutting sections in a specific fixed direction is desirable. The fakir method developed by (Kubínová and Janáček 1998) does not require randomization of sectioning, because the method is based on application of digitally designed virtual 3D probes with random orientation (Figure 3), which enables to measure surface area without bias from sections having arbitrary fixed orientations.



Figure 3:

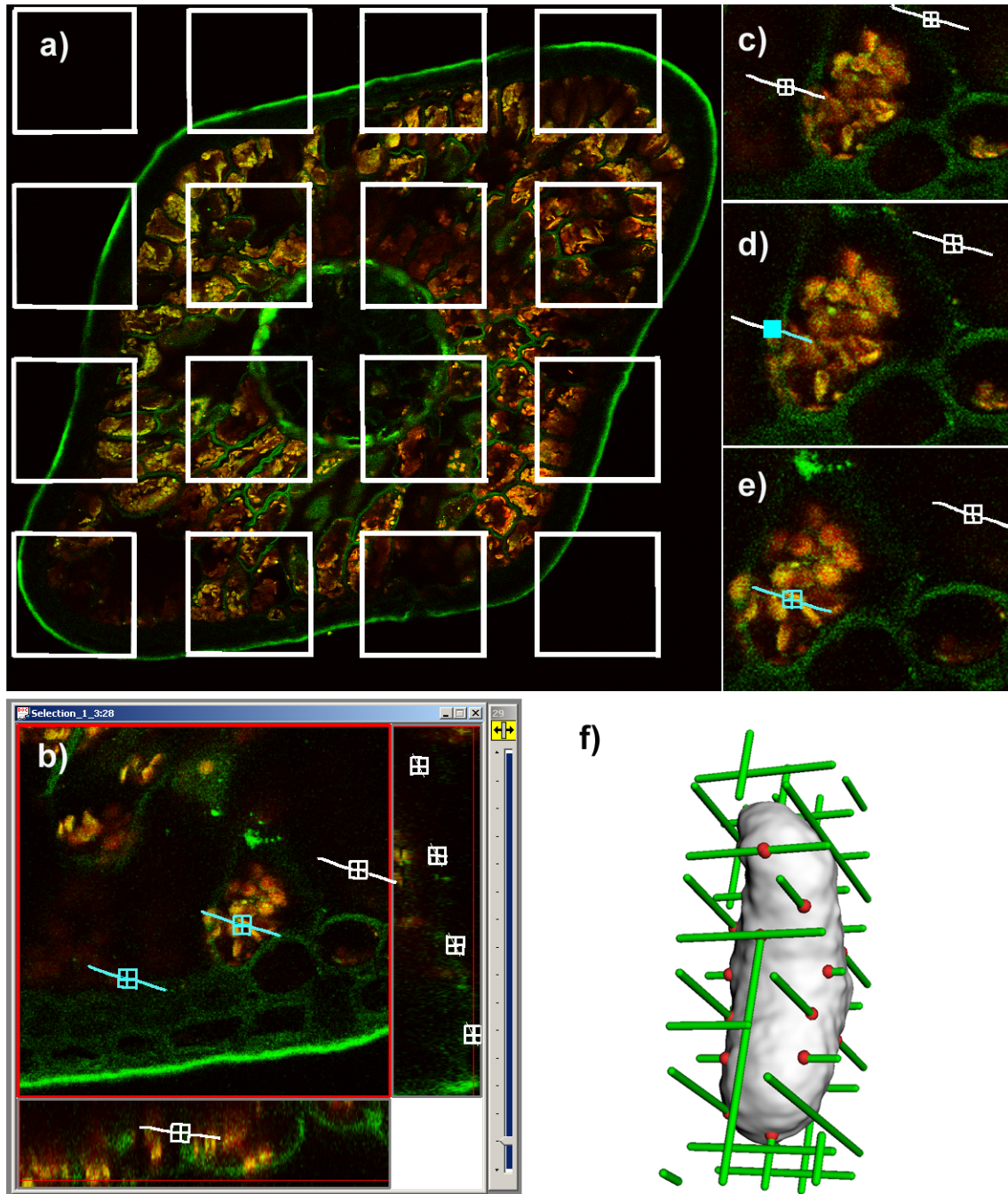


Figure 3: Measurement of the internal surface area of Norway spruce needle mesophyll.

a) Systematic uniform random sampling of unbiased sampling frames, generated in the Ellipse software. **b)** Individual sampling window with fakir probes generated in random orientation. The procedure is shown in three subsequent optical sections. The first optical section is shown in **c)**, the second one in **d)** and the third one in **e)**. The test lines of the fakir probe piercing the mesophyll cells are white at the beginning of the measurement **c)**. The centres of white squares denote the intersection points between the test lines and the current section. As soon as such a point comes into contact with the cell wall during focusing through **(d)**, it is marked by a mouse click and the square is filled with cyan **(d)**; the part of the test line above this point also becomes cyan **(d,e)**. One intersection point is marked here. **f)** Fakir probe intersecting an isolated mesophyll cell. The red balls denote the intersection points between the test lines and the cell surface.



4.2.2.1 Systematic uniform random sampling in needle anatomy

The principle of histo-physiological gradients mentioned already in work of (Prat 1948) emphasizes importance of heterogeneity of anatomical parameters within plant or even individual organs, which is very important for sampling designs of anatomical experiments. Therefore, we selected transverse sections along the needle axis according to the systematic uniform random (SUR) sampling (Kubínová, 1991) in all our anatomical studies (Albrechtová *et al.* 2007; Lhotáková *et al.* 2007; Lhotáková *et al.* 2008; Lhotáková *et al.* submitted, Papers I. – IV., Figure 4).

A common practice of evaluating structural characteristics of needles in plant studies usually uses only sections taken from the middle part of needles (Soukupová *et al.* 2000; Apple *et al.* 2002; Leal and Thomas 2003), which can be often sufficient if we are not interested in average structural parameters of the whole needle. However, a pilot study should be performed to confirm assumption about homogeneity of the middle part of needles. In case of a study by Zwieniecki *et al.* (2006), at least 5 sections along the main needle axis were selected. However, the principle of SUR was not applied in this study and therefore the estimation might have been biased. A gradient in the cross-sectional area along the Norway spruce needle axis was shown in our study (Lhotáková *et al.* 2007, Paper IV.), when the parameters of cross-sections (area of whole section, area of mesophyll on the cross section) sampled in the middle part of the needle differed from those sampled at the tip and base. However, area proportions of individual needle tissues remained constant. Similarly, neither the proportion of intercellular spaces in mesophyll, nor the internal surface density differed along the needle axis (Lhotáková *et al.* 2008, Paper II.). These findings are in agreement with measurement of Zwieniecki *et al.* (2006), who observed other needle parameters (needle circumference, stomatal density, ratio of xylem area to tracheid dimension) to be constant along 80 % of the needle length when studying three pine species. This implies an important conclusion about possible representativeness of a middle part for the whole needle and that justifies usual design of ecological studies on conifer needles when only middle part of a needle is studied. This approach is acceptable if we do not derive mean parameters for the entire needle based on observations at the middle needle segment only. The principle of systematic uniform random sampling of sections along the needle axis allows the inclusion of a potential gradient in anatomical characteristics within the needle. Additionally, in combination with the Cavalieri method, it allows an unbiased volume estimation of needle structural components (Kubínová, 1993), which cannot be derived from sections taken from the middle part of the needle only.





Figure 4: Sampling design of needle specimen preparation.

a, b) Systematic uniform random sampling of transverse free-hand sections: z = random position of the first section within $(0; T]$, where $T = 2$ mm. Positions of transverse sections along the needle longitudinal axis are denoted by a, b, c, d, e, f .

c) 2-mm-thick needle segment used for sectioning. Thickness was measured by millimetre scale.

d) 80- μm -thick free-hand section, from which the 40 μm thick stack of optical sections was acquired.

e) Stack of 40 optical sections 1 μm apart, lines refer to optical sections 5 μm apart, where proportion of intercellular spaces in mesophyll was measured.

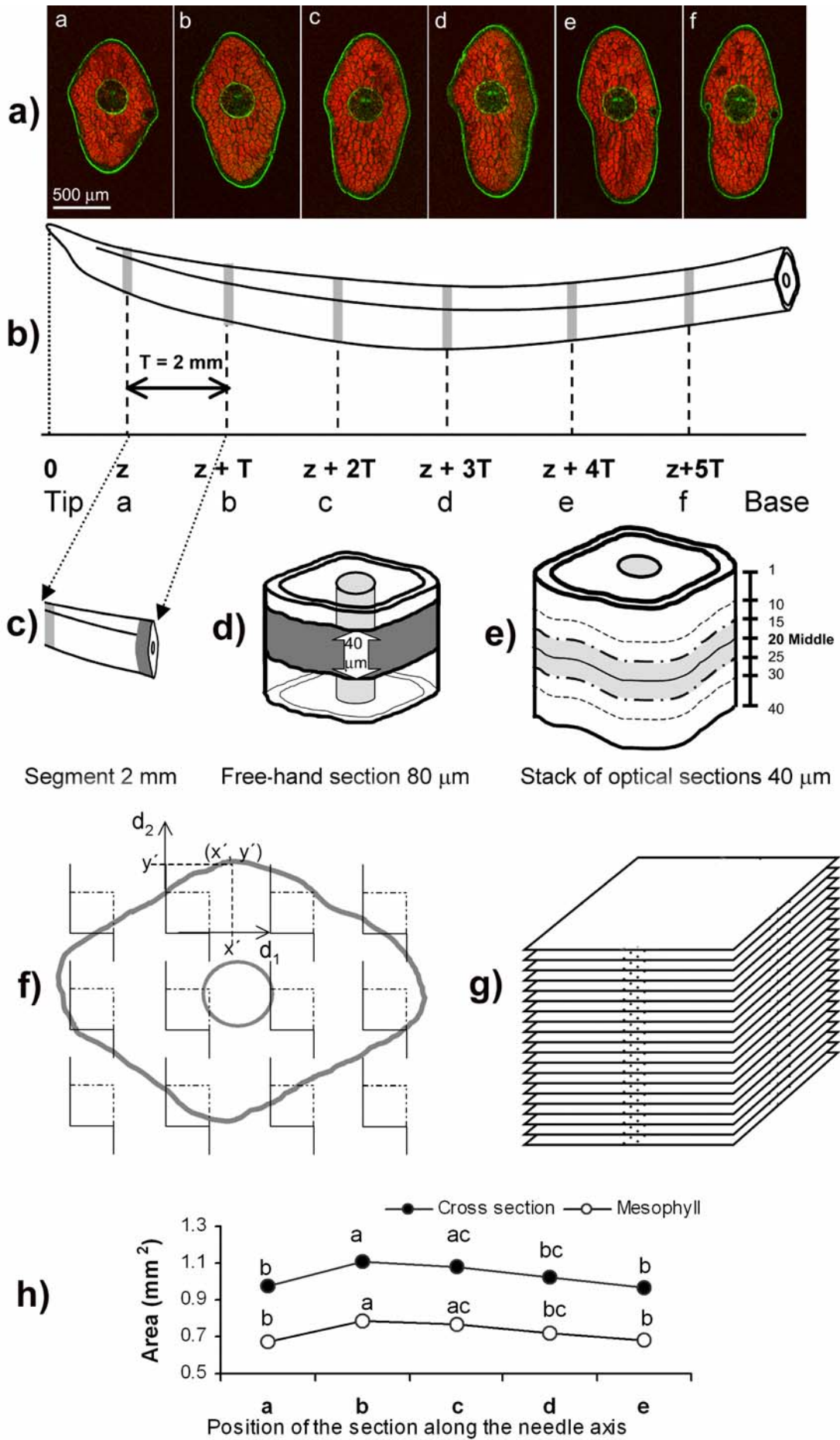
f) Systematic sampling of unbiased sampling frames, applied to confocal series used in disector and fakir methods. The system of frames was placed on the needle section so that the uppermost point of the section lay in the position (x', y') , where x', y' were uniform random points from $(0; d_1)$ or $(0; d_2)$, respectively, d_1 and d_2 being the distances between two neighbouring frames.

g) Series of optical sections acquired from the sampling frame, where stereological measurements were performed.

h) Gradient of selected structural parameters along the longitudinal needle axis. Closed circles refer to cross-section area, open circles to mesophyll area on the cross-section. Position of the cross-section: (a) tip and (e) base of a needle; Different letters above the columns show significant difference at $\alpha = 0.05$, based on one-way ANOVA. For multiple comparisons Tukey–Kramer test was used. For further detail see Lhotáková et al. 2007, Paper IV.



Figure 4.





5 Quantitative anatomy as a tool in field research in ecophysiology

As discussed in previous chapters, methods for estimation of internal leaf surface area exist for at least seven decades and their adjustment and improvement is still in progress. However, in all studies, which have dealt with internal leaf surface area, measurements have been performed on permanent leaf specimens using fixed leaf segments embedded to paraffin (Mantovani and Vieira 2000; Slaton *et al.* 2001; Turrell 1936) or resin (Miyazawa and Terashima 2001; Oguchi *et al.* 2003; Oguchi *et al.* 2008). The procedure of preparation of paraffin anatomical sections is time consuming and laborious and may bring deformations of internal structure, especially tissue shrinkage (Uwins *et al.* 1993; Dorph-Petersen *et al.* 2001). Therefore we proposed that using free-hand sections (i.e. acquired by a hand microtome) of fresh needles could eliminate such deformation problems (Albrechtová *et al.* 2007, Paper II.). Fresh sections of Norway spruce needles appeared to be very suitable for confocal laser scanning microscopy even without any special staining - due to the autofluorescence of chloroplasts containing photosynthetic pigments and also phenolic compounds localized either in cell walls or in vacuoles. Moreover, the above mentioned fakir method (Kubínová and Janáček 1998) allows to use transverse sections taken in an arbitrary direction, which is advantageous while working with thin elongated leaves as grass leaves or needles.

One of the long-term goals of Dr. Albrechtová's team was the establishment of quantitative plant anatomy involving unbiased stereological methods as powerful approach in plant ecophysiological field studies, therefore after we confirmed the technical feasibility of using fresh needles for confocal microscopy and stereological measurements (Albrechtová *et al.* 2007, Paper I.), we also needed to test sensitivity of selected geometrical parameters of mesophyll to the impact of environmental factors. During the second half of the 20th century, studying impact of acid rain and air pollution on conifer needle anatomy was an integral part of research focused on forest decline (Masuch *et al.* 1992; Moss *et al.* 1998; Viskari *et al.* 2000) and anatomical alterations in needle structure caused by those environmental factors were quite well qualitatively described. Moreover, the team of Dr. Albrechtová had already valuable experience with evaluating anatomical changes of Norway spruce needles under impact of acid rain, because of long-term monitoring of forest health in the Krušné hory (PhD. Thesis of Albrechtová 1997, Soukupová 2001; master theses of Šolcová 1999, Stejskalová 2003, Lhotáková 2004). Therefore we used simulated acid rain to induce the subtle changes in mesophyll structure. We obtained quite consistent results, e.g. increase in the volume of intercellular spaces per mesophyll cell volume (Albrechtová *et al.* 2007, Paper I.), which was previously reported by Masuch *et al.* (1992).



However, using fresh material brings further constraints to application of proposed method in extensive ecophysiological field studies, because a large number of samples is usually analysed and only few fresh needles can be processed simultaneously in one day and the trees under study should not be located too far from the place of analysis. In the subsequent study (Lhotáková et al. 2008, Paper II.), we suggested solving this problem by freezing the needles and storing them for later analysis. The effect of freezing on selected geometrical parameters (volume of intercellular spaces in mesophyll and internal surface density) was negligible and therefore the possibility to use frozen-stored needle material proves to be very promising for further research. Further, we addressed another technical problem, resulting in biased measurements, caused by the distortions of cutting surfaces of transversely cut needle sections. The arrangement of mesophyll cells in the majority of conifer needles corresponds to transversally oriented layers of cells with interconnecting cells, making the whole tissue appear like an anastomosing system with prevalingly horizontal orientation of the air spaces (Esau 1965, Cross 1940). Such arrangement of mesophyll makes the avoidance of tearing marginal mesophyll regions during hand sectioning to be quite difficult if not impossible. We found that the proportion of intercellular spaces in mesophyll may be overestimated in the marginal optical sections due this effect. Mesophyll geometrical parameters thus should be measured only in the middle region of optical section series of frozen-stored Norway spruce needles. As the next step, adjusted fakir method was applied for measuring geometrical parameters of mesophyll under impact of elevated CO₂ concentration in long-term field experiment (Lhotáková et al., submitted, Paper III.).

6 Leaf optical properties as a tool in field research in ecophysiology

Biochemical composition of leaves, e.g. content of photosynthetic pigments (Tzvetkova and Hadjiivanova 2006), and leaf structure (Nakatani *et al.* 2004; Albrechtová *et al.* 2007) are often used as indicators of the actual plant physiological status. Therefore the plant optical properties, particularly reflectance, were recognized as suitable non-invasive technique for monitoring plant stress occurrence (Horler *et al.* 1980). Moreover, reflectance of vegetation can be acquired by remote sensing techniques using air- or space-born sensors over the large spatial scales providing information at canopy or ecosystem levels, which would be extremely time and labor consuming if the information would be achieved from ground field investigation.

6.1 Leaf optical properties - definitions

The leaf optical properties, defined as the reflectance, transmittance and absorbance of the photons by and/or within the leaf tissues, are determined by the leaf



biochemical composition and leaf structure (Gates *et al.* 1965). The interaction of solar radiation with plant leaf tissues is characterized by the following processes (Fukshansky, 1981, figure 5): i) specular reflection, ii) multiple scattering iii) refraction, iv) absorption, and v) fluorescence emissions. The quality of reflected radiation by the leaf depends on its origin: radiation reflected from the leaf cuticle on the leaf surface (specular reflection) does not change spectrally; in contrary the radiation, which originates from the leaf interior (multiple scattering i.e. diffuse reflectance) changes its spectral composition due to absorption by leaf biochemical components (Gates *et al.* 1965).

In visible region of electromagnetic spectrum (400-740nm), photosynthetic pigments such as chlorophylls and carotenoids are the major absorbing substances of solar radiation with their absorption peaks in blue (445 nm) and red (645 nm) wavelengths (Gates *et al.* 1965). Thus, it is the concentration of photosynthetic pigments that determines leaf optical properties in visible spectral region.

Chlorophyll *a* also plays a major role in the fluorescence emission in red (690 nm) and far-red (735-740 nm; red edge) wavelengths (Lichtenthaler Schweiger 1998) and other leaf compounds, such as cinnamic acids derivatives covalently bound to the cell walls contribute to the fluorescence emission in green wavelengths (520-530 nm) (Lichtenthaler and Schweiger 1998). The intensity of green fluorescence is the strongest in leaf epidermis, while in mesophyll it is reabsorbed by the blue-green absorption bands of chlorophyll and carotenoids (Buschmann *et al.* 2000; Vogelmann and Evans 2002). As the solar-induced chlorophyll steady-state fluorescence emission could contribute up to 25% in red or 6% in far-red to the apparent vegetation reflectance signal (Entcheva-Campbell *et al.* 2008), it should be taken into account when dealing with leaf optical properties as indicators of plant stress response as mentioned further. Carotenoids, especially those involved in xanthophyll cycle, play a major role in photoprotective reactions induced by light excess, heat stress (e.g. Garcia-Plazaola *et al.* 2008) or drought (e.g. Haldimann *et al.* 2008). Changes in leaf reflectance in a narrow waveband centered in 531 nm result from the dynamic transitions between epoxidized and de-epoxidized forms of xanthophylls (Stylinski *et al.* 2002). Liquid water, which could represent up to 80% of leaf fresh weight, has several strong absorption bands occurring in near- and middle-infrared wavelengths (975, 1175, 1450 and 1930 nm).

As previously mentioned, leaf optical properties – particularly reflectance – in the near infrared region (750–1350 nm), are influenced by the internal leaf structure. Mesophyll cells differ in the refractive index from the air within the intercellular spaces and the light is scattered at the interfaces of cell walls and intercellular air spaces inside the leaf consisting about 30-40% of the air spaces (Vogelmann *et al.* 1996).

Figure 5:

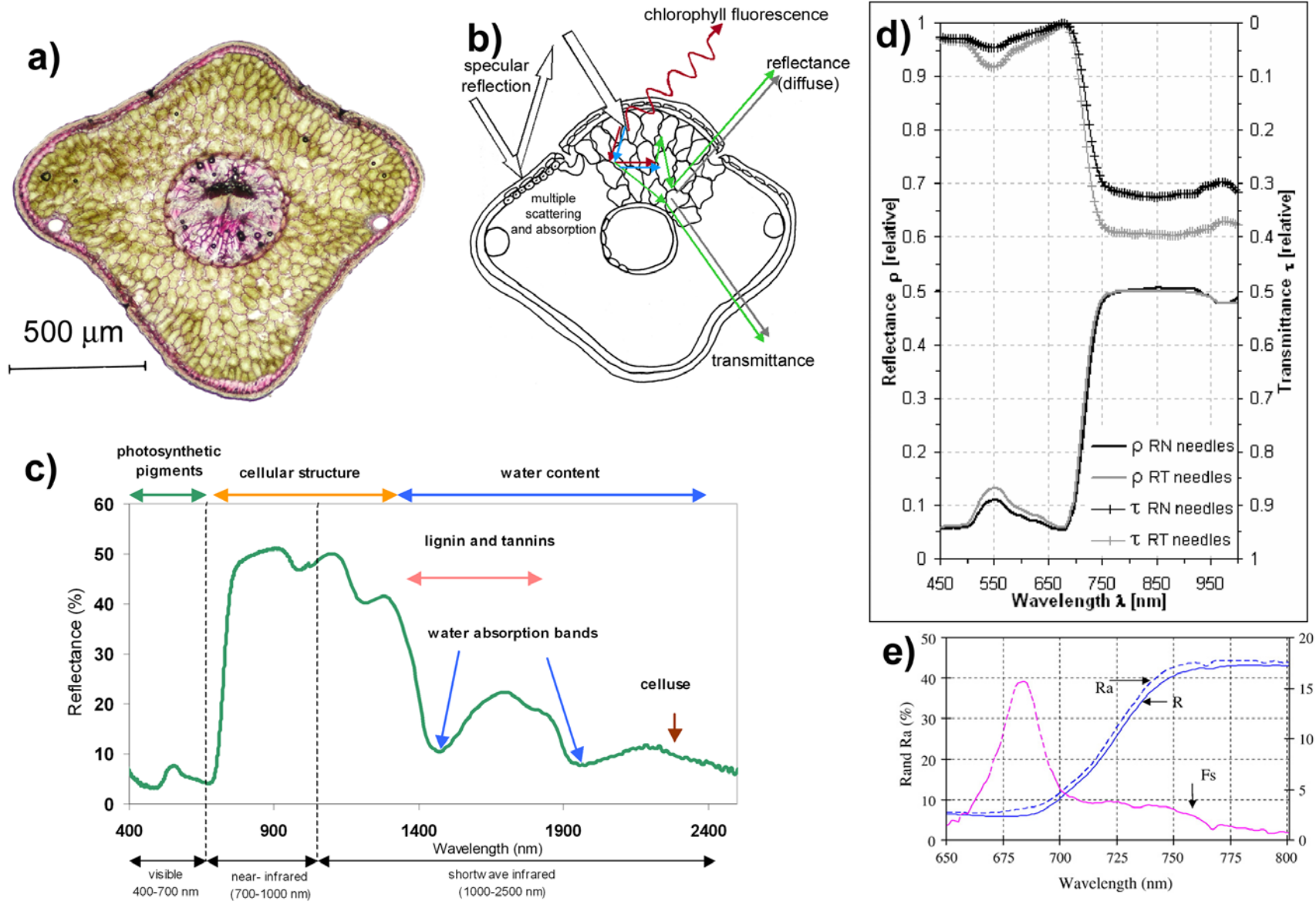




Figure 5: The interaction of solar radiation with needle tissues and needle optical properties.

a) Transverse section of Norway spruce needle, stained with phloroglucinol for lignin detection (red color in the cell walls). Bar corresponds to 500 μm .

b) The interaction of solar radiation with needle tissues. Specular reflection from the cuticle surface, which is not changed spectrally (middle white arrow). Radiation penetrating inside the leaf is absorbed by chloroplasts (blue and red wavelengths) or scattered by the cell walls within mesophyll (green, infrared) and then reflected back to the observer or transmitted through the needle (green and grey arrows). Molecules of chlorophyll a produce the fluorescence emission in red and far-red wavelengths (red wavy line).

c) Reflectance curve of Norway spruce needles. Color arrows denote spectral regions, where biochemical and structural needle properties determine the reflectance.

d) Reflectance and transmittance in visible and near-infrared for Norway spruce needles from trees with prevailing primary shoots (RN) and secondary shoots (RT), kindly provided by Zbyněk Malenovský.

Vegetation reflectance (R , solid line), apparent reflectance (R_a , dashed line) and steady state fluorescence ($F_{S\lambda} = R_a - R$, dotted line). After Entcheva-Campbell *et al.* 2008.

6.2 Progress in Remote Sensing of tree foliage

Current approaches of remote sensing operate with hyperspectral data, which in contrast to previously used multispectral data using seven wide spectral bands, consist of high number of narrow (1-5 nm) spectral bands resulting in continuous reflectance spectra. Similarly, the spatial resolution of space-borne spectroscopy images improved in last decade, e.g. from 30 meters resolution of ETM+ (Enhanced Thematic Mapper Plus) onboard of the satellite Landsat 7 in 1999 (<http://landsat.gsfc.nasa.gov/about/etm+.html>) to 20 meters resolution of CHRIS (Compact High Resolution Imaging Spectrometer) onboard of the PROBA (Project for On Board Autonomy) platform since 2004 (<http://earth.esa.int/object/index.cfm?fobjectid=4216>). For monitoring physiological status of vegetation, particularly of forest stands, hyperspectral data from air-borne sensors provide even higher spatial resolution (1.5m, Entcheva-Campbell *et al.* 2004; 1m, Moorthy 2008), or less (Malenovský *et al.* 2008). Then an individual tree crown is represented with several pixels of spectral image and a potential heterogeneity in leaf optical properties should be taken into account for proper interpretation and verification of remote sensing data. Most of the analyses from high spatial resolution spectral data are performed exclusively on pixels corresponding to sunlit part of the tree crown due to a high spectral noise in the shadowed pixels (Malenovský *et al.* 2006; Moorthy *et al.* 2008), which decrease the efficiency of leaf biochemistry (particularly chlorophyll) retrieval (Moorthy *et al.* 2008).



6.3 Heterogeneity in leaf biochemistry and related optical properties within the tree crown

O'Neill *et al.* (2002) highlighted that majority of remote sensing studies on foliar biochemistry estimation have been based on two following assumptions: i) foliar biochemistry and related leaf optical properties vary a little with leaf age or location within the crown, ii) foliar samples usually from the top of the canopy are representative for the canopy as a whole.

In evergreen species, particularly conifers, the influence of leaf age on biochemical composition, internal structure and related optical properties or reflectance spectra derived indices are known (e.g. Rock *et al.* 1988; 1994; Soukupová *et al.* 2001). Based on study of variations in several biochemicals and reflectance for different needle age classes within Sitka spruce crown, O'Neill *et al.* (2002) assumed that these variations are relatively small. On the contrary, results of Moorthy *et al.* (2008) suggest that consideration heterogeneity in chlorophyll content in differently old needles and including the age representation proportionality into chlorophyll retrieval methodologies significantly improves accuracy of pigment retrieval from reflectance spectra of coniferous canopies. Similarly, in our study on relationships between optical spectral indices, computed from shoot reflectance, and needle chlorophyll content, we found stronger correlation when analyzing younger needles (youngest three age classes against two oldest, Albrechtová *et al.* 2008, Paper V.). This finding appears to be encouraging for application of remote sensing as recent age classes of foliage dominate the upper sunlit parts of the crowns with strong reflectance signal.

In previous chapters, irradiance was discussed as the main factor driving vertical gradient of leaf structural parameters, photosynthetic capacity and also biochemical composition within the tree crown. Contribution of bottom shaded parts of the tree crown to the top-of canopy reflectance is usually negligible, but the information about potential horizontal variability of leaf optical properties within the sun-lit crown part is crucial for correct interpretation of remote sensing data using very high spatial resolution. Sampling designs in many field studies reflect assumed heterogeneity of leaf characteristics as leaves are collected from uniformly oriented branches (e.g. Tegischer *et al.* 2002; Vrchtová *et al.* 2004) in order to minimize possible microclimatic differences within the stand or canopy. We tested the hypothesis that branch azimuth orientation influence selected biochemical, structural and spectral properties of Norway spruce needles within the sun-lit upper crown part and no such a heterogeneity was revealed (Lhotáková *et al.* 2007). Similarly Zhang (unpublished, submitted to CJRS) observed only insignificant difference in chlorophyll content of black spruce needles from differently oriented branches. These findings support the assumption that information obtained from the sunlit



upper part of a coniferous tree crown can be used as ground truth or calibration input in remote sensing techniques designed for forest monitoring.

6.4 Relationships between foliar and soil chemistry, C and N fluxes monitoring based on remote sensing approach

Within intact forest ecosystems in temperate zone, nitrogen availability often stands as limiting factor for tree productivity and growth. However, since the beginning of the industrial era, atmospheric nitrogen emissions originating in human activities represent additional N source for many ecosystems including forests (Galloway *et al.* 2003), but significant portion of this N input comes in a form of acidic deposition what often may result in soil acidification and subsequent loss of mineral nutrients via leaching (e.g. Larcher 2003). For example, stream inorganic nitrogen export from the Norway spruce stands in the Czech Republic positively correlated with an average acidic deposition and also was strongly influenced by the forest floor C:N ratio (Oulehle *et al.* 2008).

In forest ecosystem many biochemical processes, such as photosynthesis, respiration, primary biomass production and litter decomposition are interconnected with the carbon and nitrogen cycles where the foliar chemistry could be understood as a linking component via plant-litter-soil interactions. On the one hand, the senescing foliage is the main contributor to the litterfall, which becomes a source for decomposition of soil organic matter by forest floor microbial societies. On the other hand, soil organic matter in the forest floor can be subsequently mineralized and contribute to the availability of inorganic nutrient forms for plant uptake, which in turn affect leaf biochemical composition (Ollinger *et al.* 2002) and primary productivity. The positive relationship between inorganic nitrogen availability and leaf chlorophyll concentration is generally established (e.g. Larcher 2003). However, in case of nitrogen, it was shown that many tree species, e.g. Norway spruce or European beech (*Fagus sylvatica* L.), are able to uptake organic nitrogen as well (in a form of free amino acids) via mycorrhizal root tips (Lipson and Nasholm 2001). According to recent investigations, dissolved organic nitrogen (DON) was detected as the predominant form of extractable N in boreal forest sites with low productivity and organic nitrogen uptake, rather than solely inorganic nitrogen, appear to contribute significantly to plant nutrition, especially in less productive, N-poor soils in arctic, alpine and boreal ecosystems (Kranabetter *et al.* 2007).

Content of lignin or other hardly degradable substances, such as tannins, in plant litterfall affect nutrient cycling by impeding decomposition (Talbot and Finzi 2008), what may result in an increased level of nitrogen in an organic form in comparison to a mineral form (Kraus *et al.* 2003). Moreover, tannins and related phenolic acids, such as ferulic, p-coumaric, p-hydroxybenzoic and vanillic acids, which are abundant in needles of conifers (e.g. Soukupová *et al.* 2000, Lhotáková submitted, Paper III.), may affect the composition



of microbial populations in soil (Kraus 2003). Considering ongoing climate changes, rising atmospheric CO₂ concentration was initially supposed to increase C:N ratios in plant biomass due to higher accumulation of recalcitrant compounds, such as lignin or other phenolic compounds (Booker and Maier 2001). This change would impede decomposition and N mineralization. However, in many woody species effect of elevated CO₂ concentration on accumulation of phenolic compounds and lignin was revealed insignificant (Asshoff and Hattenschwiler 2006; Raisanen *et al.* 2008 Lhotáková *et al.* submitted, Paper III.) and, in general, an impact of foliar chemistry change under elevated CO₂ concentration on decomposition rates was reported as insignificant (Norby *et al.* 2001).

The variation in soil N-cycling characteristics (mineralization and nitrification rates, soil C:N ratio) was found to be species-specific in Northern American (Finzi *et al.* 1998; Lovett *et al.* 2004) and European temperate forests (Vesterdal *et al.* 2008). In our study conducted in the White Mountains (New Hampshire USA) we found difference in soil C:N ratio between two dominating conifers i.e. red spruce and balsam fir (Albrechtová *et al.* 2008). The C:N ratio of foliar litterfall was suggested as a good indicator of both forest floor C and N contents and fractional annual loss of forest floor C and N in five European deciduous species and Norway spruce (Vesterdal *et al.* 2008). The relationships between the chemistry of green foliage and N cycling may differ between tree functional groups as shown by Ollinger *et al.* (2002). For deciduous species foliar N increased with increasing N mineralization suggesting a positive feedback between foliar chemistry and soil N availability, in contrast with conifers, where no variation in needle nitrogen across wide N-mineralization gradients was detected (Ollinger *et al.* 2002).

Dissolved organic matter, particularly carbon and nitrogen (DOC and DON), play a central role in nitrogen and carbon cycles in forest ecosystems (McDowell, 2003a) and also in ecosystem processes such as pedogenesis (Dawson *et al.* 1978) or microbial metabolism (Yano *et al.* 1998). Previous studies have shown that forest floor DOC production is related to forest floor C:N ratio (e.g. (Aitkenhead *et al.* 2000), which could be predicted from foliar chemistry, particularly lignin:N ratio (Ollinger *et al.* 2002; Aitkenhead-Peterson *et al.* 2006). Much less is known about forest floor DON and it seems that DON production is in many ways decoupled from DOC production (e.g. McDowell *et al.* 2004). In Paper V. (Albrechtová *et al.* 2008) we focused on the study of relationships between forest floor water-extractable DOC and DON and needle chemistry of two common Northern American coniferous species (red spruce and balsam fir). We focused on the chlorophyll content in needles, because it is closely related to leaf nitrogen content, well established as reflecting current physiological status of a plant (e.g. Larcher 2003) and it is often used as an indicator of forest health (e.g. Albrechtová *et al.* 2001; Petkovsek *et al.*



2008). Furthermore, leaf chlorophyll content determines leaf optical properties in the visible part of electromagnetic spectrum (see chapter 6.1.) and could be monitored using reflectance-based remote sensing techniques (Huber *et al.* 2008; Moorthy *et al.* 2008; Zhang *et al.* 2008b). We revealed negative relationships between chlorophyll concentration in needles and forest floor DOC and DON, however, these relationships are likely to be indirect and the mechanisms underlying them remain undisclosed.

It is desirable to establish an indicator of forest ecosystem N status, which could be monitored across large, heterogeneous landscapes. Remote sensing represents a tool with such a potential, but reliable links between spectral properties of foliage and foliar/soil chemistry have yet to be revealed and verified. Ollinger *et al.* (2002) showed that foliar lignin:N ratio is detectable from hyperspectral air-borne data and capable of prediction forest floor C:N ratio across regional scale in a mixed temperate forest in New England. We contributed to the effort of spectral monitoring of forest nitrogen status at the level of laboratory reflectance measurements and we revealed correlations between forest floor DOC and DON and selected chlorophyll-related optical indices (TCARI/OSAVI, ANMB₆₅₀₋₇₂₅ and Chl NDI) for balsam fir and red spruce foliage (Albrechtová *et al.* 2008, Paper V.). However for general use, it would be necessary to verify potential of those relationships in large scale monitoring of DOC and DON dynamics as well for other forest tree species and air-born spectral data.



SYNTHESIS – CONCLUSIONS:

This thesis combines two methodical approaches at different hierarchical levels of plant ecophysiology, which both are applied in field research and bring new valuable information for comprehension of several aspects of carbon cycle in forest ecosystem, particularly coniferous.

The quantitative anatomy represents the first approach. At the leaf level application of confocal microscopy on fresh and frozen-stored needles and subsequent stereological estimation of mesophyll geometrical parameters proved to be suitable and sensitive enough for estimation of changes in mesophyll after simulated acid rain or differentiation of sun and shade needle ecotype. Application of systematic uniform random sampling of sections along the needle axis revealed insight to heterogeneity of mesophyll geometrical parameters within the needle and confirmed representativeness of the sampling from the middle region of the spruce needles.

The second approach, laboratory measurement of shoot reflectance, represents important step in verification of relationships between leaf biochemical and optical properties, which can be subsequently used for monitoring of forest health, foliar or soil chemistry using remote sensing techniques. At the level of Norway spruce crown, samples of even-aged needles from the upper sunlit crown part proved to be representative irrespective of branch azimuth orientation regarding biochemical, structural and spectral parameters. And selected chlorophyll content related spectral indices revealed to be potential indicators of forest floor dissolved organic carbon and nitrogen pools in mixed coniferous forest.

According to determined aims and tested hypotheses, the following conclusions were drawn:

- 1. Thick free-hand sections of fresh and frozen needles proved to be suitable for estimation of important mesophyll photosynthesis-related geometrical parameters using contemporary unbiased stereological methods (fakir, disector) in combination with confocal microscopy image acquisition.**
 - Selected stereological methods, fakir and disector, revealed to be sensitive for quantitative description of changes in Norway spruce mesophyll structure induced with simulated acid rain treatment.
 - Selected geometrical parameters of mesophyll: proportion of intercellular spaces and internal surface density did not suffer from deformation by freezing during needle storage. However, stereological measurements should avoid regions of the physical sections near to cut planes.



2. The detailed study of mesophyll parameters with unbiased stereological estimators proved that an observed increase in assimilation rates in both sun and shade Norway spruce needles after eight-year exposure to elevated CO₂ concentration was not connected with structural changes.

- Neither volume proportions of individual needle tissues nor internal surface of mesophyll changed under the impact of elevated CO₂ concentration.
- Since sun and shade needle ecotypes exhibited the same pattern of differences in needle anatomy irrespective of the CO₂ enrichment, it appears that irradiance might be a higher priority environmental factor for leaf structure differentiation than an enriched CO₂ concentration in the atmosphere.

3. Selected biochemical, anatomical and spectral properties of Norway spruce needles revealed to be independent from branch azimuth orientation.

- Non-varying structural parameters, content of photosynthetic pigments and soluble phenolic compounds and selected optical vegetation indices derived from even-aged needles from branches of the four azimuth orientations suggest that the entire upper sunlit production part of the crown of Norway spruce canopy is representative in support of remote sensing studies.

4. Relationships between chlorophyll content in needles of red spruce and balsam fir and forest floor water-extractable dissolved organic C and N were revealed, which may be monitored by remote sensing techniques via selected chlorophyll-related optical indices.

- The mechanisms underlying negative correlations between needle chlorophyll content and forest floor water extractable DOC and DON concentration remain uncertain. However, the relationships were stronger for young and more physiologically active foliage rather than older senescent needles, which could indicate that the direction in the response–effect relationships is from soil to plant.
- Selected chlorophyll-related optical indices (ChINDI, TCARI/OSAVI and ANMB_{650–725}) showed potential for monitoring forest floor water-extractable DOC and DON via those relationships with chlorophyll content in needles. From the standpoint of remote sensing studies, stronger relationships between forest floor water-extractable DOC and DON and chlorophyll content in younger needles are encouraging, because young physiologically active foliage occupies the upper crowns of coniferous trees, which contributes as the main source to canopy reflectance signal.



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PAPER I.

Novel efficient methods for measuring mesophyll anatomical characteristics from fresh thick sections using stereology and confocal microscopy: application on acid rain treated Norway spruce needles.

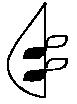
Albrechtová Jana, Janáček Jiří, **Lhotáková Zuzana**, Radochová Barbora, Kubínová Lucie. JOURNAL OF EXPERIMENTAL BOTANY 58 (6): 1451-1461, 2007



PAPER II.

**Advantages and pitfalls of using free-hand sections of frozen
needles for three-dimensional analysis of mesophyll by
stereology and confocal microscopy.**

**Lhotáková Zuzana, Albrechtová Jana, Janáček Jiří, Kubínová Lucie.
JOURNAL OF MICROSCOPY, 232: 56-63, 2008**



PAPER III.

**The impact of long-term CO₂ enrichment on sun and shade
needles of Norway spruce: photosynthetic performance, needle
anatomy and phenolic compounds.**

Lhotáková Zuzana, Urban Otmar, Dubánková Marianna, Cvikrová Milena,
Tomášková Ivana, Kubínová Lucie, Zvára Karel, Marek Michal V. and Albrechtová
Jana

Manuscript submitted to GLOBAL CHANGE BIOLOGY



PAPER IV.

**Does the azimuth orientation of Norway spruce (*Picea abies* /L./
Karst.) branches within sunlit crown part influence the
heterogeneity of biochemical, structural and spectral
characteristics of needles?**

Lhotáková Zuzana, Albrechtová Jana, Malenovský Zbyněk, Rock Barrett N.,
Polák Tomáš, Cudlín Pavel. ENVIRONMENTAL AND EXPERIMENTAL BOTANY
59 (3): 283-292, 2007



PAPER V.

Spectral analysis of coniferous foliage and possible links to soil chemistry: Are spectral chlorophyll indices related to forest floor dissolved organic C and N?

Albrechtová Jana, Seidl Zdeněk, Aitkenhead-Peterson Jacqueline, **Lhotáková Zuzana**, Rock Barrett N., Alexander Jess E., Malenovský Zbyněk, McDowell William H. SCIENCE OF THE TOTAL ENVIRONMENT 404: 424–432, 2008