Influence of different light qualities on leaf growth and biomass development of horticultural and model plants

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Susanne Tittmann

aus Karsdorf

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Zusammenfassung

Licht stellt die Grundvoraussetzung allen Lebens insbesondere des pflanzlichen Lebens dar. Pflanzen sind sessile Organismen und den damit auf sie einwirkenden Umweltbedingungen ausgesetzt. Durch Veränderung auf molekularer bis hin zur morphologischen Ebene können sie sich an die schnell verändernden Bedingungen anpassen.

Die vorliegende Dissertation ist Teil des Verbundprojektes "Innovative Gewächshäuser – Forschung für bessere Produktqualität und nachhaltige Nutzung". Die Aufgabe bestand darin, die Einflüsse verschiedener Lichtqualitäten, insbesondere der UV-B Strahlung, auf das Wachstum verschiedener Pflanzenarten (Nutz- und Modellpflanzen) zu testen. Die Gewächshäuser wurden mit unterschiedlich stark UV-B durchlässigen Materialien eingedeckt. Als Kontrolle diente das in der Gartenbaupraxis am meisten genutzte Floatglas. Eine Teflonfolie mit hoher Transmission im UV-B Bereich (ca. 80%) stellte die „hoch UV-B-Variante" dar. Lollo rosso-, Eichblattsalat-, Tomaten- sowie Tabakpflanzen wurden unter den verschiedenen UV-B Bedingungen im Gewächshaus angezogen und die Entwicklung der projizierten Blattfläche über einen artspezifisch, von der Morphologie abhängigen, Zeitraum mit Hilfe des GROWSCREEN Verfahrens (automatisierte digitale Bildaufnahme und Weiterverarbeitung) ermittelt. Als Reaktion auf erhöhte UV-B Strahlung konnte eine signifikante Reduzierung der Blattfläche sowie der Biomasse für die Salat- und Tomatenpflanzen festgestellt werden, währenddessen Tabakpflanzen keine solch eindeutige Reaktion im Wachstum auf erhöhte UV-B Strahlung zeigten. Unter zu Hilfenahme anderer Methoden, wie der Chlorophyllfluoreszenz, konnten eine erhöhte effektive Quantenausbeute und signifikant angestiegenes nicht photochemisches quenching als Reaktionen auf UV-B Strahlung festgestellt werden.

An der kontinuierlichen nicht-invasiven Messung von Biomasse besteht bis heute großes Interesse. In technischer Hinsicht stellte die Entwicklung und Etablierung eines Mikrowellenresonators ein zentrales Thema dieser Arbeit dar. Mit dieser Methode ist es möglich, den Wassergehalt von oberirdischen Pflanzenbestandteilen auch über einen längeren Zeitraum nicht-invasiv zu messen. Mit Hilfe von Kalibrierungen konnte ein Zusammenhang zwischen gemessener Änderung im elektromagnetischen Feld und dem Wassergehalt des Pflanzenmaterials hergestellt werden.

Die artspezifischen Unterschiede in Biomasse und Blattfläche unter Gewächshausbedingungen, sowie die Variabilität der abiotischen Umwelteinflüsse im

Gewächshaus, erforderten Experimente unter konstanten Umweltbedingungen. In den Expositionskammern des Helmholtz Zentrums München war es möglich, ein annähernd naturnahes Verhältnis zwischen UV-B und photosynthetisch aktiver Strahlung (PAR) herzustellen. An Tabak- und Tomatenpflanzen wurde unter Kontrollbedingungen und erhöhter UV-B Strahlung kontinuierlich die Biomasse mit Hilfe des oben erwähnten Mikrowellenresonators bestimmt. Es zeigte sich eine deutliche Reduzierung des Frischgewichts für Tomatenpflanzen unter erhöhter UV-B Strahlung, die im Laufe des Experimentes stärker ausgeprägt wurde. Im Wachstum von Tabakpflanzen zeigte sich zunächst jedoch kein Effekt in Reaktion auf UV-B Strahlung. Erst eine länger andauernde Exposition unter erhöhter UV-B Strahlung führte zu einem Anstieg in der Biomasse. Es zeigt sich hier ganz deutlich eine artspezifische Reaktion auf UV-B. Der tägliche Wasserverlust von ca. 40% bei Tabak und hingegen nur ca. 10% bei Tomatenpflanzen stellt vermutlich einen größeren Stressor für Tabakpflanzen als die UV-B Strahlung dar.

Es ist sehr gut vorstellbar, die neuentwickelte nicht-invasive Methode für Phänotypisierungen mit einem hohen Durchsatz unter verschiedensten Bedingungen, sei es Trockenstress, Untersuchungen zu Herbivor-Pflanzen Interaktionen oder Wachstum bei Pathogenbefall einzusetzen.

In einem gemeinsamen Projekt mit der Universität Bonn wurden Eichblattsalatpflanzen unter den bereits beschriebenen UV-B Bedingungen im Gewächshaus angezogen und das Wachstumsverhalten sowie sekundäre Inhaltsstoffe untersucht. Eine Reduzierung des Wachstums konnte nur im April für Pflanzen unter erhöhter UV-B Behandlung gefunden werden. Im jahreszeitlichen Verlauf konnte kein signifikanter Unterschied bezüglich der Blattfläche gefunden werden. Pflanzen, die bereits als Keimling bzw. später als Setzling UV-B Strahlung ausgesetzt waren, zeigten eine stärkere Bildung von Flavonoiden insbesondere Quercetin und Cyanidin. Bei einem Transferversuch ins Freiland konnte jedoch gezeigt werden, dass sich diese Unterschiede bereits sechs Tage nach dem Auspflanzen nivellierten.

Für die Praxis können diese hoch UV-B durchlässigen Materialien trotz allem die Zeit der Akklimatisierung vor dem Auspflanzen u.a. aufgrund der verstärkt ausgebildeten sekundären Inhaltsstoffe verkürzen. Das antioxidative Potential in den Salatpflanzen erhöht sich damit und hat somit einen fördernden Einfluss auf die menschliche Gesundheit.

Summary

Light is essential for life in particular for the life of plants and they in turn are substantial for the thriving life on Earth. Plants are sessile organisms and have to respond to a wide range of environmental factors. Their ability to adapt to a rapidly changing environment by changing their molecular and even morphological levels ensures the survival under these conditions.

The presented dissertation is part of the joint research project "Innovative greenhouses – Research for improved product quality and sustainable use". Main aim of this thesis was to investigate the influence of light quality, especially increased UV-B radiation levels, on growth of different plant species (horticultural and model plants).

Greenhouses were covered with materials showing different levels of transmittance in the UV-B spectrum band: teflon foil showed UV-B transmission rates of approximately 86%, rendering it an ideal cladding material for the high UV-B treatment. Floatglass, commonly used in horticultural practice and almost intransparent for UV-B, represented the control cladding material for low UV-B treatments. Lettuce, red oak leaf lettuce, tomato and tobacco seedlings were grown under different UV-B treatments in the above described greenhouses. The GROWSCREEN phenotyping platform was used to determine the development of projected leaf area during a species dependent adequate time interval, which also depends on the morphology of the investigated plant species. Under increased UV-B radiation salad and tomato seedlings´ projected leaf area was significantly reduced. In contrast, tobacco plants showed no distinct growth reaction to increased levels of UV-B under greenhouse conditions. Yet, other differences such as increases in quantum efficiency of chlorophyll fluorescence and in non-photochemical quenching could be detected for tobacco as well.

The non-invasive determination of plant biomass is still today in the focus of researchers. In technical respect the development and establishment of a microwave resonator was a central aim of this work. This method allows continuously determining the water content of plant shoot biomass over a longer period. *Via* calibration measurements, a correlation between measured changes in the electromagnetic field and plant water content was found.

The species-specific differences in biomass as well as the variability of the abiotic environmental influences within the greenhouse required experiments under constant

conditions. At the exposure chambers of the Helmholtz Zentrum Munich it was possible to achieve a near-natural relation between UV-B and photosynthetic active radiation (PAR). Experiments were conducted under constant environmental conditions. Tobacco and tomato plants were objects of continuous investigation of biomass in response to enhanced UV-B radiation *via* the above mentioned microwave resonator. After an acclimation period to the conditions in the resonator, tomato plants showed a distinctly reduced biomass under enhanced UV-B. The differences increased during the measurement period. In contrast to these findings tobacco plants need a much longer exposure to induce an increase in biomass in response to enhanced UV-B radiation. A species-specific reaction in response to UV-B was found. Tobacco plants showed a high diurnal water loss of roughly 40% each day during this experiment, while tomatoes lost only 10% of their fresh weight. It is likely that the water balance in tobacco plants played a greater role than the application of enhanced UV-B radiation.

It is quite obvious to use this newly developed non-invasive instrument for phenotyping at high throughput under varying conditions such as drought stress, investigation of herbivore-plant interactions or growth patterns during pathogen attack.

In a joint project with University of Bonn, growth patterns as well as the secondary components of lettuce plants, grown under the above mentioned greenhouse conditions, were investigated. A reduced growth in response to enhanced UV-B was found in April, whereas during the season no differences in leaf area development could be determined. Plants grown under high UV-B treatment showed increased accumulation of flavonoids such as cyanidine and quercetin. After transplantation to field conditions the observed differences in the greenhouses were already negligible after six days. However, the high UV-B transmitting cladding materials can provide a benefit for the acclimation of vegetable crop plants due to shortening of the acclimation period prior to transplantation to the field. Moreover, the secondary compounds increase the antioxidative potential of these vegetable crops, and this in turn has a benefit for human health.

Table of content

Citation of publications in this dissertation

The cumulative dissertation is based on the following manuscripts:

#	Citation	Journal	Status
1	Behn et al. 2010	European Journal of Horticultural Science	submitted
2	Menzel et al. 2009	Plant, Cell and Environment	published
3	Tittmann et al. 2010	Plant, Cell and Environment	submitted
4	Jansen et al. 2009	Functional Plant Biology	published

1 Introduction

Plants are essential for the thriving life on Earth. They provide the primary resource for essentially all food chains and produce oxygen for almost all organisms on Earth. Since ancient times mankind has a strong interest in plant culture and domestication, and plants are the foundation of human nutrition.

Plants have to respond to several abiotic and biotic stresses. These stresses affect plant performance, plant growth and crop production. The dimension of plant adaptation to stress factors is depending on plant genotype, developmental stage of the plant, and the degree of received stress. In general, one stress factor is followed successively by another or is received in parallel, for example heat stress is often associated with drought stress because of lack in water availability. Plants have to cope with these circumstances, and therefore they developed mechanisms to avoid damages in multiple ways (Jansen et al. 1998, Stratmann 2003, Wood 2005).

1.1 Motivation and research aims

The present dissertation was part of a larger research project concerning development of new and innovative greenhouses for horticulture (Joint project "Innovative greenhouses – Research for improved product quality and sustainable use"), which was funded by the Federal Ministry of Education and Research (*german*, BMBF). The project was divided into three subprojects, i) investigation of the influence of UV-B radiation on host/parasite interactions ("Pest resistance" project at University of Würzburg, Kuhlmann & Mueller 2009a, b), ii) effect of enhanced UV-B radiation on secondary metabolites and flavouring substances (project at University of Bonn), and finally iii) this dissertation which puts the focus on non-invasive analysis of leaf and plant biomass generation.

The central aim of the study was to investigate the influence of different light qualities on plant growth. One particular question to answer was: how do plants change their growth patterns and biomass accumulation under enhanced UV-B radiation? The focus was set on leaf development of the horticultural plants *Lycopersicon esculentum* (tomato), *Lactuca sativa* var. *crispa* (red and green bowled lettuce/ Lollo rosso) as well as the model plant for growth investigations *Nicotiana tabaccum* (tobacco). Experiments were performed under two different UV-B conditions in small greenhouses (shown in Fig. 1) and under constant environmental conditions in exposition chambers at Helmholtz Zentrum Munich.

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Figure: 1 Greenhouses at Forschungszentrum Jülich covered with cladding materials differing in transmittance of UV-B. The Float glass (control glass, first glass from the right) passes nearly no UV-B radiation, MMAR glass (a multistructured glass, the second glass from the left) is the "intermediate" treatment concerning UV-B transmittance, whereas newly developed ETFE foil (the second on the right) showed high UV-B transmittance.

Further, a particular aim of this work was to develop and use non-invasive methods for measurement of plant growth and development. For example the recently developed method GROWSCREEN (Walter et al. 2007) for 2D leaf area development was extensively used. This should finally resolve questions like:

- *How useful are newly developed cladding materials with a high UV-B transmittance for preadaptation of salad plants?*
- *Do they increase the probability of salad plant survival under field conditions after acclimation in the greenhouses?*
- *Do plants have a diurnal pattern of biomass accumulation? If yes, how does diurnal biomass accumulation of different plant species respond to different Ultraviolet-B radiation?*

1.2 Abiotic stresses

The presented study is focused on stress response of different plant species. With the help of non-invasive methods it was possible to investigate growth and biomass development of different plant species. This allowed characterization of plant growth patterns under UV-B stress or different other stress factors, which can influence plant survival. In the following section different stress factors, which strongly affect growth behaviour will be explained in more detail. Temperature, water and light conditions were in the focus of the presented investigation.

1.2.1 Temperature and the effect on growth

Temperature strongly affects plant growth dynamics. Several recent studies were conducted in greenhouses, which showed strong variation in temperature profiles during spring and summer (Behn et al. submitted, chapter 3.1). The horticultural and model plants, investigated in this thesis, displayed different growth patterns, which are affected presumably by a number of different stress factors. Temperature is one of the important environmental factors which can lead to stronger plant growth and biomass accumulation in warm months. The responses of plants to heat, water stress and several other stresses have direct relevance in the performed experiments. However, to eliminate high temperature differences and their effects on plant development, we decided to perform additional experiments in climate chambers (Tittmann et al. submitted, chapter 3.3).

For this work experiments were conducted under greenhouse conditions with cladding materials differing only in UV-B transmittance. Greenhouse-related temperature levels and fluctuations strongly depend on the season, causing different leaf growth behaviour of greenhouse-grown plants in relation to the season (Britt & Fiscus 2003). Concomitant with the different properties of the cladding materials the distribution of radiation and temperature can be influenced. Even small changes are not to disregard.

Heat stress is often associated with drought stress because of the lack of water availability. Plants have to deal with these situations in different ways (Wood 2005). They can survive in a wide range of temperatures varying from -89°C to 58°C (Schulze et al. 2002), or even higher temperatures that are found for example in deserts. To grow steadily, plants have developed adaptations to survive ranges outside optimal temperature conditions. Most of the metabolic reactions are strongly influenced by temperature, while some physical processes like light absorption are relatively temperature insensitive (Jones 1992). Photosynthetic enzymatic processes are the most temperature-sensitive part of plant growth. Plants of temperate zones tend to have their maximum of net photosynthesis

between 20°C and 30°C. Depending on the temperature to which plants are adapted, the temperature optima for photosynthesis are higher or lower. Thermal instability causes a species-specific time-dependent inactivation of the photosynthetic apparatus: Desert species, for example, are more tolerant to **high temperature**, and up to 50°C no inactivation of photosynthesis will occur. The time point and the duration of temperature stress exposure are important for survival of the plant (Larkindale et al. 2005). Photosynthesis is not only affected by temperature stress during the day; also high temperatures during the night lead to reduced productivity. Plants often have to respond to multiple stresses such as combined drought and heat stress (Larkindale et al. 2005). Higher temperatures can change the pattern of protein synthesis and above a threshold the normal protein synthesis is replaced by a rapid synthesis of a set of heat shock proteins (HSP) (Jones 1992). The induction of heat shock proteins needs acute heat stress. Several components, not HSP alone, are involved in the response to high temperatures. Increased temperature alters enzyme activity and leads to imbalances in metabolic pathways. The acquired ability to withstand higher temperatures for short-term exposure is an adaptation due to a changing diurnal temperature regime (Larkindale et al. 2005).

Plant growth intensity depends on the surrounding environment, which can have a reinforcing or inhibitory effect. The aboveground part of plants is exposed to varying temperature and light regimes while the roots are strongly affected by physicochemical and biological processes at nearly constant temperatures in soil (Walter et al. 2009). Roots respond very fast to alterations in temperature, soil water potential and even to light changes at the aboveground shoot. The growth of monocot leaves strongly depends on temperature and vapor pressure deficit. The changes in the daily growth rhythm are almost linearly related to temperature variations. In contrast to this, the growth pattern of dicot leaves is driven by endogenous rhythms (Walter et al. 2009) and factors such as root temperature: Low root zone temperatures result in distinct reduction in leaf area and biomass (Poiré et al. 2010).

In response to **low temperatures** plants stop growth, germination or reproduction. The damage after short exposure to freezing temperatures is reversible. The frozen water in the cells causes typical freezing injury. Without acclimation, temperatures of already -1 to -3°C can induce damage of the tissue while an acclimation period let plants survive up to a temperature of -40°C depending on the species and the tissue.

1.2.2 Water

In this work, water plays a crucial role in determining plant growth, beside the above-described temperature. The investigation of biomass development was followed using a new non-invasive method relying on the water content of plants. Hereby, changes of plant water content were measured using a novel microwave resonator (Menzel et al. 2009, Tittmann et al. submitted, and chapter 3.2 and 3.3 below). Moreover, changes of transpiration rates of tobacco and tomato plants under different UV-B exposure were followed in a climate chamber experiment (Tittmann et al. submitted). During the experiment tobacco plants showed a strong effect with regard to water stress. This particular result from the climate chamber experiment can also be an explanation for the varying results of tobacco *vs.* tomato plants found in plants grown under greenhouse conditions in response to UV-B radiation. The differences are likely due to different water status with a strong water loss for tobacco plants but nearly no diurnal water loss for tomato plants (Tittmann et al., submitted)

Water is essential for the life of plants, but also for all other organisms. It is a major component of plant cells and depending on species, water content can vary between 10% of fresh weight for dried seeds and 95% in some fruits and young leaves. Plants have evolved many morphological, anatomical, biochemical, developmental and physiological adaptations to cope with varying water conditions. They pursue for example a water stress avoidance strategy.

Water is liquid at normal temperatures and a strong solvent. Because of this properties water is a good medium for biochemical reactions and transport (Jones 1992, Wood 2005). Water is also needed for important processes like photosynthesis. It was shown that plants always lose water to the aerial environment. They are in disequilibrium with the surrounding atmosphere because of the great difference of water content from “wet” plants to the “dry” environment (Wood, 2005). The incompressibility of water is important for growth. Volumetric growth depends mainly on cell expansion. The water flux into a cell and growth is depending on the driving force of water uptake, the hydraulic conductivity of the cell membrane. Therefore stomata play the major role in controlling **transpiration**.

When open, the pore space of stomata equals a fraction of 0.5-5% of leaf surface. All water that is transpired by plants as well as CO_2 absorbed in photosynthesis has to pass the stomatal pore space. Stomata are very sensitive to environmental and internal physiological conditions. This allows them to optimize the balance between water loss and CO_2 uptake. Frequency and size of stomata vary depending on the leaf position and growth conditions and they can vary between different cultivars of one species. The movement of the stomata

depends on changes in turgor pressure inside the guard cells and in the adjacent epidermal cells. It happens as a result from a changing water potential of the guard cells or from active changes in osmotic potential (Jones 1992). Changes in guard cells turgor cause an opening or closure of stomata. Also an endogenous rhythm tends to affect stomatal aperture independent from the environmental conditions (even under continuous light a night-time closure can occur). Many factors like for example light, CO_2, temperature, water status and humidity influence the stomatal aperture. With decreasing leaf water potential, increasing CO_2 mole fraction in the intercellular spaces, and decreasing light the stomata tend to close. Plants under drought conditions close stomata to avoid water loss. The water loss after stomata closure occurs then only *via* diffusion through the cuticular membrane (Wood 2005). The cuticle is a protective, hydrophobic, waxy layer produced by the epidermal cells of leaves in plants. In conclusion, water is the driving force behind plant growth, thus necessary for **plant cell expansion**. Even moderate water stress reduces plant growth.

It seems that even relatively mild water stress can inhibit cell wall proliferation and expansion growth – yet not by diminishing the production of cell wall material but by inhibiting the coordinated incorporation of cell wall material (Alves & Setter 2004). Jones (1992) defined this as “stored growth”. The reduced growth during a short-term stress allows plants to recover their growth rate after re-watering (Jones, 1992). During this process, growing cells do not reach the full size and also the number of cells per leaf is reduced compared to well watered plants (Alves & Setter 2004). Whether the reduction of leaf area is caused rather by reduced cell division or by reduced cell expansion, depends on the developmental stage at which plants are stressed (Alves & Setter 2004, Schurr et al. 2000). Leaves, which are no longer involved in cell division, reduce cell expansion, which in turn leads to a reduced leaf area caused by reduced mature cell size. In younger leaves the inhibition of cell division leads to fewer cells per leaf.

1.2.3 Light - physical properties and agent for Earth's life

Solar light energy is the essential environmental resource for plants. As energy source, light is needed for the photosynthesis, rendering plants as primary producers to the starting points of almost all food chains.

In this study it was in the interest to investigate photosynthesis under different light intensities and radiation qualities. Results from the continuous measurement of biomass have shown that the identified differences in biomass accumulation are not based on differences in photosynthesis. Tomato plants accumulated more biomass under low UV-B

conditions whereas no significant increases in photosynthesis were observed (chapter 3.3). Although photosynthesis provides the essential substrates (carbohydrates) for growth, photosynthesis cannot be directly converted into shoot growth. Several other signals can also affect the growth dynamics of plants. Starch deficit mutants of *Arabidopsis thaliana* plants showed a changed diel leaf growth pattern caused by diel variations of carbohydrate metabolism (Wiese et al. 2007) and the availability of substrates. Mutants showed an increased growth rate in the afternoon induced by an excess of hexoses which are not incorporated into starch storage. The reduced growth at the end of the night is due to the inability to access carbohydrates from starch which leads to reduced growth (Wiese et al. 2007).

In general, the absorbed light energy can be used for photosynthesis, it can be dissipated within the leaf via a range of biochemical reactions or it can be re-emitted as fluorescence or as heat. Moreover, light triggers several processes such as developmental pathways, is important during the seedling development, during cotyledon expansion and chlorophyll formation (The Arabidopsis Genome Initiative 2000). A great part of this energy is converted into heat and induces other radiation exchanges and processes like transpiration. .As the wavelengths of light get shorter and the energy content increases, also the biological damage on plant material will increase (Greenberg et al. 1997).

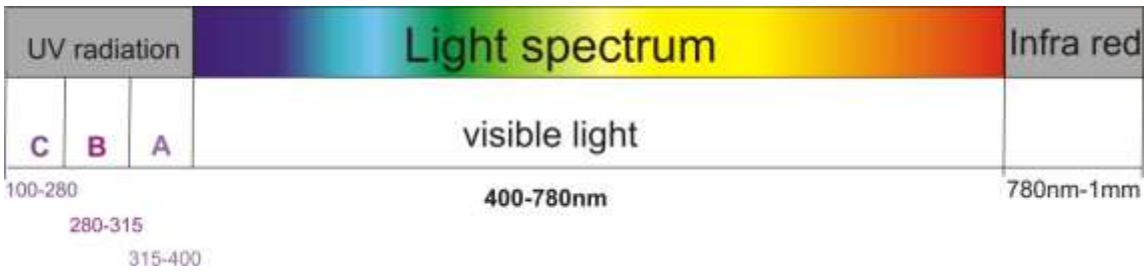

Figure: 2 : Light spectrum from ultraviolet over visible light to infra-red

The radiation, which reaches actually the Earth's surface, is modified concerning the quantity, spectral properties and angular distribution resulting from the absorption or scattering by molecules in the atmosphere and by scattering or reflection from clouds and particles (Jones 1992). Wavelengths greater than 290 nm reach the Earth surface and wavelengths less than 290 nm are absorbed by various gases in the atmosphere (Greenberg et al. 1997). The reflection and the transmission from or through for example leaves change the radiation composition.

Ultraviolet radiation is a part of the light spectrum with a wavelength range from 100-380 nm (Fig. 2). This part of the spectrum is not visible for the human eyes. It is part of the electromagnetic radiation. The most energy rich part is **Ultraviolet C** radiation (100-

280 nm). Almost none of this very short wavelength radiation reaches the Earth surface, because of the absorption in the upper atmospheric layer. UV-C radiation is technically used for sterilization and disinfection. At a wavelength of 270-300 nm (absorption peak of most proteins) the amino acid tryptophan can absorb ultraviolet radiation, and at 260 nm nucleic acids are most affected. **Ultraviolet B** belongs to short wave radiation ranging from 280 – 315 nm. Most of the UV-B radiation is absorbed by stratospheric ozone. The increasing depletion of the ozone layer enhances the fraction of UV-B radiation reaching the Earth surface. A decrease of ozone concentration of about 1% results in an increase of UV-B radiation of about 1.5% (Bavarian Environment Agency; german: Bayerisches Landesamt für Umwelt, 2009). Ultraviolet B radiation varies strongly with season (Fig. 3) and also with latitude and sun angle. **Ultraviolet A** is a longer wave length radiation (315-400 nm). These waves reach the human dermis and can cause a direct pigmentation (short-term brownness). UV-A can damage collagen and increases the risk of melanoma through accumulation of free radicals. Ultraviolet A radiation level is relatively constant in the solar spectrum and does not vary with latitude, altitude or season. UV-A will not increase with the progressive depletion of ozone layer (Greenberg et al. 1997).

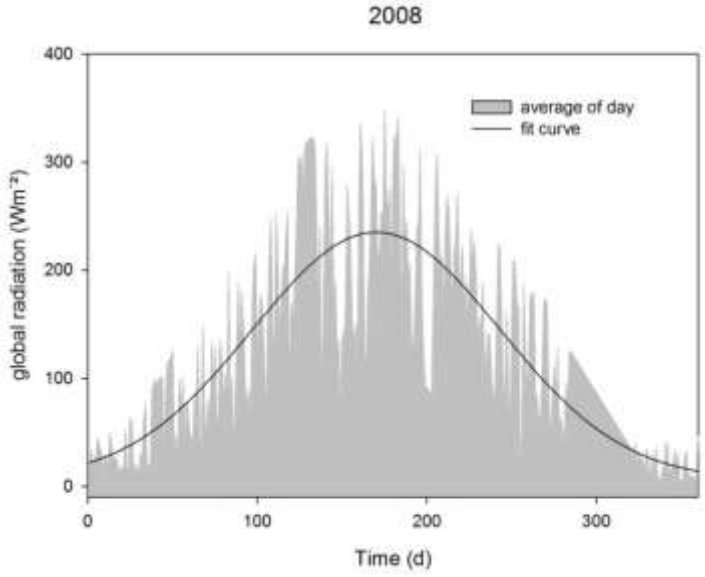

Figure: 3 Global radiation at Forschungszentrum Jülich 2008, daily mean values

The **Photosynthetic Active Radiation** (PAR; 400-700 Wm^{-2}) is the part of the spectrum, which can be used by photosynthetic active organisms. The important pigments of the photosynthetic apparatus Chlorophyll a and b absorb at 661.6 and 644.8 nm respectively. The ratio of UV-B radiation and PAR fluctuates caused by changes in solar angle and thickness of the ozone layer. The thickness of ozone layer in turn depends on seasonal meteorological conditions and on latitude (Jansen et al. 1998). This part of the spectrum is overlapping with the spectrum that is visible for humans (400-780 nm). Nearly 50% of

global radiation accounts for this visible part of the spectrum. Global radiation is the sum of direct and diffuse radiation incident on a horizontal surface (Jones 1992). On a clear day diffuse radiation contributes between 10 and 30% of the total solar irradiance. The drier the climate, the lower is the proportion of diffuse radiation. About 45% of the energy in the direct solar beam at the Earth's surface is in the photosynthetic active wavelengths. Diffuse radiation tends to enrich in the visible wavelength. This is the reason for only an average proportion of direct plus diffuse radiation in the PAR of approximately 50%.

In all three groups of the joint project (as mentioned above, chapter 1.1) one greenhouse was covered with multi structured glass (MM glass), which let pass approximately 35% of ambient UV-B radiation, a second glass with nearly no transmittance of UV-B radiation (Float glass) and a foil highly transmissible for UV-B (ETFE). All materials have a high transmittance for UV-A and PAR in common. Lettuce plants grown under diffuse radiation (MM glass) achieved sometimes higher biomass than control plants under Float glass conditions. This can be explained by the increased stress of direct incident light under control conditions (Behn et al. submitted, chapter 3.1). Absorption, reflection and transmission vary with thickness, age, water content, surface morphology and orientation of the plants. For the following experiments only the materials with the greatest difference (high and low) in UV-B transmittance were chosen.

Light as external trigger can influence growth and development of plants. It was shown that plants adapt their developmental patterns in response to changing light regimes – which is known as photomorphogenic response. This includes i) Phototropism (directional alterations in growth that occur in response to direct light stimuli), ii) Photonasty (reversible light movements that occur in response to directional and non-directional light stimuli), iii) Photoperiodism (non-directional developmental responses to non-directional but periodic light stimuli) and iv) Photomorphogenesis (other non-developmental responses to non-directional and non-periodical light stimuli) (Jones 1992). The syndrome of plant photomorphogenic reactions enables plants to establish efficient photosynthetic machinery under permanently changing light conditions (The Arabidopsis Genome Initiative 2000). Examples for these responses are the tendency to elongate stem height under shade conditions that allows escaping competitors, the development of "sun" and "shade" leaves, the biochemical and physiological characteristics and for example the induction of flowering.

1.3 UV-B - a specific stressor

1.3.1 Thinning of ozone layer and consequences for single plants and plant communities by increased UV-B radiation

After the strong reduction in stratospheric ozone layer in the 1970´s, research was focused on the effect of increased depletion of stratospheric ozone layer on plants. Recent studies direct their focus to the effects of environmentally relevant UV-B doses to investigate the plant responses (Wargent et al. 2009). The amount of UV-B radiation reaching the Earth surface increases in response to further depletion of stratospheric ozone (Rozema et al. 1997). During the last decades a decrease of ozone concentration with an average rate of 6% per decade (Björn et al. 1999) was observed, therewith the interest of the influence on plant development in response to UV-B radiation increased. The last years showed a slight recovery of ozone layer compared to the rapid and dramatic decrease during the 1970s and 1980s (Fig. 4) caused by man-made increases of chlorofluorocarbon (Searles et al. 2001, Bavarian Environment Agency; 2009). Not only human activities, also a change in the height of the tropopause, atmospheric circulation patterns or the sun activity can be an explanation for one third of the ozone loss (Bavarian Environment Agency, 2009).

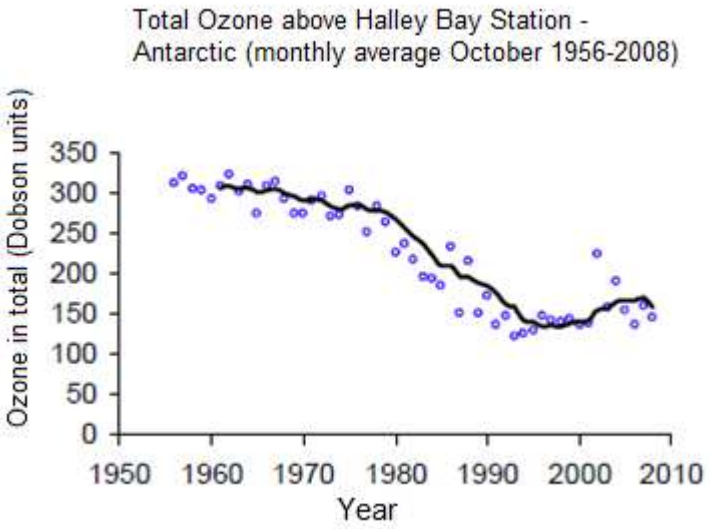

Figure: 4 Development of total Ozone during the last decades (Bavarian Environment Agency; 2009)

The study of Mäder et al. (2010) and Eyring et al. (2010) delivers the first line of evidence that the Montreal Protocol from 1987 shows a significant effect in reduction of the ozone hole. Recent studies (Mäder et al. 2010) could detect a thickening of the ozone layer over the last decade. The complete ozone layer will need the whole century to recover while the ozone hole over the Antarctic, which is built every year in September, still remains. The new findings of the reduction in ozone hole provide us with new challenges. The different studies have shown that after acclimation or under low fluence rate of UV-B, radiation does not lead to damage of plant material every time.

The study of Rozema et al. (1997) has shown, that increased UV-B radiation is not only an environmental stress but can also induce diverse other effects in plants. Direct and indirect morphological and chemical changes of plants in response to UV-B were observed. The response to UV-B radiation can differ between species and even cultivars. One crucial point is the effect of these changes on the plant community composition in ecosystems (Bornmann 1991).

The objective of this study was to understand the effect of increased UV-B radiation on plant behaviour. Leaf growth, biomass accumulation and photosynthesis are affected by UV-B to a varying extent, depending on the fluence rate (radiation per second) and duration of exposition. With novel, non-invasive techniques, it became possible recently to monitor biomass accumulation of tobacco and tomato plants with high temporal resolution (Tittmann et al., submitted). Biomass accumulation differed strongly between the two investigated species. Tomato showed inhibited growth under elevated UV-B radiation already three days after starting UV-B exposure, while tobacco plants showed initially no differences in response to UV-B radiation. A longer exposure of UV-B radiation is needed to induce an increase of biomass in treated tobacco plants.

One possible explanation for the different response of tobacco and tomato to UV-B radiation might be the different origin of the investigated species. Depending on the original latitude to which species have evolved, plant species can react more or less sensitive to UV radiation (Bornmann 1991). While plants with a genetic background of the southern latitude show a more “resistant” response to UV-B radiation, species of the same family from the northern latitude are more sensitive to enhanced UV- B radiation. A greater effect would be expected for plants of temperate and high latitude regions. Thus species of high latitudes would be more sensitive to increased UV-B radiation than plants close to the equator, which are in general adapted to high amounts of UV-B radiation (Bornmann 1991).

The UV-B environment of terrestrial plants varies strongly in time and space. For example, cloud cover has a significant influence on the amount of UV-B radiation (Bornmann 1991, Rozema et al. 1997). Thinning of ozone layer is resulting in a shift towards shorter wavelengths. If the wavelength becomes shorter this impairs the biological damage (Jansen et al. 1998). It is believed that two different UV-B perception and signalling pathways exist. One of them is triggered by longer wavelengths of the UV-B spectrum and another by shorter wavelengths, the latter negatively interfering with the former (Ulm et al. 2004). When the UV-B level is held at 1% of the visible light, Wilson & Greenberg (1993a) found a strong adaptive response to UV-B in *Brassica napus*, even at lower light intensities. The ratio of UV-B and PAR plays a major role for acclimation to UV-B (Wilson & Greenberg 1993a). Plants need a high fluence rate, Holst & Nagel 1997) of visible light to achieve optimal acclimation to UV-B radiation (Greenberg et al. 1997).

The results of the meta-analysis from Seales et al. (2001) indicate a significant decrease in shoot biomass at a simulated ozone layer depletion of minimum 20%. Otherwise no significant change of plant biomass was observed at lower simulated ozone depletion (10-20%). Since the ozone depletion beginning in the 1970´s is slightly recovering in recent years, a further significant decrease in shoot biomass is not expected (Seales et al. 2001). But monitoring of UV-B radiation was not conducted long enough to carry out a future trend of UV-B development (Björn et al. 1999). Several studies have shown that crop plants, which demonstrated a sensitive behaviour in response to UV-B under growth chamber conditions, are nearly unaffected under UV-B in the field. Plants can reduce the UV-B damage under field conditions. Nevertheless, UV-B induces plant morphogenic effects, which in turn affect the competitive relationship between plant species. Because of the higher content of more complex phenols in leaves in response to higher UV-B radiation the decomposition of leaf litter is affected. The digestion of tannins and lignin by microorganisms is decreased compared to plants grown under lower UV-B radiation (Rozema et al. 1997). Furthermore, the induction of increased secondary compounds under enhanced UV-B may change carbon cycling, herbivory and also symbiotic relationship between higher plants and microorganisms (Rozema et al. 1997, Caldwell et al. 1998). These changes in plant tissues and in the leaf components composition have impact on the following steps of terrestrial biogeochemical cycles. There are some evidences that the decomposition of plant litter was slowed down after several years of UV-B enhancement. Evergreen plants, such as *Empetrum hermaphroditum*, seem acclimated to enhanced UV-B radiation, and no changes on species composition could be established (Björn et al. 1999).

1.3.2 Plant responses and molecular effects on the regulation of plant growth caused by UV-B radiation

Leaves absorb the highest amount of UV-B radiation. Only small fractions are scattered or reflected at the leaf surface or transmitted through the entire leaf. UV-B radiation induces non-specific signals and photomorphogenic signals (Fig. 5).

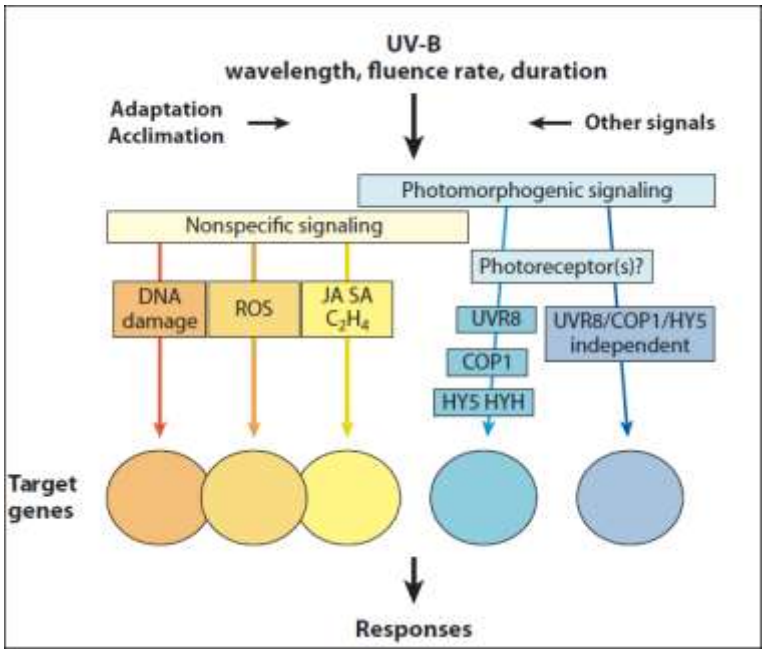

Figure: 5 Overview of UV-B signal transduction pathways (Jenkins 2009)

Increased UV-B radiation results in a wide range of changes in plants, such as inhibition of photosynthesis, damage in membranes, protein and DNA damage, delayed maturation, reduced growth and induction of flavonoid synthesis (Greenberg et al. 1997, Rozema et al. 1997, Caldwell et al. 1998). UV-B triggered changes can be distinguished between damage (e.g. inhibition of photosynthesis) and acclimation reactions (e.g. flavonoid synthesis). After exposure to high UV-B radiation stress signs were frequently observed, but under low UV-B radiation plants seem acclimate to this light condition. After adaptation, exposure to enhanced UV-B leads to a recovery of the photosynthesis. Higher UV-B fluence rates introduce permanent damage and no acclimation of the photosynthetic apparatus is possible.

Photomorphogenesis includes a wide range of light-controlled developmental responses (Fig. 5). Photomorphogenic signals induce the expression of genes involved in UV-B protection; therefore the survival of plants in a defined climate will be enhanced. Plants have evolved different mechanisms of protection and repair mechanisms against UV-B radiation. These mechanisms are effective and successful because damage of plants under natural conditions was rarely observed. General responses include different mechanisms to avoid damage of the leaf tissue. This includes thickening of the leaves, production of

waxes and hairs, increasing the surface reflectance, alteration in leaf transmittance properties and accumulation of UV-screening pigments (e.g. flavonoids, phenolic compounds etc., Behn et al. submitted, chapter 3.1). UV-B influences epicuticular wax composition and can increase the leaf wax content as a screening mechanism to reduce UV-B transmittance through the leaves (Wargent et al. 2009). Seed germination, stem elongation, leaf expansion, leaf area, leaf angle and plant architecture, the development of chloroplasts and the synthesis of chlorophyll are affected by UV-B radiation (Greenberg et al. 1997, Rozema et al. 1997, Caldwell et al. 1998, Britt 2004, Jenkins 2009, etc, Behn et al submitted, Tittmann et al .submitted, chapter 3.1 and 3.3). Changes in leaf shape can reduce the leaf area, which is exposed to UV-B radiation. Also smaller leaf areas, wrinkled morphologies and waxy leaf surface can lower the penetration with UV-B (Greenberg et al. 1997). A central aim of this thesis was to follow the development of leaf area under different UV-B radiation levels in greenhouses and climate chambers. Indeed, strong reductions of total projected leaf area of tomato and salad plants were found while tobacco plants showed no effect in response to enhanced UV-B under greenhouse conditions (Tittmann et al. submitted, chapter 3.3). A different situation was found under climate chamber conditions where tobacco plants also showed significantly reduced leaf area in response to enhanced UV-B (not published, in progress).

Plants are able to gain energy from the sun, which is then useable to fix atmospheric CO_2 into photosynthetic assimilates. UV-B exposure leads to reduced transcription and synthesis of key photosynthetic proteins and an increase in proteins, which are required for protective mechanisms. The changes in photosynthetic proteins are very fast and occur within hours and they are observable over days. These reactions are dose dependent. Degradation of the core protein of the PSII (D1) correlates with the fluence rate of UV-B radiation reaching the mesophyll. Wilson & Greenberg (1993b) found a 50% lower degradation of D1 protein and an increase in leaf flavonoid level for UV-B adapted plants. Contrary to this Rozema et al. (1997) and Searles et al. (2001) for example found out that enhanced UV-B radiation did not result in apparent reduction of **photosynthesis**, stomatal conductance, chlorophyll fluorescence and plant growth. The findings in the present study concerning the photosynthesis response support these results. However, growth was reduced for tomato plants while tobacco plants showed no difference in biomass accumulation under enhanced UV-B radiation (Tittmann et al. submitted).

During the early developmental stages of plant life growth depression can be observed in response to ambient UV-B radiation. It seems to be elusive in nature (Rozema et al. 1997). It is possible to estimate long-term net photosynthesis production. But photosynthesis is

limited by the "slowest" factor of the system, which then can slow down the whole process. This is known as the "Principle of limiting factors" (Blackmann 1905). Carbon fixation is highly affected by UV-B exposure, because of the UV-B sensitivity of Ribulose -1,5-biphosphate carboxylase (Rubisco), the central enzyme of carbon fixation pathway (Greenberg et al. 1997). Strid et al. (1990) have shown a decreased Rubisco activity of 90% caused by 8d exposure to enhanced UV-B in Pisum. Allen et al. (1998) suggested Rubisco and stomatal closure are more sensitive to UV-B radiation than photosynthesis is affected. Keiller et al. (2003) described a reduction in expression and amount of key photosynthetic proteins (including Rubisco), which resulted in a reduction in efficiency of photosynthesis. Leaf expansion is also affected by UV-B radiation, which influences the stomatal densitiy. High UV-B tends to cause stomatal closures and it thus can alter the photosynthesis rate (Keiller et al. 2003).

Photosynthesis and **transpiration** of tomato and tobacco plants were not affected by enhanced UV-B radiation during the climate chamber experiment but showed a species-specific difference in the assimilation rate (Tittmann et al., submitted, chapter 3.3). The leaf area of tobacco plants under greenhouse conditions were not affected by increased UV-B radiation but chlorophyll fluorescence showed a reduced yield (see below chapter 3.4, Jansen et al. 2009). My research aim concerning UV-B related stress in plants was to investigate the influence on growth and biomass patterns not on a molecular level, but on a phenotypic level (see below). Further, the effect on photosynthesis level and also chlorophyll fluorescence were of interest (see below, Jansen et al. 2009, Tittmann et al. submitted, chapter 3.3 and 3.4).

UV-B induced accumulation of UV-B screening pigments

Mesophyll cells of many herbaceous species are penetrated by approximately 40% of the incident UV radiation. These plants have a less effective ability to absorb UV radiation. Plants are able to change their development in several ways in response to UV-B radiation. It is possible that the exposure to UV-B radiation may enhance the tolerance to UV-B in plants but it can also result in stress symptoms (Britt 2004). The accumulation of UV-absorbing phenolic compounds reduces the penetration of UV-B radiation in epidermal tissues. Plants can perform sunscreens in the epidermis layer, which selectively absorb photons in the UV-A and UV-B range (Rozema et al. 1997, Jenkins et al. 2001). Flavonoid, a protective pigment, limits the penetration of UV-B through the plant tissues (Britt 2004). Flavonoids are involved in neutralizing radicals, which are formed by absorption of UV-B photons (Britt 1996, Rozema et al. 1997).

Abiotic and biotic stresses lead to an induction of genes encoding enzymes of the phenylpropanoid and flavonoid pathways (Jenkins 1999). The shielding properties of certain pigments and specific damage-repair systems are protective responses to enhanced UV-B radiation (Caldwell et al. 1998). Landry et al. (1995) have shown that a few specific flavonoid compounds are important in tolerance for UV-B exposure. Flavonoids have the ability to absorb not only UV-B radiation but also visible light and serve as antioxidants. The achieved "shading" protects tissues from photoinhibition during the development of the photosynthetic apparatus and degradation of senescing leaves (Britt 2004).

Our research partner at University of Bonn (subproject 2) focused on the effect of enhanced UV-B radiation on secondary metabolites and flavouring substances. In an integrated study we showed that lettuce plants reduced their biomass, which was accompanied with an increase in flavonoid content responding to enhanced UV-B radiation (Behn et al. 2010 submitted). Moreover, flavonoids are of interest in applied horticultural science as they provide nutritional benefits to humans, which are related to their antioxidative capacities. These metabolites (quercetin and kaempferol) have nutritional and pharmacological effects. The uptake of plant phenolics in human plasma has benefits for health caused by the interactions of heavily modified phenolic structures with a range of protein targets including human oestrogen receptor (Jansen et al. 2008).

The induction of the pigments is in agreement with the increased expression and enzyme activity of the phenylpropanoid pathway when photosynthetic gene expression decreases (Jenkins 1999). Experiments from Chapell & Hahlbrok (1984) showed an increase in transcription rates of the phenylpropanoid pathway after UV-B exposure. The products of these pathways are considered to be important in plant protection. They are known to have also antipathogenic properties. It is still unclear why anthocyanins and flavonoids are accumulated under stress conditions. Their antioxidant potential might be one reason. The first enzymes of the phenylpropanoid and flavonoid biosynthesis pathway are phenylalanine-ammonia-lyase (PAL) and chalcone synthase (CHS) (Jenkins 1999). PAL catalyses the deamination of phenylalanin to form trans-cinnamic acid, the first step in formation of more complex phenolic compounds like lignin. The *CHS* gene transcription in response to UV-B radiation occurs mainly in epidermal cells, the location of flavonoids generation. Chalcon synthase is a key enzyme in secondary metabolism (Jenkins et al. 2001). The *CHS* gene is also activated by several other environmental stimuli such as fungal elicitors and pathogen attack (Greenberg et al. 1997). The metabolic cost to repair damages related to enhanced UV-B radiation and the induction of protective mechanisms is not fully understood (Rozema et al. 1997).

Interaction with other stress signals

Gene expression induced by some UV-B signalling pathways overlap with other defence mechanisms such as wound-response or pathogen-defence pathways. Ultraviolet-B radiation can induce the production of phytohormones (salicylic acid, jasmonic acid and ethylene). They are involved in the wounding and/or defence pathways (Jenkins et al. 2001). Several plant signalling components are supposed to play a role in UV-B response, such as reactive oxygen species (ROS), jasmonic acid and nitric oxide but it is also likely that they form parts of a general multiple-stress response network such as regulating wound and defence signalling (Wargent et al. 2009). Our research partner at University of Würzburg (subproject 1) focused on the influence of UV-B radiation in host/parasite interaction and they found no effect of glucosinolates, which are characteristic secondary components of broccoli plants, and protease inhibitors in response to UV-B radiation, independent of the developmental stage of these plants (Kuhlmann & Müller 2009a). Furthermore, a significant increase in plant infestation by phloem feeding insects compared to UV-B excluding conditions were observed (Kuhlmann & Müller 2009a). Thus, the cell content feeders were more abundant under -UV-B conditions. It seems that radiation quality plays a more important role than the plant quality itself: Broccoli plants showed an increase in flavonoid content, which was elicited by a radiation-protection mechanism and not as part of a defence reaction against herbivores. After insect attack glucosinolate content increased. This indicates a separate stimulus-specific response (Kuhlmann & Müller 2009b). There is an overlap in gene-expression due to UV-B and herbivore attack but plants are able to differentiate between impact by UV-B or stress caused by herbivorous feeding (Pandey and Baldwin 2008).

In the presented study the morphogenic responses and the reaction of the water status relating to different UV-B conditions was the focus of my research interest. Nevertheless, it is necessary to put the findings of the presented study in the context of the current knowledge on the molecular responses of plants towards elevated UV-B which is done in the following chapter. For more information, the reader is referred to the reviews by Britt (1996), Caldwell et al. (1998) and Jenkins (2009) (and citations within).

Ultraviolet-B penetration varies with the developmental stage of plants. Younger leaves attenuate UV-B radiation less than older leaves do (Caldwell et al. 1998). **DNA** can be directly damaged at higher UV-B doses when radiation excites DNA molecules causing aberrant covalent bonds to form between adjacent cytosine bases. This results in producing a dimer. The misreading (AA not the original CC) causes a wrong replication, adding TT

on the growing strand and summing up to a mutation ("classical C-T mutation"). DNA strongly absorbs UV-B radiation, and the degree of damage depends on the type of wavelength and the length of exposure. The most abundantly occurring modifications are cyclobutan pyrimidine dimers (CPD) and (6-4) photoproducts (pyrimidine adducts) (Britt 1996, Caldwell et al. 1998, Björn et al. 1999), which inhibit DNA and RNA polymerase and subsequently disables transcription. Despite the easy repairing of these two lesion types a failure of repair will result in cell death (Björn et al. 1999). The inhibitory effects on transcription and replication are more serious as the mutagenic effects related to plant growth (Britt 1996, Britt 2004). Plants have developed a number of **repair mechanisms** such as photo-reactivation and excision repair (dark repair) to reduce the UV-B induced damages. The photoreactivation requires some photo-activated DNA repair enzymes (Britt 1995, Greenberg 1997; Björn et al. 1999, Mackerness 1999, Britt 2004). The DNA damage is repaired by DNA photoylases. The enzyme photolyase reverses the cyclobutan-type dimers, which constitute the main DNA lesion induced by UV-B radiation. To enable this repair reaction a photon in the UV-A and/or blue range of the spectrum is required (Britt & Fiscus 2003). Other DNA damages can only be repaired *via* excision. Dark repair replaces the damaged DNA with new undamaged nucleotides. There are two major dark repair mechanisms: i) base excision repair and ii) nucleotide excision repair (NER). Compared to the photo-activated mechanism of the photoylase it is a slow running and less effective mechanism. Excision repair is predominantly used for repair of minor, non-dimer UV-B induced photoproducts (Britt 1996, Greenberg et al. 1997, Britt & Fiscus 2003). Chen and coworkers (1994) reported an *Arabidopsis thaliana* CPD photolyase activity only after exposure to visible light. The transcription of the gene encoding the photolyase is regulated by white light and UV-B, but is not simply induced by light - a day and night rhythm seemed also required. Plants under continuous white light showed a successive decreasing of photolyase protein level (Watherworth et al. 2002).

Figure: 6 Overview of the influence of UV-B radiations on damage and induction of protection mechanisms on plant levels (from Caldwell et al. 1998)

Many environmental organic contaminants are activated or even enhanced by light and some have strong absorbance in the UV-B region. The damage caused by UV-B is induced *via* active oxygen (ROS), photooxidation and free radical reactions, no matter whether the UV-absorbing component is endogenous (protein, DNA) or exogenous (a xenobiotic molecule) (Fig. 6). Singlet oxygen has the potential to attack any biomolecule and therefore forms organic peroxides. Lipid peroxides, for example, can inhibit the membrane fluidity and function (Greenberg et al. 1997). Damage of macromolecules such as DNA but also the generation of ROS or the impairment of cellular processes is possible signals in response to UV-B (Jenkins 2009). Oxidative damage to DNA results in lesions, which cannot be photorepaired, but repaired by slow light independent processes (Björn et al., 1999). Damage can lead to reduction in photosynthesis, plant growth or productivity, anatomical or morphological changes (Caldwell et al. 1998; Mackerness & Thomas 1999). These responses are mediated by non-specific pathways (Fig. 5). Photomorphogenic responses on the other hand belong to the UV-B specific pathways. The thresholds of UV-B to induce photomorphogenic responses are much lower than those causing DNA damage. Responses to UV-B influence the plants architecture (morphology) or chemical composition (e.g. 10-fold induction of flavonoids) (Gitz et al. 2005; Jenkins 2009) (Fig. 6). This induction of secondary metabolites increases the adaptation of plants under ambient and enhanced UV-B radiation.

Quality and quantity of light can have complex effects on plant morphology. Seedlings grown in darkness become etiolated, which means they grow very long and pale. This is an adaptation to extend above the soil surface into the light before expanding the leaves

(Greenberg et al. 1997). The process of de-etiolation needs the activity of all three main photoreceptor systems. The transcription factor ELONGATED HYPCOTYL (**HY5**), a mediator of several photomorphogenic pathways, plays an important role in light regulated de-etiolation. HY5 is required for UV-B activated gene expression (Ulm et al. 2004, Fig. 5). The following development is also light dependent and is controlled by phytochrome and blue light system. Low irradiance causes maximum leaf development at the cost of stem extension, and also increases the length of stem per unit weight, thus resulting in larger but thinner leaves (shade avoidance) (Greenberg et al. 1997).
The CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1) protein regulates a range of low fluence UV-B mediated gene expressions that lead to flavonoid accumulation and inhibition of hypocotyl elongation. COP1 and HY5 are involved in the regulation of signal events in response to no UV-B and enhanced UV-B radiation stimuli. The UV RESISTANCE LOCUS8 (UVR8) signalling acts in the UV-B specific regulation of gene expression (Wargent et al. 2009). UVR8 is involved in the regulation of a wide range of genes. Furthermore, UVR8 regulates the transcription of genes related to terpenoid biosynthesis, photoylase activity and photooxidative repair.

Photoreceptor

Plants respond to light via a range of complex signalling cascades that are specific for certain fluence rates, wavelengths or doses of exposure (Wargent et al. 2009). The most important photoreceptors in plants are chlorophylls, that are susceptible for a range of wavelengths, while blue light is mainly perceived via cryptochrome (likely a flavoprotein) and red light via phytochrome (red/far-red reversible chromoproteins) (Britt 2004). More tolerant plants maintain normal levels of chlorophyll at higher UV-B doses. Plants grown under UV-B light showed no inhibition in net photosynthetic carbon assimilation in comparison with plants grown only under visible light (Greenberg et al. 1997). The photoylase family is branched off to produce related genes, necessary for the regulatory responses to light. These “cryptochromes” lack photoylase activity but keep their light absorbing co-factors. They are involved in blue light perception (Britt 2004). Known photoreceptors for UV-A/blue light are: cryptochrome 1 (cry1) and cryptochrome 2 (cry2), and the phototropism photoreceptor phototropin. The photoreceptor cry1 is involved in the suppression of hypocotyl or stem elongation by blue light. The extension and expansion of several organs is controlled by cry1. At low fluence rates of blue light cry2 is involved in the control of stem extension. High fluence rates result in destruction of cry2. The photoreceptor cry1 induces genes of flavonoid biosynthesis and anthocyanins (Jenkins et

al. 2001). The *CHS* expression is stimulated by UV-B and blue light, depending on species and developmental stage. It can also be stimulated by red and/or far-red light absorbed by phytochromes. Most responses of plants to UV and blue light are mediated by several additional photoreceptors. (Jenkins et al. 2001).

The UV-B acclimation is mediated by the UV-B specific morphogenic photoreceptor, the **phytochrome** and the UV-A/blue light receptor. The gene expression in plants in response to UV-B radiation is likely connected to several pathways. Because of their UV-B absorbing properties, phytochromes are required for diminishing UV-B effects (Kim et al. 1998, Jenkins 1999). In *Arabidopsis thaliana* plants only very young seedlings showed phytochrome induction of *CHS.* This is not connected to the UV-B induced expression in older leaves (Kim et al. 1998, Jenkins et al. 2001). Phytochrome deficient mutants show the induction of *CHS* expression under UV/blue light. The presumption is that plants have one or even more UV-B specific photoreceptors (Jenkins 1997). Plants use different photoreceptors depending on their developmental stage. Many organic compounds in the cell absorb UV-B. There are some indications that responses to UV-B radiation are downstream events of absorption by a specific UV-B photoreceptor (Mackerness & Thomas 1999). However, up to now no specific UV-B photoreceptor was identified in plants (Jenkins et al. 2001, Ulm et al. 2004). The UV-B UV-A /blue light signalling pathways are distinct and are different from the phytochrome signalling pathways regulating *CHS* expression (Jenkins 1999, Jenkins et al. 2001).

Changes in growth and biomass in response to UV-B

Some of the UV-B effects contribute to a stimulation of growth of a specific tissue or organ (e.g. axillary branching, leaf thickening) while other effects lead to an inhibition of organ growth (diminished hypocotyl elongation). Monocots are more responsive to UV-B than dicots are. Many morphogenic responses are induced by relatively low UV-B levels, which do not impede growth and photosynthesis (Jansen et al. 2002). Most plant responses are appropriate to neutralize the stress, repair the damage or lead to re-growing of tissue (Stratmann 2003). Flint & Caldwell (2003) found decreased plant height and first leaf insertion after exposure to enhanced UV-B radiation. Longer irradiation at higher fluxes was necessary to produce effects at longer wavelengths, while plant responses were substantial at shorter wavelengths even with short-term duration irradiation (Flint & Caldwell 2003). The effect of the applied UV-B radiation depends on developmental stage of plant growth. Growth of soybean was affected by UV-B during the transition phase between vegetative and reproductive growth. UV-B radiation is detrimental to plant growth

because some reallocation of resources to repair the UV-B related damage and induction of protection mechanisms might occur (Teramura et al. 1987). One primary cause of growth reduction can be found in the reduction of cell division rate.

Ultraviolet resistance locus 8 (UVR8) is required to maintain the leaf expansion under UV-B exposure, which can be explained by the effects on cellular differentiation. UVR8 is the only known UV-B specific signalling component (Wargent et al. 2009). Wargent et al. (2009) have shown UVR8 controls aspects of leaf expansion as a result of effects on UV-B mediated compensatory epidermal cell growth. UV-B regulates with the help of UVR8 several key developmental plant responses. This shows that UV-B is not only a damage-inducing source of stress but can act as an environmental cue in higher plants. Ultraviolet – B enables plants to regulate protection against several factors, including UV-B itself, high light, drought and herbivory (Wargent et al. 2009).

The results of this thesis, which indicate a differentiated response of plant growth to altered UV-B conditions, depending on the physiological framework of the plant, have to be taken into account in future studies when the molecular framework of UV-B response is elucidated in more detail. Mutants altered in single or multiple pathways of the above mentioned signalling cascades have to be tested for their biomass growth response in varying UV-B conditions to clarify the involvement of the signalling cascades for improvement of crop yield or other applied purposes. With respect to the applicability of the results of the above-mentioned molecular studies, two main points of criticism have to be raised: First, numerous studies only report on hypocotyl growth (mostly of *Arabidopsis thaliana*) and it is unclear, whether these results can be extrapolated to growth of other organs in other species. Second, the exposition of plants towards altered UV-B regimes is typically realized in laboratory conditions with irradiation spectra deviating strongly from natural light regimes occurring in greenhouses or in the field. The following chapter relates to this point and shows the benefit of certain greenhouse cladding materials that can modulate UV-B in a controlled way, thereby simulating spectral regimes that can occur in the field or in horticultural production.

1.4 New cladding materials and their benefit

Plants respond differentially to UV-B radiation under suboptimal PAR conditions (Rozema et al. 1997). This knowledge is important for designing UV-B related experiments in greenhouses or climate chambers. Early studies were conducted indoors under low PAR conditions which results in reduction of plant defence capacity against UV-B radiation (Teramura & Sullivan 1987, Paul & Jones 2003). An unnatural relation between PAR and

UV-B radiation can lead to enhanced UV-B effects. For example, many reported damages might be overrated in greenhouse and growth chamber experiments by using an unrealistic high UV-B:PAR ratio applied by supplemental light sources (Rozema et al. 1997). In general, plants grown under low light conditions thicken their leaves less compared to plants under high light treatment as a result of suboptimal growth conditions (Bornmann 1991). In contrast to former experiments conducted under greenhouse conditions, in the presented study innovative materials (such as ETFE foil) with a high transmittance in the UV-B and also in the visible range were used, which allows to omit any additional source of UV-B radiation. The aim was to investigate the growth behaviour under these conditions.

In nature, depending on cloud coverage the UV-B:PAR ratio can distinctly vary. This UV-B:PAR ratio is important in determining UV-B effects on plants. If UV-B to visible light ratio is held at 1:100 negative effects of stress were not often observed, and plants showed acclimation responses at this UV-B level. But at increasing UV-B radiation and thus increasing ratio the effects became stronger and inhibition of photosynthesis and leaf wrinkling often occur. Plant growth under greenhouse or climate chamber conditions can lead to an increased sensitivity to UV-B radiation, caused by the reduced acclimation ability as a consequence of low light conditions (Greenberg et al. 1997). Under field conditions only modest responses were observed in response to altered UV-B radiation in the ambient range (Paul & Jones 2003). The light and weather conditions in the field are unpredictable and if even a supplemental UV-B source is used to simulate for example ozone depletion and if UV-B is not reduced on cloudy days this can induce stress response concerning an enhanced UV-B and PAR ratio. Using control treatments with entirely excluded UV-B radiation, with a very low background UV-B and PAR, leads to unrealistic observations. As mentioned above, UV-A radiation and visible light is necessary to mitigate UV-B effects on plants (Flint & Caldwell 1996, Searles et al. 2001). The effects of UV-B radiation under more realistic field conditions are smaller than expected from the growth chamber experiments (Searles 2001). Ultraviolet-A radiation, similar to UV-B, can induce a reduction of aboveground biomass and leaf area (Krizek et al. 1998).

Therefore, we decided to use **specific climate chambers** for UV-B experiments on tomato and tobacco plants, resembling the natural spectrum of visible light (PAR) and varying proportions of UV-B radiation under constant environmental conditions (at the Helmholtz Zentrum munich, Tittmann et al. submitted).

One other aspect of the work was to investigate the influence of preadaptation of lettuce plants to UV-B radiation in greenhouses and the effect after transfer to field conditions.

Many of the above-described responses have direct impact for horticultural applications. It is preferable to have UV-B adapted plants for sale and the possibility to transfer these plants directly to field conditions with an increased likelihood of survival under ambient conditions. Together with our research partner from University of Bonn we found significantly higher quercetin and cyanindin content during the greenhouse phase in response to enhanced UV-B radiation. But six days after transfer to field conditions the secondary metabolites of UV-B pre-treated lettuce plants are levelled (Behn et. al. submitted, chapter 3.1).

Foils with a modified Red: Far red (R:FR) ratio are available. The enhanced blue light may be a useful tool in horticulture because of the increased inhibition of sporulation of many important phytopathogens in response to blue light (Paul et al. 2005). The standard films used in horticulture showed decreased transmittance the shorter the wavelength of the UV range is. Plants grown under these conditions achieve nearly zero UV-A and zero UV-B radiation. UV-B opaque films act against crop pests by interfering at the dispersal and foraging behaviour in species that use UV in their visual system (Paul et al. 2005). The intensity of infestation of some crop plants can be regulated with these films. Films with enhanced transmittance of UV-B radiation induce significant crop responses such as increased growth and yield, altered pigment composition and changes in herbivory and disease. These new developed foils are an alternative to regulate growth in commercial horticulture. UV-B transparent foils display also a possibility for growth regulation for a range of ornamental species and can be an alternative to commercially used chemical growth regulators. A crucial point in horticulture is increasing the crop quality and at the same time improving biochemical plant composition. For some crops, this aim should easily be reached by altering UV-B exposition. It is well known for example that UV-B affects biomass and the taste of salad plants; but also pigmentation, or volatile oil content in some herbs can be altered (Paul et al. 2005) (Behn et al. 2010). Using covering materials with enhanced UV transmittance can also influence the biological control of disease (Kuhlmann & Müller 2009b). The results of this study demonstrated that the usage of innovative covering materials with high transmittance in the range of UV-B radiation effected biomass accumulation, leaf area development and induction of UV-screening pigments accompanied with leaf coloration of diverse plant species (Tittmann et al. submitted).

1.5 Non-invasive determination of growth and biomass related to UV-B stress

Although plant biomass is a logical proxy to extrapolate crop yield or to rate the effect of an experimental treatment, only few experimental approaches have been elaborated to determine the biomass of a living plant with high precision. Until today biomass is often measured invasively, by harvesting parts of plants or even whole plants. To achieve a better picture of biomass accumulation, methods with higher temporal resolution are needed. Several approaches have been established to estimate non-invasive parameters of plant growth such as LIght Detection And Range (LIDAR) to determine tree height using airborn photography (Osama et al. 2003), electronic registration of tree diameter (Ceramak et al. 2007), or organ surface expansion by digital imaging sequence processing (DISP, Walter & Schurr 2005, Walter et al. 2009). In another approach, Van Ieperen (1996) used a balance system to investigate the dynamic patterns of growth and transpiration. Two communicating vessels, filled with a nutrient solution, were placed on two balances, where water uptake and transpiration were measured simultaneously. For plant researchers, an online method for the non-destructive determination of fresh and dry weight over a longer period is highly desired, to observe plant biomass changes in response to changing environmental factors (Walter et al. 2007) or to determine developmental differences of genetically different plant species (Meyer et al. 2004).

One main objective of the present dissertation was to develop a system, which can be used for non-invasive measurements of plant biomass with a high temporal resolution. The system we have developed is using a **microwave resonator**. A microwave cavity resonator, microwave electronic module and correlation libraries for different plant species are the main components of this system. The dielectric permittivity is a measure of the extent to which a substance concentrates the electrostatic lines of flux. Imposing a static electric field across a medium, the relative dielectric permittivity denotes the ratio of the amount of electric energy stored in the medium relative to vacuum. The theoretical background of microwaves and microwave resonators is described for example in the work of Zinke & Brunswig (2000). The dielectric properties of water molecules and its fast reorientation in a field were used. Transfer of plant biomass into the resonator results in a reduction of resonance frequency. The differences between empty resonator and measurement of sample material were calculated. Relative and not absolute values to estimate plant biomass development were used (Menzel et al. (2009), chapter 3.2).

The application under different light qualities was the next logical step. We were interested in the different types of biomass accumulation in response to varying UV-B radiation and therefore new ways to determine biomass were used (Tittmann et al. submitted, chapter 3.3). With the help of a microwave resonator (Fig. 7) a continuous measurement of biomass accumulation pattern over several days is possible. In combination with different other methods "real-time" biomass, transpiration, and photosynthesis were measured, and an overall picture of plant performance under distinct light and environmental conditions was determined.

Figure: 7 View inside the opened folding resonator with a tomato plant grown in agar filled magenta box under enhanced and control UV-B radiation (exposition chambers at Helmholtz Zentrum Munich)

Experiments of forb species have shown different responses related to UV-B radiation. Biomass can be stimulated, decreased or unaffected under higher levels of UV-B (Musil 1996) depending on the species with which the experiment were conducted. The biomass of tobacco plants in the presented study showed no clear difference during the first days, whereas a longer duration of exposure under enhanced UV-B radiation led to an induction of biomass accumulation for treated plants. In contrast to this the diurnal water loss was significantly higher from beginning of the experiments on for tobacco in comparison to tomato (Tittmann et al. submitted chapter 3.3, Fig. 5). Tobacco plants lose approximately 40% of their plant weight *via* transpiration each day independent of the treatment, while tomato plants showed not such a strong response. The water loss is likely the most limiting factor for growth whereas the influence of UV-B radiation plays a minor role. However, the growth of tomato plants is limited by the carbon supply in response to UV-B (Tittmann et al. submitted, chapter 3.4, Fig. 5).

Beside the importance of non-invasive determination of biomass under different environmental conditions the development of leaf growth is still of major interest. In the study of Walter et al. (2007) leaf area and growth rate were measured using GROWSCREEN. The measurement of leaf area in this method is based on automatically captured pictures from a camera (Fig.8a) placed above the tray with plants. In some cases no changes under certain environmental conditions in leaf area development were found, but initially experiments indicated differences for example in photosynthesis or metabolites. Experiments under enhanced UV - B radiation showed no induction of changes in leaf area of tobacco plants but a reduced quantum yield was detected with the newly developed GROWSCREEN FLUORO. Hereby, the photosynthesis efficiency (F_v/F_m) can be an indicator for stress responses (see chapter 3.4, Jansen et al. 2009, Fig. 8b).

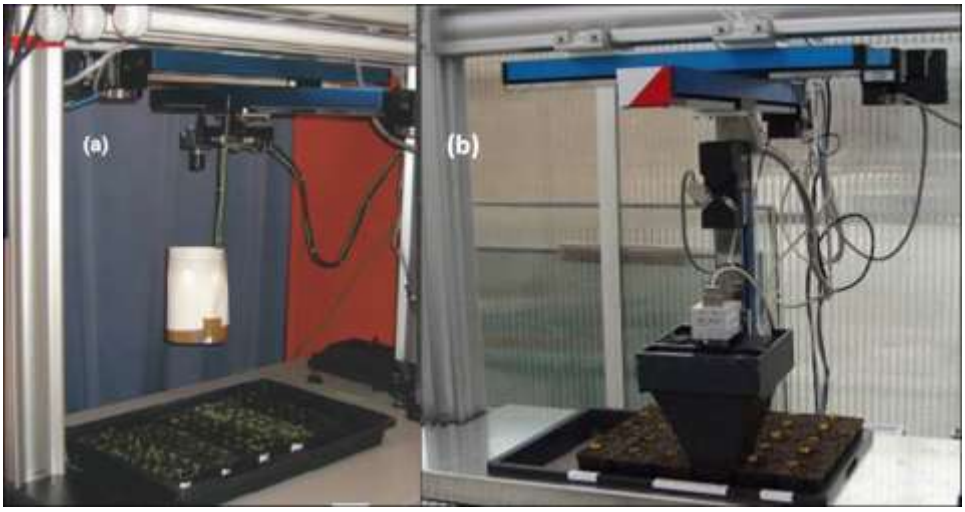

Figure: 8 Non-invasive leaf growth measurements using (a) GROWSCREEN and (b) GROWSCREEN FLUORO to measure total projected leaf area (a and b) and chlorophyll fluorescence (b).

The goal in recent and future investigations is to combine and integrate multiple different methods in single systems to estimate the effect of several factors on plants performance. These two new methods allow a high throughput screening of genetically different plant material (mutant screening), or to test plants for example on drought resistance which becomes more important in the near future because of the increasing temperature and longer periods with high temperatures. Plants tolerant to water deficit will become more necessary for the conditions which will be expected in the next decades. Tolerance is the ability to survive under certain environmental conditions (Wood 2005). Several stress applications are thinkable. In summary, these systems are interesting tools for phenotyping because of their fast and accurate measurements.

A great part of plant research focused during the last years on effects of enhanced UV-B radiation as consequences of the thinning of the stratospheric ozone layer. The following table (Table. 1) gives a short summary about the already known effects on plant behaviour and the results found in this study.

Overall this study shows that the response in relation to UV-B radiation is species specific concerning biomass accumulation and leaf growth development. With different non-invasive techniques the influence of UV-B radiation on growth was investigated. The study could also show that cultivation under newly developed UV-B transparent cladding materials resulted in reduced growth and led to increased accumulation of secondary components such as flavonoids of lettuce plants under greenhouse conditions. The transplantation to field condition induced a severe growth reduction but a strong enhancement of flavonoids. In the greenhouse observed differences were overridden within few days.

Table 1: Overview of the effects of enhanced UV-B radiation on plant performance

	"known" effects	Results of this study
Plant morphogenic responses	UV-B can induce changes in (depending on fluence rate): • leaf growth • biomass accumulation • Shoot biomass (significant reduction at simulated ozone depletion of 20%) • Thickening of leaves • Changes in leaf shape, leaf angle • Stem elongation • photosynthesis • Induction of more complex phenols in leaves which leads to changed decomposition of leaf litter • Secondary compounds (UV-screening pigments) • Production of waxes and hairs • Plant infestation by phloem feeding insects	Under enhanced UV-B the following changes were measured • Reduction of leaf area and biomass accumulation in greenhouses for horticultural plants but increased growth for tobacco plants • Diurnal differences in biomass accumulation between tomato and tobacco plants • Water loss of 40% of plant weight for tobacco plants • No changes in photosynthesis; only differences between species not related to UV-B level • Higher stomatal conductance and transpiration for tobacco plants • Increased level of flavonoids for lettuce plants in greenhouses, six days after transfer to field differences were negligible between high and low UV-B
Nonspecific signalling	• Protein, DNA damage • Delayed maturation • Degradation of core protein of PSII • ROS, Jasmonic acid, nitric oxide • Cyclobutan pyrimidine dimers (CPD) and (6-4) phot-products	**These parameters were not investigated in this study**
Molecular level **Photomorpho-genic signals**	• Induction of the expression of genes involved in UV-B protection • HY5, COP1, UVR8 • Expression and enzyme activity of the phenyl-propanoid pathway • Chalcone synthase activity • Production of phytohormones	

The project partner from the University of Bonn investigated among others the composition and yield of oil from leaves of Peppermint (*Mentha* × *piperita* L) under different PAR and UV-B conditions in exposition chambers. They reported highest yield during flowering at high PAR and ambient UV-B radiation, while low PAR and the absence of UV-B induced a reduction of menthol and an increase in menthone content accompanied with decrease in oil quality. The results of that study allow the conclusion that high level of natural light intensity is necessary to achieve qualitative high oil.

Partners from the University of Würzburg were interested in the influence of UV-B radiation on Broccoli plant performance, secondary compounds, composition of the leaf wax layer and mainly the infestation and growth of herbivores. It was shown in the end that young plants were affected by UV-B to a higher extent than older plants, but no differences in secondary metabolites such as glucosinaltes were found in response to enhanced UV-B. Herbivore insects showed species specific preferences for plants under high or low UV-B conditions respectively. Ultraviolet-B radiation hardens plants adverse herbivores and can lead to an increase in concentration of valuable herbal ingredients.

Altogether, we could show that the spectral light composition of the innovative cladding materials, with high transmittance in ultraviolet radiation and visible light spectra, influenced crop plants in terms of an optimized plant growth. Investigated plant species exhibited a more compact plant growth under enhanced UV-B radiation and showed increased content of secondary compounds compared to control plants.

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3 Articles of the dissertation

Article 1: Helen Behn*, Susanne Tittmann*, Achim Walter, Ulrich Schurr, Georg Noga & Andreas Ulbrich. UV-B transmittance of greenhouse covering materials affects growth and flavonoid content of lettuce seedlings *European Journal of Horticultural Science* (submitted)
*both authors contributed equally to this work

Article 2: Marion I. Menzel*, **Susanne Tittmann***, Jonas Bühler, Stella Preis, Norbert Wolters, Siegfried Jahnke, Achim Walter, Antonia Chlubek, Ariel Leon, Normen Hermes, Andreas Offenhäuser, Frank Gilmer, Peter Blümler, Ulrich Schurr & Hans-Joachim Krause. Non-invasive determination of plant biomass with microwave resonators. *Plant, Cell and Environment* (2009), 32, 368-379
*both authors contributed equally to this work

Article 3: **Susanne Tittmann**, Andreas Albert., Jonas Bühler. Ulrich Schurr & Achim Walter. Growth response to UV-B radiation interacts with pronouncedly differing diel water status fluctuations in tomato and tobacco plants. *Plant, Cell and Environment* (submitted)

Article 4: Marcus Jansen, Frank Gilmer, Bernhard Biskup, Kerstin Nagel, Uwe Rascher, Andreas Fischbach, Sabine Briem, Georg Dreissen, **Susanne Tittmann**, Silvia Braun, Iris De Jaeger, Michael Metzlaff, Ulrich Schurr and Achim Walter. Simultaneous phenotyping of leaf growth and chlorophyll fluorescence via GROWSCREEN FLUORO allows detection of stress tolerance in Arabidpopsis thaliana and other rosette plants. *Functional Plant Biology* (2009) 36, 902-914

3.1 First publication: UV-B transmittance of greenhouse covering materials affects growth and flavonoid content of lettuce seedlings

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* both authors are contributed equally to this work

Own contribution

- Experimental design
- Experiments
- Data Analysis
- Preparation of the manuscript (in cooperation with co-authors)

UV-B transmittance of greenhouse covering materials affects growth and flavonoid content of lettuce seedlings

H. Behn[1,2)*], S. Tittmann[2,3)*], A. Walter[2)], U. Schurr[2)], G. Noga[1)] & A. Ulbrich[1,2,4)]

[(1 Institute of Crop Sciences and Resource Conservation – Horticultural Sciences, University of Bonn, Bonn, Germany, [2)]Institute for Chemistry and Dynamic of the Geosphere (ICG-3 Phytosphere), Forschungszentrum Jülich GmbH, Jülich, Germany, [3)]Current address: Geisenheim Research Center, Institute of Viticulture, Geisenheim, Germany and [4)]Faculty of Agricultural Science and Landscape Architecture, University of Applied Science Osnabrück, Oldenburger Landstraße 24, D-49090 Osnabrück, Germany)

** both authors contributed equally to this work*

Corresponding author

Susanne Tittmann

Address:
Geisenheim Research Center, Institute for Viticulture, Von-Lade-Str. 01 D-65633 Geisenheim, Germany
Tel.: +49 6722-502-146
Fax: +49 6722-502-212
E-Mail: Susanne.Tittmann@fa-gm.de

Summary

In Europe lettuce (*Lactuca sativa* L., asteraceae) is commonly raised in greenhouses and transplanted to the field at the age of two to four weeks in order to prolong the growing season. The sudden exposure to outdoor conditions including altered temperature, radiation levels and rainfall events is extremely stressful for non-acclimated seedlings. Particularly the increase in ultraviolet-B radiation is considered a serious threat. A new approach to pre-acclimate seedlings to ambient ultraviolet-B radiation is the use of ultraviolet-B transparent covering materials. In order to estimate the benefit of UV-B pre-acclimation, lettuce plants were raised in greenhouses covered with three different materials varying in ultraviolet-B transmittance and transplanted to the field at the age of three weeks. Ultraviolet-B exposure during the greenhouse period led to a reduction in growth (leaf length, leaf area and leaf number) and an increase in flavonoid content. Transplantation to the field induced a strong enhancement in flavonoid content and a severe growth reduction overriding differences between UV-B treatment groups within a few days. At the time of harvest plant fresh weight was therefore independent from previous ultraviolet-B treatment. Effects of UV-B acclimation on plant performance immediately after transplantation require more detailed examination.

Key words: *Lactuca sativa*, biomass, flavonoids, leaf area, leaf length, lettuce, UV-B radiation

Introduction

In order to extend the growing season in Europe, sensitive horticultural crops such as lettuce (*Lactuca sativa* L., asteraceae) are commonly raised in protected cultivation and are transplanted to the field at the age of two to four weeks. Transplantation causes severe stress to the seedlings due to sudden changes in the biotic and abiotic environment coupled with possible mechanical injuries (SOUTH and ZWOLINSKI 1997; KORKMAZ and DUFAULT 2001; MITTLER 2006). Among abiotic stress factors increased UV-B (280-315 nm) radiation may be particularly harmful to non-acclimated seedlings (ROZEMA et al. 1997). UV-B radiation is a minor component of sunlight but may cause severe damage due to generation of reactive oxygen species (ROS) and absorption by biologically active molecules such as nucleic acids, proteins (aromatic amino acids) and lipids (SCHMITZ-HOERNER and WEISSENBOCK 2003; ULM and NAGY 2005). Plant UV-B responses generally aim at protecting sensitive tissues from UV-B penetration and repairing UV-B induced damage (JANSEN 1998). UV-B protection is mainly achieved by epidermal accumulation of UV-absorbing flavonoids and hydroxycinnamic acids (CALDWELL 1983; LOIS 1994). Repair of UV-B induced damage includes induction of enzymatic and non-enzymatic scavengers of ROS and activation of DNA repair mechanisms (JANSEN et al. 1998; BRITT 1999).

In horticulture diverse strategies have been developed to pre-acclimate greenhouse grown seedlings to ambient or above-ambient UV-B levels and thereby increase the plant´s stress tolerance and facilitate coping with outdoor conditions (DEL CORSO and LERCARI 1997; HOFFMANN 1999; TEKLEMARIAM and TERENCE 2003; CHALKER-SCOTT and SCOTT 2004). A new approach is the use of recently developed covering materials. These innovative foils and glasses allow for pre-acclimation due to an increased UV-B transmittance (KUHLMANN and MÜLLER 2009a). Several recent studies have addressed the effects UV-B transparent covering materials on growth and metabolism of lettuce. Biomass was reduced while flavonoid content was enhanced with increasing UV-B level (KRIZEK et al. 1998; GARCIA-MACIAS et al. 2007; TSORMPATSIDIS et al. 2008). Yet, performance of lettuce seedlings after transplantation to the field has not been examined so far.

Habitus and flavonoid content of lettuce plants are not only critical for the plant`s stress resistance at transplantation but also for product quality at harvest (RYDER 2001). Both affect optical appearance and durability of harvested lettuce (COUTURE et al. 1993). Flavonoids are supposed to exert health-promoting effects since the intake of flavonoid-rich fruit and vegetables was found to be negatively correlated with the occurrence of

cardiovascular disease and certain forms of cancer (HERTOG et al. 1993; HOLLMAN 2001; GRAF et al. 2004). This effect is attributed to the radical scavenging capacity of flavonoids (RICE-EVANS 1996). In European diet, lettuce is an important source of flavonoids (CROZIER et al. 1997; FERRERES et al. 1997; LLORACH et al. 2008). Particularly the red varieties, such as red oak leaf lettuce, are rich in antioxidants, mainly quercetin and cyanidin derivatives (CALDWELL 2003; LIU et al. 2007).

The present study was therefore based on the hypothesis that UV-B exposure during greenhouse cultivation enhances flavonoid content and reduces either leaf growth or leaf number of lettuce seedlings in a dose-dependent manner. Particular interest was also focused on effects of UV-B acclimation on plant performance after transplantation to the field.

The experiment was therefore divided into two consecutive phases: A greenhouse and a field period. Lettuce plants were first raised from seed in greenhouses covered with three materials differing in UV-B transmittance. At the age of three weeks half of the lettuce seedlings were transplanted to the field while half of them remained in the greenhouses. During the greenhouse period leaf length and area, leaf number, plant fresh weight, and flavonoid content of the seedlings were continuously determined, while during the field period plant fresh weight and flavonoid content were assessed. The experiment was performed three times in April, May and June 2007, which allowed for an estimation of the seasonal impact, as well.

Materials and Methods

Plant material and cultivation conditions

Experiments were performed in newly developed greenhouses providing ideal features for examination of plant responses to solar UV-B radiation (design: Gerhard Reisinger, University of Bonn, construction: Siedenburger Gewächshausbau, Rahden, Germany). The experimental greenhouses, installed at Marhof Experimental Station in Wesseling (Germany), were characterized by a light-weight construction in order to minimize shading and a small ground area of 4.2 x 3 m. Six greenhouses were covered with three different materials (two greenhouses each) substantially varying in UV-B transmission: ETFE film ("high UV-B" treatment, ethylene-tetrafluoroethylene, 100 µm, Asahi Glass Green-Tech, USA, China, South Korea, Japan) and MM glass ("intermediate UV-B" treatment, microstructured low iron glass, CENTROSOL MM; Centrosolar Glas, Fürth, Germany) exhibit a UV-B transparency of about 86 and 30%, respectively, whereas the conventional Float glass ("low UV-B" treatment, Siedenburger Gewächshausbau, Radhen, Germany)

almost excludes radiation in the UV-B range (Table 1) (see also KUHLMANN and MÜLLER 2009a).

Table 1: Proportion of UV-B radiation transmitted by Float glass (low), MM glass (intermediate) and ETFE foil (high UV-B).

Treatment	Covering material	Transmission (%)	
		UV-B	PAR
low UV-B	Float glass	0.7	89.1
intermediate UV-B	MM glass	30.1	91.3
high UV-B	ETFE foil	86.2	93.1

Transmission spectra of Float glass, MM glass and ETFE film, determined in the range between 280 and 750 nm by means of a UV/Vis spectrometer (LAMBDA, Perkin Elmer, Massachusetts, USA) are given in Figure 1.

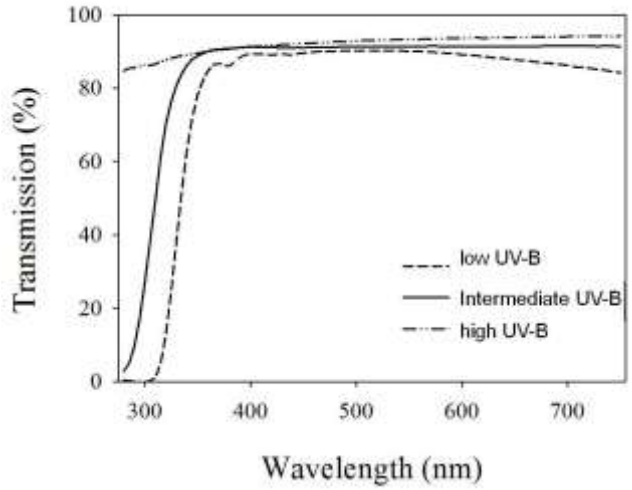

Figure 1: Transmission spectra of Float glass (low), MM glass (intermediate) and ETFE foil (high UV-B transmittance) from 280 to 750 nm, measured with a UV/Vis spectrophotometer

Red oak leaf lettuce (*L. sativa*, L. cv. Bughatti) (Hild Samen GmbH, 71672 Marbach, Germany) was grown from seed in trays with 100 small press pots placed on tables inside the greenhouses. Manual irrigation with well water was done every morning. On day twenty-four, twenty-one and twenty after sowing (in the April, May and June experiment, respectively) 200 seedlings from each greenhouse were transplanted to four field plots (50 plants to each plot), while 200 seedlings were kept in the greenhouses.

Solar radiation was monitored inside the greenhouses and in the field with an $X1_2$ Optometer (Gigahertz Optik, Puchheim, Germany). Triple radiation sensors (detecting UV-B, UV-A and PAR separately) were positioned on plant height in the centre of the greenhouses. Temperature and humidity were measured using dataloggers (ELV Elektronik AG, Leer, Germany); values were comparable under the different covering materials.

Growth monitoring

Determination of leaf area and length as well as the rate of leaf formation was restricted to the greenhouse period while assessment of plant fresh weight was continued during field cultivation.

For growth analysis three to four trays per greenhouse were screened photographically every second day and total leaf area per plant was calculated. Images of entire germination trays were acquired using a digital camera (Panasonic DMC-FZ7), installed in a fixed position on a tripod. In order to avoid any influence of the circadian rhythm pictures were always taken at the same daytime. Picture analysis was based on the method of "ColorSegmentation"- an analysis tool developed at Forschungszentrum Jülich, ICG-3. The amount of green pixels characterizing the leaf area was transformed into units of leaf area by the use of an internal standard where 2285 pixel correspond to a leaf area of one cm². Color segmentation between green leaf area and brown/black background was performed on the basis of hue, saturation and value (HSV) -formatted images. They were transformed from RGB (red, green, blue) images provided by the camera. For more details, see WALTER et al. 2007. Due to leaf curling and overlapping, photographic examination of leaf area was restricted to early stages of plant development (stage 13 according to BBCH code, FELLER et. al. 1995).

Leaf lengths of 20-day old plants were measured starting with the oldest (referred to as leaf number 1) and proceeding to the youngest leaf (number 6 or 7). 80 replicates were taken of each treatment group. The number of leaves per plant was counted between day 14 and 28 after sowing.

Determination of plant fresh weight was started during the greenhouse period and continued throughout the field period until harvest. Fresh weight of above-ground biomass was assessed by cutting the entire plant just above the roots. Six replicates per treatment were taken during the greenhouse period and eight replicates were taken during field cultivation. The calculation of relative growth rate is based on following equation:

$$\text{RGR} = \frac{(LN(FW_{t2}) - (LN - FW_{t1})}{(t_2 - t_1)}. \quad (1)$$

(FW= fresh weight, t= timepoint)

Determination of flavonoid content

Samples for flavonoid analysis were collected three times during greenhouse cultivation and twice after transplantation between day 15 and 30 after sowing. Mixed samples of three to twelve whole plants ($n_{day\ 14} = 12$, $n_{day\ 17} = 6$, $n_{day\ 20} = 6$, $n_{day\ 26} = 3$, $n_{day\ 33} = 3$) were frozen in liquid nitrogen and stored at -25°C. Frozen plant tissue was lyophilized and subsequently ground in a swing mill (MM 2000, Retsch, Haan, Germany) to fine powder. The powder (0.25 g) was extracted with 3 and 2 ml 62.5% aqueous methanol (AppliChem, Darmstadt, Germany) and centrifuged at 4000 rpm for 10 min. The combined supernatants were washed with 2 x 4 ml petrolether (AppliChem, Darmstadt, Germany). Acidic hydrolysis of flavonoid glycosides was performed by addition of 3 ml of 18.5% HCl (2 M in total) and incubation at 70°C for 2 h (see Hertog, 1992). Extracts were brought up to a volume of 10 ml with methanol. Before HPLC analysis samples were filtered though syringe filters (polypropylene membrane, 0.2 µm, VWR International GmbH, Darmstadt, Germany) and stored at -25°C.

Samples were analyzed using an Agilent (Santa Clara, California, USA) 1100 series automated liquid chromatography equipped with a MWD. A C_{18} column (LiChrosorb RP-18, 125x3 mm, 5 µm, Chromatographie Service GmbH, Langerwehe, Germany) served for reversed phase separation. The mobile phase performed a 42 min. gradient, (15-100%) of 0.1% formic acid (solvent A) and acetonitile (solvent B, both purchased at AppliChem, Darmstadt, Germany) at a flow rate of 0.8 ml min^{-1}. Compounds were identified by comparison of retention times and absorption maxima with standard substances. Utilized standard substances were cyanidin chloride and quercetin (purchased at Carl Roth GmbH & Co. KG, Karlsruhe, Germany and Fluka AG, Buchs, Switzerland, respectively).

Statistical Analysis

Significant differences in the growth parameters between plants of the three treatment groups were tested and compared by means of a one-way ANOVA (Tukey Test) using SPSS 11.5 (SPSS Inc., Chicago, USA). Analysis of flavonoid data was performed by the use of SPSS 17.0 (SPSS Inc., Chicago, USA). An overview of the statistical dataset is given in the supplemental table 1.

Results

Radiation measurements

Radition levels in the UV-B range under the three different covering materials were continuously recorded. During each of the three experiments the degree of UV-B transmission showed highest values under ETFE film (9-12 kJm^{-2}), intermediate under MM glass (2.8-3.5 kJm^{-2}) and lowest under Float glass (0.7-0.8 kJm^{-2}, Fig. 2). Minor differences were detected in the transmission of ultraviolet-A (UV-A,) and photosynthetically active radiation (PAR, data not shown). The proportion of UV-B radiation transmitted by the three covering materials was comparable in all experiments although variability was higher in May and June as compared to April (Fig. 2).

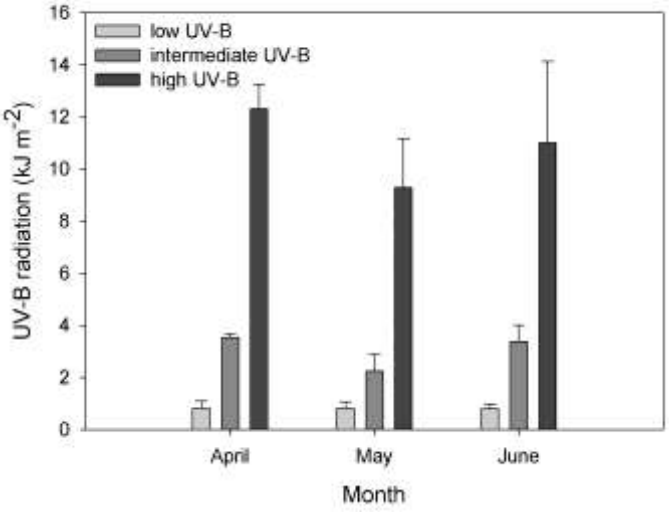

Figure 2: Mean UV-B radiation level ($kJ*m^{-2}$) transmitted by Float glass (low), MM glass (intermediate) and ETFE foil (high UV-B transmittance) in April, May and June. Radiation was recorded by means of a triple sensor (UV-B, UV-A and PAR) for five days at a frequency of one minute**.**

Growth analysis

Total leaf area per plant was gradually reduced with increasing UV-B exposure at all four time points (day 8, 12, 14 and 16) in April. The reduction in leaf area accounted for 6 and 14 % when grown at 30 and 86 % UV-B transmission, respectively, compared to the control group kept in a UV-B free environment (day 16, Fig. 3 a-c). This difference in total leaf area was found in April but not in May and June.

Leaf lengths were significantly lowered by UV-B exposure in all three experiments. In almost every leaf position, a clear reduction in leaf length was found at high UV-B treatment compared to UV-B exclusion. Plants exposed to the intermediate UV-B level ranged between the extreme treatments (Fig. 3d-f). In general, leaf area and length increased at a higher rate in May and June than in April. In May and June the projected

leaf area per plant on day 14 accounted for 7.5 cm², whereas it was only about 4.8 cm² in April (Fig. 3a-c).

Leaf number of lettuce seedlings was significantly lower at high compared to intermediate and low UV-B conditions on day 27/28 in all three experiments (Fig. 3 g-j). The previous measurements (day 14/15 and day 18/21) showed the same results except for two measurements, which didn´t show any differences.

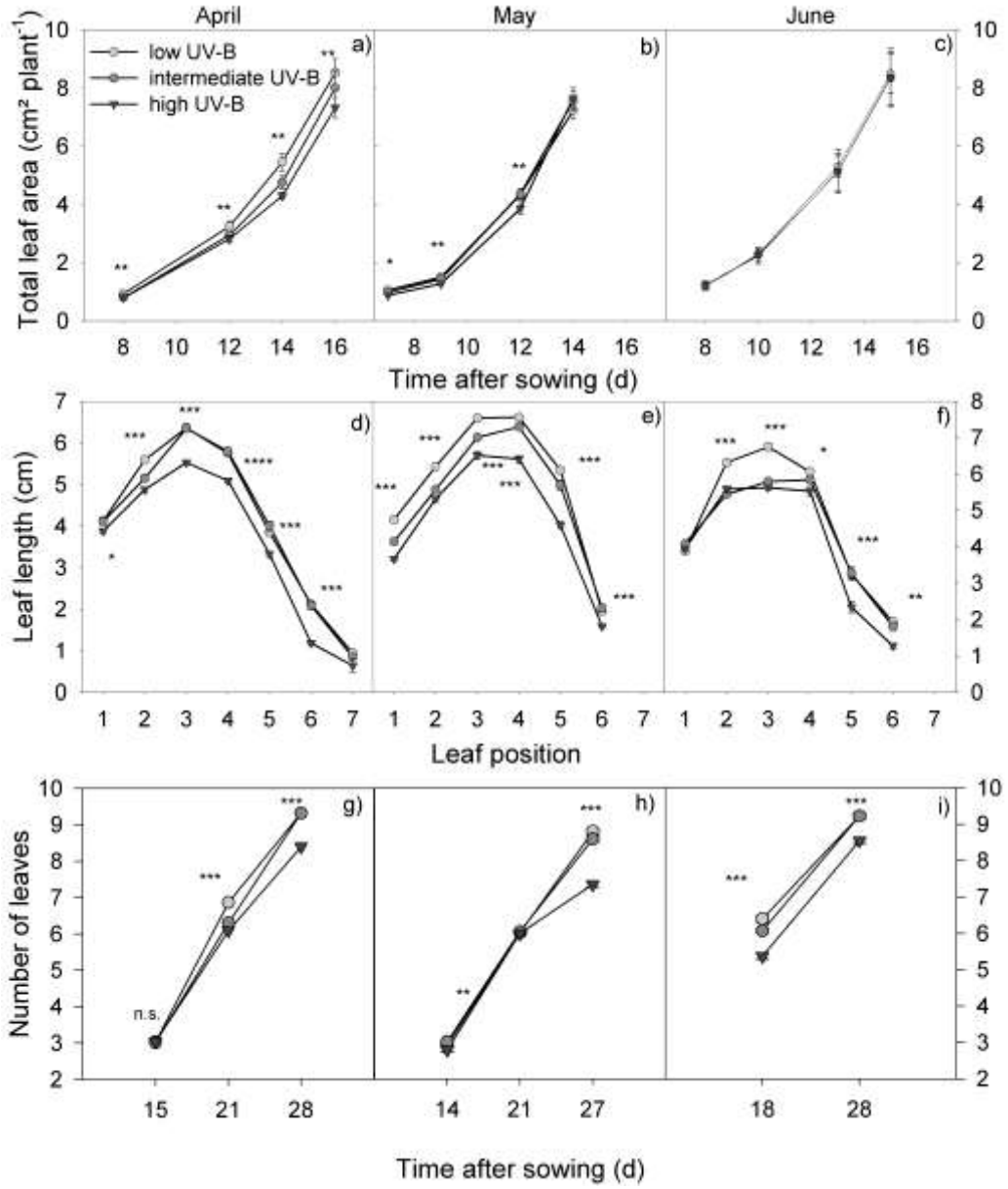

Figure 3: Morphological parameters. a-c) Total leaf area per plant (cm^2) between day 7 and 16 of seedling development in a) April, b) May and c) June, mean ±SD, n=104; d-f) Leaf lengths (cm) of leaf positions 1 (oldest) to 6/7 (youngest) of 20-day old lettuce plants in d) April, e) May and f) June, mean±SE, n=40; g-i) Total leaf number between day 14 and 28 after sowing at high, intermediate and low UV-B conditions in g) April, h) May and i) June 2007, mean±SE, n=40. Statistical analysis were performed by ANOVA, followed by a tukey post-hoc test, stars denote significant differences between low and high UV-B treatment, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$.

Table 2: Relative growth rate (% d-1), based on fresh weight data, between day 20 and 25 and between day 20 and 29 under greenhouse and field conditions.

	Day 25		Day 29	
	greenhouse	field	greenhouse	field
low UV-B	29	18	20	15
intermediate UV-B	27	21	19	15
high UV-B	25	22	19	18

Plant fresh weight did not differ significantly between UV-B treatments (Fig. 4) although the relative growth rate calculated from fresh weight data between day 20, the day of transplantation, and 25 indicates a slight growth reduction under + UV-B conditions (Tab. 2). After transplantation to field conditions, the plant fresh weight of all treatment groups was strongly reduced compared to plants kept under controlled conditions. Plant fresh weight of field grown plants was 25% lower compared to control plants in the greenhouse after four days of outdoor exposure (day 25, Tab. 2). At the stage of harvest (age: 61 days) plant fresh weight did not vary between UV-B treatment groups (see supplementary information).

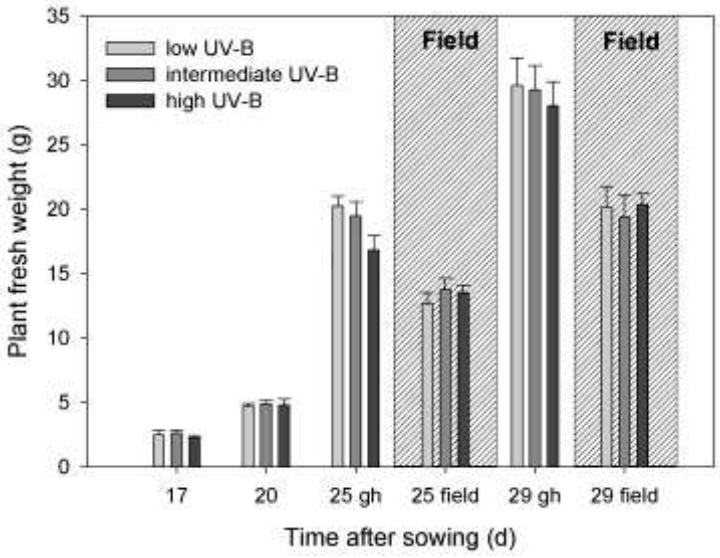

Figure 4: Plant fresh weight (g) of different UV-B treatment groups on day 17, 20, 25 and 29 in the greenhouse and on day 25 and 29 in the field (indicated by grey boxes) during June experiment. Mean±SE, n=6-8. Data were statistically analyzed by ANOVA and tukey test. No significant differences.

Flavonoid content

The main flavonoid aglycones detected in extracts of red oak leaf lettuce were quercetin and cyanidin. During greenhouse cultivation, cyanidin and quercetin content were gradually elevated with increasing UV-B level in the order low > intermediate > high UV-B transmission (Fig. 5, data obtained in June). On day 20, plants exposed to intermediate and high UV-B showed an increase in quercetin content by 19 and 45% and in cyanidin content by 23 and 78%, respectively, compared to plants kept at low UV-B. Six days after transfer to the field, quercetin content was increased by 97% and cyanidin content by 104% (average of the three treatment groups), respectively. This strong enhancement was coupled to an equalization of differences between treatment groups. Between day 26 and 33 quercetin and cyanidin content declined by 26 and 3%, respectively. These observations were comparable in all experiments, independent from season.

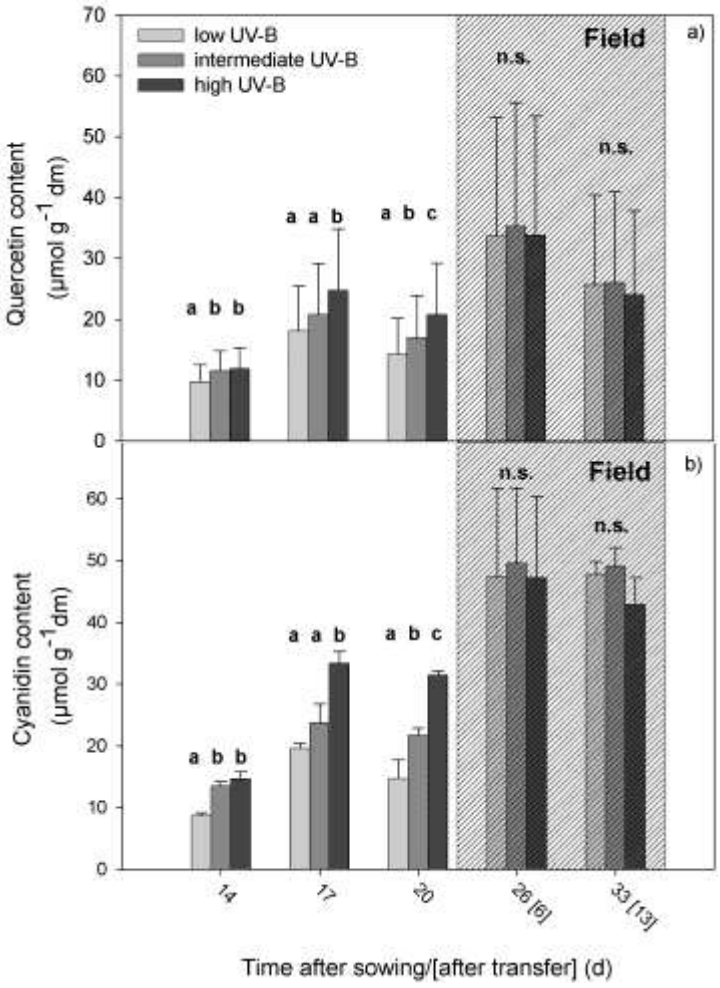

Figure 5: a) Cyanidin and b) Quercetin content (µmol/g DM) of lettuce seedlings from different UV-B treatment groups between day 14 and 33 after sowing at greenhouse and field conditions (indicated by grey boxes). Mean ± SD, $n_{day\ 14} = 12$, $n_{day\ 17/20} = 6$, $n_{day26/33} = 3$.

Discussion

Greenhouse period

During greenhouse cultivation flavonoid content as well as leaf growth and leaf number of lettuce seedlings were clearly affected by the specific proportions of solar UV-B radiation transmitted by Float glass (low), MM glass (intermediate) and ETFE film (high UV-B treatment). The induction of flavonoid accumulation observed in the intermediate and high UV-B treatment group (Fig. 5a, b) is a common response to UV-B radiation, found in numerous plant species including lettuce (CALDWELL 1981; LOIS 1994; KRIZEK et al. 1998; GARCIA-MACIAS et al. 2007). Our results are consistent with the assumption that flavonoid induction is a dose-dependent response (TSORMPATSIDIS et al. 2008) as flavonoid contents seemed to be gradually elevated with increasing UV-B level (Fig. 5). The most abundant flavonoid aglycones found in extracts of red oak leaf lettuce are quercetin and cyanidin, which in vivo are mainly represented by quercetin- 3-O-(6´´-O-malonyl)-glucoside and cyanidin-3-O-(6´´-O-malonyl)-glucoside (GARCIA-MACIAS et al. 2007; LLORACH et al. 2008). Flavonols such as quercetin provide UV-B protection as epidermally deposited UV shields (CALDWELL 1983; LOIS 1994), whereas anthocyanins such as cyanidin are supposed to contribute relatively little to total UV absorbance (WOODALL and STEWART 1998). Both, quercetin and cyaniding, possess strong antioxidant activity in vitro (RICE-EVANS 1996). Yet, their contribution to the mitigation of UV-B induced oxidative stress in vivo is difficult to estimate since they are for the most part localized in epidermal vacuoles and thereby isolated from ROS generation in the chloroplasts of the palisade mesophyll (GOULD and LISTER 2006).

The reduction in total leaf area per plant found at intermediate and high UV-B conditions is obviously due to two processes: A decrease in leaf number and a decline in leaf expansion as indicated by lower leaf lengths (Fig. 3a-i). Both is consistent with previous studies reporting UV-B treated lettuce plants to show a reduction in leaf number and leaf area which is often coupled with an increased leaf thickness (KRIZEK et al. 1998; ROUSSEAUX et al. 2004). UV-B induced changes in leaf morphology are supposed to diminish UV-B exposure of sensitive tissues (JANSEN 1998). The decline in leaf length was observed in most leaf positions indicating that this response is independent of the leaf developmental stage in 20-day-old plants. Plant fresh weight was expected to be reduced by UV-B exposure as described by several other authors (KRIZEK et al. 1998; GARCIA-MACIAS et al. 2007; TSORMPATSIDIS et al. 2008), but the differences we found were not significant (Fig. 4).

Planting month was also found to affect growth and growth responses to UV-B. The finding that leaf length was reduced by UV-B exposure in all three months whereas leaf area was only affected in April (Fig. 3 a-c) might be due to changes in leaf morphology (e.g. stronger curling) and methodological limitations. For the analysis of the projected leaf area a nearly planar leaf surface is necessary to avoid underestimation of the real leaf area. With increasing curling of leaves, the accuracy of the method decreases. In general, relative growth rate (based on fresh weight) was higher in May and June than in April, indicating a higher photosynthetic productivity in early summer (Fig. 3, Tab. 2). This effect may be due to elevated temperatures (MEDLYN et al. 2002; WALTER et al. 2009). This is in contrast with results obtained by TSORMPATSIDIS et al. (2008) who found no interaction between vegetative growth and planting month in experiments conducted in a more northern region.

At the time of transplantation lettuce seedlings grown at intermediate and high UV-B showed an increase in quercetin content by 18.7 and 45.3%, an elevation in cyanidin content by 23.2 and 78.4% (day 20, June experiment), respectively, and a decrease in total leaf area per plant by 6 and 14 % (day 16, April experiment), respectively, compared to plants grown in the absence of UV-B radiation (Fig. 5 and 3 f). The increase in epidermally deposited flavonols, such as quercetin, and the decline in leaf area reduce the penetration of harmful UV-B radiation into metabolically active tissues (BURCHARD et al. 2000). Elevated flavonoid contents contribute to the elimination of stress-induced ROS (RICE-EVANS et al. 1996). Therefore, these compositional and structural changes might possibly enhance the plant´s stress tolerance under outdoor conditions.

Field period

Transplantation to field conditions induced rapid and strong compositional and morphological responses in propagation plants. The increase in flavonoid content by 97% (quercetin) and 104% (cyanidin), respectively, and the reduction in fresh weight accumulation by 25% compared to greenhouse cultivation within four to five days indicate that sudden exposure to outdoor conditions is quite challenging for plants raised in protected cultivation (Fig. 4 and 5). Reduced biomass and elevated flavonoid content are well defined responses of field grown compared to greenhouse grown lettuce plants as previously described by ROMANI et al. (2002). These non-specific stress responses are supposed to be induced by a broad array of biotic and abiotic stress factors including pathogen attack, increased UV-B level, altered temperature, humidity, availability of water

and nutrients, wind and mechanical injury (RABINO and MANCINELLI 1986; ROZEMA et al. 1997; MITTLER 2006; TREUTTER 2006).

The coincidence of flavonoid induction and growth attenuation in lettuce has been reported in former studies (KRIZEK et al. 1998; GARCIA-MACIAS et al. 2007; TSORMPATSIDIS et al. 2008) and is generally explained by the `growth-differentiation balance hypothesis´ postulating a trade-off from growth to defense due to the induction of protecting metabolites (HERMS and MATTSON 1992). This resource allocation mechanism is particularly found in young plants with limited stock reserves (KUHLMANN and MULLER 2009b).

UV-B acclimation during the greenhouse period had no long-term effect on growth and flavonoid content under field conditions (Fig. 4 and 5). In several studies addressing pre-adaptation, UV-B treatment during seedling development had proven beneficial, e.g. in terms of an enhanced stress tolerance (DEL CORSO and LERCARI 1997; TEKLEMARIAM and TERENCE 2003; HOFFMANN 1999).

A more positive effect of UV-B pre-adaptation on plant performance under field conditions is conceivable under generally more stressful conditions. If lettuce plants would have been exposed to more adverse environmental conditions after transfer to the field, which often occurs in practice, increased flavonoid content and reduced biomass might have been beneficial for plant performance. Assessment of specifically stress-related parameters, as well as a higher temporal resolution of the data obtained immediately after transplantation might have shown clearer differences between UV-B treatment groups.

The present results confirm the hypothesis that UV-B exposure during greenhouse cultivation leads to a reduction in leaf growth and leaf number as well as to an increase in flavonoid content of lettuce seedlings in a mostly dose-dependent manner. Previous UV-B treatment had no long-term effect on the plant´s response to field conditions after transplantation. While former studies on performance of lettuce at different UV-B levels are restricted to greenhouse cultivation, our work includes subsequent transplantation to the field and therefore represents a first approach to estimate the effects of UV-B pre-acclimation on performance of lettuce seedlings under outdoor conditions. In future studies, the benefit of pre-acclimation to near-ambient solar UV-B radiation will be investigated in more detail along the lines of the experimental procedures described here, taking a closer look at stress tolerance and product quality.

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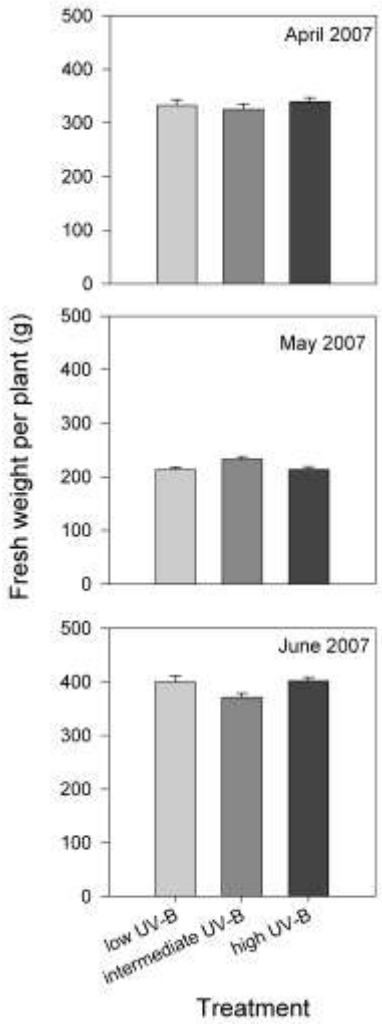
Addresses of authors: Helen Behn, Georg Noga and Andreas Ulbrich Institute of Crop Sciences and Resource Conservation – Horticultural Sciences, University of Bonn, Auf dem Hügel 6, D-53121 Bonn; Helen Behn, Susanne Tittmann (corresponding author), Achim Walter, Ulrich Schurr and Andreas Ulbrich Institute for Chemistry and Dynamic of the Geosphere (ICG-,3 Phytosphere), Forschungszentrum Jülich GmbH, D-52425 Jülich; Andreas Ulbrich Faculty of Agricultural Science and Landscape Architecture, University of Applied Science Osnabrück, Oldenburger Landstraße 24, D-49090 Osnabrück, and Susanne Tittmann Research Center Geisenheim, Institute of Viticulture, Von-Lade-Str. 1, D-65366 Geisenheim,

e-mail: susanne.tittmann@fa-gm.de

Supplemental Table 1: Significance level of leaf area, leaf length and leaf number of all three experiments.

Leaf area	**April** d.a.s 8/12/14/16		**May** d.a.s 7/9/12/14		**June** 7/9/12/14	
	Intermediate	**High UV-B**	**Intermediate UV-B**	**High UV-B**	**Intermediate UV-B**	**high UV-B**
Low UV-B	0.09/**0.047**/**0.04**/0.33	**0.05**/0.08/**0.00**/**0.017**	**0.034**/0.23/0.978/0.38	**0.00**/**0.00**/**0.012** /0.227	0.985/0.961/0.911/ 0.96	0.548/0.888/ 0.927/0.974
Intermediate UV-B		0.903/0.490/**0.039**/ 0.171		**0.006**/**0.003**/**0.0 09**/0.923		0.645/0.979/ 0.999 0.999
Leaf length	**Leaf 1-7**		**Leaf 1-6**		**Leaf 1-6**	
Low UV-B	0.994/**0.00**/0.976/ 0.849/0.485/0.96/ 0.289	**0.028**/**0.00**/**0.00**/**0.00 0.00**/**0.00**/0.68	**0.0**/**0.0**/**0.0**/**0.051**/**0.02**/ 0.685	**0.00**/**0.00**/**0.00**/ **0.00**/**0.00**/**0.001**	0.112/**0.00**/**0.00**/ 0.252/0.997/0.636	0.809/**0.00**/ **0.00**/**0.00**/**00**/ **0.001**
Intermediate UV-B		**0.037**/**0.019**/**0.00**/**0.0 0**/**0.00**/**0.00**/0.31		**0.00**/**0.01**/**0.00**/ **0.00**/**0.00**/**0.00**		0.346/0.411/ 0.308/0.052/ **0.00**/**0.01**
Leaf number	**d.a.s. 15/21/28**		**d.a.s. 14/21/27**		**d.a.s. 18/26**	
Low UV-B	0.735/**0.000**/1.0	0.926/**0.000**/**0.000**	0.286/0.756/0.135	0.144/0.756/**0.0**	**0.007**/1.0	**0.000**/**0.000**
Intermediate UV-B		0.501/**0.001**/**0.000**		**0.002**/0.331/**0.0**		**0.000**/**0.000**



Supplemental Figure 1: Fresh weight (g) of plants of different UV-B treatment groups at harvest in the a) April; b) May and c) June experiment. Mean±SE, n=70.

3.2 Second publication: Non-invasive determination of plant biomass with microwave resonator

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*both authors are contributed equally to this work

Own contribution

- Experiments
- Data Analysis
- Preparation of the manuscript (in cooperation with co-authors)

Non-invasive determination of plant biomass with microwave resonators

MARION I. MENZEL[1]*, SUSANNE TITTMANN[1]*, JONAS BÜHLER[1], STELLA PREIS[1], NORBERT WOLTERS[2], SIEGFRIED JAHNKE[1], ACHIM WALTER[1], ANTONIA CHLUBEK[1], ARIEL LEON[2], NORMEN HERMES[3], ANDREAS OFFENHÄUSER[2], FRANK GILMER[1], PETER BLÜMLER[1], ULRICH SCHURR[1] & HANS-JOACHIM KRAUSE[1]

[1]ICG-3 (Phytosphere), [2]IBN-2 (Bioelectronics) and [3]ICG-4 (Agrosphere), Forschungszentrum Jülich GmbH, D-52425 Jülich, Germany

ABSTRACT

Non-invasive and rapid determination of plant biomass would be beneficial for a number of research aims. Here, we present a novel device to non-invasively determine plant water content as a proxy for plant biomass. It is based on changes of dielectric properties inside a microwave cavity resonator induced by inserted plant material. The water content of inserted shoots leads to a discrete shift in the centre frequency of the resonator. Calibration measurements with pure water showed good spatial homogeneity in the detection volume of the microwave resonators and clear correlations between water content and centre frequency shift. For cut tomato and tobacco shoots, linear correlations between fresh weight and centre frequency shift were established. These correlations were used to continuously monitor diel growth patterns of intact plants and to determine biomass increase over several days. Interferences from soil and root water were excluded by shielding pots with copper. The presented proof of principle shows that microwave resonators are promising tools to quantitatively detect the water content of plants and to determine plant biomass. As the method is non-invasive, integrative and fast, it provides the opportunity for detailed, dynamic analyses of plant growth, water status and phenotype.

Key-words: biomass; cavity resonator; dielectric properties; microwave; non-invasive analysis; water content.

INTRODUCTION

The plant's capability to produce biomass determines crop yield and plant vigour. Procedures to determine the increase in plant biomass (growth) or the amount of standing crop biomass have thus been developed and applied throughout human history, but they are generally destructive. In parallel to an increasingly refined molecular understanding of plant growth processes (Nozue & Maloof 2006), there is a clear need for methods that allow plant growth monitoring with higher temporal resolution (Schmundt *et al.* 1998). Consequently, various approaches have been established, allowing non-invasive determination of parameters serving as a realistic proxy for overall plant growth such as LIght Detection And Range (LIDAR)-observations of tree height (Omasa *et al.* 2003), electronic registration of tree diameter (Zweifel, Item & Hasler 2001; Cermak *et al.* 2007), interferometric analysis of plant height (Jiang & Staude 1989) or organ surface expansion (Walter & Schurr 2005). Yet, non-invasive analysis of fresh weight (*FW*) or dry weight (*DW*) increase remains largely unexplored, in particular when data on the whole-plant scale are requested. Some attempts have been made to determine plant biomass non-invasively via weighing methods (Simonneau *et al.* 1993; Van Ieperen & Madery 1994) where, in the work of Van Ieperen & Madery (1994), growth of tomato plants was determined by placing two communicating vessels filled with a nutrient solution on balances. One of the vessels contained a plant whose fresh weight was determined by the difference of transpiration (summary loss of weight) and water uptake (weight difference evolving between the two balances).

Tools for non-invasive analysis of plant biomass and its automation would contribute not only to 'online' detection of single-plant fresh weight but also to the emerging field of plant phenotyping (Leister *et al.* 1999; Granier, Aguirrezabal & Chenu 2006; Walter, Scharr & Gilmer 2007). For that, it is a central aim to scan the standing biomass or the increase in biomass of a large number of individual plants at user-defined intervals to observe either reactions of plants towards altered environmental conditions (Granier *et al.* 2006; Walter *et al.* 2007) or to determine developmental differences between genetically different plant lines (Meyer *et al.* 2004). This clearly can not be achieved via differential weighing methods. Total projected leaf area extracted from automated imaging methods is a good proxy for biomass determination as long as plants are in a vegetative rosette stage, such as in *Arabidopsis thaliana* (Leister *et al.* 1999) or in young seedlings of *Nicotiana tabacum* (Walter *et al.* 2007). Yet, the correlation between visible leaf area and plant biomass is questionable for other plant architectures

Correspondence: F. Gilmer. Fax: +49 2461 61 2492; e-mail: f.gilmer@fz-juelich.de

*Both authors contributed equally to this work.

that do not allow simple, quantitative visualization of total leaf area. Hence, it was our aim to establish a non-invasive and easy-to-use method having the potential for automation, based on electromagnetic fields for determining the fresh weight of a plant body.

Nelson and co-workers discussed the 'variation of dielectric properties (of grain and soybeans) as functions of moisture content, frequency, temperature, and bulk density' (Nelson 1991); they especially focused on the measurement of dielectric properties of grain and oilseed using free-space measurements in the GHz range (Trabelsi & Nelson 2003). Although this approach has found widespread use in food processing (Hu, Toyoda & Ihara 2008), it has not yet been adapted to a useful tool in plant biology. Remote airborne active and passive microwave sensors (Ferrazzoli *et al.* 1992) were employed to relate biomass to microwave backscattering and emission; however, this approach revealed sensitivity to plant geometry in prediction of water content and hence is applicable mostly at large plot scales (Wigneron *et al.* 2003). Jones and co-workers reviewed several approaches which either derive the water content of biomaterials from comparison of unknown materials with test materials of known permittivity or correlate transmission differences (Jones *et al.* 2006). They realized a free space system operating in the range of 300–900 MHz, but needed sophisticated statistical methods to find strong correlations between signal attenuation because of water and reference measurements.

The approach taken here is to use a microwave cavity resonator to determine water content of a plant, a principle that has proven its applicability in inspection of building materials (Herrmann, Sikora & Zaage 1997; Leschnik 1999). With an appropriate resonator design, different modes can preferentially be excited, reacting with high sensitivity to changes of the dielectric properties of the resonator cavity when a plant is inserted (Krause *et al.* 2006). In order to evaluate possible impact of microwave field geometries on measurement accuracy, the spatial distribution of the electromagnetic fields inside the resonator was determined systematically using a test sample. Several series of measurements on pure water samples, harvested plant parts and intact plants were performed in order to evaluate the applicability of the method for water content measurement of plants as a proxy for biomass, fresh or dry weight. These parameters can be deduced from plant-specific calibrations with good reliability. As the presented method is non-invasive and fast, changes in growth patterns of intact plants can be monitored on a time scale ranging between minutes and days, allowing monitoring of diurnal growth patterns. Examples of such measurements are presented here and discussed in terms of future use and potential.

MATERIALS AND METHODS

The measurement system for non-invasive biomass determination with a microwave resonator consists of three main components: the microwave cavity resonator, the microwave electronic module (laboratory set-up, miniaturized version and software) and correlation libraries for different plant species. For a description of the theory of microwaves and microwave resonators, the reader is referred to the relevant literature (for instance Zinke & Brunswig 2000) as an extended review of the underlying method is beyond the scope of this paper.

Microwave resonator design

The dielectric permittivity is a measure of the extent to which a substance concentrates the electrostatic lines of flux. In the case of imposing a static electric field across a medium, the relative dielectric permittivity denotes the ratio of the amount of electric energy stored in the medium relative to vacuum. Placing a material with a high dielectric constant (i.e. water) in an electric field leads to a reduction of the field amplitude. Therefore, inserting biomass (which contains high amounts of water) into a cavity resonator yields a reduction of the resonance frequency of the resonator.

The general design and properties of the cavity resonator (wall thickness: 1.8 mm) is depicted in Fig. 1a with properties listed in Table 1. The openings of length L_2 are chosen smaller in diameter than the main body (D_1) of the resonator in order to stabilize the resonant modes. Plants and samples inserted via the openings should not exceed L_1 in height. To restrict samples to the most homogeneous part of the field, a cylinder of plastic foil (diameter: D_2 – not shown in Fig. 1) has been fixed inside the cavity resonator, which also prevent plant samples from touching the coupling coils. Inside the resonator, two coupling coils at opposite sides (to minimize direct spurious coupling) at central height couple the circulating electromagnetic field into and out of the resonator. Via SMA-connectors (SubMiniature version A, suited for high-frequencies), these coils are connected to the radio frequency (RF)-electronic module. The lowest mode, the transversal-magnetic TM_{010} mode (with lowest resonance frequency, f_{010}), is preferably excited. To first order approximation, the resonance frequency of the mode is calculated according to the theory of cavity resonators in a completely closed cylinder of diameter D_1 (see Fig. 1a), surrounded by a perfect electric conductor, according to

$$f_{010}^{r}(D_1) = \frac{c}{\sqrt{\varepsilon_r \mu_r}} \frac{x_{01}}{\pi D_1} \qquad (1)$$

Here, $x_{01} = 2.4048$ denotes the first zero-crossing of the Bessel function $J_0(x)$, $c = 2.9979 \times 10^8$ m/s is the velocity of light in vacuum, μ_r is the relative magnetic permeability and ε_r is our variable of interest referring to the dielectric permittivity of the medium inside the cavity resonator. Table 1 compares the theoretically calculated resonance frequencies f_{010} to the experimental result for the given prototype.

Sample container and pot shielding

For sample positioning during calibration, a cylindrical Perspex-container (height, 60 cm; outer diameter, 15 cm; wall thickness, 5 mm) was built (Fig. 1b); its position

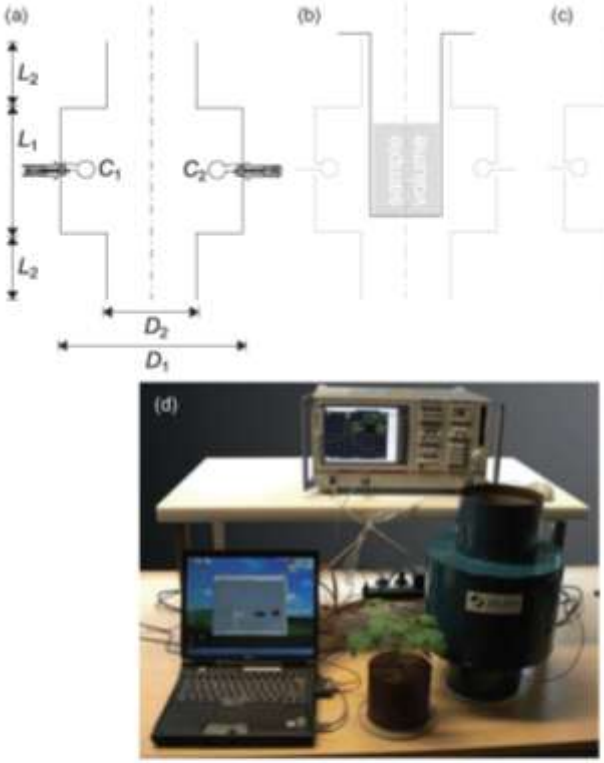

Figure 1. Design of the microwave resonator set-up. (a) Schematic of a resonator with dimensions D_1, D_2, L_1 and L_2 listed in Table 1. (b) Schematic of the Perspex-container for water calibration and cut shoots measurement. (c) Schematic of the copper shield positioning, containing pot with intact plant. (d) Photo of the measurement setup; top, network analyser; bottom, measurement computer, tomato plant with copper shield and microwave cavity resonator.

relative to the resonator is adjustable in height. For shoot biomass determination of intact plants, the plant pot, roots and possibly humid soil were shielded by a cylindrical copper shield (Fig. 1c) constructed with removable cap and feed-through for the plant stem (shielding diameter, 13.5 cm; height, 12.5 cm; material thickness, 2.5 mm). Figure 1d depicts the measurement set-up with resonator (P2, see also Table 1) and plant within the pot/soil shield. Perspex distance rings were used to ensure reproducible positioning of the plant and shielding within the resonator.

System characterization, data acquisition and analysis

The transmission characteristics of the empty cavity resonator were analysed with a network analyser (ZVB4 Vector Network Analyzer, Rohde & Schwarz, Cologne, Germany). Initially, within the range of 300 kHz up to 4 GHz, complex impedance versus frequency was scanned to identify the first stable resonance-peak (the TM_{010} mode). Based on this stable resonance, measurements of the empty or loaded resonator were conducted within a smaller range of ±25 MHz in steps of 2.5 kHz. The following variables were acquired from the measured complex spectra (see Fig. 2a): the centre or resonance frequency f_0, the bandwidth Δf (at 3 dB loss), the signal quality $Q = f_0/\Delta f$ and the resonance frequency shift, delta centre frequency (ΔCF).

$$\Delta CF = f_0(\text{empty}) - f_0(\text{sample}) = CF_1 - CF_2 \quad (2)$$

Using a LabView-based (National Instruments Corporation, Austin, TX, USA) programme, the measurement was

Prototype version	D_1 (mm)	D_2 (mm)	L_1 (mm)	L_2 (mm)	f_{010}(theo) (MHz)	f_{010}(exp) (MHz)
P2	300	180	210	126	765.0	812.8
P3	250	150	175	100	917.9	976.9
P4	180	130	90	65	1274.9	1428.6

Table 1. Resonator dimensions and resonance frequencies of three resonator prototypes (P2–P4)

The dimensions are illustrated in Fig. 1a. f_{010}(theo) refers to the calculated resonance frequency of the primary mode according to Eqn. 1 while f_{010}(exp) shows the experimental result for the given prototype.

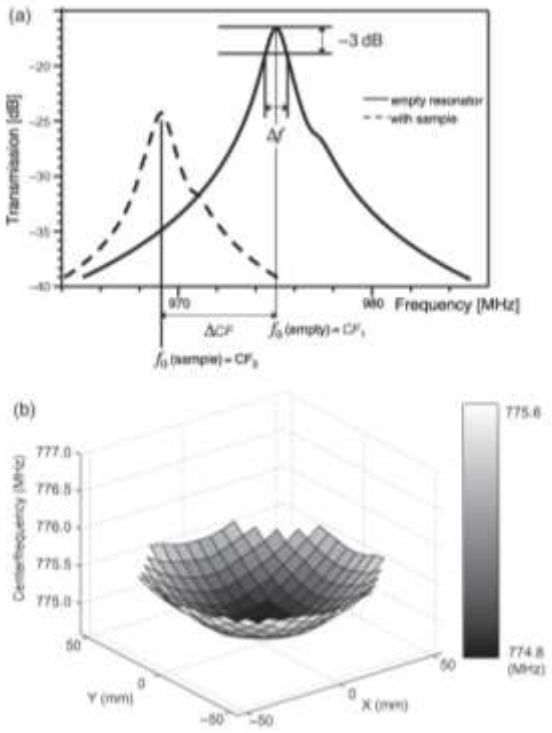

Figure 2. Schematics of measurement principle.
(a) Transmission curves of microwave resonator P2: empty (solid) and with sample (dashed). Sample causes a relative frequency shift $\Delta CF = CF_1 - CF_2$. (b) Spatial variation of first mode centre frequency f_0 of resonator P2 in xy-plane at position $z = 2.44$ mm, relative to centre of resonator ($z = 0$).

automated and the acquired complex spectra were transferred from the network analyser to the controlling PC and stored. The main part of the measurement duration was required for sample positioning, the complex spectra acquisition occurred within seconds, so that a complete measurement including positioning could be completed within approximately 20 s. By using a Matlab-based (The Mathworks, Natick, MA, USA) programme, the spectral data were analysed. From the complex spectrum, the logarithmic amplitude A_{dB} was calculated according to Eqn. 3:

$$A_{dB} = 20 \cdot \log_{10}\left(\sqrt{\text{real part}^2 + \text{imaginary part}^2}\right) \quad (3)$$

Depending on the amplitude's signal to noise ratio, low pass filtering in Fourier space was applied. The maximum of the resonance spectrum in the range of the selected mode was determined via the first derivative and its change of sign, with Q as quality factor. In a series of measurements, the resonance frequency and amplitude, the bandwidth and the quality factor were automatically stored in spreadsheet files.

This set-up with resonator P2 indicated that the first stable resonance is located in the range between 750 MHz and 850 MHz depending on the amount of water or biomass within the resonator. Based on this information, the multifunctional network analyser could be replaced by a less expensive and miniaturized electronic module. The frequency range of further resonator prototypes (P3 and P4) had to be detected accordingly.

Miniaturized electronic module and data analysis system

For routine measurements, a miniaturized electronic module was developed containing the basic functionality of the network analyser but reduced to the minimum requirements of our application. It constitutes a miniaturization in size as well as in cost. The electronic module comprises a microwave source, a splitter, an amplifier-attenuator chain and a gain and phase demodulator. As a high-frequency source, a voltage-controlled oscillator (VCO) selected according to the required frequency range is used. For frequency adjustment and stabilization, the VCO is phase-locked by a quartz-controlled fractional-N frequency synthesizer chip, SKY72302 from Skyworks Solutions Inc. (Woburn, MA, USA). The absolute frequency precision of 2 mg g^{-1} is limited by the stability of the quartz reference. The relative frequency precision of 70 Hz (i.e. better than 0.1 mg g^{-1}) is determined by the divider values programmed to the fractional-N divider of the synthesizer. The RF output is split; one branch is fed to the resonator via an adjustable attenuator and a 20 dB amplifier, the other branch is supplied to the demodulator chip after passing a second adjustable attenuator. The power level of the reference signal is adjusted to −30 dBm (1 μW). The demodulator part of the electronic module is based on the gain and phase detector chip AD8302 from Analog Devices (Norwood, MA, USA). The chip yields the amplitude and phase of the RF signal transmitted through the resonator, relative to the supplied reference signal. The amplitude range is 60 dB with a logarithmic gain scale, and the phase is measured in all four quadrants. The high-frequency section of the electronic module is mounted in an RF-tight aluminium casing.

The RF-electronics module is connected to a touch panel computer based on the microcontroller Tiny Tiger from Wilke Technology (Aachen, Germany). The implemented programme contains subroutines for setting the frequency of the synthesizer, for adjusting the RF levels for excitation and reference, and for amplitude and phase signal readout from the demodulator chip via 16-bit Analog/Digital converters. The user interface includes a routine for measuring a complete spectrum and for convenient peak tracking.

Because the resonance peak of the cavity resonator is well described by a Lorentzian peak f_L (Eqn. 4), the tracking programme matches the measured data to such a Lorentzian peak which is continuously updated in order to track the peak position (f_0 centre frequency), the peak width ($\Delta f_{\frac{1}{2}}$ half width half maximum), which also yields the quality factor, and the peak height (A the transmission loss in the peak).

$$f_L(f, f_0, A, \Delta f_{1/2}) = \frac{A \cdot \Delta f_{1/2}^2}{4 \cdot (f - f_0)^2 + \Delta f_{1/2}^2} \quad (4)$$

The tracking routine is implemented as a start routine which performs a scan over the total frequency range, a search for the maximum, and from there a search of the left and the right –3 dB points (shoulders). This routine is continuously repeated, involving the precise measurement of the three characteristic points (maximum and both shoulders), averaging, an analytic determination of the Lorentzian which runs exactly through the measured points, a calculation of the new maximum and the new –3 dB points from the Lorentz curve, and a calculation of the running average mean value and standard deviation. If the peak tracking routine loses the peak, it automatically starts again with the measurement of the whole spectrum.

This module functions like a network analyser but is reduced in its frequency range and provides only the essential measuring functions at significantly reduced costs. It is designed and built by JSQ GmbH (Jülich, Germany) for the prospective use in biomass determination experiments.

Growth conditions – plant material

Tomato (*Lycopersicon esculentum* L.) and Tobacco (*Nicotiana tabacum* L.) plants were used as model plants in this study. Plants were germinated in standard soil for cultivation (type ED73 Einheitserde, Balster Einheitserdewerk, Fröndenberg, Germany) and grown under 100–180 μmol m^{-2} s^{-1} photosynthetic active radiation in a growing room in-house with controlled relative humidity (RH; %) and temperature (T; °C). Condensation, leading to measurement artefacts, was not observed inside the resonator under the selected conditions. If plants were harvested after or prior to a measurement, the fresh weight (*FW*; g) was determined with an analytical balance (Mettler Toledo, Giessen, Germany). The plant material was then dried at 75 °C for 72 h in an oven (Heraeus Kulzer, Hanau, Germany) and the dry weight (*DW*; g) was determined. The relative water content (*RWC*; %) was determined according to

$$RWC = (FW - DW)/FW \times 100\% \quad (5)$$

The regressions for all statistical analysis were performed using SigmaPlot 10.0 (Systat Software Inc., San Jose, CA, USA).

RESULTS

Spatial electromagnetic field distribution

Because the electric field distribution varies within the resonator because of position and observed mode, the sensitivity of the experimental set-up had to be tested with respect to these parameters. To characterize the spatial field distribution of the first four modes within the resonator, a reference sample was used to scan the whole measurement volume for each mode. These experiments were carried out using a hollow Perspex cube (outer edge length: 2.5 cm) filled with 7.3 g of distilled water as a reference. The cube was attached to a computer controlled three-dimensional (3D) moving stage (Owis GmbH, Staufen, Germany) and supported in the volume of interest with a Perspex rod (diameter 8 mm). All measurements were carried out at room temperature (approximately 22 °C). Two sets of spatial distribution measurements, one with and one without the Perspex sample container, were completed by automatically positioning the reference sample on a predefined grid within the resonator via the 3D moving stage. The electromagnetic field distribution, that is, the resonance frequency sensitivity towards water, was scanned in horizontal xy-planes for a radius of 55 mm with Perspex sample container and 75 mm without sample container at different positions (step width: 6 mm with sample container, 25 mm without sample container) on the *z*-axis covering a height of 163 mm with sample container and 200 mm without sample container. The full resonance spectrum was recorded at each grid position and analysed, resulting in 3D data sets representing the spatial distribution of resonance frequency, width and amplitude for the first four modes of the resonator. Figure 2b shows an example of a plot of the data for the spatial distribution of the first mode in an xy-plane in the middle plane of the cylindrical detection volume. Over the full volume, for each vertical slice, the maximal deviations of resonance frequencies from the reference frequency defined at the centre of the resonator are summarized in Table 2. These deviations define the inherent frequency sensitivity and accuracy of the measurement. The relative frequency error due to sample position was smaller than 0.3% for the first four resonance frequencies of resonator P2 (see Table 1). The homogeneity of the spatial distribution of resonance

Table 2. Resonance frequencies of resonator P2 depending on the mode and the position in the measuring volume

Mode #	f_{org} (MHz)	f_{min} (MHz)	f_{max} (MHz)	Δf_{max} (MHz)	Δf_{rel} %
1	776.0	774.6	776.9	2.3	0.296
2	1143.5	1141.7	1144.7	3.0	0.262
3	1481.8	1480.8	1482.4	1.6	0.108
4	1812.0	1811.6	1812.3	0.7	0.039

f_{org}, Resonance frequency at origin (centre of resonator); f_{min}, minimum resonance frequency; f_{max}, maximum resonance frequency; $\Delta f_{max} = f_{max} - f_{min}$, maximum shift of resonance frequency inside the measuring volume; Δf_{rel}, shift in resonance frequency relative to frequency at origin (f_{org}).

frequencies can thus be considered to not markedly influence water content determination. All but especially the higher modes (>second mode) showed a spatially uniform resonance frequency distribution so that modes 1 to 4 have been evaluated for further experiments.

Measuring water content in the microwave resonator

Based on the spatial distribution of the resonance frequency sensitivity identified above, a central measurement position was fixed for all following experiments. This ensured a reproducible positioning of the water or plant sample. To this end, the Perspex container bottom was positioned in the geometric centre of the resonator. In order to evaluate the dynamic range, that is, the minimal and maximal water amount being measurable, the Perspex cylinder was filled successively with increasing amounts of water and the resonance spectrum was recorded for the first four modes (Fig. 3). The most noticeable observation is the presence of discontinuities in the resonance frequency as a function of the water amount for the higher modes (>1). These discontinuities can be explained as follows: with the presence of more polarizable material within the resonator – the resonance frequencies of the higher modes shift to a higher extent and therefore collide and reform with each other, delivering unreliable results. As the primary mode (TM_{010} mode) was stable over a wide range of water amount, it was chosen for further measurements. The observed linear correlation between resonance frequency shift ΔCF and water content (0–300 g) in the sensitive volume of the resonator was evaluated using SigmaPlot 10.0 (Systat Software) using a linear fit:

$$f = y_0 + a \cdot x \qquad (6)$$

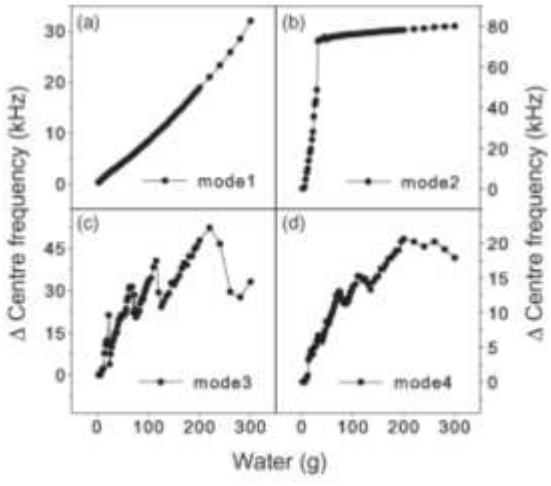

Figure 3. Correlations between water content and corresponding delta centre frequencies (ΔCF) for first to fourth microwave resonator mode in panels a, b, c and d, respectively.

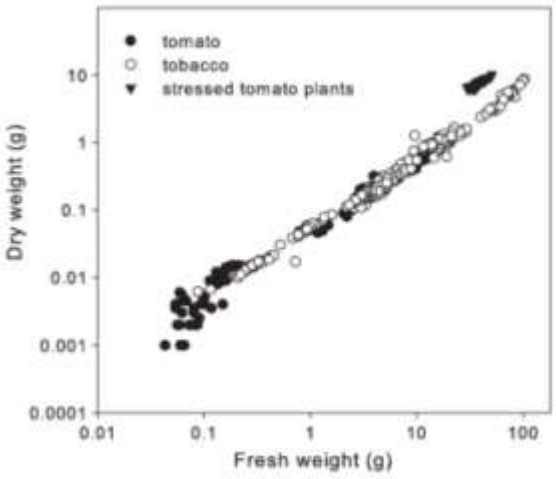

Figure 4. Correlations between fresh and dry weight of the investigated plant species tomato and tobacco. A water-stressed population shows a significantly changed correlation.

The correlation coefficient was excellent ($R^2 = 0.9897$, $P < 0.0001$) for mode 1, which proves the capability of a microwave resonator for precise determination of water content.

Measuring fresh weight of cut shoots

Shoots of tomato and tobacco plants of different developmental stages were measured in the resonator immediately after harvesting and fresh and dry weights were determined with a balance. The tomato and tobacco shoots showed a clear linear correlation between FW and DW (Fig. 4; $a = 0.05$, $y_0 = -0.01$, $R^2 = 0.97$ for tomato and $a = 0.07$, $y_0 = -0.12$, $R^2 = 0.98$ for tobacco plants) over three orders of magnitude which provided the basis to simply calculate total and relative water contents of the plant samples. For a water-stressed population of tomato plants with high fresh weights, different correlation coefficients apply (Fig. 4, black triangles, $a = 0.18$, $y_0 = 0.65$, $R^2 = 0.87$).

The correlation between ΔCF and FW of tomato shoots in the range of 1–22 g FW (Fig. 5a) is linear. Depending on plant size, this correlation (ΔCF versus FW) ranges from fair quality for very small plants ($FW < 1$ g, $a = 90.9$, $y_0 = 0.29$, $R^2 = 0.42$) plants to good quality for intermediate-sized plants (FW 1–5 g, $a = 729.3$, $y_0 = 0.68$, $R^2 = 0.93$) which are within the 95% prediction band (see Fig. 5b).

For the very small plants, the fair correlations could be explained by the fact that, depending on air humidity, they rapidly lose water through evaporation after being cut which leads to a significant error in the non-automated balance procedure. For very large plants, a significant portion of plant material is scattered in the confined resonator space, thus interacting with slightly different field strengths. Furthermore, the presence of extended conductive structures (plant stems) may lead to a distortion of the

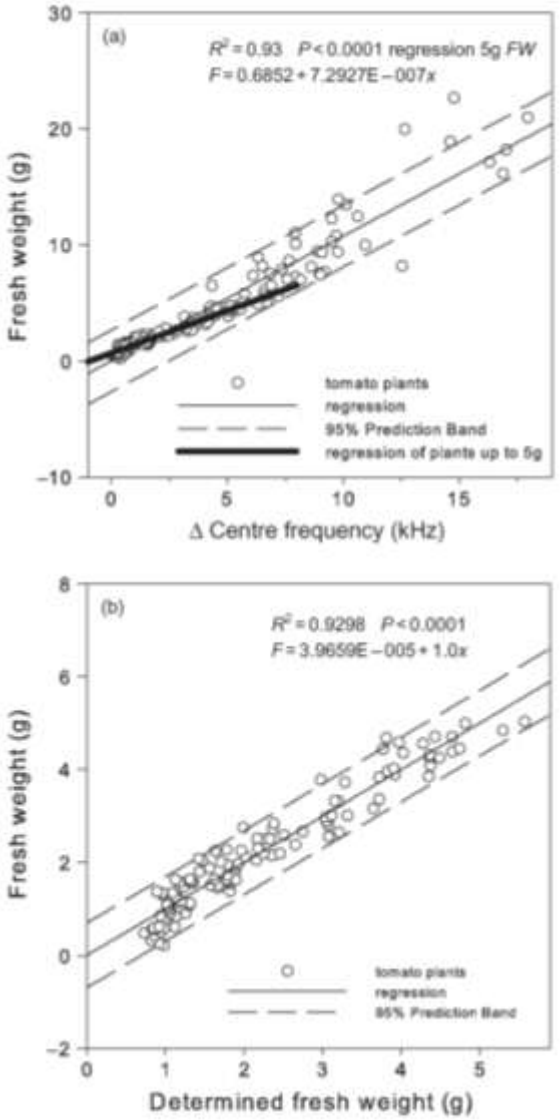

Figure 5. Calibration and testing of tomato fresh weight determination. (a) Calibration of the ΔCF signal for shoots up to 22 g of fresh weight. (b) Comparison between determined fresh weight calculated from linear regression (up to 5 g, see Fig. 5a) and balance-measured fresh weight.

resonator modes. As carbohydrates also constitute polar molecules, it could be assumed that larger carbohydrate contents in large plants also contribute to the dielectric resonance shift. We assume that all three mechanisms contribute to the observed deviation from the linear correlation. Hence, for tomatoes, the sensitive measurement range of the resonator (P2) is 1–5 g. Smaller or larger plants could be analysed more beneficially in smaller or larger resonators, respectively. Based on the fit coefficients (Eqn. 6), it is possible to calculate the FW from the ΔCF with an error of 10% ($R^2 = 0.93$).

Comparable results were obtained for tobacco plants ($FW < 10$ g, $a = 100.6$, $y_0 = 1.64$, $R^2 = 0.81$, see Fig. 6a). Plants with a fresh weight larger than 10 g are outside of the 95% prediction band (Fig. 6a). The measured and the determined fresh weight (obtained from the data fitted with SigmaPlot 10.0,) show a good correlation for plants from 1 g to 10 g fresh weight ($R^2 = 0.78$) (see also Fig. 6b).

With these experiments, we could demonstrate that it is possible to determine the FW of tomato and tobacco plants with the help of the resonator, which in turn allows determining of the DW (see Fig. 4), as the calculation of plant biomass from water content requires a known relative water content of the plant. From Fig. 4, we can suggest that relative water content shows better correlation to

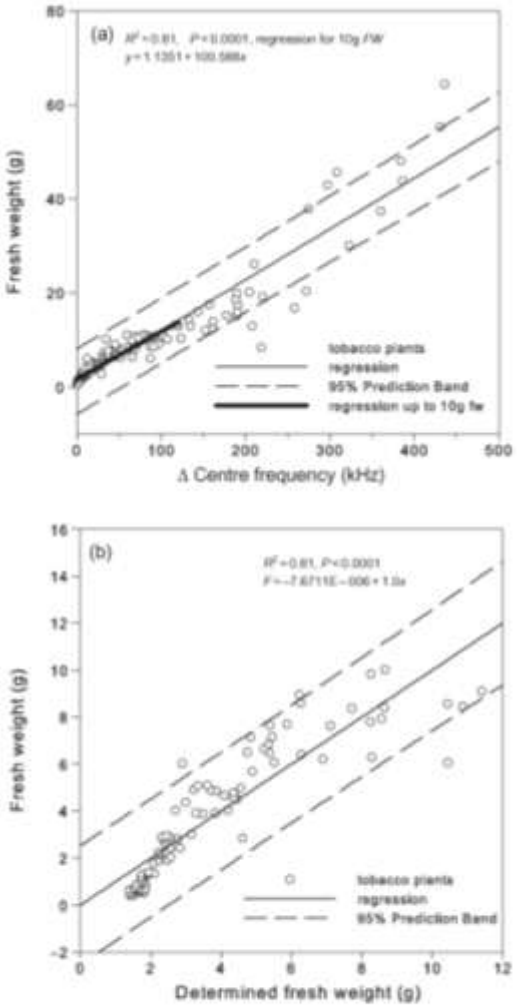

Figure 6. Calibration and testing of tobacco fresh weight determination. (a) Calibration of the ΔCF signal for shoots up to 60 g fresh weight. (b) Comparison between determined fresh weight calculated from linear regression (up to 10 g, see Fig. 6a) and balance-measured fresh weight.

environmental conditions than to developmental stages. Optimal results were obtained under most stringently controlled environmental conditions (temperature, humidity, water supply to the root).

Measuring fresh weight in intact plants (soil and pot shielded)

To detect the entire shoot of larger plants while masking the root and soil water volume, we tested the set-up with soil and pot shielded from the resonator. Previous experiments suggested that reproducible positioning of the shield is essential; therefore, we used Perspex rings differing in height to optimize pot positioning towards a detection of all shoot parts. After the measurement the plants were harvested and *DW* and *FW* were determined as above. There is good correlation between measurements performed on intact shielded plants ($R^2 = 0.59$) and harvested plants ($R^2 = 0.47$) placed inside the resonator without pot or shield (see Fig. 7). Shielded tomato plants only showed an absolute frequency offset in comparison to harvested shoots, which is irrelevant for the determination of *FW* through ΔCF. Using shielding, the biomass of the aboveground pieces of a plant can be determined.

Growth dynamics in intact plants

Repeated measurements of water content in intact plants allow monitoring of biomass increase over time (growth). Measurements of 20 intact tomato plants over 6 d and 9 intact tobacco plants over 10 d show an increasing value of

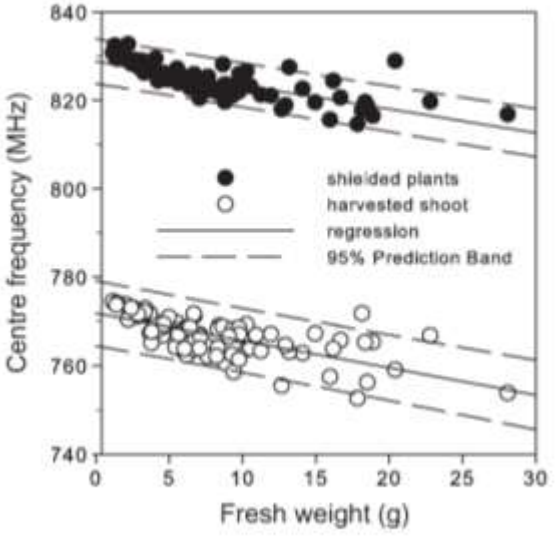

Figure 7. Centre frequency offset due to pot shielding in intact plant measurements as compared to harvested shoot measurements. Slope and ΔCF not significantly changed.

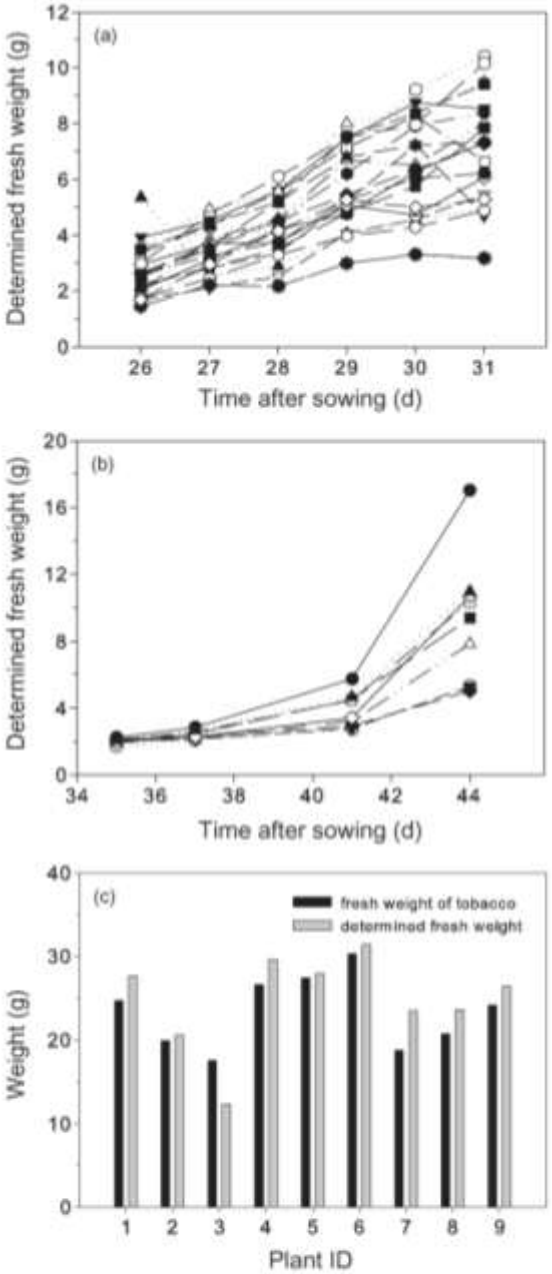

Figure 8. Increase of determined shoot fresh weight of several intact individual tomato (a) and tobacco (b) plants over time. (c) Comparison between determined and balance-measured fresh weight of tobacco shoots at day 58 after sowing.

ΔCF (see Fig. 8a,b). A clear divergence of growth between individual plants of the population was observed, which increased with time. Biomass differences reached at the end of the experiment matched harvested values very well (Fig. 8c).

To evaluate the reliability of the determined fresh weights (based on ΔCF), every second day, nine tobacco shoots were measured and at the end of the experiment, the fresh weight was measured after harvesting the whole shoot. ΔCF increased continuously with the progressing measurements, so does the dependent parameter 'determined fresh weight'. Measured and determined fresh weight for a growth experiment is compared in Fig. 8c. Figure 8a,b depicts a representative part of growth. Tobacco plants (Fig. 8c) were harvested 58 d after sowing. The biomass was determined at the end of the experiment on harvested plants. The error average is 10% (excluding plant #3). The calculated value is typically higher than the 'real' fresh weight, therefore, overestimating the plant growth.

Continuous monitoring of diel growth pattern

The continuous measurement of single plants (measurement interval 10 min) over several days under climate chamber conditions with 12 h/12 h light/dark cycle and daily irrigation (light conditions 8:00/20:00, watering at 10:00) shows a clearly rhythmic increase in fresh weight (Fig. 9). For tomato, fresh weight does not decrease markedly during the day, indicating that net loss of water due to transpiration is compensated by increase of biomass in growth processes (Fig. 9a). At night, fresh weight increases strongly, reaching a 70% higher value within 24 h at the beginning of the plant development and 20–30% at the end of the experiment. The growth conditions for the tobacco experiment were the same as described for the continuously monitored tomato plant. (For example, Fig. 9b shows records for 2 d). During the day, fresh weight decreases, indicating a loss of water due to transpiration. During the night, fresh weight increases. After 24 h, fresh weight increased from 1.92 g *FW* to 2.2 g *FW*, which corresponds to an increase of 15.5% of the initial plant biomass. The diurnal variations of temperature and humidity, to which plants of both species were exposed were similar and did not correlate directly with biomass variations. This indicates that fresh weight increase is uncoupled from the small-scale dynamics of temperature and humidity variations in the environment.

DISCUSSION

In this study, we were able to show a clear correlation between ΔCF and plant fresh weight, demonstrating the applicability of the established non-invasive method for plant biomass determination. The measurement principle allows for rapid detection of plant biomass (within several seconds) which is an important prerequisite for future high-throughput applications of the method. Here, we first demonstrated the capability of the device to register plant fresh weight evolution throughout some days with high temporal resolution. A net increase in biomass was observable throughout 24 h, corresponding to typical growth rates of the investigated plants reported before (tobacco: Walter & Schurr 1999; tomato: Nagel, Konings & Lambers 2001). Within a diel cycle (24 h), the detected fresh weight did not increase monotonically, indicating alterations in plant water status superimposed on growth: The water balance of a plant is characterized by $U - E = G + H$, where U and E are the transpiration and water uptake fluxes, and G and H are the 'storage fluxes' for growth and rehydration (or dehydration), respectively (Boyer 1985). During the day, there is a net water loss in tobacco but not in tomato. This does not imply negative growth throughout the day in tobacco, but it indicates that the relation between growth and daily change in hydration differs between the two species. The difference in the dynamics of fresh weight evolution between tobacco and tomato during the analysis interval (Fig. 9) indicates clearly that joint applications of this method and methods determining plant water status will allow differences to be resolved in growth dynamics of the investigated plants. Similar investigations based on differential weighing of peach trees have led to an improved understanding of tree physiology and to suggestions for improved irrigation management (Simonneau *et al.* 1993). A dramatic effect of altered transpiration timing on the timing of growth processes has been reported recently for the facultative Crassulacean Acid Metabolism (CAM) plant *Clusia minor* (Walter *et al.* 2008). There, maximal growth activity shifted from early night to early day when the plant switched from C_3 to CAM-mode of photosynthesis during drought stress. This indicates that the dynamics of growth processes can only be revealed, when water status and growth are investigated in parallel, requiring non-invasive, high-resolution methods for joint analyses.

A possible explanation for the observed difference in diel fresh weight increase of tobacco and tomato is that their diel growth behaviour might differ in phasing; tobacco leaves expand strongest at dawn (Walter & Schurr 2005), with maximal intensity lasting from some hours before onset of lights to some hours after onset of lights. During this time interval, shoot fresh weight first increases strongly (during the end of the night, Fig. 9) and then decreases again (beginning of the day) while at the same time, carbohydrates necessary for dry weight increase are produced again via photosynthesis (beginning of the day). The strong loss of fresh weight during the day, which is caused by transpiration being higher than the sum of water uptake and growth, might make it impossible for tobacco to keep up high growth rates. In tomato, this diurnal loss is not as pronounced as in tobacco, making high growth during the day more favourable and potentially leading to an increase of leaf expansion during daytime. Such an increase has been reported in other species before (poplar: Matsubara *et al.* 2006, soybean: Ainsworth *et al.* 2006). Also for tomato, high rates of fresh weight increase towards the end of the day and at night have been reported before (Van Ieperen & Madery 1994). A clear advantage over the method described in Van Ieperen & Madery (1994) is that our approach allows rapid analyses of several plants grown in different conditions, as the measurement device is portable,

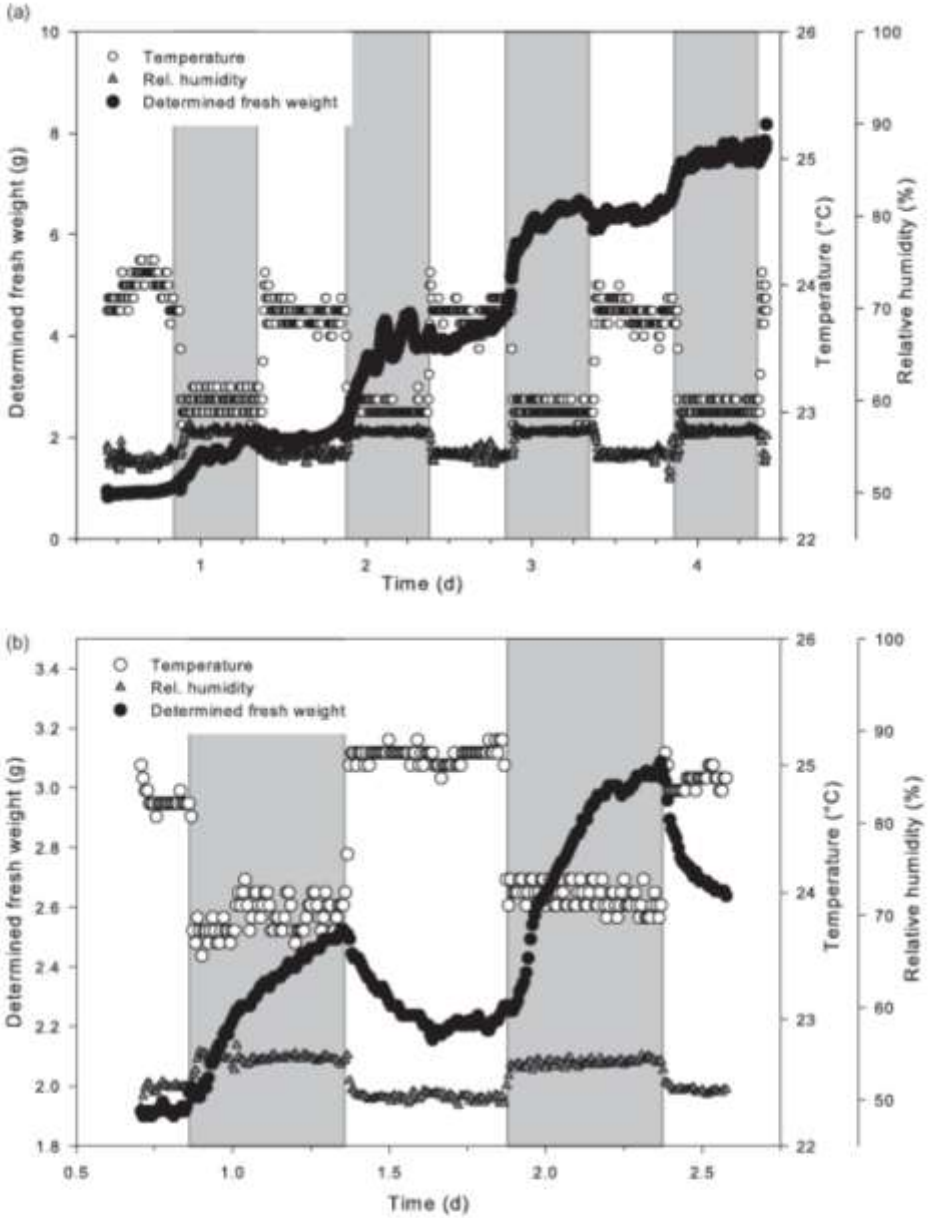

Figure 9. Continuous non-invasive measurement of fresh weight to monitor diurnal growth of (a) an intact tomato plant over a period of 4 d and (b) an intact tobacco plant over a period of 2 d. Air temperature and relative humidity were monitored in parallel. Grey areas indicate dark periods.

while the hydroponically based setup of Van Ieperen and Madery (as well as the set-up described for tree growth evaluation by Simonneau *et al.* 1993) requires the plant to be cultivated within the analysis device. This flexibility of the set-up has been utilized for the calibration procedures depicted in Figs 5–7, for which several plants were analysed sequentially. It has to be pointed out though, that the correlation between ΔCF and real plant fresh weight has to be re-evaluated at different points in time throughout the diel cycle and not only at one point in time (as performed here).

Our research plans for the future are twofold. The first aim is to establish the described method as a tool for plant phenotyping. Here, the technical challenge is the extension of the method to plant species and organs with pronounced differences in water content per tissue volume (e.g. fruits versus sklerenchymatic leaves) and the development of a reliable data base of underlying correlations.

This application would greatly benefit from multiplexing of the experiments (automated plant screening with multiple and multiplexed resonators) in order to automatically scan high replicate numbers of plants for growth analyses as well as for optimized irrigation schemes. Second, hinged resonators will be constructed, which can easily be opened, mounted around a plant and closed again. This will allow easier handling of the device and will hence facilitate, for example, field experiments. Although such devices are no real technical challenge, their application will require careful calibrations and constraints in sample positioning.

CONCLUSIONS

The measurement of dielectric properties of a cavity resonator with inserted plant material provides a novel tool to measure the water content of plants which can then be related to shoot growth under controlled environmental conditions. Although the method is indirect, relating dielectric properties of a microwave resonator to fresh weight, it is in many aspects superior to weighing procedures or other less applicable and less reliable remote-sensing-based non-destructive methods described in the introduction. In future experiments, this procedure will be applied for phenotyping of different genetic material, for analysis of fresh weight distribution within the plant (scanning single organs) and it will be developed into a field method, using larger resonators with custom-tailored design.

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3.3 Third publication: Growth response to UV-B radiation interacts with pronouncedly differing diel water status fluctuations in tomato and tobacco plants

Tittmann S, Albert A, Bühler J., Schurr U & Walter A

Status: **submitted**

Own contribution

- Experimental design
- Experiments
- Data Analysis
- Preparation of the manuscript

Growth response to UV-B radiation interacts with pronouncedly differing diel water status fluctuations in tomato and tobacco plants

SUSANNE TITTMANN[1,3], ANDREAS ALBERT [1,2], JONAS BÜHLER[1], ULRICH SCHURR[1] & ACHIM WALTER[1,4]

[1]ICG-3 (Phytosphere) Forschungszentrum Jülich GmbH D-52425 Jülich, [2]Institute of Biochemical Plant Pathology Department of Environmental Engineering, Helmholtz Zentrum München German Research Center for Environmental Health, D-85764 Neuherberg;

[3]Current address: Research Centre Geisenheim, Department Viticulture, Von-Lade-Str. 01, D- 65366 Geisenheim
[4]Current address: ETH Zürich, Agronomy and Plant Breeding, Universitätstrasse 2, CH- 8092 Zürich

Corresponding author

Achim Walter

ETH Zürich

Institute of Plant, Animal and Agroecosystem Sciences

Universitätstrasse 2

CH- 8092 Zürich

Phone: +41 44 632 32 72

Fax: +41 44 632 1143

E-Mail: walterac@ethz.ch

Keywords: UV-B, Non-invasive biomass determination, *Nicotiana tabacum L*. (tobacco), *Lycopersicon esculentum* Mill. (tomato), leaf area, gas exchange, climate chamber experiments

Abstract

Plants respond in a number of ways to ultraviolet-B (UV-B; 280-315 nm) radiation. Among other processes, UV-B affects secondary metabolite composition, cell cycle and biomass growth in many plant species. In horticultural plants, compact growth with reduced leaf expansion is often desirable, and is often detected at increased UV-B. In our greenhouse experiments, broccoli, lettuce, tomato and tobacco plants were cultivated under different types of cladding material with high and low transmittance for UV-B, respectively. The leaf area and biomass growth in young broccoli, salad and tomato plants (*Lycopersicon esculentum*), but not in tobacco (*Nicotina tabacum*), were significantly higher under low UV-B radiation. To elucidate the reason for this discrepancy between species in more detail, we investigated tomato and tobacco plant biomass non-invasively and with high temporal resolution using a newly developed microwave resonator. These experiments were performed in controlled UV-B exposure chambers in which the ratio between UV-B and photosynthetic active radiation (PAR; 400-700 nm) corresponded to that of natural light. Biomass growth differences between tomato and tobacco were confirmed under controlled conditions. The dynamics of diel (24 h) biomass accumulation differed strongly between species: Whereas a strong decrease of plant water content was observed in tobacco during the day, tomato plants grew more steadily. Together with investigations of leaf gas exchange, these results indicate that strong fluctuations in water content inhibit the growth potential of tobacco plants to a much stronger extent than detrimental effects of UV-B, which inhibit carbon accumulation, leading to reduced growth in tomato.

Introduction

Some plants show a pronounced reduction of growth when exposed to elevated UV-B radiation. This reduction can be linked to a decrease in assimilation that is reported for a lot of species (Teramura & Sullivan 1994, Correia et al. 1999) and that results from damage in photosystem II (Takahashi et al. 2010). Yet, not all species show comparable responses to elevated UV-B (Paul & Gwynn-Jones 2003) and it is controversially discussed whether an increase in UV-B – resulting from a depletion of the ozone layer – would lead to a decrease in overall plant growth and crop yield (Fiscus & Booker 1995, Allen et al. 1998). The response to enhanced UV-B radiation also depends on the extent of adaptation and prior acclimation to UV-B as well as on the fluence rate and wavelength of UV-B. Apart from damage of photosystem II, unspecific damage of DNA can occur and a number of specific signaling pathways can provoke photomorphogenic responses (Tevini & Teramura 1989, Jenkins 2009). Some years ago, the hypothesis was raised that UV-B related photomorphogenic responses are induced by flavonoid aglycones and phenol-oxidizing peroxidases that affect auxin transport and metabolism and hence plant architecture (Jansen 2002), while at the moment, the COP1- and UVR8-related expression of the transcription factor HY5 is considered as the prime element in the regulation of genes involved in photomorphogenic UV-B responses (Favory et al. 2009, Jenkins 2009).

Plant growth occurs in an ever-changing environment and – in dicotyledonous plants – its timing is strongly controlled by the circadian clock (Nozue et al. 2007, Poiré et al. 2010). As under field conditions, the degree of UV-B exposure also fluctuates strongly throughout the diel cycle (24 h), it was the aim of this study to investigate, how the dynamics of growth patterns are affected by UV-B in species that differ in their response to elevated UV-B. Rather than putting the focus on putative molecular response chains and on morphology and architecture, the physiological framework in which growth reactions occurred was investigated, when these species were exposed to different levels of UV-B that were applied with a timing and an extent that simulates field conditions.

Material and Methods

Greenhouse experiments

Plants were sown and cultivated in low and high UV-B conditions in two different greenhouses at Forschungszentrum Jülich GmbH (ICG-3; Fig. 1a). Greenhouses are covered with cladding materials differing in UV-B transmission. One of them is covered with commonly used "float glass" with low UV-B transmission (approximately 0.6%) and the other with ETFE foil, allowing a high UV-B transmission (86%; Fig. 1b).

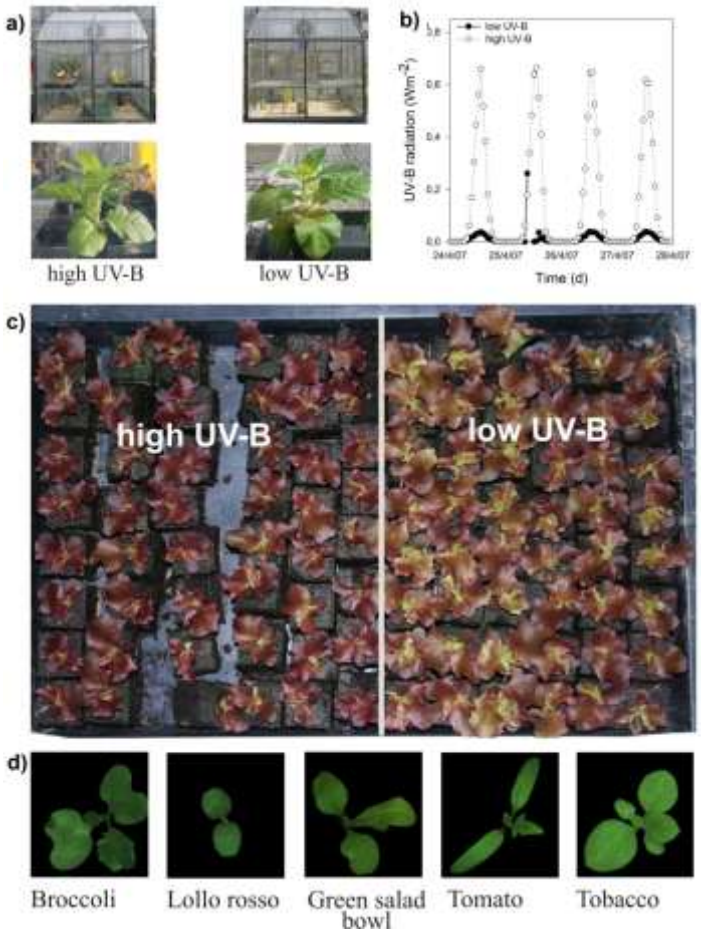

Figure 1: a) Greenhouses covered with different cladding materials from high to low transmission of UV-B radiation. Below the images of the greenhouses the corresponding views inside are shown. b) UV-B transmission of the used two different cladding materials. The ETFE foil (high UV-B; black triangle) had the highest UV-B transmission during all experiments (from April to June) followed by the float glass with lowest transmission of UV-B radiation. No differences in UV-A and PAR transmission were found for the different materials (data not shown). The image in **c)** indicates the influence of UV-B radiation on the pigmentation of lettuce plants. Plants standing under high UV-B radiation (ETFE foil) are smaller and showed strong, red color corresponding to the higher amount of scavenging pigments compared to plants under low UV-B radiation (right sight of the image). and in d) different plant species measured with the 2D GROWSCREEN and after colour segmentation.

During the growing seasons triple sensors (Gigahertz X1, Gigahertz Optik GmbH, Puchheim Germany) placed at leaf level, were used to measure photosynthetic active radiation (PAR; 400-700nm), ultraviolet-A (UV-A; 315-400 nm) and ultraviolet-B (UV-B; 280-315 nm) radiation simultaneously. These analyses confirmed that the materials only differed with respect to their transmittance in the UV-B range. The amount of UV-B radiation reaching the plant level is shown for four exemplary days in Fig. 1 b. Treated plants (high UV-B) received far more UV-B radiation compared to plants grown under control conditions (low UV-B). Lollo rosso plants (*Lactuca satviva* var.*crispa* convar. *Secalina Alef*) showed typical reactions to UV-B, such as deeper red leaf coloration and more stunted growth when cultivated at high UV-B (Fig. 1c).

Green oak leaf lettuce (*Lactuca sativa* var. *crispa*), Lollo rosso and broccoli (*Brassica oleracea* L. convar. *botrytis*) plants were sown in pressed pots produced at the University of Bonn (Versuchsgut Marhof). After sowing, broccoli pots were placed in the cooling chamber at 4°C for three days to induce germination. Afterwards pots were directly transferred to the different greenhouse conditions. . All growth experiments were repeated three times. For germination of tomato (*Lycopersicon esculentum* L. Harzfeuer) and tobacco (*Nicotiana tabacum* L. Samsun) plants ED73 soil (Balster Einheitserdewerk, Fröndenberg, Germany) was used. Plants in all greenhouses were irrigated automatically. Leaf area was determined non-invasively with 2D GROWSCREEN (Walter et al. 2007; Fig. 1d) over a period up to 22 days after sowing depending on plant species. At the end of each experiment, fresh and dry weight was determined. For dry weight analysis, fresh plant material was dried at 70°C for 5 d.

Experiments in exposure chambers

The experiments were performed from October to mid December 2008 in an exposure chamber at the Helmholtz Zentrum München (Department of Environmental Engineering). The exposure chambers provide an irradiance spectrum very close to the natural global radiation from the ultraviolet through to the infrared spectrum. They are therefore commonly referred to as “sun simulators” (Thiel et al. 1996). The natural photobiological environment was simulated using a combination of four types of lamps (metal halide lamps, quartz halogen lamps, blue fluorescent tubes, and UV-B fluorescent tubes). The lamp types were arranged in several groups to obtain the natural diurnal variations of solar irradiance by switching appropriate groups of lamps on and off.

Plants were grown in a sun simulator to study the biomass development and photosynthesis of tomato and tobacco plants in response to enhanced and low UV-B radiation at controlled environment with temperatures of 25/20°C (day/night) and with a relative humidity of 50/70% (day/night).

For the enhanced UV-B treatment, an ETFE foil (ethylene-tetrafluoroethylene, 100 µm, Asahi Glass Green-Tech, USA) of a high transmittance (86%) from the UV-B to PAR was used. A commonly used float glass (nearly no transmission in the UV-B range; 0-6%) constituted the control material of the low UV-B treatment. The two regimes were achieved by separating the sun simulator by UV-B absorbing acrylic glass into two zones of UV-B radiation: 0 and 3.6 W m^{-2}. For both treatments UV-A radiation was 6.4 W m^{-2} and PAR 700 µmol $m^{-2}s^{-1}$. The length of the day was 12 h and the duration of the UV-B exposure was set to 9 h; starting 1.5 h after the onset of day. PAR-intensity was gradually increased and decreased during periods of 3 h at the beginning and end of each day. When analyses of biomass were performed, using the microwave resonator (see below), plants inside the microwave resonator were exposed to a somewhat shaded light regime due to light reflections at the copper walls of the resonator. Control plants outside the resonator were then put under similar shading.

Plant material

Approximately one week after germination at low or high UV-B, respectively, tobacco seedlings were potted in single pots and were grown in ED73 soil (Einheitserdewerk Balster GmbH, Fröndenberg, Germany) from then on for ten to fourteen days before transferring them into the microwave resonator. The irrigation of tobacco plants was performed automatically *via* an irrigation system twice a day. Tomato plants were sown in magenta boxes filled with agar (10 mmol N). Tomato plants, which were used for sampling, were sown and cultivated in soil and grew under the same light conditions in the exposure chamber. At the end of the experiment the shoots were harvested and biomass was determined (plant material were oven dried, see above). To calculate fresh weight of plants inside the resonator from microwave resonance readings, calibration analyses were performed with plants grown in the same exposure chamber at the same settings. Afterwards the biomass was calculated using the regression of the calibration and compared with the fresh weight getting after harvest of measured plants in the resonator (for more detail of the calibration see Menzel et al. 2009).

Gas exchange measurements

Transpiration and assimilation measurements

For transpiration and assimilation measurements LI-COR6400 (Licor, Nebraska, USA) was used. The second youngest leaf of tomato and tobacco plants was measured over the course of three days at ambient light using the clear bottom chamber of the LI-COR 6400. Before starting the continuous gas exchange measurement, a light saturation curve was measured to calculate the maximum net assimilation rate (A_{max}) under different UV-B radiation regimes. Therefore, the leaf surface was irradiated in several steps from 0 to 2000 µmol $m^{-2}s^{-1}$. The measurement started one hour after switching on the UV-B lamps. Prior to gas exchange analysis, leaves were adapted to the chamber conditions of the gas analyzer. Leaf temperature within the chamber was adjusted to the ambient temperature (22-24°C). CO_2 concentration was set to 400 µmol $CO_2 m^{-2}s^{-1}$, with a flow rate of 500µmol $m^{-2}s^{-1}$.

Statistical Analyses

For comparison of averages, t-tests were made with SigmaStat 2.03 (SPSS Inc., Chicago, USA).

Results

Greenhouse experiment

Leaf area and biomass of two salad varieties, tomato and tobacco as well as broccoli plants showed a varying response to high UV-B radiation (Fig. 2): Leaf area and biomass differed considerably between treatments for some species, while for other plant species the differences between treatments were very small. Yet, in four species, except tobacco plants, enhanced UV-B induced reduction in leaf area. Correspondingly, fresh and dry weight were reduced at high UV-B with most prominent differences between treatments in tomato and least in green salad bowl. Caused by the shape of salad leaves the leaf area could be observed only throughout two weeks after sowing. Yet, already during this limited time period, a significant reduction in leaf area and biomass was measured in response to enhanced UV-B (Tab. 1). For tobacco plants, total leaf area, fresh and dry weight were increased under high UV-B (Fig. 2c,h) compared to plants grown under low UV-B. To elucidate the physiological framework of these different response patterns of species, tomato and tobacco plants were selected for an in-depth growth analysis under

controlled conditions in exposure chambers with a natural relation between PAR and UV-B radiation.

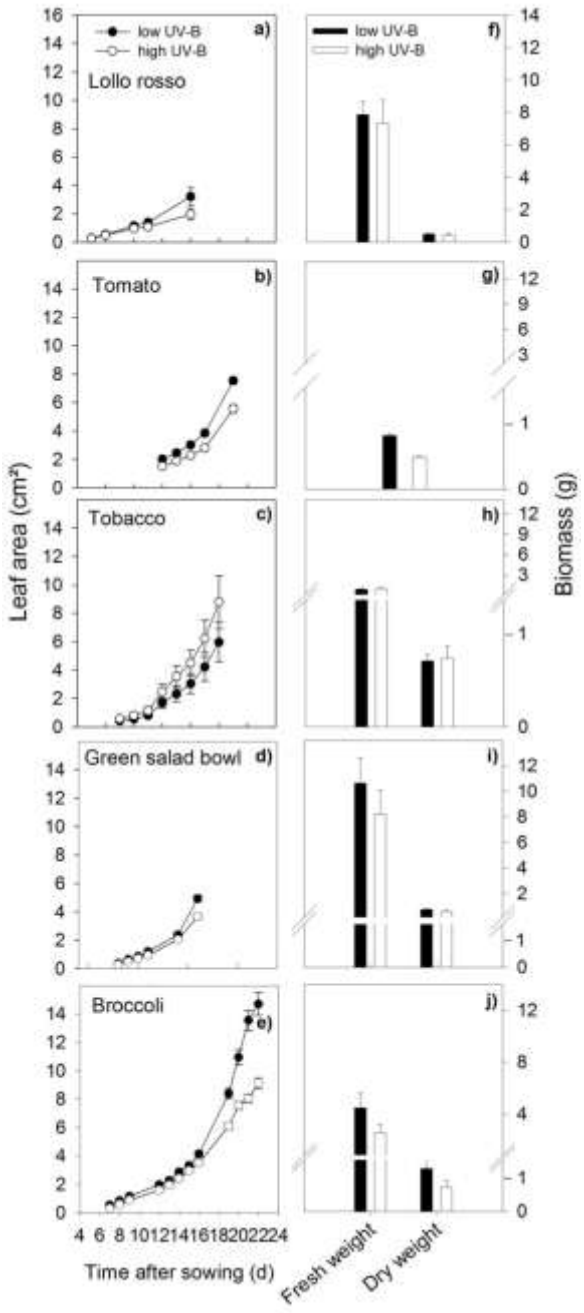

Figure 2: Different plant species were compared under greenhouse conditions in low and high UV-B conditions in a-e) total projected leaf area and f-j) Fresh and dry weight under different UV-B radiation. a) Total projected leaf area of Lollo rosso plants; b) Tomato plants; c) Tobacco plants; d) Lettuce plant and e) Broccoli plants under different UV-B conditions; f) Fresh and dry weight of Lollo rosso plants; g) Fresh weight of Tomato plants; h) Fresh and dry weight of tobacco plants; i) Lettuce plants and j) Broccoli plants under low and high UV-B conditions at harvest (n=50-100 ± SD)

Tab. 1 Development of plant leaf area of different plant species, using the 2D GROWSCREEN, measured under different UV-B radiation in differently covered greenhouses. Three categories were defined: leaf area under low UV-B treatment is greater than under high UV-B, nearly the same results were found under both treatments and thirdly leaf area of low UV-B treated plants is lower compared to plants under high UV-B conditions

	Broccoli	Lollo rosso					Lettuce			Tomato			Tobacco	
Final total projected Leaf Area	**-UV-B**	**+UV-B**	p, t-test	**-UV-B**	**+UV-B**	p, t-test	**-UV-B**	**+UV-B**	p, t-test	**-UV-B**	**+UV-B**	p, t-test	**-UV-B**	**+UV-B**
-UV-B> +UV-B	78.5±3.6 70.4±3 140.7±7.3	44.4±2.8 60.95±2.5 79.4±8.7	<0.001 0.02 <0.001	4.29±0.06 1.68±0.03 3.21±0.07	2.86±0.05 1.54±0.03 1.97±0.04	<0.001 0.002 <0.001	4.92±0.23	3.66±0.19	<0.001	6.225±0.34 7.55±0.24 5.18±0.37	3.809±0.21 5.6±0.28 4.05±0.3	<0.001 <0.001 0.03		
-UV-B ≈ +UV-B							8.55±0.47 2.76±0.13	8.71±0.47 3.1±0.14	0.8 0.09				22±0.7 1.68±0.09	20.7±0.7 1.8±0.1
-UV-B < +UV-B													5.96±0.2	8.79±0.3
n per pop. (from top to bottom)/ days after sowing	20/28 20/24 20/31	20/28 20/24 20/31		113/19 103/14 81/12	134/19 83/14 87/12		38/22 86/16 89/13	38/22 91/16 87/13		44/19 40/18 20/19	42/19 33/18 20/19		40/14 35/27 30/24	40/14 38/27 30/24

Exposure chamber experiments

Near-natural light climates were realized in specialized UV-B exposure chambers as described further above. Growth, gas exchange and hydration status of tomato and tobacco plants were monitored there.

Gas exchange measurements

Assimilation was affected by UV-B in both species to a comparable extent (Fig. 3). For example, at a light intensity of 700 µmol m^{-2}-s^{-1}, both species had assimilation rates of roughly 12 µmol m^{-2}-s^{-1} at elevated UV-B, while assimilation rates of roughly 14 µmol m^{-2}-s^{-1} were detected at low UV-B, indicating a hypothetical daily loss of assimilates of more than 10% under elevated UV-B. At low light intensity, differences between treatments were more pronounced in tobacco, while at high light intensity, assimilation was affected by UV-B to a stronger extent in tomato.

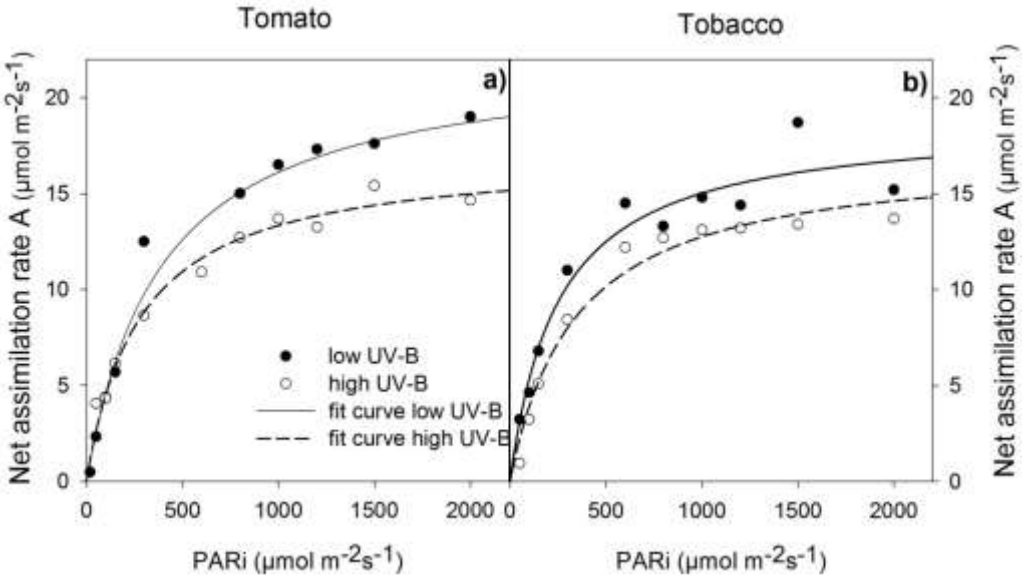

Figure 3: Assimilation in tomato and tobacco: Light response curve were measured to estimate the light saturation. a) Tomato plants showed higher photosynthesis rate under low UV-B radiation, whereas b) for tobacco plants no difference were measured under low or high U-B condition (n=2-4).

Diel analyses of gas exchange showed a much less pronounced effect of UV-B on assimilation in both species (Fig. 4a, b). Only during a few hours, plants exposed to high UV-B showed a markedly reduced assimilation rate and overall, differences between UV-B treatments were not significant and rather in the range of 1% instead of the range of 10%. Tobacco showed a more pronounced reduction than tomato during the hours of

most intense light intensity. At night, respiration did not differ strongly between treatments, but again, in tobacco potential differences between treatments seemed to exceed those found in tomato: Tobacco plants at high UV-B had a slightly decreased assimilation compared to plants at low UV-B, but at night, carbon loss via respiration was also less severe compared to carbon loss at low UV-B, thereby possibly compensating diurnal differences in carbon assimilation. Transpiration rates were comparable between treatments (Fig. 4c,d), but they were higher in tobacco compared to tomato. Growing tomato leaves transpired with rates of maximally 3.5 mmol m^2 s^{-1} while the analysed tobacco leaves reached maximal transpiration rates that were almost 50% higher (5 mmol m^2s^{-1}).

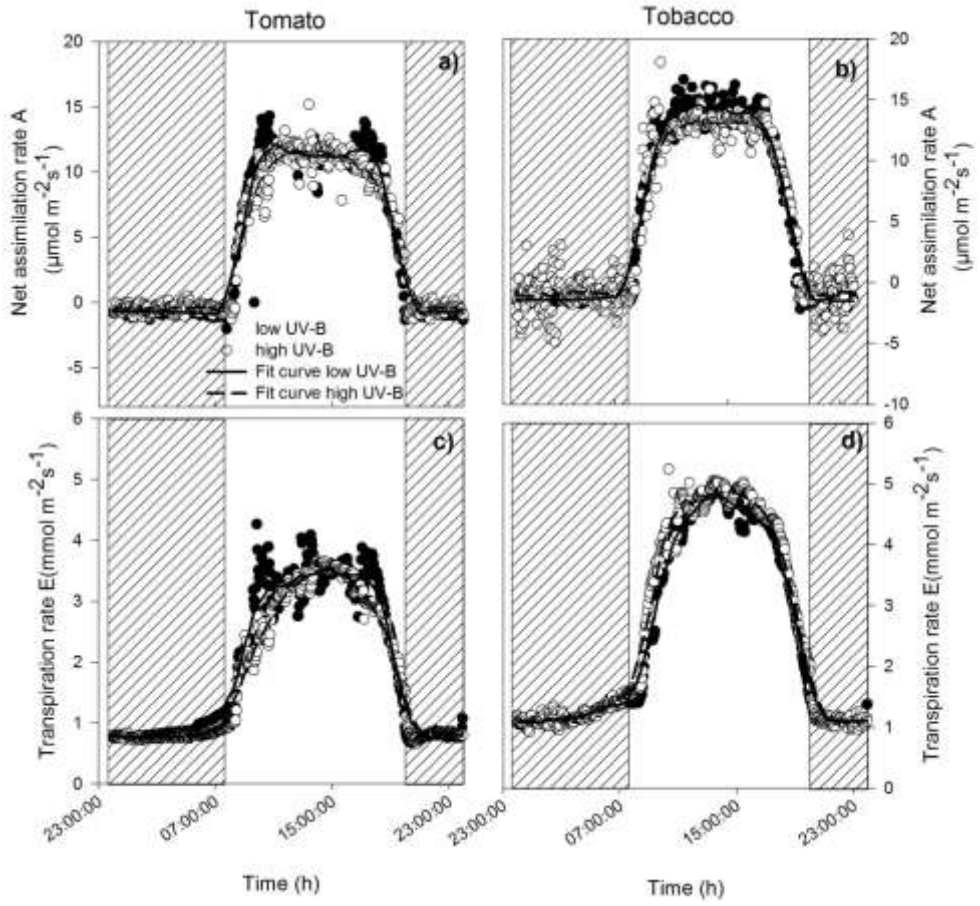

Figure 4:Transpiration and photosynthesis rate of tomato and tobacco: Daily transpiration rate were measured for a) tomato and b) tobacco plants under low and high UV-B radiation (n=3). Diurnal pattern of photosynthesis under different UV-B radiation were measured for c) tomato and d) tobacco plants (n=3). Both species showed no difference between the UV-B treatments but between species. Tobacco plants had higher photosynthesis rate than tomato plants.

Biomass determination

Finally, we determined the diel gain in fresh weight via microwave resonator analyses. These results clearly confirmed the findings of the greenhouse experiment and showed on a much finer time scale that in tomato, elevated UV-B radiation led to a decreased rate of biomass production when compared to plants treated by low UV-B radiation (Fig. 5a). In tobacco, no clear difference between treatments was observed during five to six days of analysis (Fig. 5b). During the last two days of analysis, fresh weight of the plants in high UV-B even exceeded fresh weight of low UV-B plants (inset of Fig. 5b). Moreover, a pronounced difference between biomass gain in tobacco and tomato was observed with respect to the diel cycle of fresh weight development: Tomato plants increased their fresh weight almost permanently, with only small diurnal net losses of fresh weight, while tobacco plants experienced an enormous loss of fresh weight each day (Figs. 5c, d): Tomato plants lost roughly 9% of their maximal nocturnal fresh weight during the following day, while tobacco plants in average lost 40% each day. These findings are in accordance with the results from the gas exchange measurements, where the transpiration rate of tobacco plants exceeded that of tomato plants strongly.

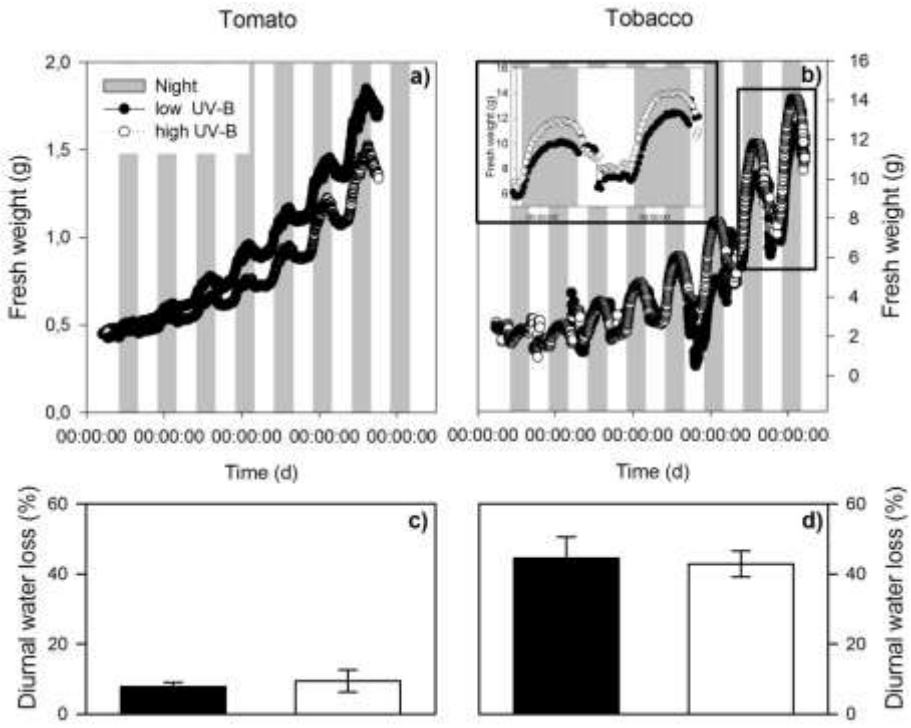

Figure 5: Non-invasive determination of plant biomass (Fresh weight) using microwave resonators. Continuous measurement of plant biomass a) of tomato and b) of tobacco plants under low and high UV - B radiation over several days with an enlargement of the last two days of measurement. c) Diurnal water loss in % of tomato plants and d) diurnal water loss of tobacco plants under different UV-B conditions (n=3).

Discussion

Greenhouse experiment

Plants investigated in the greenhouses showed species specific UV-B responses in biomass and leaf area development. Confirming the overall conclusions of Caldwell et al. (1998) the response of plant species to UV-B can differ strongly, sometimes even between varieties. In our experiments, horticultural plants, such as tomato, two salad varieties and broccoli, reduced their biomass and leaf area when exposed to elevated UV-B radiation, while tobacco did not. A lot of earlier studies reported in literature have been conducted using additional UV-B light sources in greenhouse conditions or in growth chambers. The relation between UV-B and PAR is not recorded in a lot of studies, and if reported, it is not always mimicking field conditions, to which plants have evolved. Hence, UV-B effects can be overestimated and cannot always be transferred from lab to field conditions (Krizek 2004). Lower intensities of PAR reduce the defence capacity of plants against detrimental UV-B-effects (Paul and Gwynn-Jones 2003), which can affect the final results of such studies strongly. Yet, the ratio between the intensity of PAR and UV-B radiation is not only deviating from field situations in such studies, but also in horticultural practice. There, typically cladding materials are used that exclude or reduce UV-B radiation to a stronger extent than PAR, which can result in lower yield and crop quality (Paul et al. 2005). Both experimental situations provide pitfalls that were avoided in this study by the selection of a special cladding material. The selected foil with its high transmission in the UV-B range allowed to work without an additional source of UV-B radiation, thereby making sure that two appropriate species were selected from the greenhouse experiment, for which more detailed investigations were conducted in exposure chambers with nearly natural PAR and UV-B ratio.

Exposure chamber experiment

Growth and Biomass development

The diel growth pattern of dicot plants is very dynamic, is clearly connected to the circadian clock (Dodd et al. 2005) and it has probably evolved in the context of plant adaptation to prevailing diel fluctuations of multiple environmental factors such as temperature and light (radiation) regime (Walter et al. 2009). As sessile organisms plant cannot escape and have to withstand a wide range of environmental fluctuations. While in several studies, leaf expansion is used as a best proxy for plant biomass accumulation, we

decided to analyse the diel evolution of fresh weight using a novel device based on microwave resonance principles (Menzel et al. 2009). Leaf expansion, fresh weight and dry weight, respectively, can all be considered as proxies for the increase of plant biomass, with specific advantages and disadvantages associated with all three parameters. In horticultural plants, such as tomato, the water status is an important indicator for plant development and fruit quality and it is analysed by various groups in multiple ways (Van Ieperen and Madery 1994, De Swaef and Steppe 2010). In this study it was shown that the diel water status fluctuations of tomato and tobacco plants differ enormously. This fluctuation exceeds any potential effect of UV-B radiation on biomass gain strongly (Fig. 5) and it indicates that transient water stresses in plants such as tobacco might play a far more prominent role for decreasing plant growth potential compared to plants such as tomato, which show much smaller fluctuations of water status and biomass. The latter plants might be more susceptible to an additional stress such as high – but not 'unnatural' UV-B exposure.

Gas exchange

The results of the gas exchange analyses confirm this view: Transpiration and assimilation are somewhat reduced in both species when exposed to high UV-B. Yet, in tobacco, nocturnal respiration differences might compensate for diurnal differences in assimilation, thereby leading to comparable biomasses in both treatments. In contrast to this, tomato seems to assimilate more efficiently at low UV-B and seems to allow for higher transpiration rates at low UV-B, compared to high UV-B, respectively. Overall, the gas exchange differences between treatments are small, confirming the conclusions of several authors (Banes et al. 1990, Jansen 2002, Gitz & Liu-Gitz 2003) that low UV-B radiation levels do not markedly affect photosynthesis and transpiration, but are mainly inducing photomorphogenic responses. However, the differences in leaf area and biomass development detected in the greenhouse experiment and confirming the puzzling and variable response of various species towards elevated UV-B exposure, need to be substantiated by associated differences in carbon assimilation and diel biomass evolution when treatments are compared for different species, which was successfully achieved here.

Conclusion

We conclude, that even at the moderate level of UV-B irradiation applied here, tomato biomass gain was limited by assimilation of carbohydrates, while tobacco biomass gain was rather limited by water supply. This trade-off between water- and carbohydrate-limitation of growth would explain why species differing for example in water use efficiency show differing reponses towards elevated UV-B. Moreover, it would explain why the degree to which UV-B radiation can affect growth depends strongly on environmental conditions: When water supply is severely limited, also plants such as tomato will in general reduce growth and differences in response to UV-B radiation will disappear. This is confirmed by field studies with limited water access in soybean (Teramura & Sullivan 1994).

Overall, carbon metabolism and water status interact in a complex way and the trade-off between water- and carbon-limited growth differs between species. Model assumptions, how alterations of environmental factors affect plant growth need to take this balance into account, demanding for an improved basis of our physiological understanding of plant growth.

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3.4 Fourth publication: Simultaneous phenotyping of leaf growth and chlorophyll fluorescence via GROWSCREEN FLUORO allows detection of stress tolerance in *Arabidpopsis thaliana* and other rosette plants

Jansen M, Gilmer F, Biskup B, Nagel KA, Rascher U, Fischbach A, Briem S, Dreissen G, Tittmann S, Braun S, de Jaeger I, Metzlaff M, Schurr U, Scharr H, Walter A

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Own contribution

- Data acquisition and interpretation
- Preparation of manuscript (partly)

Simultaneous phenotyping of leaf growth and chlorophyll fluorescence via GROWSCREEN FLUORO allows detection of stress tolerance in *Arabidopsis thaliana* and other rosette plants

Marcus Jansen[A], *Frank Gilmer*[A], *Bernhard Biskup*[A], *Kerstin A. Nagel*[A], *Uwe Rascher*[A], *Andreas Fischbach*[A], *Sabine Briem*[A], *Georg Dreissen*[A], *Susanne Tittmann*[A], *Silvia Braun*[A], *Iris De Jaeger*[B], *Michael Metzlaff*[B], *Ulrich Schurr*[A], *Hanno Scharr*[A] *and Achim Walter*[A,C]

[A]Institute of Chemistry and Dynamics of the Geosphere ICG-3 (Phytosphere), Forschungszentrum Jülich GmbH, 52425 Jülich, Germany.

[B]Bayer BioScience N.V., Technologiepark 38, 9052 Gent, Belgium.

[C]Corresponding author. Email: a.walter@fz-juelich.de

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Abstract. Stress caused by environmental factors evokes dynamic changes in plant phenotypes. In this study, we deciphered simultaneously the reaction of plant growth and chlorophyll fluorescence related parameters using a novel approach which combines existing imaging technologies (GROWSCREEN FLUORO). Three different abiotic stress situations were investigated demonstrating the benefit of this approach to distinguish between effects related to (1) growth, (2) chlorophyll-fluorescence, or (3) both of these aspects of the phenotype. In a drought stress experiment with more than 500 plants, poly(ADP-ribose) polymerase (PARP) deficient lines of *Arabidopsis thaliana* (L.) Heynh showed increased relative growth rates (RGR) compared with C24 wild-type plants. In chilling stress, growth of PARP and C24 lines decreased rapidly, followed by a decrease in F_v/F_m. Here, PARP-plants showed a more pronounced decrease of F_v/F_m than C24, which can be interpreted as a more efficient strategy for survival in mild chilling stress. Finally, the reaction of *Nicotiana tabacum* L. to altered spectral composition of the intercepted light was monitored as an example of a moderate stress situation that affects chlorophyll-fluorescence related, but not growth-related parameters. The examples investigated in this study show the capacity for improved plant phenotyping based on an automated and simultaneous evaluation of growth and photosynthesis at high throughput.

Additional keywords: chilling stress, drought, dynamic processes, image processing, *Nicotiana tabacum*, PARP, phenomics.

Introduction

Plant phenotypes integrate genomic features of a plant with environmental factors acting on the plant (Sultan 2000). As internal plant features and external factors fluctuate with characteristic dynamics, the selection of an appropriate temporal resolution is crucial for a meaningful analysis of plant phenotype development (Walter *et al.* 2009). Leaf growth and photosynthesis are important aspects of the plant phenotype. Growth and photosynthesis interact with each other on different temporal and organisational levels (Schurr *et al.* 2006).

Drought is the most severe abiotic stress factor reducing global crop yields (Boyer 1982). It is mediated via osmotic changes, triggering a signal transduction network in the plant cells (Knight and Knight 2001; Zhu 2002; Seki *et al.* 2007). These signalling pathways include calcium ions, protein kinase cascades, phospholipid signalling and the formation of reactive oxygen species (ROS; Xiong *et al.* 2002). Osmotic stress signalling also induces the production of abscisic acid (ABA) which activates drought-inducible genes via special transcription factors (Seki *et al.* 2007). As well as their signalling function, ROS are toxic to plant cells, and act by oxidising organic molecules, membranes and nucleic acids, which can lead to cell death (Noctor and Foyer 1998; Mittler 2006). ROS-induced DNA damage activates poly(ADP-ribose) polymerase (PARP) (Berglund 1994; Kim *et al.* 2005). PARP is a nuclear enzyme catalysing ADP-ribosylation of itself and of nuclear proteins including topoisomerase, endonuclease, and DNA polymerase (Scovassi *et al.* 1986; Lepiniec *et al.* 1995). Moreover, PARP is involved in DNA synthesis and repair (Satoh *et al.* 1994; Schreiber *et al.* 2006). Stress-induced PARP activity consumes energy by diminishing cellular concentrations of NAD^+ and ATP (Rongvaux *et al.* 2003). Recently, it was reported that silencing of PARP-genes in transgenic *Arabidopsis thaliana* (L.) Heynh and *Brassica napus* L. plants enhances tolerance to abiotic stress (De Block *et al.* 2005; Vanderauwera *et al.* 2007). Tolerance of these PARP-

deficient plants to abiotic stress was assigned to enhanced energy homeostasis. Lower energy consumption after abiotic stress, and lower frequency of cell death were found in those stress tolerant plants. In addition, changes in stress signalling and ABA levels occurred in PARP-deficient plants (Vanderauwera *et al.* 2007).

Several other abiotic stress factors, such as salinity, cold stress, ozone exposure and suboptimal light regimes, also affect plant performance and crop yield. Plant breeding approaches often take growth and photosynthesis analyses into account when assessing abiotic stress tolerance. Growth and photosynthesis can be analysed non-destructively via imaging methods, which is a necessary prerequisite for sensitive analyses of their dynamic reactions towards alterations of environmental parameters. Therefore, the development of rapid and robust methods to detect stress tolerance in realistic environmental scenarios (Granier *et al.* 2006; Montes *et al.* 2007; Walter *et al.* 2007; Rajendran *et al.* 2009) is of increasing importance for basic science and breeding for stress-tolerant plants alike (Mittler 2006). Increased process understanding will be gained only if the development of improved methods is supported by refined concepts to interpret the biological data. Moreover, it is desirable to impose relatively mild instead of extreme stress treatments in the experiments as these are more relevant for field-related questions.

Non-destructive growth analyses have become possible with the advance of imaging-based technologies throughout the last decade (Leister *et al.* 1999; Granier *et al.* 2006; Walter *et al.* 2007; Rajendran *et al.* 2009). Analysis of chlorophyll fluorescence imaging has become a widely used tool to characterise the response of photosynthesis to different environmental factors (Osmond *et al.* 1998; Chaerle and Van Der Straeten 2001; Baker and Rosenqvist 2004; Baker 2008; Woo *et al.* 2008). It is also used to screen for mutants, which are altered e.g. in non-photochemical quenching (Niyogi *et al.* 1998) or in acclimation to light environment (Walters *et al.* 2003). Yet, combined approaches to monitor growth and photosynthesis-related parameters at the same time and in meaningful environmental scenarios are still rare but urgently needed. Selection of promising lines could be accelerated enormously via non-invasive characterisation of the dynamic response of plants to altered environmental conditions.

Hence, the aim of this study was to combine and apply existing technologies of chlorophyll fluorescence imaging and of automated plant growth analysis to a tool for imaging-based plant phenotyping. Using this platform, which allows automatic analysis of roughly 60 plants h^{-1} (GROWSCREEN FLUORO; Fig. 1), we tested the hypothesis that PARP-deficient lines show a better performance than wild-type plants, even under mild drought stress conditions; and we aimed for an evaluation of the use of this technology for other stress conditions and other plant material.

Materials and methods

Plant cultivation

Plants of *Arabidopsis thaliana* (L.) Heynh ecotype C24, and transgenic plants overexpressing an RNA-interference PARP1 (PARP1-plants) or PARP2 (PARP2-plants) construct, respectively, (De Block *et al.* 2005) were grown under controlled conditions at 22/18°C, 170 µmol m^{-2} s^{-1} PAR, and a 8/16 h day/night regime. After cotyledon unfolding, single plants were transferred (pricked out) into pots filled with a mixture of potting soil and sand [67% (v) potting soil (De Ceuster Meststoffen SA/NV, Grobbendonk, Belgium; 33% (v) sand (quartz, grain size 0.7–1.4 mm, Rheinische Baustoffwerke, Weilerswist, Germany)]. Thirty pots (7 × 7 × 8 cm) were arranged on a tray and were watered thoroughly immediately after pricking out of the plants. Afterwards, water was withheld until all plant trays had lost 20% of the weight they had at the time plants were pricked out. The time until this point was reached varied between experiments: it took 1–2 weeks after pricking out the plants. Weight loss was checked daily by putting the entire plant tray on a balance whereby tray, pots and the water-drenched soil constituted the bulk of the weight (~10 kg) and the plants themselves (maximally 10 g; hence, less than 1% of the total weight) accounted only for a marginal contribution. When the trays reached 20% weight loss, control plants were watered regularly to keep this level of soil water content by replacing any further weight loss with equal amounts of water, whereas plants designated for drought stress did not receive further irrigation. Cold stress was applied by placing the pots in a cooled growth chamber (5°C, 80–100 µmol m^{-2} s^{-1} PAR; 8/16 h day/night regime).

Nicotiana tabacum L. cv. Samsun was cultivated in a sand-soil-mixture (as above) in a climate-controlled greenhouse (24–25/16–18°C, 16/8 h day/night). Drought and cold stress were applied in the same manner as for *A. thaliana* plants.

For the UV-B experiment, *N. tabacum* plants were cultivated in soil (ED73, Einheitserde, Balster Einheitserdewerk, Fröndenberg, Germany) for 3 weeks in two different greenhouses. One greenhouse was covered by float glass (CENTROSOLAR GLAS GmbH and Co. KG, Fürth, Germany) with low transparency for ultraviolet (UV)-B radiation (6% transmittance in the wavelength range 290–315 nm) and the other was covered with ETFE foil (Asahi Glass Green-Tech Co. Ltd, Chiyoda-ku, Japan) with high transparency for UV-B radiation (68% transmittance). The transmittances for PAR and UV-A were at 95–97% for both glass materials. Therefore, the only difference in both plant populations was the quantity of UV-B radiation.

Hardware setup

Measurement images were taken using a chlorophyll fluorescence imaging system (Imaging-PAM M-Series, Maxi version, Heinz Walz GmbH). This consists of a black-and-white charge-coupled device (CCD) camera (Dolphin F-145B, Allied Vision Technologies GmbH, Stadtroda, Germany) with a progressive scan CCD-sensor (Sony Corporation, 1392 × 1040 pixel physically with 4-pixel-binning, resulting in 640 × 480 pixel final resolution), a 12.5 mm C-mount objective lens (Pentax, Hamburg, Germany), and a 300 W LED-array with pulse-modulated excitation, actinic and saturation pulse light. Highly homogeneous illumination (maximal deviation ±7% from mean value) is achieved at a working distance of 185 mm resulting in an imaged area of approx. 7.8 × 10.4 cm. A conic, black, metal shading hood of 185-mm height was fitted to the LED-array in order to avoid illumination of neighbouring plants (Fig. 1).

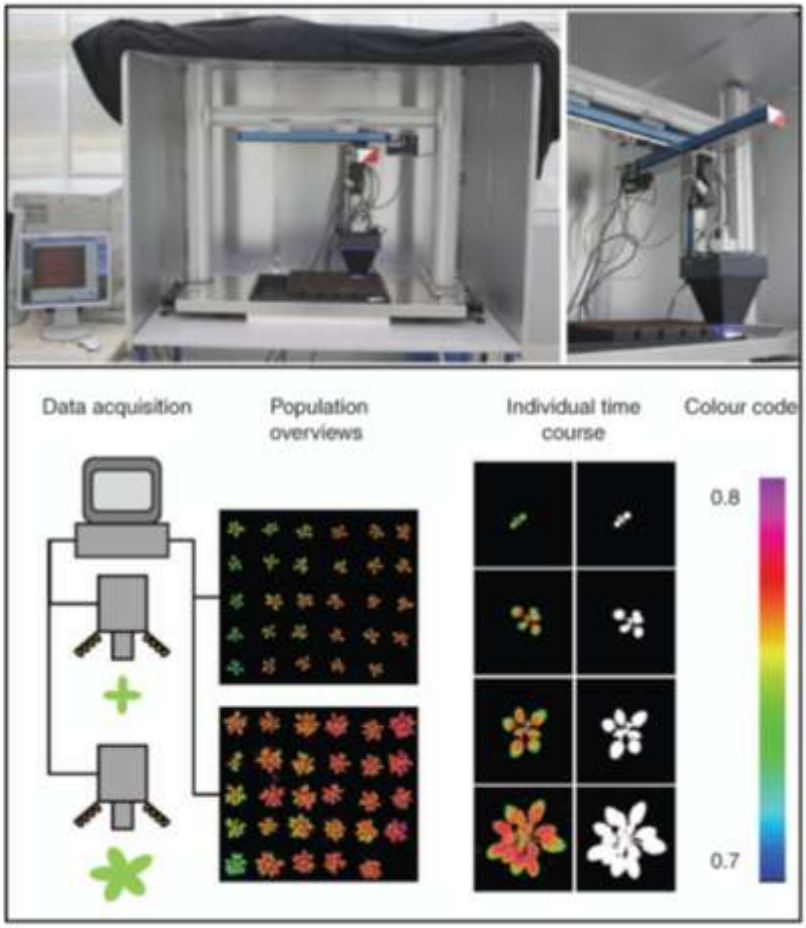

Fig. 1. GROWSCREEN FLUORO: picture of the instrument, result images and scheme of function. The instrument (left) consists of a detection head mounted on moving stages enclosed in an opaque box with a curtain and a controlling computer next to the box. The detection head (right) is a chlorophyll fluorescence imaging system equipped with illumination inside a shading hood. Chlorophyll fluorescence images are acquired of each individual plant and are evaluated for (i) total projected leaf area (A_{P1}, white insets), and (ii) potential quantum yield of PSII (F_v/F_m, colour-coded insets).

A displacement stage (Pico-Mini, Laser 2000 GmbH, München, Germany) allowed lifting and lowering the imaging system in *z*-direction. Three limit switches ensured that the stage stopped when the shading hood hit the ground. In all experiments pots were placed such that the hood touched the plastic of the pots. Plants were potted such that their average height position was approximately at the top edges of the pots. This resulted in an average working distance of 185 ± 3 mm (typical variation), where the main variations come from different leaf heights and small variations in soil fill level. Each pixel corresponded to a leaf area of 0.0266 mm^2 ±9%.

The liftable imaging system was driven to preset *x*-*y*-positions via two linear displacement stages (Pico-Mini, Laser 2000 GmbH) that were mounted to a solid metal stand (X95, Linos Photonics, Göttingen, Germany).

Software environment

Positioning of the imaging system, measurement protocol and data analysis were automated via a database-driven client server application written in QT/C++, Python, SQL and MS Visual Basic similar as the one described in more detail by Biskup *et al.* (2009). The server system consisted of two computers: a database server running Suse Linux 10.2 and a computer running Windows XP (Microsoft Corporation, Redmond, WA, USA) used as file server and for image acquisition. They were interconnected by Gigabit ethernet.

The database (MySQL, MYSQL AB, Uppsala, Sweden) stored user-defined and system-depending parameters, current system state, paths to the acquired image data, and automatically-calculated results. Parameters for positioning, imaging and data analysis as well as system state schedule were user defined via a

multiuser GUI (graphical user interface) client software running on any computer connected to the server via intranet.

On the database server two Python programs periodically checked the database for system state changes. The first one checked for positioning and imaging events and executed them. For imaging, communication with the chlorophyll fluorescence imaging system was done via Imaging Win (version 2.12a, Heinz Walz GmbH) running on the Windows computer. Imaging Win was controlled via a MS Visual Basic script triggered from the first programs on the Linux machine. Using Imaging Win ensured operation of the measurement system as intended by the manufacturer. Settings of the chlorophyll fluorescence imaging system were optimised for the plants according to the recommendations of the manufacturer. In addition, the client allows flexible setting of the fluorescence measurement protocol, where duration of dark adaptation, actinic light intensity, timing and frequency of repetition of dark and light measurements are specified for the intended application.

The second program on the database server checked the database for completed measurements and executes appropriate data analysis routines according to rules defined in the database.

Measurement protocol

Trays of up to 30 plants were placed beneath the GROWSCREEN FLUORO system for image analysis, and were removed again immediately thereafter. For analysis of F_v/F_m, plants were dark-adapted for at least 30 min; the last plants to be analysed were dark-adapted for 60 min. Via spacers, it was ensured that the position of each plant was the same in consecutive measurements throughout one experiment. The measurement protocol for each individual analysis consisted of five steps: (i) camera positioning; (ii) image acquisition; (iii) image segmentation for growth analysis; (iv) calculating morphological parameters and parameters related to chlorophyll fluorescence from single images; and (v) calculating RGR from consecutive images.

Camera positioning was realised by moving the detection head (consisting of the camera, illumination and shading hood; Fig. 1) via the *x*- and *y*-displacement stages to each predefined position. The detection head was then lowered by 150 mm until the working distance of 185 mm between pot surface and camera was reached. Then, the shading hood enclosed the brim of the pot completely, thereby preventing the light pulses from affecting neighbouring plants. When the detection head had reached its final position, F_0 and F_m (for parameter definition see Maxwell and Johnson 2000; Schreiber 2004) were acquired using the standard protocol of the chlorophyll fluorescence imaging system (saturation pulse intensity setting: 10; duration: 0.8 s). Colour-coded images of F_v/F_m can be scaled on demand using a rainbow lookup table (Fig. 1), which allows a more detailed visual depiction of differences, gradients and heterogeneities in F_v/F_m compared with the colour-code depiction options of the manufacturer. For analysis of effective quantum yield of PSII (quantum yield; for parameter definition see Genty *et al.* 1989) and non-photochemical quenching (NPQ; for parameter definition see Maxwell and Johnson 2000), the shading hood was removed and an ambient light intensity of 500 µmol PAR m^{-2} s^{-1} was realised via HPI-T plus 400 W (Philips, Köln, Germany) lamps. When image acquisition was finished, the detection head was raised again and was driven to the next position. For F_v/F_m, the analysis of each individual plant needed 40 s, whereas 6 min were needed per plant for the analysis of quantum yield and NPQ.

Image segmentation for growth analysis was performed via a global threshold segmentation of the F_m images. Values above 50 were defined as 'object' (leaf material); below 50 as 'background'. A_{PT} was calculated by multiplying the number of object pixels by 0.0266 mm^2. RGR (% day^{-1}) was calculated from consecutive images of the single plants by $RGR = 100 \times 1/t \times \ln(A_2/A_1)$ with t indicating the time between the acquisitions of A_1 and A_2.

Finally, the morphological parameters: (i) leaf number; (ii) surface coverage; and (iii) stockiness were calculated. Leaf number was determined by counting leaf centre points. Leaf centre points were calculated as local maxima of a distance map containing distances between each object pixel and the nearest background pixel, using the open source library VIGRA (Computer Vision Library, University Hamburg, Germany). Artefact maxima on petioles were suppressed via a threshold on the local distance. It was possible to count all unoccluded leaves of the rosette. Surface coverage equals the ratio between the convex hull and A_{PT} of each plant. The convex hull was calculated using the function cvConvexHull2 of the open source library OpenCV (Intel) which is based on the Sklansky algorithm. This algorithm detects the shortest line around a given object. Surface coverage illustrates the compactness of a rosette. Stockiness is another measure for the compactness, which considers leaf shape to a larger extent than surface coverage as it relates A_{PT} to the length of the borderline L of the entire leaf rosette (the length of the outline), and, hence, also to structures inside the convex hull. Stockiness equals the ratio between $4\pi A_{PT}$ and L^2, i.e. a circular object has a stockiness of 1. For example, when two plants with the same total leaf area and similar leaf shape but differing petiole lengths are compared, the rosette with short petioles will render a higher surface coverage and a higher stockiness. When, as another example, two plants with the same total leaf area and the same petiole length but differing shapes of their leaf blades, the surface coverage would be the same but the plant with more ovate leaf shape would have a higher stockiness than the plant with more incised or fractionated leaf shape.

Statistical analysis

Treatment effects were analysed using one way ANOVA or a *t*-test (SigmaStat, Systat Software Inc., Richmond, CA, USA), as specified in the text. Where indicated, *post-hoc* comparisons of treatment effects were performed within each group using the Tukey adjustment.

Results

Calibration of growth analysis

To evaluate the precision of chlorophyll fluorescence based plant images acquired with a state of the art chlorophyll fluorescence imaging machine (Imaging-PAM M-Series, Maxi

version, Heinz Walz GmbH), leaf area analysis was calibrated against an existing technology. Total projected leaf area (A_{PT}) of 540 plants from different developmental stages of *A. thaliana* and of 220 plants of *N. tabacum* was analysed from the fluorescence images captured with GROWSCREEN FLUORO (Fig. 2). The same plants were analysed some minutes later with an automated device measuring A_{PT} from colour images of plants illuminated with white light (GROWSCREEN; Walter *et al.* 2007). When the data pairs from the two instruments were compared, linear correlations were obtained with correlation coefficients of $r^2 = 0.92$ for *A. thaliana* and $r^2 = 0.99$ for *N. tabacum*, respectively. Deviation of the inclination of the fit lines from unity can be explained by the different segmentation procedures performed by the two instruments. To determine A_{PT}, GROWSCREEN FLUORO takes each pixel into account, which exceeds a certain fluorescence threshold. Nearly the entire leaf material of all plants is covered by this procedure (compare F_v/F_m images in Fig. 1), but as some pixels may not exceed the threshold value, A_{PT} has a tendency to be smaller than the true projected leaf area. GROWSCREEN in turn separates leaf area from the background via a colour-dependent segmentation procedure. Due to different colours of leaf blade, veins and trichomes that lead to a higher number of 'artefact pixels', it is necessary to fill in gaps that are smaller than a certain threshold area. This in turn can lead to an erroneous detection of background as leaf material, when leaf blades and petioles surround a small spot of background (compare the situation in Fig. 1, where these 'inclusions' are not assigned to A_{PT}). Such erroneous inclusions increase A_{PT} and they occur more often in *A. thaliana* than in *N. tabacum* due to the leaf shape and rosette structure of the plants. However, they do not affect the linearity of the fit lines. Growth comparisons between treatments were mainly done on the basis of RGR which is directly linked to the increase of A_{PT}. Therefore, the deviation of the increase of the fit lines from unity (Fig. 2) does not affect result interpretation. Generally, we note that from certain developmental stages on younger leaves can overlap with older ones and the projected leaf area (A_{PT}) is less than the total leaf area. Despite some overlap, A_{PT} correlates almost linearly with the plant biomass in the range of plant sizes investigated in this study (Leister *et al.* 1999; Walter *et al.* 2007). To date, it has not been possible to analyse plant dry weight non-invasively, so leaf area and the increase of leaf area relative to the standing leaf area, which is a RGR, is the best proxy available to quantify plant growth non-destructively and with high throughput.

Drought stress tolerance of PARP lines of A. thaliana

As a first application of combined analyses of growth and chlorophyll fluorescence, a series of drought stress experiments was performed. In these, drought stress was not intended to be close to lethality for the investigated plants. To reduce complexity, only one of six replicate drought stress experiments is reported in detail below; key results of the replicate experiments are displayed where appropriate. Growth of A_{PT} and the potential quantum yield of PSII photochemistry (F_v/F_m, Butler 1978; Schreiber 2004) were analysed in *A. thaliana* with decreased poly(ADP-ribose) polymerase-levels (PARP-lines) in the associated wild-type C24 (Fig. 3). Measurements started four days after pricking out the plants to single pots, and continued until the drought stressed plants stopped growing. In the experiment depicted in Fig. 3, plants were pricked 16 days after sowing (AS), and drought stressed plants were not watered any more from then on. Growth came to a halt 29 days AS, and in the other replicate experiments RGR decreased to zero between 25 and 42 days AS. When RGR reached zero, plants did not show any signs of wilting or necrosis. In preliminary experiments, prolonged drought induced leaf rolling in all populations, which in turn led to 'negative' RGRs as A_{PT} decreased. Plants were still able to recover completely upon rewatering in these preliminary experiments. Approximately 1 week after RGR declined to zero, the majority of plants were not able to recover from drought stress any more.

Between 20 and 25 days AS, A_{PT} was similar in all populations (Fig. 3*a*). At 26 days AS, the growth curves of well watered and drought stressed plants started to diverge. From day 27 to day 29, these differences increased, leading to significantly smaller leaf sizes of drought stressed plants compared with well watered plants ($P < 0.001$, *t*-test, for C24 and $P < 0.001$, *t*-test, for PARP2, respectively). At day 29, plants were re-watered

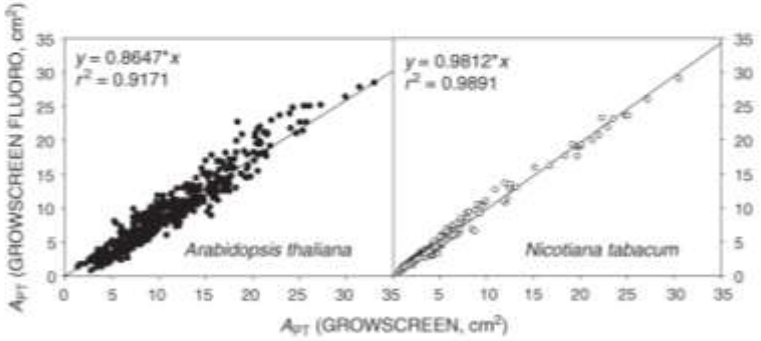

Fig. 2. Calibration of leaf area detection of GROWSCREEN FLUORO. Total projected leaf area (A_{PT}) of *Arabidopsis thaliana* and *Nicotiana tabacum* plants at different stages of development were assessed using two different analysis techniques.

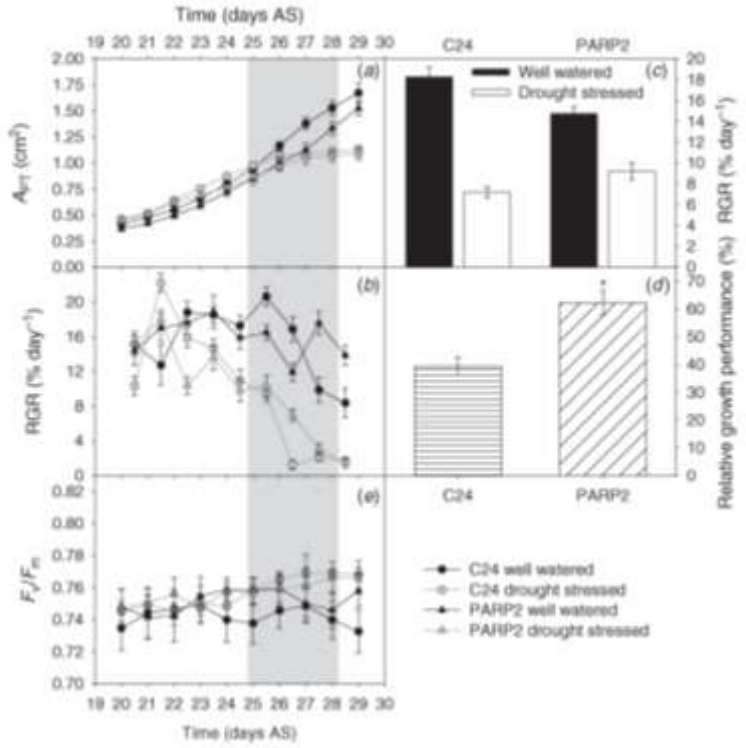

Fig. 3. Assessment of drought tolerance of *Arabidopsis thaliana* C24 and PARP2. Drought stressed plants did not receive water from 16 days AS on (*a*) total projected leaf area (A_{PT}) measured with GROWSCREEN FLUORO between 20 and 29 days AS, (*b*) relative growth rate (RGR) calculated form A_{PT} of individual plants. Grey box in (*a*, *b*, *e*), evaluation period (see text). (*c*) RGR during the evaluation period. (*d*) Growth performances of drought stressed plants. (*e*) Potential quantum yield of PSII (F_v/F_m). Asterisks mark significant differences between wild-type and transgenic plants ($P = 0.001$, *t*-test). The figure shows one representative experiment from a series of six independent experiments; $n = 15$ plants per treatment; mean values ± s.e.

and they recovered fully thereafter (data not shown). RGR time courses of the well watered plants of both genotypes showed oscillations and a tendency to decrease with time (Fig. 3*b*). The drought stressed plants showed a strong decrease of RGR from day 24 on (Fig. 3*b*). In drought stress conditions, the decrease of RGR was more pronounced for C24 than for PARP2. The decrease of RGR led to the definition of an 'evaluation period' of three measurement intervals before RGR decreased to zero (days 25–28) that was used to calculate derived parameters explained below.

RGR of the well watered PARP2 (14.8% day^{-1}) plants was lower that that of the C24 (18.3% day^{-1}) plants during the evaluation period (Fig. 3*c*). Drought stressed PARP2 plants had an enhanced RGR (9.2% day^{-1}) compared with the stressed C24 plants (7.2% day^{-1}). 'Relative growth performances' of drought stressed plants were calculated finally by taking the ratio of the RGRs of drought stressed and well watered plants: RGR_{DS}/RGR_{WW}; (Fig. 3*d*). This ratio relates RGR determined during drought stress to RGR reached at this developmental stage by well watered plants exposed to the same set of environmental conditions as the drought stressed plants. During the evaluation period, drought stressed C24 plants showed a relative growth performance of 39.4%, whereas PARP2-plants reached a significantly higher relative growth performance of 62.3% ($P = 0.001$, *t*-test).

When all six replicate experiments were taken into account, PARP1 and PARP2 showed significantly enhanced relative growth performance compared with C24 during the evaluation period ($P = 0.014$; ANOVA; Tukey test; Fig. 4). Note well that in each replicate experiment, 15 individual plants were automatically evaluated and the relative growth performance was calculated as the ratio of the mean value of two populations. This means that for each of the three plant lines, 90 well watered and 90 drought stressed plants were taken into account (in total 540 plants).

Potential quantum yield (F_v/F_m) was determined automatically for each individual plant after a dark adaptation of at least 30 min by calculating the average F_v/F_m for the entire rosette (compare Fig. 1). F_v/F_m increased slightly in all populations during the experiment (Fig. 3*e*). During the

evaluation period, drought stressed C24 plants showed significantly higher values compared with well watered C24 plants in this experiment ($P = 0.021$, t-test), whereas the difference between drought stressed and well watered PARP2 plants was not significant.

When all replicate experiments were taken into account, F_v/F_m reached values between 0.75 and 0.76 in well watered plants of all genotypes during the evaluation period (Fig. 5). Drought stressed C24, PARP1, and PARP2 plants showed F_v/F_m values between 0.77 and 0.78 during the evaluation period. However, differences were significant neither between genotypes ($P = 0.854$) nor between well watered and drought stressed plants ($P = 0.258$; ANOVA).

Within the populations, differences between individuals were small (see Fig. 1 for a typical population at two developmental stages). The average F_v/F_m of the two most extreme individuals often differed no more than the values at the base and the tip of an individual leaf (see enlargements in Fig. 1). Individual leaves of an intermediate developmental stage (not the youngest and not the oldest leaves currently present at a given plant) often showed clear base-tip gradients of F_v/F_m. Because of these developmental gradients and because the entire rosette area was taken into account, F_v/F_m values reported here did not achieve the theoretical maximum of 0.83.

To get an estimate of the remaining water in the pots relative to the initial soil-drenching upon pricking out the plants, the pot-containing trays were weighed regularly. Hereby, the increase of weight of the plants was negligible, as the plant biomass made up less than 1% of the total weight of the tray. Weight loss of the tray was related to water evaporation and transpiration and could be taken as an indicator for decreasing water content in the soil, or – from the view of the plant – increasing drought. Relations between drought tolerance and soil water content were obtained by plotting relative growth performance against relative weight loss of plant cultivation trays (Fig. 6). The growth performance is the ratio of the growth rate of the drought stressed plants to the growth rate of well watered plants at the same age. As long as relative weight loss did not exceed ~27% of the initial mass of the cultivation tray, all genotypes showed a relative growth performance of almost 100%. Relative weight loss of more than 27% was correlated with a decrease in relative growth performance. At weight losses of 27% and more, data points of C24-plants (black circles in Fig. 6) clustered at low growth performances. Data points of PARP2 (white triangles in Fig. 6) clustered at higher performances and those of PARP1 (black squares in Fig. 6) lay in between. This distribution indicates that lowest relative growth performances were obtained for C24 plants, while PARP1 and PARP2 plants showed higher relative growth performances when the soil water content was reduced.

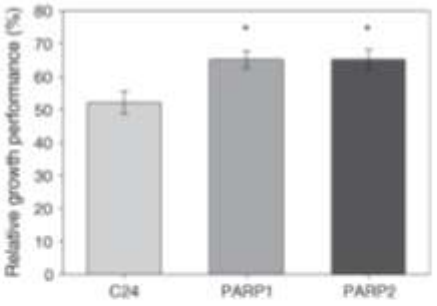

Fig. 4. Relative growth performances of drought stressed plants of *Arabidopsis thaliana* C24, PARP1, and PARP2. Asterisks mark significant differences between wild-type and transgenic plants ($P = 0.014$; ANOVA; Tukey test); $n = 5$–6 independent experiments (15 plants per treatment in each experiment); mean values ± s.e.

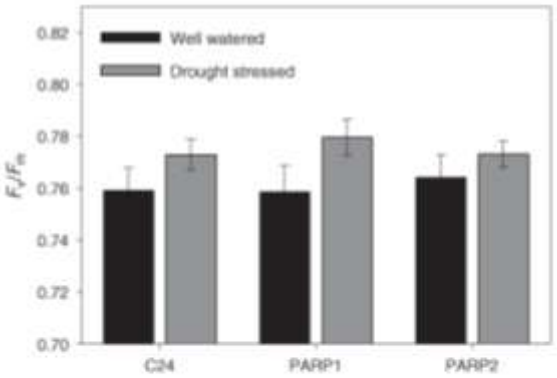

Fig. 5. Potential quantum yield of PSII (F_v/F_m) of *Arabidopsis thaliana* C24, PARP1 and PARP2. Chlorophyll fluorescence of the drought stressed and the well watered populations was assessed at the end of the drought stress evaluation period; $n = 3$ independent experiments (15 plants per treatment in each experiment); mean values ± s.e.; no significant differences between treatments ($P = 0.258$) or genotypes ($P = 0.854$; ANOVA).

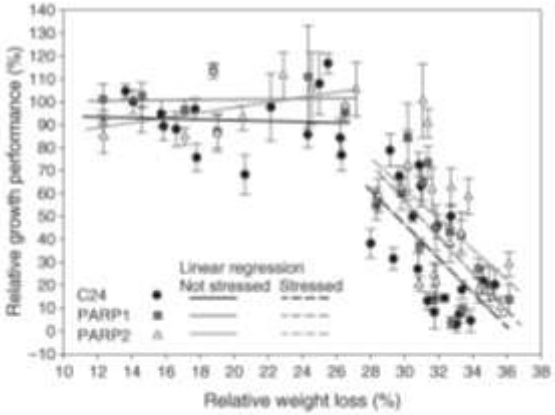

Fig. 6. Relative growth performance of drought stressed *Arabidopsis thaliana* C24 (black circles), PARP1 (grey squares), and PARP2 (white triangles) in relation to relative weight loss of plant cultivation trays. Weight loss was taken to monitor the increasing drought due to water loss (by evaporation and transpiration); $n = 4$ independent experiments (15 plants per treatment in each experiment); mean values ± s.e.

In addition to differences in performance of growth and photosynthesis, some morphological parameters characterising the shape of the rosette were analysed (Fig. 7). Three parameters calculated from the images were (i) the number of leaves per plant, (ii) surface coverage (leaf-covered area inside the convex hull of the plant), and (iii) stockiness (ratio of leaf area to the square of rosette outline).

In drought stressed plants, there was a reduced number of leaves per plant, an increase of surface coverage and an increase of stockiness, indicating reduced petiole length. In the well watered state, C24, PARP1, and PARP2 had similar numbers of leaves per plant, whereas under drought stress, PARP2 tended to have more leaves than the other genotypes; however,

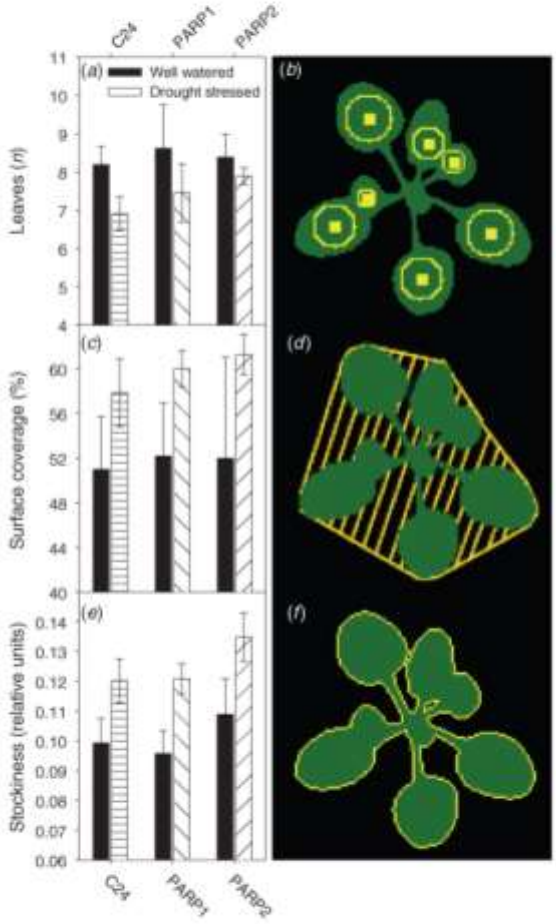

Fig. 7. Morphologic parameters of *Arabidopsis thaliana* C24, PARP1 and PARP2 in well watered and drought stressed conditions. (*a*, *c*, *e*) Parameter values of well watered and drought stressed plants at the end of the drought stress evaluation period. (*b*, *d*, *f*) Images of a representative plant. (*a*, *b*) Number of leaves per plant (yellow circles with dots). (*c*, *d*) Surface coverage [proportion of leaf area (green) inside the plant's convex hull (yellow dashed area)]. (*e*, *f*) Stockiness [ratio of leaf area (green) and square of rosette outline (yellow line)]; n = four independent experiments (15 plants per treatment in each experiment); mean values ± s.e.

this effect was not significant (ANOVA, $P = 0.448$). Surface coverage was identical for well watered plants of all genotypes. In drought stress, both PARP1 and PARP2 tended to have enhanced values compared with C24 (ANOVA, $P = 0.220$). The stockiness of PARP2 plants was higher than that of PARP1 and C24 in both well watered and drought stressed populations (ANOVA, $P < 0.050$), indicating slight differences in leaf shape.

Dynamic reaction of C24 and PARP1 in chilling stress

As PARP-lines were reported to perform better than wild type plants under a wide range of stress conditions (high light, drought, and heat), their performance was also tested in a second stress situation that acts on a different temporal scale and that was not tested hitherto. Although drought stress develops gradually in the field, chilling stress can start abruptly. When C24 and PARP1 plants were transferred to 5°C, RGR decreased to almost zero immediately, and F_v/F_m decreased more gradually (Fig. 8). For RGR, no pronounced differences between the two genotypes were obtained. F_v/F_m decreased faster in PARP1 compared with C24. Although at day 18, PARP1 already showed values around 0.70 which remained on this level throughout the next 5 days, F_v/F_m of C24 chilling-treated plants was comparable to that of control plants and only decreased to 0.72 until day 23.

Stress detection in N. tabacum

The reaction of growth and chlorophyll fluorescence to different abiotic stress situations was also evaluated for *N. tabacum*. In drought stress, a similar pattern as in *A. thaliana* emerged: although leaf growth of drought stressed wild-type plants decreased some days after water was withheld, F_v/F_m rather increased without deviating markedly from the values of well watered plants (Fig. 9). In chilling stress, a similar reaction pattern as in *A. thaliana* was observed: leaf growth declined rapidly to a constant RGR close to zero, whereas F_v/F_m showed a gradual, almost linear decline in stressed plants.

For *N. tabacum*, a third type of stress was applied throughout the ontogeny of young plants: plants cultivated under ultraviolet (UV)-B-transparent greenhouse cladding were compared with plants grown under a standard greenhouse cladding, which is almost opaque to UV-B light. Here, in contrast to the situations in drought and chilling stress, growth did not differ significantly between the two populations (Fig. 10*a*). Moreover, after a dark adaptation of 30 min, F_v/F_m showed no significant differences between plants grown at low and high UV-B transmittance (first data point in Fig. 10*b*). Yet, effective with the onset of actinic light of 500 µmol m^{-2} s^{-1}, plants grown at high UV-B exhibited a higher ΔF/Fm' (effective quantum yield of PSII) compared with plants grown at low UV-B. A similar and even more pronounced effect was found for non-photochemical quenching (NPQ): NPQ was significantly increased by up to 30% in plants exposed to high UV-B compared with plants grown at low levels (Fig. 10c, $P = 0.05$, *t*-test). This analysis demonstrated the applicability of the device for automated measurements of chlorophyll-fluorescence-related parameters other than F_v/F_m. Thus, it shows a third combination of reaction patterns of growth and

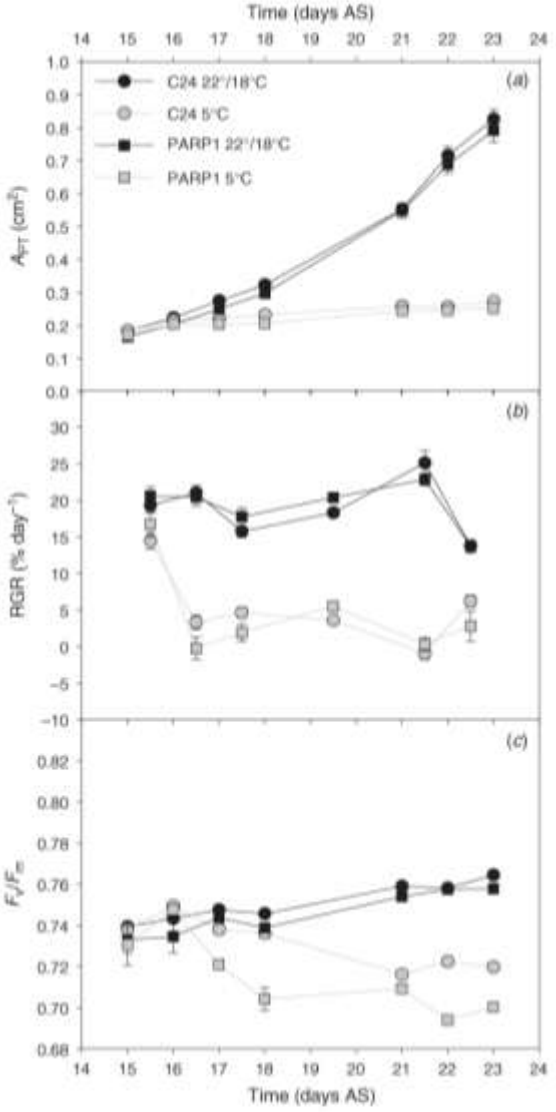

Fig. 8. Reactions of *Arabidopsis thaliana* C24 and PARP1 to chilling stress. (*a*) Total projected leaf area (A_{PT}). (*b*) Relative growth rate (RGR). (*c*) Potential quantum yield of PSII (F_v/F_m). The experiment was repeated with similar results; $n = 15$ plants per treatment; mean values ± s.e.

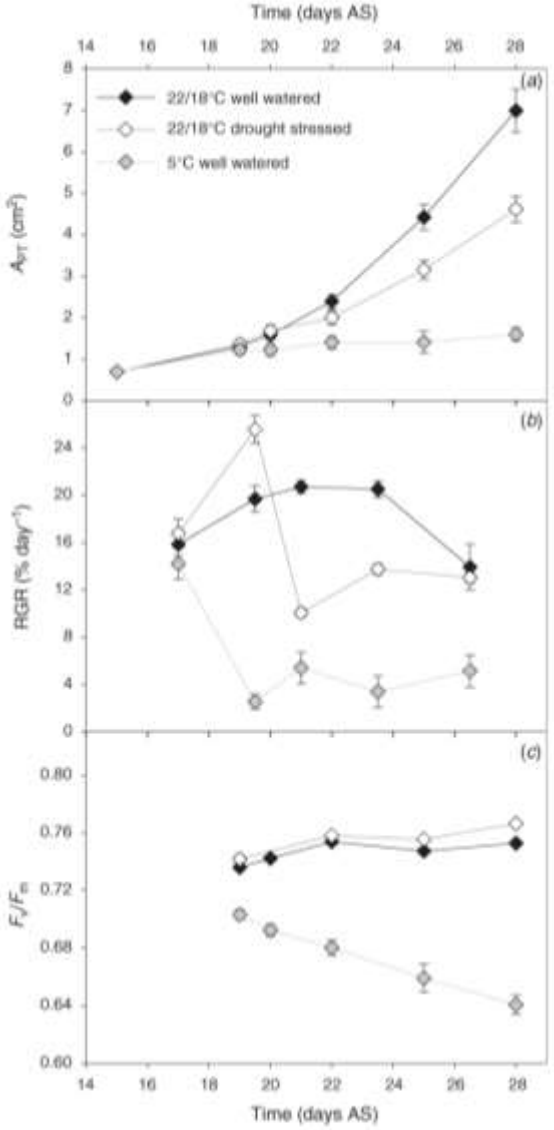

Fig. 9. Assessment of stress reactions in *Nicotiana tabacum*. (*a*) Total projected leaf area (A_{PT}). (*b*) Relative growth rate (RGR). (*c*) Potential quantum yield of PSII (F_v/F_m). The experiment was repeated with similar results; $n = 20$–30 plants per treatment; mean values ± s.e.

photosynthesis: no growth reaction, but a clear reaction in chlorophyll-fluorescence-related parameters.

Discussion

Growth and photosynthesis were affected differently by the three stress situations investigated in this study. First, drought stress reduced stomatal aperture, not leading to photoinhibition, i.e. F_v/F_m values remained unaffected. Only when very severe drought stress was imposed, were chronic photoinhibition and an effect of F_v/F_m evoked (Baker and Rosenqvist 2004). When this was detected, leaves were often lethally damaged (Woo *et al.* 2008). Growth (and crop yield) was affected more directly already in situations of moderate drought stress. Although this is a triviality that holds true for each field crop, it is difficult to quantify the reduction in growth under typical laboratory situations and it is difficult to simulate the complex syndrome of drought stress for model plants (Granier *et al.* 2006). Here, it was possible to elaborate a very simple and

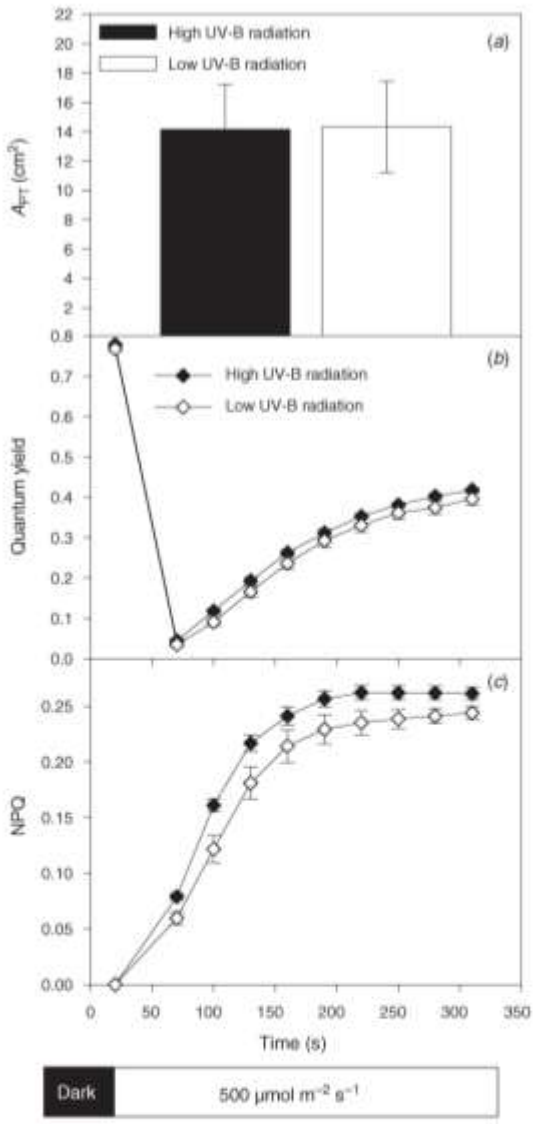

Fig. 10. Effect of light quality on leaf growth and chlorophyll fluorescence related parameters in *Nicotiana tabacum*. (*a*) Total projected leaf area (A_{PT}) at the time of fluorescence measurement. (*b*) Effective quantum yield of PSII (quantum yield). (*c*) Non-photochemical quenching (NPQ) of plants grown in greenhouses with UV-B transmissible (high UV-B radiation) and UV-B opaque (low UV-B radiation) cladding, respectively, $n = 9$–10 plants; mean values ± s.e.

flexible drought-stress protocol for tiny plants of *A. thaliana* and to demonstrate the superiority of PARP-lines compared with C24 in this situation (Figs 3–6). Only via highly sensitive growth analyses applied to a meaningful number of replicates, was it possible to demonstrate the growth effect in an automated and rapid way. Although the application of GROWSCREEN FLUORO is restricted to rosette-like developmental stages of dicot species, it is important to elaborate such a technique as a first step to joint analyses of growth and photosynthesis in crop species. On the one hand, investigations of mutants, transgenic approaches and of effects of chemical additives in the model species *A. thaliana* will elucidate principles helpful to improve the performance of crop plants. On the other hand, experiences gathered with this technology will help to devise more powerful solutions for stress detection and improved breeding pre-selection able to characterise growth and photosynthesis also in upright, often distorted leaves of monocot crop species and in more complex and self-occluded canopies of later stages of plant development.

Drought stress

The reaction mechanism altered in PARP-deficient plant lines provides more energy to the stressed plant on cost of a higher risk of DNA-damage. In *A. thaliana* and *B. napus*, it was reported that ROS-induced DNA-damage and cell death increases, although NAD^+ and ATP are increasingly available to the plant (De Block *et al.* 2005; Vanderauwera *et al.* 2007). This advantage of PARP-deficient lines over wild-type lines was clearly confirmed in our experiments by applying the flexible concept of the growth-dependant evaluation period. Moreover, the experiments clearly demonstrated a gradual and almost linear reaction of relative growth performance to soil drying (Fig. 6).

The time-course of F_v/F_m (Fig. 3*e*) revealed that F_v/F_m-values increased with plant age but did not differ between lines, confirming that F_v/F_m is only affected by severe drought stress. Although young plants exclusively consist of immature leaves showing low F_v/F_m values, older plants represent a mixture of mature and immature leaves. Older leaves, in turn, have higher F_v/F_m-values than young leaves (Walter *et al.* 2004). The observation of slightly increased F_v/F_m in drought stressed plants might correspond to their reduced growth, which, in turn, leads to an earlier leaf differentiation and to a higher fraction of leaf material with F_v/F_m values typical for full-grown leaves. This is underlined by the fluorescence images of a plant tray at two points in time and by the time course of one of these plants (Fig. 1). Here, younger plants show a higher fraction of 'green' pixels (encoding for low F_v/F_m) compared with older plants. Within individual rosettes, youngest leaves in the centre of the rosette typically show lower values than older leaves; sometimes even within single leaves clear gradients are apparent. It would exceed the scope of this manuscript to interpret and discuss these gradients in detail but the depiction of gradients of F_v/F_m in a large number of plants at different developmental stages provides a useful analytical tool in itself, which can be exploited in future studies.

Analysis of morphological parameters revealed that drought not only decreased plant size but also the number of leaves per plant (Fig. 7). In addition, drought stressed plants showed a somewhat more compact growth habit than well watered ones, which can be quantified via surface coverage and stockiness. Increased values of drought stressed plants are related to shorter leaves, a more stunted growth habit and a higher

fraction of leaf overlap. Again, the possibility to quantify such differences between phenotypes provides a powerful tool for future analyses of a variety of plants.

Chilling stress

Chilling stress affects all plant processes in a very direct way (Pearce 1999), and also decreases F_v/F_m (Öquist and Huner 2003; Ehlert and Hincha 2008). On the background of a generally reduced metabolism, it is expected that in chilling stress, an immediate, harsh growth reaction was observed (Figs 8, 9). In contrast to the drought stress reaction, F_v/F_m decreased following the onset of stress, but the reaction was slower compared with the growth reaction. Within one week, F_v/F_m decreased by only ~10%. Moreover, F_v/F_m decreased faster in PARP1 than in C24. This indicates that ROS-induced damage, which is less prevented in PARP-lines compared with C24, can act faster on the photosynthetic machinery in PARP1 compared with C24. At the same time, PARP1-plants invest less energy than C24 plants to avoid detrimental effects of stress. Therefore, the energy 'saved' by PARP-plants might be used to metabolise storage compounds important for survival of the plants if chilling stress would persist. This situation may be comparable to sustained downregulation of photosynthetic efficiency observed under low temperature (Adams *et al.* 1995; Gilmore and Ball 2000).

UV light effect

Deleterious crop reactions induced by increased UV-B have been discussed intensely in the context of stratospheric ozone depletion, coming to the overall conclusion that crop yield, plant growth and photosynthesis are not markedly affected by this kind of stress (see reviews by Fiscus and Booker 1995; Allen *et al.* 1998). Yet, increased UV-B increases the production of various secondary metabolites such as flavonoids playing a role against UV-damage (Rozema *et al.* 1997). This, in turn, is a desirable feature for horticultural plants cultivated in greenhouses. There, cladding material is typically opaque for UV-B, leading to less intensely coloured plants and to vegetables containing less valuable metabolites than field-grown plants. Hence, plant reaction was monitored here under an illumination almost exempt of UV-B, simulating an environmental scenario that should not lead to a significant decrease of growth, but to an altered way of dealing with the intercepted light (Tsormpatsidis *et al.* 2008). In this scenario, it was shown that leaf growth was not affected, but non-photochemical quenching and photosynthetic yield were reduced at low UV-B. Hence, the detected reaction pattern might be a valuable proxy for the production of secondary metabolites such as anthocyanins, carotenoids and flavonoids, which are known to differ between plants exposed to different intensities of UV-B. Because of the time needed to adapt plants to light, analysis of yield and NPQ in an automated manner requires more time per plant and the throughput of GROWSCREEN FLUORO is lower compared with the analysis of F_v/F_m (10 *v.* 60 plants h^{-1}). Nevertheless, the experiments clearly demonstrate the capability of the approach to analyse also more complex parameters of chlorophyll fluorescence and to detect small differences between populations by increasing the experimental throughput.

Methodological advances

The approach to analyse growth and chlorophyll-fluorescence-related parameters at the same time has been realised before (Barbagallo *et al.* 2003), but not in a way that allows plant cultivation in meaningful environmental scenarios. Barbagallo *et al.* (2003) used microtiter plates to cultivate plants (200 µL Murashige-Skoog-medium per plant), but the plants of our experiments were grown in soil-filled pots. The latter provided much higher experimental flexibility than other studies, as rosettes of diameters up to 10 cm can be analysed, the method will be useful not only for *A. thaliana*, but also for other plants and seedlings of several crop and model species, as has been demonstrated here with *N. tabacum*. As single images are taken of each plant, the resolution is sufficient for a detailed analysis of morphological parameters such as rosette shape, leaf number, individual leaf size and for the heterogeneity of chlorophyll-fluorescence related parameters. The degree of automation reached here allows for dynamic measurements; a feature that has been crucial for the establishment of the evaluation period for drought-stress detection.

Conclusion

Abiotic stress can act on plant growth and photosynthesis in several ways. Methods to decipher the plant stress reaction are urgently needed and should ideally combine the potential for a range of phenotypic parameters to be detected. In this study, we assessed the effect of three different stress scenarios on *A. thaliana* and *N. tabacum* plants with a single methodology that allowed for a relatively high throughput and that revealed characteristic features of the different stress types. PARP-lines indeed proved to be more stress resistant than the wild type when exposed to moderate drought stress. Furthermore, plants exposed to chilling stress and to an altered spectral composition of ambient light also showed clear differences between treatments with respect to growth and chlorophyll fluorescence.

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4 Synopsis

It was the aim of this study to investigate the influence of different light quality on biomass accumulation and growth behaviour of horticultural and model plants under varying UV-B levels. The first part of the project was conducted under greenhouse conditions using innovative cladding materials with high transmission in the UV-B (86%) and visible spectra, which allows omitting any additional UV-B source. Horticultural plant species showed a reduction in leaf area and biomass in response to UV-B, while tobacco plants did not show such a reaction. To elucidate these unexpected differences found under greenhouse conditions, experiments under controlled conditions in exposure chambers were performed. The exposure chambers of the Helmholtz Zentrum Munich provide a near-natural relation between UV-B and photosynthetic active radiation (PAR).

The non-invasive determination of biomass accumulation was investigated using a new developed method *via* microwave resonator. The development of this instrument and the establishment of this method was a central part of my PhD (Menzel et al. 2009). Under constant environmental conditions tomato and tobacco, the two investigated species, showed a different biomass accumulation pattern in response to UV-B radiation. A short time period was sufficient to induce a reduction in biomass for tomato plants but a longer duration of exposure was necessary to increase the biomass in response to UV-B in tobacco. It was found that tobacco plants lost 40% of the fresh weight each day *via* transpiration compared to 10% for tomato plants. These findings suggest that the water balance of the plant system is more important than the applied UV-B radiation. It seems that the effect of UV-B as stressor is lower compared to the influence of the plant water balance (Tittmann et al. submitted)

5 List of abbreviations

A	Net assimilation rate
BBCH	Federal Agency, Federal Office of Plant Varieties and Chemical Industry; german: **B**undesanstalt, **B**undessortenamt und **CH**emische Industrie
BMBF	Federal Ministry of Education and Research german: **B**undes**m**inisterium für **B**ildung und **F**orschung
CHS	Chalcone synthase
COP1	Constitutively photomorphogenic1
CPD	Cyclobutan pyrimidine dimer
Cry1	Cryptochrome1
Cry2	Cryptochrome 2
D1	Core protein of photosystem II
DISP	Digital imaging sequence processing
DNA	Desoxy ribulose acid
Dw	Dry weight
E	Transpiration rate
ETFE	**E**thylene-**T**etra**f**luoro**e**thylene
Fw	Fresh weight
F_v/F_m	Optimal quantum efficiency
HY5	Elongated Hypocotyl5
LIDAR	LIght Detection and Range
NER	Nucleic excision repair
PAL	Phenylalanine-ammonia lyase
PAR	Photosynthetic active radiation
PSII	Photosystem II
R:FR	Red: Far-red ratio
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Rubisco	Ribulose – 1,5-biphospphate carboxylase
UV-A/UV-B/UV-C	Ultraviolet –A; B Ultraviolet; Ultraviolet-C radiation
UVR8	Ultraviolet resistance locus8
2D	Two dimensional

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