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Sensing the response of sugar beet and spring barley to abiotic and biotic stresses with proximal fluorescence techniques

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Dipl.-Ing. agr. Georg Leufen

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Referent:	Prof. Dr. Georg Noga
Korreferent:	Prof. Dr. Heinrich W. Scherer
Korreferent:	PD Dr. Mauricio Hunsche
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Sensing the response of sugar beet and spring barley to abiotic and biotic stresses with proximal fluorescence techniques

The aim of these studies was to evaluate the potential of non-destructive fluorescence techniques for the detection and differentiation of abiotic and biotic stress situations. For this purpose, sugar beet cultivars were grown under water shortage and low nitrogen supply, and inoculated with powdery mildew (*Erysiphe betae* (Vaňha) Weltz.). With focus on drought stress, we investigated the impact of recurrent drought on the 'drought memory' and the physiological performance of the plants. Finally, spring barley cultivars of different susceptibly to powdery mildew (*Blumeria graminis* f. sp. h*ordei* (DC.) Speer) and leaf rust (*Puccinia hordei* G.H. Otth.) were used to exploit the pathogen-plant interaction and the consequences for the fluorescence signature at leaf level. Here, the major aim was to analyse genotype-specific responses using spectrally-resolved and imaging-based fluorescence techniques. Experiments were structured in individual chapters, and results can be summarized as follows:

1. Multi-parametric fluorescence recording was a valuable tool to sense abiotic and biotic stress symptoms in sugar beet plants. However, a robust differentiation of individual stresses by one specific fluorescence index was not possible. The most relevant fluorescence indices to detect water deficit and/or powdery mildew infection were the 'Simple Fluorescence Ratio' (SFR_G) and the 'Nitrogen Balance Index' (NBI_G), particularly due to their strong relationship with the chlorophyll concentration. In general, of the evaluated stress factors water deficit had the most pronounced impact on plant physiology.
2. Fluorescence indices based on the far-red chlorophyll fluorescence were reliable indicators for sensing temporary drought stress in sugar beet, regardless of whether the genotypes were cultivated in the field or under greenhouse conditions. This was particularly applicable for the 'Blue-to-Far-Red Fluorescence Ratio' (BFRR_UV) and the SFR_G. For the latter, we demonstrated that green light excitation was found to be the best suitable light source for chlorophyll fluorescence recordings, as it better reveals the responses of the stressed sugar beet plants. Besides, all evaluated cultivars had distinct responses concerning the extent of the changes during the stress and re-watering i.e. recovering phases. These findings were confirmed by gas exchange and destructive reference measurements.
3. Temporal water withholding followed by re-watering caused changes in the fluorescence lifetime (410 to 560 nm), red fluorescence intensity (FR_G) and the SFR_G; in general, the observed alterations were similar in the three consecutive drought-recovery phases. Nevertheless, the fluorescence parameters do not indicate any hints towards improved physiological response to preliminary stresses. With this, the 'memory effect' could not be confirmed. Destructive reference-analysis of the osmotic potential, proline and *total* chlorophyll concentration exhibited a different picture, as all metabolic indices showed minor changes during the second experimental phase.
4. Spectrally-resolved and image-based fluorescence techniques enabled the detection of pathogen infection, i.e. disease development in spring barley. Thereby, susceptible and resistant varieties showed distinct modifications in mean lifetime from 410 to 560 nm, both in the SFR_G and the BFRR_UV. Based on the modification of these parameters in the time-course of the experiment it was possible to characterize the varieties according to their susceptibility degree. The multispectral fluorescence imaging system provides basic information to distinguish between both diseases, since powdery mildewed leaves significantly exhibit a higher blue and green fluorescence intensity as compared to leaf rust diseased leaves. We further highlight the importance of different excitation and emission ranges for sensing and differentiation of leaf diseases, as the UV-excited blue fluorescence and the blue-excited green fluorescence offer the most promising information for further studies on these topics.

Bestimmung der Reaktion von Zuckerrüben und Sommergerste gegenüber abiotischen und biotischen Stress mittels proximaler Fluoreszenztechniken

Ziel der vorliegenden Arbeit war es, das Potenzial von zerstörungsfreien Fluoreszenzverfahren für den Nachweis und die Differenzierung von abiotischen und biotischen Stresssituationen zu beurteilen. Zu diesem Zweck wurden Zuckerrüben-Sorten einem Wassermangel und einer geringen N-Zufuhr ausgesetzt sowie mit Echtem Mehltau (*Erysiphe betae*) (Vaňha) Weltz.) inokuliert. Mit Fokus auf Trockenstress, untersuchten wir den Einfluss wiederkehrender Dürre auf den 'drought memory' Effekt und die physiologischen Eigenschaften der Pflanzen. Zum Schluss wurden Sommergerste-Sorten verwendet, die unterschiedlich anfällig gegenüber Echtem Mehltau (*Blumeria graminis* f. sp. *hordei* (DC.) Speer) und Braunrost (*Puccinia hordei* G.H. Otth.) sind, um Pflanzen-Pathogen-Interaktionen und die Folgen für die Fluoreszenzsignatur auf Blattebene zu bewerten. Das wesentliche Ziel hierbei war, Genotyp-spezifische Reaktionen mittels spektral aufgelöster und bildgebender Fluoreszenztechniken zu identifizieren. Die Experimente wurden in einzelne Kapitel untergliedert, und die Ergebnisse können wie folgt zusammengefasst werden:

1. Für die Detektion der abiotischen und biotischen Stresssymptome an Zuckerrübenpflanzen erwiesen sich multi-parametrische Fluoreszenzaufnahmen als sehr geeignet. Eine robuste Differenzierung der einzelnen Stressoren durch einen spezifischen Fluoreszenz-Index war jedoch nicht möglich. Das 'Simple Fluorescence Ratio' (SFR_G) und der 'Nitrogen Balance Index' (NBI_G) waren, insbesondere durch ihre starke Beziehung zum Chlorophyllgehalt, die wichtigsten Fluoreszenz-Indices, um Wassermangel und/oder Mehltauinfektionen zu erkennen. Generell war Wassermangel der Stressfaktor, der sich am stärksten auf die Pflanzenphysiologie ausgewirkt hat**.**
2. Fluoreszenz-Indices auf Basis der Nah-Infraroten Chlorophyllfluoreszenz waren zuverlässige Indikatoren, um temporären Wassermangel an Zuckerrüben zu erfassen, unabhängig davon, ob die Sorten im Feld oder im Gewächshaus angebaut wurden. Dies traf insbesondere für das ‚Blue-to-Far-Red Fluorescence Ratio' (BFRR_UV) und das SFR_G zu. Für letzteres haben wir zeigen können, dass Grün-Anregung die bestgeeignetste Lichtquelle für Chlorophyll-Fluoreszenz–Aufnahmen war, da es die Reaktionen der gestressten Zuckerrübenpflanzen besser aufdeckte. Zudem wiesen alle untersuchten Sorten unterschiedliche Reaktionen hinsichtlich des Ausmaßes der Änderungen auf Stress und Wiederbewässerung, d.h. der Erholungsphase, auf. Diese Ergebnisse wurden durch Gasaustausch- und destruktive Referenzmessungen bestätigt.
3. Zeitweiser Wasserentzug, gefolgt von Wiederbewässerung, verursachte Veränderungen in der Fluoreszenzlebenszeit (410 bis 560 nm), Rot-Fluoreszenzintensität (FR_G) und dem SFR_G; generell ähnelten sich die beobachteten Veränderungen in den drei aufeinanderfolgenden Trocken-und Wiederbewässerungsphasen. Dennoch zeigten die Fluoreszenzparameter keinen Hinweis in Richtung einer verbesserten physiologischen Reaktion als bei vorherigem Stress. Somit ließ sich der "Memory-Effekt" nicht bestätigen. Destruktive Referenzanalysen des osmotischen Potentials sowie der Prolin- und Gesamtchlorophyllkonzentration dagegen ließen für alle Stoffwechselindizes in der zweiten Versuchsphase gering Veränderungen erkennen.
4. Spektral aufgelöste und Bild-basierte Fluoreszenztechniken ermöglichten den Nachweis von Pathogeninfektionen, d.h. der Krankheitsentstehung an Sommergerste. Dabei zeigten anfällige und resistente Sorten unterschiedliche Veränderungen in der mittleren Lebenszeit von 410 bis 560 nm, sowie beim SFR_G und dem BFRR_UV. Basierend auf den Änderungen dieser Parameter im Zeitverlauf des Experiments war es möglich, die Sorten entsprechend ihres Anfälligkeitsgrads zu charakterisieren. Das multispektrale Fluoreszenzbild-gebungssystem lieferte grundlegende Informationen für die Unterscheidung beider Krankheiten, da mit Mehltau befallene Blätter eine deutlich höhere blaue und grüne Fluoreszenzintensität als die mit Zwergrost befallenen Blätter aufwiesen. Zusätzlich haben wir die Bedeutung der verschiedenen Anregungs- und Emissionsbereiche zur Erfassung und Differenzierung von Blattkrankheiten herausgestellt, da die UV-angeregte Blaufluoreszenz und die blau-angeregte Grünfluoreszenz die vielversprechendsten Daten für weitere Studien in diesem Bereich zeigten.

Table of Contents

A Introduction 1
1 Use of optical sensors in modern agriculture 1
2 Fluorescence sensors: Characteristics and information 3
2.1 Principles of fluorescence emission 3
2.2 Instrumental setup 4
2.3 Excitation and emission spectra 5
3 The challenge of knowledge and technology application 7
3.1 Upscaling from cell level to field level 7
3.2 Differentiation between species 8
4 Relevance of selected model systems 8
4.1 *Beta vulgaris* L. 8
4.1.1 *Erysiphe betae* 9
4.1.2 Water deficit stress 9
4.2 *Hordeum vulgare* L. 10
4.2.1 *Blumeria graminis* f. sp. *hordei* 10
4.2.2 *Puccinia hordei* 11
4.2.3 Impact of drought stress on spring barley 12
4.3 Effects of drought on leaf photosynthesis 13
5 Objectives of the study 13
6 References 15

B Fluorescence indices for the proximal sensing of powdery mildew, nitrogen supply and water deficit in sugar beet leaves 25
1 Introduction 25
2 Material and Methods 27
2.1 Plant material, growth conditions and pathogen inoculation 27
2.2 Non-destructive measurements 28
2.2.1 Portable multiparametric fluorescence sensor 28
2.2.2 Laboratory multispectral fluorescence imaging 29
2.3 Biochemical indicators 30
2.3.1 Sampling methodology 30
2.3.2 Chlorophyll concentration 30

2.3.3 Osmotic potential 30
2.4 Statistical analysis 31
3 Results 31
3.1 Multiparametric fluorescence 31
3.1.1 ‘Simple Fluorescence Ratio’ (SFR_G) 31
3.1.2 ‘Fluorescence Excitation Ratio’ (FER_RUV) 32
3.1.3 ‘Nitrogen Balance Index’ (NBI_G) 33
3.1.4 ‘Blue-to-Far-Red Fluorescence Ratio’ (BFRR_UV) 35
3.1.5 Temporal sensitivity of selected fluorescence indices 36
3.2 Fluorescence imaging 37
3.3 Reference parameters 38
4 Discussion 39
5 Conclusion 43
6 References 43

C Physiological response of sugar beet (*Beta vulgaris*) genotypes to a temporal water deficit, as evaluated with a multiparameter fluorescence sensor 49
1 Introduction 49
2 Materials and Methods 50
2.1 Greenhouse experiment 50
2.2 Field experiment 51
2.3 Non-destructive measurements 51
2.3.1 Fluorescence measurements 51
2.3.2 Gas exchange measurements 52
2.4 Analysis of reference constituents 52
2.4.1 Sampling methodology 52
2.4.2 Chlorophyll concentration 52
2.4.3 Ferulic acid 53
2.4.4 Osmotic potential 53
2.5 Statistical analysis 53
3 Results 54
3.1 Gas exchange 54

3.2 Fluorescence emission 55
3.2.1 Chlorophyll fluorescence and ‘Simple Fluorescence Ratio’ 55
3.2.2 Blue fluorescence 57
3.2.3 ‘Blue-to-Far-Red Fluorescence Ratio’ 58
3.3 Destructive reference analysis of greenhouse plants 59
3.4 Field experiment 60
4 Discussion 62
5 Conclusion 64
6 References 67

D Drought stress memory in sugar beet: mismatch between biochemical and physiological parameters 71
1 Introduction 71
2 Material and Methods 72
2.1 Plant material and growth conditions 72
2.2 Non-destructive determinations 73
2.2.1 Multiparametric fluorescence 73
2.2.2 Fluorescence lifetime 73
2.2.3 Gas-exchange 74
2.3 Reference parameters 74
2.3.1 Sampling method 74
2.3.2 Chlorophyll concentration 75
2.3.3 Proline concentration 75
2.3.4 Osmotic potential 75
2.4 Statistical analysis 76
3 Results 76
3.1 Biochemical indicators: osmotic potential, proline and chlorophyll concentration 76
3.2 Net photosynthesis and transpiration 78
3.3 Red fluorescence (RF) and ‘Simple Fluorescence Ratio’ (SFR_G) 79
3.4 Fluorescence lifetime 81
4 Discussion 82
5 Conclusion 84

6 References 89

E Proximal sensing of plant-pathogen interactions in spring barley with three fluorescence techniques 93

1 Introduction 93

2 Experimental Section 94

2.1 Plant material and growth conditions 94

2.2 Inoculation of *Puccinia hordei* and *Blumeria graminis* f. sp. *hordei* 95

2.3 Fluorescence measurements 96

2.3.1 Fluorescence lifetime 96

2.3.2 Fluorescence imaging 97

2.3.3 Portable multiparametric fluorescence sensor 98

2.4 Statistical analysis 98

3 Results 98

3.1 Fluorescence lifetime 98

3.2 Fluorescence images 101

3.2.1 Indications provided by selected spectral ranges 101

3.2.2 Green fluorescence intensity 102

3.2.3 Green fluorescence intensity of infected leaf area 103

3.3 ‘Blue-to-Far-Red Fluorescence Ratio’ and ‘Simple Fluorescence Ratio’ 105

4 Discussion 106

5 Conclusion 110

6 References 113

F Summary and conclusion 117

List of abbreviations

ABA	abscisic acid
ANOVA	analysis of variance
BBCH	German identification code to identify the phenologic growth stages of plants
BF	blue fluorescence
BGF	blue-green fluorescence
BFRR_UV	ultraviolet excitation ratio of blue-green and far-red chlorophyll fluorescence
C	control
C*a*	chlorophyll *a* content
C*b*	chlorophyll *b* content
CCD	charge-coupled device
ChlF	chlorophyll fluorescence
Ci	internal CO_2 partial pressure
Cm	centimeter
Cm²	square centimeter
CO_2	carbon dioxide
C*t*	*total* chlorophyll content
°C	degree Celsius
DAI	days after inoculation
DAS	days after sowing
DMSO	dimethyl sulfoxide
E	transpiration rate
EC	electrical conductivity
et al.	et alii (m.), et aliae (f.), and others
FAO	Food and Agriculture Organization of the United Nations
Fig	figure
FER_RUV	Fluorescence Excitation Ratio (red & UV excitation) chlorophyll fluorescence
FEP	FG perfluorethylenpropylene-copolymer
FRF_G	far-red fluorescence (green excitation)
f.sp.	formae speciales
g	gram
g	gravitational force
G	green
G	stomatal conductance
GER	Germany
GIS	Geographic Information System
GF	green fluorescence
GPS	Global Positioning System
H	hour
ha	hectare
H_2O	water
Hz	hertz
I	inoculated
i.e.	id est, that is
k	thousand

kg	kilogram
K_2O	potassium oxide
L	liter
LED	light-emitting-diodes
m	meter
m²	square meter
MgO	magnesium oxide
min	minutes
mL	milliliter
mm	millimeter
mm²	square millimeter
mmol	millimole
MPa	megapascal
mS	millisiemens
Mt	million tons
mV	millivolt
mW	milliwatt
µg	microgram
µJ	microjoule
µL	microliter
µmol	micromole
µW	microwatt
N	nitrogen
ND	nitrogen deficit
n	number of replications
n.s.	not significant
ns	nanosecond
NBI_G	Nitrogen Balance Index (green excitation)
nm	nanometer
OP	osmotic potential
%	percent
P	probability of error
P_2O_5	phosphorus pentoxide
PAM	pulse-amplitude-modulated
PAR	photosynthetic active radiation
PF	Precision Farming
PS II	photosystem II
Pn	net photosynthesis
ppm	parts per million
PM	powdery mildew
R	red
rel.	relative
RF	red fluorescence
RF_G	red fluorescence (green excitation)
SD	susceptibility degree
SE	standard error
SFR_G	Simple Fluorescence Ratio (green excitation)
UV	ultraviolet
V	volt

v/v	volume per volume
VIS	visible
W	watt
WD	water deficit

A Introduction

1 Use of optical sensors in the modern agriculture

In the last decades, basic and applied research allowed significant technological progress and positively influenced the agricultural production. Such improvements contribute, and are essential, to match the need for doubling agricultural production to feed the increasing global population estimated in 9 billion people by 2050 (Godfray *et al*. 2010). However, breeding and agronomic improvements alone will not be enough, as they increase on average food production in 32 Mt per year, which is not enough to match the Declaration of the World Summit on Food Security targeted by 44 Mt per year (Tester and Langridge 2010). Moreover, climate change, decreases in arable land, and urbanization will rise demands on new technologies to harvest more from less land, with less but more expensive manpower. Nonetheless, the need of a more efficient use of agricultural inputs such as fertilizer, pesticides, seeds, in the context of a more economic and environmental sustainable production enforces the strategy of 'Precision Agriculture' or 'Precision Farming' (PF).

Precision farming includes techniques such as global positioning system (GPS), geographic information systems (GIS), yield monitoring devices, soil, plant and pest sensors, remote sensing, and variable-rate technologies for applicators of inputs (Seelan *et al*. 2003). Usually, PF is inevitably associated with the site-specific nitrogen fertilization (Auernhammer 2001) because of the multitude of available optical sensors in this area (Fig. 1). Many of these sensors are based on spectral reflectance recordings, e.g. Yara N-Sensor, Isaria, Greenseeker. These techniques have shown promising results (Lammel *et al*. 2001; Mistele and Schmidhalter 2008), nevertheless specific practical aspects such as the type of variety, crop health and physiological age decrease the value of the information for the site-specific N-fertilization (Galambošová *et al*. 2014; Kipp *et al*. 2012; Zillmann *et al*. 2006).

Other physical principles such as transmittance, thermography and fluorescence might be used to detect non-destructively plant physiological responses to environmental stresses. Amongst them, fluorescence is one of the most promising: by recording the laser-induced fluorescence in the field, the MiniVeg®-N technique has enabled the analysis of the N-status of winter wheat (Limbrunner and Maidl 2007) and oilseed rape (Thoren and Schmidhalter 2009). As basis, chlorophyll-fluorescence is recorded in the

narrow red (F690) and far-red (F740) spectral bands to calculate specific indices; unfortunately, the commercial relevance of this system is still not expressive, yet. Differently, systems recording the multispectral fluorescence after multiple excitation opened up new perspectives for a more precise agriculture. Depending on the equipment settings, the fluorescence can be detected from the blue (F425) to the far-red spectral band (F755) with UV, blue or green, and red excitation to identify changes in chlorophyll and phenolic compounds. The wide spectrum of possibilities was recently demonstrated for the detection of drought stress in wheat (Bürling *et al*. 2013) and sugar beet varieties (Leufen *et al*. 2013), pathogen-plant interactions in barley (Leufen *et al*. 2014a), N-variability in maize (Longchamps and Khosla 2014), quality attributes in grape wine (Agati 2013) and ripening degree of tomatoes (Hoffmann *et al*. 2015).

Fig. 1. Optical techniques for the site-specific characterization of agricultural crops in 'Precision Farming' (modified after Gebbers 2013).

In the last years, considerable efforts have been made to better understand the complex interactions between plant-disease interactions and responses of genotypes to a multitude of environmental factors. In particular, a set of structural, physiological, and performance-related traits (e.g. photosynthesis, growth or biomass production) of a genotype in a given environment can be characterized and quantified by using automated,

high-throughput imaging and non-imaging methods in the field or laboratory (Dhondt *et al*. 2013; Walter *et al*. 2012).

Because of the fact that current assessments of phenotype characteristics in breeding largely depend on time-consuming visual scoring, high-throughput phenotyping may revolutionize further breeding processes (Li *et al*. 2014). Nevertheless, even if plant-phenotyping performs very well in the lab (Jansen *et al*. 2009), several factors might become limiting in the field (Li *et al*. 2014). In particular, plants are generally exposed to multiple stresses often causing similar and/or overlapping responses.

2 Fluorescence sensors: Characteristics and information

2.1 Principles of fluorescence emission

Fluorescence spectroscopy has reached considerable importance in many areas of basic and applied scientific fields, also in agricultural sciences. Fluorescence itself, its physical principle and applications, are precisely described in many scientific articles and reference books. Here, we briefly summarize the information on basis of the book edited by Lakowicz (2006).

A number of natural substances emit a characteristic light after exposure to light of different color. This process is defined as luminescence and can be divided into two categories. The first is the fluorescence in which the emitted light occurs rapidly after illumination (10^{-8} s). When the emission takes longer (10^{3} to 10^{-1} s), the process is defined as phosphorescence. The various energy levels involved in the absorption and emission of light by a fluorophore can be schematically illustrated with the classical Jablonski diagram (Fig. 2). At room temperature, fluorophores absorb photons; their electrons rise from the ground energetic state (S0) to higher vibrational levels of the first (S_1) or second excited state (S_2) in about 10^{-15} s. Mostly, the molecules relax by internal conversion in about 10^{-12} s to the lowest vibrational level (S_1). Afterwards, molecules relax to the ground stage in different ways. On the one hand, they can radiatively get to S_0 level through fluorescence emission in about 10^{-8} s, or they can reach the ground state by non-radiative relaxation. Further, the molecules in S_1 state can move to a triplet state (T_1) by spin conversion, which can then relax to the ground state by emitting a phosphorescence photon, or by a non-radiative relaxation. This type of transition is called intersystem crossing.

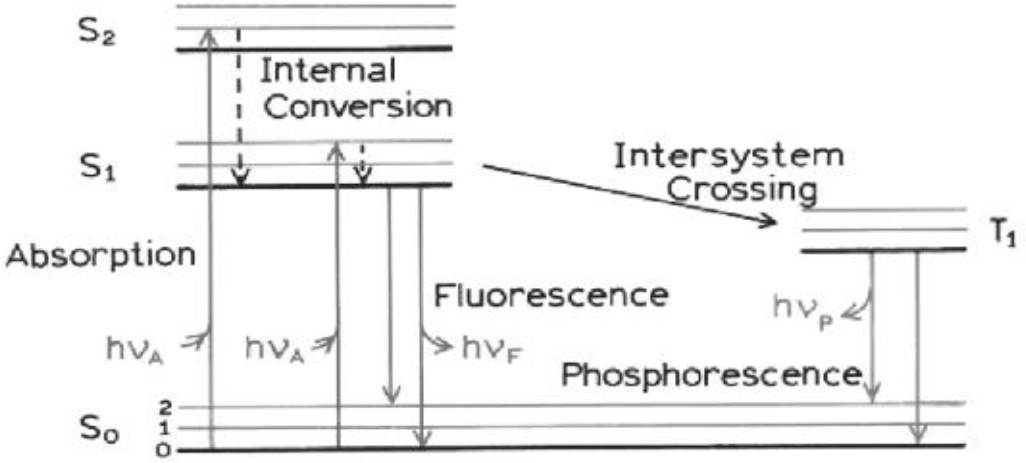

Fig. 2. Jablonski Diagram showing the energy level transitions when excitation light is incident on a molecule (source: Lakowicz 2006).

Fluorescence typically occurs in substances that exhibit an aromatic structure in its molecule. These fluorophores are characterized by specific absorption and emission spectra as well as by their respective fluorescence lifetime. The latter is defined as the time in which the fluorescence intensity decays to 1/e of the intensity immediately following excitation (Lakowicz 2006). Some important fluorophores of plant origin are anthocyanins, coumarins, cinnamic acid derivates, flavones, and chlorophylls that emit fluorescence in the visible range (Goodwin 1953). Thereby, the fluorophore has a longer wavelength than the wavelength of the exciting light because of the Stoke's Law.

2.2 Instrumental setup

Nowadays many scientific areas rely on fluorescence sensors, as passive or active systems, as imaging or non-imaging technologies, to be used under laboratory or field conditions. Both spectrometric fluorescence techniques that were used in this thesis (Muliplex® or Lambda® LF401) have some similarities with respect to their technical setup. Nevertheless, major features, such as active light for fluorescence excitation (xenon lamp, LED, or pulsed laser beam), excitation filter or monochromator, sample holder, emission filter or monochromator, photomultiplier, and equipment-specific software, differed significantly as illustrated in Figure 3.

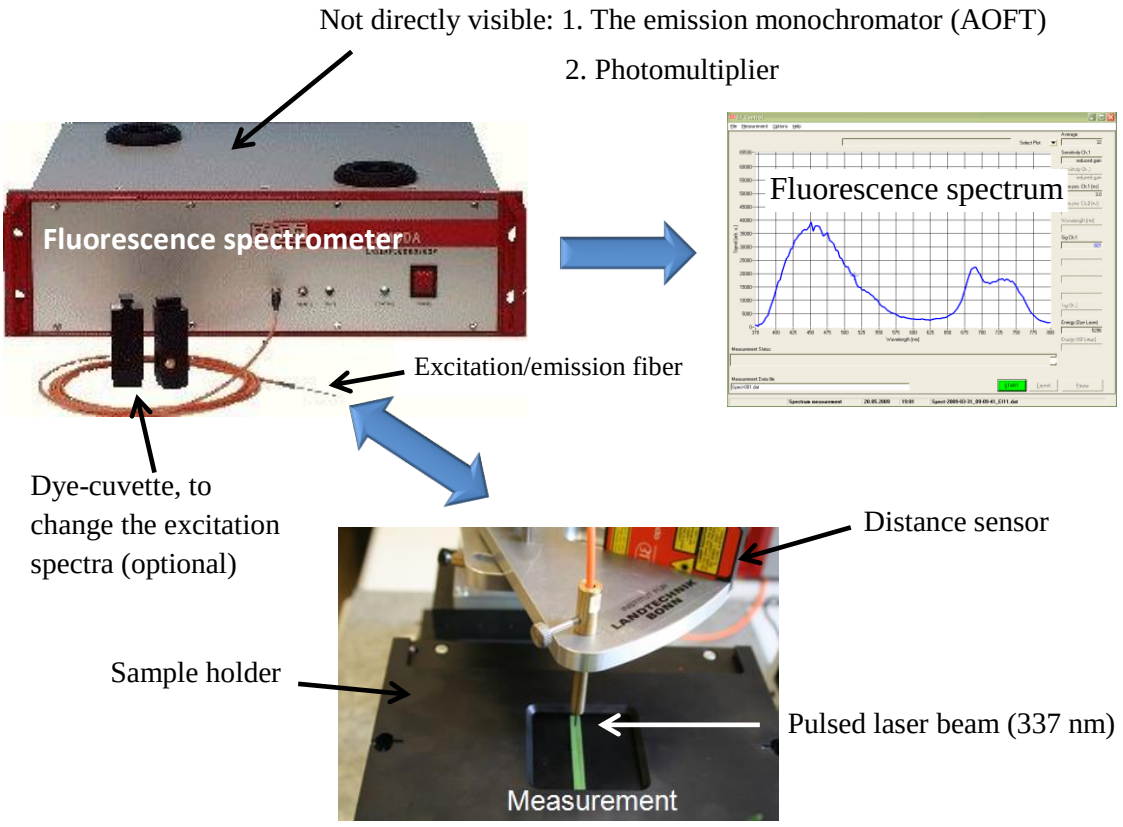

Fig. 3. Schematic setup of a fluorescence spectrometer. Here, it is illustrated how a fluorescence spectrum is recorded by using the fluorescence spectrometer Lambda® LF 401.

The determination of the laser-excited fluorescence can be explained as follows: A fiber optic probe is used to excite with a pulsed laser. If the light does not correspond to the absorption spectra of the fluorophore, a specific filter, dye-cuvettes, or an excitation monochromator might be used for adjustments. Afterwards, the emitted light of the sample is transmitted to an emission monochromator. For example, the Lambda® LF 401 (Fig. 3) is equipped with an acousto-optic tunable filter (AOTF), which helps to separate the wavelength of light from the broadband source whereupon the fluorescence signal can be detected at 1 nm intervals (Gat 2000). The fluorescence light is then transferred to a photomultiplier, which converts the optical into an electronic signal.

2.3 Excitation and emission spectra

Under UV-excitation, characteristic fluorescence might be measured in the blue (440 to 450 nm), green (520 nm), red (690 nm) and far-red (735 nm) spectral regions (Buschmann *et al*. 2000), as shown in Figure 4. The blue-green fluorescence (BGF) mainly originates from

epidermal cell walls and leaf veins, mainly from ferulic acid, which is covalently bound to the cell walls (Buschmann and Lichtenthaler 1998; Lang *et al*. 1991; Lichtenthaler and Schweiger 1998). In contrast, red and far-red fluorescence maxima are related to the chlorophyll *a* fluorescence from the mesophyll (Lichtenthaler *et al*. 1997). In this context, the wavelength and intensity of the excitation light are of particular relevance, as they mainly determine the intensity of the fluorescence emission due to re-absorption processes in the leaf.

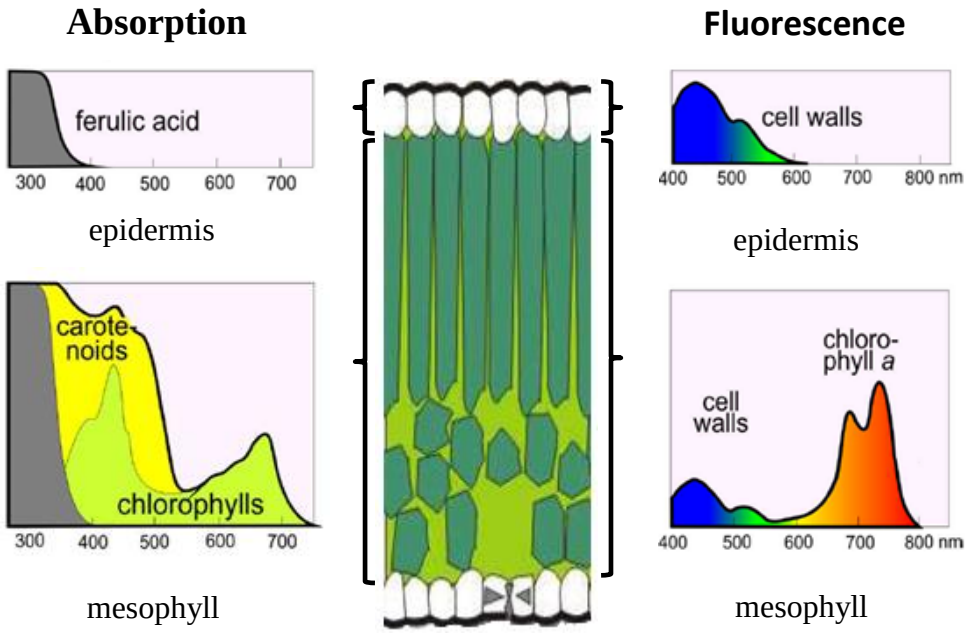

Fig. 4. UV-induced fluorescence spectra of a leaf cross-section. Cinnamic acids in the chlorophyll-free epidermis cells absorb in the UV and show a strong blue-green fluorescence. The latter is less intensive in the mesophyll, as part of it is re-absorbed by carotenoids and chlorophylls (modified after Buschmann *et al*. 2000).

The re-absorption of fluorescence light is much stronger with green and red excitation as they penetrate deeper into the leaf than blue light which is slightly absorbed by chlorophyll and carotenoids in the upper part of the leaf below the epidermis (Buschmann and Lichtenthaler 1998; Gitelson *et al*. 1998). In recent years, fluorescence ratios have been widely adopted, as they are more robust against the undesirable impact of external factors such as distance, atmospheric scattering and leaf geometry and movements (Cerovic *et al*. 1999; Schweiger *et al*. 1996). The most suited fluorescence ratios in plant physiological studies are the red to far-red (RF/FRF), blue to red (BF/RF) or blue to far-red (BF/FRF) and the blue-to-green (BF/GF), as outlined by Buschmann *et al*. 2008. Thereby, the RF/FRF is

used in a number of studies as it is a fast and non-destructive tool to assess the performance of the photosystems and to estimate the chlorophyll content of plants (D'Ambrosio *et al*. 1992; Lichtenthaler and Rinderle 1988).

An alternative to fluorescence ratios is the recording of the fluorescence lifetime. Even if this method is not as widespread as the fluorescence ratios, there are key studies demonstrating the great potential of this method in plant physiological studies (Bürling *et al*. 2011; Meyer *et al*. 2003; Morales *et al*. 1994).

3 The challenge of knowledge and technology application

Nowadays, the ChlF is eminently associated to plant ecophysiology, biophysics and photobiology in laboratory research. For remote sensing, powerful UV lasers are needed for excitation to move from cell and leaf levels to canopy level, as it is the case for LIDAR (light detection and ranging) techniques (Cerovic *et al*. 1999). There are some practical applications of fluorescence in remote sensing (Chappelle *et al*. 1984; Dahn *et al*. 1992; Günther *et al*. 1994; Hák *et al*. 1993). Remote sensing techniques enable a unique solution for mapping canopies spectrometrically on a large scale, and the response of plants to stresses. In order to ensure a high quality analysis, imaging techniques allowing high spatial and spectral resolution provide valuable information, which however is much more precise under controlled lab conditions than in the field.

3.1 Upscaling from cell level to field level

In this work, the fluorescence signature of leaves was recorded with spectral resolution at leaf level from approximately one mm^2 (laser fluoroscope used for lifetime recordings) to 40 cm^2 (portable equipment to record multiple fluorescence indices). Thereby, we found similar impact on pathogen-plant interactions despite the considerable differences in the recorded area as well as the technical specifications of equipment. This is surprising as the fluorescence of a small leaf spot providing just one piece of information per leaf is rarely representative for the whole leaf area (Buschmann and Lichtenthaler 1998). In addition, most biotic strains cause primary punctual disease symptoms before they spread over the whole plant surface; this represents a big challenge for the early stress detection with remote sensing by means of punctual spectrometric fluorescence techniques. Thus, fluorescence imaging techniques with high resolution might be more suited for such applications due to their pixel-wise analyses, but they are not yet ready for the practical use in precision farming. Under field conditions, it is quite difficult to obtain information on individual strains by using

fluorosensing techniques since different abiotic and biotic factors might cause signal overlapping (Leufen *et al*. 2014b). This effect can be explained, as most of the stresses directly or indirectly impact the photosynthetic efficiency of plants.

3.2 Differentiation between species

Leaves of monocotyledoneous and dicotyledoneous in general differ in their fluorescence signature (Stober and Lichtenthaler 1993). Especially cereals emit pronounced BGF, which is caused by their large content of ferulic acid bound to cell walls (Harris and Hartley 1980). In contrast to chlorophyll fluorescence, BGF is very stable during leaf aging because of only minor changes in the fluorophore composition (Meyer *et al*. 2003). Differences in fluorescence properties of plants can be of special interest in the site-specific sensing of weeds and their control in modern agriculture (Longchamps 2009).

4 Relevance of selected model systems

4.1 *Beta vulgaris* L.

Sugar beet (*Beta vulgaris* L.) is a biennial dicotyledonous plant species. Its life-cycle comprises the formation of the root body as storage organ in the first year of vegetative growth, followed by the generative phase in the second year. Compared to the classical cereals, sugar beet is a markedly younger crop, where its origin dates back to the 18th century (Fischer 1989). All cultivated forms of *B. vulgaris* including leaf beets, garden beets, and fodder beets are sexually compatible with the wild sea beet (*B. vulgaris* subsp. *maritima*) and belong to the family of *Amaranthaceae* (Grimmer *et al*. 2008). According to Biancardi *et al*. (2010), about one-quarter of the global sugar production (around 160 Mt) is produced from sugar beets while the remainder from sugarcane (*Saccharum officinarum* L.). Russia (39 Mt), France (33 Mt), USA (29 Mt), and Germany (22 Mt) were the world's largest sugar producer from sugar beets in 2013 (FAO 2014). In moderate climates, the biennial sugar beet is of economic importance, since it is the only sucrose accumulating crop. Because of the recent increase of global sugar production, there is a tendency to use the beets also for bio-gas and bio-fuel production.

To ensure high profitability, in Germany, the sugar beet production is established particularly in regions with proper soil and climatic conditions. In 2013, in about 315.000 ha (3% out of the total German arable land) sugar beet produce on average 11 t/ha sugar (WVZ and VdZ 2015).

Sugar beets can be affected by a multitude of serious stresses during their life-cycle, significantly influencing their yield potential. One relevant example for an abiotic stress is drought, while powdery mildew (*Erysiphe betae*) is one of the most important biotic stresses.

4.1.1 *Erysiphe betae*

Powdery mildew (*Erysiphe betae*) belongs to the most widespread foliar diseases in sugar beet production. The fungus is present in all regions where sugar beets are cultivated, and infects also other beet crops. Particularly in areas with arid climate, e.g. Middle East, Central Asia, Europe, and south-western USA, the fungus causes severe yield losses (Duffus and Ruppel 1993). As estimated by Weltzien and Ahrens (1977), sugar yield losses might reach up to 30% under strong infestation. Symptoms and life-cycle of the fungus *Erysiphe betae* are close to other mildew species as described in chapter 4.2.1.

4.1.2 Water deficit stress

Sugar beet has been credited with a rather wide range of response to drought stress (Winter 1980). However, several authors have found negative effects of drought on yield (Mohammadian 2005; Pidgeon *et al*. 2001). In this context, Richter *et al*. (2001) highlighted that drought stress is the major reason of yield losses in sugar beet production in the UK. It causes an average annual yield reduction of 10% (Jaggard *et al*. 1998), reaching under extremely severe conditions up to 30%. Thereby, timing and duration of drought influence the strength of the stress on dry matter accumulation, rot yield, top yield, sugar yield, and harvest index (AL-Jbawi and Abbas 2013). In Germany, the negative effects of drought on agricultural production were particularly noticed in 2013, when the irregular water supply and high temperatures throughout the vegetative period significantly caused yield losses, also in sugar beet plants.

Besides yield losses, drought has a strong impact on the quality of sugar beet production. Beets try to overcome dry periods with osmotic adjustment to ensure their survival and further existence (Clarke *et al*. 1993). Unfortunately, the concentration of α-amino-N compounds, glycine betaine, proline (Gzik 1996; Rover and Buttner 1999; Shaw *et al*. 2002) as well as sodium and potassium in the root body due to desiccation reduce sugar beet quality by inhibiting crystallization during processing (Clarke *et al*. 1993).

To minimize the negative effects of drought on yield and quality of sugar beet production and sugar, attempts need to be made in breeding and selection of drought tolerant genotypes. In this context, stomata density seems to be of relevance to withstand water

shortage (Bagatta *et al*. 2008). Closure of stomata can reduce leaf water potential and support water uptake to maintain photosynthesis, but it also inhibits carbon dioxide uptake and nutrient flow from the roots, which finally affect photosynthesis and carbohydrate production (Clarke *et al*. 1993).

4.2 *Hordeum vulgare* L.

Barley (*Hordeum vulgare* L.) is one of the most important cereal crops worldwide. Due to its broad adaptation to cold and dry climate, it is grown and used in areas where other essential crops (e.g. maize) cannot be produced (Ullrich 2011). Considering an area of approx. 49 million hectare, in 2013 the global production of barley was about 144 million tons, which lead to an average yield of 2.9 t/ha (FAO 2014). There are several morphological and commercial forms of barley, e.g. winter, spring, two-row, six-row, awned, awnless, hooded, covered, naked, hulless, malting, feed, and food types (Baik and Ullrich 2008). Even if barley was used in the past for human food, currently more than half of the global production is utilized for feed and most of the remaining part for malting (Ullrich 2011).

In 2014, spring and winter barley together were cultivated on about 1.6 million ha in Germany (Kleffmann 2014). Winter barley, with about 1.24 million ha, had the highest commercial importance in Germany. This is mainly due to the higher yield performance of the winter-type and the benefit of early harvest. Even if the total acreage of spring barley has been declining in the last ten years (Kleffmann 2014), its production is still relevant in Germany, as this type fits especially in unfavorable areas.

The yield outcome of barley finally depends on the number and intensity of abiotic and biotic stress factors that may prevent the plant from reaching its maximum genetic potential (Cattivelli *et al*. 2010). Some important examples are described in more detail in the chapters below.

4.2.1 *Blumeria graminis* f. sp. *hordei*

Powdery mildew is a serious disease in many crops worldwide. The following information about the fungus is based on the book edited by Agrios (2005). Briefly, the appearance of the obligate parasite is easy to distinguish from other plant diseases, as it appears as whitish to grayish spots or patches on the upper leaf side. Powdery mildew can also affect stems. It is present in almost all climates, even in warm, dry areas as long as there is a highly relative humidity to ensure sufficient spore germination. Once infested, conidia and/or ascospores germinate on the surface of the plant tissue. Thereby, they obtain nutrients

from their host by producing haustoria into the epidermal cells. The ongoing production of haustoria results in a spreading mycelium on the tissue that contains conidiophores. Each conidiophore consists of individual conidia which are spread by wind. Under unfavorable conditions or at the end of the growing season, powdery mildew produces ascospores enclosed in a fruiting body called cleistothecia (Heffer *et al*. 2006).

Powdery mildew diseases are caused by many different species of fungi of the *Erysiphaceae* family. They are grouped into several main genera, such as powdery mildew on barley - *Blumeria graminis* f. sp. *hordei*. This type of mildew is present on spring and winter barley varieties and may cause yield losses ranging from one to 10 % mainly due to a lower kernel weight (Mathre 1997). However, under strong infestation pressure the yield losses may reach higher levels (Paulitz and Steffenson 2011). Effective strategies against mildew are the application of fungicides, crop rotation, and/or the use of resistant varieties. The latter is of special importance, as resistant isolates of *Blumeria graminis* f. sp. *hordei* have developed negatively influencing the effectiveness of fungicides (Brown 2002; Wolfe 1992). In this context, resistance breeding will become even more important in the future, as most of the active substances have lost their registration, and only a few new crop protection agents have been developed in recent years.

4.2.2 *Puccinia hordei*

Leaf rust, caused by the basidiomycete *Puccinia hordei*, is an obligate parasite of high economic relevance. Due to its impact on yield and its widespread occurrence in Europe, USA, North Africa, New Zealand, Australia, some parts of Asia and of South America (Dickson 1956; Mathre 1987; Manisterski 1989), the fungus is considered as the most important rust disease of barley in the World (Woldeab *et al*. 2006). Estimates indicate that *Puccinia hordei* might cause yield losses of about 30% (Paulitz and Steffenson 2011).

The following description based on Agrios (2005) briefly summarizes the development cycle of the pathogen. The fungus survives over the winter as teliospore, which develops to basidiospores when environmental conditions become favorable. The basidiospores infect alternate hosts, where spermagonia that contain spermatia are produced. These spermatia penetrate the receptive hyphae and generate dikaryotic mycelium and dikaryotic spores that finally lead to the production of aeciospores. These aeciospores can directly infest barley and initiate uredospore. *Puccinia hordei* can perpetuate easily in areas with high density of winter and spring barley cultivation, volunteer barley and/or wild *Hordeum* species, where uredospore can directly infect the new barley plant without an alternate horst (Clifford 1985).

4.2.3 Impact of drought stress on spring barley

Water is essential for human, animal and plant life. In agricultural terms, more than 50 % of the worldwide yield losses are caused by abiotic stresses, including drought (Boyer 1982; Bray *et al*. 2000). Forecasts indicate that climate change will increase the number of water deficit periods, while drought might cause water shortages affecting about two-thirds of the World population (Ceccarelli *et al*. 2004). Even if barley better adapts to drought than other cereals (González *et al*. 1999; Voltas *et al*. 1999), drought e.g. during grain-filling negatively impacts plant performance and yield (Oukarroum *et al*. 2005; Sánchez-Díaz *et al*. 2002). For optimum plant development, proper water supply during the whole plant's life-cycle is necessary since drought can markedly influence morphology and physiological processes from germination until maturity. Thus, breeding for drought tolerance is one essential pillar for a sustainable production of agricultural food under constraining conditions. In this context, a broad genetic variability is the basis for determining positive adaptation to environmental stresses (Cattivelli *et al*. 2010). Unfortunately, modern elite lines are characterized by a lack of genetic diversity as the gene pool has declined during the process of domestication (Honsdorf *et al*. 2014). Hence, the investigation of positive traits of wild barley (*Hordeum vulgare* ssp. *spontaneum*) becomes of special interest in further breeding activities (Nevo and Chen 2011).

Breeding for drought tolerance aims to develop varieties that maintain performance and yield with less water or temporary dry conditions. However, breeding for drought tolerance is very complicate as water deficit can occur in different physiological stages, along with other stresses, turning it very difficult to exploit and understand the complex tolerance mechanisms for each individual stress (Roy *et al*. 2011). With the development of quantitative trait locus analysis (QTL), specific chromosome regions could be identified that are relevant for morphological or physiological changes in plants during stress events. This enables new possibilities in modern breeding, as there were mapped several major loci for yield under different environmental conditions (Cattivelli *et al*. 2010).

4.3 Effects of drought on leaf photosynthesis

Water deficit at any developmental stage affects morphological, physiological, and biochemical processes in plants. Particularly leaf photosynthesis responds quite sensitive to lower water availability, as indicated below. Water deficit induces the production of the phytohormone abscisic acid (ABA) in dehydrated roots, which is transported to the xylem and regulates the leaf growth in shoots as well as stomatal opening (Chaves *et al*. 2009; Zhang *et*

al. 1987). Under prolonged and severe drought, ABA can also be transported from older to younger leaves, causing stomatal closure in the latter ones (Zhang and Davies 1989). Thereby, the water loss through stomatal transpiration is stopped; however, leaf photosynthesis declines due to the restricted CO_2 diffusion to the sites of carboxylation inside the chloroplast (Flexas *et al*. 2012). This effect can lead to increased susceptibility to photo-damage, as the decrease of the CO_2 influx spares more electrons for the formation of active oxygen species (Cornic and Massacci 1996; Farooq *et al*. 2009). At the end, this strategy might cause starvation and plant death.

Biochemical limitations might appear immediately after or concomitantly with stomatal limitations. Amongst others, photosynthetic enzymes such as ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), phosphoenolpyruvate carboxylase (PEPCase), NADP-malic enzyme (NADP-ME), fructose-1,6-bisphosphatase (FBPase) and pyruvate orthophosphatedikinase (PPDK) might be severely impaired by water deficit (Farooq *et al*. 2009). In fact, photosynthesis is even limited under mild drought stress due to impaired photophosphorylation and adenosine triphosphate synthesis (Tezara *et al*. 1999). Consequently, oxidation of reduced NADP in the Calvin cycle is lower, and $NADP^+$ is not sufficiently available (Farooq *et al*. 2012). Hence, when drought stressed plants are exposed to high irradiance, overproduction of ROS causes photoinhibition and destruction of membranes (Flexas and Medrano 2002).

5 Objectives of the study

Water availability, nitrogen supply as well as foliar diseases are the most important factors influencing and limiting crop productivity worldwide. The use of irrigation systems, additional N-fertilization and crop protection programs support the primary production, but in most cases they do not match the requirements for an environmentally sustainable production. Irrigation drives the risk of soil-salinization in maritime regions, excess of nitrogen fertilization raises water, soil, and air pollutions combined with the potential of disturbing the ecosystem, whereas the use of fungicides always entails a contamination of non-target organism. However, employment of non-invasive and highly sensitive online-systems for the site-specific application of herbicides, plant growth regulators, and nitrogen fertilizer, enables product and monetary savings. Finally, recent improvements in technology resulted in the availability of numerous sensors for physiological research and practical agriculture.

Imaging and non-imaging fluorescence techniques became very popular to study perturbations of the photosynthetic apparatus, and/or the evaluation of epidermal phenolic

compounds in field and/or laboratory. Nevertheless, there are still many open questions that need to be clarified before using them more intensively in practical precision farming. Thus, we exploited the suitability of the Laser-Induced-Fluorescence (LIF), the multi-parametric fluorescence and the imaging-based multispectral fluorescence technique to sense stresses in a multi-scale approach under consideration of temporal and spatial modifications in fluorescence signals in barley (*Hordeum vulare* L.) and sugar beet (*Beta vulgaris* L.). As biotic stress factors, in barley, the impact of powdery mildew (*Blumeria graminis* f. sp. *hordei*) and leaf rust (*Puccinia hordei*), while in sugar beet only powdery mildew (*Erysiphe betae*), were investigated. In addition, the impact of water deficit stress was evaluated in differing varieties of sugar beet to address genotypic-specific responses to drought.

This thesis is composed by four individual chapters, each one having its specific aims and hypothesis, as follows:

1. In chapter 1, we aimed to assess the fluorescence signature of sugar beet leaves in response to three single (lower N-supply, powdery mildew infection, and drought stress) or interacting stresses, under similar conditions as they may arise in commercial production. Here, the working hypothesis was that a pre-symptomatic detection and differentiation of the individual stresses would be accomplished.
2. The second chapter aimed to elucidate the impact of drought on the fluorescence signature of four sugar beet genotypes. Thereby, we hypothesized that multiparametric fluorescence sensing is an appropriate method to characterize genotype-specific responses to temporary water deficit.
3. The objective of the following chapter was to exploit the occurrence of drought memory by using non-destructive methods. In this context, we hypothesized that, if existent, the ‘memory effect’ in sugar beet plants would be identified by the less accentuated changes in the fluorescence signature in recurrent stress phases.
4. In the last chapter we targeted a better understanding of the detection and excitation light for the assessment of plant-pathogen interactions in spring barley. Here, we hypothesized that specific fluorescence indices would enable the characterization and differentiation genotype-specific responses to powdery mildew (*Blumeria graminis* f. sp. *hordei*) and leaf rust (*Puccinia hordei*).

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B Fluorescence indices for the proximal sensing of powdery mildew, nitrogen supply and water deficit in sugar beet leaves[1]

1 Introduction

Agronomic crops are exposed to a number of biotic and abiotic stresses which may induce considerable yield losses (Boyer 1982; Oerke 2006). Under practical conditions, the negative impact of stresses can be restricted to a certain extent by cultivation and management practices such as appropriate use of fertilizers, irrigation, crop rotation or application of pesticides. Nevertheless, precise methods for stress sensing and differentiation are required particularly in those cases in which plants respond with similar symptoms across two or more stress conditions (Chaerle *et al.* 2009; Hsiao 1973; Krantz and Melsted 1964). As outlined by Jones and Schofield (2008) and Jones and Vaughan (2010), remote sensing techniques possess promising potential in precision farming since they allow a fast and reliable monitoring of vegetation on a large scale (Günther *et al.* 1994; Lichtenthaler *et al.* 1996). With particular regard to the sensing of N status of the plant, fluorescence emission indices, recorded in the spectral wavelengths from 440–730 nm, seem to be more precise than some of the classical pulse amplitude modulated chlorophyll fluorescence (PAM) parameters (Rambo *et al.* 2010). Thereby, the chlorophyll fluorescence (ChlF) which is exclusively emitted by chlorophyll *a* molecules, might be recorded in the red (F680) and the far-red (F730) spectral region by using different excitation light sources (Buschmann *et al.* 2000; Lichtenthaler *et al.* 1997). By using UV-irradiation, several phenolic substances, mainly located in the epidermal layers or leaf veins, emit a characteristic blue (F440) and green (F530) fluorescence (Cerovic *et al.* 1999). Moreover, the ferulic acid covalently bound to the cell walls, is a major emitter of blue-green fluorescence (BGF) (Lichtenthaler and Schweiger 1998; Meyer *et al.* 2003; Morales *et al.* 1996).

In general, fluorescence ratios are better suited for the remote sensing of plants compared to the absolute fluorescence intensities since the latter can vary considerably under changing measurement conditions or due to morpho-physiological variations of the plants (Cerovic *et al.* 1999; Schweiger *et al.* 1996). The F690/F735 is a frequently used fluorescence

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ratio which reflects the chlorophyll fluorescence in the red and far-red spectral region. It has been shown that this ChlF-ratio decreases with increasing leaf chlorophyll concentration (Lichtenthaler *et al.* 1990) and is therefore a reliable *in vivo* indicator of the chlorophyll content (D'Ambrosio *et al.* 1992; Hák *et al.* 1990). Further, Lichtenthaler and Rinderle (1988) demonstrated the suitability of this ratio for the assessment of the photosynthetic activity. Because of the positive correlation between leaf chlorophyll and N content, Bredemeier and Schmidhalter (2001) recognised this ratio as a potential tool for the side-specific N application in precision farming.

Langsdorf *et al.* (2000) showed that lower N-supply increased the blue-to-red (F440/F690) and the blue-to-far-red (F440/F730) fluorescence ratio of sugar beet leaves. Similar observations were made by Heisel *et al.* (1996) targeting detection of the nutrient deficiency of maize. Moreover, the fluorescence indices F440/F690 and F440/F730 rose in plants exposed to water deficit (Bürling *et al.* 2013; Dahn *et al.* 1999; Lang *et al.* 1996) and pathogen infection (Lüdeker *et al.* 1996). Recently, the "Nitrogen Balance Index" (NBI), a specific excitation-emission ratio which depends on epidermal phenolics and chlorophyll content (Cerovic *et al.* 2009; Tremblay *et al.* 2012), was proposed for non-destructive stress sensing. Nitrogen deficiency significantly decreases this ratio (Apostol *et al.* 2003; Cartelat *et al.* 2005). On the other hand, Bilger *et al.* (2001) proposed the fluorescence excitation-emission ratio "FER_RUV" to estimate the effect of epidermal transmission of UV-radiation in leaves, and their ability for photoprotection.

As shown in the literature, the above mentioned fluorescence indices provided reliable information about the plant physiological status in response to changing environmental conditions or occurrence of stress. However, the majority of the studies were done by choosing one single stress, the other experimental conditions remaining constant, contrasting the situation under natural conditions, where plants are generally exposed to a multitude of interacting stress factors (Chaerle *et al.* 2009). In order to overcome this shortcoming, we aimed to assess the fluorescence signature of sugar beet leaves in response to three single or interacting stresses, under similar conditions as they may happen in commercial production sites. For this purpose, we selected the factors water supply, N-supply as well as powdery mildew infection as economically and eco-physiologically relevant stresses. Based on our previous studies on sugar beet (Leufen *et al.* 2013), and being aware that all the selected stresses might at least partially influence the photosynthetic performance and the chlorophyll fluorescence, we hypothesized that a pre-symptomatic stress differentiation of the individual stresses can be accomplished. In order to improve the experimental design, we selected two

sugar beet cultivars, differing in their susceptibility to powdery mildew, which were individually grown in pots and allocated in a horticultural polytunnel.

2 Material and Methods

2.1 Plant material, growth conditions and pathogen inoculation

Seeds of two sugar beet (*Beta vulgaris* L.) cultivars, Pauletta and Cesira, differing in their susceptibility degree (SD) to powdery mildew, were provided by the company KWS (KWS Saatgut AG, Einbeck, Germany). According to the descriptive list of the German Federal Plant Variety Office 2010, in a scale ranging from 1–9, "Pauletta" (SD = 6) is characterized as more susceptible to powdery mildew than "Cesira" (SD = 4). Untreated seeds were sown in trays filled with a mixture of sand-peat as growing medium. Fourteen days after germination, uniform plants were thinned and individually transplanted into 4 liter plastic pots (0.233 m height, 0.157 m diameter), equally filled with a special nutrient-poor peat substrate (Einheitserde Typ 0, Einheitserde- und Humuswerke Gebr. Patzer GmbH & Co. KG, Sinntal-Altengronau, Germany). Plants (n = 8 per cultivar and treatment) were placed at random in two individual horticultural polytunnels (each with a size of 5.8 × 3.2 × 2.3 m) covered with UV-permeable Norton FEP-Film (FG perfluorethylenpropylene-copolymer). One tunnel was used exclusively for the pathogen inoculation in order to avoid accidental inoculation and infection of the powdery mildew-free treatments. The inoculation was done 29 days after sowing (DAS) by gently moving and slightly shaking infected sugar beet plants on top and along the experimental plants. Plants were fertilized by using commercial mineral fertilizers at rates of 100 kg P_2O_5 ha^{-1}, 400 kg K_2O ha^{-1} and 85 kg MgO ha^{-1}, after the first leaf pair was unfolded (21 DAS). Thereby, two levels of nitrogen supply were established: the lower dose of 50 kg N ha^{-1} (ND, nitrogen deficit) and a high dose of 150 kg N ha^{-1}. A lack of micronutrients was avoided by watering all plants with 50 ml nutrient solution (pH 6.5 and an EC 180 mS cm^{-1}) twice (21 and 30 DAS). Plants were regularly watered by using a drip irrigation system; water deficit was induced by withholding the water from 32 to 47 DAS. Treatments investigated in this study are shown in Table 1.

Table 1. Short description of the treatment groups and the respective abbreviations.

Abbreviation	Treatment description
C	Control plants were regularly watered and received 150 kg N ha^{-1}
ND	Lower N treated plants were regularly watered but received only 50 kg N ha^{-1}
PM	Powdery mildewed plants were inoculated at 29 DAS and handled similarly as control plants
WD	Water deficit was started at 32 DAS and plants received 150 kg N ha^{-1}
PM-ND	Combination of powdery mildew and lower N-supply
WD-ND	Combination of water deficit and lower N-supply
WD-PM	Combination of water deficit and powdery mildew
WD-PM-ND	Combination of water deficit, powdery mildew and lower N-supply

2.2 Non-destructive measurements

2.2.1 Portable multiparametric fluorescence sensor

A hand-held multiparametric fluorescence sensor (Multiplex® 3, Force-A, Orsay, France) was used to record the fluorescence at leaf level. Light-emitting-diodes excited the fluorescence at 375 nm (UV), 518 nm (green) and 630 nm (red) while the emitted fluorescence light was detected in the blue (BGF: 425–475 nm), red (RF: 680–690 nm) and far-red (FRF: 720–755 nm) spectral regions. Different excitation and emission channels result in more than 12 signals. The used fluorescence indices were derived according to the respective formula:

$$SFR\_G = FRF\_G/RF\_G$$

$$NBI\_G = FRF\_UV/FR\_G$$

$$FER\_RUV = FRF\_R/FRF\_UV$$

$$BFRR = BGF\_UV/FRF\_UV$$

A detailed technical description of the portable fluorescence sensor and the explanation of the individual fluorescence indices are provided by Ben Gholzen *et al.* (2010) and Cerovic *et al.* (2009). In our equipment a grid in front of the sensor enabled the illumination of an area of approximately 0.005 m^2 by maintaining a constant distance of 10 cm between sensor and leaf surface. Fluorescence readings were taken from leaves of the

second leaf pair at 34 and 41 DAS (BBCH stage 14, BBCH being the official German identification code to identify the phenologic growth stages of plants), and the third leaf pair at 43 and 47 DAS (BBCH stage 16), respectively.

2.2.2 Laboratory multispectral fluorescence imaging

For a better understanding of the stress related changes in the fluorescence characteristics, fluorescence images were recorded on attached leaves with the multispectral fluorescence imaging system Nuance® TM FX (Caliper Life Sciences, PerkinElmer, MA, USA). For this purpose representative plants were transported to the laboratory at 41 and 47 DAS. The fluorescence system comprises a 1.4 megapixel CCD camera and the equipment-specific software (Nuance® 2.4 imaging software, PerkinElmer, MA, USA). The camera was mounted onto a stereomicroscope (Zeiss SteREO Lumar V12, Jena, Germany) equipped with a filter wheel containing three Zeiss Lumar filters (01, 09 and 14). With this setup, the fluorescence can be excited in spectral ranges about 365 ± 12 nm (UV), 450–490 nm (blue) and 510–560 nm (green). According to the filter used, the emitted fluorescence light can be measured by long-pass emission filters at 397 nm (UV-excitation), 515 nm (blue-excitation) and 590 nm (green-excitation), respectively. Fluorescence data were acquired using a 0.8 X Zeiss Neo Lumar objective with a free working distance of 0.08 mm. A cold-light (LQ-HXP 120, Leistungselektronik Jena GmbH, Jena, Germany) equipped with a mercury short-arc lamp (Osram, HXP R 120W/45C UV, München, Germany) was used as light source. Images were recorded by using the highest light intensity (303.45 W m^{-2} at leaf level). Before measurements, intact leaves were fixed employing a vacuum sample holder, which allows the recording of clear images from a flat leaf surface. An object field of 110.25 mm^2 was illuminated using a 11× magnification and a focus of 51.4 mm. Fluorescence intensities were recorded under UV-excitation in 10 nm steps for the following spectral ranges: 420–500 nm (blue), 500–580 nm (green) and from 620–720 nm (red). Fluorescence images were always recorded under standardized conditions in the dark at 21 °C by using the full CCD frame (1392 × 1040 pixels). Before recording, the equipment automatically set the optimum exposure time in order to get reliable and strong fluorescence signals for each wavelength range.

2.3 Biochemical indicators

2.3.1 Sampling methodology

Reference parameters were obtained from leaves sampled at 41 and 47 DAS. For this purpose, the leaves previously used for the fluorescence readings were harvested and stored at −21 °C in bags for later determination of osmotic potential. Before freezing, a leaf section of 130 mm^2 was punched out from the apex of one leaf per plant for subsequent determination of chlorophyll concentration. The effects of multiple stresses on above ground biomass production were assessed by harvesting and weighting (Kern EV 4200-2NM, Balingen, Germany) the remaining sugar beet leaves at 47 DAS.

2.3.2 Chlorophyll concentration

Immediately after sampling, leaf sections were transferred into 10 mL centrifugal vials filled with 5 mL dimethyl sulfoxide (DMSO). Afterwards, vials were closed and dark-stored for 24 h under laboratory conditions to extract the chlorophyll from the tissue by avoiding its uncontrolled degradation due to light exposition. A UV-VIS spectrophotometer (Perkin-Elmer, Lambda 5, MA, USA) was used to determine the chlorophyll concentration by measuring the absorbance of extracts at 665 nm (A665) and 647 nm (A647). Chlorophyll *a* (Chl a) and chlorophyll *b* (Chl b) concentrations were calculated according to the following equations (Blanke 1992):

$$\text{Chl } a = 12.7 \times \text{A665} - 2.79 \times \text{A647}$$

$$\text{Chl } b = 20.7 \times \text{A647} - 4.64 \times \text{A665}$$

2.3.3 Osmotic potential

Freeze-stored samples were defrosted at room temperature and extruded with a hand homogenizer. Thereafter, a volume of approximately 0.5 mL was filled into 1.5 mL tubes, and the cell sap was centrifuged (Heraeus Biofuge Pico, Kendro Laboratory Products, Newtown, USA) for 5 min with a relative centrifugal force of 16,060× *g*. From the supernatant, 15 µL were transferred into 0.5 mL Eppendorf tubes and the osmolality measured with a freezing-point depression osmometer (Osmomat 030-D, Genotec GmbH, Berlin, Germany).

2.4 Statistical analysis

Data were statistically analyzed with (SPSS) statistic software (PASW statistics version 19.0, SPSS Inc., Chicago, IL, USA). ANOVA and sequential Duncan *post-hoc* analysis was used for treatment comparison within each cultivar and evaluation date. The results are expressed as mean ± standard error (SE), and the level of statistical significance of differences is $p \leq 0.05$.

3 Results

3.1 Multiparametric fluorescence

3.1.1 'Simple Fluorescence Ratio' (SFR_G)

Figure 1 displays the impact of treatments on the SFR_G for both cultivars at two leaf stages: BBCH 14 (34 and, 41 DAS) and BBCH 16 (43, and 47 DAS). As shown, in most cases the control treatment displayed the highest SFR_G. Fluorescence readings on the second leaf pair displayed a strong decrease of SFR_G from 34 DAS to 41 DAS, irrespective of the experimental treatment. In "Cesira", plants of the control and low N-supply (ND) treatments did not show any significant differences, irrespective of leaf stage. All other treatments had by trend a lower SFR_G. SFR_G of "Pauletta" displayed nearly the same trend as "Cesira". Nevertheless, slight differences were seen at 34 and 43 DAS, when significant differences between the control and the low N-supply plants were detected (Fig. 1). The strongest impact of the applied treatments was observed for both cultivars at 47 DAS. Here, water deficit (WD) and water deficit combined with powdery mildew (WD-PM) resulted in a considerable decrease of SFR_G.

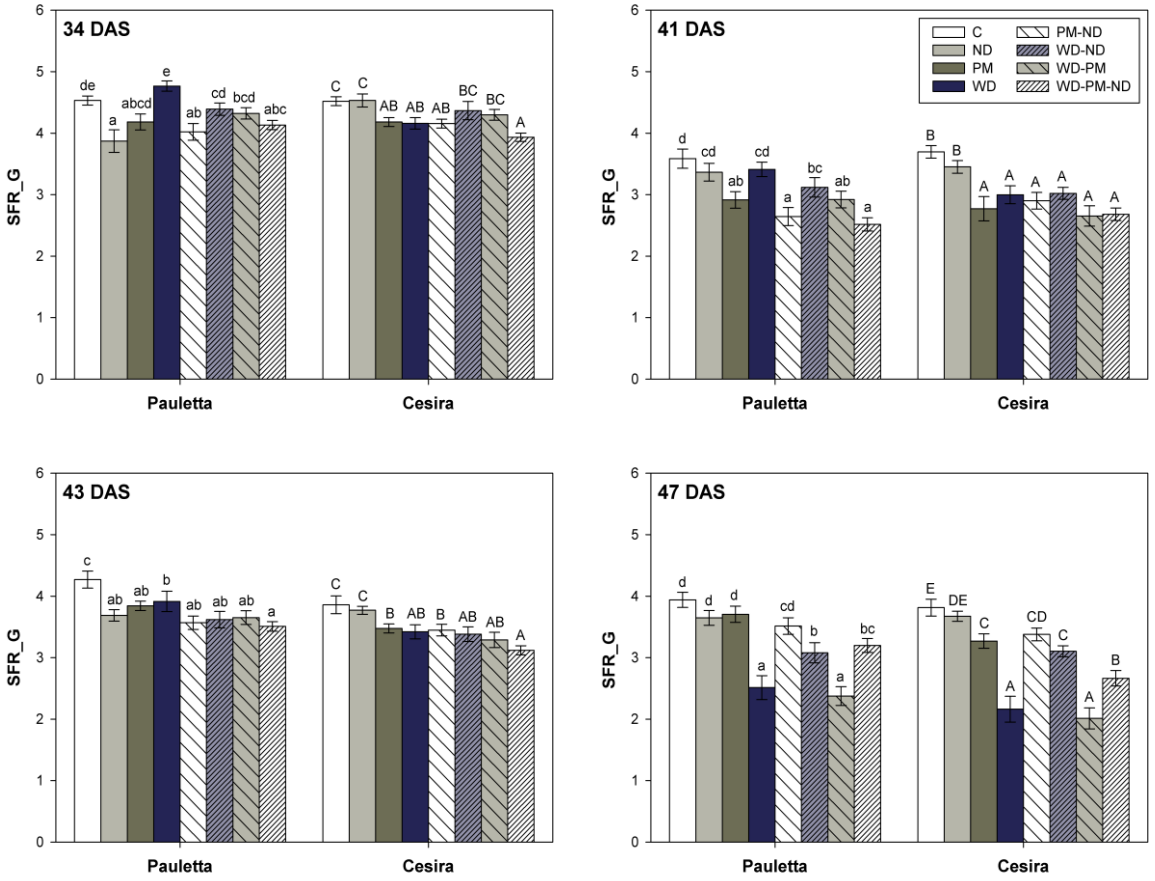

Fig. 1. Influence of N-supply (ND), powdery mildew (PM), water deficit (WD), or combined stresses (PM-ND, WD-ND, WD-PM, WD-PM-ND) on the 'Simple Fluorescence Ratio' excited with green light (SFR_G) of the sugar beet cultivars Pauletta and Cesira. Fluorescence readings were taken on the second leaf pair at 34 and 41 DAS, and on the third leaf pair at 43 and 47 DAS. Values indicate mean ± SE (n = 16). Letters (case sensitive) indicate significant differences among the treatment groups for each cultivar ($p \leq 0.05$, Duncan's test).

3.1.2 'Fluorescence Excitation Ratio' (FER_RUV)

Irrespective of the cultivar, with the FER_RUV we were not able to detect any significant differences among the treatments at 34 DAS (Fig. 2). This was also the case for "Cesira" at 41 and 43 DAS. However, the combinations of powdery mildew + lower N-supply (PM-ND) as well as water deficit + powdery mildew (WD-PM) induced a strong increase of FER_RUV in "Pauletta" at 41 DAS (Fig. 2). Revealed by higher FER_RUV

intensities at 43 and 47 DAS, fluorescence measurements confirm previous indications which indicate a stronger stress response in the leaves of “Pauletta” than in those of “Cesira”.

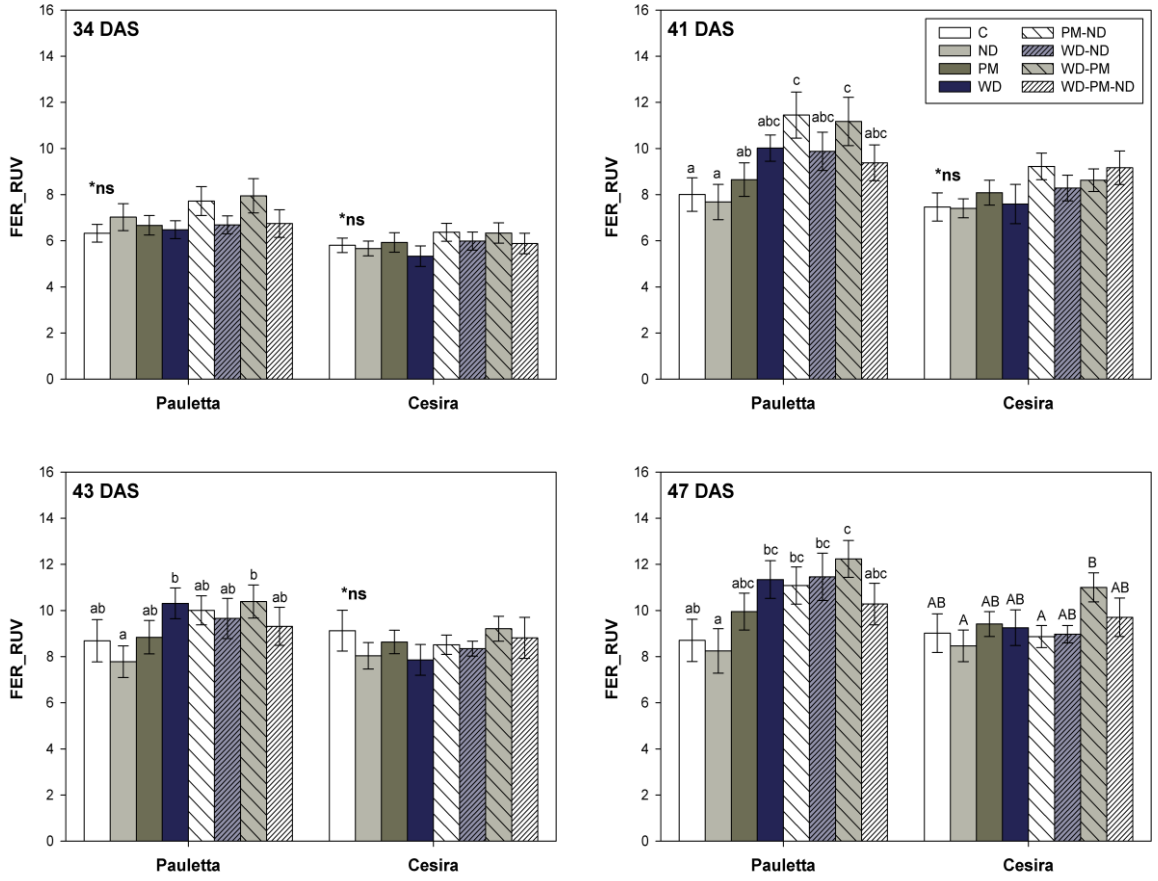

Fig. 2. Influence of N-supply (ND), powdery mildew (PM), water deficit (WD), or combined stresses (PM-ND, WD-ND, WD-PM, WD-PM-ND) on the ‘Fluorescence Excitation Ratio’ (FER_RUV) of the sugar beet cultivars Pauletta and Cesira. Fluorescence readings were taken on the second leaf pair at 34 and 41 DAS, and on the third leaf pair at 43 and 47 DAS. Values indicate mean ± SE (n = 16). Letters (case sensitive) indicate significant differences among the treatments for each cultivar ($p \leq 0.05$, Duncan’s test); * ns, non-significant according to the analysis of variance (ANOVA).

3.1.3 ‘Nitrogen Balance Index’ (NBI_G)

The NBI_G decreased in both “Pauletta” and “Cesira” from 34 DAS to 41 DAS (Figure 3). In “Pauletta”, with exception of WD all the treatments led to a decrease of NBI_G at 34 DAS; in “Cesira”, the combined stress factors PM-ND, WD-ND, WD-PM, and WD-PM-ND

induced a stronger decrease of NBI_G. Similarities in the response of both cultivars were also observed at 41 DAS. Here, the occurrence of multiple stresses decreased the NBI_G to values below the control and low N-supply (Fig. 3). Measurements at the third leaf, at 43 DAS, confirmed the trends observed before, particularly for "Pauletta". Determinations at 47 DAS indicate comparable results on the effects of single and combined stresses in both cultivars. Thereby, plants exposed to water deficit (WD) alone or combined with powdery mildew (WD-PM) had the strongest decrease of NBI_G. On the other hand, no differences could be detected between the control and low N-supply (ND) treatment (Fig. 3).

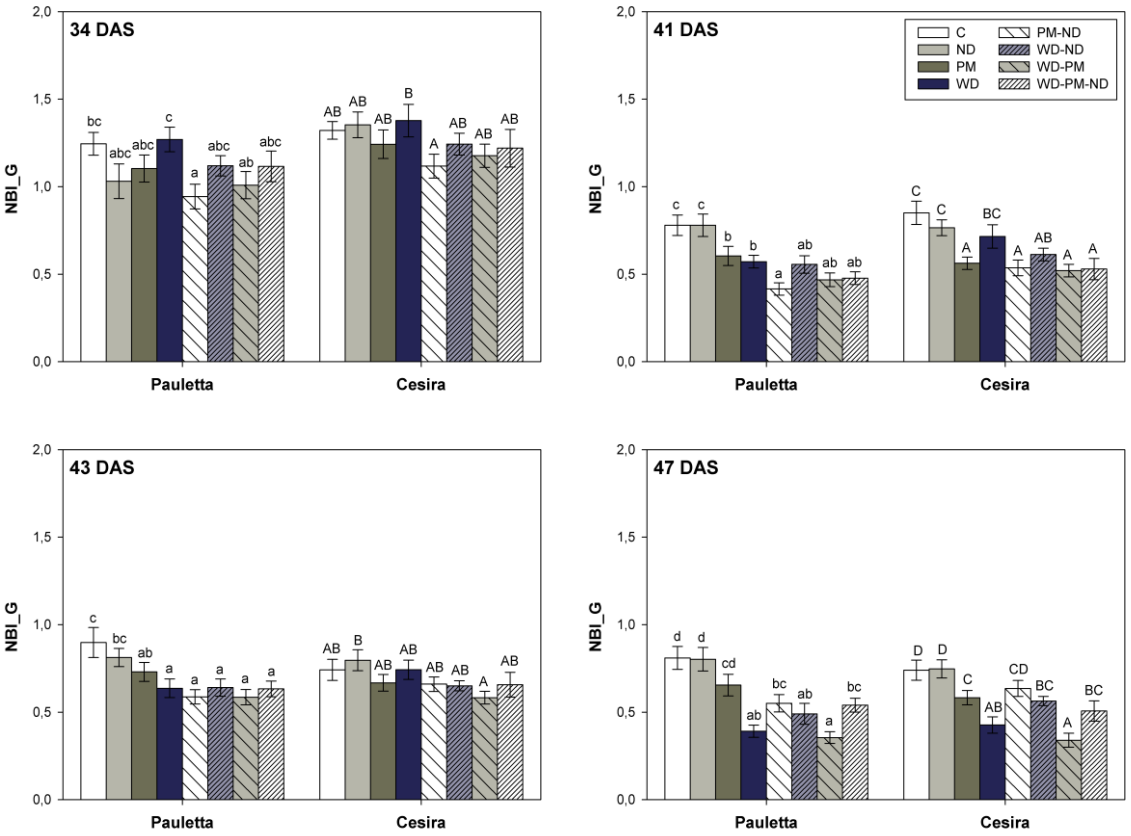

Fig. 3. Influence of N-supply (ND), powdery mildew (PM), water deficit (WD), or combined stresses (PM-ND, WD-ND, WD-PM, WD-PM-ND) on the 'Nitrogen Balance Index' excited with green light (NBI_G) of the sugar beet cultivars Pauletta and Cesira. Fluorescence readings were taken on the second leaf pair at 34 and 41 DAS, and on the third leaf pair at 43 and 47 DAS. Values indicate mean ± SE (n = 16). Letters (case sensitive) indicate significant differences among the treatments for each cultivar ($p \leq 0.05$, Duncan's test).

3.1.4 ‘Blue-to-Far-Red Fluorescence Ratio’ (BFRR_UV)

The BFRR_UV revealed a significant impact of the N-supply in leaves of “Pauletta” as well as the effect of water deficit in combination with a low N-supply in leaves of “Cesira” at 34 DAS (Fig. 4). Particularly at 41 and 47 DAS, the cultivar Pauletta responded with a stronger increase in BFRR_UV than the cultivar Cesira. Here, the powdery mildew triggered the increase of the values, especially when associated with lower N-supply (PM-ND). At 47 DAS, when the water deficit stress was at its highest, both powdery mildew (PM) and water deficit (WD), alone or in combination (WD-PM), raised the BFRR_UV in “Pauletta”. The same stress factors affected the cultivar Cesira, although at lower intensity.

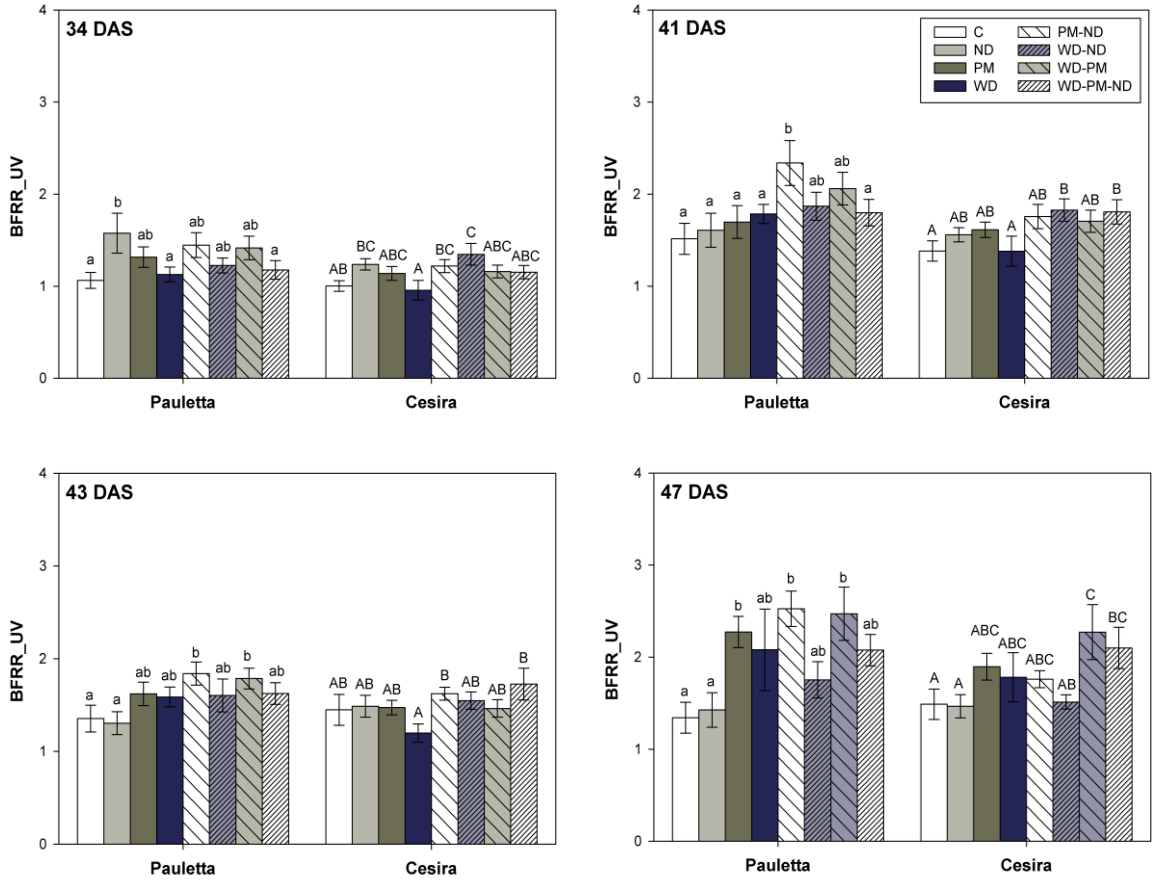

Fig. 4. Influence of N-supply (ND), powdery mildew (PM), water deficit (WD), or combined stresses (PM-ND, WD-ND, WD-PM, WD-PM-ND) on the ‘Blue-to-Far-Red Fluorescence Ratio’ (BFRR_UV) of the sugar beet cultivars Pauletta and Cesira. Fluorescence readings were taken on the second leaf pair at 34 and 41 DAS, and on the third leaf pair at 43 and 47 DAS. Values indicate mean ± SE (n = 16). Letters (case sensitive) indicate significant differences among the treatments for each cultivar ($p \leq 0.05$, Duncan’s test).

3.1.5 Temporal sensitivity of the selected fluorescence indices

In a simplified way, Table 2 displays the sensitivity of the SFR_G, FER_UV, NBI_G and BFRR_UV by presenting the occurrence of significant differences between control and stress-exposed plants at the four measurement dates. Particularly the SFR_G and the NBI_G were highly sensitive and might be adopted to indicate the effect of PM or WD and multiple stresses, such as PM-ND, WD-ND, WD-PM, WD-PM-ND. As one example, PM-ND was detected with the SFR_G on leaves of both cultivars already at 34 DAS (Table 2). In the further course of the study, nearly all stress related differences in the fluorescence signature could be sensed by using the SFR_G and NBI_G.

Table 2. Development of the temporal sensitivity of fluorescence indices for sensing single and combined abiotic and biotic stresses on sugar beet leaves of the cultivars Pauletta and Cesira. (x) indicates significant differences between control and stress-exposed leaves (ANOVA, $p \leq 0.05$).

	BBCH Stage 14								**BBCH Stage 16**							
	34 DAS Fluorescence Indices*				**41 DAS Fluorescence Indices***				**43 DAS Fluorescence Indices***				**47 DAS Fluorescence Indices***			
	SFR	**FER**	**NBI**	**BFRR**	**SFR**	**FER**	**NBI**	**BFRR**	**SFR**	**FER**	**NBI**	**BFRR**	**SFR**	**FER**	**NBI**	**BFRR**
Pauletta ND	x			x					x							
PM					x		x		x		x					x
WD							x		x	x	x		x		x	
PM-ND	x		x		x	x	x		x		x	x			x	x
WD-ND					x		x	x	x		x		x		x	
WD-PM					x	x	x		x	x	x	x	x	x	x	x
WD-PM-ND	x				x		x		x		x		x		x	
Cesira ND																
PM	x				x		x		x				x		x	
WD	x				x				x				x		x	
PM-ND	x				x		x		x				x			
WD-ND				x	x		x	x	x				x		x	
WD-PM					x		x		x				x		x	x
WD-PM-ND	x				x		x	x	x				x		x	x

* SFR = SFR_G; FER = FER_RUV; NBI = NBI_G; BFRR = BFRR_UV.

3.2 Fluorescence imaging

Fluorescence images recorded from representative plants at 41 and 47 DAS provide supporting material for a better understanding of the multiparametric fluorescence readings in the field. In general, blue and green fluorescence intensities of "Cesira" were rather weak compared to "Pauletta" (Table 3). Fluorescence signals were significantly higher in powdery mildew infected plants compared to the control or to plants receiving low N-supply. In both cultivars, the blue-to-green ratios displayed slight differences between water deficit (WD) and control plants. Particularly at 47 DAS a strong decline in the chlorophyll fluorescence intensity in low N-supply plants (ND), also when combined with powdery mildew (PM-ND), was visualized. Our images confirm that particularly the blue fluorescence was higher in the powdery mildew infected leaves, leading to higher blue-to-red fluorescence ratios (BF/RF) in those plants (Fig. 5).

Table 3. Blue (BF), green (GF) and red (RF) fluorescence intensities, as well as blue-to-green (BF/GF) and blue-to-red (BF/RF) fluorescence ratios, of sugar beet leaves at 41 and 47 DAS. Absolute fluorescence intensities were recorded under UV excitation with the Nuance® fluorescence imaging system. Absolute fluorescence intensities (BF, GF, RF) are represented as average signal (scaled_counts/s * 1000).

Treatment		41 DAS					47 DAS				
		BF	GF	RF	BF/GF Ratio	BF/RF Ratio	BF	GF	RF	BF/GF Ratio	BF/RF Ratio
Pauletta	C	0.3	3.8	53.4	0.09	0.01	0.3	2.6	68.1	0.13	0.00
	ND	0.5	1.4	42.5	0.34	0.01	0.4	5.4	1.3	0.08	0.34
	PM	42.4	117	65.4	0.36	0.65	185.2	286.1	76.5	0.65	2.42
	WD	3.2	31.5	48.8	0.10	0.07	3.2	46.2	65.4	0.07	0.05
	PM-ND	13.6	62.2	52.3	0.22	0.26	50.9	116.2	7.8	0.44	6.53
	WD-ND	3.0	8.1	1.0	0.37	3.03	3.6	23.5	54.3	0.15	0.07
	WD-PM	18.3	47.1	24.0	0.39	0.76	137.7	141.9	61.6	0.97	2.23
	WD-PM-ND	32.3	84.4	44.1	0.38	0.73	37.3	83.0	57.3	0.45	0.65
Cesira	C	0.4	5.7	99.9	0.07	0.00	0.8	3.8	48.8	0.21	0.02
	ND	0.6	1.9	51.7	0.33	0.01	0.5	1.8	10.4	0.63	0.05
	PM	38.9	115.8	72.6	0.34	0.54	30.7	81.9	62.5	0.37	0.49
	WD	2.8	46.8	29.4	0.06	0.10	3.4	33.6	61.5	0.10	0.06
	PM-ND	37.7	79.7	60.0	0.47	0.63	12.3	30.4	32.5	0.40	0.38
	WD-ND	3.2	22	47.6	0.14	0.07	2.9	6.4	49.3	0.45	0.06
	WD-PM	12.5	48.8	63.5	0.26	0.20	4.4	42.0	74.7	0.10	0.06
	WD-PM-ND	5.7	18.5	49.8	0.31	0.11	6.2	36.7	60.0	0.17	0.10

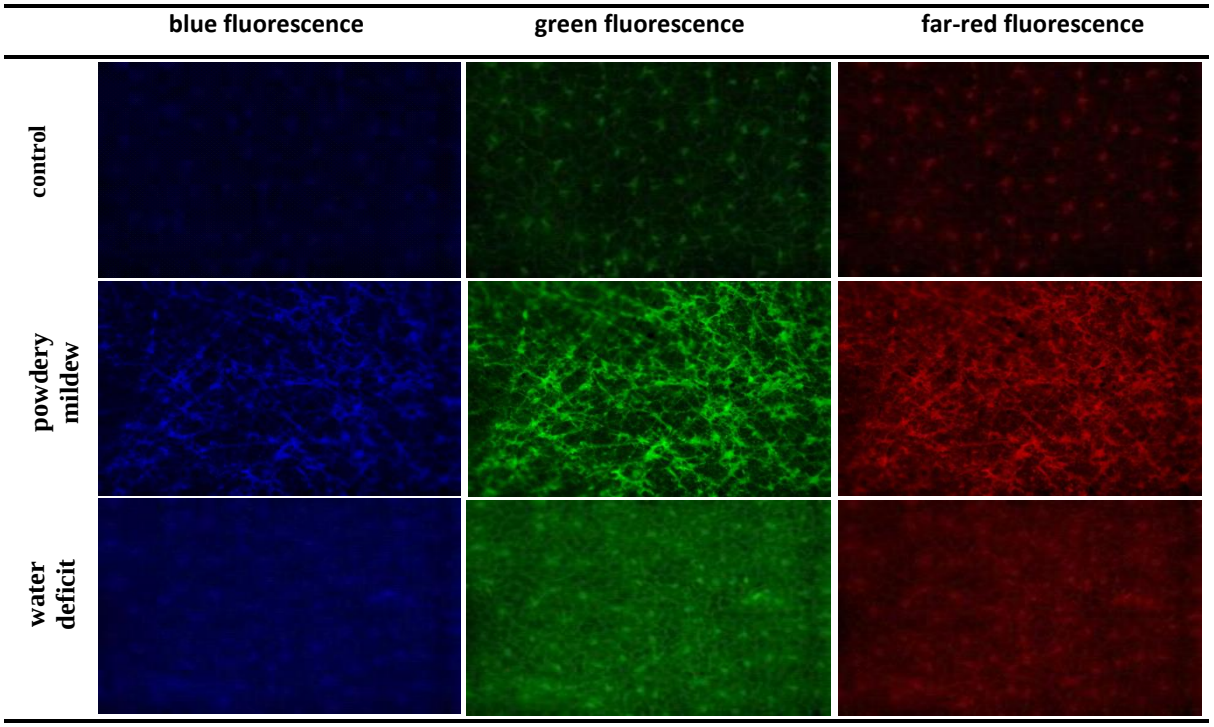

Fig. 5. Blue, green and far-red (chlorophyll) fluorescence images of representative sugar beet leaves of the cultivar Cesira: control, powdery mildewed and water deficit leaves at 47 DAS. Images were recorded under UV excitation with a Zeiss ApoLumar S objective (focus of 17.29, magnification of 85×, light intensity of 98429 μW cm^{-2} at leaf level).

3.3 Reference parameters

The osmotic potential and the total chlorophyll concentration were analyzed from leaves sampled at 41 and 47 DAS, while the above ground biomass was assessed at 47 DAS (Figure 6). The analyses at 41 DAS indicate that the osmotic potential was affected in both cultivars when plants were exposed to water deficit (WD) or water deficit + powdery mildew (WD-PM). A significant rise of the osmotic potential in these treatments was also detected at 47 DAS. In contrast to the other experimental treatments, lower N-supply (ND) reduced only slightly the total chlorophyll concentration in leaves of “Pauletta” and “Cesira”. In general, “Pauletta” had a more pronounced decrease in the total chlorophyll concentration as compared to “Cesira” (Figure 6). Also, water deficit (WD) considerably impacted the total chlorophyll concentration in a negative way at 47 DAS in both cultivars. Further comparisons showed no significant effect of powdery mildew (PM) on the above

ground biomass production. In contrast, all other treatments restricted the biomass accumulation in both cultivars (Fig. 6).

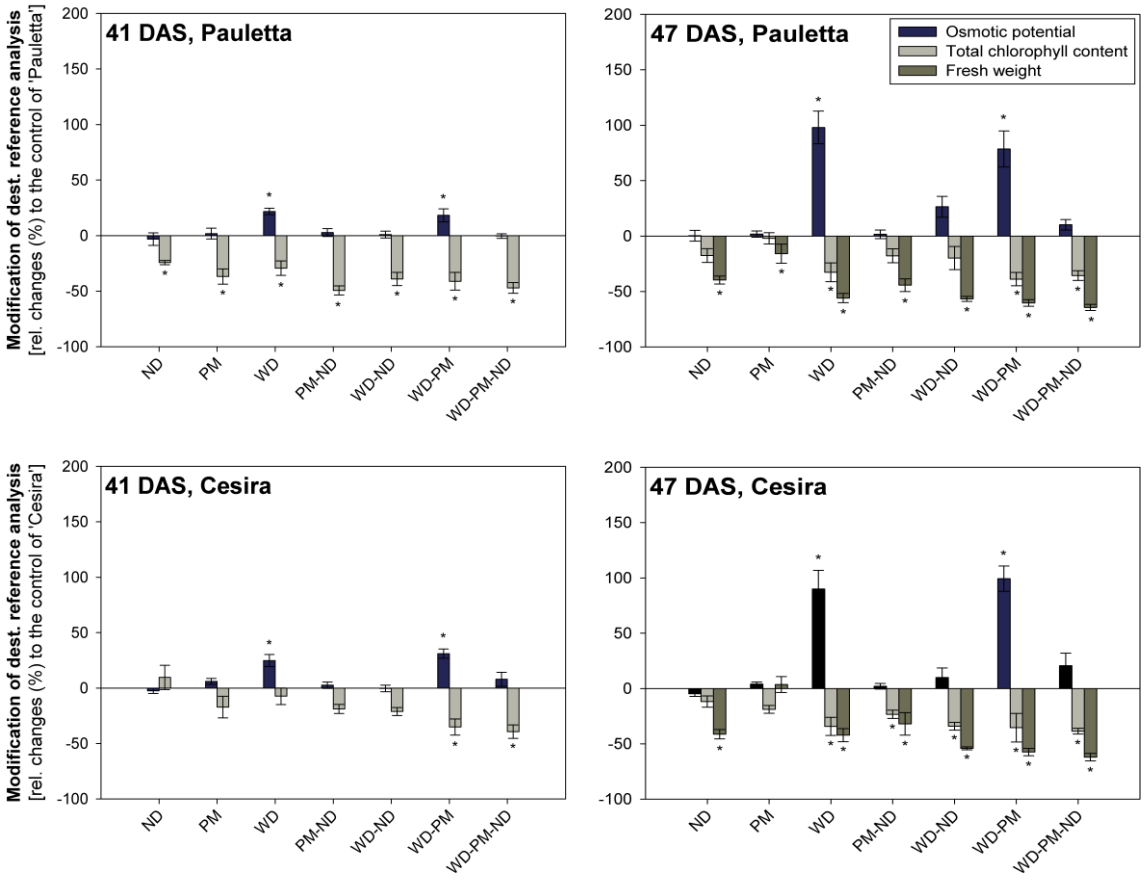

Fig. 6. Influence of single or combined stresses on the osmotic potential, total chlorophyll content and above ground biomass production at 41 and 47 DAS of the cultivar Pauletta (top) and Cesira (bottom). Treatment groups: N-level (ND), powdery mildew (PM), water deficit (WD), or combined stresses. Figures illustrate the relative changes (%) of the stressed to the control plants of each cultivar. Values indicate mean ± SE (n = 8); asterisks indicate significant differences among the treatments and the control (ANOVA, $p \leq 0.05$).

4 Discussion

In this study we investigated, whether the fluorescence indices SFR_G, NBI_G, BFRR_UV, and FER_RUV are suited to assess the impact of multiple stresses on two sugar beet cultivars. Thereby, we hypothesized that these indices allow a differentiation of the

individual stresses despite the potential similarity of their effects on the plant physiology and fluorescence signature. Besides interesting and promising results, our working hypothesis could not be confirmed.

The impact of single stresses, as water deficit or powdery mildew, could be detected in the cultivar Cesira by using the SFR_G at 34 DAS (Table 2). However, the similar decrease in the fluorescence intensity hindered the differentiation of both stresses. This fact was an overall obstacle in our study and was also observed for combined stresses such as PM-ND, WD-ND and WD-PM-ND. One possible explanation for these observations is that all chosen stresses reduce plant photosynthetic efficiency (Evans 1989; Flexas and Medrano 2002; Gordon and Duniway 1982), and the differentiation becomes more complicated due to the interaction of specific processes of the individual stresses (Bainbridge 1974; Oerke and Schönbeck 1986).

Among all evaluated fluorescence indices, the SFR_G and the NBI_G were quite sensitive to stress and strain intensity (Table 2). The SFR_G roughly corresponds to the inverse ratio of F680/F730 ratio, and allows information about the chlorophyll content *in vivo* (D'Ambrosio *et al.* 1992; Hák *et al.* 1990). However, in our studies an overall significant correlation between SFR_G and total chlorophyll concentration was only found in non-irrigated (WD) plants of "Cesira" at 41 and 47 DAS, as well as for "Pauletta" at 47 DAS (*data not shown*). This suggests that decrease of SFR_G was mainly caused by alterations of the electron flow and other processes in the photosynthetic apparatus (Lichtenthaler and Rinderle 1988; Tremblay *et al.* 2012). Later, with increasing dehydration, SFR_G values dropped stronger due to a decline in the chlorophyll concentration resulting from damages to the chloroplasts caused e.g., by active oxygen species (Lawlor 1995). Our assumption was supported by the stronger increase of the red fluorescence intensity than the far-red fluorescence intensity (*data not shown*), indicating that decline in the SFR_G was initiated by an inhibition of PSII (Agati *et al.* 1995). In contrast to Valentini *et al.* (1994), who challenged the effectiveness of this ratio as an early stress indicator, we have clearly shown that modifications in the SFR depended also on the tested cultivars. The higher susceptibility of "Cesira" to water deficit, here starting after our first recording, was also noticed in previous findings and confirms the usefulness of SFR_G (Leufen *et al.* 2013).

Since N plays an important role for the chlorophyll synthesis (Bredemeier and Schmidhalter 2003; Peng *et al.* 1996), we expected an influence of the N-supply on the ChlF. To our surprise, destructive chlorophyll analysis and recordings of the SFR did not show an overall effect of the lower N-supply (ND) on the chlorophyll concentration. Minor changes in

the SFR_G were only noted on leaves of “Pauletta” at 34 and 43 DAS (Fig. 1), indicating that this cultivar was more sensitive to a lower N-supply. These changes were driven by a decline in both the red fluorescence and the far-red fluorescence (*data not shown*), and are in agreement with the findings of Chappelle *et al.* (1984). Contrasting this, above ground biomass was significantly influenced by N-supply at 47 DAS (Fig. 6). This unexpected result can be explained by the lower sensitivity of younger leaves to a nitrogen shortage (Heisel *et al.* 1996), or due to the reallocation of N from older to younger leaves (Leuning *et al.* 1991). The higher N availability for the control plants enabled the production of more aboveground biomass, which may have influenced the leaf area (Pearman *et al.* 1977; Spiertz and Ellen 1978). Finally, it can be assumed that the real nitrogen deficit was not as strong as expected.

Cultivar specific differences against powdery mildew were only shown by a strong decline in the chlorophyll concentration in mildewed leaves of “Pauletta” at 41 DAS (Fig. 6). Moreover, SFR_G values of mildew-infected leaves show a negative trend, irrespective of the cultivar (Fig. 1). As outlined by Gordon and Duniway (1982), net photosynthesis in mildewed sugar beet leaves is reduced by an affected mesophyll conductance, accelerating senescence. In general reduced chlorophyll concentration limits the energy transfer between the photosystem II and photosystem I, and induces alterations in the ChlF intensity, as also shown in our group for winter wheat (Bürling *et al.* 2011).

Analogous to the SFR_G, NBI_G values of both cultivars enabled the sensing of stress and its intensity (Table 2). This fact is not surprising, as the NBI_G is influenced by both chlorophyll and flavonol content. The main advantage of this ratio is the utilization of UV and green light for fluorescence excitation. Since the later penetrates deeper in the leaf tissue, as compared to UV-light, it enables information about the ChlF which also comes from deeper tissue layers (Buschmann and Lichtenthaler 1998). In our trials, water deficit significantly reduced the NBI_G of “Pauletta” (Fig. 3) driven by a continuous decline in the UV excited far-red fluorescence (*data not shown*). Cartelat *et al.* (2005) outlined that several strains may influence the synthesis of polyphenols, including hydroxycinnamic acids and flavonoids. Hence, the reduced ChlF can be attributed to an accumulation of such compounds which operate as a photoprotection-system that absorbs the UV-radiation in the epidermal layers (Schweiger *et al.* 1996). Water deficit or water deficit combined with powdery mildew (WD-PM) caused a strong decline of the NBI_G in both cultivars, till harvest at 47 DAS (Fig. 3). Analytical determinations confirm the induced drought-stress. The high osmotic potential (Fig. 6) was caused by the osmotic adjustment through accumulation of solutes in order to maintain a constantly high turgor (Bagatta *et al.* 2008).

Sensing the impact of low N-supply with the NBI_G was not successful in our study, irrespective of the cultivar (Table 2). However, powdery mildew significantly changed the NBI_G, in a similar way to that described for the SFR_G. In addition to the negative impact of the fungus on the photosynthesis and chlorophyll content, the accumulation of blue-green fluorescing defense substances such as salicylic acid and phenylpropanoid compounds (Chaerle *et al.* 2007; Lenk *et al.* 2007; Nicholson and Hammerschmidt 1992; Scholes *et al.* 1994), as well as the shielding of the plant tissue by the whitish mycelium of the fungus, may have contributed to a decline in the NBI_G (Lüdeker *et al.* 1996). Indications for the shielding effect were also observed in the fluorescence imaging recordings (Fig. 5), resulting also in a strong increase of the blue and green fluorescence (Table 3). This supports the observed effects on BFRR_UV, particularly in the susceptible cultivar Pauletta at 47 DAS (Fig. 4). Moreover, the clear impact of low N-supply in "Pauletta" is explained by smaller changes in the blue fluorescence as compared to the chlorophyll fluorescence at 34 DAS (*data not shown*). Similar results were reported by Heisel *et al.* (1996) showing that the BGF is less influenced by the N-supply than the chlorophyll fluorescence.

The potential of the FER_RUV in proximal and remote sensing was previously illustrated by Apostol *et al.* (2003), who used this parameter for estimation of the plant N status. In our study, individual stress detection by using this excitation ratio was not successful. However, we could show that the tested cultivars responded in a different way to the multiple stress situations (Fig. 2). The more relevant stresses here were water deficit (WD) and powdery mildew (PM). Higher blue-green fluorescence intensities in sugar beet plants of Pauletta (Table 3) explain the differences between the cultivars.

In summary, the evaluated fluorescence indices provide reliable information about plant physiological modifications during and after stress exposure. While the hand-held sensor is a tool for practical use in the field, the fluorescence camera providing spectrally and spatially resolved information enables a more precise understanding of physiological and biochemical processes, particularly the interaction plant-pathogens. As one important outcome, we showed that WD led to a proportionally strong rise of the green fluorescence as compared to the blue fluorescence intensity (Table 3). Similar results were found by Chappele *et al.* (1984) on water stressed soybean leaves. Such increase of green fluorescence might be caused by the accumulation of specific green fluorescing compounds. Amongst others, the flavonol quercetin is one possible emitter of the green fluorescence (Lang and Lichtenthaler 1991), also responsible for the BGF in sugar beet plants (Morales *et al.* 1994). On this basis, we recognize the need for a more detailed exploration of the green fluorescence in future activities.

5 Conclusions

Our results confirm the suitability of SFR_G, NBI_G, BFRR_UV, and FER_RUV to sense stress symptoms triggered by water deficiency, low N-supply, and powdery mildew. However, a robust differentiation by using only one of the proposed indices was not possible. The most relevant fluorescence indices to detect water deficit and or powdery mildew infection were the SFR_G and the NBI_G, particularly due to their strong relationship to the chlorophyll concentration. In general, water deficit was the stressor with a stronger influence on plant physiology. Nonetheless, stress intensity and duration, as well as the cultivar-specific responses, are relevant for the interpretation of such data especially in proximal and remote sensing. Our results elucidate the potential for using fluorescence-based indices in precision farming, including fertilization measures.

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C Physiological response of sugar beet (*Beta vulgaris*) genotypes to a temporal water deficit, as evaluated with a multiparameter fluorescence sensor[2]

1 Introduction

Agricultural productivity is limited worldwide by various biotic and abiotic stresses. Drought is of particular importance, since it is the main abiotic stress factor which causes the highest yield losses (Boyer 1982). Recently, strong efforts have been made to improve drought tolerance of commercial varieties aiming to maintain high production level under adverse conditions (Ober *et al.* 2004, 2005). Conventional breeding programs are long-term and cost-intensive projects, which comprise the evaluation of several hundreds of crossing-lines. Hence, there is a rising demand for precise and objective evaluation methods to support a selection of promising genotypes. As proven, photosynthesis is highly sensitive to drought, since its efficiency decreases with increasing water deficit (Bloch *et al.* 2006). Thereby, the reduced soil water content triggers the stomatal closure leading to a lower internal CO_2 concentration, which consequently limits photosynthesis (Cornic and Masacci 1996). Furthermore, specific metabolic impairments may limit photosynthesis during drought (Flexas and Medrano 2002). Gas exchange measurements can contribute to elucidate the physiological mechanisms underlying the drought tolerance. However, this method is not practical for comparing a large number of genotypes due to its sensitiveness to external environmental influences.

In contrast, several studies point out the potential of optical non-invasive methods as reliable tools to characterize plant physiological changes under stress situations (Berger *et al.* 2010; Chaerle 2001; Jones and Schofield 2008). Especially, the pulse-amplitude modulated (PAM) chlorophyll fluorescence (ChlF) has become a more and more frequently used method to determine the effects of adverse conditions on plant photosynthesis. Thereby, chlorophyll *a* fluorescence readings indicate that the photosystem II may react insensitively to water deficit (Havaux 1992). When recorded with spectral resolution, the chlorophyll fluorescence has two peak emission maxima i.e., in the red (680 nm) to far-red (730 nm) spectral region. Both emission maxima are related to the chlorophyll *a* fluorescence (Lichtenthaler *et al.* 1997).

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Progressive desiccation of plant leaves can lead to an increase of the red fluorescence (Lichtenthaler and Rinderle 1988). Furthermore, excitation of green leaves by UV-light enables the recording of the species-characteristic (Lichtenthaler and Schweiger 1998; Stober and Lichtenthaler 1993a, b) blue and green fluorescence (BGF). This fluorescence signal is primarily emitted by various phenolic compounds mainly located in the cell walls (Cerovic *et al.* 1999; Lang *et al.* 1991). In this context it has been shown, that plants exposed to water deficit may accumulate more phenolic compounds such as ferulic acid, which is reflected in higher fluorescence intensity (Hura *et al.* 2007, 2009a, b; Lichtenthaler and Schweiger 1998).

In general, the fluorescence intensity is strongly influenced by the technical equipment and measuring conditions (Cerovic *et al.* 1994; Morales *et al.* 1998), and therefore the use of fluorescence ratios is highly recommended (Cerovic *et al.* 1999; Schweiger *et al.* 1996). An example is the ratio F690/F735, which is a good *in vivo* estimator of chlorophyll content (D'Ambrosio *et al.* 1992; Hák *et al.* 1990). Others are the blue-to-red or blue-to-far-red fluorescence ratios, which rise up with increasing drought stress (Buschmann *et al.* 2000; Cerovic *et al.* 1999). In contrast to the BGF, which needs to be excited by UV-light, the selection of the excitation source is of relevance for the chlorophyll fluorescence readings. Amongst others the light colour impacts the penetration profile of the light in the leaf tissue and enables to collect information either from more superficial or deeper tissue layers (Brodersen and Vogelmann 2010).

Investigations of BGF and ChlF for an *in vivo* detection of drought susceptibility in sugar beet plants are scarce. In our studies we hypothesized that the multiparameter fluorescence sensing is a suitable method to characterize genotype-specific responses of sugar beet to temporary water deficit conditions. Furthermore, we expected that with increasing desiccation photosynthesis is reduced and the secondary plant metabolism raised, both together influencing the fluorescence signals used to sense the plant response to water deficit. In order to prove this, greenhouse experiments under semi controlled conditions were carried out using sugar beet genotypes with unknown drought susceptibility. The obtained data were subsequently validated in a field experiment to prove whether the method used is an effective screening technique or not.

2 Materials and Methods

2.1 Greenhouse experiment

The experiments were carried out from October 2010 to January 2011 in a heated greenhouse. Seeds of the sugar beet (*Beta vulgaris* L.) cultivars Pauletta (a), Berenika (b),

Cesira (c) and Mauricia (d) distinctly differing in leaf morphology and performance under common agricultural practices were provided by the company KWS Saat AG (Einbeck, Germany). Pelleted seeds of each genotype were sown in trays filled with sand as growing medium. One week after germination, uniform plants were transplanted into 2 l plastic pots (0.20 m height, 0.10 m diameter) filled with a commercial peat substrate (Typ 5, Brill, Georgsdorf, Germany). Plants (n = 8 per genotype and treatment) were placed at random on two benches (10.5 x 1.65 m) with controlled nutrient supply (pH 6.5 and an EC 180 mS cm^{-1}) at 20/16 °C day/night temperature and 16 h photoperiod. Plants received supplemental light by using high-pressure sodium lamps (Philips SON-T Agro 400 W) providing 250-350 µmol m^{-2} s^{-1} PAR at the leaf level. The water deficit was accomplished by withholding the nutrition solution and was induced on the same plants in two consecutive phases. Between the two drought periods plants were allowed to recover under full irrigation for 29 days.

2.2 Field experiment

The field experiment was conducted at the Institute of Crop Science and Resource Conservation (INRES), Department of Horticultural Science, University of Bonn. Crop establishment and management during plant's development followed recommendations of good practice. Accordingly, seeds were sown in the spring when soil temperature was on average higher than 5°C. Seeds were sown by using a commercial three row plot drill targeting a density of 90,000 plants per hectare (0.50 m distance between rows, 0.18 m between plants within the row). The plots (9.8 m x 3 m) were randomized (n = 4 for each genotype and treatment) in the blocks. Water supply was assured by a drip irrigation system along the central row of plants. During the season, the soil moisture was measured at 0.40 m depth with digital tensiometers (Blumat Digital BD2, LM-GL, Bambach GbR, Geisenheim, Germany) in five non-irrigated plots and four irrigated plots. Fluorescence measurements and sampling for destructive reference analysis were performed at random along the central row of each plot on the youngest fully expanded leaves.

2.3 Non-destructive measurements

2.3.1 Fluorescence measurements

The hand-held optical fluorescence sensor Multiplex® 3 (Force-A, Orsay, France) was used to record the auto fluorescence of leaves under ambient light conditions, as described by Ghozlen *et al*. (2010). Briefly, the fluorescence is excited by light-emitting-diodes (LED) in spectral ranges about 375 nm, 518 nm, and 630 nm. Fluorescence signals are measured in the

blue (425-475 nm), red (680-690 nm) and far-red (720-755 nm) spectral regions. A grid in front of the sensor enabled a constant distance of 0.10 m between sensor and leaves. The fluorescence signals were always recorded at leaf level and an area of approximately 50 cm². In the greenhouse study, at day 61 after sowing, two upper, fully-expanded opposing leaves of each plant were labelled, and fluorescence readings were taken up to 105 days after sowing (DAS).

2.3.2 Gas exchange measurements

Gas exchange measurements were conducted during the first experimental phase with a portable infrared gas analyzer (CIRAS-1, PP Systems, United Kingdom) equipped with a leaf cuvette (PLC B, PP Systems, United Kingdom) covering an area of 2.5 cm². Net photosynthetic rate (Pn), stomatal conductance (G), internal CO_2 partial pressure (CI) and transpiration rate (E) were measured at the leaf tip by avoiding major veins. In order to standardize the measurement conditions and to minimize the effect of the environment, plants were taken to a defined measuring site established in the greenhouse. For the measurements, CO_2 concentration was set to 350 ± 5 ppm, light irradiation on the leaf surface was about 250–350 µmol m^{-2} s^{-1} PAR, and the air flow entering the chamber was 200 ± 5 ml min^{-1}.

2.4 Analysis of reference constituents

2.4.1 Sampling methodology

The osmotic potential from leaves of the greenhouse experiment, as well as their chlorophyll and ferulic acid concentration, were determined at day 100 after sowing. From each sugar beet plant, one of the labelled leaves was harvested from which two leaf sections (1 cm² each) were punched out from the apex. Of these samples, chlorophyll and ferulic acid concentration were determined. The remaining part of the harvested leaf was used for determination of the osmotic potential. Analysis of chlorophyll concentration and osmotic potential of sugar beet plants grown in the field were carried out at 104 and 108 DAS, following a procedure similar to the greenhouse study. Thereby, eight leaves were randomly collected from all plots, including each genotype and treatment.

2.4.2 Chlorophyll concentration

For the determination of the chlorophyll concentration leaf disks were transferred into 10 ml centrifugal glasses filled with 5 ml dimethyl sulfoxide (DMSO). The glasses were closed and dark-stored for 24 h under laboratory conditions. The chlorophyll concentration

was determined with a UV-VIS spectrophotometer (Perkin-Elmer, Lambda 5, Massachusetts, USA) by measuring the absorbance of extracts at 665 nm (A665) and 647 nm (A647). Chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and *total* chlorophyll (Chl *t*) concentrations were calculated according to the following equations:

$$\text{Chl } a = 12.7 \times A665 - 2.79 \times A647$$
$$\text{Chl } b = 20.7 \times A647 - 4.64 \times A665$$
$$\text{Chl } t = \text{Chl } a + \text{Chl } b$$

2.4.3 Ferulic acid

Ferulic acid concentration was determined according to the method described by Morales *et al.* (1996), with specific modifications. Before analyzing with a UV-VIS spectrophotometer (Perkin-Elmer, Lambda 5, Massachusetts, USA), ferulic acid was extracted with 5 ml of methanol / water (4:1, v/v), the mixture was shaken for one minute. Ferulic acid (4 hydroxy - 3 methoxycinnamic acid, Merck, Hohenbrunn, Germany) at a purity of ≥ 98% was used as standard.

2.4.4 Osmotic potential

In both greenhouse and field studies, leaf disks were used for the determination of osmotic potential serving as reference for the intensity of drought stress. The samples were placed in bags (Bioreba, Switzerland) and extruded with a hand homogenizer. Thereafter, a volume of 1.5 ml was filled and the cell sap centrifuged (Eppendorf, Centrifuge 5417 R, Hamburg, Germany) for 10 minutes at 25000 min^{-1} at 4 °C. From the supernatant, 15 µl were pipetted into tubes and the osmolality measured with a freezing-point depression osmometer (Osmomat 030-D, Genotec GmbH, Berlin, Germany). At the beginning of the measurements, the osmometer was calibrated by using preformed Genotec vials (850 mmol kg^{-1} H_2O) and distilled water (0 mmol kg^{-1} H_2O).

2.5 Statistical analysis

Data were statistically analyzed with SPSS statistic software (PASW statistics version 19.0, SPSS Inc., Chicago, USA). For each genotype and evaluation date, means of well-watered and water-deficit plants were compared by analysis of variance and paired t-test or Mann-Whitney U test ($p \leq 0.05$).

3 Results

3.1 Gas exchange

Gas exchange measurements were conducted under defined conditions in the greenhouse for the first experimental period between 61 and 71 DAS. Therein focus was on Pn and E of irrigated (control) and temporarily non-irrigated (stressed) plants. Immediately after the water supply was withheld in the non-irrigated plants, E continuously decreased up to day 67 (Fig. 1).

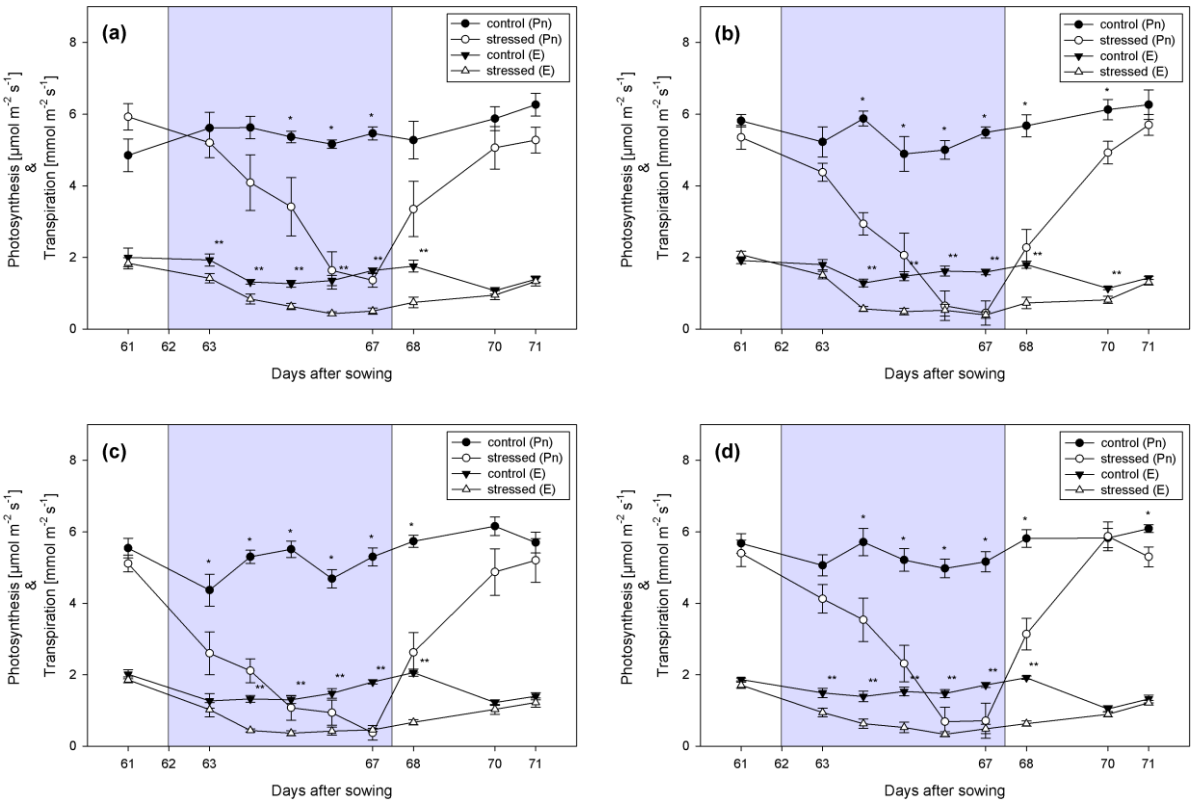

Fig. 1. Development of net photosynthesis [µmol m^{-2} s^{-1}] and transpiration [mmol m^{-2} s^{-1}] for the sugar beet cultivars Pauletta (a), Berenika (b), Cesira (c) and Mauricia (d) influenced by water supply. Measurements took place under semi-controlled conditions in the greenhouse between 61 and 71 DAS on leaves of irrigated (control) and temporarily non-irrigated (stressed) sugar beet plants; water withholding for the 'stressed' plants was between 62 and 67 DAS. Asterisks indicate significant differences (t-test, $P \leq 0.05$) between irrigated and temporarily non-irrigated plants: * indicate significant differences for photosynthesis (Pn), ** for transpiration (E). Mean ± SE (n=8).

Significant differences between both treatments could be detected for the cultivars Pauletta and Mauricia already one day after the water supply was stopped (63 DAS). As soon as the stressed treatment groups were re-watered, E progressively recovered. The effects of the temporary water shortage followed a similar trend for Pn, whereas here changes were even more accentuated in non-irrigated plants as compared to control plants. Similarly, Pn reduction was significantly impaired on the first or second day of the water deficit, thereby, the smallest decline of Pn observed in the cultivar Pauletta. The recovery phase allowed Pn and E to reach nearly the normal level some days after re-watering of plants.

3.2 Fluorescence emission

3.2.1 Chlorophyll fluorescence and 'Simple Fluorescence Ratio'

With the portable sensor for multiple fluorescence excitation, the fluorescence intensity in three spectral regions was determined. Based on the fluorescence intensities, several independent or correlated fluorescence parameters (fluorescence ratios) were calculated. The development of selected chlorophyll fluorescence readings at leaf level is illustrated for each cultivar, treatment and experimental stage (59-71 DAS and 94-105 DAS, respectively) in Figs. 2 to 4. Especially during the first experimental period a very late and small increase of the red fluorescence emission for non-irrigated plants of the cultivar Pauletta could be determined (Fig. 2a), whereas the red fluorescence of the cultivars Cesira (Fig. 2c) and Berenika (Fig. 2b) rose shortly after the water supply was stopped. A similar trend could be observed during the second experimental phase. Here, the red fluorescence emission of all water deficit plants immediately increased when the water supply was withheld. Especially for the genotype Pauletta, the values recovered much earlier during and after the second stress phase as compared to the other cultivars (Fig. 2a). In contrast, the stressed plants of Cesira could not recover during the experimental phase due to strong damage. In general, similar trends could be established for all cultivars when recording the far-red fluorescence (Supplementary Fig. S1) over the whole experimental period.

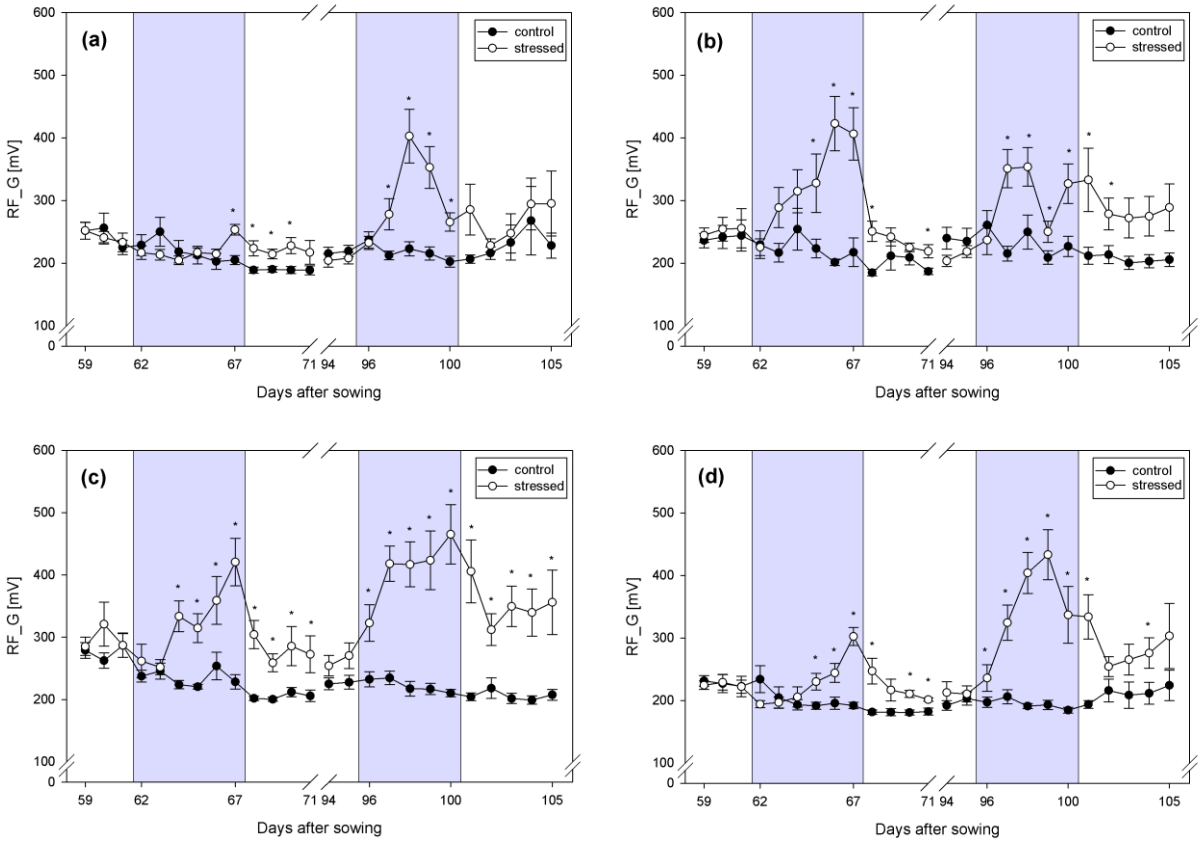

Fig. 2. Influence of water supply on the red fluorescence (RF) recorded after green excitation light (G) on the sugar beet cultivars Pauletta (a), Berenika (b), Cesira (c) and Mauricia (d) cultivated in the greenhouse. Measurements were regularly taken on marked leaves between 59 and 105 DAS. Grey regions in the graphs indicate the periods where water supply in the water deficit treatment group was stopped. Values indicate mean ± SE ($n \geq 8$). Asterisks indicate significant differences with a $P \leq 0.05$ (t-test) between leaves of irrigated (control) and seasonal non-irrigated (stressed) plants for each cultivar and measuring day.

Water withholding from 62 to 67 DAS, and 96 to 100 DAS, caused significant alterations in the 'Simple Fluorescence Ratio' (SFR, far-red emission divided by the red emission) measured after green (G) or red (R) excitation (Fig. 3). Comparisons indicate that the most pronounced differences in the absolute values between irrigated and non-irrigated plants were obtained when using green excitation light. Temporary water withholding led to a reduction of the SFR in the affected plants. Concerning the differences between genotypes during the first experimental phase, the cultivars Pauletta and Mauricia showed a less pronounced susceptibility to the water shortage than the cultivars Cesira and Berenika (Fig. 3).

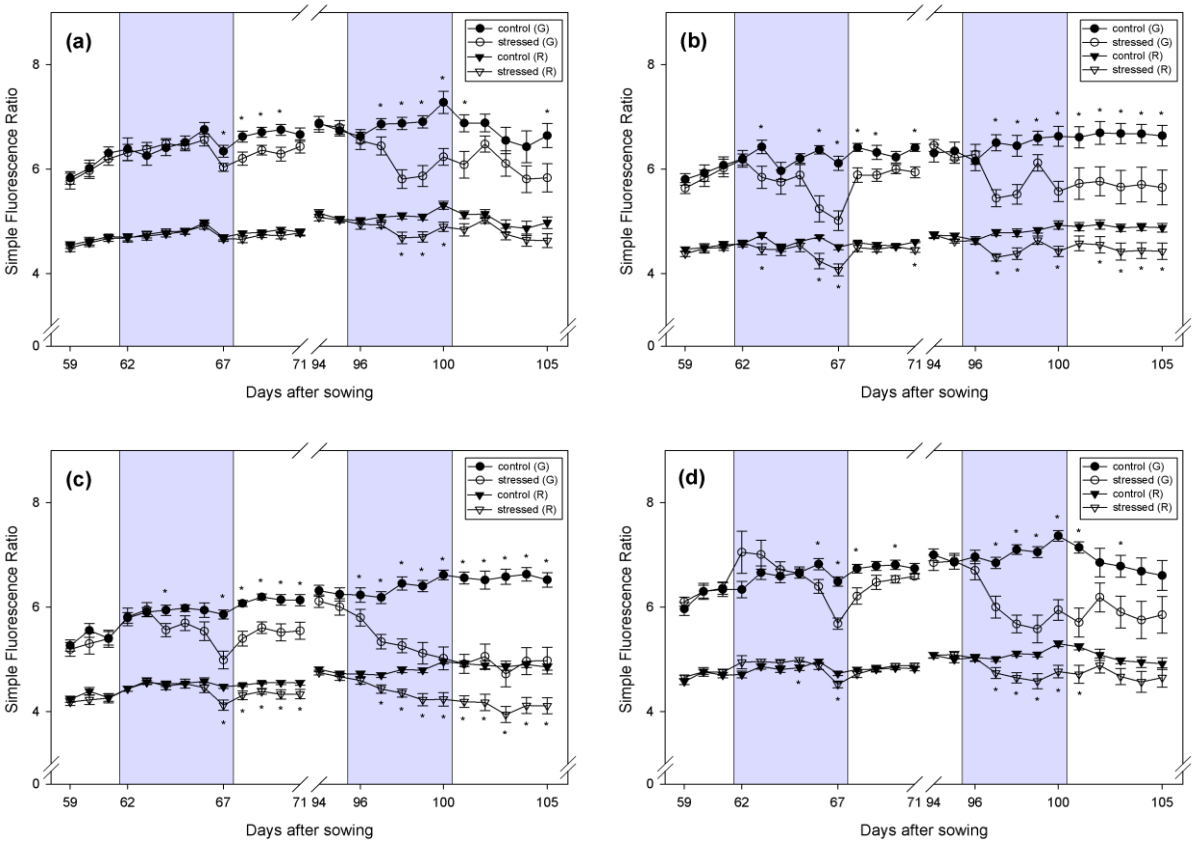

Fig. 3. Influence of water supply on the 'Simple Fluorescence Ratio' (SFR) on the sugar beet genotypes Pauletta (a), Berenika (b), Cesira (c) and Mauricia (d) measured after excitation with green (G) and red light (R). Fluorescence recordings were taken in greenhouse plants at leaf level between 50 and 105 DAS. Grey regions in the graph illustrate the periods where water supply was stopped for the stress treatment. Values indicate mean ± SE ($n \geq 8$). Asterisks indicate significant values with a $P \leq 0.05$ (t-test) between leaves of irrigated (control) and seasonal non-irrigated (stressed) plants for each cultivar and measuring day.

3.2.2 Blue fluorescence

Changes in the BGF intensity were marginal during the experimental periods. Of all tested cultivars, significant differences between the treatments could be ascertained only for Pauletta (Fig. 4). Thereby, a slight increase of the BGF of temporarily non-irrigated plants in comparison to well watered plants could be identified between 64 up to 71 DAS. Furthermore, all cultivars show a significantly higher BGF during the second experimental period than in the first one.

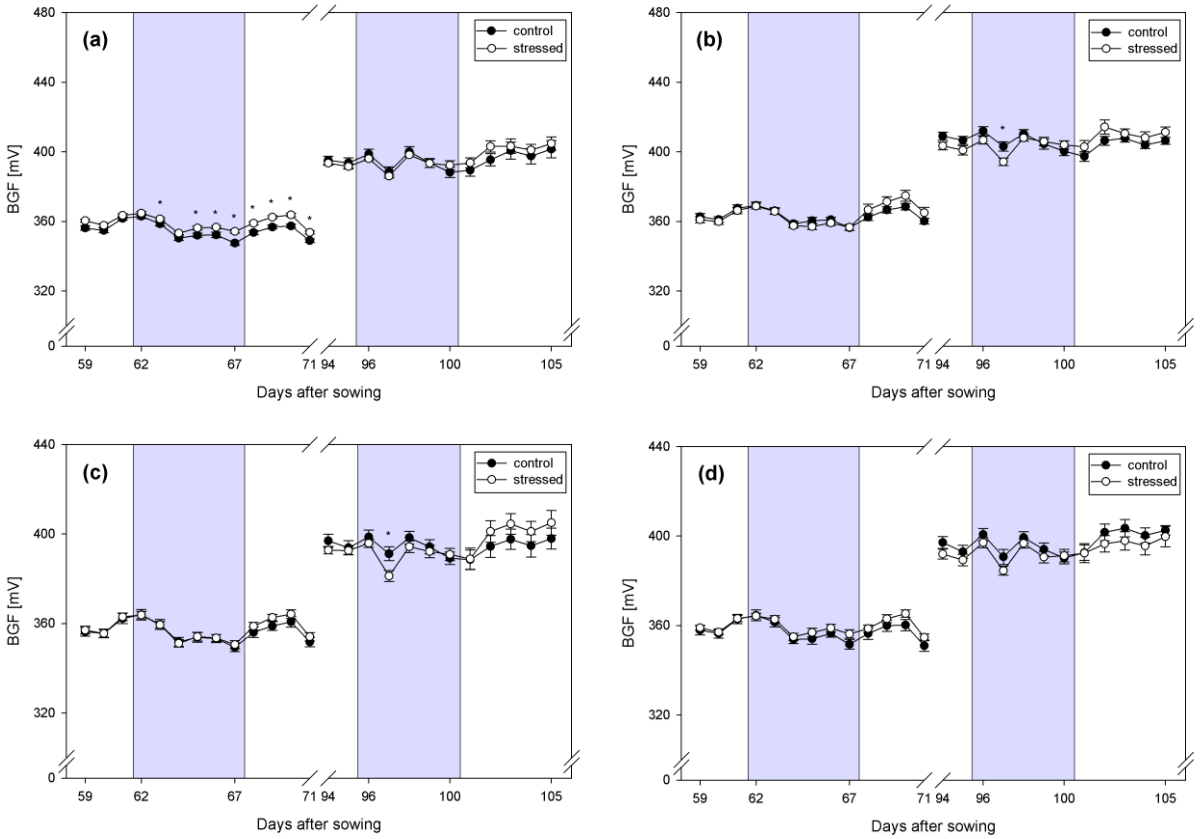

Fig. 4. Influence of water supply on the blue fluorescence (BGF) recorded after UV excitation light on the sugar beet cultivars Pauletta (a), Berenika (b), Cesira (c) and Mauricia (d) cultivated in the greenhouse. Measurements were regularly taken on marked leaves between 59 and 105 DAS. Grey regions in the graphs illustrate the periods where water supply was stopped in the case of the non-irrigated plants. Values indicate mean ± SE ($n \geq 8$). Asterisks indicate significant differences with a $P \leq 0.05$ (t-test) between leaves of irrigated (control) and seasonal non-irrigated (stressed) plants for each cultivar and measuring day.

3.2.3 'Blue-to-Far-Red Fluorescence Ratio'

Based on the blue fluorescence (BGF) and the far-red fluorescence, the parameter blue to far-red fluorescence emission ratio (BFRR_UV) was calculated. Similar to the 'Simple Fluorescence Ratios', in all cultivars the BFRR_UV decreased when the water supply was stopped in the first or second experimental phase. Thereby, the BFRR_UV was more strongly affected in the cultivar Mauricia and less affected in leaves of the cultivar Pauletta (Fig. 5). At the beginning of the second experimental phase, significant differences between the treatments could be ascertained for the cultivar Mauricia. This suggests that the recovery period of 29 days (67-96 DAS) was insufficient for a complete regeneration of this cultivar.

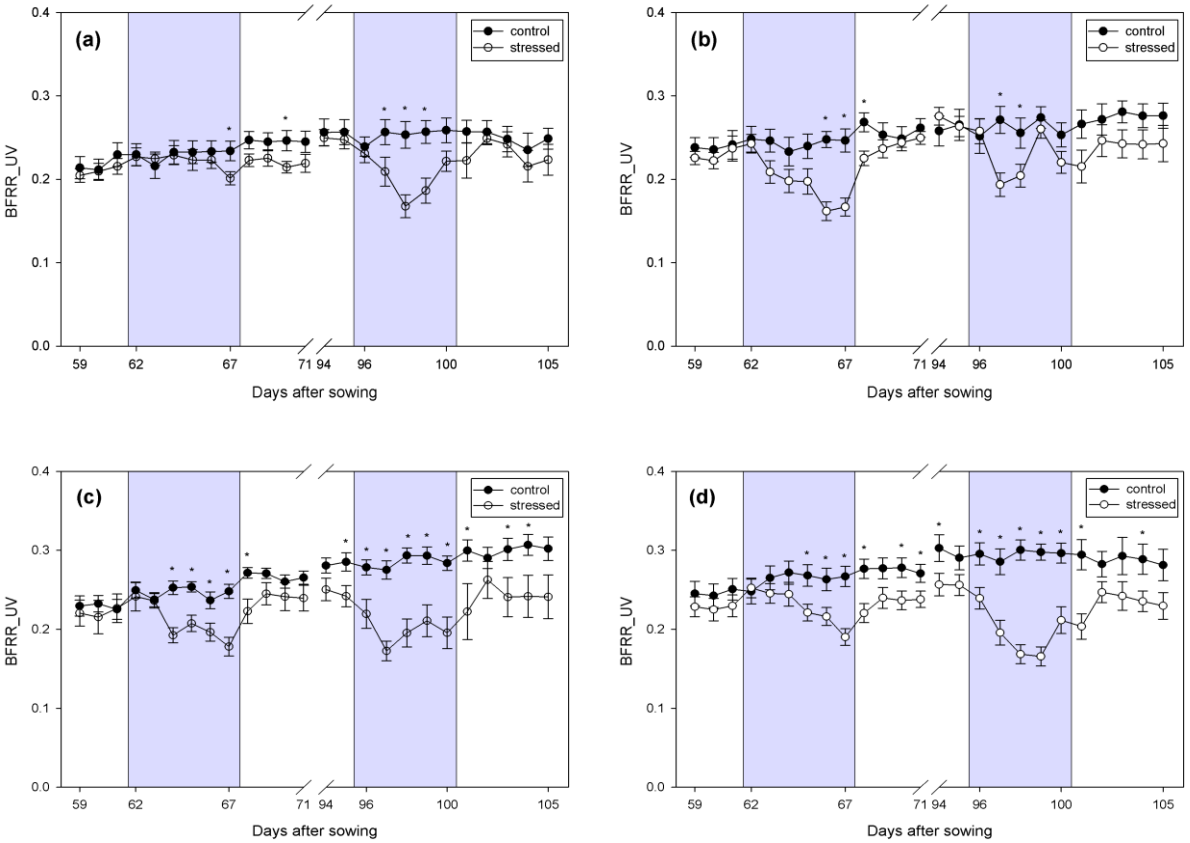

Fig. 5. Influence of water supply on the 'Blue-to-Far-Red Fluorescence Ratio' (BFRR_UV) recorded on the sugar beet cultivars Pauletta (a), Berenika (b), Cesira (c) and Mauricia (d) cultivated in greenhouse. Measurements were regularly taken on marked leaves between 59 and 105 DAS. Grey regions in the graphs illustrate the periods where water supply was stopped in the case of the non-irrigated plants. Values indicate mean ± SE ($n \geq 8$). Asterisks indicate significant differences with a $P \leq 0.05$ (t-test) between leaves of irrigated (control) and seasonal non-irrigated (stressed) plants for each cultivar and measuring day.

3.3 Destructive reference analysis of greenhouse plants

Changes in the concentration of chlorophyll *a*, *total* chlorophyll and ferulic acid, as well as osmotic potential were analyzed at 100 DAS, as displayed in Table 1. As comparisons show, the osmotic potential of the stressed plants was significantly higher than that of the respective control plants. Osmotic adjustment in the cultivar Pauletta was less accentuated as compared to the other genotypes. Considering the behavior of each cultivar, no differences between the treatments could be observed when referring to the concentrations of chlorophyll *a*, *total* chlorophyll and ferulic acid. However, irrespective of the treatments, the cultivar

Cesira revealed significantly lower chlorophyll *a*, *total* chlorophyll and ferulic acid concentrations than the other cultivars (Table 1).

Table 1. Osmotic potential [-MPa], chlorophyll *a*, *total* chlorophyll and ferulic acid concentration [µg cm^{-2}] in leaves of irrigated (control) and temporarily non-irrigated (stressed) sugar beet cultivars grown under greenhouse conditions and sampled at 100 DAS

Sugar beet cultivars	Treatment	Osmotic Potential [-MPa]	Chlorophyll *a* [µg cm^{-2}]	*Total* chlorophyll [µg cm^{-2}]	Ferulic acid [µg cm^{-2}, 10^{-4}]
Pauletta	Control	0.99 ± 0.04*	9.89 ± 0.7	12.83 ± 0.8	0.83 ± 0.07
	Stressed	1.42 ± 0.07	9.24 ± 0.8	12.01 ± 1.0	0.83 ± 0.07
Berenika	Control	1.16 ± 0.02*	8.91 ± 0.4	11.69 ± 0.4	0.70 ± 0.04
	Stressed	1.80 ± 0.03	9.73 ± 1.1	12.61 ± 1.4	0.71 ± 0.10
Cesira	Control	1.09 ± 0.02*	8.34 ± 0.9	10.91 ± 1.1	0.51 ± 0.05
	Stressed	1.77 ± 0.07	8.50 ± 0.8	10.81 ± 1.0	0.53 ± 0.07
Mauricia	Control	0.98 ± 0.02*	9.73 ± 0.5	12.51 ± 0.6	0.93 ± 0.14
	Stressed	1.57 ± 0.05	10.4 ± 0.6	13.54 ± 0.7	0.86 ± 0.06

*Significant differences (t-test, $P \leq 0.05$) between the water supply treatments for each cultivar, Mean ± SE (n = 8)

3.4 Field experiment

In the field experiment, a longer rainless period resulted in an increase of tensiometer readings to about 750 mbar (maximum value) in all rain fed plots, whereas the soil moisture tension of the irrigated plots reached 50 mbar between 103 and 108 DAS (*data not shown*). Similar fluorescence parameters were used in the field, in order to validate the results of the greenhouse study. Relative changes to control plants showed that soil desiccation led to an increase of absolute fluorescence readings and a decrease of fluorescence ratios at 104 to 108 DAS (Table 2). However, in the case of the cultivar Pauletta, all fluorescence parameters showed no significant differences between irrigated and rain fed plots even during the rainless period. A completely different trend was observed for ‘Berenika’, where all fluorescence parameters of both treatments were significantly different at 104 DAS, already. When comparing the four cultivars, variations of the BGF intensity were relatively small. Nevertheless, the drought under field conditions led to significant differences for ‘Berenika’ and ‘Mauricia’ at 104 DAS, and for ‘Pauletta’ and ‘Cesira’ at 108 DAS, respectively (Supplementary Fig. S2).

Table 2. Influence of water supply on selected fluorescence parameters measured 104 and 108 DAS in sugar beet leaves developed under field conditions. The following data represent relative changes (%) of control plants

Sugar beet cultivars	Days after sowing	Modification of fluorescence parameters [% of control]				
		RF_G	FRF_G	BFRR_UV	SFR_G	SFR_R
Pauletta	104	4.9	2.6	-8.7	-1.9	-0.9
	108	5.8	5.2	-12.2	-2	-1
Berenika	104	16.1*	10.1*	-8.7*	-4.4*	-1.3
	108	25.6*	15.7*	-16.3*	-7.4*	-4.2*
Cesira	104	9.1	3.7	2.4	-3.6*	-2.2*
	108	9.6*	5.5*	-14.3	-4.1*	-3.0*
Mauricia	104	2.6*	0.6	0.1	-3.0*	-1.7
	108	14.9*	10.1*	-13.6*	-3.7*	-1.8*

*Significant differences (fluorescence ratios analyzed by t-test, $P \leq 0.05$, absolute fluorescence parameters by Mann-Whitney U-Test, $P \leq 0.05$) between irrigated and rainfed plants for each cultivar and measuring day (n=96)

The analytical determination of chlorophyll *a*, *total* chlorophyll, as well as osmotic potential provided reference parameters for the fluorescence readings. The rainless period showed only slight effects on chlorophyll *a* and *total* chlorophyll concentration (Table 3).

Table 3. Chlorophyll *a*, *total* chlorophyll concentration [µg cm^{-2}] and osmotic potential [-MPa] analyzed 104 and 108 DAS in sugar beet leaves developed under temporary water deficit and field conditions

Sugar beet cultivar	Treatment	104 DAS			108 DAS		
		Chlorophyll *a* [µg cm^{-2}]	*Total* chlorophyll. [µg cm^{-2}]	Osmotic Potential [-MPa]	Chlorophyll *a* [µg cm^{-2}]	*Total* chlorophyll. [µg cm^{-2}]	Osmotic Potential [-MPa]
Pauletta	Control	42.8 ± 1.3	49.8 ± 2.4	1.05 ± 0.02*	32.8 ± 0.9	42.5 ± 1.1	1.04 ± 0.03*
	Stressed	44.0 ± 1.0	49.4 ± 1.2	1.21 ± 0.03	34.2 ± 1.0	43.9 ± 1.3	1.30 ± 0.05
Berenika	Control	43.1 ± 0.9	47.7 ± 1.0	1.03 ± 0.03*	37.9 ± 1.3*	49.1 ± 1.5*	1.10 ± 0.03*
	Stressed	42.3 ± 1.1	47.4 ± 1.3	1.42 ± 0.02	33.9 ± 1.1	45.0 ± 1.3	1.48 ± 0.05
Cesira	Control	35.9 ± 0.9	40.4 ± 1.1	1.03 ± 0.03*	30.5 ± 0.9*	40.2 ± 1.1*	1.00 ± 0.03*
	Stressed	35.9 ± 1.9	40.4 ± 2.2	1.31 ± 0.04	28.0 ± 0.8	36.3 ± 1.0	1.44 ± 0.05
Mauricia	Control	44.3 ± 0.9	50.6 ± 1.0	1.03 ± 0.05*	36.0 ± 1.1	46.3 ± 1.4	1.12 ± 0.03*
	Stressed	42.5 ± 1.2	48.6 ± 1.8	1.29 ± 0.03	38.6 ± 1.1	49.5 ± 1.4	1.39 ± 0.02

*Significant differences (t-test, $P \leq 0.05$) between irrigated and rainfed plants for each cultivar and measurement day (Mean ± SE, n=32)

A significant decline of both parameters in the rainfed treatments could be determined at 108 DAS for the cultivars Berenika and Cesira. In contrast, irrigated and rainfed plants of all tested cultivars displayed differences in the rate of osmotic potential as early as 104 DAS. Increasing soil desiccation led to a progressive decline of osmotic potential in all rainfed plants between both sampling dates.

4 Discussion

The present paper aims to elucidate the impact of drought on the fluorescence signature of four sugar beet genotypes. Thereby, our hypothesis was that multiparameter fluorescence sensing is an appropriate method to characterize genotype-specific responses to temporary water deficit conditions. Here, we demonstrate that this technique provides reliable information about plant physiological constitutions resulting from transient shortage of water supply. Further, essential fluorescence parameters for detection of drought-induced stress in sugar beet plants were identified.

In contrast to Clover *et al.* (1999), our results clearly show that chlorophyll fluorescence is strongly affected by drought. In our independent experiments under greenhouse and field conditions the values of F690 and F730 rose with increasing water deficit. A number of studies on drought stress point out that changes in chlorophyll fluorescence intensities originate mainly from disruptions in the photosynthetic performance, resulting in damage to the photosystems and light-harvesting complexes (Buschmann and Lichtenthaler 1998; Lang *et al.* 1996, Schweiger *et al.* 1996). Thereby, inhibited carbon metabolism and reduced utilization of light-phase products are responsible for the damage to PSII, since the harvested radiation cannot be converted into chemical energy (Cornic and Masacci 1996). In our trials, increasing chlorophyll fluorescence suggest that the light utilization by the stressed plants was temporarily lower than in the well-watered plants, which was confirmed by the leaf gas exchange measurements. Soil moisture deficit also changed the ‘Simple Fluorescence Ratio’ due to a strong decline of its values in non-irrigated sugar beet plants. These decreases were caused by higher far-red fluorescence rather than by the decrease of red fluorescence. Higher far-red fluorescence intensities (Supplementary Fig. S1) is subjected to a strong re-absorption of the ChlF near 690nm, since *in vivo* chlorophyll *b* and carotenoids transmit the absorbed energy to chlorophyll *a* (Cerovic *et al.* 1999). As previous studies pointed out, the FR/R ratio corresponding to the SFR might be used to estimate the chlorophyll content *in vivo* (D’Ambrosio *et al.* 1992; Hák *et al.* 1990). However, our findings indicate that changes in the

SFR do not necessarily correlate with changes of the chlorophyll content. This effect becomes obvious for all tested genotypes in the greenhouse study where both treatments did not display any significant differences in the chlorophyll *a* and *total* chlorophyll concentration. Better matching between SFR and destructive chlorophyll values could be determined under field conditions, indicating that this ratio is influenced by the cultivation system and environmental conditions. Further, the selection of the right excitation light source was of particular relevance for the data quality. Green excitation light provides significantly better information about the ChlF than red excitation light. As compared to red, green excitation light is less absorbed by chlorophylls and penetrates into lower leaf layers before the light is absorbed and transferred into red fluorescence (Buschmann and Lichtenthaler 1998).

In order to prove the sensitivity of the tested fluorescence technique, gas exchange measurements were carried out during the first drought period in the greenhouse study. Similar to the fluorescence readings, water withdrawal negatively affected all gas exchange parameters. As soon as the water supply was stopped, a continuous decrease in Pn became obvious. This effect is explained by a reduced CO_2 diffusion caused by a reduction in stomatal conductance, since root-sourced abscisic acid lead to stomatal closure (Davies and Zhang 1991). However, Pn of 'Pauletta' was characterized by a lower sensitivity to changing water supply conditions. The differences between the cultivars regarding their photosynthesis during desiccation and re-watering periods highlight that drought tolerant species can efficiently control their stomatal function and permit some carbon fixation at drought stress (Yordanov *et al.* 2003). Despite the fact that both fluorescence and gas exchange devices have different work principles, they indicated the same genotype-specific responses to drought. Consequently, the present knowledge can be used in further water deficit studies with fluorescence measurement as a potential substitute of gas exchange measurements, which are frequently limited by environmental influences.

Calculations of leaf osmotic potential (OP) are an additional important parameter to estimate plant specific responses to desiccation. All temporarily non-irrigated genotypes, independent of the cultivation system, were characterized by lower OP than control plants. This follows the osmotic adjustment which leads to the accumulation of solutes to maintain a constantly high turgor (Bagatta *et al.* 2008; Chimenti *et al.* 2002). Our results indicate genotype-specific differences, as non-irrigated plants of 'Pauletta' always have the lowest OP. As indicated before, the blue fluorescence (BF) provides relevant information to characterize cultivar specific responses to unfavourable conditions (Cerovic *et al.* 1999; Hura *et al.* 2009a; Lichtenthaler *et al.* 1997). In the greenhouse trial, differences only occurred for 'Pauletta'

during the first experimental period. This might be explained by the pronounced accumulation of phenolic compounds in plant leaves under water deficit. From a physiological point-of-view, these phenolics operate as a light-filter for the photosynthetic apparatus (Schweiger *et al.* 1996). Thereby, the harmful radiation is transferred into blue-green fluorescence, and in this way PSII is protected (Buschmann and Lichtenthaler 1998). Due to the fact that ferulic acid is the main component responsible for the blue fluorescence in sugar beet leaves (Morales *et al.* 1996, 1998), an increase of its emission could be expected in temporarily non-irrigated plants. Unfortunately, destructive reference analysis of ferulic acid concentrations were only performed 100 DAS. Our results confirm that the evaluated genotypes differ in their ferulic acid concentration. 'Cesira' and 'Berenika', which reacted more sensitively in their ChlF to temporary water deficit, were characterized by a considerable lower ferulic acid concentration than 'Pauletta' and 'Mauricia'. Nevertheless, similar to the BF, there were no significant differences between the treatments. This effect can be associated with genotype as well as growth stage dependence of the BF (Dahn *et al.* 1999; Meyer *et al.* 2003). As our results displayed, the B/FR ratio is also an appropriate parameter. Thereby, changes in the blue to far-red fluorescence ratio were mainly caused by the increase of the ChlF. Thus, we proved in this study that ChlF by using green excitation light was the predominant parameter to identify unfavorable conditions in sugar beet plants.

5 Conclusion

Specific parameters of the multispectral fluorescence signature, and especially those indices based on the far-red chlorophyll fluorescence, are reliable indicators for sensing a temporary water deficiency stress in sugar beet. The four evaluated cultivars had distinct responses concerning the extent of the changes during the stress and re-watering induced recovery phases. These findings were confirmed by gas exchange and destructive reference measurements. Regardless of whether the genotypes were cultivated in the field or under greenhouse conditions, the most promising fluorescence parameter was far-red fluorescence induced by green excitation. Perspectively, our results support the potential of the multiparametric fluorescence technique for the objective screening of sugar beet genotypes to drought stress tolerance.

The following pages of this chapter display the supplementary figure S1 and S2.

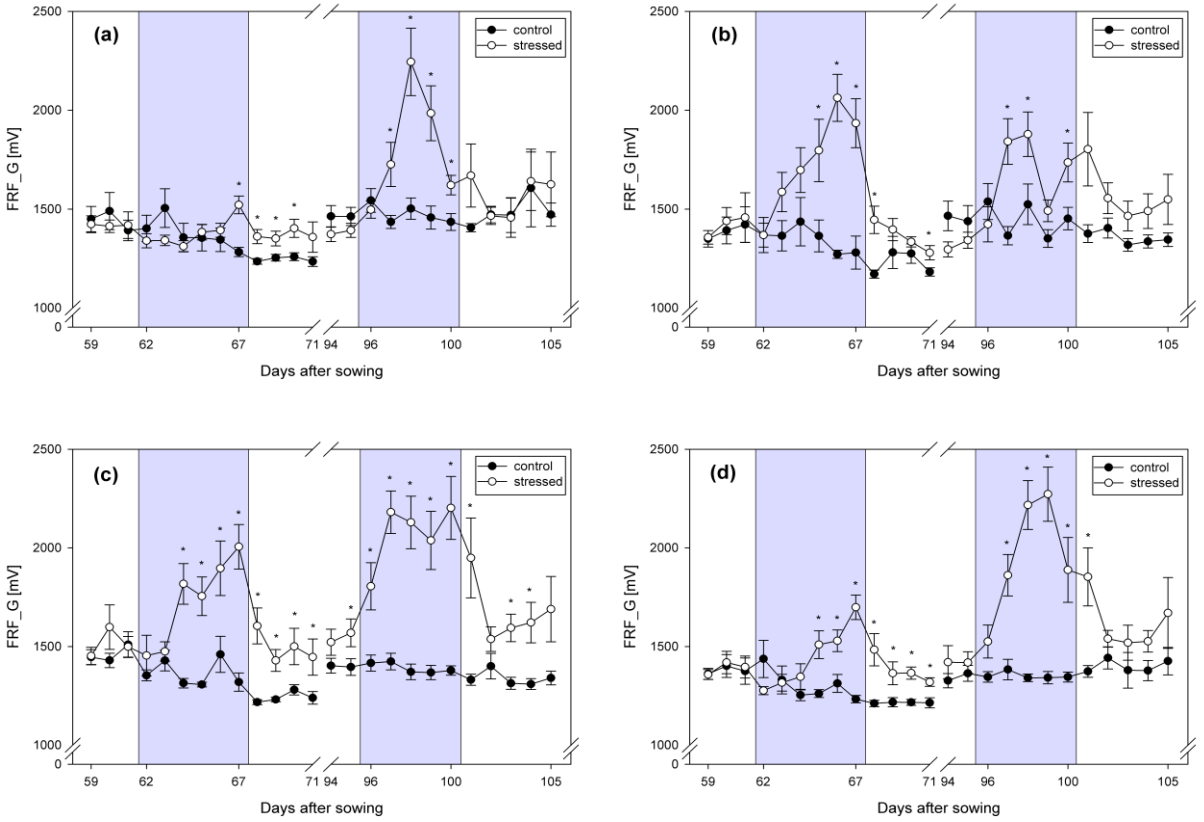

Fig. S1. Influence of water supply on the far-red fluorescence (FRF) recorded after green excitation light (G) on the sugar beet cultivars Pauletta (a), Berenika (b), Cesira (c) and Mauricia (d) cultivated in greenhouse. Measurements were regularly taken on marked leaves between 59 and 105 DAS. Grey regions in the graphs illustrate the periods where the water supply was stopped in the case of the non-irrigated plants. Values indicate mean ± SE ($n \geq 8$). Asterisks indicate significant differences with a $P \leq 0.05$ (t-test) between leaves of irrigated (control) and seasonal non-irrigated (stressed) plants for each cultivar and measuring day.

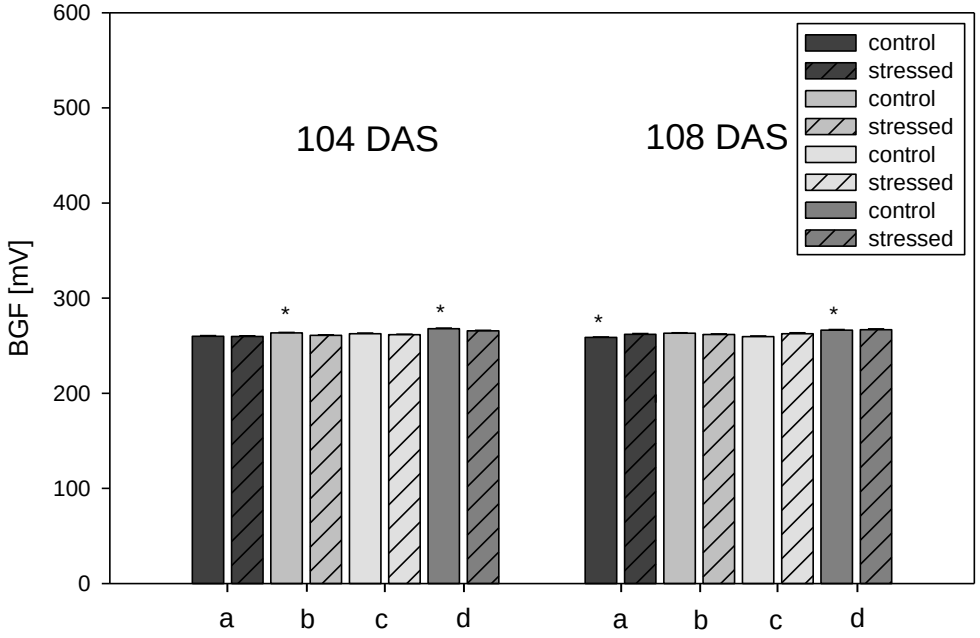

Fig. S2. Influence of a seasonal water shortage on the blue fluorescence (BF) excited with UV-light on the four sugar beet genotypes Pauletta (a), Berenika (b), Cesira (c) and Mauricia (d). Leaves were measured at day 104 and 108 after sowing of plants cultivated under field conditions. Columns indicate mean ± SE (n=96). Asterisks mean significant values with a $P \leq 0.05$ (t-test) between leaves of irrigated (control) and rainfed (stressed) plants for each cultivar and measuring day.

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D Drought stress memory in sugar beet: mismatch between biochemical and physiological parameters[3]

1 Introduction

In their life-cycle, plants commonly face a number of stress situations which negatively influence their development and agronomic performance. In general, water deficit is one of the main environmental factor limiting growth and productivity of crops (Montesinos-Pereira *et al*. 2014). Under drought, photosynthetic efficiency is decreased as stomatal closure restricts the CO_2 uptake (Pantin *et al*. 2013). Under extended drought, several non-stomatal mechanisms inhibit essential cellular processes required to maintain photosynthesis (Flexas and Medrano 2000). Alterations in the photosynthetic activity are also associated with a decrease of chlorophyll concentration as consequence of membrane disturbances in the mesophyll cells (Cornic and Masacci 1996). Finally, several physiological, biochemical and molecular responses occur to maintain plant´s vitality and survival during drought (Reddy *et al*. 2004).

In general terms, a better understanding of the response mechanisms of plants to water deficit is essential to enhance their drought tolerance (Thapa *et al*. 2011). In their evolution, plants have developed morphological, physiological and biochemical mechanisms to adapt and overcome stress phases (Biswal *et al*. 2011; Conde et *al*. 2011), as reported for different plant species under different conditions. Moreover, plants might have some kind of stress 'memory', which could support their fitness in response to recurrent environmental stresses (Thellier and Lüttge 2013; Thellier *et al*. 1982). Although the precise mechanisms involved in regulation of physiology and molecules is poorly understood (Hu *et al*. 2015), epigenetic changes and sustained alterations of important metabolites or transcription factors support the transgeneration of stress memory in plants (Bruce *et al*. 2007; Hu *et al.* 2015; Kinoshita and Seki 2014; Molinier *et al*. 2006). In Arabidopsis, the involvement of abscisic acid (ABA)-dependent pathways for the stress memory was proven (Goh *et al*. 2003). Specifically on water deficit, plants with transcriptional stress memory displayed an increase in the rate of transcription, and elevated transcript levels of a subset of the stress–response genes (Ding *et al*. 2012). Similarly, it had been shown that even perennial plants and long-lived trees

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(Thellier and Lüttge 2013) as well as grasses (Hue *et al*. 2015; Walter *et al*. 2011) might store and recall stress imprints.

While the absolute majority of the 'stress memory' studies focus on responses at transcript level, there are only a few publications on the effects on plant physiology or in a more applied sense, the agronomic performance of crops. In one of the rare examples, it has been proposed that the grass *Arrhenatherum elatius* might have a drought-memory over an entire vegetation period, as demonstrated using chlorophyll *a* fluorescence as indicative techniques (Walter *et al*. 2011). In this context it is also not clear if stress imprints might also be stored in roots, such as the pronounced storage root of sugar beet.

In our previous studies, we demonstrate the potential of non-invasive fluorescence techniques for the characterization of physiological responses of different plant species such as tomatoes (Kautz *et al*. 2014), cereals (Bürling *et al*. 2013) and sugar beet (Leufen *et al*. 2013, 2014) when exposed to water deficit. In this scope, instead of using the classical pulse-amplitude chlorophyll fluorescence we adopted either the spectrally resolved fluorescence, the fluorescence lifetime, or the multiparametric fluorescence technique, all of them providing precise indications on stress-induced alterations of plant physiology as well as the type and content of pigments in the cells. Particularly in sugar-beet (Leufen *et al*. 2013, 2014), physiological measurements as well as visual observations clearly demonstrate a fast recovery of the stressed plants when the optimum growing conditions were re-established.

In commercial cultivations, sugar beet is grown over a comparatively long period (until 8 months), and has a prominent root system which could, in addition to the leaves, store stress imprints for posterior recall. However, to our knowledge, it is still unexploited if sugar beet plants possess such a 'memory' which could enable them to better overcome recurrent stresses. The objective of the trial was to study the existence of drought stress memory in sugar beet in a long-term greenhouse experiment under semi-controlled conditions, with focus on physiological parameters as evaluated. Based on our previous work and indications from the literature we hypothesized that, if existent, the memory effect in sugar beet plants would be identified by the less accentuated changes in the fluorescence signature in recurrent stress phases.

2 Materials and Methods

2.1 Plant material and growth conditions

The experiment was conducted from October 2011 to March 2012 in a heated greenhouse. Seeds of the sugar beet (*Beta vulgaris* L.) genotypes Pauletta, OVK and 8GK

were provided by the company KWS Saat AG (Einbeck, Germany). These genotypes were selected because of their differences in leaf morphology and general plant performance as indicated by the plant breeder (personal communication Dr. Britta Schulz, KWS Saat AG, Einbeck, Germany), and confirmed in our preliminary trials. Seeds without any agrochemical treatment were germinated in a sowing tray, and after one week uniform plants were transplanted into 4 l plastic pots (0.233 m high, 0.157 m diameter) evenly filled with peat substrate (Einheitserde Typ VM, Einheitserde- und Humuswerke Gebr. Patzer GmbH & Co.KG, Sinntal-Altengronau, Germany). Plants were assigned to the experimental treatments (n = 4 per genotype, treatment and evaluation date) and placed at random on two benches (10.5 x 1.65 m) with automatic nutrient supply (pH 6.5 and an EC 180 mS cm^{-1}). Photoperiod (16 h) and photosynthetic active radiation (250-350 $\mu mol^{-2}s^{-1}$) were enabled by supplemental light from high-pressure sodium lamps (Philips SON-T Agro 400W, Philips Electronics N.V., Hamburg, Germany). Water deficit was induced by withholding the irrigation in three consecutive phases, considering the number of days after sowing (DAS) as time-reference: 35-54 DAS, 86-102 DAS and 135-151 DAS. The full stop of irrigation caused increasing water deficit stress in the time course of the experiment until rewatering of plants. In the periods between two water deficit phases, plants were allowed to recover under full irrigation.

2.2 Non-destructive determinations

2.2.1 Multiparametric fluorescence

Fluorescence recordings were done in the laboratory at leaf level by using a hand-held optical fluorescence sensor (Multiplex® 3, Force-A, Orsay, France), as previously described (Leufen *et al*. 2013, 2014). Briefly, light-emitted-diodes (LED) excite sequentially the fluorescence with UV (peak at 375 nm), green (peak at 518 nm) and red (peak at 630 nm) light and the fluorescence signals are recorded in the blue (425-475 nm), red (680-690 nm) and far-red (720-755 nm) spectral regions. A grid in front of the sensor enabled a constant distance of 0.10 m between sensor and leaves; thereby, an area of approximately 50 cm^2 was illuminated. Recordings were always taken on the two upper, fully-expanded opposite leaves of each plant. As target parameters we selected the red fluorescence (RF_G), and the 'Simple Fluorescence Ratio' (SFR) after excitation with green light.

2.2.2 Fluorescence lifetime

Leaves were fixed horizontally on a sample holder by maintaining a constant distance (3.95 mm) between sample and fiber-optical probe. Fluorescence lifetimes were recorded on

the leaf tip, about 2 cm from the leaf margin, by avoiding major veins. Fluorescence lifetime was recorded with a compact fiber-optic spectrometer (IOM GmbH, Berlin Germany), as described elsewhere (Buerling *et al*. 2011, 2012). Briefly, a pulsed nitrogen laser (MNL 100, LTB Lasertechnik Berlin GmbH, Berlin, Germany) excites the plant tissue (337 nm, repetition rate of 30 Hz). The pulse energy at the probe exit was adjusted to be 8-8.5 µJ. A photomultiplier (PMT, H5783-01, Hamamatsu, Hamamatsu City, Japan), with a sensitivity of 800 Volt, was used as detector. Fluorescence lifetime was recorded in a range of 410 to 560 nm in the interval of 30 nm. The detection gate was opened from 0.0 to 16 ns following excitation and the step width of the integrator gate was set to 0.4 ns. Each single data point was averaged from 16 pulse counts. Fluorescence decays were analyzed by using deconvolution software (DC4, V. 2.0.6.3, IOM GmbH, Berlin, Germany).

2.2.3 Gas exchange

Gas exchange was measured with a portable infrared gas analyzer (CIRAS-1, PP Systems, United Kingdom) equipped with a leaf cuvette (PLC B, PP Systems, United Kingdom) covering an area of 2.5 cm^2. Net photosynthetic rate (Pn), stomatal conductance (G), internal CO^2 partial pressure (CI) and transpiration rate (E) were measured at the leaf tip by avoiding major veins. Readings were performed in the greenhouse under standardized conditions at a measuring station to minimize the effect of the environment. Recordings were done from 17:00h to 19:30h to avoid midday-depression and minimize the impact of external light. Equipment settings were adjusted at an internal CO^2 concentration of 350 ± 5 ppm, light irradiation on the leaf surface was about 250–350 µmol m^{-2} s^{-1} PAR and the air flow entering the chamber was 200 ± 5 ml min^{-1}. For the measurements, pots of plants were taken randomly to avoid systematic errors concerning the measuring time, and transported to the measuring station. Although the light intensity for photosynthesis determination was not saturating, the conditions corresponded to those find on the cultivation tables. Values of photosynthesis were recorded when steady-state was attained.

2.3 Reference parameters

2.3.1 Sampling method

Leaves were harvested at irregular intervals in the timeframe from 43 to 159 DAS. Thereby, the leaves previously used for the fluorescence recordings were stored in plastic bags at -21 °C for the posterior quantification of chlorophyll and proline contents. The underlying leaf pair was stored for the consecutive determination of osmotic potential.

Samples for determination of chlorophyll and proline were lyophilized, grounded and stored in the dark at room temperature.

2.3.2 Chlorophyll concentration

Chlorophyll was extracted from 50 mg lyophilized material by 5 ml methanol, and filled up to 50 ml. After extraction the absorbance of extracts was measured at 665 nm (A665) and 650 nm (A650) with a UV-VIS spectrophotometer (Perkin-Elmer, Lambda 5, Massachusetts, USA). The concentration of chlorophyll *a* (C*a*), *b* (C*b*) and total chlorophyll content (C*t*) was calculated with the following equations, as published by Hoffmann *et al*. (2015):

$$C\mathit{a}\ (\mu g\ g^{-1}) = ((16.5*A665 - 8.3*A650)/\text{dry mass})*50$$
$$C\mathit{b}\ (\mu g\ g^{-1}) = ((33.8*A650 - 12.5*A665)/\text{dry mass})*50$$
$$C\mathit{t}\ (\mu g\ g^{-1}) = ((25.5*A650 + 4.0*A665)/\text{dry mass})*50$$

2.3.3 Proline concentration

Determination of proline followed the method established in our group and recently published by Hoffmann *et al*. (2015). Of each sample, 20 mg lyophilized material was mixed with 3 ml sulfosalicylic acid (3 %), homogenised and afterwards centrifuged at 4,000 × g for 15 min. under room temperature. Thereafter, 0.4 ml of the supernatant was added to 1.6 ml sulfosalicylic acid (3 %) by gently shaking while adding 2 ml of glacial acetic and ninhydrin acid. The solution was placed for 1 h in a 100 °C water bath; after cooling down, 4 ml toluene was added. The upper part of the solution was pipetted and the absorbance of the extracts was analysed at 520 nm with a UV-VIS spectrophotometer (Perkin-Elmer, Lambda 5, Massachusetts, USA). The concentration of proline was calculated with the following equation:

$$\text{Proline}\ (\mu g\ g^{-1}) = ((\mu g\ \text{proline} * ml^{-1}\ \text{sample}) * 10\ ml) / \text{sample mass [g]}$$

2.3.4 Osmotic potential

Osmotic potential was determined according to Kautz *et al*. (2014). Samples were placed in bags (Bioreba, Switzerland) and extruded with a hand homogenizer. Thereafter, 2 ml of the extract was collected, filled and centrifuged (Eppendorf, Centrifuge 5417 R, Hamburg, Germany) for 10 minutes (25,000 *g min^{-1} at 4 °C). From the supernatant, 15 µl were pipetted into tubes and the osmolality measured with a freezing-point depression

osmometer (Osmomat 030-D, Genotec GmbH, Berlin, Germany). At the beginning of the measurements, the osmometer was calibrated by using preformed Genotec vials (850 mmol kg^{-1} H_2O) and distilled water (0 mmol kg^{-1} H_2O).

2.4 Statistical analysis

In several cases, results are presented as percent of modification (as compared to the respective control group) enable more precise comparisons between genotypes and evaluations over the time. Data were statistically analyzed with SPSS statistic software (PASW statistics version 19.0, SPSS Inc., Chicago, USA). For each genotype and evaluation date, control and temporary non-irrigated plants were compared by analysis of variance and paired t-test ($p \leq 0.05$).

3 Results

3.1 Biochemical indicators: osmotic potential, proline and chlorophyll concentration

Modifications of the leaf osmotic potential, proline concentration and total chlorophyll concentration to temporary water deficit and recovery are shown in figure 1. As compared to control plants, water deficit caused a strong increase of leaf osmotic potential (OP) during and immediately after the first drought period (53-65 DAS), reaching a maximum of 150% as compared to control plants; this alteration was most pronounced in the cultivar OVK (Fig. 1a). At the same time, proline content increased in a much higher extent (Fig. 1b) reaching max of 1000%, while chlorophyll concentration decreased in the worst case in about 60% (Fig. 1c). In the recovery phase, values of proline approached those of the control plants while osmotic potential and chlorophyll content still remained altered. In the second stress cycle, alterations during water deficit were in general less accentuated while the recovery of the parameters to the ‘normal’ values was also observed not only for proline (Fig. 1b) but also for the osmotic potential (Fig. 1 a). In the third cycle, biochemical response of plants was higher than in the second phase, with exception for the chlorophyll content. As observed in the three consecutive cycles, decrease in chlorophyll content was stronger immediately after rewatering the stressed plants, followed by a slight recovery in the following days. In general, we observed significant discrepancies in the intensity and speed of changes of the three parameters (osmotic potential, proline and chlorophyll content) when plants were exposed to drought stress and recovery. The strongest variations were ascertained for ‘OVK’ during the first and second phase.

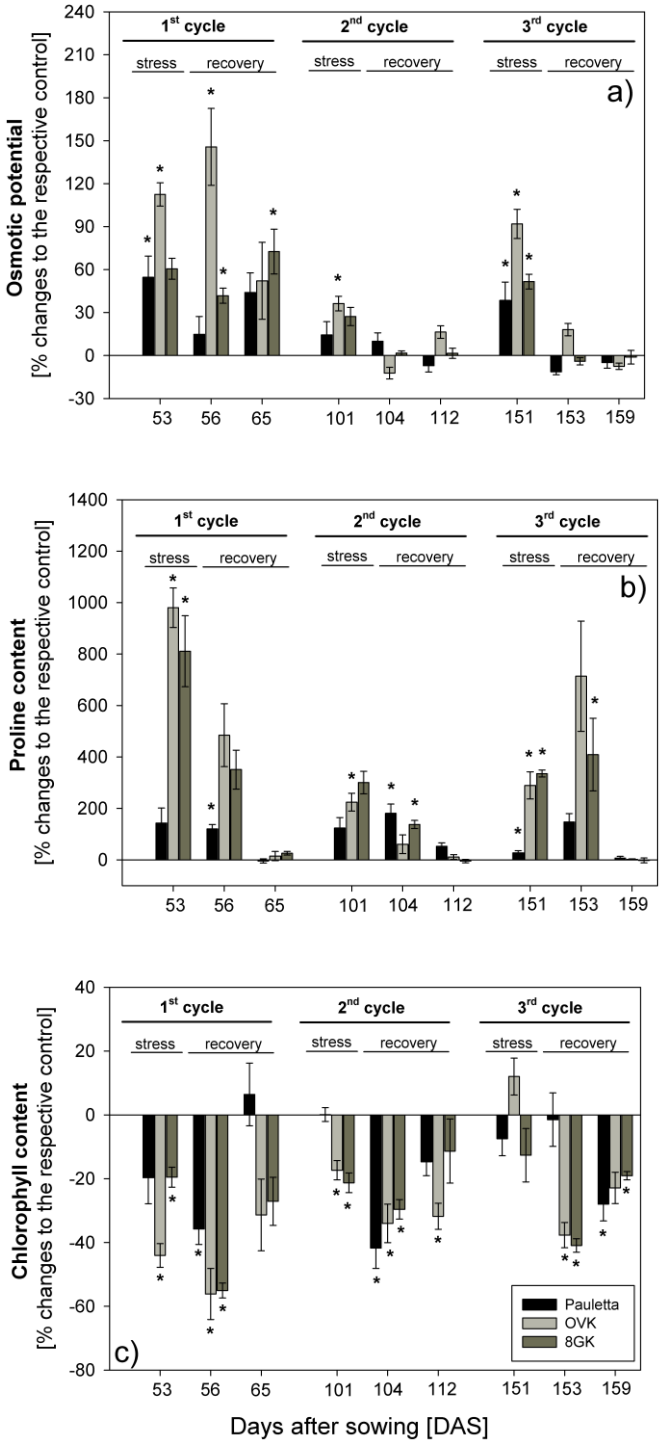

Fig. 1. Influence of temporary water deficit and re-watering on the osmotic potential (a), proline concentration (b), and the total chlorophyll concentration (c) of the cultivars Pauletta, OVK and 8GK. Recordings (here, displayed as relative percent to the control) were done on selected days (53, 56, 65, 101, 104, 112, 151, 153 and 159 DAS). Asterisks indicate significant differences with a $P \leq 0.05$ (t-test), between irrigated and non-irrigated plants for each cultivar and measuring day (n = 4).

3.2 Net photosynthesis and transpiration

Gas exchange was measured to assess the progress and intensity of drought on plant´s most basic process, the photosynthesis. In all three stress cycles, photosynthesis decreased very strongly, reaching values close to zero shortly before the recovery phase (Figs. 2a-i).

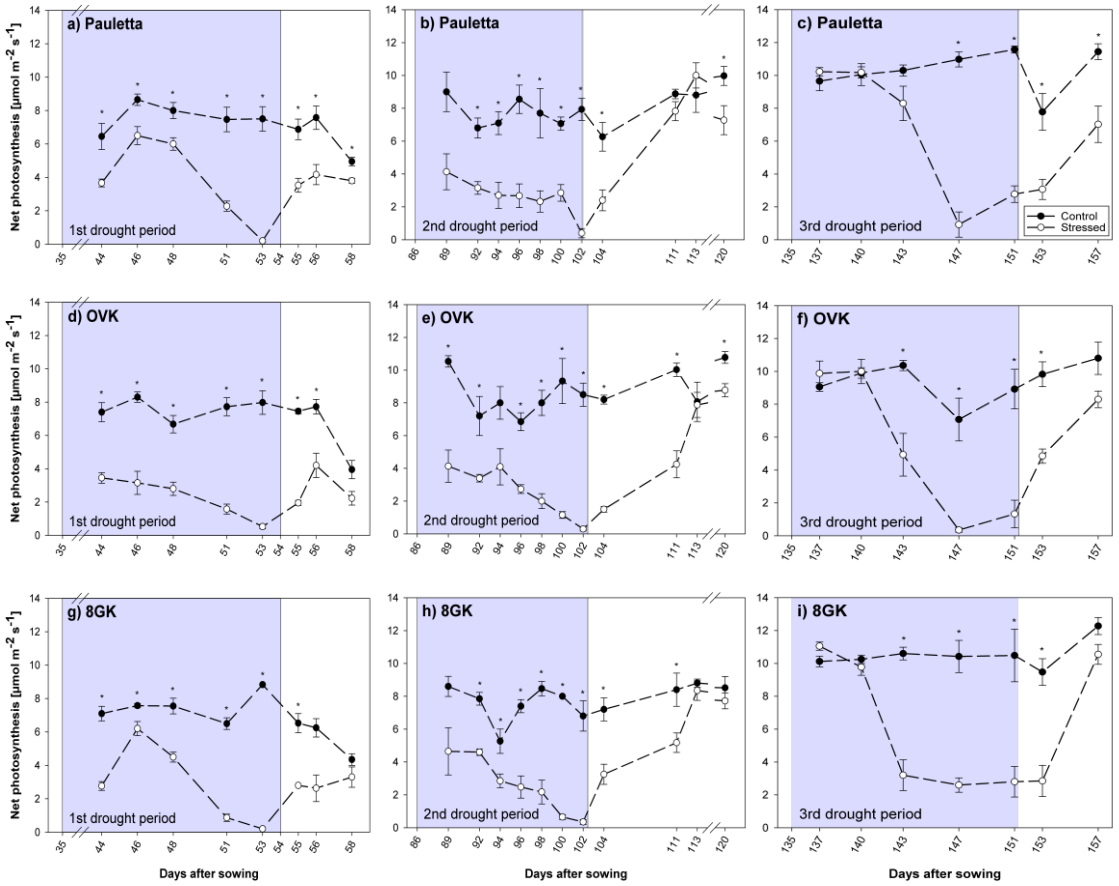

Fig. 2. Leaf net photosynthesis [µmol m^{-2} s^{-1}] of the sugar beet cultivars Pauletta (a-c), OVK (d-f) and 8GK (g-i) influenced by water supply. Measurements took place under semi-controlled conditions in the greenhouse in three consecutive phases, 35-58 DAS, 86-120 DAS and 135-157 DAS, on leaves of irrigated (control) and temporarily non-irrigated (drought) plants; Asterisks indicate significant differences (t-test, $P \leq 0.05$) between irrigated and non-irrigated plants. Mean ± SE (n = 4).

Transpiration was affected in a similar way (Fig. 3a-i), with less pronounced stress-driven alterations in the second phase. As general pattern, following the rewatering plants reached values similar to those measured on control plants, except in the third phase. In general, no concrete hints of drought-memory could be observed here; one exception, however, is found in the third stress cycle for the cultivar 8GK. Here, the minimum net

photosynthesis, although very low (about 3 µmol m^{-2} s^{-1}) was still significant higher than both the values observed for the other cultivars in the same phase (below 1 µmol m^{-2} s^{-1}) and the values of the same cultivar in the previous stress cycles (below 0.5 µmol m^{-2} s^{-1}).

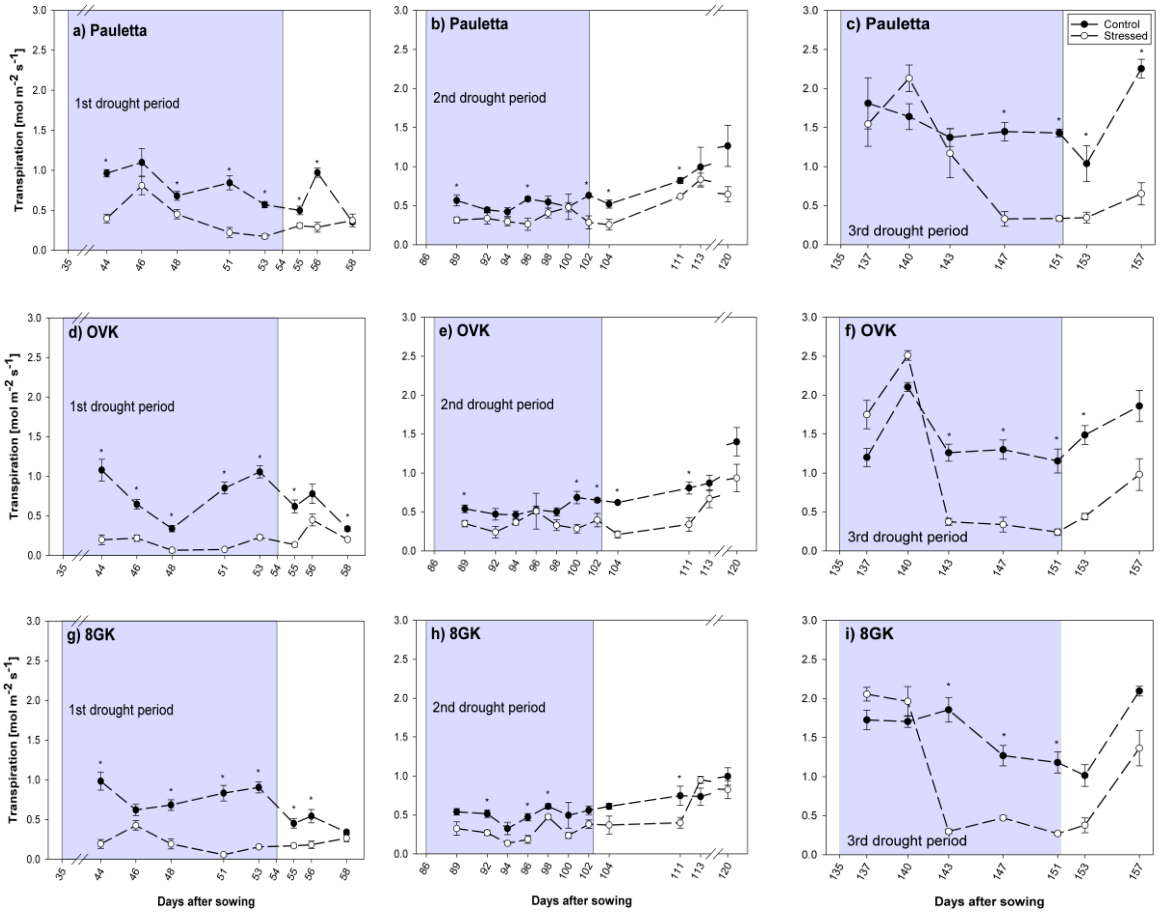

Fig. 3. Development of leaf transpiration [mol m^{-2} s^{-1}] on the sugar beet cultivars Pauletta (a-c), OVK (d-f) and 8GK (g-i) influenced by water supply. Measurements took place under semi-controlled conditions in the greenhouse in three consecutive phases, 35-58 DAS, 86-120 DAS and 135-157 DAS, on leaves of irrigated (control) and temporarily non-irrigated (drought) plants; Asterisks indicate significant differences (t-test, $P \leq 0.05$) between irrigated and temporarily non-irrigated plants. Mean ± SE (n = 4).

3.3 Red fluorescence (RF) and 'Simple Fluorescence Ratio' (SFR_G)

The development of the green-excited red fluorescence (RF_G, Fig. 4 a-c) and the 'Simple Fluorescence Ratio' (SFR, Fig. 4 d-f) over the three experimental phases (35-65 DAS, 86-121 DAS and 135–159 DAS) is displayed exemplarily the cultivar Pauletta. In all

experimental cycles, starting at the third recording date, RF_G of stressed plants was higher than RF_G of control plants; in the sequence, values reached or at least approached the level of control plants at the end of the recovery phase (Fig. 4a-c). Nevertheless, highest increase of RF_G was observed during the second cycle (Fig. 4b), which was, according to the biochemical indicators, the phase with lower stress (Fig. 1). A detailed analysis of the SFR demonstrates a stress-induced decrease of the values, however, following similar trends as observed for RF_G (Fig. 4a-c). Both parameters were also recorded for the cultivars OVK and 8GK (Figs. S1 and S2). In general, the chlorophyll fluorescence parameters (FR_G and SFR) of these cultivars follow the similar pattern as the trends reported for Pauletta. Nevertheless, 'OVK' responded more sensitively to desiccation as the other varieties, as RF_G and SFR values decline immediately after the water supply was stopped during the first experimental period (Fig. S1). Irrespective of that, a clear response to the water deficit in the second or third stress cycle could not be related to the plant response in the preceding stress cycle.

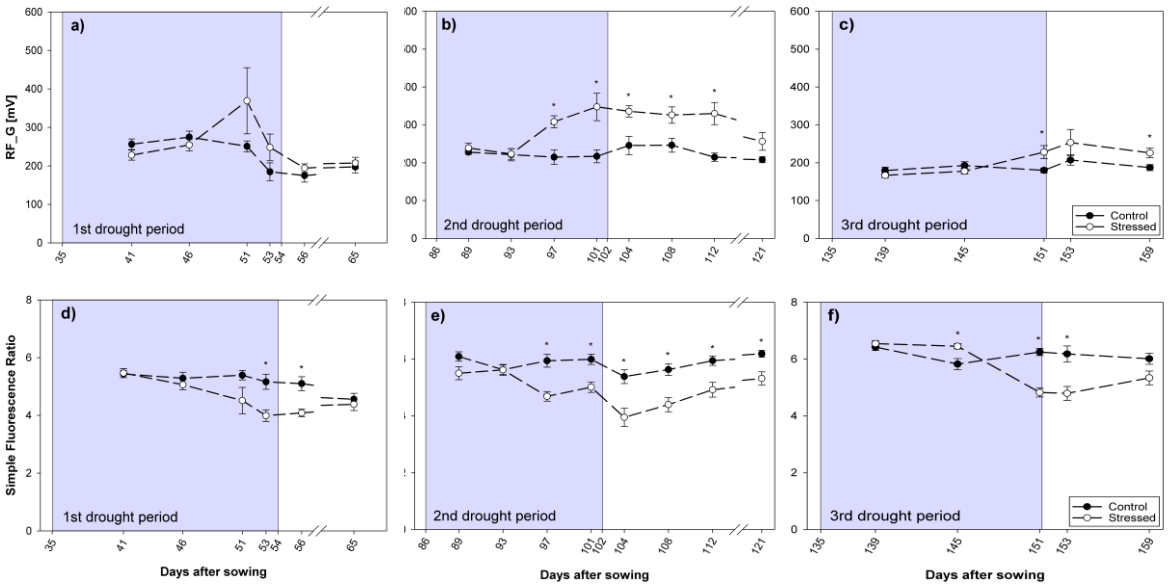

Fig. 4. Influence of water supply on the red fluorescence (RF_G, a-c) and on the 'Simple Fluorescence Ratio' (d-f) of the sugar beet genotype Pauletta measured after excitation with green light. Fluorescence recordings were taken at leaf level between 41 and 159 DAS in three consecutive phases. Grey regions in the graph illustrate the periods where water supply was stopped for the stress treatment. Values indicate mean ± SE (n = 8). Asterisks indicate significant values with a $P \leq 0.05$ (t-test) between leaves of irrigated (control) and non-irrigated (stressed) plants for each cultivar and measuring day.

3.4 Fluorescence lifetime

Fluorescence mean lifetime was lower in plants exposed to water deficit as compared to control plants, mainly in the blue spectral region (410 nm and 440 nm). Numerical differences were also observed in the green region (500 nm and 560 nm), but in most cases statistical significance ($p < 0.05$) was not asserted. This was observed for the cultivar Pauletta (Table 1) as well as for the cultivars OVK (Table S1) and 8GK (Table S2). As observed, in the recovery phase the values measured in the stressed plants could not reach those levels recorded in control plants. For all cultivars, the most suited wavelength to distinguish the experimental treatments was at 410 nm (Table 1, supplemental material table S1 and S2). Nevertheless, precise indications of drought memory could not be detected using this spectroscopic method.

Table 1. Mean fluorescence lifetime at selected wavelength of control (c) and non-irrigated plants (d) of the sugar beet cultivar Pauletta from 46 to 159 days after sowing (DAS). In this table, always the first two readings (e.g., 46 and 53 DAS) were conducted during drought stress phase, whereas the following two (56 and 65 DAS) were done during re-watering. Similar is displayed for the second (93 – 112 DAS) and third experimental phase (145 – 159 DAS).

DAS	Wavelength [nm]							
	410		440		500		560	
	c	d	c	d	c	d	c	d
46	1.19*	0.83	1.07	0.94	1.39	1.36	1.54	1.46
53	1.22*	0.78	1.26*	0.82	1.42*	1.23	1.59*	1.43
56	1.20*	0.83	1.12*	0.87	1.45	1.31	1.63	1.53
65	1.20*	0.79	1.03*	0.92	1.41	1.31	1.60	1.57
93	1.07*	0.91	1.16	1.01	1.40	1.32	1.71*	1.58
101	0.99*	0.69	1.13*	0.85	1.54	1.28	1.65*	1.43
104	0.93	0.73	1.17	0.78	1.42	1.25	1.51	1.46
112	0.99*	0.75	1.06	0.91	1.43	1.31	1.61	1.56
145	1.22*	0.83	1.07	0.94	1.39	1.36	1.54	1.46
151	1.22	0.78	1.26	0.82	1.42	1.23	1.59	1.43
153	1.20*	0.83	1.12*	0.87	1.45*	1.31	1.63	1.53
159	1.2	0.79	1.03	0.92	1.41	1.31	1.6	1.57

* Significant differences (t test, $p \leq 0.05$; $n = 4$) between control (c) and non-irrigated (d) plants for each variety and measuring day

4 Discussion

In our experiments we used selected non-invasive methods to study plant responses to transient and recurrent water deficit, aiming to exploit and better understand mechanisms of drought memory. Due to the fast setup of the storage organ in beets, starting two weeks after emergence (Rapoport and Loomis 1986), we expected improved stress response by adjusted sink-source regulations in beets, which should become visible through lower stress-related changes in biochemical and physiological parameters during the second and/or third stress period.

When drought stress begins, stomatal closure reduces CO_2 assimilation (Cornic and Massacci 1996), in our experiments causing a decline in leaf net photosynthesis in all evaluated varieties (Fig. 2). A decrease in photosynthesis is usually accompanied by increase in heat dissipation and chlorophyll fluorescence as immediate mechanisms to avoid damages to the photosystems. Extended and severe water deficit led to further structural and functional disturbances in the photosynthetic apparatus, illustrated by strong alterations in chlorophyll fluorescence indices (Fig. 4, supplemental material Fig. S1 and S2). Thereby, the increase in the red fluorescence and the simultaneous decrease in the SFR_G throughout the individual stress periods can be linked to an impairment of the photosynthetic quantum conversion and a lower capacity for light-harvesting (Iturbe-Ormaetxe *et al.*1998; Lichtenthaler and Rinderle 1988; Mafakheri *et al*. 2010). Similar results were also obtained in previous studies (Leufen *et al*. 2013, 2014). Nevertheless, the new information is that these changes follow a similar trend in all three experimental phases, despite the different development stages of the beets.

Even if the physiological readings of drought-exposed plants indicate similar trends in the three cycles (Figs. 2-4), biochemical parameters respond significantly less intensive to desiccation during the second period (Fig. 1). This effect might be explained by improved osmotic adjustment in beets. It is known that proline acts as a stress indicator but also significantly contributes to osmotic adjustment (Molinari *et al*. 2007); this adjustment happens together with other compounds, increasing the osmotic potential during drought (Ingram and Bartels 1996). The decline in the total chlorophyll concentration is caused by a general lower chlorophyll synthesis, as well as the formation of reactive oxygen species which induce oxidative stress in proteins, membrane lipids, and other cellular components (Farooq *et al*. 2009; Molinari *et al*. 2007).

In contrast to the chlorophyll content, net photosynthesis and all chlorophyll fluorescence indices recover very soon after re-watering. It is known that severe drought stress might increase synthesis of ABA preventing excessive water loss through excessive

transpiration as well as accelerating senescence due to recycling of vital nutrients (Munné-Bosch and Alegre 2004; Wingler and Roitsch 2008). On the other hand, during recovery of plants cytokinins might delay senescence and/or induce stomatal opening (Vomáčka and Pospíšilová 2003).

Mean fluorescence lifetime recordings in the blue-green spectral range did not show any hints of improved stress tolerance in recurrent stress cycles. Thereby, lifetime was numerically and in some cases statistically lower in drought stressed plants than in control plants leaves (Tables 1, S1 and S2). Differences might be associated with a decrease of blue-green fluorescing compounds, e.g. ferulic acid, p-cumaric acid, through dehydration, which finally caused lower mean fluorescence lifetimes (Cerovic *et al.* 1994; Morales *et al*. 1994; Sgherri *et al*. 2004).

Even if we could assess non-invasively drought-related stress patterns in all three sugar beet cultivars, a ‘drought memory’ as indicated on annual grasses using classical chlorophyll fluorescence (Walter *et al*. 2011), could not be proven in our study. Amongst others, morphological and physiological differences between mono and dicotyledonous might play a significant role, also to explain the weaker stress responses in the second period besides comparable experimental conditions in the three cycles (Stober and Lichtenthaler 1993; Cerovic *et al*. 1999). Further, the sink-source relations in the different stress phases might have had influenced both the response of the plants detected with physiological and biochemical parameters. In this context, there is a higher relevance to maintain existing structures in beets under stress than the storage process (Shaw *et al*. 2002). With our experimental setup, plants exposed to drought could effectively accumulate substances either before the trial or during the recovery phases after stress. Particularly in the second stress cycle, the comparatively lower stress-induced alteration of the biochemical parameters might be explained by a decrease in the concentration of sucrose and other compounds in the storage root (Bloch *et al*. 2006). If the storage substances are not available anymore, and starvation happens in the leaves, biochemical parameters might be stronger affected than physiological parameters, as observed again in the third stress cycle. In this context, the hypothesis of accelerated senescence at the end of the third phase can be excluded since in each cycle new fully developed leaves were selected for the recordings, while the plant of sugar beet, a bi-annual species under natural environments, can continuously produce new leaves.

Taking this fact into consideration, the detection and elucidation of a ’memory effect’ in species which build up a pronounced storage organ during their life cycle, as sugar beets do, is particularly difficult. Unfortunately, in our experimental setup we had no plants exposed to

only a single drought event at any one of the stress cycles. Thus, we cannot differentiate if the lower sensitivity to drought during the second period was caused by growth-dependent alterations and/or changes in levels of key signaling metabolites or transcription factors initiated by previous stress (Bruce *et al*. 2007). In this context, a more precise elucidation requires extended destructive analysis of shoot and root components such as ABA, carbohydrates, phenolic compounds and soluble constituents on top of our determinations.

5 Conclusion

Our study indicates that the ‘Simple Fluorescence Ratio’ is a reliable parameter to assess the physiological state of sugar beet plants to changing water supply conditions. Nevertheless, similar to the leaf net photosynthesis, fluorescence parameters did not provide strong indications towards a ‘drought memory’. In general, we observed no clear relation in the different cycles between results of biochemical and physiological parameters. Thus, further studies are needed to clarify the details about plant physiological mechanism to changing water supply situations, involving also investigations of the root-body, as main source for providing reserve substances under harmful growth conditions.

The following pages of this chapter display the supplementary figures S1 and S2 as well as tables S1 and S2.

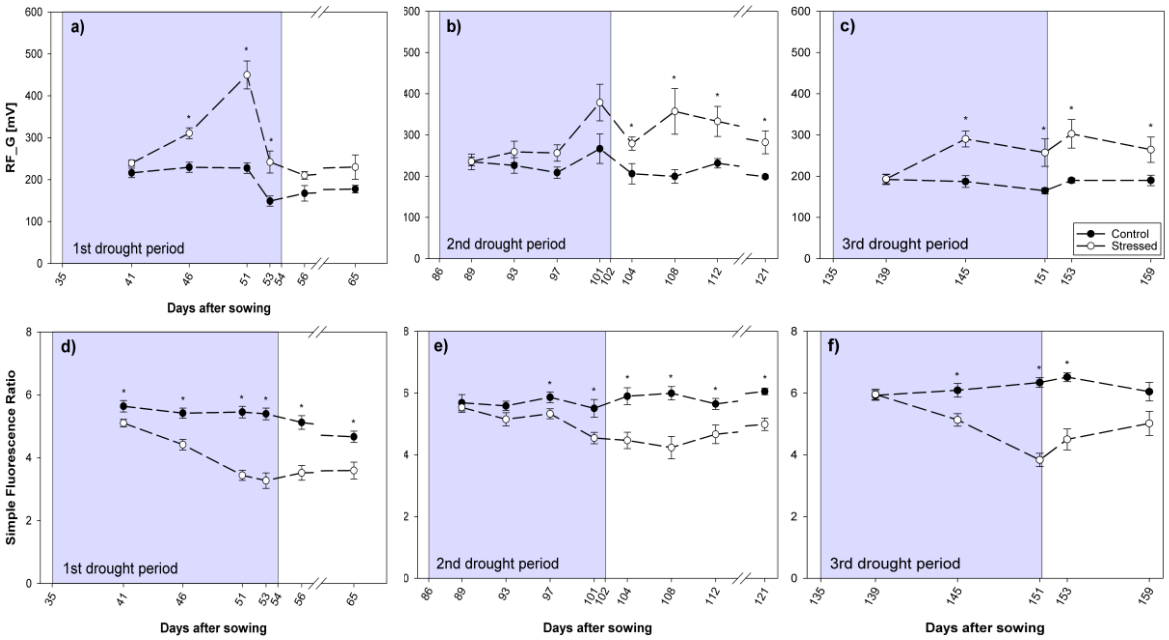

Fig. S1. Influence of water supply on the red fluorescence (RF_G, a-c) and on the 'Simple Fluorescence Ratio' (d-f) of the sugar beet genotype OVK measured after excitation with green light. Fluorescence recordings were taken at leaf level between 41 and 159 DAS in three consecutive phases. Grey regions in the graph illustrate the periods where water supply was stopped for the stress treatment. Values indicate mean ± SE (n = 8). Asterisks indicate significant values with a $P \leq 0.05$ (t-test) between leaves of irrigated (control) and non-irrigated (stressed) plants for each cultivar and measuring day.

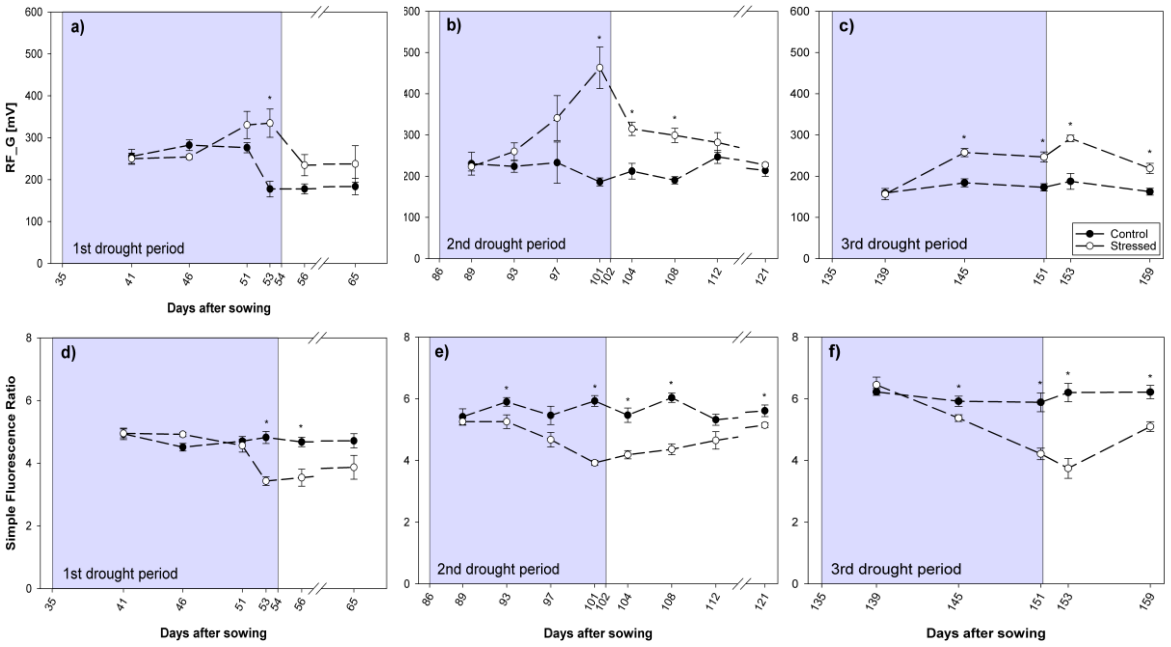

Fig. S2. Influence of water supply on the red fluorescence (RF_G, a-c) and on the 'Simple Fluorescence Ratio' (d-f) of the sugar beet genotype 8GK measured after excitation with green light. Fluorescence recordings were taken at leaf level between 41 and 159 DAS in three consecutive phases. Grey regions in the graph illustrate the periods where water supply was stopped for the stress treatment. Values indicate mean ± SE (n = 8). Asterisks indicate significant values with a $P \leq 0.05$ (t-test) between leaves of irrigated (control) and non-irrigated (stressed) plants for each cultivar and measuring day.

Table S1. Mean fluorescence lifetime at selected wavelength of control (c) and non-irrigated plants (d) of the sugar beet variety OVK from 46 to 159 days after sowing (DAS). In this table, always the first two readings (e.g. 46 and 53 DAS) were conducted during drought stress phase, whereas the following two (56 and 65 DAS) were done during re-watering. Similar is displayed for the second (93 – 112 DAS) and third experimental phase (145 – 159 DAS).

DAS	Wavelength [nm]							
	410		440		500		560	
	c	d	c	d	c	d	c	d
46	1.15*	0.89	1.21	0.98	1.66*	1.39	1.72*	1.53
53	1.24*	0.80	1.16*	0.95	1.57*	1.36	1.78*	1.45
56	1.36	1.16	1.40	1.13	1.61	1.61	1.78	1.75
65	1.47	1.30	1.66	1.44	2.16	1.68	2.14	1.78
93	1.35	1.09	1.47	1.26	1.69	1.47	1.76	1.64
101	1.12*	0.92	1.40	1.41	1.91	1.74	1.88	1.57
104	1.07	1.01	1.29	1.37	1.81	1.69	1.76	1.56
112	1.05*	0.85	1.18	1.34	1.51	1.76	1.65	1.59
145	1.15*	0.89	1.21*	0.98	1.66*	1.39	1.72*	1.53
151	1.24*	0.80	1.16*	0.95	1.57*	1.36	1.78*	1.45
153	1.36*	1.16	1.40	1.13	1.61	1.61	1.78*	1.75
159	1.47*	1.30	1.66*	1.44	2.16*	1.68	2.14*	1.78

* Significant differences (t test, $p \leq 0.05$; $n = 4$) between control (c) and non-irrigated (d) plants for each variety and measuring day.

Table S2. Mean fluorescence lifetime at selected wavelength of control (c) and non-irrigated plants (d) of the sugar beet variety 8GK from 46 to 159 days after sowing (DAS). In this table, always the first two readings (e.g. 46 and 53 DAS) were conducted during drought stress phase, whereas the following two (56 and 65 DAS) were done during re-watering. Similar is displayed for the second (93 – 112 DAS) and third experimental phase (145 – 159 DAS).

DAS	Wavelength [nm]							
	410		440		500		560	
	c	d	c	d	c	d	c	d
46	0.89	1.22	0.97	1.12	1.48	1.49	1.49	1.56
53	1.32	1.15	1.23	1.33	1.56	1.61	1.57	1.57
56	1.32*	0.80	1.41*	0.83	1.67*	1.31	1.74*	1.55
65	1.31	1.13	1.25	1.31	1.49	1.57	1.74	1.70
93	1.16*	0.87	1.17	1.00	1.41	1.34	1.70	1.65
101	1.08*	0.67	1.24	0.81	1.53	1.26	1.62	1.40
104	0.99*	0.71	1.30	1.02	1.53	1.41	1.60	1.52
112	0.89	0.79	0.98	1.03	1.42	1.48	1.51	1.47
145	0.89*	1.22	0.97*	1.12	1.48	1.49	1.49*	1.56
151	1.32	1.15	1.23	1.33	1.56	1.61	1.57	1.57
153	1.32*	0.80	1.41	0.83	1.67	1.31	1.74*	1.55
159	1.31	1.13	1.25	1.31	1.49	1.57	1.74	1.70

* Significant differences (t test, $p \leq 0.05$; $n = 4$) between control (c) and non-irrigated (d) plants for each variety and measuring day.

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E Proximal sensing of plant-pathogen interactions in spring barley with three fluorescence techniques[4]

1 Introduction

During their whole lifecycle, agricultural crops are exposed to a multitude of harmful organisms *i.e.*, pathogens, that cause considerable yield losses. Obligate biotrophic parasites, like powdery mildew and leaf rust, cause the most serious and widespread diseases in agronomic crops (Ridout *et al*. 2006). Despite crop protection activities, estimations indicate that more than 10 per cent of the worldwide wheat losses can be attributed to pathogens (Oerke 2006). The adoption of varieties which are resistant to the pathogens is one promising and environment-friendly attempt to mitigate this problem. Nevertheless, the development of new varieties in traditional breeding programs is an expensive and time-consuming process, and requires many expensive field studies and validations over several years (Schnabel *et al*. 1998). In breeding programs new and promising lines are classified and rated to their susceptibility to diseases after a visual monitoring done by trained specialists. However, precise classifications on the susceptibility to pathogens are often difficult, amongst others due to interpretations of the operators (Lüdeker *et al*. 1996).

In recent studies, the potential of non-invasive techniques for the detection of plant diseases was demonstrated (Mahlein *et al*. 2012; Chaerle and Van Der Straeten 2000). In particular, chlorophyll fluorescence (ChlF) could be adopted as a reliable tool to estimate plant responses to different types of pathogens (Kuckenberg 2008; Scholes and Rolfe 1996). On wheat leaves, pathogen attacks raise the ChlF Red/Far-Red ratio, indicating photosynthetic impairments and a possible decrease of chlorophyll content (Bürling *et al*. 2011). Besides the ChlF, which is primary emitted by chlorophyll *a* molecules (Lichtenthaler *et al*. 1997), several phenolic substances and other fluorophores emit a characteristically blue (F440) or green (F530) fluorescence when excited with UV radiation (Lang *et al*. 1991; Cerovic *et al*. 1999). Bürling *et al*. (2011a, 2011b, 2012) have highlighted the potential of selected fluorescence ratios, such as the blue-to-green (F451/F522), the blue-to-red (F451/F687) and the blue-to-far-red ratio (F451/F736) for a pre-symptomatic or at least early detection of powdery mildew and leaf rust in susceptible and resistant wheat varieties. An

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alternative to these well-established fluorescence ratios is the determination of fluorescence lifetime (Cerovic *et al*. 1999). Modifications in the lifetime, as a result of pathogen infection, might result from the accumulation of defence-related secondary compounds leading to longer fluorescence decay (Bürling *et al*. 2011b, 2012). Substances like salicylic acid and phenylpropanoid compounds were previously identified as key ones in plant disease resistance (Lenk *et al*. 2007).

In the last decades significant advances were made in understanding the *in vivo* and *in situ* pigment fluorescence, and the relevance of the several influencing factors for the quality and reliability of the results. Despite the promising perspectives for applied research in plant sciences and practical use in agriculture (Cerovic *et al*. 1999), extensive agronomic and phytopathological studies aiming to explore the potential of different types of fluorosensing devices are still scarce.

The studies done by Bürling *et al*. (2011a, 2011b, 2012) served as a basis for our current work targeting the potential of the imaging-based spectrally resolved fluorescence and fluorescence-indices of a portable multiparametric device to assess the impact of powdery mildew (*Blumeria graminis* f. sp. *hordei*) and leaf rust (*Puccinia hordei*) on the fluorescence signature of four spring barley varieties. In this context we aimed a better understanding concerning the detection area and the excitation light for the assessment of plant-pathogen interactions. Moreover, we hypothesized that specific fluorescence indices would enable the characterization and differentiation genotype-specific responses to the diseases. With this background, we set the experiments under controlled conditions using four spring barley varieties with different susceptibility degrees to powdery mildew and leaf rust. Fluorescence lifetime, image-based spectrally resolved fluorescence intensity and several fluorescence-indices of a handheld sensor were recorded from a pre-symptomatic stage (3 days after inoculation, dai) until the stage where strong disease symptoms became visible (9 dai).

2 Experimental Section

2.1 Plant material and growth conditions

Experiments were conducted sequentially in environment-controlled growth cabinets simulating a 16 h photoperiod with 150 µmol·m^{-2}·s^{-1} photosynthetic active radiation (PAR; Philips PL-L 36W, Hamburg, Germany), a day/night temperature of 20/18 ± 2 °C and a relative humidity of 70/80 ± 5%. The spring barley (*Hordeum vulgare* L.) varieties Belana

and Marthe (Saaten Union GmbH, Isernhagen, Germany) and Conchita and Tocada (KWS Saat AG, Einbeck, Germany), differing in their susceptibility degree (SD) to powdery mildew and leaf rust (Table 1), were selected for the experiments. Untreated seeds were sown into 0.27 l plastic pots (0.08 m height, 0.07 m diameter), evenly filed with commercial peat substrate (Einheitserde Typ VM, Einheitserde- und Humuswerke Gebr. Patzer GmbH & Co.KG, Sinntal-Altengronau, Germany). Plants were regularly watered with tap water. One week after germination, seedlings were thinned out to maintain one plant per pot. Plants (n = 5 per genotype and treatment) were placed at random into the growth chambers. Twenty-one days after sowing the inoculation of the pathogens was performed at the second fully expanded leaf (BBCH stage 12).

Table 1. Susceptibility degree of the selected varieties to powdery mildew and leaf rust. Classification follows the descriptive variety list of the German Federal Plant Variety Office 2013 in a scale ranging from 1–9 (from less to more susceptible), 5 represents medium susceptibility.

Susceptibility Degree Against	**Belana (*Saaten Union*)**	**Marthe (*Saaten Union*)**	**Conchita (*KWS*)**	**Tocada (*KWS*)**
Powdery mildew	6	2	2	7
Leaf rust	4	5	4	6

2.2 Inoculation of *Puccinia hordei* and *Blumeria graminis* f. sp. *hordei*

The inoculation of *Puccinia hordei* was done according to the method described by Bürling *et al*. (2011b), with minor modifications. Briefly, *Puccinia hordei* spores (courtesy of the Department of Phytomedicine, University of Bonn, Bonn, Germany) were suspended in a solution of distilled water and Tween 20 (0.01%, Merck-Schuchardt, Hohenbrunn, Germany). After estimating the spore concentration with a Fuchs-Rosenthal counting chamber, the spore density was adjusted to 3.8×10^4 spores·mL^{-1}. Afterwards, leaves were fixed horizontally on a sample holder and twelve 6 µL droplets of spore suspension were evenly distributed on the adaxial side, starting at seven centimetres downwards from the leaf tip. Thereby, the inoculated area (approx. 4.5 cm^2) was labelled with a felt tip pen for the subsequent fluorescence determinations. Plants were kept for 24 h into a closed environment with a relative humidity ≥95% to provide optimum environment for spore germination and the

establishment of disease. Control plants were handled in similar way, but were treated with droplets of distilled water and Tween 20 (0.01%) only.

The inoculation of *Blumeria graminis* followed the method described in the literature (Bürling *et al*. 2012). Thereby, conidia of powdery mildew (Department of Phytomedicine, University of Bonn) were removed with a fine brush from infected plants and evenly distributed over the whole adaxial leaf surface of the experimental plants, particularly in a section of seven centimetres from the leaf tip. The inoculated area was marked by felt tip pen; twenty-four hours after inoculation visible conidia were removed by gently blowing over the leaf surface.

2.3 Fluorescence measurements

Fluorescence measurements were conducted at leaf level by using a compact fiber-optic spectrometer (IOM GmbH, Berlin, Germany), a multispectral fluorescence imaging system (Nuance TM^{FX}, Caliper Life Sciences, PerkinElmer, MA, USA) and a hand-held optical fluorescence technique (Multiplex3®, Force-A, Orsay, France). As important characteristic, the size of detection area was significantly different between the used methods ranging from approximately 1 mm^2 (laser fluoroscope used for lifetime recordings) to 40 cm^2 (portable equipment to record multiple fluorescence indices), as shown in the Supplemental Material (Figure S1). With exception of the fluorescence images which were taken under dark conditions, fluorescence recordings were done in the lab (average temperature 21 °C) under ambient light. Spectroscopic analysis of leaves inoculated with powdery mildew and leaf rust were done separately in two consecutive phases to avoid multiple stress caused by both pathogens.

2.3.1 Fluorescence lifetime

Settings and instrumental setups of the laser spectrometer were similar as described elsewhere (Bürling *et al*. 2012). In our experiments the fiber-optic spectrometer was used to record the fluorescence lifetime in in a range of 410 to 560 nm with an interval of 30 nm. For this purpose a pulsed nitrogen laser (MNL 100, LTB Lasertechnik Berlin GmbH, Berlin, Germany) with excitation at 337 nm and repetition rate of 30 Hz was used. The pulse energy at the probe exit was adjusted to be 2–3.5 µJ. A photomultiplier (PMT, H5783-01, Hamamatsu, Japan), with a sensitivity of 800 Volt, was used as detector. The detection gate

was opened from 0.0 to 16 ns following excitation, and the step width of the integrator gate was set to 0.4 ns. Each single data point was calculated by an average of 16 pulse counts. Before measuring, leaves were placed on a horizontal sample holder by keeping a constant distance (3.95 mm) between sample and the optical probe. Fluorescence decay was analyzed by using deconvolution software (DC4, V. 2.0.6.3, IOM GmbH, Berlin, Germany). Fluorescence lifetime readings were taken from control and pathogen inoculated leaves eight centimetres from the leaf tip. Particularly on leaf rust inoculated leaves, efforts were made to ensure that readings were always taken over the inoculated area.

2.3.2 Fluorescence imaging

Fluorescence images were recorded with a 1.4 megapixel CCD camera mounted onto a stereomicroscope (Zeiss Stereo Lumar V12, Jena, Germany). Three Zeiss Lumar filters (01, 09 and 14) enabled the fluorescence excitation in spectral ranges about 365 ± 12 nm (UV), 450–490 nm (blue) and 510–560 nm (green). Fluorescence data were acquired using a 0.8 X Zeiss Neo Lumar objective. A mercury short-arc lamp (HXP R 120W/45C UV, Osram, München, Germany) installed into a cold-light (LQ-HXP 120, Leistungszentrum Jena, Jena, Germany), was used as illumination source. To ensure clear images with a high data quality, images had to be recorded by using the highest light intensity, reaching 111,170 $\mu W \cdot cm^{-2}$ (UV filter), 128,882 $\mu W \cdot cm^{-2}$ (blue filter), and 30,346 $\mu W \cdot cm^{-2}$ (green filter) at leaf level. Leaves were fixed on a specially developed sample holder; here, a vacuum device produces a controllable negative pressure so that leaves lay flat on the surface. Settings were adjusted by 11x magnification and a focus of 51.4 mm to evaluate an object field of 110.25 mm^2. Fluorescence intensities were recorded under different excitation light sources in 10 nm steps for the following spectral ranges: 420–500 nm (blue), 500–580 nm (green) and from 620–720 (red) nm; signals were detected where at least 100 adjacent pixels had the same signature. Exposure time was automatically defined for each sample. Finally, the images were analyzed by using Nuance 2.4 imaging software. This software performs an automatic unmixing of fluorescence intensities and enables the determination of the corresponding fluorescing area. To increase the data quality, a spectral library was created for each excitation/emission range of control and pathogen inoculated varieties at 3, 6 and 9 dai.

2.3.3 Portable multiparametric fluorescence sensor

The hand-held fluorescence technique enables to record multiple fluorescence indices (Ben Ghozlen *et al*. 2010) to sense the response of plants to environmental factors under semi-controlled (Kautz *et al*. 2014) and field conditions (Leufen *et al*. 2014). Briefly, light-emitting-diodes excite the fluorescence with UV excitation (peak at 375 nm), green light (peak at 518 nm) and red light (peak at 630 nm) while the emitted fluorescence is detected in the blue (425–475 nm), red (680–690 nm) and far-red (720–755 nm) spectral region. Recordings were conducted at leaf level; here, the area of approximately 12.56 cm^2 was illuminated by maintaining a constant distance of 0.10 m between sensor and leaf surface. In a preliminary screening, the 'Simple Fluorescence Ratio' (SFR) and the 'Blue-to-Far-Red Fluorescence Ratio' (BFRR_UV) yielded the most promising results to sense both fungal diseases. The SFR is the inverse fluorescence ratio of the chlorophyll fluorescence ratio F680/F730 recorded with green excitation, whereas the BFRR depends on the blue and far-red fluorescence, recorded with UV excitation.

2.4 Statistical analysis

Data were statistically analyzed with SPSS statistic software (PASW statistics version 19.0, SPSS Inc., Chicago, IL, USA). For each genotype, pathogen and evaluation date, means of five control and five pathogen inoculated plants were compared by analysis of variance and paired t-test ($p \leq 0.05$).

3 Results

3.1 Fluorescence lifetime

Fluorescence lifetime in healthy and inoculated leaves at 410, 440, 470, 500, 530 and 560 nm displayed in Figures 1 and 2. The impact of powdery mildew (Fig. 1) and leaf rust (Fig. 2) is shown for each variety on the third and ninth day after inoculation. In the most cases, inoculation of the leaves led to higher mean fluorescence lifetime as compared to the control (healthy) leaves, but each situation (combination of variety, pathogen, dai, wavelength) has to be considered separately.

A detailed analysis indicate that 'Conchita' had the most pronounced differences between inoculated and control plants at 3 dai at the wavelengths 560 nm, followed by 500, 470 and 440 nm (Fig. 1).

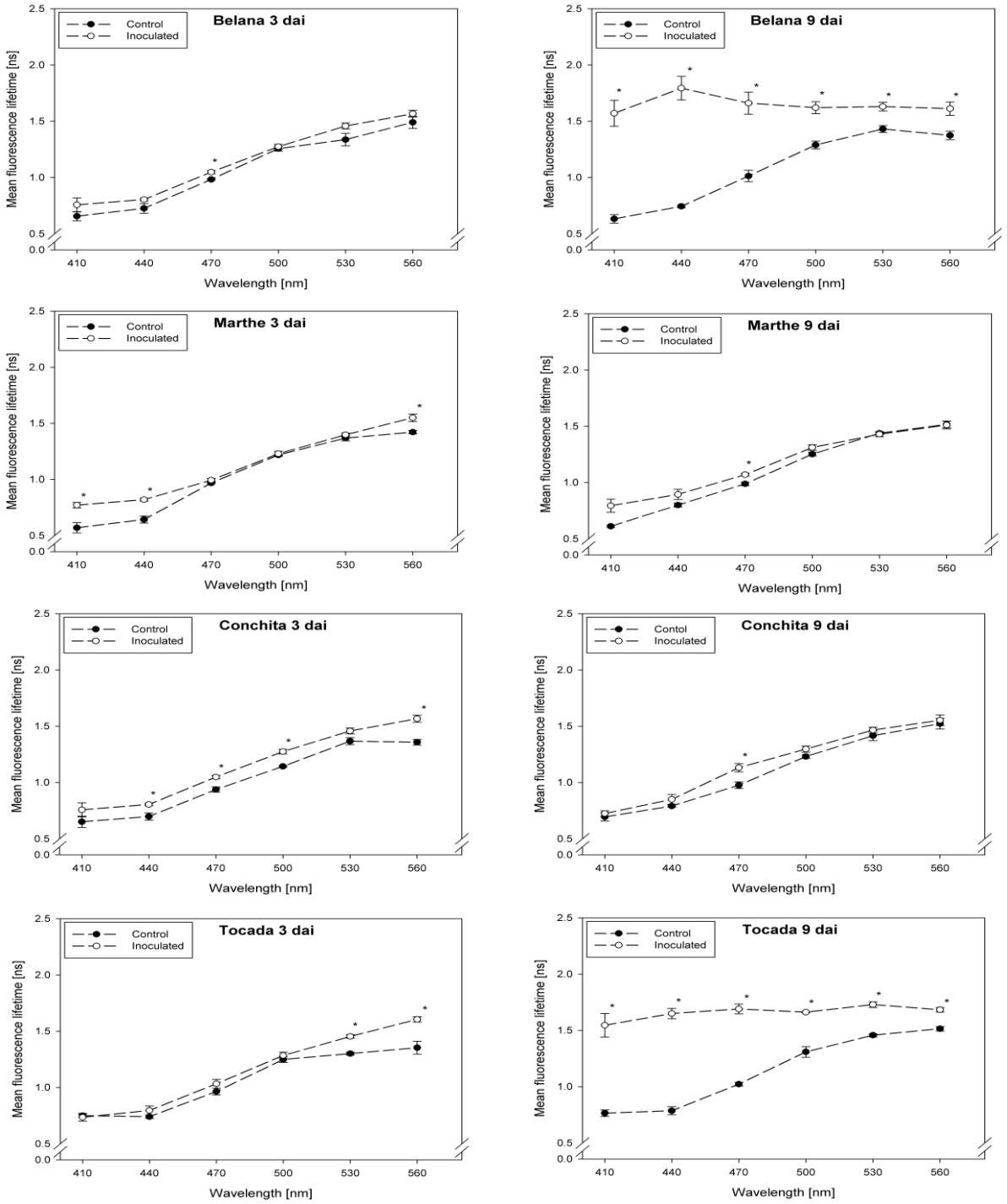


Fig. 1. Mean fluorescence lifetime at selected wavelength (410–560 nm) recorded from control and powdery mildewed leaves of the barley varieties Belana, Marthe, Conchita and Tocada (from top to bottom) at 3 and 9 days after inoculation (dai). Values indicate mean ± standard error (n = 5). Significant differences (t-test *, $p \leq 0.05$) between control and inoculated leaves for each variety, wavelength, and measuring day are shown.

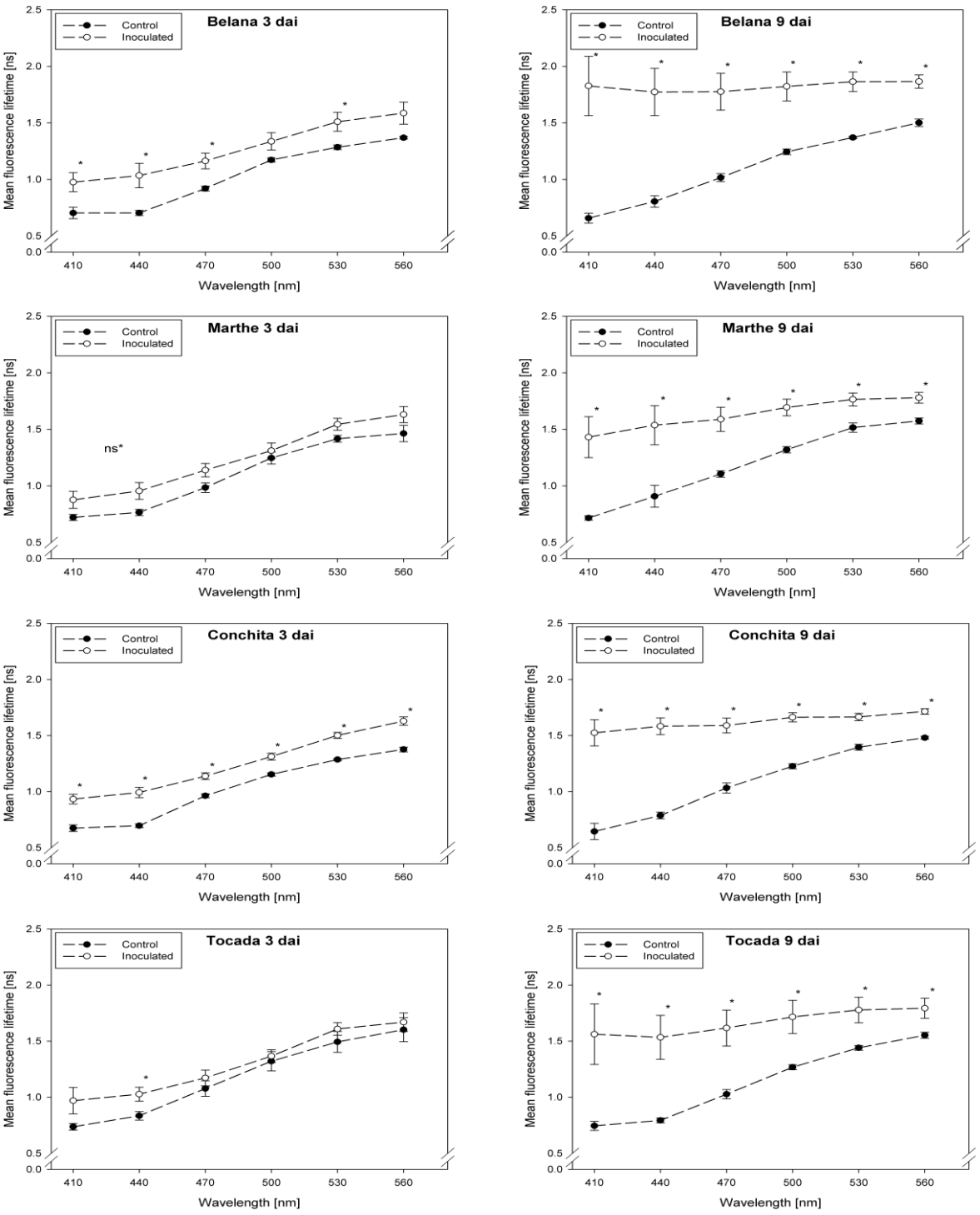


Fig. 2. Mean fluorescence lifetime at selected wavelength (410–560 nm) of control and leaf rust inoculated leaves of the barley varieties Belana, Marthe, Conchita and Tocada (from top to bottom) at 3 and 9 days after inoculation (dai). Values indicate mean ± standard error (n = 5). Significant differences (t-test *, $p \leq 0.05$) between control and inoculated leaves for each variety, wavelength, and measuring day are shown.

At the same time, significant differences between healthy and diseased leaves were ascertained for the varieties Belana (470 nm), Marthe (410, 440 and 560 nm) and Tocada (530 and 560 nm). Recordings at 6 dai *(data not shown*) and 9 dai indicate that mean fluorescence lifetime in 'Conchita' and 'Marthe' had a similar pattern as observed at 3 dai. In 'Tocada' significant differences were observed at 6 dai particularly in the spectral range from 440 to 530 nm (*data not shown*). At 9 dai, fluorescence lifetime in the range of 410–560 nm was significantly higher in the diseased tissues of both 'Belana' and 'Tocada', whereas 'Marthe' and 'Conchita' exhibited only slight alterations at 470 nm (Fig. 1).

In general, inoculation of plants with leaf rust raised the fluorescence mean lifetime in all varieties and wavelengths, even if not always statistically significant. Evaluations at 3 dai indicate that 'Marthe' and 'Tocada' were less affected while 'Belana' and 'Conchita' were more sensitive by leaf rust (Fig. 2). At 6 dai (*data not shown*) significant differences between both treatment groups, control and inoculated leaves, were determined in the four varieties nearly in all wavelengths. Thereby, the numerical difference between healthy and diseased leaves was more pronounced in the blue spectral range between 410 and 470 nm. This trend was confirmed at the end of the experiment (9 dai), when leaves infected with rust caused a strong increase of mean fluorescence lifetime in comparison to the control plants, but also to the previous evaluation dates.

3.2 Fluorescence images

3.2.1 Indications provided by selected spectral ranges

Divided into three spectral ranges (420–500 nm, 500–580 nm, 620–720 nm), we recorded the fluorescence intensity from 420 to 720 nm by using a spectral camera with different excitation sources. To enable a fast and precise overview of our major findings we summarize the outcomes in a simplified manner by indicating the significant differences between control and inoculated plants (Table 2). With exception of 'Belana' at 6 dai, powdery mildew inoculation led to significant differences between control and mildewed leaves in the UV excited blue fluorescence (420–500 nm) in all varieties (Table 2). This trend was also observed for the blue excited green fluorescence (500–580 nm), irrespective of the susceptibility degree. Differently, leaf rust led to more frequent and pronounced differences at 6 and 9 dai. In general, both diseases caused only minor changes in the ChlF (620–720 nm). However, green induced ChlF responded quite sensitively to pathogen infection

(Table 2). Particularly the mildewed leaves of ‘Marthe’ were characterized by a significant higher ChlF (*data not shown*).

Table 2. Temporal development of the blue (420–500 nm), green (500–580 nm) and ChlF (620–720 nm) for mildewed and rust infected spring barley leaves of the varieties Belana, Marthe, Conchita and Tocada. (x) indicates significant differences between control and pathogen inoculated leaves (*t*-test, $p \leq 0.05$).

Barley Variety	420–500 nm			500–580 nm						620–720 nm								
	UV Excited			UV Excited			Blue Excited			UV Excited			Blue Excited			Green Excited		
	3 dai	6 dai	9 dai	3 dai	6 dai	9 dai	3 dai	6 dai	9 dai	3 dai	6 dai	9 dai	3 dai	6 dai	9 dai	3 dai	6 dai	9 dai
Powdery mildew																		
Belana	x		x	x			x	x	x									x
Marthe	x	x	x	x		x	x	x	x			x		x	x	x	x	x
Conchita	x	x	x				x	x	x				x					
Tocada	x	x	x	x			x	x	x									x
Leaf rust																		
Belana					x			x	x	x						x		
Marthe	x		x			x			x								x	x
Conchita							x	x	x				x	x				
Tocada				x	x	x		x	x							x		

3.2.2 Green fluorescence intensity

Irrespective of variety and measuring day, the blue excited green fluorescence in powdery mildewed leaves was significantly higher as compared to the respective control plants (Fig. 3). Moreover, in the time-course from 3 to 9 dai, green fluorescence intensity in powdery mildewed leaves of ‘Belana’ and ‘Tocada’ decreased to a larger extent than in ‘Marthe’ and ‘Conchita’.

In contrast, leaves inoculated with leaf rust had distinctly lower green fluorescence intensity as compared to control leaves (Fig. 4). Thereby, ‘Conchita’ displayed strong differences between control and inoculated leaves from 3 to 9 dai. Differences of lower magnitude were observed for ‘Belana’ and ‘Tocada’ (6 and 9 dai) as well as ‘Marthe’ at 9 dai.

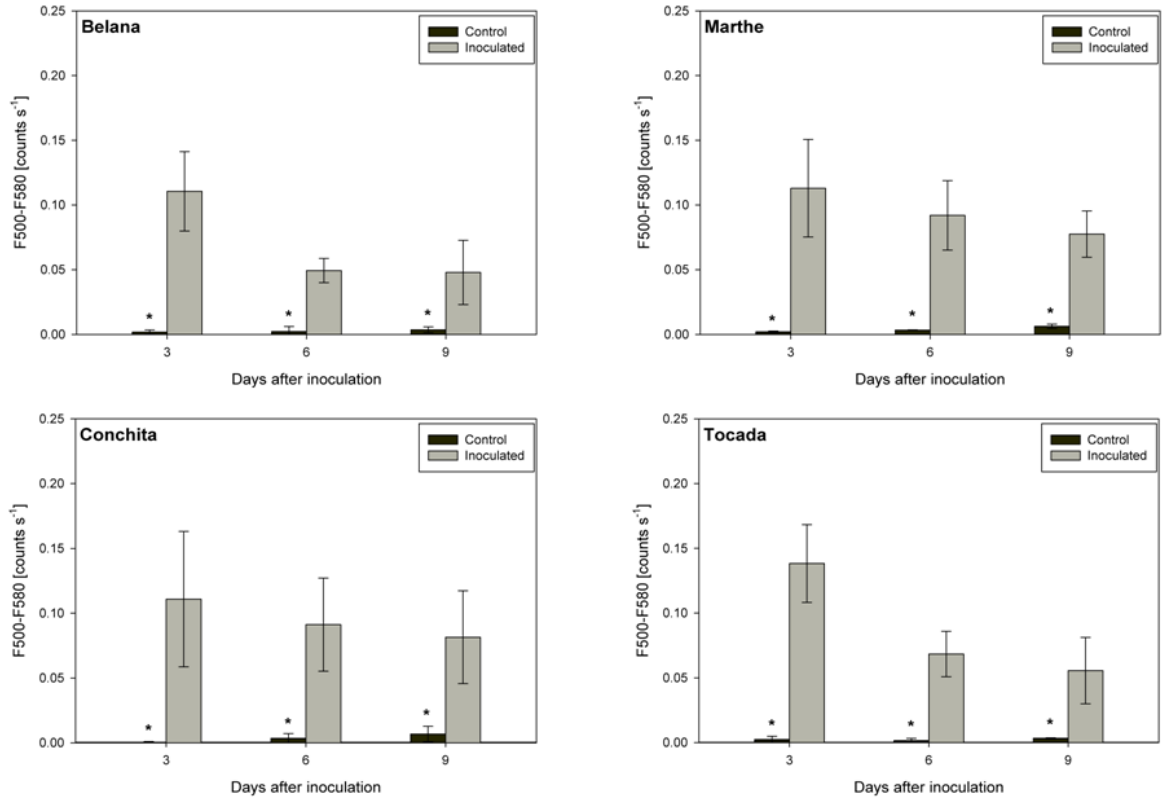


Fig. 3. Green fluorescence intensity (500–580 nm scaled as counts s^{-1}) recorded under blue excitation. Leaves of the healthy control and powdery mildewed plants of the barley varieties Belana, Marthe, Conchita and Tocada were studied at 3, 6, 9 days after inoculation. Values indicate mean ± standard error (n = 5). Asterisk (*) indicate significant differences (t-test, $p \leq 0.05$) between control and inoculated leaves for each variety and measuring day.

3.2.3 Green fluorescence intensity of infected leaf area

Due to strong effect of the pathogen inoculation on the blue excited green fluorescence (500–580 nm), we calculated the leaf area with similar emission properties in order to identify the area of the tissue affected by the pathogens. In the time-course from 3 to 9 dai, the area of green fluorescence intensity in powdery mildewed leaves of ‘Belana’ and ‘Tocada’ increased to a larger extent than in ‘Marthe’ and ‘Conchita’. As shown, we recorded significant differences between control and powdery mildewed leaves for all varieties and measuring days (Table 3). Similar results were obtained in the leaf rust study; however, here differences were only observed in ‘Marthe’ and ‘Conchita’ at 3 and 6 dai (Table 3).

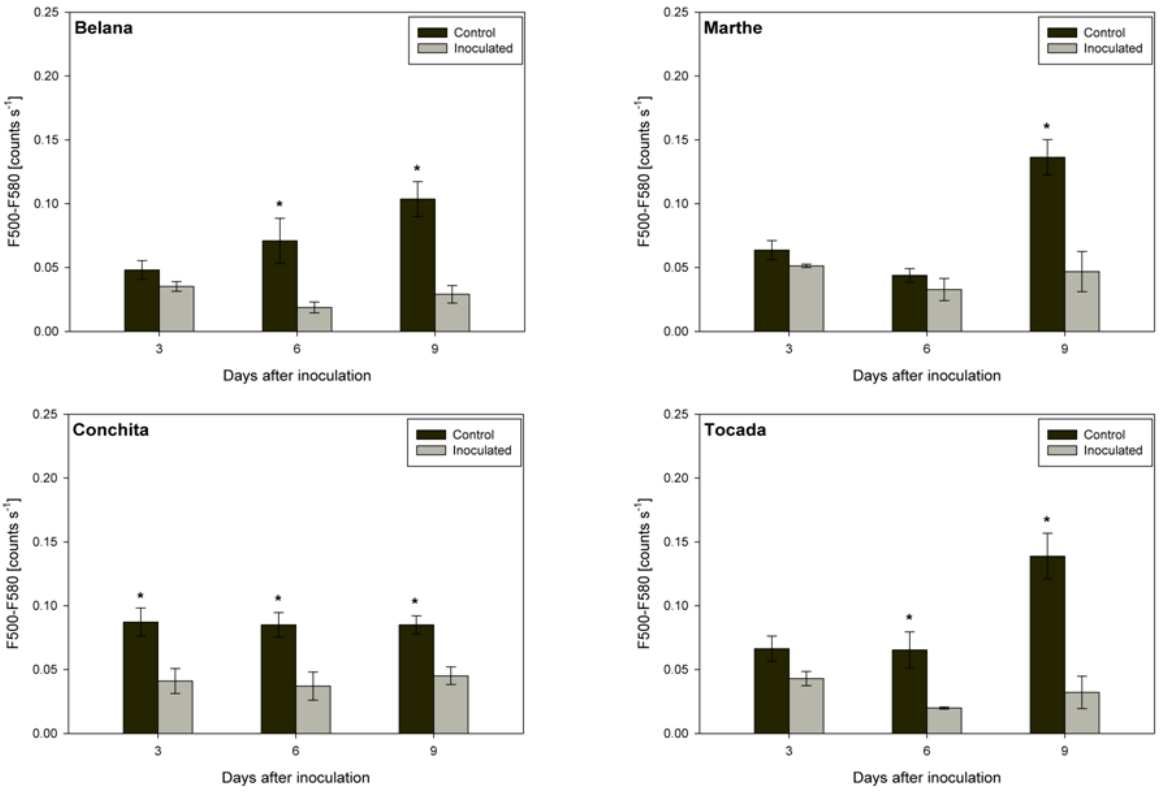


Fig. 4. Green fluorescence intensity (500–580 nm scaled as counts s^{-1}) recorded under blue excitation. Leaves of the healthy control and leaf rust inoculated plants, of the barley varieties Belana, Marthe, Conchita and Tocada were studied at 3, 6, 9 days after inoculation. Values indicate mean ± standard error (n = 5). Asterisks (*) indicate significant differences (t-test, $p \leq 0.05$) between control and inoculated leaves for each variety and measuring day.

Table 3. Area of green fluorescence intensity (mm^2) of control (C), powdery mildew or leaf rust inoculated (I) barley leaves of the varieties Belana, Marthe, Conchita and Tocada at 3, 6 and 9 days after inoculation (dai).

Barley variety	3 dai		6 dai		9 dai	
	C	I	C	I	C	I
Powdery Mildew						
Belana	0.05 ± 0.05 *	15.88 ± 5.58	0.28 ± 0.48 *	26.92 ± 6.03	0.85 ± 1.30 *	37.29 ± 4.78
Marthe	0.07 ± 0.02 *	18.65 ± 5.57	0.14 ± 0.02 *	19.14 ± 5.88	0.98 ± 0.62 *	19.51 ± 5.98
Conchita	0.01 ± 0.02 *	20.58 ± 5.93	0.30 ± 0.35 *	19.67 ± 5.97	0.44 ± 0.38 *	19.97 ± 6.00
Tocada	0.09 ± 0.10 *	17.08 ± 3.75	0.11 ± 0.09 *	27.27 ± 4.94	0.12 ± 0.00 *	37.72 ± 3.52
Leaf Rust						
Belana	1.95 ± 0.18 *	13.31 ± 1.09	2.99 ± 1.07 *	23.89 ± 1.88	21.55 ± 1.09 *	28.68 ± 0.80
Marthe	3.19 ± 0.77 *	12.60 ± 0.98	2.30 ± 0.50 *	16.70 ± 3.02	17.47 ± 2.74	20.95 ± 2.09
Conchita	3.76 ± 0.80 *	15.25 ± 2.26	6.23 ± 0.86 *	19.01 ± 2.53	23.15 ± 4.17	24.12 ± 1.75
Tocada	2.77 ± 1.39 *	12.27 ± 2.13	3.69 ± 1.59 *	19.15 ± 2.36	18.49 ± 2.04 *	26.22 ± 1.59

Significant differences (*t*-test *, $p \leq 0.05$) between control (C) and inoculated (I) leaves for each variety and measuring day are shown, values indicate mean ± standard error ($n = 5$).

3.3 'Blue-to-Far-Red Fluorescence Ratio' and 'Simple Fluorescence Ratio'

Powdery mildew led to significant alterations of the 'Blue-to-Far-Red Fluorescence Ratio' (BFRR_UV) and the 'Simple Fluorescence Ratio' (SFR_G) in the varieties Belana and Tocada (Table 4). Thereby, a considerable increase of BFRR_UV could be determined in the inoculated leaves of 'Belana' and 'Tocada' while no differences between control and inoculated leaves were observed for both SFR_G and BFRR_UV on 'Marthe' and 'Conchita'. On the other hand, leaf rust inoculation significantly changed the BFRR_UV in inoculated leaves of 'Belana' and 'Tocada' but not of 'Marthe' and 'Conchita' (Table 4). In addition, at 3 and 6 dai the SFR_G displayed significant differences between control and inoculated leaves in 'Belana' and 'Marthe', and for 'Tocada' at 6 and 9 dai. In contrast, no differences were found for 'Conchita'.

Table 4. 'Blue-to-Far-Red Fluorescence Ratio' (BFRR_UV) and 'Simple Fluorescence Ratio' (SFR_G). The fluorescence signals were recorded from control (C), powdery mildew and leaf rust inoculated (I) leaves of the barley varieties Belana, Marthe, Conchita and Tocada at 3, 6 and 9 days after inoculation (dai).

Fluorescence Ratio	Barley Variety	3 dai		6 dai		9 dai	
		C	I	C	I	C	I
Powdery Mildew							
BFRR_UV	Belana	1.19 ± 0.06 *	1.60 ± 0.08	1.13 ± 0.03 *	1.76 ± 0.10	1.12 ± 0.06 *	3.42 ± 0.30
	Marthe	1.26 ± 0.11	1.23 ± 0.02	1.33 ± 0.07	1.25 ± 0.02	1.36 ± 0.06	1.28 ± 0.04
	Conchita	1.18 ± 0.04	1.23 ± 0.05	1.24 ± 0.04	1.26 ± 0.05	1.27 ± 0.03	1.23 ± 0.05
	Tocada	1.36 ± 0.03	1.63 ± 0.17	1.35 ± 0.04 *	1.87 ± 0.19	1.32 ± 0.01 *	5.21 ± 0.61
SFR_G	Belana	4.27 ± 0.02 *	4.74 ± 0.15	4.74 ± 0.11 *	4.31 ± 0.08	4.91 ± 0.28 *	3.58 ± 0.16
	Marthe	4.35 ± 0.20	4.39 ± 0.04	4.42 ± 0.05	4.26 ± 0.08	4.50 ± 0.05	4.31 ± 0.16
	Conchita	4.14 ± 0.17	4.61 ± 0.16	4.59 ± 0.10	4.79 ± 0.15	4.88 ± 0.26	4.42 ± 0.15
	Tocada	4.51 ± 0.25	4.79 ± 0.17	4.54 ± 0.06 *	4.19 ± 0.04	4.39 ± 0.06 *	2.97 ± 0.08
Leaf Rust							
BFRR_UV	Belana	1.43 ± 0.02 *	1.59 ± 0.06	1.49 ± 0.05 *	1.79 ± 0.06	1.54 ± 0.02 *	2.09 ± 0.03
	Marthe	1.33 ± 0.03	1.35 ± 0.03	1.34 ± 0.03 *	1.49 ± 0.05	1.37 ± 0.05	1.47 ± 0.13
	Conchita	1.48 ± 0.06	1.49 ± 0.04	1.49 ± 0.04	1.64 ± 0.06	1.52 ± 0.09	1.91 ± 0.18
	Tocada	1.46 ± 0.04 *	1.60 ± 0.03	1.53 ± 0.04 *	1.75 ± 0.07	1.52 ± 0.02 *	2.03 ± 0.10
SFR_G	Belana	4.56 ± 0.07 *	4.23 ± 0.04	4.89 ± 0.15 *	4.19 ± 0.13	4.61 ± 0.14 *	3.73 ± 0.18
	Marthe	4.30 ± 0.07 *	3.88 ± 0.12	4.61 ± 0.10 *	4.10 ± 0.16	4.31 ± 0.06 *	3.57 ± 0.17
	Conchita	4.29 ± 0.12	4.11 ± 0.08	4.62 ± 0.13	4.37 ± 0.16	4.38 ± 0.13	3.90 ± 0.19
	Tocada	4.27 ± 0.07	4.32 ± 0.15	4.79 ± 0.08 *	4.18 ± 0.19	4.36 ± 0.13 *	3.67 ± 0.12

Significant differences (*t*-test *, $p \leq 0.05$) between control (C) and inoculated (I) leaves for each variety and measuring day are shown, values indicate mean ± standard error ($n = 5$).

4 Discussion

Our results demonstrate the potential of three techniques - the fluorescence lifetime, the image-resolved multispectral fluorescence and selected indices of a portable multiparametric fluorescence sensor-for the proximal sensing of plant-pathogen interactions in spring barley. Irrespective of the remarkable technical differences between the sensors, in particular with respect to the analysed area and the spectral characteristics for excitation and detection, the fluorescence devices used here enabled to sense the impact of powdery mildew and leaf rust, and indicated some genotype-specific responses to these pathogens. The selected fluorescence signals and indices reflect changes in the amount and chemical composition of different compounds and substance groups including chlorophyll *a* and *b*, plant polyphenols, and pathogen-originated fluorophores.

In our studies we adopted a commercially available fluorescence imaging system originally developed to screen the efficacy of medicinal products in the pharmaceutical industry (Fig. S1C). Similar to the findings of Rousseau *et al*. (2013) and Pineda *et al*. (2008), our studies showed temporal and spatial changes in the fluorescence of control and pathogen inoculated leaves. Thereby, *Blumeria graminis* f. sp. *hordei* and *Puccinia hordei* caused only minor alterations in the ChlF as compared to the BGF (Table 2); this can be attributed to the biotrophic relationship of the pathogens with their host (Mendgen and Hahn 2002). As shown, both foliar diseases led to variations in the UV-excited blue (420–500 nm) as well as in the UV and blue excited green fluorescence (500–580 nm). With this technique, which is designed for operation in the laboratory under dark conditions, it is possible to differentiate the impact of both leaf diseases (Table 2). In particular, powdery mildew significantly influenced the UV-excited blue fluorescence, irrespective of the susceptibility degree of the genotypes (Table 2). While control leaves displayed a characteristic blue fluorescence which mainly originates from trichomes and/or leaf veins (Meyer *et al*. 2003), the higher values recorded on mildewed leaves arise from the blue fluorescing inoculum, e.g., conidiophores (Fig. S2). Residues of the inoculum as well as newly formed fungal structures overlap and partially shield the plants' natural fluorescence. In this context our findings confirm previous observations (Poutaraud *et al*. 2007) suggesting that the development cycle of other obligate biotrophs is accompanied by a characteristically blue autofluorescence.

As reported by Lüdeker *et al*. (1996), fungal infection led to a stronger increase in the green (F520) than in the blue fluorescence (F440), that can be either caused by the fungi or due to accumulation of intercellular substances. According to our results, the inoculum of *Blumeria graminis* f.sp. *hordei* exhibits a typical blue-green fluorescence under UV excitation, whereas spores of *Puccinia hordei* produce a rather green-orange spectral range. On barley leaves, blue excitation caused a significantly rise of the fluorescence intensities, and on powdery mildew spores to a stronger shift towards the green-orange spectral range (Fig. S2). Spore specific fluorescence patterns might explain why the blue excited green fluorescence was the most useful parameter to identify the temporal and spatial development of both diseases (Table 2).

Differences concerning the inoculation method might explain the strong variations in the green fluorescence intensity when comparing both pathogens. Powdery mildew spores were inoculated across the leaf surface, whereas rust spores were spot inoculated by placing droplets of a spore suspension. After inoculation, green fluorescence intensity measured on the powdery mildew resistant varieties Marthe and Conchita dropped slower as compared to

the susceptible varieties Belana and Tocada. Two processes might have contributed for these results: firstly, the mycelium at the surface of susceptible varieties changed the optical properties in the time course of our study; secondly, the reduced ability of susceptible varieties to overcome the pathogen attack. Increasing area of green fluorescence intensity in the time course of our study supports our first assumption (Table 3). Moreover, minor changes in the green fluorescence intensity of the resistant varieties suggest the accumulation of pathogen or resistance specific compounds, such as lignin, and/or the production of waxes, affecting the fluorescence emission (Chaerle *et al*. 2004; Swarbrick *et al*. 2006; Jenks *et al*. 1994; Hooker *et al*. 2002; Liakopoulos *et al*. 2001). To this point our findings confirm and support previous studies which focussed on the process of fungal influencing the autofluorescence of leaves (Liu *et al*. 2012), even if we did not record the fluorescence of single cells. A completely different trend was shown in rust inoculated leaves which exhibited a significantly lower green fluorescence as their respective control leaves. Control leaves might undergo a significantly faster aging resulting in a higher blue-green fluorescence (Chappelle *et al*. 1984), whereas rust infection can cause a delay of normal senescence, due to an increase in the concentration of cytokinins (Scholes and Farrar 1987).

Differently than the time-consuming spectroscopic technique used by Bürling *et al*. (2011b, 2012), we assessed the most promising fluorescence ratios (SFR_G and BFRR_UV) with a significantly faster operating hand-held fluorometer. Leaves inoculated with *Blumeria graminis* f. sp. *hordei* demonstrate a first increase after pathogen inoculation (3 dai), which was followed by a pronounced decrease (6–9 dai) of the ChlF-ratio F730/F685, here referred as SFR_G (Table 4). These changes were more pronounced in the susceptible varieties Belana and Tocada, as compared to the resistant varieties Conchita and Marthe. Similar results are reported in the literature (Kuckenberg 2008; Bellow *et al*. 2013). Modifications of the SFR_G were caused by a first disruption of the photosynthetic quantum conversion and consequently in a later decrease of the chlorophyll content (Lichtenthaler and Rinderle 1988). Leaf rust causing punctual diseased spots significantly influenced the chlorophyll fluorescence (displayed as SFR_G) in all four varieties (Table 4). Similar results were reported by Scholes and Rolfe (1996) and Bürling *et al*. (2010), who showed that the photosynthesis of regions which were not invaded by the fungal mycelium was severely impaired. The analogous trend observed for all varieties is explained amongst others by their comparable susceptibility degree in the range from 4 to 6 (Table 1). In this context, either the higher susceptibility degrees of the four barley varieties to powdery mildew or different patterns in the infection and development cycles of mildew and rust, might explain the immediate decline of SFR_G

values in rust infected leaves at 3 dai. While *Blumeria graminis* f. sp. *hordei* exclusively affects the epidermal cell layer (von Röpenack *et al*. 1998), *Puccinia hordei* infects also mesophyll cells and modifies the chloroplasts (Bolton *et al*. 2008). Contrasting the limited potential of the ChlF recorded with the imaging system, the extensive results recorded with the hand-held sensor can be explained by its wider spectral range which covers completely both chlorophyll emission ranges. Both fungal diseases were also indicated by BFRR_UV index. In case of powdery mildewed leaves, BFRR_UV followed the same trend as described for SFR_G, indicating that these changes were mainly caused by chlorophyll degradation (Buschmann 2007; Gitelson *et al*. 1998). Comparable results were ascertained by Lüdeker *et al*. (1996) on rust infected wheat leaves. Finally, both foliar diseases led to a considerable increase in the mean fluorescence lifetime. Due to their comparable susceptibility degree to leaf rust, the results observed for the four varieties followed the same trend. Nevertheless, at 3 dai 'Belana' and 'Conchita' (less susceptible to rust) exhibit a significant higher mean lifetime from 410–560 nm than 'Marthe' and 'Tocada' (higher susceptibility). In case of powdery mildew, we confirmed previous observations of Bürling *et al*. (2012). Thereby, we indicate higher mean lifetime starting from 500 nm (3 dai) and a later (6–9 dai) rise in the spectral region of 410–500 nm; these results correlate with the appearance of disease symptoms and the strong blue fluorescence intensity of *Blumeria graminis* spores (Fig. S2).

Compared to previous studies on the impact of pathogens on the laser-induced spectrally resolved fluorescence (Bürling *et al*. 2011a, 2011b, 2012), our findings show two significant improvements. Firstly, we did not work only with UV-excitation to assess BGF and ChlF, but we highlight the benefits of multiple fluorescence excitation as essential tool basic and applied studies (Table 2). Secondly, we adopted and tested techniques which are appropriate for different situations, starting with the highly time-resolved fluorescence spectroscopy (punctual measurement with micrometric scale), the spectrally-resolved imaging system (measurements on square centimetre level), and a robust equipment suitable for field evaluations (Fig. S1). Besides the wide range concerning the detection area, we show that all tested methods enable the detection of spectral modifications caused by leaf diseases. In addition to the system-specific excitation and the spectral detection ranges, there are other pertinent differences which influence the efficiency of these methods for larger scientific studies or practical applications. Up to the hand-held fluorescence device, the other techniques can only be run in the laboratory, sometimes requiring extensive preparation time before and after the fluorescence readings. Changes in the amount and or composition of fluorophores might be better recorded with the laser-induced fluorescence spectrometer.

Visualization of dynamic plant-pathogen interactions, as shown with our high-resolution fluorescence camera, is also not possible with the field-suitable technique. At this point we see the main benefit of imaging methods since foliar diseases differ in their development cycle and appearance (Stubbs *et al*. 1986), also influencing the spectral signature (Mahlein *et al*. 2012). To the best of our knowledge, this is the first study where fluorescence images were automatically analysed based on a previously created spectral library. Here, we recognise promising perspectives if a broad spectral library comprising several pathogens, plants, and development stages can be set. If successful, the range of possible assignments include pre-breeding programs and physiological studies in different pathogen-host systems.

5 Conclusions

The fluorescence techniques adopted in our studies enabled the detection of pathogen infection and disease development in barley. Susceptible and resistant varieties inoculated with pathogens showed distinct modifications in mean lifetime from 410 to 470 nm, as well in the indices SFR_G and the BFRR_UV. Following the modification of these parameters in the time-course of the experiment it was possible to differentiate the varieties according to their susceptibility degree. The used multispectral fluorescence imaging system provides basic information to distinguish between both diseases, since powdery mildewed leaves significantly exhibit a higher blue and green fluorescence intensity as leaf rust diseased leaves. Finally, we highlight the importance of different excitation and emission ranges for sensing and differentiation of diseases as well as the screening for tolerant and susceptible genotypes. The UV-exited blue fluorescence and the blue-excited green fluorescence offer the most promising information for further studies on these topics.

The following pages of this chapter display the supplementary figure S1 and S2.

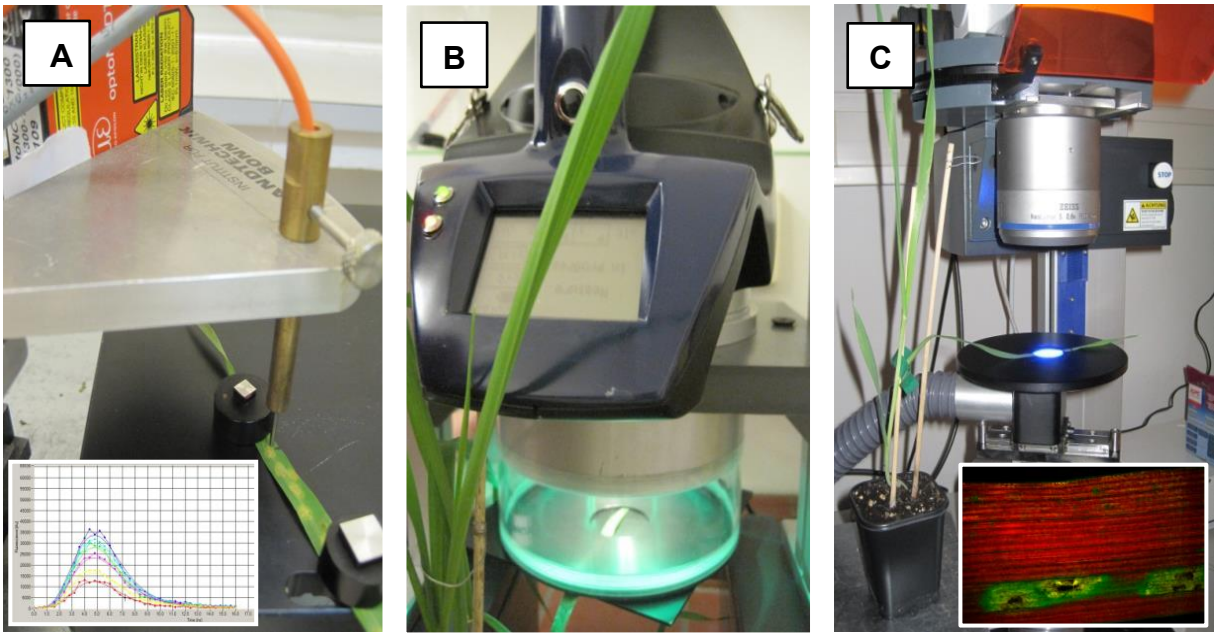


Fig. S1. Fluorescence devices used in this experiment: A) excitation and emission fiber of the time resolved fluorescence spectrometer Lambda 401. Insert at the bottom displays typical fluorescence lifetime curves; B) multiparametric fluorescence spectrometer, Multiplex®; C) lens of the stereomicroscope on which the multispectral fluorescence imaging system Nuance® is mounted. Insert shows a false colour image of foliar diseases on a barley leaf.

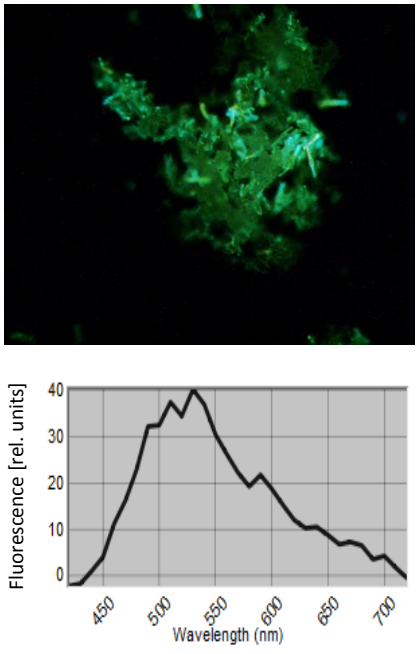
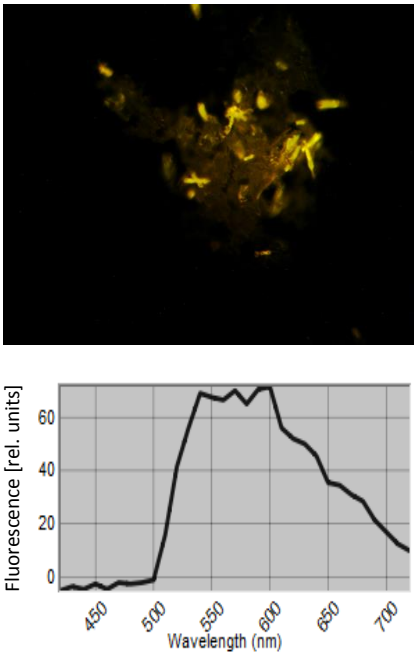
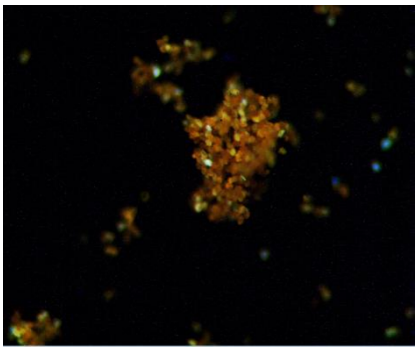
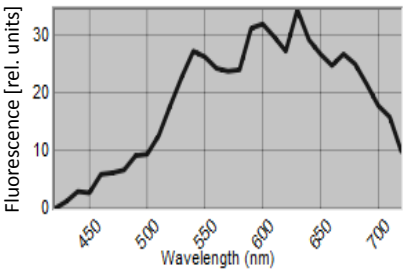
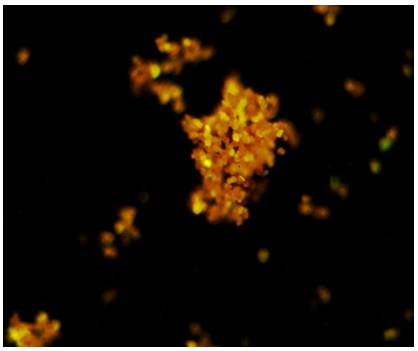
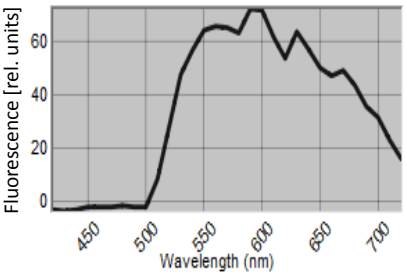
Pathogen	UV excitation	Blue excitation
Blumeria graminis f.sp. *hordei*		
Puccinia hordei	 	 

Fig. S2. Autofluorescence of *Blumeria graminis* f. sp. *hordei* and *Puccinia hordei* spores and the corresponding spectra recorded from 420-720 nm. Fluorescence was excited with UV and blue light and recorded with a multispectral fluorescence camera mounted on a stereomicroscope equipped with a Zeiss ApoLumar S objective (focus of 17.1 and magnification of 120x). Images were taken from the spores only after powdering them on a black plate as background.

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F Summary and conclusions

The aim of the present thesis was to assess the potential of non-destructive fluorescence techniques for the detection and differentiation of abiotic and biotic stress situations. For this purpose, sugar beet cultivars were temporarily exposed to water shortage and low nitrogen supply as well as inoculated with powdery mildew (*Erysiphe betae*). With focus on water deficit stress, we investigated the impact of recurrent drought in the scope of greenhouse and field experiments. Moreover, we studied for the first time if recurrent drought caused a 'drought memory' leading to enhanced physiological performance in previously stressed plants. Finally, spring barley cultivars of different susceptibly to powdery mildew (*Blumeria graminis* f. sp. *hordei*) and leaf rust (*Puccinia hordei*) were used to exploit pathogen-plant interaction and the consequences for the leaf fluorescence signature. Here, the major aim was to analyse genotype-specific responses using spectrally-resolved and imaging-based fluorescence techniques. Experiments were structured and arranged as individual chapters, and the major outcome can be summarized as follows:

1. Multiparametric fluorescence recording was a valuable tool to sense abiotic and biotic stress symptoms in sugar beet plants. However, a robust differentiation of individual stresses by just one specific fluorescence index was not possible. The most relevant fluorescence indices to detect water deficit and or powdery mildew infection were the 'Simple Fluorescence Ratio' (SFR_G) and the 'Nitrogen Balance Index' (NBI_G), particularly due to their strong relationship to the chlorophyll concentration. In general, water deficit was the stress factor with the most pronounced impact on plant physiology.

2. Fluorescence indices based on the far-red chlorophyll fluorescence were reliable indicators to sense temporary water deficiency stress in sugar beet, regardless of whether the genotypes were cultivated in the field or under greenhouse conditions. This was appropriately demonstrated by the 'Blue-to-Far-Red' (BFRR_UV) and the SFR_G. For the latter, we demonstrated that green excitation proved to be the best suitable light source for chlorophyll fluorescence recordings, as it better reveals the responses of the stressed sugar beet plants. Besides, all evaluated cultivars showed distinct responses concerning the extent of the changes during the stress and re-watering i.e. recovery

phases. These findings were confirmed by gas exchange and destructive reference measurements.

3. Temporal water withholding followed by re-watering caused changes in the fluorescence lifetime (410 to 560 nm), red fluorescence intensity (FR_G) and the SFR_G. In general, the observed alterations were similar in the three consecutive drought-recovery phases. Nevertheless, the fluorescence parameters do not indicate any hints towards improved physiological response following preliminary stresses. With this, the ‘memory effect’ could not be confirmed. Destructive reference-analysis of the osmotic potential, proline and total chlorophyll concentration have shown a different picture, as changes in all metabolic indices were lower during the second experimental phase.

4. Spectrally-resolved and image-based fluorescence techniques enabled the detection of pathogen infection i.e. disease development in spring barley. Thereby, susceptible and resistant varieties showed distinct modifications in mean lifetime from 410 to 560 nm, as well in the SFR_G and the BFRR_UV. Based on the modification of these parameters in the time-course of the experiment it was possible to characterize the varieties according to their susceptibility degree. The multispectral fluorescence imaging system provides basic information to distinguish between both diseases, since powdery mildewed leaves exhibit a significantly higher blue and green fluorescence intensity than leaf rust diseased leaves. We further highlight the importance of different excitation and emission ranges for sensing and differentiation of leaf-diseases, as the UV-exited blue fluorescence and the blue-excited green fluorescence offer the most promising information for further studies on these topics.

In summary, the results obtained in our studies demonstrate the huge potential of spectral and imaging-based fluorescence techniques for plant physiology, environmental research, plant breeding, and precision farming. Among the multitude of detected and calculated fluorescence indices, we were able to select specific parameters to evaluate sugar beet varieties according to their drought resistance level, as well as spring barley varieties to their susceptibility degree against leaf rust and powdery mildew. Our studies highlight the importance of the proper selection of the spectral range and excitation wavelength. In particular, we have shown that green-excited ChlF as well as blue-excited green fluorescence

are the most suitable parameters to detect drought stress in sugar beet, or to differentiate pathogen infections in spring barley, respectively. With this knowledge, extensive and in depth studies might be conducted using automated high-throughput systems to screen a big number of genotypes in the field or under controlled conditions. Nevertheless, the differentiation of interacting multiple stresses needs to be further investigated, as this is currently the biggest challenge in precision farming.

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