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**Study of Quantitative Parameters of Norway Spruce Needle Structure under the Effect
of Elevated CO₂ Concentration and Different Irradiance**

Studium kvantitativních parametrů struktury jehlic smrku ztepilého pod vlivem zvýšené
koncentrace CO₂ a rozdílné ozáření

Doctoral Thesis

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1. Abstract

Atmospheric concentration of CO₂ is increasing, while its influence on plants is still not fully elucidated. Norway spruce (*Picea abies* L. Karst.) is an abundant conifer tree in European temperate and boreal forests, which behave as carbon sink in the global carbon cycle. The physiological response to elevated CO₂ concentration may be interconnected with changes in leaf anatomy and morphology. Needle structure is also determined by other factors in addition to CO₂ concentration, irradiance being the most important one. Thus, effect of irradiance was also included in our studies.

The effects of elevated CO₂ concentration and irradiance on Norway spruce needle structure were studied using new applications of well-established quantitative methods and novel methods enabling effective and unbiased analysis of needle structural traits. The General Procrustes analysis showed to be effective for needle shape on cross section comparison and the disector method proved to be suitable for chloroplast number estimates.

The influence of elevated CO₂ concentration and different irradiance on needle structure was studied at two hierarchical levels: At the level of needle morphology, irradiance was stronger morphogenic factor than elevated CO₂ concentration, while at the level of cell structure, the chloroplast density was enhanced by CO₂ concentration.

Irradiance microgradient within a shoot caused by needle self-shading was measured and shape differences among the needles within the same shoot were observed: Upper needles, i.e. needles growing from the upward side of the shoot, resembled sun needles by having larger cross-section area and less flat shape. However, the needle length was in counteraction as upper needles were rather shorter. Thus, needle volume differed following macroscale light gradient – needles from sun shoots had larger volume than needles from shade shoots regardless of their orientation on a shoot.

The main effect of elevated CO₂ concentration was stimulation of light-saturated CO₂ assimilation rate causing production of larger amount of starch in sun needles, which was accumulated in starch grains in chloroplasts. Larger starch grain area and starch areal density on cross section were observed in sun in comparison with those in shade needles in elevated CO₂ concentration. However, our observations may be influenced by the effect of the season on starch areal density under elevated CO₂ concentration.

In conclusion, anatomical studies contributed to integration of findings obtained by various types of analyses; thus, quantitative anatomy is inevitably important in the synthesis of knowledge how elevated CO₂ concentration may affect Norway spruce in the future.

2. Abstract in Czech – abstrakt v českém jazyce

Koncentrace CO₂ v atmosféře vzrůstá, zatímco její vliv na rostliny stále ještě není zcela objasněn. Smrk ztepilý (*Picea abies* L. Karst.) je hojně rozšířeným jehličnanem v evropských severských lesích a lesích mírného pásma, které jsou jedním ze sinků uhlíku v jeho globálním cyklu. Fyziologické odpovědi rostlin na zvýšenou koncentraci CO₂ mohou být vzájemně propojeny s anatomickými a morfologickými změnami listu. Struktura listu je také ovlivněna dalšími faktory, přičemž nejvýznamnější z nich je ozáření, která byla proto zařazena do našich studií.

Vlivy zvýšené koncentrace CO₂ a ozáření na strukturu jehlic smrku ztepilého byly studovány pomocí nově uzpůsobených zavedených efektivních metod kvantitativní analýzy, které poskytují nevychýlené odhady daných parametrů. Metoda všeobecné Prokrustovy analýzy byla efektivní pro porovnání tvarů jehlic na příčném řezu a metoda disektoru umožnila přesný odhad počtu chloroplastů ve smrkových jehlicích.

Vliv zvýšené koncentrace CO₂ a ozáření na strukturu jehlic byl zkoumán na dvou hierarchických úrovních: Na úrovni morfologie jehlice byla ozáření silnějším morfogenním faktorem než zvýšená koncentrace CO₂, zatímco na úrovni buněčné struktury vlivem zvýšené koncentrace CO₂ narostla hustota chloroplastů.

V rámci výhonu byla naměřena rozdílná ozáření způsobená vzájemným stíněním jehlic. Plocha a tvar příčného řezu středem jehlice závisely na orientaci jehlice na výhonu. Horní jehlice připomínaly slunné jehlice tím, že jejich řezy měly větší plochu a byly méně zploštělé. Naproti tomu však byly kratší a tudíž objem jehlic závisel na rozdílné ozáření v rámci stromu – jehlice slunných výhonů byly objemnější než jehlice výhonů stinných nezávisle na jejich orientaci na výhonu.

Efekt zvýšené koncentrace CO₂ se projevil hlavně zvýšením maximální rychlosti světlem saturované asimilace CO₂, které způsobilo tvorbu většího množství škrobu ve slunných jehlicích. Ten se nahromadil ve škrobových zrnech v chloroplastech. Škrobová zrna měla na řezu větší plochu ve slunných než ve stinných jehlicích ve zvýšené koncentraci CO₂. Nicméně naše pozorování může být ovlivněno sezónními změnami v poměrném zastoupení škrobu na řezu chloroplastem.

Anatomické studie přispěly k propojení poznatků z jiných typů analýz a jsou tudíž nevyhnutelně důležitou součástí syntézy vědomostí o budoucím působení zvýšené koncentrace CO₂ na smrk ztepilý.

3. Abbreviations

AC	ambient air CO ₂ concentration
A _{max}	light-saturated CO ₂ assimilation rate
AQE	apparent quantum efficiency
EC	elevated air CO ₂ concentration
GPA	Generalized Procrustes analysis
LMA	leaf mass per area
PPFD	photosynthetic photon flux density
ppm	parts per million
PSII	photosystem II
R _D	dark respiration rate
Rubisco	ribulose-1,5-bisphosphate carboxylase/oxygenase
RuBP	ribulose-1,5-bisphosphate
STAR _{max}	maximum ratio of shoot silhouette area to total needle surface area
SUR	systematic uniform random

4. Introduction

The ever increasing atmospheric concentration of CO₂, which is occurring in many last decades (Etheridge *et al.*, 1998; ESLR, 2019), affects plant physiological processes and anatomical structure (e.g. Körner, 2006; Leakey *et al.*, 2009; Domec *et al.*, 2017; Kurepin *et al.*, 2018). To reveal its influence on plants, changes at all hierarchical levels should be analysed – from the ecosystem and population, through the individual plant characteristics and the organ anatomy to the subcellular structure level. The findings from all these levels and their synthesis will enable to understand the complex response of plants to elevated CO₂ concentration.

Temperate and boreal forests play an important role in the global carbon cycle (Calfapietra *et al.*, 2010). Forests act as a carbon sink (Goodale *et al.*, 2002; Luysaert *et al.*, 2008; Pan *et al.*, 2011), evapotranspire and create surface roughness (Bonan, 2016) and the increased leaf area index (occurring i.e. due to afforestation) contributes to mitigation of the increase in global land-surface air temperature (Zeng *et al.*, 2017). On the other hand, forests decrease surface albedo and therefore may contribute to local warming, thus their role is complex and their research demands interdisciplinary approach (Bonan, 2016).

Norway spruce (*Picea abies* L. Karst.) is an abundant conifer tree species in European temperate and boreal forests. It belongs to the most important timber tree species and thus it is plentifully planted even outside its original biotope (Spiecker, 2000). Because of its abundance, Norway spruce was chosen as the model plant species in this study.

Leaf anatomy and morphology are interconnected with the ability of plants to absorb light and CO₂ and tree leaves usually adapt their anatomy to surrounding environmental conditions to enable more efficient photosynthesis (Terashima *et al.*, 2011). Therefore it is necessary to study not only the physiological response to elevated CO₂, but also the changes in leaf anatomy in plants growing under elevated CO₂ together with irradiance effect to reveal their adaptations to these conditions. The presented studies mainly focused on adjusting methods to unbiasedly evaluate needle anatomy to complement the physiological studies.

Concerning leaf anatomy, an important morphogenic factor is irradiance (Stahl, 1883; Gaba and Black, 1983; Niinemets, 2007). Sun needles are longer, thicker, wider, heavier and denser than shade needles as it was noticed in mature Scots pine (*Pinus sylvestris* L.) (Gebauer *et al.*, 2015a). Norway spruce sun needles are wider and have higher dry weight per area (Niinemets and Kull, 1995), larger volume and internal mesophyll surface (Lhotáková *et al.*, 2012) and larger tracheid lumen area (Gebauer *et al.*, 2012) than shade needles.

Besides irradiance, turgor pressure may influence needle morphology. In crowns of very tall conifers (above 90 m height), *Pseudotsuga menziesii* (Mirb.) Franco, *Picea sitchensis* (Bong.) Carrière and *Sequoia sempervirens* (D.Don) Endl., the needle length decreased with height due to turgor pressure limitation of cell expansion and leaf mass per area (LMA) increased with height probably due to smaller intercellular space (Chin and Sillet, 2019). Moreover, Yáñez *et al.* (2017) claim that the differences in needle morphological parameters even within a crown of young and up to 4,5 meter tall *Pinus taeda* L. trees may not be driven solely by light and that the height of the tree where needles are sampled may play a role, too. They observed seasonal differences in morphological parameters between crown positions and thus supposed that other factors may contribute such as soil moisture, nutrients availability and light quality (Yáñez *et al.*, 2017). Variability in foliage morphology was also observed in connection with geographic elevation, where the shoots in Norway spruce crowns in higher elevation were shorter, the crowns were more transparent and needles had lower dry mass than in lower elevation (Gottardini *et al.*, 2016).

To study the influence of elevated CO₂ concentration (EC) on plants, different experimental designs were established, while the common approach is to compare plants growing under ambient atmosphere with plants growing in atmosphere with elevated CO₂ concentration. One issue complicating the synthesis of results from different studies is that the ambient CO₂ concentration (AC) in the atmosphere is continually increasing and thus this value is increasing in the conducted experiments during the years, too. Moreover, the CO₂ concentration elevation in the experiment may be in different extent. Therefore, ambient CO₂ concentration was considered e.g. as 350 ppm and elevated as 750 ppm (Lin *et al.*, 2001), while in later studies, the concentrations may be e.g. 384–466 and 524–605 ppm (Klein *et al.*, 2016), respectively. Material for the publications presented as a part of this thesis was sampled in the adjustable glass domes with controlled atmosphere at the Bílý Kříž experimental site in the Beskid Mountains, Czech Republic, where the EC was always kept 700 ppm regardless the changes in the ambient one (Urban *et al.*, 2001).

Another fact to consider when comparing different experiments aimed on elevated CO₂ concentration is duration of the experiment, because buds and needles developing in previous years may be influenced by the then atmosphere. The season of the year when the measurements are done should be also considered, as some parameters (e.g. chloroplast ultrastructure) seasonally change (Sutinen, 1987, Senser *et al.*, 1975). The tree age may have influence on leaf anatomical parameters and, thus, has to be considered too, because seedlings may respond differently, such as in *Pinus sylvestris* L., where the needles of 7-year-old trees

after 4 years of treatment were thicker in EC than in AC (Lin *et al.*, 2001), while no effect of EC on needle thickness was observed in 20-year-old trees after 3 years of treatment (Luomala *et al.*, 2005). Needle age may also influence the anatomical parameters, as current needles had larger internal mesophyll volume area than 3-year-old needles (Lhotáková *et al.*, 2012). When the needle length was influenced in EC, it was significantly higher only in the three youngest age classes (Pokorný *et al.*, 2011).

Changes of needle anatomy under elevated CO₂ have been studied and observed in various conifer species (Lin *et al.*, 2001; Eguchi *et al.*, 2004; Kurepin *et al.*, 2018) and the response to EC may be species specific (Kurepin *et al.*, 2018).

The conditions, in which the parent plants grow influence the progeny. *Pinus yunnanensis*, Franch. and *Pinus sylvestris* L. seedlings planted from seeds taken from different locations in China and Northern Europe respectively, and grown at experimental site had needles with some anatomical characteristics corresponding to the climatic conditions, in which the parent trees lived (Huang *et al.*, 2016; Jankowski *et al.*, 2017). However, the adult trees were adapted to the new site (Jankowski *et al.*, 2017). Thus, the needle anatomy is probably genetically based, but it is phenotypically plastic and can adapt during ontogenesis.

Similarly, Mašková *et al.* (2017) found great intraspecific differences in starch accumulation and soluble carbohydrate levels in Norway spruce needles of trees grown both in AC in EC, thus there may be a genetically based variability in other than anatomical parameters. In our studies, we used samples from a mix of genetically different trees and we supposed that the intraspecific differences within the groups were overcome by the influence of the environment.

In most of the past needle anatomy studies on Norway spruce (e.g. Niinemets and Kull, 1995; Gebauer *et al.*, 2012; Homolová *et al.*, 2013; Gebauer *et al.*, 2015b), the possible heterogeneity of structural parameters was usually considered mostly in sun versus shade shoots or needles and it was not always taken into account that heterogeneity may occur even within the same shoot or needle. In order to fully understand the effect of irradiance and elevated CO₂ concentration on needle morphology and anatomy, it is essential to be aware of the possible heterogeneity or gradients of measured parameters within the plant organs, and thus to apply proper sampling and use precise methods of measurement.

The anatomical parameters should be evaluated precisely. The fundamental stereological studies presented the methods for unbiased sampling and estimation of particle number in 3D space (Sterio, 1984; Mayhew and Gundersen, 1996). In the field of plant anatomy, the unbiased sampling and stereological methods are still rarely applied

(Kubínová *et al.*, 2017). Despite the fact that the stereological methods enabling estimation of mesophyll anatomical characteristics had been established (Kubínová and Janáček, 1998; Albrechtová *et al.*, 2007; Lhotáková *et al.*, 2008; Albrechtová *et al.*, 2014), they were further used by only a few other plant biology researchers, i.e. by Bandaru *et al.* (2010).

This study should help to elucidate how elevated CO₂ concentration influence sun and shade needle structure of Norway spruce. The response of needle anatomy could then be related to responses of physiological characteristics revealed in previous studies at the same experimental stand and the synthesis of knowledge will help to predict the future reaction of Norway spruce on expected elevated CO₂ concentration.

5. Norway spruce tree aboveground morphology and needle anatomy

In this chapter, Norway spruce morphology and anatomy is summarised to explain the terms used later in the text. The presented studies were done on three different hierarchical levels: *a)* on the level of a needle (Kubínová *et al.*, 2014, 2018, 2019), *b)* on the level of a mesophyll cell (Kubínová *et al.*, 2014, 2019) and *c)* on the level of a chloroplast (Kubínová *et al.*, 2019).

In Norway spruce trees, shoots are accruing from buds set during the previous season. During one vegetative season, new regular buds are set and remain dormant until the next vegetative season, when new primary shoots are flushed and elongated (Polák *et al.*, 2006). Apart from these regular buds, dormant buds with remaining growth potential and aborted buds may be also present (Polák *et al.*, 2006). Shoots form whorls, while new whorl accrues from the buds formed on the stem every year. Whorls are numbered from the top, thus the first whorl is the youngest one. Branches in the whorl consist of shoots, which accrue every year from the buds located at the previous year terminal shoot and to some extent also from the side shoots (Fig. 1a). Needles are growing all around the shoot (Fig. 1b).

Norway spruce needle (Fig. 2a) has a central cylinder (comprising vascular bundle and transfusion tissue) bordered by endodermis and surrounded by mesophyll tissue. Plates of mesophyll cells and intercellular spaces between them are visible on needle longitudinal cross section (Fig. 2b). Mesophyll cells contain chloroplasts and form several layers. In our studies, the first mesophyll layer is defined as the outermost layer (Fig. 2a). Mesophyll is surrounded by sclerified hypodermis and the outermost needle tissue is one-cell-layer epidermis. Stomata are pores formed by guard cells in epidermis connecting intercellular space with the outside; hypodermis is not present under them. Resin ducts (if present) are located under the epidermis (Fig. 2a).

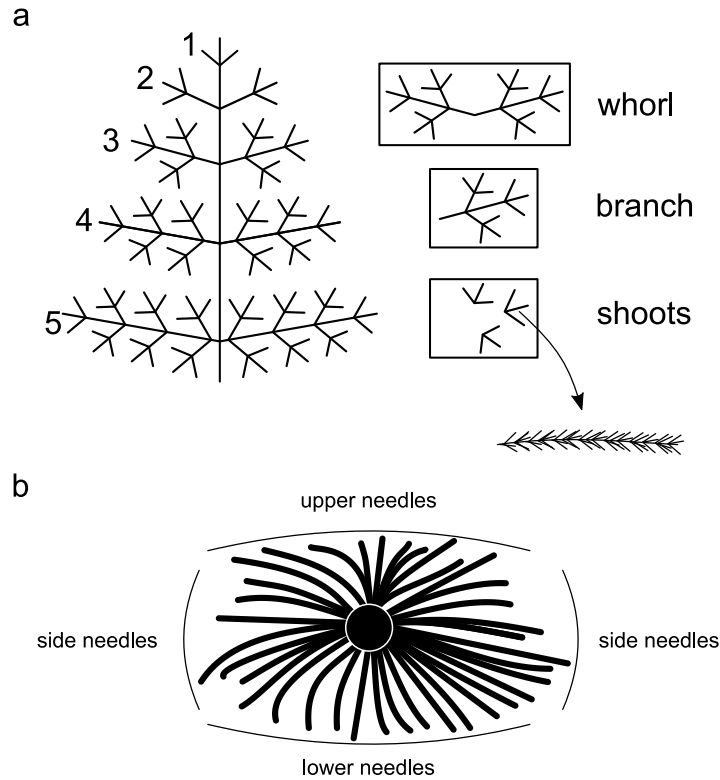


Fig. 1 Simplified schemes of Norway spruce tree and shoot. a – whorls, whorl numbers, branches and shoots; b – scheme of a shoot protruding towards the viewer showing that needles are growing all around the twig (white circle). Orientation of needles on a shoot: Upper needles grow from the upward facing side of the twig, lower needles grow from the downward facing side of the twig and side needles grow from sides of the twig.

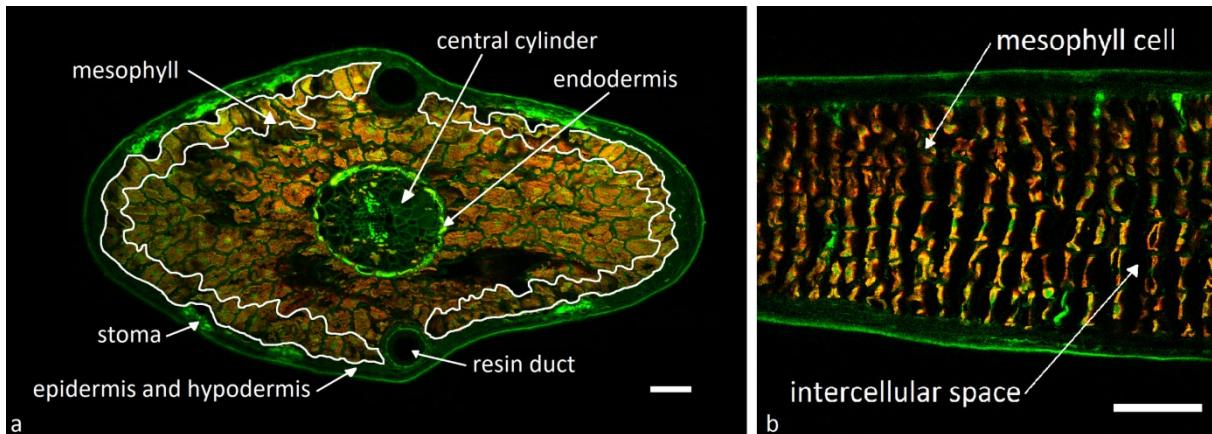


Fig. 2 Norway spruce needle. a – transverse cross section with contoured first mesophyll layer (white line); b – longitudinal cross section. Maximum projection of a stack of cross sections acquired by a Leica SP2 AOBS confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany) with Ar laser excitation of 488 nm. Red - chlorophyll autofluorescence in chloroplasts detected in the red channel (emission 625–710 nm), green – phenolics autofluorescence detected in the green channel (emission 494–577 nm). The bars correspond to 100 μm .

6. Objectives and hypotheses

The main objectives of this thesis were 1) to study the effects of elevated CO₂ concentration and irradiance on Norway spruce needle structure and 2) to use unbiased methods to quantify the studied needle parameters because that was necessary to fulfil the first objective. These methods included both well-established and novel quantitative methods for needle characteristics evaluation.

The influence of elevated CO₂ concentration and different irradiance on Norway spruce needle structure was studied at three hierarchical levels: *a*) the whole needle (needle cross-section shape), *b*) mesophyll (number of chloroplasts) and *c*) chloroplast ultrastructure (chloroplast area, starch grain area, and starch areal density).

Firstly, the effects of elevated CO₂ concentration, irradiance and needle spatial orientation on a shoot were studied. The hypotheses were that (H1) a microscale gradient in irradiance caused by needle self-shading exists within a shoot, and therefore needle spatial orientation on a shoot determines (H2) needle size and (H3) needle cross section shape similarly to sun and shade needle ecotypes differentiation; and (H4) irradiance is stronger morphogenic factor than elevated CO₂ concentration.

Additionally, the effects of elevated CO₂ concentration and irradiance on chloroplast number and chloroplast ultrastructure within a needle mesophyll were studied. Plants under elevated CO₂ concentration may invest into building more chloroplasts, as was reported previously. Thus the hypothesis was that (H5) the chloroplast density is enhanced by elevated CO₂ concentration. Photosynthesis is often stimulated under elevated CO₂ concentration, thus the hypotheses were that (H6) starch grain area and (H7) starch areal density are larger and lead to (H8) larger chloroplast area under elevated CO₂ concentration.

Finally, the effects of elevated CO₂ concentration, irradiance and spatial variability of structural parameters within needle mesophyll were studied. The hypotheses were that (H9) the first mesophyll layer is representative for the whole needle cross section regarding chloroplast density in mesophyll and that the first mesophyll layer is representative for the whole needle cross section regarding (H10) starch grain area, (H11) starch areal density and (H12) chloroplast area in the cross section.

7. Critical review of results

7.1 Critical review of methods used for anatomical characteristics quantification

The sampling method is an important part of the plant anatomical analysis. After sampling, methods for evaluation of the selected characteristics, such as shape analysis and methods for estimation of particle number and area are applied. Strengths and weaknesses of the aforementioned methods are discussed in this section.

7.1.1 Sampling

In order to fully understand the effect of irradiance and elevated CO₂ concentration on needle morphology and anatomy, the possible heterogeneity or gradients of measured parameters within the plant organs have to be taken into account, thus the suitable unbiased sampling and precise methods of measurement should be used.

The unbiased sampling may be done using the methods of simple random sampling, proportionator (Gardi *et al.*, 2008) or systematic uniform random sampling (Sterio, 1984). The systematic uniform random sampling (SUR) ensures that all selected particles have the same probability to be sampled (Sterio, 1984). In Norway spruce needle anatomy research, the SUR sampling have already been used in previous studies of our team (Albrechtová *et al.*, 2007; Lhotáková *et al.*, 2008) and showed to be effective and suitable for plant anatomy analysis. Therefore, it was used in the current studies.

One of the SUR methods, the optical disector (Sterio, 1984; Gundersen, 1986) enables unbiased estimation of particle number within 3D object. That is important, because gradients of many anatomic characteristics exist within plant organs (Pazourek, 1966). We have used the disector method implemented in a special plug-in module developed in the Ellipse software environment (Tomori *et al.*, 2001) to count chloroplasts in a stack of serial optical sections using virtual 3D probe. This method was applied in our study of chloroplast number within a needle (Kubínová *et al.*, 2014) and in the study of heterogeneity of chloroplast characteristics within the needle (Kubínová *et al.*, 2019). The use of disector for chloroplast number estimation is further discussed in section 7.1.3.

Sampling effectivity in the field was enhanced by formation and usage of a special belt for microtubes (Fig. 3). Belt was made from straps undid from a worn rucksack and a new

elastic, which were hand sewed together by a polyester thread. It was important to determine the right size of the loop, so that the microtube is both fixed enough not to fall from the belt and loose enough to be easily pulled out. Spaces between the loops need to be wide enough to enable the microtubes to fit next to each other without mutual pushing out. It was proven that the belt is useful.

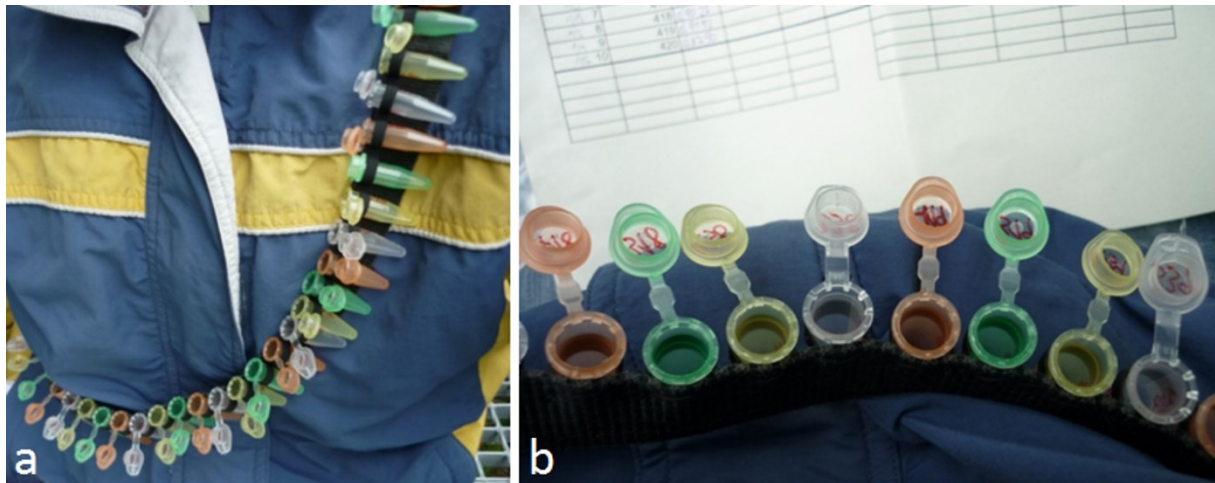


Fig. 3 Field belt for microtubes. a – detail of a person wearing the belt at the experimental site; b – detail showing spaces between the loops with microtubes for needle samples.

7.1.2 Analysis of the needle cross section shape

The main morphological characteristics of the needle cross section can be described by the ratio of needle thickness to needle width, which is used in the majority of studies (Sellin, 2001; Apple *et al.*, 2002; Palmroth *et al.*, 2002; Homolová *et al.*, 2013; Gebauer *et al.*, 2015b). However, this ratio may not cover the complex information about the shape, such as the lateral and or adaxial/abaxial asymmetry of the needle cross section (Fig. 4 A in Kubínová *et al.*, 2018). These parameters may differ, while the ratio of needle thickness to needle width may remain the same.

For precise cross section shape description, we applied the geometric morphometric method of Generalized Procrustes analysis (GPA) and it showed to be effective to estimate and compare the needle cross section shape (Kubínová *et al.*, 2018). Nevertheless, the major parameter responding to the irradiance gradient observed in our study was the needle flatness, which may be also described by the ratio of needle thickness to needle width. Therefore, it seems plausible that the traditional method enables to recognize the main morphological traits of Norway spruce needles and their response to studied environmental factors. On the other hand, the GPA provides comparison of the complex shape and may reveal subtle changes,

such as the size and shape of flower parts in *Iris pumila* L. (Tucić *et al.*, 2018), conceivably omitted or hardly detectable by the simple analysis.

7.1.3 Chloroplast number estimation

Previously used methods of chloroplast number estimation include methods applied in two-dimensional space, such as (a) macerating and flattening the cells, which reduces the risk of overlap of the chloroplasts during observation under the microscope and enables to count the chloroplasts in one layer, and (b) cutting the leaf and counting profiles of chloroplasts on the cross section. Newly introduced method for chloroplast counting is the optical disector method.

When considering the method to be used for chloroplast number estimation, it is important to choose a proper method, which is unbiased, that means that all counted particles (in this case chloroplasts) have the same probability to be sampled (Sterio, 1984).

The chloroplast counting in flattened mesophyll cells of herbaceous plants was described by Possingham and Saurer (1969) on *Spinacia oleracea* L. leaves: At first, the leaves were fixed, then macerated to separate the mesophyll cells, which were then flattened by a cover slip while making microscopic slide. That enabled the chloroplasts to appear in a single plane of focus so that their counting under an optical microscope was feasible. This method was further developed for other herbaceous plants. Regarding unbiasedness, as far as proper sampling of mesophyll cells is ensured, the maceration method provides unbiased results. It may be applied under the condition that it is possible to separate individual mesophyll cells, which may not be possible in coniferous needles with lignified cell walls (Kubínová *et al.*, 2014).

The chloroplast number per mesophyll cell is often estimated as the number of chloroplast profiles in leaf cross section using light or transmission electron microscopy (Boffey *et al.*, 1979; Wang *et al.*, 2004; Jin *et al.*, 2011; Du *et al.*, 2019). This method is based on the assumption that the number of chloroplast profiles on a 2D cross section corresponds with the real chloroplast number in the entire 3D cell. However, we claim that the chloroplasts are spread in cytoplasm of the cell and that it is not likely that chloroplast profiles on a single optical section would unbiasedly represent the total chloroplast number per cell – for illustration see our 3D model of a mesophyll cell and its cross sections (Fig. 5 in Kubínová *et al.*, 2014). A comparison of counted profiles of chloroplasts and chloroplasts counted in

a whole single isolated cell could be made from the data in the study of Stata *et al.* (2014): From the diagram in the Supplementary figure S6 could be deduced that C3 and C4 plant species in the study had approximately 46 and 20 chloroplasts per cell, respectively. However, in the same study in Table 1. the “chloroplast number per planar cell per area 1000 μm^2 ”, which may correspond to a single cell cross section, were 17.4 ± 2.7 and 8.1 ± 1.6 for C3 and C4 plant species, respectively.

In contrary to the above mentioned methods, the optical disector method based on 3D unbiased sampling probe enables unbiased estimation of the number of particles in 3D (Sterio, 1984; Gundersen, 1986). It has already been used for estimation of mesophyll cell number (Albrechtová and Kubínová, 1991; Kubínová, 1991, 1993, 1994; Kubínová *et al.*, 2002; Albrechtová *et al.*, 2007) and we have adapted it and used it for chloroplast number estimation (Kubínová *et al.*, 2014).

We conclude that the disector method of counting chloroplasts in 3D showed to be more accurate than the frequently used profile counting in 2D cell cross sections when the number of chloroplasts is immensely underestimated (Kubínová *et al.*, 2014).

7.1.4 Chloroplast area, starch grain area, and starch areal density estimation

In ultrastructural studies on TEM images, the area measurement of organelles or structures within them may be tedious as they cannot be automatically segmented. Starch grain area was previously measured using the point counting method (Kutík *et al.*, 2004; Kubínová and Kutík, 2007). Another method is the interactive segmentation method based on drawing a line along the borders of the object under study. Glanc (2016) compared these methods and found out that both methods provide comparable results and claimed the interactive segmentation method as faster and more suitable for compact structures, thus it was used to determine the chloroplast area, starch grain cross-section size and starch areal density (Kubínová *et al.*, 2019).

7.2 Critical review of measured characteristics

To reveal the effect of elevated CO₂ concentration and irradiance, the following assumptions support the selection of studied characteristics.

The thicker is the needle, the larger is its internal mesophyll area and the easier is the CO₂ diffusion into chloroplasts (Terashima *et al.*, 2006). Therefore we measured the needle volume to discover whether it is influenced by either CO₂ concentration or irradiance.

Shade needles of Norway spruce were reported to be shorter than sun needles (Gebauer *et al.*, 2012), hence we included needle length measurement in our study.

Elevated CO₂ enhances photosynthetic carbon gain (Leakey *et al.*, 2009). That may be caused by a higher rate of CO₂ assimilation. The course of photosynthesis is influenced by structural parameters within needle mesophyll (Terashima *et al.*, 2006), therefore we measured the needle outer surface area and chloroplast number per cell.

In order to reveal whether the supposed microscale irradiance gradient within the shoot exists, the relative light penetration was measured and subsequently the potential spatial variability of needles within a shoot was evaluated by determining the needle size and cross-section shape parameters.

Finally, number of chloroplasts in the first mesophyll layer was compared with the number within the whole needle cross section to determine whether the measurement in the first layer may be representative for the whole cross section. That is important for sampling. However, technical feasibility and time consumption of the method have to be considered too.

7.2.1 Effect of elevated CO₂ concentration and irradiance on needle structure

In this section, the effects of the studied factors on needle structure are discussed. They may affect the needle morphology, needle anatomy and even chloroplast ultrastructure. Chloroplast ultrastructure is thoroughly discussed in section 7.2.5 together with results of physiological measurements.

7.2.1.1 At the level of whole needle, irradiance was stronger morphogenic factor than elevated CO₂ concentration

In a previous study, needle volume was significantly influenced by irradiance – it was larger in sun than in shade needles, while it was not affected by elevated CO₂ (Lhotáková *et al.*, 2012). In accordance with that, we observed that sun needles had larger volume than shade needles no matter the CO₂ concentration (Kubínová *et al.*, 2018). However, we observed that the EC sun needles had larger volume than AC sun, while the EC shade needles had lower volume than AC shade (Kubínová *et al.*, 2018). In our study, needle volume was estimated using needle length and needle cross-section area, thus those two parameters are discussed below.

Regarding needle length, main differences were that sun needles were longer than shade needles and EC sun needles were longer than AC sun needles (Kubínová *et al.*, 2018). Shorter shade than sun needles have been already recorded (e.g. Gebauer *et al.*, 2012) and sun needle length decreased with canopy depth (Pokorný *et al.*, 2011). Pokorný *et al.* (2011) compared only sun needles and observed significantly longer EC than AC needles only in the year when more drought periods occurred and only in the three youngest needle age classes. However, needle length may be influenced not only by light, but also by nitrogen and soil-water availability, and temperature (Roberntz, 1999, Pokorný *et al.*, 2011). In EC, the trees were better adapted to drought due to larger total surface area of fine-absorbing roots (Pokorný *et al.*, 2011). Thus, AC trees may be more affected by water stress. However, their needles' relative water content was not significantly different from that of EC needles (Pokorný *et al.*, 2011). Therefore, shorter needles in AC may be caused by different water relations and adaptations in AC and EC trees.

Regarding needle cross-section area, the sun needles' cross-section area was generally larger in EC, with the exception of middle and tip cross sections of side needles (Fig. 4), while shade needles' cross-section area was smaller in EC as compared to AC (Kubínová *et al.*, 2018). Our results on sun needles were confirmed by the results of Kurepin *et al.* (2018), who observed larger cross-section area in Norway spruce sun needles under EC than under AC. That study was conducted on seedlings and our study on juvenile trees, thus it seems that this difference is not limited to seedlings.

As the irradiance had consistent influence on needle volume and thickness, while the effect of CO₂ was not detected or ambiguous, the hypothesis (H4) that irradiance is stronger morphogenic factor than elevated CO₂ concentration was accepted (Kubínová *et al.*, 2018).

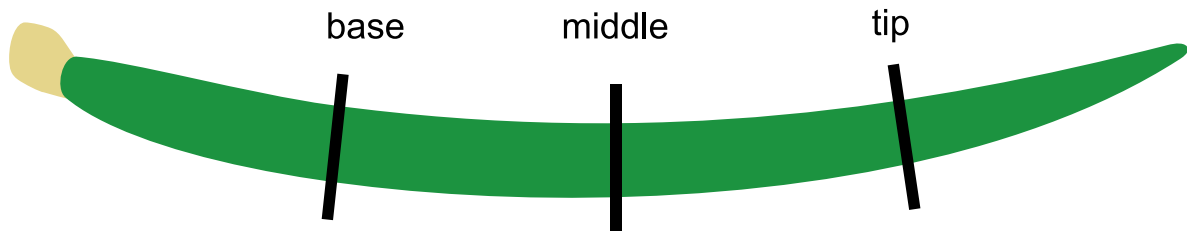


Fig. 4 Cross section positions along the needle termed base, middle and tip cross section.

7.2.1.2 At the level of mesophyll, chloroplast number was higher under elevated CO₂

In general, the chloroplast number per plant cell varies due to both environmental and internal factors. The environmental factors enhancing chloroplast number include increased light intensity (Possingham and Smith, 1972), specific light quality (red and blue light) (Possingham, 1973), and elevated CO₂ concentration (Wang *et al.*, 2004; Teng *et al.*, 2006). On the other hand, chloroplast number may decrease for example under nitrogen deficiency (Antal *et al.*, 2010), manganese deficiency (Henriques, 2004), water deficiency (Wang and Zhang, 2002), and elevated temperature (Kivimäenpää *et al.*, 2014).

The internal factors enhancing chloroplast number per cell include higher ploidy (Mochizuki and Sueoka, 1955), larger cell size (Tymms *et al.*, 1983) and chloroplast number may be also influenced by signalling compounds such as sugars (Butterfass, 1979, Van Digenen *et al.*, 2016). Cole (2016) claims that there may exist an optimal organelle number per cell determined by the ratio of nuclear- and organelle-produced subunits forming organellar complexes. In my opinion, the translation of these subunits will probably depend on external factors and may change with time.

Higher number of chloroplasts per mesophyll cell in EC was previously observed in herbaceous plants and attributed to stimulated chloroplast biogenesis (Wang *et al.*, 2004; Teng *et al.*, 2006). To my knowledge, no such studies for conifers exist, neither about influence of EC on chloroplast number, nor about the mechanisms how EC is affecting chloroplast number. In our study, the chloroplast number per mesophyll volume based on SUR measurement was significantly higher in needles growing in EC in comparison to needles growing in AC. However, it was not significantly different between sun and shade needles (Kubínová *et al.*, 2019). Thus the hypothesis (H5) that the chloroplast density is enhanced by CO₂ concentration was accepted.

7.2.2 The first mesophyll layer was not representative for the whole needle cross section regarding chloroplast density, starch grain area and starch areal density

The chloroplast number measured in SUR sampled locations on the needle cross section showed significant influence by the level of CO₂ concentration, while the same parameter measured from the same needle cross sections from locations sampled solely in the first mesophyll layer showed no significant difference between AC and EC (Kubínová *et al.*, 2019). Therefore, the hypothesis (H9) that the first mesophyll layer is representative for the whole needle cross section regarding chloroplast density in mesophyll was rejected.

Sampling approach considering possible differences in anatomy was applied in a study of the impact of ozone on Norway spruce needles (Kivimäenpää *et al.*, 2004), where the needle cross section was divided into five regions to perform an anatomical study. The first mesophyll layer on the sky-facing side of the needle was most affected by the air pollution (Kivimäenpää *et al.*, 2004). In such kind of studies, it may be advantageous to analyse just the most affected part of the mesophyll providing that the purpose of the study is to assess the scale of air pollution damage. However, in studies focused on overall reaction of needle anatomy to some factor, the possible heterogeneity of structural characteristics within the needle should be considered. For example, light penetration may differ within the needle.

The starch grain area and starch areal density of sun needles in AC were significantly larger in SUR locations than in the first layer of mesophyll (Kubínová *et al.*, 2019). Therefore, the hypotheses (H10) that the first mesophyll layer is representative for the whole needle cross section regarding starch grain area and (H11) regarding starch areal density were rejected. The hypothesis (H12) that the chloroplast area in the cross section in the first mesophyll layer is representative for the whole needle cross section regarding starch grain area was not clearly rejected (Kubínová *et al.*, 2019). However, in SUR there was a trend to larger chloroplast area in shade than in sun AC needles, while that was not the case in the first mesophyll layer (Kubínová *et al.*, 2019), thus I would not consider the first mesophyll layer as representative for the chloroplast area.

In conclusion, the SUR sampling is recommended to apply in anatomical studies. However, regarding the TEM measurement, the technical aspects of sample preparation must be taken in account. In some cases, such as when the contrast on TEM sample in deeper layers of mesophyll is so low that it is not possible to recognise the structures of interest, the SUR sampling is not feasible to perform (Kubínová *et al.*, 2019).

7.2.3 Needle self-shading caused irradiance microscale gradient within a shoot

Vertical irradiance gradient within a canopy and within an individual tree caused by shoots and needles overlapping is taken into account in ecophysiological studies. This gradient is often pronounced: up to only one third of the photosynthetic photon flux density (PPFD) present at the top was present at the bottom of Norway spruce tree crown (Reiter *et al.*, 2005) and similarly, only a few percent of the value of PPFD at the fully exposed locations were present at the lowest branches of selected conifers (Wyka *et al.*, 2012).

These distinct differences in light availability influence the conifer shoot architecture. That may be described by the parameter STAR_{max} (maximum ratio of shoot silhouette area to total needle surface area, measured when the shoot axis is perpendicular to the direction of irradiance; Carter and Smith, 1985; Stenberg, 1996). On a sun shoot, the needles appear all around the shoot axis, while on a shade shoot the needles grow prevalently in the horizontal direction (Stenberg, 1996; Cescatti and Zorer, 2003; Ishii *et al.*, 2012; Fig. 3 in Kubínová *et al.*, 2018). Higher STAR_{max} of shade than sun shoots was noticed in *Picea engelmannii* Parry ex Engelm. (Carter and Smith, 1985), it was probably caused by less needles growing around the shade shoot and thus lower needle surface area of shade shoots, which act as a denominator in the STAR_{max} formula.

Despite the fact that the conifer shoot architecture is commonly examined and thus irradiance gradient is taken into account at the tree level (Stenberg *et al.*, 1998; Suzaki *et al.*, 2003; Gebauer *et al.*, 2019), to our knowledge, the probable irradiance gradient within an individual shoot and its possible effect on needles within the same shoot have not yet been examined.

In our study, we have measured the relative light penetration under artificial laboratory conditions and found such within-shoot gradient in Norway spruce sun and shade shoots. Lower needles (i.e. growing on the bottom of the shoot) received $34.29\% \pm 0.19$ and $53.76\% \pm 0.20$ of the light received by upper needles on the same shoot in sun and shade shoots, respectively (Kubínová *et al.*, 2018). Thus, we concluded that needle self-shading caused irradiance microscale gradient within a shoot and accepted H1 hypothesis.

7.2.4 Microscale gradient in irradiance caused needle morphology differentiation around the shoot

In species with dorsiventral leaves, sun and shade leaves develop under the irradiance gradient (Stahl, 1883; Wylie, 1949). Similarly in conifers, differences in needle structural parameters between sun and shade needles were observed: Sun needles were thicker than shade ones (Korstian, 1925; Niinemets, 2007).

We have studied parameters of individual Norway spruce needles within a shoot and revealed that needle orientation on a shoot had significant influence on needle cross-section area (Tables 1 and 2 in Kubínová *et al.*, 2018). Upper needles had larger cross-section areas than did side needles, and side needles had larger cross-section areas than did lower needles (Kubínová *et al.*, 2018). Sun needle cross-section areas were larger than shade needle cross-section areas, thus upper needles' areas corresponded to sun needles' ones regardless of the shoot position. However, mean needle length was significantly greater and needle volume larger in sun needles as compared to shade needles, while upper needles were shorter than needles in other orientations and thus needle volume was not dependent on needle orientation (Kubínová *et al.*, 2018).

Therefore, the hypothesis (H2) that needle spatial orientation on a shoot determines needle size similar to sun and shade needle ecotypes differentiation was rejected: The needle spatial orientation around the shoot determined the cross-section area similarly to sun and shade needle ecotypes, while the needle length was in counteraction, thus the needle volume corresponded to the shoot position.

In the middle cross section of the needle, the sun needles were significantly less flat than shade needles. Similarly, upper needles were significantly less flat than needles from other orientations in both CO₂ experimental conditions, irrespective of irradiance. Therefore, the hypothesis (H3) that needle spatial orientation on a shoot determines needle cross-section shape similar to sun and shade needle ecotypes differentiation was accepted.

In conclusion, there are differences between needles not only within the canopy and within the individual tree, but even within the shoot. Therefore, the individual position of the needle on a shoot may have impact on its analysed parameters in anatomical studies and should to be taken into account.

7.2.5 Elevated CO₂ concentration enhanced the light-saturated CO₂ assimilation rate and lead into higher amount of starch and thus larger starch grain cross-section area in sun needles

In this section, the chloroplast ultrastructure is discussed together with the photosynthetic parameters measured on the trees from the same experimental site.

Firstly, the hypothesis (H8) that chloroplast cross section area is larger under elevated CO₂ concentration was rejected in our study on needles sampled in October (Kubínová *et al.*, 2019). Similarly, needles of *Pinus palustris* P. Mill. sampled in autumn had no significant differences in chloroplast area in the cross section between AC and EC treatments (Pritchard *et al.*, 1997). However, EC needles sampled in spring in the same study had in most cases larger chloroplast area in the cross section than AC needles, probably due to larger starch grains' area (Pritchard *et al.*, 1997). Senser *et al.* (1975) observed seasonal differences and noticed largest chloroplast area in *Picea abies* in AC in winter. Thus the effect of season probably mitigated the difference between AC and EC chloroplast area in our study.

Larger starch grain area and starch areal density were observed in sun EC needles in comparison with sun AC needles, however, in shade needles, no significant difference was observed; needles were sampled in October (Kubínová *et al.*, 2019). Therefore, the hypotheses that (H6) starch grain area and (H7) starch areal density are larger under elevated CO₂ concentration were rejected. In agreement with our anatomical analysis results, higher amounts of starch in EC needles were detected biochemically for sun shoots of the same trees in August two years before our sampling (Mašková *et al.*, 2017). The increased amount of starch may be a result of increased light-saturated CO₂ assimilation rate (A_{\max}) and it is discussed later. However, proportion of starch in chloroplast and number of starch grains in cross sections in EC were not different from AC and chloroplast cross section area was not significantly different between AC and EC (Mašková *et al.*, 2017). Measurements conducted on the same trees after next two years of treatment revealed generally more starch in July than in October in AC needles, while in EC needles the amount of starch remained similar until October, probably due to postponed winter hardening in EC (Holá *et al.*, manuscript in preparation). Thus, the increased amount of starch may be a result of increased A_{\max} but it may be also a result of other processes. The proportion of starch in chloroplast cross sections depended on the season and thus the month of sampling has to be considered.

The A_{\max} values of following current sun and shade Norway spruce shoots of trees of different age, month of measurement and EC experiment duration from the Bílý Kříž

experimental site are discussed further: Shoots of 12-year-old spruces being under EC for 7 years, measured in September (Kubínová *et al.*, 2018), shoots of 12-year-old spruces being under EC for 4 years, measured in September (Urban *et al.*, 2012a) and finally, shoots of 18-year-old spruces being under EC for 8 years, measured in August (Lhotáková *et al.*, 2012).

A_{\max} in current sun EC shoots was about 47% higher than in current sun AC shoots (Kubínová *et al.*, 2018). However, Urban *et al.* (2012) measured approximately 70% increase of A_{\max} and Lhotáková *et al.* (2012) measured approximately 100% increase of A_{\max} in current sun shoots being under EC than in current sun shoots under AC. In current shade shoots, A_{\max} in EC was about 97% higher than A_{\max} of the corresponding AC shoots (Kubínová *et al.*, 2018). However, Lhotáková *et al.* (2012) measured approximately only 50% increase of A_{\max} in EC. Generally, A_{\max} is increased in EC (Stinziano and Way, 2014; Dusenge *et al.*, 2019). Thus, the result that A_{\max} was higher in EC was in accordance with previous studies, but the magnitude of the enhancement differed. Other factors included in determining A_{\max} besides irradiance and elevated CO_2 treatment duration may be e.g. nutrient availability and sink strength (Dusenge *et al.*, 2019). Regarding the role of irradiance, diurnal changes in A_{\max} occurred (Bader *et al.*, 2016) and the photosynthesis was enhanced under cloudy conditions (Urban *et al.*, 2012b). The photosynthesis regulation is very complex and sensitive. The factors such as preceding weather condition during the season may play an important role. Therefore, further studies should be performed to determine the relationship between the factors studied so far.

In the above mentioned studies, A_{\max} was higher in EC than in AC. The selected results from the studies were done on current shoots. However, acclimation (decreasing A_{\max} after long-term EC) may occur in older shoots (Marek *et al.*, 2002; Holišová *et al.*, 2012). Lamba *et al.* (2017) observed that mature trees in EC were affected by acclimation. However, her study was done on one-year-old shoots, thus the effects of tree and shoot age may be hard to distinguish. Additionally, in one-year-old shoots A_{\max} was also higher in EC than in AC (Urban *et al.*, 2012a). Thus, the relationship between A_{\max} , EC and tree and shoot age remains to be explained by further studies.

The higher A_{\max} occurred in current year needles in EC in spite of lower increase in Rubisco content and in one-year-old needles in spite of the steeper decrease of Rubisco content in EC than in AC during the vegetation season, because higher proportion of Rubisco active form was present in EC at the end of the vegetation season (Urban *et al.*, 2012a). In EC, the dark respiration rate (R_D) and apparent quantum efficiency (AQE) were lower than in AC (Lhotáková *et al.*, 2012). In July, the quantum yield of photosystem II

(PSII) photochemistry (the proportion of light absorbed by PSII chlorophyll) was higher in EC samples than in AC samples when measured in EC (Holá *et al.*, manuscript in preparation). However, no significant differences were observed when measured in AC and under any conditions in current needles in October in 11-year-old spruces being under EC for 6 years. Moreover, not only season, but also needle age plays role in response to EC (Holá *et al.*, manuscript in preparation).

In general, photorespiration (i.e. the consequence of oxygenase activity of Rubisco) may be lower due to higher proportion of CO₂ leading to increased probability that Rubisco will fix CO₂ and that its oxygenase activity will be reduced (Dusenge *et al.*, 2019). However, in EC the ratio of leaf internal to atmospheric CO₂ concentration may not significantly differ from AC as it was observed e.g. in *Eucalyptus* (Gimeno *et al.*, 2016).

Stimulation of photosynthesis in EC possibly enhanced growth of needles, while the number of needles per unit shoot length was not affected in 17- to 19-year-old spruces grown in the domes for 6–8 years (Pokorný *et al.*, 2011). Thus, trends to longer sun needles, larger projected sun needle area and larger leaf mass per sun needle projected area in EC than in AC were noticed, while these parameters differed significantly only in the youngest needles in the year when drought periods occurred (Pokorný *et al.*, 2011). Similarly, we found current sun EC needles longer than sun AC needles (Kubínová *et al.*, 2018). It may be caused by the fact that spruces in EC had higher absorbing root area and thus enhanced root to conductive stem area proportions, which, accompanied by early seasonal stimulation of photosynthesis, lead to advanced needle development (Pokorný *et al.*, 2011). Moreover, EC induced higher growth of the leading shoot and longer 6th whorl branches (Lhotáková *et al.*, 2012). In accordance with that, Mašková *et al.* (2017) found out that total soluble carbohydrate levels and composition did not differ between the AC and EC treatments despite of the increased CO₂ assimilation rate with no increase in dark respiration in EC current shoots observed by Holišová *et al.* (2012). Both studies were conducted on the same 9-year-old spruces grown 4 years in the domes. Mašková *et al.* (2017) supposed that the surplus carbohydrates were used to support growth and sink organs development. This assumption based on the results of biochemical analysis and physiological measurements was supported by anatomical studies.

In conclusion, the synthesis of knowledge from different types of analyses may enable deeper insight into complex processes, such as the reaction of Norway spruce on elevated CO₂ concentration.

8. Summary

The main results of this thesis are that 1) Norway spruce needle morphological and anatomical parameters are influenced not only by macroscale irradiance gradient, but also by microscale irradiance gradient around the single shoot (H1 and H3 were accepted) and that 2) the anatomical characteristics are not homogenous even within an individual needle (H9–H11 were rejected; however, H12 was not clearly rejected). Therefore, systematic uniform random sampling and precise analytical methods, which were presented in the enclosed publications, should be used to obtain unbiased results. The presented methods are universal and can be used in other anatomical studies.

In the studies presented in this thesis, irradiance was stronger morphogenic factor than elevated CO₂ concentration (H4 was accepted). Shape differences among the middle needle cross sections within the same shoot were observed (H3 was accepted). Upper needles, i.e. needles growing from the upward side of the shoot, were significantly less flat than side needles, while lower needles were significantly flatter than side needles. Similarly, the needles from shade shoots were flatter than needles from sun shoots. The needle spatial orientation determined the cross-section area similarly to sun and shade needle ecotypes, while upper needles were shorter than needles of other orientations. Thus the needle volume did not depend on needle orientation (H2 was rejected).

Regarding the effect of elevated CO₂ concentration, light-saturated CO₂ assimilation rate was stimulated and photosynthetic acclimation was not observed. However, we measured the current shoots and the acclimation probably occurs mostly in older shoots.

The chloroplast density was found to be enhanced by EC (H5 was accepted). Larger starch grain area and starch areal density on chloroplast cross section in EC, significant in sun needles only (H6–H8 were rejected), were probably caused by stimulated light-saturated CO₂ assimilation rate. However, our observation may be influenced by the seasonal dynamics of starch areal density in EC.

In conclusion, the response of needle quantitative parameters to elevated CO₂ concentration and irradiance was related to responses of physiological characteristics and revealed that the changes in anatomy were interconnected with physiological changes. Thus it was proven that the anatomical studies help in the synthesis of knowledge and may support the findings made from other types of analyses. Moreover, the presented methods enable to capture even subtle changes in the needle morphology and anatomy and thus may stimulate new research questions, which may not arise from merely physiological research.

9. Summary in Czech – Závěry práce v českém jazyce

Hlavním výsledkem disertační práce jsou poznatky, že 1) morfologické a anatomické vlastnosti jehlic smrku ztepilého jsou ovlivňovány ozářeností nejen ve větším měřítku, ale i v malém měřítku v případě rozdílné ozářenosti v rámci výhonu (H1 a H3 byly přijaty) a že 2) anatomické vlastnosti nejsou homogenní ani v rámci jedné jehlice (H9–H11 byly zamítnuty; nicméně H12 nebyla jednoznačně zamítnuta). Z toho vyplývá, že k získání nevychýlených výsledků je potřeba používat systematicky rovnoměrně náhodné vzorkování a přesné metody analýzy obrazu, které byly prezentovány v příložených publikacích. Uvedené metody jsou univerzální a mohou být použity i v jiných anatomických studiích.

V předložených publikacích působila ozářenost jako morfogenní faktor silněji než zvýšená koncentrace CO₂ (H4 byla přijata). U jehlic z téhož výhonu byly pozorovány rozdíly v jejich tvaru na prostředním řezu (H3 byla přijata). Horní jehlice, tj. jehlice vyrůstající z horní části výhonu, byly méně zploštělé než postranní jehlice, zatímco spodní jehlice byly významně více zploštělé než postranní jehlice. Obdobně jehlice ze stinných výhonů byly více zploštělé než jehlice z výhonů slunných. Orientace jehlice na výhonu ovlivnila plochu řezu podobně jako u slunných a stinných jehlic, zatímco horní jehlice byly kratší než ostatní jehlice. Objem jehlice proto nezávisel na její orientaci na výhonu (H2 byla zamítnuta).

Efekt zvýšené koncentrace CO₂ se projevil hlavně zvýšením maximální rychlosti světlem saturované asimilace CO₂, přičemž fotosyntetická aklimace nebyla pozorována, což mohlo být proto, že byly výhony narostlé v roce měření.

Hustota chloroplastů byla zvýšena při zvýšené koncentraci CO₂ (H5 byla potvrzena). Větší plocha škrobových zrn a její poměrné zastoupení na řezu chloroplastem při zvýšené koncentraci CO₂, signifikantní pouze u slunných jehlic (H6–H8 byly zamítnuty), byly pravděpodobně způsobeny zvýšenou maximální rychlostí světlem saturované asimilace CO₂. Nicméně naše pozorování může být ovlivněno sezónními změnami v poměrném zastoupení škrobu na řezu chloroplastem v EC.

Změny v kvantitativních parametrech jehlic v reakci na zvýšenou koncentraci CO₂ a ozářenost odpovídaly změnám fyziologických parametrů a odhalily propojení anatomických změn s fyziologickými. Bylo tak prokázáno, že anatomické studie pomáhají v syntéze znalostí a mohou podpořit poznatky z jiných typů analýz. Předložené metody navíc umožňují zachytit i méně patrné změny v morfologii a anatomii jehlic, čímž mohou podnítit nové výzkumné otázky, které by nemusely vyvstat z čistě fyziologického výzkumu.

10. List of publications

Following publications are presented as part of the thesis. Their full texts are in the appendix, which is not publicly available on-line. However, the full texts may be available under the hypertext links below each citation.

The participation of the thesis author, Zuzana Kubínová (ZK), on each publication is described below.

10.1 Kubínová Z, Janáček J, Lhotáková Z, Kubínová L, Albrechtová J. 2014. Unbiased estimation of chloroplast number in mesophyll cells: advantage of a genuine three-dimensional approach. *Journal of Experimental Botany* 65, 609–620.

<https://doi.org/10.1093/jxb/ert407>

ZK participated in establishing the design of the study, acquired and analysed the confocal microscopic data and contributed to the manuscript writing, figures processing and final editing.

10.2 Kubínová Z, Janáček J, Lhotáková Z, Šprtová M, Kubínová L, Albrechtová J. 2018. Norway spruce needle size and cross section shape variability induced by irradiance on a macro- and microscale and CO₂ concentration. *Trees* 32(1), 231–244.

<https://doi.org/10.1007/s00468-017-1626-3>

ZK had the idea to evaluate Norway spruce needle shapes, participated in material collection, processed the needles, acquired the microscopic data, placed the landmarks, finalised the figures and contributed to writing and final editing of the manuscript.

10.3 Kubínová Z, Glanc N, Radochová B, Lhotáková Z, Janáček J, Kubínová L, Albrechtová J. 2019. Unbiased estimation of Norway spruce (*Picea abies* L. Karst.) chloroplast structure: Heterogeneity within needle mesophyll under different irradiance and [CO₂]. *Image Analysis & Stereology* 38(1), 83–94.

<https://doi.org/10.5566/ias.2005>

ZK participated in establishing the design of the study, participated in material collection, acquired and analysed the confocal microscopic data and contributed to the manuscript writing, figures processing and final editing.

10.4 Kubínová L, Radochová B, Lhotáková Z, Kubínová Z, Albrechtová J. 2017.

Stereology, an unbiased methodological approach to study plant anatomy and cytology: past, present and future. *Image Analysis & Stereology* 36(3), 187–205.

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ZK contributed to chloroplast counting part, participated in editing and did final references processing.

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