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Sensitivity of photosynthesis and stomatal conductance in mature beech (*Fagus sylvatica* L.) to ambient or experimentally enhanced ozone regimes

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Part of the results and data of this doctoral thesis have previously been published. Publications included in this thesis are marked by * (see Appendix A).

* **Löw M**, Herbinger K, Nunn AJ, Häberle K-H, Leuchner M, Heerdt C, Werner H, Wipfler P, Pretzsch H, Tausz M, Matyssek R (2006) "Extraordinary drought of 2003 overrules ozone impact on adult beech trees (*Fagus sylvatica*)". Trees 20: 539-548

* **Löw M**, Häberle K-H, Warren C, Matyssek R "O_3 flux-related responsiveness of photosynthesis, respiration and stomatal conductance of adult *Fagus sylvatica* to experimentally enhanced free-air O_3 exposure". Plant Biology (in press)

* Warren CR, **Löw M**, Matyssek R, Tausz M (2007) "Internal conductance to CO_2 transfer of adult *Fagus sylvatica*: variation between sun and shade leaves and due to free-air ozone fumigation". Environmental and Experimental Botany 59: 130-138

Blumenröther MC, **Löw M**, Matyssek R, Oßwald W "Flux-based response of sucrose and starch in leaves of adult beech trees (*Fagus sylvatica* L.) under chronic free-air O_3 fumigation". Plant Biology (in press)

Deckmyn G, Op De Beeck M, **Löw M**, Then C, Verbeeck H, Wipfler P, Ceulemans R "Simulating ozone effects on adult beech trees through simulation of defense, damage and repair costs: implementation of the CASIROZ ozone model in the ANAFORE forest model". Plant Biology (in press)

Herbinger K, Then C, **Löw M**, Haberer K, Alexou M, Remele K, Rennenberg H, Grill D, Matyssek R, Wieser G, Tausz M "Gas exchange and antioxidative compounds in young beech trees under free-air ozone exposure and comparisons to adult trees". Plant Biology (in press)

Herbinger K, Then C, **Löw M**, Haberer K, Alexous M, Koch N, Remele K, Heerdt C, Grill D, Rennenberg H (2005) "Tree age dependence and within-canopy variation of leaf gas exchange and antioxidative defence in *Fagus sylvatica* under experimental free-air ozone exposure". Environmental Pollution 137: 476-482

Nunn AJ, Kozovits AR, Reiter IM, Heerdt C, Leuchner M, Lütz C, Liu X, **Löw M**, Winkler JB, Grams TEE, Matyssek R (2005) "Comparison of ozone uptake and sensitivity between a phytotron study with young beech and a field experiment with adult beech (*Fagus sylvatica*)". Environmental Pollution 137: 494-506

Matyssek R, Wieser G, Nunn A, **Löw M**, Then C, Herbinger K, Blumenröther MC, Jehnes S, Reiter I, Heerdt C, Koch N, Häberle K-H, Haberer K, Werner H, Tausz M, Fabian P, Rennenberg H, Grill D, Oßwald W (2005) "How sensitive are forest trees to ozone - new research on an old issue". In: Omasa K, Nouchi I, De Kok L (eds) Plant Responses to Air Pollution and Global Change. Springer Verlag, Tokyo

Matyssek R, Le Thiec D, **Löw M**, Dizengremel P, Nunn AJ, Häberle K-H (2006) "Drought stress in the presence of O_3 impact on forest trees". Plant Biology 8: 11-17

Op de Beeck M, **Löw M**, Verbeeck H, Deckmyn G "Suitability of a combined stomatal conductance and photosynthesis model for calculation of leaf-level ozone fluxes". Plant Biology (in press)

Table of contents

Zusammenfassung

Über die Wirkungen tropospherischer Ozonkonzentrationen (O_3) auf Altbäume unter natürlichen Standortsbedingungen in Mitteleuropa ist bisher nur wenig bekannt. Chronisch erhöhte, tropospherische O_3-Belastung kann biologische Prozesse auf Zell-, Blatt-, Baum- und Bestandsebene verändern und letztendlich zu Wachstumseinbußen führen. Zentrale, experimentell zu klärende Fragen der vorliegenden Studie waren a) sind adulte Buchen durch erhöhte O_3-Konzentrationen an einem Waldstandort gefährdet? b) werden O_3-Wirkungen durch die Witterung modifiziert? c) werden Buchen aufgrund von chronischen O_3-Konzentrationen anfälliger gegenüber weiterem Umweltstress, insbesondere Trockenheit? d) ist die aufgenommene O_3-Dosis maßgeblich für O_3-Effekte? und e) haben O_3-induzierte Blattschäden eine größere Wirkung auf die Photosynthese als aufgrund der Größe der sichtbaren Schäden erwartet werden kann?

Diese Studie besteht aus zwei Teilen. Im ersten Teil wird die Interaktion von Trockenheit mit O_3 sowie der Zusammenhang zwischen O_3-Dosis und O_3-effekten untersucht. Die wesentlichen Hypothesen sind:

- Die von den Blättern aufgenommene O_3-Dosis ist das bessere Maß für die Abschätzung des Gefährdungspotentials durch O_3 als die externe O_3-Konzentration
- Die Wirkung der Trockenheit wird durch O_3 verstärkt
- Die O_3-Effekte hängen von der O_3-Dosis ab und sind mit der O_3-Aufnahme positiv korreliert.

Im zweiten Teil werden makroskopische O_3-Schäden auf Blättern anhand von „Chlorophyllfluoreszenz Imaging“ in Kombination mit Lichtmikroskopie untersucht. Zentrale Hypothesen sind:

- Die Chlorophyllfluoreszenz zeigt den Schädigungsgrad von Geweben an
- Der Parameter F_v/F_m ist ein geeignetes Maß, um die O_3-Schädigung zu visualisieren
- Der Einfluss von O_3 auf die Photosynthese ist größer als anhand von sichtbaren O_3-Schäden erwartet werden kann.

Auf der Forschungsfläche „Kranzberger Forst“ bei Freising wurden die Auswirkungen eines experimentell chronisch erhöhten O_3-Regimes auf die Photosynthese und stomatäre Leitfähigkeit ca. 60 Jahre alter Buchen (*Fagus sylvatica* L.) im Rahmen des EU-Projektes „CASIROZ“ untersucht: In einem Teil des Buchen-Fichten-Mischbestandes wurde im Vergleich zur unveränderten Umge-

bungsluft die O_3-Konzentration mit Hilfe einer „Free-Air-Begasung“ kontinuierlich während der Vegetationsperioden verdoppelt ($2xO_3$). Buchen des natürlichen**,** unveränderten O_3-Regimes dienten als Kontrolle ($1xO_3$). Um akute O_3-Konzentrationen zu vermeiden, wurde $2xO_3$ auf maximal 150 nl O_3 l^{-1} begrenzt. Im Sommer 2003, dem Beginn der vorliegenden Studie, war die „Free-Air-Begasung“ schon im vierten Jahr in Betrieb. Der Vergleich der O_3-Effekte in beiden untersuchten Gruppen erlaubte die Erfassung und Quantifizierung der O_3-Wirkungen.

Der trocken-heiße Sommer 2003 ermöglichte es, die Auswirkungen erhöhter O_3-Belastung bei gleichzeitiger Trockenheit im Vergleich zu einem Jahr ohne Wasserlimitierung (2004) zu untersuchen. Trockenheit wird möglicherweise im Rahmen der sich verändernden Umweltbedingungen („Global Change“) in Zukunft häufiger auftreten. Da Trockenheit im Allgemeinen zu Stomataschluß führt, nehmen Blätter potentiell wenig O_3 auf, was 2003 tatsächlich der Fall war. Somit ist nicht die O_3-Konzentration in der Luft, sondern die vom Blatt aufgenommene O_3-Dosis physiologisch wirksam. Die erhöhte O_3-Konzentration der Luft in 2003 führte daher nicht per se zu vermehrter Schädigung in Buche.

Als Maß für die O_3-Dosis wurde die „kumulative O_3-Aufnahme“ (COU) der Blätter nach Parametrisierung auf der Basis gemessener Gaswechseldaten modelliert, und mit dem für politische Empfehlungen gebräuchlichen Expositionsindex AOT40 („akkumulierte Ozondosis oberhalb eines Grenzwertes von 40 ppb“) verglichen. Es zeigte sich, dass in Jahren mit nicht-limitierendem Niederschlag beide O_3-Indices positiv miteinander korrelieren, mit zunehmender Trockenheit in 2003 wurde jedoch weniger O_3 von den Blättern aufgenommen als die externe O_3-Konzentration erwarten ließ.

Im Vergleich der Jahre 2003 und 2004 (einem Jahr mit durchschnittlichem Niederschlag), war die Nettophotosyntheserate unter sättigenden Lichtbedingungen (A_{max}), die stomatäre Leitfähigkeit für Wasserdampf (g_s) und die Elektronentransportrate des Photosystem II (ETR) stärker durch die Trockenheit beeinflusst als durch O_3. Der Befund war konsistent mit der Reaktion des Stammzuwachses. Die Hypothese, dass erhöhte O_3-Belastung die Anfälligkeit von *F. sylvatica* für zusätzliche Stressoren, wie z.B. Trockenheit, erhöht, bestätigte sich nicht, obwohl anhand von Wasserpotentialmessungen und dem Verhältnis ETR/g_s gezeigt werden konnte, dass die Trockenheit am Standort „Kranzberger Forst“ im Jahr 2003 tatsächlich extrem war.

Des weiteren wurde der Zusammenhang zwischen COU und verschiedenen ökophysiologischen Parametern untersucht. Hypothesen dieser Untersuchung waren, dass 1) die Parameter A_{max}, g_s, maximale Carboxylierungsrate (Vc_{max}), Ribulose-1,5-bisphosphat-limitierte Photosyntheserate (J_{max}), CO_2-Kompensationspunkt (CP), apparente Quantenausbeute der CO_2 Aufnahme (AQ), Carboxlierungseffizienz CE, Atmung bei Tag (r_{day}) und bei Nacht (r_{night}) und ETR durch O_3 beeinträchtigt werden können und 2) solche O_3-Effekte dosisabhängig sind, so dass Unterschiede in den genannten Parametern zwischen den beiden O_3-Regimen mit steigender O_3-Aufnahme größer werden. Die Trockenheit spielte bei diesen beiden Fragestellungen ebenfalls eine entscheidende Rolle, denn die Abhängigkeit der O_3-Effekte von COU war unterschiedlich zwischen den beiden Jahren: In 2004, dem „feuchten" Jahr, waren A_{max}, g_s und ETR unter $2xO_3$ bei steigendem COU erwartungsgemäß niedriger als unter $1xO_3$ und die Unterschiede zwischen den beiden O_3-Regimen wurden mit steigendem COU größer. Im Jahr 2003 dagegen wurden die Unterschiede in allen drei Parametern (A_{max}, g_s, ETR) zwischen den beiden O_3-Regimen mit steigender O_3-Aufnahme geringer.

J_{max} und CE zeigten 2003 keine signifikanten Unterschiede zwischen den beiden O_3-Regimen bei Trockenheit, waren im September 2004 unter $2xO_3$ dagegen um 10 bis 30 % niedriger als unter $1xO_3$. Vc_{max} war bei hohem COU (> 25 mmol O_3 m^{-2}) im Jahr 2003 nicht erniedrigt, während in 2004 eine signifikante Erniedrigung schon bei > 20 mmol O_3 m^{-2} ermittelt wurde. Eine hohe O_3-Dosis muss daher nicht unbedingt negative Folgen für den Blattgaswechsel haben, wofür wohl die biochemische Streßabwehr entscheidend ist. Der Zusammenhang zwischen O_3-Dosis und -Wirkung erwies sich als variabel und witterungsbestimmt.

Sichtbare Symptome von O_3-Schädigung und Wirkungen anderen Stressoren in Blättern sind oftmals kleinflächige Chlorosen und Nekrosen. Schädigungen im Umfeld O_3-induzierter Nekrosen wurden mit Hilfe von bildgebenden Verfahren der Chlorophyllfluoreszenz-Messung („Imaging-PAM") und Lichtmikroskopie in Zusammenarbeit mit dem „Ozone Validation Centre" (Dr. P. Vollenweider, Birmensdorf, Schweiz) analysiert. Zur Klärung des Zusammenhangs zwischen makroskopischem Symptombild des Blattes und anatomischen Ursachen wurden Chlorophyllfluoreszenzaufnahmen der Blattspreite angefertigt und anschließend Blattquerschnitte einzelner Regionen innerhalb der Aufnahmen mikroskopisch untersucht. Der direkte Vergleich ergab, dass der häufig bei Chlorophyllfluoreszenzmessungen verwendete Parameter F_v/F_m (maximale Quantenausbeute des PSII) kein geeignetes Maß ist, um O_3-Schädigung zu visualisieren. Trotz massiver, ozonbedingter Schädigung des Palisadenparenchyms zeigte sich keine signifikante Verminderung in F_v/F_m. Bei an-

deren Streßwirkungen (wie z.B. mechanischen Schädigungen, Insektenfraß, Trockenheit) war F_v/F_m ein geeignetes Maß für die Intaktheit des Palisadenparenchyms. Offensichtlich bestimmt der geschädigte Gewebetyp im Blatt (Palisadenparenchym, Schwammparenchym) das resultierende Chlorophyllfluoreszenzbild. Liegen Schäden in epidermisnahen Zellschichten vor, können darunter liegende, intakte Zellschichten nach wie vor hohe F_v/F_m-Signale verursachen. Damit Reduktion in F_v/F_m messbar wird, müssen weitreichende, massive Mesophyllschäden im gesamten Blattvolumen vorliegen. Zur Verbesserung der chlorophyllfluoreszenzbasierten Diagnostik von O_3-Symptomen wurden Φ_{PSII} (Effizienz der Quantenausbeute unter Lichtbedingungen), NPQ (nichtphotochemisches Quenching) und q_L (photochemisches Quenching) in die Bewertung einbezogen und entsprechende Methoden erarbeitet.

Die Schädigung der Photosynthese im Blatt kann ausgedehnter sein als die makroskopisch erkennbar zerstörte Fläche. Die zeitlich-räumliche Entwicklung von O_3-induzierten Blattschädigungen wurde mit Nekrosenentwicklungen infolge Insektenfraß oder Pilzinfektion als Referenz verglichen. Die „indirekt“ durch Insektenfraß betroffene Blattfläche mit verminderter Photosyntheseleistung war größer als die „direkt“ durch Fraß verloren gegangene Fläche. Pilzverursachte Nekrosen waren von einem Ring erhöhter Photosyntheseleistung umgeben, dessen Fläche meist ähnlich groß wie die Nekrosenfläche selbst war. Im Falle von O_3-induzierten Blattflecken war die „indirekt“ betroffene Fläche zumeist kleiner oder gleich groß wie die sichtbar betroffene Fläche. Allerdings war die Konsistenz zwischen sichtbarem Symptom und zugehörigem Chlorophyllfluoreszenzbild oftmals gering, so dass eine neue Methode für die Visualisierung der betroffenen Flächen entwickelt wurde. Hierzu wurden Bilder des absoluten Fluoreszenzsignals F_s mit Bildern von q_L kombiniert. F_s ist u.a. abhängig von der Dicke der Zellschichten im Blatt, die das Licht durchdringen muss. Somit kann F_s zur Lokalisation des Fluoreszenzsignals und damit des verbliebenen, intakten Blattgewebes herangezogen werden, während q_L den physiologischen Status des dort aktiven Photosyntheseapparates wiedergibt. Die Diagnose und Visualisierung von Bereichen mit eingeschränkter Photosyntheseleistung kann somit nach Zellschichten getrennt durchgeführt werden (wie z.B. speziell für das Palisadenparenchym im Falle von O_3-Einwirkung in dem der O_3-Einfluss lichtmikroskopisch nachgewiesen werden konnte).

Die auf diese Weise erfolgende Quantifizierung von O_3-Effekten erlaubte eine schnelle, nicht-invasive und umfassende Diagnostik. Die potentiell betroffene Blattfläche war nach dieser Methode deutlich größer als die nekrotische Fläche. Für die O_3-Diagnostik und Visualisierung haben sich im

Rahmen dieser Studie insbesondere Φ_{PSII}, ETR, NPQ und q_L bewährt, während der häufig verwendete F_v/F_m Parameter nur wenig durch O_3 beeinflusst war.

Zusammenfassend zeigte sich, dass Photosynthese und stomatäre Leitfähigkeit der untersuchten Buchen durch O_3-Streß beeinflusst sind. Allerdings sind die Streßreaktionen nicht konsistent, da witterungsabhängig. Darüber hinaus ist die aufgenommene O_3-Dosis, als biologisch wirksame Größe, nicht unbedingt von der externen O_3-Konzentration abhängig.

F. sylvatica ist im „Kranzberger Forst“ in ihrer Existenz durch realitätsnah chronisch erhöhte O_3-Regime nicht akut gefährdet, obwohl sich vielfältige, zeitlich-räumlich variable O_3-Schädigungen mit potentiell langfristigen Auswirkungen zeigten.

Summary

Little is known about the effects of tropospheric ozone concentrations (O_3) on mature trees under natural site conditions in Central Europe. Increased tropospheric O_3 levels can impair biological processes at cell, leaf , tree and stand level and finally lead to growth reduction. Main questions of the study were a) Are adult beech trees at a forest site at risk from increased O_3 concentrations? b) are O_3 effects modified by climatic conditions? c) are beech trees, growing under enhanced O_3 concentrations, more susceptible to additional environmental stressors, in particular to drought? d) are O_3 effects dependent on the O_3 dose? and e) is the impact of visible O_3-induced leaf injuries on the photosynthesis larger than expected from the size of the injuries?

The thesis is divided in two main parts: First, the interaction of O_3 and drought, and the relationship between the O_3 dose and O_3 effects are investigated. Main hypotheses are:

- O_3 uptake rather than O_3 exposure reflects the risk of O_3 induced injury
- The effect of drought on adult beech trees is aggravated by enhanced O_3 levels
- Effects of O_3 are dose dependent and are positively correlated with O_3 uptake

Second, the macroscopic O_3 injuries are investigated by chlorophyll fluorescence imaging to clarify whether

- chlorophyll fluorescence resembles the degree of injury of leaf tissues
- F_v/F_m is a suitable tool to assess O_3 injury
- the impact of O_3 on photosynthesis is larger than can be estimated from visible O_3 induced necroses, and whether the area affected by O_3 can be quantified.

Within the scope of the EU project "CASIROZ", the impact of chronic O_3 concentrations on photosynthesis and stomatal conductance of 60-year-old beech trees (*Fagus sylvatica* L.) was investigated at the research site "Kranzberger Forst" near Freising, Germany. By means of a "Free-air" O_3 fumigation system, the ambient O_3 concentrations were doubled ($2xO_3$) during the growing seasons. Beech trees of the ambient, unchanged O_3 regime served as control ($1xO_3$). To prevent acute O_3 injury, the doubling was limited to a maximum of 150 nl O_3 l^{-1}. In summer 2003, the beginning of this study, the "Free-Air" fumigation was employed in the fourth year already. The comparison between the two O_3 regimes allowed the identification and quantification of O_3 effects.

The dry and hot summer of 2003 provided the opportunity to investigate the impact of increased O_3 concentrations with concurrent drought. O_3 effects encountered during 2003 were compared to the effects of 2004, a year without limited water supply. Such a severe drought as in 2003 might occur more frequently within the scope of the changing environmental conditions ("Global Change") in the future. As drought generally leads to stomatal closure, the O_3 uptake of the leaves might be limited, as was confirmed in 2003. Therefore, the actual O_3-dose encountered by a leaf rather than the external O_3 concentration in the air, is biologically meaningful.

To assess the O_3 dose, the "cumulative O_3 uptake" (COU) of the leaves was modelled after parametrization on the basis of measured gas exchange rates, and was compared to the exposure index AOT40 ("accumulated O_3 dose above a threshold of 40 ppb"), an O_3-index commonly used for environmental assessments. It appeared that during years with non-limiting precipitation both O_3 indices correlate positively with each other. Nevertheless, with increasing drought in 2003 less O_3 was taken up by the leaves as was suggested by the external exposition.

In the comparison of the years 2003 and 2004, the net photosynthetic rate under saturating light conditions (A_{max}), the stomatal conductance for water vapour (g_s) and the electron transport rate of photosystem II (ETR) where influenced by drought rather than by O_3. These findings were consistent with the response of the stem increment. The hypothesis, that increased O_3 enhances the susceptibility of *F. sylvatica* to additional stressors like drought, was not confirmed, although the actual water-potential and the ETR/g_s ratio indicated an extraordinary drought at "Kranzberger Forst".

In addition, the relation between COU and different ecophysiological parameters was examined. Hypotheses of this investigation were that 1) the parameters A_{max}, g_s, maximum carboxylation rate (Vc_{max}), Ribulose-1,5-bisphosphat-limited rate of photosynthesis (J_{max}), CO_2 compensation point (CP), apparent quantum yield of the CO_2 uptake (AQ), carboxylation efficiency (CE), respiration at day (r_{day}) and night time (r_{night}) and ETR are affected by O_3 and 2) such O_3 effects are dependent on the dose, so that differences in the investigated parameters become larger between the O_3-regimes with increasing dose. Again, drought was a major factor, because the dependency of the O_3 effects on COU was different between both years: In 2004, the "humid" year, A_{max}, g_s and ETR were lower under $2xO_3$ than under $1xO_3$ with rising COU, as expected, and the differences between both O_3-regimes became larger with rising COU. In 2003 however, the differences in all three parameters (A_{max}, g_s, ETR) between both O_3-regimes became smaller with rising O_3 dose .

In 2003, J_{max} and CE showed no significant differences between both O_3-regimes, but were 10 to 30% lower in $2xO_3$ compared to $1xO_3$ in September 2004. Vc_{max} was not decreased at high COU (> 25 mmol O_3 m^{-2}) in 2003, whereas in 2004 a significant decrease was already evident at > 20 mmol O_3 m^{-2}. Hence, a high O_3 dose does not necessarily have negative impact on the photosynthesis, as the biochemical defence against O_3 plays an important role. The relation between O_3 dose and O_3 effect turned out to be variable and weather-dependent.

Visible symptoms of O_3 injury and symptoms of other stressors on leaves are often small scale patterns of chloroses and necroses. Impairment of photosynthesis in the vicinity of O_3-induced necroses were analysed by chlorophyll fluorescence imaging ("ImagingPAM") and light microscopy in collaboration with the "Ozone Validation Centre" (Dr. P. Vollenweider, Birmensdorf, Switzerland). To clarify the relation between macroscopic symptoms on the leaf and their cause on the cellular level, chlorophyll fluorescence images of the leaf blade were compared to microscopic cross sections of selected areas. The comparison showed that the parameter F_v/F_m (maximum quantum yield of the PSII), a parameter frequently used in chlorophyll fluorescence assessments, is not suitable to visualise O_3 injury. Despite massive, O_3-induced injuries in the palisade parenchyma, no significant decrease was found in F_v/F_m. In the assessment of other stressors (as for example mechanical injury, insects, drought), F_v/F_m was a suitable measure for the vitality of the palisade parenchyma. Obviously, the injured tissue type in the leaf (palisade parenchyma, spongy parenychma) determines the resulting chlorophyll fluorescence image. If injuries are located close to the epidermis, intact cell layers underneath can still cause high F_v/F_m signals. For a reduction in F_v/F_m, the injuries must cover major parts of te mesophyll. To improve diagnostics of O_3 symptoms by means of chlorophyll fluorescence imaging, Φ_{PSII} (efficiency of the quantum yield under light conditions), NPQ (non-photochemical quenching) and q_L (photo-chemical quenching) were included in the assessments and new methods were developed.

The impairment of photosynthesis in the leaf can be larger than recognizable by the macroscopically destroyed surface. The spatio-temporal development of O_3-induced leaf injuries were compared to the development of necroses caused by insects and fungal infections. The "indirectly" affected leaf area (i.e. the area of reduced photosynthesis) was larger than the area "directly" removed by the insect. Necroses caused by fungi were surrounded by a ring of increased photosynthetic performance and its area was mostly similarly large as the necrotic areas themselves. In the case of O_3-induced leaf necroses the "indirectly" affected area was mostly smaller or equally in size to the visibly af-

fected area. But the consistency was small between the visible symptoms and the corresponding chlorophyll fluorescence image, so that a new method was developed to visualize the affected areas. Therefore, images of the absolute fluorescence signal F_s were combined with images of q_L. F_s depends in parts on the thickness of the cell layers passed by the light. Therefore, F_s can be used for the localisation of the fluorescence origin and thus of remaining, intact tissue layers. q_L, at the same time, indicates the physiological status of the photosynthetic apparatus in these cell layers. Therefore, the diagnosis and visualisation of areas of decreased photosynthetic performance can now focus on specific cell layers (e.g. especially for the palisade parenchyma which is the tissue layer mainly affected by O_3 as can be shown by light microscopy).

The quantification of O_3 effects based on this new method allowed quick, non-invasive and comprehensive diagnostics. The area, indicated by this method to be potentially affected, was much larger than the area estimated from the necroses. For the diagnostics and visualisation of O_3 impact, Φ_{PSII}, ETR, NPQ and q_L were especially useful, while the often used F_v/F_m parameter was hardly affected by O_3.

In summary, the photosynthesis and stomatal conductance of the investigated beech trees are affected by O_3-stress. However, the responses are not consistent between years, as they are depending on the climatic conditions. In addition, the O_3 dose, as the biologically meaningful factor, is not dependent on the external O_3 concentration.

F. sylvatica at "Kranzberger Forst" is not at high risk from increased chronic O_3-regimes, although varying, spatio-temporal O_3 injuries were detected, which might influence the trees in the long term.

1 Background and concept

1.1 Tropospheric O_3

Tropospheric ozone (O_3) is a secondary air pollutant of highest risk potential for plants (Ashmore 2005, Giles 2005). Formation of O_3 increased since the industrial revolution due to rising concentrations of primary anthropogenic air pollutants like hydrocarbons, NO_x and volatile organic compounds (VOCs).

Photolysis of NO_2 leads to NO and O. O_2 and O then form the oxidizing agent O_3 (Stockwell *et al.* 1997, Fabian 2002). This reaction is reversible, (Matyssek & Sandermann 2003). In polluted air, the back reaction is not favoured. NO is oxidised to NO_2 by organo-peroxy ($RO_2\cdot$) and hydro-peroxy ($H_2O\cdot$) without consumption of O_3, resulting in high O_3 concentrations (Stockwell *et al.* 1997).

The reaction from N + O back to NO_2 is not favoured (Matyssek & Sandermann 2003). Furthermore, O_3 can be transported from the stratosphere to the troposphere, and is produced locally in wetlands and swamps.

The build-up of O_3 is favoured under high light conditions in polluted air, so O_3 levels can increase rapidly especially in the morning hours of sunny summer days. Pre-industrial O_3 levels of around 10 – 20 nl O_3 l^{-1} (Matyssek *et al.* 2003, Vingarzan 2004) have increased steadily to 30 – 40 nl O_3 l^{-1} in 2001 (Vingarzan 2004). Whether or not the O_3 concentrations will stabilize at this level is still an open question (Fabian 2002; Ashmore 2005) but some models predict annual mean O_3 concentrations of above 60 nl O_3 l^{-1} after 2060 (Vingarzan 2004). As O_3 is closely connected to "Global Change" (IPCC 2001), actual and further increases in tropospheric NO_x will lead to rising O_3 concentrations on a global scale (Richter *et al.* 2005). Additionally, O_3 might counteract the suggested increased carbon sink strength of plants under increased CO_2 levels ("fertilization effect"), therefore O_3 has to be considered as a major player in global change scenarios (Long *et al.* 2005). Within global change scenarios, "warmer and more frequent hot days" are "virtually certain" for the 21st century (IPCC 2007) so that the frequency of drought combined with high insolation will increase. These conditions are conducive to the formation of high tropospheric O_3 levels (Stockwell *et al.* 1997, Fabian 2002). In the future, this high O_3 risk poses a major threat to plants and might lead to a decreased crop yield worldwide (Giles 2005).

1.2 Effects of O_3 on plants

After O_3 enters the plant through the stomata, it reacts with leaf internal surfaces and with H_2O of the apoplast (LAISK *ET AL.* 1989) and produces various reactive oxygen species (ROS, KANGASJÄRVI *ET AL.* 1994), which decrease the permeability of the membranes (HEATH *ET AL.* 1997). Additionally, O_3 triggers similar biochemical pathways as fungi and other pathogens (SANDERMANN *ET AL.* 1998) on the molecular and biochemical level leading to programmed cell death and protein degradation, but also to the increased formation of defence metabolites (SANDERMANN 1996, MATYSSEK *ET AL.* 2003). Especially antioxidants (ascorbate, glutathion, α-tocopherol) and chloroplast pigments are utilized as radical-scavenging-systems to defend against O_3 (DE TEMMERMAN *ET AL.* 2002, HERBINGER *ET AL.* 2002).

On the leaf blade, chronic O_3 impact becomes visible within days or weeks as mild chlorosis, necrosis, pigmentation, stippling, and premature senescence (INNES *ET AL.* 2001). The main tissue affected by O_3 is the palisade parenchyma, where the ROS cause lesions (SCHRAUDNER *ET AL.* 1997, BUSSOTTI *ET AL.* 2005). But O_3 can also affect the lower mesophyll layers (PELLINEN *ET AL.* 1999). O_3 impact on the cellular level can be investigated by light microscopy using cellular markers for oxidative stress, antioxidants, accelerated cell senescence, and the induction of defence responses (VOLLENWEIDER *ET AL.* 2003).

Many tree species, e.g. Birch (MATYSSEK *ET AL.* 1991, UDDLING *ET AL.* 2004), Aspen (KING *ET AL.* 2005), Pine (BEYERS *ET AL.* 1992); LE THIEC *ET AL.* 2003) and Beech (GRAMS *ET AL.* 1999, NUNN *ET AL.* 2005b), are known to be susceptible to O_3 impact. However, the individual responses of the tree species can be variable (SKÄRBY *ET AL.* 1998) and might depend on different growth strategies (MATYSSEK 2001).

1.3 Critical levels of O_3

Of major concern is the economic impact of O_3 as crop yield and plant growth might be impaired (ASHMORE *ET AL.* 1999, KARLSSON *ET AL.* 2005). To protect vegetation from O_3 impact, different "Critical levels" have been suggested, which were determined on the basis of the O_3 concentration in the air. A simple measure for O_3 exposure is the "Sum of all O_3 concentrations > 0" (SUM0). But this approach did not yield sufficient relationships with plant responses (c.f. PANEK *ET AL.* 2002). This index is still often used to express the total "amount" of O_3 encountered at a specific site.

The most commonly used index, which is currently employed by UNECE, is the AOT40, the "accumulated ozone exposure over a threshold of 40 nl O_3 l^{-1}" calculated during daytime (FUHRER *ET AL.* 1994, FUHRER *ET AL.* 1997). It was suggested that O_3 exposure above this critical level will cause a

growth reduction of 10% compared to pre-industrial O_3 levels. The threshold for AOT40 was set at 10 µl O_3 l^{-1} h, and was later lowered to 5 µl O_3 l^{-1} h (KARLSSON *ET AL.* 2004b, CLRTAP 2004). However, as the O_3 in the air can not enter the plant when the stomata are closed, a stomatal uptake based critical level might be superior over an exposure based index like AOT40 (KARLSSON *ET AL.* 2003), especially when plants from different environmental conditions are compared (VANDERHEYDEN *ET AL.* 2001). Also, the AOT40 concept showed major discrepancies between observed patterns in forests and the suggested threshold (MATYSSEK *ET AL.* 1999). Therefore, many studies employed and further developed the O_3 uptake approach (EMBERSON *ET AL.* 2000, WIESER *ET AL.* 2004, KARLSSON *ET AL.* 2004a, NUNN *ET AL.* 2005a). The uptake of O_3, i.e. the dose encountered by the leaves, provides a biologically more meaningful basis for O_3 risk assessment (MATYSSEK *ET AL.* 2003, MASSMAN *ET AL.* 2000), especially under drought conditions, when stomata are closed (MATYSSEK *ET AL.* 2006b).

The current, provisional critical level for the "cumulative stomatal ozone uptake" (COU) or "accumulated O_3 flux through the stomata" (AFst) for forests was established during a recent UNECE workshop in Obergurgl, Austria at 4 mmol m^{-2} (for a COU only summarizing O_3 concentrations above a threshold of 1.6 nmol O_3 m^{-2} s^{-1}, c.f. WIESER *ET AL.* 2005).

COU can be modelled successfully (c.f. NUNN *ET AL.* 2005a, UDDLING *ET AL.* 2004) based on a multiplicative model (EMBERSON *ET AL.* 2000) integrating stomatal conductance to water vapour, O_3 concentration, climatic data and stand specific parameters. However, the COU model itself is being further improved (BÜKER *ET AL.* 2007).

Basically, COU is a function of the maximum stomatal conductance for O_3 and the external O_3 concentration (Equations 1 and 2):

$$F_{O_3} = g_{O_3}[O_3] \qquad \text{Eq. (1)}$$

$$COU = \sum_{SGS}^{EGS} F_{O_3} \qquad \text{Eq. (2)}$$

FO_3 is the stomatal O_3 flux, gO_3 is the stomatal conductance for O_3, SGS is the date of the start of the growing season and EGS is the end of the growing season. gO_3 is a function of maximum and minimum stomatal conductance, light, phenology, temperature, VPD, soil moisture, day and night time (see NUNN *ET AL.* 2005a for further details).

Still, the COU approach needs some improvement, because detailed site specific parametrisation is extensive, especially as data for adult trees under natural site conditions are still scarce and difficult to obtain. To improve the application of the COU critical levels, the modelling of stomatal O_3 uptake can be accompanied and improved by supporting methods, like sap-flow measurements which provides a simple measure of the whole crown transpiration (MATYSSEK *ET AL.* 2004, NUNN *ET AL.* 2006), eddy-flux measurements (CIESLIK 2004) or the direct measurement of O_3 flux into the leaf (GRULKE *ET AL.* 2007).

1.4 The EU Project "CASIROZ"

This doctoral thesis was part of the EU Project "CASIROZ", The **Ca**rbon **Si**nk Strength of Beech in a Changing Environment: Experimental **R**isk Assessment of Mitigation by Chronic **Oz**one Impact". In a highly integrated approach, groups from different biological disciplines, from molecular biology to modelling, worked simultaneously at one forest site. This approach allowed to integrate and cross compare findings on the O_3 impact on beech trees between different "scaling levels" (see Figure 1.1) to investigate patterns of O_3 responses and to develop a mechanistic stand model and a database of all findings. The major aim of the project was to clarify the vulnerability of adult beech trees to chronic O_3 impact. Due to this joint research, several datasets from the "ecophysiology" scaling level (Figure 1.1) which are presented in this thesis have already been published in cooperation with several "CASIROZ" project partners (BLUMENRÖTHER *ET AL.* 2007, DECKMYN *ET AL.* 2007, HERBINGER *ET AL.* 2005, HERBINGER *ET AL.* 2007, OP DE BEECK *ET AL.* 2007, WARREN *ET AL.* 2007).

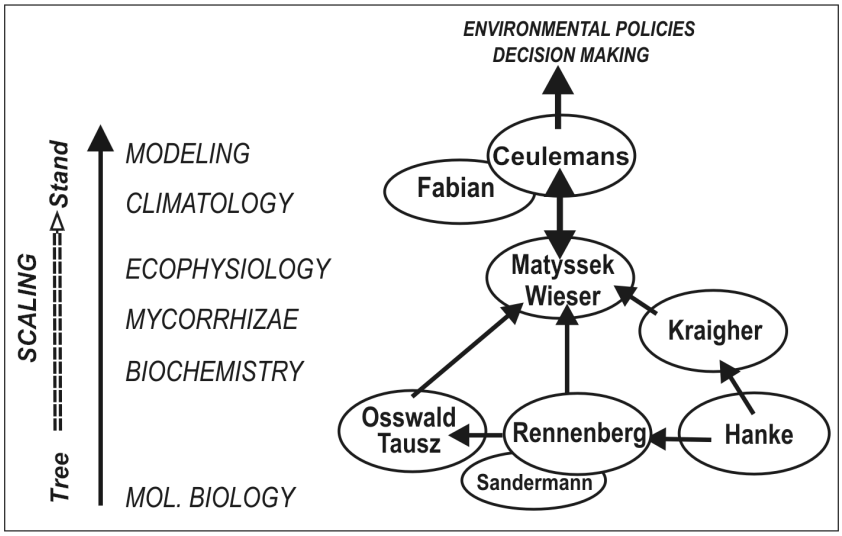


Figure 1.1: "CASIROZ" scaling levels and group structure (name of the principal scientist of each participating group is given). Arrows and circles indicate cooperation on and integration of data (after www.casiroz.de).

1.5 Experimental design

The study site is located within a mixed beech/spruce forest ("Kranzberger Forst") in southern Germany near Freising, Germany (48°25 N, 11°39 E, elevation 485m a.s.l., size ca. 0.5 ha). The soil at the site is a luvisol derived from loess over tertiary sediments.

Scaffoldings (up to 28 m high) and a research crane provided access to the sun and shade crowns of ten representative (REITER *ET AL.* 2005) about 62-year-old beech trees (*Fagus sylvatica* L.) and ten about 52-year-old spruce (*Picea abies* [L.] KARST) trees (WIPFLER *ET AL.* 2005). Within the plot, five beech and five spruce trees were exposed to an experimentally enhanced O_3 regime, employed by a "free-air canopy O_3 exposure system" (NUNN *ET AL.* 2002, WERNER *ET AL.* 2002) since the year 2000. The parameters presented in this doctoral thesis were measured during 2003 and 2004, i.e. in the fourth and fifth year of free-air O_3 fumigation.

Air from the site was used to produce the O_3 needed for the canopy fumigation. The oxygen from dry air was enriched to 90% and passed through an molecular sieve to prevent NO_x formation. From this purified air, O_3 was produced by electrical discharge. The free-air fumigation system covers a volume of about 2000 m^3. Within this volume, O_3 was released into the canopy from a grid of 120 equally distributed PTFE tubes (hanging from wires above the canopy) by calibrated outlets every 33 cm (WERNER *ET AL.* 2002, FABIAN *ET AL.* 2005). Unavoidable high O_3 concentrations were limited to about 4 – 5 cm around each outlet, and due to dilution the employed O_3 regime was homogeneous without "hot spots" (NUNN *ET AL.* 2002, HEERDT 2007). Also, to prevent acute O_3 injury, the O_3 levels of this regime were restricted to a maximum of 150 nl O_3 l^{-1}.

Based on the natural background O_3 concentration the O_3 regime was continuously doubled ($2xO_3$) in the area of the fumigation throughout the growing seasons. Five adjacent *F. sylvatica* trees under the unchanged ambient regime ($1xO_3$) prevailing at the site served as control.

1.6 Climatic characteristics

Long-term regional annual air temperature is 7.5°C, and annual precipitation amounts to 788 mm (monitored by DWD at climate station "Weihenstephan", at 4 km distance from the research site; DWD Offenbach, Germany). The weather conditions at the research site of the years 2003 and 2004 are given in Figure 1.2 and Table 1.1. The year 2002 was included as an additional reference.

This study covered climatically contrasting years. The mean daily air temperature within the canopy was conspicuously higher during the growing season of 2003 as compared to the respective time period in the previous or succeeding year. Annual and growing season precipitation varied between the years as the cumulative rainfall in 2002 exceeded that in 2003 by a factor of almost two (Table 1.1). Rainfall of 2004 was equal to the long term average at the site. The high temperature and low precipitation during 2003 were not restricted to "Kranzberger Forst", but were common for Central Europe at that time (CIAIS *ET AL.* 2005, SCHÄR *ET AL.* 2004).

Volumetric soil water content hardly fell below 30% in 2002, but decreased to about 15% in August of 2003 and remained that low until early October of the same year (Figure 1.2 D). At this low level hardly any water was left for plant uptake, indicating severe drought (RASPE *ET AL.* 2004). The soil water content did not fully recover during winter after the drought of 2003, so that water supply was limited again during summer 2004, however, did neither impair photosynthesis nor stomatal conductance.

Table 1.1: Weather conditions at the site "Kranzberger Forst" near Freising / Germany, during the years 2002, 2003 and 2004. Precipitation was measured in a forest clearing at 1 km distance from the site. After *LÖW ET AL. (2006)*

		Year		
		2002	2003	2004
annual sum of global radiation above canopy	[GJ m^{-2}]	4.1	4.6	4.4
mean annual air temperature at canopy height, 24 m above ground	[°C]	8.8	9.1	9.4
mean daily air temperature during growing season (May to October) at canopy height, 24 m above ground	[°C]	14.1	16.2	14.9
annual sum of precipitation	[mm]	1015	557	786
precipitation during growing season (May to October)	[mm]	626	359	448

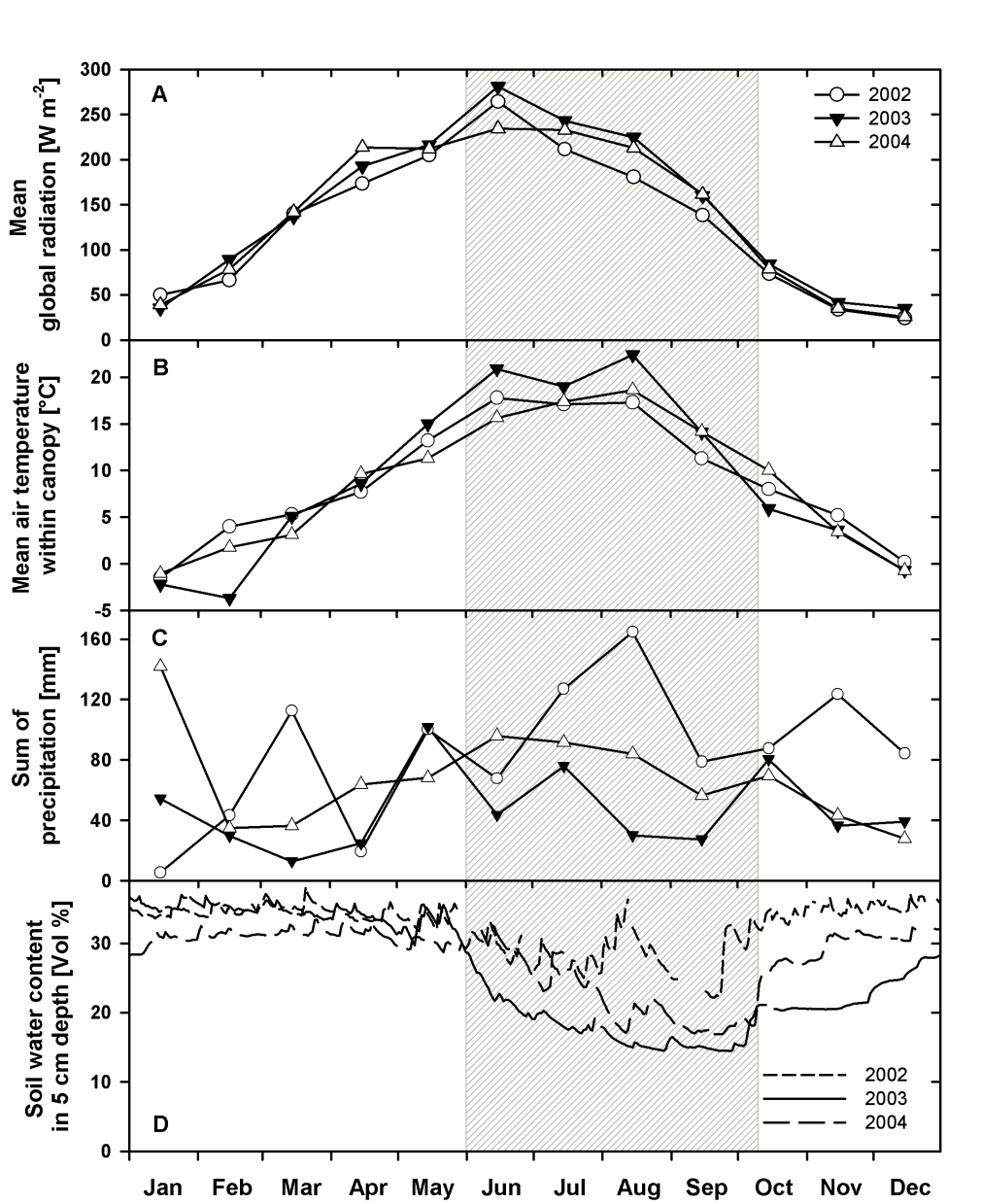


Figure 1.2: Monthly sum of global radiation (A), air temperature at canopy height, i.e. 24 m above ground (B), precipitation (C) and soil water content (D) during the years 2002, 2003 and 2004 at the research site "Kranzberger Forst" in southern Germany. Symbols given in (A) also apply for (B) and (C). Hatched area indicates drought period in 2003. Data in (C) and (D) by courtesy of LWF (G. Gietl and W. Grimmeisen). From *LÖW ET AL. (2006)*.

1.7 Chlorophyll fluorescence imaging

As chlorophyll fluorescence *imaging* is not a common technique for ecophysiological assessments in the crowns of adult trees, a short introduction is given here. Chlorophyll fluorescence based measurements of the physiological status of plants give insight in the conversion of absorbed light energy to photochemical energy. Light absorbed by chlorophyll molecules can either drive photo-

synthesis, can be dissipated as heat or can be emitted as chlorophyll fluorescence (MAXWELL *ET AL.* 2000). These three processes (and further processes which will not be described here, VON WILLERT *ET AL.* 1995) compete with each other at all times, so that information on photosynthetic energy yield and heat dissipation can be derived by measuring chlorophyll fluorescence (MAXWELL *ET AL.* 2000). At room temperature, chlorophyll fluorescence is mainly emitted from photosystem II (PSII), but the contribution of PSI can not be neglected, but is, as of today, difficult to measure in the field, (c.f. PETERSON *ET AL.* 2001, OXBOROUGH 2004). However, new commercial instruments are becoming available.

In a leaf in darkness, all PSII reaction centres are open, ready for light energy. This fluorescence level is called F_o (Figure 1.3). If a flash of light (i.e. saturating light pulse) is applied, photosynthesis becomes activated, at first a fast incline and then a slower decline of chlorophyll fluorescence is recorded within less than 1 s. The maximum encountered chlorophyll florescence is denominated F_m. At this point, no light energy can be used for photochemical purposes, therefore, the now excessive light energy is mainly used up by fluorescence to avoid injury to the photosynthetic apparatus. The fast increase from F_o to F_m is connected to PSII reaction centres getting activated and therefore becoming closed. This increase is called the “photochemical quench” of fluorescence (MAXWELL *ET AL.* 2000).

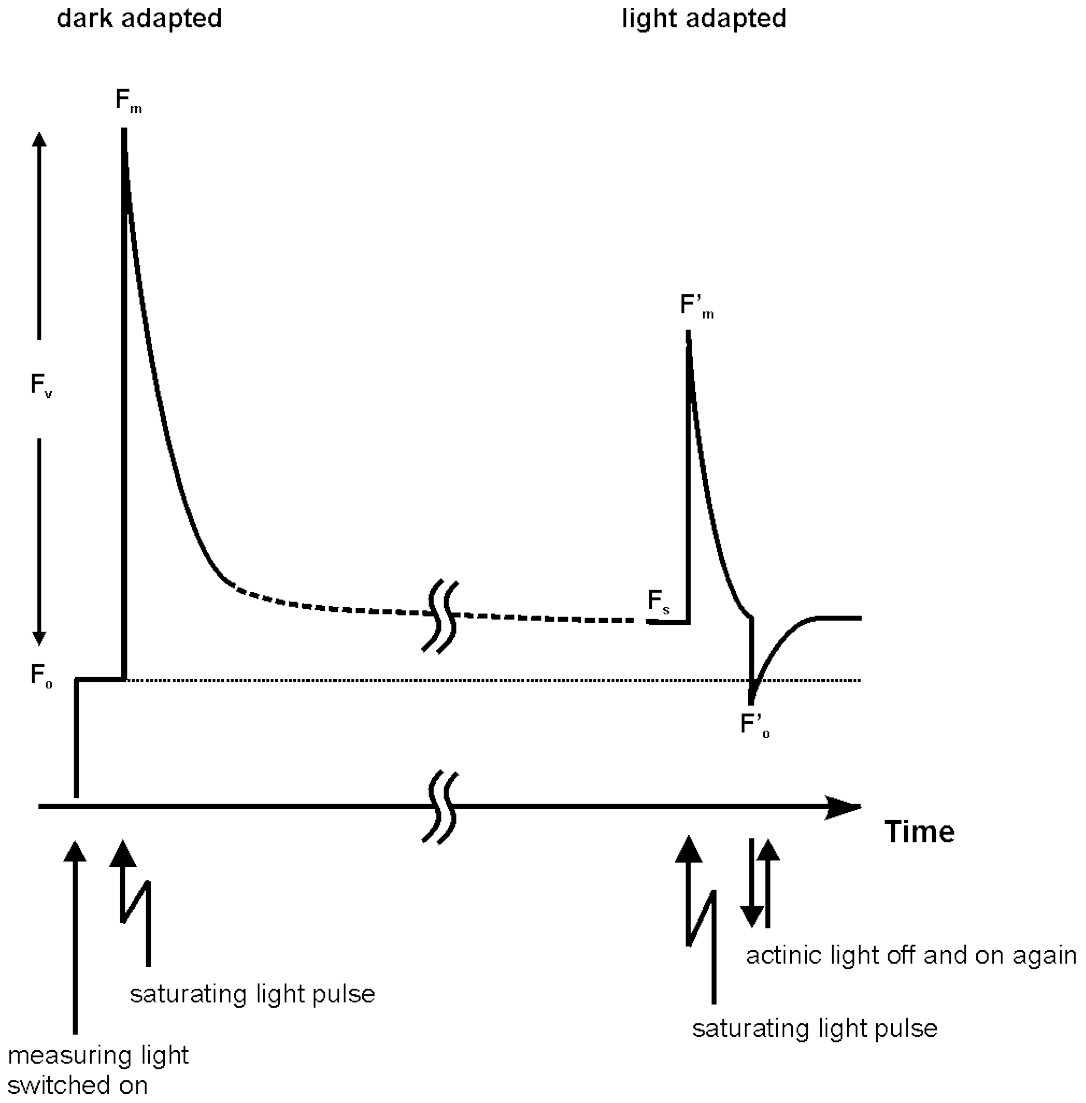


Figure 1.3: Basic chlorophyll fluorescence scheme and nomenclature as used in this study.

The ratio F_v/F_m (maximum quantum yield), measured after dark adaptation, is a commonly used parameter in for the assessment of the physiological status of leaves (LICHTENTHALER *ET AL.* 2005).

Under light conditions, the fluorescence signal decreases again to a steady value F_s, which is above F_o, as some PSII reaction centres are closed while driving photosynthesis (Figure 1.3). An additional light pulse yields another increase of the fluorescence signal, but F'_m is lower than F_m as dissipation of energy ("non-photochemical quenching" NPQ) is high under light conditions .

As the nomenclature of chlorophyll fluorescence parameters is still not standardized and names can be confusing (c.f. GENTY *ET AL.* 1989, BAKER *ET AL.* 2004, KRALL *ET AL.* 1992, KRAMER *ET AL.* 2004, WALZ 2005), the name, the description and the equations of the parameters, as used throughout this thesis, are given in Table 1.2

Table 1.2: List of measured and calculated chlorophyll fluorescence parameters and related factors. If not stated otherwise, units are arbitrary, scaled from 0 to 1. Adapted from different sources, references given below.

Name	Description	Equation	Reference
	measured parameters		
F	fluorescence		
F_o	dark adapted minimal fluorescence		1, 6, 8
F_m	dark adapted maximal fluorescence		1, 6, 8
F_s	light adapted fluorescence in steady state		4
F'_m	light adapted maximal fluorescence		1, 8
	calculated parameters		
F'_o	light adapted minimal fluorescence (can be measured, but not with the instrument used in this study i.e. Imaging PAM, WALZ, Effeltrich, Germany)	$F'_o = \frac{F_o}{(\frac{F_v}{F_m}) + (\frac{F_o}{F'_m})}$	6
F_v	variable fluorescence	$F_v = F_m - F_o$	8
F_v/F_m	maximum quantum yield (in darkness)	$F_v / F_m = \frac{(F_m - F_o)}{F_m}$	5
Φ_{PSII}	effective quantum yield (in light)	$\Phi_{PS_{II}} = \frac{F'_m - F_s}{F'_m}$	2, 4
q_L	proportion of open photosystems II = coefficient of photochemical quenching based on “lake model” [a]	$q_L = \frac{F'_m - F_s}{F'_m - F'_o} \cdot \frac{F'_o}{F_s}$	4
NPQ	non-photochemical quenching (heat dissipation) [arbitrary units, scale 0-4]	$NPQ = \frac{F_m - F'_m}{F'_m}$	4
ETR	apparent electron transport rate [µmol electrons m^{-2} s^{-1}]	$ETR = \Phi_{PS_{II}} \cdot PPFD \cdot Abs \cdot 0.5$ Abs = 0.84 if not measured	3
Abs	absorptivity (i.e. fraction of absorbed incident red light, Red = sample in red light, NIR = sample in near infrared light)	$Abs = 1 - \frac{Red}{NIR}$	7

References: 1 = Baker *et al.* (2004), 2 = Genty *et al.* (1989), 3 = Krall *et al.* (1992), 4 = Kramer *et al.* (2004), 5 = Maxwell *et al.* (2000), 6 = Oxborough *et al.* (1997b), 7 = Walz (2005), 8 = van Kooten *et al.* (1990)

[a] the “lake” model assumes that the photosystem (PS) II reaction centrers are connected by shared antenna in contrast to the “puddle” model (c.f. q_p) in which each PSII centre has independent antenna systems (Kramer *et al.,* 2004).

The visualisation of chlorophyll fluorescence is still an emerging technique (OXBOROUGH 2004), but is widely used to assess and diagnose spatio-temporal patterns in photosynthesis of different origin on plants (OMASA *ET AL.* 1987, CHAERLE *ET AL.* 2001). Applications for chlorophyll fluorescence imaging cover e.g. assessment of stomatal patchiness and internal CO_2 concentration C_i (SIEBKE *ET AL.* 1995, MEYER *ET AL.* 1998), plant-pathogen-interactions (ROLFE *ET AL.* 1996, SCHARTE *ET AL.* 2005), early-detection of stress (CHAERLE *ET AL.* 2000), assessment of fruit quality (NEDBAL *ET AL.* 2000), "screening" (BARBAGALLO *ET AL.* 2003), visualisation of electrical signals (KOZIOLEK *ET AL.* 2004) and assessment of O_3 impact (LEIPNER *ET AL.* 2001).

The scaffolding at "Kranzberger Forst" enabled to use a chlorophyll fluorescence imaging system ("Imaging PAM", WALZ, Effeltrich, Germany) within the tree crowns to examine the patterns and potential impact of O_3 injuries and other stressors on photosynthesis. For comparisons with microscopical assessments, the high resolution IMAG-MIC Micro Head (WALZ, Effeltrich, Germany) was used.

Image processing and calculation of the parameters shown in Table 1.2 was conducted with a customized version of ImageJ 1.37v (RASBAND 1997-2006) and several self-written scripts. Further details are given in section 3 of this thesis.

1.8 Aim of the study

The aim of this study was to examine, quantify, and visualize the O_3 impact on photosynthesis and stomatal conductance of mature beech trees, a species of major importance in Central Europe. The extraordinary drought encountered in 2003, the first year covered by this study, allowed to assess O_3 impact with concurrent drought, so that an insight in possible future climatic conditions was possible.

Main questions of the study were a) Are adult beech trees at a forest site at risk from increased O_3 concentrations? b) Are O_3 effects modified by climatic conditions? c) Are beech trees, growing under enhanced O_3 concentrations, more susceptible to additional environmental stressors, in particular to drought? d) Are O_3 effects dependent on the O_3 dose? and e) Is the impact of visible O_3-induced leaf injuries on the photosynthesis larger than expected from the visible size of the injuries?

The following hypotheses were tested:

(1) Cumulative ozone uptake (COU) rather than exposure-based O_3 indices like the accumulated ozone above a threshold of 40 nl O_3 l^{-1} (AOT40) reflects risk of O_3-induced damage (e.g. in stem production).

(2) The effect of drought on adult *F. sylvatica* trees is aggravated by enhanced ozone levels.

(3) Ecophysiological parameters (e.g. photosynthesis, stomatal conductance, chlorophyll fluorescence) are negatively affected by chronic O_3 stress.

(4) Deleterious effects of O_3 are dose-dependent and thus the size of differences between treatments in photosynthetic measurements are positively related to COU.

(5) Chlorophyll fluorescence resembles findings of microscopy in a way that the degree of injury of leaf tissue correlates with the fluorescence signal, and that the signal mainly originates from the palisade parenchyma of beech, the tissue of high photosynthetic potential which is likely to be affected by O_3.

(6) F_v/F_m is a suitable measure for O_3 injury in symptomatic laminae of beech leaves from "Kranzberger Forst".

(7) The impact of O_3 and other stressors on photosynthesis is larger than can be estimated from the visible size of these injuries and photosynthesis is decreased in an area surrounding the leaf injuries.

(8) Chlorophyll fluorescence imaging allows to quantify the leaf area affected by O_3 and allows the identification of specific patterns before corresponding injuries can be detected by the naked eye.

1.9 Structure of the thesis

This doctoral thesis is divided in two main parts:

In the first part (chapter 2), questions regarding the interactions of ozone and drought, and the relationship between the O_3 dose and O_3 effects are addressed (hypotheses 1 – 4). In this section, results from three publications are summarized: (1) LÖW *ET AL.* (2006), (2) LÖW *ET AL.* (2007), (3) WARREN *ET AL.* (2007), see Appendix A. In (1) climatic data from "Kranzberger Forst" were contributed by Dr. M. Leuchner and C. Heerdt, COU was calculated in cooperation with Dr. A.J. Nunn and stem diameter data was contributed by P. Wipfler. In (2), all the presented parameters were measured by my-

self, except one measurement in August, which was also part of (3). Data shown in (3) were measured together in a joint campaign with Dr. C.R. Warren. Data on SLA and N data were additionally contributed by myself.

In the second part of this thesis (chapter 3), examples for the application of chlorophyll fluorescence imaging at adult forest trees are presented and discussed (covering hypotheses 5 – 8). Main aspects are the visualisation of O_3 injuries on the macroscopic and microscopic scale, to quantify to which extent photosynthesis is changed by different stressors (including O_3), and to compare the visible and invisible impact of different stressors on photosynthesis. Additionally, a new method based on chlorophyll fluorescence imaging analysis is presented, which improves the diagnosis, quantification and visualization of O_3 impact on leaves.

2 Ozone impact on *Fagus sylvatica* under contrasting climatic conditions

2.1 Introduction

Within "Global Change" scenarios, predictions include a rise in temperature driven by the anthropogenic release of CO_2 and other "climate-effective" gases (including O_3, IPCC 2001, IPCC 2007), into the atmosphere, which increases the probability of drought at regional scale (SCARASCIA-MUGNOZZA *ET AL.* 2001).

Compared to the long term record, the summer of 2003 was characterised by high air temperatures and low precipitation in Central Europe (DWD 2003, LUTERBACHER *ET AL.* 2004, SCHÄR *ET AL.* 2004, CIAIS *ET AL.* 2005). This climatic constellation was conducive for the build-up of high tropospheric O_3 levels (STOCKWELL *ET AL.* 1997), therefore the AOT40 index, an exposure based index for O_3 stress (FUHRER *ET AL.* 1994), indicated an enhanced risk for O_3 injuries on forest trees (MATYSSEK *ET AL.* 1999). However, the actual dose of O_3 encountered by the plant determines O_3 stress and drives stress responses (MATYSSEK *ET AL.* 2003). Therefore, the uptake of O_3 through the stomata of the leaf rather than the exposure determines the O_3 risk (WIESER *ET AL.* 2003, MATYSSEK *ET AL.* 2004). As drought induces stomatal closure (SCHULZE 1994), by this limiting the the O_3 uptake (PÄÄKKÖNEN *ET AL.* 1998ab), plants might be protected from O_3 impact (DOBSON *ET AL.* 1990; MAIER-MAERCKER 1998). In fact, there is now considerable evidence that assessment based on O_3 uptake (i.e., the O_3 dose in phytomedical terms; MATYSSEK *ET AL.* 2006a) is biologically more meaningful (MATYSSEK *ET AL.* 2003, UDDLING *ET AL.* 2004). Still, there is some uncertainty, as most of the knowledge on O_3-dose-response interactions is derived from studies with young trees, which might not respond the same way as adult trees (KOLB *ET AL.* 2001).

It has been suggested, that O_3 stress predisposes plants to further stressors like drought and forest pests which eventually causes break-down of trees and forests (c.f. MILLER *ET AL.* 1999). So, several studies primarily focused on the O_3 impact on stem increment (FUHRER *ET AL.* 1997, KARLSSON *ET AL.* 2004b, PLEIJEL *ET AL.* 2004, UDDLING *ET AL.* 2004), an economic damage relevant for policy decisions (CLRTAP 2004), but as was shown, the physiology of trees can be affected by O_3 (BLUMENRÖTHER *ET AL.* 2007, HABERER *ET AL.* 2007) without impairment of growth at the same time (WIPFLER *ET AL.* 2005, c.f. MATYSSEK *ET AL.* 2007). Still, responses on the leaf and cell level can affect the fitness of trees

through changes in whole plant carbon allocation (MATYSSEK *ET AL.* 2005) and ontogenetic development (SANDERMANN *ET AL.* 2004).

The following hypotheses were examined:

(1) The Cumulative ozone uptake (COU) rather than exposure-based O_3 indices like AOT40 reflects the risk of O_3-induced damage (e.g. in stem production).

(2) The effect of drought on adult *F. sylvatica* trees is aggravated by enhanced ozone levels.

(3) Ecophysiological parameters (e.g. photosynthesis, stomatal conductance, chlorophyll fluorescence) are negatively affected by chronic O_3 stress.

(4) Deleterious effects of O_3 are dose-dependent and thus the size of differences between treatments in photosynthetic measurements are positively related to COU.

The examined ecophysiological parameters were light-saturated CO_2 uptake rate (A_{max}), stomatal conductance to water vapour (g_s), maximum rate of carboxylation (Vc_{max}), ribulose-1,5-bisphosphate turnover limited rate of photosynthesis (J_{max}), CO_2 compensation point (CP), apparent quantum yield of net CO_2 uptake (AQ), carboxylation efficiency (CE), day- and nighttime respiration) and chlorophyll fluorescence (electron transferrate, ETR).

Additionally to the stomatal conductance, the impact of O_3 on the internal conductance to CO_2 movement (g_i) was examined. g_i might limit photosynthesis to the same extent as the stomata movement (WARREN *ET AL.* 2003) as g_i is not infinite as previously suggested (LONG *ET AL.* 2003). Additionally, as O_3 affects the permeability of membranes (HEATH *ET AL.* 1997), alters the lignification of cell walls and can cause cell death (MATYSSEK *ET AL.* 2003), g_i might be lower under enhanced O_3 levels due to structural changes in the leaf, and, therefore, causing a significant draw-down from C_i (intercellular CO_2 concentration) to C_c (chloroplastic CO_2 concentration). The calculation of Vc_{max}, a parameter widely used for mechanistic modelling of photosynthesis (VON CAEMMERER 2000), based on C_c might therefore improve the modelling accuracy, especially when information on the impact of O_3 on g_i can be incorporated.

The impact of drought in 2003 was assessed and characterised by measurements of the twig and soil water potentials and additionally by the ratio of ETR/g_s as suggested by MEDRANO *ET AL.* (2002).

2.2 Material and Methods

At the research site "Kranzberger Forst", stomatal conductance, photosynthetic characteristics, water potential, chlorophyll levels and stem growth of the beech trees were examined during the exceptional drought of 2003 and compared to findings from the year 2004, which represented the long-term average at the site (See section 1.6).

2.2.1 Assessment of leaf gas exchange

Gas exchange and chlorophyll fluorescence parameters were assessed using a Licor 6400 CO_2/H_2O diffusion porometer equipped with a "Leaf Chamber Fluorometer 6400-40" (Li-Cor, Lincoln, USA). Measurements were made at 40 – 60% relative air humidity, 360 µmol mol^{-1} CO_2 of the ambient air and saturating light conditions (1500 µmol $m^{-2}s^{-1}$ photosynthetic photon flux density, PPFD) within the chamber. Leaves were acclimated to the chamber conditions for at least 2 min until gas exchange readings became stable. Measurements were then recorded every 10 s for at least 3 min. A saturating light flash (duration 0.8 s, PPFD > 7000 µmol $m^{-2}s^{-1}$) concluded each measurement to determine the electron transport rate of PSII (ETR, see Table 1.2). g_i was assessed after HARLEY *ET AL.* (1992), c.f. WARREN (2006)). For the assessment of light and CO_2 responses of leaf gas exchange, as well as day- and night-time respiration in sun and shade leaves a LI-6400-02(B) chamber was used.

Relative humidity within the chamber was set close to ambient conditions (40 – 60%), while leaf temperature was kept constant around 25°C. Due to climatic conditions encountered at the site, this temperature was not always achieved, but did not exceed 30°C during measurements.

The IR gas analyser of the Licor 6400 had been calibrated before measurements by means of Wösthoff gas mixing pumps (Wösthoff oHG, Bochum, Germany) and a dew point generator (Kr-Kw30/7, Walz, Effeltrich, Germany).

The O_3-responses under $2xO_3$ of A_{max}, g_s, and ETR to COU during the years 2003 and 2004 years were expressed relative to respective responses under $1xO_3$ measured on the same days (cf. BORTIER *ET AL.* 2000; NOVAK *ET AL.* 2005). The response level at $1xO_3$ was set as 100% for each respective day. As COU differed on each respective day between the two O_3 regimes, the COU at $1xO_3$ is given for as a reference in Figures 2.4 and 2.5.

2.2.2 Assessment of climatic data, water potential and stem increment

The ozone indices AOT40 and COU (parametrised and based on measurements from the site and adapted for drought conditions (NUNN *ET AL.* 2005a) were calculated as described previously (see section 1.3). Micro-climatic data were either measured at the research site or at a forest clearing at 1 km distance. Regarding weather conditions and O_3 regimes, 2002 was included additionally for comparison.

In June, August and October 2003 and additionally in July 2004 (as a reference under non-limiting water supply) the pre-dawn twig water potential was measured. Foliated twigs of 30 cm length were cut, stored in plastic bags until measurement (Scholander pressure bomb, model 3000, Soilmoisture Equipment Corporation, Santa Barbara, USA).

The annual increment of radial stem growth at breast height was monitored on each study tree with permanent girth measurement tapes (model D1, UMS, München, Germany) across the years 2001 through 2004 (2001 as reference for the subsequent years).

2.2.3 Statistics

The statistical tests employed, "General linear model", Mann-Whitney-U test, Wilcoxon test, linear and hyperbolic regression analysis, are described in detail in LÖW *ET AL.* (2006) and LÖW *ET AL.* (2007), see Appendix A.

2.3 Results

The years 2002, 2003 and 2004 had contrasting weather conditions (see Figure 1.2). As described previously (see section 1.6), the sunny and dry weather conditions during the summer of 2003 favoured the formation of O_3 and therefore had highest values for the exposure based indices SUM0 and AOT40, but not for COU (Table). During each of the three study years, AOT40 exceeded the "Critical Level of Ozone" of 10 µl O_3 l^{-1} h which is proposed by UNECE (FUHRER *ET AL.* 1997) – or 5 µl O_3 l^{-1} h as suggested recently by KARLSSON *ET AL.* (2004b) even at 1xO_3. But, COU under 1xO_3 was lower in 2003 (by a factor of 0.8) than during the two more humid years, although annual SUM0 and AOT40 were highest (Table 2.1). In 2xO_3, COU of 2003 only slightly exceeded the COU levels of each of the two humid years (by a factor of 1.1).

Table 2.1: O_3 regimes at "Kranzberger Forst" during the years 2002, 2003 and 2004. SUM0 is the "Sum of all O_3 concentrations", AOT40 is the "Accumulated exposure over a threshold of 40 nl O_3 l^{-1}, COU is "cumulative ozone uptake". After LÖW *ET AL.* (2006).

		Year		
		2002	2003	2004
SUM0 1xO_3	[µl O_3 l^{-1} h]	126.0	193.6	142.7
SUM0 2xO_3	[µl O_3 l^{-1} h]	234.0	357.0	232.6
AOT40 1xO_3	[µl O_3 l^{-1} h]	16.0	33.0	17.3
AOT40 2xO_3	[µl O_3 l^{-1} h]	67.0	117.0	63.0
COU 1xO_3	[mmol m^{-2}]	22.5	18.9	24.3
COU 2xO_3	[mmol m^{-2}]	28.2	32.2	28.0

In 2003, the relationship between COU and AOT40 was approximately linear until July 31 at 1xO_3 and August 2 at 2xO_3 (Figure 2.1 B, breakpoints indicated by arrows), the dates when the soil water content approached its minimum (c.f. Figure 1.2). The slope declined significantly at this point, as COU decreased per unit AOT40 under water limitation (Figure 2.1 B). In the years with unlimited water supply, no such breakpoints were identified (Figure 2.1 A,C).

The change of the slopes at the breakpoints in 2003 was driven by stomatal regulation as g_s was strongly reduced (> 70%) under drought conditions (Figure 2.2) when pre-dawn twig water potential reached -1.3 MPa (see Appendix A). Throughout the summer, g_s stayed at a low level. Remarkably, g_s under 2xO_3 was already low in June, before the onset of drought. Comparing g_s between 2003 and 2004, g_s of 2xO_3 was not as sensitive to drought as under 1xO_3. During 2004, the overall annual

course of g_s was significantly decreased under $2xO_3$ ($p = 0.039$). A_{max} was significantly higher during 2004 in both O_3 regimes compared to 2003 ($p < 0.05$ for each O_3 regime), however, no overall effect of O_3 on A_{max} was found, except for September 2004 (decreased A_{max}, $p = 0.048$, Figure 2.2). ETR was very sensitive to drought in both O_3 regimes as a steep continuous decrease was found between July and October 2003, however, no difference between the $1xO_3$ and $2xO_3$ was identified (Figure 2.2C). During 2004, ETR was significantly reduced by O_3 only in May, but was lower throughout July and September. The relationship between ETR and stomatal conductance distinctly differed between 2003 and 2004, as a correlation was apparent only during the humid year ($r^2 = 0.63$, $p < 0.001$), but not under the drought of 2003 ($r^2 < 0.01$, see Appendix A).

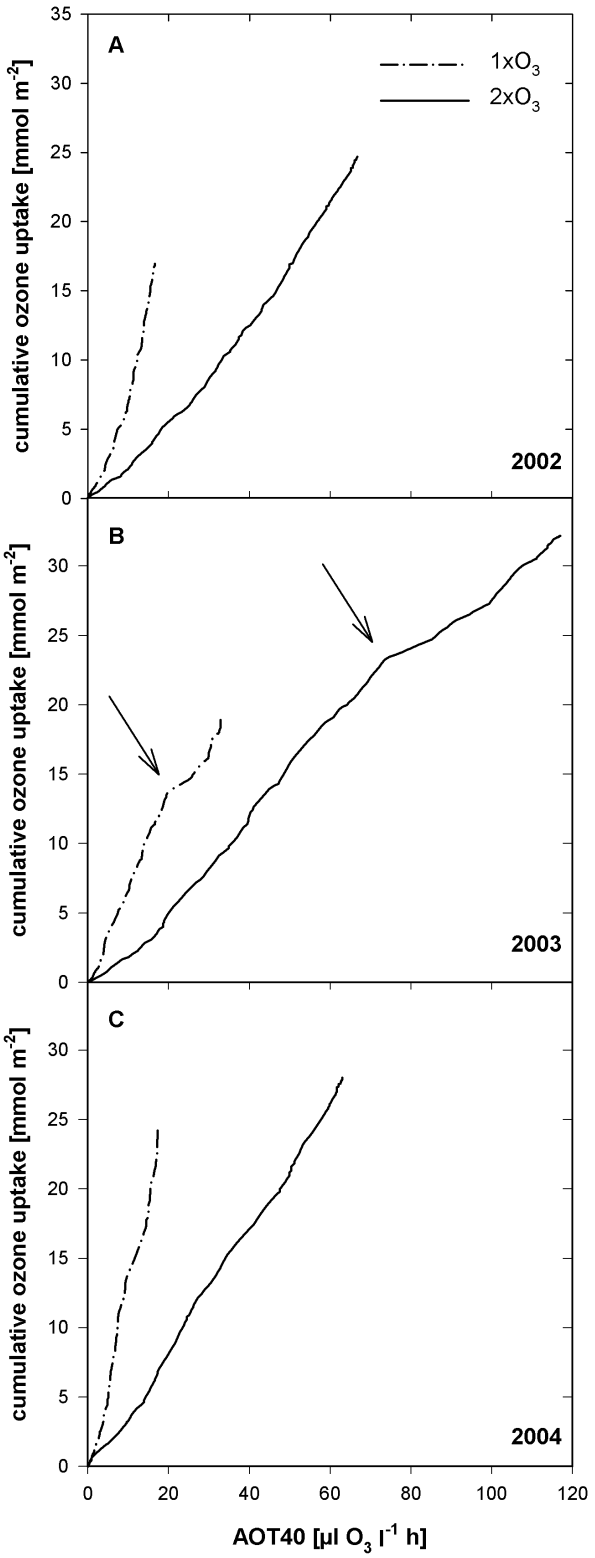


Figure 2.1: Relationship between AOT40 and cumulative ozone uptake during 2002 (A), 2003 (B) and 2004 (C). Arrows in (B) indicate July 31, 2003 in $1xO_3$ and August 2, 2003 in $2xO_3$, when soil moisture became limiting. From *LÖW ET AL. (2006)*.

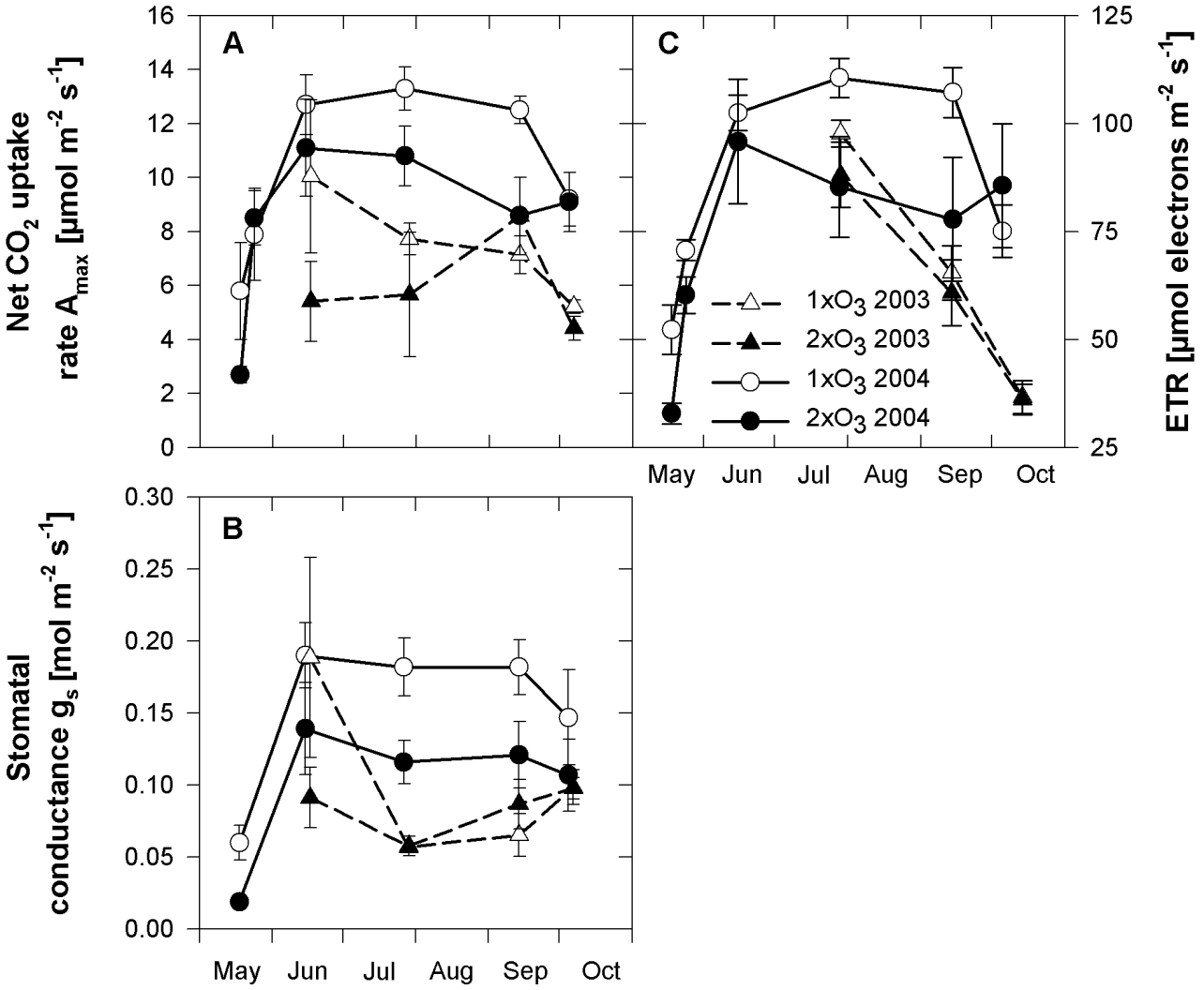


Figure 2.2: Net CO_2 uptake rate (A_{max}) (A), stomatal conductance to water vapour (g_s) (B), electron transport rate (ETR) (C) (all measured at saturating light conditions) during 2003 and 2004 (means ± one standard error of the mean of n = 3–5 trees in each pair of values). Symbols given in (C) are valid also for (A) and (B). After *LÖW ET AL. (2006)*

In 2003, O_3 had no significant effect on photosynthetic and respiratory parameters (Vc_{max}, J_{max}, CP, AQ, CE, r_{day}, r_{night} see Appendix A) even at high COU, whereas in September 2004, Vc_{max} (Figure 2.3, $p = 0.007$), J_{max}, and CE were significantly lower under 2xO_3.

Relationships of parameters with COU were variable. At COU > 20 mmol m^{-2}, in 2004, Vc_{max} was significantly lower under 2xO_3, whereas such an effect was not found in 2003 at even higher COU (Figure 2.3). Vc_{max} was slightly enhanced throughout 2003 in 2xO_3 compared to 1xO_3, which was confirmed in 2004 at COU < 20 mmol m^{-2}, whereas in the shade crowns, mean Vc_{max} was slightly higher in 1xO_3 compared to 1xO_3 in both years.

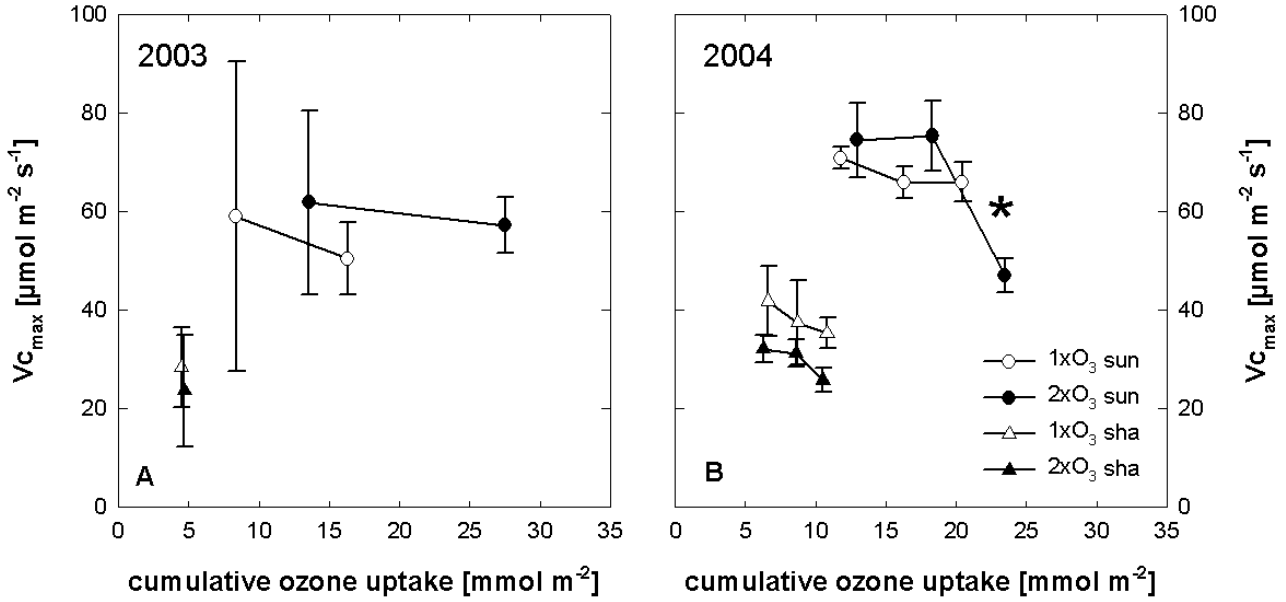


Figure 2.3: The maximum rate of carboxylation (Vc_{max}) during the years 2003 (A) and 2004 (B) of leaves in the sun (sun) and shade (sha) crown of adult *Fagus sylvatica* at "Kranzberger Forst". Means ± standard deviation of 4–5 trees. (*) indicates a statistically significant treatment effect at p = 0.007. Symbols in A as given in B. Second assessment in 2004 from Warren et al. (2006). Vc_{max} was calculated on the basis of C_i. Modified after *LÖW ET AL. (2007)*.

There was no indication, that g_i was affected by O_3, even though C_c tended to be lower in $2xO_3$ compared to $1xO_3$ (see Appendix A). Due to the internal conductance g_i, C_c was lower by 55 µmol $m^{-2}s^{-1}$ compared to C_i in August 2004. Therefore Vc_{max} was increased by 22 – 39 % when calculated on the basis of C_c instead of C_i. However, at the time of the assessment of g_i (i.e. the second assessment of Vc_{max} in 2004, Figure 2.3 B), no reduction in the C_i-based Vc_{max} was found.

The "absolute" and "relative" relationships of A_{max}, g_s, and ETR with COU were generally similar in all the investigated parameters (Figures 2.4, 2.5 B, D, see Appendix A for ETR), but contrasted strongly between 2003 and 2004. However, in each year the "relative" data of mid-May and October were different from the intervening measurements (late May, June, July, August, and September). To avoid confounding physiological responses to O_3 with possible ontogenetic effects (due to incomplete leaf differentiation in spring and senescence in autumn), the mid-May and October data were regarded as non-representative of the summer months so that the first-order linear regression only used late May, June, July, August, and September measurements within the "relative" relationships (Figures 2.4, 2.5 C,D). Remarkably, these ontogenetic effects were not evident in "absolute"

data (Figures 2.4, 2.5 A,B), thus demonstrating the usefulness of "relative"data for examining differences between treatments.

The course of "absolute" A_{max} during 2004 reached a maximum in mid summer (Figure 2.4 B), whereas, such a comparable course was not observed during the drought of 2003. Over the whole range of COU, A_{max} did not change much (Figure 2.4 B). The seasonal course of "relative" A_{max} was significantly different ($p = 0.015$) between the two years. Remarkably, a negative correlation of parameter differences (i.e. the differences between O_3 regimes getting smaller with increasing dose) between the two O_3 regimes and COU ($r^2 = 0.88$, $p < 0.05$) was found in 2003, in the absence of an overall O_3 effect on "absolute" A_{max} (Figure 2.4 C, cf. Figure 2.2 A). g_s confirmed the findings on A_{max}, the overall patterns are more or less equal.

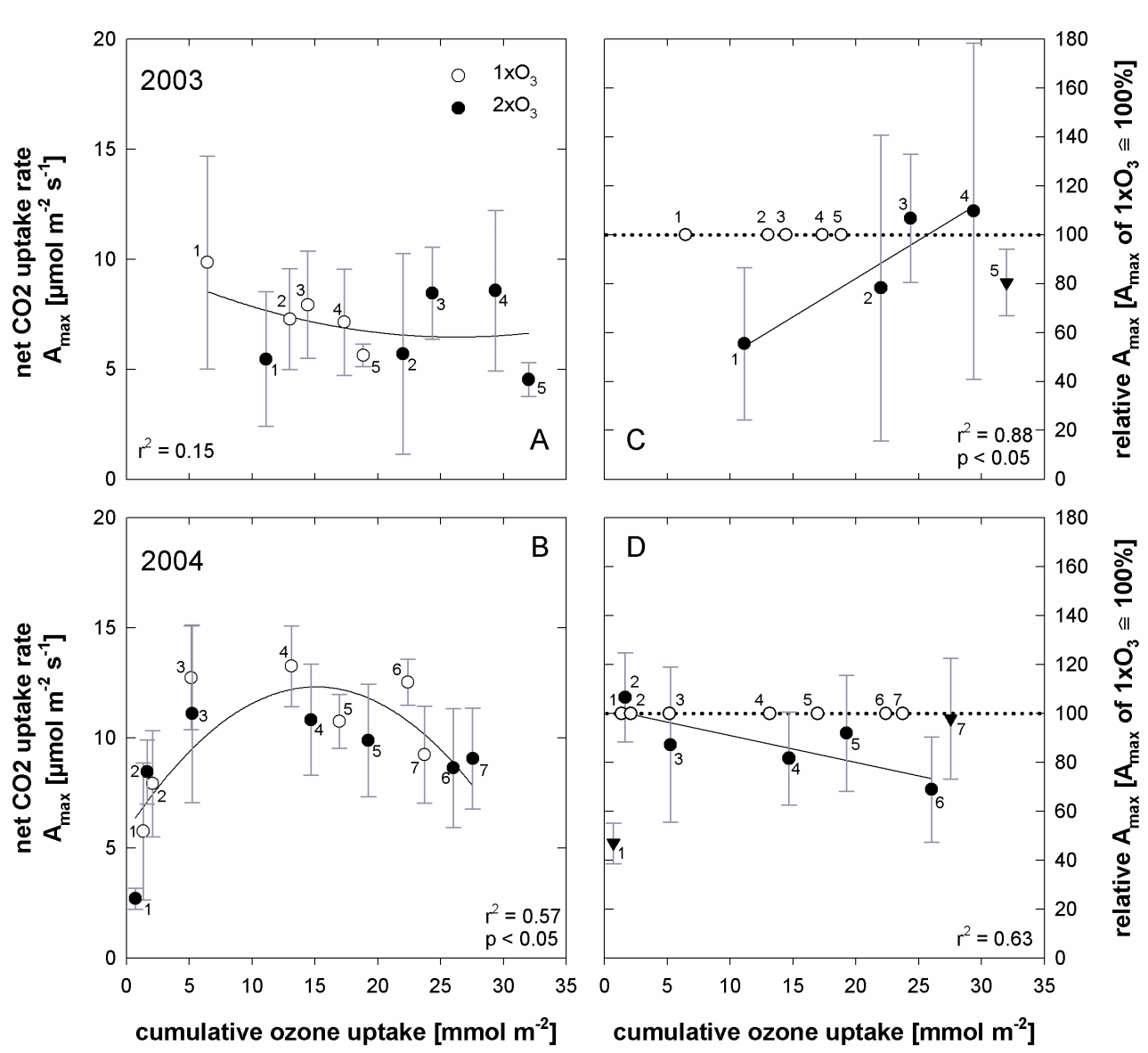


Figure 2.4: "Absolute" light-saturated net CO_2 uptake rate A_{max} during the years 2003 (A) and 2004 (B) and "relative" A_{max} (see "Materials and Methods", section 2.2) during 2003 (C) and 2004 (D). Symbols in B – D as given in A. Triangular symbols in C,D indicate that data were not included in linear regression. Numbers next to the symbols indicate the date of measurement: for 2003 (A,C) 1 = June 17, 2003; 2 = July 29, 2003; 3 = August 11, 2003; 4 = September 14,2003; 5 = October 14, 2003; for 2004 (B,D) 1 = May 18, 2004; 2 = May 24, 2004; 3 = June 16, 2004; 4 = July 27, 2004; 5 = August 11, 2004; 6 = September 14, 2004; 7 = October 5, 2004. In A,B the solid line represents second-order linear regression. In C,D the solid line represents first-order linear regression and the dashed line represents the 100% reference, i.e., $1xO_3$ (see "Materials and Methods"). From LÖW *ET AL.* (2007)

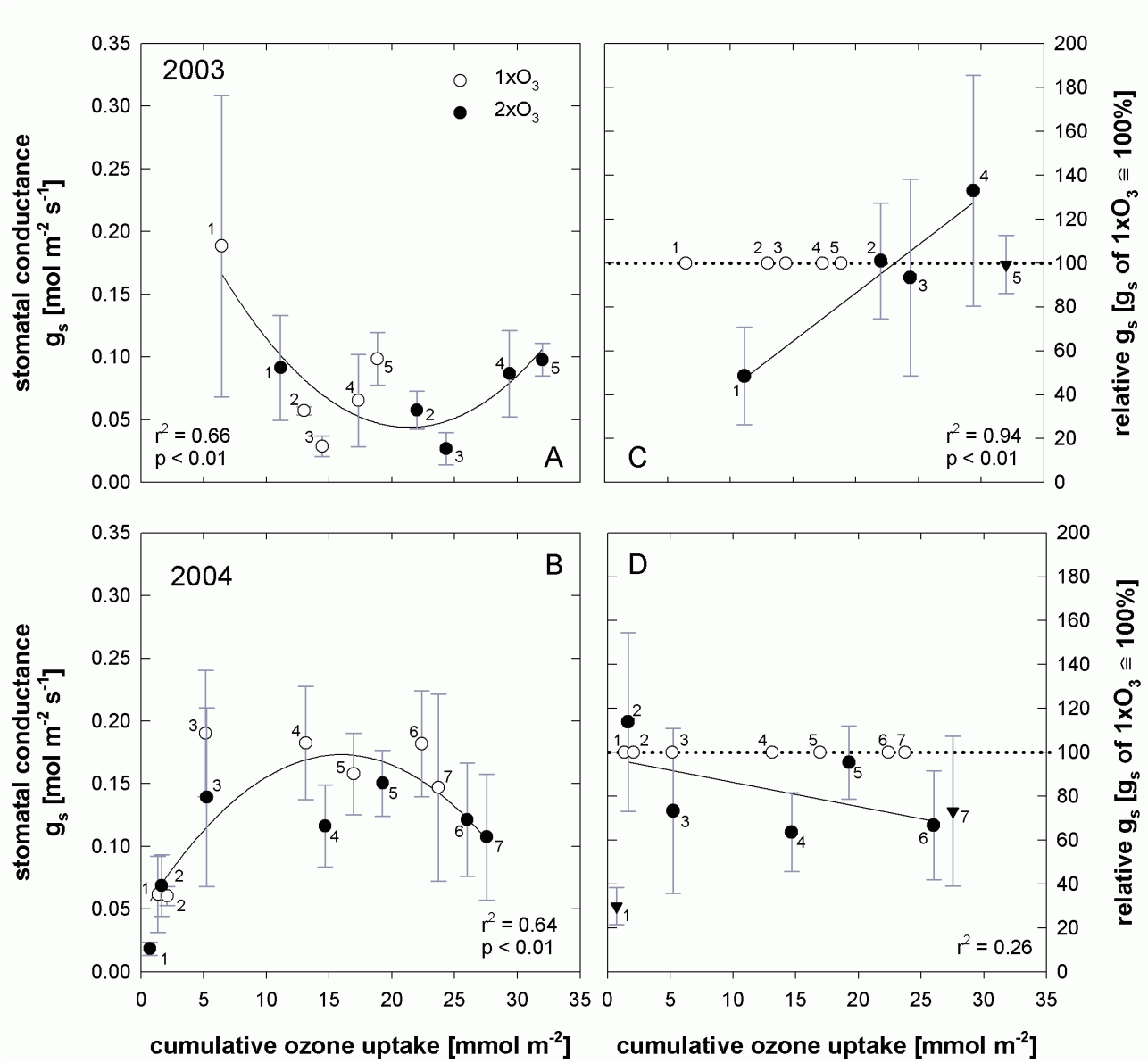


Figure 2.5: "Absolute" stomatal conductance g_s during 2003 (A) and 2004 (B) and "relative"g_s (see "Materials and Methods", section 2.2) during 2003 (C) and 2004 (D). Symbols in B – D as given in A. Triangular symbols in C,D indicate that data were not included in linear regression (see "Results" and "Discussion"). Numbering of dates and meaning of lines as given in Figure 2.4. From LÖW *ET AL.* (2007).

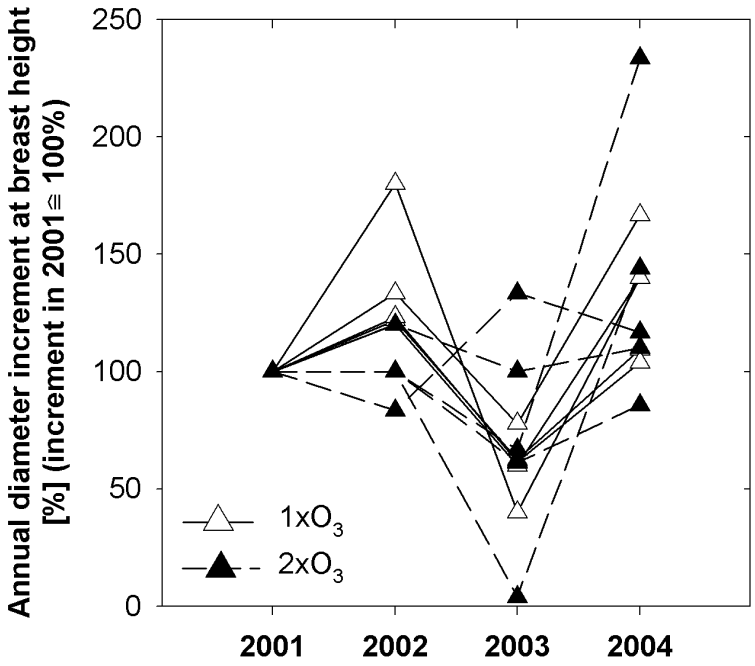


Figure 2.6: Relative annual stem diameter increment at breast height of the ten adult *F. sylvatica* trees examined in this study during the years 2001, 2002, 2003 and 2004 (increment in 2001≙ 100%). From *LÖW ET AL. (2006).*

For the radial stem growth at breast height no O_3 effect was identified, due to the high variability between the ten individual *F. sylvatica* trees. Still, stem growth was limited under the drought of 2003, as nine out of the ten examined trees displayed a reduced annual increment compared with the increment of the year 2001 (Figure 2.6).

2.4 Discussion

The higher external O_3 exposure, as expressed through SUM0 or AOT40, did not lead to an increased uptake of the leaves, which confirmed hypothesis (1) that ozone uptake (COU) rather than exposure-based O_3 indices like AOT40 and SUM0 reflects the risk of O_3-induced damage in 2003. Drought induced stomatal closure and therefore simultaneously limited the possibility of O_3 uptake into plants (MATYSSEK *ET AL.* (2003). Stomatal responses to moisture deficits can limit the O_3 uptake to the extent that the influx of O_3 may be less during sunny, dry days with high O_3 levels than under the lower O_3 regimes of overcast, humid days (WIESER *ET AL.* 1993). The severe drought at "Kranzberger Forst", documented in this study, obviously protects from O_3 impact (RETZLAFF *ET AL.* 2000). Already before the onset of the drought period in 2003, the stomata were closed to some extent in the $2xO_3$ regime. This can be attributed to an O_3-induced change of sensitivity (MATYSSEK *ET AL.* 2003) or inefficient control of the stomatal movements as has been reported before (KELLER *ET AL.* 1984, BARNES *ET AL.* 1990, PEARSON *ET AL.* 1993, KARLSSON *ET AL.* 2004b, PAOLETTI *ET AL.* 2005). Such O_3-induced changes may be mediated through altered "mechanics" in the stomatal apparatus upon reduced cell wall lignification (MAIER-MAERCKER 1998), or the disturbed osmotic control of the guard cells upon membrane impairment (HEATH *ET AL.* 1997). However, it appears to some extent that stomatal regulation stayed intact under O_3 stress, as the low stomatal aperture was maintained throughout the drought period, so that the daily maximum in water tension, represented through Ψ_{min}, was stabilized, which may prevent catastrophic cavitation in the xylem vessels under persisting drought (cf. TYREE *ET AL.* 2002). Additionally, the high ETR with simultaneous low g_s during drought may indicate photorespiration to avoid photoinhibition (GUAN *ET AL.* 2004, MEDRANO *ET AL.* 2002).

During years without limiting water supply, the relationship between O_3 exposure and O_3 uptake was linear (Figure 2.1 A,C). Stomatal closure during the drought of 2003 underlined the shortcoming of exposure-based indices like SUM0 and AOT40 in assessing the O_3 risk to trees (PANEK 2004) as the relationship between COU and AOT 40 was changed (Figure 2.1 B). The maximum O_3 uptake does not necessarily correlate with periods of peak external O_3 levels. SUM0 and AOT40 may adequately correlate with O_3 uptake only when soil moisture is not limiting (PANEK *ET AL.* 2002), which can lead to erroneous conclusions about possible risks of O_3 injury in dry years, as it was exemplified in this study. The modelling of stomatal O_3 uptake by adequate algorithms (EMBERSON *ET AL.* 2000), provides a suitable tool for the assessment of O_3 impact, especially, if the model takes

drought into account (NUNN *ET AL.* 2005a). The improvement of the COU model for drought significantly increased modelling precision of O_3 uptake.

The avoidance of O_3 uptake by stomatal closure is accompanied by impaired CO_2 fixation (PANEK *ET AL.* 2001), so that limitations in biomass production and resource allocation may be the "price" of the exclusion of ozone (MATYSSEK *ET AL.* 2003). However, the beech trees at "Kranzberger Forst" were limited by drought rather than O_3 (Figure 2.6), additionally, no O_3 induced biomass reduction was reported (WIPFLER *ET AL.* 2005). Although Hypothesis (1) was accepted in that COU was uncoupled from AOT40, the stem growth was highly buffered (c.f. KÖRNER 2003) against O_3 stress, so that no damage occurred.

As drought did not enhance the susceptibility of trees to O_3 (cf. MATYSSEK *ET AL.* 2006b) at "Kranzberger Forst", suggested reductions in stem growth over the years (DITTMAR *ET AL.* 1999, DITTMAR *ET AL.* 2003) or even a decline of trees and forests (MILLER *ET AL.* 1999) were not confirmed. The effects of ozone and drought led to differential effects on A_{max}, g_s and stem growth, therefore hypothesis (2) was to be rejected.

The size of the O_3 impact became bigger with increasing dose as Vc_{max}, J_{max} and CE were reduced by around 30% only at high COU levels in 2004. Such levels are far above any suggested thresholded CLRTAP (2004). Also, g_i was not affected by O_3 in August 2004 at > 15 mmol m^{-2}, but it cannot be ruled out that the reduction of Vc_{max} in September 2004 (Figure 2.3) might be related to cell collapse under increased O_3 levels (MATYSSEK *ET AL.* 1991) which would impair g_i rather than Rubisco activity. On the other hand, Vc_{max} was not decreased even by the higher O_3 dose encountered in 2003. Therefore, hypotheses (3) had to be rejected as the different investigated ecophysiological parameters were only affected by O_3 under non-limiting water supply. In 2004 there was evidence that the effects of O_3 on relative A_{max}, and g_s were dose-dependent (Figures 2.4, 2.5 D), resulting in bigger differences between the two O_3 regimes with rising dose. Drought turned the relation between the "relative" parameters and COU (Figures 2.4, 2.5 C,D). This might be explained by an increased defence capacity. The high resistance of ETR to O_3 stress at the encountered COU levels of this study (see Appendix A) may well indicate that high concentrations of α-tocopherol and other antioxidative substances (HABERER *ET AL.* 2006) protected the photosynthetic apparatus (KRONFUSS *ET AL.* 1998, De TEMMERMAN *ET AL.* 2002). The absence of a correlation during 2003 between COU and "absolute" A_{max} and g_s (Figures 2.4, 2.5 A) suggests that drought overruled the O_3 impact as O_3 intake was low

under high O_3 exposure (PANEK *ET AL.* 2002). The effects of O_3 on gas exchange were not consistently dose dependent, even though the O_3 doses where large. Hypothesis (4) is therefore rejected in total, but confirmed in a year with average precipitation. In other words, drought changed the O_3 sensitivity per unit of O_3 uptake, i.e., the effective O_3 dose (MATYSSEK *ET AL.* 2004; MUSSELMAN *ET AL.* 2006). In neither year were the responses of photosynthesis and stomatal conductance linearly related to COU throughout the whole season. Early and late in the growing season the relationships were different, as leaf ontogeny might interfere with O_3 impact.

The inconsistency of responses to O_3 at the leaf level between 2003 and 2004 (cf. MATYSSEK *ET AL.* 2007) and the absence of an effect on stem growth in the short term (WIPFLER *ET AL.* 2005) supports the view that growth in adult trees is highly buffered against O_3 stress. Nevertheless, adverse effects of O_3 on future stem growth (DECKMYN *ET AL.* (2007) as shown by modelling) and, in particular, on tree fitness (cf. MATYSSEK *ET AL.* 2005) cannot be ruled out during the extended life spans of trees (MATYSSEK *ET AL.* 2007).

3 Visualisation and diagnosis of leaf injury by chlorophyll fluorescence imaging

3.1 Detection of leaf injury by chlorophyll fluorescence imaging and microscopical survey – a comparison

3.1.1 Introduction

Injuries on the leaf blade by biotic and abiotic factors influence the photosynthetic capacity of individual leaves and therefore may lead to reductions in the overall carbon balance of the plant (CYR *ET AL.* 1993). Injurious agents can be air pollutants, such as O_3, or e.g. insect herbivory, fungal infections or mechanic impacts. Tissue may become impaired or lost during symptom development leading to more or less specific patterns on the leaf blade. In addition, the leaf can respond to injury by compensatory regulation of photosynthesis in the surrounding tissue. These patterns might be detected by light microscopy and chlorophyll fluorescence imaging analysis already during early stages of symptom development (SCHREIBER *ET AL.* 1978, FAORO *ET AL.* 2005, HJORTH *ET AL.* 2006).

In general, assessment of chlorophyll fluorescence may detect injuries before macroscopic symptoms appear, using imaging or non-imaging approaches (CHAERLE *ET AL.* 2001, OBENLAND *ET AL.* 2005). This holds true also in view of O_3 impact (GUIDI *ET AL.* 1997, SOJA *ET AL.* 1998, LEIPNER *ET AL.* 2001, GIELEN *ET AL.* 2006a). Likewise, it is possible to evaluate O_3 injuries by microscopic means before they become macroscopic (FAORO *ET AL.* 2005).

Light microscopy can distinguish between different causes of injuries due to different cellular markers, although the leaf needs to be destructively harvested and prepared for investigation at high magnification which is required for detailed symptom validation. Conversely, chlorophyll fluorescence measurements on intact leaf blades is a fast, typically non-destructive tool, however, distinction between stress agents is limited in leaves which are exposed to field conditions with their multitude of stressors even if the magnification of the chlorophyll fluorescence imaging is high. A combined chlorophyll fluorescence / microscopical approach might allow to diagnose the cellular cause of patterns visible on the leaf blade, and the two-dimensional imaging can give insight into the impact of injuries in specific cell layers.

Visible O_3 symptoms are known to originate from the upper (adaxial) leaf structures (LEIPNER *ET AL.* 2001, VOLLENWEIDER *ET AL.* 2003), especially from the palisade parenchyma (PASQUALINI *ET AL.* 2003, BUSSOTTI *ET AL.* 2005), the tissue mainly driving photosynthesis. Injuries in the palisade parenchyma are also common for other the stressors (due to the multitude of stressors at a natural forest site). The chlorophyll fluorescence signal of leaf cross-sections corresponds to the chlorophyll concentration (VOGELMANN *ET AL.* 2002) so that disturbance is related to the functional status of the available PSII. I hypothesize chlorophyll fluorescence to resemble findings of microscopy in a way that the degree of injury of leaf tissue correlates with the fluorescence signal (Hypothesis 5a), and that the signal mainly originates from the palisade parenchyma of beech, the tissue which is likely to be affected by O_3 (Hypothesis 5b).

The most commonly used parameter of chlorophyll fluorescence analysis is the maximum quantum yield F_v/F_m (LICHTENTHALER *ET AL.* 2005, see Table 1.2). Some studies report on no significant decrease under O_3 influence (NUNN *ET AL.* 2005b, GIELEN *ET AL.* 2006b), some other studies report on such O_3 responses but not throughout the whole experiment (BORTIER *ET AL.* 2001, GRAMS *ET AL.* 1999), perhaps depending on the O_3 dose, although negative effects of O_3 on the quantum yield of PSII can be distinct (CARRASCO *ET AL.* 2001). It is investigated in the present study, whether F_v/F_m is a measure of microscopically validated O_3 injury in symptomatic blades of beech leaves from "Kranzberger Forst" (Hypothesis 6). Focus was on sun crown leaves of adult beech. Whereas most other studies have concentrated on plants grown under controlled conditions, only few of them dealing with adult trees, to assess O_3 impact on leaf blades in detail.

3.1.2 Material and Methods

Sampling

Five leaves were sampled from the sun crowns of four beech trees on September 2, 2003, stored in moist plastic bags and transported to the laboratory within one hour. Sampling focused on different kinds of macroscopic injury on leaf blades independent of its origin. The injuries were typical for the beech trees investigated in this study. No visible indication of beginning autumnal senescence was detected in the leaf blades.

Imaging and microscopy

After sampling, leaves were kept in darkness or under low-light conditions (during processing in the laboratory, PPFD < 5 µmol m^{-2} s^{-1}) for at least 1 hour. In the laboratory, pictures of the whole leaves were taken immediately upon arrival (Film Fuji Sensia 100, camera Pentax Z1, flash Braun mecablitz LZ 5 positioned at a 45° angle relative to the lens axis, Tokina lens 35–70 mm in macro position, f8-22, 1/125 s). All photographs were digitized by a slide scanner later.

Two samples were punched out of each leaf blade with a round chisel (1.5 cm diameter), including the symptomatic area. Detailed photographs of the resulting leaf discs were taken with the above camera equipment (f22, no flash) using additional extension tubes to allow magnification of around 1:1. The leaf discs were then mounted to the Imaging PAM "Micro" head (IMAG-MIC, WALZ, Effeltrich, Germany), covered with an additional microscope slide from the top and pressed down to keep the sample in focus of the Imaging PAM camera. The imaged area was 3.5 mm x 4.5 mm. The chlorophyll fluorescence parameters F_o, F_m and F_v/F_m were determined by applying one saturating light flash.

Immediately after chlorophyll fluorescence imaging, the leaf discs were fixed in glutaraldehyde, stored in closed vials and sent to Dr. P. Vollenweider (Ozone Validation Center, WSL, Birmensdorf, Switzerland) for microscopical assessment and validation of the different symptoms.

On the basis of the photographs and the corresponding chlorophyll fluorescence images, transect lines were marked along which microscopical cuttings were to be performed. Two microscopical cuttings per sample covered different areas of the same symptom, providing two cuts of each leaf disc for microscopical analysis. Therefore, the resulting overall sample size is 10 microscopical cuts from 5 leaves collected from 4 trees.

Preparation of transect cuts, embedding of leaf stripes and microscopical analysis were conducted as described by VOLLENWEIDER *ET AL.* (2003, 2005).

Along each transect cut, the status of the palisade parenchyma and spongy parenchyma was assessed every 100 to 200 µm, looking for O_3-specific "structural markers" of apoplastic and symplastic oxidative stress, accelerated cell senescence, hypersensitive responses (VOLLENWEIDER *ET AL.* 2003, VOLLENWEIDER *ET AL.* 2005) or other leaf injury. The degree of tissue intactness at each position was expressed as a "degree of injury" index (Table 3.1). In the bifacial leaves of beech, two cell layers form the upper palisade parenchyma (c.f. Figure 3.2). Both of them were assessed individually.

Throughout this study, the upper layer of the palisade parenchyma is denominated PP_1, the lower cell layer of the palisade parenchyma is denominated PP_2, whereas the spongy parenchyma is abbreviated SP.

At the 20 to 30 positions (see above) within each microscopical transect, the tissues were classified as given in Table 3.1. The investigated part at each position was as wide as 3 – 5 palisade cells.

Table 3.1: Five-graded classification of tissue status used in the microscopic assessment of the "degree of injury" (DOI) of first and second palisade cell layer of upper palisade parenchyma and spongy parenchyma cells. Adapted from *VOLLENWEIDER ET AL. (2005).*

degree of injury (DOI)	tissue status
4	uninjured
3	slightly injured
2	strongly injured
1	partly necrotic
0	necrotic

The overall "degree of injury" (DOI_{Sum}) summarizes the individual tissue status of the different layers (Table 3.1) with respect to the proportion in the mesophyll occupied by each tissue (Equation 3). Leaf thickness was assessed several times within the transect to calculate the proportion of mesophyll (VOLLENWEIDER *ET AL.* 2005).

$$DOI_{Sum} = DOI_{PP_1} \cdot PIM_{PP_1} + DOI_{PP_2} \cdot PIM_{PP_2} + DOI_{SP} \cdot PIM_{SP} \qquad \text{(Eq. 3)}$$

In equation 3, DOI is „degree of injury" (see Table 3.1), PIM is the „proportion in mesophyll" of the respective tissue, PP_1 is upper layer within the palisade parenchyma, PP_2 is lower layer within the palisade parenchyma and SP is spongy parenchyma.

The comparative analysis of microscopy and chlorophyll fluorescence imaging concentrated on one transect of a leaf which was injured by O_3. Other factors were excluded (VOLLENWEIDER *ET AL.* 2005). The additional samples are part of Appendix B.

The F_o and F_m images were exported from the WALZ ImagingWin Software (Version 1.00b, WALZ, Effeltrich, Germany) as 8bit Tiff-images. Images of F_v/F_m were calculated from F_o and F_m images with ImageJ 1.37v (RASBAND 1997-2006) using a self-programmed plug-in. The resulting F_v/F_m images were stored as 32bit Tiff-images with pixel intensities ranging between 0 and 1.

The exact position of the DOI assessments on the chlorophyll fluorescence images were determined by trigonometric methods (accuracy < 20 µm) relative to the site of microscopic assessment.

Actual DOI levels were compared to the corresponding means of pixel squares (size: 3x3 pixels) in the chlorophyll fluorescence images.

Statistics

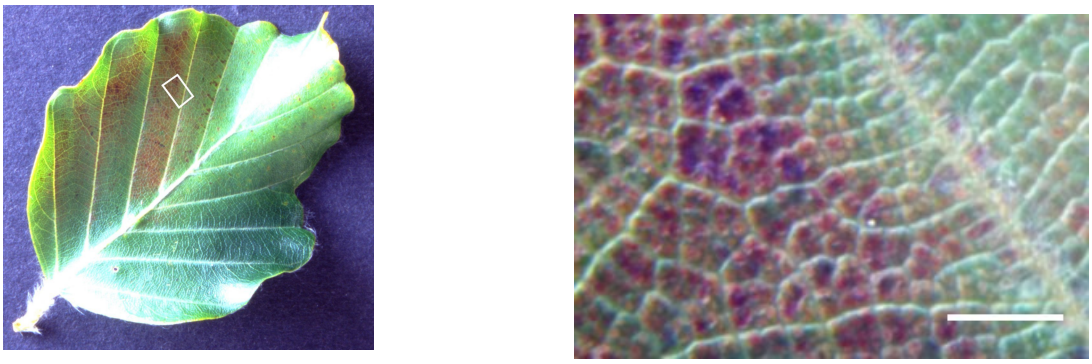
The relationship between the parameters derived from microscopy and chlorophyll fluorescence imaging were examined via Pearson correlation analysis (SPSS 14, SPSS Inc, Chicago, USA). To identify possible driving factors which account for the majority of the variance in the obtained data, "Principal component analysis" using "Kaiser criterions" (SPSS 14) was conducted for the transects individually and for the samples altogether. To obtain a clear pattern of the resulting loadings per component, the factors were rotated by the "varimax" method.

Mean levels of chlorophyll fluorescence parameters from "symptomatic" leaf parts of the transect were compared with those from "asymptomatic" parts by Student´s T-test (SPSS 14).

3.1.3 Results

The leaf samples from "Kranzberger Forst" displayed several symptoms of different origin. The most abundant causes (determined by light microscopy) of leaf injury were: insect stitching, drought, microbial infection, mechanical impact and O_3. Eight out of the ten samples were "multi-symptomatic" so that injuries of different origins were found along each transect. The detailed results of all samples are given in Appendix B. In this section, only one sample from the leaf with distinct O_3 symptoms ("mono-symptomatic") is exemplified in detail (other stressors than O_3 excluded: VOLLENWEIDER *ET AL.* 2005, see Materials and Methods section 3.1.2).

The picture of the whole leaf in Figure 3.1 (A) shows brown, O_3-induced stippling on the upper left side of the primary vein and unaffected green tissue in other parts of the leaf blade. The O_3 symptoms are characterised by confluent, strictly intercostal stippling (Figure 3.1 B) extending throughout PP_1 and PP_2 in some cases (Figure 3.2 A). Indicative of O_3 impact are the hyper-sensitive-like reactions in cells of the palisade parenchyma layer and accelerated senescence in the surrounding cells (VOLLENWEIDER *ET AL.* 2005).



A B

Figure 3.1: (A) Photograph of a leaf with brown stippling caused by O_3. The white frame marks the area further investigated by microscopy and chlorophyll fluorescence imaging. (B) Magnification of the investigated area. Length of white scale bar: 5 mm. See Figure 2 for further details.

The individual positions of the microscopical DOI assessments were assigned to the corresponding positions in the Imaging PAM images and are displayed on the F_o, F_m and F_v/F_m images in Figure 3.2 (bcd) as small squares. From right to left, the investigated areas follow a gradient of tissue without any O_3 injury towards severely degraded vitality across a transect of about 4000 µm. F_o and F_m (Figure 3.2, BC) show highest observed levels in the area marked with a white bar above Figure 3.2 A (right of the secondary leaf vein and within 500 µm left of the vein). In the area marked by the black bar above Figure 3.2 A, both parameters display significantly lower levels in the symptomatic part of the transect ($p < 0.001$ for F_o and F_m each, t-Test,) compared to the remaining area. The low F_o and F_m levels match with the dark red-brown areas on the real-colour photograph of the leaf (Figure 3.1 B, Figure 3.2 a) and the collapsed palisade cells in Figure 3.2 A.

F_v/F_m does not show a similar change along the transect (Figure 2, D; absence of statistically significant differences, i.e. $p = 0.347$, between compared leaf areas; levels stay at 0.5 to 0.7 across the transect). These F_v/F_m levels were representative for the sun foliage of the investigated trees (data not shown). The standard deviation of F_v/F_m increased in the area with severe stippling (cf. Figure 3.2 A). The image of F_v/F_m (Figure 3.2 d) was not that detailed as the images of F_o and F_m in that leaf veins and the area of O_3 stipples were barely visible. The area of lowered F_v/F_m is smaller and more diffuse than the brown area in the real-colour photograph or the distinct areas of lowered F_o and F_m. These findings are confirmed by a second transect (see Appendix B, Figure 6.8).

To clarify the relationship between the investigated microscopic parameters (DOI of PP_1, PP_2, SP, DOI_{Sum}) and fluorescence indices (F_o, F_m, F_v/F_m) Pearson correlations were calculated (Table 3.2). F_o and F_m are significantly correlated with DOI_{PP1}. F_m correlated with F_o , however, F_v/F_m was neither correlated with any DOI, nor with any other chlorophyll fluorescence parameter (Table 3.2). All individual DOIs of the three tissues were significantly correlated with DOI_{Sum}.

Pooling all ten microscopical transect cuts and chlorophyll fluorescence assessments of this study, which cover a broad range of different leaf injury (see Appendix B), reveals many statistically significant relationships (Table 3). The DOI of one tissue is always highly correlated with that of the two other tissue types each. Again, all three individual tissue DOIs are closely correlated with DOI_{Sum}. The chlorophyll fluorescence parameter F_m was significantly correlated with the DOI of all tissue types, but F_o and F_v/F_m were not. F_o is only correlated with the DOI of PP_1, and F_v/F_m only with that of PP_2.

The major influential components, which explain most of the variance within the investigated parameters, were described by "Principal component analysis". Two major components were extracted from the six investigated parameters (DOI of PP_1, PP_2 and SP each, F_o, F_m and F_v/F_m). The initial eigenvalue of component 1 explained 39% of the total variance (Table 3.4 A) and had high loadings on the chlorophyll fluorescence parameters (Table 3.4 B). The eigenvalue of component 2 explained around 22% (Table 3.4 A) of the overall variance and was mainly driven by the DOI of the individual tissues (Table 3.4 B). Thus, 39% of the overall variability in the description of the sampled leaf tissues can be explained by the chlorophyll fluorescence parameters.

Figure 3.2 (next page): Comparison of a microscopical cut with results of F_o, F_m and F_v/F_m assessments in a leaf with O_3 stipples in the absence of other injury (further details on next page).

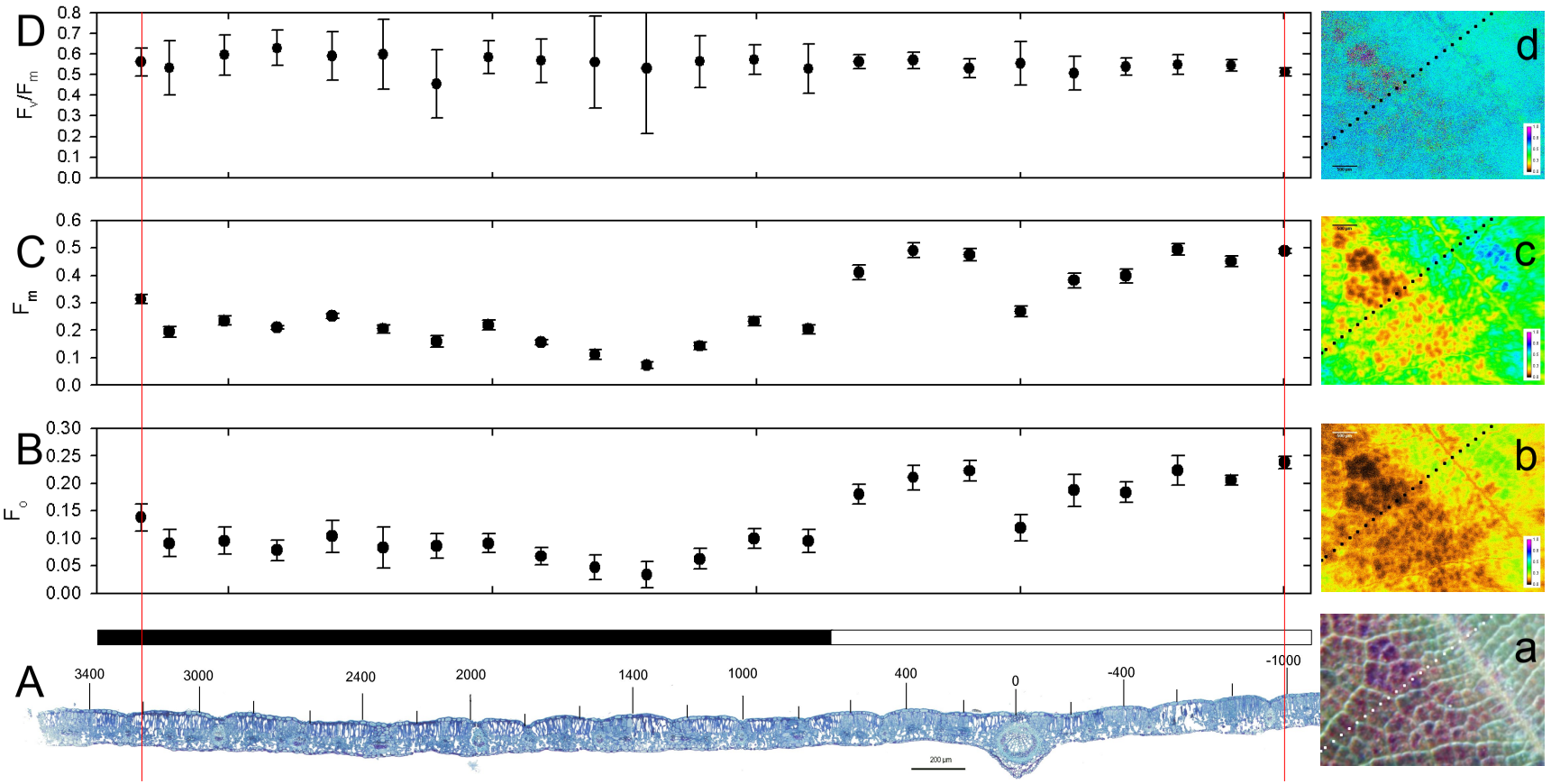


Figure 3.2: Comparison of a microscopical cut with results of F_o, F_m and F_v/F_m assessments in a leaf with O_3 stipples in the absence of other injury. (A) microscopical cut. Numbers in (A) give the distance to the secondary leaf vein at position 0 [µm]. Bars above (A) mark the asymptomatic (white) and symptomatic (black, severe O_3 injury) parts of the transect. (a) real-colour photograph of the investigated leaf area, white squares indicate the positions of microscopical DOI assessments. F_o (B), image of F_o (b), F_m (C), image of F_m (c), F_v/F_m (D), image of F_v/F_m (d). Data points in (B), (C), (D) represent means ± SD of 9 pixels from the positions indicated by black squares in (b), (c), (d).

Table 3.2: Pearson correlation table summarizing the relationships between the individual DOIs of PP_1, PP_2 and SP, the overall DOI (DOI_{Sum}) assessed by microscopy, and chlorophyll fluorescence measurements (F_o, F_m, F_v/F_m) for a leaf with distinct O_3 injury. Data derived from two transects.

		DOI_{PP1}	DOI_{PP2}	DOI_{SP}	DOI_{Sum}	F_o	F_m	F_v/F_m
DOI_{PP1}	Pearson Correlation	1	0.265	-0.370(*)	0.524(**)	0.763(**)	0.765(**)	0.020
	Sig.		0.166	0.034	0.001	0.000	0.000	0.914
	N	39	29	33	39	33	33	33
DOI_{PP2}	Pearson Correlation	0.265	1	0.067	.591(**)	0.212	0.211	0.192
	Sig.	0.166		0.736	0.001	0.309	0.312	0.359
	N	29	29	28	29	25	25	25
DOI_{SP}	Pearson Correlation	-0.370(*)	0.067	1	0.390(*)	-0.258	-0.284	-0.002
	Sig.	0.034	0.736		0.023	0.185	0.143	0.993
	N	33	28	34	34	28	28	28
DOI_{Sum}	Pearson Correlation	0.524(**)	0.591(**)	0.390(*)	1	0.327(*)	0.359(*)	0.254
	Sig.	0.001	0.001	0.023		0.045	0.027	0.124
	N	39	29	34	45	38	38	38
F_o	Pearson Correlation	0.763(**)	0.212	-0.258	0.327(*)	1	0.990(**)	0.039
	Sig.	0.000	0.309	0.185	0.045		0.000	0.802
	N	33	25	28	38	43	43	43
F_m	Pearson Correlation	0.765(**)	0.211	-0.284	0.359(*)	0.990(**)	1	0.157
	Sig.	0.000	0.312	0.143	0.027	0.000		0.313
	N	33	25	28	38	43	43	43
F_v/F_m	Pearson Correlation	0.020	0.192	-0.002	0.254	0.039	0.157	1
	Sig.	0.914	0.359	0.993	0.124	0.802	0.313	
	N	33	25	28	38	43	43	43

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Table 3.3: Pearson correlation table summarizing the relationships between the individual DOIs of the tissues (PP_1, PP_2 and SP), the overall DOI (DOI_{Sum}) assessed by microscopy, and chlorophyll fluorescence measurements (F_o, F_m, F_v/F_m). The table integrates all ten microscopical cuttings and chlorophyll fluorescence assessments. The ten samples covered a broad range of leaf injury (see Appendix B).

		DOI_{PP1}	DOI_{PP2}	DOI_{SP}	DOI_{Sum}	F_o	F_m	F_v/F_m
DOI_{PP1}	Pearson Correlation	1	0.442(**)	0.337(**)	0.790(**)	0.203(**)	0.454(**)	0.065
	Sig.		0.000	0.000	0.000	0.002	0.000	0.326
	N	267	242	235	267	234	234	234
DOI_{PP2}	Pearson Correlation	0.442(**)	1	0.387(**)	0.703(**)	-0.050	0.228(**)	0.223(**)
	Sig.	0.000		0.000	0.000	0.467	0.001	0.001
	N	242	243	223	243	213	213	213
DOI_{SP}	Pearson Correlation	0.337(**)	0.387(**)	1	0.741(**)	0.090	0.233(**)	0.020
	Sig.	0.000	0.000		0.000	0.193	0.001	0.773
	N	235	223	239	239	209	209	209
DOI_{Sum}	Pearson Correlation	0.790(**)	0.703(**)	0.741(**)	1	0.152(*)	0.400(**)	0.105
	Sig.	0.000	0.000	0.000		0.018	0.000	0.104
	N	267	243	239	276	241	241	241
F_o	Pearson Correlation	0.203(**)	-0.050	0.090	0.152(*)	1	0.657(**)	0.176(**)
	Sig.	0.002	0.467	0.193	0.018		0.000	0.002
	N	234	213	209	241	300	300	300
F_m	Pearson Correlation	0.454(**)	0.228(**)	.233(**)	0.400(**)	0.657(**)	1	0.439(**)
	Sig.	0.000	0.001	0.001	0.000	0.000		0.000
	N	234	213	209	241	300	300	300
F_v/F_m	Pearson Correlation	0.065	0.223(**)	0.020	0.105	0.176(**)	0.439(**)	1
	Sig.	0.326	0.001	0.773	0.104	0.002	0.000	
	N	234	213	209	241	300	300	300

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 3.4: Initial eigenvalues (A) of the six individual parameters (DOI_{PP1}, DOI_{PP2}, F_o, F_m and F_v/F_m) extracted from all transects (n = 10). Rotated component matrix (B) of the two extracted components with the individual loads of the six investigated parameters.

A

	Initial Eigenvalues		
Component	Total	% of Variance	Cumulative %
1	2.36	39.38	39.38
2	1.35	22.45	61.83
3	0.97	16.13	77.96
4	0.68	11.27	89.22
5	0.45	7.41	96.64
6	0.2	3.36	100

B

	Component	
	1	2
DOI_{PP1}	0.259	0.710
DOI_{PP2}	-0.056	0.841
DOI_{SP}	0.148	0.699
F_o	0.875	-0.062
F_m	0.892	0.291
F_v/F_m	0.504	0.119

3.1.4 Discussion

The distinct areas of cells with increased DOI shown in the microscopical cut and in the photograph of the leaf section are not detectable by all chlorophyll fluorescence parameters. But, there is a close relationship between the DOI of leaf tissues and chlorophyll fluorescence signals (Table 3.2) in the case of beech leaves, which confirms hypothesis (5a). Hence, the chlorophyll fluorescence signal (F) is not only correlated with the amount of chlorophyll (VOGELMANN *ET AL.* 2002, LICHTENTHALER *ET AL.* 1998), it can also express the "DOI" (as defined in this study) of a leaf cross-section. Of course, the DOI score incorporates information on the photosynthetic tissues (VOLLENWEIDER *ET AL.* 2005) and therefore on the chlorophyll content. Nevertheless, the defined DOI is a measure based on anatomy.

The lowered F_o in the symptomatic parts of the transect might be due to photoinhibition (DEMMIG *ET AL.* 1987, MAXWELL *ET AL.* 2000), a result of injured or fully closed (reduced) Q_A in the necrotic areas. Hence, the photosynthetic energy conversion is severely degraded. F_o and quenching of F_o is closely related to the antenna pigments (LHC, LICHTENTHALER *ET AL.* 2005, MAXWELL *ET AL.* 2000) and should be lowered in areas of impaired PSII reaction centres, as it is the case in Figure 3.2 (B) in the symptomatic parts of the transect. Only in rare cases F_o seems to be unaffected by O_3 (CIOMPI *ET AL.* 1997). F_m is closely related to the chlorophyll concentration (VOGELMANN *ET AL.* 2002), and is lowered in the symptomatic area due to collapsed cells (Figure 3.2 A,C).

In the example of an O_3-stressed leaf, there was neither a correlation between the commonly used parameter F_v/F_m and the DOI of individual leaf tissues nor with DOI_{Sum} (Figure 3.2, Table 3.2). This might explain why in many cases O_3 injury cannot be detected by F_v/F_m measurements alone (c.f. LEIPNER *ET AL.* 2001). O_3 affects cells in PP_1 in the first place, as can be seen by the collapsed cells and disrupted cell contents (Figure 3.2), and which is reflected by significant correlations between F_o and F_m with the DOI_{PP1} (Table 3.2). Also, LEIPNER *ET AL.* (2001) reports on a O_3 induced decrease of Φ_{PSII} measured by chlorophyll fluorescence imaging in the areas surrounding stomata on the upper leaf side of *Phaseolus vulgaris*. But the conclusions given there are not applicable to beech leaves, as no O_3 induced injuries were identified surrounding the stomata (i.e. only at the lower leaf side). Additionally, the O_3 induced injuries of this study where much larger than reported by LEIPNER *ET AL.* (2001). The PP_2 cell layer and SP remained unharmed or were only slightly affected.

Remarkably, F_v/F_m was not closely related to the tissues which correlated with F_o and F_m. F_v/F_m is a measure for the maximum efficiency at which absorbed light reduces Q_A. Therefore, F_v/F_m should be low in leaf areas where cells are injured and the cell content is disrupted (Figure 3.2). Unchanged F_v/F_m might be due to the fact that both F_o and F_m are low in the area of O_3 stipple and high in the asymptomatic area of the transect, as O_3 induces photoinhibition and affects the chlorophyll concentration. Thus, the ratio of the two parameters, F_v/F_m, remains constant as displayed in Figure 3.2 C. Additionally, the F_v/F_m calculation suffers from a low signal-to-noise ratio in the necrotic parts of the transect, as both F_o and F_m are close to the suggested lower resolution limit of the fluorescence equipment (WALZ 2005). This might explain to some extent the large standard deviations of F_v/F_m in the symptomatic part. Still, this problem cannot be avoided as the basic settings of the Imaging PAM have to cover the symptomatic and asymptomatic parts of the investigated leaf so that inevitably the fluorescence signal from necrotic parts is low.

Due to these biological and technical reasons, F_v/F_m cannot detect O_3 injury in the leaf blade in a satisfactory way so that additional methods need to be used in investigations on the O_3 impact on leaves. Hypothesis 6 is therefore rejected.

For the assessment of O_3 impact on trees, CLARK *ET AL.* (2000) proposed the use of a chlorophyll fluorescence "performance index" (PI, CLARK *ET AL.* 1999, cf. GRAVANO *ET AL.* 2004) as an "accurate method of O_3 damage diagnosis that can be used regionally". This kind of test is based on the fast kinetics of the chlorophyll fluorescence rise ("JIP" test, reviewed by LAZAR 2006). But, this index is still not sufficient, however, in detecting leaf discolourations even though it "performed" better than

F_o, F_m and F_v/F_m (ROSSINI *ET AL.* 2006) in the case of *Quercus robur*. Thus, the usage of the chlorophyll fluorescence parameters remains difficult in the field of O_3 injury diagnosis. An improvement might be to include additional parameters from chlorophyll fluorescence into measurements which describe the quenching of fluorescence (e.g. NPQ, q_L) and allow better insights in O_3 related responses (see following sections).

Nevertheless, viewing all microscopical transects featuring various leaf injuries in total, F_v/F_m is an adequate measure of the DOI_{PP2} (Table 3.1). Obviously, if cells of the PP_1 layer are injured or "dead", the fluorescence signal may originate from the lower layer (PP_2) which might be unharmed resulting in high F_v/F_m, even though there might be visible injuries on the leaf blade. Of course, lower cell layers within the palisade parenchyma and the spongy parenchyma also contain chlorophyll (VOGELMANN *ET AL.* 2002), and contribute therefore to net photosynthesis to some extent. Due to a relatively uniform decline of the fluorescence signal from the adaxial to abaxial cell layers within a leaf blade (VOGELMANN *ET AL.* 2000), the signal originating from the PP_2 and SP cannot be neglected. These tissues might compensate or overrule the signal from the upper cell layers in the case of injured PP_1. As chlorophyll fluorescence imaging could not detect the severe O_3 impact in PP_1 sufficiently, it must remain open in the case of beech leaves from "Kranzberger Forst", whether the fluorescence signal mainly originates from the tissue of highest chlorophyll content (upper side of the palisade parenchyma), and hence, highest photosynthetic capacity. Hypothesis 5b is therefore rejected.

The results derived from "Principle component analysis" (Table 3.4) indicate that chlorophyll fluorescence indices are valuable tools to describe the degree of leaf intactness. In summary, these indices account for a major part of the variance in the investigated parameters (i.e. the three individual DOI of the tissues and F_o, F_m and F_v/F_m) and therefore can be used independently of microscopy, as exemplified in the following chapters.

3.2 Impact of stressors on the leaf blade visualized by chlorophyll fluorescence imaging

3.2.1 Introduction

At "Kranzberger Forst", additional causes of injury on leaf blades, other than O_3, are herbivorous insects and fungi. The leaf area affected by such pests leads to reduced net primary production in forests and cannot be neglected (CYR *ET AL.* 1993), especially when trees are predisposed to additional stressors like O_3 (MILLER *ET AL.* 1999).

In contrast to air pollutants like O_3 (see previous chapter), tissue is actively removed or injured, whereas in the case of O_3 specific biochemical pathways, which cause cell degradation and cell death, are triggered from within the leaf. This chapter compares the "direct" and "indirect" impact of common stressors at natural stands on photosynthesis, visualized by chlorophyll fluorescence imaging.

ZANGERL *ET AL.* (2002) distinguishes between the "direct" and "indirect" impact of herbivores on photosynthesis. The "direct" impact is the actual amount of removed or affected leaf tissue (consumed leaf area) which is visible to the bare eye. This area is surrounded by the "indirectly" affected area, i.e. parts of the leaf with decreased or changed photosynthesis. The "indirectly" affected leaf area is not visible to the naked eye in most cases. Therefore, the "impact of folivory on photosynthesis is greater than the sum of its holes" (ZANGERL *ET AL.* 2002). This concept also applies to fungal infections (ALDEA *ET AL.* 2006) and eventually to O_3 impact as will be investigated in this study.

I hypothesize, that in the case of beech leaves from "Kranzberger Forst" the "indirectly" affected area is bigger than the area removed by an insect (beech weevil, *Rhynchaenus fagi*), occupied by a plant pathogen (fungi, *Apiognomonia errabunda*) or injured by O_3 (Hypothesis 7a), and that this "indirectly" affected area is characterised by decreased photosynthesis (Hypothesis 7b). Each of the investigated chlorophyll fluorescence parameters (F_m, F_v/F_m, Φ_{PSII}, NPQ, q_L) covers a specific aspect of the electron transport chain and shows individual spatio-temporal patterns. Therefore it is possible to quantify the O_3-affected leaf area (Hypothesis 8, see also previous chapter).

As chlorophyll fluorescence imaging is a non-invasive tool, this method can be used to monitor the status of leaves at their natural positions throughout the growing season. This allows to follow the development of leaf injuries to eventually visualize possible defence reactions and to detect leaf injuries before they are visible to the human eye.

3.2.2 Material and Methods

Experimental design

During the growing seasons 2003 and 2004 chlorophyll fluorescence images were taken of leaves with injures by O_3, herbivory and fungal infection.

During May and June leaves (in some cases specific injured leaves) were selected for repeated chlorophyll fluorescence imaging analysis and marked by small wires (n = 20 leaves in each year). Due to the natural stand conditions with their multitude of natural stressors (e.g. wind, insects, drought) many of these leaves were lost during the growing season or did not display injuries in the selected leaf area, therefore the number of complete time lines was limited (four time lines are shown in this chapter). Measurements were taken throughout the summer to visualize potential symptom development. However, every leaf represents an unique, individual case study and is therefore treated independently. Additionally, leaves with characteristic, distinct symptoms were chosen for detailed analysis.

The spatio-temporal patterns of photosynthesis were visualized by the WALZ Imaging PAM (IMAG-K in combination with IMAG-USH, WALZ, Effeltrich, Germany). The LED-ring and sample holder were modified to allow dark adaptation of the imaged leaf area (17 x 22 mm). A portable table was constructed to set the Imaging PAM equipment up at the natural position of each respective leaf. Calibration with the "red standard" was conducted before the measurements. Due to the heterogeneity within the crowns, the optimal settings for gain, damping and actinic light (WALZ 2005) were chosen individually for each leaf before the dark adaptation. The frequency of the measurement light was always set to 8 Hz. The IMAG-MIC Micro Head (WALZ, Effeltrich, Germany) was used for detailed assessments of fungal necroses.

As the leaves and branches at "Kranzberger Forst" move with the wind even at calm days, dark adaptation was allowed for 5 min, which is sufficient for the following diagnostic test, a light response assessment at ca. 0, 4, 40, 80, 135, 175, 365, 585, 745 µmol $m^{-2}s^{-1}$ PPFD. Each PPFD level was ap-

plied for 2 min as previous tests showed that the chlorophyll fluorescence signal was stable after this time.

For each leaf, an overview and a detailed photographic image were taken (FujiFilm Sensia 100, camera Pentax Z1, flash Braun mecablitz LZ8 at 45° angle relative to lens axis, macro extension tubes depending on desired magnification, f22, 1/125 s). Digitized images were scaled and rotated to match with the chlorophyll fluorescence images either manually (The Gimp 2.2.13, www.gimp.org) or semi-automatically ("Turboreg" PlugIn for ImageJ 1.37v, RASBAND, 1997-2006). Small distortions between the photographs and the fluorescence images were unavoidable due to different cameras, lenses, and distances, but did not interfere with area assessments as angular distortions were smaller than 3% and each photograph had its own known scale.

Fungi: *Apiognomonia errabunda*

The endophytic ascomycete *Apiognomonia errabunda* (Roberge ex Desm.) Höhn (Diaporthales, Valsaceae) is a quite common companion for *F. sylvatica*, especially in the shade leaves (BAHNWEG *ET AL.* 2005). The fungi induces a necrotic lesion of round or irregular shape on the leaf blade caused by destroyed palisade cells. This necrosis is visible to the naked eye.

Herbivore insect: Beech weevil (Rhynchaenus fagi L.)

The egg of a Beech weevil (*Rhynchaenus fagi* L., Coleoptera, Curculionidae) is layed in the primary vein. After eclosion (approx. four weeks after bud burst), the three larval instars mine through parenchymatic leaf tissue to the periphery for about six weeks (BALE 1984, DOCHERTY *ET AL.* 1996). The mine is typically a serpentine tunnel and later a blotch which is visible as brown necrotic tissue.

Calculation of chlorophyll fluorescence parameters

Images of F_o and F_m were exported from the ImagingWin software (Version 1.00b, WALZ, Effeltrich, Germany) as 8bit tiff stacks. From these files, several chlorophyll fluorescence parameters were calculated using a self-modified version of ImageJ 1.37v and several self-built macros. The calculated parameters are given in Table 1.2. The resulting images were either stored as 8bit gif-images (F_o, F_m) or as 32bit tiff-images (F_v/F_m, Φ_{PSII}, NPQ, q_L). F'_o can not be measured by the Imaging PAM, therefore it was calculated after OXBOROUGH *ET AL.* (1997b) and WALZ (2005), see Table 1.2.

Calculation of "directly" and "indirectly" affected leaf area

Areas were calculated on the basis of thresholded images. Every pixel below a specific chlorophyll fluorescence value (= threshold) was set to black, therefore the sum of all these black pixels represents the area below that threshold. As the size of each image, and therefore of each pixel, was known, the black areas could be calculated.

A new method was developed which allows the comparison of images taken under a broad range of settings (the instrument settings for each measurement were varied to ensure optimal conditions, see above). Therefore, absolute values for thresholds, as used by LÖW *ET AL.* (2004), were not sufficient for the broad range of images in this study. However, relative thresholds, based on the distribution of pixels within each image (i.e. the amount of pixels per colour, histogram), can be compared between different images without limitations.

The distribution of pixels was assessed on the basis of 256 bins (i.e. the chlorophyll fluorescence scale was divided in 256 parts) and the number of pixels within each bin was calculated. The relative thresholds were set empirically to match all images of this study based on the following principles:

1. the lower threshold was set at the mean value (+ standard deviation SD) of the lower 2% of all pixels in the image. Obviously, this area contains regions with a low signal-to-noise ratio WALZ (2005) and severely degraded photosynthesis. Especially holes in the leaf are part of this percentage.
2. pixels which form the area of depressed or changed photosynthesis (= "total affected leaf area", (cf. ALDEA *ET AL.* 2006) are located between this lower threshold and the mean chlorophyll fluorescence value (- SD) of the image.
3. the mean of the upper 5% (± SD) serves as control if needed, as this leaf area is unaffected by the investigated stressors.
4. The "directly affected area" (i.e. the area apparently altered by specific stressors) within the "total affected leaf area" is known from either the true-colour photographs, or the absorptivity or "RED" images of the Imaging PAM (see Table 1.2). This area is identified based on the semi-automatic selection of all symptomatic pixels in the image (one area of symptomat-

ic pixels is selected manually, then this selection is extended to include all pixels of similar properties).

5. The "indirectly" affected leaf area is the part of the "total affected leaf area" outside of the "directly" affected area.
6. In the case of tissue removal by herbivores, step 1 is omitted because holes are part of the "directly affected leaf area".
7. Major leaf veins were excluded (masked) from processing if needed, as the chlorophyll fluorescence of leaf veins is distinctly different from the rest of the leaf blade (GIELEN *ET AL.* 2006b). Masks were created by binary operations (erosion, dilation, outline; ImageJ 1.37v).

Differential diagnosis of O_3 impact on leaf blades based on the combination of the absolute fluorescence signal and functional analysis

Additionally to the assessment of the "(in)directly" affected leaf area, chlorophyll fluorescence imaging allows additional statements on the status of individual cell layers within the leaf (as exemplified in the previous chapter 3.1 on the comparison of microscopy and chlorophyll fluorescence) which can be used in stress diagnosis. A new differential diagnostic approach was developed and tested on leaf samples injured by O_3.

As known from VOGELMANN *ET AL.* (2002), the absolute fluorescence signal F_s is a measure for the absolute amount of fluorescence which is mainly dependent on the amount of chlorophyll molecules. F_s is lower in abaxial (lower) cell layers because of the absorption of light in layers above. Due to this loss and scatter of light in the upper cell layers, less light reaches the chlorophyll molecules in lower cell layers, so they are protected to some extent from light stress. These unstressed chlorophyll molecules yield a high fluorescence signal, but again, some fluorescence light is lost during the passage through several cell layers, therefore the final signal detected by the Imaging PAM is lower than expected from unstressed chlorophyll molecules. F_s might therefore be used to determine the origin of the signal (e.g. upper or lower leaf layer).

The coefficient of photosynthetic chlorophyll fluorescence quenching (proportion of open PSII, q_L) is a measure for the physiological status of plastoquinone (Q_A) and gives insight in the condition of the electron transport chain under light. High q_L indicates that some chlorophyll fluorescence can be used to drive photosynthesis, increasing the photosynthetic yield by avoiding excessive fluorescence.

Two images of the same sample can be compared by image correlation and "colocalization", a method widely used in microscopy (see review by BOLTE *ET AL.* 2006). This technique was adapted and extended for the combination of F_s and q_L chlorophyll fluorescence images (see Figure 3.3) to improve the visualization of O_3 impact on leaf blades. In this "colocalization" graph, each F_s value is related to its corresponding q_L value. Based on this graph, specific features of both images are visualized simultaneously (e.g. only pixels with high F_s and high q_L are displayed in a new image) and used for diagnosis.

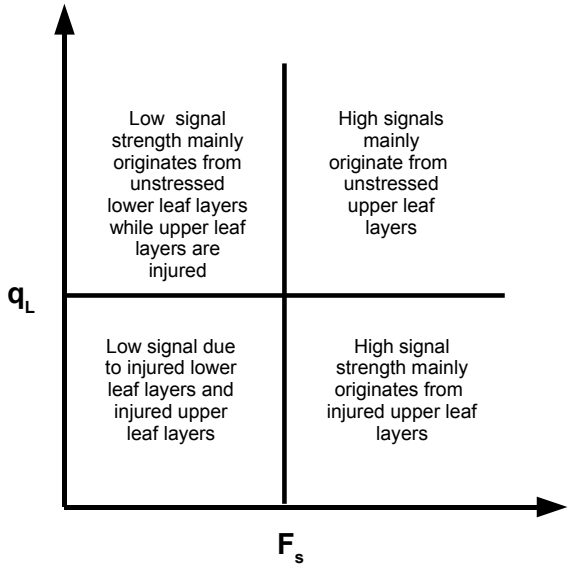


Figure 3.3: Basic aspects of the "diagnostic square" based on q_L and F_s. See text for description.

The resulting graph ("diagnostic square", Figure 3.3) was divided into four quadrants and images of each quadrant were calculated. The borders between the four quadrants were based on the histograms (i.e. distribution of pixel intensities) of the two original images. For F_s and q_L each, the border was defined by the mean pixel intensity of the image plus ½ SD. Again, pixels with very low pixel intensities (i.e. low chlorophyll fluorescence values) were excluded from the analysis of the quadrants. For these low values, the signal-to-noise ratio is low and some unspecific aspects of the leaf, which are not linked to O_3 impact but are inevitable present in leaves from a forest site, contribute to the sum of these pixels (e.g. holes in the leaf blade, major veins, mechanical damage). The pixels of minimal intensities forming the lower 2% of all pixels were calculated for each image and discarded.

The “upper right” area of the graph represents parts of the leaf (i.e. pixels) with high F_s and high q_L values. As F_s is high, the signal must originate from the upper leaf layers. At the same time, q_L indicates an unstressed photosynthetic apparatus for the pixels within this quadrant. When calculating an image from the pixels in this quadrant, the unstressed and unharmed parts of the leaf should be visualized.

The “lower right” quadrant is characterised by low q_L and simultaneous high F_s values. The low q_L is a hint for an injured PSII (disturbed electron transport chain) in the upper cell layers of the leaf, whereas F_s indicates that chlorophyll molecules are present and basically operational.

Pixels located in the “upper left” quadrant, display a low absolute fluorescence signal (F_s) which originates from the lower cell layers of the leaf. F_s is low as the actinic and the measurement light has to pass several cell layers to reach operational chlorophyll molecules (as indicated by high q_L) in the lower cell layers.

In areas represented by the “lower left” quadrant, PSII is not functional (= low q_L) and low F_s indicates injured or missing chlorophyll molecules. This is indicative for either severe injuries or down-regulation of PSII in these leaf areas.

The main impact site of O_3 injury are the upper cell layers of the leaf, mainly the palisade parenchyma (see previous chapter). Therefore, I hypothesize, that the leaf area affected by O_3 can be visualized (c.f. hypotheses 8) by either images of the “upper left” or “lower right” quadrant of the proposed “diagnostic square”.

The calculation of the four images of the “diagnostic square” (“upper left”, “upper right”, “lower left”, “lower right”) was automated by several custom-built programs (based on the ImageJ 1.37v software). The programs calculate the mean and SD of the q_L and F_s image each, use these information to create the borders of the quadrants and calculate the four corresponding black and white images. Additionally, images including only the discarded pixels were calculated for F_s and q_L, and combined to one image integrating all pixels with low intensities. This image was subtracted from each of the images representing the quadrants.

The only input variables needed for the programs are a) a modifying factor for the width of the standard deviation SD (default 0.5) which is used to fine-tune the border between the four quadrants and b) the percentage of pixels with low intensities to be excluded from analysis (default 2%). If needed, the scatterplot of q_L vs F_s was created (Gnuplot 4.0, www.gnuplot.info) to visualize the rela-

tion between these two parameters and to review the positions of the borders between the four quadrants and the discarded pixels. The frequency (occurrence) of each F_s - q_L data point was derived using the "Image Correlator" plugin for ImageJ.

3.2.3 Results

Injury caused by herbivory

During 2003, the development of a *Rhynchaenus fagi* (beech weevil) leaf mine was followed in the shade crown of a $1xO_3$ beech tree. The first assessment was in June 2003, when the parenchymatous leaf mine was already several centimetres long (Figure 3.4A, B). The larva was still inside the leaf at the time of assessment. The path of the mine traces the typical serpentine pattern expected for *R. fagi* (Figure 3.4B) and was defined as the "directly affected" leaf area. No other leaf injuries were found on the leaf blade.

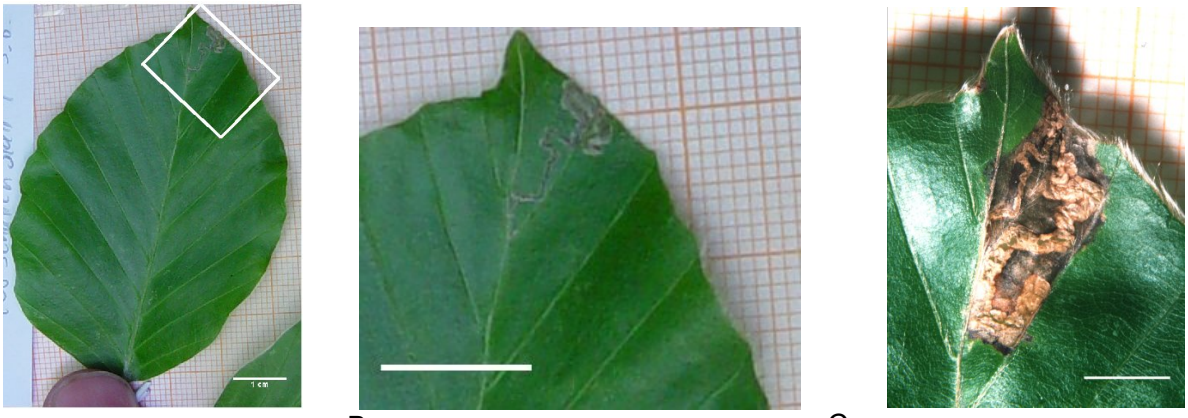


Figure 3.4: True colour photographs of a shade crown leaf with a mine caused by *Rhynchaenus fagi*. (A) Overview of the whole leaf blade, white frame marks the area further investigated. (B) magnification of the leaf tip with the leaf mine. (A) and (B) taken at June 6, 2003. (C) the same leaf mine at September 8, 2003. Scale bar in (A) and (B) is 1 cm, scale bar in (C) is 0.5 cm.

The second assessment was in September 2003 (Figure 3.4C), after the leaf mine was abandoned by the larvae. The total necrotic area had increased since June and was then a blotch instead of the previous serpentine path. Now the tissue directly affected by mining (i.e. removed) was completely necrotic (orange colour in Figure 3.4C) and surrounded by a black and brown necrotic area, which was not caused by the feeding larvae ("indirectly" affected). The complete pathway of the larvae,

from eclosion to leaving the leaf is visible. Again, no further injuries but the mine were present on this leaf blade.

Results from the light response assessment in June 2003 are shown in Figure 3.5. With increasing PPFD, the mean of Φ_{PSII} and q_L decreases whereas NPQ increases. After a rapid decrease, Φ_{PSII} and q_L reach a stable plateau between 80 – 135 PPFD. Additionally, NPQ shows only minor changes in the same PPFD range after a steep initial increase.

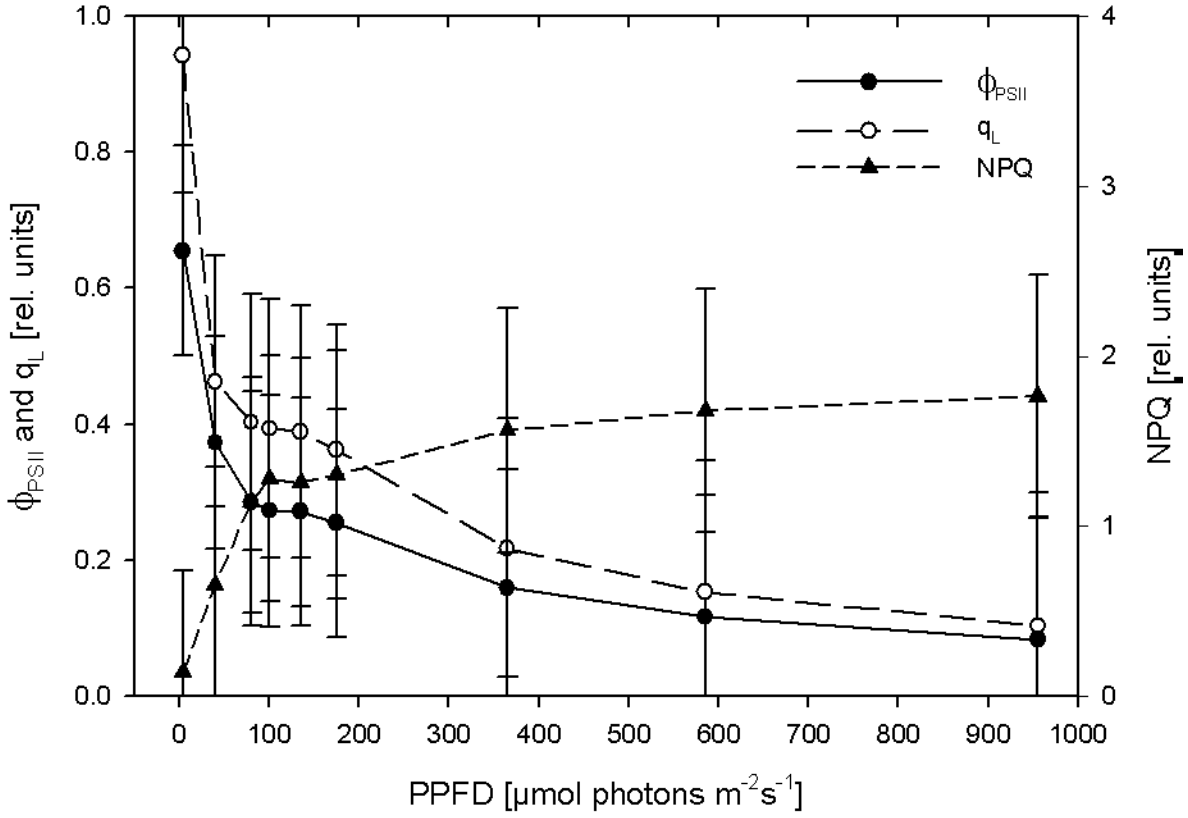


Figure 3.5: Light response of Φ_{PSII} , q_L and NPQ measured by chlorophyll fluorescence imaging on June 3, 2003 for the leaf shown in Figure 3.4 B. Means ± standard deviation (n = 307200).

In chlorophyll fluorescence images taken at light intensities above 135 µmol photons $m^{-2}s^{-1}$ (i.e. beyond the plateau) the leaf mine is only barely visible in Φ_{PSII}, q_L and NPQ (see Appendix B 6.1). To standardize the assessment, only images taken at 4 and 135 µmol photons $m^{-2}s^{-1}$ were investigated in detail. The complete set of images is shown in Appendix B 6.1.

In June 2003, F_m and Φ_{PSII} display high values except for the area of the leaf mine (Figure 3.6 AB) and an additional area of low Φ_{PSII} in the upper left corner of the leaf. NPQ (measured at 135 µmol photons $m^{-2}s^{-1}$) is lowest in the area of the mine, but additional patches of low NPQ are visible on the leaf blade.

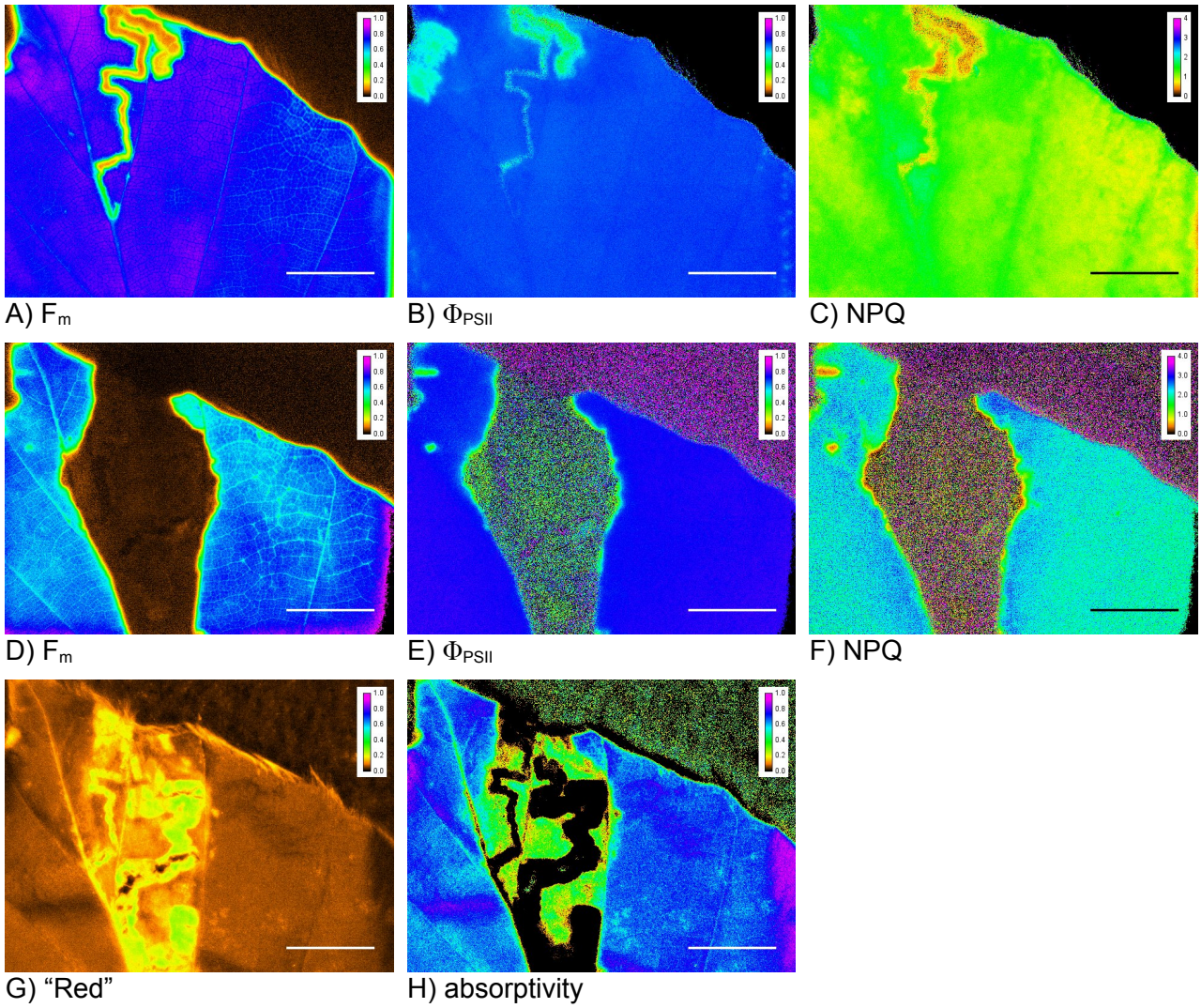


Figure 3.6: Chlorophyll fluorescence, "red" and absorptivity images of the leaf mine (see Figure 3.4). (A), (B) and (C) taken on June 3, 2003; (D), (E), (F), (G) and (H) measured September 4, 2003. Φ_{PSII} image taken at 4 µmol photons $m^{-2}s^{-1}$, NPQ image taken at 135 µmol photons $m^{-2}s^{-1}$. Scale bar is 0.5 cm each.

In September, a large part of the leaf displayed very low values for F_m, Φ_{PSII} and NPQ as if the leaf mine was converted into a hole (Figure 3.6 D,E,F). Also, the area missing for photosynthesis was much bigger than the area affected by the larvae in June. The actual leaf mine only covered a small fraction of the leaf area in question as the photograph, the "red" and the absorptivity images show

(Figures 3.4 C, 3.6 G,H). The areas surrounding this necrosis had high F_m, high Φ_{PSII} and medium NPQ values.

The "directly" affected leaf area, (i.e. tissue removed by the *R. fagi* larvae along the serpentine mine) is clearly visible in Figure 3.6 G,H. From the detailed photographs of the leaf mine (Figure 3.4) and the chlorophyll fluorescence images (Figure 3.6), the "directly" and "indirectly" affected leaf area was calculated (Table 3.5). In all investigated chlorophyll fluorescence images of June, the area of reduced photosynthesis was smaller than estimated from the photograph, therefore, no "indirectly" affected leaf area was found. In September, however, the total area of reduced chlorophyll fluorescence (affected area) was always slightly greater than the area estimated from the photograph (Table 3.5). The area "indirectly" affected were only 5 % (F_m), 7 % (Φ_{PSII}) and 1 % (NPQ), smaller then the area "directly" affected.

Table 3.5: "Directly" affected (tissue removed or consumed by the larvae) and "indirectly" affected (area of altered chlorophyll fluorescence outside of the mine) leaf area on June 6, 2003 and September 4, 2003. "Affected area" is the sum of the "directly" and "indirectly" affected leaf area. Φ_{PSII} image measured at 4 µmol photons $m^{-2}s^{-1}$, NPQ image measured at 135 µmol photons $m^{-2}s^{-1}$.

	June 2003		September 2003		
image type	"directly" affected area [cm^2]	"indirectly" affected area [cm^2]	affected area [cm^2]	"directly" affected area [cm^2]	"indirectly" affected area [cm^2]
true colour image	0.178	0	0.874	0.361	0.513
F_m	0.144	0	0.885	0.454[a]	0.431
Φ_{PSII}	0.162	0	0.876	0.454[a]	0.422
NPQ	0.131	0	0.904	0.454[a]	0.450
Abs	[b]	[b]		0.454	

[a] value for "directly" affected area taken from absorptivity image.
[b] not available.

Injury by Apiognomonia errabunda

Necrotic patches (anthracnoses) caused by the fungi *Apiognomonia errabunda*, similar to those described by BAHNWEG *ET AL.* (2005), were found on leaves of the shade crown of a $2xO_3$ tree in August 2003 (Figure 3.7 A, 3.8 A). Several fungal infections were established on the leaf blades, one isol-

ated necrosis was selected from each leaf for detailed assessment (Figure 3.7 B, 3.8 B). No further injuries were found in the close vicinity of the investigated patch.

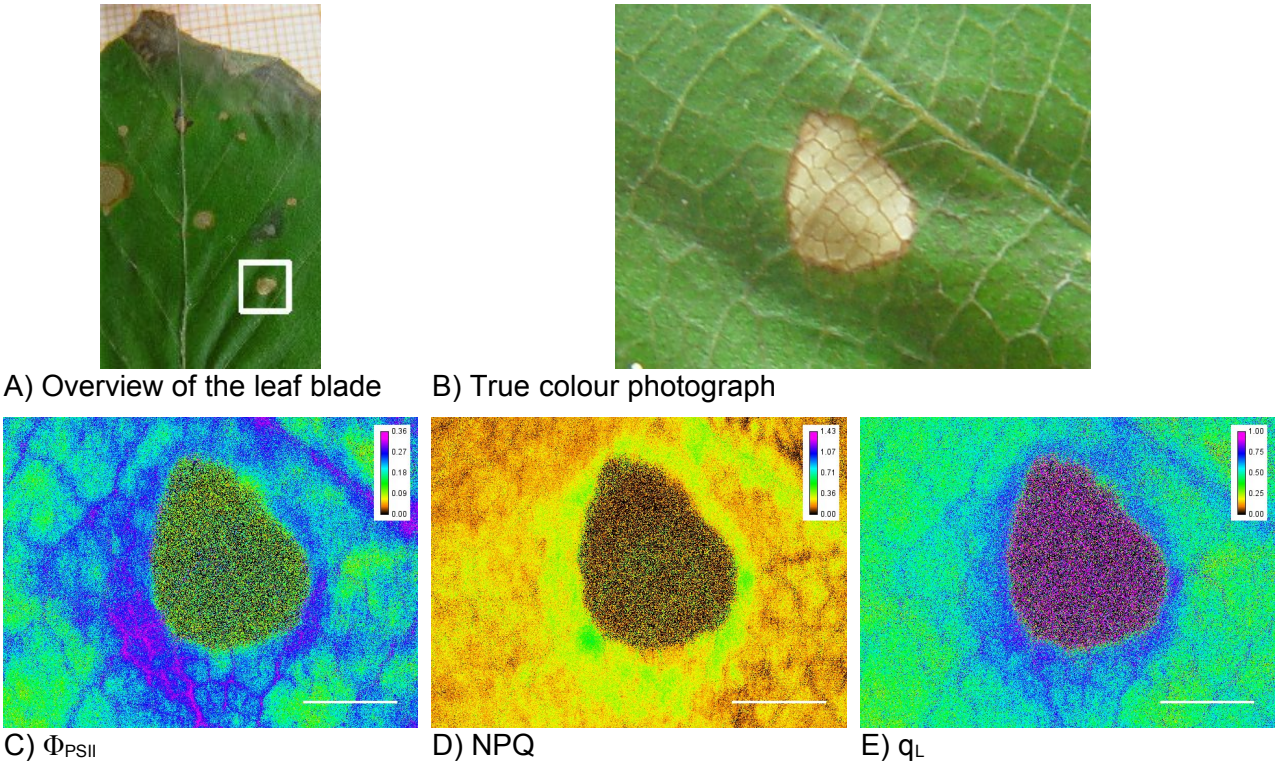


Figure 3.7: (A) Overview of a shade crown leaf from a $2xO_3$ tree in August 2003 with several necrotic patches caused by *Apiognomonia errabunda*. Area of detailed assessment is enclosed by white frame. (B) Detailed view (magnification 40x) of the area investigated by chlorophyll fluorescence imaging. Chlorophyll fluorescence images (C), (D) and (E) captured at 60 µmol $m^{-2}s^{-1}$ PPFD. Please note the individual colour ranges ranges in C,D,E.

Table 3.6: Summary of the "directly" (necrotic area) and "indirectly" (area of increased chlorophyll fluorescence outside of the necrosis) affected leaf area for a *Apiognomonia errabunda* necrosis (see Figure 3.7). Area of increased chlorophyll fluorescence was defined by pixels with higher intensities than the mean + SD of the image (see Material and Methods). "Total affected area" is the sum of the "directly" and "indirectly" affected leaf area.

image type	total affected area [mm^2]	"directly" affected area [mm^2]	"indirectly" affected area [mm^2]
true colour image	2.5	2.5	
Φ_{PSII}	6.2	2.5[a]	3.7
NPQ	6.8	2.5[a]	4.3
q_L	4.6	2.5[a]	2.1

[a] value for "directly" affected area estimated from the true colour image.

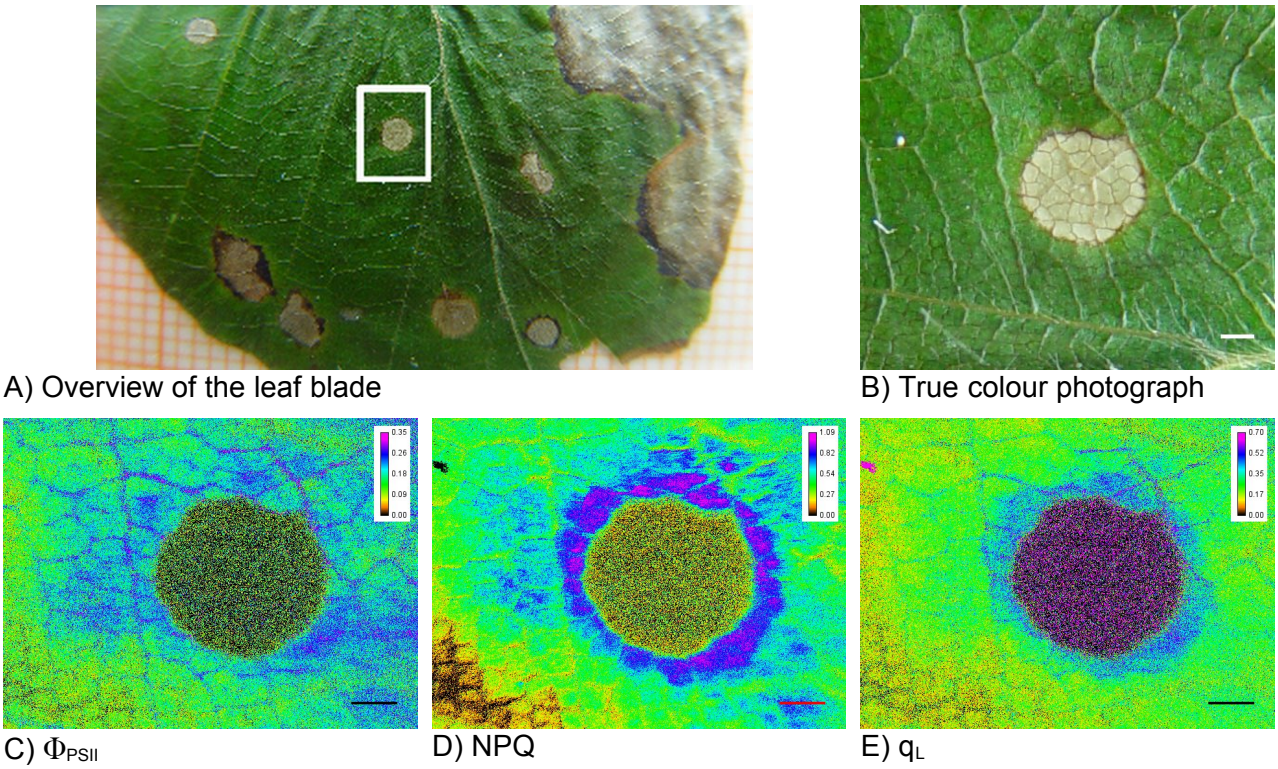


Figure 3.8: Second example of a leaf injury caused by the fungi *Apiognomonia errabunda*. The necrosis is surrounded by a ring of enhanced Φ_{PSII}, NPQ and q_L. (A) Overview of the leaf blade, white frame marks the area investigated in detail. (B) True colour image of the necrotic patch. Chlorophyll fluorescence images (C,D,E) taken at 60 µmol photons $m^{-2}s^{-1}$. Please note the individual colour ranges ranges in C,D,E. Scale bars in B,C,D,E represent 500 µm.

Table 3.7: Summary of the "directly" (necrotic area) and "indirectly" (area of increased chlorophyll fluorescence outside of the necrosis) affected leaf area for a *Apiognomonia errabunda* necrosis (see Figure 3.8). Area of increased chlorophyll fluorescence was defined by pixels with higher intensities than the mean + SD of the image (see Material and Methods). "Total affected area" is the sum of the "directly" and "indirectly" affected leaf area.

image type	total affected area [mm^2]	"directly" affected area [mm^2]	"indirectly" affected area [mm^2]
true colour image	2.5	2.5	
Φ_{PSII}	4.7	2.5	2.2
NPQ	4.8	2.5	2.3
q_L	3.2	2.5	0.7

[a] value for "directly" affected area estimated from the true colour image.

Chlorophyll fluorescence images of both investigated leaves showed a decrease in the photosynthetic parameters within the necrosis caused by *A. errabunda* and an increase in the surrounding (Figure 3.7 and 3.8 C,D,E). These "coronas" were not visible to the naked eye (Figure 3.7 and 3.8 A,B). Especially at a light level of 60 µmol photons $m^{-2}s^{-1}$ PPFD the "coronas" of stimulated, enhanced Φ_{PSII}, NPQ and q_L (Figure 3.7 and 3.8 C,D,E) were evident. However, this stimulation was statistically significant at all light levels ($p < 0.001$ in all parameters, regression analysis).

In the first example (Table 3.6), the "coronas" of increased Φ_{PSII} and NPQ (i.e. the area of pixel intensities above the mean + SD of the image) around the necrosis were bigger in size than the necrosis. The area of increased q_L was smaller than the necrotic area.

In the second example (Table 3.7), the "coronas" of increased NPQ and q_L were smaller than the area "directly" (i.e. the visible brown necrosis) affected by the fungi. q_L was only increased in a very small area.

The area of increased Φ_{PSII} was rather patchy in both examples and not clearly linked to the necrotic leaf area (Figure 3.7 C, 3.8 C), but still formed a circle around the fungi, and was therefore included in the analysis. The ring of enhanced fluorescence around the necrosis was not visible in any image of the investigated parameters taken below a light level of around 60 µmol $m^{-2}s^{-1}$, and only NPQ images displayed the ring clearly above that light level (data not shown). Only two detailed examples are shown in this chapter, additional examples with comparable results are not shown.

Yellow intercostal necroses and O_3 injury

During 2003 and 2004 leaves in the sun crown of "Kranzberger Forst" showed light green to yellow necroses of abiotic origin which had previously been validated as O_3-related symptoms (GÜNTHARDT-GOERG 2001). Examples are given in Figure 3.9.

A B

Figure 3.9: Examples of typical yellow intercostal necroses and chloroses of sun crown leaves at "Kranzberger Forst". A leaf from a 1xO_3 tree in June 2004 (A) and another typical leaf in July 2003 (B).

The F_v/F_m image (Figure 3.10 A) of a leaf with typical O_3-related necroses (Figure 3.9 A) showed mainly high values (mean 0.729 ± 0.04). Some patches of decreased F_v/F_m are scattered over the leaf blade. The "affected" areas with pixels below the mean minus the standard deviation (but above the lower 2% of all pixels, see Materials and Methods) does not correspond well with the yellow necrotic area (Figure 3.10 C). Mean Φ_{PSII}, measured at 138 µmol $m^{-2}s^{-1}$ PPFD, was 0.473 ± 0.04. The area of decreased Φ_{PSII} was distinctly larger than for F_v/F_m and did align better with the necrotic pattern, even though not every yellow spot was covered. The affected area of the yellow necroses was 0.082 cm^2 (estimated from Figure 3.9 A), 0.227 cm^2 for F_v/F_m and 0.640 cm^2 for Φ_{PSII}. As the visible pattern did neither match with F_v/F_m nor with Φ_{PSII}, and additionally omitted several of the necroses, the "directly" and "indirectly" affected areas were not calculated, even though the potentially affected area was much larger than estimated from the true colour image.

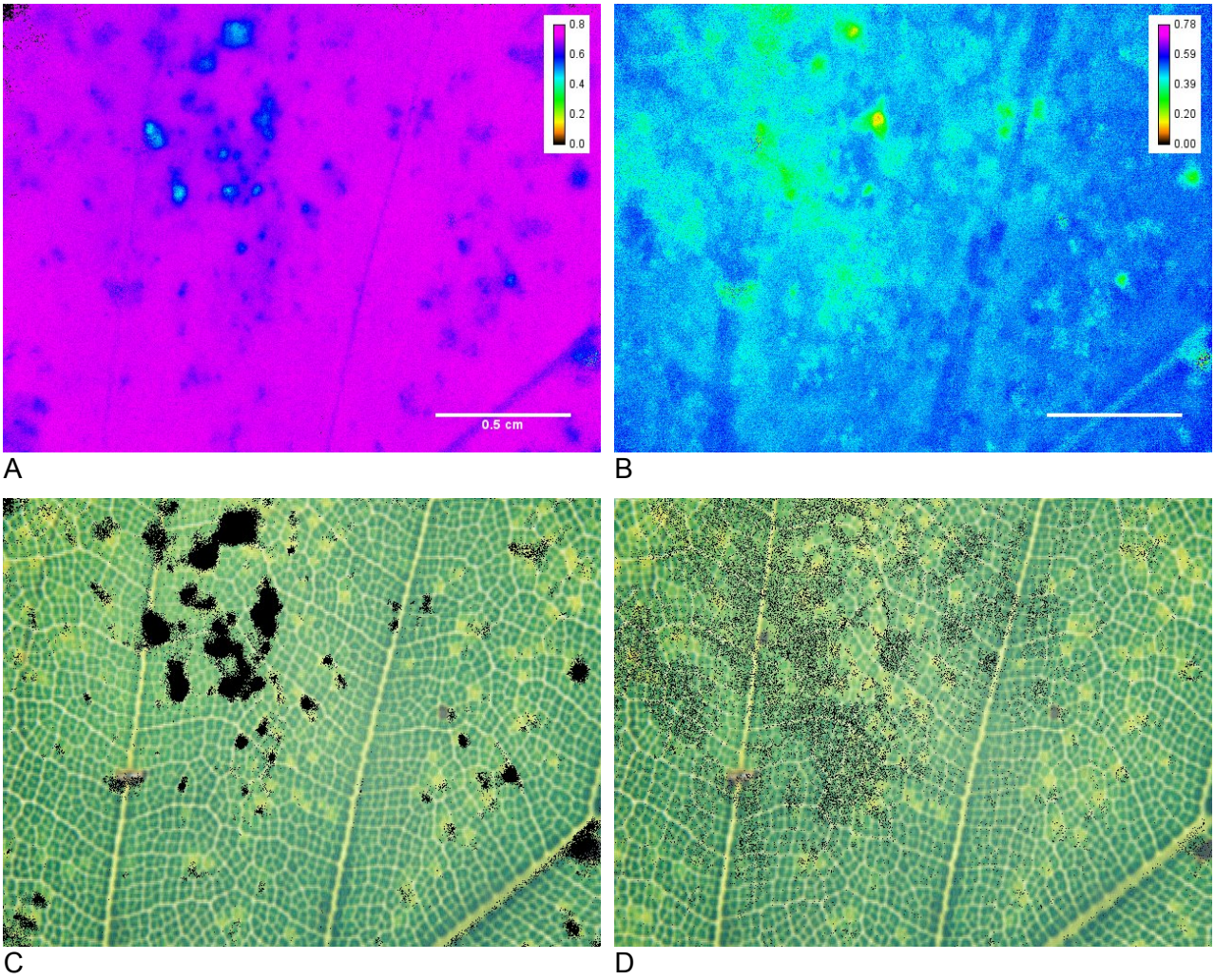


Figure 3.10: F_v/F_m (A) and Φ_{PSII} (B) image a leaf with typical yellow intercostal necroses (see Figuren 3.9 A) and comparison with the area of decreased values (C) and (D). Black areas in (C) and (D) had pixel intensities lower than the mean (-SD) of the respective image. (C) black area calculated from F_v/F_m, (D) black area calculated from Φ_{PSII} at 138 µmol $m^{-2}s^{-1}$ PPFD.

During the growing season of 2003, a leaf close to a O_3 release tube (distance to O_3 outlet < 2 cm) was assessed several times to follow the development of the yellow O_3 induced necroses (Figure 3.11). Already in June, this leaf had yellow necrotic patches at the leaf tip (Figure 3.11 A). In the following months, these patches became more dense and formed a distinct yellow blotch by September (Figure 3.11 B,C). Additionally, the leaf showed some mechanical injury.

Within the light response assessment, the plateau of stable chlorophyll fluorescence (c.f. Figure 3.5) was found again (not shown). The images taken at light levels within this plateau had the best contrast within the assessment and therefore the images captured at about 138 µmol photons $m^{-2}s^{-1}$ PPFD were selected for analysis.

A B C

Figure 3.11: Development of yellow intercostal necroses during 2003. The leaf was located in the close vicinity of a O_3 release tube. (A) On June 5, (B) on July 25, (C) on September 19, 2003.

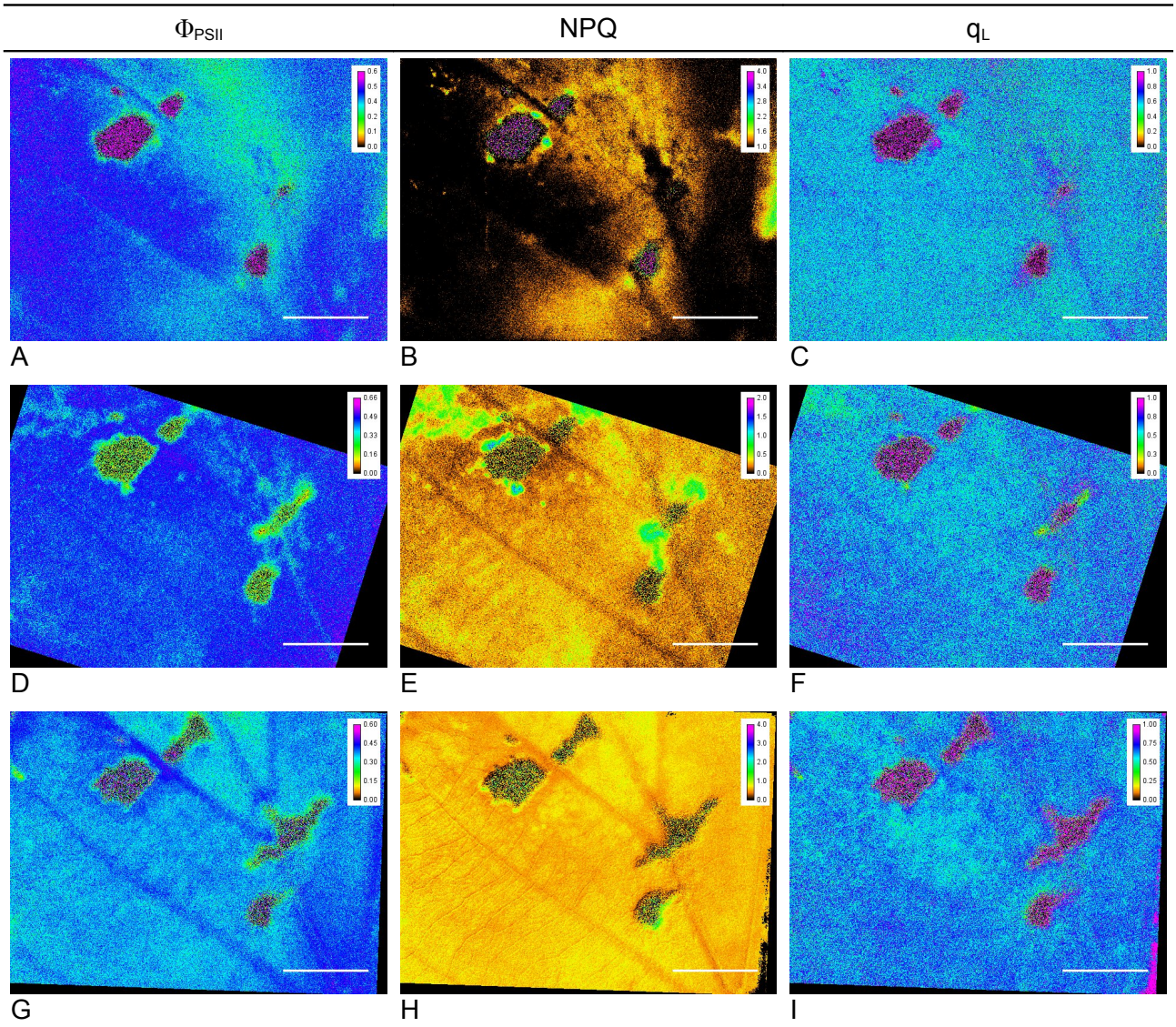


Figure 3.12 (previous page): Φ_{PSII}, NPQ, and q_L images of the leaf shown in Figure 3.11. First column (ADG) Φ_{PSII}, second column (BEH) NPQ and third column (CFI) q_L images. Images in the first row (ABC) measured on June 5, second row (DEF) on July 25 and third row (GHI) on September 19, 2003. All images were aligned to the images of June 5th. If possible, the colour scale was adjusted (as indicated by the colour scale) to visualize the necrotic leaf area. All measurements were performed at 138 – 142 µmol photons m^{-2} s^{-1} PPFD. Scale bars are 0.5 cm.

The necrotic areas of the leaf (Figure 3.11) were only visible in Φ_{PSII} and NPQ images taken in May (Figure 3.12 A,B). In July and September no distinct areas of lowered chlorophyll fluorescence were found despite the apparent yellow necroses. The mean Φ_{PSII}, of the leaf increased from June (mean ± SD was 0.39 ± 0.1, Figure 3.12 A) to July (0.54 ± 0.1, Figure 3.12 D), while NPQ dropped simultaneously from a mean of 1.03 (± 0.34) in June to 0.32 (± 0.35) in July. q_L did not show major changes from June to September and never displayed a pattern comparable to the visible necrotic areas (Figure 3.12 C,F,I).

Yellow intercostal discolourations were also present on a leaf blade from a $1xO_3$ tree (Figure 3.13) throughout 2004. In May, after the leaf was fully expanded, a yellow patch was clearly visible (Figure 3.13 A). The corresponding Φ_{PSII}, NPQ and q_L images indicate reduced photosynthesis in the related areas (Figure 3.13 B, C, D) however, not as an large patch, but as smaller stipples instead. By June and September, the discoloured area decreased and showed several distinct yellow spots (Figure 3.13 E, I) which had been found in NPQ already in May (Figure 3.3). But in June and September, none of the investigated chlorophyll fluorescence parameters (Figure 3.13 F, G, H, J, K, L) displayed a pattern comparable to the discolourations visible in the photographs (Figure 3.13 E, I). NPQ measured in May and the symptoms visible in June match to some extent in the area indicated by in Figure 3.3,

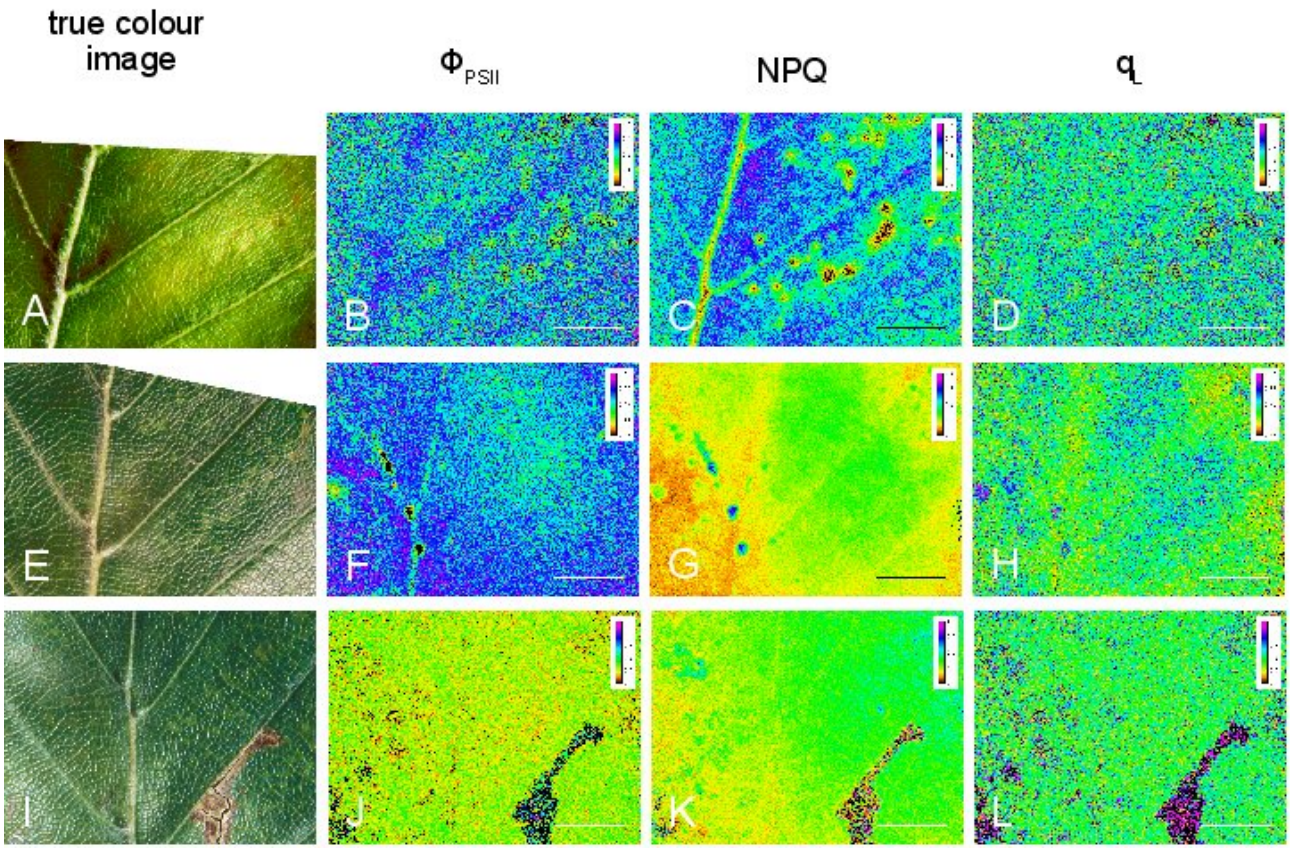
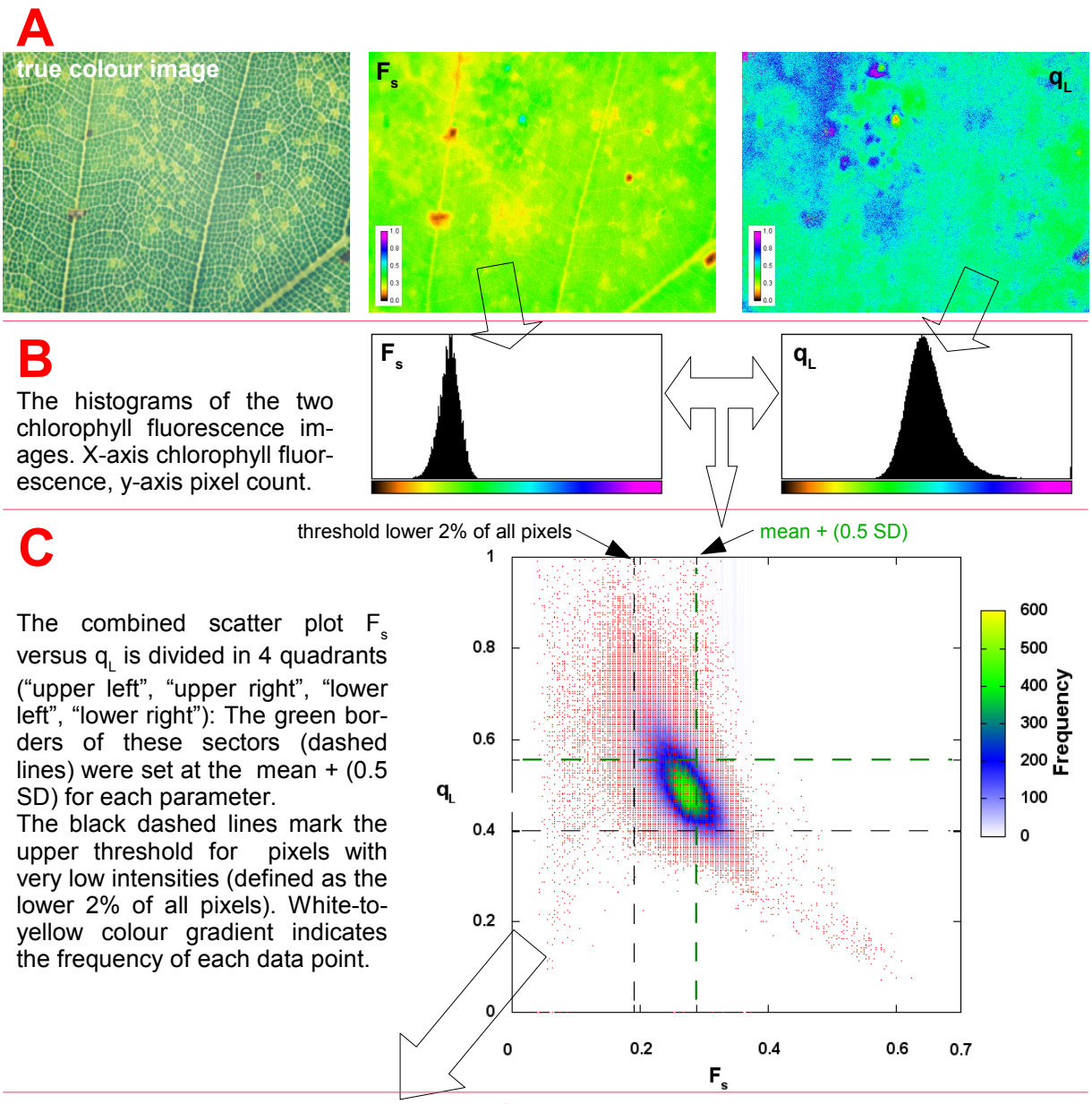


Figure 3.13: Development of intercostal discolourations in a sun crown leaf of a tree from $1xO_3$. Images in the first row (ABCD) captured on May 15, in the second row (EFGH) on June 29 and in the third row (IJKL) on September 7, 2004. Φ_{PSII}, NPQ and q_L images taken at 139 µmol photons $m^{-2}s^{-1}$. Please note the individual colour scales given in the chlorophyll fluorescence images. Scale bar in Φ_{PSII}, NPQ and q_L images is 0.5 cm.

Figures 3.14 (next page) and 3.15 (following page): Examples for the application of the "diagnostic square". Please follow the white arrows from A to E. The F_s and q_L images were captured at 138 µmol photons $m^{-2}s^{-1}$ PPFD. See text for description.

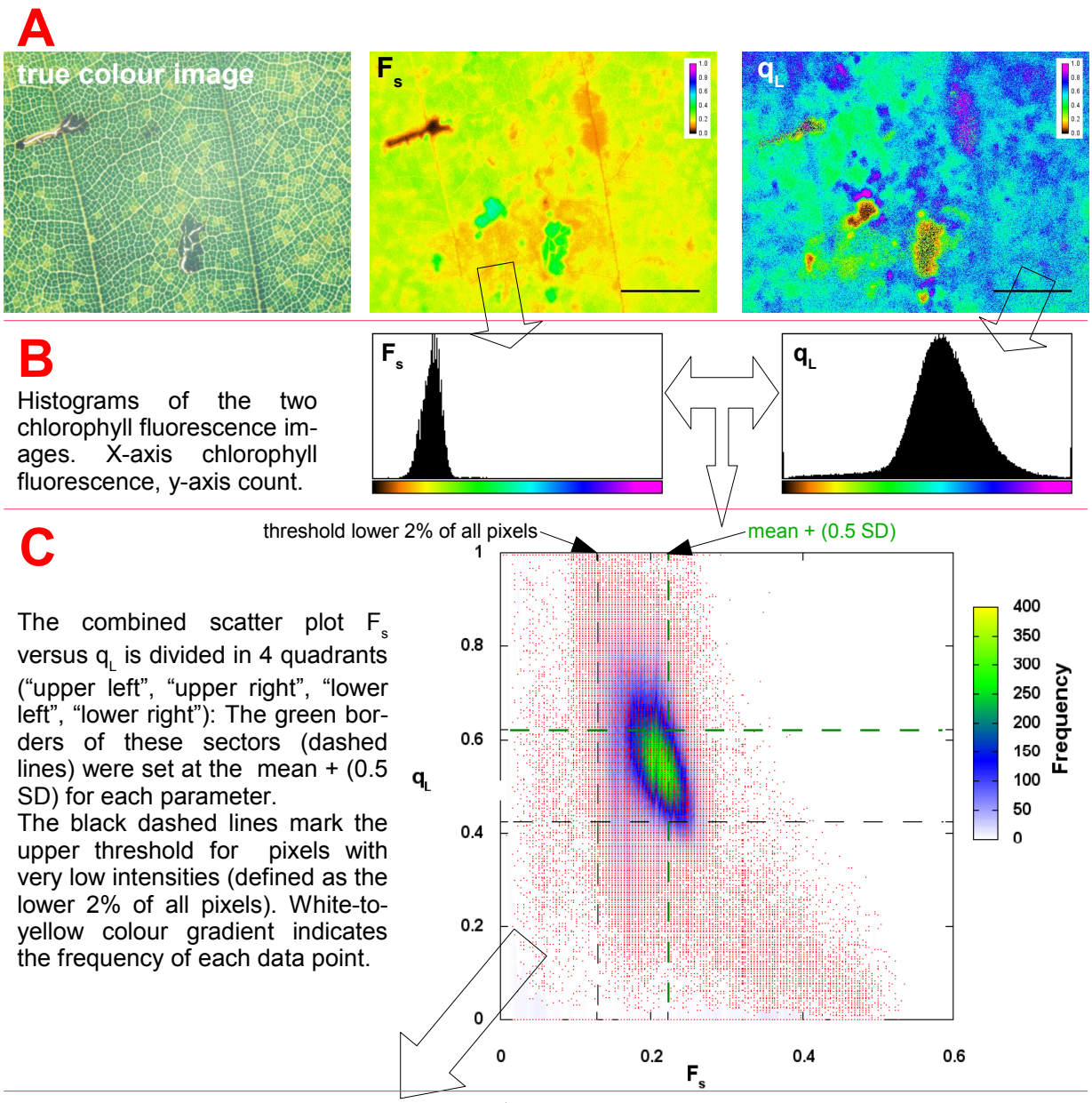


The histograms of the two chlorophyll fluorescence images. X-axis chlorophyll fluorescence, y-axis pixel count.

The combined scatter plot F_s versus q_L is divided in 4 quadrants ("upper left", "upper right", "lower left", "lower right"): The green borders of these sectors (dashed lines) were set at the mean + (0.5 SD) for each parameter.
The black dashed lines mark the upper threshold for pixels with very low intensities (defined as the lower 2% of all pixels). White-to-yellow colour gradient indicates the frequency of each data point.

The individual images of the four quadrants (lower 2% excluded)

The combination of the real-colour image with the "upper left" sector (black pixels).


A
true colour image
F_s
q_L
B
Histograms of the two chlorophyll fluorescence images. X-axis chlorophyll fluorescence, y-axis count.
F_s
q_L
C
threshold lower 2% of all pixels
mean + (0.5 SD)
The combined scatter plot F_s versus q_L is divided in 4 quadrants ("upper left", "upper right", "lower left", "lower right"): The green borders of these sectors (dashed lines) were set at the mean + (0.5 SD) for each parameter.
The black dashed lines mark the upper threshold for pixels with very low intensities (defined as the lower 2% of all pixels). White-to-yellow colour gradient indicates the frequency of each data point.
q_L
F_s
Frequency
D
high q_L
low q_L
low F_s
high F_s
F_s
individual images of the four quadrants (lower 2% excluded)
E
combination of the true-colour image with the "upper left" sector (black pixels).

Application of the "diagnostic square"

The application of the "diagnostic square" to chlorophyll fluorescence imaging analysis is shown in Figures 3.14 and 3.15. In both examples, neither the F_s nor the q_L image showed an acceptable approximation of the yellow intercostal necroses (section "A" in each Figure). In both images, some areas revealed patterns as expected from the photograph, in other parts of the same image no such compliances were found. However, the resulting combined F_s - q_L image (Figure 3.14, E) showed a better estimation than images of F_v/F_m alone for the leaf areas potentially affected by O_3.

In the F_s image of the first example (Figure 3.14, A, F_s), the necrotic parts of the leaf display more or less similar pixel intensities as the leaf veins, therefore, the binary image-processing methods used to mask the leaf veins, did not work properly. This finding is also visualized in the histogram of F_s (Figure 3.14 B, F_s) as only a small part of the available chlorophyll fluorescence range from 0 to 1 is used. On the other hand, the q_L image contains no information on the basic leaf anatomy (Figure 3.14 A, q_L), as the leaf veins were not visible. Also, the used colour range of q_L is wider than in the F_s image (Figure 3.14 B, q_L).

The combination of the F_s and q_L images (Figure 3.14 C) shows, that the majority of all pixels aggregate in a very small area of the graph, as depicted by the colour gradient. Some F_s and q_L values were excluded as indicated by the black dashed lines. The remaining pixels were divided into the four quadrants based on the mean pixel intensities as indicated by the green dashed lines, resulting in four new images (Figure 3.14 D). The "upper right" quadrant contains only few pixels (shown in black), whereas the pixel number increases steadily from the "lower right", to "upper left", "to "lower right". The image depicting low F_s and high q_L ("upper left" quadrant) was comparable to the necrotic leaf area (Figure 3.14 E).

The "diagnostic square" method worked equally well for the leaf shown in Figure 3.15 (more comparable examples not shown). As in the previous example, the "lower right" quadrant represented the visible yellow intercostal necroses best and did not include the brown necrotic areas (Figure 3.15 E). All the findings from Figure 3.14 were confirmed. The area "directly" affected (estimated from the true colour image) was 0.082 cm^2 in the first (Figure 3.14 A), and 0.139 cm^2 in the second example (Figure 3.15 A). The areas of low F_s and simultaneously high q_L ("upper left quadrant") where 0.721 cm^2 (Figure 3.14) and 0.883 cm^2 (Figure 3.15).

3.3 Discussion

The impact of different stressors on photosynthesis were visualized and displayed unique patterns for folivory, fungal infections and O_3-related impact. However, the findings can not be generalized, because of huge differences in amount and direction of the changes in photosynthesis.

In September, the impact of the leaf miner *Rhynchaenus fagi* on photosynthesis was greater than expected from the removed area, confirming findings by ZANGERL *ET AL.* (2002) and ALDEA *ET AL.* (2006). In June, however, when the mine was still occupied by the larvae, the decrease in photosynthesis did not exceed the removed leaf area. Therefore Hypothesis (7), that the affected area is larger and decreased in photosynthesis, can not be accepted for the herbivore *R. fagi*. Obviously, the impact of folivory on photosynthesis changes with time, and in controversy to ZANGERL *ET AL.* (2002), was not always "greater than the sum of its holes". It can not be clarified whether the "indirectly" affected leaf area was primarily induced by the plant as a defence reaction, or if it was a result of structural damage and disturbance of leaf internal pathways for water and nutrients (TANG *ET AL.* 2006). The chewing damage analysed in this study decreased F_m, Φ_{PSII} and NPQ to zero in the vicinity of the mine, indicating the absence of chlorophyll molecules and therefore of working PSII reaction centres. The area of reduced photosynthesis was larger than 1 mm around the "directly" affected area as reported by ALDEA *ET AL.* (2006), however, the overall impact of *R. fagi* on the photosynthesis of the investigated beech trees is rather small due to its limited abundance at the site (NUNN 2004, KLOTSCHE 2005). Given the choice, *R. fagi* prefers leaves grown under ambient O_3 concentrations to those grown under charcoal filtered conditions (HILTBRUNNER *ET AL.* 1992), but at "Kranzberger Forst" more Beech weevil mines have been found in the sun crowns of $2xO_3$ trees (as compared to sun crowns of $1xO_3$ trees) in 2004 (KLOTSCHE 2005), but a general trend over several years was not detected (NUNN *ET AL.* 2005b). Also, no overall difference was found with respect to the position within the canopy (sun or shade).

For fungal infections, a "corona" of increased Φ_{PSII}, NPQ and q_L was found in the close vicinity of the visible necroses. The opposite, a decrease of Φ_{PSII} by 25 % was reported for several fungi in a loblolly pine forest understory, but NPQ on the other hand, increased in the same way (ALDEA *ET AL.* 2006). The simultaneous increase of non-photochemical (NPQ) and photochemical (Φ_{PSII}) processes is only rarely reported. An increase in NPQ indicates that absorbed light is emitted as heat instead of being used for photosynthesis (GARCIA *ET AL.* 2002). This process is related to zeaxanthin formation (BJÖRKMAN *ET AL.* 1994) and helps to omit photoinhibition (LICHTENTHALER *ET AL.* 2005). Therefore,

Φ_{PSII} was also high in the "corona". This simultaneous increase of quantum yield and avoidance of photoinhibition might be indicative for ongoing defence reactions against the pathogen. The total extent of the "coronas" around fungal infections of *Apiognomonia errabunda* was much smaller than reported by ALDEA *ET AL.* (2006), but the area of increased Φ_{PSII} and NPQ nearly covered the same area as was lost for photosynthesis. The stimulated area was not always bigger than the affected area, so hypothesis (7a) had to be rejected again. *A. errabunda* infections did not only cause an increase in non-photochemical processes but additionally stimulated photosynthesis in the "indirectly" affected leaf area. Hypothesis (7b) was therefore rejected.

The visualization of the photosynthetic response patterns of herbivory and fungal infection worked well. But, in the case of O_3 the recognized spatio-temporal heterogeneities did not always comply with visible macroscopic symptoms. The pattern found for Φ_{PSII} was closer related to the actual necroses than the pattern found for F_v/F_m (Figure 3.10). Also, even when following the development of distinct O_3 stipples, patterns of decreased chlorophyll fluorescence did not mirror the shape of the necroses, except for measurements in May or June. In these cases, NPQ showed a good approximation for the necrotic areas. However, in one example, NPQ was increased in the O_3 affected leaf area (Figure 3.12 B), and decreased in another example (Figure 3.13 C) while Φ_{PSII} was decreased in both cases. A slight decrease of NPQ due to O_3 was also hypothesized by SHAVNIN *ET AL.* (1999), but did not substantiate, however, CARRASCO *ET AL.* (2001) reports on a decreased NPQ in *Avena sativa* leaves with established O_3 symptoms.

The reason for the differences between O_3 stress and the other investigated stressors might be, that O_3 triggers physiological, cell internal processes (e.g. induced cell death), whereas fungi and insects target and destroy the structure of the leaf. Thus, the impact of O_3 is less pronounced and closer related to cell internal reactions. Generally, the possible defence plays an important role in O_3 research and is even incorporated in the structure of "Critical Level for O_3" concepts (c.f. FUHRER *ET AL.* 1994). These critical levels assume, that the pollutant can be defended against to some extent. This defence might explain, why the macroscopic impact of O_3 seems to be larger than can be visualized by simple chlorophyll fluorescence assessments. On the other hand, O_3 as a gaseous pollutant, is not targeting specific structures, so its biological impact can spread over a large area and only be detectable by the visualization of secondary responses like the increase in NPQ or q_L (i.e. increased demand for defence related metabolites, increased demand for the avoidance of photoinhibition), but not by the detection of "dead" cells. No area of altered photosynthesis was found in the immediate,

distinct vicinity of the symptoms, so no zone of enhanced photosynthesis existed around the necrosis as it was the case for fungal infections. Defence reactions against O_3 take place at the same cellular level as the O_3 impact (i.e. within the affected cells, c.f. MATYSSEK *ET AL.* 2003), so no spread of altered photosynthesis beyond the injured cells can be expected based on the findings from this study. Injuries in young leaves might be repaired or delayed, as shown in Figure 3.13, but they do not vanish. The visible discolouration between the secondary leaf veins was later transformed in distinct light green areas which were distributed in a pattern typical for O_3 impact at "Kranzberger Forst". Chlorophyll fluorescence imaging can be used as a tool for the early detection of O_3 related leaf symptoms. The pattern found in NPQ in May already indicated the distribution which became visible to the naked eye later in the year. Astonishingly, this pattern in NPQ was not detectable any more, when the typical pattern for the intercostal chloroses and necroses showed up. The "affected" area therefore does not mirror the macroscopic O_3 injuries. For leaves investigated at a forest site with a multitude of possible stress factors, microscopy might still be needed for clarification of the exact cause of an injury. For monosymptomatic leaves and to assess the overall vitality, this validation can be omitted (see previous chapter).

As the impact of O_3 is potentially small, the detection is even more challenging and needs improved methods. The widely used F_v/F_m ratio (LICHTENTHALER *ET AL.* 2005) detects stressinduced changes rather late, especially in the case of O_3 (MAURER *ET AL.* 1997), and can only assess photoinhibition which might not be triggered by O_3. One advantage of F_v/F_m is, that the mandatory dark adaptation puts the leaf in a specific, standardized and optimal state. But, such a standardized state can also be established by a light response assessment, as employed in this study, which takes advantage of additional chlorophyll fluorescence parameters available under light conditions. Especially, the quenching parameters, which are known to be sensitive for O_3, become available and can improve interpretation of photoinhibition (MAXWELL *ET AL.* (2000).

Also, the hereby induced light stress can be used for diagnostic purposes. Within the light response assessments at "Kranzberger Forst" a typical plateau was identified, in which chlorophyll fluorescence did not change much with increasing light (Figure 3.5). Such a "shoulder" within the light response was also reported by SHAVNIN *ET AL.* (1999) and might be related to a shift in energy conversion from the slow relaxation of NPQ with oxidized Q_A to reduced Q_A and photophosphorylation of the light harvesting complexes. Images captured at this light levels took benefit of an excellent signal-to-noise-ratio while the leaves encountered some light stress, so these images showed more pro-

nounced patterns than F_v/F_m images.

Another methodological problem of the application of chlorophyll fluorescence is, that the exact origin of an chlorophyll fluorescence signal can not be traced in the field easily. As shown in the previous chapter on the combination of microscopy and chlorophyll fluorescence imaging, the different layers of a leaf yield different signals (c.f. VOGELMANN *ET AL.* 2002, LICHTENTHALER *ET AL.* 2005). Therefore, a new method was developed which uses information on the passage of light through the leaf, i.e. the absolute fluorescence signal (F_s), and a biologically meaningful parameter from quenching analysis (q_L) and combines these two parameters in a "diagnostic square" (Figure 3.3) for easy interpretation. Images resulting from this method showed patterns comparable to the visible O_3-related yellow intercostal necroses, even though the calculated area from the "upper left" quadrant of the "diagnostic square", and therefore the *potentially* O_3-affected area, was six to eight times larger than estimated from visual assessments (Figure 3.14, 3.15). The "upper left" quadrant combines low F_s and high q_L values, a constellation which was hypothesized to be affected by O_3, as O_3 injuries are located in the upper cell layers, especially within the palisade parenchyma close to the epidermis (see previous chapter, cf. VOLLENWEIDER *ET AL.* 2003, GRAVANO *ET AL.* 2004, BUSSOTTI *ET AL.* 2005). If the PSII reaction centres of cells in these layers are injured, they will not contribute much to photosynthetic quenching, but the measurement light will reach additional cells below the injured ones. As chlorophyll molecules in lower leaf layers are protected to some extent from light stress by the layers above (as some light has already been absorbed and scattered in the upper layers resulting in low F_s, cf. VOGELMANN *ET AL.* 2002) they can yield high photosynthetic quenching to avoid energy loss by excessive fluorescence. Hypothesis (8b) is therefore accepted. Less pronounced injuries, which were supposed to be indicated by the "lower right" quadrant of the "diagnostic square", were not connected to the visible yellow necroses. High F_s in this quadrant suggests that the actinic and the assessment light did not pass many cell layers, so the low q_L indicates a changed redox state of Q_A (KRAMER *ET AL.* 2004) or a downregulation of the PSII reaction centres. Still, in the case of simultaneous injuries by several stressors, interpretations based on the "diagnostic square" will be difficult.

The "diagnostic square" might be improved by using the F'_o parameter instead of F_s, as F'_o holds additional, biologically more meaningful information about dynamic and chronic photoinhibition (OSMOND *ET AL.* 1995) while including information on the absolute fluorescence. Hence, it was refused to use F'_o as a stand-alone parameter because it is not measured by the Imaging PAM but derived from measurements of F_o, F'_m, and F_v/F_m instead (OXBOROUGH *ET AL.* 1997b).

Additionally, the final images derived from the "diagnostic square" might be optimised by introducing basic mathematical and morphological image operations (i.e. noise reduction, binary erosion and closing, BURGER *ET AL.* 2005) to reduce the amount of isolated pixels. This would reduce the scatter within the image and the focus would be more on distinct large areas, but informations on smaller areas will be lost.

On the other hand, the original images might be modified before the start of the analysis by contrast or histogram stretching which would maximise the use of the dynamic range of the available colour gradient while maintaining the shape of the pixel distribution (OXBOROUGH *ET AL.* 1997a). Even though these modified images improve the detection of barely visible patterns, information on the absolute amount of the respective chlorophyll fluorescence parameter is changed and the images might not be comparable any more between individual assessments.

Also, it can be considered not to discard pixels with very low pixel intensities, as the "diagnostic square" is quite robust and can differentiate between yellow intercostal necroses and mechanical injuries (as shown by the brown necroses in the true colour image of Figure 3.15 A, E), as these factors are represented by different quadrants (i.e. yellow intercostal necroses are represented in the "upper left", while brown necroses contribute to the "lower left" quadrant).

The next step in the assessment of spatio-temporal patterns might be to quantify and visualize the heterogeneity, and therefore the dynamics, a method which can be applied on different scales (SCHURR *ET AL.* 2006). Methods based on the "wavelet heterogeneity" were already successfully applied to quantify and visualize the spatio-temporal O_3 impact on beech leaves from a climate chamber study (LANZINGER 2007).

In conclusion, the impact of pathogens on photosynthesis of beech leaves depends on the actual stressor and varies with time. Hypothesis (7a), that the "indirect" impact of folivory, fungi and O_3 on photosynthesis is always bigger than the area visibly affected, was not accepted. Also, the impact of these stressors on photosynthesis was not always negative (hypothesis 7b), as Φ_{PSII} and q_L increased in the surrounding of fungal necroses, hypothesis (7) was therefore rejected in total. Hypothesis (8), assuming that O_3 impact can be visualized by chlorophyll fluorescence imaging was confirmed, as the new "diagnostic square" showed a good and biologically meaningful approximation of the leaf area *potentially* affected by O_3.

4 Conclusions

The dry summer of 2003 provided the unique opportunity to study the combined impact of ozone (O_3) and drought on adult F*agus sylvatica* trees at the forest site "Kranzberger Forst" (Freising, Germany).

In conclusion, the photosynthesis and stomatal conductance of the investigated beech trees are affected by O_3-stress. However, the responses are not consistent between years, as they are strongly depending on the climatic conditions. O_3 decreased stomatal conductance in the two study years, but the extraordinary drought of 2003 caused a larger decrease in photosynthesis and stomatal conductance than the chronic O_3 regime. Drought changed the O_3 sensitivity per unit of O_3 uptake as the relationships between the O_3 dose and the effect of O_3 were reversed under limiting water supply. In 2004 on the other hand, there was evidence that the O_3 effects on several ecophysiological parameters (A_{max}, g_s, and ETR) were dose-dependent resulting in larger differences between the ambient and experimentally enhanced O_3 regime with rising O_3 dose. However, during 2003 the differences between the O_3 regimes diminished with rising COU. But this confirmed the hypothesis, that O_3 uptake rather than O_3 exposure reflects the risk of O_3 induced damage, even though stem increment was not affected at the site. In addition, the O_3 dose, the biologically meaningful factor, does not depend on the external O_3 concentration, as despite high O_3 exposure during 2003, the O_3 dose was not enhanced. Drought therefore protected from O_3 impact. Also, O_3 did not predispose the trees to stress, the drought effect was not aggravated under the enhanced O_3 regime.

Additionally, the macroscopic impact of O_3 on the leaf blades was visualized by chlorophyll fluorescence imaging and compared to microscopical findings. Chlorophyll fluorescence generally resembled the findings by microscopy, but not in the case of O_3 injury. Despite of massive, O_3-induced injuries in the palisade parenchyma, no significant decrease was found in F_v/F_m. To improve the diagnosis of O_3 impact on photosynthesis by chlorophyll fluorescence imaging, a new method was developed and tested. The joint assessment of F_s (absolute fluorescence signal) and q_L (coefficient of photochemical quenching) gave suitable results and indicated, that the area affected by O_3 is larger than can be estimated from visible O_3 induced necroses on the leaf blade.

In summary, *F. sylvatica* at "Kranzberger Forst" is not at high risk from increased chronic O_3-regimes, although varying, spatio-temporal O_3 injuries were detected, which might influence the trees in the long term.

5 Appendix A

Publications used in this doctoral thesis

* **Löw M**, HERBINGER K, NUNN AJ, HÄBERLE K-H, LEUCHNER M, HEERDT C, WERNER H, WIPFLER P, PRETZSCH H, TAUSZ M, MATYSSEK R (2006) "Extraordinary drought of 2003 overrules ozone impact on adult beech trees (*Fagus sylvatica*)". Trees 20: 539-548

* **Löw M**, HÄBERLE K-H, WARREN C, MATYSSEK R "O_3 flux-related responsiveness of photosynthesis, respiration and stomatal conductance of adult *Fagus sylvatica* to experimentally enhanced free-air O_3 exposure". Plant Biology (in press)

* WARREN CR, **Löw M**, MATYSSEK R, TAUSZ M (2007) "Internal conductance to CO_2 transfer of adult *Fagus sylvatica*: variation between sun and shade leaves and due to free-air ozone fumigation". Environmental and Experimental Botany 59: 130-138

Extraordinary drought of 2003 overrules ozone impact on adult beech trees (*Fagus sylvatica*)"

Abstract

The extraordinary drought during the summer of 2003 in Central Europe allowed to examine responses of adult beech trees (*Fagus sylvatica*) to co-occurring stress by soil moisture deficit and elevated O_3 levels under forest conditions in southern Germany. The study comprised tree exposure to the ambient O_3 regime at the site and to a twice-ambient O_3 regime as released into the canopy through a free-air O_3 fumigation system. Annual courses of photosynthesis (A_{max}), stomatal conductance (g_s), electron transport rate (ETR) and chlorophyll levels were compared between 2003 and 2004, the latter year representing the humid long-term climate at the site. ETR, A_{max} and g_s were lowered during 2003 by drought rather than ozone whereas chlorophyll levels did not differ between the years. Radial stem increment was reduced in 2003 by drought but fully recovered during the subsequent, humid year. Comparison of AOT40, an O_3 exposure-based risk index of O_3 stress, and cumulative ozone uptake (COU) yielded a linear relationship throughout humid growth conditions, but a changing slope during 2003. Our findings support the hypothesis that drought protects plants from O_3 injury by stomatal closure, which restricts O_3 influx into leaves and decouples COU from high external ozone levels. High AOT40 erroneously suggested high O_3 risk under drought. Enhanced ozone levels did not aggravate drought effects in leaves and stem.

Introduction

"Global Change" scenarios are basically driven by the anthropogenic release of CO_2 and other "climate-effective" gases into the atmosphere (IPCC 2001). Predictions include rise in temperature coupled with altered precipitation, which regionally increase the probability of drought (Scarascia-Mugnozza et al. 2001). Compared to the long-term record, the summer of 2003 showed extraordinarily high air temperature and low precipitation regimes in Central Europe (Luterbacher et al. 2004; Schär and Jendritzky 2004; DWD 2003; Ciais et al. 2005). As seasonal drought is typically associated with high insolation, as was the case in 2003, conditions are conducive to the formation of high tropospheric ozone (O_3) levels (Stockwell et al. 1997; Fabian 2002). Hence, exposure-based indices of O_3 stress (e.g. the AOT40 concept of "Critical Levels for Ozone", Fuhrer and Achermann 1994) indicate enhanced risks of O_3 injury to forest trees (Matyssek and Innes 1999). In view of current Global Change scenarios, O_3 exposure might become exacerbated during the upcoming decades (Fowler et al. 1999; Ashmore 2005). However, it is the actual dose of O_3 uptake through leaf stomata rather than exposure that determines the O_3 stress and drives the stress response in plants (Matyssek and Sandermann 2003; Wieser et al. 2003; Matyssek et al. 2004). Drought usually leads to a decrease in stomatal conductance (Schulze 1994) and limits the O_3 flux into plants (Pääkkönen et al. 1998a; b). In fact, drought might "protect" against O_3 stress, although findings conflict about interacting drought-ozone effects in trees (Dobson et al. 1990; Chappelka and Freer-Smith 1995; Maier-Maercker 1998; Nali et al. 2004). The uncertainty on such interactions is substantial, in particular in adult forest trees, because the large majority of studies have focused on O_3 stress in juvenile woody plants under chamber conditions (Kolb and Matyssek 2001). As chronic O_3 stress bears the risk of predisposing trees to other stresses like drought (or frosts or pests), severe episodes of such stressors can eventually cause break-down of trees and forests (cf. Miller and McBride 1999).

The present study was conducted on adult beech trees (*Fagus sylvatica*) growing at a forest site in Central Europe, where they are exposed to ambient or an experimentally enhanced O_3 regime imposed by free-air canopy fumigation (Nunn et al. 2002; Werner and Fabian 2002). Trees were examined for their responsiveness to O_3 across the tree-internal scaling levels of cells, leaves, branches and the whole tree (Nunn et al. 2005a; b). We assessed stomatal conductance, photosynthetic characteristics, chlorophyll levels and stem growth during the exceptional drought of 2003 and the humid year of 2004 which represented long-term climatic average conditions. Potential O_3-induced reduction in radial stem growth was regarded as an indication of economic damage (sensu CLRTAP 2004). The following hypotheses were tested: (1) Cumulative ozone uptake (COU) rather than exposure-based O_3 indices like AOT40 reflects risk of O_3-induced damage (e.g. in stem production). (2) The effect of drought on adult *F. sylvatica* trees is aggravated by enhanced ozone levels.

Material and Methods

Study site and experimental design: The study site is located within a mixed beech / spruce forest ("Kranzberger Forst") in southern Germany near Munich (48°25'08" N, 11°39'41" E, elevation 485 m a.s.l.). The soil at

the site is a luvisol derived from loess over tertiary sediments. Beech trees (*Fagus sylvatica* L.) used in this study were about 60 years old and up to 28 m high (Pretzsch et al. 1998). Scaffolding and a research crane provided access to the crowns of 10 representative *F. sylvatica* trees (Reiter et al. 2005). A free-air ozone fumigation system (Werner and Fabian 2002; Nunn et al. 2002) was employed since the year 2000 to expose the joint canopies of five of the 10 study trees to a twice-ambient ozone regime ($2xO_3$). To prevent acute O_3 injury, the O_3 levels of this regime were restricted to a maximum of 150 nl O_3 l^{-1}. Five adjacent *F. sylvatica* trees under the unchanged ambient regime ($1xO_3$) prevailing at the site served as control (Nunn et al. 2002). Long-term regional annual air temperature is 7.5°C, and annual precipitation amounts to 788 mm (monitored by DWD at climate station "Weihenstephan", at 4 km distance from the research site; DWD Offenbach, Germany). The weather conditions at the research site of the years 2002 through 2004 which were covered in this study are given in Table 1. Ecophysiological assessments during the humid year 2004 served as the reference of those in the dry year 2003. Regarding weather conditions and O_3 regimes, 2002 was included additionally for comparison.

Assessment of micro-climate, ozone and radial stem growth: Global radiation above the canopy was measured with a pyranometer (type CM 11; Kipp & Zonen, Delft, Netherlands), and air temperature along with air humidity at 24 m height within the canopy with an aspiration psychrometer (model Assmann; Theiss, Göttingen, Germany). Rainfall was recorded by a rain gauge (model Pluvio; Ott Messtechnik, Kempten, Germany) in a forest clearing at 1 km distance from the site. O_3 levels were monitored throughout growing seasons by O_3 analysers (TML 8811; Teledyne Monitor Labs, Englewood, USA) within the canopy, both under the $1xO_3$ and $2xO_3$ regime. O_3 regimes were expressed as "Sum of all ozone concentrations" (SUM0) and "Accumulated exposure over a threshold of 40 nl O_3 l^{-1}" (AOT40, Fuhrer and Achermann 1994), as currently adopted by UNECE. All O_3 indices have been calculated for the time period between leaf expansion in spring and senescence in autumn, which is the time span relevant for ozone uptake. Phenological data have been assessed separately in each O_3 regime. Cumulative ozone uptake (COU) during the growing season was calculated according to Emberson et al. (2000), accounting for drought effects according to Nunn et al. (2005a). COU is a function of maximum stomatal conductance for O_3 and external O_3 concentration (Eq. 1 & 2):

$$F_{O_3} = g_{O_3} \cdot [O_3] \qquad (1)$$

$$COU = \sum_{SGS}^{EGS} F_{O_3} \qquad (2)$$

with F_{O3} being the stomatal O_3 flux, gO_3 the stomatal conductance for O_3, and SGS and EGS the beginning and end of the growing season, respectively. gO_3 is a function of maximum and minimum stomatal conductance, light, phenology, temperature, VPD, soil moisture, day and night time (see Nunn et al. 2005a for further details about the employed model and its parameterisation).

Annual increment of radial stem growth was monitored in each study tree at breast height with permanent girth measurement tapes (model D1, UMS, München, Germany) across the years 2001 through 2004 (2001 as reference for the subsequent years).

Assessment of leaf gas exchange: Gas exchange and chlorophyll fluorescence parameters were assessed using a Licor 6400 CO_2/H_2O diffusion porometer equipped with a "Leaf Chamber Fluorometer 6400-40" (Li-Cor, Lincoln, USA). Measurements were made at 40 – 60% relative air humidity, 360 µmol mol^{-1} CO_2 of the ambient air and saturating light conditions (1500 µmol m^{-2} s^{-1} photosynthetic photon flux density, PPFD) within the chamber. PPFD of light-saturated photosynthesis (A_{max}) had been previously determined by separate assessments (data not shown). Contrasting with 2004, the leaf temperature of 25°C could not always be maintained in 2003 due to high ambient air temperatures; nevertheless, leaf temperature never exceeded 30°C in 2003 during measurements. Leaves acclimated to the chamber conditions for at least two minutes until gas exchange readings became stable. Measurements were recorded then every 10 seconds for at least three minutes. A saturating light flash (duration 0.8 sec, PPFD > 7000 µmol m^{-2} s^{-1}) concluded each measurement to determine the electron transport rate (ETR). ETR was calculated after Krall and Edwards (1992):

$$ETR = \Delta F / Fm' \times PPFD \times a \times f \qquad (1)$$

with the absorptivity (a) set to 0.84 and light distribution between Photosystem I and II (f) to 0.5.

Assessment of water potential: Pre-dawn twig water potential was measured in June, August and October 2003 and in July 2004 with a Scholander pressure bomb (Model 3000, Soilmoisture Equipment Corporation, Santa Barbara, USA) on detached foliated twigs of 30 cm length, preventing water loss through enclosure in plastic bags prior to measurement.

Biochemical analysis: Leaf samples taken for biochemical analysis were frozen at once in liquid nitrogen and kept at –70 °C until lyophilisation. Lyophilised leaves were ground in a dismembrator (Retsch, Haan, Germany), and stored in humidity-proof plastic vials at –25 °C prior to HPLC analysis. Pigments were analysed in acetone extracts using the HPLC gradient method according to Pfeifhofer (1989) and Tausz et al. (2003). About 60 mg lyophilised plant powder and some calcium carbonate were extracted in 1 ml acetone in dark 1.5 ml reaction tubes. The extract was shaken with the Vortex-shaker (Heidolph, Reax 2000) for half a minute and centrifuged (Beckman, Avanti 30 Centrifuge, 14000 rpm) for 10 minutes at 4°C. The supernatant was filled in calibrated tubes and kept closed in the dark at 4°C. The pellet was re-extracted in 1 ml acetone, shaken and centrifuged. The supernatants were merged and the resulting volumes of the acetone extract were noted. Aliquots of the extracts were centrifuged for 30 minutes at 4°C and subjected to the HPLC analysis consisting of: SunFlow 100 pump with gradient former GF (SunChrom) and online degasser (Knauer), LKB2151 UV/VIS detector (440 nm), integrator PC software ChromStar, Midas Spark Holland autosampler cooled at 4°C, Chrom Spherisorb S5 ODS-2 250 x 4.6 mm column with Chrom Spherisorb S5 ODS-2 10 x 4.6 mm precolumn; gradient setting, solvent A: acetonitril:methanol:water = 100:10:5 (v/v/v); solvent B: acetone:ethylacetate = 2:1 (v/v); linear gradient from 90% (v) A to 20% (v) A in 18 min, 5 min at 20%, back to 90% A in 3 min; total run time of 30 min, at flow rate of 1 ml min^{-1}. Calibration was done with pure pigment standards.

Sampling protocol: Leaf gas exchange and chlorophyll fluorescence of sun-exposed *F. sylvatica* leaves were assessed four times in 2003 (June, July, September and October) and five times in 2004 (May, June, July, September and October). To ensure comparable light and temperature conditions, sampling of leaves took place around solar noon on sunny days of similar insolation.

Statistics: Statistical analysis was performed by means of the "general linear model" (GLM) of the SPSS 13 software (SPSS Inc., Chicago, USA). For each sampling date, univariate analysis was employed for comparison of responses to O_3 regimes. The overall effect of ozone, integrating all sampling dates, was analysed through the "repeated measurements" sub-category of GLM. To meet prerequisites for GLM, data had been tested for homogeneity using "Levene Test for Equality of Variances". Given the fact that the study trees at "Kranzberger Forst" are of same age but not same size, and that size might influence water and carbon relations of trees (Ryan et al. 2000), stem diameter at breast height was used as covariate in all statistical tests.

Analysis of linear and hyperbolic regressions was performed using the SigmaPlot 9 software package (Systat Software, Erkrath, Germany) in combination with SPSS 13 (SPSS Inc., Chicago, USA) and Graphpad Prism 4 (GraphPad Software Inc., San Diego, USA).

Results

The years 2002 through 2004 slightly varied in the cumulative annual insolation and mean air temperature as recorded inside the canopy at the study site (Table 1), with the annual maxima within this 3-year period occurring 2003 in global radiation and 2004 in air temperature. The mean daily air temperature at the canopy position determining tree transpiration, i.e. the sun-exposed foliage, was conspicuously higher during the growing season of 2003 as compared to the respective time period in the previous or succeeding year. The minor annual variation was reflected in rather similar seasonal courses of global radiation (as affected by changing cloudiness) and mean monthly air temperature (Fig. 1A,B), although the latter reached maxima of above 20°C during the summer of 2003 (these levels not being reached during the respective periods in 2002 and 2004). Precipitation varied between years, both during the growing season and on an annual basis, as the cumulative rainfall in 2002 exceeded that in 2003 by a factor of almost two (Table 1, Fig. 1C), in addition to high precipitation in March and November of 2002, abundant rainfall occurred throughout the summer (maximum in August), whereas precipitation was remarkably low across the entire year of 2003 (in particular during summer). 2004 was intermediate in precipitation between 2002 and 2003, both during summer and on an annual basis (Table 1; Fig. 1C). Volumetric soil water content hardly fell below 30 % in 2002, but decreased to about 15 % in June of 2003 and stayed that low until early October of the same year (Fig. 1D). At 14 % no water is left available to plant uptake in the given soil (Raspe et al. 2004). The soil water content did not fully recover during winter after the drought of 2003 so that a water deficit was occurring again during summer 2004 compared to 2002; nevertheless, effects on the trees were much less pronounced in 2004. As a consequence of the persisting soil drought in 2003, the predawn water potential (Ψ_{pre}) of *F. sylvatica* branches dropped to minima of about –1.3 to –1.4 MPa in August, regardless of the O_3 regime (Fig. 2). Such levels are extraordinarily low for Central-European forest conditions (cf. Larcher 2001). The daily minimum water potential (Ψ_{min}), as assessed during early afternoon, never dropped below –2 MPa

(means ± SD of Ψ_{min} during 2003 were -1.9 ± 0.38 MPa in $1xO_3$ and 2.0 ± 0.37 MPa in $2xO_3$) – these minimum levels are in consistency with findings on deciduous forest trees of the temperate climate zone (cf. Larcher 2001). In contrast, Ψ_{pre} was high at ample rainfall (around –0.1 MPa), as exemplified in July 2004 (Fig. 2).

The sunny and dry weather conditions during the summer of 2003 favoured the formation of ozone so that SUM0 of $1xO_3$ during the growing season amounted to 194 µl O_3 l^{-1} h (Table 1), exceeding the respective levels of both 2002 and 2004 by a factor of 1.5. Across the three-year period, SUM0 of the $2xO_3$ regime was not enhanced by a factor of 2 relative to $1xO_3$ (i.e. about 1.6-fold in 2004 and 1.8-fold in 2003 and 2002), because the experimentally imposed O_3 regime was restricted to maximum O_3 levels of 150 nl O_3 l^{-1} (s. Materials & Methods). This relative increase of SUM0 at $2xO_3$ was similar across the three study years (Table 1). AOT40 of $2xO_3$ during the growing season 2003 did reach, however, about twice the levels of 2002 and 2004 (Table 1). During each of the three study years, AOT40 exceeded the "Critical Level of Ozone" of 10 µl O_3 l^{-1} h which is proposed by UN-ECE (Fuhrer et al. 1997 – or 5 µl O_3 l^{-1} h as suggested recently by Karlsson et al. 2004) even at $1xO_3$. Across the three years, AOT40 under $2xO_3$ was between 3.5 and 4.2 times the levels that were reached annually at $1xO_3$. Remarkably, COU under $1xO_3$ was lower in 2003 (by a factor of 0.8) than during the two more humid years, although annual SUM0 and AOT40 were highest in 2003 (Table 1). Regarding $2xO_3$, COU in 2003 only slightly exceeded the levels that amounted during each of the humid years (by a factor of 1.1).

In 2003, the relationship between COU and AOT40 stayed approximately linear until July 31 at $1xO_3$ and August 2 at $2xO_3$ (Fig. 3B, see arrows), when a breakpoint was reached as the soil water content approached its minimum (Fig. 1). The slope of the linear regressions ($r^2 > 0.9$, $p < 0.001$ each) before and after these dates differed significantly ($p < 0.001$) from each other under both O_3 regimes. The slope declined, as COU per unit decreased under water limitation (Fig. 3B). The relationship between COU and AOT40 during the years 2002 and 2004 did not display such a breakpoint (Fig. 3A,C), as COU nearly linearly increased with AOT40 ($r^2 > 0.99$, $p < 0.001$) in both years and O_3 regimes. All three study years differed significantly in these slopes under $1xO_3$ and $2xO_3$ ($p < 0.001$, in 2003 only data points used prior to the breakpoint).

The cause of the change in slope between COU and AOT40 between dry and humid years was stomatal regulation, as g_s was strongly reduced during drought to less than one third of the levels that were reached under the humid conditions of 2004 (Fig. 4B). During the period of lowest Ψ_{pre} (Fig. 2), g_s did not differ anymore between the two O_3 regimes, although in June 2003, g_s was reduced under $2xO_3$ already prior to the onset of drought (Fig. 4B). In addition, g_s under $2xO_3$ appeared to be less sensitive to drought than under $1xO_3$. In contrast, the seasonal course of g_s was fairly constant under humid conditions, as exemplified during 2004, although levels of g_s were lower and less stable under the $2xO_3$ than $1xO_3$ regime (Fig. 4B). g_s was significantly reduced under $2xO_3$ conditions in July 2004 ($p = 0.047$) as compared to $1xO_3$. Analysis of the annual course (s. Materials & Methods) showed that g_s was significantly reduced during 2004 in $2xO_3$ ($p = 0.039$). The course of photosynthetic performance resembled that of g_s (Fig. 4A) in each year, however, A_{max} did not recover in October 2003. A_{max} was reduced significantly under the $2xO_3$ regime in September 2004 ($p = 0.048$). The annual course of A_{max} in 2003 was significantly different from that in 2004 in both O_3 regimes ($p < 0.001$ at $1xO_3$ and $p < 0.02$ at $2xO_3$).

Beginning in June 2003, ETR declined gradually until October, irrespective of the O_3 regime (Fig. 4C), as opposed to 2004, when under $1xO_3$ ETR was significantly higher in May ($p = 0.04$) and slightly enhanced in July and September as compared to $2xO_3$. Due to reduced ETR levels under $2xO_3$, differences between the two years under this O_3 regime were smaller than under $1xO_3$, although ETR was significantly lower in October 2003 than in October 2004 ($p = 0.04$). The relationship between ETR and stomatal conductance (Fig. 5) distinctly differed between 2003 and 2004, as correlation was apparent during the humid year ($r^2 = 0.63$, $p < 0.001$), but not so under the drought of 2003 ($r^2 < 0.01$). Correlation coefficients for hyperbolic rectangular regressions were significantly different between both years ($p = 0.0016$). Even when the regression was applied only to the data range covered by both years ($g_s < 0.15$ mol m^{-2} s^{-1}), the hyperbolic regression rendered a similar result in 2004 ($r^2 = 0.60$, $p < 0.01$) so that still both years could not be described ($p = 0.018$) by one regression. Levels of dry mass-related chlorophyll a and b (Fig. 4D) neither differed with respect to the annual water availability nor to the O_3 regimes.

Radial stem growth at breast height (Fig. 6) was limited under the drought of 2003. Nine out of the ten examined trees displayed reduced annual increment as compared with that in 2001. Given the high variability between the individual *F. sylvatica* trees in this stem response, the mean radial stem increment did not significantly differ between the two O_3 regimes.

Discussion

In general, drought is viewed to counteract adverse O_3 effects through, at least, partial closure of the stomata (Schulze 1994) which determine the pathway of O_3 uptake into plants (Matyssek and Sandermann 2003). Stomatal responses to moisture deficits can limit the O_3 uptake to the extent that the influx of O_3 may be less during sunny, dry days with high O_3 levels than under the lower O_3 regimes of overcast, humid days (Wieser and Havranek 1993). Moisture-related contrasts in O_3 influx can also be observed at the annual time scale, as shown by the reduced O_3 uptake of adult *F. sylvatica* under the high O_3 exposure during the sunny and dry growing season of 2003 relative to the high uptake under the humid summer conditions and lower O_3 levels of 2002 and 2004. Although sunny and dry weather favours O_3 formation (Stockwell et al. 1997; Fabian 2002), the higher external O_3 exposure, as expressed through SUM0 or AOT40, was not translated into increased uptake into the trees, which leads us to accept hypothesis (1) in 2003. In our study, g_s and O_3 uptake declined under both O_3 regimes at "Kranzberger Forst" as Ψ_{pre} of twigs decreased, along with soil moisture (Panek 2004), to levels that were exceptionally low for trees of the Central-European climate (Larcher 2001). However, only severe drought – as encountered in the present field study – tends to protect from O_3 impact (Retzlaff et al. 2000). In our study, this was supported by the absence of major macroscopic leaf injury in adult *F. sylvatica* in 2003. Mild soil moisture deficit, on the contrary, can exacerbate O_3 injury (Grulke et al. 2003) and accelerate leaf loss (cf. Pääkkönen et al. 1998b).

O_3 impact may change sensitivity in stomatal regulation (Matyssek and Sandermann 2003), as indicated by sluggishness in stomatal movements or inefficient control of transpiration (Keller and Häsler 1984; Barnes et al. 1990a,b; Pearson and Mansfield 1993; Karlsson et al. 2004; Paoletti and Grulke 2005) so that trees may be predisposed to drought injury. Remarkably, g_s under $2xO_3$ conditions already was lower before the onset of drought and seemed to be less sensitive to drought (cf. Paoletti and Grulke 2005). Such O_3-induced changes may be mediated through altered "mechanics" in the stomatal apparatus upon reduced cell wall lignification (Maier-Maercker 1998; cf. Kivimäenpää et al. 2003). Another explanation is the disturbed osmotic control of the guard cells upon membrane impairment (Heath and Taylor 1997; cf. Nali et al. 2004), although stomatal responsiveness to ABA has not yet been clarified under O_3 stress (Torsethaugen et al. 1999). In adult *F. sylvatica*, the relative decrease in g_s in response to drought was smaller under $2xO_3$ than $1xO_3$, however, it is remarkable that during the remainder of the drought period g_s stayed at similarly low levels in both O_3 regimes. This may relate to Ψ_{min} which was maintained at around -2 MPa regardless of the O_3 regime, although Ψ_{pre} had dropped to unusually low levels as a consequence of soil drought. It appears that stomatal regulation stayed intact under O_3 stress to the extent that the daily maximum in water tension, represented through Ψ_{min}, was stabilized, which may prevent catastrophic cavitation in the xylem vessels under persisting drought (cf. Tyree and Zimmermann 2002). O_3 stress per se did not significantly affect water potentials.

Stomatal closure during the drought of 2003 underlined the shortcoming of exposure-based indices like SUM0 and AOT40 in assessing the O_3 risk to trees (Panek 2004). Maximum O_3 uptake does not necessarily correlate with periods of peak external O_3 levels. SUM0 and AOT40 may adequately correlate with O_3 uptake only when soil moisture is not limiting, which can lead to erroneous conclusions about risks of O_3 injury in dry years (Panek et al. 2002). Until the end of July 2003, the relationship between cumulative ozone uptake and AOT40 was linear, although such correlations may be rather variable, and linearity cannot be generalized (Karlsson et al. 2004; Matyssek et al. 2004). However, the relationship changed its slope around the end of July 2003, when g_s decreased as a consequence of the continuing drought. This effect was more pronounced under the $2xO_3$ than the $1xO_3$ regime. Panek et al. (2002) suggested to weight O_3 levels by g_s or soil moisture. One approach, which was adopted in the present study, is to extend g_s-based models of O_3 uptake (Emberson et al. 2000) by adequate algorithms, such as the model extension by Nunn et al. (2005a). The extension given there significantly increased modelling precision of O_3 uptake.

However, stomatal exclusion of O_3 is accompanied by restricted CO_2 fixation (Panek and Goldstein 2001), and the latter effect may even be exacerbated through synergism between O_3 and drought (Grulke et al. 2002). In adult *F. sylvatica* of our study, drought-driven decline in A_{max} in parallel to decreasing g_s was observed under $1xO_3$ during the year 2003, but not so under $2xO_3$, where A_{max} and g_s had been low since early summer. Nevertheless, it is reported that O_3 may even seasonally increase g_s and A_{max}. This may be a consequence of sink induction for carbon in relation to O_3 defence (Kolb and Matyssek 2001; cf. Körner 2003) or resource retranslocation from old injured into newly formed leaves (Beyers et al. 1992; Maurer et al. 1997).

ETR showed a distinct drought effect in 2003 (cf. Fig. 4C) in the *F. sylvatica* trees at "Kranzberger Forst". Other studies reported only slight drought effects on ETR (Ogaya and Penuelas 2003, Flexas et al. 1999).

Furthermore, ETR was only poorly related to g_s at "Kranzberger Forst" in the dry year 2003 ($r^2 < 0.01$; Fig. 5). This relationship was well established under the humid conditions of 2004 ($r^2 = 0.63$). The correlation was similar to that reported by Flexas et al. (2002) from *Vitis vinifera* which was grown both under irrigated and non-irrigated conditions in the Mediterranean region, characterized by regular summer drought. In addition, this kind of relationship between ETR and g_s has been reported by Medrano et al. (2002) for several plant species from different climatic zones. The absence of such a correlation in *F. sylvatica* during 2003 apparently underlines, in Central Europe, the anomaly of the severe drought in that year (Luterbacher et al. 2004). Nevertheless, high levels of ETR at low g_s in 2003 are consistent with the conceptual scheme by Medrano et al. (2002, Fig. 3 given there) to the extent that this scheme may be applicable to *F. sylvatica* at "Kranzberger Forst". High ETR at low g_s during drought may indicate ongoing photorespiration as counteracting photoinhibition (Guan et al. 2004, Medrano et al. 2002). Despite the variation in ETR, chlorophyll levels in *F. sylvatica* were neither affected by drought nor ozone, suggesting – in accordance with findings by Herbinger et al. (2002) in *Triticum durum* and *T. aestivum* – intactness of the photosynthetic apparatus.

Given the trade-off between protection against O_3 injury at high O_3 exposure and reduced A_{max} through the drought-driven closure of stomata, limitations in biomass production, resource allocation and structural differentiation may be the "price" of the exclusion of ozone (Matyssek and Sandermann 2003). On mesic sites, O_3 impact resulted, in *Pinus jeffreyi*, in thinner branches but earlier needle loss than on xeric sites (Grulke et al. 2003). In *F. sylvatica*, Stribley and Ashmore (2002) reported decrease in shoot growth to be mainly related to drought and concluded that, depending on the site, also O_3 can contribute to the decline of *F. sylvatica*. Nutrition was affected in *Picea abies* through drought rather than O_3 (Wallin et al. 2002; Kivimäenpää et al. 2003), and even though O_3 limited stem growth, the pollutant was regarded less important than drought in this latter respect. Also in the adult *F. sylvatica* trees of "Kranzberger Forst", drought rather than O_3 limited radial stem growth in 2003. The absence of an O_3 effect on stem growth under $2xO_3$ relative to $1xO_3$ was consistent with the observation during humid years. Hence, no O_3-induced damage on stem production was substantiated (Wipfler et al. 2005). Although Hypothesis 1 was corroborated in that COU was uncoupled from AOT40, the low O_3 sensitivity of stem growth prevented, in the given case of beech at "Kranzberger Forst", the damage to materialize. Apparently, stem growth of tall trees is highly buffered against O_3 stress, which appears to be in contrast to juvenile trees (cf. Kolb and Matyssek 2001). This difference may be due to a high reserve storage capacity (backing processes of detoxification and repair) or stress compensation already within the crown of tall trees. The metabolic responsiveness at the crown level leads to conclude that risks of chronic O_3 stress still cannot be ruled out (Nunn et al. 2005a; b). Suggestions that chronic O_3 stress may enhance the susceptibility of trees to drought (cf. Matyssek et al. 2006), resulting in reduced stem growth during subsequent years (Dittmar and Elling 1999; Dittmar et al. 2003) or even decline of trees and forests (Miller and McBride 1999), were not confirmed in our study. Hence, irrespective of the analysed tree parameter, hypotheses (2) was to be rejected, the more so as drought and ozone led to differential effects on g_s, A_{max}, ETR and stem growth of the study trees.

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Table 1: Weather conditions and ozone regimes at the site "Kranzberger Forst" near Freising / Germany, during the years 2002, 2003 and 2004. Precipitation was measured in a forest clearing at 1 km distance from the site. SUM0 is the "Sum of all O_3 concentrations", AOT40 is the "Accumulated exposure over a threshold of 40 nl O_3 l^{-1}", COU is "cumulative ozone uptake".

		Year		
		2002	2003	2004
annual sum of global radiation above canopy	[GJ m^{-2}]	4.1	4.6	4.4
mean annual air temperature at canopy height, 24 m above ground	[°C]	8.8	9.1	9.4
mean daily air temperature during growing season (May to October) at canopy height, 24 m above ground	[°C]	14.1	16.2	14.9
annual sum of precipitation	[mm]	1015	557	786
precipitation during growing season (May to October)	[mm]	626	359	448

[a] data by courtesy of LWF, G. Gietl.

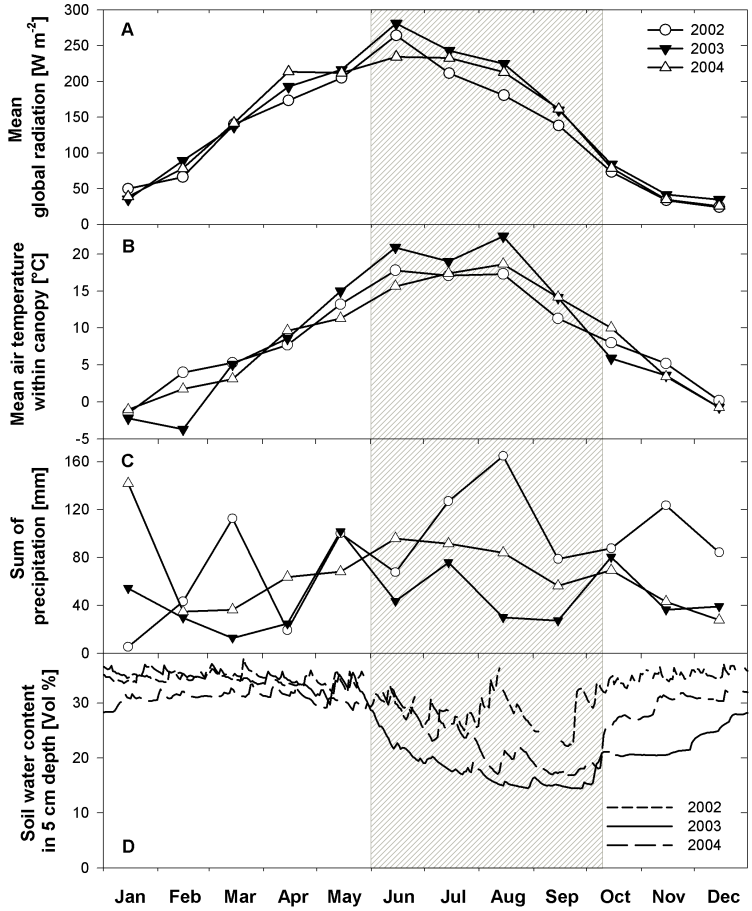

Fig. 1: Monthly sum of global radiation (A), air temperature at canopy height, i.e. 24m above ground (B), precipitation (C) and soil water content (D) during the years 2002, 2003 and 2004 at the research site "Kranzberger Forst" in southern Germany. Symbols given in (A) also apply for (B) and (C). Hatched area indicates drought period in 2003. Data in (C) and (D) by courtesy of LWF (G. Gietl and W. Grimmeisen).

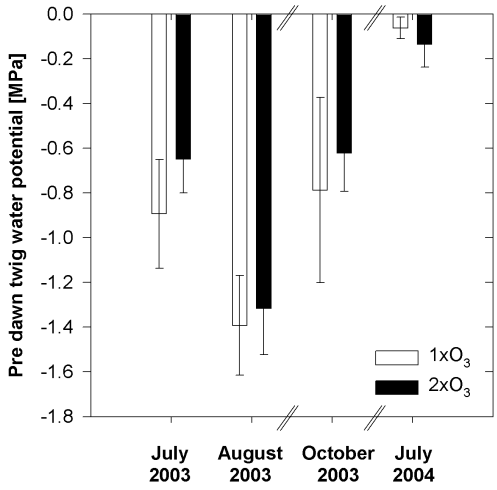

Fig. 2: pre-dawn twig water potential in July, August, October 2003 and July 2004. Columns represent mean values ± standard deviation (n = 5 trees in each bar).

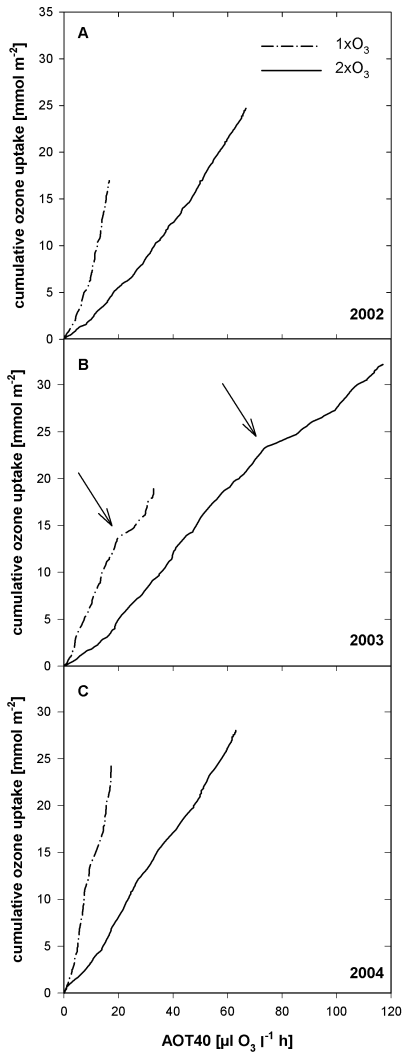

Fig. 3: Relationship between AOT40 and cumulative ozone uptake during 2002 (A), 2003 (B) and 2004 (C). Arrows in (B) indicate July 31, 2003 in $1xO_3$ and August 2, 2003 in $2xO_3$, when soil moisture became limiting. (B) re-evaluated after Matyssek et al. (2006).

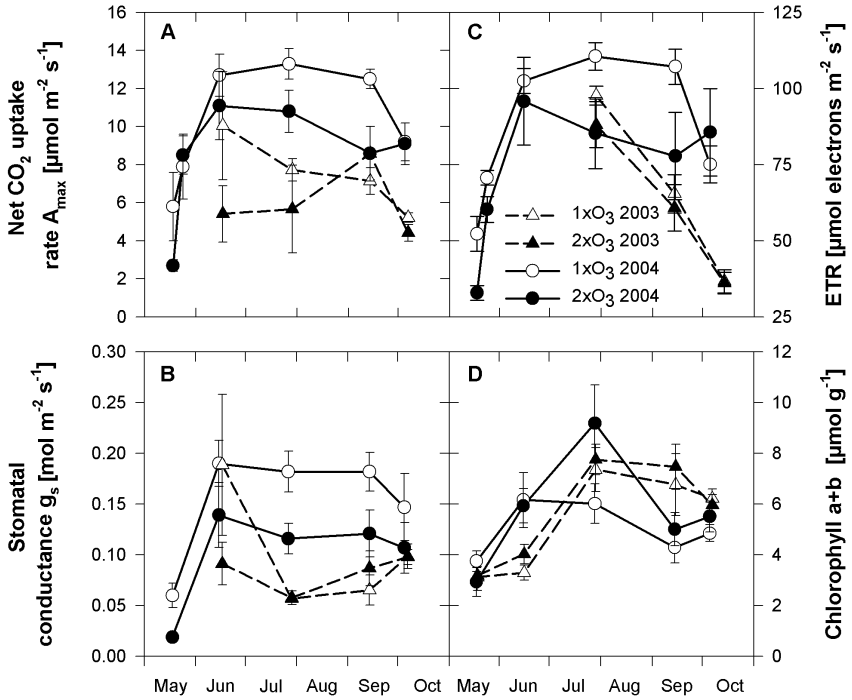

Fig. 4: Net CO_2 uptake rate (A_{max}) (A), stomatal conductance to water vapour (g_s) (B), electron transport rate (ETR) (C) (all measured at saturating light conditions) and dry mass related chlorophyll levels (D) during 2003 and 2004 (means ± one standard error of the mean of n = 3 – 5 trees in each pair of values). Symbols given in (C) are valid also for (A), (B) and (D).

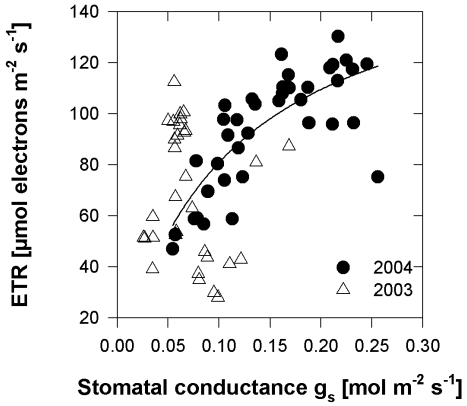

Fig. 5: Relationship between electron transport rate (ETR) and stomatal conductance for water vapour (g_s) during 2003 and 2004. Hyperbolic regression (of single rectangular type) at $r^2 = 0.63$ only in 2004; $r^2 < 0.01$ in 2003 (regression line not shown).

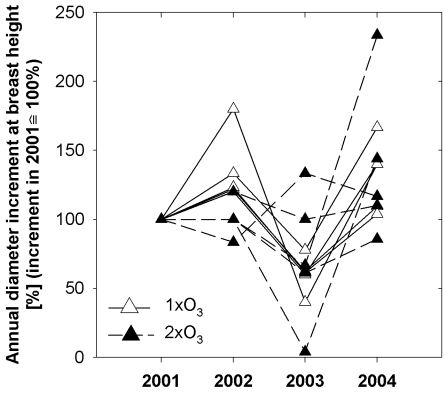

Fig. 6: Relative annual stem diameter increment at breast height of the ten adult *F. sylvatica* trees examined in this study during the years 2001, 2002, 2003 and 2004 (increment in 2001≙ 100%).

O_3 flux-related responsiveness of photosynthesis, respiration and stomatal conductance of adult *Fagus sylvatica* to experimentally enhanced free-air O_3 exposure

Abstract

Knowledge of responses of photosynthesis, respiration and stomatal conductance to cumulative ozone uptake (COU) is still scarce, and this is particularly the case for adult trees. The effect of ozone (O_3) exposure on trees was examined with 60-year-old beech trees (*Fagus sylvatica*) at a forest site of southern Germany. Trees were exposed to the ambient O_3 regime ($1xO_3$) or an experimentally elevated twice-ambient O_3 regime ($2xO_3$). The elevated $2xO_3$ regime was provided by means of a free-air O_3 canopy exposure system. The hypotheses were tested that (1) gas exchange is negatively affected by O_3 and (2) the effects of O_3 are dose-dependent and thus the size of differences between treatments are positively related to COU. Gas exchange (light-saturated CO_2 uptake rate A_{max}, stomatal conductance g_s, maximum rate of carboxylation Vc_{max}, ribulose-1,5 bisphosphate turnover limited rate of photosynthesis J_{max}, CO_2 compensation point CP, apparent quantum yield of net CO_2 uptake AQ, carboxylation efficiency CE, day and nighttime respiration) and chlorophyll fluorescence (electron transfer rate, ETR) were measured *in situ* on attached sun and shade leaves. Measurements were made periodically throughout the growing seasons of 2003 (an exceptionally dry year) and 2004 (a year with average rainfall).

In 2004 Vc_{max}, J_{max} and CE were lower in trees receiving $2xO_3$ compared with the ambient O_3 regime ($1xO_3$). Treatment differences in Vc_{max}, J_{max}, CE were rather small in 2004 (i.e. parameter levels were lower by 10 – 30% in $2xO_3$ than $1xO_3$) and not significant in 2003. In 2004 COU was positively correlated with the difference between treatments in A_{max}, g_s and ETR (i.e. consistent with the dose-dependence of O_3's deleterious effects). However, in 2003, differences in A_{max}, g_s and ETR between the two O_3 regimes were smaller at the end of the dry summer 2003 (i.e. when COU was greatest). The relationship of COU with effects on gas exchange can apparently be complex and, in fact, varied between years and within the growing season. In addition, high doses of O_3 did not always have significant effects on leaf gas exchange. In view of the key findings, both hypotheses were to be rejected.

Introduction

Responses of trees to chronic ozone (O_3) stress are inconsistent because of different growing seasons, sites, species, and differences in ontogeny (e.g. Kolb and Matyssek, 2001; Karnosky et al., 2005; Nunn et al., 2005b; Riikonen et al., 2005; Velikova et al., 2005; Matyssek et al., 2006c). Research has focused on the O_3-induced reduction of biomass production, largely because it is an indicator of economic damage relevant for policy decisions (CLRTAP, 2004). A growing number of studies are showing that high levels of tropospheric O_3 can affect the physiology of plants (Matyssek and Sandermann, 2003; Ashmore, 2005), even in cases where growth is unaffected. Therefore, O_3 provides additional uncertainty to global-change scenarios (Giles 2005). In adult European Beech (*Fagus sylvatica*), for example, annual stem increment was unaffected by enhanced chronic O_3 exposure over three years (Wipfler et al. 2005), although a range of biochemical tree parameters were affected by O_3 (Herbinger et al., 2005; Blumenröther et al., 2006; Haberer et al., 2006; Matyssek et al., 2006b). An important implication of these and other studies is that examining annual stem increment alone may ignore O_3 responses at the leaf and cell level, even though leaf and cell level responses can affect fitness through changes in whole-plant carbon allocation (Matyssek et al., 2005) and ontogenetic development (Sandermann and Matyssek 2004).

Data on the photosynthetic response to O_3 uptake are still scarce for adult forest trees because biomass reduction has been the focus of research (Fuhrer et al., 1997; Karlsson et al., 2004; Pleijel et al., 2004; Uddling et al., 2004). The present study examines the relevance of O_3 in adult beech trees under Central-European forest conditions for photosynthesis. Photosynthesis is used as an indicator of leaf performance under chronic O_3 stress rather than as a determinant of tree growth (Bazzaz 1997; Körner 2003).

One reason for contradictory results in studies on O_3 effects on trees is the difficulty in quantifying O_3 stress in a biologically meaningful way. Different indices may give different results and examination of the suitability of O_3 exposure versus uptake indices is advocated in risk assessment on forest trees (Wieser and Tausz, 2005). In fact, there is now considerable evidence that assessment based on O_3 uptake (i.e. the O_3 dose in phytomedical terms; Matyssek et al., 2006b; cf. Medical Dictionary, 2003) is biologically more meaningful (Matyssek and Sandermann 2003; Uddling et al. 2004). If the effects of O_3 are truly dose-dependent then we would predict a correlation

between the size of O_3 effects and cumulative O_3 uptake (COU). Therefore, one aim of this study was to determine whether there is a correlation between reductions in gas exchange and COU.

The study used a novel free-air canopy O_3 fumigation system as described elsewhere (Werner and Fabian, 2002; Nunn et al., 2002; Karnosky et al., 2006). The hypotheses tested were (1) that photosynthetic parameters (light-saturated CO_2 uptake rate A_{max}, electron transfer rate ETR, maximum rate of carboxylation Vc_{max}, ribulose-1,5 bisphosphate turnover limited rate of photosynthesis J_{max}, CO_2 compensation point CP, apparent quantum yield of CO_2 uptake AQ, carboxylation efficiency CE), day and nighttime respiration as well as stomatal conductance (g_s) are negatively affected by chronic O_3 stress**,** and (2) the deleterious effects of O_3 are dose-dependent and thus the size of differences between treatments in gas exchange are positively related to COU.

Materials and Methods

Research site: The research site "Kranzberger Forst" was near Freising (Germany) within a mixed beech / spruce forest (*Fagus sylvatica* (L.) / *Picea abies* (L.) Karst., 485 m a.s.l., 48°25' N, 11°39' E). Ten adult beech trees were selected, i.e. five adjacent individuals to be studied under the unchanged ambient O_3 regime prevailing at the forest site ($1xO_3$ = control), or under an experimentally enhanced O_3 regime ($2xO_3$). To prevent acute O_3 injury, the $2xO_3$ regime was limited to a maximum of 150 nl O_3 l^{-1}. $2xO_3$ was generated through a free-air canopy O_3 exposure system , continuously tracking the $1xO_3$ regime (for details on the site and fumigation see Pretzsch et al., 1998; Karnosky et al., 2006). Weather conditions at the site during this study are presented in Table 1. Long-term regional annual air temperature is 7.5°C, and annual precipitation amounts to 788 mm (monitored by DWD at climate station "Weihenstephan", at 4 km distance from the research site; DWD Offenbach, Germany).

Assessment of leaf gas exchange and chlorophyll fluorescence: Gas exchange and chlorophyll fluorescence of beech leaves were measured using an open-path gas exchange system (LI-6400, Li-Cor, Lincoln, USA). Two different chambers were used depending on measurements. A Li-Cor LI-6400-40 "Leaf Chamber Fluorometer" was used (cf. Löw et al. 2006) for determining net CO_2 uptake rate (A_{max}), stomatal conductance to water vapour (g_s) and electron transport rate (ETR) at saturating light (1500 µmol photons m^{-2} s^{-1}) in the sun crown. A LI-6400-02(B) chamber was used for assessing light and CO_2 responses of leaf gas exchange as well as day and night-time respiration in sun and shade leaves. The IR gas analyser was calibrated with Wösthoff gas mixing pumps (Wösthoff oHG, Bochum, Germany) and a dew point generator (Kr-Kw30/7, WALZ, Effeltrich, Germany).

Dependence of the gas exchange of sun and shade leaves on irradiance and CO_2 concentration along with daytime respiration were assessed twice in 2003 (July and September) and three times in 2004 (July, August and September) per each tree and crown position. Relative humidity within the chamber was 40 – 60% while leaf temperature was 25°C. The irradiance response of photosynthesis was determined under 20, 40, 60, 80 and 1500 µmol photons m^{-2} s^{-1} at 360 µmol CO_2 mol^{-1}, and the CO_2 response under saturating light of 1500 µmol photons m^{-2} s^{-1} and five CO_2 concentrations (80, 180, 360, 740 and 2000 µmol mol^{-1}) following procedures described by Reiter et al. (2005).

Apparent quantum yield of net CO_2 uptake (AQ) was calculated as the linear slope between the net CO_2 uptake rate and photosynthetic photon flux density (PPFD) at < 80 µmol m^{-2} s^{-1}, and carboxylation efficiency (CE) as the linear slope in relation to the CO_2 concentration of the leaf-intercellular air space at CO_2 levels < 360 µmol mol^{-1}. The latter regression also allowed for assessing the CO_2 compensation point (CP, the point within the CO_2 response assessment with zero net CO_2 exchange). The response of photosynthesis to CO_2 was fitted to the biochemical model of C_3 photosynthesis described by Farquhar et al. (1980). The maximum rate of carboxylation (Vc_{max}) and the electron transport-related and ribulose-1,5-bisphosphate turnover-limited rate of photosynthesis (J_{max}) were derived as demonstrated by Reiter et al. (2005). A possible limitation by triose phosphates was not included because there was no evidence for reductions in photosynthesis at high CO_2 levels (data not shown). Leaf temperatures varied somewhat from 25°C, and thus it was necessary to normalise Vc_{max} and J_{max} to 25°C according to Harley et al. (1992) and Bernacchi et al. (2001).

The daytime respiration rate (r_{day}) was measured as the net CO_2 release rate after exposing the leaf blade to darkness for 20 minutes at 360 µmol CO_2 mol^{-1} in the gas exchange cuvette. Night-time respiration rate (r_{night}) was assessed before sunrise. Conditions inside the gas exchange cuvette were set to 360 µmol CO_2 mol^{-1} of the ambient air, and leaf temperature and relative humidity were set close to night-time ambient conditions at 17°C and 60%, respectively.

Gas exchange (A_{max}, g_s) and chlorophyll fluorescence (ETR) of sun leaves under light saturation were assessed five times during the growing season of 2003 (June 17, July 29, August 11, September 14, October 14) and seven times during 2004 (May 18, May 24, June 16, July 27, August 11, September 14 and October 5). Leaves were already fully expanded at the first measurement in May and did not show autumnal discolouration at the last assessment date in October. Measurements were conducted around noon of sunny days, and leaves were acclimated to the chamber conditions for at least two minutes until gas exchange readings became stable. The chamber conditions were set to 40 – 60% relative air humidity (close to ambient conditions), leaf temperature of 25°C, 360 µmol CO_2 mol^{-1} of the ambient air, and saturating light conditions (1500 µmol photons m^{-2} s^{-1}) for standardization of measurements. During the hot summer of 2003, the leaf temperature during gas exchange measurements at saturating light could not always be maintained at 25°C but did not exceed 30°C except for the assessment during August. A single pulse of > 7000 µmol photons m^{-2} s^{-1} concluded each gas exchange measurement to assess light-saturated ETR.

O_3 flux calculation: Cumulative O_3 uptake (COU) was calculated based on a multiplicative model described by Emberson et al. (2000). The model was extended so as to consider the effect of soil drought on O_3 uptake (Nunn et al. 2005a). The extended model was parametrised separately for each O_3 regime and sun and shade leaves. The model states that COU is a function of maximum stomatal conductance for O_3 and external O_3 concentration (Eq. 1 & 2):

$$F_{O_3} = g_{O_3}[O_3] \qquad (1)$$

$$COU = \sum_{SGS}^{EGS} F_{O_3} \qquad (2)$$

with F_{O3} being the stomatal O_3 flux, gO_3 the stomatal conductance for O_3, and SGS and EGS the beginning and end of the growing season, respectively. To determine the O_3 flux into leaves gO_3 was modified by the model as a function of the external O_3 concentration, VPD, temperature, phenology, senescence, daytime, soil moisture and PPFD (for more information see Nunn et al., 2005a). All input variables were measured at the site.

Statistics and regression analysis: Statistical analysis was performed with the SPSS 13 software package (SPSS Inc., Chicago, USA). A two-factorial ANOVA based on "General Linear Model" was used to analyse the effect of O_3 ($1xO_3$ / $2xO_3$) and leaf types (sun crown / shade crown) on the leaf parameters derived from the light and CO_2 dependences of leaf gas exchange. Homogeneity was proved by the "Levene Test for Equality of Variances". If this test failed (indicated in Table 2), the non-parametric Mann-Whitney-U test, using "exact probabilities", instead of GLM, was employed for sun and shade leaves each. Data comparisons between years were performed through the Wilcoxon test. Analysis of first and second-order linear regressions was performed using SigmaPlot 9 (Systat Software, Erkrath, Germany) in combination with Prism 4 (GraphPad Software Inc., San Diego, USA).

Responses of the examined tree parameters assessed several times during both study years (A_{max}, g_s and ETR) under $2xO_3$ to COU were expressed relative to respective responses under $1xO_3$ measured within same days (cf. Bortier et al., 2000; Novak et al., 2005). The response level at $1xO_3$ was set as 100% at each respective day. COU differed at each respective day between the two O_3 regimes, therefore, COU at $1xO_3$ is given each as a reference in Figs. 2, 3 and 4.

For clarification, the term "relative" will be used for results as derived from the above method, otherwise, results will be referred to as "absolute". Each pair of "relative" data in Figs. 2,3,4 C,D each represent the mean across the examined trees under $2xO_3$ as related to the corresponding mean under $1xO_3$ within same days. Data measured at the same date are labelled with the same number in Figs. 2,3,4.

Results

Most parameters were consistently lower in shade leaves than sun leaves (Fig. 1, Table 2). In 2003 O_3 had no significant effect on photosynthetic and respiratory parameters (Vc_{max}, J_{max}, CP, AQ, CE, r_{day}, r_{night}, Fig. 1, Table 2), whereas in September 2004 Vc_{max} (Fig. 1, $p = 0.007$), J_{max} and CE (Table 2) were significantly lower under $2xO_3$. r_{day} measured in July 2004 was lower in sun leaves under $2xO_3$ ($p = 0.032$). Relationships of parameters with COU were inconsistent. At COU > 20 mmol m^{-2} in 2004 Vc_{max} was significantly lower under $2xO_3$, whereas such an effect was not found in 2003 at even higher COU (Fig. 1A,B). At COU < 20 mmol m^{-2}, Vc_{max} even tended to be enhanced in 2004 and throughout 2003 in sun leaves under $2xO_3$ (Fig.1). Shade leaves did not differ in Vc_{max}

between the O_3 regimes in 2003, but tended to be lower under $2xO_3$ in 2004. Both Vc_{max} and J_{max} were statistically significantly different under $1xO_3$ in the dry September 2003 compared to September 2004 (Table 3), whereas no such effect was found under $2xO_3$. AQ was significantly lower under $2xO_3$ in sun leaves in September 2003 in comparison to September 2004 ($p = 0.043$). r_{day}, CP and CE did not differ between the two study years.

The "absolute" and "relative" relationships of A_{max}, g_s and ETR with COU were generally similar to one another (Figs. 2,3,4 B, D), but differed strongly between 2003 and 2004. In fact, relationships contrasted between 2003 and 2004. However, in each year the "relative" data of mid-May and October were markedly different from the intervening measurements (late May, June, July, August and September). This may be because differentiation of leaves was incomplete in May, and by October leaves had begun senescence (although in the absence of discoloration). To avoid confounding physiological responses to O_3 with ontogenetic effects, mid-May and October data were regarded as non-representative of summer months so that first-order linear regression only used summer measurements within the "relative" relationships (Figs. 2,3,4 C,D). It is noteworthy that these ontogenetic effects were not evident in "absolute" data (Figs. 2,3,4 A,B), thus demonstrating the utility of "relative" data for examining differences between treatments.

The annual course of "absolute" A_{max} reached a maximum during 2004 ($r^2 = 0.57$, $p < 0.05$ for a second-order regression; Fig. 2B), whereas during the drought of 2003 no such time course was observed and the correlation between "absolute" A_{max} and COU was poor (Fig. 2A). The maximum COU during 2003 was higher than during 2004. Under $1xO_3$, COU of 2003 stayed below the maximum of 2004, whereas highest COU was reached under $2xO_3$ in 2003. The seasonal courses of "relative" A_{max} differed between 2003 and 2004 ($p = 0.015$, Fig. 2C,D). Remarkably, a negative correlation of parameter differences (i.e. differences between O_3 regimes getting smaller with rising dose) between the two O_3 regimes and COU ($r^2 = 0.88$, $p < 0.05$) was found in 2003, in the absence of an overall O_3 effect on "absolute" A_{max} (Fig. 1C). The annual course of g_s and its relationships with COU resembled that of A_{max} in 2003 and 2004 (Fig 3, under both "absolute" and "relative" perspective). Additionally, there was no statistically significant effect of O_3 and drought (2003 versus 2004) on the stomatal limitation of photosynthesis (Farquhar and Sharkey, 1982), data not shown (cf. Warren et al., 2006). Relationships of ETR with COU were weak and inconsistent. The relationship between "absolute" ETR and COU displayed a maximum in 2004 ($r^2 = 0.62$, $p < 0.05$, Fig. 4B), as was also observed for A_{max} and g_s. During 2003 the relationship of ETR with COU differed between treatments (Fig. 4A), which contrasts with the consistent responses observed for A_{max} and g_s. In neither 2003 nor 2004 was "relative" ETR related to COU (Fig. 4C,D).

Discussion

Data lend tentative support to hypothesis (1) that chronic O_3 stress reduces gas exchange of *Fagus sylvatica*. However, the effects of O_3 were inconsistent and rather modest, especially in comparison with the effects of drought in 2003. O_3 only reduced Vc_{max} by 29% ($p = 0.007$) when COU was greater than 20 mmol m^{-2} during 2004. Such COU levels are above suggested O_3 thresholds to protect vegetation from O_3-induced damage (Fuhrer et al., 1997; CLRTAP, 2004), which highlights the O_3 tolerance of adult *F. sylvatica*. The effect of O_3 exposure on Vc_{max} was evident in 2004, but not in 2003 at even higher COU (Fig. 1). The minor and inconsistent effects of chronic O_3 stress contrast strongly with the more consistent reductions throughout the season in Vc_{max} and J_{max} due to episodic drought stress (Table 3, cf. Wilson et al., 2000; Grassi and Magnani, 2005). Given the contrasting findings in 2003 and 2004, hypothesis (1) in total is to be rejected. Photosynthetic parameters were negatively affected by chronic O_3 exposure only when seasonal water conditions were not limiting.

In mid-summer 2004, r_{day} was reduced by elevated O_3, but similar reductions were not observed later in the growing season. Such reductions in respiration under O_3 stress were reported by Edwards et al. (1994) for mature *Quercus rubra* and by Peñuelas et al. (1994) for *Picea sitchensis* seedlings, but these results contrast with other studies showing O_3 increases respiration (e.g. Reich, 1983; Amthor, 1988; Maurer et al., 1997; Dizengremel, 2001). Reductions in r_{day} in this and other studies might be because the effect of enlarging necroses (Klotsche and Häberle, unpublished) overrode the effect of stimulated respiration in the remaining living cells. Such latter interrelationships may be one reason why the observed reduced respiration rate in this present study vanished by September, i.e. at high COU. Overall, consistency appears to exist with conclusions about high carbon buffering capacity of adult beech trees (cf. Körner, 2003).

A key finding is that the effect of O_3 on gas exchange differed between an atypical dry year (2003) and a year with average precipitation (2004). An earlier investigation at the same site found similar between-year differences (Nunn et al., 2005b). The drought during the summer of 2003 (Schär and Jendritzky 2004) affected how plants responded to O_3 (Löw et al., 2006), hence, the relationships of COU with "absolute" A_{max}, g_s and ETR differed substantially between 2003 and 2004 (Figs. 2,3,4). In years of average rainfall (e.g. 2004), A_{max}, g_s and ETR reached a distinct maximum during summer (Koike, 1990; Leuschner et al., 2001; Uemura et al., 2004), but maxima were not observed in 2003 in *F. sylvatica* (this study) or in *Quercus robur* and *Fraxinus oxyphylla* (Grassi and Magnani, 2005). ETR was not affected by O_3 (Fig. 4A), which appears at least partially at odds with the finding that gas exchange parameters were affected. The most likely explanation for this discrepancy is that gas exchange parameters are determined on a leaf area basis and thus are affected by the O_3-induced decline in leaf structure (Matyssek et al., 1991), whereas ETR is independent of changes in leaf structure. The high resistance of ETR to O_3 stress under the COU encountered in this study may well indicate that high concentrations of α-tocopherol and other antioxidative substances (Haberer et al., 2006) protected the photosynthetic apparatus (Kronfuss et al., 1998; De Temmerman et al., 2002). The absence of a correlation during 2003 between COU and "absolute" A_{max} and g_s (Figs. 1,2) suggests that drought overruled the O_3 impact as O_3 intake was low under high O_3 exposure (Panek et al., 2002; Löw et al., 2006).

The effects of O_3 on gas exchange were not consistently dose dependent. Hypothesis (2) is therefore rejected in total, but confirmed in a year with average precipitation. In 2004 there was evidence that the effects of O_3 on relative A_{max}, g_s and ETR were dose-dependent (Figs. 2,3,4 D each) resulting in bigger differences between the two O_3 regimes with rising dose. However, during 2003 there was a reversed relationship of O_3 effect on dose, so that differences between the O_3 regimes diminished with rising COU. In other words, drought changed the O_3 sensitivity per unit of O_3 uptake, i.e. the effective O_3 dose (Matyssek et al., 2004; Musselman et al., 2006). In neither year were the responses of photosynthesis and stomatal conductance linearly related to COU throughout the whole season. Early and late in the growing season relationships were somewhat different. The reason for "atypical" findings at the beginning and end of the growing season might be that O_3 caused delayed leaf differentiation and premature senescence under $2xO_3$ (Matyssek et al., 2006a). Changing sensitivity of net photosynthesis and stomatal conductance throughout the growing season (cf. Polle et al., 2001) in particular to O_3 (cf. Figs. 2,3,4 C,D) was also shown by Novak et al. (2005).

Leaf-level parameters were sensitive to other seasonal internal and external factors (drought, leaf ontogeny) in parallel to O_3 impact. The effects of O_3 on photosynthesis, respiration and stomatal conductance were not statistically significant throughout the growing seasons, and this is despite the fact that O_3 doses were large. The inconsistency of responses to O_3 at the leaf level between 2003 and 2004 (cf. Matyssek et al., 2006a) and the absence of effect on stem growth at a short term scale (Wipfler et al., 2005) supports the view that growth in adult trees is highly buffered against O_3 stress. Nevertheless, adverse effects of O_3 on the long term scale on future stem growth (Deckmyn et al., 2006, as shown by modeling) and, in particular, on tree fitness (cf. Matyssek et al., 2005) cannot be ruled out during the extended life spans of trees (Matyssek et al., 2006a).

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Table 1: Air temperature within the canopy and precipitation at the site "Kranzberger Forst" near Freising (Germany), during the years 2003 and 2004 (adapted from Löw et al., 2006).

		Year	
		2003	2004
mean annual air temperature at canopy height, 24 m above ground	[°C]	9.1	9.4
mean daily air temperature during growing season (May to October) at canopy height, 24 m above ground	[°C]	16.2	14.9
annual sum of precipitation	[mm]	557	786
precipitation during growing season (May to October)	[mm]	359	448

Table 2 (next page): Electron transport-related ribulose-1,5-bisphosphate-limited rate of photosynthesis (J_{max}, µmol m^{-2} s^{-1}), CO_2 compensation point (CP, µmol CO_2 mol^{-1}), apparent quantum yield of net CO_2 gas exchange (AQ, µmol CO_2 $µmol^{-1}$ photons), carboxylation efficiency (CE, µmol CO_2 m^{-2} s^{-1} $µl^{-1}$ CO_2), respiration during daytime (r_{day}, µmol CO_2 m^{-2} s^{-1}) and respiration during night-time (r_{night}, µmol CO_2 m^{-2} s^{-1}) of adult *Fagus sylvatica* trees at "Kranzberger Forst" during 2003 and 2004. Means of five trees. S.D. is standard deviation. "Sun" = sun crown leaves, "Sha" = shade crown leaves. p-values of effects calculated by General linear model ANOVA (GLM) for main effects leaf type sun crown "Sun" / shade crown "Sha", O_3-treatment ($1xO_3$ / $2xO_3$) and leaf type x O_3-treatment or Mann-Whitey-U test respectively. The Mann-Whitney-U test was used when preconditions of GLM were not met (Levene test) indicated by letter 'a'. Significant differences at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) are indicated by bold numbers.

2003		July											September									
leaf type	Treatment	J_{max}	S.D.	CP	S.D.	AQ	S.D.	CE	S.D.	r_{day}	S.D.	r_{night}	S.D.	J_{max}	S.D.	CP	S.D.	AQ	S.D.	CE	S.D.	r_{day}	S.D.	r_{night}	S.D.
Sun	$1xO_3$	102	53	48	5	0.05	0.03	0.06	0.03	1.6	0.3	-		90	17	47	2	0.03	0.02	0.05	0.02	1.6	0.6	-	
Sun	$2xO_3$	97	26	54	10	0.05	0.02	0.09	0.02	1.0	0.6	-		98	6	49	4	0.01	0.01	0.07	0.01	1.1	0.2	-	
Shade	$1xO_3$	-	-	-	-	-	-	-	-	-	-	-		46	14	46	0	0.01	0.00	0.04	0.01	0.5	0.3	-	
Shade	$2xO_3$	-	-	-	-	-	-	-	-	-	-	-		49	27	45	1	0.02	0.01	0.03	0.02	1.0	0.7	-	
p-values of differences between above parameters																									
leaf type		-		-		-		-		-		-		**0.000**		a		0.225		**0.010**		a		-	
Treatment		0.845		a		0.489		0.186		0.075		-		0.528		a		0.272		0.688		a		-	
leaf type x Treatment		-		-		-		-		-		-		0.800		a		**0.022**		**0.038**		a		-	
Treatment sun Mann-Whitney-U		-		0.662		-		-		-		-		-		0.762		-		-		0.257		-	
Treatment shade Mann-Whitney-U		-		-		-		-		-		-		-		1.000		-		-		0.250		-	

2004		July											September									
leaf type	Treatment	J_{max}	S.D.	CP	S.D.	AQ	S.D.	CE	S.D.	r_{day}	S.D.	r_{night}	S.D.	J_{max}	S.D.	CP	S.D.	AQ	S.D.	CE	S.D.	r_{day}	S.D.	r_{night}	S.D.
Sun	$1xO_3$	133	7	46	1	0.06	0.00	0.07	0.01	1.5	0.2	0.6	0.3	126	8	47	2	0.05	0.01	0.06	0.01	1.5	0.3	0.5	0.2
Sun	$2xO_3$	131	31	46	1	0.05	0.01	0.08	0.02	1.0	0.3	0.5	0.2	95	25	46	1	0.05	0.01	0.05	0.01	1.2	0.5	0.5	0.3
Shade	$1xO_3$	78	24	46	0	0.03	0.01	0.04	0.02	0.4	0.2	0.3	0.2	77	20	45	0	0.03	0.01	0.04	0.01	0.5	0.1	0.3	0.1
Shade	$2xO_3$	50	9	46	1	0.03	0.00	0.04	0.01	0.4	0.1	0.3	0.2	57	8	45	0	0.02	0.00	0.03	0.01	0.5	0.1	0.2	0.1
p-values of differences between above parameters																									
leaf type		a		0.630		a		**0.000**		a		0.070		**0.000**		a		**0.000**		**0.000**		a		**0.008**	
Treatment		a		0.437		a		0.734		a		0.624		**0.004**		a		0.133		**0.012**		a		0.538	
leaf type x Treatment		a		0.648		a		0.169		a		0.595		0.480		a		0.734		0.672		a		0.845	
Treatment sun Mann-Whitney-U		1.000		-		0.310		-		**0.032**		-		-		0.222		-		-		0.421		-	
Treatment shade Mann-Whitney-U		0.190		-		0.421		-		0.690		-		-		0.247		-		-		0.792		-	

Table 3: Wilcoxon test results (p-values of differences) of drought effects comparing July and September of 2003 with the respective months of 2004. Maximum rate of carboxylation (Vc_{max}), electron transport-related ribulose-1,5-bisphosphate-limited rate of photosynthesis (J_{max}), CO_2 compensation point (CP), apparent quantum yield of net CO_2 uptake (AQ), carboxylation efficiency (CE), respiration during daytime (r_{day}) in sun crown (Sun) and shade crown (Shade). Significant differences at $p < 0.05$ (*) are indicated by bold numbers.

		July					
Treatment	leaf type	Vc_{max}	J_{max}	CP	AQ	CE	r_{day}
$1xO_3$	Sun	0.893	0.345	0.686	0.500	0.500	0.686
$1xO_3$	Shade	-	-	-	-	-	-
$2xO_3$	Sun	0.225	0.138	0.273	0.465	1.000	1.000
$2xO_3$	Shade	-	-		-	-	-
		September					
Treatment	leaf type	Vc_{max}	J_{max}	CP	AQ	CE	r_{day}
$1xO_3$	Sun	**0.043**	**0.043**	0.893	0.800	0.800	0.138
$1xO_3$	Shade	**0.043**	**0.043**	0.249	0.116	0.463	0.463
$2xO_3$	Sun	0.144	0.465	0.225	**0.043**	0.080	0.345
$2xO_3$	shade	0.109	0.593	0.655	0.180	0.655	0.655

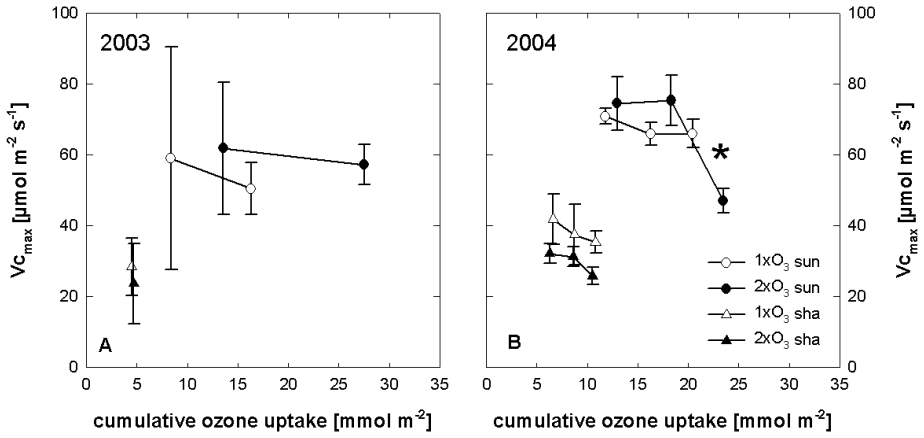

Fig. 1: The maximum rate of carboxylation (Vc_{max}) during the years 2003 (A) and 2004 (B) of leaves in the sun (sun) and shade (sha) crown of adult *Fagus sylvatica* at "Kranzberger Forst". Means ± standard deviation of 4 - 5 trees. (*) indicates a statistically significant treatment effect at p = 0.007. Symbols in (A) as given in (B). Second assessment in 2004 from Warren et al. (2006).

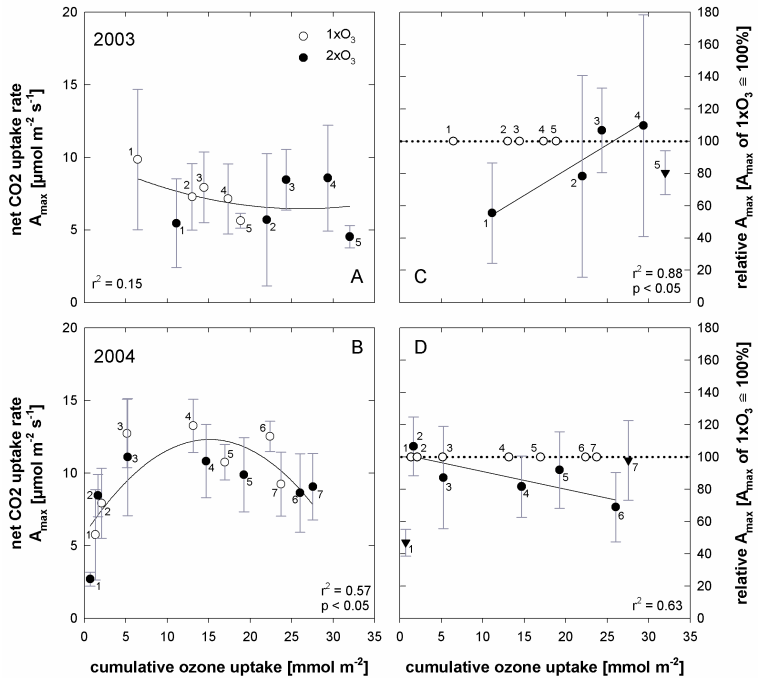

Fig. 2: "Absolute" light-saturated net CO_2 uptake rate A_{max} during the years 2003 (A) and 2004 (B) and "relative" A_{max} (see Materials and Methods) during 2003 (C) and 2004 (D). Symbols in (B), (C) and (D) as given in (A). Triangular symbols in (C) and (D) indicate that data were not included in linear regression (see Results and Discussion). Numbers next to the symbols indicate the date of measurement: For 2003 (A,C) 1 = June 17, 2003, 2 = July 29, 2003, 3 = August 11, 2003, 4 = September 14, 2003 and 5 = October 14, 2003. For 2004 (B,D) 1 = May 18, 2004, 2 = May 24, 2004, 3 = June 16, 2004, 4 = July 27, 2004, 5 = August 11, 2004, 6 = September 14, 2004 and 7 = October 5, 2004. In (A),(B) the solid line represents second-order linear regression. In (C),(D) the solid line represents first-order linear regression and the dashed line represents the 100% reference i.e. $1xO_3$ (see Materials and Methods).

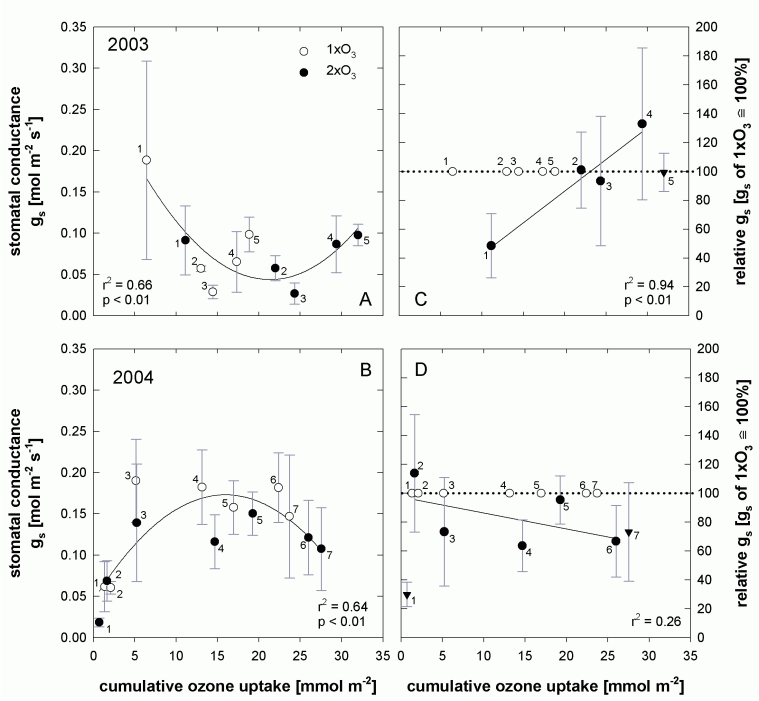

Fig. 3: "Absolute" stomatal conductance g_s during 2003 (A) and 2004 (B) and "relative" g_s (see Materials and Methods) during 2003 (C) and 2004 (D). Symbols in (B), (C) and (D) as given in (A). Triangular symbols in (C) and (D) indicate that data were not included in linear regression (see Results and Discussion). Numbering of dates and meaning of lines as given in Fig. 2.

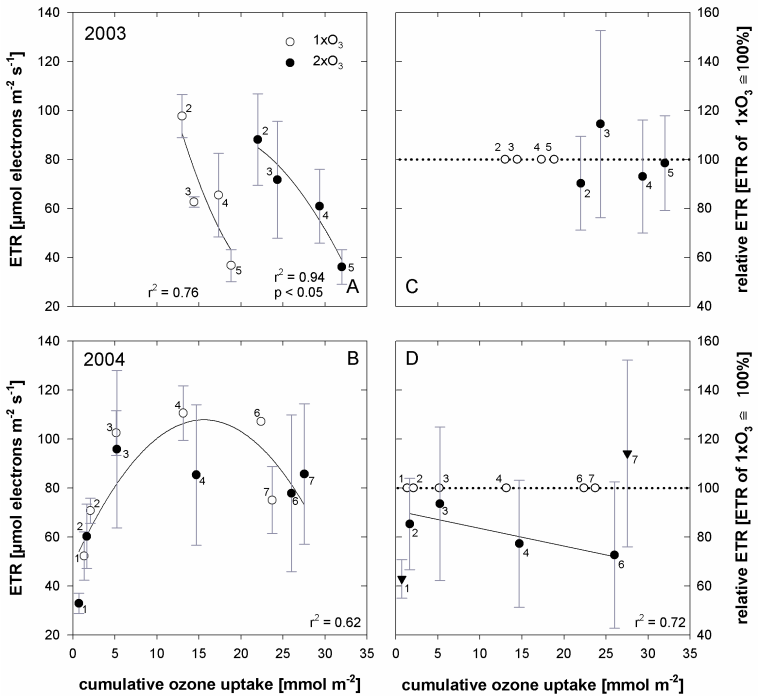

Fig. 4: "Absolute" electron transfer rate ETR during 2003 (A) and 2004 (B) and "relative" ETR (see Materials and Methods) during 2003 (C) and 2004 (D). Symbols given in (A) also valid for (B), (C) and (D). Triangular symbols in (D) indicate that data were not included in linear regression (see Results and Discussion). Numbering of dates and meaning of lines as given in Fig. 2.

Does free-air ozone fumigation affect the internal conductance to CO_2 transfer in sun and shade leaves of adult *Fagus sylvatica*?

Abstract

Two separate objectives were considered in this study. We examined 1) the variation in internal conductance to CO_2 (g_i) and photosynthetic limitations between sun and shade leaves of 60-year-old *Fagus sylvatica*, and 2) whether free-air ozone fumigation affects g_i and photosynthetic limitations. g_i and photosynthetic limitations were estimated from simultaneous measurements of gas exchange and chlorophyll fluorescence on attached sun and shade leaves of *Fagus sylvatica*. The field grown trees were exposed to ambient air ($1xO_3$) and air with twice the ambient ozone concentration ($2xO_3$) in a free-air ozone canopy fumigation system in southern Germany (Kranzberg Forest).

g_i varied between 0.12 and 0.24 mol m^{-2} s^{-1} and resulted in a draw-down from intercellular CO_2 concentrations (C_i) to chloroplastic CO_2 concentrations (C_c) of approximately 55 µmol mol^{-1}. The maximum rate of carboxylation (V_{cmax}) was 22 to 39% lower when calculated on a C_i basis compared with a C_c basis. g_i was approximately twice as large in sun leaves compared to shade leaves. Relationships among net photosynthesis, stomatal conductance and g_i were very similar in sun and shade leaves. This proportional scaling meant that neither C_i nor C_c varied between sun and shade leaves.

Rates of net photosynthesis and stomatal conductance were about 25% lower in the $2xO_3$ treatment compared with $1xO_3$, while V_{cmax} was unaffected. There was no evidence that g_i was affected by ozone.

Introduction

Ozone (O_3) is a phytotoxic air pollutant widely considered a risk factor for forest trees (Pye 1988, Matyssek and Innes 1999). Toxic effects of O_3 on trees include decreases in photosynthesis (A) (Reich 1987, Pye 1988, Dizengremel 2001, Matyssek and Sandermann 2003). The O_3-related decrease in A is commonly attributed to either a decrease in stomatal conductance (e. g. Pearson and Mansfield 1993), or increases in biochemical limitations, such as a decrease in ribulose-1,5-diphosphate-carboxylase-oxygenase (Rubisco) activity (e. g. Lütz et al. 2000, Dizengremel 2001), and/or O_3-driven decline in mesophyll structure (Matyssek et al. 1991). Decreases in Rubisco activity are often inferred from decreases in the maximum rate of carboxylation (V_{cmax}) as indicated by a decrease in the initial slope of an A/C_i curve, e.g. in adult beech exposed to O_3 (Matyssek et al. 2005).

The traditional calculation of V_{cmax} from A/C_i curves (Long and Bernacchi 2003) assumes an infinite internal conductance to CO_2. The internal conductance to CO_2 movement (g_i) describes the draw-down in CO_2 concentration between intercellular spaces (C_i) and chloroplast (C_c) as a function of the rate of photosynthesis ($g_i = A_n/(C_i-C_c)$). For a long time the assumption of an infinite internal conductance was accepted; however, this has now been brought into question by studies showing that g_i is finite, causes a significant draw-down from C_i to C_c and poses a significant limitation to photosynthesis, often almost as large as that due to stomata (e.g. Epron et al. 1995; Warren et al. 2003a). One consequence of finite g_i is that V_{cmax} calculated from C_i is not solely biochemical but also contains "information" pertaining to g_i (e.g. Epron et al. 1995; Ethier and Livingston 2004). Normally reductions in the slope of the A/C_i relationship are purported to be "*prima facie* evidence of inhibition of A by altered metabolism" (Lawlor 2002, see also Boyer 1971; Quick *et al.* 1992; Lawlor 1995); however, such an approach is flawed if g_i changes. For example, we do not know if the observed reduction in V_{cmax} (calculated from A/C_i) due to O_3 is due to a reduction in Rubisco activity or a reduction in g_i. To date, we do not know if g_i is affected by O_3 and this severely limits our ability to interpret the reported effects of O_3 on V_{cmax}.

The mechanistic basis of g_i is currently being disputed, but recent suggestions are that carbonic anhydrase and/or aquaporins are responsible for rapid responses of g_i to environmental variables (Gillon and Yakir 2000; Terashima and Ono 2002). Given that O_3 may reach the cell membrane (Heath and Taylor 1997), direct effects of ozone or its oxidative derivatives on carbonic anhydrase and/or aquaporins are possible. Furthermore, O_3 stimulates phosphoenolpyruvate carboxylase (Landolt et al. 1997; Lütz et al. 2000), which catalyses anaplerotic processes that compete with chloroplasts for CO_2. This would decrease apparent g_i and also changes the $\delta^{13}C$ signature in a way that mimics increase in the photosynthetic water-use efficiency (Saurer et al. 1995; Matyssek & Sandermann 2003). To date, there are no studies of O_3-related variation in g_i, although it is possible that decreases in g_i contribute to O_3-induced decrease in A.

The overwhelming majority of studies on g_i have examined interspecific variation among juvenile and/or glasshouse-grown specimens (von Caemmerer and Evans 1991; Epron et al. 1995). Intraspecific variation be-

tween sun and shade foliage of tree species is not well known and the handful of studies have reported conflicting results. In *Prunus persica* and *Citrus paradisi* internal conductance of sun leaves was only 20-25% greater than in shade leaves (Lloyd et al. 1992), while similar 25-35% differences between sun and shade needles were reported for *Pseudotsuga menziesii* (Warren et al. 2003a). These modest differences between sun and shade leaves contrast with the situation in *Juglans regia* where internal conductance approximately doubled between shade leaves and sun leaves (Piel et al. 2002). In canopies of *Fagus sylvatica* there is a large decrease in light intensity with depth in the canopy. For example, in a canopy of 60-year-old *F. sylvatica* only 3% of the daily irradiance reaches the shade canopy (Herbinger et al. 2005). The leaves acclimate to this light environment anatomically, morphologically and physiologically (e.g. by differences in specific leaf area, photosynthesis rates, pigments, or antioxidant concentrations - Wieser et al. 2003). We might expect, therefore, that g_i would vary between sun and shade leaves of *Fagus sylvatica*, yet this hypothesis has not been tested and the potential effects remain unquantified.

Most studies examining the effects of O_3 on forest trees have used chamber studies with small seedlings (e. g. Reich, 1987; Sandermann et al., 1997; Matyssek and Innes, 1999). This is problematic because seedlings are uncertain surrogates for adult forest trees given ontogenetic changes in morphological and physiological characteristics (Kolb and Matyssek, 2001). The present study makes use of a unique free-air O_3 fumigation facility (Nunn et al., 2002; Werner and Fabian, 2002) to make measurements on tall trees in the field. Two separate objectives were considered in this study. We 1) examined the variation in internal conductance and photosynthetic limitations between sun and shade leaves of large field-grown *Fagus sylvatica* trees, and 2) asked whether free-air O_3 fumigation affects internal conductance and photosynthetic limitations. Measurements of photosynthesis (A, V_{cmax}), stomatal conductance (g_s) and g_i were used to apportion limitations to biochemical, internal and stomatal factors.

Materials and methods

Study site, treatments and experimental design

The study was carried out at the Kranzberg Forest near Freising, Germany (485 m a.s.l., 48°25'08'' N, 11°39'41'' E; Pretzsch et al., 1998). Throughout the growing seasons of 2003 and 2004, 60-year-old beech trees (*Fagus sylvatica* L.) were exposed to the O_3 regime of the unchanged ambient air at the site ($1xO_3$, control) or an experimental, twice-ambient O_3 regime ($2xO_3$). The latter regime was generated by a free-air canopy O_3 exposure system (Nunn et al., 2002; Werner and Fabian, 2002). To prevent acute O_3 injury under $2xO_3$, maximum O_3 levels were restricted to 150 nl l^{-1}. Measurements were made on sun (at above 22 m canopy height) and shade leaves (at about 17 - 20 m) from five trees in each of the O_3 regimes.

Gas exchange and fluorescence measurements

Gas exchange and fluorescence were measured between the 6th and 10th of August, 2004. All measurements were made *in situ* on attached leaves. The CO_2 response of gas exchange and fluorescence was determined using an open gas exchange system (LI-6400, LI-COR, Lincoln, NE, USA) with an integrated fluorescence chamber (LI-6400-40). Leaf temperature ranged between 25 and 29 °C, depending on air temperature levels during summer afternoons in relation to the cooling capacity of the LI-6400. Nevertheless, for individual leaves the variation in leaf temperature stayed <1°C. Relative humidity varied between 50 and 70%.

For each leaf a complete A-C_i curve was generated at 1500 µmol m^{-2} s^{-1} PPFD, with two partial A-C_i curves measured at lower PPFDs. Leaves were exposed to 400 µmol mol^{-1} CO_2 in air and a PPFD of 1500 µmol m^{-2} s^{-1} until rates of photosynthesis and transpiration were steady. After this, an A-C_i curve was generated by increasing C_a, stepwise, to 2000 µmol mol^{-1}. Following measurements at 2000 µmol mol^{-1}, C_a was decreased to 400 µmol mol^{-1} and the curve was continued by decreasing C_a, stepwise, to 50 µmol mol^{-1}. Two additional partial A-C_i curves from 50 to 150 µmol mol^{-1} were generated at lower PPFDs. For sun leaves we used PPFDs of 300 and 100 µmol m^{-2} s^{-1}, while for shade leaves we used PPFDs of 200 and 100 µmol m^{-2} s^{-1}. These light intensities were chosen following preliminary trials to ensure maximum separation of the response curves while maintaining a similar degree of inhibition of dark respiration in the light (data not shown). At each C_a, photosynthesis was allowed to stabilise for at least four minutes, or until gas exchange and fluorescence were steady. Each full A-C_i curve was comprised of 14, while partial A-C_i curves were comprised of 5 data pairs.

Data were corrected for diffusion of CO_2 into and out of the leaf chamber, according to LI-COR (2001). Diffusion leaks are proportional to the diffusion gradient in CO_2 between inside and outside of the chamber and the flow rate of air through the chamber. This is accounted for by a diffusion coefficient which was determined by measur-

ing the diffusion of CO_2 into an empty chamber (C_a = 0 μmol mol^{-1}) as a function of the flow rate of air through the chamber. The CO_2 concentration of the sample cell was measured by the reference IRGA using the match valve, and the assessed diffusion coefficient was used then to re-calculate the gas exchange data as described previously (LI-COR 2001).

Calculation of Γ, R_d and internal conductance*

Internal conductance was determined by the "constant J" method (Harley et al. 1992). At high CO_2 concentrations photosynthesis is limited by the regeneration of ribulose-1,5-diphosphate and the rates of electron transport (J) are constant across a range of CO_2 concentrations. Variation in rates of photosynthesis in this "constant J" region are due to changing proportions of Rubisco carboxylation and oxygenation. This is itself a function of C_c and the relative CO_2/O_2 specificity of Rubisco, which is described by the chloroplastic CO_2 compensation point Γ*. This relationship of J with A may be expressed in terms of C_i:

$$J = (A + R_d)\frac{4\left(\left(C_i - \frac{A}{g_i}\right) + 2\Gamma^*\right)}{\left(C_i - \frac{A}{g_i}\right) - \Gamma^*} \qquad (1)$$

If A is measured at various C_i at which J is constant, then the equation may be solved for the value of g_i that minimises variance in J. One complication is that this method requires measurements of Γ* and mitochondrial respiration in the light (R_d). Directly measuring Γ* is difficult, whereas the intercellular compensation point (C_i*) is easily measured. C_i* is related to Γ* by R_d and g_i (von Caemmerer and Evans 1991; Peisker and Apel 2001):

$$C_i^* = \Gamma^* - \frac{R_d}{g_i} \qquad (2)$$

C_i* and R_d were estimated from the intersection of the initial linear portion of the three A-C_i curves generated at different light intensities (Laisk 1977). Equation (2) was substituted into (1) and the value of g_i that minimised variance in J was found. This was done for 5 or 6 measurements made at high C_i where J was constant. Quantum yield of photosystem 2 (PSII) as derived from assessments of chlorophyll fluorescence was used to identify when J was constant. The values of g_i and Γ* that minimised variance in J were found iteratively using the solver add-in of Microsoft Excel.

Calculation of V_{cmax} and the relative limitation on photosynthesis imposed by g_i and g_s

Using estimated g_i and measured A and C_i, C_c was calculated as:

$$C_c = C_i - \frac{A}{g_i} \qquad (3)$$

V_{cmax} was determined from measured A and C_c fitted to the photosynthesis model of Farquhar et al. (1980), essentially as described previously (Warren et al. 2003b). For $[K_c (1 + O_c/K_o)]$, describing the biochemical characteristics of Rubisco, a value of 549 μmol mol^{-1} was used (von Caemmerer et al. 1994).

The limitation of A imposed by finite g_i and g_s was based on estimates of the potential rate of photosynthesis assuming these conductances were either infinite or as assessed (Farquhar and Sharkey 1982; Warren et al. 2003). Estimates of A were based on CO_2-response curves and assessed g_i and g_s. Rates of net photosynthesis were estimated assuming g_i and g_s were as assessed (A, the light-saturated rate of photosynthesis at C_a = 360 μmol mol^{-1}), assuming g_i was infinite and g_s as assessed (A_{il}, the light-saturated rate of photosynthesis at $C_c = C_i$), or assuming g_i was as assessed and g_s was infinite (A_{sl}, the light-saturated rate of photosynthesis at C_i = 360 μmol mol^{-1}). The relative limitations due to internal conductance (L_i) and stomatal conductance (L_s) were calculated as:

$$L_i = \frac{A_{il} - A}{A_{il}} \qquad (4)$$

$$L_s = \frac{A_{sl} - A}{A_{sl}} \qquad (5)$$

Specific leaf area and N

The specific area of leaves (SLA, m^2 projected area kg^{-1} dry mass) was determined as the ratio of projected area

determined with a flatbed scanner (LiDE 20, Canon, Krefeld, Germany) over leaf dry mass (72 h at 60ºC). Dried leaves were subsequently ground to a fine powder in a mixer mill (MM2, Retsch, Haan, Germany) and analysed for N using an Eurovector elemental analyzer (EUROEA3028-HT, Eurovector, Milan, Italy).

Statistics

Two-way ANOVA evaluated the effects of canopy position (sun versus shade leaves) and ozone exposure ($1xO_3$ versus $2xO_3$) on variables. ANOVA assumptions were verified by testing for deviations from homogeneous variances (using Levene's test), by plotting means against variances to ensure they were not correlated across groups, and by visually checking the distribution of residuals.

Results

Specific leaf area and N

SLA of sun leaves (10.8 – 12.7 $m^2\ kg^{-1}$) was less than half that of shade leaves (25 – 33 $m^2\ kg^{-1}$)(Table 1). There was no significant difference in nitrogen content per unit mass between sun and shade leaves, whereas there was a larger (and significant) difference in nitrogen per unit area. SLA was greater in the $2xO_3$ leaves compared to $1xO_3$; however, this difference was only marginally significant (P = 0.058). Nitrogen content per unit mass was not affected by O_3 treatment (Table 1). Nitrogen content per unit area was slightly, but significantly lower under the $2xO_3$ treatment (Table 1).

Gas exchange at ambient CO_2

The rate of net photosynthesis (A), stomatal conductance (g_s) and internal conductance (g_i) were all approximately twice as high in sun leaves than in shade leaves (Figure 1, Table 2). Net photosynthesis, for example, was 11 – 14 μmol $m^{-2}\ s^{-1}$ in sun leaves and 5-7 μmol $m^{-2}\ s^{-1}$ in shade leaves. Neither the intercellular CO_2 concentration (C_i) nor the chloroplastic CO_2 concentration (C_c) were significantly different between sun and shade leaves. C_i was, on average, about 140 μmol mol^{-1} lower than atmospheric CO_2, while the C_c was a further 55 μmol mol^{-1} lower than C_i (Table 2, Figure 1). The drawdown from C_i to C_c did not vary as a function of g_i and was the same for sun and shade leaves (Figure 2). The relative limitation of photosynthesis due to stomatal conductance (L_s) did not differ significantly between sun and shade leaves with the average among all treatments being 0.34 (out of 1). The relative limitation due to internal conductance also did not vary among treatments and was considerably lower with an average among all treatments of 0.24. The chloroplastic CO_2 compensation point (Γ*) did not vary significantly between sun and shade leaves (or O_3 treatments) and was, on average, 48 ± 3 μmol mol^{-1} (mean ± SD) (data not shown).

Rates of net photosynthesis were significantly lower, by approximately 25%, in leaves from the $2xO_3$ treatment compared to the $1xO_3$ treatment. Stomatal conductance was lower in $2xO_3$ versus $1xO_3$, while there was no significant difference in internal conductance. There was a non-significant trend (P = 0.064) for C_i to be lower in $2xO_3$ than $1xO_3$, and this was especially evident in sun leaves. Similarly, C_c tended to be lower in $2xO_3$ compared with $1xO_3$, but once again this was not significant (P = 0.108).

The maximum rate of carboxylation

The maximum rate of carboxylation was 22 to 39% lower when calculated on a C_i ($V_{cmax,Ci}$) basis compared with a C_c ($V_{cmax,Cc}$) basis (Table 3). $V_{cmax,Cc}$ of sun leaves varied between 92 and 109 μmol $m^{-2}\ s^{-1}$ and was approximately twice as great as shade leaves ($V_{cmax,Cc}$ = 40 and 61 μmol $m^{-2}\ s^{-1}$) (Figure 1). Ozone treatment did not affect $V_{cmax,Ci}$ or $V_{cmax,Cc}$.

Discussion

Internal conductance of Fagus sylvatica

In 60-year-old *Fagus sylvatica* the internal conductance to CO_2 transfer varied between 0.12 and 0.24 mol $m^{-2}\ s^{-1}$ and resulted in a draw-down from C_i to C_c of approximately 55 μmol mol^{-1} (Table 2). There are no other measurements of g_i for adult *Fagus sylvatica*, but our data may be compared with results for pot-grown two-year-old seedlings (Epron et al. 1995). Compared with our results for 60-year-old trees, g_i of seedlings was smaller (0.1 mol $m^{-2}\ s^{-1}$) and the draw-down from C_i to C_c was larger (92 μmol mol^{-1}). These findings suggest that the limitation due to g_i is smaller in 60-year-old trees than in pot-grown two-year-old seedlings.

The finite internal conductance of *Fagus sylvatica* results in a significant draw-down from C_i to C_c and imposes a large limitation on photosynthesis (Table 2). We estimated the relative limitation due to internal conductance (L_i) as 0.24 or 24%, indicating internal conductance is an important limiting factor for photosynthesis and carbon bal-

ance of this species. The limitation due to internal conductance is not as large as the limitation due to stomatal conductance (L_s = 0.34 or 34%), but the magnitudes are comparable. The relative limitations reported here are similar to those reported for other tree species (e.g. Epron et al. 1995; Warren et al. 2003a) and underline that internal conductance nevertheless poses a large limitation on CO_2 fixation and thus should be considered in process-based models of C exchange (Ethier and Livingston 2004). In addition to limiting photosynthesis, finite internal conductance affects estimates of V_{cmax} and their interpretation. Most published estimates of V_{cmax} underestimate the true V_{cmax} by not considering the draw-down from C_i to C_c. In *Fagus sylvatica* $V_{cmax,Ci}$ under-estimates $V_{cmax,Cc}$ by 22-39% (Table 3, see also Epron et al. 1995)

Variation in photosynthetic limitations and g_i between sun and shade leaves

Consistent with our hypothesis we found a large and significant difference in g_i between sun and shade leaves (Table 2). g_i was approximately twice as large in sun leaves than shade leaves of *Fagus sylvatica*, which is similar to the two-fold difference between sun and shade leaves of *Juglans regia* (Piel et al. 2002), but much larger than the modest 20-35% differences reported for *Prunus persica*, *Citrus paradisi* (Lloyd et al. 1992) and *Pseudotsuga menziesii* (Warren et al. 2003a). Leaf anatomical and morphological traits correlate with g_i (e.g. Nobel 1991; Evans et al. 1994; Syvertsen et al. 1995), vary significantly between sun and shade leaves (e.g. in *Fagus sylvatica*, Table 1, see also Wieser et al. 2003) and this may well explain the large differences in g_i between sun and shade leaves of *Fagus sylvatica*. Accordingly, species with smaller differences in anatomical and morphological traits exhibit smaller differences in g_i between sun and shade leaves (e.g. *Pseudotsuga menziesii*, Warren et al. 2003a).

The observation that g_i is greater in thick sun leaves than thin shade leaves provides clues as to whether g_i is primarily a gas-phase or liquid-phase resistance. If mesophyll porosity is small and foliage is hypostomatous, gas phase conductance is correspondingly small and may be a significant component of g_i (Syvertsen *et al.* 1995). However, in the case of *Fagus sylvatica* it seems unlikely that gas phase conductance is limiting because if it was then g_i should be lower in thick sun leaves compared to thin shade leaves, whereas the opposite was in fact observed. Hence, we tentatively conclude that liquid phase conductance is the dominant component of g_i in *Fagus sylvatica*, as has also been reported for other species (Evans et al. 1994). CO_2 diffusion in the liquid phase is a function of cell wall thickness and the surface area of mesophyll cells or chloroplasts exposed to the intercellular air spaces (Nobel 1991; Evans *et al.* 1994) and/or of variations in related enzyme activities (Gillon and Yakir 2000; Terashima and Ono 2002). At present we cannot deduce which of these varies between sun and shade leaves.

Relationships among A, g_s and g_i were very similar in sun and shade leaves (Figures 1 and 2, Table 2). This proportional scaling meant that none of C_i, C_c, L_s or L_i varied between sun and shade leaves, and indicates that in *Fagus sylvatica* the limitations due to stomatal and internal conductance are conservative. This result is in accordance with other studies, which show that the approximate scaling of g_i and g_s with A makes the relative limitations to photosynthesis rather conservative within canopies of tree species (e.g. *Pseudotsuga menziesii*, Warren et al 2003a, compare also von Caemmerer and Evans 1991; Loreto et al. 1992; Epron et al. 1995; Warren et al. 2003a).

Does O_3 affect photosynthesis, photosynthetic limitations and g_i?

There was no evidence that internal conductance was affected by ozone (Table 2). It is, however, premature to generalize given that our study is the first to examine whether ozone affects g_i – and that only part of the growing season was covered in this study. This is especially the case as effects on photosynthesis were rather small and ozone concentrations were capped at 150 nl l^{-1} to prevent acute injury. Furthermore, lower g_s of $2xO_3$ trees decreased O_3 uptake and reduced the difference in cumulative uptake between $1xO_3$ and $2xO_3$ (compare AOT40 with COU, Table 1). It may well be the case that g_i is reduced by more severe ozone impact, resulting in larger effect on photosynthesis.

In light of the large effect of g_i on V_{cmax} and its interpretation (see above), the fact remains that previous reports of reductions in V_{cmax} might indicate reductions in g_i (perhaps also relating to incipient cell collapse under O_3 stress, cf. Matyssek et al. 1991) rather than in Rubisco activity. The danger of interpreting V_{cmax} has been illustrated by studies showing that reductions in the slope of the A/C_i relationship due to drought stress can indicate reduced g_i rather than an effect on Rubisco activity (e.g. Warren et al. 2004). Therefore, despite the fact that we did not observe an effect of ozone on g_i, we argue that g_i is a key parameter that should be measured in gas exchange studies reporting V_{cmax}.

Rates of net photosynthesis of *Fagus sylvatica* were about 25% lower in the $2xO_3$ treatment compared with $1xO_3$ (Table 2). This finding is also consistent with other reports of O_3-induced reductions in photosynthesis (Reich

1987, Pye 1988, Dizengremel 2001, Matyssek and Sandermann 2003) and confirms that stomatal conductance is reduced under chronic O_3 stress (e.g. Pearson and Mansfield 1993; Matyssek et al. 1995; Matyssek & Sandermann 2003). However, unlike some previous studies we did not observe a significant effect of ozone treatment on V_{cmax} (Table 3, c.f. Farage and Long 1995; Matyssek et al. 2005), which might have been functionally related to lower nitrogen content per unit area in $2xO_3$ trees (Table 1). Ozone effects on adult trees in the field can be quite inconsistent, and even at the very same study site (Kranzberg Forest), significant differences between $1xO_3$ and $2xO_3$ were observed in some, but not all, previous seasons (Matyssek et al. 2005, Nunn et al. 2005).
We conclude that irrespective of the actual degree of O_3 impact (or other kinds of stress), g_i should be included into response assessments for ensuring a mechanistic view on gas exchange performance and its alterations.

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Table 1: The effect of ozone treatment on specific leaf area (SLA), the content per unit area and mass of nitrogen (N) of sun and shade leaves of 60-year-old beech trees (*Fagus sylvatica* L.). The field-grown trees were exposed to ozone at ambient concentrations ($1xO_3$, control) or two-times ambient concentrations ($2xO_3$). Data are the mean (standard deviation) of leaves from five replicate *Fagus sylvatica* trees. ANOVA indicated the significance of difference (P) between ozone treatments, sun *vs*. shade leaves (canopy), and their interaction.

Canopy	Treatment	AOT40 (µl l^{-1} h^{-1})	COU (mmol m-2)	SLA (m^2 kg^{-1})	N (mol kg^{-1})	N (mmol m^{-2})
Sun	$1xO_3$	12.46	15.64	10.8 (0.6)	1.72 (0.11)	159 (11)
Sun	$2xO_3$	41.13	17.46	12.7 (0.5)	1.82 (0.24)	144 (18)
Shade	$1xO_3$	12.19	8.46	25 (4)	1.92 (0.17)	74 (13)
Shade	$2xO_3$	30.24	8.26	33 (7)	1.90 (0.34)	57 (10)
	Canopy			<0.001	0.212	<0.001
	Treatment			0.058	0.742	0.017
	Canopy x Treatment			0.261	0.571	0.815

Table 2 (next page): The effect of ozone treatment on gas exchange parameters of sun and shade leaves of 60-year-old beech trees (*Fagus sylvatica* L.). The field-grown trees were exposed to ozone at ambient concentrations ($1xO_3$, control) or two-times ambient concentrations ($2xO_3$). Measurements were made at an ambient CO_2 concentration of 360 µmol mol^{-1}, a photosynthetic photon flux density of 1500 µmol m^{-2} s^{-1} and a leaf temperature between 25 and 29 ºC. Parameters determined are the rate of net photosynthesis (A_n), stomatal conductance to water (g_s), internal conductance to CO_2 (g_i), intercellular CO_2 concentration (C_i), chloroplastic CO_2 concentration (C_c), the relative limitation due to stomatal conductance (L_s) and the relative limitation due to internal conductance (L_i). Data are the mean (standard deviation) of leaves from five replicate *Fagus sylvatica* trees. ANOVA indicated the significance of difference (P) between ozone treatments, sun *vs*. shade leaves (canopy), and their interaction.

Canopy	Treatment	A_n (μmol m^{-2} s^{-1})	g_s (mol m^{-2} s^{-1})	g_i (mol m^{-2} s^{-1})	C_i (μmol mol^{-1})	C_c (μmol mol^{-1})	L_s	L_i
Sun	1xO$_3$	14 (3)	0.17 (0.07)	0.24 (0.1)	236 (28)	179 (21)	0.24 (0.10)	0.24 (0.03)
Sun	2xO$_3$	11 (2)	0.10 (0.04)	0.21 (0.02)	194 (35)	144 (25)	0.38 (0.14)	0.27 (0.05)
Shade	1xO$_3$	7 (3)	0.07 (0.02)	0.14 (0.07)	216 (31)	165 (41)	0.34 (0.09)	0.26 (0.16)
Shade	2xO$_3$	5 (1)	0.05 (0.01)	0.12 (0.08)	206 (20)	158 (26)	0.39 (0.06)	0.19 (0.05)
	Canopy	<0.001	0.001	0.028	0.730	0.969	0.298	0.410
	Treatment	0.046	0.026	0.533	0.064	0.108	0.069	0.539
	Canopy x Treatment	0.536	0.196	0.970	0.252	0.276	0.340	0.198

Table 3: The effect of ozone treatment on the maximum rates of carboxylation (V_{cmax}) of sun and shade leaves of 60-year-old beech trees (*Fagus sylvatica* L.) exposed to ozone at ambient concentrations (1xO$_3$, control) or two-times ambient concentrations (2xO$_3$). The maximum rates of carboxylation were estimated from A/C_i ($V_{cmax,Ci}$) or A/C_c ($V_{cmax,Cc}$) responses measured at a PPFD of 1500 μmol m^{-2} s^{-1} and a leaf temperature between 25 and 29 ºC. Data are the mean (standard deviation) of leaves from five replicate *Fagus sylvatica* trees. ANOVA indicated the significance of difference between ozone treatments, sun *vs*. shade leaves (canopy), and their interaction.

Canopy	Treatment	$V_{cmax,Ci}$ (μmol m^{-2} s^{-1})	$V_{cmax,Cc}$ (μmol m^{-2} s^{-1})
Sun	1xO$_3$	66 (7)	92 (15)
Sun	2xO$_3$	75 (18)	109 (33)
Shade	1xO$_3$	37 (17)	61 (49)
Shade	2xO$_3$	31 (6)	40 (11)
	Canopy	<0.001	0.002
	Treatment	0.798	0.881
	Canopy x Treatment	0.229	0.185

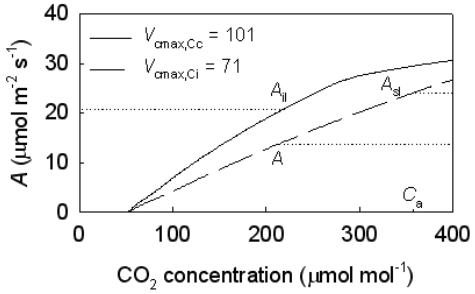


Fig. 1: The mean response of net photosynthesis (A) to CO_2 concentration in sun leaves of 60-year-old *Fagus sylvatica*. The response of photosynthesis is shown for CO_2 concentration in the chloroplast (C_c, solid line) and intercellular spaces (C_i, dashed line). Response curves were calculated according to Farquhar et al. (1980) using the mean V_{cmax} and J_{max} (on C_i- or C_c-bases) of sun leaves (N = 10). The 1xO$_3$ and 2xO$_3$ treatments were not significantly different and thus were combined. Rates of net photosynthesis are indicated: A, the light-saturated rate of photosynthesis at C_a = 360 μmol mol^{-1}; A_{il}, the light-saturated rate of photosynthesis at $C_c = C_i$; A_{sl}, the light-saturated rate of photosynthesis at $C_i = C_a$ (360 μmol mol^{-1}). The relative limitations due to internal conductance (L_i) and stomatal conductance (L_s) were calculated as: $L_i = (A_{il} - A)/A_{il}$; $L_s = (A_{sl} - A)/A_{sl}$. See materials and methods for further details.

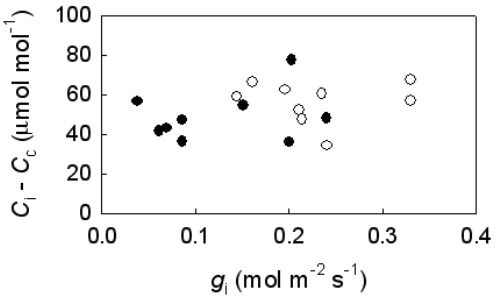


Fig. 2: The relationship between internal conductance to CO_2 transfer (g_i) and the draw-down in CO_2 concentration from intercellular spaces to chloroplasts (C_i-C_c). The 1xO$_3$ and 2xO$_3$ treatments were not significantly different and thus were combined. Data are for sun leaves (○) and shade leaves (●) of 60-year-old *Fagus sylvatica*.

6 Appendix B

Example of a light response assessment

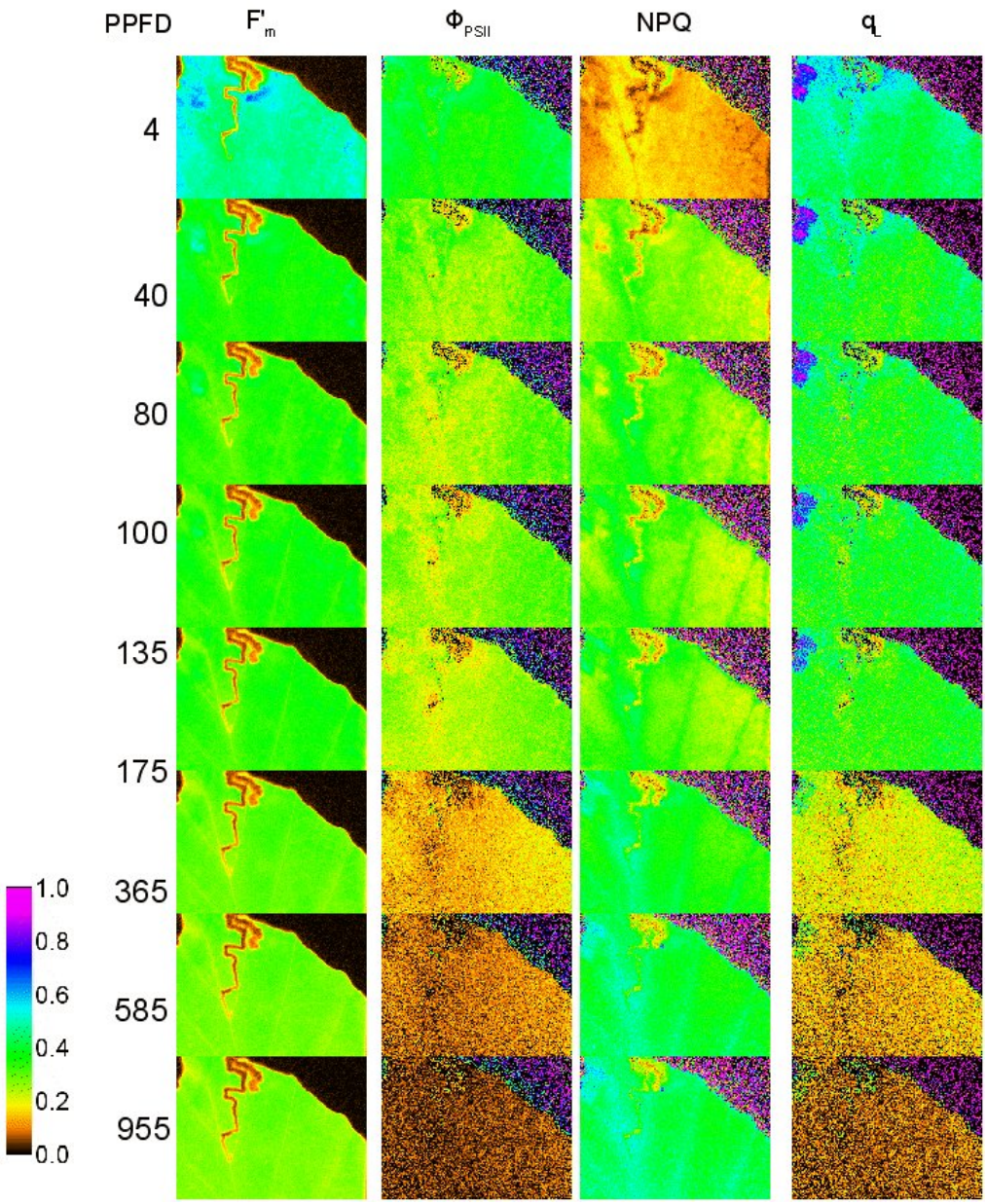


Figure 6.1: Light response of Φ_{PSII}, q_L and NPQ measured by chlorophyll fluorescence imaging on June 3, 2003 for the leaf shown in Figure 3.4 B. PPFD given in [µmol m^{-2} s^{-1}]. NPQ is given as NQP/4.

Visualisation and diagnosis of leaf injury by chlorophyll fluorescence imaging

Additional samples (c.f. chapter 3.1)

Legend for all Figures of Appendix B:

Comparison of a microscopical cut with results of F_o, F_m and F_v/F_m assessments. (A) microscopical cut. Numbers in (A) indicate the distance to the secondary leaf vein at position 0 [µm]. (a) real-colour photograph of the investigated leaf area, white squares indicate the positions of microscopical DOI assessments. F_o (B), image of F_o (b), F_m (C), image of F_m (c), F_v/F_m (D), image of F_v/F_m (d). Data points in (B), (C), (D) represent means ± SD of 9 pixels from the positions indicated by black squares in (b), (c), (d).

Descriptions of main symptoms encountered in samples shown in Appendix B:

Table 6.1: Description of microscopical cuts of the Figures in Appendix B (after VOLLENWEIDER *ET AL.* 2005).

Figure	General symptom description
6.2	insect stitching, little or no O_3 symptoms
6.3	insect stitching, little or no O_3 symptoms
6.4	destruction of lower epidermis, mechanical damage, wound tissue, no stomata, biotic infections
6.5	destruction of lower epidermis, mechanical damage, wound tissue, no stomata, biotic infections
6.6	necrotic spots of biotic origin, possible insect stitching, oxidative stress, moderate O_3- and light stress
6.7	necrotic spots of biotic origin, possible insect stitching, oxidative stress, moderate O_3- and light stress
6.8	most typical O_3 symptoms
6.9	multisymptomatic: drought, O_3, microbial infection, insect stitching
6.10	multisymptomatic: drought, O_3, microbial infection, insect stitching

Figure 6.2: insect stitching, little or no O_3 symptoms. See page 122 for description.

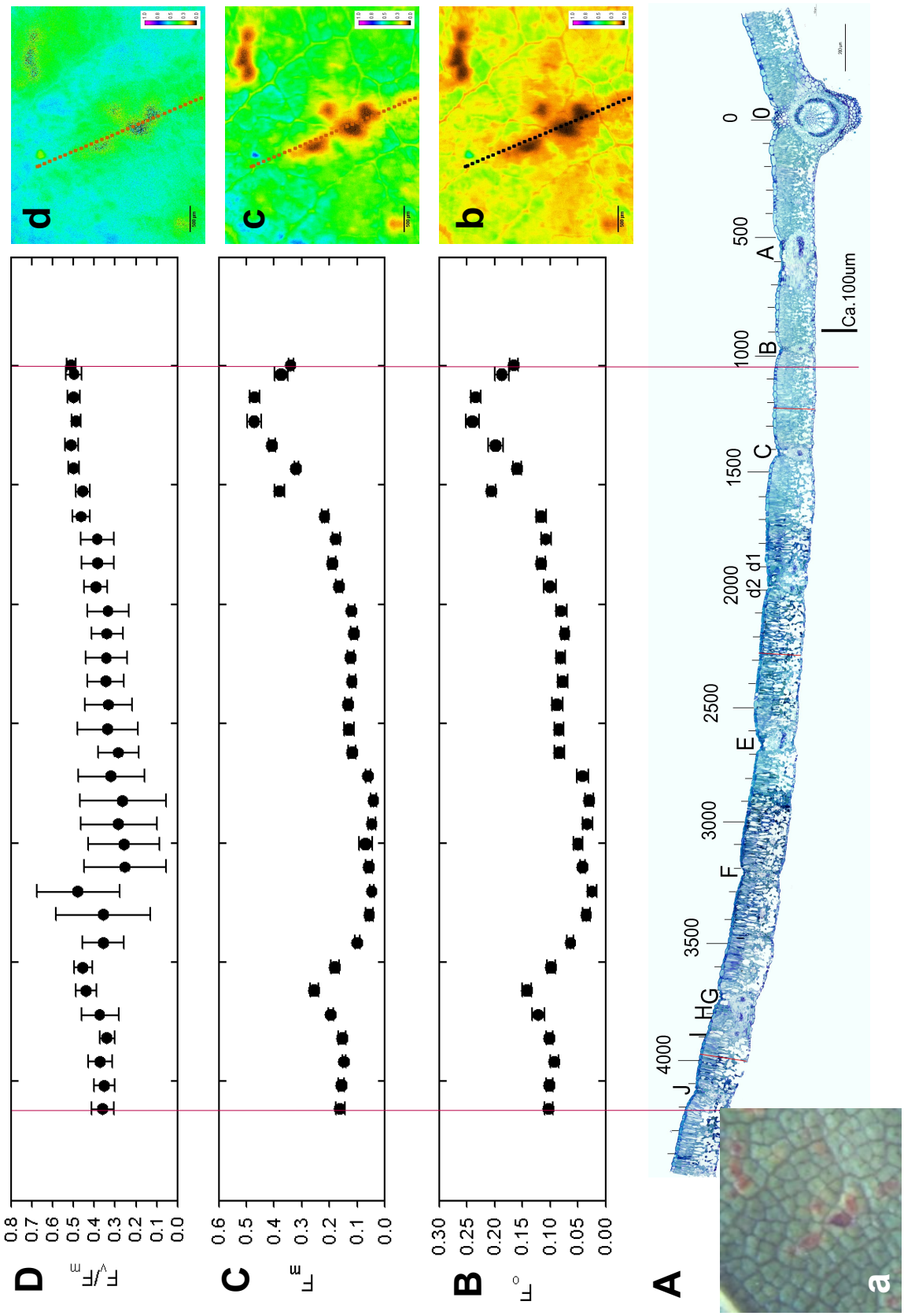


Figure 6.3: insect stitching, little or no O_3 symptoms. See page 122 for description.

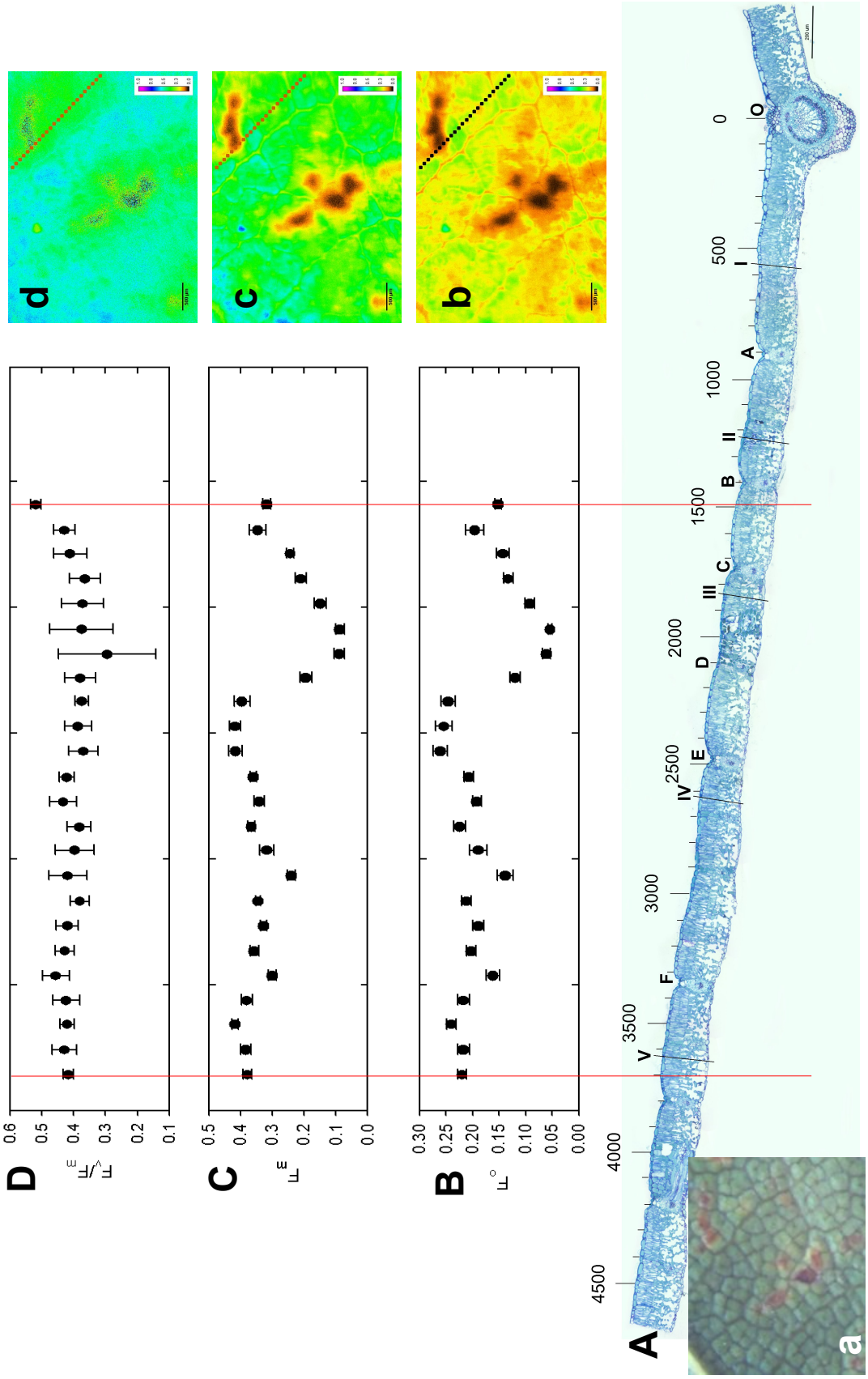


Figure 6.4: destruction of lower epidermis, mechanical damage, wound tissue, no stomata, biotic infections. See page 122 for description.

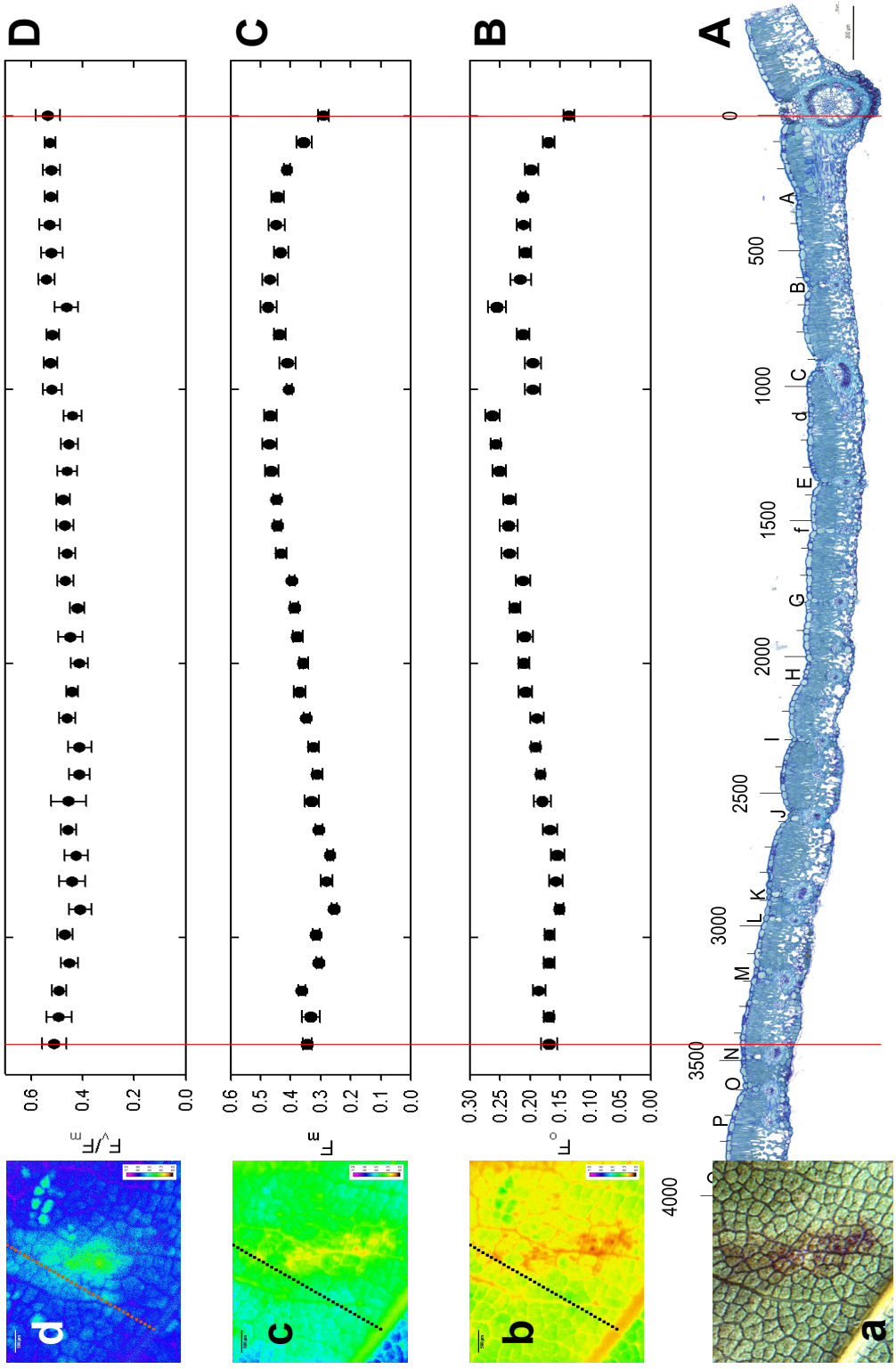


Figure 6.5: destruction of lower epidermis, mechanical damage, wound tissue, no stomata, biotic infections. See page 122 for description.

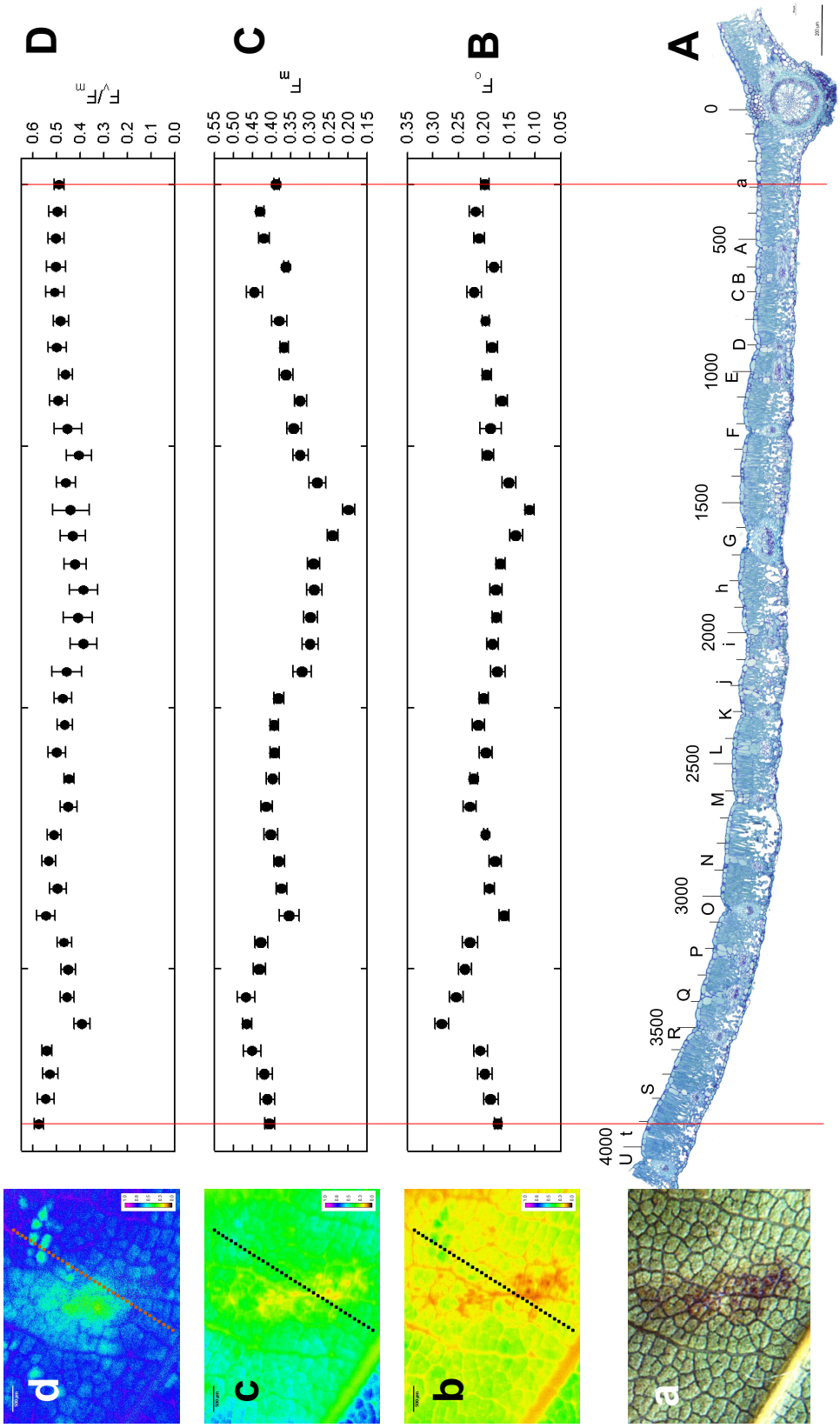


Figure 6.6: necrotic spots of biotic origin, possible insect stitching, oxidative stress, moderate O_3- and light stress. See page 122 for description.

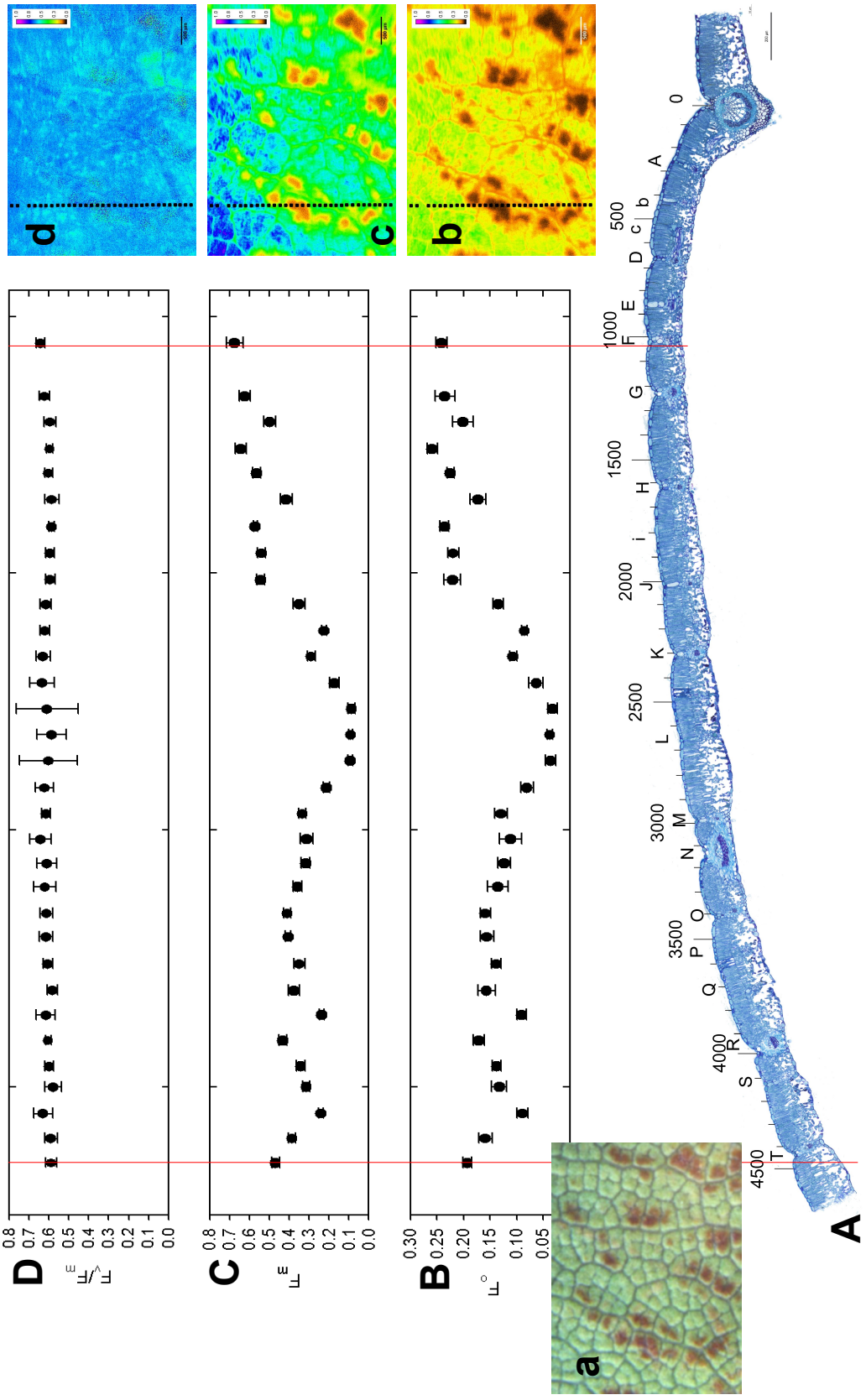


Figure 6.7: spots of biotic origin, possible insect stitching, oxidative stress, moderate O_3- and light stress. See page 122 for description.

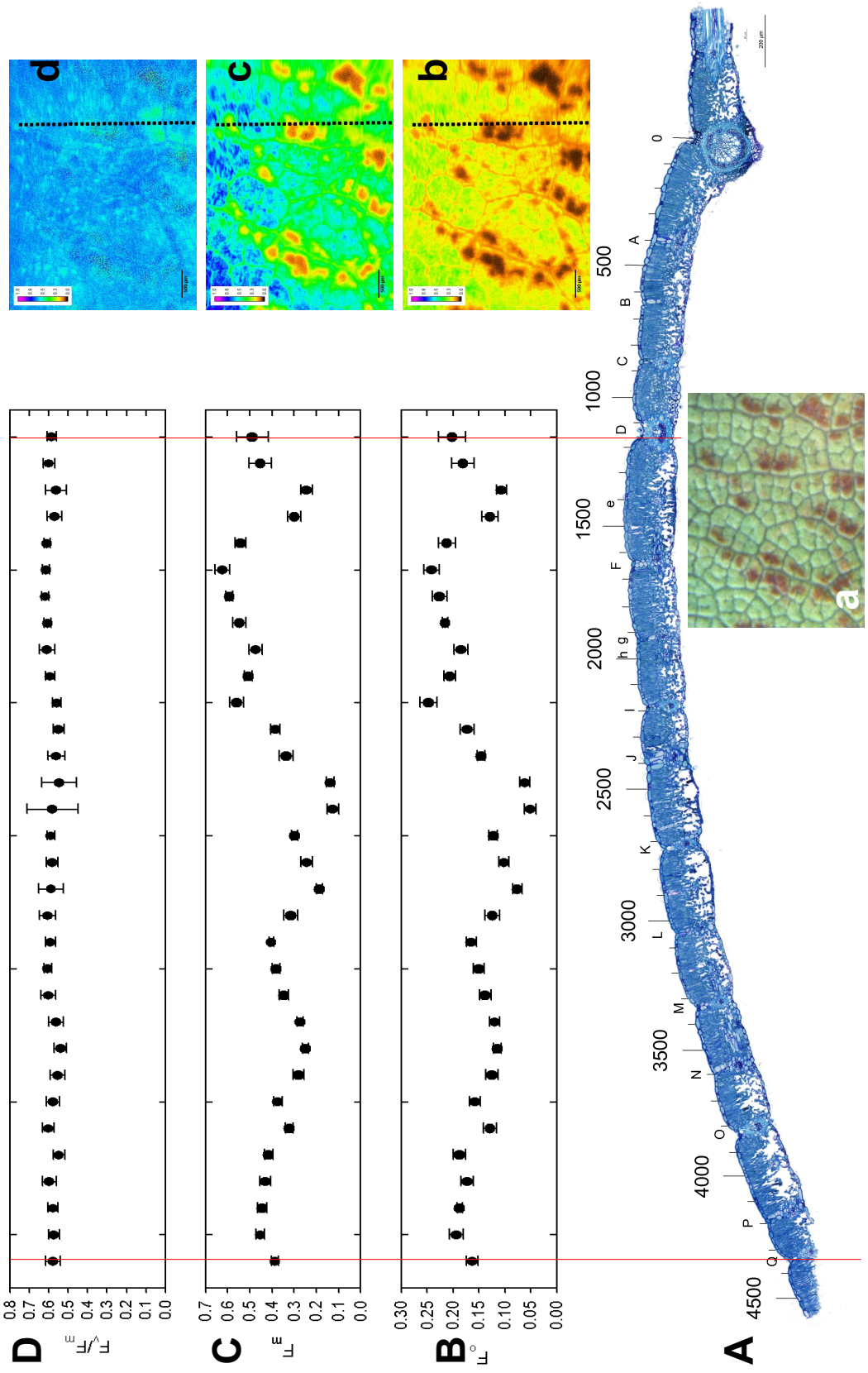


Figure 6.8: most typical O_3 symptoms. See page 122 for description.

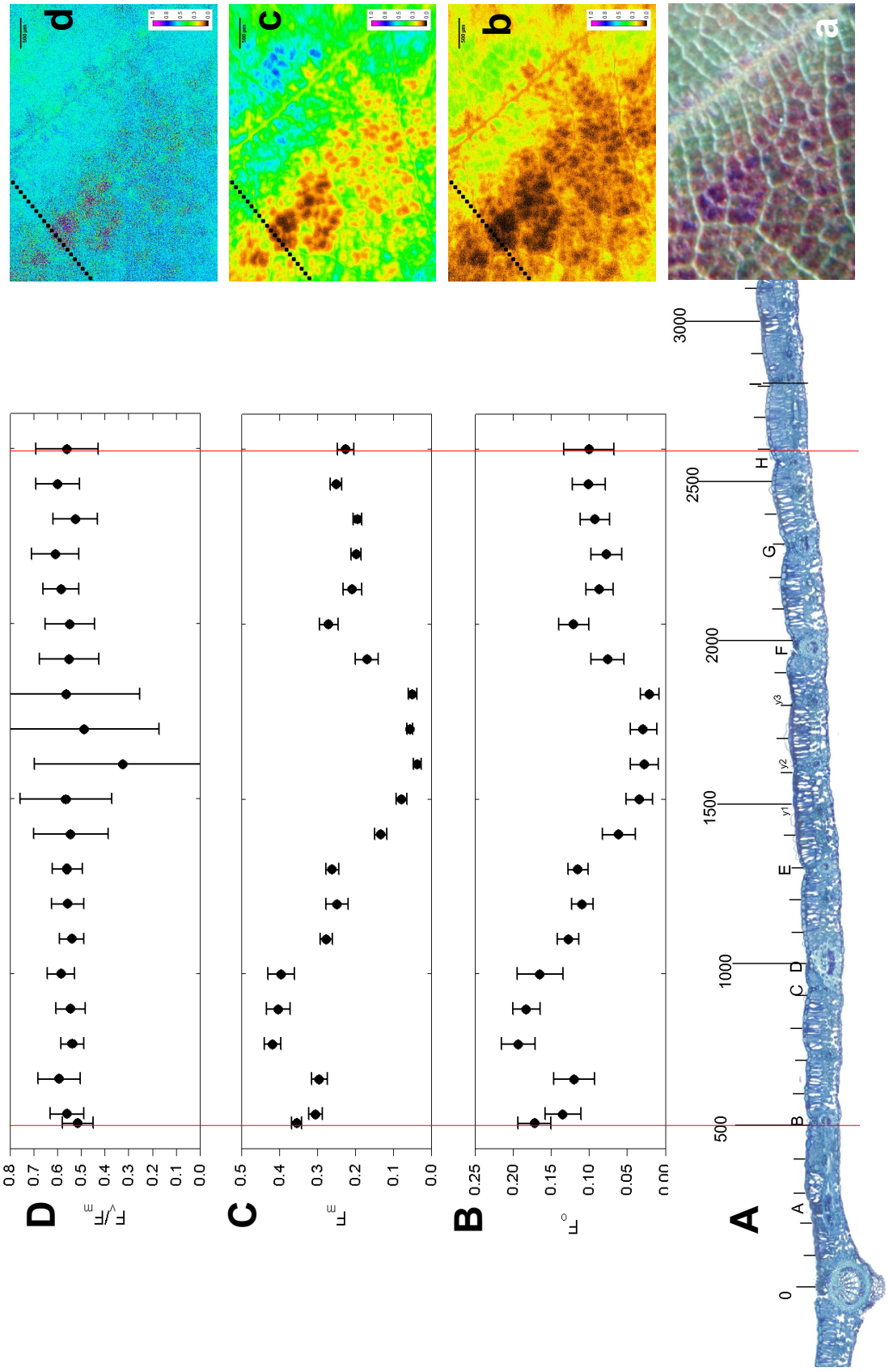


Figure 6.9: multisymptomatic: drought, O_3, microbial infection, insect stitching. See page 122 for description.

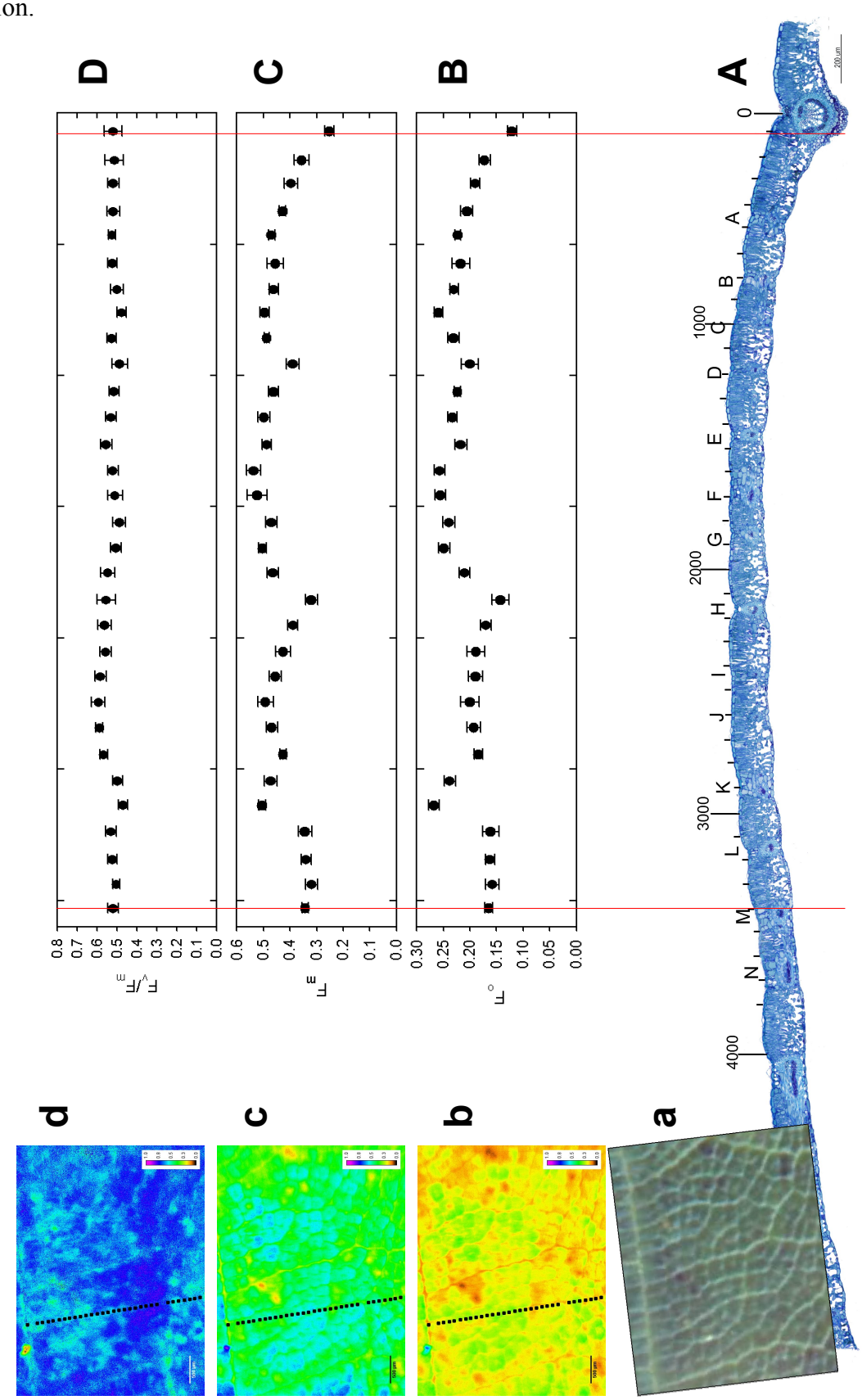
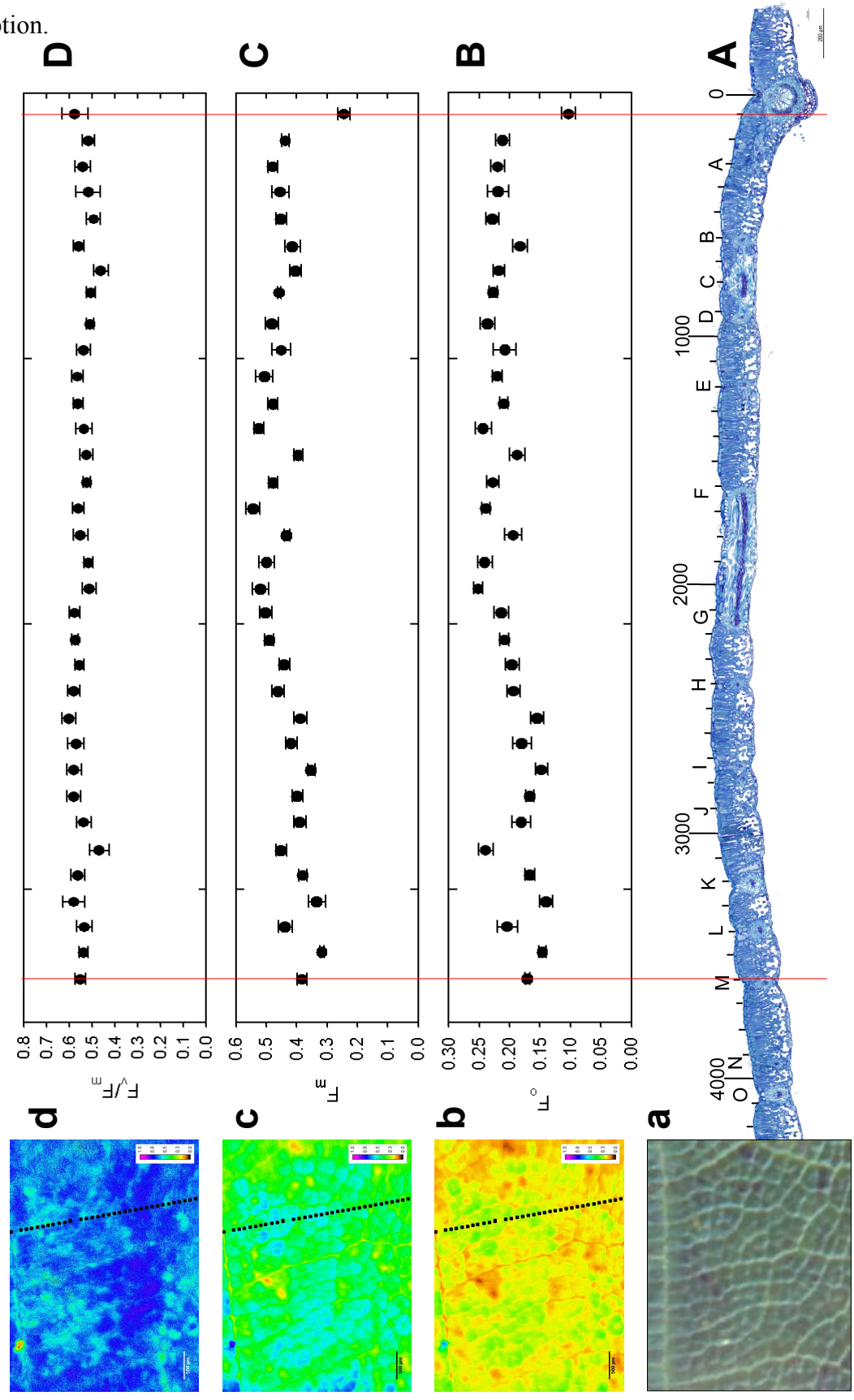


Figure 6.10: multisymptomatic: drought, O_3, microbial infection, insect stitching. See page 122 for description.



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Curriculum vitae

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