Photoprotective Isoprenoids as Indicators for Stress Responses in Forest Trees

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Cell and Systems Biology
University of Toronto

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2017

Abstract

For long-lived forest tree species, intraspecific variation among populations in their response to environmental conditions can reveal their ability to cope with and adapt to climate change. Plants constantly adjust the pigment composition of the photosynthetic apparatus to environmental conditions. Under abiotic stress conditions (e.g. drought), plants induce isoprenoid-mediated photoprotective mechanisms such as non-photochemical quenching (NPQ) and increased biosynthesis of antioxidants to minimize photooxidative stress. This thesis investigated the intraspecific variation in the isoprenoid metabolism in Douglas-fir (*Pseudotsuga menziesii*) comparing Douglas-fir provenances, which represent populations from locations with contrasting environmental conditions. Furthermore, the influence of foliar photosynthetic pigments on leaf optical properties was studied using senescing sugar maple (*Acer saccharum*) leaves. First, I established a simple and cost-effective protocol for the rapid analysis of isoprenoids using high-performance liquid chromatography (HPLC) (Chapter 2). Three experiments were conducted to evaluate 1) the adjustments of the isoprenoid metabolism when photosynthesis is limited in response to environmental conditions (Chapter 3 and 4); 2) provenance-specific adjustments of
photoprotective isoprenoids in response to drought (Chapter 3 and 4); and 3) the influence of senescence-associated changes in isoprenoid levels on leaf optical properties in sugar maple (Chapter 5). In chapter 3, photosynthesis and photosynthetic pigments in seedlings of two Douglas-fir provenances were compared under controlled drought conditions. In chapter 4, intraspecific variation in photosynthesis and photosynthetic pigments in response to changing environmental conditions were studied in mature trees of four provenances over the course of two years. Both experiments revealed that the more drought-tolerant interior provenances exhibit enhanced carotenoid-chlorophyll ratios, and larger pools of xanthophyll cycle pigments and β-carotene compared to coastal provenances from mesic habitats. This provenance-specific variation demonstrated the importance of the isoprenoid metabolism for the adaptation of provenances to drought. In chapter 5, the leaf optical properties of senescing sugar maple leaves were studied. The degradation of photosynthetic pigments as indicator for the progress of senescence was reflected by spectral reflectance measurements and digital image analysis. Isoprenoid metabolism may thus be a potential trait for selection of provenances for future forest management and indicator for remote-sensing of the plant physiological status.
Acknowledgments

I thank my supervisor Professor Ingo Ensminger, for taking me on as a graduate student and making me part of his groups in Freiburg and Toronto. I appreciate his enthusiasm, ideas and guidance. I am especially grateful that I was allowed to expand the topic of my PhD by conducting the additional project on maple leaves.

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I acknowledge Prof. Andreas Hamann for taking on my thesis as my external examiner and Prof. Katharina Bräutigam for serving on my final oral examination committee.

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<tr>
<td>$\alpha$Car</td>
<td>$\alpha$-carotene</td>
</tr>
<tr>
<td>$\alpha$Toc</td>
<td>$\alpha$-tocopherol</td>
</tr>
<tr>
<td>$\beta$Car</td>
<td>cis-$\beta$-carotene</td>
</tr>
<tr>
<td>$\Delta^{13}$Car</td>
<td>discrimination against $^{13}$C in water soluble organic matter</td>
</tr>
<tr>
<td>$\Phi$PSII</td>
<td>effective quantum yield of photosystem II</td>
</tr>
<tr>
<td>$\Psi$</td>
<td>water potential</td>
</tr>
<tr>
<td>A</td>
<td>rate of photosynthetic carbon assimilation</td>
</tr>
<tr>
<td>ABA</td>
<td>abscisic acid</td>
</tr>
<tr>
<td>Acetyl-CoA</td>
<td>acetyl coenzyme A</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>Ant</td>
<td>antheraxanthin</td>
</tr>
<tr>
<td>ARI</td>
<td>anthocyanin reflectance index</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>bCC</td>
<td>blue chromatic coordinate</td>
</tr>
<tr>
<td>CAM</td>
<td>coastal Douglas-fir provenance Cameron Lake</td>
</tr>
<tr>
<td>Car</td>
<td>carotenoids</td>
</tr>
<tr>
<td>Chl</td>
<td>chlorophyll</td>
</tr>
<tr>
<td>Chl a</td>
<td>chlorophyll a</td>
</tr>
<tr>
<td>Chl a+b</td>
<td>total chlorophylls</td>
</tr>
<tr>
<td>Chl a/b</td>
<td>ratio of chlorophyll a to chlorophyll b</td>
</tr>
<tr>
<td>Chl b</td>
<td>chlorophyll b</td>
</tr>
<tr>
<td>cis-$\beta$Car</td>
<td>cis-$\beta$-carotene</td>
</tr>
<tr>
<td>COA</td>
<td>coastal Douglas-fir provenance Snoqualmie</td>
</tr>
</tbody>
</table>
CON  coastal Douglas-fir provenance Conrad Creek
DEPS  de-epoxidation state of the xanthophyll cycle
DMAPP  dimethylallyl diphosphate
DXP  1-deoxy-D-xylulose 5-phosphate
DW  dry weight
E  transpiration rate
ExG  Excess green index derived from digital image analysis
ExG\textsubscript{M}  Excess green index derived from spectral reflectance measurements
FPP  farnesyl diphosphate
F\textsubscript{m}  dark-adapted maximum fluorescence of photosystem II
F\textsubscript{m}'  light-adapted maximum fluorescence of photosystem II
F\textsubscript{o}  dark-adapted minimum fluorescence of photosystem II
F\textsubscript{o}'  light-adapted minimum fluorescence of photosystem II
F\textsubscript{t}  transient fluorescence
F\textsubscript{v}/F\textsubscript{m}  maximum quantum yield of photosystem II
G3P  glyceraldehyde 3-phosphate
g\textsubscript{CC}  green chromatic coordinate
GGPP  geranyl-geranyl-diphosphate
GPP  geranyl-diphosphate
GRVI  green red vegetation index
g\textsubscript{s}  stomatal conductance
HPLC  high-performance liquid chromatography
INT  interior Douglas-fir provenance Fehr Lake
IWUE  intrinsic water use efficiency
HMG-CoA  3-hydroxy-3-methylglutaryl-coenzyme A
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPP</td>
<td>isopentenyl diphosphate</td>
</tr>
<tr>
<td>LHC</td>
<td>light-harvesting complexes</td>
</tr>
<tr>
<td>LMA</td>
<td>leaf mass per area</td>
</tr>
<tr>
<td>Lut</td>
<td>lutein</td>
</tr>
<tr>
<td>LutEpox</td>
<td>lutein epoxide</td>
</tr>
<tr>
<td>MEP</td>
<td>2-C-methylerythritol 4-phosphate</td>
</tr>
<tr>
<td>MVA</td>
<td>mevalonic acid</td>
</tr>
<tr>
<td>ND</td>
<td>not determined</td>
</tr>
<tr>
<td>NDI</td>
<td>Normalized difference index</td>
</tr>
<tr>
<td>NDVI</td>
<td>Normalized difference vegetation index</td>
</tr>
<tr>
<td>Neo</td>
<td>neoxanthin</td>
</tr>
<tr>
<td>n.s.</td>
<td>not significant</td>
</tr>
<tr>
<td>NPQ</td>
<td>nonphotochemical quenching</td>
</tr>
<tr>
<td>pF</td>
<td>soil water tension expressed in log(-ψ(cm H₂O))</td>
</tr>
<tr>
<td>phytlyl-PP</td>
<td>phytlyl-diphosphate</td>
</tr>
<tr>
<td>PRI</td>
<td>photochemical reflectance index</td>
</tr>
<tr>
<td>PSI</td>
<td>photosystem I</td>
</tr>
<tr>
<td>PSII</td>
<td>photosystem II</td>
</tr>
<tr>
<td>PTFE</td>
<td>polytetrafluoroethylene</td>
</tr>
<tr>
<td>R²</td>
<td>coefficient of determination</td>
</tr>
<tr>
<td>r(CC)</td>
<td>red chromatic coordinate</td>
</tr>
<tr>
<td>Rd</td>
<td>rate of cellular respiration in the absence of light (dark respiration rate)</td>
</tr>
<tr>
<td>rETR₁</td>
<td>electron transport rates at PSI</td>
</tr>
<tr>
<td>rETR₂</td>
<td>electron transport rates at PSII</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>------</td>
<td>------------</td>
</tr>
<tr>
<td>Rubisco</td>
<td>ribulose-1,5-bisphosphate carboxylase/oxygenase</td>
</tr>
<tr>
<td>SAL</td>
<td>interior Douglas-fir provenance Salmon Arm</td>
</tr>
<tr>
<td>SAN</td>
<td>coastal Douglas-fir provenance Santiam River</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SE</td>
<td>standard error</td>
</tr>
<tr>
<td>Sun</td>
<td>sunshine duration</td>
</tr>
<tr>
<td>T</td>
<td>temperature</td>
</tr>
<tr>
<td>TAW</td>
<td>total available soil water</td>
</tr>
<tr>
<td>USGS</td>
<td>U.S. Geological Survey</td>
</tr>
<tr>
<td>Vio</td>
<td>violaxanthin</td>
</tr>
<tr>
<td>VAZ</td>
<td>xanthophyll cycle pigments per total carotenoids</td>
</tr>
<tr>
<td>Zea</td>
<td>zeaxanthin</td>
</tr>
</tbody>
</table>
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Table 2.1: Solvent system developed for separation of chlorophylls, carotenoids and tocopherols using a C30-column. A) the standard gradient was developed to include all pigments. B) A modification of the standard method with a shorter run-time. This modified method can be used when cis-β-carotene the most abundant nonpolar carotenoid and uses less MTBE. Solvent A: methanol; Solvent B: methyl-tert-butyl-ether (MTBE); Solvent C: water buffered with 20 mM ammonium acetate, pH 6.

Table 3.1: The effect of provenance and treatment on biomass, photosynthetic gas exchange and isoprenoid metabolism. For each parameter, the best model including Provenance, Treatment and the interaction thereof was chosen according to the Akaike information criterion (AIC). The effects of Treatment and Provenance were assessed by pairwise comparison of models with and without each factor with and without each factor using a log-likelihood ratio test. The significance of Significance levels are given as p-values which are bolded if p<0.05. IWUE = Intrinsic water use efficiency. Chlorophylls a+b = total chlorophyll per fresh weight, Carotenoids = total carotenoids per total chlorophyll, VAZ = xanthophyll cycle pigments per total carotenoids, DEPS= de-epoxidation status of the xanthophyll cycle pigments, Lutein = Lutein per total carotenoids, Neoxanthin = Neoxanthin per total carotenoids, β-carotene = β-carotene per total carotenoids, α-carotene = α-carotene per total carotenoids, Stored monoterpenes = total stored monoterpenes per dry weight, stored sesquiterpenes = total stored sesquiterpenes per dry weight, Emission of monoterpenes = total monoterpenes emissions.

Table 3.2: Cardinal points of the light response curves of an interior and a coastal Douglas-fir provenance under control conditions, drought and recovery. Maximum assimilation rate, light intensity at half maximum assimilation rate and light compensation point were estimated from exponential curve-fitting of gas exchange measurements obtained from N=5 (±SE) seedlings per provenance and treatment. Significant differences (p < 0.05 using Kruskal-Wallis-rank-sum-test) are indicated by different letters.

Table 3.3: Composition of volatile isoprenoid pools and emissions. The percentage of the five most abundant stored monoterpenes and sesquiterpenes as well as emitted monoterpenes per provenance are listed. For control plants data from all samplings was averaged (N=15, ±SE),
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**Table 4.1:** Climatic and geographical details of the field sites (elevation and mean annual parameters taken from Kenk and Ehring (2004).

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**Table 4.3:** Weather conditions and twig water potential during measurement campaigns. 14-day-cumulative precipitation, mean daily temperature, sunshine duration (Sun), and total available soil water (TAW) were averaged over the duration of field campaign (±SD). Twig water potential of Douglas-fir (*Pseudotsuga menziesii*) was assessed at the end of each campaign in 2011 in n = 12 trees, equally distributed between provenance (±SD; no provenance-specific differences were detected). NA = Not assessed.

**Table 4.4:** Effect of Provenance and Site on photosynthetic gas exchange, isotope discrimination, chlorophyll fluorescence, and isoprenoids assessed by two-way ANOVA, as well as correlation between these physiological parameters to environmental conditions revealed by Pearson-correlation. Section ANOVA shows the p-values from two-way ANOVA between Provenance and Site including interactions (Prov:Site). Section Pearson-Correlation shows the correlation coefficients of the Pearson's product-moment correlation test for each physiological parameter to the three environmental variables total available soil water (TAW), mean daily temperature (Temp) and sunshine hours per day as proxy for solar radiation (Sun). Significance with p < 0.05 is indicated by bolded numbers. A = assimilation rate, gs = stomatal conductance, IWUE = Intrinsic water-use efficiency, Rd = dark respiration, Δ^{13}\text{C}_{WSOM} = discrimination against $^{13}$C in water soluble organic matter, Fv/Fm = maximum quantum yield of dark-adapted needles, ΦPSII = yield, NPQ = non-photochemical quenching, Chl a+b = total chlorophyll per fresh weight, Carotenoids = total carotenoids per total chlorophyll, VAZ = xanthophyll cycle pigments per total chlorophyll, DEPS = de-epoxidation status of the xanthophyll cycle pigments, monoterpenes = total stored volatile isoprenoids per dry weight, emitted monoterpenes = total monoterpane emissions.
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**Fig. 1.1:** Schematic representation of the two isoprenoid biosynthesis pathways, the cytosolic mevalonate pathway and the plastidal non-mevalonate pathway. ABA, abscisic acid; Acetyl-CoA, Acetyl coenzyme A; DMAPP, dimethylallyl diprophosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; FPP, farnesyl diprophosphate; G3P, glyceraldehyde 3-phosphate; GPP, geranyl-diphosphate; GGPP, geranyl-geranyl-diphosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; IPP, isopentenyl diprophosphate; MEP, 2-C-methylerythritol 4-phosphate; MVA, mevalonic acid. Scheme was drawn following Rodríguez-Concepción et al. (2004).

**Fig. 1.2:** The carotenoid biosynthetic pathway of higher plants diverges into two branches (scheme was drawn following García-Plazaola et al. (2007) and modified according to Havaux (2014)).

**Fig. 1.3:** The habitat of coastal (*Pseudotsuga menziesii* var. menziesii, shown in blue) and interior Douglas-fir (*P. menziesii* var. glauca, shown in green) along the North American west coast. Map adapted from Aas (2008) using data from Little (1971).

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**Fig. 2.1:** Schematic biosynthesis pathway of isoprenoids in higher plants. Molecular structures of carotenoids and tocopherols separated by the described methods are shown. DMAPP, dimethylallyl diprophosphate; GGPP, geranyl-geranyl-diphosphate; IPP, isopentenyl diprophosphate; phytol-PP, phytol-diphosphate.

**Fig. 2.2:** HPLC chromatograms of photosynthetic pigments and isoprenoids from leaves of *Arabidopsis thaliana*. Chromatograms were recorded at A) 290 nm, B) 450 nm, C) 656 nm. Ant, antheraxanthin; βCar, β-carotene; cis-βCar, cis-β-carotene; Chl a, chlorophyll a; Chl b, chlorophyll b; Lut, lutein; Neo, neoxanthin; αToc, α-tocopherol; Vio, violaxanthin; Zea, zeaxanthin.

**Fig. 2.3:** Comparison of HPLC chromatograms of photosynthetic pigments and isoprenoids from A) shade-grown and B) light-acclimated avocado leaves (*Persea americana*) recorded at 450 nm.
Ant, antheraxanthin; αCar, α-carotene; βCar, β-carotene; cis-βCar, cis-β-carotene; Chl a, chlorophyll a; Chl b, chlorophyll b; Lut, lutein; LutEpox, lutein epoxide; Neo, neoxanthin; Vio, violaxanthin; Zea, zeaxanthin.

**Fig. 2.4:** HPLC chromatograms showing seasonal changes of photosynthetic pigments and isoprenoids in deciduous and evergreen trees recorded at 450 nm. A) Green sugar maple (*Acer saccharum*) leaf sampled in summer, B) senescent (light green) sugar maple leaf sampled in October, C) summer-acclimated Douglas-fir (*Pseudotsuga menziesii*) needles sampled in September, and D) winter-acclimated Douglas-fir needles sampled in February. Ant, antheraxanthin; αCar, α-carotene; βCar, β-carotene; cis-βCar, cis-β-carotene; Chl a, chlorophyll a; Chl b, chlorophyll b; Lut, lutein; Neo, neoxanthin; Vio, violaxanthin; Zea, zeaxanthin.

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**Fig. 3.1:** Soil water availability and drought stress of interior (INT) and coastal (COA) Douglas-fir seedlings under control and drought conditions. A) Soil water tension (pF) and B) pre-dawn twig water potential (Ψ). Data show mean of $n = 3$ measurements (±SE). Light grey background indicates period of reduced watering, dark grey background indicates period of withheld watering, followed by rewatering.

**Fig. 3.2:** Growth and needle morphology of interior (INT) and coastal (COA) Douglas-fir seedlings under control and drought conditions. A) Biomass gain (average dry weight at harvest compared to average dry weight at initial harvest of seedlings at day 0), B) leaf mass per area (LMA). Data show mean of $n = 5$ measurements (±SE). Significant differences ($p < 0.05$ using Kruskal-Wallis-rank-sum-test) are indicated by different letters. Light grey background indicates period of reduced watering, dark grey background indicates period of withheld watering, followed by rewatering.

**Fig. 3.3:** Gas exchange parameters of interior (INT) and coastal (COA) Douglas-fir seedlings under control and drought conditions. A) Stomatal conductance ($g_s$), B) transpiration rate ($E$), C)
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Fig. 3.4: Light response curve of interior (INT) and coastal (COA) Douglas-fir seedlings under control and drought conditions taken on A) day 0, B) day 41, and C) day 56. Data show mean of n = 3-5 measurements (±SE). Significant differences of the maximum assimilation rate (p < 0.05 using Kruskal-Wallis-rank-sum-test) are indicated by different letters.

Fig. 3.5: Chlorophylls and carotenoids of interior (INT) and coastal (COA) Douglas-fir seedlings under control and drought conditions. A) Total chlorophylls per fresh weight (Chlorophylls FW\(^{-1}\), B) chlorophyll a to chlorophyll b ratio (Chl a Chl b\(^{-1}\)) and C) carotenoids per total chlorophyll (Carotenoids Chl\(^{-1}\)). Data show mean of n = 5 samples (±SE). Significant differences (p < 0.05 using Kruskal-Wallis-rank-sum-test) are indicated by different letters. Light grey background indicates period of reduced watering, dark grey background indicates period of withheld watering, followed by rewatering.

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Statement of Coauthorship

Chapter 2 is authored by Laura Verena Junker and Ingo Ensminger. I collected samples and performed the laboratory work. I developed and prepared the manuscript under supervision from Ingo Ensminger. The manuscript has been published by *Physiologia plantarum*.

Chapter 3 was written by myself following discussion between myself and Ingo Ensminger, and including comments from Arthur Gessler, Kirstin Jansen, Marc Johnson and Nicholas Provat. The manuscript is in preparation for submission to *Tree Physiology*. The described experiment was carried out in context of the DougAdapt-project, which additionally studied the diversity of volatile isoprenoids, carbon isotope signatures, transcriptomics as well as growth and morphology of mature trees, aiming to develop functional genetic markers for drought tolerance in Douglas-fir.

Chapter 4 is authored by Laura Verena Junker, Anita Kleiber, Kirstin Jansen, Henning Wildhagen, Moritz Hess, Zachary Kayler, Bernd Kammerer, Jörg-Peter Schnitzler, Jürgen Kreuzwieser, Arthur Gessler, and Ingo Ensminger. Arthur Gessler, Jürgen Kreuzwieser and Ingo Ensminger conceived the experiment as part of the DougAdapt project. Field work was performed by myself, Anita Kleiber, Kirstin Jansen, Henning Wildhagen, and Moritz Hess. Analysis of essential isoprenoids was conducted by myself. Analysis of non-essential isoprenoids was conducted by Anita Kleiber. Analysis of carbon isotope signature was conducted by Kirstin Jansen. I wrote the manuscript under supervision by Ingo Ensminger. All authors contributed to the data interpretation and final editing of the manuscript, which was accepted for publication by *Scientific Reports*.

Chapter 5 is authored by Laura Verena Junker and Ingo Ensminger. I designed the experiment, conducted the analyses, interpreted the data and prepared the manuscript under supervision from Ingo Ensminger. The manuscript has been published by *Tree physiology*. 
Chapter 1
Introduction

1 Introduction

Plants exhibit a wide range of adjustments of their metabolism in response to environmental conditions. Adaptation to different environmental conditions lead to variation in plant metabolism and responses to environmental stress conditions between species, but there is also considerable variation within species, e.g. among populations. With increases in drought and heat globally as a consequence of climate change, intraspecific variation in response to water availability may make the survival of some species dependent on populations that are inherently pre-adapted to drier and hotter climate. For long-lived forest tree species the understanding of intraspecific variation among populations and their response to water availability can reveal their potential to cope with and adapt to climate change. Environmental stresses such as drought limit photosynthesis and enhance the demand for defense mechanisms such as photoprotective mechanisms, which are mediated to a large extend by isoprenoids. Photoprotective isoprenoids are adjusted in response to environmental conditions, and consequently can be used as indicators for plant performance under stress. Due to their light-absorbing properties, they affect leaf optical properties which can be sensed remotely. Remote-sensing of forest ecosystems is an increasingly used method to monitor ecosystem productivity and the timing of phenological events, both of which are affected by climate change.

This thesis investigates the adjustments of essential and nonessential isoprenoids in response to drought stress and during senescence. First, intraspecific variation in adjustments of photoprotective isoprenoids in response to drought was studied in Douglas-fir provenances that originated from contrasting habitats. Second, the influence of foliar isoprenoid pigments on leaf optical properties as indicator for the progress of senescence was studied by spectral reflectance measurements and digital image analysis in relation to photosynthesis and photosynthetic pigments in senescing sugar maple leaves. This following introduction provides the background information about photosynthesis, function and biosynthesis of photoprotective isoprenoids as well as the study species, Douglas-fir and sugar maple, and gives an overview about the subsequent chapters.
1.1 The photosynthetic apparatus of plants

Photosynthesis allows plants to convert light energy into chemical energy used for metabolism and growth. The photosynthetic apparatus of plants, consisting of photosystem II (PSII), photosystem I (PSI) and the proteins of the electron transport chain, is localized in the thylakoid membranes of the chloroplasts (Eckardt, 2001). PSII and PSI are protein complexes, which each comprise a reaction centre consisting of several protein subunits at the core surrounded by multiple light-harvesting complexes (Esteban et al., 2015a). In the light dependent reaction of photosynthesis, light energy is absorbed by chlorophylls and carotenoids bound to the light-harvesting complexes (LHC) and transferred to the reaction centre of the photosystems (Cheng & Fleming, 2009). The core pigments of both reaction centres are two chlorophyll molecules, which form a dimer (Mamedov et al., 2015). One of the chlorophyll molecules is excited to the singlet state and passes on one electron to the proteins of the electron transport chain (Fassioli et al., 2014). The final electron acceptor of the electron transport chain is NADP+ that is reduced to NADPH (Rochaix, 2011). Simultaneously, at PSII, water is split into oxygen, protons and electrons (McEvoy et al., 2005). While electrons are used to regenerate chlorophylls and protons contribute to a proton gradient driving adenosine triphosphate (ATP) synthesis by ATPases, oxygen is released. ATP and NADPH are subsequently used in the Calvin-Benson cycle (Rochaix, 2011).

The Calvin-Benson cycle fixes carbon dioxide and forms glucose using ATP and NADPH generated by the light reactions. During the initial step of the Calvin-Benson cycle, carbon dioxide is bound to the C$_5$-sugar ribulose 1,5-biphosphate by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) (Igamberdiev, 2015). Following this carboxylation step, the resulting C$_6$-sugar is immediately split into two C$_3$-molecules of 3-phosphoglycerate that are partly used for the biosynthesis of sucrose and starch. In the following reduction step, 3-phosphoglycerate is reduced by NADPH (Takahashi & Murata, 2006). Additionally, ATP produced during the light reactions of photosynthesis is used. Finally, ribulose 1,5-biphosphate is regenerated, using ATP. The enzymes of the Calvin-Benson cycle are regulated by an reversible redox modification. In contrast to the long used term ‘dark reaction’ to describe the Calvin-Benson-Cycle, the activity of the Calvin-Benson cycle is indeed stimulated by light, giving rise to the term ‘carbon reaction’ (Buchanan, 2016). The activity of the Calvin-Benson-cycle is regulated in response to environmental conditions, mainly by feedback
regulation by the availability of carbon dioxide and the intermediates of the Calvin-Benson-cycle (Paul & Foyer, 2001). The availability of carbon dioxide is limited when stomatal conductance is decreased under drought stress (Chaves et al., 2009).

The uptake of light energy for photosynthesis as well as quenching of excess light energy is regulated by the composition of light-harvesting complexes surrounding the reaction centers of the photosystems (Croce & van Amerongen, 2014). The proteins of the light-harvesting complexes have specific binding sites for pigments that are involved in absorption of light energy and transfer of excitation energy to the photosystem reaction centers but also non-photochemical quenching of light energy (Croce et al., 1999). PSII and PSI vary in the number of associated light-harvesting protein complexes and consequently photosynthetic pigments bound to them (Esteban et al., 2015a). Furthermore, the pigment composition, pigment organization and antenna size of light-harvesting complexes are adjusted to environmental conditions (Croce & van Amerongen, 2014). Since photosynthetic pigments are bound to specific binding sites of the proteins and convey various functions in light-harvesting and non-photochemical quenching, pigment content and composition indicate the plant physiological status (Esteban et al., 2015a).

The main photosynthetic pigments in higher plants are chlorophyll a and b, and the carotenoids lutein, β-carotene, neoxanthin, violaxanthin, antheraxanthin and zeaxanthin, which serve as accessory pigments in all species (Pogson et al., 1998). Some species have additional carotenoids such as α-carotene, lutein epoxide, or β-cryptoxanthin (Mukherjee et al., 2008; Esteban et al., 2009a; Matsubara et al., 2009). The core of PSII binds chlorophyll a and β-carotene, while the antennae bind to chlorophyll a, chlorophyll b and the xanthophylls lutein, neoxanthin, violaxanthin, antheraxanthin or zeaxanthin (Bassi et al., 1993). The latter three are involved in the xanthophyll cycle and are easily converted into each other, but occupy the same binding site (Caffarri et al., 2001). Similarly, the core of PSI contains chlorophyll a and β-carotene, while the light harvesting antennae contain chlorophyll a, chlorophyll b, β-carotene, lutein and violaxanthin, but no neoxanthin (Amunts et al., 2010).

These accessory pigments do not only enhance uptake of light emitted at wavelengths not absorbed by chlorophylls, but are also involved in non-photochemical quenching (NPQ) mechanisms that protect plants from excess light energy (Niyogi, 2000). Often, the absorbed
light energy exceeds the energy needed for photosynthesis, and only a fraction of absorbed light energy is quenched photochemically (Murchie & Niyogi, 2011). When the electron acceptors downstream the electron transport chain are already reduced, chemical energy and electrons generated by light reactions can be donated directly to oxygen and therefore lead to formation of reactive oxygen species (ROS), which can lead to photooxidative damage (Foyer et al., 1994). Electrons of the excited state of chlorophyll a in the reaction centers of PSII and PSI cannot be passed on, and singlet chlorophyll spontaneously transforms to triplet chlorophyll (Mozzo et al., 2008). Triplet chlorophyll cannot provide energy for photosynthesis, but enhances the formation of singlet oxygen ($^1$O$_2$), which is a highly reactive molecule belonging to the ROS (Jahns & Holzwarth, 2012). Reduced photochemical quenching of absorbed light energy consequently enhances the need for non-photochemical quenching of excess light energy, which is especially when photosynthetic gas exchange is reduced in response to abiotic stresses (Martínez-Ferri et al., 2000). NPQ mechanisms such as the dissipation of excess light energy in terms of heat, mediated by the xanthophyll cycle, structural changes of the PSII-LHCII supercomplexes and aggregation of LHC proteins quench excess light energy and thus reduce photooxidative damage (Goss & Lepetit, 2015). NPQ is induced by a low pH of the thylakoid lumen, which occurs when the absorbed light energy exceeds the capacity of the subsequent photochemical reactions (Muller et al., 2001).

Furthermore, plants can scavenge ROS by a tightly regulated antioxidant machinery including enzymatic components such as ascorbate peroxidase and superoxide dismutase, as well as non-enzymatic antioxidants such as carotenoids, tocopherols, flavonoids, ascorbic acid and reduced glutathione (Das & Roychoudhury, 2014). Antioxidants scavenge ROS physically by excitation energy transfer or chemically by an electron transport reaction (Yadav et al., 2010).

The whole photosynthetic apparatus is thus under constant adjustment to optimize photosynthetic capacity but minimize photooxidative stress. The risk of photooxidative damage is enhanced under abiotic stresses such as drought (Niyogi, 2000) or during the reorganization of the photosynthetic apparatus during leaf senescence (Juvany et al., 2013). Under abiotic stresses, plants adjust the pigment composition of the photosynthetic apparatus, including accessory pigments derived from the isoprenoid metabolism, to enhance photoprotective NPQ mechanisms and scavenging of ROS (Dall’Osto et al., 2014).
1.2 Isoprenoids

Isoprenoids are a diverse class of chemical compounds essential for plants, mammals, fungi and many bacteria and are composed of five-carbon units of diphosphorylated isoprene (Chappell 1995). Isoprenoids in plants, which are also referred to as terpenoids or prenol lipids, usually range in size from C$_5$ to C$_{40}$ (Vranová et al. 2012). They comprise volatile isoprenoids such as isoprene (C$_5$), monoterpenes (C$_{10}$) and sesquiterpenes (C$_{15}$), plant hormones such as abscisic acid (C$_{15}$) and gibberellins (C$_{20}$), antioxidants such as tocopherols (C$_{20}$), sterols (C$_{30}$), and plant photosynthetic pigments such as carotenoids (C$_{40}$). Isoprenoids are also fused with products of other pathways, such as the phytol side chain of chlorophyll which is derived from the isoprenoid metabolism (Lichtenthaler et al. 1997; Owen and Peñuelas 2005). Up to 30,000 naturally occurring isoprenoids have been identified so far, which differ in size, chemical properties and fulfill diverse roles in plant metabolism (Owen and Peñuelas 2005).

The volatile isoprenoids isoprene, mono- and sesquiterpenes are non-essential for plant metabolism and do not occur in all plant species (Owen & Peñuelas, 2005). Volatile isoprenoids are involved in biotic interactions and have antioxidant properties (Vickers et al. 2009). Many of the C$_{15}$ to C$_{40}$ isoprenoids are essential for plants. Plant hormones are signal molecules involved in the regulation of plant growth and development (Vanstraelen and Benková 2012). Tocopherols are involved in membrane stabilization and antioxidant reactions (Munné-Bosch and Alegre 2002). Sterols are membrane components which influence membrane fluidity and permeability and are involved in signal transduction (Hartmann 1998). Carotenoids are pigments which are essential for photosynthesis and important for floral colour (Lichtenthaler et al. 1997).

1.3 Isoprenoids biosynthesis

All isoprenoids larger than C$_5$ are formed by enzymatic linear or cyclic addition of the diphosphorylated isoprene subunits isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Additionally, isoprenoids can be modified by oxygenation, acetylation and esterification (Ogura & Koyama, 1998; Dewick, 2002). IPP and DMAPP are synthesized in two distinct pathways (Fig. 1.1). The mevalonate pathway takes place in cytoplasm; the non-mevalonate pathway, which is often named MEP-/ DXP-pathway after its first products 1-deoxy-
D-xylulose 5-phosphate or 2-C-methylerythritol 4-phosphate, occurs in plastids (Vranová et al., 2012) (Fig. 1). Although DMAPP and IPP and its derivatives can be exchanged between cytosol and plastids, both pathways mainly yield different classes of isoprenoids (Lange, 2000; Laule et al., 2003). The cytosolic mevalonate pathway provides mainly sesquiterpenes and sterols, while the plastidal non-mevalonate pathway provides monoterpenes, plant hormones, tocopherols, and carotenoids (Laule et al., 2003).

![Diagram of isoprenoid biosynthesis pathways](image)

**Mevalonate pathway (cytosolic)**
- Acetyl-CoA
- HMG-CoA
- MVA
- DMAPP ↔ IPP
- C<sub>5</sub> Sesquiterpenes ↔ FPP → Sterols

**Non-mevalonate pathway (plastidal)**
- G3P, Pyruvat
- DXP
- MEP
- C<sub>5</sub> DMAPP ↔ IPP
- C<sub>10</sub> GPP → Monoterpenes
- C<sub>20</sub> GGPP → Phytol chain → Tocopherols
- C<sub>40</sub> Carotenoids
- C<sub>15</sub> ABA

Fig. 1.1: Schematic representation of the two isoprenoid biosynthesis pathways, the cytosolic mevalonate pathway and the plastidal non-mevalonate pathway. ABA, abscisic acid; Acetyl-CoA, Acetyl coenzyme A; DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; G3P, glyceraldehyde 3-phosphate; GPP, geranyl-diphosphate; GGPP, geranyl-geranyl-diphosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; IPP, isopentenyl diphosphate. Size of compounds is indicated by C<sub>x</sub>. Scheme was redrawn and modified following Rodríguez-Concepción et al. (2004).

In plastids, IPP and DMAPP derived from the non-mevalonate-pathway are condensed to the C<sub>10</sub>-isoprenoid geranyl-diphosphate (GPP), which is the precursor of monoterpenes (Fig. 1). The
The next key precursor is the \( \text{C}_{20} \)-isoprenoid geranyl-geranyl-diphosphate (GGPP), which is the precursor for the biosynthesis of tocopherols and carotenoids, respectively (Rodríguez-Concepción et al., 2004). The \( \text{C}_{15} \) plant hormone abscisic acid is derived from the breakdown of carotenoids (Nambara & Marion-Poll, 2005).

In the cytosol, three units of IPP or DMAPP derived from the mevalonate pathway are used to form the \( \text{C}_{15} \)-isoprenoid farnesyl diphosphate (FPP) via intermediates. FPP is then transformed to diverse sesquiterpenes or further condensed to \( \text{C}_{30} \) sterols (Rodríguez-Concepción et al., 2004).

![Carotenoid Biosynthetic Pathway](image)

**Fig. 1.2:** The carotenoid biosynthetic pathway of higher plants diverges into two branches. The light grey boxes indicate the classification as carotenes and xanthophylls, respectively. The dark grey box emphasizes the lutein epoxide and xanthophyll cycle, respectively. (Scheme was drawn following García-Plazaola et al. (2007) and modified according to Havaux (2014)).

The biosynthesis of carotenoids diverges from its first substrate lycopene into an \( \alpha \)- and a \( \beta \)-branch (Fig. 1.2). The \( \alpha \)-branch leads to the formation of \( \alpha \)-carotene, lutein and lutein epoxide, while the \( \beta \)-branch leads to \( \beta \)-carotene, zeaxanthin, antheraxanthin, violaxanthin and neoxanthin (Caliandro et al., 2013). The unoxygenated carotenoids such as lycopene, \( \alpha \)- and \( \beta \)-carotene are
called carotenes, while the oxygen-containing carotenoids like lutein, neoxanthin, violaxanthin, antheraxanthin, zeaxanthin are considered xanthophylls (Havaux, 2014).

### 1.4 Photoprotective functions of isoprenoids

Isoprenoids are of key importance for photosynthetic life forms. Essential isoprenoids are associated with the photosynthetic apparatus and involved in diverse photoprotective functions. Chlorophyll a and b are the main photosynthetic pigments involved in light-harvesting. Under abiotic stress, plants can decrease their chlorophyll content to minimize light absorption, which has been observed in *Quercus*, *Pinus* and *Picea* species (Duan *et al.*, 2005; Ba quedano & Castillo, 2006). Furthermore, plants decrease the size of antenna complexes, which are comprised of proteins and pigments, under stress to minimize light uptake (Croce & van Amerongen, 2014). Chlorophyll a is part of the core complexes and antennae complexes, while chlorophyll b only occurs in the antennae complexes (Esteban *et al.*, 2015a). Therefore, the ratio between the chlorophyll a and chlorophyll b, which can be determined by simple biochemical analyses, is used as an indicator for the size of the peripheral antenna complexes (Tanaka *et al.*, 2001). Although the size of antennae complexes is thought to decrease under stress to reduce light absorption, the opposite was indicated by a decreased chlorophyll a/b ratio in drought-stressed trees of Aleppo pine (*Pinus halapensis* Miller), Phoenicean juniper (*Juniperus phoenicea* L.) and beech (*Fagus sylvatica* L.) which indicates an to date unknown physiological function (Garcia-Plazaola & Becerril, 2000; Ba quedano & Castillo, 2006).

In contrast to chlorophylls, carotenoids are not only involved in light uptake, but also in quenching of excess light energy. Therefore, the ratio between carotenoids and chlorophylls is an important indicator for the adjustment of the photosynthetic apparatus to stress conditions (Peñuelas *et al.*, 1995). An enhanced carotenoid-chlorophyll ratio under drought was previously shown for various tree species including *Picea asperata*, *Pinus halepensis* and *Quercus pubescens* (Duan *et al.*, 2005; Ba quedano & Castillo, 2006; Gallé *et al.*, 2007). Carotenoids are involved in different photoprotective mechanisms, as is summarized below.

The xanthophyll cycle is the best studied pigment-mediated photoprotective mechanism in plants (Demmig-Adams & Adams, 2006). Under low light conditions, the epoxidized xanthophyll
Violaxanthin is associated with the light-harvesting antenna complexes, enhancing light absorption. Under high light conditions, low pH in the thylakoid lumen activates the enzyme violaxanthin deepoxidase, which converts violaxanthin to the deepoxidized xanthophylls antheraxanthin and zeaxanthin (Fig. 1.2) (Fufezan et al., 2012). Zeaxanthin enables the dissipation of absorbed light energy as heat. If light energy is no longer occurring in excess as indicated by decreasing thylakoid pH, zeaxanthin is reverted to antheraxanthin and violaxanthin by zeaxanthin epoxidase (Eskling et al., 1997).

The xanthophyll lutein is the dominant carotenoid in most plant species, localized in the light-harvesting antennae. Lutein is important for correct folding of the antennae proteins and is involved in light harvesting (Jahns & Holzwarth, 2012). Although lutein is not essential for photosynthesis (Pogson et al., 1996), it is important for photoprotection, especially by the deactivation of triplet chlorophyll (Dall'Osto et al., 2006). In some plant species, lutein and its epoxidized counterpart lutein epoxide form a photoprotective cycle similar to the xanthophyll cycle, which likely also involves violaxanthin deepoxidase and zeaxanthin epoxidase (Esteban et al., 2009b). Lutein epoxide has been found in tropical shade-acclimated plants (Matsubara et al., 2009), avocado plants (Persea americana Mill.) (Förster et al., 2009), the parasitic plants species dodder (Cuscuta reflexa Roxb.) (Snyder et al., 2005), and plants of the genus Cucurbitaceae (Esteban et al., 2009a). Neoxanthin is another xanthophyll localized in the light-harvesting antenna complexes (Ruban et al., 2001; Dall'Osto et al., 2007b). It is involved in photoprotective processes around PSII, and functions include preventing oxygen from binding to the PSII reaction center as well as detoxifying superoxide anions (O$_2^-$). O$_2^-$ is produced by the Mehler reaction, which is enhanced under abiotic stress conditions (Dall'Osto et al., 2007a; Mozzo et al., 2008).

α- and β-carotene are the major carotenes in higher plants. β-carotene occurs in all plants species where it is localized in the core of the reaction centers of PSII and PSI (Telfer, 2002; Morosinotto et al., 2005). β-carotene protects the D2 reaction center proteins of PSII from singlet oxygen (Telfer, 2005), and is important for photoprotection of PSI (Cazzaniga et al., 2012). β-carotene has the potential to bind ROS, and therefore often undergoes specific and unspecific oxidation (Havaux, 2014). In contrast to other accessory pigments, which are mainly synthesized de novo to adjust pool sizes, β-carotene pools are constantly replenished and thus undergo
constant turnover (Beisel et al., 2010). Under low light conditions, α-carotene can partly replace β-carotene in some species (Young & Britton, 1989). For example, a decrease of the α-carotene/β-carotene ratio under light exposure has been observed in various woody tropical species (Matsubara et al., 2009) and the conifer species Norway spruce, *Picea abies* L. (Siefermann-Harms, 1994), while the contrary is expected under abiotic stress.

Aside from carotenoids, other isoprenoids such as tocopherols and volatile isoprenoids are involved in photoprotective processes (Peñuelas & Munné-Bosch, 2005). Tocopherols are C\textsubscript{20}-isoprenoids with antioxidant characteristics. They protect the envelope and thylakoid membranes of the chloroplast from lipid oxidation by their chain-breaking action during lipid oxidation caused by ROS (Fryer, 1992). They are also directly involved in photosynthetic reactions, by binding to the quinones of the cyclic electron transport chain (Munné-Bosch and Falk 2004) and quenching of singlet oxygen produced in PSII (Kriege-Liszkay & Trebst, 2006).

Many plant species also produce non-essential volatile isoprenoids which are thought to play important roles in abiotic and biotic stress defense (Loreto & Schnitzler, 2010; Loreto et al., 2014). Volatile isoprenoids enhance abiotic stress tolerance by stabilization of membranes, exhibit an antioxidant function and reduce oxidative damage by the use of excess energy during their biosynthesis (Possell & Loreto, 2013; Palmer-Young et al., 2015). In addition to their photoprotective functions, volatile isoprenoids are involved in tritrophic interactions, act as pollinator attractants, and are involved in the defense against herbivores, competitors and pathogens (Gershenzon & Dudareva, 2007; Loreto et al., 2014).

Volatile isoprenoids can be either produced *de novo* and directly emitted or stored within specialized leaf structures. The emission of volatile isoprenoids is enhanced under heat stress conditions and in response to high light (Loreto et al., 1998; Peñuelas & Munné-Bosch, 2005). However, emission of volatile isoprenoids can also contribute to a significant loss of previously fixed carbon. Transgenic tobacco plants emitting isoprene showed lower ROS levels and lipid oxidation compared to non-isoprene-emitting tobacco but at the expense of lower biomass accumulation (Ryan et al., 2014). Beech seedlings showed decreased growth due to decreased rates of photosynthesis and enhanced monoterpene emission rates under drought (Šimpraga et al., 2011).
1.5 Regulation of photosynthesis and adjustments of the photosynthetic apparatus under drought

Plants respond to drought and limited soil water availability by closing leaf stomata to minimize water loss. When stomatal conductance is decreased, photosynthetic gas exchange is reduced and consequently the uptake of carbon dioxide is limited (Chaves et al., 2003). Many plant species acclimate to drought by an enhanced water use efficiency, measured as the ratio between photosynthetic carbon assimilation and stomatal water loss. Nevertheless, drought has a major impact on photosynthetic carbon assimilation (Chaves et al., 2009). In addition to directly limiting plant productivity, reduced photosynthetic gas exchange under drought limits the fraction of absorbed light energy that is photochemically quenched (Martínez-Ferri et al., 2000). Consequently, drought-stressed plants often experience high light stress that enhances the formation of ROS and thus increases the risk of photooxidative damage (Miller et al., 2010).

Generally, an enhanced ratio of carotenoids to chlorophylls indicates an adjustment to stress conditions, as carotenoids are involved in diverse NPQ mechanisms (Peñuelas et al., 1995; Demmig-Adams & Adams, 1996). The adjustment of the carotenoid-chlorophyll ratio under drought can occur by a reduction of the chlorophyll content, as observed in drought-stressed Picea asperata seedlings (Duan et al., 2005), or by enhanced carotenoid synthesis, as observed under prolonged stress in Cistus creticus (Munne-Bosch et al., 2009). Among carotenoids, individual carotenoids vary in their response to stress conditions.

Xanthophyll cycle pool sizes (sum of violaxanthin (V), antheraxanthin (A) and zeaxanthin (Z)) are increased under drought to minimize photooxidative damage when photochemical quenching of light energy is reduced under drought (Demmig-Adams & Adams, 1992; Demmig-Adams et al., 1995). The increase of xanthophyll cycle pool sizes in response to drought has been observed in many plant species (Esteban et al., 2015a). Furthermore, the deepoxidation state of the xanthophyll cycle measured as (0.5 A+Z)/(V+A+Z) is rapidly upregulated if absorbed light energy exceeds the photosynthetic capacity (Demmig-Adams & Adams, 2006).

Lutein content is typically increased in relation to chlorophylls under drought, as a meta-analysis of photosynthetic pigment composition revealed (Esteban et al., 2015a). Nevertheless, the opposite pattern was observed in rosemary, Rosmarinus officinalis (Munné-Bosch & Alegre, 2002). Neoxanthin is usually unaffected by drought (Esteban et al., 2015a). The effect of drought
stress on α- and β-carotene was rarely studied. Nevertheless, a decrease of β-carotene was reported for the evergreen shrubs Phillyrea angustifolia and Arbutus unedo (Peñuelas et al., 2004; Ripullone et al., 2009), and decreased α-carotene was observed in Norway spruce, Picea abies (Kronfuss et al., 1998). Since carotenes quench ROS by chemical quenching, this results in an unspecific oxidation (Ramel et al., 2013; Havaux, 2014). Carotene content thus decreases, if de novo formation is insufficient to replace oxidized carotenones (Ramel et al., 2013). This is in contrast to xanthophylls that quench ROS physically and are thus unaltered (Cazzaniga et al., 2016). The changes of the pigment composition of the photosynthetic apparatus under drought can thus be assumed to be results of adjustments of the photosynthetic apparatus to enhanced photoprotection as well as for some pigments, degradation of pigments by oxidation.

Volatile isoprenoids have been shown to exhibit rather diverse responses under drought. In conifers, the monoterpene concentrations in needles were increased under drought (Picea abies: Kainulainen et al. 1992; Pinus ponderosa: Johnson et al. 1997; Pinus halepensis: Llusia & Peñuelas 1998; Llusia et al. 2006). Similarly, monoterpene emission was increased in drought-stressed Fagus sylvatica (Šimpraga et al., 2011). Pool sizes and emission of volatile isoprenoids have been shown to differ between species (Llusia & Peñuelas, 1998). The general assumption is, that monoterpene biosynthesis and emission remain unaffected or are stimulated under moderate drought, but are impaired under severe drought as a consequence of decreased photosynthetic assimilation rates (Peñuelas et al., 2009; Šimpraga et al., 2011; Nogués et al., 2015a; Nogués et al., 2015b).

1.6 Evidence for intraspecific variation in photoprotective isoprenoids

Although the composition of the photosynthetic apparatus is highly conserved, species vary in chlorophyll and carotenoid content and composition (Pogson et al., 1998). Enhanced potential for photoprotection may confer an advantage under adverse environmental conditions such as drought and could thus be affected by local adaptation (Ramírez-Valiente et al., 2015). For example, xanthophyll cycle pigment levels in different tree species range from 40 to 220 mmol per mol chlorophyll (Niinemets et al., 1999; McKinnon & Mitchell, 2003; Ensminger et al., 2004). The xanthophyll cycle pool size is also increased in response to environmental stresses.
which may contribute to variation in drought tolerance (Alonso et al., 2001; Gallé et al., 2007; Galmés et al., 2007). Differences in adjustments of isoprenoid metabolism in response to environmental conditions have been observed among species from the genus *Quercus* (Peguero-Pina et al., 2009), and among provenances of *Picea asperata*, which are adapted to different habitats (Duan et al., 2005), and suggest intraspecific variation. Nevertheless, evidence for intraspecific variation as indicator for the involvement of the isoprenoid metabolism in adaptation of species or genotypes is scarce, although isoprenoid-mediated photoprotective mechanisms might prevent photooxidative stress and enhance productivity under drought stress. In this thesis, Douglas-fir was chosen as study species for intraspecific variation in photoprotective isoprenoids, because Douglas-fir has a large habitat and thrives under diverse conditions, and many provenances which differ in drought tolerance have been identified (Montwé et al., 2015).

### 1.7 Regulation of photosynthesis and adjustments of the photosynthetic apparatus during leaf senescence

Deciduous trees remobilize nutrients from leaves prior to their abscission in fall (Wilson et al., 2001). Nutrient remobilization in senescing leaves mainly relies on the breakdown of enzymes to remobilize the incorporated nitrogen. When enzymes involved in carbon fixation are degraded, photosynthetic capacity is reduced (Hörtensteiner & Feller, 2002). Consequently, light absorption needs to be minimized to avoid photooxidative stress. Therefore, the degradation of enzymes of the photosynthetic apparatus is well regulated (Biswal et al., 2003; García-Plazaola et al., 2003a; Ougham et al., 2005). Leaf senescence is regulated in response to temperature, photoperiod, as well as abiotic and biotic stress to maximize nutrient recovery, but avoid photooxidative damage (Keskitalo et al., 2005; Lim et al., 2007).

Most deciduous trees show a total degradation of chlorophylls (Lee et al., 2003; Ougham et al., 2005) but only an incomplete degradation of carotenoids (Biswal, 1995). Among carotenoids, β-carotene and the xanthophyll cycle pigments violaxanthin, antheraxanthin and zeaxanthin are relatively enhanced compared to other carotenoids (García-Plazaola et al., 2003a; Ougham et al., 2005). These antioxidant and photoprotective accessory pigments likely contribute to regulation of reactive oxygen species (ROS) which accumulate during senescence but need to be tightly
regulated due to their toxicity (Juvany et al., 2013). Additionally, many tree species accumulate anthocyanins during autumn senescence (Archetti et al., 2009). Anthocyanins can convey protection from photo-oxidative stress due to their antioxidant characteristics (Hoch et al., 2001; van den Berg & Perkins, 2007), are involved in screening of UV-B light (Ferreira da Silva et al., 2012) and have a potential role in biotic interactions (Archetti et al., 2009).

1.8 Role of isoprenoids in monitoring of ecosystems

The described changes in pigment composition during leaf senescence reflect the physiological performance of plants. As they strongly influence the color of leaves, pigments can serve as indicators for autumn phenology and the progress of senescence (Ustin et al., 2009). Monitoring of pigment dynamics by remote sensing is increasingly used to assess primary production and phenological events of ecosystems (Yang et al., 2014). Remote-sensing of ecosystems is typically carried out by airborne spectral reflectance measurements (Treitz & Howarth, 1999; Pettorelli et al., 2005). In addition, near-surface remote sensing by digital cameras (“phenocams”), which take images from a canopy at regular intervals, has recently been established (Richardson et al., 2013). These methods can be used to detect optical properties of plants on the leaf-, canopy- and ecosystem-level (Garbulsky et al., 2011).

The detected optical properties can be used to calculate vegetation indices, which were established to represent the plant physiological status, productivity or phenology. While the estimation of gross primary production and general seasonal changes using vegetation indices are well established on the canopy level for both methods, little is known about the representation of physiological processes during autumn leaf senescence (Sonnentag et al., 2012; Yang et al., 2014). In addition to the major physiological changes during autumn leaf senescence, many tree species also accumulate anthocyanins (Archetti et al., 2009). Anthocyanins have been shown to affect many of the widely used vegetation indices (Gitelson & Merzlyak, 1994). In this thesis, sugar maple was chosen as study species to investigate the suitability of vegetation indices to reflect essential isoprenoids as indicators for the progress of senescence, because sugar maple accumulates large amounts of anthocyanins during leaf senescence (Schaberg et al., 2008; Moy et al., 2015).
1.9 Climate change

Climate change is caused by anthropogenic emissions of carbon dioxide and other greenhouse gases into the atmosphere. As a consequence, globally increased temperatures, rising sea levels, changing precipitation patterns, and increasing frequencies of severe weather events such as droughts, heat waves and storms have been observed (IPCC, 2013). In the future, climate change will lead to elevated air temperature and reduced precipitation in most of the northern hemisphere, particularly in summer (Sheffield & Wood, 2008; IPCC, 2013). Extended periods of summer drought, as experienced in Europe in 2003, are expected to occur more often and constrain forest productivity (Rennenberg et al., 2006). Drought stress directly impacts trees by decreased photosynthetic gas exchange, irreversible damage to the xylem and enhanced risk of mortality (Bréda et al., 2006). To assess forest productivity and response to stresses, monitoring techniques such as remote-sensing of leaf optical properties are increasingly used to estimate the effect of climate change on forest ecosystems (Weiskittel et al., 2011).

On the long term, species need to migrate to more suitable environments or adapt to future climate conditions to avoid extinction (Aitken et al., 2008). Trees species typically have long generation times and slow migration rates (Bréda et al., 2006). Many tree species that are adapted to current climate conditions will not be able to adapt fast enough to rapid climatic changes, and an increasing mismatch between climatic conditions and local adaptation of provenances might exacerbate the vulnerability of forest ecosystems (St Clair & Howe, 2007; Aitken et al., 2008). Forest management therefore aims to mitigate the negative impacts of climate change by planting species and provenances adapted to future climate conditions (St Clair & Howe, 2007; Bolte et al., 2009). Although many provenance trials have been conducted, our knowledge of traits contributing to intraspecific variation in stress tolerance and resilience is scarce. This thesis investigates intraspecific variation in isoprenoid metabolism of Douglas-fir as well as the role of essential isoprenoids as indicators for autumn phenology of Sugar maple leaves, thus aiming to contribute to a better understanding of the effects of climate change on forest ecosystems.
1.10 Study species

1.10.1 Douglas-fir

Douglas-fir, *Pseudotsuga menziesii* Mirb., is native to the west coast of North America and its natural range extends from Canada to Mexico. Thus, Douglas-fir is found under diverse climatic conditions and shows high phenotypic and genetic diversity (Krutovsky & Neale, 2005). Two subspecies have been defined, coastal (*Pseudotsuga menziesii* var. *menziesii*) and interior Douglas-fir (*P. menziesii* var. *glauca*), which are common east and west of the Rocky Mountains, respectively (Bartlein *et al.*, 1998) (Fig. 1.3).

In each of the subspecies, provenances can be defined which show local adaptation to the climatic conditions of their habitat (Rehfeldt, 1989). Local adaptation as a result of natural selection results in a higher relative fitness of a provenance in its local habitat compared to provenances from other habitats (Boshier *et al.*, 2015). Generally, coastal provenances are adapted to moist maritime climates, while interior provenances are adapted to drier conditions with extended summer droughts (Aas, 2008; Sergent *et al.*, 2014). Nevertheless, variation in climatic conditions within the coastal and interior habitats lead to adaptation to moist or dry conditions also within the coastal and interior subspecies, respectively (Aitken *et al.*, 1995; Anekonda *et al.*, 2002; Montwé *et al.*, 2015). Provenances within both subspecies reveal variation in growth potential and productivity under drought (Eilmann *et al.*, 2013; Montwé *et al.*, 2015). Aitken et al. (1995) also observed provenance-specific differences in water-use efficiency. Variation in photosynthetic carbon assimilation and isoprenoid metabolism may contribute to intraspecific variation in response to drought. Because of its fast growth and of its high quality wood properties, Douglas-fir is used in reforestations across Europe.
Fig. 1.3: The habitat of coastal (*Pseudotsuga menziesii* var. *menziesii*, shown in blue) and interior Douglas-fir (*P. menziesii* var. *glauca*, shown in green) along the North American west coast. Map adapted from Aas (2008) using data from Little (1971).

### 1.10.2 Sugar maple

Sugar maple, *Acer saccharum* Marsh., is a deciduous tree species originating in northeastern North America (Godman *et al.*, 1990) (Fig. 1.4). Sugar maple dominates many northern hardwood forest ecosystems and has a wide distribution in the northeastern US and southeastern Canada (Lovett & Mitchell, 2004). Sugar maple is also of economic importance in North America, because it is used for the production of maple syrup and has a high wood quality (Godman *et al.*, 1990; Moore *et al.*, 2014). Sugar maple thrives in regions with cool, moist climates, where growing season length ranges between 80 and 260 days (Godman *et al.*, 1990). Sugar maple shows an enhanced susceptibility to global warming due to the reduced snow cover and enhanced frost damage of roots (Auclair *et al.*, 2010). Recently, a decline of sugar maple has been monitored throughout its natural habitat (Groffman *et al.*, 2012).
Fig. 1.4: The habitat of sugar maple (*Acer saccharum*) at the North American east coast. Map provided by the U.S. Geological Survey (USGS), based on data from Little (1971).

1.11 Thesis framework

1.11.1 Hypotheses

My thesis investigates adjustments of photoprotective isoprenoids when photosynthesis is limited due to adverse environmental conditions or during senescence. Specifically, I have studied intraspecific differences in amount and composition of photoprotective isoprenoids in Douglas-fir provenances in response to soil water availability and their contribution to provenances’ drought tolerance. Furthermore, I investigated the changes of isoprenoid content during autumn senescence of sugar maple leaves and how isoprenoids and pigments can be used as indicators to monitor autumn phenology and physiology during senescence in sugar maple. For a fast and reliable isoprenoid analysis, I established an HPLC method, which is applicable for a wide range of plant species.

I hypothesized that 1) isoprenoid-mediated photoprotective mechanisms are induced in response to environmental conditions that limit photosynthesis or during senescence to match an enhanced demand for non-photochemical quenching of light energy; 2) interior Douglas-fir provenances avoid water loss by low stomatal conductance, and consequently show enhanced photoprotection of the photosynthetic apparatus mediated by essential and non-essential isoprenoids, and 3)
essential isoprenoids are indicators of physiological performance and phenological events in remote-sensing applications because plants adjust the composition of the photosynthetic apparatus in response to environmental conditions.

1.11.2 Chapter 2

Chapter 2 describes the high-performance liquid chromatography (HPLC) method which I used to quantify the abundance of isoprenoids in plant tissue. At the beginning of my PhD, I needed to establish an HPLC method to quantify all major essential isoprenoids, chlorophylls and carotenoids, which are part of the photosynthetic apparatus of plants. I was further able to separate the non-ubiquitous carotenoids lutein epoxide and α-carotene as well as α- and δ-tocopherol. In this chapter, I provide the analytical information and demonstrate the applicability of the developed method for pigment extracts from diverse plant species and tissue types.

1.11.3 Chapter 3

Chapter 3 investigates the adjustments of isoprenoid metabolism of Douglas-fir seedlings in response to experimental drought stress. The aim of this study was to assess provenance-specific differences in photosynthesis, photoprotective mechanisms and isoprenoid metabolism in seedlings of a coastal and an interior Douglas-fir provenance, respectively, under controlled drought conditions. Douglas-fir seedlings of the interior and coastal subspecies were exposed to limited watering for six weeks, followed by a two week recovery phase. I hypothesized that seedlings of the interior provenance show rather drought avoiding characteristics including earlier stomatal closure and consequently stronger induction of photoprotective mechanisms to combat lower photochemical quenching of light energy compared to the coastal provenance originating from a humid environment. I expected furthermore, that provenances vary in photoprotective mechanisms, showing either enhanced photoprotection mediated by essential isoprenoids or enhanced accumulation and emission of non-essential volatile isoprenoids.
1.11.4 Chapter 4

Chapter 4 investigates the intraspecific differences in photosynthesis, photoprotective mechanisms and isoprenoid metabolism in 50-year-old field-grown Douglas-fir trees of four divergent provenances at two field sites under different climatic conditions. The aim of this study was to assess intraspecific differences in photosynthetic gas exchange and photoprotective mechanisms in response to limitations in water availability. I hypothesized that all provenances show enhanced NPQ, increased xanthophyll cycle de-epoxidation state and increased emission of non-essential volatile isoprenoids as short-term (milliseconds to minutes) response to reduced soil water availability. Furthermore, drought avoiding provenances are expected to show more pronounced long-term adjustments (hours to months) of the photosynthetic apparatus compared to drought tolerant provenances, including increased pools of xanthophyll cycle pigments and stored volatile isoprenoids. Gas exchange and chlorophyll fluorescence measurements of the sun-exposed crown of fifty-year-old trees of four Douglas-fir provenances that originated from contrasting habitats grown at two provenance trials were taken and combined with the isoprenoid analysis of needle samples.

1.11.5 Chapter 5

Chapter 5 addresses the influence of isoprenoids on leaf optical properties. Specifically I studied the degradation of isoprenoids during autumn senescence and how autumn phenology and physiology can be assessed remotely spectral reflectance measurements and digital image analysis. I aimed to assess how efficiently several widely used vegetation indices capture leaf senescence and reflect the associated changes in physiology and photosynthetic pigment content during autumn. To correlate pigment content and photosynthetic efficiency with vegetation indices derived from spectral reflectance measurements and digital images, I measured chlorophyll fluorescence, P700 absorbance, photosynthetic pigment and anthocyanins content in green sugar maple leaves sampled during summer, and green, yellow, orange and red leaves, and conducted leaf-level spectral reflectance measurements and color analysis of digital images.
1.11.6 Chapter 6

In chapter 6 the adjustments of isoprenoid metabolism in response to drought stress in Douglas-fir and intraspecific variation in photosynthesis and isoprenoid metabolism are discussed. Furthermore I discuss the influence of isoprenoids on leaf optical properties and their use as indicator to monitor forest phenology.
Chapter 2

Fast detection of leaf pigments and isoprenoids for ecophysiological studies, plant phenotyping and validating remote-sensing of vegetation

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2 Fast detection of leaf pigments and isoprenoids for ecophysiological studies, plant phenotyping and validating remote-sensing of vegetation

2.1 Abstract

Rapid developments in remote-sensing of vegetation and high-throughput precision plant phenotyping promise a range of real-life applications using leaf optical properties for non-destructive assessment of plant performance. Use of leaf optical properties for assessing plant performance requires the ability to use photosynthetic pigments as proxies for physiological properties and the ability to detect these pigments fast, reliably and at low cost. We describe a simple and cost-effective protocol for the rapid analysis of chlorophylls, carotenoids and tocopherols using high-performance liquid chromatography (HPLC). Many existing methods are based on the expensive solvent acetonitrile, take a long time or do not include lutein epoxide and α carotene. We aimed to develop an HPLC method which separates all major chlorophylls and carotenoids as well as lutein epoxide, α carotene, and α tocopherol. Using a C$_{30}$-column and a mobile phase with a gradient of methanol, methyl-tert-butyl-ether (MTBE) and water, our method separates the above pigments and isoprenoids within 28 minutes. The broad applicability of our method is demonstrated using samples from various plant species and tissue types, e.g. leaves of Arabidopsis and avocado plants, several deciduous and conifer tree species, various crops, stems of parasitic dodder, fruit of tomato, roots of carrots and Chlorella algae. In comparison to previous methods, our method is very affordable, fast and versatile and can be used to analyse all major photosynthetic pigments that contribute to changes in leaf optical properties and which are of interest in most ecophysiological studies.

2.2 Introduction

Remote-sensing and plant phenotyping allow the non-destructive characterization of plant performance and productivity (Walter et al., 2015). Photosynthetic pigments are important indicators for the physiological status of plants, as they reflect the adjustment of the photosynthetic apparatus to seasonal variation of climatic conditions and in response to abiotic and biotic stresses (Ustin et al., 2009). Due to their optical properties, photosynthetic pigments
affect leaf color, and pigment dynamics can be monitored by spectral reflectance measurements and camera-based techniques across spatial and time scales (Hufkens et al., 2012). Various methodologies are applied to validate information derived from remote-sensing and plant phenotyping, among them the analysis of foliar photosynthetic pigment levels (Gitelson et al., 2009; Walter et al., 2015). The pigments associated with the photosynthetic apparatus of higher plants vary in their specific function. Therefore, the analysis of foliar pigment content and composition indicates plant performance and adjustments to environmental conditions.

Chlorophyll a and b are the main photosynthetic pigments involved in light absorption. Carotenoids are accessory pigments which contribute to light absorption but are also involved in photoprotective processes to quench excess light energy (Jahns & Holzwarth, 2012). The major carotenoids associated with the photosynthetic apparatus of all higher plants are lutein, β-carotene, violaxanthin, antheraxanthin, zeaxanthin and neoxanthin. Some species accumulate additional carotenoids, such as α-carotene, β-cryptoxanthin, lycopene (Demmig-Adams et al., 1996; Tanaka et al., 2008) or lutein epoxide ( Förster et al., 2011). The composition of chlorophylls and major carotenoids of the photosynthetic apparatus is remarkably conserved (Pogson et al., 1998). Nevertheless, plants regulate the total amount of these pigments and the ratio between carotenoids and chlorophylls in response to environmental conditions (Esteban et al., 2015a).

Carotenoids are synthesized in the isoprenoid biosynthetic pathway (Fig. 2.1) which also generates tocopherols and precursors for the phytol side chain of chlorophyll (Chappell, 1995; Lichtenthaler et al., 1997). Lycopene is the first substrate of the carotenoid biosynthetic pathway which diverges into an α- and β-branch leading to the unoxygenated carotenes α- and β-carotene, respectively (Caliandro et al., 2013). α-carotene is further oxygenated to form the xanthophylls lutein and lutein epoxide (Fig. 2.1; García-Plazaola et al., 2007), while β-carotene is a precursor for the xanthophylls β-cryptoxanthin, zeaxanthin, antheraxanthin, violaxanthin and neoxanthin (Fig. 2.1; da Silva Messias et al., 2014).

Carotenoids are associated with specific pigment-binding proteins of the photosynthetic apparatus, where they serve different functions. Cis- and trans-isomers of β-carotene are antioxidants which protect the reaction centers of the photosystems from oxidative damage (Nayak et al., 2002; Telfer, 2005). α-carotene is not ubiquitous, but confers shade tolerance
(Matsubara et al., 2009) and also shows seasonal fluctuations (Siefermann-Harms, 1994; Ottander et al., 1995). Xanthophylls are associated with proteins of the light-harvesting complexes and minimize the formation of reactive oxygen species (ROS), e.g. neoxanthin detoxifies superoxide anions (O$_2^-$) (Dall’Osto et al., 2007a).

![Schematic biosynthesis pathway of isoprenoids in higher plants. Molecular structures of carotenoids and tocopherols separated by the described methods are shown. DMAPP, dimethylallyl diphosphate; GGPP, geranyl-geranyl-diphosphate; IPP, isopentenyl diphosphate; phytol-PP, phytol-diphosphate.](image-url)

Fig. 2.1: Schematic biosynthesis pathway of isoprenoids in higher plants. Molecular structures of carotenoids and tocopherols separated by the described methods are shown. DMAPP, dimethylallyl diphosphate; GGPP, geranyl-geranyl-diphosphate; IPP, isopentenyl diphosphate; phytol-PP, phytol-diphosphate.
In higher plants, two xanthophyll cycles have been identified which confer rapid adjustments of the photosynthetic apparatus to changing light conditions. The xanthophyll cycle involving violaxanthin, antheraxanthin and zeaxanthin is ubiquitous, while the lutein epoxide cycle formed by lutein epoxide and lutein is found only in some species (Esteban et al., 2009a; Matsubara et al., 2009). The epoxidized xanthophylls, violaxanthin and lutein epoxide, increase the light harvesting efficiency (Matsubara et al., 2007), while the de-epoxidized xanthophylls zeaxanthin and lutein have a photoprotective function. Zeaxanthin dissipates excess light energy in terms of heat (Demmig-Adams & Adams, 2006), and lutein quenches triplet chlorophyll (Dall'Osto et al., 2006). Although lutein epoxide typically resolves well in standard carotenoid analyses, it is rarely reported because it is not of interest for the purpose of the study, or because it is absent in the studied species.

Tocopherols are C20-isoprenoids which are also involved in photoprotective processes. They have an antioxidant function and stabilize the chloroplast membranes (Havaux et al., 2003; Peñuelas & Munné-Bosch, 2005). α-tocopherol is increased in response to high light, but it is not routinely determined in studies that focus on isoprenoids and photosynthetic pigments (Esteban et al., 2015a). δ-tocopherol has been shown to have a role in ROS scavenging in tobacco leaves (Matringe et al., 2008) and to accumulate in senescing sugar maple leaves (Junker and Ensminger, 2016), but its exact function is not well understood yet.

The structure of the photosynthetic apparatus and composition of pigments are adjusted in response to environmental conditions (Croce & van Amerongen, 2014). Many ecophysiological studies are based on the detailed analysis of plant photosynthetic pigments because their response to environmental stress can provide insight into the structure and functionality of the whole photosynthetic apparatus (Esteban et al., 2015a). Nevertheless, a comprehensive literature review of photosynthetic pigment composition in response to environmental conditions (Esteban et al., 2015a) revealed considerable inaccuracy in pigment measurements (Fernández-Marín et al., 2015).

Plant photosynthetic pigments are commonly analysed using reversed-phase high-performance liquid chromatography (HPLC). A large number of methods for the extraction and separation of plant pigments and essential isoprenoids using different types of HPLC-columns and solvent systems have been described. A disadvantage of some earlier methods is the insufficient or
challenging separation of isomers such as lutein and zeaxanthin (Lichtenthaler et al., 1982; Gilmore & Yamamoto, 1991). Another disadvantage of many methods is the use of rather complex and expensive solvents such as acetonitrile (Gilmore & Yamamoto, 1991). More recently many HPLC methods for the separation of photosynthetic pigments have used C\textsubscript{30}- columns which provide better specificity for carotenoids and which can be used with affordable solvents such as methanol and methyl-tert-butyl-ether (MTBE) as a mobile phase (Sander et al., 2000). Nevertheless, protocols based on these types of columns and solvents have a runtime of one hour per sample or do not include non-ubiquitous pigments such as lutein epoxide and \(\alpha\)-carotene (Fraser et al., 2000; Taylor et al., 2006).

We aimed to develop a fast HPLC-method which can separate major isoprenoids and photosynthetic pigments that contribute to changes in leaf optical properties and which are of interest in ecophysiological studies.

The method presented here separates and allows for fast and reliable quantification of lutein and lutein epoxide, \(\alpha\)- and \(\beta\)-carotene, \(\alpha\)-tocopherol and \(\delta\)-tocopherol, as well as all major photosynthetic pigments of higher plants, including the violaxanthin-zeaxanthin xanthophyll cycle pigments.

2.3 Material and Methods

2.3.1 Plant tissue samples

A variety of samples from different species and environments was used in order to demonstrate the broad applicability of our method. Leaf samples from Arabidopsis thaliana were obtained from plants growing in plant growth chambers under long-day conditions (16 h day/ 8 h night, 22/18 °C, 70 % humidity). Leaves from the sun-exposed canopy of red maple (Acer rubrum), white oak (Quercus alba), and current year needles from the sun-exposed canopy of white pine (Pinus strobus) were sampled in August 2015 in Turkey Point, ON, Canada. Sun-exposed leaves of sugar maple (Acer saccharum) were sampled in July and October of 2013 in Huron Park, Mississauga, ON, Canada. Current year needles from the sun-exposed canopy of field-grown Douglas-fir (Pseudotsuga menziesii var. menziesii) were sampled in September of 2010 and February of 2011 in Reilingen, Germany. Needles of well-watered and drought stressed Douglas-
fir seedlings were taken from one-year-old seedlings of an interior (P. menziesii var. glauca) provenance subjected to a control or a drought-stress treatment for six weeks under controlled conditions. Leaves of well-watered and drought-stressed (following a three week drought treatment) common bean plants (Phaseolus vulgaris) were taken from greenhouse-grown plants. Wheat (Triticum aestivum), corn (Zea mays), and soybean (Glycine max) were grown in a greenhouse, and individual leaves were harvested from about three-week-old plants. The parasitic plant dodder (Cuscuta gronovii) was grown in a rooftop garden using coleus (Plectranthus scutellarioides) as a host plant. An avocado plant (Persea americana) was grown from seed for one year under ambient light conditions and subsequently transferred to low light conditions (35 µmol photons m⁻² s⁻¹ photosynthetic active radiation) to foster the development of shade leaves which are enriched in lutein epoxide (Förster et al., 2009). The tip of the second youngest leaf was sampled early morning in darkness. Subsequently, the same leaf was exposed to 125 µmol photons m⁻² s⁻¹ photosynthetic active radiation and sampled after one hour. Ripe red tomato fruits (Solanum lycopersicum) and orange carrot roots (Daucus carota) were obtained from a local grocery store. All plant samples were frozen in liquid nitrogen upon harvesting, ground to a frozen powder in liquid nitrogen using a mortar and pestle and stored at -80 °C. The green algae Chlorella variabilis was cultured in modified Bold’s Basal Medium (MBBM; Van Etten et al. 1983) in climate chambers (22 °C, continuous light). An aliquot of the culture was sampled, and algae were harvested by filtering the medium through a paper filter. The algae were retained on the filter and transferred into a microtube. The sample was dried under vacuum and frozen at -80 °C. To suspend the pellet during extraction, the sample was sonicated for ten seconds during extraction.

2.3.2 Extraction of essential isoprenoids

Approximately 50 mg of frozen ground plant material was transferred to an amber 2 ml-microtubes filled with 700 µl of 98 % methanol and 2 % distilled water buffered with 0.5 M ammonium acetate (adjusted to pH 7.1 with acetic acid). After 2 h of incubation in a thermomixer at 4 °C and rotating at 900 rpm, extracts were centrifuged for 5 min at 14000 g. The supernatant was transferred to a new amber 2 ml-microtube and kept on ice. The pellet was washed twice using 700 µl pure methanol. All supernatants were combined, vortex-mixed and
centrifuged for 5 min at 14000 g to remove any suspended particles from the combined supernatant. The same protocol, but using 100% acetone buffered with sodium bicarbonate as an extraction solvent (Ensminger et al., 2004), was used to compare the yield of our methanolic extraction with another established protocol. Prior to the HPLC analysis, the extracts were filtered through syringe filters (0.2 µm PTFE membrane; Target® National Scientific Co., TN, USA). During extraction all materials and extracts were kept on ice and in dim light conditions.

2.3.3 HPLC Method

High-performance liquid chromatography (HPLC) was conducted using an Agilent Infinity Quaternary LC System (Agilent Technologies Deutschland GmbH & Co. KG, Waldbronn) consisting of a quaternary pump (model 1260), a thermosta Table 2.autosampler (model 1260), a column oven (model 1260), a photodiode array detector (model 1290) and a thermosta Table 2.fraction collector (model 1290). Separation of isoprenoids was achieved using a YMC C\textsubscript{30}-column (5 µm, 250*4.6 mm; YMC Inc., Wilmington, NC, USA), protected by a guard column (C\textsubscript{30}, 5 µm, 4.0*23 mm; YMC Inc., Wilmington, NC, USA).

A combination of three solvents was used to obtain a gradient of decreasing polarity in the mobile phase (Table 2.1a). Solvent A was methanol, solvent B was methyl-tert-butyl-ether (MTBE) and solvent C was ultrapure water buffered with 20 mM ammonium acetate and set to pH 6 using acetic acid (Table 2.1). The initial solvent composition was 92 % solvent A, 5 % solvent B and 3 % solvent C for 3 min. Following the injection of the sample with a volume of 100 µl, solvent A was gradually replaced by solvent B, with the concentration of solvent B at 17 min being 33.6 %, at 22 min being 81.3 % and from 23-27 min at a maximum of 94 %. During the next minute, the concentration of solvent B was reduced to the initial concentration of 5 %. Elution of all pigments was completed within 28 minutes, and the column was reconditioned to the initial solvent concentrations by the end of the run after 35 min. A time of 7 min was sufficient to re-establish the initial solvent conditions and recondition the column, as indicated by stabilizing backpressure of about 100 bar. The flow rate was 1 ml min\textsuperscript{-1}, and column temperature was maintained at 25 °C.
Table 2.1: Solvent system developed for separation of chlorophylls, carotenoids and tocopherols using a C\textsubscript{30}-column. A) the standard gradient was developed to include all pigments. B) A modification of the standard method with a shorter run-time. This modified method can be used when cis-\(\beta\)-carotene the most abundant nonpolar carotenoid and uses less MTBE. Solvent A: methanol; Solvent B: methyl-\text{tert}-butyl-ether (MTBE); Solvent C: water buffered with 20 mM ammonium acetate, pH 6.

a) Standard method

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b) Modified standard method with decreased run-time using less MTBE (Solvent B)

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A full wavelength scan from 250 nm to 680 nm was performed and chromatograms at 290 nm, 450 nm and 656 nm were recorded and analysed using ChemStation B.04.03 software (Agilent Technologies, Böblingen, Germany). Peak quantification was performed using standards for chlorophyll a, chlorophyll b, \(\beta\)-carotene, \(\alpha\)-tocopherol and \(\delta\)-tocopherol from Sigma-Aldrich (Oakville, ON, Canada), and antheraxanthin, \(\alpha\)-carotene, \(\beta\)-carotene, \(\beta\)-cryptoxanthin, lutein, lycopene, neoxanthin, violaxanthin and zeaxanthin were obtained from DHI Lab products (Hørsholm, Denmark). The \(\beta\)-carotene standard was synthetically produced and contained only the trans-isomer of \(\beta\)-carotene. Since \(\beta\)-carotene in photosynthetic tissues occurs as cis- and trans-isomers, the same calibration factor obtained from trans-\(\beta\)-carotene was used for cis-\(\beta\)-carotene. Peak identification of lutein epoxide was conducted using the fraction of all-trans-lutein epoxide extracted from \textit{Taraxacum officinale} petals following a protocol from Meléndez-
Martínez (2006). All-trans-lutein is the lutein epoxide isomer exhibiting photoprotective function in the chloroplast (Shizue Matsubara, personal communication). The retention times of all pigments are summarized in Table A6.1. The retention factor (k) was calculated as \( k = (t_R - t_0) / t_0 \), with \( t_R \), retention time of pigment and \( t_0 \), retention time of solvent peak (3min), respectively. The ratio between the retention factors of a pigment and its predecessor was used to calculate the separation factor (\( \alpha \); Taylor et al. 2006). The level of detection (LOD) and level of quantification (LOQ) for each standard were determined as three and ten times the standard deviation of the calibration curve divided by the slope of the calibration curve, respectively (Şengül, 2016). The reproducibility of the extraction and HPLC method was estimated by the average percent coefficient of variation (CV) between six technical replicates extracted from one Douglas-fir needle sample.

2.3.4 Chemicals

All chemicals and solvents used were of HPLC grade. Methanol was obtained from Caledon (Georgetown, ON, Canada), MTBE was obtained from Sigma-Aldrich (Oakville, ON, Canada), water was purified using a MilliQ® water purification system (EMD Millipore, Etobicoke, ON, Canada). Buffers were prepared using ammonium acetate with a purity >99% (Fluka Chemie AG, Buchs, Switzerland). The pH of buffers was adjusted with glacial acetic acid (EMD Millipore).

2.4 Results and Discussion

We aimed to develop a fast HPLC-method for separating major photosynthetic pigments and isoprenoids that can be used to assess plant performance and to validate remote-sensing of vegetation and high throughput precision plant phenotyping. We particularly aimed to improve previous methods (Fraser et al., 2000; Taylor et al., 2006) by shortening the run time while targeting isoprenoids contributing to phenotypic plasticity and acclimation to changing environmental conditions. Our method provides a fast and reliable separation of all major carotenoids of higher plants within a run time of only 35 minutes per sample. This is only one third to half of the time required by previously published methods (Fraser et al., 2000; Taylor et
al., 2006). Furthermore, we achieve excellent parallel detection of the photoprotective xanthophylls including violaxanthin, antheraxanthin and zeaxanthin, lutein epoxide and lutein, as well as a separation of α- and β-carotene, and α-tocopherol. The separation of the isomers lutein and zeaxanthin is challenging in many protocols (Lichtenthaler et al., 1982; Gilmore & Yamamoto, 1991), but we achieved an excellent baseline separation. This is also indicated by a separation factor of 1.13 (Table A6.1) which is similar to several more recent methods (Fraser et al., 2000: 1.15; Gupta et al., 2015: 1.14; Taylor et al., 2006: 1.36) and beyond earlier methods (Gilmore and Yamamoto, 1991: 1.07). We can thus separate all major and minor isoprenoids involved in photoprotective non-photochemical quenching and scavenging of ROS in plants in a single run.

Although the proposed HPLC method can be used to separate pigments dissolved in various solvents, a methanol-based extraction was used due to its compatibility with our C30-column. Using methanol, the extraction solvent can be directly injected and good peak shapes are achieved and baseline separation promoted (Zapata & Garrido, 1991). Originally, (aqueous) acetone has been used to extract chlorophylls and carotenoids (Thayer & Björkman, 1990; Gilmore & Yamamoto, 1991). Recently, the use of methanol for extraction of chlorophylls was recommended, because of its lower volatility, its contribution to an improved peak shape of polar pigments, and a more representative chlorophyll a/b ratio (Porra et al., 1989; Wright & Jeffrey, 2006). Nevertheless, chlorophylls are less stable than chlorophylls in pure methanol compared to the extraction solvent acetone (van Leeuwe et al., 2006). Therefore, we used 98 % methanol buffered with ammonium acetate which enhances the stability of chlorophylls (Mantoura et al., 1997). Acetonic and methanolic pigment extractions from higher plants have shown that methanol extracts most pigments as efficiently as acetone, with the exception of a slightly lower efficiency for β-carotene (Dunn et al., 2004). We compared the yield of our methanolic extraction to an extraction with acetone and confirmed that the yield of extraction with methanol is as high as with aceton, except for an about 6% lower extraction of carotenes (Fig. A6.3). Longer extraction times or an additional sonication step did not improve the yield of extractions (data not shown) and were therefore omitted. The reproducibility of the method was estimated by the average percent coefficient of variation (CV) between six samples of Douglas-fir needles. CV values lower than 5 % indicate a high reproducibility of the extraction and analysis of all pigments (Table A6.1).
The spectral characteristics of the studied isoprenoids are summarized in Table A6.1. Chlorophylls, carotenoids and tocopherols vary in their absorbance maxima. In order to achieve a high sensitivity of the method for all substance classes, HPLC chromatograms were recorded at wavelengths close to the respective absorbance maxima. The absorption spectra of chlorophyll a showed absorption maxima at 431.5 nm and 665.5 nm, and the absorption spectra of chlorophyll b showed maximum absorbance at 466.0 nm and 649.5 nm. We therefore used a chromatogram recorded at a wavelength of 656 nm to quantify chlorophylls. Absorbance spectra of carotenoids are characterized by three absorbance maxima between 400-500 nm and were therefore quantified using chromatograms recorded at 450 nm. Tocopherols have a narrow absorbance maximum between 280-305 nm and were quantified using chromatograms obtained at 290 nm as described previously (Fraser et al., 2000; Guzman et al., 2012). The limit of detection (LOD) and limit of quantification (LOQ) are therefore very low, indicating a high sensitivity of the method (Table A6.1).

Figure 2 shows chromatograms recorded at 290 nm, 450 nm and 656 nm for an Arabidopsis pigment extract. The chromatogram at 290 nm shows a solvent peak at 2.5-3.5 min, α-tocopherol at 12.4 min and peaks corresponding to carotenoids and chlorophylls which also absorb at 290 nm (Fig. 2.2a, Table A6.1). The chromatogram at 450 nm represents all carotenoids in the order of decreasing polarity, starting with violaxanthin (11.1 min), neoxanthin (11.8 min), antheraxanthin (14.5 min), chlorophyll b (15.8 min), lutein (16.5 min), zeaxanthin (17.9 min), chlorophyll a (19.4 min), β-carotene (24.7 min) and cis-β-carotene (25.0 min) (Fig. 2b, Table A6.1). The chromatogram at 656 nm shows chlorophyll a at 15.8 min and chlorophyll b at 19.4 min (Fig. 2.2c, Table A.1). Chlorophylls were also quantified at 450 nm, but chromatograms at 656 nm reflect exclusively chlorophylls and their degradation products. Chromatograms at 656 nm wavelength are therefore ideal to validate sample integrity and chlorophyll derivatives.
Fig. 2.2: HPLC chromatograms of photosynthetic pigments and isoprenoids from leaves of Arabidopsis thaliana. Chromatograms were recorded at A) 290 nm, B) 450 nm, C) 656 nm. Ant, antheraxanthin; βCar, β-carotene; cis-βCar, cis-β-carotene; Chl a, chlorophyll a; Chl b, chlorophyll b; Lut, lutein; Neo, neoxanthin; αToc, α-tocopherol; Vio, violaxanthin; Zea, zeaxanthin.

The dynamics of the photoprotective violaxanthin-zeaxanthin xanthophyll cycle as well as the lutein epoxide cycle can be accurately determined by our method, as can be seen in Fig. 2.3 showing the chromatograms obtained from avocado (Persea americana) leaves. Recently, Förster et al. (2011) demonstrated the accumulation of lutein epoxide in shade-acclimated avocado leaves. We observed a sharp lutein epoxide peak in Fig. 2.3a. In comparison, Fig. 2.3b reveals a decrease in the amount of lutein epoxide, but an increased amount of lutein in light-acclimated avocado leaves, demonstrating the increased de-epoxidation state of the photoprotective lutein epoxide cycle pigments upon illumination. Similarly, the light-acclimated leaf sample exhibited a smaller amount of violaxanthin but a higher amount of zeaxanthin compared to the shade-acclimated leaf sample (Fig. 2.3), indicating an enhanced de-epoxidation state of the violaxanthin-zeaxanthin xanthophyll cycle. In addition, the chromatograms also
reveal high amounts of $\alpha$-carotene which is considered an indicator of shade acclimation (Matsubara et al., 2009).

Fig. 2.3: Comparison of HPLC chromatograms of photosynthetic pigments and isoprenoids from A) shade-grown and B) light-acclimated avocado leaves (*Persea americana*) recorded at 450 nm. Ant, antheraxanthin; $\alpha$Car, $\alpha$-carotene; $\beta$Car, $\beta$-carotene; cis-$\beta$Car, cis-$\beta$-carotene; Chl a, chlorophyll a; Chl b, chlorophyll b; Lut, lutein; LutEpox, lutein epoxide; Neo, neoxanthin; Vio, violaxanthin; Zea, zeaxanthin.

The ability of our method to detect changes in the composition of photosynthetic pigments in response to environmental conditions and in different species and tissues is demonstrated in Fig. 2.4-5 and Fig. A6.1-A6.2. Pigment composition can be accurately assessed from leaves of trees, including deciduous species and conifers such as maple and Douglas-fir, common crop species (wheat, corn and soy) and green algae, fruits and roots. The separation of chlorophyll a, chlorophyll b, violaxanthin, neoxanthin, lutein epoxide, antheraxanthin, lutein, zeaxanthin, $\beta$-carotene, cis-$\beta$-carotene in all species was obtained using our standard protocol for pigment extraction and our standard HPLC protocol without any modifications (Fig. 2.4-2.5 and Fig. A6.1). In accordance with the conserved composition of carotenoids of the photosynthetic
apparatus including lutein, violaxanthin, antheraxanthin, zeaxanthin, neoxanthin, and β-carotene (Pogson et al., 1998), the ratio of these pigments to each other is quite similar across tree and crop species. The only exception are the variable fractions of the xanthophyll cycle pigments violaxanthin, antheraxanthin and zeaxanthin. In contrast, the amounts of α-carotene and lutein epoxide are highly variable across species. Interestingly, the leaf samples of white oak (Quercus alba), corn (Zea mays) and soy (Glycine max) also contained lutein epoxide (Fig. A6.1b,e,f). Lutein epoxide is widely distributed across different plant taxa including monocotyledon plants and has also been described in oak trees (García-Plazaola et al., 2003b; Esteban et al., 2009b). However, few studies have reported lutein epoxide when pigments were analysed, although it might affect vegetation indices derived from remote-sensing in a similar way as the violaxanthin-zeaxanthin xanthophyll cycle pigments. The absorption maximum of violaxanthin is 439.0 nm, while the absorption maxima of zeaxanthin is 450.5 nm. Therefore, the de-epoxidation of the xanthophyll cycle pigments causes a shift of the absorption maxima by about 10 nm towards longer wavelengths (Britton et al., 2004). Changes of the xanthophyll cycle de-epoxidation state can be detected as a change in the photochemical reflectance index (PRI) (Wong & Gamon, 2015). Since the de-epoxidation of lutein epoxide to lutein causes a similar shift in the absorption spectra (Britton et al., 2004), PRI is likely also affected by changes in the lutein epoxide cycle.

For remote-sensing and plant phenotyping approaches, variation in photosynthetic pigment content is a valuable indicator for plant performance and phenological events. Fig. 2.4 demonstrates the seasonal variation in foliar pigment levels in deciduous and conifer trees. A senescent sugar maple leaf sampled in autumn reveals decreased chlorophyll and carotenoid content compared to a non-senescent leaf sampled in summer (Fig. 2.4a,b; equal amounts of fresh weight were used for the extraction of pigments). In addition to decreased chlorophylls and carotenoids, the autumn leaf sample also reveals several peaks with a late retention time that are absent in the summer sample. These peaks are caused by carotenoid esters which can typically be observed in senescing leaf tissue and indicate carotenoid degradation during leaf senescence (Biswal, 1995). Evergreen conifers retain their leaves during winter. This is paralleled by a reorganization of the thylakoid membrane and changes in energy partitioning that primes the violaxanthin-zeaxanthin xanthophyll cycle for sustained non-photochemical quenching (Ensminger et al., 2006; Fréchette et al., 2015). This acclimation to winter stress conditions is revealed in the chromatogram of Douglas-fir needles sampled in February. Compared to summer
samples, winter-acclimated needles show an enhanced carotenoid-chlorophyll ratio, largely caused by decreases in chlorophyll content, but also indicate an increase in the xanthophyll cycle de-epoxidation state that facilitates sustained non-photochemical quenching (Fig. 2.4c,d).

Fig. 2.4: HPLC chromatograms showing seasonal changes of photosynthetic pigments and isoprenoids in deciduous and evergreen trees recorded at 450 nm. A) Green sugar maple leaf sampled in summer, B) senescent (light green) sugar maple leaf sampled in October, C) summer-acclimated Douglas-fir needles sampled in September, and D) winter-acclimated Douglas-fir needles sampled in February. Ant, antheraxanthin; αCar, α-carotene; βCar, β-carotene; cis-βCar, cis-β-carotene; Chl a, chlorophyll a; Chl b, chlorophyll b; Lut, lutein; Neo, neoxanthin; Vio, violaxanthin; Zea, zeaxanthin.

The ability to monitor the effect of environmental stress on plant performance through changes in foliar pigment composition is shown in Fig. 2.5. Drought-stressed common bean (*Phaseolus vulgaris*) leaves show decreased photosynthetic pigment content and a shift from violaxanthin.
towards zeaxanthin, indicating an increased xanthophyll cycle de-epoxidation state (Fig. 2.5a,b). In comparison, drought-stressed Douglas-fir seedlings (*Pseudotsuga menziesii*) show a less plastic response to drought, but still reveal an increased de-epoxidation of the xanthophyll cycle pigments. This is seen by a higher proportion of zeaxanthin and antheraxanthin but lower proportion of violaxanthin among the xanthophyll cycle pigments (Fig. 2.5c,d).

![HPLC chromatograms of photosynthetic pigments and isoprenoids from well-watered and drought-stressed plants recorded at 450 nm.](image)

**Fig. 2.5:** Comparison of HPLC chromatograms of photosynthetic pigments and isoprenoids from well-watered and drought-stressed plants recorded at 450 nm. A,B) Common bean (*Phaseolus vulgaris*), and (C,D) Douglas-fir. Ant, antheraxanthin; αCar, α-carotene; βCar, β-carotene; cis-βCar, cis-β-carotene; Chl a, chlorophyll a; Chl b, chlorophyll b; Lut, lutein; Neo, Neoxanthin; Vio, violaxanthin; Zea, zeaxanthin.

Our method can also be applied to accurately detect pigments in green algae, and in tissues from higher plants other than leaves. Fig. A6.2a shows the photosynthetic pigments extracted from the
green algae *Chlorella variabilis*, revealing a pigment composition similar to higher plants (Grumbach *et al.*, 1978). *Cuscuta* is a genus of parasitic plants devoid of functional leaves which has reduced chloroplasts and shows only low photosynthetic rates (Choudhury & Sahu, 1999). *Cuscuta* shows almost no chlorophylls and a complete lack of neoxanthin (Snyder *et al.*, 2004), but produces additional carotenoids like β-cryptoxanthin and carotenoid esters with unknown function (Mukherjee *et al.*, 2008). The chromatogram obtained from a sample of *Cuscuta gronovii* stem tissue confirms this unusual composition (Fig. A6.2b). The unidentified peaks occurring between 25-28 min represent carotenoid esters which are characterized by carotenoid-like absorption spectra, but generally have a later retention time (Zonta *et al.*, 1987). The pigment extract of ripe red tomato fruits (*Solanum lycopersicum*) contains little lutein and α-carotene, but large amounts of β-carotene and lycopene (Fig. A6.2c). The sample of carrot roots (*Daucus carota*) contains large amounts of α- and β-carotene (Fig. A6.2d). Since fresh carrots typically lack the cis-isomer of β-carotene (Lessin & Schwartz, 1997), this confirms that the commercially available β-carotene standard and the major β-carotene peak in the samples obtained from photosynthetic tissue represents the trans-isomer of β-carotene, while the peak at 24.9 min represents the cis-isomer of β-carotene (e.g. *Arabidopsis*, Fig. 2.2b) which is involved in photosynthesis (Bialek-Bylka *et al.*, 1996).

A major advantage of our method is the precise baseline separation of all major photosynthetic pigments and rare carotenoids such as lutein epoxide and α-carotene in a single run within a total runtime of only 35 min. A second major advantage of our method over previously published methods (e.g. Taylor *et al.*, 2006) is the low and constant water content in the mobile phase. This has two important implications. First, the low water content of only 3 % lowers the column backpressure which in our experience never exceeds 105 bar. This low pressure further allows for higher flow rates in most HPLC systems and hence decreases the runtime and increases sample throughput. The other important implication is that although water is required to improve the column specificity, variations in water content effect the column hydration status. Using a constant water content throughout the entire run allows to maintain the hydration status of the stationary phase constant. This minimizes the reconditioning time of the column at the end of each run, when the initial solvent concentration is re-established and residual effects of the prior solvents on the stationary phase are abolished following each run (Gilmore & Yamamoto, 1991).
Due to the unchanged hydration status, reconditioning of the column is achieved after only 7 min compared to e.g. 10-30 minutes in other protocols (Fraser et al., 2000; Taylor et al., 2006).

Furthermore, methanol is an inexpensive solvent compared to acetonitrile, and only small volumes of MTBE are used in our method. High concentrations of MTBE (94 %) are only necessary to elute the non-polar carotenoids such as lycopene and carotenoid esters during the last 4 minutes of each sample run. To reduce costs, for samples lacking extremely non-polar carotenoids such as lycopene, the method can be even adjusted to a maximum of only 81.3 % MTBE for one minute compared to 94 % for four minutes (Table 2.1b). This can save 35 % of MTBE compared to our standard method and is sufficient to elute all major carotenoids. The solvent gradient is the same until minute 22 which corresponds to a retention time of 25 min due to the delay volume between pump and column. Since cis-β-carotene elutes after 24.9 min, the described adjustment does not affect the retention times of all other isoprenoids but the rare lycopene. The analysis of photosynthetic pigments could furthermore be extended to include carotenoid esters, as have been observed in senescing leaves or in *Cuscuta* (Fig. 2.4, Fig. A6.1). To achieve separation of carotenoid esters, the gradient from min 20 onwards can be decreased to slow down the elution of esters and improve their separation. If tocopherols need to be detected with higher sensitivity, an additional fluorescence detector could be used. When results of the method should be compiled using different systems, canthaxantin and α-tocopherol acetate can be used as internal standards (Lashbrooke et al., 2010).

### 2.5 Conclusions

We developed a fast and reliable HPLC method to separate all major plant photosynthetic pigments including chlorophylls, carotenoids and tocopherols. The short run time of only 35 min together with the use of the relatively inexpensive organic solvents methanol and MTBE allows for a cost-effective and high-throughput analysis of samples. The simultaneous analysis of all pigments involved in the regulation of light-harvesting and energy dissipation upon exposure to changing environmental conditions, including pigments of the xanthophyll and lutein epoxide cycle, α- and β-carotene and α-tocopherol, have to our knowledge not been achieved by previously published methods. Since the method is broadly and easily applicable, we propose it
as a robust tool to validate information derived from remote-sensing and plant phenotyping applications.

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Chapter 3

Douglas-fir provenances vary in isoprenoid-mediated photoprotective mechanisms under controlled drought conditions

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3 Douglas-fir provenances vary in isoprenoid-mediated photoprotective mechanisms under controlled drought conditions

3.1 Abstract

Essential and volatile isoprenoids are involved in plant abiotic stress responses, as they protect plants from excess light energy and mitigate oxidative stress. Here we assess the important question: Do Douglas-fir (Pseudotsuga menziesii) provenances that originate from habitats with contrasting water availability reveal intraspecific variation of the isoprenoid metabolism? For this purpose we compared photosynthetic capacity, photoprotection by accessory pigments and volatile isoprenoid pool sizes as well as emission of volatile isoprenoids of two Douglas-fir provenances originating from contrasting habitats under controlled drought conditions. One-year-old seedlings of an interior and a coastal Douglas-fir provenance were subjected to reduced watering for six weeks. We further assessed performance of the seedlings following a two-week recovery from drought stress. We observed variation in the regulation of photosynthetic gas exchange and metabolism of essential and non-essential isoprenoids. The interior provenance exhibited a more conservative regulation of stomatal conductance, indicated by reduced water loss but lower assimilation rates. Additionally, the interior provenance showed enhanced photoprotection of the photosynthetic apparatus by increases of the essential isoprenoids including the xanthophyll cycle pigments, β-carotene and neoxanthin. In contrast, the coastal provenance exhibited generally higher assimilation rates, but an enhanced biosynthesis and emission of non-essential volatile isoprenoids. Our results demonstrate that there is intraspecific variation in isoprenoid-mediated photoprotective mechanisms among Douglas-fir provenances. Enhanced photoprotection might be an important trait to mitigate the negative effects of prolonged drought periods expected under future climate conditions.

3.2 Introduction

Many tree species that are growing well under current climate conditions will not be able to withstand the changing climatic conditions expected in future (St Clair & Howe, 2007). Climate change imposes increased abiotic stress on trees, especially by prolonged drought periods.
Drought is thought to limit forest productivity and increase tree mortality (Bolte et al., 2009; Allen et al., 2010). Nevertheless, there is also considerable variation in drought tolerance between and within species (e.g. George et al. 2015). To enhance resistance and resilience of forests to drought, tree species or provenances are suggested to be promoted by forest management which are better adapted to the predicted future drier and warmer climatic conditions (Aitken et al., 2008; Bolte et al., 2009).

The North American conifer species Douglas-fir is an economically important tree species at its origin and in plantations across Europe due to its high productivity and moderate drought tolerance (Hermann & Lavender, 1999; Sergent et al., 2014). A coastal subspecies (var. menziesii) occurs along the west coast under mainly moist maritime climatic conditions, and an interior subspecies (var. glauca) inhabits mountainous regions under rather dry continental climatic conditions (Ferrell & Woodard, 1966; Aas, 2008; Lavender & Hermann, 2014). Variation in climatic conditions within the coastal and interior habitats lead to local adaptation of provenances to moist or dry conditions also within the coastal and interior subspecies, respectively (Aitken et al., 1995; Anekonda et al., 2002; Montwé et al., 2015). Douglas-fir provenances from contrasting habitats are known to vary in radial and height growth under drought (Eilmann et al., 2013; Jansen et al., 2013; Neophytou et al., 2016). Provenances originating from dry habitats typically exhibit a lower productivity, but are generally considered to be better adapted to drought (Sergent et al., 2014; Bansal et al., 2015; Montwé et al., 2015).

Adaptation to drought is conferred either by drought avoidance and drought tolerance mechanisms. Drought avoiding tree species prevent low water potentials, e.g. by reduced rates of water loss due to lower stomatal conductance (Brunner et al., 2015). In contrast, drought tolerant tree species are able to maintain higher gas exchange rates because they tolerate higher water loss and reduced water potential. In drought-avoiding trees, the regulation of stomatal conductance in response to drought is rather isohydric, e.g. early stomatal closure to maintain water potential, while drought-tolerant trees are rather anisohydric, e.g. late stomatal closure to sustain photosynthesis at the increased risk of leaf damage and xylem cavitation (Hoffmann et al., 2011). Anisohydric stomatal regulation has been shown to contribute to faster growth of anisohydric compared to isohydric poplar genotypes under controlled drought conditions (Attia et al., 2015). Therefore, intraspecific variation in stomatal regulation may thus contribute to variation in productivity under drought (Sade et al., 2012).
Species furthermore vary in the intrinsic water use-efficiency (IWUE) (Lévesque et al., 2014). IWUE is the ratio between CO$_2$ assimilation rate and stomatal conductance, which is often assessed by the discrimination of $^{13}$C (Farquhar et al., 1982). IWUE can be enhanced under drought to foster CO$_2$ assimilation (Chaves et al., 2009). Intraspecific variation in IWUE has been observed in various tree species including Douglas-fir (Aitken et al., 1995; Jansen et al., 2013). Although IWUE was not directly correlated to productivity of poplar provenances under drought, it was seen as a promising trait to select tree species which need less water to sustain productivity under drought (Monclus et al., 2006).

Generally, the reduction of stomatal conductance under drought leads to reduced CO$_2$ assimilation which thus limits the photochemical quenching of absorbed light energy and increases the demand for non-photochemical quenching (NPQ). Excess light energy otherwise promotes the formation of reactive oxygen species (ROS) which lead to photooxidative damage of the photosystems and membranes (Chaves et al., 2009). Reduced light absorbance by the photosystems, increased NPQ and enhanced scavenging of ROS can minimize photooxidative stress caused by drought (Baroli & Niyogi, 2000; Munekage et al., 2002). Accessory photosynthetic pigments and many antioxidants involved in these photoprotective mechanisms are essential isoprenoids, which are ubiquitous in all plant species (Esteban et al., 2009b). In addition, many plant species including Douglas-fir also produce non-essential volatile isoprenoids which contribute to plant stress tolerance (Owen & Peñuelas, 2013).

The essential isoprenoids associated with the photosynthetic apparatus are carotenoids which serve a dual function in photosynthetic light-harvesting. Under low light conditions, these accessory pigments enhance light uptake. When light energy exceeds the photosynthetic capacity, they are involved in NPQ and scavenging of ROS (de Bianchi et al., 2010). The major plant carotenoids of the photosynthetic apparatus are lutein, neoxanthin, the xanthophyll cycle pigments (violaxanthin, antheraxanthin and zeaxanthin), and β-carotene (Pogson et al., 1998). These carotenoids serve different functions in photosynthesis and photoprotection and are adjusted in response to drought stress (Esteban et al., 2015a).

The xanthophyll cycle pigments violaxanthin, antheraxanthin and zeaxanthin are directly involved in quenching of excess light energy. While violaxanthin increases the light harvesting efficiency under light-limited conditions, zeaxanthin can dissipate excess light energy via heat
The deepoxidation of violaxanthin via antheraxanthin to zeaxanthin in response to excess light energy provides an instantaneous mechanism to facilitate NPQ, while the pool size of the xanthophyll cycle pigments is adjusted on the long-term and determines the photoprotective capacity (Niinemets et al., 1998; Demmig-Adams & Adams, 2006). Lutein is important for correct folding of the antennae proteins, involved in light harvesting (Jahns & Holzwarth, 2012) and can protect plants from photooxidative stress by the deactivation of triplet chlorophyll (Dall'Osto et al., 2006). Neoxanthin is another xanthophyll involved in protection of PSII from oxidation by detoxifying superoxide anions (O$_2^-$) (Dall'Osto et al., 2007a). While the abovementioned xanthophylls are bound to the antenna complexes of the photosystems, β-carotene protects the reaction centers of the photosystems from oxidative damage (Nayak et al., 2002; Telfer, 2005). Some plant species additionally biosynthesize α-carotene which occurs especially in shade-tolerant species (Matsubara et al., 2009) and also varies seasonally (Ottander et al., 1995; Siefermann-Harms, 1994). Furthermore, carotenoids are important antioxidants for scavenging of ROS (Esteban et al., 2015b).

Although the composition of the photosynthetic apparatus is remarkably consistent among species (Pogson et al., 1998), adjustments of the photoprotective isoprenoids contribute to the acclimation of plants to different environmental conditions (Esteban et al., 2015a). Under drought, when photochemical quenching of light energy is decreased and the demand for NPQ is enhanced, an increased carotenoid-to-chlorophyll ratio has been observed in many species, e.g. in Mediterranean trees, and the spruce Picea asperata (Faria et al., 1998; Duan et al., 2005). Furthermore, increased pool sizes of xanthophyll cycle pigments have been observed in response to drought in many species, including many forest tree species (Wujeska et al., 2013). Other plant species also exhibit increased amounts of lutein in response to drought (Esteban et al., 2015a).

Many plant species also produce non-essential volatile isoprenoids, consisting of hemi-, mono- and sesquiterpenes, that are involved in mitigation of abiotic stress (Peñuelas & Munné-Bosch, 2005). While some volatile isoprenoids can be stored within specialized leaf structures, others are emitted instantly following their biosynthesis (Ghirardo et al., 2010). Volatile isoprenoids are thought to enhance abiotic stress tolerance by the use of excess energy during biosynthesis, stabilization of membranes and their antioxidant function (Possell & Loreto, 2013; Palmer-
Young et al., 2015). The emission of volatile isoprenoids occurs constitutively, but can also be induced by abiotic or biotic stress conditions (Loreto et al., 1998; Peñuelas et al., 2005). Volatile isoprenoid emissions thus partly originate from pools of stored isoprenoids and partly originate from de novo synthesis (Ghirardo et al., 2011).

The suit of photoprotective processes that are employed in response to drought contribute to mitigate photooxidative stress under drought. Tree provenances that vary in drought tolerance might thus reveal intraspecific differences in photoprotective mechanisms in response to drought. Nevertheless, evidence for intraspecific variation in photoprotective mechanisms is scarce. Intraspecific differences in chlorophylls and energy quenching have been observed in two Picea asperata populations from contrasting climates (Duan et al., 2005). In contrast, provenances from Quercus coccifera L. varied in energy quenching, but did not differ in photosynthetic pigments (Balaguer et al., 2001). Intraspecific variation in isoprenoid-mediated photoprotective mechanisms among Douglas-fir provenances was suggested by a study with field-grown mature Douglas-fir trees of four provenances (Junker et al., in press). Seedlings of the two provenances used in this experiment have been shown to differ in levels of antioxidants ascorbate and α-tocopherol induced by drought (Du et al., 2016). Intraspecific variation in the regulation of stomatal conductance and photoprotective isoprenoids in response to drought might reveal different strategies to cope with drought stress among provenances and identify traits which contribute to drought and could thus facilitate the selection of provenances suitable to grow under future climate conditions.

The aim of this study was to assess if two Douglas-fir provenances, that originate from contrasting habitats, vary in isoprenoid-mediated photoprotective mechanisms and induction thereof in response to drought. Photosynthetic gas exchange, photosynthetic pigments as well as volatile isoprenoid pools and emission were studied in seedlings of an interior and a coastal provenance exposed to limited watering for six weeks, followed by a two week recovery phase in comparison to well-watered control seedlings. Seedlings from both provenances are expected to reduce photosynthetic gas exchange in response to limited soil water availability to avoid water loss. Consequently, we expect an induction of isoprenoid-mediated photoprotective mechanisms to mitigate photooxidative stress. We hypothesized that interior seedlings will show lower stomatal conductance to minimize water loss which is needed to withstand prolonged drought periods which are frequently occurring in their natural habitat. Consequently, we expect
enhanced NPQ of excess energy by essential isoprenoids in the interior provenance, to combat lower photochemical quenching of light energy. In contrast, the coastal provenance is thought to sustain higher assimilation rates at the expense of higher water loss, but a lower need for photoprotective mechanisms. We furthermore expect that provenances differ in the photoprotective mechanisms mediated by volatile isoprenoids, because they are under much less selective pressure compared to essential isoprenoids. Nevertheless, differences in capacity of the antioxidant machinery, as previously observed for the provenances studied in our experiment, may reduce the demand for enhanced photoprotection mediated by essential isoprenoids or enhanced accumulation and emission of non-essential volatile isoprenoids.

3.3 Materials and Methods

3.3.1 Plant material

One-year-old seedlings of an interior (var. glauca) and a coastal (var. menziesii) Douglas-fir provenance were obtained from nurseries. Seedlings of the interior provenance Fehr Lake (INT) (seedlot: FDI 39841, N50.71, W120.86) were obtained from BC Timber Sales (Vernon, Canada). INT originates from a dry habitat (800 m above sea level, 5.8 °C mean annual temperature) in British Columbia with an annual precipitation of 333 mm, and 162 mm precipitation during the growing season (May to September). Seedlings of the coastal provenance Snoqualmie (COA) (seedlot: pme 07(797) 412-10) were obtained from Forestry Commission, Wykeham Nursery (Sawdon, England). COA originates from a humid habitat (457-610 m above sea level, 7.9 °C mean annual temperature) with an annual precipitation of 2134 mm, and 365 mm precipitation during the growing season. Seedlings of Snoqualmie were grown from seeds collected from a group of trees in seed zone Snoqualmie, Washington (Zone 412 of the Tree Seed Zone Map, 1973). Seedlings of Fehr Lake were grown from seed collected in a seed orchard, which was established using seeds collected in the respective seed zones. Upon arrival in March/ April, seedlings were planted in 3l pots with medium-fibrous peat soil (Container substrate 1 medium + GreenFibre basic, pH = 5.3; Klasmann-Deilmann GmbH, Geeste, Germany) and fertilized with NPK fertilizer (N170 + P200 + K230 + Mg100 + S150 mg l⁻¹).
3.3.2 Growth conditions

The experiment was carried out at the Leibniz Centre for Agricultural Landscape Research in Müncheberg, Germany from June to September of 2011. Two walk-in environmental chambers (VB 8018, Vötsch Industrietechnik GmbH, Germany) equipped with metal halide lamps (Powerstar HQI-BT 400 W/D PRO Daylight, Osram GmbH, Munich, Germany). Temperature was maintained at 21 °C during day and night, with a relative humidity of 70%. Light intensity at canopy height was 500 μmol m⁻² s⁻¹ for 16 h per day. Seedlings were randomly distributed among both chambers and watered daily with 100 ml water (50% tap water, 50% distilled water). The drought treatment for half of the seedlings per chamber started after more than three months of acclimation under growth conditions as mentioned above on July 20th after the second flush in most seedlings of COA and some seedlings of INT had occurred.

3.3.3 Control and drought treatment

Control seedlings were watered daily from July 20th (day 0) onwards with 200 ml water, while watering of the seedlings of the drought treatment was only 65 ml until August 10th (day 21). From August 10 to August 31 watering was entirely withheld. On the evening of August 31 (day 42), seedlings of the drought treatment were rewatered generously. All seedlings were grown for another two weeks with daily supply of 200 ml water. Daily soil moisture measurements with ECH2O sensors (EC5, Decagon Devices, Inc., Pullman, USA) in the upper 10 cm of substrate were used as guidance for consistent soil water availability of control and drought stress seedlings. Soil moisture tension was calculated according to Schindler et al. (2010). COA seedlings consistently required about ten percent more water in order to keep soil moisture comparable to interior seedlings.

Pre-dawn twig water potential was monitored throughout the experiment on days 7, 17, 26, 36, 42, 43 and 52 using twigs of N=3 seedlings. Twig water potential was determined using a pressure chamber (Model 3015G4, Soil moisture Equipment Corp., Santa Barbara, CA, USA) according to Scholander et al. (1965).
3.3.4 Sampling

Samples of current-year needles (excluding second flush) were taken prior to the drought treatment on June 28th (shown as day 0), and on days 28, 41 (drought), 56 (rewatered) for the determination of photosynthetic pigments and stored volatile isoprenoids. Needles were cut from the twigs and immediately frozen in liquid nitrogen. Samples were stored at -80°C and ground immersed in liquid nitrogen using mortar and pestle.

3.3.5 Photosynthesis measurement

Photosynthesis measurements were conducted on June 28th (shown as day 0), day 20, 41, 43 and 56. Gas exchange was measured on N=5 seedlings per provenance and treatment in current-year needles of the uppermost whirl using a LI-COR 6400 XT portable gas exchange system (LI-COR Biosciences, Lincoln, NE, USA). About 10-15 needles on an intact twig were placed into the cuvette to form a flat area. Measurement conditions in the closed cuvette were set to a 400 ml min$^{-1}$ flow rate, 25 °C block temperature, 40% relative humidity, and a CO$_2$ concentration of 400 ppm. Prior to starting the gas exchange measurements, needles were dark-adapted for 25 minutes. Measurements of the steady state of photosynthetic CO$_2$ gas exchange were taken at 0, 400, 500, 1000, 1500 and 2000 µmol photons m$^{-2}$ s$^{-1}$ light intensity after a minimum of 5 min acclimation time at each light intensity. Light response curves were modelled using a nonlinear least square fit (function nls; Chambers and Bates, 1992) of the function $y=A*(1-exp(-b*L))+R$ with $A$ = maximum net photosynthesis rate, $b$ = coefficient of curvature, $L$ = light intensity, and $R$ = rate of respiration. Maximum photosynthetic rate ($A_{max}$) was calculated as $A_{max}= A+R$, light compensation point ($I_0$) as $I_0=-1/b*ln((1+R)*A^{-1})$ and light intensity at half-maximum rate of photosynthesis ($I_{1/2}$) as $I_{1/2}=-1/b*ln(0.5+0.5 A*R^{-1})$. Light exposed needle surface area was then determined using WinSeedle software and scanner (Regents Instruments Inc., Québec, Canada). The rate of photosynthetic gas exchange was expressed per projected needle area exposed to the light. Intrinsic water-use efficiency was calculated as the ratio of net CO$_2$ assimilation rate to stomatal conductance (IWUE= $A/g_s$).
3.3.6 Analysis of photosynthetic pigments

Pigments were extracted using 98% methanol buffered with 0.5 M ammonium acetate and analysed by HPLC-DAD following the protocol described in Junker and Ensminger (2016). A high-performance liquid chromatography (HPLC) system (model 1260, Agilent Technologies, Böblingen, Germany) with a quaternary pump (model 1260), autosampler (model 1260, set to 4 °C), column oven (model 1260, set to 25 °C) and photodiode array detector (model 1290, recording absorption at 290 nm, 450 nm and 656 nm wavelength) was used for reverse-phase chromatography using a C₃₀-column (5 µm, 250*4.6 mm; YMC Inc., Wilmington, NC, USA). Three solvents (A: 100% methanol, B: 100% methyl-tert-butyl-ether, C: water buffered with 20 mM ammonium acetate) were used to run a gradient starting with 92% A, 5% B, and 3% C. After 3 minutes, Solvent A was gradually replaced by solvent B, while solvent C remained constantly at 3%. The amount of B increased to 33.6% at 17 minutes, 81.3% B at 22 minutes, and reached a maximum of 81.3% B from 23 to 27 minutes. Afterwards, the initial solvent concentration was re-established within one minute, and the column reconditioned for seven minutes prior to the next run. Peaks were quantified using standards for chlorophyll a, chlorophyll b and β-carotene from Sigma Aldrich (Oakville, ON, Canada). Standards for antheraxanthin, α-carotene, lutein, neoxanthin, violaxanthin and zeaxanthin were obtained from DHI Lab products (Hørsholm, Denmark). ChemStation B.04.03 software (Agilent Technologies, Böblingen, Germany) was used for peak integration.

3.3.7 Sampling of emitted volatile isoprenoids

Emitted volatile isoprenoids of current-year samples were collected on day 21, 25 (drought) and 53 (rewatered) independent from other measurements using customized one liter glass enclosure consisting of a lower half with a wide opening to insert seedlings and an upper half which was set atop enclosing the whole seedling without physical contact. To avoid any effects from young second-flush needles, second-flush twigs were removed two days prior to the start of volatile isoprenoid sampling (day 19). One day prior to the measurements, seedlings were carefully inserted into the lower parts of the enclosures to avoid a contamination of measurements by needle injuries. The opening around the stem was sealed using sealing tape (Terostat VII Henkel Teroson GmbH, Heidelberg, Germany). Before the actual measurement, the airtight enclosure
was closed and supplied with a mixture of synthetic air (Air Liquide, Ludwigshafen, Germany) and 400 ppm CO₂. Temperature in the cuvette was maintained at 24.5 ±1.5 °C, with an illumination of 690 ±50 µmol m⁻² s⁻¹. After 5 min acclimation time, air was drawn from the outlet of the cuvettes with a flow rate of 200 ml min⁻¹ using an air sampling pump (Analyt-MTC, Müllheim, Germany). Emitted volatiles were trapped using air sampling tubes packed with adsorbent beds of 20 mg Tenax TA 60/80 and 30 mg Carbotrap B 20/40 (Supelco, Bellafonte, PA, USA) between glass wool. After 40 min, air sampling tubes were disconnected and stored in airtight glass vials at 4 °C until analysis. To determine the needle mass in the enclosure, needles within the enclosure were sampled after the end of the experiment and their dry weight was determined. Monoterpene emission rates were calculated per dry weight and over time. Zero references using an empty cuvette were used to correct for background emission.

3.3.8 Extraction of stored volatile isoprenoids
Volatile isoprenoids stored in sampled needles were extracted using 500 µl methanol per 25 mg fresh weight. After 20 min of stirring at 30 °C followed by centrifugation, extracted isoprenoids were bound by stirring at 1400 rpm with pre-conditioned polydimethylsiloxane (PDMS) coated Twisters® (10 mm length, 1 mm PDMS coat; Gerstel, Germany) for 60 min at 30 °C. A control sample (pure methanol instead of the extract) was run with every set of samples to control for background contamination. Twisters® were dried with a lint free paper tissue and placed into glass cartridges for immediate analysis with TDU-CIS and GC-EI/MS.

3.3.9 Volatile isoprenoid analysis
Emitted and stored volatile isoprenoids were analysed using gas chromatograph (GC, model 7890A, Agilent, Germany), coupled to a mass-selective detector (MS, 5975C, Agilent, Germany) and equipped with a thermodesorption/cold injection system (TDU-CIS; Gerstel, Germany) according to Müller et al. (2013). Cartridges/ Twisters® were desorbed with the TDU at 240°C, cryofocused at -100°C and heated to 240°C in the CIS prior to injection into the GC-MS: Separation on a DB-624 column (Agilent, Germany) occurred during an oven temperature programme beginning at 40 °C, increasing at a rate of 6 °C min⁻¹ for 3 min to 100 °C, when the
temperature ramp speed up to 16 °C min⁻¹ until the column reached 230 °C. Volatile isoprenoids were identified by comparison of peaks and de-convoluted fragmentation spectra to external standards and to the NIST database using the AMDIS software (National Institute of Standards and Technology (NIST), Gaithersburg, MD, USA). Monoterpenes include camphene, (+)-carene, 3-carene, limonene, β-myrcene, ocimene, β-phellandrene, α-pinene, β-pinene, sabinene, α-terpinene, γ-terpinene, and tricyclene. Sesquiterpenes include γ-cadinene, α-caryophyllene, β-caryophyllene, α-cubebene, β-elemene, δ-elemene, and (+)-longifolene. Conversion from fresh to dry weight was determined by drying approximately 10 g of frozen needles for 4 h at 200 °C.

3.3.10 Statistics

All statistical tests were performed using R 3.0.3 (R Development Core Team, 2010). A linear mixed-effect model using Provenance and Treatment as fixed factors and sampling day as random factor were used to evaluate if provenance or treatment specific differences occurred during the drought stress (function lmer, package lme4, Bates et al. 2013). Models using Provenance, Treatment, Provenance + Treatment and Provenance x Treatment were compared to a null model considering only an intercept and sampling day as random factor. Based on lowest Akaike Information Criterion (AIC; Akaike 1973) the best-fit model was chosen. Significance of the fixed factors was assessed by a pairwise comparison of the best-fit model to the best-fit model minus one of each of its fixed effects (function anova, Zuur 2009).

Differences between provenances and treatments on each sampling day were determined using the parametric Kruskal-Wallis-rank-rum-Test (function kruskal.test) followed by a Tukey-type pairwise comparison using the function nparcomp (package nparcomp). Due to only minor variation in the composition of stored and emitted volatile isoprenoids, amounts were averaged per provenance and treatment and summarized in Table 3.3.

Differences in responses between provenances as shown in Figures 8 and 9 were determined comparing linear models (function lm) describing the response of a provenance to a parameter as Provenance x Parameter by an Tukey-type comparison using the function lsmeans (package lsmeans). It must be noted that gas exchange parameters were repeatedly assessed from the same set of plants. Therefore, provenance-specific effects may be overestimated. Nevertheless, we
consider our approach valid, since the only parameter which revealed provenance-specific differences in response to the gas exchange parameter assimilation, sesquiterpenes, revealed an obvious difference.

3.4 Results

3.4.1 Water availability and plant growth

For control seedlings, a target soil water tension (pF) of 2.4 log(-ψ(cm H₂O)) was maintained, corresponding to 37% volumetric soil water content (Fig. 3.1a). For drought-stressed seedlings, pF increased to a maximum of 7.7 log(-ψ(cm H₂O)) in both provenances by day 42, which corresponds to a volumetric water content as low as 2%. Seedlings of the coastal provenance (COA) received about 10% more water than seedlings of the interior provenance (INT) received, to keep soil water availability at similar levels in both provenances and to be able to compare adjustments of photoprotective isoprenoids between provenances despite differences in water use. When seedlings were rewatered on the evening of day 42, soil water tension recovered to control levels within one day. Predawn twig water potential of drought stressed seedlings of both provenances decreased only after day 36, with lowest water potential of -2.4 MPa in INT and -2.7 MPa in COA on day 42 (Fig. 3.1b). After rewatering, twig water potential of drought stressed seedlings recovered to control plant levels within one day.

INT seedlings had an initially higher total biomass of 18.6±0.5 g dry weight (DW) compared to COA with only 6.0±0.9 g DW. During the control treatment, COA gained 16.5 g biomass and INT gained 13.0 g biomass (Fig. 3.2a). Biomass gain of both provenances was reduced under drought and still delayed after rewatering, resulting in a total biomass gain of 8.1 g in COA drought seedlings and 3.5 g in INT drought seedlings. Leaf mass per area (LMA) showed a slight increase in control seedlings of both provenances (Fig. 3.2b). Needles of INT seedlings were thicker and more rigid compared to needles of COA, which is expressed in a slightly higher LMA (Fig. 3.2b). LMA of both provenances were significantly lower during the drought treatment (Table 3.1), but were comparable to control seedlings two weeks after rewatering.
Fig. 3.1: Soil water availability and drought stress of interior (INT) and coastal (COA) Douglas-fir seedlings under control and drought conditions. A) Soil water tension (pF) and B) pre-dawn twig water potential (Ψ). Data show mean of n = 3 measurements (±SE). Light grey background indicates period of reduced watering, dark grey background indicates period of withheld watering, followed by rewatering.

Fig. 3.2: Growth and needle morphology of interior (INT) and coastal (COA) Douglas-fir seedlings under control and drought conditions. A) Biomass gain, B) leaf mass per area (LMA). Data show mean of n = 5 measurements (±SE). Significant differences (p < 0.05 using Kruskal-Wallis-rank-sum-test) are indicated by different letters. Light grey background indicates period of reduced watering, dark grey background indicates period of withheld watering, followed by rewatering.
Table 3.1: The effect of provenance and treatment on biomass, photosynthetic gas exchange and isoprenoid metabolism. For each parameter, the best model including Provenance, Treatment and the interaction thereof was chosen according to the Akaike information criterion (AIC). The effects of Treatment and Provenance were assessed by pairwise comparison of models with and without each factor using a log-likelihood ratio test. Significance levels are given as p-values which are bolded if p<0.05. IWUE = Intrinsic water use efficiency, Chlorophylls a+b = total chlorophyll per fresh weight, Carotenoids = total carotenoids per total chlorophyll, VAZ = xanthophyll cycle pigments per total carotenoids, DEPS= de-epoxidation status of the xanthophyll cycle pigments, Lutein = Lutein per total carotenoids, Neoxanthin = Neoxanthin per total carotenoids, β-carotene = β-carotene per total carotenoids, α-carotene = α-carotene per total carotenoids, Stored monoterpenes = total stored monoterpenes per dry weight, stored sesquiterpenes = total stored sesquiterpenes per dry weight, Emission of monoterpenes = total monoterpene emissions.

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3.4.2 Photosynthetic gas exchange

COA control seedlings showed an initial stomatal conductance (g_s) of 0.18 mmol H_2O m^{-2} s^{-1} which decreased over the course of the experiment to 0.11 mmol H_2O m^{-2} s^{-1}. g_s of INT control
seedlings was consistently lower and ranged between 0.1 to 0.15 mmol H$_2$O m$^{-2}$ s$^{-1}$ (Fig. 3.3a). $g_s$ decreased to a minimum of 0.03 mmol H$_2$O m$^{-2}$ s$^{-1}$ in COA drought seedlings and 0.01 mmol H$_2$O m$^{-2}$ s$^{-1}$ in INT drought seedlings. Upon rewatering, $g_s$ increased to 0.04 mmol H$_2$O m$^{-2}$ s$^{-1}$ in both provenances within one day and reached $g_s$ comparable to control seedlings within two weeks after rewatering. COA thus showed a significantly higher $g_s$ compared to INT, and both provenances were equally impacted by the drought treatment (Table 3.1).

Transpiration rates ($E$) exhibited a pattern similar to $g_s$, with generally higher values in COA compared to INT and reduced $E$ during the drought treatment in both provenances (Fig. 3.3b, Table 3.1). COA control seedlings reached maximum $E$ of 3.7 mol H$_2$O m$^{-2}$ s$^{-1}$ compared to 3.0 mol H$_2$O m$^{-2}$ s$^{-1}$ in INT control seedlings (Fig. 3.3b). $E$ in both provenances decreased during the drought treatment to a minimum of 0.6 mol H$_2$O m$^{-2}$ s$^{-1}$ in COA which was twice as high compared to INT seedlings of the drought treatment with 0.3 mol H$_2$O m$^{-2}$ s$^{-1}$. Therefore, $E$ was significantly affected by Treatment as well as Provenance (Table 3.1).

Assimilation rates ($A$) were 10-12 µmol CO$_2$ m$^{-2}$ s$^{-1}$ for control seedlings of both provenances, with a drop to lower values of 8 µmol CO$_2$ m$^{-2}$ s$^{-1}$ at the end of the experiment (Fig. 3.3c). During the drought treatment, both provenances showed significantly lowered $A$, with minimum values of 3.5 µmol CO$_2$ m$^{-2}$ s$^{-1}$ in COA and 1.1 µmol CO$_2$ m$^{-2}$ s$^{-1}$ in INT at the end of the drought treatment (Fig. 3.3c, Table 3.1). One day after rewatering, $A$ was increased by 3.2 ±0.9 µmol CO$_2$ m$^{-2}$ s$^{-1}$ in INT but only by 1.6 ±0.6 µmol CO$_2$ m$^{-2}$ s$^{-1}$ in COA. Within 14 days after rewatering, $A$ of both provenances recovered to control plant levels.

The intrinsic water use efficiency (IWUE), calculated as the ratio between assimilation rate and stomatal conductance ($A/g_s$), did not vary in control seedlings of both provenances. INT control seedlings had an initially higher IWUE of 98 µmol CO$_2$ mol$^{-1}$ H$_2$O compared to 75 µmol CO$_2$ mol$^{-1}$ H$_2$O in COA control seedlings, but did not significantly vary over the course of the experiment (Fig. 3.3c, Table 3.1). Both provenances revealed significantly increased IWUE under drought, with maximum values of about 115 µmol CO$_2$ mol$^{-1}$ H$_2$O in both provenances at the end of the drought treatment.
Fig. 3.3: Gas exchange parameters of interior (INT) and coastal (COA) Douglas-fir seedlings under control and drought conditions. A) Stomatal conductance ($g_s$), B) transpiration rate ($E$), C) assimilation rate ($A$), and D) intrinsic water use efficiency (IWUE). Data show mean of $n=5$ measurements (±SE) taken at a light intensity of 1000 µmol photons m$^{-2}$ s$^{-1}$. Significant differences ($p<0.05$ using Kruskal-Wallis-rank-sum-test) are indicated by different letters. Light grey background indicates period of reduced watering, dark grey background indicates period of withheld watering, followed by rewatering.

Measurements of light response curves allowed additionally to estimate the maximum assimilation rate ($A_{max}$), the half-saturation light intensity and the light compensation point (Fig. 3.4; Table 3.2). On day 0 and day 41, control seedlings of COA showed insignificantly higher $A_{max}$ at light intensities exceeding 750 µmol photons m$^{-2}$ s$^{-1}$ compared to INT control seedlings (Fig. 3.4a,b, Table 3.2). At the end of the drought treatment (day 41), $A_{max}$ was significantly reduced in both provenances (Fig. 3.4b; Table 3.2). After recovery (day 56), $A_{max}$ of control and previously drought exposed seedlings of both provenances were comparable (Fig. 3.4c,
Table 3.2). Treatment-induced differences in $A_{\text{max}}$ on day 42 also affected the half-saturation light intensity and the light compensation point which were increased in seedlings of the drought treatment compared to seedlings of the control treatment in both provenances (Table 3.2).

Fig. 3.4: Light response curve of interior (INT) and coastal (COA) Douglas-fir seedlings under control and drought conditions taken on A) day 0, B) day 41, and C) day 56. Data show mean of $n = 3-5$ measurements (±SE). Significant differences ($p < 0.05$ of the maximum assimilation rate using Kruskal-Wallis-rank-sum-test) are indicated by different letters.
Table 3.2: Cardinal points of the light response curves of an interior and a coastal Douglas-fir provenance under control conditions, drought and recovery. Maximum assimilation rate, light intensity at half maximum assimilation rate and light compensation point were estimated from exponential curve-fitting of gas exchange measurements obtained from n=5 (±SE) seedlings per provenance and treatment. Significant differences (p < 0.05 using Kruskal-Wallis-rank-sum-test) are indicated by different letters.

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<th>COA Control</th>
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<tr>
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<td>9.4 ±2.5</td>
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<td>83 ±57</td>
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3.4.3 Photosynthetic pigments

The chlorophyll content decreased over the course of the experiment in both provenances. COA showed a significantly higher chlorophyll content with initial values of 2.1 µmol g⁻¹ FW compared to 1.6 µmol g⁻¹ FW in INT (Fig. 3.5a, Table 3.1). Both provenances showed non-significant increased chlorophyll content during the drought treatment, but recovered to the level of control seedlings by day 56. The chlorophyll a/b ratio was stable in control seedlings of both provenances, with higher chlorophyll a/b ratio in INT compared to COA (Fig. 3.5b). During the drought treatment, the chlorophyll a/b ratio decreased in both provenances, but to a stronger extent in INT compared to COA, thus exhibiting a significant Provenance x Treatment interaction (Table 3.1). The carotenoid/chlorophyll ratio in both provenances increased as chlorophylls decreased in control seedlings over the course of the experiment, and was significantly higher in INT compared to COA (Fig. 3.5c;Table 3.1). Although we did not observe a significant Treatment effect, we observed a nonsignificant increase of the carotenoid/chlorophyll ratio in INT seedlings at the end of the drought treatment and significantly higher carotenoid/chlorophyll ratios in recovered INT seedlings compared to recovered COA seedlings.
Fig. 3.5: Chlorophylls and carotenoids of interior (INT) and coastal (COA) Douglas-fir seedlings under control and drought conditions. A) Total chlorophylls per fresh weight (Chlorophylls FW⁻¹), B) chlorophyll a to chlorophyll b ratio (Chl a Chl b⁻¹) and C) carotenoids per total chlorophyll (Carotenoids Chl⁻¹). Data show mean of n = 5 samples (±SE). Significant differences (p < 0.05 using Kruskal-Wallis-rank-sum-test) are indicated by different letters. Light grey background indicates period of reduced watering, dark grey background indicates period of withheld watering, followed by rewatering.
The pigments of the xanthophyll cycle in relation to total carotenoids showed an increase from 0.2 to 0.35 mol mol\(^{-1}\) Car in both provenances during the first month of the experiment (Fig. 3.6a). During the whole experiment, xanthophyll content of COA seedlings of the drought treatment was equal to control seedlings, with a decrease during the last two weeks of the experiment. In contrast, INT seedlings showed an increased xanthophyll content at the end of the drought treatment compared to control seedlings and had not recovered to control plant levels two weeks after rewatering. The deepoxidation state of the xanthophyll cycle (DEPS) also showed a general increase during the first month of the experiment (Fig. 3.6b). Both provenances showed a significant treatment effect, with increased DEPS at the end of the drought treatment, which was more pronounced for INT (Fig. 3.6b, Table 3.1).

\(\beta\)-carotene per carotenoids was relatively constant during the experiment, with a slight decrease by day 41 followed by an increase towards the end in both provenances (Fig. 3.6c). \(\beta\)-carotene was with 0.141 mol mol\(^{-1}\) Car in INT significantly higher compared to 0.12 mol mol\(^{-1}\) Car in COA, but did not vary between control and drought stressed seedlings (Table 3.1). Neoxanthin decreased in relation to total carotenoids over the course of the experiment, from initial values of 0.13 mol mol\(^{-1}\) Car to 0.05 mol mol\(^{-1}\) Car in control seedlings of both provenances (Fig. 3.6d). INT showed a slight, but significant higher neoxanthin content compared to COA, while no treatment effect was observed (Table 3.1). \(\alpha\)-carotene decreased in all seedlings over the course of the experiment. \(\alpha\)-carotene decreased from 0.10 to 0.03 mol mol\(^{-1}\) Car in COA and 0.13 to 0.01 mol mol\(^{-1}\) Car in INT, respectively (Fig. 3.6e, Table 3.1). Lutein per carotenoids increased in both provenances over the course of the experiment. COA had a significantly higher lutein content, which increased from 0.47 to 0.61 mol mol\(^{-1}\) Car, compared to INT, which had a lutein content increasing from 0.43 to 0.56 mol mol\(^{-1}\) Car (Fig. 3.6f). Lutein content was not affected by the drought treatment (Table 3.1).
Fig. 3.6: Essential isoprenoids of interior (INT) and coastal (COA) Douglas-fir under control and drought conditions. A) Xanthophyll cycle pigments per total carotenoids (VAZ Chl⁻¹), B) de-epoxidation state of the xanthophyll cycle (DEPS, calculated as (0.5A+Z)/(V+A+Z)⁻¹), C) β-carotene per total carotenoids, D) neoxanthin per total carotenoids, E) α-carotene per total carotenoids and F) lutein per total carotenoids. Data show mean of n = 5 samples (±SE). Significant differences (p < 0.05 using Kruskal-Wallis-rank-sum-test) are indicated by different letters. Light grey background indicates period of reduced watering, dark grey background indicates period of withheld watering, followed by rewatering.
3.4.4 Volatile isoprenoid pools and emissions

The monoterpene pool sizes in seedlings of both treatments remained stable over the course of the experiment, but revealed a significant Provenance effect (Fig. 3.7a, Table 3.1). INT pool sizes were 187 µmol g⁻¹ FW and COA stored about 138 µmol g⁻¹ FW monoterpenes. Sesquiterpene pool sizes of control seedlings of both provenances were relatively stable around 1.5 µmol g⁻¹ over the course of the experiment (Fig. 3.7b). While INT seedlings of the drought treatment did not deviate from control seedlings, COA seedlings of the drought treatment showed increased sesquiterpene pool sizes with a maximum of 3.5 µmol g⁻¹, indicating a significant Provenance x Treatment interaction (Table 3.1). Emission levels of monoterpenes were quite stable and did not vary among treatments, but showed a high tree-by-tree variation. Monoterpene emissions of COA were significantly higher compared to INT (Fig. 3.7c; Table 3.1).

The composition of volatile isoprenoid pools and emission showed little variation between treatments but varied between provenances (Table 3.3). COA revealed higher amounts of stored β-pinene and 3-carene, but lower amounts of α-pinene and camphene compared to INT. Sesquiterpene pools were much more similar between provenances, but COA exhibited slightly higher amounts of β-caryophyllene and lower amounts of (+)-longifolene compared to INT (Table 3.3). Monoterpene emissions exhibited high variation between individual trees, but did not significantly differ between provenances.
Fig. 3.7: Volatile isoprenoids of interior (INT) and coastal (COA) Douglas-fir seedlings under control and drought conditions. A) stored volatile monoterpenes, B) stored volatile sesquiterpenes and C) monoterpane emission. Data show mean of n = 5 samples (±SE). Significant differences (p < 0.05 using Kruskal-Wallis-rank-sum-test) are indicated by different letters. Light grey background indicates period of reduced watering, dark grey background indicates period of withheld watering, followed by rewatering.
Table 3.3: Composition of volatile isoprenoid pools and emissions. The percentage of the five most abundant stored monoterpenes and sesquiterpenes as well as emitted monoterpenes per provenance are listed. For control plants data from all samplings was averaged (N=15, ±SE), while drought plants exclude samples taken after recovery (N=10, ±SE). Significant differences (p < 0.05 using Kruskal-Wallis-rank-sum-test) are indicated by different letters.

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<td>β-caryophyllene</td>
<td>8.6 ±2.4&lt;sup&gt;a&lt;/sup&gt; 7.2 ±2.1&lt;sup&gt;ab&lt;/sup&gt; 13.1 ±1.4&lt;sup&gt;ab&lt;/sup&gt; 15.5 ±1.6&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>β-elemene</td>
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<tr>
<td>δ-elemene</td>
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<tr>
<td>(+)-longifolene</td>
<td>17.1 ±7.5&lt;sup&gt;a&lt;/sup&gt; 7.3 ±2.1&lt;sup&gt;a&lt;/sup&gt; 5.2 ±2.1&lt;sup&gt;ab&lt;/sup&gt; 0.0 ±0.0&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Limonene</td>
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</table>

3.4.5 Physiological adjustments in response to decreasing water availability

The twig water potential of both provenances was upheld as soil water tension increased up to a pF of 7 log(-ψ[cm H₂O]) during the drought treatment (Fig. 3.8a). When soil water tension further increased towards the end of the drought treatment and volumetric water content of the soil was as low as 2%, twig water potential steeply decreased to extremely low values. Consequently, gs in both provenances was downregulated before twig water potential dropped (Fig. 3.8b). Nevertheless, minimum stomatal conductance in both provenances was observed in response to minimum twig water potential. The downregulation of gs correlated well with the decrease in A (Fig. 3.8c). We did not observe significant variation between provenances, which represents similar IWUE (represented by the slope of the regression line) between provenances.
Fig. 3.8: Relationship between A) soil water tension (pF) and pre-dawn twig water potential ($\Psi$; lowest values were excluded from the correlation), B) pre-dawn twig water potential ($\Psi$) and stomatal conductance ($g_s$), C) stomatal conductance ($g_s$) and assimilation rate ($A$). Each data point represents mean of $n = 3$-5 measurements (±SE) taken per sampling day. Lines represent a linear regression fit per provenance across seedlings of the control (closed circles) and drought treatment (open circles). $R^2$-values indicate the coefficient of determination per provenance, n.s. denotes non-significant variation estimated by ANOVA comparison of linear models describing the independent variable interaction. $A$ and $g_s$ were measured at a light intensity of $1000 \mu$mol photons m$^{-2}$ s$^{-1}$. 
Fig. 3.9: Adjustments of isoprenoids involved in protective mechanisms in response to decreasing assimilation rate (A). A) xanthophyll cycle pigments per total carotenoids (VAZ Chl⁻¹), B) de-epoxidation state of the xanthophyll cycle (DEPS), C) stored volatile monoterpenes, D) stored volatile sesquiterpenes in relation to assimilation rate (A). Each data point represents mean of n = 5 measurements (±SE) taken per sampling day. Lines represent a linear regression fit per provenance across seedlings of the control (closed circles) and drought treatment (open circles). R²-values indicate the coefficient of determination per provenance, p-values indicate significant differences estimated by ANOVA comparison of linear models describing the independent variable interaction, n.s. denotes non-significant differences. A was measured at a light intensity of 1000 µmol photons m⁻² s⁻¹.

The adjustments of photoprotective mechanisms in response to decreased assimilation rates under drought visualizes the effects of Treatment and Provenance. The xanthophyll cycle pool sizes in both provenances were increased when A were decreased, but revealed generally higher values and stronger variability in INT compared to COA (Fig. 3.9a). Both provenances showed a marked increase of DEPS in response to decreasing A, confirming the significance of DEPS in
response to *Treatment* (Fig. 3.9b, Table 3.1). While monoterpenes pool sizes were largely invariable in both provenances, sesquiterpene pool sizes of both provenances increased with decreasing assimilation rate (Fig. 3.9d). COA showed higher sesquiterpene pool sizes and stronger induction as A decreased, confirming the significant *Provenance x Treatment* interaction in adjustments of sesquiterpenes in response to drought (Table 3.1).

### 3.5 Discussion

#### 3.5.1 Provenances differ in water use, biomass accumulation and needle morphology

Our results revealed differences in photosynthetic gas exchange and photoprotection by essential and non-essential isoprenoids between the two Douglas-fir provenances. Seedlings of both provenances were exposed to increasing soil water tension, until a minimum soil water content of about 2% was reached (Fig. 3.1a). Only then, both provenances exhibited a sudden drop of twig water potential (Fig. 3.1b). This is in accordance with observations that Douglas-fir effectively minimize water loss to maintain twig water potential for weeks without watering and at very low soil water availability (Khan *et al.*, 1996; Dinger & Rose, 2009). We only observed a slightly earlier increase of soil water tension and decrease of twig water potential in COA compared to INT, but significantly higher stomatal conductance ($g_s$) in COA, which lead to enhanced water loss by transpiration.

INT seedlings revealed a reduction of biomass gain by 70% during the drought treatment, compared to only 50% reduction in COA (Fig. 3.2a). In comparison, control seedlings gained comparable dry matter over the course of the experiment, although the initial weight of INT seedlings was threefold higher compared to COA. This difference can be attributed to upbringing in different nurseries and intraspecific differences in seedling morphology (Rehfeldt, 1989). For logistical reasons, seedlings had to be purchased from different nurseries. Acclimation of provenances to different conditions at the nurseries might have exacerbated differences between provenances due to genotypic variation. We minimized variation due to phenotypic plasticity by acclimating seedlings of both provenances for more than three months to experimental conditions. Nevertheless, differences between provenances may not exclusively reflect genotypic variation, but also variation in the range of phenotypic plasticity and greenhouse effects which
might be especially obvious in the size of seedlings. Needle development of seedlings mainly occurred when seedlings were already growing under controlled conditions, and thicker and more rigid needles of the interior compared to the coastal provenance likely represent an adaptive mechanism to dry habitats (Zhang & Marshall, 1995; Niinemets, 2001). Nevertheless, this did not lead to significant variation in leaf mass per area (LMA; Fig. 3.2b). The effect of drought on carbon metabolism were also implied by reduced LMA during the drought treatment in both provenances, which can be attributed to reduced amounts of nonstructural carbohydrates in needles when photosynthetic carbon assimilation is limited (Bertin & Gary, 1998; Poorter et al., 2009). This was also observed in drought-stressed Pinus sylvestris (Bansal et al., 2013).

Higher stomatal conductance (g_s) in COA compared to INT under control and drought conditions contributed to increased water loss and higher productivity of COA (Fig. 3.3a, Table 3.1). The limiting effect of g_s on transpiration rates (E) and assimilation rates (A) (Fig. 3.3b,c) is well established, especially when g_s is below 0.15 mol m\(^{-2}\) s\(^{-1}\) (Flexas et al., 2004). Both provenances maintained a high twig water potential by an early decrease of g_s in response to drought (Fig. 3.1b), which indicates an isohydric regulation of stomatal conductance that is typical for conifers (Warren et al., 2003; McCulloh et al., 2014). Contrary to differences in the tendency to anisohydry among seedlings of Sequoiadendron giganteum populations from contrasting habitats (Ambrose et al., 2015), we did not observe differences in the regulation of stomatal conductance among the Douglas-fir provenances studied here.

Higher E of COA lead to an enhanced water use of this provenance which was met by about 10 % more watering compared to INT. Although this did not negatively affect COA during the short-term drought applied here, it increases the susceptibility of COA to prolonged drought periods (McDowell, 2011). In contrast, the generally lower E of INT indicates a rather conservative water use, which is thought to increase survival and allow for a faster recovery (Attia et al., 2015). On the other hand, higher g_s in COA compared to INT also enabled higher A and might have contributed to the non-significantly higher growth of COA (Fig. 3.3a,c). Both provenances increased the intrinsic water use efficiency (IWUE) by about 40 % in response to drought (Fig. 3.3d), as was previously observed in coastal Douglas-fir (Smit & van den Driessche, 1992). Previous studies on IWUE in Douglas-fir provenances showed a negative correlation between IWUE and altitude of origin (Zhang et al., 1993; Aitken et al., 1995; Zhang & Marshall, 1995). We rather observed slightly higher IWUE in INT compared to COA
(significant on day 0), although INT originates from higher altitudes compared to COA. This variation is within the range that could be expected from earlier studies (Zhang et al., 1993; Aitken et al., 1995).

Reduced maximum assimilation rates and higher half-saturation light intensities under drought as derived from light response curve modelling revealed that photosynthesis is not light limited, but light energy is available in excess and cannot be used in photochemical reactions (Fig. 3.4, Table 3.2). Reduced photochemical quenching of absorbed light energy consequently enhanced the need for photoprotective mechanisms which quench excess light energy non-photochemically. An efficient protection of the photosynthetic apparatus is also indicated by the fast recovery of photosynthetic assimilation rate in both provenances upon rewatering (Fig. 3.3).

3.5.2 Essential isoprenoids indicate enhanced photoprotection of the photosynthetic apparatus in the interior provenance

Provenances revealed significant differences in the composition of essential isoprenoids associated with the photosynthetic apparatus. INT showed significantly lower chlorophyll content, higher chlorophyll a/b ratio and higher carotenoid/chlorophyll content compared to COA (Fig. 3.5, Table 3.1). These characteristics have also been displayed by Pinus species exposed to high irradiance (Sánchez-Gómez et al., 2006). Lower chlorophyll content reduces the uptake of light energy (Lei et al., 1996; Murchie & Horton, 1997). A high chlorophyll a/b ratio indicates reduced antennae complex size which reduces light absorbance (Bailey et al. 2001). Higher carotenoid-chlorophyll ratio together with a significantly increased ratio at the end of the drought treatment in INT, can be attributed to enhanced stress tolerance and photoprotection (Baquedano & Castillo, 2006). Besides these constitutive differences between provenances, a more pronounced decrease of the chlorophyll a/b ratio in response to drought in INT compared to COA revealed a significant Treatment x Provenance (genotype by environment) effect (Table 3.1). The physiological function of a decreased chlorophyll a/b ratio under drought is unknown, although it has been previously observed in different tree species including Aleppo pine (Pinus halapensis Miller), Phoenicean juniper (Juniperus phoenicea L.) and beech (Fagus sylvatica L.) (Garcia-Plazaola & Becerril, 2000; Baquedano & Castillo, 2006). The overall decrease in chlorophyll content in all seedlings was likely related to decreasing nitrogen content
of the soil over the course of the experiment, as seedlings were last fertilized before the drought treatment to avoid tampering of soil moisture measurements (see Ripullone et al. 2003).

Provenance-specific variation was also observed in the composition of carotenoids, suggesting differences in the regulation of carotenoids biosynthesis among provenances (Fig. 3.6). Interestingly, we did not observe significant treatment- or provenance-specific differences in xanthophyll cycle pigments (Table 3.1), although the xanthophyll cycle is well-known to contribute to NPQ by thermal dissipation of excess light energy (Demmig-Adams et al., 2014). Nevertheless, a significant increase of xanthophyll cycle pool size in INT seedlings at the end of the drought treatment (Fig. 3.6a) and significantly increased de-epoxidation status of the xanthophyll cycle pigments (DEPS) in both provenances indicate an enhanced demand for photoprotection under drought (Fig. 3.6b, Table 3.1; see Demmig-Adams and Adams 2006). Furthermore, we observed significantly higher proportion of β-carotene and neoxanthin, but lower proportion of the most abundant carotenoid, lutein, in INT compared to COA (Fig. 3.6c,d,f). Carotenoids biosynthesis starts from the precursor lycopene and diverges into the α-branch, which leads to α-carotene and lutein, and the β-branch, which leads to β-carotene, the xanthophyll cycle pigments and neoxanthin (Ruiz-Sola & Rodríguez-Concepción, 2012). The α- and β-branch carotenoids serve different but complementary photoprotective functions (Dall’Osto et al., 2007b), e.g., higher levels of β-carotene at the expense of α-carotene as observed in INT are considered an adjustment to high light (Telfer, 2005; Matsubara et al., 2009). The observed provenance-specific differences suggest an enhanced biosynthesis of β-branch carotenoids in INT.

3.5.3 The coastal provenance shows enhanced protection by non-essential isoprenoids

Both provenances exhibited substantial variation in amount and composition of stored and emitted monoterpenes and sesquiterpenes (Fig. 3.7). Monoterpene pool sizes and emissions did not vary in response to the drought treatment, but COA showed higher monoterpene emissions despite lower monoterpene pool sizes compared to INT (Fig. 3.7a,c). Intraspecific variation in amount and composition of stored monoterpenes was previously observed within species of the genus Quercus, but could not be related to abiotic stress tolerance (Staudt et al., 2004). Emitted
monoterpenes either originate from stored pools or are synthesized *de novo* (Ghirardo et al., 2011). In conifer species, namely *Pinus sylvestris* and *Picea abies*, a third to half of emitted monoterpenes were synthesized *de novo* (Ghirardo et al., 2010). Furthermore, discrepancies between the composition of stored and emitted monoterpenes in both provenances (Table 3.3) suggest, that substantial amounts of monoterpenes emitted originate from *de novo* biosynthesis. We conclude that COA must have maintained an enhanced biosynthesis of monoterpenes compared to INT. The enhanced emission of volatiles implies a loss of previously fixed carbon and might result in reduced growth (Šimpraga et al., 2011; Ryan et al., 2014). However, we observed comparable biomass gain between control seedlings of COA and INT despite lower initial biomass of COA, and less reduction of biomass gain under drought in COA (Fig. 3.2a). Enhanced protection from oxidative stress by volatile isoprenoids in COA may have entailed higher assimilation rates observed in COA.

COA also revealed strongly induced sesquiterpene pool sizes in COA during the drought treatment (Fig. 3.7b, Table 3.1). An increased emission of sesquiterpenes in Douglas-fir has been observed in coastal Douglas-fir in response to heat (Joó et al., 2011). This indicates an involvement of sesquiterpenes in abiotic stress responses, although their exact function is still unknown (Palmer-Young et al., 2015).

### 3.5.4 Intraspecific variation in the physiological response to drought

The observed variation in photosynthetic gas exchange and essential and non-essential isoprenoids mainly described constitutive variation between seedlings and showed little provenance-specific responses to drought. Both provenances maintained a high and nearly unchanged twig water potential even when soil water tension was high (Fig. 3.8a), which was facilitated by a tight regulation of $g_s$ to maintain nearly unchanged twig water potential (Fig. 3.8b). This indicates an isohydric regulation of stomatal behavior, which is typical for conifers (McDowell et al., 2008). We did not observe intraspecific variation in the regulation of stomatal conductance or IWUE in response to drought, which is also indicated by similar $A / g_s$ ratios in INT and COA, respectively (Fig. 3.3d, Fig. 3.8c).
In contrast to our hypothesis, that INT shows a stronger induction of photoprotective mechanisms, we have only little evidence for provenance-specific differences in the physiological response to drought. This is in accordance with the observation by Hess et al. (2016), that two Douglas-fir provenances showed substantial variation in gene expression, but only a few genes were differentially regulated in response to environmental conditions. We observed a slightly stronger adjustment of xanthophyll cycle pool sizes per chlorophylls in INT compared to COA (Fig. 3.9a), which suggests a higher potential for NPQ in INT (Fig. 3.9a, see Murchie and Niyogi, 2011). Both provenances increased the DEPS of the xanthophyll cycle in response to reduced assimilation rates under drought (Fig. 3.9b), displaying the importance of protection of the photosynthetic apparatus from excess light energy under drought (Niyogi, 2000). Stable monoterpenes pool sizes despite variation in assimilation rate in both provenances indicate that decreased assimilation during drought did not cause a depletion of monoterpenes pools (Fig. 3.9c). This is interesting, since data on other conifers suggested that a substantial amount of emitted monoterpenes is synthesized de novo (Ghirardo et al., 2010), which is hypothesized to be hampered during severe drought stress (Nogués et al., 2015). Most interestingly, we observed strongly induced levels of stored sesquiterpenes in COA when A was low (Fig. 3.9d). Although the function of sesquiterpenes in abiotic interactions is not yet known (Palmer-Young et al., 2015), we demonstrate their involvement in abiotic stress response in a coastal Douglas-fir provenance.

3.5.5 Conclusions

Seedlings of a rather drought avoiding interior provenance and a rather drought tolerant coastal provenance of Douglas-fir varied in photosynthetic gas exchange and the amount and composition of essential and non-essential isoprenoids under control and drought conditions. The interior provenance exhibited generally lower stomatal conductance, consequently avoiding water loss. Therefore, a higher demand for NPQ was met by higher levels of essential carotenoids. In contrast, the coastal provenance showed generally higher stomatal conductance under drought despite higher loss of previously fixed carbon by monoterpenes emissions. In addition to these constitutive differences, provenances revealed variation in response to the drought treatment. The interior provenance exhibited significantly increased pool sizes of
xanthophyll cycle pigments at the end of the drought treatment. The coastal provenance showed a stronger induction of sesquiterpene pools in response to drought, revealing a genotype by environment effect. We conclude that Douglas-fir reveals intraspecific variation in isoprenoid-mediated photoprotective mechanisms which might be caused by local adaptation to habitats with contrasting water availability.

3.6 Acknowledgments

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Chapter 4
Variation in short-term and long-term responses of photosynthesis and isoprenoid-mediated photoprotection to soil water availability in four Douglas-fir provenances

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This chapter is in press in the journal Scientific Reports:

Variation in short-term and long-term responses of photosynthesis and isoprenoid-mediated photoprotection to soil water availability in four Douglas-fir provenances

4.1 Abstract

For long-lived forest tree species, the understanding of intraspecific variation among populations and their response to water availability can reveal their ability to cope with and adapt to climate change. Dissipation of excess excitation energy, mediated by photoprotective isoprenoids, is an important defense mechanism against drought and high light when photosynthesis is hampered. We used 50-year-old Douglas-fir trees of four provenances at two common garden experiments to characterize provenance-specific variation in photosynthesis and photoprotective mechanisms mediated by essential and non-essential isoprenoids in response to soil water availability and solar radiation. All provenances revealed uniform photoprotective responses to high solar radiation, including increased de-epoxidation of photoprotective xanthophyll cycle pigments and enhanced emission of volatile monoterpenes. In contrast, we observed differences between provenances in response to drought, where provenances sustaining higher CO2 assimilation rates also revealed increased water-use efficiency, carotenoid-chlorophyll ratios, pools of xanthophyll cycle pigments, β-carotene and stored monoterpenes. Our results demonstrate that local adaptation to contrasting habitats affected chlorophyll-carotenoid ratios, pool sizes of photoprotective xanthophylls, β-carotene, and stored volatile isoprenoids. We conclude that intraspecific variation in isoprenoid-mediated photoprotective mechanisms contributes to the adaptive potential of Douglas-fir provenances to climate change.

4.2 Introduction

The response of plant species to limited water availability varies widely, but there is also considerable variation within species and among populations (Chaves et al., 2002; Quero et al., 2006). With increases in drought and heat globally as a consequence of climate change, the survival of species may become dependent on populations that are inherently pre-adapted to drier and hotter climate (Aitken et al., 2008; Bansal et al., 2015). For long-lived forest tree species,
intraspecific variation in response to water availability contributes to their ability to cope with and adapt to climate change. Douglas-fir (Pseudotsuga menziesii) is one of the most ecologically and economically important tree species in Europe and in its origin, the Western USA and Canada (Kleinschmit, 1973). Douglas-fir thrives under diverse climatic conditions and thus has a wide distribution (Sergent et al., 2014). The coastal subspecies (var. menziesii) which originates from the humid maritime climate along the West Coast typically shows higher wood productivity compared to the interior subspecies (var. glauca), which occurs under dry conditions in the continental mountain regions (Sergent et al., 2014). Provenances of both varieties show high genetic and phenotypic diversity (Krutovsky & Neale, 2005) and vary in their drought tolerance and productivity under drought (Eilmann et al., 2013). Provenances that originate from dry environments are known to cope better with water limitations and drought stress conditions, but little is known about the physiological mechanisms contributing to drought tolerance (Bansal et al., 2015).

Conifers are generally well drought-adapted and exhibit a conservative regulation of stomatal conductance (g_s) to minimize water loss (McDowell et al., 2008). Intraspecific variation in morphological traits, such as a root-shoot ratio and xylem resistance to cavitation, as well as physiological traits, such as the efficiency of the regulation of g_s and intrinsic water use efficiency (IWUE) contributes to provenances’ drought tolerance and allows to maintain higher assimilation rates (A) under drought conditions. Nevertheless, reduced A under drought conditions leads to the formation of reactive oxygen species (ROS), which impose photooxidative stress (Niyogi, 2000). Therefore, drought enhances the demand for photoprotective mechanisms such as non-photochemical quenching (NPQ), scavenging of ROS, or production and emission of volatiles. These mechanisms are often mediated by isoprenoids (Baroli & Niyogi, 2000; Peñuelas & Munné-Bosch, 2005).

Essential isoprenoids include the photosynthetic pigments, chlorophylls and carotenoids (Esteban et al., 2015a). The pigment composition of the photosynthetic apparatus reflects long-term adjustments in response to environmental conditions and varies across species due to adaptation to different environments (Ensminger et al., 2004; Croce & van Amerongen, 2014; Fréchette et al., 2015; Junker & Ensminger, 2016). The xanthophyll cycle pigment pool size in relation to chlorophylls determines the photoprotective capacity of a plant and is increased in response to drought in many plant species (Esteban et al., 2015a), e.g. in species of the genus Quercus.
In contrast to these long-term adjustments, the de-epoxidation of the xanthophyll cycle pigments in response to excess energy provides an instantaneous mechanism to quench excess light energy and facilitate NPQ (Niyogi, 2000; Demmig-Adams & Adams, 2006). In addition to the well-known xanthophyll cycle, β-carotene protects the photosystem reaction centers by chemical scavenging of ROS and undergoes oxidation by an electron transport reaction (Telfer, 2005; Ramel et al., 2013). The breakdown of β-carotene results in metabolites that are involved in plant signalling (Ramel et al., 2013; Havaux, 2014). In line with this finding, β-carotene has the highest turnover rates among the major carotenoids in Arabidopsis, indicating a constant replenishment of β-carotene pool sizes (Beisel et al., 2010). β-carotene per chlorophylls can increase in response to irradiance, but the adjustments of β-carotene pool sizes under drought stress are unknown (Esteban et al., 2015a).

In addition to essential isoprenoids, many tree species including Douglas-fir produce non-essential volatile isoprenoids (Joó et al., 2011). Pools of stored volatile monoterpenes and sesquiterpenes provide a source for emission in response to biotic and abiotic stress and are adjusted to prevailing environmental conditions (Owen & Peñuelas, 2013). The emission of non-essential isoprenoids is a rapid response mechanism to protect plants from thermal and oxidative damage (Vickers et al., 2009). The biosynthesis of non-essential isoprenoids also serves as an important metabolic sink for electrons that result from the uptake of excess energy (Owen & Peñuelas, 2005). However, emission of volatile isoprenoids can also contribute to a significant loss of previously fixed carbon. Transgenic tobacco plants emitting isoprene showed lower ROS levels and lipid oxidation compared to non-isoprene-emitting tobacco, but also exhibited reduced growth (Ryan et al., 2014). In beech seedlings, monoterpene emissions resulted in decreased growth when seedlings were exposed to drought (Šimpraga et al., 2011).

Differences in short- and long-term responses of isoprenoid metabolism might contribute to intraspecific variation in photosynthetic carbon assimilation and photoprotective mechanisms under drought. Variation in the response of photosynthetic gas exchange from different Douglas-fir provenances is indicated by provenance-specific variation in growth performance (height and radial growth) in response to water-limiting conditions (Eilmann et al., 2013; Jansen et al., 2013), provenance-specific variation in carbon isotope composition (Aitken et al., 1995; Jansen et al., 2013) and differences in provenance susceptibility to xylem cavitation (Kavanagh et al., 2013).
Consequently, Douglas-fir provides an ideal model to study provenance-specific variation of the isoprenoid-mediated photoprotective mechanisms.

The aim of this study was to assess which isoprenoid-mediated photoprotective mechanisms are induced in response to drought stress in different Douglas-fir provenances. We hypothesized that all provenances show short-term responses such as enhanced NPQ, increased xanthophyll cycle de-epoxidation state and emission of non-essential volatile isoprenoids. Furthermore, provenances that exhibit lower assimilation rates are expected to show pronounced long-term adjustments compared to provenances which are able to maintain higher assimilation rates, including increased pools of xanthophyll cycle pigments and stored volatile isoprenoids.

The short- and long-term adjustments of photosynthesis, photosynthetic pigments as well as emission and pool sizes of monoterpenes in response to soil water availability were studied in 50-year-old trees of four Douglas-fir provenances grown in two provenance trials in south-western Germany.

4.3 Material and Methods

4.3.1 Field sites and plant material

We compared trees at two field sites in south-western Germany which are part of an international Douglas-fir (*Pseudotsuga menziesii*) provenance trial established in 1958 (Kenk & Thren, 1984). “Schluchsee” is located in the southern Black Forest (1050 m a.s.l) and represents a moderately cool, humid climate with 1345 mm mean annual precipitation and a mean annual temperature of 6.1 °C. “Wiesloch” is located in the Rhine valley (105 m a.s.l.) and is characterized by warmer and drier climatic conditions with 9.9 °C mean annual temperature and 660 mm mean annual precipitation (Kenk & Ehring, 2004). Meteorological data for 2010 and 2011 were obtained from nearby weather stations (Table 4.1).
Table 4.1: Climatic and geographical details of the field sites (elevation and mean annual parameters taken from Kenk & Ehring (2004)).

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<th>Wiesloch</th>
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<td>N49°16’41”, E8°34’37”</td>
</tr>
<tr>
<td>Elevation</td>
<td>1050 m</td>
<td>105 m</td>
</tr>
<tr>
<td>Mean annual temperature</td>
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<tr>
<td>Mean annual precipitation</td>
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<td>660 mm</td>
</tr>
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<td>Location of weather station</td>
<td>Schluchsee, 6 km distance N47°49’16”, E8°11’08” (elevation 992 m), privately operated</td>
<td>Waghäusel-Kirrlach, 4 km distance N49°15’0”, E8°32’24” operated by Deutscher Wetterdienst (DWD)</td>
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</table>

This included air temperature, precipitation and daily sunshine duration measured as the sum of hours when irradiance exceeds 120W m$^{-2}$ (World Meteorological Organisation, 2008). Sunshine duration has been shown to be a suitable proxy for solar irradiance due to the linear relationship between both parameters (Trnka et al., 2005; Bakirci, 2009). The 1961-1990 climate reference (Deutscher Wetterdienst, DWD) was used to characterize the long term climate at the sites. Soil water availability was calculated using the forest hydrological water budget model WBS3. The model estimates daily total available soil water (TAW) using temperature, precipitation, latitude, soil type, plant cover, slope, and slope aspect (Keitel et al., 2006). At both sites, we compared the interior provenance Salmon Arm (SAL) (var. glauca, originating from a dry habitat in British Columbia) and three coastal provenances Conrad Creek (CON), Cameron Lake (CAM), and Santiam River (SAN) (var. menziesii, all originating from humid habitats, see Table 4.2).

Table 4.2: Climatic and geographical details of the origin of the four Douglas-fir (Pseudotsuga menziesii) provenances used in the present experiments (Kenk & Ehring, 2004).

<table>
<thead>
<tr>
<th>Provenance</th>
<th>Elevation</th>
<th>Mean annual precipitation</th>
<th>Mean annual temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmon Arm (SAL)</td>
<td>650 m</td>
<td>500 mm</td>
<td>7.8 °C</td>
</tr>
<tr>
<td>Conrad Creek (CON)</td>
<td>280 m</td>
<td>2300 mm</td>
<td>9.5 °C</td>
</tr>
<tr>
<td>Cameron Lake (CAM)</td>
<td>210 m</td>
<td>1475 mm</td>
<td>10.0 °C</td>
</tr>
<tr>
<td>Santiam River (SAN)</td>
<td>800 m</td>
<td>1780 mm</td>
<td>9.5 °C</td>
</tr>
</tbody>
</table>
4.3.2 Measurement campaigns

Field work was conducted in May and July of 2010 and 2011 at both sites and included a total of eight measurement campaigns. Each campaign lasted two weeks. Because of the higher elevation of the Schluchsee site, the growing season begins two to three weeks later than at the Wiesloch site. Field measurements in Wiesloch were therefore carried out prior to the measurements in Schluchsee. Phenology of bud development was assessed in eight samples per provenance using an index with five classes according to Bailey and Harrington (2006) (Table A7.1). Gas exchange and chlorophyll fluorescence measurements were conducted in the sun-exposed crown of the trees at heights between 24 to 29 meters in 5-6 trees per provenance using a platform on a hydraulic lift. Measurements were carried out between 10:00 am and 6:00 pm. At the end of each campaign, needle material of the sun-exposed crown of 6 trees per provenance was sampled nearly simultaneously between 12 pm and 2 pm using shotguns or slingshots. Previous year needles were sampled from the twigs, immediately frozen in liquid nitrogen and stored at -80 °C. In 2011, pre-dawn and midday twig water potential was determined for 4 trees one day before or after the needle sampling day to assess xylem water tension. Water potential from freshly cut two-year-old twigs was determined between 6:00 and 8:00 am and 1:00 and 3:00 pm using a pressure chamber (Model 3015G4, Soil moisture Equipment Corp., Santa Barbara, CA, USA) according to Scholander et al. (1965).

4.3.3 Photosynthesis measurements

Chlorophyll fluorescence and gas exchange were measured in previous year needles on branches within the sun-exposed crown using a LI-COR 6400 XT with an integrated 6400-40 leaf chamber fluorometer (LI-COR Biosciences, Lincoln, NE, USA). To ensure that all measurements are comparable despite variation in environmental light conditions, measurements followed a standardised protocol and were carried out between 10:00 am and 6:00 pm using the internal light source of the leaf chamber fluorometer. Prior to starting the gas exchange measurements, needles on an intact twig were dark-adapted using the LI-COR dark adapting clip kit. After 25 min, about 10-15 needles forming a flat area were enclosed by the cuvette. Measurement conditions in the closed cuvette were set to a flow rate of 400 ml min⁻¹, 25 °C block temperature, 35 % relative humidity, and a CO₂ concentration of 400 ppm. Prior to starting
the gas exchange measurements, needles were dark-adapted for 25 minutes. Maximal and minimal fluorescence of the dark-adapted sample (F₀ and Fₘ) as well as dark respiration (Rₐ) were then assessed. Subsequently, gas exchange and chlorophyll fluorescence were measured at 1000 µmol photons m⁻² s⁻¹ light intensity after steady state of photosynthetic CO₂ gas exchange was achieved, typically after 10-12 min. After the measurement, light exposed needle surface area was then determined using WinSeedle software and scanner (Regents Instruments Inc., Québec, Canada). The rate of photosynthetic gas exchange was expressed per projected needle area exposed to the light. Intrinsic water-use efficiency (IWUE) was calculated as the ratio of net CO₂ assimilation rate to stomatal conductance (A/gₛ). The maximum quantum yield of dark-adapted needles was calculated as the ratio of variable to maximum chlorophyll fluorescence (Fᵥ/Fₘ = (Fₘ-F₀)/Fₘ), yield was calculated from light-adapted needles as (Φₚₛᵲ = (Fₘ′-Fᵢ)/Fₘ′), and non-photochemical quenching was calculated as NPQ = (Fₘ-Fₘ′)/Fₘ′, following Maxwell & Johnson (2000). Furthermore, the external quantum sensor of the LI-COR 6400XT was used to record photosynthetic photon flux density (PPFD). Since measurements were strongly affected by angle towards the sun and shading, maximum values per day were averaged per campaign (Table 1).

4.3.4 Analysis of photosynthetic pigments

Pigments were extracted using 98 % methanol buffered with 0.5 M ammonium acetate and analysed by HPLC-DAD according to a protocol modified from Ensminger et al. (2004). An Agilent high-performance liquid chromatography (HPLC) system (Böblingen, Germany) with a quaternary pump (model 1260), autosampler (model 1260, set to 4 °C), column oven (model 1260, set to 25 °C), and photodiode array detector (model 1290, recording absorption at 290 nm, 450 nm and 656 nm wavelength) was used for reverse-phase chromatography using a C₃₀-column (5 µm, 250*4.6 mm; YMC Inc., Wilmington, NC, USA). Three solvents (A: 100 % methanol, B: 60 % methanol buffered with 0.2% ammonium acetate, C: 100% methyl-tert-butyl-ether) were used to run a gradient starting with 40 % A and 60 % B. Solvent B was gradually replaced by solvent A to a minimum of 5 % B; afterwards solvent A was gradually replaced by solvent C until the solvent mixture consisted of 45 % A, 5% B and 50 % C. Peaks were quantified using standards for chlorophyll a, chlorophyll b and β-carotene from Sigma Aldrich.
(Oakville, ON, Canada). Standards for violaxanthin, antheraxanthin and zeaxanthin were obtained from DHI Lab products (Hørsholm, Denmark). ChemStation B.04.03 software (Agilent Technologies, Böblingen, Germany) was used for peak integration.

4.3.5 Analysis of monoterpene pools and monoterpene emissions

The 8-cm²-cuvette of a portable gas exchange analyser (GFS-3000, Walz, Effeltrich, Germany) was closed around previous-year-needles of an intact sun-exposed twig. Care was taken to prevent needle injury causing emission of stored monoterpenes. The observed monoterpene emission was similar to that observed in other studies on Douglas-fir (Constable et al., 1999), and the composition of emitted monoterpenes was considerably different from that of stored monoterpenes (data not shown), clearly indicating that emissions from injured needles were negligible in our study. The cuvette was flushed with 650 ml min⁻¹ of compressed air (Air Liquid, Ludwigshafen, Germany) at 35% relative humidity, 400 ppm CO₂ concentration, 30°C leaf temperature and 1000 µmol m⁻² s⁻¹ light intensity. After equilibration of photosynthesis to these conditions, air was drawn from the cuvette through an air sampling tube packed with 20 mg Tenax TA 60/80 and 30 mg Carbotrap B 20/40 (Supelco, Bellafonte, PA, USA) for 40 min at a flow rate of 150 ml min⁻¹ using an air sampling pump (Analyt-MTC, Müllheim, Germany). Air sampling tubes were then stored in glass vials at 4 °C. The area of the needles enclosed in the cuvette was determined as described above. Monoterpene emission rates were calculated per leaf area and over time and corrected by subtracting zero references which were taken frequently using an empty cuvette to correct for background contaminations.

Monoterpenes stored in needles were extracted in 500 µl methanol per 25 mg frozen ground needle material for 20 min while the suspension was agitated and kept at 30°C. Extracted monoterpenes were diluted and quantitatively bound to polydimethylsiloxane (PDMS) coated Twisters® (Gerstel, Mülheim, Germany) by stirring them at 1400 rpm for 60 min at 30 °C. Twisters were dried with a lint free paper tissue and placed into glass tubes.

Analysis of emitted and stored monoterpenes was performed by gas chromatography-electron impact mass spectrometry (GC-EI/MS) according to Ghirardo et al. 2010 (Ghirardo et al., 2010). Peaks were identified and quantified with external standards and by comparison of the de-
convoluted fragmentation spectra with the NIST database using the AMDIS software (National Institute of Standards and Technology (NIST), Gaithersburg, MD, USA). Needle monoterpeno concentrations were calculated per gram needle dry weight.

4.3.6 $^{13}$C isotope discrimination measurements

Following Gessler et al. (2009) and Ruehr et al. (2009), the isotopic composition ($\delta^{13}$C$_{WSOM}$) of the water-soluble organic matter (WSOM) fraction of the needles (mainly sugars, but also some amino acids and organic acids) was analysed with an elemental analyzer coupled to an isotope ratio mass spectrometer (Delta V Advantage, ThermoFisher, Bremen, Germany). Carbon isotopic values were expressed in δ notation relative to the Vienna Pee Dee Belemnite (VPDB) standard. The precision for measurements as determined by repeated measurements of standards (N=10) was better than 0.1 ‰. $\delta^{13}$C$_{WSOM}$ values were corrected for the effect of reduced O$_2$ partial pressure at higher elevation assuming an increase in $\delta^{13}$C of 0.22 ‰ per 100 m (Körner et al., 1991; Keitel et al., 2006). From $\delta^{13}$C$_{WSOM}$ and tropospheric CO$_2$ ($\delta^{13}$C$_{atm}$), we calculated the photosynthetic carbon stable isotope discrimination ($\Delta^{13}$C$_{WSOM}$).

$\delta^{13}$C$_{atm}$ was based on averaged monthly data from long-term measurements at the station Schauinsland (Freiburg, Germany) between the years 1977-1996 (Levin & Kromer, 1997), and corrected for a mean decrease in $\delta^{13}$C$_{atm}$ by 0.017 ‰ yr$^{-1}$ and for a methodology based offset of 0.2 ‰ as reported by Levin & Kromer (1997). $\Delta^{13}$C$_{WSOM}$ is a proxy for IWUE (Farquhar et al., 1982). $\Delta^{13}$C$_{WSOM}$ of leaves and needles is known to integrate IWUE over a period of hours to days (Keitel et al., 2003; Brandes et al., 2006).

4.3.7 Statistics

All statistical tests were performed using R 3.0.3 (R Development Core Team, 2010). The effect of site (environment effect) and provenance (genotype effect) and the interaction thereof on physiological parameters for photosynthetic performance and stored and emitted isoprenoids across all sampling time points were assessed using two-way ANOVA including time of campaign as random effect (function aov, see Table 4.4). Homogeneity of variance and
normality of distribution were tested by Levene’s test and Shapiro-Wilk-Test, respectively (function `levene` from the library `car` and `shapiro.test`). Differences between provenances across field sites and across time points (see Fig. 4.2, 4.3) were estimated using a corresponding linear mixed-effect model (Site x Provenance, time as random effect; function `lmer`, package `lme4`, Bates et al. (2013)) followed by the determination of least-squares means (function `lsmeans`) between provenances for all physiological parameters where `provenance` was significant, using the R package `lsmeans` (Lenth & Herve, 2014). Pairwise differences between provenances were estimated and the significance of the contrasts was assessed using Tukey's multiple comparison test (Fig. 4.2, 4.3).

The correlation between physiological parameters and the three environmental factors `TAW` (total available soil water), `Sun` (sunshine duration on the day of measurement), and `Temperature` (mean daily temperature on the day of measurement) on the day of measurement or sampling, respectively was performed using Pearson's product-moment correlation coefficient (function `cor`, method `pearson`). When interaction with any environmental parameter was significant, physiological parameters were plotted against the environmental factor that showed the highest correlation. To enhance readability of the graphs, data was averaged per campaign. Differences among provenances are displayed by linear regression and significances were estimated using the corresponding linear model followed by Tukey's multiple comparison test of least-squares means.

For all abovementioned statistics, data obtained in May 2010 in Schluchsee were omitted due to the drastically different environmental conditions at the beginning of the growing season. The start of the growing season can be marked by the first day when mean daily temperature consistently exceeds 5 °C (Menzel et al., 2003). In Schluchsee in May 2010 this threshold was exceeded only two days before our measuring campaign began. For all other May campaigns, the growing season had started already 30-50 days earlier. The late start of the growing season in May 2010 in Schluchsee was also revealed by the phenology data for bud development (Table A7.1), and in Figure S1-S4, where the physiological data are presented by campaign and field site. For Figure S1-S4, the differences between provenances within sites and at each sampling time point was determined by a separate one-way ANOVA (function `aov`), followed by Tukey’s post hoc test (function `TukeyHSD`).
Inter-annual and site-specific differences in bud development as shown in Table A7.1 were estimated using Kruskal-Wallis-rank-rum-test (function \textit{kruskal.test}).

4.4 Results

4.4.1 Environmental conditions

The field site Wiesloch was generally warmer, drier and sunnier than Schluchsee, indicated by higher temperatures, lower total available soil water (TAW), and higher sunshine duration per day (Fig. 4.1A-C). Differences in TAW and precipitation were reflected in lower pre-dawn and midday water potential at the drier site Wiesloch compared to Schluchsee (Table 4.3). We also observed inter-annual differences, with approximately 2 °C higher mean temperature and 30% lower mean precipitation in 2011 compared to 2010 at both field sites. Warmer and drier spring conditions in 2011 resulted in an earlier onset of the growing season, indicated by an earlier budburst in 2011 (Table A7.1).
Fig. 4.1: Annual climate at the field sites in 2010 and 2011 and for a 30-year reference period (1961-1990). A) 5-day running average of mean daily temperature ($T_{mean}$) and mean daily temperature for the reference period ($T_{1961-1990}$), B) mean daily total available soil water for 2010 and 2011 ($TAW_{mean}$) and the reference period ($TAW_{1961-1990}$), and C) sunshine duration in hours per day in 2010 and 2011 ($Sun_{mean}$) and the reference period ($Sun_{1961-1990}$) at the two field sites Schluchsee and Wiesloch. Gray bars indicate time of the 8 measurement campaigns.
Table 4.3: Weather conditions and twig water potential during measurement campaigns. 14-day-cumulative precipitation, mean daily temperature, sunshine duration (Sun), and total available soil water (TAW) were averaged over the duration of field campaign (±SD). Twig water potential of Douglas-fir (*Pseudotsuga menziesii*) was assessed at the end of each campaign in 2011 in n = 12 trees, equally distributed between provenance (±SD; no provenance-specific differences were detected). NA = Not assessed.

<table>
<thead>
<tr>
<th></th>
<th>Schluchsee</th>
<th></th>
<th>Wiesloch</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2010</td>
<td>2011</td>
<td>2010</td>
<td>2011</td>
<td></td>
</tr>
<tr>
<td></td>
<td>May</td>
<td>July</td>
<td>May</td>
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<td>May</td>
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<td></td>
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<td></td>
<td></td>
</tr>
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<td>14 days (mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature (°C)</td>
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<td>14.7±3.1</td>
<td>13.0±2.5</td>
<td>11.6±1.6</td>
<td>10.4±2.0</td>
</tr>
<tr>
<td>Sun (h)</td>
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<td>8.2±4.1</td>
<td>4.0±1.9</td>
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</tr>
<tr>
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<td>33.2±3.0</td>
<td>96.7±2.7</td>
<td>45.7±6.5</td>
</tr>
<tr>
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<td>NA</td>
<td>-0.68±0.10</td>
<td>-0.53±0.13</td>
<td>NA</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
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<td>(MPa)</td>
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<td></td>
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<td>potential (MPa)</td>
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</tbody>
</table>

4.4.2 Site- and provenance-specific variation in photosynthesis and isoprenoid metabolism

The comparison of four Douglas-fir provenances at two field sites revealed provenance- and site-specific variation of photosynthetic gas exchange and chlorophyll fluorescence (Fig. 4.2, Table 4.4). Net CO₂ assimilation rates (A) and stomatal conductance (gₛ) were generally higher in Schluchsee than in Wiesloch, and showed provenance-specific variation (Fig. 4.2A,B, Table 4.4). All provenances showed low A and gₛ in Wiesloch, but the Salmon Arm (SAL) provenance showed significantly higher A and gₛ compared to Santiam River (SAN), with intermediate rates for Conrad Creek (CON) and Cameron Lake (CAM), especially at the Schluchsee site. In contrast, intrinsic water-use efficiency (IWUE, measured as A/gₛ) and dark respiration (R) were variable and showed no consistent difference between sites and provenances (Fig. 4.2C,D, Table 4.4). As a proxy that integrates IWUE over several days we also estimated discrimination against stable carbon isotopes in water-soluble organic matter of needles (Δ₁³C_WSOM). Low Δ₁³C_WSOM in Wiesloch compared to Schluchsee demonstrated significantly increased IWUE, and revealed considerable variation among provenances (Fig. 4.2E). Overall, CON showed the lowest
Δ\(^{13}\)C\(_{WSOM}\) values and thus highest IWUE, compared to highest Δ\(^{13}\)C\(_{WSOM}\) in SAL and intermediate values for CAM and SAN (Fig. 4.2E, Table 4.4).

**Fig. 4.2:** Photosynthesis parameters of four Douglas-fir provenances averaged over May and July of 2010 and 2011 for two field sites, Schluchsee and Wiesloch. A) Assimilation rates (A, significantly affected by Provenance and Site), B) stomatal conductance (g\(_s\), significantly affected by Provenance and Site), C) intrinsic water-use efficiency (IWUE), D) dark respiration rate (R\(_d\)), E) carbon stable isotope discrimination (Δ\(^{13}\)C\(_{WSOM}\), significantly affected by Provenance and Site), F) maximum quantum yield of dark-adapted needles (F\(_v\)/F\(_m\), significantly affected by Site), G) effective quantum yield of light-adapted needles (Φ\(_{PSII}\)), and H) non-photochemical quenching (NPQ, significantly affected by Provenance and Site). Data obtained from Schluchsee in May 2010 was excluded (see
material and methods for further details). Significant differences between provenances (p < 0.05) are indicated by different letters.

Table 4.4: Effect of Provenance and Site on photosynthetic gas exchange, isotope discrimination, chlorophyll fluorescence, and isoprenoids assessed by two-way ANOVA, as well as correlation between these physiological parameters to environmental conditions revealed by Pearson-correlation. Section ANOVA shows the p-values from two-way ANOVA between Provenance and Site including interactions (Prov:Site). Section Pearson-Correlation shows the correlation coefficients of the Pearson's product-moment correlation test for each physiological parameter to the three environmental variables total available soil water (TAW), mean daily temperature (Temp) and sunshine hours per day as proxy for solar radiation (Sun). Significance with p < 0.05 is indicated by bolded numbers. A = assimilation rate, gs = stomatal conductance, IWUE = Intrinsic water-use efficiency, Rd = dark respiration, Δ13CWSOM = discrimination against 13C in water soluble organic matter, Fv/Fm = maximum quantum yield of dark-adapted needles, ΦPSII = yield, NPQ = non-photochemical quenching, Chl a+b = total chlorophyll per fresh weight, Carotenoids = total carotenoids per total chlorophyll, VAZ = xanthophyll cycle pigments per total chlorophyll, DEPS= de-epoxidation status of the xanthophyll cycle pigments, monoterpenes = total stored volatile isoprenoids per dry weight, emitted monoterpenes = total monoterpene emissions.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ANOVA</th>
<th>Pearson-Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Provenance</td>
<td>Site</td>
</tr>
<tr>
<td>A</td>
<td>0.02</td>
<td>0.00</td>
</tr>
<tr>
<td>gS</td>
<td>0.04</td>
<td>0.00</td>
</tr>
<tr>
<td>IWUE</td>
<td>0.07</td>
<td>0.49</td>
</tr>
<tr>
<td>R</td>
<td>0.54</td>
<td>0.09</td>
</tr>
<tr>
<td>Δ13C</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Fv/Fm</td>
<td>0.13</td>
<td>0.00</td>
</tr>
<tr>
<td>ΦPSII</td>
<td>0.15</td>
<td>0.69</td>
</tr>
<tr>
<td>NPQ</td>
<td>0.73</td>
<td>0.00</td>
</tr>
<tr>
<td>Chl a+b</td>
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</tr>
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<td>Carotenoids</td>
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<td>VAZ</td>
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</tr>
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</tr>
<tr>
<td>Emitted monoterpenes</td>
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</tr>
</tbody>
</table>
Maximum quantum yield of dark-adapted needles ($F_v/F_m$) was generally higher at Wiesloch compared to Schluchsee but did not exhibit differences among provenances (Fig. 4.2F, Table 4.4). The effective quantum yield ($\Phi_{PSII}$) of light-adapted needles revealed minor differences between provenances at the field site Schluchsee, with higher values in SAL compared to the coastal provenances (Fig. 4.2G, Table 4.4). Non-photochemical quenching (NPQ), an indicator of photoprotective quenching of excess light energy, was higher in Wiesloch compared to Schluchsee, but the differences between provenances were not significant (Fig. 4.2H, Table 4.4).

Chlorophyll content was higher in Wiesloch compared to Schluchsee and varied between provenances (Fig. 4.3A, Table 4.4). SAL showed the lowest chlorophyll content, CON was intermediate and CAM and SAN showed the highest content. Similarly, carotenoids per chlorophyll were influenced by site and provenance (Fig. 4.3B, Table 4.4). Carotenoid content was generally higher in Schluchsee (Fig. 4.3B), and significantly higher in SAL compared to the coastal provenances CON, CAM and SAN. $\beta$-carotene was highest in SAL, and lowest in SAN and intermediate in CON and CAM (Fig. 4.3C, Table 4.4). The pool of xanthophyll cycle pigments (VAZ) varied significantly between provenances, with highest VAZ pools in CON, intermediate pools in SAL and lowest pools in CAM and SAN at both field sites (Fig. 4.3D, Table 4.4). The de-epoxidation state of the VAZ-pool (DEPS) did not vary among provenances and sites (Fig. 4.3E, Table 4.4).

The needle concentrations of stored monoterpenes varied slightly among sites, but the overall differences between provenances with about three times higher monoterpenes in the interior provenance SAL than in any of the coastal provenances exceeded site-specific differences by far (Fig. 4.3F, Table 4.4). Monoterpene emission rates did not significantly vary between provenances and field sites (Fig. 4.3G, Table 4.4).
Fig. 4.3: Photosynthetic pigments and volatile isoprenoid parameters averaged over May and July of 2010 and 2011 for two field sites, Schluchsee and Wiesloch. A) total chlorophyll a and b per fresh weight (chlorophylls FW$^{-1}$, significantly affected by Provenance and Site), B) carotenoids per total chlorophyll (Carotenoids Chl$^{-1}$, significantly affected by Provenance and Site), C) β-carotene per total chlorophyll (β-carotene Chl$^{-1}$, significantly affected by Provenance and Site), D) xanthophyll cycle pigments per total chlorophyll (VAZ Chl$^{-1}$, significantly affected by Provenance), E) de-epoxidation status of the xanthophyll cycle pigments (DEPS), F) total stored monoterpenes per dry weight (monoterpenes, significantly affected by Provenance and Site), and G) total monoterpenes emissions (emitted monoterpenes). Data obtained from Schluchsee in May 2010 was excluded (see material and methods for further details). Significant differences between provenances (p < 0.05) are indicated by different letters.
4.4.3 Variation in photosynthesis and isoprenoid metabolism in response to environmental factors

As a second step of analysis, we aimed to illustrate the response of Douglas-fir to changes in environmental conditions and assessed the correlation of each physiological parameter with the three environmental factors total available soil water (TAW), sunshine duration on the day of measurement (Sun), and mean temperature of the day of measurement (Temperature). In order to assess the response of Douglas-fir to changes in environmental conditions, physiological parameters were plotted against the environmental factor that showed the highest correlation.

For A and $g_s$ we observed a strong limitation of all provenances by low TAW (Fig. 4.4A,B). Maximum rates of photosynthetic gas exchange were observed in all provenances when TAW exceeded 90% in July 2011 in Schluchsee (Fig. A7.1A,B). When TAW was below 20 % as well as in May 2010, when low temperature conditions were prevailing, A and $g_s$ were low in all provenances (Fig. 4.4A,B, Fig. A7.1a). All provenances showed a decrease in A and $g_s$ in response to decreased TAW, but varied significantly in their response to environmental conditions, as indicated by significant genotype by environment interaction (Table 4.4). Especially when TAW was low, Salmon Arm (SAL) showed significantly higher rates compared to Santiam River (SAN), with intermediate rates for Conrad Creek (CON) and Cameron Lake (CAM) (Fig. 4.4A,B).

$\Delta^{13}C_{WSOM}$ decreased in all provenances in response to decreases in TAW, demonstrating increased IWUE when TAW was low (Fig. 4.4C). $\Delta^{13}C_{WSOM}$ was significantly lower in CON and SAN, indicating higher IWUE compared to SAL and CAM. Such provenance-specific differences were especially apparent in July, e.g. when the highest $\Delta^{13}C_{WSOM}$ in SAL trees occurred in July 2010 in Schluchsee, or in SAL and CAM trees in July 2010 and July 2011 in Wiesloch (Fig. A7.1D).
Fig. 4.4: Photosynthetic gas exchange parameters of four Douglas-fir provenances in response to total available soil water. A) assimilation rates (A), B) stomatal conductance ($g_s$), and C) carbon stable isotope discrimination ($\Delta^{13}C_{WSOM}$) were measured in May and July of 2010 and 2011 at two field sites, Schluchsee and Wiesloch. Data for A and $g_s$ show means of $n = 5$-6 measurements (±SE) at a light intensity of 1000 µmol m$^{-2}$ s$^{-1}$, data for $\Delta^{13}C_{WSOM}$ was obtained from $n = 5$-6 samples of previous year needles (±SE). Data obtained from Schluchsee in May 2010 was excluded (see material and methods for further details). Significant differences between provenances ($p < 0.05$) are indicated by different letters.
$F_v/F_m$ was generally around 0.8 and only decreased during the exceptionally warm and dry July 2010 (Fig. A7.2A). $F_v/F_m$ was slightly lower at the moist site Schluchsee which contributed to a negative relationship between TAW and $F_v/F_m$ (Fig. 4.5A). $\Phi_{PSII}$ was the only parameter that correlated best with temperature, with lower values as temperature increased (Fig. 4.5B), and a minimum under hot conditions in July 2010 (Fig. A7.2B). NPQ increased in response to low TAW in all provenances (Fig. 4.5C). High NPQ also occurred under low temperature conditions in May 2010 at the beginning of the growing season (Fig. A7.2C).

![Graphs showing chlorophyll fluorescence parameters](image)

Fig. 4.5: Chlorophyll fluorescence parameters of four Douglas-fir provenances in response to total available soil water and temperature, respectively. A) maximum quantum yield of dark-adapted needles ($F_v/F_m$), B) effective quantum yield of light-adapted needles ($\Phi_{PSII}$), and C) non-photochemical quenching (NPQ) were measured in May and July of 2010 and 2011 at two field sites, Schluchsee and Wiesloch. Data show means of n = 5-6 measurements ($\pm$SE) at a light intensity of 1000 $\mu$mol m$^{-2}$ s$^{-1}$. Data obtained from Schluchsee in May 2010 was excluded (see material and methods for further details). Significant differences between provenances (p < 0.05) are indicated by different letters.
Fig. 4.6: Photosynthetic pigment content of four Douglas-fir provenances in response to total available soil water. A) total chlorophyll a and b per fresh weight (chlorophylls FW⁻¹), B) carotenoids per total chlorophyll (Carotenoids Chl⁻¹), C) β-carotene per total chlorophyll (β-carotene Chl⁻¹) were measured in May and July of 2010 and 2011 at two field sites, Schluchsee and Wiesloch. Data was obtained from n = 5-6 samples of previous year needles (±SE). Data obtained from Schluchsee in May 2010 was excluded (see material and methods for further details). Significant differences between provenances (p < 0.05) are indicated by different letters.
Chlorophyll content was higher at the dry site Wiesloch compared to Schluchsee and thus was higher when TAW was low and showed significant variation between provenances (Fig. 4.6A). During all campaigns, SAL and CON showed the lowest chlorophyll content, whereas CAM showed the highest content and SAN intermediate values (Fig. A7.3A). Lower chlorophyll content under low TAW also affected the ratio of carotenoids per chlorophyll. This ratio was typically slightly lower as TAW decreased (Fig. 4.6B). In response to a decrease in TAW, the ratio of carotenoids per chlorophylls remained highest in SAL, followed by CON and CAM and lowest in SAN. However, it must be noted that the highest carotenoid content was observed under cold conditions early in the growing season in May 2010 in Schluchsee (Fig. A7.3B). The β-carotene content of needles also showed a decrease in response to soil water availability, which was less pronounced in SAL compared to the coastal provenances (Fig. 6C). Provenance-specific variation in β-carotene was especially pronounced under dry conditions in July 2010 in Wiesloch, but decreased with TAW in CON, CAM and SAL which significantly differed from SAL (Fig. 4.6C, S4A).

The pool of xanthophyll cycle pigments (VAZ) increased in response to sunshine duration per day which is a proxy for solar radiation (Fig. 4.7A). CON showed consistently highest VAZ-pools, followed by SAL, CAM and SAN. Highest VAZ-pools sizes under summer conditions were observed in July 2010 in Wiesloch when we observed 12 h of daily sunshine duration combined with lowest TAW. Here, VAZ-pools of all provenances were about 15-20% larger than in Schluchsee (Fig. A7.4B). Nonetheless, highest VAZ-pools in Schluchsee were not observed in response to water limitations and high light episodes, but during early season low temperature conditions in May 2010 (Fig. A7.3D). The de-epoxidation state (DEPS) of the VAZ-pool, which reflects the photoprotective conversion of violaxanthin into zeaxanthin and antheraxanthin, was strongly increased under sunny conditions, but we did not observe any variation among provenances (Fig. 4.7B). High VAZ-pool sizes observed under very sunny and dry conditions in July 2010 in Wiesloch were paralleled by a threefold increase in DEPS, resulting from the photoprotective conversion of violaxanthin into zeaxanthin and antheraxanthin (Fig. A7.4B).
Fig. 4.7: Photosynthetic pigment content of four Douglas-fir provenances in response to sunshine duration per day as proxy for solar radiation. A) Xanthophyll cycle pigments per total chlorophyll (VAZ Chl^{-1}), and B) de-epoxidation status of the xanthophyll cycle pigments (DEPS) were measured in May and July of 2010 and 2011 at two field sites, Schluchsee and Wiesloch. Data was obtained from n = 5-6 samples of previous year needles (±SE). Data obtained from Schluchsee in May 2010 was excluded (see material and methods for further details). Significant differences between provenances (p < 0.05) are indicated by different letters.

The needle concentrations of stored monoterpenes did not significantly correlate with any environmental factor, and they were consistently higher in SAL than in any of the coastal provenances (Fig. 4.8A, Fig. A7.4A). Monoterpene emission rates were highly variable among individual trees, but significantly correlated with sunshine duration per day (Fig. 4.8B, Fig. A7.5B).
Fig. 4.8: Monoterpene pools and emission of four Douglas-fir provenances in response to sunshine duration per day as a proxy for solar radiation. A) monoterpene pool sizes (monoterpenes DW\(^{-1}\)), and B) monoterpenes emitted per needle area (monoterpene emissions) were measured in May and July of 2010 and 2011 at two field sites, Schluchsee and Wiesloch. Data was obtained from \(n = 5-6\) samples of previous year needles (±SE). Monoterpene pools include L-\(\alpha\)-bornyl acetate, camphene, 3-carene, (+)-4-carene, \(\beta\)-citronellal, \(\beta\)-citronellol, citronellyl acetate, geraniol acetate, isoprene, (-)-isopulegol, limonene, linalool, \(\beta\)-myrcene, nerolidol, ocimene, \(\beta\)-phellandrene, \(\alpha\)-pinene, \(\beta\)-pinene, sabinene, \(\alpha\)-terpinene, \(\gamma\)-terpinene, \(\alpha\)-terpineol, L-4-terpineol and tricyclene. Data obtained from Schluchsee in May 2010 was excluded (see material and methods for further details). Significant differences between provenances (\(p < 0.05\)) are indicated by different letters.

4.5 Discussion

We assumed that Douglas-fir provenances vary in photosynthesis and isoprenoid-mediated photoprotective mechanisms in response to drought. Specifically, we expected that all provenances employ short-term photoprotective responses to drought, but reveal provenance-specific differences in long-term adjustments. Our results showed that provenances vary in photosynthetic gas exchange under intermediate to high TAW at the Schluchsee site (Fig. 1.2). In contrast, at the Wiesloch site, photosynthesis in all provenances was strongly limited by generally low TAW. Under these predominantly drier soil conditions, provenances revealed variation in long-term adjustments of the composition of essential isoprenoids (Fig. 2,3).

The observed good correlation of net \(\text{CO}_2\) assimilation (\(A\)) and stomatal conductance (\(g_s\)) with TAW (Fig. 4A,B) is typical for conifers, which control water loss through transpiration by
decreasing g<sub>s</sub> in response to decreasing soil water availability (McDowell et al., 2008; Brodribb et al., 2014). Trees with an increased drought-tolerant manage water loss better and are thus able to maintain higher assimilation rates under drought (Sade et al., 2012). Consequently, slightly higher A and g<sub>s</sub> in SAL suggests increased drought tolerance of this interior provenance compared to the three coastal provenances. The effect of local adaptation on photosynthetic performance, as was previously shown e.g. for poplar (Monclus et al., 2006) and eucalyptus (Osório & Pereira, 1994), was furthermore revealed by provenance-specific variation in discrimination against stable carbon isotopes (Δ<sup>13</sup>C<sub>WSOM</sub>). Δ<sup>13</sup>C<sub>WSOM</sub> is a time-integrated proxy of the intrinsic water use efficiency (IWUE) that exacerbates potential differences in A/g<sub>s</sub> (Brandes et al., 2006; Bogelein et al., 2012). Although the A/g<sub>s</sub> ratio was rather invariable (Fig. 2C), a positive correlation between Δ<sup>13</sup>C<sub>WSOM</sub> and TAW indicated enhanced IWUE (Farquhar et al., 1989; Jansen et al., 2013) in response to drought (Fig. 4C, Table 2). Lower Δ<sup>13</sup>C<sub>WSOM</sub> in CON and SAN furthermore suggests enhanced IWUE in these two coastal provenances compared to SAL and CAM (Fig. 4C). Similar to the observations by Jansen et al. (2013) and Zhang et al. (1995) our data reveal intraspecific variation in IWUE with coastal provenances generally showing higher IWUE compared to interior provenances.

Provenance-specific differences in photosynthetic gas exchange were not reflected by chlorophyll fluorescence parameters. For example, the optimum quantum yield of photosynthesis F<sub>v</sub>/F<sub>m</sub>, is typically a sensitive indicator for photoinhibition under high light stress and in response to drought (Adams et al., 2013). However, we only observed decreases in F<sub>v</sub>/F<sub>m</sub> under extremely dry conditions in July 2010, when yield of PSII was also minimal (Supplementary Fig. S2A,B). High F<sub>v</sub>/F<sub>m</sub> values in combination with a low yield of PSII (Fig. 5A,B) indicates that the photosynthetic apparatus remains intact during drought and the downregulation of yield of PSII reflects dynamic short-term response to mitigate acute excess energy and increased photoprotection against oxidative stress (Demmig-Adams & Adams, 2006). Since this coincided with enhanced non-photochemical quenching (NPQ) in response to decreased TAW (Fig. 5C) we likely observed photoprotective feedback response (Poulson et al., 2002) to decreases in A and g<sub>s</sub> that occurred under dry conditions.

We had hypothesized, that provenance-specific differences in photosynthesis go along with differences in a suite of isoprenoid-mediated photoprotective mechanisms. We expected that
provenances with low photosynthetic performance during drought, e.g. SAL and CAM, will show enhanced photoprotection by essential isoprenoids, including chlorophylls and carotenoids.

In contrast to our prediction, SAL showed low chlorophyll content and enhanced carotenoid-chlorophyll ratio (Fig. 6A,B, Table 2) which were previously attributed to enhanced photoprotection in various tree species including *Picea asperata*, *Pinus halepensis* and *Quercus pubescens* (Duan *et al.*, 2005; Baquedano & Castillo, 2006; Gallé *et al.*, 2007). SAL also exhibited higher amounts of β-carotene compared to coastal provenances, but revealed high plasticity (Fig. 6C, Supplementary Fig. 4A). β-carotene pool sizes are affected by long-term adjustments of the photosynthetic apparatus to prevailing light conditions (Beisel *et al.*, 2010; Esteban *et al.*, 2015a) as well as by short-term oxidation reactions (Ramel *et al.*, 2013; Havaux, 2014). The decrease of β-carotene in the coastal provenances CON, CAM and SAN in response to low TAW thus suggests, that β-carotene biosynthesis was not sufficient to combat the oxidation of β-carotene caused by chemical scavenging of ROS (Telfer, 2005). Overall, the enhanced pools of essential isoprenoids in SAL suggests enhanced photoprotection of the photosynthetic apparatus, suggesting that essential isoprenoids are important contributors to the overall higher assimilation rates of this provenance.

We also determined the involvement of the xanthophyll cycle, an ubiquitous photoprotective mechanism (Chaves *et al.*, 2002; Demmig-Adams & Adams, 2006; Esteban *et al.*, 2015a) in the adjustment of the photoprotective capacity of Douglas-fir in response to drought. The long-term adjustment of the xanthophyll cycle pigment (VAZ) pool size in response to sunshine duration per day (used here as a proxy for global radiation (Trnka *et al.*, 2005; Fig. 7A) provides evidence for an upregulation of the photoprotective capacity in response to abiotic stress conditions in Douglas-fir. Although differences in pool sizes among provenances seem to be small, with largest VAZ pools in CON and smallest pools in SAN, the pattern was consistent and revealed significant provenance-specific variation (Table 2) and likely reflects local adaptation to contrasting habitats. Provenance-specific variation in essential isoprenoids was most pronounced under extremely dry, hot and sunny conditions in July 2010 at Wiesloch when the demand for photoprotection was highest throughout all our campaigns (Table 3, Supplementary Fig. S3,4). During the same campaign at Wiesoch we also observed in all provenances a maximum in the de-epoxidation state of VAZ (DEPS, Fig. 7B), which is an indication of a short-term and
instantaneous response to mitigate photo-oxidative stress induced by high light (Faria et al., 1998; Ripullone et al., 2009).

Compared to carotenoids, which are essential and conserved in higher plants (Pogson et al., 1998), non-essential isoprenoids are highly variable across species (Kesselmeier & Staudt, 1999) and within species (Loreto et al., 2009; Welter et al., 2012). This variability was reflected by the observed provenance-specific differences in monoterpane pool sizes, which exceeded the observed variations in essential isoprenoids by far (Fig. 3F, Fig. 8A).

Stored monoterpenes indicate long-term adjustments to environmental conditions (Litvak & Monson, 1998; Snow et al., 2003). In contrast, the emission of monoterpenes is instantaneously driven by prevailing environmental conditions to mitigate acute abiotic stress (Owen & Peñuelas, 2005; Vickers et al., 2009; Possell & Loreto, 2013). Emission of monoterpenes correlated with sunshine duration per day, but showed large variability among trees and campaigns, without provenance-specific differences (Fig. 3G, Fig. 8B, Supplementary Fig. S5B). This is likely due to a high percentage of de novo biosynthesized monoterpenes which have been shown to constitute up to 58% of emitted monoterpenes in Pinus sylvestris (Ghirardo et al., 2010). Emitted monoterpenes represent a mix of stored (temperature dependent) and de novo synthesized (light and temperature dependent) monoterpenes (Loreto & Schnitzler, 2010) which may consequently contribute to the lack of a relationship between pool sizes and rates of emission and large variation in emission rates (Fig. 8A, B).

Despite their possible function in mitigating photooxidative stress, the emission of monoterpenes implies a loss of previously fixed CO₂. This loss in previously fixed CO₂ can be substantial and for beech seedlings it was shown that it can even result in reduced growth (Šimpraga et al., 2011). Previously it was shown that interior provenances reveal reduced growth compared to coastal provenances (Sergent et al., 2014), an observations that is also confirmed for the provenances studied here and at our field sites (Neophytou et al., 2016). Based on our observations, increases in the allocation of previously fixed CO₂ to the biosynthesis of monoterpenes for storage and emission in the provenance SAL might therefore affect its growth performance compared to coastal provenances.

In conclusion, our results reveal provenance-specific variation in gₛ and IWUE, indicating local adaptation to habitats with contrasting soil water availability. All provenances shared similar
short-term photoprotective responses when photosynthetic CO$_2$ uptake was decreased due to low TAW. These photoprotective responses involved higher rates of NPQ, as well as increased de-epoxidation of the xanthophyll cycle pigments and enhanced emission of volatile isoprenoids under high light conditions. In contrast to these provenance-wide responses, we also observed provenance-specific differences in long-term adjustments represented by differences in the pool sizes of xanthophyll cycle pigments, β-carotene and stored monoterpenes in response to drought and high light. Provenance-specific variation in essential and non-essential isoprenoids therefore seem to reflect local adaptation in isoprenoid-mediated photoprotective mechanisms. Most importantly, we did not observe the highest photoprotection in the provenances, that exhibit lower assimilation rates, as suggested by our hypothesis, but rather in the provenances, which were able to maintain higher assimilation rates. Therefore, isoprenoid-mediated photoprotective mechanisms seem to contribute to better adaptation of species to warmer and drier climate and to serve as an important trait to enhance forest ecosystem resilience.

4.6 Acknowledgments

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Chapter 5
Relationship between leaf optical properties, chlorophyll fluorescence and pigment changes in senescing Acer saccharum leaves

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5 Relationship between leaf optical properties, chlorophyll fluorescence and pigment changes in senescing *Acer saccharum* leaves

5.1 Abstract

The ability of plants to sequester carbon is highly variable over the course of the year and reflects seasonal variation in photosynthetic efficiency. This seasonal variation is most prominent during autumn, when leaves of deciduous tree species such as sugar maple undergo senescence which is associated with downregulation of photosynthesis and a change of leaf color. The remote sensing of leaf color by spectral reflectance measurements and digital repeat images is increasingly used to improve models of growing season length and seasonal variation in carbon sequestration. Vegetation indices derived from spectral reflectance measurements and digital repeat images might not adequately reflect photosynthetic efficiency of red-senescenting tree species during autumn due to the changes in foliar pigment content associated with autumn phenology. In this study, we aimed to assess how effectively several widely used vegetation indices capture autumn phenology and reflect the changes in physiology and photosynthetic pigments during autumn. Chlorophyll fluorescence and pigment content of green, yellow, orange and red leaves were measured to represent leaf senescence during autumn and used as a reference to validate and compare vegetation indices derived from leaf-level spectral reflectance measurements and color analysis of digital images. Vegetation indices varied in their suitability to track the decrease of photosynthetic efficiency and chlorophyll content despite increasing anthocyanin content. Commonly used spectral reflectance indices such as the Normalized difference vegetation index (NDVI) and Photochemical reflectance index (PRI) showed major constraints arising from a limited representation of gradual decreases in chlorophyll content and an influence of high foliar anthocyanin levels. Excess green index (ExG_M) and Green red vegetation index (GRVI) were better suitable to assess the process of senescence. Similarly, digital image analysis revealed that vegetation indices such as Hue and Normalized difference index (NDI) are superior compared to the often used Green chromatic coordinate (g_CC). We conclude that indices based on red and green color information generally represent autumn phenology most efficiently.
5.2 Introduction

Long-term changes in climate have been shown to affect the length of the growing season and to impact ecosystem productivity and carbon cycling (Boisvenue & Running, 2006). Phenology and the length of the growing season have been traditionally assessed by observations of bud burst, flowering time, changes in leaf color and leaf abscission (Sparks & Menzel, 2002). In deciduous trees, autumn phenology includes leaf senescence with changes in leaf color from green to yellow and/or red and eventually leaf abscission. Leaf senescence also includes downregulation and cessation of photosynthesis and eventually indicates the end of the growing season (Wilson et al., 2001). The onset and duration of senescence is variable and triggered by cues such as temperature, photoperiod, as well as abiotic and biotic stress (Keskitalo et al., 2005; Lim et al., 2007).

Autumn senescence is a highly regulated process which allows the remobilisation of nutrients while avoiding pathological effects of cell death (Hörtensteiner, 2004; Keskitalo et al., 2005; Ougham et al., 2005). Nutrient remobilization in leaves involves breakdown of the enzymes involved in carbon fixation which in turn causes a loss of photosynthetic capacity (Hörtensteiner & Feller, 2002). As photosynthetically active chloroplasts transform to gerontoplasts (Biswal et al., 2003), most deciduous trees show a total degradation of chlorophylls (Lee et al., 2003; Ougham et al., 2005). In contrast to chlorophylls, carotenoid degradation is incomplete in most plant species (Biswal, 1995), causing an increasing ratio of carotenoids per chlorophyll. Additionally, β-carotene and the xanthophyll cycle pigments violaxanthin, antheraxanthin and zeaxanthin are relatively enhanced (García-Plazaola et al., 2003a; Ougham et al., 2005). These antioxidant and photoprotective accessory pigments likely contribute in regulation of reactive oxygen species (ROS) which accumulate during senescence but need to be tightly regulated due to their toxicity (Juvany et al., 2013).

Anthocyanins are another group of pigments that accumulate in many tree species during autumn senescence (Archetti, 2009). In addition to their potential role in biotic interactions (Archetti et al., 2009), anthocyanins are involved in screening of UV-B light, protection from photo-oxidative stress and have an antioxidant function (Hoch et al., 2001; van den Berg & Perkins, 2007; Ferreira da Silva et al., 2012). Synthesis of anthocyanins during senescence has been linked to delayed leaf abscission and enhanced nutrient resorption (Hoch et al., 2003; Schaberg...
Accumulation of anthocyanins results in the characteristic red autumn colors, is enhanced in response to low temperature and high light (Hoch et al., 2001) and can vary between species (Lev-Yadun & Holopainen, 2009), progenies (Townsend, 1977) and among trees within a stand with different foliar nitrogen concentrations (Schaberg et al., 2003). In the evergreen tree species Pistoria lentiscus, red leaf coloration in winter indicates vulnerability to photoinhibition (Nikiforou & Manetas, 2010). Such variation in anthocyanin synthesis indicates its adaptive value, and anthocyanin concentrations have been suggested as an indicator for assessing forest stress (Schaberg et al., 2003).

Additionally, tocopherols accumulate in senescing leaves, partly as a product of chlorophyll breakdown (Rise et al., 1989; Mène-Saffrané & DellaPenna, 2010). They have an antioxidant function (Fryer, 1992; Fryer et al., 1998), enhancing e.g. the redox buffer capacity in senescing tobacco leaves, Nicotiana tabacum L. (Abbasi et al. 2009), and plastid membrane stability in barley, Hordeum vulgare L. (Chrost et al., 1999).

Altogether, variation in pigment levels and thus leaf color reflects the physiological status of plants and indicates the progress of autumn phenology and senescence (Ustin et al., 2009). Monitoring of pigment dynamics by remote sensing is increasingly used to assess primary production and phenological events of ecosystems (Ustin et al., 2009), and a range of airborne and spaceborne platforms using spectral reflectance measurements are available for estimating canopy color to monitor ecosystem function and growing season length (Treitz & Howarth, 1999; Pettorelli et al., 2005). Recently, near-surface remote sensing has seen improvements by the relatively simple and cost-effective use of web-based digital cameras (“phenocams”) which take images from a canopy at regular intervals. Changes in the red–green–blue (RGB) signal of these images over time have been used to monitor phenology and validate canopy scale photosynthetic CO$_2$ fluxes (Richardson et al., 2007; Ahrends et al., 2009).

Various vegetation indices derived from spectral reflectance were developed to monitor ecosystems (Ustin et al., 2009). The Normalized difference vegetation index (NDVI) is the most commonly used vegetation index to estimate ecosystem productivity (Pettorelli et al., 2005). However, it has been shown that NDVI has limitations for monitoring canopy conditions in autumn (Nagai et al., 2010), and increased anthocyanin levels seem to impair the ability of NDVI for tracking greenness and chlorophyll content adequately (Viña & Gitelson, 2011). Other
indices have been developed particularly aiming to assess anthocyanin content, e.g. the Anthocyanin reflectance index (ARI) or the Red/green reflectance ratio (RGR) (Gitelson et al., 2009). For digital imaging, fewer vegetation indices have been derived, and often only the Green chromatic coordinate (g_{CC}; also referred to as strength of green) is used to assess canopy greenness (Mizunuma et al., 2011). Promising indices for assessing canopy phenology might be e.g. the Excess green index (ExG) and Normalized difference index (NDI). ExG is also called 2G_RBi (Richardson et al., 2007) or GEI (Inoue et al., 2014), while NDI (Pérez et al., 2000) is also referred to as NDVI_{GR} (Sakamoto et al., 2010), VARI (Sakamoto et al., 2012) and GRVI (Mizunuma et al., 2011). These vegetation indices can be derived from spectral reflectance measurements and digital image analysis and could serve to better link remote sensing and near-surface methods (Hufkens et al., 2012).

A major challenge of using many of the described vegetation indices for tracking leaf phenology during autumn is a lack of detailed understanding of how physiological changes at the leaf level directly affect variations in leaf optical properties. Several aspects of the physiological changes associated with autumn phenology have been well characterised for many tree species. For sugar maple (Acer saccharum Marsh.), a commercially and ecologically important tree species in Eastern North America, Vogelmann et al. (1993), Lee et al. (2003) and Moy et al. (2015) characterised changes in chlorophylls and carotenoids, while changes in anthocyanin content were studied by van den Berg and Perkins (2007) and Schaberg (2003, 2008). Downregulation of photosynthetic efficiency during autumn senescence was demonstrated by Königer et al. (2000). However, none of these reports provides a comprehensive overview that integrates all three important aspects of leaf autumn senescence, and none of them includes leaf optical properties.

We aimed to assess how efficiently several widely used vegetation indices capture leaf senescence and reflect the associated changes in physiology and photosynthetic pigment content during autumn. For this purpose we sampled green, yellow, orange and red sugar maple leaves representing various stages of autumn leaf senescence in comparison to green leaves sampled during summer. We measured chlorophyll fluorescence, P700 absorbance, photosynthetic pigment content and anthocyanins content to monitor the process of leaf senescence and used these physiological measurements as reference to validate and compare vegetation indices derived from spectral reflectance measurements and color analysis of digital images.
5.3 Material and Methods

5.3.1 Plant material and samplings

We chose three sugar maple trees (*Acer saccharum*) which were growing in the field in Huron Park, Mississauga (ON), Canada (N43°33’34”, W79°38’11”). The trees were about 5-7 m tall, in an open stand and fully exposed to sunlight (see Fig. 5.3a). Within canopy variation in the progress of senescence (see Fig. 5.3b) allowed to sample leaves of various color classes representing various stages of senescence. On the mornings of October 13, 2012 and October 13, 2013, green, yellow, orange and red leaves were simultaneously sampled. As a reference for non-senescent leaves, green summer leaves were taken at noon of July 2, 2013. For each color category, sample size per time point and tree was N = 5-6. All leaves were picked from sun-exposed twigs of the lower part of the canopy at a height between 2-3 m of each tree. Leaves were stored in plastic bags with a damp tissue and kept on ice in the dark to minimize changes in leaf water content and pigments during transport to the nearby laboratory. Upon arrival in the laboratory, digital images were obtained from the leaves for RGB-analysis followed by chlorophyll fluorescence, P700 absorbance and spectral reflectance measurements (for details see below). Following these measurements, the leaves were further processed for pigment analysis. For this purpose major veins were removed from the leaves using razor blades and the remaining leaf tissue was shock-frozen in liquid nitrogen and stored at -80°C.

5.3.2 Chlorophyll fluorescence and P700 measurements

Energy use at Photosystem II (PSII) was estimated by chlorophyll fluorescence, and energy use at Photosystem I (PSI) was estimated by the redox state of P700, the primary donor of PSI. Both measurements were obtained using a fiberoptic version of the Dual-PAM-100 (Walz, Effeltrich, Germany). The fiberoptic was kept at a distance of 5 mm above the leaf surface at an angle of 60° using a custom made leaf clip. Measurements were taken after 25 min of dark-adaptation under low light conditions at room temperature using the internal light source of the Dual-PAM-100.

Minimum chlorophyll fluorescence (F_o) of the dark-acclimated sample was assessed as mean of three consecutive measurements, followed by a measurement of maximum fluorescence (F_m).
Subsequently, leaves were exposed to 1200 \text{\mu \text{mol} \text{photons m}^{-2} \text{s}^{-1}} light intensity for 280 s, and measurements of \( F_t, F_m' \) and \( F_o' \) were taken after 200 s, 240 s and 280 s. The maximum quantum yield of photosystem II (PSII) of dark-adapted leaves was calculated (2000) as \( \frac{F_v}{F_m} = \frac{F_m - F_o}{F_m} \). The excitation pressure on PSII (1–qp) was calculated according to Huner et al. (1998) as \( 1 - \text{qp} = 1 - \left( \frac{F_m' - F_t}{F_m' - F_o'} \right) \), yield of PSII (\( \Phi_{\text{PSII}} \)) of light-adapted leaves was calculated as \( \Phi_{\text{PSII}} = \frac{F_m' - F_t}{F_m'} \), non-regulated energy dissipation (\( \Phi_{\text{NO}} \)) was calculated (2004) as \( \Phi_{\text{NO}} = 1 / \left( \left( \frac{F_m - F_m'}{F_m'} \right) + 1 + \left( \frac{F_m' - F_t}{F_m' - F_o'} \right) \right) \), and non-photochemical quenching (\( \Phi_{\text{NPQ}} \)) was calculated as \( \Phi_{\text{NPQ}} = 1 - \Phi_{\text{PSII}} - \Phi_{\text{NO}} \).

Simultaneously, the redox state of P700, the reaction center chlorophyll of photosystem I (PSI), was measured as the difference in transmittance between 875 nm and 830 nm. After dark adaptation, P700 is fully reduced, and the signal \( P_o \) is minimal. After a saturation pulse, P700 is fully oxidized and \( P_m \) as the maximum signal is determined. \( P \) is the steady-state signal of light-adapted leaves. \( P_m' \) is determined after a saturation pulse of light-adapted leaves. Yield of PSI (\( \Phi_{\text{PSI}} \)) of light-adapted leaves was calculated as \( \Phi_{\text{PSI}} = \frac{P_m' - P}{P_m - P_o} \), non-photochemical quantum yield of PSI due to donor side limitation (\( \Phi_{\text{ND}} \)) was calculated as \( \Phi_{\text{ND}} = \frac{P - P_o}{P_m - P_o} \), and non-photochemical quantum yield of PSI due to acceptor side limitation (\( \Phi_{\text{NA}} \)) was calculated as \( \Phi_{\text{NA}} = \frac{P_m - P_m'}{P_m - P_o} \), with \( \Phi_{\text{PSI}} + \Phi_{\text{ND}} + \Phi_{\text{NA}} = 1 \) (2008). Due to low signal, for 12\% of yellow leaves, and 34\% of orange and red leaves, energy use at PSI could not be calculated.

From \( \Phi_{\text{PSII}} \) and \( \Phi_{\text{PSI}} \), electron transport rates of PSII and PSI were calculated. The calculation of ETR usually involves a fixed factor of 0.84 standing for the fraction of light absorbed by leaves as well as an equal energy distribution of light energy between PSII and PSI (Schreiber, 2004). Since we need to assume that light absorption of leaves changed during senescence due to decreasing content of photosynthetic pigments (Carter et al., 2000), we calculated relative electron transport rates (Schreiber, 2004). The energy distribution between PSII and PSI, dII and dI, was calculated from \( \Phi_{\text{PSII}} \) and \( \Phi_{\text{PSI}} \) measurements of summer leaves as unstressed reference according to Huang (2012). dI and dII were calculated from 14 summer leaves using the formula \( \Phi_{\text{PSII}} \ast dII = \Phi_{\text{PSI}} \ast dI \) with \( dII + dI = 1 \). Average \( dII = 0.5369 \) (±0.0077) and \( dI = 0.4631 \) (±0.0077). Consequently, relative electron transport rate of PSII (rETR_{II}) was calculated as \( \text{rETR}_{II} = \Phi_{\text{PSII}} \ast 0.5369 \ast 1200 \mu\text{mol photons m}^{-2} \text{s}^{-1} \) and relative electron transport rate of PSI
was calculated as $r_{ETR_I} = \Phi_{PSI} \times 0.4631 \times 1200 \, \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Ensminger et al., 2001). We furthermore calculated the ratio between $r_{ETR_I}$ and $r_{ETR_{II}}$ which was suggested to indicate the induction of relative cyclic electron transport when values are greater than one according to Yamori et al. (2011).

### 5.3.3 Spectral reflectance measurements

Spectral reflectance of leaves illuminated for 4 min was measured simultaneously to the Dual-PAM-100 measurements taken in 2013. A UniSpec-SC spectrometer (UNI007, PP Systems, Haverhill, MA, USA) connected to a bifurcated fiberoptic (UNI400) was held at a distance of 5 mm above the leaf surface at an angle of 90°. Leaf reflectance of wavelengths between 310 and 1130 nm was corrected for the dark current noise of the instrument and measured in relation to the radiance of a white reflectance standard (Spectralon, Labsphere, North Sutton, NH, USA). Wavelengths from 710-895 nm were excluded from further analysis to avoid interference with the Dual-PAM-100-measuring light. Spectral reflectance indices representing chlorophyll and anthocyanin content were calculated from an average of three measurements per leaf as followed, with $R_x$ representing the reflectance at light of $x$ nm wavelength:

1. **Normalized difference vegetation index**
   
   $$\text{NDVI} = \frac{R_{900} - R_{680}}{R_{900} + R_{680}}$$

   Penuelas et al. (1997)  

2. **Photochemical reflectance index**

   $$\text{PRI} = \frac{R_{531} - R_{570}}{R_{531} + R_{570}}$$

   Gamon et al. (1997)  

3. **Excess green index**

   $$\text{ExG}_M = \frac{2 \times R_{545-565} - (R_{620-670} + R_{459-479})}{R_{620-670} + R_{459-479}}$$

   Hufkens et al. (2012)  

4. **Green red vegetation index**

   $$\text{GRVI} = \frac{R_{500-570} - R_{620-700}}{R_{500-570} + R_{620-700}}$$

   Motohka et al. (2010)  

5. **Anthocyanin reflectance index**

   $$\text{ARI} = \frac{1}{R_{550}} - \frac{1}{R_{700}}$$

   Gitelson et al. (2009)  

6. **Red/green reflectance ratio**

   $$\text{RGR} = \frac{R_{600-700}}{R_{500-600}}$$

   Gamon and Surfus (1999)
5.3.4 RGB-analysis of images

Photographs of all leaves were taken on a grey cardboard background under natural light conditions, using a digital camera (Canon Powershot SX 150 IS, Canon Inc., Tokyo, Japan). The image processing program ImageJ (Abramoff, 2004) was used to define an oval region of interest (ROI) in the centre of each leaf and the red, blue and green color channel information was extracted (digital numbers R, G, B; 0-255). Background color information was used to correct for minor variation in light conditions. The chromatic coordinates representing the strength of each color were calculated as the ratio of each digital number to the sum of all digital numbers:

Green chromatic coordinate \( g_{cc} = \frac{G}{R + G + B} \) Gillespie et al. (1987) eq. 7

Blue chromatic coordinate \( b_{cc} = \frac{B}{R + G + B} \) Gillespie et al. (1987) eq. 8

Red chromatic coordinate \( r_{cc} = \frac{R}{R + G + B} \) Gillespie et al. (1987) eq. 9

Hue was calculated according to the Preucil color circle (Preucil, 1953):

\[
\text{Hue} \begin{cases} 
\text{if } R \geq G \geq B: & \text{Hue}_{\text{Red-Yellow}} = 60^\circ \ast (G - B) \ast (R - B)^{-1} \quad \text{Gunther (2012)} \quad \text{eq. 10-15} \\
\text{if } G \geq R \geq B: & \text{Hue}_{\text{Yellow-Green}} = 60^\circ \ast (2 - (R - B) \ast (G - B)^{-1}) \\
\text{if } G \geq B \geq R: & \text{Hue}_{\text{Green-Cyan}} = 60^\circ \ast (2 + (B - R) \ast (G - R)^{-1}) \\
\text{if } B \geq G \geq R: & \text{Hue}_{\text{Cyan-Blue}} = 60^\circ \ast (4 - (G - R) \ast (B - R)^{-1}) \\
\text{if } B \geq R \geq G: & \text{Hue}_{\text{Blue-Magenta}} = 60^\circ \ast (4 - (R - G) \ast (B - G)^{-1}) \\
\text{if } R \geq B \geq G: & \text{Hue}_{\text{Magenta-Red}} = 60^\circ \ast (6 - (B - G) \ast (R - G)^{-1}) 
\end{cases}
\]

Digital image indices were calculated from the color channel information as:

Excess green index \( \text{ExG} = 2 \ast G - (R + B) \) Richardson et al. (2007) eq. 16

Normalized difference index \( \text{NDI} = \frac{R - G}{R + G} \) Pérez et al. (2000) eq. 17
5.3.5 HPLC-analyses of chlorophylls and carotenoids

Prior to pigment extractions, leaves were immersed in liquid nitrogen in a mortar and ground to a fine powder using a pestle. Pigment extractions were performed according to Ensminger et al. (2004), but using 98% methanol buffered with 0.5 M ammonium acetate. Separation and quantification of peaks was carried out using a high-performance liquid chromatography (HPLC) system (Agilent 1260, Böblingen, Germany) following the method described in Junker et al. (unpublished). Chlorophyll a, chlorophyll b, α-tocopherol and δ-tocopherol were quantified using standards from Sigma Aldrich (Oakville, ON, Canada) and antheraxanthin, β-carotene, lutein, neoxanthin, violaxanthin and zeaxanthin were quantified using standards from DHI Lab products (Hørsholm, Denmark). In addition to the calibrated pigments, we observed many additional peaks in the HPLC chromatograms of senescing leaves. Based on their carotenoid-like absorption spectra along with late retention time, we assume that these peaks represented non-polar carotenoid esters (Zonta et al. 1987) which are typically observed in senescing leaf tissue (Biswal, 1995).

5.3.6 Spectrophotometric analysis of anthocyanins

Anthocyanins in maple leaves consist to about 82% of cyanidin-3-glucoside (Ji, 1992). We used a protocol from Esteban et al. (2008) in order to estimate anthocyanins as cyanidin-3-glycoside equivalents. Approximately 25 mg of the homogenized frozen sample were suspended in 1 ml of 3 M HCl:H2O:MeOH (1:3:16 v:v:v) and incubated for 2 hours at 4 °C and 900 rpm. Subsequently, the extract was centrifuged for 5 minutes at 30,000 RCF and 4 °C. The absorbance of the supernatant was determined at 524 nm and 653 nm. Anthocyanin concentration was calculated using a molar extinction coefficient of 33,000 M⁻¹ cm⁻¹ (Gould et al., 2000) and corrected for pheophytins using the formula $A_{524} - 0.24 A_{653}$ (Murray et al., 1994).

5.3.7 Microscopy

Cross sections of autumn leaves were prepared in order to identify the localisation and distribution of pigments. Green, yellow, orange and red leaves were cut by hand and studied using a Nikon AZ100 microscope equipped with a polarising lambda filter (Nikon Instruments
Inc., Melville, NY). Images from leaf sections were taken using a DS-Fi2 camera mounted on the microscope. The z-stacking function of the NIS-Elements BR program (Nikon Instruments Inc., Melville, NY) was used to merge several pictures to create a focused image.

5.3.8 Statistics

All statistical tests were performed using R 2.12.1 (R Development Core Team 2010). Differences between leaf classes were assessed using a mixed-effect linear model to avoid pseudoreplication. The function lmer from the package lme4 (Bates et al., 2013) was used to calculate mean and standard error of the fixed model parameters Color using the maximum-likelihood method. Tree and Year of measurements were treated as random factors. For spectral reflectance measurements which were only conducted in 2013, Tree was used as random factor. Significant differences between leaf color classes were estimated calculating the least squares means between leaf color classes using the function lsmeans from the package lsmeans (Lenth & Herve, 2014) followed by Tukey's multiple comparison test using the function cld from the package multcompView (Graves, 2012).

The relationship between vegetation indices and pigment content and rETR\textsubscript{II}, respectively, as shown in Fig. 5.8, 5.10, was characterised using regression analysis. The function wrapnls from the package nlmrt was used to perform linear, logarithmic and exponential model fits for all vegetation indices. The best fitting model was chosen based on highest pseudo-R\textsuperscript{2} which was estimated as $R^2 = 1 - (\text{sum of squared residuals} / \text{sum of squared deviations})$ according to Eisenhauer (2003). All R\textsuperscript{2}-values are summarized in Table A8.1.

5.4 Results

5.4.1 Photosynthetic pigments and antioxidants

Green non-senescent summer leaves were used as a reference for comparisons with green, yellow, orange and red autumn leaves (Fig. 5.1a). Chlorophyll content ranged from about 1.7 μmol g\textsuperscript{-1} in green summer leaves, to 0.4 μmol g\textsuperscript{-1} in green autumn leaves and was barely detectable in yellow, orange and red leaves (Fig. 5.1b). Despite the significant changes in overall
chlorophyll content, the ratio of chlorophyll a to chlorophyll b remained constant (Fig. 5.1b). Carotenoid content was about 0.5 µmol g⁻¹ in green summer leaves, about 0.2 µmol g⁻¹ in green autumn leaves and decreased further in yellow, orange and red leaves (Fig. 5.1c). However, the relative decrease in carotenoids compared to chlorophylls was smaller, resulting in an increasing ratio of carotenoids per chlorophyll in yellow, orange and red autumn leaves compared with summer and green autumn leaves (Fig. 5.1d).

Fig. 5.1: Pictures of green sugar maple leaves sampled in summer and green, yellow, orange and red leaves sampled in autumn (a) and chlorophyll a and b (b), carotenoids (c), carotenoid-chlorophyll ratio (d), anthocyanins (e), δ-tocopherol (f), and α-tocopherol (g). Data show modelled means of 5-6 leaves sampled from 3 trees in 2 consecutive years (±SE). Significant differences (p<0.05 using linear-mixed effect modelling followed by Tukey’s multiple comparison) are indicated by different letters.
Fig. 5.2: Carotenoid composition of green sugar maple leaves sampled in summer and green, yellow, orange and red leaves sampled in autumn. Xanthophyll cycle pigments (violaxanthin, antheraxanthin and zeaxanthin) (a), de-epoxidation state of the xanthophyll cycle, DEPS (b), lutein (c), β-carotene (d), neoxanthin (e). Data show modelled means of 5-6 leaves sampled from 3 trees in 2 consecutive years (±SE). Significant differences (p<0.05 using linear-mixed effect modelling followed by Tukey’s multiple comparison) are indicated by different letters.
The composition of carotenoids varied with advancing senescence (Fig. 5.2). The relative amount of xanthophyll cycle among all carotenoids increased to a maximum in orange leaves (Fig. 5.2a), and shifts in the composition of xanthophyll cycle pigments towards increased antheraxanthin and zeaxanthin content resulted in an enhanced deepoxidation status (DEPS) of the xanthophyll cycle pigments (Fig. 5.2b). Lutein showed little variation among green summer, green autumn, yellow and orange leaves but was increased in red leaves (Fig. 5.2c). This was in contrast to β-carotene and neoxanthin which decreased from green to red leaves (Fig. 5.2d,e).

The amount of anthocyanins and tocopherols increased considerably in senescing leaves compared to green summer leaves, (Fig. 5.1e-g). Anthocyanins were as low as 55 nmol g⁻¹ in green summer leaves, but increased up to 2400 nmol g⁻¹ in red leaves (Fig. 5.1e). δ-tocopherol was not detectable in summer leaves, but was present in senescent leaves with 30-45 nmol g⁻¹ (Fig. 5.1f). α-tocopherols were as low as 5 nmol g⁻¹ in summer leaves, but gradually increased in senescent leaves up to 250 nmol g⁻¹ (Fig. 5.1g).
Fig. 5.3: Pictures of one of the trees sampled for this experiment (a) including a close-up of within-canopy variation in leaf color (b). Sections of green (c), yellow (d,e), orange (f) and red (g) sugar maple leaves. Pictures were taken at 40x magnification.

The localisation of pigments was confirmed by microscopy analysis of senescent leaves (Fig. 5.3a). A typical sampling tree is shown in Fig. 5.3a with within-canopy variation of leaf senescence (Fig. 5.3b). Mesophyll cells of green autumn leaves contained green chloroplasts (Fig. 5.3c). The color of yellow leaves did result from yellow plastids and the visual absence of other pigments (Fig. 5.3d), although in some sections of yellow leaves cells with green chloroplasts were still present (Fig. 5.3e). Orange leaves showed a combination of yellow plastids and red coloration of the vacuoles of the palisade cells (Fig. 5.3f), whereas in red leaves, the red coloration of the palisade cells was dominant (Fig. 5.3g).
5.4.2 Chlorophyll fluorescence and P700 measurements

Minimum fluorescence ($F_o$) was about 0.2 in green summer and green autumn leaves and 0.02 to 0.04 in yellow, orange and red leaves (Fig. 5.4a). Maximum fluorescence ($F_m$) was about 1.0 in green summer leaves, 0.7 in green autumn leaves and between 0.02 to 0.07 in yellow, orange and red leaves (Fig. 5.4b). Consequently, the maximum quantum yield ($F_v/F_m$), was about 0.75 in green summer leaves and green autumn leaves and decreased to 0.4 in yellow, 0.25 in orange and 0.15 in red leaves (Fig. 5.4c). This gradual decrease in $F_v/F_m$ from green summer over green autumn, to yellow, orange and red leaves illustrates the increasing loss of photosynthetic capacity. $1-\Phi_P$, the excitation pressure on PSII, was slightly higher in autumn leaves compared to green summer leaves (Fig. 5.4d).

![Fig. 5.4: Minimum fluorescence, $F_o$ (a), maximum fluorescence, $F_m$ (b), maximum quantum yield of dark-adapted leaves, $F_v/F_m$ (c) and excitation pressure at PSII, $1-\Phi_P$ (d) of green sugar maple leaves sampled in summer and green, yellow, orange and red leaves sampled in autumn. Data show modelled means of measurements of 5-6 leaves sampled from 3 trees in 2 consecutive years (±SE) taken at a light intensity of 1200 μmol m$^{-2}$ s$^{-1}$. Significant differences (p<0.05 using linear-mixed effect modelling followed by Tukey’s multiple comparison) are indicated by different letters.](image)

Excitation energy partitioning at PSII also revealed a transient decrease of regulated energy dissipation from green summer, to green autumn, yellow, orange and red leaves (Fig. 5.5a). The proportion of light energy dissipated via photochemical processes ($\Phi_{PSII}$) and regulated energy dissipation ($\Phi_{NPQ}$) decreased, entailing a rise of non-regulated energy dissipation ($\Phi_{NO}$).
Excitation energy partitioning at PSI showed a similar trend (Fig. 5.5b). The photochemical quantum yield of PSI ($\Phi_{\text{PSI}}$) was about 0.17 in green summer leaves, but decreased to about 0.11 and 0.08 in green, yellow, orange and red leaves. The decrease of $\Phi_{\text{PSI}}$ caused an increased non-photochemical quantum yield at PSI (sum of $\Phi_{\text{ND}}$ and $\Phi_{\text{NA}}$), with a shift from donor side-dominated limitations ($\Phi_{\text{ND}}$) to acceptor side-dominated limitations ($\Phi_{\text{NA}}$).

![Diagram](image)

**Fig. 5.5:** Variation in energy use at PSII (a) and PSI (b) between green sugar maple leaves sampled in summer and green, yellow, orange and red leaves sampled in autumn. $\Phi_{\text{PSII}}$ = effective quantum yield of PSII, $\Phi_{\text{NO}}$ = quantum yield of non-regulated energy dissipation, $\Phi_{\text{NPQ}}$ = quantum yield of regulated energy dissipation, $\Phi_{\text{PSI}}$ = photochemical quantum yield of PSI, $\Phi_{\text{ND}}$ = non-photochemical quantum yield at PS I due to donor side limitation, and $\Phi_{\text{NA}}$ = non-photochemical quantum yield at PS I due to acceptor side limitation. Data show modelled means of measurements of 5-6 leaves sampled from 3 trees in 2 consecutive years ($\pm$SE) taken at a light intensity of 1200 $\mu$mol m$^{-2}$ s$^{-1}$. Significant differences (p<0.05 using linear-mixed effect modelling followed by Tukey’s multiple comparison) are indicated by different letters.

Relative electron transport rates of PSI (rETR$_{\text{I}}$) were 90 $\mu$mol e$^{-}$ m$^{-2}$ s$^{-1}$ in green summer leaves and decreased to 40 $\mu$mol e$^{-}$ m$^{-2}$ s$^{-1}$ in red leaves (Fig. 5.6a). In contrast, relative electron
transport rates of PSII (rETR$_{II}$) showed a stronger decrease from 80 µmol e$^{-}$ m$^{-2}$ s$^{-1}$ in green summer leaves to 5 µmol e$^{-}$ m$^{-2}$ s$^{-1}$ in red leaves (Fig. 5.6a). Consequently, the ratio between rETR$_{I}$ and rETR$_{II}$ as an indicator for cyclic electron transport increased from 1 µmol µmol$^{-1}$ in green summer leaves to 4.5 µmol µmol$^{-1}$ in red leaves (Fig. 5.6b). rETR$_{II}$ showed a logarithmic relationship to chlorophyll content (Fig. 5.6c).

![Graph showing electron transport rates at PSII, rETR$_{II}$, and PSI, rETR$_{I}$ (a), the ratio of rETR$_{I}$ to rETR$_{II}$ as an indicator for cyclic electron transport (b) and the relationship between chlorophyll content and rETR$_{II}$ (c) of green sugar maple leaves sampled in summer and green, yellow, orange and red leaves sampled in autumn. Data show modelled means of measurements of 5-6 leaves sampled from 3 trees in 2 consecutive years (±SE) taken at a light intensity of 1200 µmol m$^{-2}$ s$^{-1}$. Significant differences (p<0.05 using linear-mixed effect modelling followed by Tukey’s multiple comparison) are indicated by different letters.](image)

**Fig. 5.6:** Electron transport rates at PSII, rETR$_{II}$, and PSI, rETR$_{I}$ (a), the ratio of rETR$_{I}$ to rETR$_{II}$ as an indicator for cyclic electron transport (b) and the relationship between chlorophyll content and rETR$_{II}$ (c) of green sugar maple leaves sampled in summer and green, yellow, orange and red leaves sampled in autumn. Data show modelled means of measurements of 5-6 leaves sampled from 3 trees in 2 consecutive years (±SE) taken at a light intensity of 1200 µmol m$^{-2}$ s$^{-1}$. Significant differences (p<0.05 using linear-mixed effect modelling followed by Tukey’s multiple comparison) are indicated by different letters.

### 5.4.3 Spectral reflectance

Spectral reflectance was affected by the variation in pigment content. Spectral reflectance spectra obtained from green summer leaves revealed a reflectance peak at 550 nm and maximum reflectance in the near-infrared (NIR, 780-1000 nm) (Fig. A8.1). Green autumn leaves exhibited a similar spectrum in the visible spectrum but a lower reflectance in the NIR spectrum. Yellow leaves had a reflectance maximum at 615 nm and a second peak at 550 nm, while orange leaves had a single peak at 615 nm. Reflectance in red leaves increased over the whole range of the
yellow-red and NIR spectrum. These differences in reflectance spectra were reflected in vegetation indices calculated based on spectral reflectance (Fig. 5.7).

![Vegetation Indices](image-url)

**Fig. 5.7:** Vegetation indices calculated from spectral reflectance data for green sugar maple leaves sampled in summer and green, yellow, orange and red leaves sampled in autumn. Normalized difference vegetation index, NDVI (a), Photochemical reflectance index, PRI (b), Excess green index, ExG<sub>M</sub> (c), Green red vegetation index, GRVI (d), Anthocyanin reflectance index, ARI (e) and Red/green reflectance ratio, RGR (f). Data show modelled means of measurements of 5-6 leaves sampled from 3 trees (±SE) of light-adapted leaves (5 min at 1200 µmol m<sup>-2</sup> s<sup>-1</sup>). Significant differences in vegetation indices between leaf classes are indicated by different letters (p<0.05 using linear-mixed effect modelling followed by Tukey’s multiple comparison).

The Normalized difference vegetation index (NDVI) was as high as 0.8 and 0.75 in green summer and green senescent leaves, respectively, but was very low (0.1) in yellow and orange leaves and intermediate (0.3) in red leaves (Fig. 5.7a), thus being unable to reflect the variation in chlorophyll content or rETR<sub>II</sub> adequately (Fig. 5.8a,b). The Photochemical reflectance index (PRI) was highest in green summer leaves (-0.02), and decreased to -0.05 in green autumn leaves, -0.25 in orange leaves, and -0.15 in yellow and red leaves (Fig. 5.7b). The gradual changes allow for adequate representation of chlorophyll content and rETR<sub>II</sub> (Fig. 5.8c,d). The
Excessive green index (ExG$_M$) showed highest values of 0.14 in summer and green autumn leaves, intermediate values in yellow leaves and then lowest values for orange and red leaves with values of -0.08 and -0.06, respectively (Fig. 5.7c). Therefore, ExG$_M$ is not indicative for chlorophyll content, but correlated linearly with rETR$_{II}$ (Fig. 5.8e,f). The Green-red vegetation index (GRVI) revealed similar values for green summer and autumn leaves which were contrasted by much lower values for yellow and lowest values for orange and red leaves (Fig. 5.7d). The differences in chlorophyll content are not represented, but the gradual decay of rETR$_{II}$ is well represented (Fig. 5.8g,h). The Anthocyanin reflectance index (ARI) was around 0 and 1 for green summer and green autumn leaves, and then increased from 3 in yellow leaves to more than 20 in orange leaves and peaked at 40 in red leaves (Fig. 5.7e). A similar pattern was observed for the Red/green reflectance ratio (RGR) which showed a similar response to anthocyanin content. Low values of 0.65 were obtained in green summer and autumn leaves, whereas values increased from 1.5 in yellow and 3.3 in orange leaves to a maximum of 3.9 in red leaves (Fig. 5.7f). Both indices showed a close relation to leaf anthocyanin content and were negatively correlated with rETR$_{II}$ (Fig. 5.8i-l).
Fig. 5.8: Relationship between vegetation indices derived from spectral reflectance measurements and foliar pigment content (a,c,e,g,i,k) and electron transport rate at PSII, rETR_{II} (b,d,f,h,j,l). Normalized difference vegetation index, NDVI (a,b), Photochemical reflectance index, PRI (c,d), Excess green index, ExG_{M} (e,f), Green red vegetation index, GRVI (g,h), Anthocyanin reflectance index, ARI (i,j) and Red/green reflectance ratio, RGR (k,l). Each point represents the average (±SE) of 5-6 green sugar maple leaves sampled in summer and green, yellow, orange and red leaves sampled in autumn from 3 trees in 2 consecutive years. Spectral reflectance measurements were taken at a light intensity of 1200 µmol m\(^{-2}\) s\(^{-1}\). Lines represent either linear, exponential and logarithmic regression fit based on highest pseudo-R\(^2\), with solid lines in the left column representing regression with chlorophyll content (a,c,e,g), dashed lines representing regression with anthocyanin content (i,k) and solid lines in the right column representing regression with rETR_{II} (b,d,f,h,j,l).
5.4.4 Digital picture analysis

Analysis of red, green and blue (RGB) signals derived from digital images also depicted the change of leaf optical properties with advancing senescence (Fig. 5.9). The color change from green over yellow to red caused a gradual decrease in the green chromatic coordinate (\(g_{cc}\)) from 0.5 in green summer and green autumn leaves to 0.2 in red leaves, while the red chromatic coordinate (\(r_{cc}\)) gradually increased from 0.3 in green leaves to 0.6 in orange and red leaves (Fig. 5.9a). Consequently, the blue chromatic coordinate (\(b_{cc}\)) was lowest in green autumn and yellow leaves and was highest in green summer and red leaves (Fig. 5.9a). \(g_{cc}\) and \(r_{cc}\) were unable to distinguish between leaves with high foliar chlorophyll and anthocyanin indices, respectively, but correlated well with rETR II (Fig. 5.10a-d). The change of leaf color was also well described by the gradual change of Hue from 95° in green summer leaves to 2° in red leaves and showed a good representation of chlorophyll content and rETR II (Fig. 5.9b, 5.10e,f). The Normalized difference index (NDI) showed a gradual decrease from 0.2 in green summer leaves to -0.4 in red leaves (Fig. 5.9c) which allowed a good representation of chlorophyll content and rETR II (Fig. 5.10g,h). The Green excess index (ExG) was 165 in green summer and green autumn senescent leaves, but decreased gradually to about -100 in red leaves (Fig. 5.9d). ExG did not represent chlorophyll content well, but did reflect rETR II (Fig. 5.10i,j).

Fig. 5.9: Color and vegetation indices calculated from color channel information derived from digital images in green sugar maple leaves sampled in summer and green, yellow, orange and red leaves sampled in autumn. RGB chromatic coordinates, \(r_{cc}, g_{cc}, b_{cc}\) (a), Hue (b), Normalized difference index, NDI (c) and Excess green index ExG (d). Data show modelled means of measurements of 5-6 leaves sampled from 3 trees in 2 consecutive years (±SE). Significant differences (\(p<0.05\) using linear-mixed effect modelling followed by Tukey’s multiple comparison) are indicated by different letters.
Fig. 5.10: Relationship between vegetation indices derived from digital image analysis and foliar pigment content (a,c,e,g,i) and electron transport rate at PSII, rETR_{II} (b,d,f,h,j). Green chromatic coordinates, g_{CC} (a,b), red chromatic coordinate, r_{CC} (c,d), Hue (e,f), Normalized difference index, NDI (g,h) and Excess green index ExG (i,j). Each point represents the average (±SE) of 5-6 green sugar maple leaves sampled in summer and green, yellow, orange and red leaves sampled in autumn from 3 trees in 2 consecutive years. Lines represent either linear, exponential and logarithmic regression fit based on highest pseudo-R^2, with solid lines in the left column representing regression with chlorophyll content (a,e,g,i), dashed lines representing regression with anthocyanin content (c) and solid lines in the right column representing regression with rETR_{II} (b,d,f,h,j).
5.5 Discussion

5.5.1 Autumn leaf senescence reflected by changes in pigment and antioxidant content

Measurements of chlorophyll fluorescence and photosynthetic pigment and anthocyanin content were used as indicators for the progress of senescence and served as a reference for vegetation indices derived from spectral reflectance measurements and color analysis of digital images.

The color change of sugar maple leaves during autumn reflects the well-known senescence-associated changes in photosynthetic pigment content (Fig. 5.1a). Chlorophylls are nearly completely degraded as photosynthetically active chloroplasts transform into carotenoid-dominated gerontoplasts (Fig. 5.1a,b, Fig. 5.3c,d). The overall increase in the ratio of carotenoids per chlorophyll (Fig. 5.1d) contributes to balancing of oxidative stress in senescing tissue (Munné-Bosch & Peñuelas, 2003; Ougham et al., 2005). The composition of carotenoids during senescence was variable, with increases in xanthophylls, rather stable lutein, and decreases in β-carotene and neoxanthin content (Fig. 5.2a,c,d,e). Increases of the de-epoxidised xanthophylls antheraxanthin, zeaxanthin and lutein have also been observed in yellow-senescent tree species such as birch, alder, poplar and hazel during autumn senescence and were attributed to enhanced photoprotection (García-Plazaola et al., 2003a). Concurrently, we also observed an increased de-epoxidation state (Fig. 5.2b) which typically indicates an increased capacity for non-photochemical quenching of excess energy (Demmig-Adams & Adams, 2006). In senescent leaves with low photosynthetic activity, xanthophylls, including zeaxanthin, instead contribute as antioxidants to the protection of lipid membranes from oxidative damage (Havaux et al., 2007).

Anthocyanins drastically increased in orange and red leaves and were localized in vacuoles of the palisade mesophyll (Fig. 5.1e, Fig. 5.3f,g). The role of anthocyanins during senescence is not entirely clear (Hoch et al., 2001; Archetti et al., 2009), but mesophyll-localized anthocyanins have been shown to have an antioxidant function due to their close proximity to chloroplasts as source of reactive oxygen species (Kytridis & Manetas, 2006). Additionally, the antioxidants α- and δ-tocopherol were more abundant in senescent leaves (Fig. 5.1f,g). α-tocopherol is a membrane constituent within the chloroplast (Fryer et al., 1998; Abbasi et al., 2009). δ-tocopherols are localized in plastoglobuli of tobacco (Matringe et al., 2008) which are increasingly formed when chloroplasts differentiate to photosynthetically inactive gerontoplasts.
While α- and δ-tocopherol levels were already induced in green senescent maple leaves, anthocyanins were induced once chlorophyll degradation was nearly completed and can thus serve as an indicator of late senescence.

5.5.2 Changes in photosynthetic performance during autumn leaf senescence

Minimum and maximum fluorescence (F₀ and Fₘ) are mainly influenced by chlorophyll content (Fig. 5.4a,b, Fig. 5.1b), with equal F₀ in green summer and autumn leaves likely due to higher reabsorption of fluoresced light by neighbouring chloroplasts in summer leaves (Lichtenthaler & Babani, 2004). Nonetheless, the ratio of variable over maximum fluorescence (Fᵥ/Fₘ) showed less variation between green and yellow, orange and red leaves (Fig. 5.4c), indicating that chlorophylls retained in green cells of autumn leaves were still organized in functional PSII complexes. This has been shown for yellow leaves of plane, *Platanus hybrida* L. and *Platanus occidentalis* L. (Adams *et al.*, 1990; Lichtenthaler & Babani, 2004). Cells containing yellow gerontoplasts interspersed with cells containing green chloroplasts demonstrated this within-leaf heterogeneity during senescence (Fig. 5.3e). Such intercellular differences in the progress of senescence have been observed for aspen, *Populus tremula* (Keskitalo *et al.*, 2005).

The downregulation of yield of PSII (Φₚₛᵢ) concurred with a shift from regulated Φₙₚₒ to non-regulated energy dissipation Φₙₒ (Fig. 5.5a) and affected the overall energy balance in PSII and PSI. Although decreasing Φₚₛᵢ limited the electron transport to PSI, yield of PSI (Φₚₛᵢ) remained relatively high (Fig. 5.5b). Non-photochemical energy-quenching at PSI showed a shift from donor-side limitations (Φₙ₉) to acceptor-side limitations (Φₙₐ; Fig. 5.5b) (Klughammer & Schreiber, 2008).

Higher Φₚₛᵢ than Φₚₛᵢ is also reflected in higher relative transport rates at PSI (rETRᵢ) than PSII (rETRᵢ, Fig. 5.6a). PSI increasingly acts as an efficient sink for electrons, suggesting that cyclic electron transport might be enhanced during senescence when PSII is increasingly impaired (compare Yamori *et al.* 2001; Fig. 5.6b). Enhanced cyclic electron transport at PSI was shown in senescing leaves of tree spurge, *Euphorbia dendroides* L., when photochemical reactions were limited (Kotakis *et al.*, 2014). The role of cyclic electron transport during senescence might
include generation of ATP (Rumeau et al., 2007) and a contribution to NPQ (Miyake et al., 2005) and thus minimize unregulated formation of ROS during senescence.

5.5.3 Autumn leaf senescence reflected by spectral reflectance vegetation indices

In order to represent leaf senescence on the leaf level, vegetation indices should be sensitive to the degradation of chlorophyll which is one of the initial steps during leaf senescence (Hörtensteiner & Lee, 2007) and linked to photosynthetic capacity (Ustin et al. 2009; see Fig. 5.6c). Furthermore, vegetation indices should show a continuous and gradual change as leaves senesce, to avoid an asymptotic and thus meaningless representation of chlorophyll content. Additionally, vegetation indices need to be unaffected by the increasing anthocyanin content.

The normalized difference vegetation index, NDVI which is calculated from near infrared and red wavelength regions, did not gradually respond as leaves senesce. Instead, it was high in green leaves, low in yellow and orange leaves but intermediate in red leaves (Fig. 5.7a) which might be due to a low sensitivity of the bands used for NDVI for the color change from green to yellow (Motohka et al., 2010). This non-continuous response to senescence does not represent chlorophyll content and rETR\textsubscript{II} well, despite high R\textsuperscript{2} (Fig. 5.8a,b). Additionally, higher NDVI in red leaves might be a consequence of the presence of anthocyanins which have been shown to contribute to increased NDVI values (Viña & Gitelson, 2011).

The photochemical reflectance index, PRI which is calculated from the green wavelength region, decreased gradually during initial senescence (Fig. 5.7b) most likely due to its sensitivity to carotenoid-chlorophyll ratio (Sims and Gamon 2002; Wong and Gamon 2015; see Fig. 5.1d). The increase in PRI in red leaves might be a consequence of the large increases in anthocyanins. It has been shown that anthocyanins affect light absorption in maple leaves at wavelengths around 550 nm which in turn results in variations in leaf spectral reflectance of wavelengths used for estimation of PRI (Merzlyak et al., 2008).

In contrast to NDVI and PRI which are used to assess canopy “greenness” or photosynthetic efficiency, indices such as the Excess green index (ExG\textsubscript{M}) and Green-red vegetation index (GRVI) were developed to monitor phenology of forest canopies (Motohka et al., 2010; Hufkens
et al., 2012). While both indices were not responsive to the initial chlorophyll degradation, they exhibited a continuous change from green via yellow to red leaves (Fig. 5.7c,d), thus showing a rather asymptotic relationship with chlorophyll content, but nearly linear relationship with rETR_{II} (Fig. 5.8e-h). Although both indices cannot be used as indicators for start and end of senescence processes on the leaf level, they might be valuable on a canopy scale, when many leaves contribute to the signal, e.g. a GRVI of zero has been suggested as an indicator for the middle phase of autumn coloring of a deciduous forest on a canopy scale (Motohka et al., 2010). Furthermore, these indices correspond to two vegetation indices derived from digital images. Thus, ExG and NDI and might provide an important link between near-surface and satellite remote sensing (Hufkens et al. 2012; see below).

The Anthocyanin reflectance index (ARI) and the Red/green reflectance ratio (RGR) were developed to track anthocyanins as indicators for plant stress and senescence (Schaberg et al. 2003, Gitelson et al. 2009). Both anthocyanin sensitive indices were low in green leaves and increased to a maximum in red leaves, therefore not capturing the variation in chlorophyll content, but anthocyanin content on the leaf level well (Fig. 5.6e,f, 8i,k), while being negatively correlated with rETR_{II} (Fig. 5.8j,l). Therefore, ARI and RGR might be suitable to identify maximum anthocyanin content as indicator for forest stress (Schaberg et al., 2003).

Overall, leaf spectral indices such as NDVI and PRI using either green or red wavelength regions seemed to have limitations when measurements were obtained from autumn leaves and in particular from red leaves (Fig. 5.7a-b). Other sources causing variation of spectral reflectance index measurements in senescing tissue include differences in leaf water content as well as structural differences such as the collapse of the spongy-mesophyll layer, as was reported for senescing sweetgum leaves, Liquidambar styraciflua L. (Jensen, 2007; Salama, 2011). In contrast, vegetation indices using both green and red wavelength regions such as ExG_{M}, GRVI, ARI and RGR are better suited to track senescence involving anthocyanin formation, since major changes in the spectral reflectance spectra during senescence involve the decrease of reflectance in the green wavelength regions (540-560 nm) relative to the red wavelength region (605-625 nm, see Fig. A8.1). In particular, if these wavelength regions prove characteristic in other species and on the canopy-level, indices might be further improved by restricting green and red wavelength regions to 540-560 nm and 605-625 nm, respectively.
5.5.4 Autumn leaf senescence reflected by digital repeat photography

All tested digital imaging vegetation indices showed gradual changes without being negatively affected by high anthocyanin content, therefore representing autumn phenology and physiology on the leaf level reasonably well. The green chromatic coordinate $g_{CC}$ is the most commonly used digital imaging vegetation index and has been used previously to assess spring and autumn phenology at the canopy scale, e.g. in a Japanese beech forest (*Fagus crenata* Blume) and a northern hardwood forest (Richardson *et al.*, 2009; Mizunuma *et al.*, 2011). In our study, $g_{CC}$ was similar in green summer and green autumn leaves (Fig. 5.9a), thus having a rather asymptotic relationship with chlorophyll content and not representing the initial loss of chlorophylls during senescence well (Fig. 5.10a). However, $g_{CC}$ reflected the rather gradual decrease of $r_{ETR_{II}}$ quite well (Fig. 5.10b). On a canopy scale, $g_{CC}$ showed a gradual decay during autumn, likely since pictures represent a mixture of several leaves (Dillen *et al.*, 2012; Yang *et al.*, 2014). In our study, the blue chromatic coordinate ($b_{CC}$) showed little variation, thus the red chromatic coordinate ($r_{CC}$) increased as $g_{CC}$ decreased and was representative for anthocyanin content and negatively correlated with $r_{ETR_{II}}$ (Fig. 5.10a, 11c,d). On a canopy scale, $r_{CC}$ has been shown to correlate well with the anthocyanin reflectance index ARI for a deciduous white oak (*Quercus alba* L.) forest canopy (Yang *et al.*, 2014) which was confirmed by our leaf level measurements (data not shown).

For digital imaging, relatively little vegetation indices have been proposed yet. The Green excess index (ExG) and the Normalized difference index (NDI) have been developed in analogy to GRVI and ExG$_{M}$ derived from spectral reflectance measurements and their computation follows a similar concept. ExG was equally high for green summer and green senescent leaves, intermediate in yellow leaves and low in orange and red leaves (Fig. 5.10d). Therefore, chlorophyll was not well represented, but $r_{ETR_{II}}$ was (Fig. 5.10c, Fig. 5.11e,f). These values are beyond values from about 40 during the onset of leaf senescence to 10-20 after leaf abscission for canopies dominated by red maple (*Acer rubrum* L.) and red oak (*Quercus rubra* L.) (Sonnentag *et al.*, 2012). Such differences between leaf level and canopy level measurements are likely due to canopy structure, illumination, and the canopy signal being composed of signals from numerous leaves differing in color, especially during autumn senescence (Sonnentag *et al.*, 2012).
The Normalized difference index (NDI; Fig. 5.9d) and Hue (Fig. 5.9e) showed gradual changes with significant differences between all leaf classes, thus representing chlorophyll content and rETR_II very well (Fig. 5.11g,h,i,j). Mathematically, positive NDI indicates a stronger green than red signal. It has been rather rarely used, e.g. to monitor the growing season of yellow-senescing Japanese beech (*Fagus crenata* Blume) on the canopy level (Mizunuma *et al.*, 2011). Negative NDI might as well be used to indicate high anthocyanin content and thus autumn coloration, hence making it a strong indicator for autumn phenology of red-senescing species. Hue also has not been widely used, but proved to be superior in assessing autumn phenology of trees (Mizunuma *et al.*, 2011; Lei *et al.*, 2013).

Generally, the vegetation indices were superior to the often used chromatic coordinates in assessing autumn phenology on the leaf scale. Additionally, similar trends for indices such as NDI and ExG, and their analogues GRVI and ExG_M, respectively, indicate the comparability of spectral reflectance measurements and digital imaging. Despite variation in the absolute numbers, these indices seem to be adequate links between near-surface and remote sensing of vegetation (Hufkens *et al.*, 2012).

### 5.5.5 Conclusions

The decay of photosynthetic capacity and the decrease in chlorophylls and carotenoids together with the accumulation of anthocyanins are important indicators of the progress of leaf senescence and the autumn phenology of sugar maple. Vegetation indices derived from spectral reflectance and digital repeat photography varied in their ability to represent the autumn phenology at the leaf level. We observed that spectral reflectance vegetation indices were partially unable to represent the gradual decrease of chlorophylls during senescence. Additionally, vegetation indices developed to assess canopy “greenness” such as NDVI were also influenced by high foliar anthocyanin levels. By contrast, spectral reflectance indices based on green and red wavelength regions such as GRVI and RGR showed improved representation of the continuous changes in pigment composition and quantity during senescence. Generally, digital repeat photography indices proved superior in assessing autumn phenology at the leaf level. Hue and NDI in particular were superior to the commonly used g_CC and should be considered in further studies for assessing seasonal variation in forest phenology.
5.6 Acknowledgments

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Chapter 6
Conclusions and future directions

6 Conclusions and future directions

In this thesis, I studied intraspecific variation in adjustments of photoprotective isoprenoids in response to drought stress in Douglas-fir and how adjustments of foliar isoprenoids isoprenoid are reflected by leaf optical properties of senescing Sugar maple leaves. I hypothesized that 1) isoprenoid-mediated photoprotective mechanisms are induced in response to environmental conditions that limit photosynthesis or during senescence to match an enhanced demand for non-photochemical quenching of light energy; 2) interior Douglas-fir provenances avoid water loss by low stomatal conductance, and consequently show enhanced photoprotection of the photosynthetic apparatus mediated by essential and non-essential isoprenoids, and 3) essential isoprenoids are indicators of physiological performance and phenological events in remote-sensing applications, because plants adjust the composition of the photosynthetic apparatus in response to environmental conditions. In this chapter, I will separately discuss the studies on intraspecific variation of Douglas-fir and studies of leaf optical properties in relation to autumn physiology of Sugar maple and point out questions arising from my work.

6.1 Isoprenoid-mediated photoprotective mechanisms are induced in response to environmental conditions that limit photosynthesis or during senescence to match an enhanced demand for non-photochemical quenching of light energy

6.1.1 Adjustments of photoprotective isoprenoids in response to drought in Douglas-fir

In this subchapter, I discuss the observed adjustments of essential and non-essential isoprenoids in response to reduced photosynthesis. Without focusing at intraspecific variation at this point, I will discuss the observed adjustments of the major classes (carotenoids, monoterpenes,
sesquiterpenes) as well as individual photoprotective isoprenoids and link these results to the isoprenoid biosynthetic pathway.

In both experiments with Douglas-fir, the drought stress experiment with seedlings of two provenances and the field experiment with adult field-grown trees of four provenances, plants were affected by limited water availability, as indicated by strongly reduced photosynthetic gas exchange (Fig. 3.3, Fig. 4.4). The reduction of stomatal conductance in response to drought is a common mechanism to minimize water loss, but limits photosynthetic carbon fixation (Ensminger et al., 2015). Reduced photochemical quenching of absorbed light energy consequently leads to excess energy, which must be safely dissipated to protect the photosynthetic apparatus from photooxidative damage (Niyogi, 2000). Reduced functionality of the photosystems as indicated by a reduced maximum quantum yield of dark-adapted needles (Fv/Fm, see Adams et al., 2013) was only observed in field-grown Douglas-fir after a prolonged summer drought (see Fig. A7.2, July 2010 at both field sites). This indicates that trees were able to efficiently employ photoprotective mechanisms.

In both experiments, I observed stable amounts of stored monoterpenes (Fig. 3.7a, Fig. 4.8a). Monoterpene emissions did not increase in Douglas-fir seedlings in response to drought (Fig. 3.7c), but increased emissions in response to sun hours (a proxy for global radiation) in mature trees suggest an involvement of monoterpene emissions in photoprotection (Fig. 4.8b). Specifically, emissions were highest under hot and dry conditions in field-grown trees (Fig. A7.4b). Stable monoterpene pools thus indicate, that monoterpene biosynthesis must be induced when emission is increased. This is in accordance with the assumption, that monoterpene biosynthesis can be stimulated to mitigate mild stress (Šimpraga et al., 2011; Nogués et al., 2015a; Nogués et al., 2015b). Although other studies reported variation in the composition of monoterpenes in response to abiotic heat stress in Douglas-fir (Joó et al., 2011), I did not observe variation in the composition of monoterpene pools or emissions (Table 3.3).

Sesquiterpene pool sizes were significantly increased in seedlings of the coastal provenances (Fig. 3.7b; sesquiterpenes in mature field-grown trees were not assessed). An increased emission of sesquiterpenes has previously been observed in heat-stressed Douglas-fir seedlings (Joó et al., 2011) and during summer in *Pinus virginiana* (Geron & Arnts, 2010). Sesquiterpenes are hypothesized to enhance stress tolerance by their antioxidant properties or stabilization of
membranes (Palmer-Young et al., 2015). The observed significant TreatmentxProvenance interaction confirms the involvement of sesquiterpenes in drought stress response of coastal Douglas-fir. I did not observe variation in their composition (Table 3.3).

Carotenoid pool sizes in relation to chlorophylls were only non-significantly enhanced in the interior provenances INT and SAL under severe drought (Fig. 3.5c, Fig. 4.6b). High carotenoid-chlorophyll ratios are generally considered to enhance stress tolerance (Peñuelas et al., 1995). The individual carotenoids, which vary in their role to mediate stress (Demmig-Adams et al., 1996), revealed adjustments of the photosynthetic apparatus to drought stress.

The xanthophyll cycle pool size revealed only significant response to severe drought stress in seedlings of the interior provenances, but no correlation with soil water availability in field grown trees (Fig. 3.6a, Fig. 4.7a). This is surprising, since it is the best known NPQ mechanisms and a meta-analysis summarising responses in many species revealed a significance upregulation in response to drought stress (Esteban et al., 2015a). Furthermore, conifers are capable to greatly increase the xanthophyll cycle pool sizes in winter (Adams & Demmig-Adams, 1994; Ensminger et al., 2004). An increased de-epoxidation state of the xanthophyll cycle in response to high light intensities, as observed in field-grown Douglas-fir (Fig. 4.7b), indicates enhanced dissipation of excess energy via heat (Demmig-Adams & Adams, 2006). The enhanced demand for quenching of excess light energy during drought is evident from the increased xanthophyll cycle deepoxidation state in drought-stressed seedlings, which were grown under constant light intensity (Fig. 3.6b).

The pool sizes of β-carotene revealed a ProvenancexSite interaction in field-grown Douglas-fir trees. In coastal provenances, β-carotene content decreased in response to low soil water availability. β-carotene serves two different functions in photoprotection: it protects the reaction center proteins of the photosystems from ROS (Telfer, 2005; Cazzaniga et al., 2016), and it scavenges ROS by chemical interaction (Havaux, 2014). β-carotene oxidation products are often volatile isoprenoids such as β-cyclolocitral and β-ionone, which are involved in intracellular signalling processes (Ramel et al., 2012). We did not investigate these volatile isoprenoid, but due to the important role of β-carotene in the drought response of the supposedly drought-adapted provenance SAL, this might be a promising approach to further elucidate the role of β-carotene in drought stress tolerance. Furthermore, β-carotene occurs in a cis- and trans-isomer
which are hypothesized to convey different functions in photoprotection (Nayak et al., 2002), and a more detailed analysis of photosynthetic pigment data might reveal differences in the regulation of the biosynthesis of these isomers.

My observation of strongly increased sesquiterpene pool sizes in COA indicates an enhanced induction of the cytosolic mevalonate pathway, but only minor induction of the plastidal non-mevalonate pathway under drought (see Fig. 1.1). Furthermore, the β-carotene data suggest an enhanced induction of the β-branch of carotenoid biosynthesis in SAL. These hypotheses have not been confirmed in this thesis and would require further analyses. Furthermore, although all major carotenoids were analysed in field-grown trees, the dataset was not yet analysed.

6.1.2 Isoprenoid-mediated protection from photooxidative stress during senescence-associated downregulation of photosynthesis in Sugar maple

In this subchapter, I very briefly discuss the relevance of the main findings regarding essential isoprenoids in senescing sugar maple leaves. During leaf senescence, most enzymes are degraded to remobilize nitrogen from leaves, and chloroplasts including the photosynthetic apparatus are reorganized (Hörtensteiner, 1999; Juvany et al., 2013). As the photosynthetic capacity decreases due to enzymatic limitations, light absorption is minimized by degradation of chlorophylls to avoid unregulated formation of ROS (Hörtensteiner & Feller, 2002). As chlorophylls are degraded, I observed two isoprenoid-mediated mechanisms which contributed to minimize unregulated energy dissipation: relative increase in xanthophyll cycle pigments and increased amounts of antioxidant tocopherols.

Carotenoid degradation was much delayed compared to chlorophyll degradation, leading to an increased ratio of carotenoids per chlorophyll (Fig. 5.1b-d), which contributes to balancing of oxidative stress in senescing tissue (Munné-Bosch & Peñuelas, 2003; Ougham et al., 2005). Individual carotenoids varied in the rate of degradation, with pigments of the xanthophyll cycle exhibiting a relative increase among carotenoids (Fig. 5.2a). A relative increase of xanthophyll cycle pigments was previously observed during leaf senescence of birch, alder, poplar and hazel and was attributed to enhanced photoprotection (García-Plazaola et al., 2003a). A relatively faster decrease of β-carotene (Fig. 3.2d) might be explained by chemical scavenging of ROS.
Furthermore, I observed increased levels of \(\alpha\)- and \(\delta\)-tocopherols (Fig. 5.1e-g). Since these isoprenoids must have been synthesized \textit{de novo}, it emphasizes the importance of antioxidants during senescence (Munné-Bosch & Alegre, 2002; Juvany \textit{et al.}, 2013).

6.2 Interior Douglas-fir provenances avoid water loss by low stomatal conductance, and consequently show enhanced photoprotection of the photosynthetic apparatus mediated by essential and non-essential isoprenoids

In both experiments, the drought stress experiment with seedlings and the field experiment with field-grown trees, Douglas-fir provenances varied in the regulation of photosynthetic gas exchange in response to drought, suggesting variation in drought response strategies between provenances. Lower stomatal conductance in potted INT seedlings shows a stronger drought avoidance mechanism compared to COA (Fig. 3.3a). Low stomatal conductance is a water-saving mechanism reducing the danger of hydraulic failure and mortality in seedlings (McDowell \textit{et al.}, 2008), and interior seedlings have been shown to survive drought longer compared to coastal provenances (Ferrell & Woodard, 1966). Mature Douglas-fir trees have higher rates of survival compared to seedlings (Moore \textit{et al.}, 2004), likely due to increased bole water reserves, enhanced resistance to xylem cavitation or variation in root morphology (Kavanagh \textit{et al.}, 1999; Phillips \textit{et al.}, 2003). Higher stomatal conductance of SAL compared to coastal provenances in mature trees can be interpreted as higher drought tolerance (Fig. 4.4b).

Provenance-specific variation in photosynthetic gas exchange was accompanied by variation in the composition of chlorophylls and carotenoids of the photosynthetic apparatus, indicating adaptation of the photosynthetic apparatus to different environmental conditions. The interior provenances INT and SAL exhibited generally lower chlorophyll content and higher carotenoid-chlorophyll ratios (Fig. 3.5a,c, Fig. 4.6a,b), suggesting lower uptake of light energy and enhanced photoprotection compared to the coastal provenances (see Peñuelas \textit{et al.} 1995). Furthermore, interior provenances of both experiments exhibited significantly higher \(\beta\)-carotene content compared to coastal provenances (Fig. 3.6c, Fig. 4.6c).

In mature trees, I also observed significant variation in xanthophyll cycle pool sizes (Fig. 4.7a). Interestingly, the highest xanthophyll cycle mediated photoprotection was observed in CON,
which maintained the highest assimilation rates among coastal provenances and revealed fastest growth at the dry field site (Neophytou et al., 2016). This contradicts the assumption, that highest photoprotection should be observed in trees with lowest assimilation rates (Martínez-Ferri et al., 2000), and suggests a positive impact of improved photoprotection on plant productivity, as hypothesized by Murchie & Niyogi (2011). Intraspecific variation in xanthophyll cycle pigments within tree species has so far only been reported in Fagus sylvatica, but could not be linked to drought tolerance (Garcia-Plazaola & Becerril, 2000). In contrast, provenances from Quercus coccifera did vary in drought tolerance, but not xanthophyll cycle pool sizes (Balaguer et al., 2001). Therefore, further studies are needed to confirm, if xanthophyll cycle mediated photoprotection may contribute to drought tolerance.

The experiment with Douglas-fir seedlings furthermore revealed significantly higher neoxanthin content in INT compared to COA (Fig. 3.6d). β-carotene, the xanthophyll cycle pigments and neoxanthin are derived from the β-branch of carotenoid biosynthesis, indicating differences in the regulation of the altered α- and β-branch carotenoid biosynthesis between interior and coastal provenances.

I also showed significant differences in the amounts of stored and emitted volatile isoprenoids (Fig. 3.7, Fig. 4.8). Interestingly, the observed variation in non-essential isoprenoids extended the variation observed in essential isoprenoids. This is likely due to the highly conserved composition of essential isoprenoids associated with the photosynthetic apparatus (Pogson et al., 1998; Morosinotto et al., 2003), and a consequently lower selective pressure on non-essential isoprenoids.

Provenances revealed intraspecific variation in xanthophyll cycle pigments, β-carotene and volatile isoprenoid metabolism, which have been also shown to be adjusted in response to drought (see Chapter 6.1). Constitutive differences in the same suit of photoprotective isoprenoids that have been shown to be adjusted in response to drought or high light suggest that adaptation of provenances to dry habitats may have affected the isoprenoid metabolism. Nevertheless, my study does not provide evidence for a causal link between induced isoprenoid-mediated photoprotective mechanisms and drought stress tolerance. Further studies are needed to confirm, if isoprenoid-mediated photoprotective mechanisms contribute to drought tolerance. To evaluate the importance of photoprotective isoprenoids in drought tolerance of plants, drought
experiments with model organisms with altered isoprenoid biosynthesis such as hampered biosynthesis of individual isoprenoids, or genotypes with altered α-/β-branch biosynthesis of carotenoids (see e.g Dall’Osto et al. 2007a,b, Calandro et al. 2013) should be conducted. For a better understanding, if intraspecific variation in photoprotective isoprenoids contributes to higher productivity and enhanced resilience to drought stress in Douglas-fir, isoprenoid analyses could be linked with precise non-invasive growth measurements of seedlings and trees. Furthermore, associative studies linking DNA polymorphisms or RNA expression levels with isoprenoid analysis could be used to confirm differences in regulation of key enzymes. For example, lycopene ε-cyclase and lycopene β-cyclase could be investigated to confirm the difference in biosynthesis of α- and β-branch carotenoids (Fig. 3.6), or the induction of the cytosolic mevalonate pathway could be quantified to specify the regulatory pathway leading to enhanced sesquiterpene pools in the coastal provenance (Fig. 3.7).

Manipulation of photoprotective mechanisms has been suggested to improve plant photosynthetic efficiency (Murchie & Niyogi, 2011). In the model organisms tobacco, the accelerated adjustment of the photoprotective xanthophyll cycle has recently been shown to enhance plant productivity (Kromdijk et al., 2016). Similarly, selection of tree provenances, which are well protected from photooxidative damage, may lead to increased productivity and enhanced resilience of species to abiotic stress expected under future climate conditions. Therefore, the photoprotective mechanisms that were identified to exhibit intraspecific variation (pool sizes of xanthophyll cycle pigments, β-carotene, mono- and sesquiterpenes) might be valuable traits for the selection of tree provenances, which are able to cope with future climate conditions.

6.3 Essential isoprenoids are indicators of physiological performance and phenological events in remote-sensing applications, because plants adjust the composition of the photosynthetic apparatus in response to environmental conditions

Essential isoprenoids associated with the photosynthetic apparatus are tightly regulated in response to environmental conditions and abiotic and biotic stresses, and also reflect
developmental and seasonal changes in plant physiology (Busch et al., 2009; Esteban et al., 2015a). Individual photosynthetic pigments specifically influence leaf optical properties because of their specific absorption maxima (Ustin et al., 2009). Therefore, photosynthetic pigments are indicators of phenological events in remote-sensing applications such as spectral reflectance measurements and digital image analysis (Yang et al., 2014). Many vegetation indices have been established to represent autumn phenology of forests (Motohka et al., 2010; Mizunuma et al., 2011; Hufkens et al., 2012; Yang et al., 2014). Nevertheless, none of these studies refined the use of vegetation indices to reflect gradual changes in autumn phenology which is important to study the effects of changing climate conditions on forest phenology. The presented work on the leaf level revealed, that vegetation indices based on green and red wavelength regions/ color information are most suitable to represent pigment changes during senescence (Fig. 5.8, Fig. 5.10).

The identification of vegetation indices suitable to represent plant physiological status and phenology on the leaf-level provides the basis for further remote-sensing studies. Further assessments of vegetation indices based on green and red wavelength regions/ color information on the canopy and ecosystem level in combination with pigment analyses and measurements of plant photosynthetic efficiency should be conducted to confirm, that the suggested vegetation indices are suitable to track autumn phenology remotely. Selection of appropriate indices for monitoring of phenology (e.g. Hufkens et al., 2012; Sonnentag et al.2012; Yang et al., 2014) as well as development of improved vegetation indices (e.g. Gamon et al., 2016) will lead to refined monitoring of ecosystem phenology, which is especially important to globally assess plant and ecosystem phenology under future climate conditions.

6.4 Conclusions

Seedlings and mature trees of Douglas-fir revealed adjustments of photoprotective isoprenoids in response to drought. Adjustments in pool sizes and emission of non-essential volatile isoprenoids as well as adjustments in content and composition of essential isoprenoids, mainly β-carotene and the xanthophyll cycle pigments, contributed to photoprotection in drought-stressed Douglas-fir. Provenances, which were adapted to habitats with contrasting water availability as indicated by differences in stomatal regulation, exhibited significant differences in the suit of employed
isoprenoid-mediated photoprotective mechanisms including the xanthophyll cycle pigments, β-carotene and volatile isoprenoids. I conclude that enhanced isoprenoid-mediated photoprotective mechanisms contribute to the drought tolerance of Douglas-fir provenances. In a second experimental approach, studying the influence of senescence-associated changes in essential isoprenoids in Sugar maple leaves on leaf optical properties, I was able to show that isoprenoids are valuable indicators of plant physiological performance when suitable vegetation indices are employed. Therefore, plant isoprenoid metabolism may be a potential trait for selection of provenances that are able to cope with future climate conditions, as well as an indicator for remote-sensing of the plant physiological status.


Dillen SY, de Beeck MO, Hufkens K, Buonanducci M, Phillips NG. 2012. Seasonal patterns of foliar reflectance in relation to photosynthetic capacity and color index in two co-occurring tree species, Quercus rubra and Betula papyrifera. Agricultural and Forest Meteorology 160(0): 60-68.


Garbulsky MF, Peñuelas J, Gamon J, Inoue Y, Filella I. 2011. The photochemical reflectance index (PRI) and the remote sensing of leaf, canopy and ecosystem radiation use


Appendix 1: Experimental conditions and plant material

Climate chamber experiments with Douglas-fir seedlings (Chapter 3)

The study presented in chapter 3 with 2-year-old Douglas-fir seedlings was conducted at the Leibniz Centre for Agricultural Landscape Research in Müncheberg, Germany, from June to September of 2011. Two walk-in environmental chambers (VB 8018, Vötsch Industrietechnik GmbH, Germany), equipped with a mixture of sodium-vapour lamps (NC 1000-00, -01 and -62, Narva, Plauen, Germany) were set to 21 °C temperature during day and night, a relative humidity of 70 %, and light intensity at canopy height was 500 µmol m⁻² s⁻¹ for 16 h per day (6, 8 and 10-11% red light). Seedlings were randomly distributed among both chambers and watered daily with 100 ml 50 % tap water, 50 % distilled water. After more than three months of acclimation under growth conditions, a drought treatment was started July 20th for half of the trees of each provenance in both chambers.

Control seedlings were watered from July 20th (day 0) onwards with 200 ml water. Watering of seedlings of the drought treatment was gradually decreased until August 10th, when watering was withheld until Aug 31st (day 42). On that day, seedlings were rewatered generously, and all seedlings were grown for another two weeks with daily supply of 200 ml water. Daily soil moisture measurements with ECH2O sensors (EC5, Decagon Devices, Inc., Pullman, USA) were used as guidance for consistent watering of control and drought stress plants. Coastal seedlings consistently required about ten percent more water in order to keep soil moisture comparable to interior seedlings.

Photosynthesis measurements and samples for biomass, volatile and essential isoprenoid analysis were taken after three (four) and six weeks of drought treatment and after one day and two weeks after rewatering, respectively. These dates cover mild and severe drought stress and recovery over time. For each measurement and sampling, n=5 plants were randomly chosen.
Field work with 50-year old Douglas-firs at the sites Schluchsee and Wiesloch, Germany (Chapter 4)

The study presented in chapter 4 with 50-year-old field-grown trees of four Douglas-fir provenances was conducted at two field sites in south-western Germany, which are part of an international Douglas-fir (*Pseudotsuga menziesii*) provenance trial established in 1958 (Kenk & Thren, 1984). The field site “Schluchsee” was located in the southern Black Forest (N47°50’31”, E8°6’54”) at an elevation of 1050 m and represented a moderately cool, humid climate with 1345 mm mean annual precipitation and a mean annual temperature of 6.1 °C. The field site “Wiesloch” was located in the Rhine valley (N49°16’41”, E8°34’37”) at an altitude of 105 m and was characterized by warmer and drier climatic conditions with 9.9 °C mean annual temperature and 660 mm mean annual precipitation (Kenk & Ehring, 2004). For 2010 and 2011, climate data were obtained for Schluchsee from a private meteorological station located 6 km away from the site (N47°49’16”, E8°11’08”, elevation 992 m), and for Wiesloch from a weather station operated by Deutscher Wetterdienst (DWD) in Waghäusel-Kirrlach at a distance of 4 km from the site (N49°15’0”, E8°32’24”, elevation 105 m). Climatic and geographical details of the field sites are summarized in Table 4.1. Water availability at both sites was affected by differences in precipitation and soil properties; the loamy soils in Schluchsee generally had a higher water-holding capacity compared to the rather sandy soils in Wiesloch. The forest hydrological water budget model WBS3 was used to calculate daily water availability in terms of usable soil water capacity based on temperature, precipitation, latitude, soil type, plant cover, slope and slope aspect (Keitel *et al*., 2006).

The difference in elevation and mean annual temperature between field sites caused a later start of the growing season in Schluchsee. Therefore, measurements in Wiesloch were carried out prior to the measurements in Schluchsee. Our field work was conducted over two weeks at both sites, each in May and July of 2010 and 2011 (climatic conditions during measurement campaigns are summarized in Table 4.3). Gas exchange and chlorophyll fluorescence were measured simultaneously using a LI-COR 6400 XT (LI-COR Biosciences, Lincoln, NE, USA). To reach the sun-exposed crowns at heights of about 24 to 29 meters, a platform on a hydraulic lift was used. Typically, zero to six measurements were conducted between 10 am and 6 pm per day, depending on the weather conditions and functionality of equipment. At one day at the end of each campaign, needle material of the sun-exposed crown of N=6 trees was sampled pre-
dawn, at noon and in the evening, using shotguns or slingshots. Needles from the previous year were cut from twig and immediately frozen in liquid nitrogen and after transport to the laboratory stored at -80 °C. In addition, in 2011, every two to three days twig samples were taken between 6 and 8 am and 1 and 3 pm using a slingshot to determine pre-dawn and midday twig water potential of N=4 trees. Water potential of a freshly cut two-year-old twig was immediately determined using a pressure chamber (Model 3015G4, Soilmoisture Equipment Corp., Santa Barbara, CA, USA) after Scholander et al. (1965).

Douglas-fir provenances (Chapter 3 and 4)

For the experiments with Douglas-fir, six different provenances were used. In the climate chamber experiment described in Chapter 3, the interior provenance Fehr Lake (INT) was compared to the coastal provenance Snoqualmie (COA). For the field campaigns described in chapter 4, the interior provenance Salmon Arm (SAL) was compared to the three coastal provenances Conrad Creek (CON), Cameron Lake (CAM) and Santiam River (SAN). Mean annual temperature and mean annual precipitation varied among the habitats of the six provenances (Table A1.1).

Table A1.1: Climate conditions at the original provenance habitats as provided by the nurseries (for 1, 2) and after Kenk and Ehring (2004) and overview of geographical origin.

<table>
<thead>
<tr>
<th>Provenance</th>
<th>Elevation</th>
<th>Mean annual precipitation</th>
<th>Mean annual temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Fehr Lake (INT)</td>
<td>800 m</td>
<td>333 mm</td>
<td>5.8 °C</td>
</tr>
<tr>
<td>2 Snoqualmie (COA)</td>
<td>457-610 m</td>
<td>2134 mm</td>
<td>7.9 °C</td>
</tr>
<tr>
<td>3 Salmon Arm (SAL)</td>
<td>650 m</td>
<td>500 mm</td>
<td>7.8 °C</td>
</tr>
<tr>
<td>4 Conrad Creek (CON)</td>
<td>280 m</td>
<td>2300 mm</td>
<td>9.5 °C</td>
</tr>
<tr>
<td>5 Cameron Lake (CAM)</td>
<td>210 m</td>
<td>1475 mm</td>
<td>10.0 °C</td>
</tr>
<tr>
<td>6 Santiam River (SAN)</td>
<td>800 m</td>
<td>1780 mm</td>
<td>9.5 °C</td>
</tr>
</tbody>
</table>
Field site and sampling of Sugar maple trees in Huron Park, Mississauga (Chapter 5)

The leaves used for the study presented in chapter 5 were sampled from three field-grown sugar maple trees (Acer saccharum). The trees were chosen because of the accessibility of sun-exposed branches and close proximity between field site and laboratory. The trees were growing in an open stand at Huron Park, Mississauga (ON), Canada (N43°33’34”, W79°38’11”) and about 5-7 m tall and fully exposed to sunlight (see Fig. Y.3a). Within each canopy, leaves vary in the progress of senescence (see Fig. Y3b). This within canopy variation allowed to sample leaves of various color classes representing various stages of senescence. On the mornings of October 13, 2012 and October 13, 2013, green, yellow, orange and red leaves were simultaneously sampled. As a reference for non-senescent leaves, green summer leaves were taken at noon of July 2, 2013. For each color category, sample size per tree was N = 5-6 for each sampling. All leaves were picked from sun-exposed twigs of the lower part of the canopy at a height between 2 3 m of each tree. Leaves were stored in plastic bags with a damp tissue and kept on ice in the dark to minimize changes in leaf water content and pigments during transport to the nearby laboratory. Upon arrival in the laboratory, digital images were obtained from the leaves for RGB-analysis. Afterwards measurements of chlorophyll fluorescence and P700 absorbance using a Dual-PAM-100 (Walz, Effeltrich, Germany) and measurements of spectral reflectance measurements using a UniSpec (UNI007, PP Systems, Haverhill, MA, USA) were taken simultaneously using a custom-made leaf clip. Following these measurements, the leaves were further processed for pigment analysis. For this purpose major veins were removed from the leaves using razor blades and the remaining leaf tissue was shock-frozen in liquid nitrogen and stored at -80°C.
Appendix 2: Photosynthesis measurements

Gas exchange and chlorophyll fluorescence measurements using the LI-COR 6400XT (Chapter 3 and 4)

For all experiments with Douglas-fir, chlorophyll fluorescence and gas exchange were measured using a LI-COR 6400 XT with an integrated 6400-40 leaf chamber fluorometer (LI-COR Biosciences, Lincoln, NE, USA). For the study with Douglas-fir seedlings described in Chapter 3, current-year needles of the first flush were used. In this experiment, chlorophyll fluorescence measurements could not be analysed due to a technical problem with the leaf chamber fluorometer. For experiments with field-grown Douglas-fir trees, gas exchange and chlorophyll fluorescence of previous-year needles of the sun-exposed crowns were measured (Chapter 4). For both studies, about 10-15 needles of an intact twig were placed into the cuvette to form a flat area. Measurement conditions in the closed cuvette were set to a 400 ml min\(^{-1}\) flow rate, 25 °C block temperature, 35-40 % relative humidity, and 400 ppm CO\(_2\) concentration. Prior to starting the gas exchange measurements, needles were dark-adapted for 25 minutes. Maximal and minimal fluorescence of the dark-acclimated sample (\(F_0\) and \(F_m\)) were then assessed, as well as dark respiration of the needles. Subsequently, gas exchange and chlorophyll fluorescence measurements were stepwise obtained at 400, 0, 500, 1000, 1500 and 2000 µmol photons m\(^{-2}\) s\(^{-1}\) light intensity when a steady state of photosynthetic CO\(_2\) gas exchange was achieved, typically after 5-7 min per step. After the gas exchange was measured, needles were collected and scanned to determine the light exposed needle area using WinSeedle software and scanner (Regents Instruments Inc., Québec, Canada). The rate of photosynthetic gas exchange was expressed per cm\(^2\) surface area exposed to the light. Intrinsic water use efficiency (IWUE) was calculated as the ratio of CO\(_2\) assimilation rate to stomatal conductance (IWUE = \(A/g_s\)). The maximum quantum yield of dark-adapted needles was the ratio of variable chlorophyll fluorescence to maximum chlorophyll fluorescence of dark adapted needles (\(F_v/F_m = (F_m-F_0)/F_m\)), yield was calculated from light-adapted needles as (\(\Phi_{PSII} = (F_m\,'-F_t)/F_m\,'\)), and non-photochemical quenching was NPQ = (\(F_m-F_m\,'\))/\(F_m\,'\), following Maxwell & Johnson (2000).
Chlorophyll fluorescence and P700 absorbance measurements using the Dual-PAM-100 (Chapter 5)

To estimate photosynthetic capacity of senescing autumn leaves (Chapter 5), a Dual-PAM-100 (Walz, Effeltrich, Germany) was used. The energy use at Photosystem II (PSII) was estimated by chlorophyll fluorescence, and energy use at Photosystem I (PSI) was assessed by the redox state of P700, the primary donor of PSI. For simultaneous measurements, a fiberoptic version of the Dual-PAM-100 was used. The fiberoptic was kept at a distance of 5 mm above the leaf surface at an angle of 60° using a custom made leaf clip. Measurements were taken after 25 min of dark-adaptation under low light conditions at room temperature using the internal light source of the Dual-PAM-100.

Minimum chlorophyll fluorescence ($F_0$) of the dark-acclimated sample was assessed as mean of three consecutive measurements, followed by a measurement of maximum fluorescence ($F_m$). Subsequently, leaves were exposed to 1200 µmol photons m$^{-2}$ s$^{-1}$ light intensity for 280 s, and measurements of $F_v$, $F_m'$ and $F_o'$ were taken after 200 s, 240 s and 280 s. The maximum quantum yield of photosystem II (PSII) of dark-adapted leaves was calculated as $F_v/F_m = (F_m - F_o) / F_m$.

The excitation pressure on PSII ($1 - q_P$), as a measure of the redox state of the QA pool was calculated according to Huner et al. (1998) as $1 - q_P = 1 - [(F_m' - F_i) / (F_m' - F_o')]$, yield of PSII ($\Phi_{PSII}$) of light-adapted leaves was calculated as $\Phi_{PSII} = (F_m' - F_i) / F_m'$, non-regulated energy dissipation ($\Phi_{NO}$) was calculated as $\Phi_{NO} = 1 / \{[(F_m - F_m') / F_m'] + 1 + [(F_m' - F_i) / (F_m' - F_o')]* (F_o' / F_i) * [F_m / (F_o - 1)]\}$, and non-photochemical quenching ($\Phi_{NPQ}$) was calculated as $\Phi_{NPQ} = 1 - \Phi_{PSII} - \Phi_{NO}$ (Maxwell & Johnson, 2000; Kramer et al., 2004; Dahal et al., 2014).

Simultaneously, the redox state of P700, the reaction center chlorophyll of photosystem I (PSI), was measured as the difference in transmittance between 875 nm and 830 nm. After dark adaptation, P700 is fully reduced, and the signal $P_o$ is minimal. After a saturation pulse, P700 is fully oxidized and $P_m$ as the maximum signal is determined. P is the steady-state signal of light-adapted leaves. $P_m'$ is determined after a saturation pulse of light-adapted leaves. Yield of PSI ($\Phi_{PSI}$) of light-adapted leaves was calculated as $\Phi_{PSI} = (P_m' - P) / (P_m - P_o)$ non-photochemical quantum yield of PSI due to donor side limitation ($\Phi_{ND}$) was calculated as $\Phi_{ND} = (P - P_o) / (P_m - P_o)$, and non-photochemical quantum yield of PSI due to acceptor side limitation ($\Phi_{NA}$) was calculated as $\Phi_{NA} = (P_m - P_m') / (P_m - P_o)$, with $\Phi_{PSI} + \Phi_{ND} + \Phi_{NA} = 1$ (Klughammer & Schreiber,
2008). Due to low signal, for 12% of yellow leaves, and 34% of orange and red leaves, energy use at PSI could not be calculated.

From $\Phi_{\text{PSII}}$ and $\Phi_{\text{PSI}}$, electron transport rates of PSII and PSI were calculated. The calculation of ETR usually involves a fixed factor of 0.84 standing for the fraction of light absorbed by leaves as well as an equal energy distribution of light energy between PSII and PSI (Schreiber, 2004). Since we need to assume that light absorption of leaves changed during senescence due to decreasing content of photosynthetic pigments (Carter et al., 2000), we calculated relative electron transport rates (Schreiber, 2004). The energy distribution between PSII and PSI, $d_{\text{II}}$ and $d_{\text{I}}$, was calculated from $\Phi_{\text{PSII}}$ and $\Phi_{\text{PSI}}$ measurements of summer leaves as unstressed reference according to Huang (2012). $d_{\text{II}}$ and $d_{\text{I}}$ were calculated from 14 summer leaves using the formula $\Phi_{\text{PSII}} \times d_{\text{II}} = \Phi_{\text{PSI}} \times d_{\text{I}}$ with $d_{\text{II}} + d_{\text{I}} = 1$. Average $d_{\text{II}} = 0.5369 (\pm 0.0077)$ and $d_{\text{I}} = 0.4631 (\pm 0.0077)$. Consequently, relative electron transport rate of PSII ($r_{\text{ETR}_{\text{II}}}$) was calculated as $r_{\text{ETR}_{\text{II}}} = \Phi_{\text{PSII}} \times 0.5369 \times 1200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and relative electron transport rate of PSI was calculated as $r_{\text{ETR}_{\text{I}}} = \Phi_{\text{PSI}} \times 0.4631 \times 1200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. We furthermore calculated the ratio between $r_{\text{ETR}_{\text{I}}}$ and $r_{\text{ETR}_{\text{II}}}$ that was suggested to indicate the induction of relative cyclic electron transport when values are greater than one according to Yamori et al. (2011).
Appendix 3: Assessment of leaf optical properties

Spectral reflectance measurements using the UniSpec-SC spectrometer

Spectral reflectance of senescing autumn leaves (Chapter 5) was measured simultaneously to Dual-PAM-100 measurements, following a protocol from Frechette et al. (2015). Spectral reflectance of leaves illuminated for 4 min was measured during the short breaks following each Dual-PAM-100 measurement, which were taken every 40 seconds. A UniSpec-SC spectrometer (UNI007, PP Systems, Haverhill, MA, USA) connected to a bifurcated fiber optic (UNI400) was held at a distance of 5 mm above the leaf surface at an angle of 90°. Leaf reflectance of wavelengths between 310 and 1130 nm was corrected for the dark current noise of the instrument and measured in relation to the radiance of a white reflectance standard (Spectralon, Labsphere, North Sutton, NH, USA). Spectral reflectance indices representing chlorophyll and anthocyanin content were calculated from an average of three measurements per leaf as followed, with \( R_x \) representing the reflectance at light of \( x \) nm wavelength:

\[
\text{Normalized difference vegetation index} \quad \text{NDVI} = \frac{R_{900} - R_{680}}{R_{900} + R_{680}} \quad \text{Penuelas et al. (1997)} \quad \text{eq. 1}
\]

\[
\text{Photochemical reflectance index} \quad \text{PRI} = \frac{R_{531} - R_{570}}{R_{531} + R_{570}} \quad \text{Gamon et al. (1997)} \quad \text{eq. 2}
\]

\[
\text{Excess green index} \quad \text{ExG}_M = \frac{2 * R_{545-565} - (R_{620-670} + R_{459-479})}{R_{620-670} + R_{459-479}} \quad \text{Hufkens et al. (2012)} \quad \text{eq. 3}
\]

\[
\text{Green red vegetation Index} \quad \text{GRVI} = \frac{R_{500-570} - R_{620-700}}{R_{500-570} + R_{620-700}} \quad \text{Motohka et al. (2010)} \quad \text{eq. 4}
\]

\[
\text{Anthocyanin reflectance index} \quad \text{ARI} = \frac{1}{R_{550}} - \frac{1}{R_{700}} \quad \text{Gitelson et al. (2009)} \quad \text{eq. 5}
\]

\[
\text{Red/green reflectance ratio} \quad \text{RGR} = \frac{R_{600-700}}{R_{500-600}} \quad \text{Gamon and Surfus (1999)} \quad \text{eq. 6}
\]

These indices were chosen based on their suitability to respond to changing leaf color as well as the wavelength regions they use. Due to the simultaneous measurements of spectral reflectance
and chlorophyll fluorescence, the Dual-PAM-100 fluorescence measuring light had an unwanted influence on the spectral reflectance spectra. Therefore, spectral reflectance measurements in the wavelength region from 710-895 nm were excluded from further analysis. In future measurements, I would recommend to measure the spectral reflectance of dark-adapted leaves, pursue the Dual-PAM-100 measurement protocol, which illuminates the leave, and measure spectral reflectance of the illuminated leaf immediately after.

Digital image analysis

For color analysis of sugar maple leaves in Chapter 5, photographs of all sampled leaves were taken on a grey cardboard background under natural light conditions. A digital camera (Canon Powershot SX 150 IS, Canon Inc., Tokyo, Japan) was used and images afterwards processed using the image processing program ImageJ (Abramoff, 2004). In the center of every leaf, an oval region of interest (ROI) was defined and its red, blue and green color channel information was extracted (digital numbers: R, G, B on a scale of 0-255). Background color information was used to correct for minor variation in light conditions. The chromatic coordinates representing the strength of each color were calculated as the ratio of each digital number to the sum of all digital numbers:

Green chromatic coordinate \[ g_{CC} = \frac{G}{R + G + B} \] Gillespie et al. (1987) eq. 7

Blue chromatic coordinate \[ b_{CC} = \frac{B}{R + G + B} \] Gillespie et al. (1987) eq. 8

Red chromatic coordinate \[ r_{CC} = \frac{R}{R + G + B} \] Gillespie et al. (1987) eq. 9

Hue was calculated according to the Preucil color circle (Preucil, 1953):

\begin{align*}
\text{Hue} & \quad \text{if } R \geq G \geq B: \quad \text{Hue}_{\text{Red-Yellow}} = 60^\circ \times (G - B) \times (R - B)^{-1} \quad \text{Gunther (2012)} \quad \text{eq. 10-15} \\
& \quad \text{if } G \geq R \geq B: \quad \text{Hue}_{\text{Yellow,Green}} = 60^\circ \times (2 - (R - B) \times (G - B)^{-1}) \\
& \quad \text{if } G \geq B \geq R: \quad \text{Hue}_{\text{Green-Cyan}} = 60^\circ \times (2 + (B - R) \times (G - R)^{-1}) \\
& \quad \text{if } B \geq G \geq R: \quad \text{Hue}_{\text{Cyan-Blue}} = 60^\circ \times (4 - (G - R) \times (B - R)^{-1}) \\
& \quad \text{if } B \geq R \geq G: \quad \text{Hue}_{\text{Blue-Magenta}} = 60^\circ \times (4 - (R - G) \times (B - G)^{-1}) \\
& \quad \text{if } R \geq B \geq G: \quad \text{Hue}_{\text{Magenta-Red}} = 60^\circ \times (6 - (B - G) \times (R - G)^{-1})
\end{align*}
Digital image indices were calculated from the color channel information as:

<table>
<thead>
<tr>
<th>Index Type</th>
<th>Formula</th>
<th>Reference</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excess green index</td>
<td>$\text{ExG} = 2 \times G - (R + B)$</td>
<td>Richardson et al. (2007)</td>
<td>eq. 16</td>
</tr>
<tr>
<td>Normalized difference index</td>
<td>$\text{NDI} = \frac{R - G}{R + G}$</td>
<td>Pérez et al. (2000)</td>
<td>eq. 17</td>
</tr>
</tbody>
</table>
Appendix 4: Biochemical measurement protocols

HPLC-analysis of essential isoprenoids

When I started my PhD project, I developed new methods for extraction and analysis of essential isoprenoids using a methanol-based reversed-phase high pressure liquid chromatography (HPLC) method. This initial method allowed the analysis of chlorophylls, carotenoids and tocopherols within one hour per sample and was used for the analysis of all my samples. In the meantime I have been able to further improve the previous HPLC-method to enhance accuracy and shorten the runtime to 30 minutes. This method is presented in Chapter 2.

The extraction of essential isoprenoids was the same for both HPLC-methods that are used to subsequently analyse extracted essential isoprenoids. Essential isoprenoids were extracted from approximately 50 mg of frozen and finely ground leaf material using 700 µl 98% methanol buffered with 0.5 M ammonium acetate following a modified protocol from Ensminger et al. (2004) combined with a buffer suggested by Jeffrey et al. (1997). After 2 h incubation at 4 °C under inversion (900 rpm) in amber microtubes, the supernatant was transferred to a new amber microtube and the pellet was washed twice using 650 µl pure methanol. All supernatants were pooled, centrifuged and the supernatant was filtered prior to the HPLC analysis.

An Agilent HPLC System (Böblingen, Germany) consisting of a quaternary pump (model 1260), autosampler (model 1260, cooling samples to 4 °C), column oven (model 1260, warming column to 25 °C) and photodiode array detector (model 1290, recording absorption at 290 nm, 450 nm and 656 nm wavelength) was used for reverse-phase chromatography using a C$_{30}$-column (5 µm, 250*4.6 mm; YMC Inc., Wilmington, NC, USA). Three solvents were used, solvent A was 100 % methanol, solvent B was 100 % ultrapure water buffered with 20 mM ammonium acetate and solvent C was 100% methyl-tert-butyl-ether).

To obtain a gradient of decreasing polarity for the initial method, the initial solvent composition for each run was 64 % solvent A, 36 % solvent B and 0 % solvent C (Fig. A1b). Solvent B was gradually replaced by solvent A for 15min, to 95.2 % A and 4.8 % B. During the next 3 min, Solvent C was gradually introduced up to 5 %, while solvent B decreased to its minimum of 3 %. Subsequently, the concentration of solvent B was unchanged, while solvent A was replaced by solvent C to a concentration of 59.3 % A, 3 % B, 37.7 % C at minute 34 and a maximum of
15.7 % A, 3 % B, 81.3 % C at minute 39, which stayed constant for 3 min. After this time, all essential isoprenoids have eluted, and the column was reconditioned by maximum amounts of 97 % solvent A from minute 43 to 45, followed from the initial solvent concentration of 64 % solvent A, 36 % solvent B and 0 % solvent C for 15 minutes. The run ended after 60 min. During the run, the backpressure varied between initial 195 bar to a minimum of about 80 bar.

Pigment separation and quantification is the same between both methods (Fig. A1).

Fig. A1.1: Chromatograms of pigment extracts from white pine, Pinus strobus (A, C) and solvent gradients of my initial and improved HPLC method (B,D). Solvent A: 100% Methanol; Solvent B: 100% water buffered with 20 mM ammonium acetate to pH 6; Solvent C: 100% tert-methyl-butyl-ether.

This method was later on improved, by omitting the initial 15 to 20 minutes and minor adjustments to the original solvent concentration. A major improvement was the constant use of solvent B, which minimizes the necessary reconditioning time of the column. The improved method, as described in chapter 2, starts with an initial solvent concentration of 92 % solvent A, 3 % solvent B and 5 % solvent C for a total of 3 min (Fig. A1d). Solvent A was gradually replaced by solvent C, with the concentration of solvent C at 17 min being 33.6 %, at 22 min being 81.3 % and from 23 to 27 min at a maximum of 94 %. During the next minute, the
concentration of solvent C was reduced to the initial concentration of 5 % and the run had ended after 35 min, which provided sufficient time to recondition the column as indicated by stabilizing backpressure of about 100 bar.

Both methods yielded similar relative retention times (Fig. A1.1a,c). All chromatograms were analysed using ChemStation B.04.03 (Agilent Technologies, Böblingen, Germany). Peaks were identified and quantified using commercially available pigment standards. Chlorophyll a and b, β-carotene, α-tocopherol and δ-tocopherol standards were obtained from Sigma Aldrich (Oakville, ON, Canada). Antheraxanthin, α-carotene, β-cryptoxanthin, lutein, lycopene, neoxanthin, violaxanthin and zeaxanthin were obtained from DHI Lab products (Hørsholm, Denmark).

Spectrophotometric determination of anthocyanins

Anthocyanin content of sugar maple leaves as shown in Chapter 5 was spectrophotometrically analysed following a protocol from Esteban et al. (2008). Approximately 25 mg of the homogenized frozen sample were suspended in 1 ml of 3 M HCl:H2O:MeOH (1:3:16 v:v:v) and incubated for 2 hours at 4 °C and 900 rpm. Subsequently, the extract was centrifuged for 5 minutes at 30,000 RCF and 4 °C. The absorbance of the supernatant was determined at 524 nm and 653 nm. Anthocyanin concentration was calculated using a molar extinction coefficient of 33,000 M⁻¹ cm⁻¹ (Gould et al., 2000) and corrected for pheophytins using the formula A₅₂₄ – 0.24 A₆₅₃ (Murray et al., 1994).
Appendix 5: Statistics

All statistical tests were performed using R 3.0.3 (R Development Core Team, 2010).

ANOVA

In chapter 4, a two-way-ANOVA was used to estimate the effect of site (environment effect) and provenance (genotype effect) and the interaction thereof on physiological parameters (function \texttt{aov}). Since we included all sampling time points in this initial analysis, time was used as random effect. Homogeneity of variance and normality of distribution were tested by Levene’s test and Shapiro-Wilk-Test, respectively (function \texttt{levene} from the library \texttt{car} and \texttt{shapiro.test}). Independence of observations was implemented by the experimental design. Only for volatile isoprenoid pool data, the normality of distribution was not given, and data were rank-transformed to normality using the function \texttt{rntransform} of the package \texttt{GenABEL}). To allow for PostHoc determination of differences between provenances across field sites, a linear-mixed effect model was applied (see below). Additionally, for data shown in the supplemental, the differences between provenances within sites and at each sampling time point was determined by a separate one-way ANOVA (function \texttt{aov}), followed by Tukey’s post hoc test (function \texttt{TukeyHSD}).

Kruskal-Wallis

In chapter 3, the parametric Kruskal-Wallis-rank-sum-test (function \texttt{kruskal.test}) was used to compare differences between provenances and treatments per sampling day, followed by a Tukey-type pairwise comparison using the function \texttt{nparcomp} (package \texttt{nparcomp}).

In chapter 4, the Kruskal-Wallis-rank-sum-test was used to estimate inter-annual and site-specific differences in bud development.
Linear mixed-effect models

In chapter 3, a linear mixed-effect model using Provenance and Treatment as fixed factors and sampling day as random factor was used to evaluate if provenance or treatment specific differences occurred during the drought stress (function lmer, package lme4, Bates et al. 2013). Models using Provenance, Treatment and both with and without the interaction were compared to a null model considering only an intercept and sampling day as random factor. Based on lowest Akaike Information Criterion (AIC; Akaike 1973) the best-fit model was chosen. Significance of the fixed factors was assessed by pairwise comparison of the best-fit model to the best-fit model minus one of each of its fixed effects (function anova, Zuur 2009).

In chapter 4, a linear mixed-effect model (Provenance x Site) was used to estimate differences between provenances across field sites (function lmer, package lme4, Bates et al. 2013). As a Posthoc evaluation, least-squares means of provenances were determined using the function lsmeans (package lsmeans, Lenth 2014) for all physiological parameters where Provenance was significant. Pairwise differences between provenances were estimated and the significance of the contrasts was assessed using Tukey's multiple comparison test (Fig. 2, 3).

In chapter 5, a linear mixed-effect model was used to estimate the average for sugar maple leaf classes to avoid pseudoreplication due to sampling of several leaves per tree in two years. The function lmer from the package lme4 (Bates et al., 2013) was used to calculate mean and standard error of the fixed model parameters Color using the maximum-likelihood method. Tree and Year of measurements were treated as random factors. For spectral reflectance measurements, which were only conducted in 2013, Tree was used as random factor. Significant differences between leaf color classes were estimated calculating the least squares means between leaf color classes using the function lsmeans from the package lsmeans (Lenth & Herve, 2014) followed by Tukey's multiple comparison test using the function cld from the package multcompView (Graves, 2012).

Nonlinear least square regression

In chapter 5, nonlinear least square regression was used to estimate the relationship between vegetation indices and pigment content and rETR_{II}, respectively. The function wrapnls from the
package *nlmrt* was used to perform linear, logarithmic and exponential model fits for all vegetation indices. The best fitting model was chosen based on highest pseudo-$R^2$, which was estimated as $R^2 = 1 - \text{(sum of squared residuals / sum of squared deviations)}$ according to Eisenhauer (2003).

**Spearman’s Rank correlation**

In chapter 4, the correlation between the three environmental factors total available soil water (*TAW*), sunshine duration on the day of measurement (*Sun*), and mean daily temperature on the day of measurement (*Temperature*) with every physiological parameter, was estimated using Spearman's rank correlation coefficient (function *cor*, method *spearman*).
Appendix 6: Supplemental data for chapter 2

Chapter title: Fast detection of leaf pigments and isoprenoids for ecophysiological studies and to validate leaf optical properties for plant phenotyping and remote-sensing

Figure A6.1: Comparison of HPLC chromatograms of photosynthetic pigments and isoprenoids from several trees and crop species recorded at 450 nm. (A) red maple (Acer rubrum), (B) white oak (Quercus alba), (C) white pine (Pinus strobus), (D) wheat (Triticum aestivum), (E) corn (Zea mays), and (F) soy (Glycine max). αCar, α carotene; βCar, β carotene; cis βCar, cis β carotene; Chl a, chlorophyll a, Chl b, chlorophyll b; Lut, lutein; LutEpox, lutein epoxide; Neo, Neoxanthin; Vio, violaxanthin, Zea, zeaxanthin.
Figure A2.2: HPLC chromatograms of photosynthetic pigments and isoprenoids from various species and tissues recorded at 450 nm. (A) the unicellular green algae *Chlorella variabilis*, (B) yellow-orange stems of the parasitic plant *Cuscuta gronovii*, (C) red tomato fruit (*Solanum lycopersicum*) and (D) orange root of carrot (*Daucus carota*). αCar, α carotene; βCar, β carotene; cis βCar, cis β carotene; Chl a, chlorophyll a, Chl b, chlorophyll b; βCry, β cryptoxanthin; Lut, lutein; Neo, Neoxanthin; Vio, violaxanthin, Zea, zeaxanthin.
Figure A6.3: The yield of isoprenoids sorted by decreasing polarity using our methanolic extraction in comparison to a standard acetonic extraction. Data represent mean of variation between N=12 Douglas-fir (*Pseudotsuga menziesii*) samples extracted using 100% acetone and 98% methanol, respectively. Lutein might be underestimated in acetonic extractions due to an insufficient baseline separation from Chl b. αCar, α carotene; βCar, β carotene; Ant, Antheraxanthin; Chl a, chlorophyll a, Chl b, chlorophyll b; Lut, Lutein; Neo, Neoxanthin; Vio, violaxanthin, Zea, zeaxanthin.
Table A6.1: Plant chlorophylls, carotenoids and tocopherols and their respective retention times, spectral characteristics in the described solvent system, wavelength of detection, limit of detection, and limit of quantification using the described HPLC-method.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time (min)</th>
<th>k</th>
<th>α</th>
<th>CV (%)</th>
<th>Spectral characteristics (λ_{max}; nm)</th>
<th>Detection at (nm)</th>
<th>LOD (ng/ml)</th>
<th>LOQ (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>δ-Tocopherol</td>
<td>10.4</td>
<td>2.47</td>
<td>2.47</td>
<td>ND</td>
<td>297.5</td>
<td>290</td>
<td>0.45</td>
<td>1.52</td>
</tr>
<tr>
<td>Violaxanthin</td>
<td>11.1</td>
<td>2.70</td>
<td>1.09</td>
<td>2.03</td>
<td>415.0, 439.0, 468.5</td>
<td>450</td>
<td>0.30</td>
<td>0.99</td>
</tr>
<tr>
<td>Neoxanthin</td>
<td>11.8</td>
<td>2.93</td>
<td>0.94</td>
<td>2.40</td>
<td>412.5, 436.0, 464.0</td>
<td>450</td>
<td>0.31</td>
<td>1.03</td>
</tr>
<tr>
<td>Lutein epoxide</td>
<td>12.3</td>
<td>3.10</td>
<td>1.06</td>
<td>ND</td>
<td>415.0, 439.5, 468.0</td>
<td>450</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>12.4</td>
<td>3.13</td>
<td>1.16</td>
<td>1.15</td>
<td>292.5</td>
<td>290</td>
<td>1.49</td>
<td>4.97</td>
</tr>
<tr>
<td>Antheraxanthin</td>
<td>14.5</td>
<td>3.83</td>
<td>1.24</td>
<td>1.84</td>
<td>(416.5), 439.0, 466.0</td>
<td>450</td>
<td>0.25</td>
<td>0.83</td>
</tr>
<tr>
<td>Chlorophyll b</td>
<td>15.8</td>
<td>4.27</td>
<td>1.11</td>
<td>1.69</td>
<td>466.0, 649.5</td>
<td>656</td>
<td>1.51</td>
<td>5.04</td>
</tr>
<tr>
<td>Lutein</td>
<td>16.2</td>
<td>4.40</td>
<td>1.03</td>
<td>1.81</td>
<td>(422.0), 444.5, 472.5</td>
<td>450</td>
<td>0.29</td>
<td>0.95</td>
</tr>
<tr>
<td>Zeaxanthin</td>
<td>17.9</td>
<td>4.97</td>
<td>1.13</td>
<td>0.98</td>
<td>(425.0, 450.5, 477.5)</td>
<td>450</td>
<td>0.28</td>
<td>0.92</td>
</tr>
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<td>Chlorophyll a</td>
<td>19.4</td>
<td>5.47</td>
<td>1.10</td>
<td>1.74</td>
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<td>656</td>
<td>0.74</td>
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<tr>
<td>β-cryptoxanthin</td>
<td>22.3</td>
<td>6.43</td>
<td>1.18</td>
<td>ND</td>
<td>(427.0), 451.0, 476.5</td>
<td>450</td>
<td>2.97</td>
<td>9.89</td>
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<tr>
<td>α-carotene</td>
<td>24.5</td>
<td>7.17</td>
<td>1.11</td>
<td>4.91</td>
<td>416.5, 446.0, 473.5</td>
<td>450</td>
<td>0.80</td>
<td>2.66</td>
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<tr>
<td>β-carotene</td>
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<td>7.23</td>
<td>1.01</td>
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<td>450</td>
<td>1.12</td>
<td>3.73</td>
</tr>
<tr>
<td>cis-β-carotene</td>
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<td>7.33</td>
<td>1.01</td>
<td>3.96</td>
<td>424.0, 426.0, 473.5</td>
<td>450</td>
<td>1.12</td>
<td>3.73</td>
</tr>
<tr>
<td>Lycopene</td>
<td>28.1</td>
<td>8.37</td>
<td>1.16</td>
<td>ND</td>
<td>424.0, 446.5, 472.0</td>
<td>450</td>
<td>0.87</td>
<td>2.91</td>
</tr>
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</table>

Parentheses indicate a shoulder. LOD: Limit of detection; LOQ: Limit of quantification; CV: coefficient of variation, k: retention factor, α: separation factor; ND: not determined.
Appendix 7: Supplemental data to chapter 4

Chapter title: Variation in short-term and long-term responses of photosynthesis and isoprenoid-mediated photoprotection to soil water availability in four Douglas-fir provenances

Table A7.1: Bud development of Douglas-fir provenances revealing differences in phenology during spring measurement campaigns. Bud development of four Douglas-fir provenances at sampling day in May differed significantly between field sites in 2010 (p<0.001) and between years 2010 and 2011 (p<0.001). Provenances only differ in May 2010 in Wiesloch (p=0.004). Bud development was categorized according to Bailey and Harrington (2006) as mean of n=6-8 sampled twigs (±standard error) with 0 = dormant winter bud, 0.5 = slightly swollen and elongated bud with lighter tip, 1 = swollen buds of overall lighter colour with green tips, 1.5 = extremely swollen buds with visible green tissue and 2 = fully ruptured buds with protruding needles. Kruskal-Wallis analysis of variance was used to reveal significant differences between samples.

<table>
<thead>
<tr>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Salmon Arm</td>
<td>0.88±0.13</td>
<td>2.00±0.00</td>
<td>1.81±0.19</td>
<td>2.00±0.00</td>
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<tr>
<td>Conrad Creek</td>
<td>0.69±0.09</td>
<td>2.00±0.00</td>
<td>0.69±0.09</td>
<td>1.88±0.08</td>
</tr>
<tr>
<td>Cameron Lake</td>
<td>0.75±0.09</td>
<td>1.94±0.06</td>
<td>1.63±0.21</td>
<td>1.94±0.06</td>
</tr>
<tr>
<td>Santiam River</td>
<td>0.69±0.09</td>
<td>1.94±0.06</td>
<td>1.19±0.21</td>
<td>2.00±0.00</td>
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</tbody>
</table>
Table A7.2; p-values of Pearson-Correlation as shown in Table 4.4 as well as adjusted p-values using Posthoc correlation analyses after Benjamini and Hochberg (1995) to correct for multiple hypothesis testing. A = assimilation rate, g_s = stomatal conductance, IWUE = Intrinsic water-use efficiency, R_d = dark respiration, Δ^{13}C_WSOM = discrimination against $^{13}$C in water soluble organic matter, F_v/F_m = maximum quantum yield of dark-adapted needles, Φ_{PSII} = yield, NPQ = non-photochemical quenching, Chl a+b = total chlorophyll per fresh weight, Carotenoids = total carotenoids per total chlorophyll, VAZ = xanthophyll cycle pigments per total chlorophyll, DEPS= de-epoxidation status of the xanthophyll cycle pigments, monoterpenes = total stored volatile isoprenoids per dry weight, emitted monoterpenes = total monoterpene emissions.

<table>
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<tr>
<th>Parameter</th>
<th>p-values Pearson-Correlation</th>
<th>Adjusted p-values Pearson-Correlation</th>
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</thead>
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<td>TAW</td>
<td>Temperature</td>
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<td>A</td>
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<td>0.000000</td>
</tr>
<tr>
<td>g_s</td>
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<tr>
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<tr>
<td>R</td>
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<td>Δ^{13}C</td>
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<td>F_v/F_m</td>
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<td>Φ_{PSII}</td>
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</tr>
<tr>
<td>NPQ</td>
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</tr>
<tr>
<td>Chl a+b</td>
<td>0.000036</td>
<td>0.237162</td>
</tr>
<tr>
<td>Carotenoids</td>
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<td>0.546930</td>
</tr>
<tr>
<td>β-carotene</td>
<td>0.000000</td>
<td>0.000494</td>
</tr>
<tr>
<td>VAZ</td>
<td>0.351430</td>
<td>0.000016</td>
</tr>
<tr>
<td>DEPS</td>
<td>0.007566</td>
<td>0.000000</td>
</tr>
<tr>
<td>Monoterpenes</td>
<td>0.718067</td>
<td>0.819992</td>
</tr>
<tr>
<td>Emitted monoterpenes</td>
<td>0.060149</td>
<td>0.454295</td>
</tr>
</tbody>
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Fig. A7.1: Photosynthetic gas exchange parameters of four Douglas-fir provenances. A) assimilation rates (A), B) stomatal conductance (g_s), C) intrinsic water-use efficiency (IWUE), D) carbon stable isotope discrimination ($\Delta^{13}$C_WSOM), and E) dark respiration rate ($R_d$) were measured in May and July of 2010 and 2011 at two field sites, Schluchsee and Wiesloch. Data of A, g_s, IWUE and $R_d$ show means of n = 5-6 measurements (±SE) at a light intensity of 1000 µmol m$^{-2}$ s$^{-1}$, data of $\Delta^{13}$C_WSOM was obtained from n = 5-6 samples of previous year needles (±SE). Significant differences between provenances per campaign (p < 0.05 using one-way ANOVA followed by Tukey’s HSD test) are indicated by different letters. Blue and yellow bars above figure show average total available soil water (TAW) and average sunshine duration (Sun) during measurement campaigns, respectively.
Fig. A7.2: Chlorophyll fluorescence parameters of four Douglas-fir provenances. A) maximum quantum yield of dark-adapted needles ($F_v/F_m$), B) effective quantum yield of light-adapted needles ($\phi_{PSII}$), and C) non-photochemical quenching (NPQ) were measured in May and July of 2010 and 2011 at two field sites, Schluchsee and Wiesloch. Data of show means of $n = 5$-6 measurements ($\pm SE$) at a light intensity of 1000 µmol m$^{-2}$ s$^{-1}$. Significant differences between provenances per campaign ($p < 0.05$ using one-way ANOVA followed by Tukey’s HSD test) are indicated by different letters. Blue and yellow bars above figure show average total available soil water (TAW) and average sunshine duration (Sun) during measurement campaigns, respectively.
Fig. A7.3: Photosynthetic pigments of four Douglas-fir provenances. A) total chlorophyll a and b per fresh weight (chlorophylls FW$^{-1}$), B) chlorophyll a/b ratio (Chl a Chl b$^{-1}$), C) carotenoids per total chlorophyll (Carotenoids Chl$^{-1}$) were measured in May and July of 2010 and 2011 at two field sites, Schluchsee and Wiesloch. Data show means of n = 5-6 samples of previous year needles (±SE). Significant differences between provenances per campaign (p < 0.05 using one-way ANOVA followed by Tukey’s HSD test) are indicated by different letters. Blue and yellow bars above figure show average total available soil water (TAW) and average sunshine duration (Sun) during measurement campaigns, respectively.
Fig. A7.4: Photosynthetic pigments of four Douglas-fir provenances. A) \( \beta \)-carotene per total chlorophyll (\( \beta \)-carotene Chl\(^{-1} \)), B) xanthophyll cycle pigments per total chlorophyll (VAZ Chl\(^{-1} \)), and C) de-epoxidation status of the xanthophyll cycle pigments (DEPS) were measured in May and July of 2010 and 2011 at two field sites, Schluchsee and Wiesloch. Data show means of \( n = 5-6 \) samples of previous year needles (±SE). Significant differences between provenances per campaign (\( p < 0.05 \) using one-way ANOVA followed by Tukey’s HSD test) are indicated by different letters. Blue and yellow bars above figure show average total available soil water (TAW) and average sunshine duration (Sun) during measurement campaigns, respectively.
Fig. A7.5: Monoterpene pools and emission of four Douglas-fir provenances. A) monoterpene pool sizes (monoterpene DW⁻¹), and B) monoterpenes emitted per needle area (monoterpene emissions) were measured in May and July of 2010 and 2011 at two field sites, Schluchsee and Wiesloch. Isoprenoid pool sizes were measured. Volatile isoprenoid pools were measured in n = 5-6 samples of previous year needles (±SE). Volatile isoprenoid emission was measured at 30 °C and 1200 µmol m⁻² s⁻¹ light intensity for n = 5-6 previous year needles (±SE). Significant differences (p < 0.05 using one-way ANOVA followed by Tukey’s HSD test) are indicated by different letters. Upper (blue) and lower (yellow) bars above figure show average total available soil water (TAW) and average sunshine duration (Sun) during measurement campaigns, respectively.
Appendix 8: Supplemental data to chapter 5

Chapter title: Relationship between leaf optical properties, chlorophyll fluorescence and pigment changes in senescing *Acer saccharum* leaves

Fig. A8.1: Spectral reflectance spectra of light-adapted (a) and dark-adapted (b) green sugar maple leaves sampled in summer and green, yellow, orange and red leaves sampled in autumn. Data show means of n=15-18 measurements of dark-adapted leaves (5-6 leaves sampled from 3 trees). Grey background indicates wavelength regions in which reflectance was affected by the Dual-PAM-100 measuring light (710-785 nm for fluorescence measuring light, 765-910 nm for P700 measuring light).
Table A8.1: Comparison of pseudo-$R^2$ values calculated for linear, exponential and logarithmic relationship between vegetation indices and pigment content and rel. ETR(II), respectively, as shown in Fig. 5.8 and Fig. 5.10. Regression analysis was carried out by non-linear least square fitting using the function \textit{wrapnls} (package \textit{nlmrt} in R, R Development Core Team 2010) and estimating pseudo-$R^2$ as $R^2 = 1 - (\text{sum of squared residuals} / \text{sum of squared deviations})$ (Eisenhauer, 2003). Best fitting models are bolded, NA characterised models which could not be calculated.

<table>
<thead>
<tr>
<th>Pigment content</th>
<th>Rel. ETR(II)</th>
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<tbody>
<tr>
<td></td>
<td>linear</td>
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<tr>
<td>Fig. 5.8</td>
<td></td>
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<tr>
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<tr>
<td>ExG_M</td>
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<td>PRI</td>
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<td>GRVI</td>
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<td>ARI</td>
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<tr>
<td>RGR</td>
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<tr>
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<tr>
<td>g_CC</td>
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<td>NDI</td>
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<td>ExG</td>
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