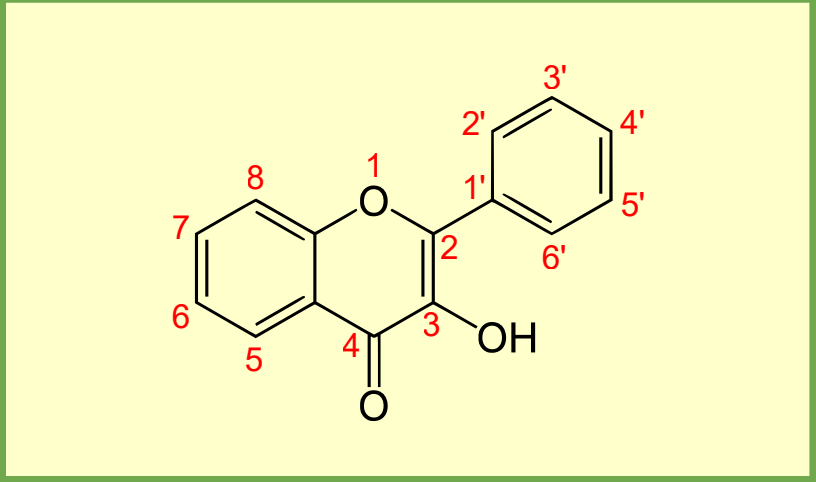
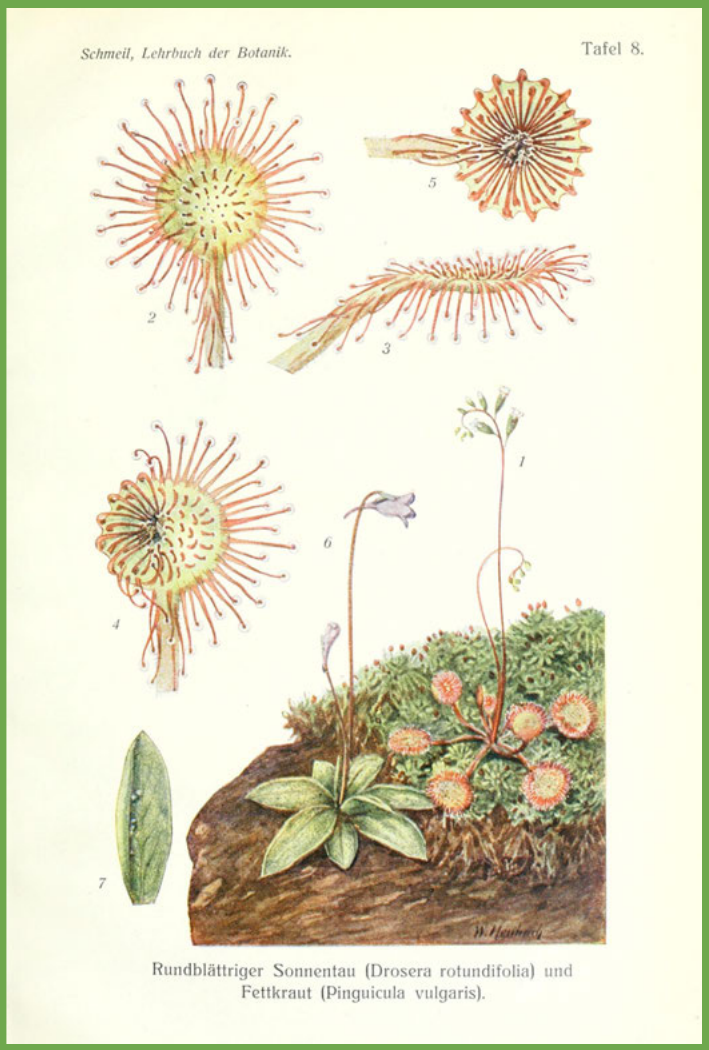
UV4Plants Bulletin 2017: 1

Brno, Czech Republic

UV4Plants workshop: *Modulation of plant UV-responses by environmental factors*
June 27 and 28, 2017





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Cover illustrations
Top: panorama of Brno, Czech Republic (Image: P. J. Aphalo). Middle left: Participants in the Brno workshop (Image: CzechGlobe). Lower left: Flavonol skeleton (Image: public domain, from Wikimedia). Lower right: Page from book Quelle & Meyer (1911) "Lehrbuch der Botanik", Leipzig (Image: public domain, Image courtesy of BHL, http://biodiversitylibrary.org).

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Contents

From the editors' desk

A moment of reflection

Welcome to the first issue of the 2017 *UV4Plants Bulletin*. When I first started thinking about this editorial it was the middle of summer. While it is now autumn for those of us living in the northern hemisphere, the weather here in New Orleans is still very much like summer. So, please bear with me as my mind is still very much on summer time activities.

Traditionally, summer is a time when schools are out and families go on vacation or holiday, people travel or spend time on the beach (sunscreen reminder seems appropriate here), and we have some degree of change in our normal daily routines. For many faculty and graduate students, summer is a season of long periods of uninterrupted time that can be devoted to research activities and, at least in northern temperate locations, ecologists are furiously trying to complete field work before inclement weather arrives and study organisms decide it is time to stop cooperating with their investigators. In New Orleans, summer is a time when life generally slows down as the heat, humidity and afternoon thunderstorms settle in and it becomes less than comfortable to pursue vigorous outdoor activities. Thus, in this part of the world, summer is a good time to relax, eat and drink good food and beverages, and ponder (Figure 1.1).

So, in this spirit I've been reflecting on the *UV4Plants Bulletin* and wondering what we seem to be doing well and in what areas we might improve upon (I decline to mention what food and drink are being consumed during this process). Beginning with its inception in December 2015, the "Bulletin" has continued to support six key aims of The International Association for Plant UV Research. These aims are itemized at the end of each issue and won't be reiterated here but in general, one can place these goals into the following broad categories or topics: 1) research; 2) news; 3) outreach; 4) product development; 5) funding; and 6) community building. After reviewing the contents of the previous Bulletins, it seems to me that we are doing a good job addressing the research, news and community building parts of our mission. The vast majority of articles published so far have focused on research (including historical accounts, profiles of UV researchers and opinion pieces), methodology (including tutorials and FAQs), book reviews, news items and information on previous and upcoming meetings. So far, a total of 18 individuals have contributed to the Bulletin, including faculty and students, and the list of new contributors grows with each issue. In my view, these are all positive signs that the Bulletin is vibrant, relevant and serving an important role in supporting many of the original aims of our association. However, it does seem that we could improve upon our efforts in communicating ideas and information in several areas—most notably, outreach and education, interfacing with industry and product developers, and advocating for increased research funding.

In the current issue, several articles address some of these "under-represented" topics while others further add to, and enhance, our current strengths. The article by Gareth Jenkins is a wonderful example of the development of a teaching lab that introduces students to molecular aspects of plant responses to UV-B, while also engaging students in the process of scientific discovery. Many of us are involved in the teaching of undergraduate and graduate students, and we encourage others who have developed

Figure 1.1: A sampling of typical New Orleans cuisine that is quite suitable to ponder over. (A) Shrimp and grits; (B) Bread pudding; (C) Char-grilled oysters; and (D) Gumbo. Photos by Andrew Barnes.

educational materials in UV photobiology to submit them to the Bulletin for publication. Marcel Jansen explores the linkages between carnivorous plants and UV radiation in a thought-provoking opinion. Carnivorous plants have fascinated naturalists and scientists as far back as Charles Darwin (1875) and they provide a wonderful way to attract and excite students and the general population about botany and, in so doing, increase people's appreciation for the plant world and those who study plants. The outreach and education about UV and plants is further addressed in Pedro Aphalo's review of David Prutchi's book *Exploring Ultraviolet Photography*. This integration of art with science is an additional avenue for UV photobiologists to connect with non-scientists, which then further contributes to the broader efforts to make science relevant and valued in today's society where "fake news" and misinformation are widespread. A methodological review/tutorial on the analysis of phenolic compounds by Susanne Neugart provides a comprehensive overview of technical approaches to sampling and quantifying phenolic compounds in plants and addresses, from a methodological perspective, the various environmental and biological factors that can influence the phenolic composition of plant tissue. The methods and recommendations outlined in this review will greatly aid all of us who wish to accurately and precisely characterize UV effects on flavonoids and related phenolic compounds. A report by Marcel Jansen on the Workshop "Modulation of Plant UV-Responses by Environmental Factors" held at Brno, Czech Republic, two reports on lab visits by students (Neha Rai, Sari Siipola and Yan Yan, and Rozenn Pineau), several news items and a letter from our President, Gareth Jenkins, round out this issue.

So, enjoy this mid-summer issue of the UV4Plants Bulletin and take a moment or two to ponder things of your own (It was grilled shrimp and red wine if you are interested).

Paul W. Barnes (editor)

New Orleans, August–October 2017.

References

Darwin, C. (1875). *Insectivorous plants*. London: John Murray. 462 pp.

Letter from the President

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When I was a student I thought I should try to understand economics. It was only a brief flirtation. I tried hard to read a few books, but economics seemed more about theory, politics and personalities than evidence, which didn't fit with my scientific outlook. However, I was attracted by some of the ideas in E. F. Schumacher's famous book on economics, Small is Beautiful, which took a swipe at the globalisation strategy of multinational conglomerates and advocated locally focused, sustainable, people-orientated economics. The reason I mention this here is that 'small' and 'beautiful' perfectly describe UV4Plants and I think Schumacher would have approved of our ethos. Unequivocally our association is relatively small, because the focus of our interest is tightly defined and our membership is largely drawn from European research groups. Nevertheless, I think we can be confident about the future strength of our organization because numbers of publications and citations show there is considerable, and growing, interest in the effects of UV radiation on plants. Of course, 'beauty is in the eye of the beholder', but there is certainly good reason to think that UV4Plants is 'beautiful'. Our aims are well defined, we try to serve the needs of our members and we don't have ambitions to change our scope. Schumacher would commend us for being focused. Moreover, Schumacher put people at the heart of his economic ideas and UV4Plants is very much focused on its membership. We have a core of interactive, collaborative members, and, being small, we can promote a 'family' identity to our organization. Consequently our meetings are enjoyable and productive and provide an excellent environment to introduce young researchers to the benefits of scientific interaction. This is well illustrated by the UV4Plants sessions we recently had in Pisa. They were very well attended and provided a great opportunity for networking and discussion. We assembled two very interesting programmes and there was the added opportunity to attend relevant ESP sessions and to experience the delights of Pisa (not least authentic Gelato!). In addition, we have recently announced that the next UV4Plants Congress will be held in Bled, Slovenia, in April 2018, and that promises to be a great 'family reunion'. Several members will remember the EU COST Action meeting we had in Bled in 2014, so we know it is a very attractive location and that our local organizer, Alenka Gaberščik and her team will organize a great meeting.

Back to economics; in order to organize conferences and provide bursaries to facilitate the attendance of younger researchers, UV4Plants needs income, which comes principally through members' subscriptions. Moreover, it is much easier to plan ahead if we have a consistent, predictable income. Our Treasurer, Matthew Robson, recently e-mailed a reminder to everyone to renew subscriptions for 2017. Please subscribe every year, even if you are not attending one of our meetings. Also, please encourage PhD students, postdocs and others to join UV4Plants;

there are numerous UV researchers in Europe who are not members and it would be good if we could engage their commitment.

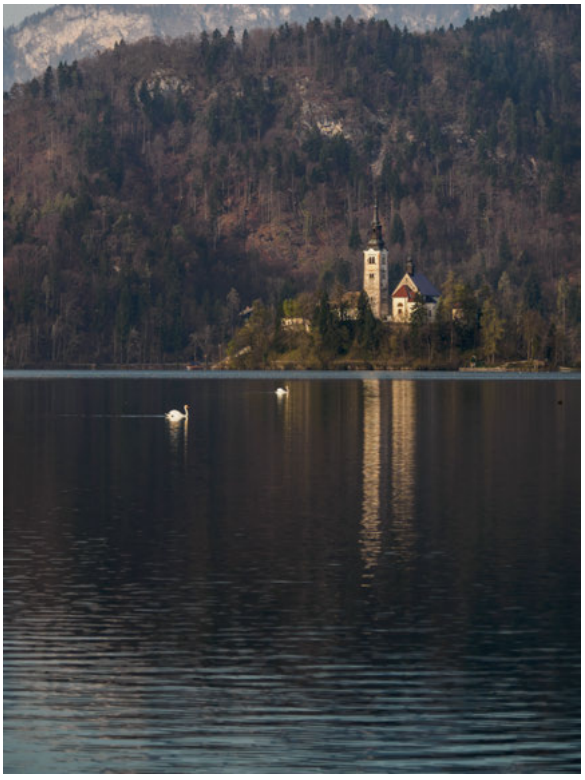
Best wishes,

Gareth Jenkins
(President UV4Plants)

News

The second UV4Plants conference is planned for April 2018

The *2018 UV4Plants Conference*, will be held in Bled, Slovenia. Provisional dates: 15–18 April, 2018. To be preceded by a Training Workshop. Local organiser: Alenka Gaberščik. The First Announcement will appear later this year.

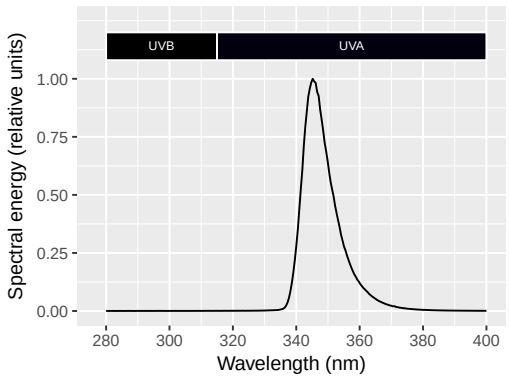


Bled, Slovenia in April. The venue of the *2018 UV4Plants Conference*.

New 340 nm power LEDs from Marktech

Not only these new LEDs from Marktech Optoelectronics (Latham, NY, USA) emit more radiation than earlier available power LEDs emitting at this same wavelength, but they are a lot cheaper (49 € vs. 4 400 €). Type is MTSM340UV-F5120, peak wavelength 340 nm, half band width 11 nm, radiant power 55 mW, at a typical power dissipation of 2.15 W.

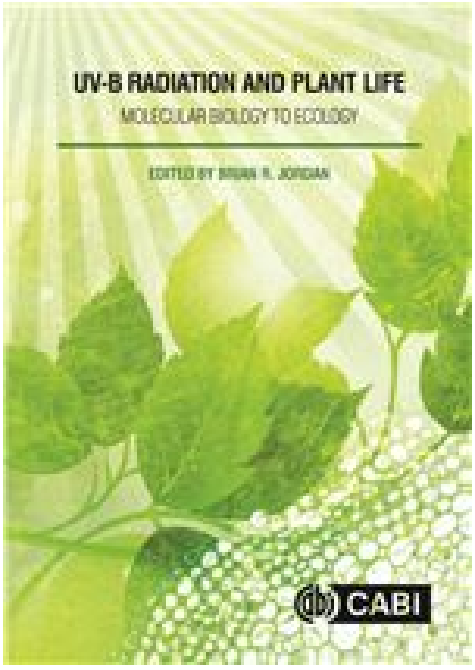
An UV-A LED type MTSM340UV-F5120, from Marktech Optoelectronics.



Emission spectrum from UV-A LED, type MTSM340UV-F5120, from Marktech Optoelectronics. Measured with a Maya2000 Pro spectrometer. Data and figure by P. J. Aphalo.

Efficiency and output of these LEDs is still far from that of the best power LEDs emitting at 365 nm, such as type LZ1-10UV00 (radiant power 1200 mW at a power dissipation of 2.7 W) from LED Engin (Jan Jose, CA, USA).

Book: UV-B Radiation and Plant Life



Publication of the book "UV-B Radiation and Plant Life: Molecular Biology to Ecology", edited by Brian R. Jordan, is scheduled for October 2017. The publisher is (CABI Centre for Agriculture and Biosciences International), a not-for-profit organization. Several members of UV4Plants have authored or co-authored chapters. The book's web page is at http://www.cabi.org/bookshop/book/9781780648590. From the table of contents we can see that the book covers, as promised by its subtitle from molecular biology to ecology of terrestrial plants.

Part 1: The UV-B Environment

1. Towards an Understanding of the Implications of Changing Stratospheric Ozone, Climate and UV Radiation
2. Quantification of UV Radiation
3. UV Radiation and Terrestrial Ecosystems: Emerging Perspectives

Part 2: UV-B Induced Changes to Plant Physiology, Morphology and Secondary Metabolism

4. UV-B Changes in Secondary Plant Metabolites
5. UV-B Induced Morphological Changes — an Enigma
6. Plant Responses to Fluctuating UV Environments

Part 3: The Biochemistry and Molecular Biology of UV-B responses

7. The Effects of UV-B on the Biochemistry and Metabolism of Plants
8. Discovery and Characterization of the UV-B Photoreceptor UVR8
9. UV-B Signal Transduction from Photoperception to Response

Part 4: UV-B Impact on Agriculture and Horticulture

10. The Effects of Ultraviolet-B on *Vitis vinifera* — How Important is UV-B for Grape Biochemical Composition?
11. Turning UV Photobiology into an Agricultural Reality

The publisher's description of the book at http://www.cabi.org/bookshop/book/9781780648590 is:

> Ultraviolet-B radiation (UV-B) has profound effects on plant growth and development, and exposure varies with ozone depletion and across geographic regions, with ecosystem and agricultural consequences. This book deals with large-scale impacts but also how UV-B affects plants at the molecular level is also fascinating, and the UV-B photoreceptor has only recently been characterised. While UV-B radiation can be damaging, it also has a more positive role in plant photomorphogenesis. Consequently UV-B treatments are being developed as innovative approaches to improve horticulture. This book is a timely synthesis of what we know and need to know about UV-B radiation and plants.

A review will be published in a future issue of the Bulletin.

■ Organizers' report

Workshop "Modulation of plant UV-responses by environmental factors"

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DOI: 10.19232/uv4pb.2017.1.01

A group of 21 plant UV-researchers came together on June 27 and 28, 2017 for a discussion-intensive workshop at CzechGlobe in the south Moravian town of Brno in the Czech Republic. The workshop was organised by Drs. Otmar Urban (CzechGlobe—Global Change Research Institute, Czech Republic) and Marcel Jansen (University College Cork, Ireland) under the auspices of UV4Plants, with sponsorship by the Czech Ministry of Education (grants LO1415 and CZ.02.1.01/0.0/0.0/16_013/0001608) and Science Foundation Ireland (grant 11/RFP.1/EOB/3303). The workshop brought together a very nice mix of "young" and "not-so-young" researchers from 10 countries, and included some familiar faces, as well as researchers new to the UV community. Brno was a very appropriate host-town, being the place where Gregor Mendel did most of his pioneering research on pea genetics. Indeed, in the evening of the 27th of June we visited Mendel's old monastery for a guided tour learning, among others, about the great man's poor track record in passing exams, as well as his lesser known research on plant taxonomy, meteorology (tornados and their geometry) and honey-bee crossbreeding (Figure 4.1), and his banking activities arising from his position as abbot of Augustinian monastery. It was also good to see Mendel's original glasses, so well-known from the portraits.

Figure 4.1: Building where Gregor Mendel used to keep his bee hives. Old Augustinian Monastery, Brno. Photo: Pedro J. Aphalo.

The objective of the workshop was to bring together plant scientists with an interest in cross-talk between UV-B and other environmental drivers. Over the last two decades, extensive data have been generated on plant responses to UV-radiation. Experimental set-ups vary, but in general plants are kept under near-optimal conditions, in the field, glasshouse or growth room, where they are exposed to supplemental UV radiation. However, a more environmentally realistic situation is where plants are simultan-

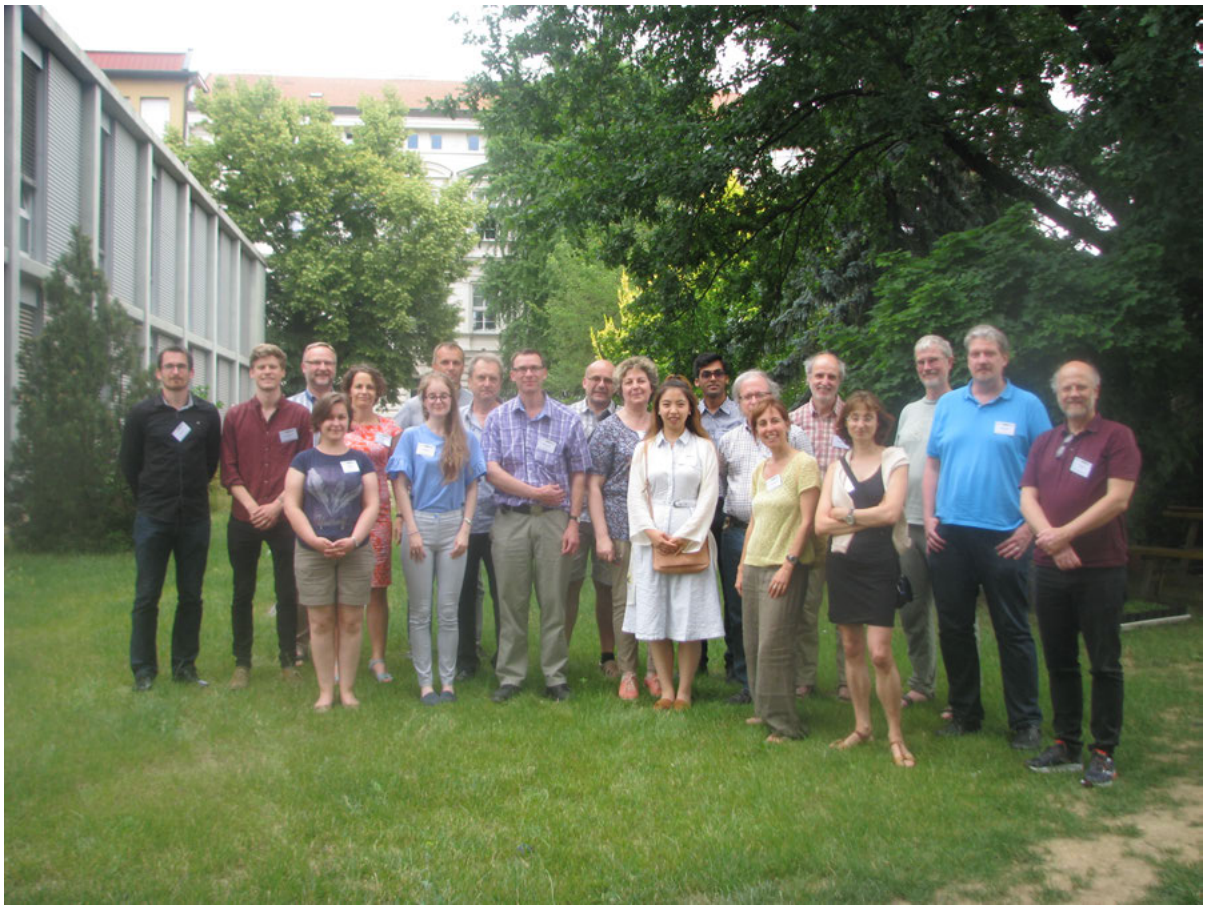

Figure 4.2: Workshop participants at the grounds of the Old Augustinian Monastery, Brno.

eously exposed to multiple environmental signals and/or stressors. In nature high levels of UV-B radiation are commonly accompanied by high levels of photosynthetic radiation (i.e. risk photoinhibition), while heat and drought are also likely to be relevant under such conditions. Appropriately, several presentations focused on the interaction between UV-B and drought. Presentations by Laura Llorens (Girona) and Anikó Mátai (Pécs) demonstrated interactive effects of UV and drought on plant morphology, carotenoids, flavonoids, total antioxidant activity, and photosynthetic energy dissipation. Regulation of flavonoid accumulation, in particular, seems to be a target of interactive effects of UV-B and other environmental factors. Dirk Schenke (Kiel) revealed some of the highly complex interactions between UV-B and pathogens, with pathogens suppressing the UV-induced accumulation of flavonoids, while a role for flavonoids in pathogen suppression is being explored. Karel Klem (Brno) showed that flavonoid levels are associated with tissue C:N ratios, and thus ultimately soil conditions. Wolfgang Bilger (Kiel) emphasised the role of low temperatures in controlling flavonoid-mediated UV-screening by epidermal cells. In agreement, Marcel Jansen (Cork) showed that accumulation of flavonoids in *Arabidopsis* grown outdoors peaks under low winter temperatures, with no discernible solar UV-effect noted. Line Nybakken (Ås) showed that higher temperatures were associated with lower concentrations of phenolic compounds. Furthermore, these studies also showed the full complexity of interactions between temperature

Figure 4.3: Craig Brelsford (Helsinki) giving his presentation to an attentive audience.

and UV-B with consequent effects on secondary metabolites, plant growth and phenology. Thus, although the induced accumulation of flavonoids is one of the "classic" plant UV-B responses, several presentations revealed how interactions with "other" environmental factors can moderate, or completely mask, UV-induced flavonoid accumulation.

Interactions between UV-B and other parts of the solar spectrum were discussed by several authors, including Ashutosh Sharma (Bristol) who reported on the role of UV-B and UVR8 in shade avoidance, i.e. interactions with the red / far-red sensing phytochrome system. Craig Brelsford (Helsinki) had studied plant responses to blue and UV-A radiation in the dynamic light environment of forest understories, and revealed that the UVR8 mediated induction of phenolic acid derivatives can be driven by UV-A. Yan Yan (Helsinki) showed flavonoid accumulation in the epidermis induced by short UV and blue light. Jakub Nezval (Ostrava) showed that UV-A (and to a lesser extent UV-B) shielding can be induced by blue light in combination with high intensity of photosynthetically active radiation (PAR). These studies clearly show the interactions between the different spectral regions in controlling biosynthesis of plant flavonoids. Knut Solhaug (Ås) emphasised the complex accumulation patterns of secondary metabolites, and showed that some of these UV-induced compounds don't apparently contribute to UV-protection, but rather to protection against high PAR intensities.

Perhaps one of the most important reasons to explore interactions between plant response to UV-and other environmental factors is the increasing reality of climate change. Otmar Urban (Brno) reported on the interactive effects of elevated CO_2 and UV-B on photosynthetic performance of beech saplings. UV reduces the positive effects of elevated CO_2 on photosynthesis, and this would have substantial impacts on predictions of plant productivity in future climate scenario's.

A plenary lecture by Jacques Roy (Montpellier) addressed some of the limitations of our current approaches in studying interactions between multiple environmental factors. The "reproducibility crisis" and the "local truth" refer to over-standardised approaches that do not take in consideration the complexities of the natural environment. To resolve this, Roy emphasised the importance of shared international infrastructures, and large, collaborative experiments. The examples of the Montpellier Ecotron and the European infrastructure AnaEE (Analysis and Experimentation on Ecosystems) were presented and enabled us to start interconnecting UV4Plants and AnaEE research communities.

Presentations were accompanied by discussion sessions that focussed on many of the aspects presented in the talks. One major issue concerned the terminology to describe and discuss data (stress, acclimation, adaptation and especially cross-talk and cross-tolerance). Cross-talk was considered a mechanistic concept that refers to reciprocal interactions whereby two streams of information (signalling pathways) influence each other. Cross-tolerance is an outcome, whereby a cell or organism that has gained protection against one environmental factor is also more tolerant towards another factor. The assembled group aims to draft a dis-

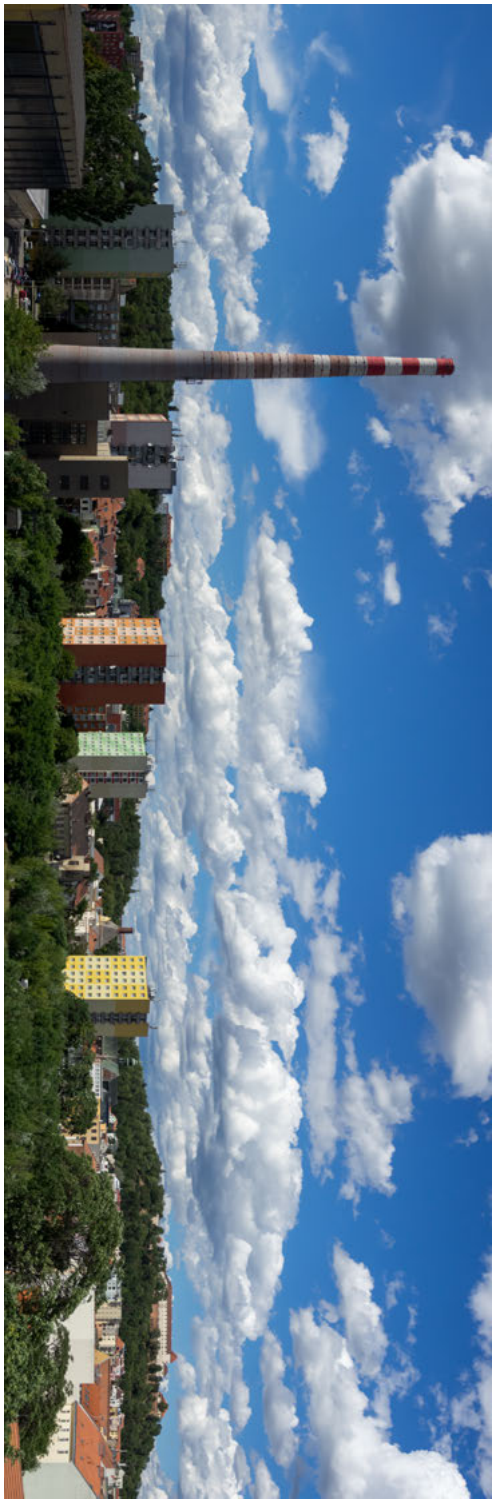

Figure 4.4: Panoramic view of Brno and its castle from the roof of Czech Globe. Photo: Pedro J. Aphalo.

cussion paper detailing the issues and putting forward appropriate terminology. It is intended to publish this discussion paper as part of a special issue of the journal Plant Physiology and Biochemistry, focussed on the theme of "Modulation of plant UV-responses by environmental factors". UV-researchers interested in contributing a paper please contact Marcel Jansen on mailto:brno17.papers@uv4plants.org.

A central question in the discussion was whether the UV-sensing capability of plants is simply about UV-B protection, or underpins a more comprehensive priming of plant protective responses. It was agreed that UVR8-mediated signalling / changes to gene-expression should guide us when discussing the ecological role of UV-B / UVR8. Although there are no direct answers to this question (yet!), several contributors emphasised the regulatory role of UV-B and the commonly reported lack of UV-stress. Pedro Aphalo (Helsinki) introduced the concept of "pre-emptive cross-acclimation" to, among others, restricted water supply, and argued that a main role of UV perception by plants is to acquire advance information about changes in the environment that are correlated to UV-doses. In fact, presentations at the workshop showed that a broad range of plant environment responses (among others to drought, spectral-composition, nitrogen, temperature, carbon dioxide, and bacterial pathogens) is moderated by UV-B, and vice versa. In conclusion, it was argued by several contributors that UV-B has a ubiquitous, modulating effect on all plant-environment responses. This sweeping generalisation has not (yet) been proven, but triggers important and novel questions about the ecological function of plant UV-B sensing.

Editorial-board-reviewed article.
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Opinion

Carnivorous plants and UV-radiation: a captivating story?

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DOI: 10.19232/uv4pb.2017.1.12

Carnivorous plants are "different", and this fascinates people. The "appetite" of carnivorous plants has, on occasion, taken on mythical proportions. In "The Day of the Triffids" giant, man-eating plants go on a rampage, while in "Ice Age 3: Dawn of the Dinosaurs" a carnivorous plant gobbled up an entire mammoth. On a slightly less violent note, we have observed that carnivorous plants on our University College Cork recruitment stand help attract high school students during open days, giving us a chance to advertise our undergraduate degree in plant biology. A psychologist could probably write a PhD thesis on the fascination of humans with carnivorous plants. However, let me just say that if you are interested in these plants, you are in good company. Back in 1875, Charles Darwin wrote "Insectivorous Plants", a book focussing heavily on *Drosera sp* (sundew) (Fig. 1A). In fact, Charles Darwin was so fascinated by these carnivorous plants that he once stated that "at this present moment, I care more about the *Drosera* than the origin of all the species in the world" (Darwin 1860). I surmise that Charles Darwin's interest in carnivorous plants was inspired by his family. In fact, Dr. Erasmus Darwin, his grandfather, was already investigating how the tentacles of *Drosera* species respond to stimuli (Cheers 1992). Another carnivorous plant aficionado was Joseph Hooker (of Bentham and Hooker taxonomic fame) who as director of Kew Gardens, London, was able to collect carnivorous species from across the world. Hooker studied the digestive system of carnivorous plants and concluded that "a substance, acting as a pepsin is given off from the inner wall of a pitcher [of a *Nepenthes sp*], but chiefly after placing the animal matter in the acid fluid" (Cheers 1992). Taken together, these early observations summarise the main characteristics of carnivorous plants: their ability to capture and digest prey (insects, arthropods, and even small mammals) for the purpose of plant nourishment. In fact, there is not a lot else that the different taxa of carnivorous plants share. The nearly 600 species of carnivorous plants that occur across nine families and different taxa are not necessarily closely related. Indeed, there is strong evidence that carnivory in plants has evolved independently on at least nine separate occasions (Givnish 2014). Their trapping structures are highly diverse, and these include the sticky leaves and/or responsive tentacles (like those observed by Erasmus Darwin), pitfall traps or pitchers with digestive juices (as studied by Joseph Hooker), hinged-trapping leaves in *Dionaea sp* (Venus fly trap) and bladder-traps in *Utricularia sp* (bladderwort). In general, there is good understanding of the actual mechanical responses involved in capturing prey as well as the subsequent digestive processes involved in extracting nutrients from the prey. What is typically less clear is why any insect (or other prey) would venture near the trapping-structure of a carnivorous plant. Secreted nectar, scent and trap

Figure 5.1: *Drosera rotundifolia* plants in visible light (A) and under UV radiation (B) showing reflectance of tentacles. Copyright O. Holovachov (http://www.holovachov.com).

shape and colour are often listed as key attractants for insects (Joel et al. 1985), with in some cases flowers emitting different scents as the trapping-structure to avoid potential pollinator-prey conflicts (Ho et al. 2016). Yet, it has also been suggested that UV-radiation plays a role in prey attraction (Joel et al. 1985). In fact, in 2012/2013 several major news outlets reported that "These Carnivorous Plants Glow Under Ultraviolet Light to Attract Prey" (Smithsonian.com, December 11, 2013), "Carnivorous Plants Glow to Attract Prey" (National Geographic, February 25, 2013), and "Carnivorous plant species glow blue to lure prey" (BBC Nature, February 19, 2013). These stories referred to strong UV-induced fluorescence, which was reputed to attract prey, and an example of which is shown in figure 2. Here, I will explore the evidence for a role of UV-radiation in attracting prey, and identify some of the gaps in our understanding of this putative role of UV-radiation.

It has long been known that floral patterns of UV-reflection or absorbance play a key role in pollination biology (Brock et al. 2016; Cronin and Bok 2016). Such floral UV patterns are common. Indeed, the *UV4Plants bulletin* (Issue 1, 2016) displayed a photograph which showed UV-patterns in dandelion flowers. These patterns are thought to contribute to attracting or deterring of specific insects, serve as plant-species specific markers and/or as orientation cues. Joel et al. (1985) noted the conceptual similarities between flowers attracting pollinators and carnivorous plants attracting prey. Using UV-photography, Joel et al. (1985) surveyed UV-patterns in the trapping structures of carnivorous plants. The authors took their photos under natural sunlight conditions using filters that transmit between 305 and 385 nm. Their study showed that various carnivorous plants have "conspicuous UV patterns" on or near their traps. The pitchers of *Heliamphora* display a clear UV-reflecting entrance to the beaker-structure. In contrast, *Sarracenia* pitchers contain UV-absorbing nectar, while in *Drosophyllum* species the old leaves are UV-reflecting, whilst the young, carnivorous leaves in the centre of the plant are UV-absorbing and appear

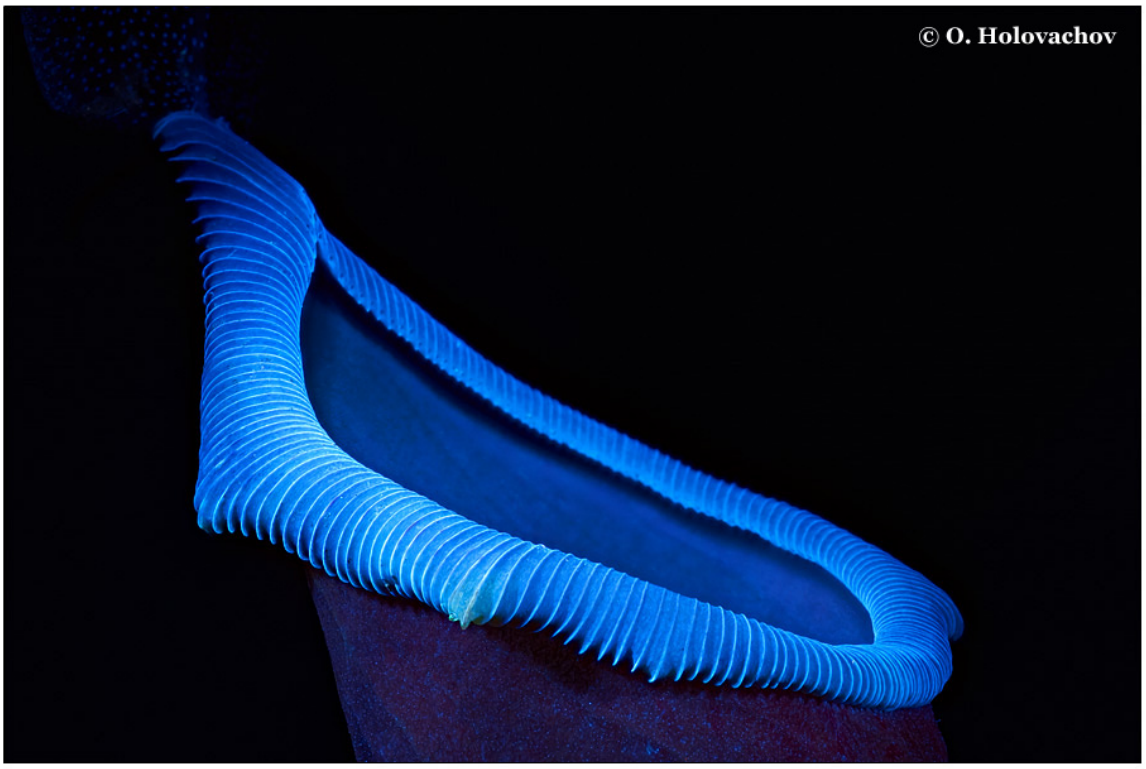

Figure 5.2: Blue fluorescence emitted by the rim of a *Nepenthes alata* beaker. Copyright O. Holovachov (http://www.holovachov.com).

as a relatively dark environment. A recent photograph of UV-reflectance in *Drosera rotundifolia* by Oleksandr Holovachov shows reflectance of tentacles (Figure 1B). Kurup et al. (2013) took the study of UV-patterns a step further by scanning the fluorescence of trapping structures using a densitometer. Kurup et al. (2013) focussed on blue fluorescence, following excitation with 366 nm radiation. In this context it is worth pointing out that the technology used by Joel et al. (1985) showed reflectance and absorbance in the UV-B and UV-A part of the spectrum, while Kurup et al. (2013) measured UV-induced blue fluorescence. Not surprisingly, different UV-patterns were noted by the two groups. Thus, technology plays a key role in what UV-pattern is observed, and this is a major consideration when interpreting the literature. Kurup et al. (2013) explored the blue fluorescence of *Nepenthes sp* peristomes (the ring of tissue that surrounds the entrance to the digestive tube). The authors stated that "The peristomes of *Nepenthes* species flashed like well-designed blue fluorescent tracks".

The markings in or near traps have been hypothesised to have a functional role in prey capture (Joel et al. 1985; Kurup et al. 2013). Moran, Clarke, Greenwood, et al. (2012) showed that an insectivorous species of *Nepenthes* displayed a distinct colour pattern compared to a closely related species that harvests tree-shrew excreta. Light does play a role in creating this pattern. Shading experiments by Moran, Clarke, and Gowen (2012), showed that reductions in visible and UV light resulted in a substantial decrease in the capture of *Drosophila* by *Nepenthes aristolochioides* pitchers. However, these results were interpreted in the context of light

(of all wavelengths) transmitted through the translucent pitcher, rather than as a specific role for UV-patterns. Kurup et al. (2013) experimentally tested whether UV-induced blue fluorescence has a functional role in prey capture. The authors found that removal of the peristome (rim) from *Nepenthes* pitchers led to a dramatic decrease in captured prey. Masking the peristome with acetone-extracts had a similar negative effect on prey capture. These results might be interpreted as supporting a role of UV-induced fluorescence in prey-capture (which is exactly what popular scientific journals did), but are far from conclusive. Clearly, excising tissue will not just remove UV-induced fluorescence, but also cells that are important for the production of nectar and scent, while causing massive tissue disruption. Similarly, acetone-extracts will have multiple effects on cells and tissues. Furthermore, basic photobiological questions should be asked concerning UV-induced blue fluorescence. How realistic is it that the sensitivity of the insect eye is such that it can perceive small changes in UV induced blue fluorescence, against a background of solar blue radiation? In this context, it is also important to be aware of the background of blue autofluorescence emitted by cell-wall-bound ferulic acid and other plant secondary metabolites following excitation with UV wavelengths (Buschmann et al. 2000; García-Plazaola et al. 2015). Thus, although pictures of UV-induced blue fluorescence look great, doubts remain concerning the functional role of such fluorescence. In fact, these doubts also apply to the common UV-induced blue fluorescence in, for example, flowers. Holovachov (2015) states that "despite considerable research efforts, the function of ultraviolet-induced visible fluorescence in the world of plants remains poorly understood", and indeed is unlikely to play a key role in the interactions between insects and plants.

Phenomena such as UV-reflectance, and UV-absorbance are more likely candidates for attracting insect prey. Both UV-reflectance, and UV-absorbance are known to be involved in the well characterised process of pollinator attraction in flowers (Guldberg and Atsatt 1975; Silberglied 1979). Unfortunately, the monitoring of UV reflectance and absorbance is often subject to technical limitations. UV-enabled cameras are equipped with filters that transmit in the UV-range of the spectrum, thus omitting the visible wavelengths. Yet, changes in UV absorbance and reflectance need to be interpreted in terms of the contrast with other wavelength zones. Exploring whether UV-radiation per se has a role in carnivory can be straightforward, for example by comparing prey capture in the presence or absence of UV-radiation. However, interpreting the precise role of UV radiation is complex as any UV-effect can be mediated either through the insect (i.e. vision) or through the plant (i.e. absorbance or reflectance). If the duration of the experiment is long enough, UV acclimation responses will further modify the biochemical make-up of the plant, and therefore potential UV-patterns. Clearly, prising apart the complex interaction between prey and carnivorous plant will be highly complex. Nevertheless, the application of the principles and terminology of photobiological research and UV-manipulation, as commonly practised in the UV4Plants community, can potentially contribute to the understanding of the role of UV radiation in prey capture. Exploring the wavelength dependency of UV reflectance and absorbance vis-á-vis insect vision can consolidate the link between these processes; for instance, local excitation with UV lasers can trigger local UV-reflectance whilst avoiding a direct effect on the insect. The use of artificial "model traps" together with the application of UV-absorbing pigments can similarly be used to experimentally test attraction traits. Nevertheless, it is unlikely that such work will generate more than "correlations" between UV-exposure and prey capture. Genetic manipulation is a more likely

strategy to conclusively proof a role for UV radiation in prey capture. Manipulation of UV-patterning through, for instance, breeding (Moyers et al. 2017), or manipulation of the spectral vision of the prey, for example of the model species *Drosophila melanogaster* (Feiler et al. 1992), are both realistic. Studies using genetically modified material should be able to reveal the relative importance of UV patterns, relative to other attractants such as secreted nectar, scent and trap shape and colour.

For now, UV-patterns exist, caused by UV-reflectance, UV-absorbance and UV-induced blue fluorescence. But although the story of UV-and carnivorous plants may be captivating, the truth about the functional role of UV-patterns is still to be captured!

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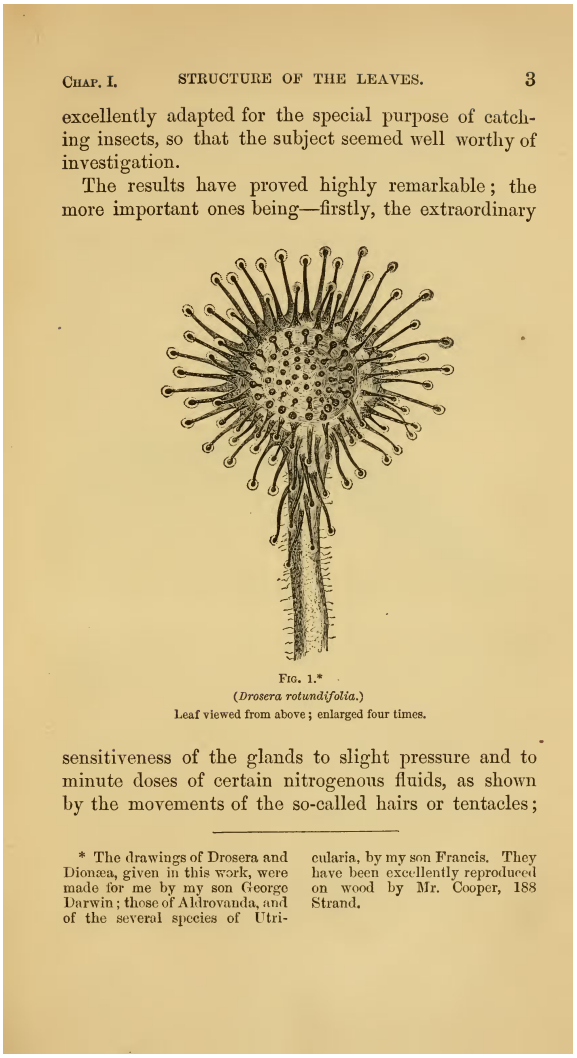

CHAP. I. STRUCTURE OF THE LEAVES. 3

excellently adapted for the special purpose of catching insects, so that the subject seemed well worthy of investigation.

The results have proved highly remarkable; the more important ones being—firstly, the extraordinary

FIG. 1.*
(*Drosera rotundifolia.*)
Leaf viewed from above; enlarged four times.

sensitiveness of the glands to slight pressure and to minute doses of certain nitrogenous fluids, as shown by the movements of the so-called hairs or tentacles;

* The drawings of Drosera and Dionæa, given in this work, were made for me by my son George Darwin; those of Aldrovanda, and of the several species of Utricularia, by my son Francis. They have been excellently reproduced on wood by Mr. Cooper, 188 Strand.

Figure 5.3: Page 3 from the book *Insectivorous plants* (Darwin 1875).

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■ Teaching

Learning about the molecular basis of plant responses to UV-B: a laboratory class at the University of Glasgow

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Abstract

Plant responses to UV-B provide an excellent system for students to learn about the regulation of gene expression following stimulus perception. This article concerns a laboratory class for undergraduate students at the University of Glasgow that is based on molecular responses to UV-B in *Arabidopsis thaliana*. During the class students design and plan experiments, interpret and discuss their results with other students and present the findings. Hence they learn valuable research skills. Some examples of students' work are presented and students' perceptions of the class are summarized.

Introduction

Ultraviolet-B (UV-B) wavelengths (280–315 nm) have numerous regulatory effects on plant growth and development (Jenkins 2009; Jordan 1996; Robson et al. 2014; Vanhaelewyn et al. 2016). It is well established that these effects result from UV-B-stimulated differential expression of large numbers of genes (Heijde and Ulm 2012b; Jenkins 2009). Responses to UV-B may involve several different perception and signal transduction processes, but many are mediated by the UV-B photoreceptor UV RESISTANCE LOCUS 8 (UVR8) (Jenkins 2014, 2017; Ulm and Jenkins 2015). Responses to UV-B are important because they modify biosynthesis, chemical composition and nutritional quality of plants, resistance to attack by pests and pathogens, and various aspects of development (Wargent and Jordan 2013). Moreover, UV-B responses affect both agriculturally important species (Wargent and Jordan 2013) and plants growing in natural ecosystems (Robson et al. 2014). Given their wide-ranging impact, it is important to raise awareness of plant responses to UV-B, and this is one of the aspirations of UV4Plants, the international association for plant UV research. Furthermore, it is vital to train and enthuse the next generation of researchers who will extend understanding of plant responses to UV-B and apply the knowledge gained in crop production, crop improvement and biotechnology. There is a partic-

ular onus on university teachers to do this.

This article concerns a laboratory class for undergraduate students at the University of Glasgow that is based on plant responses to UV-B. In Glasgow, life sciences students normally study for a BSc Honours degree over four years (http://www.gla.ac.uk/schools/lifesciences/undergrad/). The first two years provide a broad foundation in biological subjects and the final two years are dedicated to a particular degree subject. Most life sciences students encounter plant biology at some point in their courses and students can opt to take plant science as a specialism in the degree of Molecular and Cellular Biology (with Plant Science). In the University of Glasgow, most research in plant science concerns the molecular basis of responses to the environment, and several research groups are focused on plant photobiology. Responses to UV-B provide a good vehicle for students to learn about plant environmental perception and differential gene expression.

Students taking the degree courses in Genetics (http://www.gla.ac.uk/undergraduate/degrees/genetics/) and Molecular and Cellular Biology (http://www.gla.ac.uk/undergraduate/degrees/moleculercellularbiology/) take a number of extensive laboratory classes in their third year. The laboratory class described here occupies 2.5 days per week for four weeks and is usually taken by over 80 students. The class is intended to introduce students to methods used to study gene regulation and also to working with *Arabidopsis*, but a major aim is to develop skills in planning and designing experiments. The students are given scope to select genes for study, to choose questions to address in their experiments and to plan their work. They work in teams to design, execute and interpret their experiments, which encourages discussion and promotes learning through experience. This article provides information about what the laboratory class involves, examples of outcomes, and the experiences and perceptions of students who take it.

Outline of the laboratory class

The focus of the class is to investigate the regulation of gene expression in response to UV-B exposure of *Arabidopsis*. Students examine the expression of selected genes and the role of the UVR8 photoreceptor in mediating these responses. UVR8 detects UV-B radiation and triggers responses to UV-B in plants (Jenkins 2014; Ulm and Jenkins 2015). The processes involved in UVR8 action are outlined in Figure 6.1. In the absence of UV-B, UVR8 protein forms homodimers that do not initiate UV-B signal transduction. The dimer subunits are held together by salt bridges between charged amino acid residues at the dimer interface, in particular between arginine, aspartate and glutamate amino acids (Christie et al. 2012; Wu et al. 2012). The UV-B stimulus converts UVR8 to the monomeric state (Rizzini et al. 2011). Differently to other photoreceptors, which detect radiation with chromophores, UVR8 perceives UV-B through specific tryptophans in the dimer interface (Christie et al. 2012; Wu et al. 2012). When stimulated, the tryptophans transfer excited electrons to specific charged amino acids, which become neutralized, resulting in destabilization of salt bridges and subsequent UVR8 monomerization (Christie et al. 2012; Mathes et al. 2015). In its monomeric form, UVR8 binds to CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) (Rizzini et al. 2011). In darkness, COP1 is part of an E3 ubiquitin ligase complex that targets proteins involved in the UV-B response for proteolysis, especially the ELONGATED HYPOCOTYL 5 (HY5) transcription factor. However, when bound to UVR8 monomer, COP1 is not involved in ubiquitin ligase activity allowing HY5 to accumulate (Huang et al. 2013). UVR8 and COP1 together regulate transcription of numerous UV-B response genes (Favory et al.

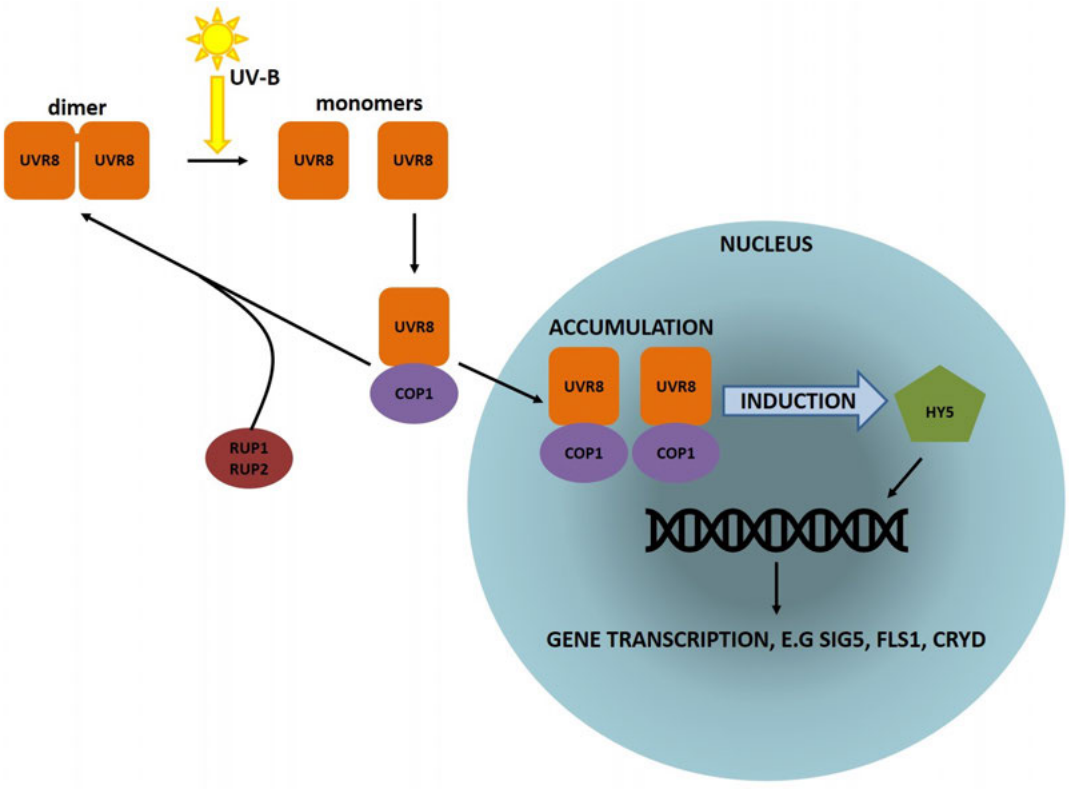

Figure 6.1: Schematic representation of UVR8 function in plant cells upon illumination with UV-B. Upon excitation with UV-B light, detected by tryptophan chromophores, the UVR8 dimer dissociates to produce monomers. In the cytoplasm, the UVR8 monomers can be bound by COP1 to initiate downstream signalling. UVR8 accumulates in the nucleus, where, together with COP1, it induces rapid accumulation of the HY5 transcription factor. This results in transcription of over 100 genes regulated by the UVR8 signalling pathway, such as *SIGMA FACTOR 5* (*SIG5*), *FLAVONOL SYNTHASE* (*FLS1*) and *CRYPTOCHROME DASH* (*CRYD*). RUP1 and RUP2, also induced by UVR8 signalling, disrupt the UVR8-COP1 interaction and promote re-dimerisation of UVR8.

2009; Jenkins 2014), including those encoding the HY5 and HY5 HOMOLOG (HYH) transcription factors. When the UV-B stimulus ceases, UVR8 re-dimerizes, re-establishing the initial conditions. UVR8 re-dimerization is facilitated by binding of REPRESSOR OF PHOTOMORPHOGENESIS (RUP) 1 and RUP2 (Heijde and Ulm 2012a). RUP protein expression is stimulated by UV-B, detected by UVR8, resulting in a negative feedback mechanism (Gruber et al. 2010). Furthermore, RUP proteins compete with COP1 for binding to the C27 region of UVR8, which comprises residues 397 to 423 in the C terminus of UVR8 (Cloix et al. 2012). This way, RUP1 and RUP2 not only repress the UV-B response by promoting re-dimerization, but also by diminishing the binding of UVR8 to COP1.

In the first, computer based session, students examine transcriptome analysis data from wild-type and *uvr8* mutant *Arabidopsis* exposed, or not, to UV-B (Brown et al. 2005; Brown and Jenkins 2007) to identify potential genes to study, and search for publications to find further information. Students then form teams based on which gene(s) they want to study. Members of the teams must work together to plan and execute experiments, initially using RT-PCR to examine gene expression. The students can use wild-type and mutant plants (such as *uvr8*, *hy5*, and *hy5 hyh*) and expose them to treatments such as

broadband or narrowband UV-B for different times and at different fluence rates; stresses such as salt treatment, or high/low temperature, to find out how these conditions influence the expression of their gene of interest. Based on the initial results, teams then plan and perform a second set of experiments. After they have the results of all their RT-PCR assays, they select a number of RNA samples to quantify the transcripts of specific genes through the use of real-time qPCR. To investigate UV-B signal transduction by UVR8, students carry out a yeast 2-hybrid assay to examine interaction with COP1. This allows them to test the importance of particular domains/amino acids of UVR8, especially the C-terminal region, in the interaction with COP1. In addition, the students use transgenic Arabidopsis *uvr8* mutant plants expressing wild-type or mutant forms of UVR8 fused to Green Fluorescent Protein (GFP). They visualize the protein by immunodetection using anti-GFP antibodies on western blots, enabling them to monitor the dimer/monomer status of UVR8 following UV-B exposure. Finally, students expose purified wild-type UVR8 protein to UV-B or other selected treatments, and examine dimer/monomer status by gel electrophoresis. The students keep records of their experiments and write a lab report, which is assessed.

Examples of student work

Gene expression

In the gene expression experiments undertaken by the 2017 class, over 10 different genes and a variety of treatments were studied. The data below are illustrative of the results obtained.

Expression of *RUP1* and *RUP2* genes was examined in response to different levels of UV-B exposure. Wild-type Landsberg erecta (L*er*) and *uvr8*-1 mutant *Arabidopsis thaliana* plants were grown in a growth cabinet at 20°C for 21 days in a low fluence rate (25 $\mu mol\,m^{-2}\,s^{-1}$) of fluorescent white light lacking UV-B, essentially as described by Brown and Jenkins (2007). Plants were then exposed to 0, 5 and 10 $\mu mol\,m^{-2}\,s^{-1}$ of narrowband UV-B for 4 hours (total doses[1] of 0, 72 and 144 $mmol\,m^{-2}$ respectively). The narrowband source has a peak emission at 312 nm and is effective in activating UVR8 (Favory et al. 2009). Plants were harvested, RNA isolated and *RUP1* and *RUP2* transcript levels quantified relative to control *ACTIN2* transcripts, which are unaffected by UV-B exposure, using RT-qPCR with gene-specific primers. Figure 6.2 shows increased expression of *RUP1* and *RUP2* in a UVR8-dependent manner at 72 $mmol\,m^{-2}$. At 144 $mmol\,m^{-2}$ expression drops drastically in wild-type plants suggesting that fluence rate plays a key role in *RUP1* and *RUP2* expression. A 4-fold greater increase of *RUP2* transcript levels compared to *RUP1* was observed in the wild-type plants at 72 $mmol\,m^{-2}$. Such a difference in levels of expression was not observed by Gruber et al. (2010). The observations add to previous studies showing that *RUP1* and *RUP2* expression is transient and induced by different light qualities (Gruber et al. 2010), but the experiments need to be repeated and extended to learn more about the regulation of the *RUP* genes.

ZINC FINGER OF ARABIDOPSIS THALIANA12 (ZAT12) is a zinc finger protein that has been functionally characterized to play a role in response to abiotic and biotic stress factors (Davletova 2005). *ZAT12* gene expression increases rapidly in response to a range of stress treatments, including UV-B (Kilian et al. 2007), and regulation in response to oxidative stress may underpin these responses Hahn et al. 2013. The aim of the experiment was to determine whether *ZAT12* expression was induced by UV-B and oxidative stress independently and in combination. Wild-type

[1] Editor's note: although frequently used when *photon exposure* is meant, according to the IUPAC Gold Book, this use is discouraged as dose describes photons or energy absorbed per unit volume or mass (see https://goldbook.iupac.org/)

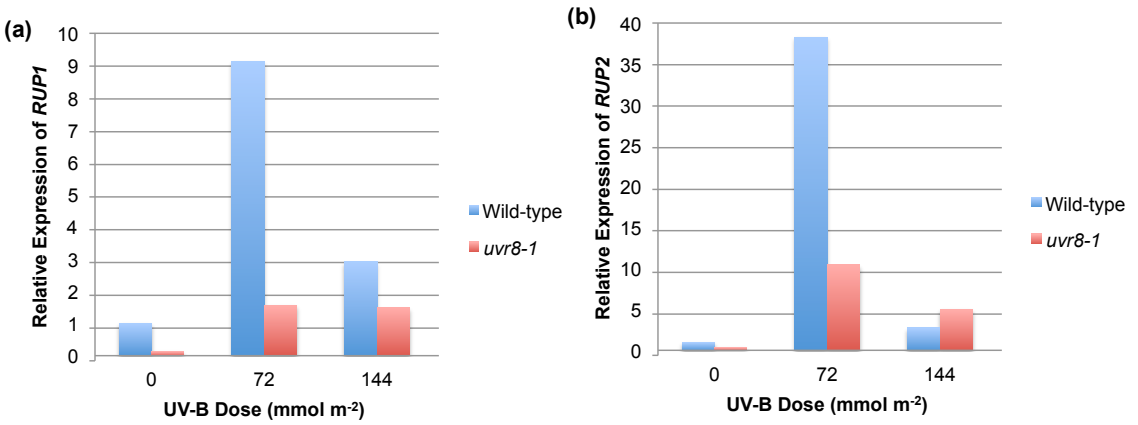

Figure 6.2: *RUP1* and *RUP2* gene activation in response to narrowband UV-B in wild-type and *uvr8*-1 mutant *Arabidopsis thaliana* plants. Wild-type L*er* and *uvr8*-1 mutant plants grown in fluorescent white light lacking UV-B were illuminated with 0, 5 or 10 $\mu mol\, m^{-2}\, s^{-1}$ narrowband UV-B for 4 hours, corresponding to doses of 0, 72 and 144 $mmol\, m^{-2}$ UV-B. Transcript levels of *RUP1* (a) and *RUP2* (b) were determined by quantitative RT-PCR analysis and normalized to the level of control *ACTIN2* transcripts. Transcript levels are presented relative to expression in wild-type without UV-B illumination.

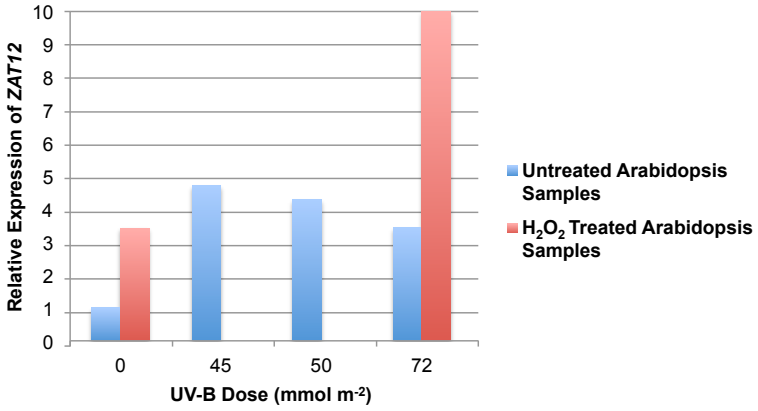

Figure 6.3: Relative *ZAT12* expression in UV-B treated and H_2O_2 treated *Arabidopsis thaliana* plants. Wild-type L*er Arabidopsis* plants grown in fluorescent white light lacking UV-B were given different doses of broadband UV-B by varying fluence rate and duration of exposure. Where indicated, H_2O_2 treatment was provided by spraying the plants 3 times with a 1% (v/v) solution. *ZAT12* transcript levels in RNA samples were assayed by quantitative RT-qPCR and normalised to levels of control *ACTIN2* transcripts. *ZAT12* expression in the different samples is expressed relative to that in the non-treated sample.

A. thaliana plants grown as above were exposed to different doses of UV-B provided by a broadband source (spectrum described by Cloix et al. 2012) by altering the fluence rate or duration of exposure. *ZAT12* transcripts were assayed by RT-qPCR using gene-specific primers, and normalized against transcripts of the control *ACTIN2* gene. Figure 6.3 shows that a significant increase of *ZAT12* expression was observed following exposure to all UV-B doses used. Additionally, under oxidative stress (caused by spraying plants with hydrogen peroxide), it was found that *ZAT12* expression was much higher, both in the presence and absence of UV-B. This is in line with previous studies which show that *ZAT12* expression is induced by UV-B stress and oxidative stress. It is likely that the moderate UV-B doses used here did not generate a sufficient level of reactive oxygen species to induce maximal *ZAT12* expression, which would explain why additional expression occurred when hydrogen peroxide was applied.

Interaction between UVR8 and COP1

A yeast 2-hybrid assay was used to examine protein-protein interactions between wild-type UVR8, a deletion mutant of UVR8 lacking amino acids 397-423 (termed the C27 region) in the C-terminus (UVR8$^{\Delta C27}$), and COP1. The methods of Cloix et al. (2012) were used. UVR8 and the UVR8$^{\Delta C27}$ mutant were each cloned as a fusion with the DNA-binding domain of the yeast GAL4 transcription factor, whereas COP1 was fused to the activation domain of GAL4 in a separate plasmid vector. Expression of both fusion proteins in yeast and interaction between them resulted in the reconstruction of GAL4 from two separate polypeptides, which enabled growth on a selective medium; no interaction resulted in the absence of growth. Mammalian T antigen and p53 proteins that interact strongly were used as a positive control, whereas empty vectors were used as a negative control.

As shown in Table 6.1, all colonies grew in non-selective media, confirming viability of the yeast cells. Yeast cell growth was also observed in over 90% of selective plates containing the positive control, both in darkness and under illumination with UV-B. On the contrary, no growth was observed for the negative control. Under UV-B exposure, yeast cell colonies transfected with UVR8 and COP1 plasmids grew in approximately 81% of plates (Table 6.1) while in darkness, no growth was observed. In contrast, when illuminated, 92% of yeast co-transfected with UVR8$^{\Delta C27}$ and COP1 did not form colonies (Table 1). Similarly, in darkness, growth was observed in only 1 out of 26 plates.

Since yeast colony growth reflects interactions between the proteins of interest, the results indicated that UVR8 interacts with COP1 in the presence of UV-B light. However, the mutant UVR8 protein lacking the C27 region did not interact with COP1, indicating that C27 is required for UV-B dependent interactions of UVR8 and COP1 in yeast. This finding is in agreement with that of Cloix et al. (2012), who additionally found that C27 is required for interaction with COP1 in plants. They further reported that transgenic plants expressing UVR8$^{\Delta C27}$ had impaired responses to UV-B radiation, including *HY5* expression, which has a key role in mediating UVR8 responses. However, another study (Yin et al. 2015) reported that COP1 can interact with UVR8 lacking the C-terminal amino acids that include C27. These researchers discovered two distinct domains of UVR8 interacting with COP1, the first being the C27 region and the second the β-propeller domain, which interacts with the WD40 region of COP1 in a UV-B dependent manner. Nevertheless, it should be mentioned that this study employed less stringent selection to test interaction than used here.

Table 6.1: Yeast 2-hybrid assay of the interaction between UVR8 and COP1. Yeast 2-hybrid plasmids containing the GAL4 DNA binding domain (BD) or activation domain (AD) fused to the proteins indicated were co-transformed in yeast. Negative control: plasmid vectors with no inserts (-); positive control: plasmid vectors containing mammalian p53 and antigen T; test interactions: plasmid vectors containing either wild-type UVR8 or a mutant with a deletion of the C27 region (UVR8$^{\Delta C27}$) and COP1. Yeast growth was tested on non-selective media (for viability) and selective media (for interaction) in darkness or under 0.1 $\mu mol\,m^{-2}\,s^{-1}$ narrowband UV-B. The numbers in the table indicate growth (+) or no growth (−) and are the data of 26 groups of students.

		Darkness				UV-B			
Culture Medium		Non-sel.		Selective		Non-sel.		Selective	
Yeast growth		+	−	+	−	+	−	+	−
BD	AD								
-	-	26	0	3	23	26	0	2	24
P53	T-Ant	26	0	24	2	26	0	25	1
UVR8	COP1	26	0	1	25	26	0	21	5
UVR8$^{\Delta C27}$	COP1	26	0	1	25	26	0	2	24

Effect of UV-B radiation on UVR8

An experiment was undertaken to examine the effect of UV-B exposure on wild-type and mutant UVR8 proteins expressed in *Arabidopsis uvr8*-1 as GFP fusions. Extracts were prepared from plants and illuminated on ice with UV-B as described by Cloix et al. (2012). Following UV-B exposure, plant extract samples were run on a SDS-PAGE gel without boiling, followed by immunoblotting with a GFP-specific antibody. This method permits detection of the dimer and monomer forms of UVR8 (Rizzini et al. 2011). The western blot (Figure 6.4) showed an increase in the intensity of the monomer band following UV-B illumination of plant extracts with 1 $\mu mol\,m^{-2}\,s^{-1}$ broadband UV-B for 15 to 60 minutes (approximate monomer size was identified by complete UVR8 denaturation in a boiled control). Quantification of band intensity with ImageJ showed that monomer proportion correlates with UV-B dose (Figure 6.5); while 10% of total UVR8 was present as monomer in the non-illuminated control, monomer proportion had increased to 65% following a 1-hour UV-B treatment. This observation is consistent with research demonstrating that UV-B exposure induces UVR8 monomerization. Monomers then interact with proteins downstream in the signalling pathway (Rizzini et al. 2011).

Deletion of the C27 region did not alter UVR8 response to increasing dose of UV-B as compared to GFP-UVR8 (an unpaired *t*-test confirmed an insignificant difference). Despite the demonstrated importance of this region for interaction with COP1 and the induction of UVB-mediated photomorphogenic responses (Cloix et al. 2012; Yin et al. 2015), our results indicate it that it does not influence dimer formation or UV-B induced monomerization (Figures 6.4 and 6.5). Interestingly, C-terminal deletion has been observed to hinder re-dimerization (Heilmann and Jenkins 2012) as the C27 region is necessary for binding of RUP proteins (Cloix et al. 2012), which facilitate this process (Heijde and Ulm 2012a). It would therefore have been interesting to leave illuminated samples in the dark and inspect their rate of re-dimerization as compared to GFP-UVR8.

On the other hand, replacement of tryptophan 285 with phenylalanine prevented UVR8 monomerization upon UV-B exposure (Figure 6.4), demonstrating the critical im-

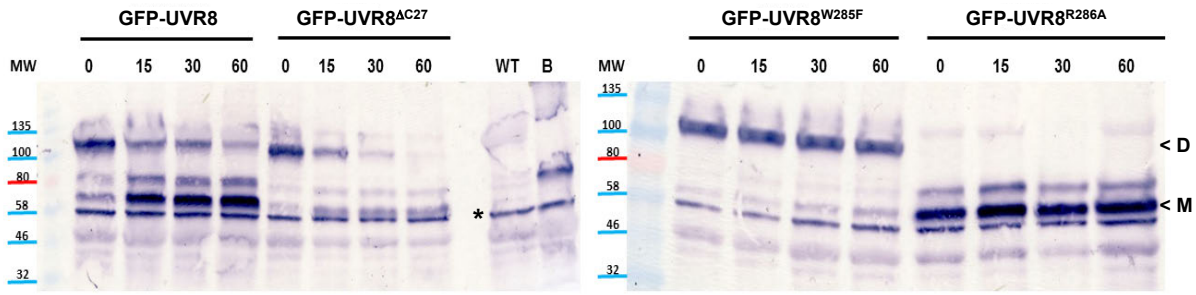

Figure 6.4: Effects of UV-B illumination on GFP-UVR8 mutants. Protein extracts of *Arabidopsis uvr8-1* expressing GFP-UVR8, GFP-UVR8$^{\Delta C27}$, GFP-UVR8^{W285F} or GFP-UVR8^{R286A}, were left untreated or illuminated for 15, 30 or, 60 min with broad-band UV-B of 1 $\mu mol\,m^{-2}\,s^{-1}$. Samples were run on SDS-PAGE without boiling and a western blot was performed for detection of GFP-UVR8 protein in dimer (D) and monomer (M) states using anti-GFP antibody. A non-illuminated wild-type (WT) sample was used to detect non-specific antibody binding (indicated by an asterisk). A boiled sample (B) was used as a control for monomerization which occurs after boiling UVR8 in SDS. MW: molecular weight marker proteins, in kDa.

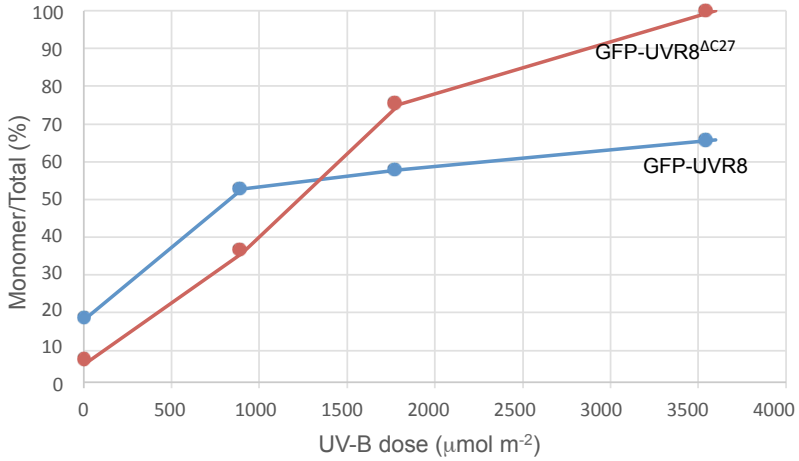

Figure 6.5: GFP-UVR8 and GFP-UVR8$^{\Delta C27}$ monomerization following UVB exposure. ImageJ was used to quantify UVR8 dimer and monomer band intensities.

portance of this residue for UV-B photoreceptor activity (Christie et al. 2012; Rizzini et al. 2011; Wu et al. 2012). UVR8 therefore differs from other photoreceptors, which absorb light using cofactors as chromophores (Jenkins 2014).

Replacement of arginine 286 with alanine resulted in UVR8 monomerization even in the absence of UV-B light (Figure 6.4), demonstrating a key role of this residue in the maintenance of the UVR8 homodimer. R286 is located at the interface of interaction between the two monomers and forms two hydrogen bonds with D107 and a hydrogen bond with D96 on the opposing monomer. The monomer interaction interface contains many charged amino acids, which similarly form salt bridges with residues of complementary charge on the opposing monomer (Jenkins 2014). Due to the denaturing properties of the SDS buffer, we would expect disruption of all weak interactions, so weakened salt bridges could be disrupted in the gel but remain intact in vivo. However, size ex-

clusion chromatography demonstrates that salt bridges are indeed disrupted following a R286A mutation, causing constitutive monomerization (Christie et al. 2012; Heilmann et al. 2016; Wu et al. 2012). Therefore, R286 is critical for formation of salt bridges that maintain the dimer.

The monomerization of purified UVR8 was also monitored, to draw conclusions on the dose of UV-B required to initiate and complete the monomerization of photoreceptor dimers. The purified protein (Christie et al. 2012) was exposed on ice to a broadband UV-B source at different fluence rates (1 to 5 $\mu mol\,m^{-2}\,s^{-1}$) for different durations, for a maximum of 3 hours. The samples were then run, without boiling, on a SDS-PAGE gel to resolve the UVR8 dimer and monomer (Christie et al. 2012). After the gels were stained, ImageJ was used to quantify the band intensities and the [$UVR8^{monomer}$] / [$UVR8^{total}$] percentages were calculated and plotted against increasing UV-B dose.

As can be observed from Figure 6.6a, UVR8 monomerization in response to increasing UV-B dose follows a logarithmic trend. UVR8 monomerization is initiated at relatively low exposures of UV-B, and the first monomers are detected after a UV-B dose of 30 $\mu mol\,m^{-2}$ (Figure 6.6b). The relative level of monomer reaches 50% at a dose of less than 200 $\mu mol\,m^{-2}$, while complete dimer dissociation requires a minimum dose of approximately 5200 $\mu mol\,m^{-2}$ of UV-B (Figure 6.6a). Moreover, a plot of the data on a semi-log graph reveals that the UV-B dose-response relationship is linear (Figure 6.6c). Our findings are consistent with those of Christie et al. (2012), who treated purified UVR8 with 450, 1350, 2700 and 5400 $\mu mol\,m^{-2}$ of narrowband UV-B and showed a rise in monomer formation with increasing UV-B doses, and those of Wu et al. (2012) and Zeng et al. (2015) who used undefined doses. The results of this study extend previously published findings on UVR8 in that a set of quantitative data points was used to determine a dose-response relationship.

Student experiences and perceptions

Most laboratory classes that students take in the early years of their undergraduate studies are designed to reinforce theoretical concepts and teach practical skills, so there is limited opportunity for investigation. The present laboratory class was intended to introduce students to some of the skills used in research, which potentially would help them in their final year project work and after graduation. Nevertheless, having to formulate questions and design experiments was a new and challenging experience for the students.

To discover students' perceptions, anonymous questionnaires were collected from over 120 students who took the class in years 2016 and 2017. Interestingly, 85% agreed, or strongly agreed, that the laboratory class helped them to understand how to plan and design experiments (0% disagreed; 15% neutral). Similarly 74% agreed, or strongly agreed, that it helped them think how to ask scientific questions (5% disagreed). Several students stated that the class encouraged active engagement by allowing them the freedom to choose a gene of interest and create an appropriate aim. Moreover, it fostered a lot of collaboration amongst students as it was vital to work together to discuss results of their experiments, and the results obtained sometimes forced changes in approaches and feasible objectives. Students commented that the approach promoted independence, confidence and an ability to communicate ideas to other students, and that receiving frequent feedback helped them further improve their experiments and realize their strengths and limitations. In addition, some felt they were encouraged to learn throughout the duration of the class.

In the questionnaire, 72% of students said

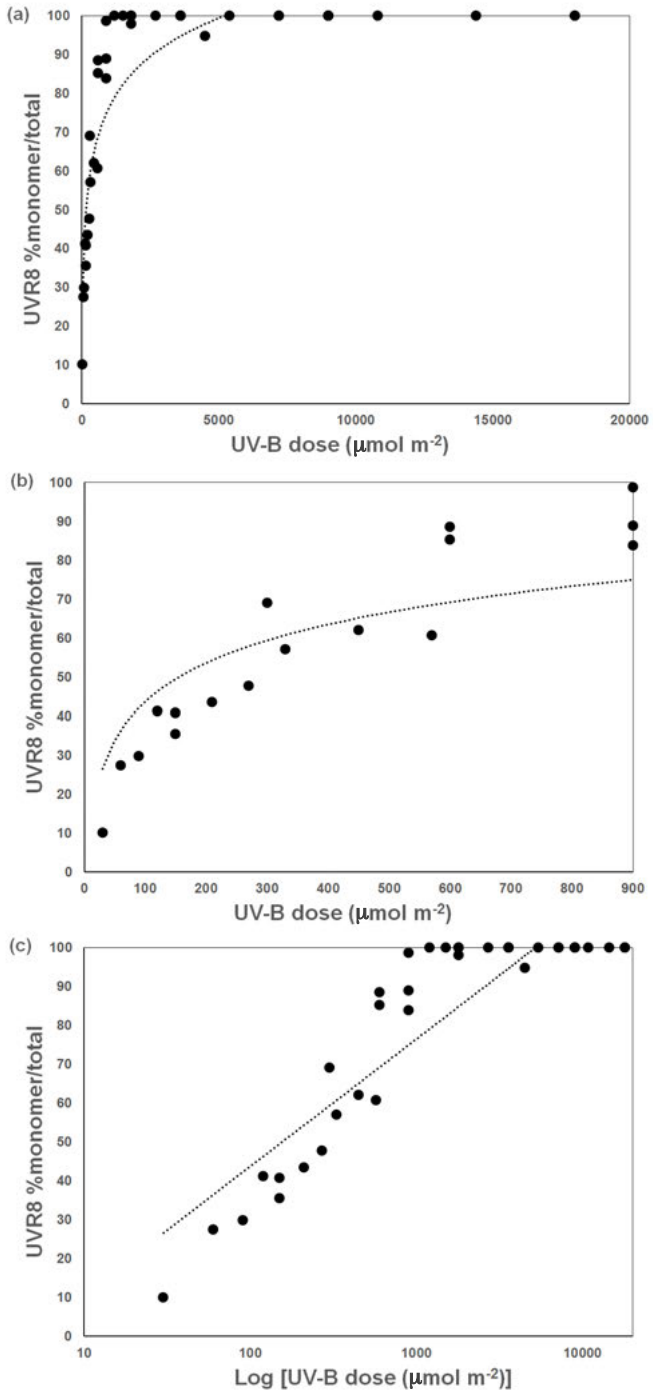

Figure 6.6: Monomerization of purified UVR8 with increasing dose of UV-B. Aliquots of purified UVR8 were exposed to increasing doses of broadband UV-B, produced by varying fluence rate and duration, and harvested. Non-boiled samples were run on SDS-PAGE gels and stained with Coomassie Blue to display dimer and monomer bands. For each sample, the relative abundance of the monomer and dimer bands was analyzed using ImageJ. The data are plotted and lines fitted to the data points using Excel. (a) Linear-scale plot of the increase in [UVR8$^{\text{monomer}}$] / [UVR8$^{\text{total}}$] (shown as 'UVR8 % monomer-/total') of the whole data set. (b) Detail on the increase in [UVR8$^{\text{monomer}}$] / [UVR8$^{\text{total}}$] (shown as 'UVR8 % monomer/total') from 0 to 900 $\mu\text{mol}\,\text{m}^{-2}$, using a subset of the data. (c) Semi-log plot of the whole data set. Data compiled from experiments done by 4 teams of students.

that the class helped them to interpret data. Some stated that they helped each other and discussed how they were analysing the data and that they were able to compare and evaluate the methods they used. Due to the freedom to work on genes and aspects of regulation that were sometimes not fully characterized in the literature, students said that they felt their work had significance. This is likely why 76% stated that the laboratory class gave them a taste of what it would be like to do research, and 82% liked the idea that some of their experiments may not have been done previously. Overall, 89% agreed or strongly agreed that the class was a valuable learning experience, and 75% thought it would help them after they had completed their degree. Some felt the class allowed them to develop problem-solving and teamwork skills essential for future research work. UV4Plants members will be pleased to learn that the class generated interest in plant responses to UV-B. As expected, most (59%) students said they had little knowledge of how plants sense and respond to UV-B prior to doing the laboratory, but 72% that they learnt about it as a result. It was mentioned that the focus on analyzing their own data fostered a deeper interest in plant biology, specifically on the role of UVR8 and the chosen genes.

Concluding remarks

The laboratory class helps students develop practical skills in molecular biology but, more importantly, they start to develop key research skills that cannot easily be taught, including: formulating questions, having ideas, working collaboratively in a team, acquiring the confidence to express an opinion, developing independence. Moreover, they develop their ability to interpret data and evaluate observations in relation to the methods used to obtain them. Being given the scope to select genes and treatments for study promotes engagement and a taste for research. Importantly, gaining an appreciation of how new knowledge is generated encourages students to critically appraise published data. However, while many students relish the freedom this type of class gives, others find it difficult. For instance, some students commented that they prefer classes focused on learning techniques and some felt the time for team discussion slowed down the work. Nevertheless, most found the class enjoyable and realized that they gained from the experience. From the class leader's perspective, there are organizational challenges that arise in undertaking an investigative approach with a large class. In addition, it can be difficult achieving the correct balance between giving students independence and intervening to ensure their experiments are well designed. But ultimately all the students produce data and learn from the experience, and it is satisfying that the approach generates an interest in the subject.

Plant responses to UV-B provide an excellent system for students to learn about the regulation of gene expression. The stimulus is easily applied and a wide range of genes can be studied. Moreover, *Arabidopsis* mutants are available lacking UVR8 and the downstream transcription factors HY5 and HYH. Activity of the photoreceptor itself can readily be monitored both with respect to monomerization and interaction with COP1, and mutants in the UVR8 protein are available. Hence there are numerous questions that students can define and address. Many of the experiments undertaken have not been reported in the literature and some of the findings are interesting and generate ideas for further research. Furthermore, when the whole class work on the same task valuable data can be generated, as illustrated with the dose-response experiment presented in Figure 6.6, where the findings extend published information. Evidently, the class is facilitated by the availability of resources and expertise generated in research projects in the University of Glasgow, consistent with the University's strategy that teaching should be

Figure 6.7: A number of authors of the paper enjoying the Glasgow sunshine.

research-led.

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■ Methods—*invited article*

Analysis of phenolic compounds: which factors to consider?

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Introduction

Phenolic compounds are of special interest as antioxidants and shielding compounds accumulated in response to ultraviolet-B radiation (UVB; λ = 280-315 nm) and other abiotic factors. The majority of phenolic compounds are based on phenolic acid or flavonoid aglycones (Schmidt et al. 2010a), but mainly occur in plants as glycosides (Calderon-Montano et al. 2011) and some of these compounds are acylated (Calderon-Montano et al. 2011; Schmidt et al. 2010b). Additionally, in case of flavanols, polymerization leads to tannins, also known as proanthocyanidins (Gadkari and Balaraman 2015). The compounds can vary from simple to highly complex structures, which makes their identification and quantification challenging.

To date, there are three main approaches to measure phenolic compound concentrations in plants.

1. The spectrophotometric measurements of total phenolic content, total flavonoid content, total flavonol content or others. However, this approach does not allow for the identification of single phenolic compounds within the extract. Moreover, to quantify content, standards like gallic acid are used that do not necessarily occur in the sample. A benefit is that the results are comparable to other data due to the intensive usage of this method worldwide, which is of high interest to compare plants and results of different labs.

2. The measurement of flavonoid aglycones after acid, alkaline or enzymatic hydrolysis of the flavonoid glycosides is best performed with high-pressure liquid chromatography (HPLC). A number of aglycone standards are already available, and thus, the identification and quantification of these compounds are both possible.

3. The identification and quantification of flavonoids glycosides require an analytical platform including HPLC coupled to a mass spectrometer (MS). For the identification of new compounds, nuclear magnetic resonance (NMR) would also be mandatory. This approach is mainly semi-quantitative due to the fewer standards that are currently available.

Of note is that there is so far no standard procedure for the measurement of phenolic compound profiles and concentrations in plants. However, the review of Julkunen-Tiitto et al. (2015) summarizes possible techniques to investigate the effect of UVB radiation on plant phenolics.

This article provides a comprehensive overview on how sampling, drying and storage as well as extraction and measurement affect the quantification of phenolic compounds in plants (Fig. 7.1). Furthermore, the need of

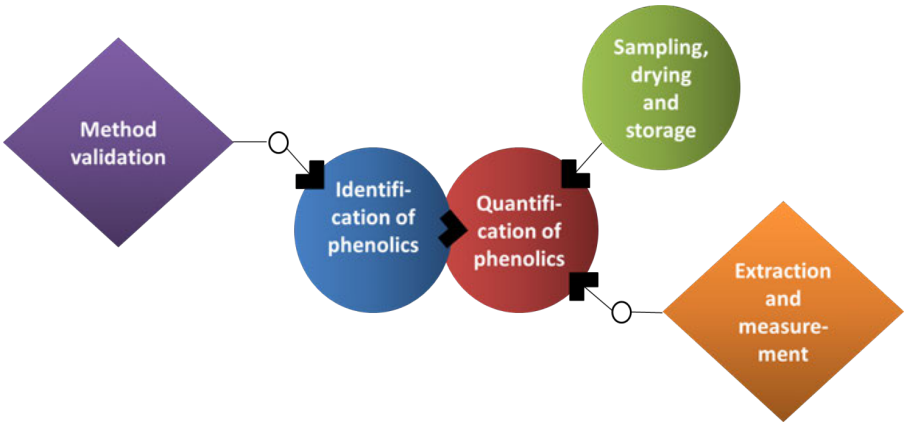

Figure 7.1: How sampling, drying and storage as well as extraction and measurement affect the quantification of phenolic compounds and the need of the method validation for the identification and quantification of phenolics.

method validation to be able to obtain reliable results regarding the identification and quantification of these compounds will be highlighted. Here, results from our research, done at the IGZ and TU Berlin, on kale's phenolic compound profile and concentrations will be used as examples (Neugart et al. 2013, 2014; Neugart, Kläring, et al. 2012; Neugart, Zietz, et al. 2012; Schmidt et al. 2010a,b; Zietz et al. 2010). The major benefit of using these experiments as examples is the possibility of comparing the same cultivar under different abiotic factors based on metabolite measurements obtained with the exact same validated method, including freeze-drying, extraction and measurement which would not be the case in a broad literature review.

This article does not provide a widely applicable standardized working protocol. Instead, it describes all the steps required for reliable quantification, highlighting the difficulties we encounter when comparing and interpreting phenolic compound measurements reported in the scientific literature, as there are enormous differences that may not only be dependent on the genotype and environment, but also depend on the various analytic approaches and protocols used in different labs. Below, we discuss the different possible sources of uncertainty at different steps of the quantification protocols and how we can control them.

Sampling

The sampling of the plants is a crucial step in measuring the concentration of phenolic compounds and should be considered carefully. Sampling protocols determine how the results of a study can be interpreted, and which conclusions can be validly drawn. The objective of the study and the desired range of validity of the results determine the design of the sampling protocol to be used. Plants' chemical composition is affected by both genotype and environment, while the amount of uncontrolled variation and avoidance of bias depend on the design of experiments, surveys and sampling protocols from an statistical point of view.

A valid approach for sampling the plants is to repeat the same experiment 3–4 times and generate independent results. This is the standard for climate chamber experiments requiring the use of multiple chambers or replication in time with random re-allocation of treatments to chambers and positions within chambers. In order that field experiments can generate truly interpretable data, the climate conditions should be monitored,

allowing the relationships between environmental variables and metabolite concentrations to be examined. In field experiments, broad validity will require replication in time and/or space at a scale matching the intended validity of the results: varying from replicate plots at one site and time—e.g. a randomized block design with at least 3–4 biological replicates–to temporal (years) and/or spatial (localities) replication—e.g. a regional or national network of field sites with replication over several years.

Generally, when comparing species or cultivars, the harvested plants or parts of plants should be grown and treated under the same conditions as far as possible to be able to obtain accurate results. More generally, random variation should be either controlled or quantified.

Other factors which can affect profiles and concentrations of phenolic compounds should be also considered: cultivar and genotype, developmental stage, plant organ, nutritional status of the plant, temperature and photosynthetically active radiation (PAR), UVB radiation, diurnal changes, as well as wounding. All these factors need to be considered when designing a sampling protocol and randomization applied to the sampling protocol so as to ensure that no bias is introduced. It is important to be aware that concentrations per unit dry matter can change rapidly both through fast synthesis, transformation or degradation of the metabolites under study, and by changes in the accumulation of other metabolites such as starch (e.g. starch concentration in leaves varies through the day as well as in response to any factor affecting photosynthesis or respiration—in the case of respiration, even after their collection, the dry mass of samples decreases, unless they are rapidly frozen or dried so as to stop metabolism.)

As a final consideration, data analysis should be performed taking into account what units in an experiment are true replicates and which ones are sub-samples within such replicates. In many cases valid analysis requires the use of nested designs to take into account the properties of the sampling scheme used.

Species or Cultivar

It is known that there is strong variation in phenolic compound profiles and concentration among species (Häkkinen and Törrönen 2000; Klepacka et al. 2011; Neugart et al. 2017, 2015; Wu et al. 2004). However, it is also now known that such variation exists between different cultivars of the same species. For example, various studies have shown that plants of different cultivars grown under the same conditions have remarkable differences in their phenolic compound concentration as described in the literature (Buendía et al. 2010; Castillo-Muñoz et al. 2010; Flanigan and Niemeyer 2014; Luo et al. 2013; Y.-W. Lv et al. 2011; Pérez-Gregorio et al. 2010; Wang et al. 2008; Zheng et al. 2012). For examples of kale, see sections Flavonoid Aglycones and Flavonoid Glycosides. If species or cultivars should be compared it is mandatory to do that in one experiment with the same environmental conditions regarding developmental stage, temperature, radiation and plant nutrition.

Developmental Stage

For sampling, plants should be at the same developmental stage, unless developmental effects are being studied. When interpreting results, one should take into account that environmental conditions, including the treatments under study, can affect the timing of plant development, such as reproductive induction. Moreover, variation of the phenolic compounds concentration with age has been described for several species (Edwards et al. 1997; Reifenrath and Müller 2007; Schoedl et al. 2012; Vogt and Gul 1994). As an additional example, in *Arabidopsis thaliana* and other plant species, an increase of anthocyan-

ins with age has been attributed to the higher concentration of reactive oxygen species in older plants (J. Huang et al. 2010; Kovinich et al. 2014; Stracke et al. 2010; Yonekura-Sakakibara et al. 2011). Data from one of our experiments, are used to exemplify how kale plants grown under consistent environmental conditions (250 µmol m^{-2} s^{-1} and 10°C) show a decrease in flavonoids with ongoing plant development (Fig. 7.2).

In addition to the plant's age, leaves at different positions and/or different ages within a single plant can also differ enormously in their phenolic compound profile and concentration. For example, in adult kale plants (12 weeks), the younger, light-green coloured leaves from the top of the plant contained higher concentrations of quercetin than the intermediate-aged or old leaves (Fig. 7.3), and similar results were found for strawberry fruits (Tsormpatsidis et al. 2011). Therefore, it is important to harvest parts of the plants that are at comparable developmental stage if one is to get meaningful and robust data. This does not necessarily mean a single developmental stage, but rather that samples from different individuals should be comparable in this respect in all cases—i.e. sampling of a single developmental stage of the plant or organ, multiple stages as separate samples, or as a pooled "stratified" sample.

Variation Within and Between Organs

Different plant organs vary in their flavonoid profile and concentration (El Morchid et al. 2014) and even within a single organ, variation has been found (e.g. Julkunen-Tiitto et al. 2015). As a further example, we found that the concentration of flavonoids in the midrib of kale leaves is at most 20% of what is found in the rest of the leaf, consistently accross different cultivars (Fig. 7.4). To minimize the effect on the results of differences between cultivars size of the leaves' midribs, one solution is to cut out the midrib where possible or sample the whole leaf and quantify the contribution of the midrib to each leaf's dry mass. Consequently, the harvested organs should be as equal as possible regarding developmental stage.

Nutritional Status of the Plants

Another factor affecting the phenolic compound concentration is the nutritional status of the plants. For example, several articles have described a negative correlation of phenolics, especially flavonoids, and nitrogen supply in the plants (Fallovo et al. 2011; Groenbaek et al. 2016, 2014; Han et al. 2010; Løvdal et al. 2010; Nguyen and Niemeyer 2008; K. M. Olsen et al. 2009; Strissel et al. 2005). We exemplify these effects, with data from one of our experiments on kale, where we observed that quercetin greatly decreased while kaempferol and isorhamnetin slightly decreased with increasing nitrogen fertilization (Fig. 7.5). However, even though other nutrients can also affect the concentration of phenolic compounds, they are less frequently discussed: e.g. sulphur fertilization can lead to a species-specific increase of phenolic acids in *Brassica rapa* subsp. *sylvestris* (De Pascale et al. 2007). Consequently, in pot experiments the same batch of well mixed soil should be used for all plants that are compared in an experiment, and pots assigned at random to different treatments. In field experiments the soil should be monitored for the nutrient status. Fertilizer applications during an experiment should be considered while interpreting the results and should be described in enough detail as part of the experimental methods.

Temperature and PAR

Temperature and photosynthetically active radiation (PAR) can affect the profile and concentration of phenolic compounds (Bernal et al. 2013; Chennupati et al. 2012; Mølmann et al. 2015; Mori et al. 2007; H. Olsen et al. 2009; Uleberg et al. 2012; Zandalinas et al. 2017). In

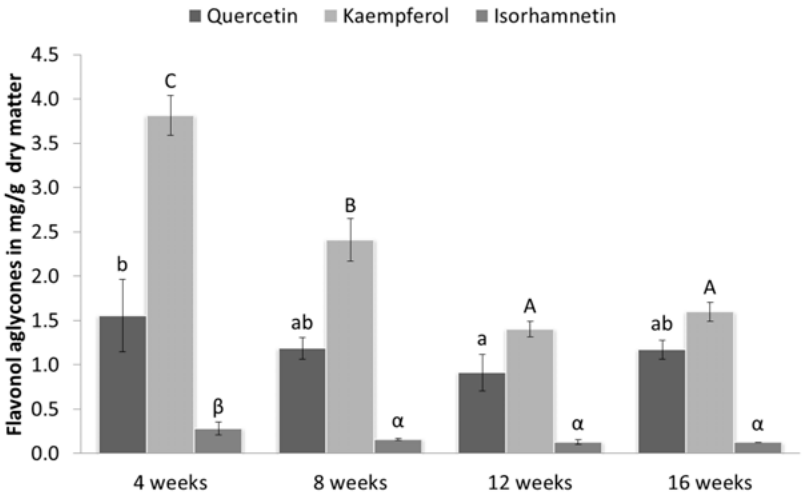

Figure 7.2: Flavonol aglycone concentrations of kale cv. 'Winterbor' dependent on the plant's developmental stage in plants grown at 10°C and 250 µmol/m² s. Different letters indicate significant differences between plants at different developmental stage for each flavonol aglycone ($p \leq 0.05$ by Tukey's HSD test). Each value represents the mean of three replicates ± SD. Susanne Neugart, unpublished data.

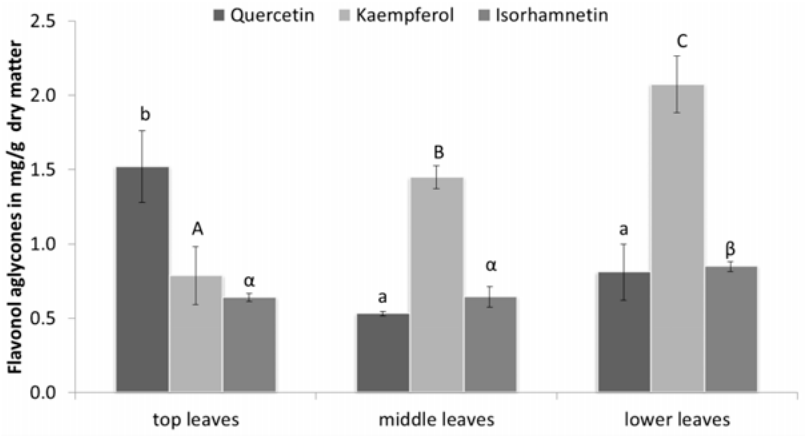

Figure 7.3: Flavonol aglycone concentrations of 12-week-old kale cv. 'Winterbor' dependent on the leaves' age in 12 week old plants grown at 10°C and 250 $\mu mol\,m^{-2}\,s^{-1}$. Different letters indicate significant differences between leaves of different age for each flavonol aglycone ($p \leq 0.05$ by Tukey's HSD test). Each value represents the mean of three replicates ± SD. Susanne Neugart, unpublished data

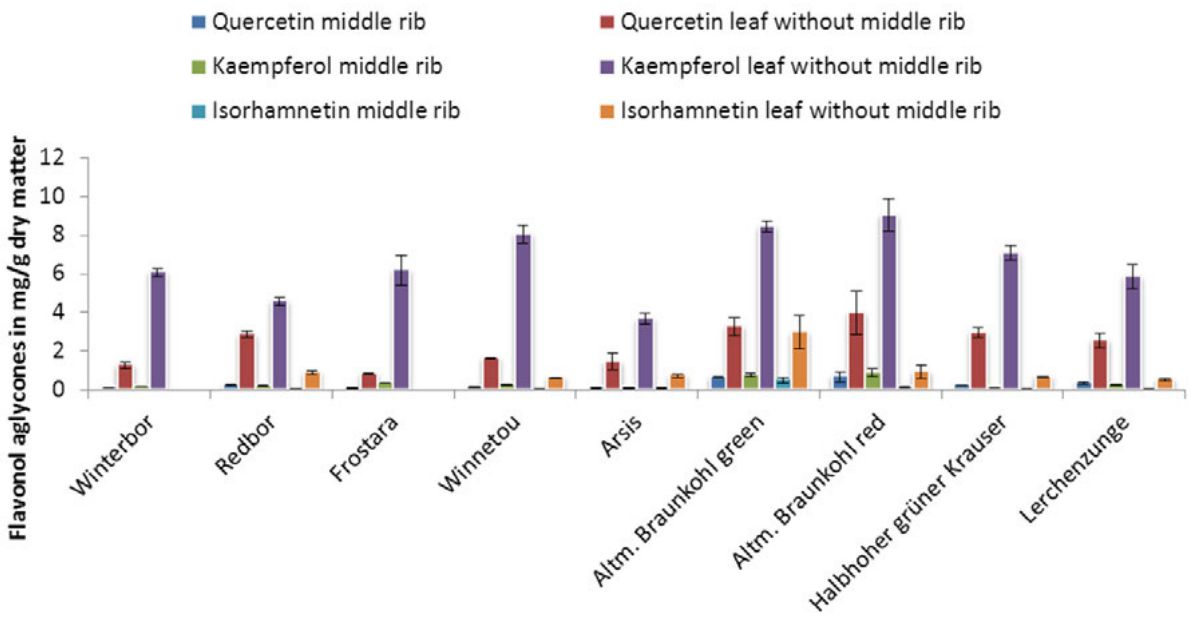

Figure 7.4: Flavonol aglycone concentrations of eight different kale cultivars grown in the field. The leaf was separated into midrib and leaf blade without midrib. The midrib contained remarkably lower concentrations of each flavonol aglycone. Susanne Neugart, unpublished data. For the statistical contrasts between the cultivars please refer to Schmidt et al. (2010).

several articles on kale, we have shown that the flavonoid glycoside concentrations are affected by temperature and photosynthetically active radiation (Neugart et al. 2013; Neugart, Kläring, et al. 2012; Neugart et al. 2016). Moreover, after acid hydrolysis, quercetin, kaempferol and isorhamnetin concentrations were observed to increase under low temperature (Fig. 7.6) whereas strong photosynthetically active radiation led to higher quercetin but lower kaempferol concentrations (Fig. 7.7). In more detail, blue and red light gained strong attention in horticulture production due to different effects on plant growth and metabolism (Demotes-Mainard et al. 2016; Huché-Thélier et al. 2016). While blue light induces mainly caffeic acid derivatives, quercetin and kaempferol glycosides as well as anthocyanins (Johkan et al. 2010; Nascimento et al. 2012; Siipola et al. 2014; Taulavuori et al. 2016) red light is not discussed as an inducer of phenolic compounds (Demotes-Mainard et al. 2016). However, synergistic effects of blue and red light are found (Johkan et al. 2010). In consequence, during sampling, one of the major challenges is to sample plants that have experienced the same conditions in temperature and radiation. Thus, one should harvest plants or plant parts from plants that are either from the middle of a block to avoid "border effects", or from one side of a row, or alternatively separately from each side of a row.

UVB Radiaition

Numerous researchers found effects of UVB radiation on the biosynthesis of phenolic compounds (Guidi et al. 2016; Jansen 2012; Lavola et al. 2013; Luis et al. 2007; Luthria et al. 2006; Z. Lv et al. 2013; Martínez-Lüscher et al. 2013; Morales et al. 2010; Nascimento et al. 2012; K. M. Olsen et al. 2009; Ryan et al. 1998; Suzuki et al. 2005; Tegelberg and Julkunen-Tiitto 2001). In more detail, UVB radiation is known to enhance the synthesis of B-Ring polyhydroxylated flavonoids such as quercetin and its glycosides (Becker et al.

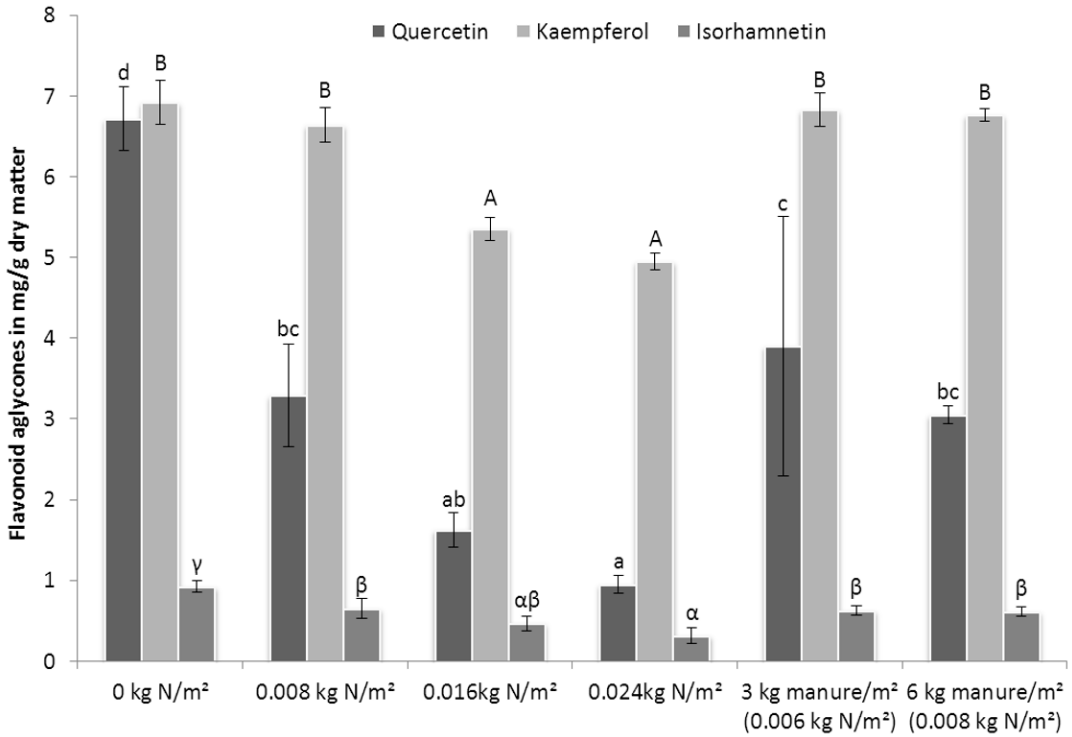

Figure 7.5: Flavonol aglycone concentrations of kale cv. 'Winterbor' dependent on the fertilization with mineral nitrogen (N) 0 to 0.024 kg/m² and organic manure 3 to 6 kg/m² (equals 0.006 to 0.008 kg N/m²). The plants were grown in the field. Different letters indicate significant differences between leaves of plants from different fertilization treatment for each flavonol aglycone ($p \leq 0.05$ by Tukey's HSD test). Each value represents the mean of three replicates ± SD.

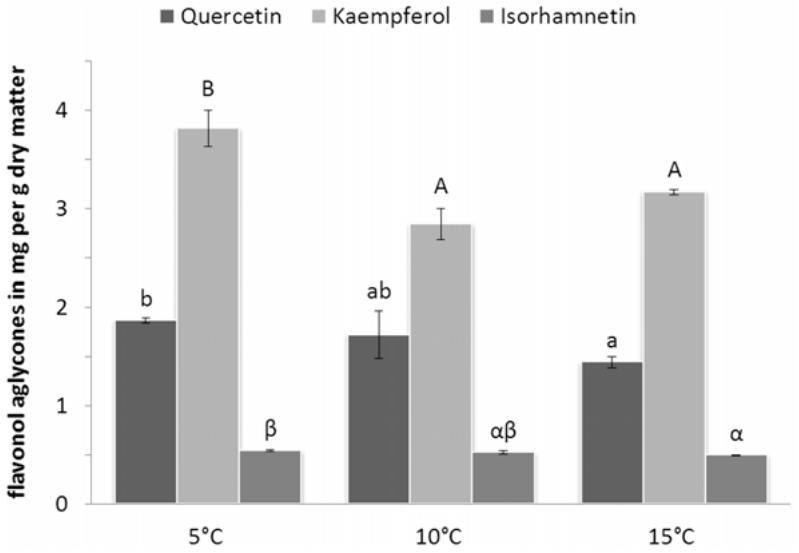

Figure 7.6: Flavonol aglycone concentrations of kale cv. 'Winterbor' dependent on the temperature in plants grown at 250 µmol/m² s. Different letters indicate significant differences between plants grown at different temperatures for each flavonol aglycone ($p \leq 0.05$ by Tukey's HSD test). Each value represents the mean of three replicates ± SD. Susanne Neugart, unpublished data

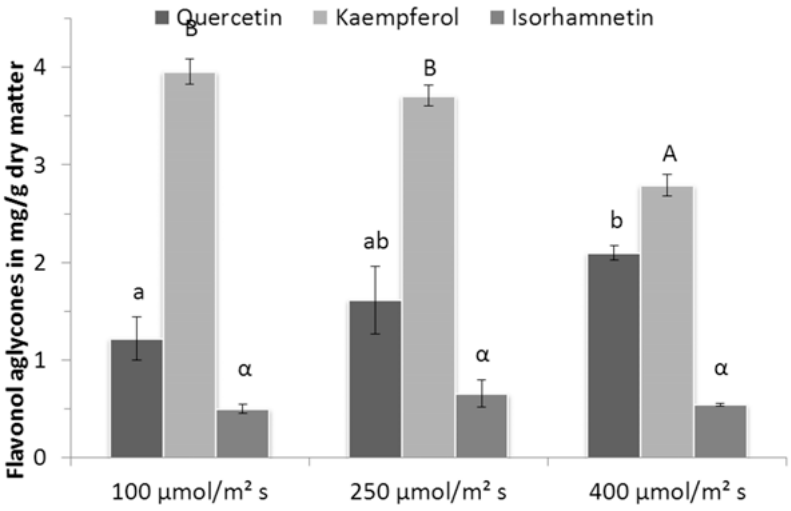

Figure 7.7: Flavonol aglycone concentrations in kale cv. 'Winterbor' plants grown at 10°C under different irradiances of photosynthetically active radiation. Different letters indicate significant differences in concentration between irradiance treatments, separately for each flavonol aglycone ($p \leq 0.05$ by Tukey's HSD test). Each value represents the mean of three replicates ± SD. Susanne Neugart, unpublished data.

2013; Bilger et al. 2007; Gliszczyńska-Świgło et al. 2007; Jaakola et al. 2004; Klem et al. 2015; Neugart, Zietz, et al. 2012; Tattini et al. 2005). Recently the interaction of UVB and temperature or water availability gained special interest (Bernal et al. 2015; Halac et al. 2014; Neugart et al. 2014). It is therefore extremely important to harvest plants or plant organs that have faced the same UVB conditions so either from the same side of the row in field experiments or of the same distance to artificial light sources in climate chambers. In case of UVB the exact measurement and monitoring of the UVB physiological quantities and the biological effective doses of UVB is mandatory for the interpretation of the data.

Diurnal Changes

Besides the accumulation of phenolic compounds as a response to environmental changes there is evidence that some plants exhibit diurnal changes in their flavonoid profiles and concentrations (reviewed by Julkunen-Tiitto et al. 2015). Therefore, the harvest should be as fast as possible and controls for each time point that plants are harvested (day or hour) should be taken if more than one harvest is necessary for the experiment.

Wounding

Sometimes sampling leads to wounding of the plants or single leaves. In such cases, the samples should be frozen in liquid nitrogen immediately to minimize any reaction caused by wounding even though that might need hours or even days (Surjadinata and Cisneros-Zevallos 2012). Sequential sampling of organs from the same individual plants should be avoided unless done within a very short length of time, such as a few minutes. One should also consider whether earlier, even non-destructive, measurements could have affected the organ or plant being sampled. In the case of wounding by herbivores, one should take into account how this relates to the objectives of the study, and if appropriate avoid sampling damaged plants, and in all cases taking note of any abnormal fea-

tures of individual plants or organs sampled.

In conclusion, the design of a sampling protocol must take into account the aims of the study. In climate chamber experiments it is simple to have defined conditions and change exclusively one or two factors. However, it is much more complicated in field experiments. It is recommended to sample plants at the same developmental stage and that were grown under the same conditions regarding exposure to various abiotic factors such as temperature and radiation. If different developmental stages or abiotic conditions are part of the experiment, these differences should be accurately measured, e.g. temperature and irradiance as well as leaf number and size. If plants are sampled from different growing locations, it can be useful to have soil samples to also allow for the assessment of the plants' nutritional status. All of the above components that make up the sampling method affect what conclusions can be validly drawn from the measurements. If single plants or leaves are sampled the biological variability among them can be directly estimated. If pooled samples are used, such estimates of variability need to be estimated indirectly. Relying on a minimum of 5 samples facilitates the detection of outliers. From the statistical point of view, what matters is the number of true replicates, which usually are neither individual plants nor leaves. Pooling of samples from different plant parts discards information but can be used if the mass of individual samples is too low. A non-random sampling sequence can be a source of bias. The order in which each plant is sampled should always be random, within blocks if present in the design of the experiment. Randomization should ensure that the sampling of individuals from different treatments is interspersed in time. The comparability and reproducibility of results reported is much improved when authors describe the sampling protocol in detail.

Drying and Storage

A comprehensive summary of drying methods and storage conditions is provided by Julkunen-Tiitto et al. (2015). In general, samples should be as dry as possible which is easily achieved by freeze-drying. If the humidity of the sample is less than 3%, samples can be stored at room temperature (Pérez-Gregorio et al. 2010). Additionally, the samples should be stored in desiccators if needed e.g. in summer or tropical climate. Otherwise the samples can be stored at -20°C to avoid the effect of humidity (Syamaladevi et al. 2011). This is the same for anthocyanins (Pérez-Gregorio et al. 2010; Syamaladevi et al. 2011). It is known that, in solution, quercetin readily photodegrades (Dall'Acqua et al. 2012), while degrading at a slower rate in dry samples. Nevertheless, samples should be analysed as soon as possible to minimize the effect of degradation on metabolite quantification.

Extraction and Measurement

Currently, there are numerous methods for extracting phenolic compounds (reviewed by Julkunen-Tiitto et al. 2015). However, a major challenge still remains in the identification and quantification of phenolic compounds in the plant matrix. In detail, the end measurement is strongly influenced by the extraction method, e.g. acid hydrolysis vs. methanolic extraction. It is important to always remember that the size of particles in a sample affects extraction efficiency—the smaller the size, the better. The second crucial point that should be taken into account is that when using a portion of a larger sample for an extraction, any lack of homogeneity in the sample compromises quantification through decreased accuracy (see section Reproducibility on page 50). If samples are difficult to grind, or inhomogeneous, it is a good trick-of-the-trade to increase the sample mass that is extracted to 1 g or more

and proportionally increase the volume of the extraction solution. The extraction generally is more efficient at higher temperatures (up to 40°C) and with longer extraction time (see section Optimization of the Extraction on page 49). If a large number of plants should be compared, it can be of use to be less specific and measure the total phenolic content (TPC) or the flavonoid aglycones and other phenolic compounds' aglycones. If the response of one plant to a biotic or abiotic factor is investigated, it can be helpful to discuss structure-activity-relationships based on a very detailed analysis of flavonoid glycosides and other phenolic compounds' glycosides.

Below is a summary of extraction methods and measurement of phenolic compounds we have previously successfully used for several species after optimization for each species. Results from our studies on kale are used to exemplify what kind of data can be generated and how these can be interpreted.

Total Phenolic Content

This approach is a fast method to quantify the total phenolic content, or TPC (for more details on this and other assays see Julkunen-Tiitto et al. 2015) in plants but was originally developed to measure proteins (Lowry et al. 1951). It yields a single measured value per sample analysed, which is assumed to behave as an approximate joint quantifier for a large group metabolites. The potential interaction with proteins is one of the disadvantages of this method. Some researchers also consider vitamin C and other antioxidants as these compounds can also react with the Folin-Ciocalteu regent and influence the results. However, the method has the advantage that different plants can be compared due to the reference gallic acid. Additionally correlations to the antioxidant activity are often also measured. Plants differ in their phenolic profiles and this might lead to over- or underestimation of the real concentrations of these phenolic compounds. Consequently, when treatments affect the phenolic profile, or when genotypes differ in their metabolite composition, bias in TPC measurements can be introduced by the use of this method. One solution is to additionally measure the plant's main phenolic compound as a standard reference and then to quantify the TPC based on this. If the antioxidant activity is to be correlated to the total phenolic content, it is recommended to measure other antioxidants of relevance in the plant as well e.g. vitamin C or carotenoids. Nevertheless, different extraction methods regarding kind of solvent, volume of solvent, extraction time, extraction temperature and other possible influencing factors such as pH can affect the quantification of the TPC, and therefore, should be optimised in order to accurately validate the different methods used. The same is true for other chemical reactions often used to estimate total flavonoid or anthocyanin content. Note that the method for the extraction and measurement of phenolic compounds needs to be validated see section Method Validation on page 46.

In kale, we analysed the TPC using the following method published in Zietz et al. (2010). For extraction, 2 g of ground sample were dissolved in 25 ml of 62.5% aqueous methanol and stirred at 500 rpm for 1 h. The mixture was then filtered and aliquots were used for further analysis. The total phenolic content TPC of kale extracts was determined using the Folin-Ciocalteu colourimetric method. In brief, 400 µl of a 20-fold dilution of each extract was mixed with 2.5 ml of distilled water, 1 ml of Na_2CO_3 (7.5% w/v) and 100 µl of Folin-Ciocalteu reagent. The thoroughly mixed solution was incubated at 35°C for 15 min. After the solution had cooled to room temperature, the absorbance was measured at $\lambda = 736$ nm (SPECORD 40, Analytik Jena AG, Jena, Germany). The results were expressed as millimoles of gallic acid equivalents per gram of dry matter (mmol GAE/g dry matter). All extracts were ana-

lysed in duplicate. In the eight investigated kale cultivars the gallic acid equivalent concentration (GAE) ranged between 0.18 and 0.31 mmol/g dry matter. The genotypic variation shows that especially the traditional, old cultivars 'Altmärker Braunkohl', 'Halbhoher gruner Krauser' and 'Lerchenzunge' as well as the red hybrid 'Redbor' are characterized by relatively high TPC, while the cultivars 'Winterbor', 'Frostara', 'Winnetou' and 'Arsis' have lower values. Those genotypes investigated in our study had higher TPC values than those found by other researchers, which ranged from 0.08 to 0.11 mmol GAE/g dry matter (Hagen et al. 2009; Heimler et al. 2006; H. Olsen et al. 2009). This difference might result from different extraction efficiency (see section Optimization of the Extraction on page 49).

Flavonoids as Flavonol Aglycones

As several flavonoids present in a given sample may share the same aglycone, and differ only in the attached residues, removing these residues decreases the number of compounds remaining in the extract. This is achieved through de-acylation and de-glycosylation of flavonoid glycosides to flavonoid aglycones. The advantage of this method is the smaller number of compounds being quantified. Of these aglycones, many are available as reference standards, which allows to accurately identify phenolic aglycones with HPLC by direct comparison against such reference standards. This identification can then be verified by mass spectrometry (then MS grade solutions for extraction solvents and measurement eluents are mandatory). Quantification of each peak compared to a standard reference is possible, i.e. the samples are measured and the peak areas under the curve for the aglycones from both samples and standards are computed. Calibration curves can then be produced with a minimum of four known concentrations of pure aglycones (see section Calibration Curves on page 51). The peak areas of the calibration curves should be in the same range as the peak areas of the samples. With the help of the calibration curves and the dilution factors (during extraction and measurement of the samples), the areas of the samples can be re-expressed as concentrations. Whether to give the concentrations in dry matter or fresh matter depends on the scientific question being addressed. For example, if the treatment of the plants or the general plant development leads to morphological changes, it is recommended to work with concentrations in dry matter due to a different water content expected.

In kale, we analysed the flavonol aglycones quercetin, kaempferol and isorhamnetin using the following method published in (Schmidt et al. 2010a). A lyophilized kale sample (0.5 g) was hydrolysed with 50% aqueous methanol and 1.6 M HCl in double determination experiments. After refluxing at 90°C for 2 h, the extract was cooled down to room temperature, adjusted to 100 ml and then sonicated for 5 min. After which, the extract was filtered through a 0.45 µm PTFE filter for HPLC analysis. With this method, phenolic acids and anthocyanidins can be measured. However, higher concentrations of HCl are needed to measure anthocyanidin concentrations (Merken et al. 2001). The extracts were separated on a Prodigy (ODS 3, 150x3.0 mm, 5 µm, particle size 100 Å) column (Phenomenex, Aschaffenburg, Germany) with a security guard C18 (ODS 3, 4 3.0 mm, 5 lm, 100 Å), at a temperature of 25°C using a water/acetonitrile gradient. Solvent A consisted of 99.5% water and 0.5% acetic acid; solvent B contained 100% acetonitrile. The following gradient was used for eluent B: 30–35% (0–5 min), 35–39% (5–17 min), 39–90% (17–21 min), 90% isocratic (21–26 min), 90–30% (26–29 min), 30% isocratic (29–34 min). Flow was performed using 0.3 ml min^{-1}, and the measured detector wavelength was $\lambda = 370$ nm. The standards dihydroquercetin, kaempferol and isorham-

netin (Carl Roth GmbH, Karlsruhe, Germany) were used to obtain an external calibration curve in the range of 0.01–10 mg/100 ml. The total flavonol concentration was calculated as the sum of the concentration of the individual flavonol aglycones quercetin, kaempferol and isorhamnetin. Quercetin, kaempferol and isorhamnetin in kale (Fig. 7.8) were identified by comparison to standards (Fig. 7.9) as deprotonated molecular ions and characteristic mass fragment ions by HPLC–DAD–ESI–MS2 with an ion trap mass spectrometer. The mass optimization was performed for quercetin [M-H]- m/z 301. The ESI source potential on capillary was 3.5 kV. The declustering voltage was -40 V and the focusing voltage was 153 V. The automated collision energy was 1 V (30–200%).

In eight kale cultivars including hybrid and traditional cultivars, high concentrations of the flavonol aglycones kaempferol and quercetin, followed by isorhamnetin were identified and quantified by comparison to standards. The total concentration of these flavonol aglycones was between 6.0 and 14.8 mg/g dry matter, which corresponds to 97.4–298.5 mg / 100 g fresh matter (Schmidt et al. 2010a). The genotypic variation revealed that traditional, old cultivars 'Altmärker Braunkohl', 'Halbhoher grüner Krauser' and 'Lerchenzunge' are characterized by relatively high flavonoid concentrations, while lower flavonoid concentrations were found in the hybrids 'Arsis' and 'Winterbor', as well as in the cv. 'Frostara'. Comparable concentrations to our results were also determined by (Z. Huang et al. 2007) in curly kale (*Brassica oleracea* var. *acephala*), with 90.5, 31.8 and 23.6 mg/100 g fm for kaempferol, quercetin and isorhamnetin, respectively. Furthermore, similar quercetin concentrations (7.7-24.4 mg/100 g fm) were detected in curly kale, but the kaempferol concentrations were much lower (21–47 mg/100 g fm) compared to our investigated cultivars, whereas isorhamnetin was not detected in these kale varieties (Hertog et al. 1992; Zhang et al. 2003) underlining the effect of strong photosynthetically active radiation during plant growth (Zhang et al. 2003) see section Temperature and Radiation on page 34. Note that phenolic acids and anthocyanindins were not investigated in these kale samples.

Flavonoids as Flavonol Glycosides

In contrast to methods described in the previous two sections, here the aim is to quantify the individual flavonoids as they are in the plant. With this method, one can investigate detailed structure-activity relationships and it is also the most precise approach to identifying phenolic compound profile and concentration. However, the reliable identification of these compounds is complex and time-consuming. To start with, an HPLC instrument coupled to a mass spectrometer (MS) as detector is needed. For precise structural determination other methods (such as nuclear magnetic resonance, i.e. NMR) are needed, e.g. to distinguish between isomers differing only in the position where the same substituent is bound. Very sophisticated MS methodology such as ion mobility could be useful for that purpose as well. Julkunen-Tiitto et al. (2015) summarize which extraction solvents, columns, eluents and wavelength as well as mass spectrometric parameters and NMR approaches can be used in the identification and quantification of flavonoids.

Due to the small number of available reference standards for complex glycosylated and acylated phenolic compounds, often only a semi-quantitative quantification based on related standards is possible. Further, it is not common to calculate response factors since reference standards are missing that would be required to do that. Generally, the quantification works as described for the flavonoid aglycones, but with one standard being used for different glycosides, e.g. quercetin-3-glucoside for several quercetin glycosides.

In kale, we analysed the flavonol glycosides

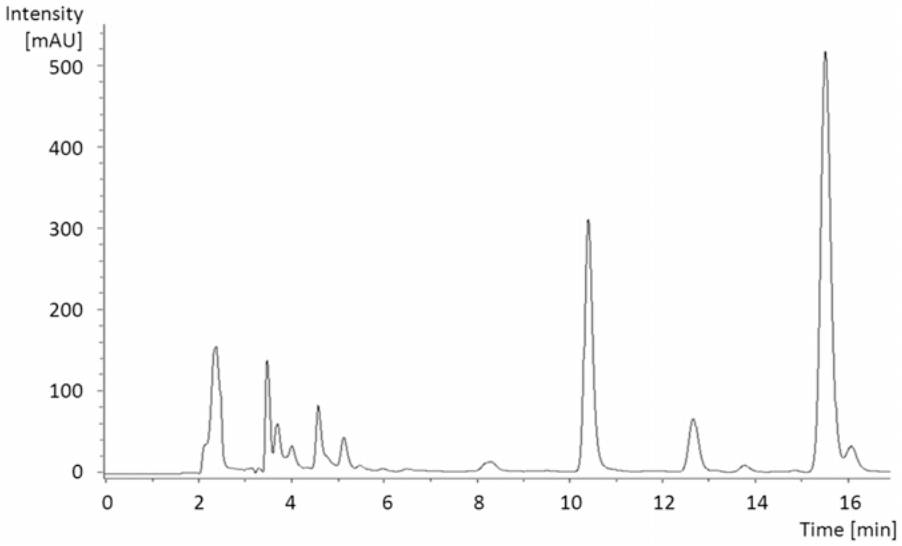

Figure 7.8: Cromatogram of flavonol aglycones in kale cv 'Winterbor' after acid hydrolysis at λ = 370 nm. Susanne Neugart, unpublished data.

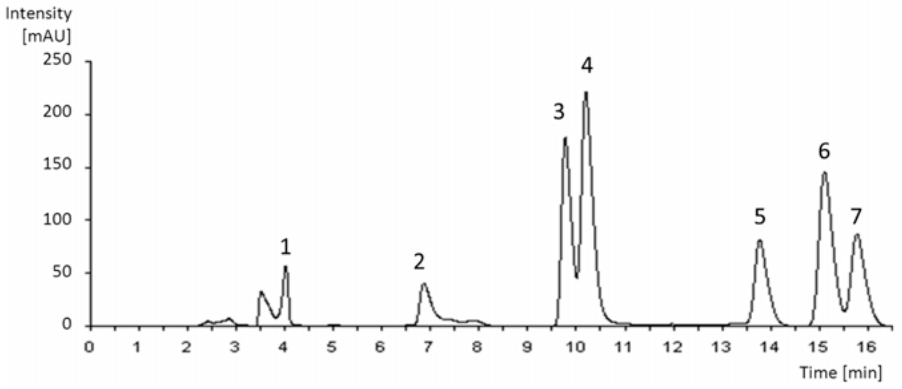

Figure 7.9: Chromatogram of a standard flavonoid glycoside and aglycone mixture at λ = 370 nm: 1-quercetin-3-rutinoside, 2-myricetin, 3-luteolin, 4-quercetin, 5-apigenin, 6-kaempferol, 7-isorhamnetin. Susanne Neugart, unpublished data.

and hydroxycinnamic acid derivatives using the following method published in (Schmidt et al. 2010b). Lyophilized kale (0.5 g) was extracted with 15 ml of 60% aqueous methanol on a magnetic stirrer plate for 1.5 h in a double determination approach. The extract was filtered through a fluted filter and subsequently evaporated to dryness. The residue was dissolved in 5 ml of distilled water and then filtered through a cellulose-mixed ether-membrane (CME) filter for HPLC analysis.

A modification for smaller volumes has been recently published in (Neugart et al. 2017). Lyophilized, ground plant material (0.02 g) was extracted with 600 µl of 60% aqueous methanol on a magnetic stirrer plate for 40 min at 20°C. The extract was centrifuged at 4500 rpm for 10 min at the same temperature and the supernatant was collected in a reaction tube. This process was repeated twice with 300 µl of 60% aqueous methanol for 20 min and 10 min, respectively. The three corresponding supernatants were then pooled. The extract was subsequently evaporated until it was dry and was then sus-

pended in 200 µl of 10% aqueous methanol. The extract was centrifuged at 3000 rpm for 5 min at 20°C through a Corning® Costar® Spin-X® plastic centrifuge tube filter (Sigma Aldrich Chemical Co., St. Louis, MO, USA) for HPLC analysis. Each extraction was carried out in duplicate. This method can also be used for hydroxycinnamic acid derivatives and proanthocynaidins. However, to date, no method validation for these compounds has been performed. Finally to extract anthocyanins, acidified methanol (0.1% v/v formic acid) is commonly used to stabilize the cations (H. Olsen et al. 2010). The flavonol glycosides were analysed using a Prodigy (ODS 3, 150 3.0 mm, 5 lm, 100 Å) column (Phenomenex, Aschaffenburg, Germany) with a security guard C18 (ODS 3, 4 3.0 mm, 5 lm, 100 Å) at a temperature of 25°C using a water/acetonitrile gradient. Solvent A consisted of 99.5% water and 0.5% acetic acid; solvent B contained 100% acetonitrile. The following gradient was used for eluent B (100 % acetonitrile) at a temperature of 30°C: 5–7% (0–12 min), 7–9% (12–25 min), 9–12% (25–45 min), 12–15 % (45–100 min), 15% isocratic (100–150 min), 15–50 % (150–155 min), 50 % isocratic (155–165 min), 50–5% (165–170 min), 5% isocratic (170–175 min). The flow was performed using 0.4 ml min^{-1}, and the measured detector wavelength for the quantification was set at $\lambda = 370$ nm for non-acylated flavonol glycosides and $\lambda = 330$ nm for acylated flavonol glycosides. The standards quercetin-3-O-glucoside and the corresponding 3-O-glucosides of kaempferol and isorhamnetin (Carl Roth GmbH, Karlsruhe, Germany) were used in a semi-quantitative approach to obtain an external calibration curve in the range of 0.1–10 mg/100 ml. Mass optimization for the ion optics of the mass spectrometer was performed for quercetin m/z 301 for the low mass flavonol glycosides. In addition, due to the lack of standards, arbitrary m/z 1000 was used as the target mass in auto-mode to include higher mass fragments for higher mass flavonol glycosides. The ESI source potential on capillary was 3.5 kV. The declustering voltage was -40 V and the focusing voltage was 153 V at mass optimization m/z 301 and 200 V at mass optimization m/z 1000. The automated collision energy was 1 V (30–200%). The MSn experiments were performed in auto- or manual mode up to MS4 in a scan from m/z 200 to 2000. Note that anthocyanins were not investigated in these kale samples. In kale, 71 flavonol glycosides have been tentatively identified by HPLC-DAD-MSn. Of these, 27 non-acylated, 30 monoacylated and 14 diacylated glycosides have been found based on the flavonol aglycones quercetin, kaempferol and isorhamnetin. Seven of these 71 compounds have been further identified with NMR in a previous study (Fiol et al. 2012). The main flavonol glycosides in kale are non-acylated and monoacylated quercetin and kaempferol glucosides, with the majority of flavonol glucosides being acylated with hydroxycinnamic acids. A presentation of selected quercetin and kaempferol glycosides is depicted in Fig. 7.10. Of the non-acylated (Fig. 7.10-A) and monoacylated (Fig. 7.10-B) compounds, the kaempferol glycosides were in higher concentration in the plants and traditional cultivars had higher concentrations than the hybrid cultivars-except for the cultivar 'Redbor'. Interestingly, this is different in the complex diacylated tetraglycosides (Fig. 7.10-C) which are in high concentrations only in cultivar 'Redbor'. Finally, our results on the identification and quantification of flavonoid glycosides in kale are supported by findings of other groups (Ferreres, Fernandes, Oliveira, et al. 2009; Lin and Harnly 2009; H. Olsen et al. 2009). Of note is that the precision had a variation coefficient up to 8% and the accuracy has a variation coefficient of up to 8% for the main phenolic compounds and up to 20% for the minor compounds which is higher than that for flavonol aglycones in kale see section Reproducibility (precision and accuracy) on page 50.

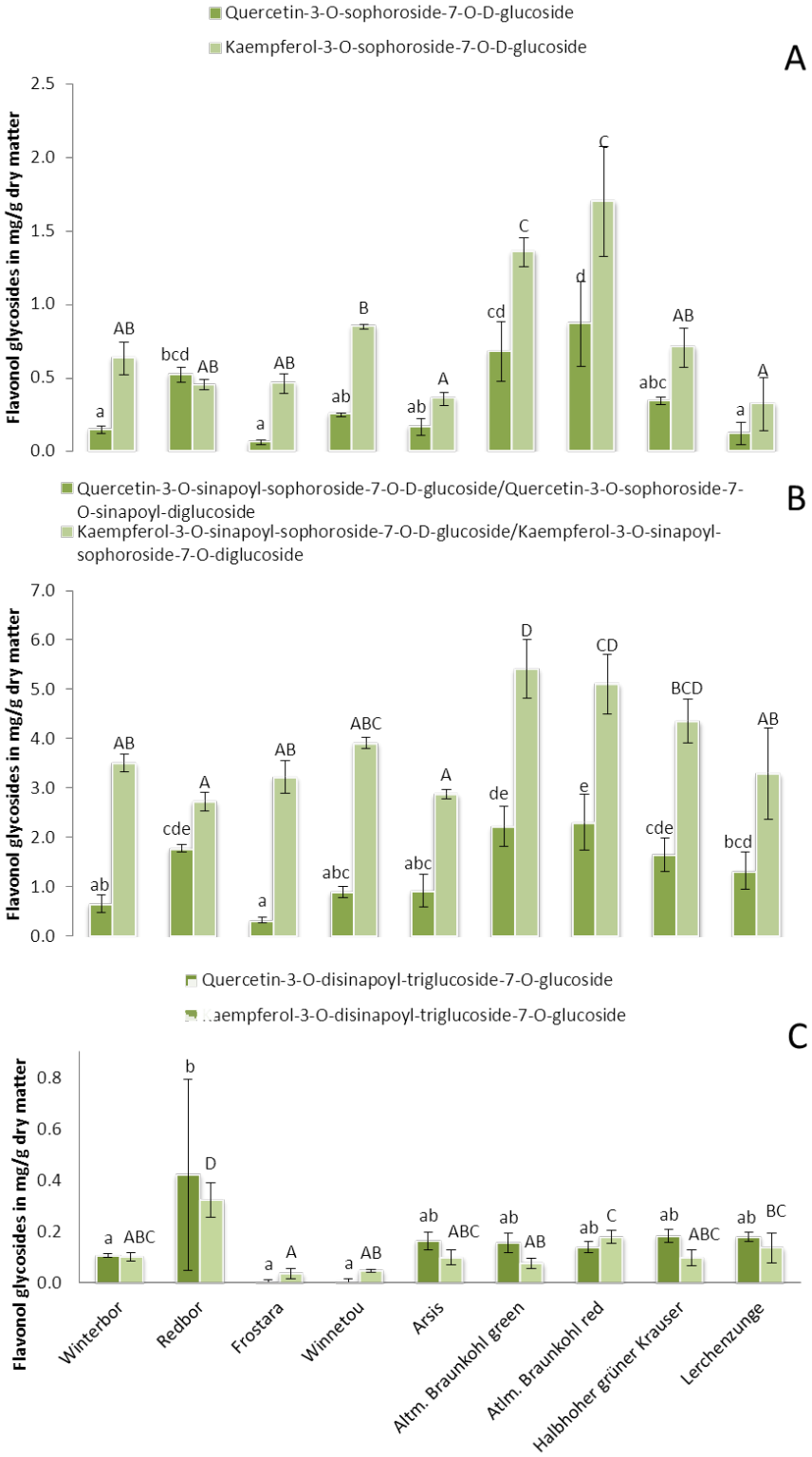

Figure 7.10: Structurally different flavonoid glycoside concentrations in kale cv. 'Winterbor'. A: non-acylated triglycosides; B: monoacylated triglycosides; C: diacylated tetraglycosides. The plants were grown in the field. Different letters indicate significant differences between the cultivars for each flavonol glycoside ($p \leq 0.05$ by Tukey's HSD test). Each value represents the mean of three replicates ± SD. Susanne Neugart, unpublished results.

Identification of Hydroxycinnamic Acid Derivatives

The identification of phenolic compounds is one of the major challenges in analytics today as there is a number of compounds and species differ enormously in their profiles and concentrations. The following is an example on the hydroxycinnamic acid derivatives in kale to highlight the process of the identification of phenolic compounds by (1) do a general literature research and find general fragmentation pattern for phenolics, (2) interpreting the fragmentation pattern measured and (3) compare to the literature if these compounds have been described before for the same genotype or species. From studies with HPLC-DAD-ESI-MSn in auto-mode to MS3, 30 hydroxycinnamic acid derivatives and a hydroxybenzoic acid glycoside (diprotuchatechuic acid-gentiobioside) were tentatively identified in kale (Fig. 7.11 and Table 7.1). The hydroxycinnamic acid derivatives can be classified into four aglycones, four quinic acid esters, four monoacylated hydroxycinnamic acid glycosides (mono-, di- and triglycosides), 12 diacylated hydroxycinnamic acid glycosides (di- and triglycoside) and six triacylated hydroxycinnamic acid glycosides (di- and triglycosides). For all identified glycosides, glucose was exclusively glycosylated – a finding that is also confirmed in the literature for other *Brassica* species (Ferreres, Fernandes, Sousa, et al. 2009; Harbaum et al. 2007; H. Olsen et al. 2009). In addition to the cleavage of 324 Da for diglucosides, the glycosides did not show the cleavage of 180 Da (characteristic of sophoroses). Thus, the diglucosides can be identified as gentiobioses, which is the case for kale (Ferreres, Fernandes, Sousa, et al. 2009; Lin and Harnly 2009).

As an example, the identification of hydroxycinnamic acid derivatives' based on fragmentation patterns is described. In the case of the triacylated hydroxycinnamic acid glycosides, three substances have the same fragmentation pattern. Starting from the deprotonated molecular ions m/z 929 (H30), m/z 945 (H25) and m/z 959 (H29), a sinapic acid (224 Da) was first cleaved in MS2 followed by the loss of a second sinapic acid or a sinapic acid residue. MS3 is characterized by the fragment ions [MH-224-224]- and [MH-224-206]-. In addition, the loss of a ferulic acid by the fragmentation [M-H-224-176]- was observed in the MS3 of substance H30. For all substances, MS3 showed the deprotonated molecular ions of hyroxyferulic acid (m/z 209) or sinapic acid (m/z 223). The substances were identified as disinapoyl-feruloyl gentiobioside (H30), disinapoyl-hydroxyferuloyl gentiobioside (H25) and trisinapoyl gentiobioside (H29). The substances disinapoyl-feruloyl gentiobioside (H30) and trisinapoyl gentiobioside (H29) have already been found in various *Brassica oleracea* (Ferreres, Fernandes, Oliveira, et al. 2009; Lin and Harnly 2009; H. Olsen et al. 2009, 2010) and *Brassica rapa* (Harbaum et al. 2007). The disinapoyl-hydroxyferuloyl gentiobioside (H25) has hitherto only been found in tronchuda cabbages (Ferreres, Fernandes, Oliveira, et al. 2009). A further substance shows in the MS the deprotonated molecular weight m/z 899. In MS2, the loss of a ferulic acid is characterized by the fragment ion [M-H-194]-. The MS3 shows both the cleavage of a sinapic acid residue ([M-H-194-206]-) as well as the cleavage of a ferulic acid ([M-H-194-194]-) and a ferulic acid residue. The substance is identified as sinapoyl-diferuloyl gentiobiose and has already been identified by (Ferreres, Fernandes, Sousa, et al. 2009) in tronchuda cabbage. For the exact identification measurements on high resolution mass spectrometry followed by NMR measurements would be necessary.

Method Validation

Which extraction method or measuring method is used depends on the scientific

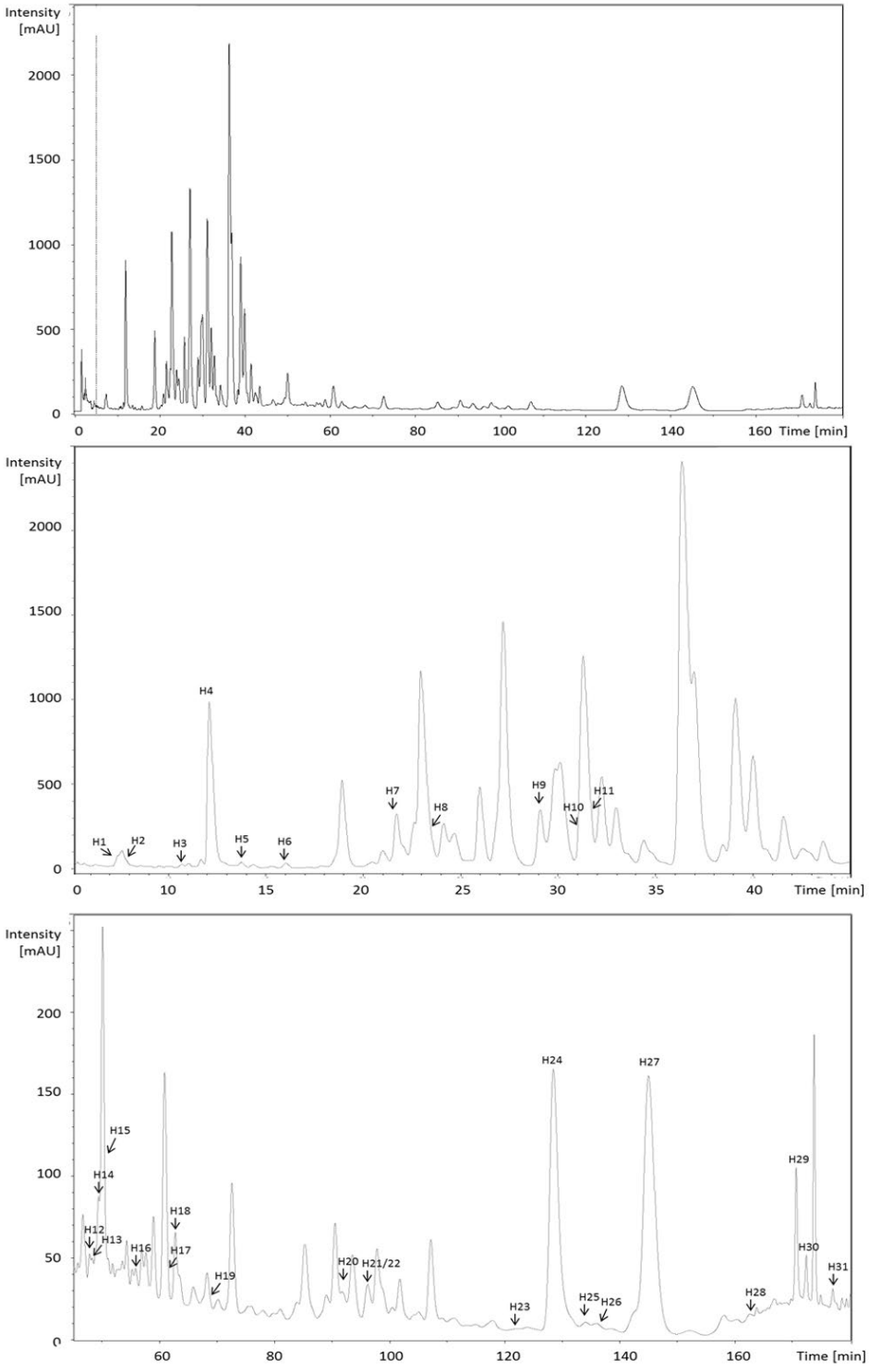

Figure 7.11: Chromatogram of flavonol glycosides and hydroxycinnamic acid derivatives of kale cv. 'Winterbor' at λ = 320 nm H1-H31 represent the tentatively identified hydroxycinnamic acid derivatives and a hydroxybenzoic acid glycoside (diprotuchatechuic acid-gentiobioside). The two lower panels are enlarged views for the initial 45 min of the run, and remaining of the run, respectively. Scale limits differ among panels. See Table 7.1 for additional details. Susanne Neugart, unpublished data.

Table 7.1: Fragmentation patterns of 30 hydroxycinnamic acid derivatives and one hydroxybenzoic acid derivative in kale. Susanne Neugart, unpublished data

	Tentative name	MS	MS2	MS3	Source
H01	feruloyl diglucoside	517	353		—
H02	diprotucatechuic acid gentiobiose	631	315	153	—
H03	feruloyl triglucoside	517, 677	179, 353, 341		—
H04	chlorogenic acid derivative	353	191		Lin and Harnly 2009; H. Olsen et al. 2009, 2010
H05	caffeic acid glucoside	341	179		Harbaum et al. 2007
H06	hydroxyferulic acid glucoside	371	209		—
H07	chlorogenic acid derivative	353	191		Lin and Harnly 2009; H. Olsen et al. 2009, 2010
H08	caffeic acid	179			Lin and Harnly 2009
H09	hydroxyferulic acid	209			Lin and Harnly 2009
H10	sinapic acid glucoside	385	223		Ferreres, Fernandes, Sousa, et al. 2009
H11	chlorogenic acid derivative	353	191		Lin and Harnly 2009; H. Olsen et al. 2009, 2010
H12	ferulic acid	193			Lin and Harnly 2009
H13	disinapoyl gentiobioside	753	529	223	
H14	feruloyl quinic acid	367	191		Lin and Harnly 2009
H15	sinapic acid	223			Lin and Harnly 2009
H16	sinapoyl-caffeoyl triglucoside	871	709	485	—
H17	sinapoyl-coumaroyl triglucoside	855	693	469	—
H18	sinapoyl-feruloyl triglucoside	885	723	499	H. Olsen et al. 2009
H19	diferuloyl triglucoside	855	693	499	H. Olsen et al. 2009
H20	sinapoyl-hydroxyferuloyl gentiobioside	739	515	191	—
H21	disinapoyl-feruloyl triglucoside	1091	929	705	H. Olsen et al. 2009
H22	sinapoyl-caffeoyl gentiobioside	709	485	161	—
H23	trisinapoyl triglucoside	1121	959	735	H. Olsen et al. 2009
H24	disinapoyl gentiobioside	753	529	223	Ferreres, Fernandes, Sousa, et al. 2009; Harbaum et al. 2007; Lin and Harnly 2009; H. Olsen et al. 2009, 2010
H25	disinapoyl-hydroxyferuloyl gentiobioside	945	721	515	—
H26	sinapoyl-coumaroyl gentiobioside	693	469	163	—
H27	sinapoyl-feruloyl gentiobioside	723	499	193	Ferreres, Fernandes, Sousa, et al. 2009; Harbaum et al. 2007; Lin and Harnly 2009; H. Olsen et al. 2009, 2010
H28	diferuloyl-gentiobiose	693	499	193	Ferreres, Fernandes, Sousa, et al. 2009; Lin and Harnly 2009
H29	trisinapoyl gentiobioside	959	735	529	Ferreres, Fernandes, Sousa, et al. 2009; Harbaum et al. 2007; Lin and Harnly 2009; H. Olsen et al. 2009, 2010
H30	disinapoyl-feruloyl gentiobioside	929	705	481, 499, 529	Ferreres, Fernandes, Sousa, et al. 2009; Harbaum et al. 2007; Lin and Harnly 2009; H. Olsen et al. 2009, 2010
H31	sinapoyl-diferuloyl gentiobiose	899	705	499	Ferreres, Fernandes, Sousa, et al. 2009

question that should be answered. Nevertheless, a method validation is the basis of reliable results. This includes optimization of the extraction, selectivity, peak purity, reproducibility (precision and accuracy), recovery, detection limit and quantification limit (if applicable), as well as calibration curve (linearity) (Bayram et al. 2013; Fucina et al. 2012; Gouveia and Castilho 2012; Li et al. 2012; Schoedl et al. 2011). If the quantification of compounds is performed with a mass spectrometer, other factors, such as ionization stability and ion suppression by the matrix, should also be considered. Importantly, a method validation should be conducted at the beginning of the establishment of an extraction or measuring method. To ensure that the established method is still correct, a double or triple determination of each sample is recommended and one or two reference standards should be measured with each sequence. Most of the parameters can then be rated in comparison with the validation conducted at the beginning. A new validation is necessary when something changed in the method, e.g. lower amounts of solutions during extraction, a new column of the same or other packing material, changes in the gradient or changes in the ionization process in the mass spectrometer.

The example shown here is the method validation performed for the flavonol aglycones in kale in which the compounds were quantified by HPLC.

Optimization of the Extraction

For the optimization of the extraction there are several factors to consider dependent on the extraction method (for different possible extraction methods please see Julkunen-Tiitto et al. 2015): extraction solution (including mixtures of polar and non-polar solution), extraction time and number of extractions, extraction temperature, sample weight, volumes of the extraction solution, shaking or mixing of the sample (Table 7.2). Exemplarily for kale the concentrations of HCl and methanol were not changed for the optimization of the kale extracts as these were established during previous experiments for broccoli (Krumbein et al. 2007). The investigation of the hydrolysis time (1, 2, 3, 4 and 6 h) on the flavonol aglycones quercetin, kaempferol and isorhamnetin occurring in kale showed that 2 hours were sufficient for acid hydrolysis to take place (50% methanol with 1.6 M HCl at 100°C).

Selectivity

The selectivity of the method is the sum of HPLC parameters that are optimized for the measurement of the phenolic compounds including choice of eluents, gradient, oven temperature, and detection wavelength. Therefore (1) sample extracts of kale (Fig. 7.8) and (2) standard mixtures of the available flavonoid aglycone standards (Fig. 7.9) were used to validate the separation of peaks and detection wavelength (chosen based on compounds' absorption spectrum) and the possible partial overlap of peaks due to elution times. This led to the selection of the eluents solvent A (99.5% water and 0.5% acetic acid) and solvent B (100% acetonitrile). The gradient and oven temperature were optimized. The detection wavelength for quercetin, kaempferol and isorhamnetin was chosen nearest to their measured absorption maxima and set at $\lambda = 370$ nm. For the method details, see section Flavonol Aglycones on page 41.

Peak Purity

The peak purity was verified by DAD (λ = 190-600 nm) by comparing peak shapes and the absorption spectrum at the key locations of a peak (base, before and after the peak; turning point, in the increasing and decreasing slope; and apex). For the relevant peaks of quercetin, kaempferol and isorhamnetin, no impurities or co-elutions were detected in

Table 7.2: Parameters of an extraction and their optimization.

Parameters of extraction	Optimization
solvent/extractant[a]	highly depends on the polarity of the target compounds, generally 50-70% methanol or ethanol are sufficient for the extraction of a wide number of phenolic compounds
length of time and number of extractions	the extraction time should be as long as necessary but as short as possible to avoid oxidation processes, the number of extractions for one extract can vary from 1 to 5
temperature	glycosides are more sensitive to temperature and might be degraded to aglycones above 40°C
sample mass	the more sample weight (5–500 mg) is used the lower is the variation coefficient of the reproducibility
volume of solvent/extractant	should be in relation to the sample weight, but the higher the volume the less concentration of phenolic compounds is found per ml so a concentration step for the extracts may be useful
shaking or mixing	for the better extraction shaking or mixing is essential during the whole extraction process

[a] Including mixtures of polar and non-polar solvents

kale, the species used here as example.

Reproducibility

To determine reproducibility, the precision (variation dependent on the HPLC measurement procedure by itself) as well as the accuracy (variation dependent on HPLC measurement procedure plus sample preparation, after acid hydrolysis) for the flavonol aglycones quercetin, kaempferol and isorhamnetin were investigated for kale. To determine precision, the *same* hydrolysed extract was injected 10 times, and average, standard deviation, as well as variation coefficient were calculated. Such calculations should be performed intra-day (within one day) and inter-day (over several days). To determine accuracy, 10 times 0.5 g of the freeze-dried kale was hydrolysed with 50% aqueous methanol and 1.6 M HCl as previously described (see section Flavonol Aglycones on page 41). The variation coefficient of accuracy was 3% for quercetin and kaempferol and 10% for isorhamnetin of which the variation coefficient of precision was below 1 % for all.

Stability

The stability of solutions is of special interest for polyhydroxylated flavonoids. Especially aglycones are degraded quickly both as standards as well as in the samples. For the standard stability, stock solutions of quercetin, kaempferol and isorhamnetin were prepared in the concentrations 1 mg/100 ml each. Aliquots of these were stored at 4°C and measured each day up to five days. The stability after one day was 96% for quercetin, 99% for kaempferol and 95% for isorhamnetin (Fig. 7.12). After five days, the stability was 52% for quercetin, 96% for kaempferol and 53% for isorhamnetin. The comparable results were observed for the samples of kale after acid hydrolysis. These highly differing percentages highlight that samples should be

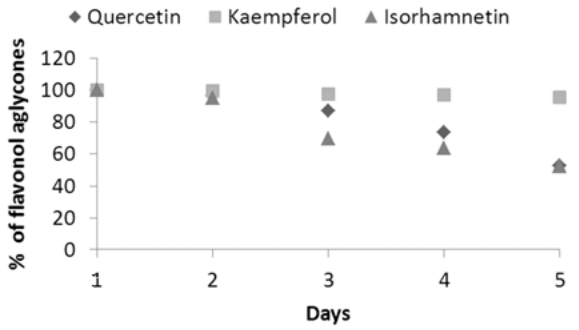

Figure 7.12: Stability of quercetin, kaempferol and isorhamnetin aglycones in kale extracts after acid hydrolysis.

prepared freshly and measured within one day after acid hydrolysis.

Flavonoid glycoside and hydrocycinnamic acid derivative standards (quercetin-3-O-glucoside, kaempferol-3-O-glucosides and isorhamnetin-3-O-glucosides, 3-chlorogenic acid) were stable (≥ 95%) under the same conditions for up to three months. Such stability was also observed for kale samples. Thus, these samples can be stored for weeks at 4°C.

Recovery

The recovery serves the review of the method concerning the quantitative evaluation. The aim is to increase the areas of the sample's flavonoids by 50% by addition of an appropriate standard. The recovery rate describes how much of the known added amount of standard contained in the measured sample is detected by the measuring procedure after extraction. To determine the recovery rate of the aglycones quercetin, kaempferol and isorhamnetin, three samples each of 0.5 g of freeze-dried kale alone, standards alone (0.5 ml each of quercetin and kaempferol (concentration 1 mg/10 ml) as well as 2 ml of isorhamnetin (concentration 15 mg/15 ml) and 0.5 g of freeze-dried kale plus the stated amounts of standards were hydrolysed with 50% aqueous methanol and 1.6 M HCl as previously described (see section Flavonol Aglycones on page 41). The aglycones were determined quantitatively using the HPLC-DAD method as previously described (see section Flavonol Aglycones on page 41). The recovery rate is defined as the ratio of the area of kale sample plus standards (x%) to the sum of the areas from the kale sample alone and the standards alone (100%). The recovery rate for quercetin (108%), kaempferol (112%) and isorhamnetin (110%) were higher than 100% which would lead to an overestimation of the results. Higher or lower recovery rates are a result of interactions of the standard compound with the matrix e.g. due to antioxidants in the matrix that stabilizes the standards. These recovery rates need to be included in the quantification to avoid over- or underestimation of compounds.

Detection Limit and Quantification Limit

The detection limit is the lowest concentration of detection of a target compound and was determined for the aglycone isorhamnetin as it occurs at low concentrations in kale. For this purpose, the signal-to-noise ratio was used to estimate the detection limit. The detection limit is reached when the noise (baseline) detected is exceed by a signal (peak) by a factor of 3. After acid hydrolysis, the kale sample was diluted and measured. The dilution for measuring the smallest signal was 1:20 and the dilution for measuring the noise was 1:100 (several others are measured). The detection limit was 276 ng/g dry matter with a peak at the dilution 1:20 that was approximately 3 times higher than the baseline of the dilution 1:100. The quantification limit is calculated based on the detection limit and should exceed the detection limit by 3 times. Thus, for isorhamnetin, the quantification limit is 828 ng/g dry matter.

Calibration Curves (linearity)

After the method validation the calibration curves for the quantification of compounds

are measured. Therefore, a range of minimum 4 known concentrations of reference standards are measured and the areas are used to generate a function. The most precise way is to use isotopic labeled standards. For quercetin, kaempferol and isorhamnetin for flavonoid analysis in kale the calibration curves were prepared with available reference standards. For the quantification of the aglycones quercetin, kaempferol and isorhamnetin standards (solved in 50% aqueous methanol) were measured externally. The concentrations were adjusted using pre-experiments with kale and measured in a range of 0.01–10 mg/100 ml. For quercetin (302.2 g/mol), the standard was quercetin dihydrate (338.3 g/mol). For the initial weight of 1 mg of quercetin to 10 ml, 1.12 mg of quercetin dihydrate was used. The equation of the reference standards were used for the calculation of the flavonoid concentration in kale.

Conclusion

The accurate analysis of phenolic compound profiles and concentrations in plants has been of interest for many years. However, to date, a standard procedure does not exist.

This article highlights the effect of abiotic factors on flavonoids and recommends that these should be considered while planning and conducting experiments. It is of high importance to equalize the growth conditions for plants under different treatments with only the factor(s) under study varying systematically. Proper randomization and precisely monitoring the experimental conditions helps ensure reproducibility of studies. We here have taken advantage that we have conducted a number of experiments on kale cv. 'Winterbor' covering responses to various abiotic factors. Using data from a single cultivar makes it easier to demonstrate the range and complexity of the responses. In kale, indoor experiments in climate chambers resulted in concentrations of kaemp-

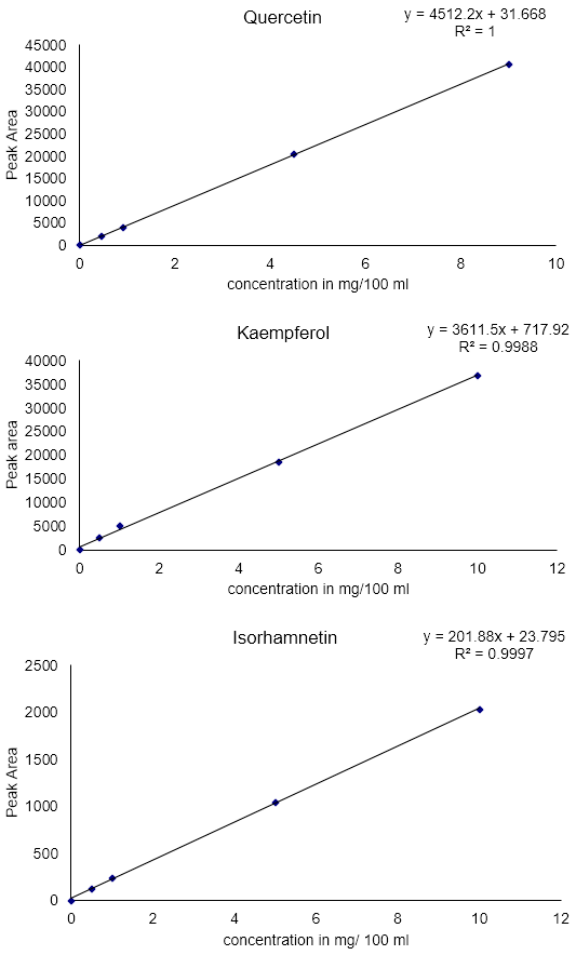

Figure 7.13: Calibration curves for the three aglycones quercetin, kaempferol and isorhamnetin. The integrated area under the peaks of the absorbance vs. time curves, plotted against the known concentration of the standard samples that were injected. The fitted equations, shown in the top right corner of each panel, are later used to convert peak areas from samples of unknown concentration into actual concentrations in the extracts.

ferol, the main aglycone of kale, ranging from 0.7 to 4.0 mg/g dry matter while quercetin varied from 0.6 to 2.1 mg/g dry matter dependent on the factor that was investigated. In the outdoor experiment on nitrogen fertilization the concentrations were much higher at 5.0–6.8 mg/g dry matter for kaempferol and 1.0–6.5 mg/g dry matter for quercetin. Of note is that the quercetin to kaempferol ratio changed dramatically. The observed pattern was that the quercetin to kaempferol ratio increased with increasing plant age, in young leaves, with low nitrogen supply, with high irradiance (photosynthetically active and UVB radiation). A ranking of which of these factors has a stronger impact is difficult, if not impossible, to establish as this requires setting a "reference" condition for each factor. Interactions among factors can be expected as well. In conclusion, in studies of mechanisms, controlled environment experiments should be favored to exclude uncontrolled variation in biotic and abiotic factors. Field experiments provide more realistic conditions, but are subject to temporal and spatial variation. This means that continuous monitoring of environmental conditions must be routinely done and the resulting data reported. For example, daily irradiance during growth up to the harvest needs to be reported as plants accumulate phenolic compounds dependent on the accumulated exposure to radiation (Del-Castillo-Alonso et al. 2016). Furthermore concentrations at the time of metabolite measurement, also depend on sample storage: i.e. low humidity of the samples is more important than the temperature during storage in the case of phenolic metabolites.

Several different methods can be used to measure profiles and concentrations of phenolic compounds. The decision of which method to use should be related to the scientific question. The total phenolic content is a fast and cheap method to gain preliminary information about the extracts. However, specific identification and quantification of phenolic compounds is not obtained. A more detailed analysis of flavonoid aglycones and aglycones of other phenolic compounds is useful for a number of questions related to the beneficial effects of plant phenolic compounds in humans and how large an effect can be expected. However, the most suitable method to answer questions related to metabolism and function in plants is the analysis of flavonoid glycosides and glycosides of other phenolic compounds. This method is time-consuming, expensive and needs good analytical skills to be able to achieve a correct identification and quantification based on HPLC and mass spectrometric data. Nevertheless, one should be aware that structurally different phenolic compounds might respond differently to biotic and abiotic factors. It is not possible to rank the methods as all of them are useful for different research questions, consequently the question under study should drive the selection of the analytical method.

Nevertheless, method validation is the basis of reliable results and should be performed in advance of the measurement of samples from experiments so as to establish the quality of the data to be acquired. A new validation is required whenever the plant species, the extraction method and/or any other significant aspect of the protocol are changed. As validation is the basis of reliable and consistent results, one should take the time and do a proper valid as frequently as needed. One of the most important aspect is reproducibility, which can be monitored by means of double or triple parallel determinations of each sample or by including one or two reference standards in each batch of samples measured in the laboratory.

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Report on short term missions

UV4Plants: our experience from visiting two research centers in Germany

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We have been studying how solar UV and visible radiation affect plant growth, performance, gene expression and accumulation of metabolites as part of our Ph.D. studies. To monitor the responses in natural light conditions, we performed several outdoor experiments in the experimental field at the Viikki campus of the University of Helsinki. Challenges of these experiments are changes in weather conditions, such as from clear to cloudy sky, rainy to completely dry days, and even in the summer, temperature occasionally dropping to nearly zero degrees at night or rising to thirty degrees in full sunlight. In order to create different solar light treatments, we use Plexiglas and polycarbonate filters which exclude selected wavelengths of UV and visible radiation. Plants are either germinated and grown under filters, or transferred from growth rooms to outdoors for solar light treatments. In both cases, fluctuation in environmental conditions causes high variation in the collected data, requiring ample replication within each experiment and in time to ensure reproducibility. With the high number of uncontrolled variables, it is also difficult to pinpoint one specific factor causing the variation in responses among replicates. Day length is one of the other major limitations of outdoor summer experiments in Helsinki (60° North) where days are very long during summer. *Arabidopsis thaliana* is one of our model plants which flowers much earlier under long-day conditions than under short-day conditions. Therefore, performing a complete growth cycle outdoor experiment with this species in Helsinki during summer is difficult.

Figure 8.1: View of the hall where the sun simulators are located, in a redeployed building that used to be the home of a small nuclear reactor.

In order to perform experiments in stable environmental conditions, we collaborated with Dr. Andreas Albert, a physicist, and Dr. Barbro J. Winkler, a biologist, from Prof. Jörg-Peter Schnitzler's research group at the Research Unit Environmental Simulation (EUS), Helmholtz Zentrum, Munich. This collabor-

Figure 8.2: View of the sun simulator used in the experiments at EUS.

ation gave us an opportunity to use the sun simulators at the EUS facility (Figs. 8.1 and 8.2). They are growth chambers with lighting conditions very similar to natural sunlight and yet they provide a controlled environment, enabling stable conditions for both short- and long-term experiments. During our visit, we familiarized ourselves with the EUS facility and got acquainted with other researchers working at the Helmholtz Zentrum Munich. Most of the work related to the sowing of seeds, transplantation, irrigation and setting up the solar simulator chamber was carried out by the personnel of the EUS facility under the supervision of Dr. Andreas Albert.

Our experiment was performed as a series of four replications in time (February–April 2015 and October–November 2015). The main aim of this experiment was to compare the short-term (6 h) and long-term (21 days) effects of UV and blue components

Figure 8.3: *Arabidopsis* plants from the experiments at EUS.

of simulated solar radiation on the growth, gene expression, and metabolite accumulation and composition of *Arabidopsis thaliana* plants (Fig. 8.3). We used four genotypes of Arabidopsis: wild type Landsberg *erecta*, UV-B photoreceptor (UVR8) mutant *uvr8*-2, UV-A and blue light photoreceptor (Cryptochromes 1 and 2) mutant *cry1cry2* and transparent testa 4 (*tt4*) mutant which has a mutation in the flavonoid biosynthesis pathway.

Plants were randomly distributed under five light treatments to study the effect of UV-B (wavelength 280–315 nm), UV-A (315–400 nm), blue (400–500 nm), short UV-A (315–350 nm) and long UV-A (350–400 nm). For the treatments, we used similar optical filters (glass and Plexiglas) to those used in our field experiments. The chamber consisted of two cuvette systems one for short– term treatment and another for long–term treatment. Photographs of the plants were taken at the end of each round to quantify the projected rosette area. At the end of the experiment, plants were harvested in liquid nitrogen and stored at −80°C for gene expression and metabolite accumulation analysis.

The European Plant Phenotyping Network (EPPN) funded this experiment, which gave us an opportunity to experience a different environment and work culture in a laboratory abroad. This collaboration was essential for producing high-quality research

by combining the expertise of Dr. Pedro J. Aphalo's research group at the University of Helsinki with Prof. Jörg-Peter Schnitzler's research group at the Helmholtz Zentrum.

The Helmholtz Zentrum is located in a pleasant and nature-surrounded area, away from the city noise. It has regular public transport connections to different parts of Munich, but sometimes the frequency of the transportation was lower in the evenings and weekends, which caused us some problems. In addition, working in a completely new environment required some time to adjust, to learn the location of the equipment and the way things work. However, after a few days, we integrated well into the system and managed to work more efficiently. Dr. Andreas Albert and Dr. Barbro Winkler, who made us feel welcome, gave us a lot of advice and helped us in the practical issues related to our experiment and stay in Munich.

During each round, we ground the frozen samples and transported them to the University of Helsinki. The gene expression study is in progress and being carried out at the University of Helsinki. To assess the metabolite accumulation and composition, we are collaborating with Dr. Susanne Neugart from Prof. Monika Schreiner's research group at the Leibniz Institute of Vegetable and Ornamental Crops (IGZ), Großbeeren, Germany. This collaboration was one of the many positive outcomes from our 1st Network Conference of UV4Plants held on 30–31 May 2016 in Pécs, Hungary.

In early 2017 Neha Rai travelled to Großbeeren, Germany, staying as a visiting researcher for one month at Prof. Monika Schreiner's lab. Großbeeren is a small town located on the outskirts of Berlin. Both the institute and guesthouse were located on the same campus, hence very convenient for work purposes. Prof. Monika Schreiner's research group has excellent expertise and equipment for the identification and quantification of plant secondary metabolites such as flavonoid glycosides and glucosinolates

Figure 8.4: The lab bench at Großbeeren, Germany

(Fig. 8.4). There was also an opportunity to quantify the hormone abscisic acid (ABA) under the guidance of Prof. Susanne Baldermann and PhD student David Schröter. For extracting metabolites and ABA, we used freeze dried samples and followed standard protocols. This research visit provided an opportunity to learn the principles behind HPLC-mass spectrometry and also hands-on experience with different sample extraction methods and data processing. The visit was funded by the EMBO short-term fellowship (Ref: ASTF 570-2016) granted to Neha Rai.

From our experience, we highly recommend the use of solar simulators in plant UV research and in general any plant research requiring a steady, easily adjustable environment with natural-like light conditions. We also encourage all young researchers to take advantage of available opportunities for visiting other laboratories, which allow not only training but also the development of a network of contacts and collaborators for the future.

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Neha Rai is a doctoral student in Sensory Photobiology and Ecophysiology of Plants (SenPEP) group working under Dr. Pedro J. Aphalo at University of Helsinki, Finland. She has been studying the molecular responses of plants exposed to solar and simulated UV and blue radiation. She is also assessing the molecular responses of UV- drought interaction in plants. Previously, she has worked as a research assistant at the Laboratory of Photosynthetic membranes at the Biological Research Centre, Szeged, Hungary. She holds her Master's degree in Plant Biology and Biotechnology from University of Hyderabad, India. She did her master's thesis on understanding the role of osmolytes in rescuing the damage of photosynthetic apparatus of salt induced *Chlamydomonas reinhardtii*. She did her Bachelor's degree in Botany Honors from Banaras Hindu University, India. In addition to her research activities she is keenly interested in creative writing and photography

Sari Siipola completed her Masters thesis in Plant Biology in 2011 at the University of Helsinki. In her thesis, she studied effects of UV and blue light on pea plants' secondary metabolism and growth. Currently Sari Siipola is studying towards her PhD. In her Doctoral thesis, she will discuss effects of UV and blue light on plant physiology and structure, and the possible use of these responses in plant production. In addition to Plant Biology, Sari Siipola has studied Environmental Biology and Science communication.

Yan Yan has specialized and completed a Master programme in Ecology during September 2012 to June 2015 in the school of Life Science of Lanzhou University, China, during which she focused on functional analysis of the UDP-Glucose Pyrophosphorylase gene family from *Populus euphratica* and *Populus pruinosa*. Currently, she is doing PhD studies in DPPS doctoral programme of the University of Helsinki. Her study subject are the different responses to solar blue and UV radiation of different UV and drought sensitive cultivars of the legumes species *Vicia faba* and *Medicago truncatula*: looking at connections between responses from whole plant, physiological and molecular levels. She is currently visiting the lab in Großbeeren, in relation to the analysis of samples from an experiment carried out in Helsinki.

UV4Plants Association's and UV4growth COST Action's role. This experiment was imagined and planned during UV4growth and UV4Plants meetings. It directly involves members from three different institutions, and was funded through various sources. In part by a large grant from the Academy of Finland to Pedro J. Aphalo, additional funding for use of the solar simulators from EPPN (European Plant Phenotyping Network) and for a planning visit by Andreas Albert and Barbro Winkler to Helsinki, from EMBO for N.R.'s visit to Susanne Neugart's lab in Großbeeren, from the doctoral programme in plant science (DPPS) of the university of Helsinki for other travel expenses. Not only the visits, but the whole experiment were made possible by the UV4Plants association and its predecessor, the UV4growth COST Action.

■ Report on two internships

A first step into Plant Research

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As part of my bachelor degree program in Biology and Applied Mathematics in France, I had to study abroad for one year. I wanted to be able to follow my courses in English but also to learn a new language. I wanted to discover life in another European country and I was fascinated by the light, weather and the level of education of the Nordic countries…I landed in Helsinki in late August, one year ago.

What I really valued in Finland was the opportunity for the students to fully create their program by choosing their courses. My two first years of a B.Sc. in France, where we follow compulsory courses, gave me a broad view of the different areas in Biology. In Helsinki, I had the new and vertiginous liberty to create my own education. This was interesting and formative, as it forced me to decide what I wanted to do. I chose to study mostly Plant Biology and Mathematical Modeling.

I had the chance to attend a lab course in Molecular Plant Biology. This course consisted in different experiments supervised by researchers from several teams. This kind of lab course where you work directly in the university labs with the researchers who are not necessarily teachers is not common in France. It is however a great chance for the students, as it provides the opportunity to meet the researchers working at the university and to discover their areas of expertise. I learned to apply useful tools in plant molecular biology research, and to present the results in the usual ways for scientific research, by writing a report or doing oral presentations.

Another interesting and sometimes arduous point of this year abroad was the work in pairs or in teams. The English language sometimes seemed to be different between countries. So speaking of details of an experiment, or discussing results often turned out to be a complex exercise. We had to overcome our differences in culture and background, to fully understand and agree with each other.

At the end of the first semester, I contacted Pedro J. Aphalo and T. Matthew Robson to ask them for an internship opportunity, and happily obtained two positive answers. I definitely appreciated the fact that the labs were open to the students interested in their work and in gaining research experience.

Supervised by Luis O. Morales in the SenPEP (Sensory and Physiological Ecology of Plants) research group, I modestly contributed to a bigger project studying the impacts of UV and blue light on *Arabidopsis thaliana* gene expression and its dependence on different photoreceptors. I did all the stages of the experimental research from the sowing of seeds and application of light treatments, to the harvest and lab work. Being able to do the experiment from the very start, the seed, right through to the qPCR on the leaf samples of the treated plants was very exciting and gratifying. However, I had to leave before I could analyze the data, and missed one of the most important steps in the research.

I spent my last month in Finland at Lammi Biological Station, helping PhD students from

Figure 9.1: The author and Craig Brelsford in a forest near Lammi, Finland.

CanSEE (Canopy Spectral Ecology and Ecophysiology) research group, and running my own small experiment. I lived with the other students and researchers, trapped in the heart of Finland, a lake and a forest as my playing field (Fig. 9.1). I experienced spring's everyday changes by helping the PhD students Craig Brelsford and Marta Pieristè doing their measurements in the forest, which was rapidly waking up after the cold and snowy months. In parallel, I also set up a small field experiment. I chose to work on the effect of sunlight and shade on leaf defense response to herbivores. I sampled *Sorbus aucuparia* trees, common in Finland and attractive to herbivores, growing in either North- or South-exposed forest stands and hedgerows. I could discuss the different questions that were raised as I set up the experiment with Craig, who provided valuable help. Back in France, I had the chance to write an article about my results. It was the first time I did this kind scientific writing, and it turned out to be a truly intriguing but complex exercise to complete at a distance.

I realized how hard it is to obtain the best conditions for an experiment when you work out in the field. I realized how many factors you have to take into account, from the time of the day and sun exposure, to the soil quality…I realized how important it is to have a strong scientific background in your research domain when you do an experiment, and how crucial it is to be able to think about the results obtained by presenting the data in different ways to identify any mistake or interesting behaviour. I also realized the importance of communication in science, the interest to share and discuss your results with the others, and to keep up with the results of your peers.

Living in a research station was a great experience. It enables you to be directly in touch with the different work going on there and to discover other research areas, by attending conferences, or asking questions to the scientists working at the station. I also really appreciated that in both of my internships, my supervisors were available if I had any questions, but let me work independ-

ently. It was not easy, but really formative and it helps in getting to know oneself better and in gaining self-confidence.

Amongst my courses, I also followed Mathematical Modeling classes, given by Eva Kisdi and Stefan Geritz, which I really enjoyed. Mathematics and Biology are two domains that we usually separate from each other, but I am truly convinced that mathematical tools are powerful for biological research, and will be of a great help as we try to understand and grasp complex systems. As part of one course, I carried out a small project on the evolution of seed dormancy in a fluctuating (stochastic) environment. I studied the probability that a seed germinates, according to the local environmental conditions over several years. Modeling biological processes such as plant colonization, development, or nutrient fluxes within the plant gives another and more mechanistic understanding of plant functioning. Simulations test many hypotheses without practically doing the experiment, something that would cost both time and money. Simulations also raise new questions and new ideas, and allow not only a better comprehension of the system but also a prediction of the outcome.

In Finland, I gained research experience. Most bachelor programs are largely theoretical in France, and practical experience was not something I was expecting from this year of study, but I am truly happy to have been able to spend time in the lab and in the field doing research. Overall, my stay in Finland really helped me to find myself in science and confirmed my desire to continue into plant research. I had the chance to make a first step into plant science research, and I am willing to carry on along this path.

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Rozenn Pineau. I obtained my Bachelor degree in Biology and Applied Mathematics in June, 2016 from the University of Pierre et Marie Curie, Paris. I started a Master in Plant Biology at the University of Montpellier in September, 2016. The first year of the program is composed of courses during the first semester, and of a research internship for the second semester. I completed this internship in Yuelin Zhang lab, working on actors of the plant immunity activated downstream pathogen perception, in Vancouver, at the University of British Columbia. This novel experience was challenging, but I still would like to pursue a carrier as a Plant Biology researcher. I am planning to take a gap year to gain more research experience from September, 2017. I will be working in Plant Conservation, Ethnobotany and Phytochemistry with Cassandra Quave, at the University of Emory, in Atlanta.

Book Review

Exploring Ultraviolet Photography

by David Prutchi

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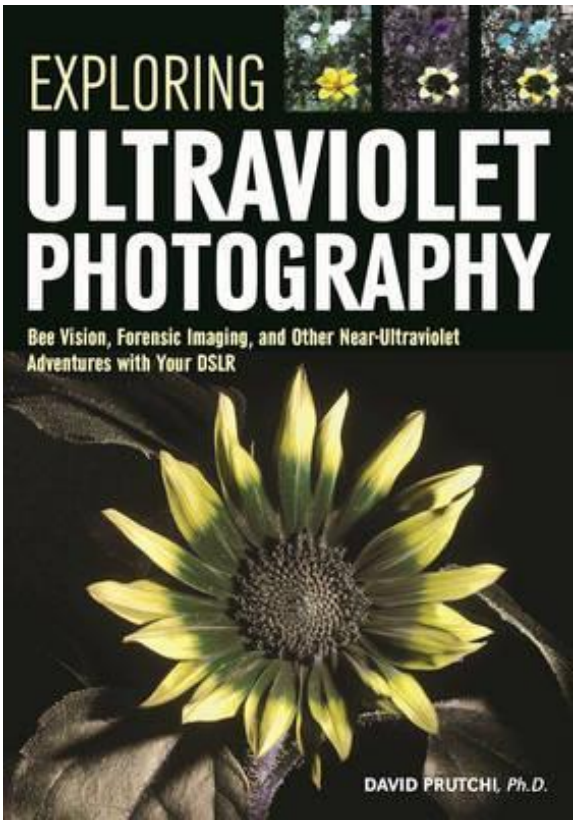

I am writing this review after discussing my impressions about the book with Lasse Ylianttila. The book authored by Prutchi (2017), fills a void as there is no other book on UV photography with digital cameras. This introductory book is written in a way that is easy to understand and concise (128 pages). Rather surprisingly, a second book describing methods for UV and IR photography will be published in October: *Digital Ultraviolet and Infrared Photography* Davies 2017.

The first impression and the expectations raised by Prutchi's book were very positive. Both myself and Lasse Ylianttila would have liked a more formal treatment, with citations or explanations about the origin of the data presented in plots and more elaborate arguments and explanations to justify some of the recommendations given. It is not a scientific or technical presentation of methods. However, this does not mean that the book is not good, but rather that we, and other UV photographers with an understanding of radiation physics and/or aiming at producing UV images in a consistent and reproducible way are not the main audience the book is aimed at. That it is published in the same series as books on wedding photography and on starting a photography business reveals the intended audience. The book is an introduction to the subject and will be very useful to anyone getting started in UV photography.

The book is structured in a logical way, and divided into chapters titled: Introduction, 1. DSLR Cameras for Ultraviolet Photography, 2. Lenses for Ultraviolet Photography, 3. Filters for Ultraviolet Photography, 4. Ultraviolet Light Sources, 5. Technique, 6. The World Through the Eyes of Birds, Bees and Butterflies, 7. Applications in Science, Medicine, Forensics and Art. The titles of the chapters provide a good description of the contents of the book, with some exceptions: chapter 1, not only describes cameras, but also gives detailed instructions on how to convert a DSLR (digital single lens reflex)

camera for use in UV photography. Chapter 2 describes lenses designed for UV photography and "accidental" UV capable lenses. Chapter 3, about filters, is very short but describes in enough detail the main problem of many ionic UV-pass filters: that they transmit IR radiation. Chapter 4, on light sources describes, in addition to sunlight, both traditional discharge lamps and state-of-the-art LEDs. In the case of LEDs, the book even describes how to build a portable light source. Chapter 5, on technique, it is more heterogeneous, covering different aspects of the capture of digital UV photographs, including brief treatment of some techniques like focus stacking and HDR (high dynamic range) based on merging multiples images, and which are also useful in other contexts. Chapters 6 and 7 could have been more thorough both in covering different use cases, and also in explaining the principles involved. With respect to biology I noticed some inaccuracies, and over simplifications. What is missing in the book are detailed descriptions of image processing and of the use of wired or wireless tethering of cameras to a computer, or nowadays alternatively to a tablet or even a phone. Having live view images on a larger screen is very useful, as is for the photographer to be able to control and trigger the camera from a distance, and in this way avoid unnecessary exposure to UV radiation.

A book this short cannot be comprehensive in relation to camera options. My major quibble is that mirror-less cameras are barely mentioned although nowadays are a very good, if not a better alternative to DSLRs. The book will be most useful to readers who have little previous experience with UV photography, providing a much faster learning curve than that possible by reading here and there in the internet the different scattered pieces of information and advice. More experienced readers will find some new ideas and recommendations, but much of the content will not be new to them. As an example, after reading the instructions for building an UV source based on LEDs, I revised the design and built a UVA light source for myself.

The recommendation of disassembling and turning around the Baader-U filter surprised me because in theory the transmittance of a filter does not depend on its orientation. I even measured transmittance of our Baader-U obtaining almost identical spectra independently of which side of the filter faced the light source. In the end I found a possible explanation. Apparently, reversing the filter can ameliorate reflections and reduce flare in certain cases. I found the explanation in a post about the book (Blum 2017) at a site very useful to those interested in UV photography and plants (http://www.ultravioletphotography.com/).

All in all a very useful introduction to the subject that focuses on *how* to take UV photographs with a modified digital single lens reflex camera, with limited explanations of why some approaches work and others do not.

Acknowledgements I wish to thank Lasse Ylianttila, not only for sharing his impressions about the reviewed book, but also for getting me interested in UV photography some years ago and sharing his knowledge on the subject and his photographs, some of which can be viewed in the image gallery at the UV4Plants web site (http://uv4plants.org/).

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Key aims of the UV4Plants international association are to

- promote and foster research-excellence and good practice in plant UV research through the organisation of innovative events in research, public engagement and education
- provide channels for members to inform the plant UV research community about relevant activities or events of common interest
- enhance the usefulness of plant UV research by facilitating the transfer of knowledge from academia to stakeholders and the general public
- initiate and foster stakeholder contacts as part of an agenda of product development
- liaise with scientific funding bodies to influence their research agenda
- develop with its members the benefits of membership and the relevance of the Association

The Rules of the UV4Plants association, information on membership, management committee and up-to-date news are available at http//www.uv4plants.org.

A new association with a history The origin of UV4Plants was the very successful COST Action FA0906 'UV4Growth' which was active from 2009 to 2014. It brought together photobiologists, molecular biologists, ecologists, meteorologists and stakeholders from agriculture and industry. Many new collaborations were started and new ideas developed.

Three large conferences, and several workshops and training events were organized. Four special journal issues were produced: *Physiologia Plantarum* **145**, 4, *Emirates Journal of Food and Agriculture* **24**, 6, *Plant Physiology and Biochemistry* **93**, and *Plant, Cell & Environment* **38**, 5.

Most participants, the members of the managing committee and the external evaluator all agreed in that a way of continuing and furthering the achievements of 'UV4Growth' was needed.

Invitation to Join UV4Plants UV4Plants welcomes a whole spectrum of members from both academia and industry, applied and basic research. Membership fees for 2016 are EUR 25.00 for students and retired staff, EUR 50.00 for academic members, and EUR 250.00 for industry members. See http://www.uv4plants.org/news/invitation-to-join-our-association/ or contact mailto:secretary@uv4plants.org for details.

UV4Plants

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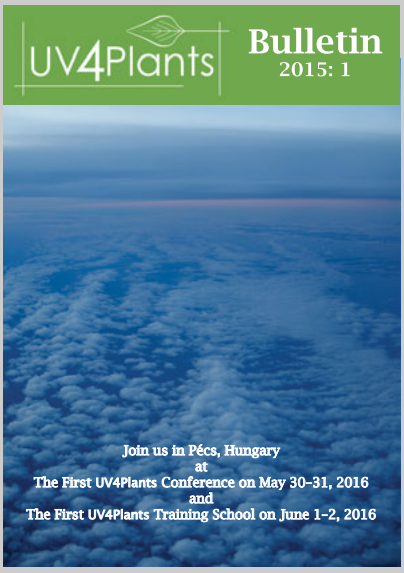
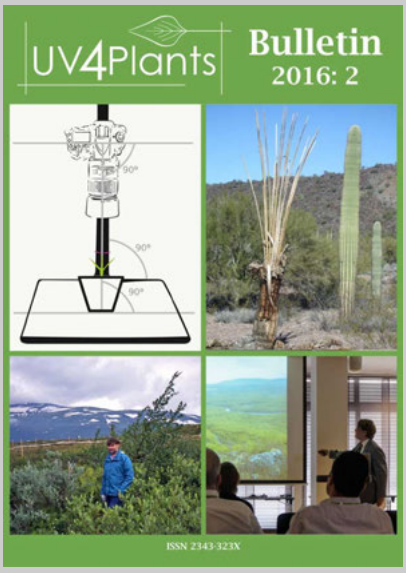
In the current issue

Past issues





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