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Potential and limitations for the practical use of fluorescence sensors to detect physiological adaptations of crops

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Potential and limitations for the practical use of fluorescence sensors to detect physiological adaptations of crops

The key objective of the present work was to evaluate the potential and limitations for the practical use of fluorescence sensors to detect physiological responses of crops to abiotic stresses, and the crop adaptations to these constrain factors. For this purpose, we investigated apple plants (*Malus domestica* Borkh.) grown under water deficit or different lighting systems, and barley cultivars (*Hordeum vulgare* L.) cultivated in the field and fertilised with different nitrogen levels. The outcome of the three single chapters is as follows:

1. In the first study, we investigated the influence of water shortage and light quality provide either by light emitting diodes (LED) or compact fluorescence lamps (CFL) on physiological and biochemical parameters of apple seedlings. Stress responses were assessed by fluorescence indices to non-destructively evaluate the suitability and practicability of portable sensors. Nitrogen Balance Index (NBI) and Nitrogen Balance Index with Red light excitation (NBI_R) showed similar patterns as chlorophyll (Chl) content, with higher values for plants under CFL. Flavonol Indices (Flav_Dx and Flav_Mx) were higher on plants cultivated under LEDs. Stomatal conductance (Gs) and maximal photochemical efficiency (Fv/Fm) were lower on plants grown under LED. Particular attention should be given to fluorescence indices related to nitrogen status and flavanol content as promising parameters to sense physiological impairments under the given conditions.
2. The aim of the second study was to investigate whether two-years-old apple plants, cultivars ‘Pinova 10’ and ‘Gala Galaxy’, show different physiological reactions when grown under water deficit. Plants were grown under two watering regimes with 100% and 50% of the substrate field capacity (W100 and W50) and a subsequent water deficit phase with no water supply (WD). ‘Gala Galaxy’ showed higher tolerance to water deprivation than ‘Pinova 10’, indicated especially by increased chlorophyll fluorescence indices on single measurement days throughout the experimental course. Fluorescence-based indices, related to chlorophyll content and nitrogen balance, are useful parameters to estimate physiological status of young apple trees cultivated under water restriction regimes.
3. In the third study, fluorescence-based sensors were employed to portray plant physiological conditions and estimate the yield performance of four cultivars of summer barley (‘Beatrix’, ‘Eunova’, ‘Sebastiana’, and ‘Victoriana’) in response to three levels of nitrogen fertilisation (0, 40, and 80 kg/ha). Highest chlorophyll content and grain yield were observed in all cultivars when 80 kg/ha N was applied. Grain yield strongly correlated with leaf chlorophyll concentration. Further studies should concentrate on more cultivars and should also consider further fluorescence indices and approaches to estimate plant physiological status in a non-destructive way during growing and pre-generative periods of barley.

Das Potential und die Einschränkungen der praktischen Anwendung von Fluoreszenzsensoren zur Detektion physiologischer Anpassungen bei Nutzpflanzen

Das Hauptziel dieser Studie bestand darin, das Potenzial und die Einschränkungen der praktischen Anwendung von Fluoreszenzsensoren zur Erkennung physiologischer Reaktionen von Nutzpflanzen auf abiotischen Stress sowie deren Anpassung unter suboptimalen Wachstumsbedingungen zu untersuchen. Zu diesem Zweck wurden Apfelpflanzen (*Malus domestica* Borkh.), die unter Wassermangel und unterschiedlichen Beleuchtungssystemen kultiviert wurden, sowie Gerstensorten (*Hordeum vulgare* L.) gedüngt mit unterschiedlichen Stickstoffgaben, betrachtet. Die Ergebnisse der drei Einzelkapitel lauten wie folgt:

1. In der ersten Studie wurde der Einfluss von Wassermangel in Kombination mit Lichtqualität durch Leuchtdioden (LED) und Kompaktfluoreszenzlampen (CFL) auf den physiologischen und biochemischen Zustand von Apfelsämlingen untersucht. Die Stressreaktionen wurden anhand von Fluoreszenzindizes bewertet. Der Stickstoffbilanzindex (NBI) und der Stickstoffbilanzindex mit Rotlichtanregung (NBI_R) zeigten in Bezug auf die Lichtquelle ein ähnliches Muster wie die Blatt-Chl-Ergebnisse, mit einer höheren Effizienz der CFL. Flavonol (Flav_Dx und Flav_Mx) erbrachte höhere Indexwerte bei Pflanzen, die unter LED kultiviert wurden. Der stomatäre Leitwert (Gs) und die maximale photochemische Effizienz (Fv/Fm) waren bei LED beleuchteten Pflanzen niedriger als bei Pflanzen unter CFL. Besondere Aufmerksamkeit sollte den Fluoreszenzindizes in Verbindung mit dem Stickstoffstatus und dem Flavonolgehalt als vielversprechende Parameter zur Erfassung physiologischer Beeinträchtigungen unter den gegebenen Bedingungen gewidmet werden.
2. Ziel der zweiten Studie war die Untersuchung der physiologischen Entwicklung zweijähriger Apfelbäume der Sorten 'Pinova 10' und 'Gala Galaxy' in Reaktion auf Wassermangel (zwei Bewässerungsmodule mit 100% und 50% Feldkapazität (W100 und W50) und einer anschließenden Wasserdefizitphase mit hundertprozentiger Bewässerungseinstellung (WD)). ‚Gala Galaxy' zeigte eine höhere Toleranz gegenüber Wassermangelzuständen als "Pinova 10", was sich insbesondere durch erhöhte Chlorophyllfluoreszenzindizes an bestimmten Messtagen während des gesamten Versuchsverlaufs zeigte. Die Fluoreszenzindizes in Bezug auf den Chlorophyll-Gehalt (Chl_Index und SFR_R) und die Stickstoffbilanz (NBI und NBI_R) zeigten ähnliche Kurven. Fluoreszenz basierende Indizes, die sich auf den Chlorophyllgehalt und die Stickstoffbilanz beziehen, bewiesen sich als geeignetes nicht-destruktives Mittel zur Erfassung des physiologischen Zustands junger Apfelbäume, kultiviert unter wasserdefizitären Bedingungen.
3. In der dritten Studie wurden fluoreszenzbasierte Sensoren zwecks Erfassung pflanzenphysiologischer Reaktionen und Ertragsleistungsbewertung vier verschiedener Sommergerstensorten ('Beatrix', 'Eunova', 'Sebastiana' und 'Victoria') in Reaktion auf drei Stufen der Stickstoffdüngung (0, 40 und 80 kg/ha) eingesetzt. Der höchste Chlorophyllgehalt und Kornertrag wurden bei allen Sorten bei einer Ausbringung von 80 kg/ha N beobachtet. Der Kornertrag korrelierte stark mit der Chlorophyllkonzentration im Blatt. Folgestudien sollten weitere Sorten, sowie zusätzliche Fluoreszenzindizes und Methoden um die nicht-destruktive Erfassung des physiologischen Zustands von Gersten in der Wachstum- und vorgenerativen Phase in Betracht ziehen.

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List of abbreviations

ΔΨ – Water potential gradient

ATP – Adenosine triphosphate

BF – Blue fluorescence

BFRR_UV – Blue-to-far-red fluorescence with UV excitation

CFL – Compact fluorescence lamps

Chl - Chlorophyll

ChlF – Chlorophyll fluorescence

Fo – Minimal chlorophyll fluorescence intensity measured in the dark-adapted state, when all PSII reaction centres are open

Fm – Maximal chlorophyll fluorescence intensity measured in the dark-adapted state during the application of a saturating pulse of light

Fm' – Maximal chlorophyll fluorescence intensity measured in the light-adapted state during the application of a saturating pulse of light

Fv/Fm - Maximum quantum yield of PSII photochemistry measured in the dark-adapted state

Flav – Flavonol index

FRF – Far-red fluorescence

FRF_G – Far-red fluorescence with green excitation

FRF_R – Far-red fluorescence with red excitation

FRF_UV – Far-red fluorescence with UV excitation

GF – Green fluorescence

LED – Light-emitting diodes

LHC II – Light-harvesting complex

LICF – Laser-induced chlorophyll fluorescence

N – Nitrogen

NADPH – Nicotinamide adenine dinucleotide phosphate (reduced form)

NBI_R – Nitrogen balance index with red excitation

NBI_G – Nitrogen balance index with green excitation

NH_4^+ - Ammonium

NO_3^- - Nitrate

PAM – Pulse-amplitude modulation

PAR – Photosynthetic active radiation

PSI – Photosystem I (Plastocyanin-ferredoxin oxidoreductase)

PSII – Photosystem II (Water-plastoquinone oxidoreductase)

RF – Red fluorescence

RF-R – Red fluorescence with red excitation

SFR_G – Simple fluorescence ratio with green excitation

SFR_R – Simple fluorescence ratio with red excitation

UV – Ultraviolet

A Introduction

1 Abiotic stress factors

Crops are constantly exposed to different abiotic and biotic stress factors during their cultivation, which can impair not only general growth and physiology, but also ultimately lead to yield loss. Biotic stress is defined as a stress caused to plants due to damage by other living organisms, including fungi, bacteria, viruses, parasites, weeds, insects, and other native or cultivated plants (Dorantes-Acosta et al., 2012). In contrast, abiotic stress is not caused by living organisms, rather than, for instance, by water deficiency, salinity, heat, coldness or light conditions. Amongst others, these might cause series of morphological, physiological, biochemical and molecular changes that unfavourably affect plant growth, development and productivity (Msanne, 2011). After all, abiotic stress is one of the main factors of crop yield deprivation worldwide, reducing normal yields of major food crops by more than 50 percent, raising thereby enormous economic losses in several countries (Shanker and Venkateswarlu, 2011).

Plant growth and development is controlled by a variety of physiological, biochemical, and molecular processes. To that effect, photosynthesis is a key mechanism, providing them, to a large extent, energy and essential organic molecules (Ashraf and Harris, 2013). Due to the incidence of different stressors, in the best-case scenario, plants might alter the photosynthetic rate to adapt themselves to the new imposed stressful condition. Therefore, plants have evolved several adaptive strategies to cope with potentially harmful environmental influences (Chaves et al., 2002). However, the development of defence strategies might not be short-termed reliable to overcome the constraint factors. Thus, one important step to circumvent them can be reflected by the necessity to accurately assess photosynthetic efficiency to better screen and select more tolerant genotypes.

Besides various biotic factors, the abiotic factors, such as light quality, water and nutrient supply, play a crucial role during cultivation and also hugely impact the photosynthetic process (Farquhar and Sharkey, 1982; Taiz and Zeiger, 2006). Essentially, considering the quality and intensity of the incident light, damages on photosynthetic appa-

ratus and, in extreme cases, chlorophyll photooxidation might occur when its supply exceeds normal taxes. On the other hand, when lack of light happens, it can conduct to plant etiolation and decrease of photosynthetic activity (Solymosi and Schoefs, 2010).

Addressing damages caused by irregular water supply, its deficiency is responsible for lower growth, smaller leaf area and, subsequently, less photosynthesis. In contrast, excess can affect proper root oxygenation and favour the incidence of vascular pathogen agents. Nitrogen shortage can lead to impairment of leaf elongation by lowering turgor and/or cell wall extensibility, while its excess can stimulate growth of foliage, over flowering, fruiting and/or formation of storage organs such as tubers and roots (Radin et al., 1982; Palmer et al., 1996; Dodd et al., 2002; Taiz and Zeiger, 2006).

In general, both deficit and surplus of specific resources can lead to growth and development impairment, resulting in stress responses. Therefore, the importance of a balance in light, water and nitrogen, being the most important mineral nutrient in terms of quantity, are described in more detail in the following sections.

1.1 Light quality

The light-dependent reactions of the photosynthesis represent a photobiochemical process using light energy to produce ATP and NADPH, ultimately consumed in the assembly of carbon atoms in organic molecules. Functionally, photons are harvested by protein–chlorophyll (*Chl*)–carotenoid complexes, that form the light harvesting antenna of photosystems. From there, they are transferred to the photosystem reaction centre, where electrons are generated; these processes take place in the chloroplast (Fig. 1). In the absence of light or under deep shade conditions plants might develop etiolation symptoms, absence of Chl, reduced leaf size and hypocotyl elongation (Taiz and Zeiger, 2006). In contrast, excessive light generates excessive oxygen radicals and causes photoinhibition. Both phenomena strongly limit plant productivity (Darkó et al., 2014). Though, as described in the literature, most of the studies are focused on high light stress rather than low light irradiance, because of the deleterious effects of photoinhibition and photodamage on plants (Nouri et al., 2015).

In addition to the quantity, light quality also influences photosynthesis, as changes in light intensity can cause imbalance in light capture by the photosystems. Nouri et al.

(2015) explain that, to ensure optimal photosynthesis efficiency, plants adjust the relative abundance of PSI and PSII according to the light quality. According to the authors, a rapid repression may happen in genes encoding light-harvesting complex, PSI and PSII reaction centre subunits, if plants are exposed to high light intensity. While PSII is highly susceptible to photodamage, PSI is efficiently protected against it. Foyer et al. (2012) explained the mechanisms that regulate reactions in the photosynthetic electron transport chain in which the rate of production of ATP and NADPH is coordinated with the rate of their utilisation in metabolism. This mechanism optimises light use efficiency at low irradiance or dissipates excess excitation energy as heat at high light conditions. The energy absorbed by plants under high irradiance exceeds the capacity of light utilisation in photosynthesis and this causes photoinhibition. Although PSII is a primary site of inhibition, there is evidence that under certain circumstances, PSI can be photoinhibited even faster than PSII (Foyer et al., 2012).

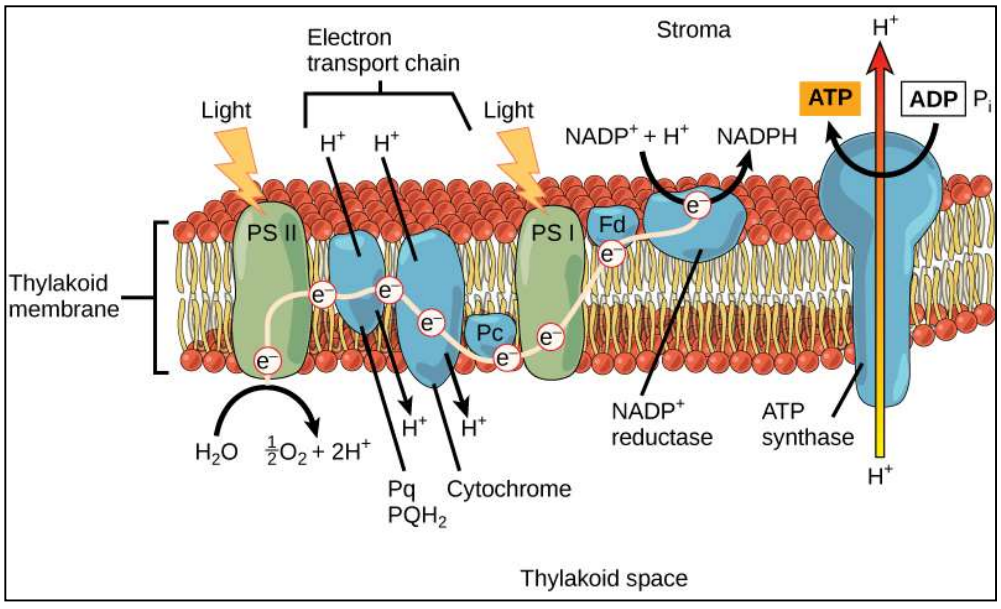

Fig. 1 The light-dependent reactions of photosynthesis - electron transport chain. From photosystem II, the electron travels along a series of proteins. This electron transport system uses the energy from the electron to pump hydrogen ions into the interior of the thylakoid. A pigment molecule in photosystem I accepts the electron (Source: Molnar and Gair, 2015).

Especially under protected cultivation (growth chambers and greenhouses), the natural sun light is usually not enough for crop cultivation, so that additional, artificial light has to be provided. In horticultural production compact fluorescence lamps (CFL) and high-pressure sodium lamps are commonly used to provide photosynthetic active radiation (PAR) to the plants. The spectrum of these artificial lighting systems is often characterised by comparatively wide-band peaks containing small amounts of blue and high amounts of green and red light (Hoffmann et al., 2015). In terms of both economics and sustainability, innovative lighting technologies, such as light-emitting diodes (LEDs) have been developed, providing narrow peaks (some nanometres) with high reproducibility and spectral resolution. This enables to design optimal species-specific light supply and precise investigation of spectral-dependent plant responses (Massa et al., 2008).

Besides the technological properties, LEDs should be compatible with the photosynthesis and light-signalling requirements of plants, which are tightly linked with the two main characteristics of light: wavelength and fluence. Artificial lighting must also be able to provide plants with energy and information required for their development. Several studies report that plants perform better under the blue and red spectra (Hoffmann et al., 2015; Darkó et al., 2014; Şenol and Taşdelen, 2014; Hemming, 2011).

1.2 Water shortage

Water is not only the universal solvent of living cells, but it is also substrate in cellular metabolism, e. g. as the electron and proton donor in photosynthesis (Kadereit et al., 2014). Plants are comprised by 80 – 95% of water in their fresh biomass of non-woody tissues (Gindaba, 1993), being clear that this element is crucial factor for plant growth and development. Whereas plants incorporate more than 90% of the absorbed nutrients into new tissues, less than 1% of the water absorbed is retained in the biomass while the remainder is lost by transpiration (Gindaba, 1993).

Terrestrial vascular plants mainly absorb water via the roots, since the cuticle on the leaves opposes the water with strong diffusion resistances. The uptake by the root from the soil is only possible if there is a corresponding water potential gradient ($\Delta\Psi$). As the water content of the soil decreases, its water potential becomes more negative. It can easily reach values of -2 MPa and below. The water potential in the root is essentially

determined by the osmotic potential of the cell sap. If the soil dries out to such an extent that the entire root system can no longer absorb any or sufficient water, or even loose water to the soil because of the reversal of the water potential differences, the plant withers, which becomes irreversible from a certain water potential of the soil (Kadereit et al., 2014). Besides wilting, cessation of growth or even the complete death of the whole plant or parts of it can result from deficit water availability (Fig. 2).

Water scarcity strongly affects stomatal conductance, influencing transpiration rate, leading to turgor loss (Kadereit et al., 2014). When stomata are less active or closed due to drought, both photosynthesis and transpiration may reduce as much as 40% before the leaves show any wilting, and over 90% during wilting. When soil dryness is severe, stomata may not open at all to prevent water loss from the leaves. Hydraulic signals and chemical signals, such as abscisic acid, can cause partial or total stomatal closure, maintain turgor and partially carry on photosynthesis even under water stress conditions (Gindaba, 1993).

The water balance goes through several phases, which increase burdens to the vegetal organism. These burdens concern defence or response mechanisms, such as root growth stimulation, leaf shedding and even death of individual plants (Kadereit et al., 2014). At molecular level, drought stress also leads to striking changes in nitrogen metabolism through the accumulation of free proline as a result of net *de novo* synthesis of glutamic acid (Tully et al., 1979). It is generally accepted that under conditions of water deprivation, proline accumulation serves as a defence against osmotic challenge by acting as a compatible solute. The accumulation of organic osmolytes is a well-characterised biochemical response of plant cells to water deficit (Hare and Cress, 1997).

In crop production, the frequency of periods of soil and atmospheric water deficit during plants' life cycle is likely to increase in the future even outside today's arid/semi-arid regions. As mentioned, plant responses to water scarcity are complex, involving deleterious and/or adaptive changes, and under field conditions these responses can be synergistically or antagonistically modified by the superimposition of other stresses, such as heat, salinity, or photoinhibition or photodamage due to incident light excess (Chaves et al., 2002).

Fig. 2 Symptoms of drought stress on potted young apple (*Malus domestica Borkh.*) plants in a water deficit experiment. Cessation of growth and wilting are some of the most common symptoms of water scarcity (Photo: Hamann, 2017).

Plant strategies to cope with drought normally involves coordination of stress avoidance and stress tolerance mechanisms that vary according to plant species and genotype. Therefore, to maintain or increase the productivity of crops, it is crucial to understand plant water relations and the consequences of inadequate water supply for growth and development. Optimisation of water supply is one of the most challenging issues to irrigate crops more precisely according to their real physiological needs and to avoid water profusion. Well-structured and implanted irrigation systems in controlled cultivation areas, such as greenhouses, portray an efficient way to validate precision irrigation in water shortage scenarios, mainly improving horticultural production on behalf of good agricultural practices (FAO, 2013)

1.3 Nitrogen fertilisation

From all mineral elements which plants require in their lifecycle, nitrogen (N) is needed in the largest amounts. Nitrogen exists in organic and inorganic forms and the highest N content can be found in seeds, leaves, shoots and roots (Ali et al., 2011). Deficiency of nitrogen leads to loss of green colour in the leaves, decreased leaf area and lower intensity of photosynthesis, since N integrates the chlorophyll molecules (Taiz and Zeiger, 2006). Understanding the processes that govern N uptake and distribution in crops is of major importance with respect to both environmental concerns and the quality of agricultural products. Nitrogen uptake and accumulation in crops represent two major components of the N cycle in the agrosystem (Bojović and Marković, 2009). Plants absorb nitrogen as a mineral nutrient mainly from soil, either as ammonium (NH_4^+) or nitrate (NO_3^-) (Tremblay et al., 2012).

However, overfertilisation seems to be an underrated topic in the administration of N doses for plants growing yet can likely occur when farmers are worried about their soil fertility levels. This practice, besides entailing unnecessary expenditure, conducts to nitrate leaching, soil denitrification, and volatilisation as the main processes for N-fertiliser losses, contributing to environmental pollution. Consequently, when there is a high N supply in leafy vegetable crops, concentration of N mobile forms i.e., nitrate and ammonium, increases in leaves, thus sometimes becoming also hazardous to human health (Tremblay et al., 2012).

Naturally, administering N according to the plant's actual needs would be profitable for both the farm and the environment. Several strategies exist to give fertilisation recommendations, such as soil tests which are capable of estimating the intensity of N release at any point in time. In contrast, the nutritional status of the plant itself might be a good indicator for fertilisation strategies and N use efficiency in general (Verhulst et al., 2014).

2 Non-destructive assessment of plant status

Traditional approaches to assess plant physiological status, such as leaf chlorophyll content, are frequently time-consuming and mostly not suitable for field applications. Despite their local precision on direct assessment and recognition of physiological disturbances in the plant tissue, they are of limited value for numerous examinations required within heterogeneous fields (Schmidhalter et al., 2008). They are essentially based on destructive sampling methods, demanding selection and harvest of plant parts, if not whole plants, to be subsequently processed wet-chemically in the laboratory. The following analysis normally consists of determining quantitatively the cellular components, such as pigments and secondary metabolites, as indicators of stress responses. In order to overcome this laborious process, several research activities in Precision Farming have been focusing on non-destructive techniques to elucidate physiological processes in plants. Major aim in these activities is to maintain plant integrity, whereas a quick and qualitative determination of crop physiological status is provided.

For this purpose, optical methods have been introduced as tools for genotype screening and plant phenotyping. Amongst others, the principle of transmittance has been applied at the development of chlorophyll meters, such as SPAD-502, one of the most used instruments in plant N determination studies. Its operation consists in enclosing a leaf section in a small chamber to expose it to red and infrared light sources positioned just above the leaf, so that the difference in transmission of the filtered wavelengths is the chlorophyll content indicator per unit leaf area (Demotes-Mainard et al., 2008). In order to detect greater field areas, ground-based sensors not depending on sunlight due to their own light sources, such as GreenSeeker®, Yara N-Sensor® and CropCircle® have been developed. These are characterised, for instance, by capturing more biomass per unit of soil surface, recording a wide waveband or describing the variation in the crop canopy according to the crop's N status, even close to N saturation (Muñoz-Huerta et al., 2013).

To that effect, research in concepts and applications of optical equipment have been expanded and optimised to gradually spare assessment of plant physiological status by destructive and sample pre-processing requirements. In this way, principles that can improve recordings and eliminate erroneous signals, while detecting N deficiency and, at the same time, responses due to light and water stress, have been tested (Muñoz-Huerta et al., 2013). Thus, sensor analysis based on reflectance measurements with spectrometers

have been used for decades, as more than 90% of the spectral information about the crop canopy status are contained in the red and near-infrared spectral bands. Consequently, nitrogen limitation in plants results in higher reflections in the red spectral region because of lower chlorophyll content in the plant cells (Gitelson et al., 2003; Mistele, 2006; Mistele and Schmidhalter, 2008; Mistele and Schmidhalter, 2010).

Nevertheless, some models are less suitable to be used *in situ*, due to the necessity to connect them to a data receiver during the recordings. Others might require white reference measurements in changing illumination conditions and, consequently, stops for calibration might be required when measurements are performed (Zecha et al., 2017). Hence, fluorescence sensors have shown their practical potential on matching these requirements, whilst special attention has been given to methods based on the detection of leaf chlorophyll fluorescence (ChlF), owing to the advantages of registering fluorescence signals directly from plant's green biomass (leaves and canopies), without interference from other substrates (Mistele and Schmidhalter, 2010; Cerovic et al., 2012; Tremblay et al., 2012; Goffart et al., 2013).

2.1 Chlorophyll fluorescence

Under normal physiological conditions, the major part of the light absorbed by the photosynthetic pigments, chlorophylls and carotenoids, is used for photosynthetic quantum conversion, while only a small proportion is de-excited via emission as heat or as red and far-red chlorophyll fluorescence (Lichtenthaler and Miehé, 1997) (Fig. 3). Plants use energy from the environment by absorbing sunlight by their leaf pigments. Light absorption transfers the pigments from their ground state to a more energetic excitation state. Subsequently, part of the absorbed energy is radiated as heat. Chlorophylls can also emit energy as fluorescent light or, at extremely low temperatures, to a very small extent as phosphorescent light. The previously excited pigment molecule is deactivated during energy release and returns to its ground state (Buschmann, 1986).

In contrast, under many stress conditions, the photosynthetic quantum conversion declines, with a concomitant increase in red and far-red chlorophyll fluorescence (ChlF). Blue and green fluorescence emissions also change under stress conditions (Bürling et

al., 2011). Analysis of chlorophyll fluorescence and its photochemical and non-photochemical components, under stress conditions, shows a disturbance in the photochemical reactions of photosynthesis, with a blockage of electron transfer between LHC II and PSII. Angelopoulos et al. (1996), for example, have shown that chlorophyll fluorescence in olive trees increases at noon, especially for plants under water stress. Ultimately, changes in the chlorophyll concentration can be detected on the basis of changes in the plant's fluorescence spectra (Schmidhalter et al., 2008).

The information on the leaf Chl content is crucial in many domains of plant research with agronomical and horticultural application. Chlorophyll is an important parameter for cultivar selection and phenotyping. It is the basis for the expression of the rate of photosynthesis and, consequently, plant productivity (Taiz and Zeiger, 2006). For large-scale canopy productivity estimation, often assessed through remote sensing, the use of Chl meters is crucial for ground verification (Tremblay et al., 2012). Therefore, proximal fluorescence sensors – here not only based on chlorophyll fluorescence, but also on fluorescence of other pigments and secondary metabolites - encompass a more located, fast and easy-to-repeat technique, that can be directly applied in protected cultivation and in the field. Simultaneously, a large amount of data can be recorded during the measurements of singles leaves or the whole canopy. Considering its' non-destructively operation, monitoring the samples – leaves, fruits or whole plants – along an entire crop season is possible (Agati et al., 2013a).

2.2 Fluorescence sensors

Besides the technical feature of crop sensors on assessing plant physiological status, new developed models have been taking into consideration the influence of environmental variability and temporal analysis with regards to their validation and applicability in production areas. On these terms, Pajares (2011) reviewed the efficiency of image-based sensors on the analysis and quantification of crop damage, residual soil coverage in the field - with purposes on fertilisation management, as well as on the differentiation between crop and weeds. The author exposed some practical examples, such as the combination of laser and image cameras as efficient way to control the quality of apples under

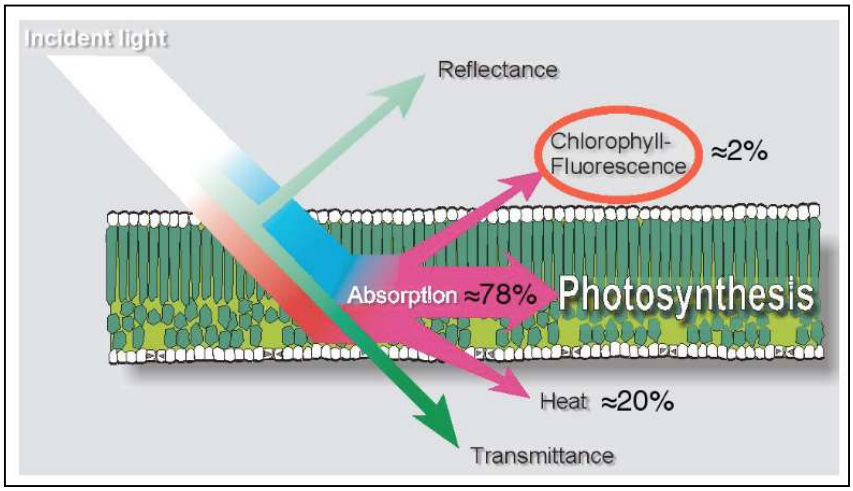

Fig. 3 Chlorophyll fluorescence on the leaf layer. The incident energy (sun light) can be directed to different energy pathways: Reflectance and transmittance – not absorbed by chlorophyll molecules, or photosynthesis, fluorescence or loss by heat – absorbed first by chlorophyll molecules (Source: © Dr. Claus Buschmann).

storage. Thereby the authors highlighted the advantage on using hyperspectral and chlorophyll fluorescence imaging due to the facility on analysing integrally infected areas.

The use of chlorophyll fluorescence in plant physiology studies is not new, since this method has been used for many years as a tool for photosynthesis research and for stress detection in plants (Buschmann et al., 2000; Yu et al., 2013; Leufen et al., 2014; Kautz et al., 2014; Kautz et al., 2015). Fernandez-Jaramillo et al. (2012) have reviewed chlorophyll fluorescence sensing methods and the need for an embedded sensor system to reduce measurement efforts. Along these lines, the development of portable fluorescence sensors with rapid and instant assessment without necessity of interpretation by means of images, instead of, by indices, have been underlined as a potential method. The potential applications cover a wide range including fruit shape and leaf size, making it possible to be carried out extensively *in situ* (Eyletters et al., 2010; Ben Ghozlen et al., 2010; Cerovic et al., 2012; Tremblay et al., 2012).

Optical fluorescence sensors represent the practical example of promising fluorescence-based tools with extended application possibilities on the detection of plant physiological stress responses caused by unbalanced nutritional and/or water supply, in view of precision and site-specific application of resources, as well selection of more tolerant varieties (Baker, 2004; Pajares, 2011; Kautz et al., 2015; Hamann et al., 2018).

The active methods of fluorescence-based sensors use LEDs or lasers to excite the chlorophyll molecule before beginning the measurements. In cases where no dark-adaptation of leaves before measurements is done, only the fluorescence under light conditions is recorded. The second part of the procedure is the measurement of the chlorophyll fluorescence. This can include several procedures and equipment, each with different characteristics such as cost, resolution, ease of processing or portability. These characteristics primarily take into account the cameras, photodiodes, optical fibre and satellite images used during the process (Fernandez-Jaramillo et al., 2012). Excitation of a leaf with blue or red light enables the recording of the ChlF. Irradiation of a green leaf with UV-light (~370 nm) allows the determination of a fluorescence emission spectra typically showing four fluorescence peaks: the blue peak (BF) (~450 nm) and the green (GF) shoulder (~520 nm) as well as the chlorophyll (Chl) peaks in the red (RF) (~690 nm) and the far-red (FRF) (~735 nm) spectral regions (Buschmann et al., 2000; Talamond et al., 2015), as demonstrated in Figure 4. In green leaves, epidermal screening of UV radiation works only when the Chl is situated in a layer below the epidermal cell layer in which the flavonoids are located (Bengtsson et al., 2006). Nevertheless, as shown on grape berries (Agati et al., 2007), the screening effect can still be used even when there is overlap between Chl and a flavonoid absorber. In that case, the absorber was anthocyanin and the method was extended also to the screening of visible (green) light by anthocyanins (Ben Ghozlen et al., 2010).

In general, fluorescence sensors are still not suitable to be used as ground-based remote sensors, such as those that capture a spread amount of biomass per unit of soil surface. In spite of this, they can be applied for monitoring crop areas more site-specifically (Muñoz-Huerta et al., 2013). High expenses to often limit acquisition of such sensors by end-users. However, the necessity of less environmental-impacted crop and horticultural production with optimised allocation of resources could contribute to reduce production costs and increase use of such sensors in near future. With this background, specific fluorescence-based sensors applied in this study are overviewed in the following sections. Their operating principles, as well as their potential and limitations are also described.

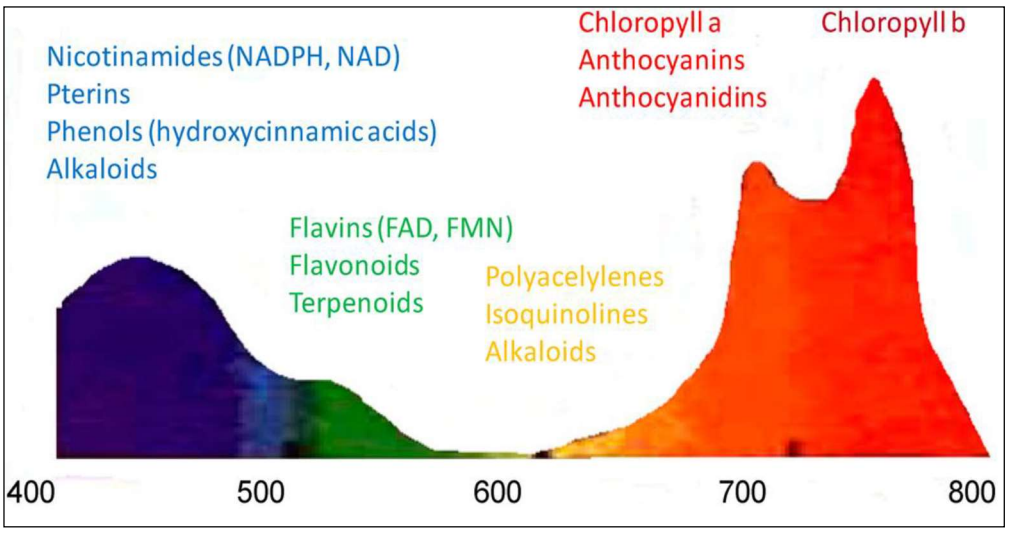

Fig. 4 Fluorescence emission spectrum of a typical green leaf under UV-radiation. (Source: Talamond et al., 2015).

2.2.1 Dualex®

The Dualex® 4 Scientific (FORCE-A, Orsay, France) (Fig. 5A) is a device which focuses on measuring polyphenolic compound content in leaves by means of chlorophyll fluorescence. It has two excitation wavelengths: (1) a chlorophyll fluorescence excitation light source at 375 nm (UV), and (2) a reference light source at 650 nm (red). These beams are activated sequentially. UV light is absorbed by polyphenols, mainly by flavonols (Flav) - according to their concentration. Differently, the red light passes through the epidermis without being absorbed before reaching the chlorophyll in the mesophyll. Fluorescent light emitted at 695 nm by chlorophyll excited with UV and red light sources is measured by the Dualex®, which calculates a ratio between both fluorescence responses (Muñoz-Huerta et al., 2013).

Previous works focused on the relationships between polyphenols and chlorophyll contents by means of two different prototypes to assess these two compounds. For instance, Meyer et al. (2006) showed the importance of quantifying leaf dry mass per area in order to understand resource allocation to epidermal polyphenol and protein from area-based optical measurements, using two leaf-clips – SPAD-502 and Dualex® – for chlorophyll and epidermal polyphenol estimation, respectively. On that note, the concept of Dualex® 4 is based on those leaf-clips, displaying three values on the screen: ‘Chl’ for the

Chl index, ‘Flav’ for the flavonol index and ‘NBI’ for the nitrogen balance index. NBI is the ratio of Chl and Flav (Cerovic et al., 2012). The same authors showed that Dualex® is precise and accurate sensor to simultaneously assess on the same leaf spot Chl and Flav, with a linear response to leaf Chl content, delivering readings on units of $\mu g.cm^{-2}$. They also stated its usefulness of non-destructive measurement of Chl, since reproducibility is granted, especially for mature leaves of crop plants. This is probably less true for exotic species with high Chl contents and thick leaves (Cerovic et al., 2012).

In this view, leaf-clip chlorophyll meters such as Dualex® are valuable tools for the estimation of Chl thanks to their capacity for many measurements allowing detection of the natural heterogeneity of leaves. In addition to the application in agriculture, the availability of a calibrated sensor with a linear response to Chl helps to generalise the sensor use in ecophysiology and plant protection (Hawkins, 2009). The combination of Chl and Flav in the Dualex® allows a more widespread use of the NBI index that has been shown superior to Chl alone for N-fertilisation in several crops (Tremblay et al., 2012). This advantage constitutes one of the most favourable features of the new models of Dualex®, along with its portability and easy-to-manage characteristic and, certainly, the capacity to take records directly in production areas – field or greenhouses, without necessity of previous dark adaption of samples before measurements are taken.

2.2.2 Multiplex®

Multiplex® (FORCE-A, Orsay, France) (Fig. 5B) is a hand-held, multi-parametric fluorescence sensor based on light-emitting-diode (LED) excitation and filtered-photodiode detection that is designed to work in the field under daylight. Having four excitation channels (Blue, Green, Red and UV) as referential light sources, and three detection channels (Blue or Yellow, Red and Infrared), this device measures 12 individual signals (with standard deviation estimation) for a multiparametric analysis. The generated fluorescence ratios, as related to flavonols, anthocyanins and chlorophyll content, are calculated and registered along with the individual signals. These signals might be used to conclude on nitrogen nutrition, crop and product quality, or crop response to different abiotic stresses. However, the use of blue light as referential light source may limit the utility of ChlF, when it is used to determine epidermal UV transmittance in cold environments, since UV

and low temperatures may stimulate the production of blue-green absorbing pigments (Andersen and Kasperbauer, 1971; Barnes et al., 2000). This constriction is avoided when source light in the blue spectral region is turned off, not occurring the same for the light sources in the red region (Pfündel et al., 2007).

The combinations of excitation and fluorescence signals result in a big number of fluorescence ratios potentially useful to interpret the Chl (SFR_R and SFR_G), flavonoid (FLAV), and N (NBI_R and NBI_G) content status of the sample. Ratios are preferred over independent signals for their reduced dependency on measurement distance (Ben Ghozlen et al., 2010; Tremblay et al., 2012). SFR is defined as the simple fluorescence emission ratio FRF_R/RF_R (FRF and RF, respectively, under red excitation). FLAV is defined as log (FRF_R/FRF_UV) (FRF under red and UV excitations, respectively). NBI is the Chl-to-flavonoids ratio computed by the ratio FRF_UV/RF_R (FRF under UV excitation and RF under red excitation). There is also the ratio ANTH which is computed as the log (FRF_R/FRF_G) under red and green excitations, and the B to FR fluorescence ratio after UV-light excitation (BFRR_UV), which is well known to be a sensitive indicator for drought impact (Buschmann and Lichtenthaler, 1998). Logarithm of the ratio of FR fluorescence after R light excitation to the FR fluorescence after excitation with UV-light [Flavonol-Index (FLAV)] is related to the concentration of flavonols in the epidermis (Cerovic et al., 2012). The Multiplex® sensor is insensitive to ambient light, as the LED sources are pulsed and synchronised to the detection (Agati et al., 2013b).

Multiplex® can be employed as a detecting sensor for in-season assessment of nitrogen balance, as well as physiological disorders caused, for instance, by water deficit (Leufen et al., 2014, Hamann et al., 2018). This sensor is convenient for larger field measurement areas, requiring only one calibration step before start of the measurements (Zecha et al., 2017). Recordings are made at a 10-cm distance from the light sources, and the measurement surface corresponds to a circle of 8 cm in diameter. Plant fluorescence can be recorded by shots of the excitation lights on the sample area or as continuous mode, when the fluorometer is fixed to a moving equipment and plants pass directly within the 8 cm dimeter opening of the sensor (Ben Ghozlen et al., 2010). This may be seen as a limitation particularly in high-throughput systems mounted on carriers where uneven heights in crop stands would call for the device to occasionally touch the leaf, or for sophisticated guiding system (Tremblay et al., 2012).

Zhang et al. (2012) firstly employed Multiplex® for the assessment of corn nitrogen status, in order to select the parameters of greatest interest, and to compare those selected Multiplex® indices with other recognised biological indicators. SFR_G (simple fluorescence ratio under green excitation) and a fluorescence excitation ratio to determine anthocyanin relative index were found to be reliable indicators for monitoring corn N status at early stages. In this context, Multiplex® indices were recorded from the leaf or from above the plant were strong influenced by applied N dose in between the second and sixth collar emerged – from V2 to V6. In addition, further research has also directed attention to the chlorophyll ratio SFR due to the high correlation with the yield of winter wheat fertilised with different nitrogen dosages (Zecha et al., 2017). In other cases, N-supply was combined with water shortage and powdery mildew, so that besides SFR, NBI indices could likewise demonstrated a strong sensitivity to chlorophyll concentration in sugar beet leaves, though a more robust stress differentiation by using only one fluorescence ratio could not be accomplished (Leufen et al., 2014). Furthermore, overlapping in specific cases with the use of blue light as reference light can be considered as a limitation of Multiplex®, as determination of the epidermal UV transmittance of plants in cold environments can be suppressed, yet this limitation can be solve by switching off the blue light in the emission channels.

2.2.3 Miniveg N

The prototype fluorescence sensor MiniVeg-N (Fritzmeier Umwelttechnik GmbH, Großhelfendorf, Germany) (Fig. 5C) uses laser-induced chlorophyll fluorescence (LICF) to estimate crop N status. Core of the device is an internal laser diode (red light laser - R), inducing the chlorophyll molecules in plant cells to emit fluorescence light. The intensity of fluorescence light is detected with highly sensitive optical components at the wavelengths of 690 nm (red - R; F690) and 730 nm (far-red - FR; F730) and the vegetation index ratio is calculated (F690/F730) (Schmidhalter et al., 2008).

The ratio F690/F730 demonstrated optimistic results to estimate the total areal nitrogen in wheat canopies (Mistele and Schmidhalter, 2010). As a tractor- or implement-mounted device, the LICF readings of canopies can be performed independent of external conditions, even during the night, since only chlorophyll molecules are induced to emit

fluorescence light, so that no effects of soil reflection have to be considered. Thus, the application of the Miniveg N-sensor is practicable at the tillering of cereals, under conditions with low leaf area index ground cover of plants. Furthermore, a detection in row cultivars such as maize, potatoes, and sugar beet also reveals a promising field application (Schmidhalter et al., 2008). Nevertheless, limitations on the application of LICF technique might be related to the influence of sunlight and temperature on the detected fluorescence signals, influencing therefore the results calculated by the ratio F690/F30 (Mistele and Schmidhalter, 2010).

Fundamentally, all the other optical sensors analysed in this study encompasses the ultimate attempts in Precision Faming and Plant Physiology research to develop modern systems and new models, that can be directly applied in greenhouses and field. While light is shed on the efficiency and suitability of fluorescence sensors, a deeper glance into the potential and limitations of those devices has to be taken. Based on it, table 1 exposes summarily the principle, potential and limitations of the fluorescence-based sensors applied in this study.

2.2.4 Imaging-PAM

The Imaging-PAM Chlorophyll Fluorometer (Heinz-Walz GmbH, Effeltrich, Germany) (Fig. 5D) is designed for the study of two-dimensional heterogeneities of photosynthetic activity at leaf level. The Imaging-PAM applies pulse-amplitude-modulated light for assessment of chlorophyll fluorescence yield. The same LEDs serve for generation of the actinic illumination driving photosynthesis and for Saturation Pulses transiently saturating energy conversion at Photosystem II (PS II) reaction centres. The Saturation Pulse method provides a non-destructive means of analysing the photosynthetic performance of plants. It allows to assess the quantum yield of energy conversion at PS II reaction centres, which is affected by numerous intrinsic and environmental parameters, like the physiological health, light conditions and various stress factors (Baker, 2008).

With Imaging-PAM, the characteristic fluorescence levels Fo, Fm and Fm' can be assessed and quenching coefficients derived. Also, the PS II quantum yield Fv/Fm (maximum quantum yield of PSII) can be determined and induction curves, as well as light

saturation curves with quenching analysis, can be measured. Under stress conditions, Fv/Fm tends to decrease, indicating dysfunctions in the photosynthetic process, which for example can be caused by photodamage to PSII reaction centres (Maxwell and Johnson, 2000; Baker, 2004; Mishra et al., 2012). Furthermore, this parameter has been proposed as a reliable indicator to evaluate light acclimation of leaves to sunny or shaded conditions (Lichtenthaler et al., 2013). However, despite of Fv/Fm can be a good indicator for photosynthetic activity (Maxwell and Johnson, 2000; Hofmann et al., 2015), it is not considered a robust indicator of nutrient status or relative growth rate (Rodríguez-Román and Iglesias-Prieto, 2005; Kruskopf and Flynn, 2006). Furthermore, stress-induced limitations, which eventually will lead to damage, are not evenly distributed over the whole leaf area. Fluorescence imaging may serve as a convenient tool for early detection of such stress induced damage. Hence, favourite fields of application of fluorescence imaging are academic and scientific plant stress physiology and plant pathology (Heinz Walz, 2014).

Imaging-PAM has been applied in wide range of chlorophyll fluorescence studies for several decades as precursor for the development of subsequent sensors. One of its major limitations though consists in the difficulty for its transportation and, certainly, in the requirement of dark adaption of samples for at least 20 minutes to allow the rapidly reversible components of non-photochemical quenching to relax (Harbinson, 2013).

Although the pulse-amplitude-modulated (PAM) chlorophyll fluorescence is a reliable method for monitoring of light-induced modifications in the PSII photochemistry (Sarijeva et al., 2007) and stress-related changes in energy utilisation (Lichtenthaler and Miehé, 1997; Maxwell and Johnson, 2000) the technique does not provide reliable data on the pigment composition of leaves and fruits (Buschmann, 2007). At leaf level, the pigment composition is of central importance to better understand the mechanisms affecting the photosynthetic functions whereas at fruit level the pigment composition allows estimations on ripening progress and quality attributes. Nevertheless, non-destructive optical sensors are commercially available and allow monitoring on pigment accumulation in leaves and fruits and represent an alternative to the expensive and time-consuming wet chemical analysis. The techniques are based on the optical properties of leaves and fruits affecting both the absorption and emission (reflection, remission and fluorescence) of light.

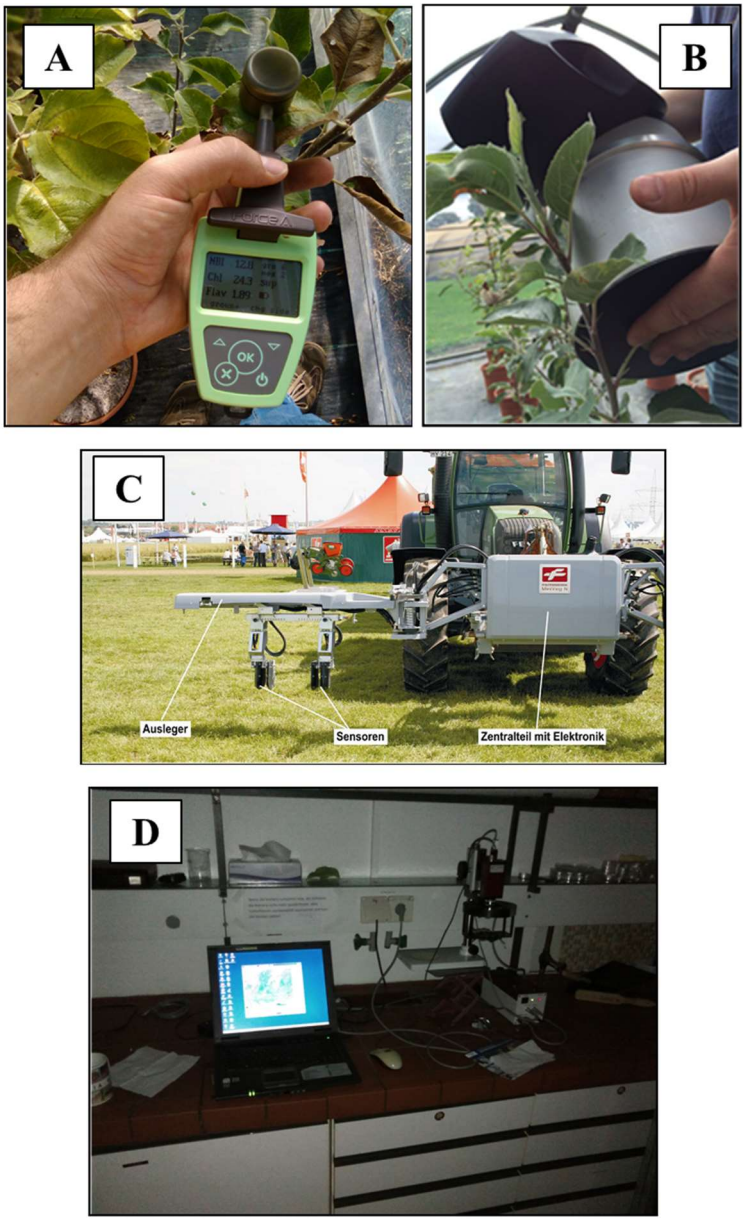

Fig. 5 Commercial and prototypes of fluorescence sensors. (A) Dualex® 4 Scientific (FORCE-A, Orsay, France), (B) Multiplex® (FORCE-A, Orsay, France), (C) MiniVeg-N (Fritzmeier Umwelttechnik GmbH, Großheldendorf, Germany), (D) Imaging-PAM Chlorophyll Fluorometer (Heinz Walz GmbH, Effeltrich, Germany). (Photos: A, B and D – Hamann 2017, C – umwelt.fritzmeier.de/miniveg)

Table 1. Potential and limitations of the sensors used in this study.

Technique	Principle	Potential	Limitations
Dualex®	Transmittance and screening effect on ChlF Light sources: UV (Measuring) and red (Reference) Determination of Chlorophyll (Chl_Index), Flavonols (Flav_Dx), and Nitrogen Balance Index (NBI)	Indication of nitrogen level, detection of water stress, light stress, leaf greening, plant senescence, protection of plants against UV radiation, cultivar selection Portability Recorded data can be followed on LCD screen Easy data transfer to a personal computer	Not able to be used as ground-based remote sensor Precocious detection of physiological disturbs not accurate
Multiplex®	Generation of fluorescence signals after LED excitation - in UV, Blue (B), Green (G) and Red (R) wavelengths Detected fluorescence signals : Blue (B), Red (R) and Far-Red (FR) fluorescence Multiparametric analysis: ChF and screening effect of polyphenols on ChF. Generation of 12 individual signals	Elimination of erroneous signals from bare soil and no sun light dependency Application of calculated ratios as indicator of a large number of plant physiological status: nutrient deficiency, presence of pathogens, grape maturation, chlorophyll, flavonol, nitrogen and anthocyanin contents	Acquisition costs Overlapping effects with blue excitation light
Miniveg N	Induction of ChlF by an internal laser diode in red wavelength	Ability to detect N deficiency	Influence of temperature and sun light
Imaging-PAM	Pulse-amplitude-modulated measuring light for assessment of chlorophyll fluorescence yield	Detection of variable, ground and maximal fluorescence as a suitable indicator of photosynthetic activity – PSII maximum quantum yield High application on fundaments of ChlF research	Not portable Previous dark adaption of samples

3 Objectives of this study

Abiotic stress factors impair crops irrespectively of cultivation method, growth or development stages. Water deficiency, imbalanced nutrient availability and light provision are major limiting factors, causing crop losses in different species. For this purpose, timed and accurate detection of the effects caused by those abiotic stresses, the discrimination between those stressors, and the differentiation between tolerant and susceptible genotypes, becomes indispensable in cropping activities. Hence, in order to optimise and accelerate the process of evaluating the physiological status of plants, the use of non-destructive fluorescence-based sensors has been proposed. In addition, existing techniques require quite often adaptations and improvements according to crop features and eventually particularities related to the cultivation area, if records can be promoted by punctual or in continuous mode measurements. In the present study, fluorescence-based sensors indices were used to evaluate the impact of abiotic stresses on the fluorescence signature of apple (*Malus domestica* Borkh.) leaves from seedlings and two-years-old trees, and barley (*Hordeum vulgare* L.) leaves, an annual culture, whose growing phases can be monitored in one cultivation cycle. Thereby, changes in the fluorescence signature as influenced by watering regimes, light quality and nitrogen fertilisation were related to physiological changes in chlorophyll concentration, water potential and secondary metabolism.

In detail, aim of this study was to verify the following hypotheses:

1. Fluorescence indices determined *in situ* under light conditions provide information about the physiological status of apple plants. Therefore, we hypothesised that multiparametric fluorescence indices reveal the onset and intensity of drought stress in young apple seedlings, as well as the effects of re-watering. On this basis, we wanted to investigate if the multiparametric fluorescence indices are supportive for the fast screening of apple seedlings regarding drought tolerance and light quality.
2. Appropriate indices of fluorescence sensors to evaluate the response of two-years-old apple trees to different watering regimes. In this regard, we hypothesised that fluorescence-based indices can be used to sense the impact of reduction of water supply in two different apple cultivars.

3. Fluorescence sensors are able to distinguish nitrogen fertiliser levels as early as in the vegetative growth stages, serving as a reliable tool to predict leaf chlorophyll contents and yield performance in the further pre-ripening and harvesting stages of cereals. Along these lines, the aim in this chapter was to evaluate the efficiency of fluorescence-based sensors on the differentiation of nitrogen levels on the fertilisation of summer barley cultivars, with inferences to leaf chlorophyll content and grain yield.

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B Influence of water shortage on apple seedlings growth under different light qualities [1]

1 Introduction

Improper light sources and limitation of water supply can significantly impair the developmental stage of seedlings, when there is a high susceptibility of cellular molecules - such as lipids, proteins, and nucleic acids - to damage and degradation, resulting in various biochemical and physiological disorders on photosynthetic processes, and consequently in irreversible yield and economical loses at the upcoming reproductive and harvest stages (Osakabe et al., 2014; Nouri et al., 2015).

In controlled (climate-chamber) and semi-controlled (greenhouse) environments, a broad range of artificial light, e. g. sodium vapour lamps, compact fluorescence lamps (CFL) and light emitting diodes (LED), are commonly used to reach optimal photosynthetic active radiation (PAR). Most LED systems provide mainly red and blue light, which – in comparison to green light - is the most absorbed spectrum by photosynthetic pigments and provides most energy for photosynthesis (Kim et al., 2008; Massa et al., 2008; Astolfi et al., 2012). Nevertheless, under strong white light conditions, green light might drive leaf photosynthesis even more efficiently than red light (Terashima et al., 2009). Excessive blue light might initiate a chloroplast avoidance response as well as a decrease in mesophyll conductance resulting in decreased photosynthetic efficiency (Loreto et al., 2009). In summary, these studies suggest that a fine balance in choosing the right light spectrum for plant cultivation is essential.

Light quality might also support plants in protecting them against water shortage by mediating stomatal aperture and controlling transpiration (Zeiger and Field, 1982; Chen et al., 2012). On these terms, Hoffmann et al. (2015) demonstrated that blue light caused structural and functional acclimations at pepper plants (*Capsicum annuum* L.) at leaf and cellular levels. In the same study, morphological acclimations contributed to different water requirements, while photosynthetic acclimations, affected by the composition of chloroplasts, reduced susceptibility to a short-term water deficit. Generally, wide-

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spread information on the interaction between light quality and physiological responses of plants to water deficit is still missing. A more precise knowledge of such interactions could contribute to promote a new outlook for purposeful applications of light quality to reduce negative impacts of stress conditions on ornamental and horticultural plants.

Methods to assess plant responses to the effect of light and water supply conditions mostly depend on destructive and time-consuming determination of biochemical indicators, such as chlorophyll, nitrogen and proline content, and physiological indicators, such as water and osmotic potentials, as well as relative water content. However, non-destructive sensing methods have also been applied in plant breeding systems to minimise these limitations on evaluating stress responses. Among others, the use of fluorescence approaches with Pulse-Amplitude-Modulation (PAM) has gained attention (Roháček et al., 2008; Lichtenthaler et al., 2013).

Fluorescence of chlorophyll molecules, emitted in the red (R) and far-red (FR) spectral regions of the light spectrum, is the basis of a widely applied technique with the purpose to evaluate impacts of adverse environmental conditions on plant physiology (Bürling et al., 2013; Lichtenthaler et al., 2013; Kautz et al., 2014). Chlorophyll (Chl a and Chl b) emits fractions of absorbed light energy as fluorescence light (Buschmann and Lichtenthaler, 1999). Besides traditional methods based on the PAM method, chlorophyll fluorescence (ChlF) can also be detected through multi-indices fluorescence excitation approaches (Tremblay et al., 2012). Variations in the fluorescence signature caused by alterations in the amount and composition of fluorescing pigments besides chlorophyll can be used as additional indicators to broaden the assessment of the environmental impact on plant physiology (Gitelson et al., 1999). Portable fluorescence sensors have been developed to facilitate the assessment of secondary metabolites, such as phenolic compounds in the leaf epidermis. Their synthesis is frequently triggered as a protective mechanism against environmental stresses as imbalance reaction to the primary metabolism. Different ratios and indices can be calculated depending on the incident light excitation and collected fluorescence signals to estimate a plant's physiological status by means of chlorophyll, flavonol and nitrogen content (Ghozlen et al., 2010; Cerovic et al., 2012; Tremblay et al., 2012).

In addition, studies with different species are important to understand how, for example, the morphological structure will influence fluorescence measurements. Species, such as sweet peppers, with a shorter growing phase, low lignified stems and thicker leaf

layer, might have a different fluorescence signature than species with a longer growing phase, higher lignified stem and thinner leaf layer, such as apple plants (*Malus domestica* Borkh.) (Kautz et al., 2015; Hamann et al., 2018). Our study hypothesises that specific light-mediated acclimations at plant and leaf levels reduce the vulnerability of apple seedlings to water deficit. In particular, the aim of this study was to investigate the physiological responses of apple seedlings cultivated either under CFL or under LEDs and additionally subjected to a period of water shortage by fluorescence technique. As reference parameters, leaf relative water content, proline and chlorophyll concentration, as well as stomatal conductance, were analysed.

2 Material and methods

2.1 Plant material, growth conditions and experimental setup

The trial was conducted in a custom-built climate chamber. *Malus domestica* Borkh., cultivar 'Golden Delicious', seeds were stratified for 28 days at 4 °C in the dark. Subsequently, they were sown in sterilised trays filled with a mixture of peat (60 %), sand (20 %) and perlite (20 %) and allocated under white compact fluorescence lamps (CFL) with main radiation peaks at 435 nm, 545 nm and 612 nm (MASTER PL-L 4P, Cool Daylight, 30 °C, Philips, Amsterdam, Netherlands) (Hoffmann et al., 2015). The photosynthetic photon fluence rate of CFL was set to 95 µmol m^{-2} s^{-1} whereby 14 % of the light energy was provided by blue, 40 % by green and 46 % by red light.

Five weeks after sowing, seedlings were transplanted into pots (11 cm, ES round 8, Göttinger, Lamprecht-Verpackungen GmbH, Göttingen, Germany) filled with 250g of the above described peat-sand-perlite mixture and cultivated under the same environmental conditions for three additional weeks. Next, half of the plants were kept under CFL while the remaining plants were allocated under LED modules (a prototype optimised for our research purposes; Ushio Lighting Inc., Tokyo, Japan). The LED-modules were characterised by a 2:1 combination of red and blue LEDs with single peaks at 665 and 445 nm, respectively. The photosynthetic photon fluence rate of the LED modules was also set to 95 ± 5 µmol m^{-2} s^{-1}, whereby 36 % of the energy was provided by blue light and 64 % by red light. Plants were cultivated under a photoperiod of 12 h, with day/night

temperatures of 21/20°C and relative humidity of 80 %. One week after the plants were assigned to the respective lighting systems (CFL or LED), the water supply treatments were initiated (63 days after sowing - DAS). After the substrate was saturated with a Hoagland nutrient solution (pH 6.2, electrical conductivity 1.4 mS cm^{-1}), drought stress was induced by withholding water and nutrient solution for 15 days (63–78 DAS), followed by a recovery period for 8 days (78–86 DAS). Control plants were irrigated daily according to their needs throughout the experiment with the nutrient solution. The experimental treatments were identified as follows:

— CFL = Compact fluorescence lamps (control)

— CFL_WD = Compact fluorescence lamps, water deficit

— LED = Light-emitting diodes (control)

— LED_WD = Light-emitting diodes, water deficit

The experiment was separated into three different watering phases: well-watered – when all plants were completely watered to soil saturation, water deficit – when watering of the plants was withheld, and recovery – when all plants were re-watered to pot saturation.

2.2 Physiological and biochemical indicators

The so-called reference parameters were assessed in a destructive way (wet chemical) to compare with, and validate against, the parameters assessed by fluorescence devices. Leaves were harvested and sampled to undergo the laboratorial analysis. The evaluated parameters comprise leaf relative water content as well as chlorophyll and proline concentrations, which were measured on 71, 76, and 83 DAS.

2.2.1 Relative water content (RWC)

Relative water content (RWC), i.e. a measurement of leaf hydration status (actual water content) relative to its maximal water holding capacity at full turgidity (Mullan and Pietragalla, 2012), was measured according to the method described by Barrs and Weatherley (1962) with few adaptations. One disc of 2.10 cm^2 was excised from a detached leaf

with a sharp cork borer. The samples were taken from the most expanded leaves in the middle part of the canopy. Sample sizes were large enough to avoid leaf veins. Discs were then weighed to obtain leaf fresh weight (W), after which the samples were immediately hydrated to full turgidity in Petri dishes overnight under laboratory room light and temperature. After hydration, the samples were taken out of water, quickly dried of any surface moisture with filter/tissue paper and immediately weighed to obtain fully turgid weight (TW). Samples were then oven dried at 80 ºC overnight and weighed again to determine dry weight (DW). The obtained values for each sample were placed into the following calculation: described by González and González-Vilar (2001).

2.2.2 Chlorophyll content

Chlorophyll content was assessed applying the method described by Strobl and Türk (1990). Three fully expanded leaves of each seedling on each measurement day were collected and cold-transported to the lab, immediately frozen (-20 ºC ± 2), freeze-dried, ground for 1 minute using a ball mill (MM 2000, Retsch GmbH, Haan, Germany) and stored at room temperature (20 ºC ± 5) for approximately one week with silica gel to reduce air humidity and prevent chlorophyll degradation. Briefly, 5 ml of methanol were added to 50 mg of the dried and ground sample, mixed and centrifuged at 4,000 rpm for 15 min (Varifuge 3 OR, Heraeus Sepatech GmbH, Hanau, Germany). Supernatants were then decanted into 50 ml flasks and the pellet was extracted three more times until the extract was colourless. The collected supernatant was filled up to 50 ml with methanol. Absorbance of the extracts was quantified by a UV-VIS spectrophotometer (Perkin-Elmer, Lambda 35, Waltham, MA, USA) at 650 nm and 665 nm. Chlorophyll content was calculated using formulas given in Hoffmann et al. (2015).

2.2.3 Proline concentration

Proline concentration was determined colourimetrically according to the method described by Ábrahám et al. (2010) with slight modifications. Briefly, 3 ml sulfosalicylic acid (3 % w/v) were added to 0.1 g dried and ground leaf material, and the mixture was

homogenised and centrifuged at 4,000 rpm for 15 min (Varifuge 3.0R, Heraeus Sepatech GmbH, Hanau, Germany). Next, 0.2 ml of the supernatant were added to 1.8 ml sulfosalicylic acid, 2 ml glacial acetic acid and 2 ml ninhydrine acid and incubated in a hot water bath (100 °C) for 1 h. After cooling to 20 °C, 4 ml toluene were added and mixed. The absorbance of the supernatant was measured at 520 nm with a UV-spectrophotometer (Lambda 35 UV/VIS Spectrophotometer, Perkin-Elmer, Waltham, USA). Proline concentrations were calculated from a standard curve.

2.3 Fluorescence measurements

Fluorescence indices were assessed by two portable fluorescence sensors, Dualex®4 Scientific and Multiplex®3 (Force-A, Orsay, France). They were adopted to record plant physiological conditions during the experiment. These devices can be used for instant evaluation of the plant status both under natural light irradiation in the field or with artificial lighting in controlled environments. Three different leaves per plant distributed in the lower, middle and upper level of the seedling were selected for fluorescence evaluations. The recordings by Dualex® and Multiplex® were taken on 63, 66, 69, 71, 73, 76, 78, 83, and 86 DAS.

The handheld sensor Dualex®4 Scientific combines the use of fluorescence and light transmission of a leaf (Cerovic et al., 2012). It determines the optical absorbance of the leaf epidermis in the ultraviolet (UV) optical range through the differential measurement of the chlorophyll fluorescence and can also estimate the chlorophyll content of the leaf using different wavelengths in the red (R) and in the far-red (FR) region. In this study, chlorophyll index (Chl _INDEX), flavonol index (Flav _Dx), and nitrogen balance index (NBI) were measured. The calculation details of these parameters can be found in the literature (Cerovic et al., 2012; Tremblay et al., 2012).

The portable multi-parametric fluorescence sensor Multiplex®3 (Force-A, Orsay, France) uses light emitting diodes (LED) which excite the plant material at three excitation channels in spectral regions of higher energy, i.e. at 375 nm (UV), 518 nm (green) and 630 nm (red). The plant fluorescence was detected in the red (RF: 680–690 nm) and far-red (FRF: 720–755 nm) spectral regions. A disc with an aperture of 4 cm Ø was used

in front of the optical unit to enable the illumination and measurement of an area of approximately 12.5 cm^2 by maintaining a constant distance of 10 cm between the light source in the device and the measured leaf surface. The analysed indices obtained by Multiplex®3 were SFR _R, NBI _R, and Flav _Mx. The description of these parameters and their calculations can be found in the literature (Bürling et al., 2011; Zhang et al., 2012; Leufen et al., 2014; Hoffmann et al., 2015) and in Table 1.

Table 1 Fluorescence indices recorded by fluorescence-based portable sensors and conventional dark-adapted-sample technique used for non-destructive assessment of plant physiological status of apple seedlings cultivated in a climate chamber under different light systems (CFL and LED) and watering regimes (fully watered and water deficit)

Index	Sensor	Description	Formula
Chl Index	Dualex®	Chlorophyll content estimation	$FRT^a - RT^b$/ RT
SFR_R	Multiplex®	Simple Fluorescence Ratio (red light excitation)	FRF_R^c/RF_R^d
NBI	Dualex®	Nitrogen Balance Index	Chl Index/Flav_Dx
NBI_R	Multiplex®	Nitrogen Balance Index (red light excitation)	FRF_UV^e/RF_R
Flav_Dx	Dualex®	Epidermal flavonol content	Log FRF_R/FRF_UV
Flav_Mx	Multiplex®	Epidermal flavonol content	Log (FER_UV^f)
F_v^g/F_m	Imaging-PAM®	Maximum quantum yield PSII	$(F_m^h - F_o^i)/F_m$

a. FRT = far-red transmission

b. RT = red transmission

c: FRF_R = far-red fluorescence with red excitation light

d: RF_R = red fluorescence with red excitation light

e: FRF_UV = far-red fluorescence with UV excitation light

f: FER_UV = fluorescence excitation ratio with red and UV excitation lights

g: F_v = variable fluorescence yield

h: F_m = maximal fluorescence yield

i: F_o= dark fluorescence yield

Maximum quantum efficiency of PSII (Fv/Fm) was recorded once a week (63, 71, 76, and 83 DAS) using an imaging pulse-amplitude-modulated fluorometer (Imaging PAM, Heinz-Walz GmbH, Effeltrich, Germany). Fluorescence images (640 × 480 pixels) were taken by a black and white CCD camera on fully expanded leaves at the third leaf level. To standardise measuring conditions and to make sure that all PSII reaction centres were open when the maximal photochemical quenching was determined, plants were dark-adapted for 30 min prior to the evaluations (see review of Maxwell and Johnson 2000). After recording the ground fluorescence (F_0), a light saturation pulse was given to determine the maximum fluorescence yield (Fm). Measurements were performed on 63, 71, 76 and 83 DAS.

Correlation between maximum quantum efficiency of PSII (Fv/Fm) and chlorophyll fluorescence indices, represented by the fluorescence indices (Chl _INDEX and SFR _R), were portrayed in diagrams with linear regression and coefficient of determination (R^2).

2.4 Stomatal conductance (G_s)

Stomatal conductance (G_s) was recorded with a portable infrared gas analyser (CI-RAS-1, PP Systems, Amesbury, USA) equipped with a standard 2.5 cm^2 leaf cuvette (PLC B, PP Systems, Amesbury, USA). Measurements were carried out under the corresponding CFL and LED lighting conditions with adopted standardised settings: CO_2 concentration 350 ± 5 ppm, photon fluence rate 100 ±5 µmol m^{-2} s^{-1} PAR, a boundary layer resistance (Rb) of 0.27 m^2 s mol^{-1} and a leaf chamber air flow rate of 200 ml min^{-1}.

2.5 Statistics

Statistical procedure was performed with the SPSS statistic software (PASW statistics version 25.0, SPSS Inc., Chicago, USA). For this, a one-way ANOVA for each measuring event and a bi-factorial analysis (2x2) for each watering phase ($p \leq 0.05$), determining the impact of light quality (lighting) and water supply (watering), as well as the interaction of both, was performed. A linear relation with the coefficient of determination

(R^2) between chlorophyll-based fluorescence indices, maximum photochemical efficiency of PSII and the leaf chlorophyll content was also performed.

3 Results

3.1 Physiological and biochemical indicators

Mean values of RWC, Chl content, and proline content determined during the water deficit and recovery phases can be seen in Fig. 1. Leaf relative water content showed no significant differences during the early water deficit phase (71 DAS). However, higher RWC was seen in well-watered plants on 76 DAS compared to the water deficit treatments. During the recovery phase (83 DAS), water restricted plants subjected to LED treatment (LED_WD) presented less RWC mean values (Fig. 1A).

Light quality and watering regimes significantly affected total leaf chlorophyll content during water deficit and recovery phases. Lowest leaf chlorophyll concentrations were measured in LED_WD plants, followed by their well-watered control plants. In contrast, highest values were seen in CFL well-watered plants during the course of the experiment. Interestingly, chlorophyll concentration slightly decreased on 76 DAS for CFL_WD plants (Fig. 1B).

Lowest proline concentrations were recorded for CFL plants throughout the experimental period (Fig. 1C). Even though there were no significant differences, both LED treatments – LED and LED_WD - showed higher proline concentrations on 71 DAS (early water deficit phase). On 76 DAS, higher proline concentrations were seen in both water deficit treatments. These differences faded at 83 DAS (recovery period).

3.2 Non-destructive parameters

3.2.1 Chlorophyll fluorescence indices

Chlorophyll estimation by Chl_INDEX and SFR R are represented in Fig. 2. Both indices show increasing values throughout the treatment period. In addition, lowest Chl INDEX values were recorded for CFL well-watered plants during the whole course of the

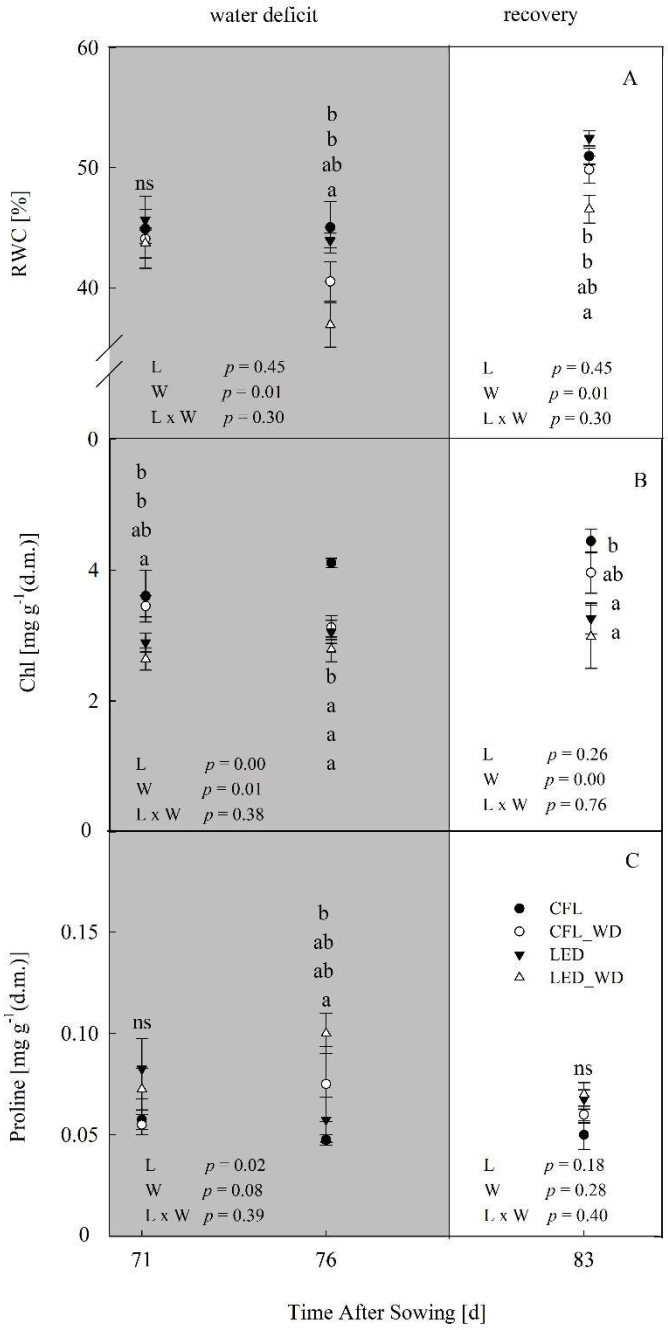

Fig. 1 A) Leaf Relative Water Content (RWC, %), B) Leaf chlorophyll content (Chl) [mg g^{-1} (dm)], and C) Leaf proline content [mg g^{-1} (d.m.)] of apple seedlings cultivated in a climate chamber under different light qualities (CFL – compact fluorescence lamps, and LED – light emitting diodes) during water deficit and recovery phases. Treatments: CFL – control plants grown under CFL, CFL_WD – water deficit plants grown under CFL, LED – control plants grown under LED, and LED_WD – water deficit plants grown under LED. Data are means of 15 replicates, different letters indicate significant differences between treatments on each measurement day. Statistical analysis by two-way ANOVA, $p \leq 0.05$, n=60, mean values ± SE. L – Lighting. W – Watering. L*W – Interaction Lighting and Watering.

experiment (Fig. 2A). In contrast, significantly higher Chl INDEX means were recorded for LED_WD plants on 76 DAS (water deficit period) and for LED well-watered plants on 78 DAS (water deficit period) as well as 83 and 86 DAS (recovery period).

Interestingly, the SFR R index showed more pronounced differences between the treatments. Already on 69 DAS, significantly higher values were recorded for both water deficit treatments (CFL and LED) when compared to the well-watered plants (Fig. 2B). On 76 DAS, higher values were seen in both LED treatments and this trend continued throughout the recovery phase, with significantly lowest values recorded for CFL plants on 78, 83 and 86 DAS.

3.2.2 Nitrogen balance indices

Nitrogen balance status was assessed by two indices, NBI and NBI R, as a factor of the mesophilic chlorophyll content and the epidermal phenolic compounds content of the leaf (Fig. 3). Irrespectively of the watering regime, highest values for both indices were recorded for plants grown under CFL. For NBI, this effect was seen from 66 DAS onwards (start of the water deficit phase, Fig. 3A), whereas NBI R recorded significant differences already on 63 DAS (Fig 3B). The NBI was not able to identify significant differences between the control and respective water deficit treatments (Fig. 3A). In contrast, the NBI R showed highest values for CFL_WD plants (significant on 78 DAS, last day of water deficit period). On this day, LED_WD plants also showed significantly higher values when compared with their well-watered plants. This effect faded during the recovery phase.

3.2.3 Flavonol fluorescence indices

Figure 4 displays the fluorescence indices estimating the epidermal flavonols. In contrast to the NBI indices, significantly highest values (both Flav Dx, Fig. 4A and Flav Mx, Fig. 4B) were recorded for plants grown under LED lighting from 66 DAS onwards. Interestingly, Flav Dx values for both LED treatments increased until the end of the experimental course, whereas values from CFL plants remained stable (Fig. 4A). The Flav_Mx index

behaved similarly, even though there was a dip in values on 78 DAS (end of water deficit period) for LED_WD plants (Fig. 4B).

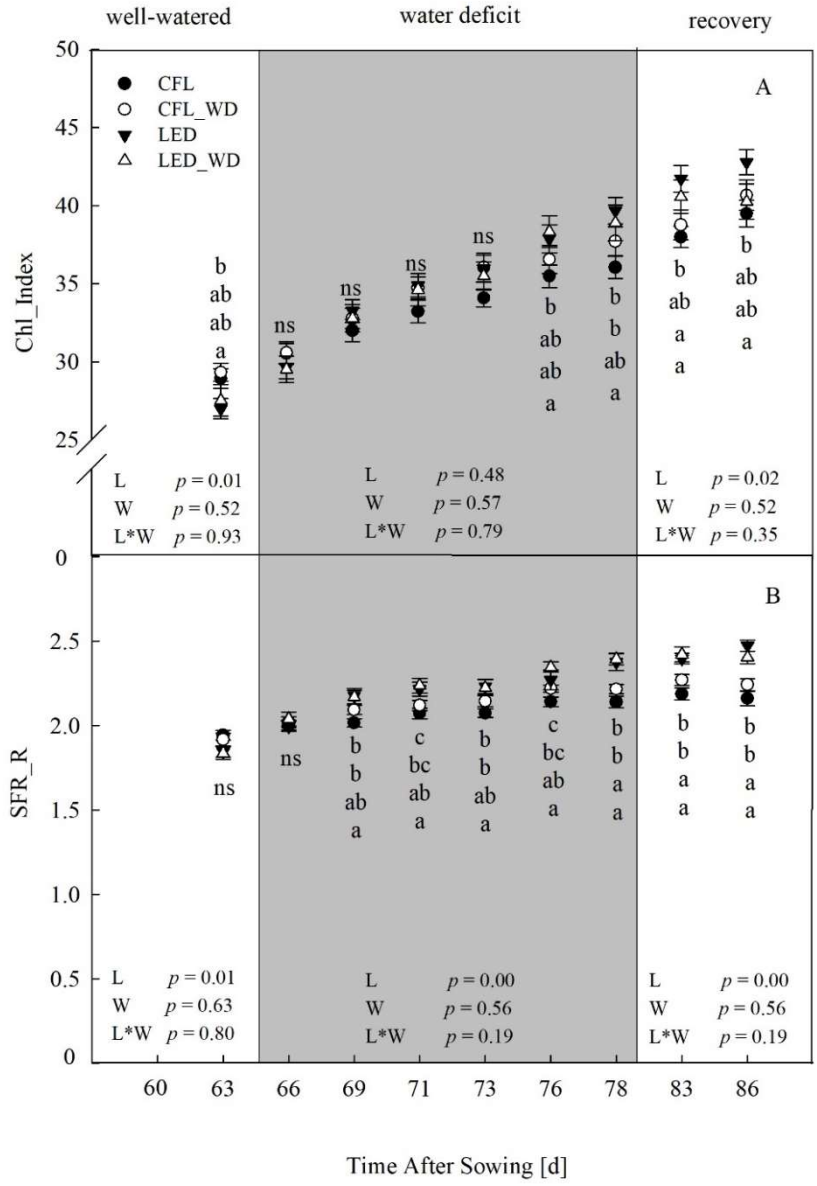

Fig. 2 A) Chlorophyll fluorescence indices Chl_Index and B) SFR_R assessed, respectively, by Dualex® and Multiplex® in apple seedlings cultivated in a climate chamber under different light qualities (CFL – compact fluorescence lamps; LED – light emitting diodes) during three watering phases: well-watered, water deficit and recovery. Treatments: CFL – control plants grown under CFL, CFL_WD – water deficit plants grown under CFL, LED – control plants grown under LED, and LED_WD – water deficit plants grown under LED. Data are means of 15 replicates; different letters indicate significant differences between treatments on each measurement day. Statistical analysis by two-way ANOVA, $p \leq 0.05$, n=60, mean values ± SE. L – Lighting. W – Watering. L*W – Interaction Lighting and Watering.

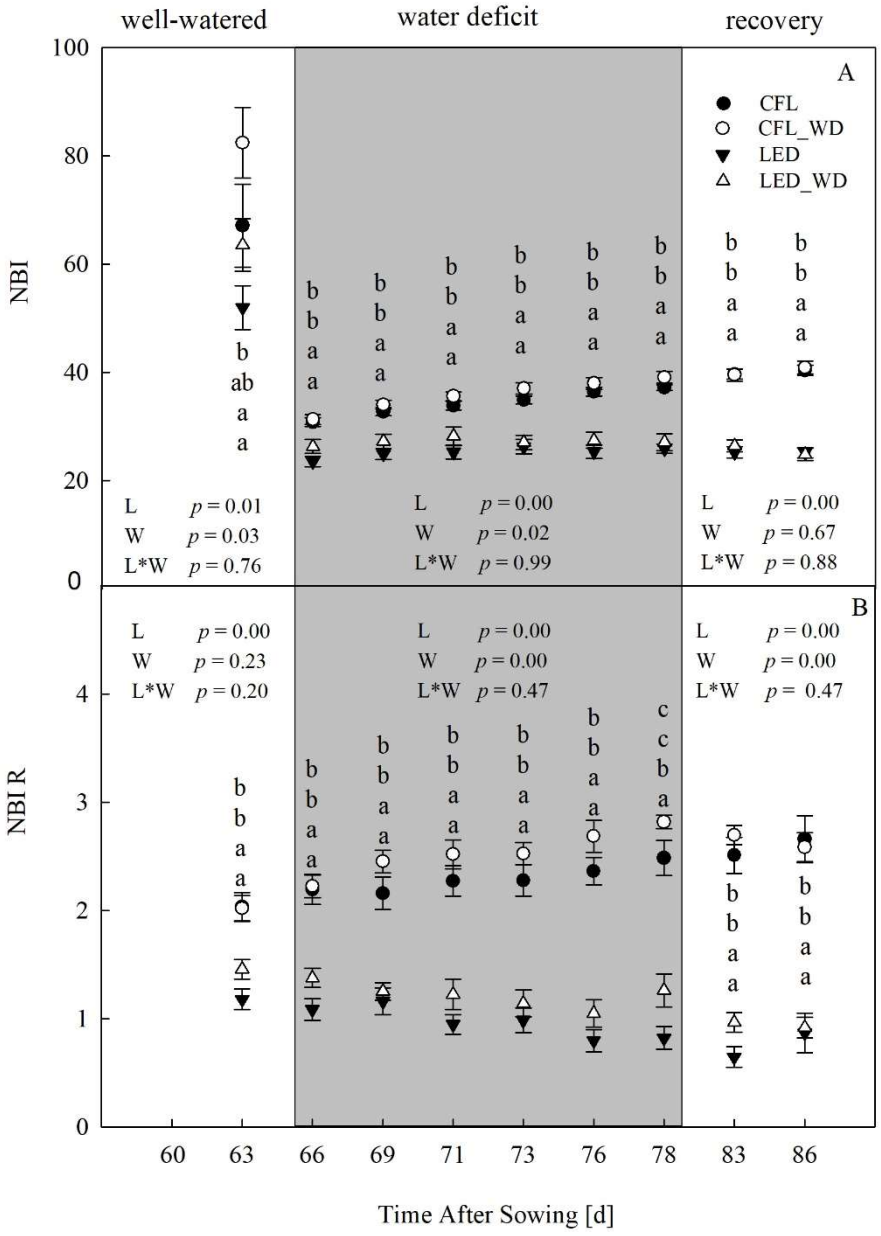

Fig. 3 A) Nitrogen balance indices NBI and B) NBI_R assessed, respectively, by Dualex® and Multiplex® in apple seedlings cultivated in a climate chamber under different light qualities (CFL – compact fluorescence lamps, and LED – light emitting diodes), during three watering phases: well-watered, water deficit and recovery. Treatments: CFL – control plants grown under CFL, CFL_WD – water deficit plants grown under CFL, LED – control plants grown under LED, and LED_WD – water deficit plants grown under LED. Data are means of 15 replicates; different letters indicate significant differences between treatments on each measurement day. Statistical analysis by two-way ANOVA, $p \leq 0.05$, n=60, mean values ± SE. L – Lighting. W – Watering. L*W – Interaction Lighting and Watering.

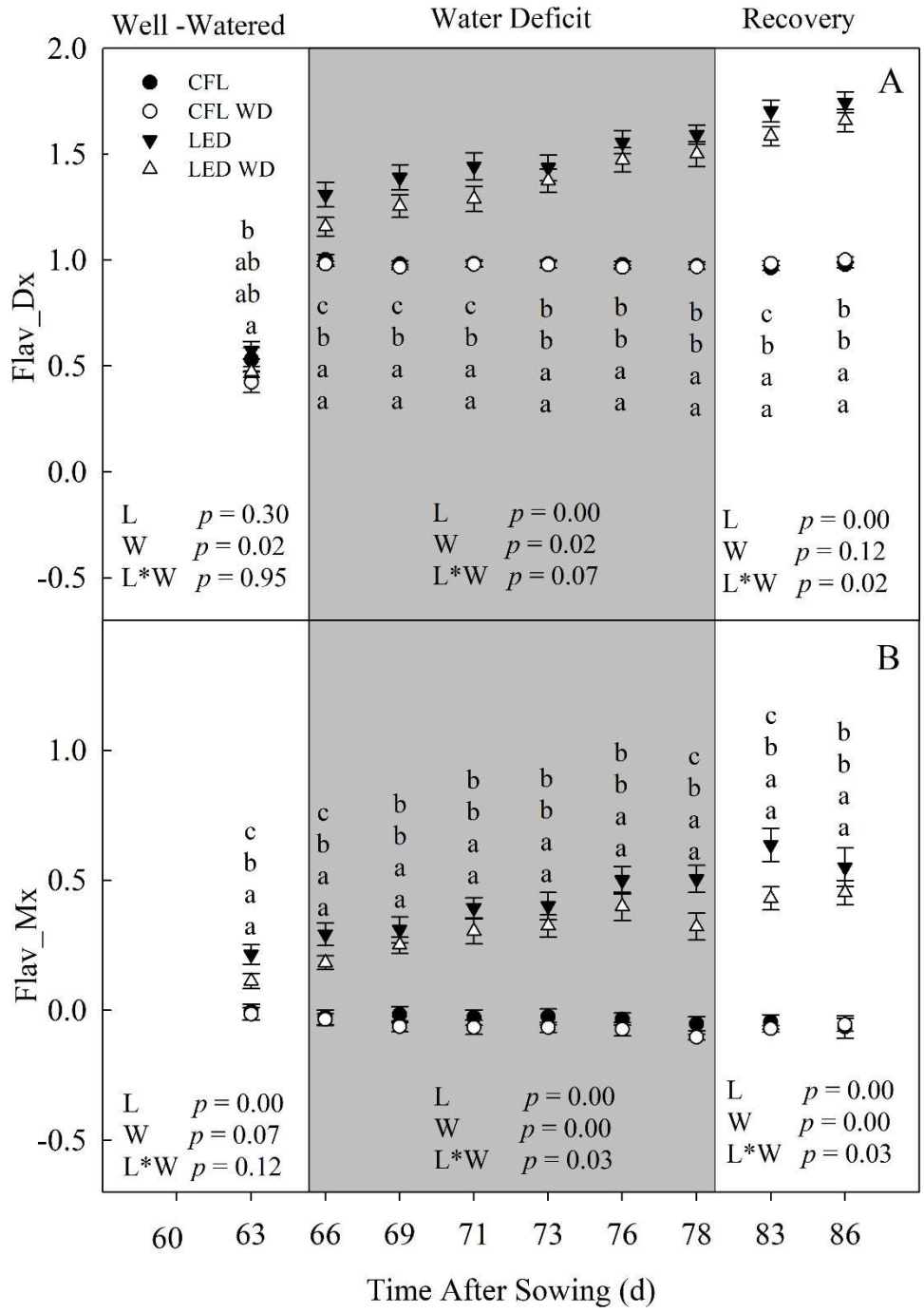

Fig. 4 A) Flavonol indices Flav_Dx and B) Flav_Mx assessed, respectively, by Dualex® and Multiplex® in apple seedlings cultivated in a climate chamber under different light qualities (CFL – compact fluorescence lamps, and LED – light emitting diodes), during three watering phases: well-watered, water deficit and recovery. Treatments: CFL – control plants grown under CFL, CFL_WD – water deficit plants grown under CFL, LED – control plants grown under LED, and LED_WD – water deficit plants grown under LED. Data are means of 15 replicates; different letters indicate significant differences between treatments on each measurement day. Statistical analysis by two-way ANOVA, $p \leq 0.05$, n=60, mean values ± SE. L – Lighting. W – Watering. L*W – Interaction Lighting and Watering.

3.2.4 Stomatal conductance (G_s) and maximum photochemical efficiency (F_v/F_m)

Significant differences in stomatal conductance (G_s) were seen especially during the water deficit phase (Fig. 5A), with a lower G_s for water stressed plants grown under LED on 71 DAS. On 76 DAS, both CFL and LED plants which were subjected to water deficit decreased their stomatal conductance even further. During the recovery period, there were no significant differences between the treatments.

The fluorescence parameter F_v/F_m indicates the maximal photosynthetic yield of PSII when all reaction centres are open and is shown in Fig. 5B. During the well-watered period, lowest values were recorded for both water deficit treatments, while highest maximum quantum yield was seen in CFL well-watered plants. There were no significant differences between all four treatments during the water deficit phase. However, LED_WD plants showed lowest values on 83 DAS (recovery period) and highest values were recorded for both CFL treatments.

3.2.5 Maximum quantum yield x chlorophyll content

Figure 6 compares the maximum photosynthetic efficiency (F_v/F_m) with the performance of chlorophyll fluorescence indices (Chl INDEX and SFR R). On 63 DAS, the treatments cluster around a lower Chl INDEX and low to medium F_v/F_m value. Coefficient of determination shows a high to very high correlation, respectively, between Fv/Fm and Chl INDEX and F_v/F_m and SFR R (Fig. 6A, E). In contrast, higher Chl INDEX values are seen for all treatments on 71 DAS (Fig. 6B) and F_v/F_m values increase as well on 76 DAS (water deficit period, Fig. 6C), though a lower relation between the indices was observed. No clear correlation (i.e. very low coefficient of determination) was seen between F_v/F_m and SFR_R during the water deficit phase (Fig. 6F and G). During the recovery period (83 DAS), all treatments show high Chl INDEX and SFR R values. Interestingly, both CFL treatments also have a high maximal photosynthetic efficiency. Coefficient of determination presented in the two correlations were significant (Fig. 6D and H).

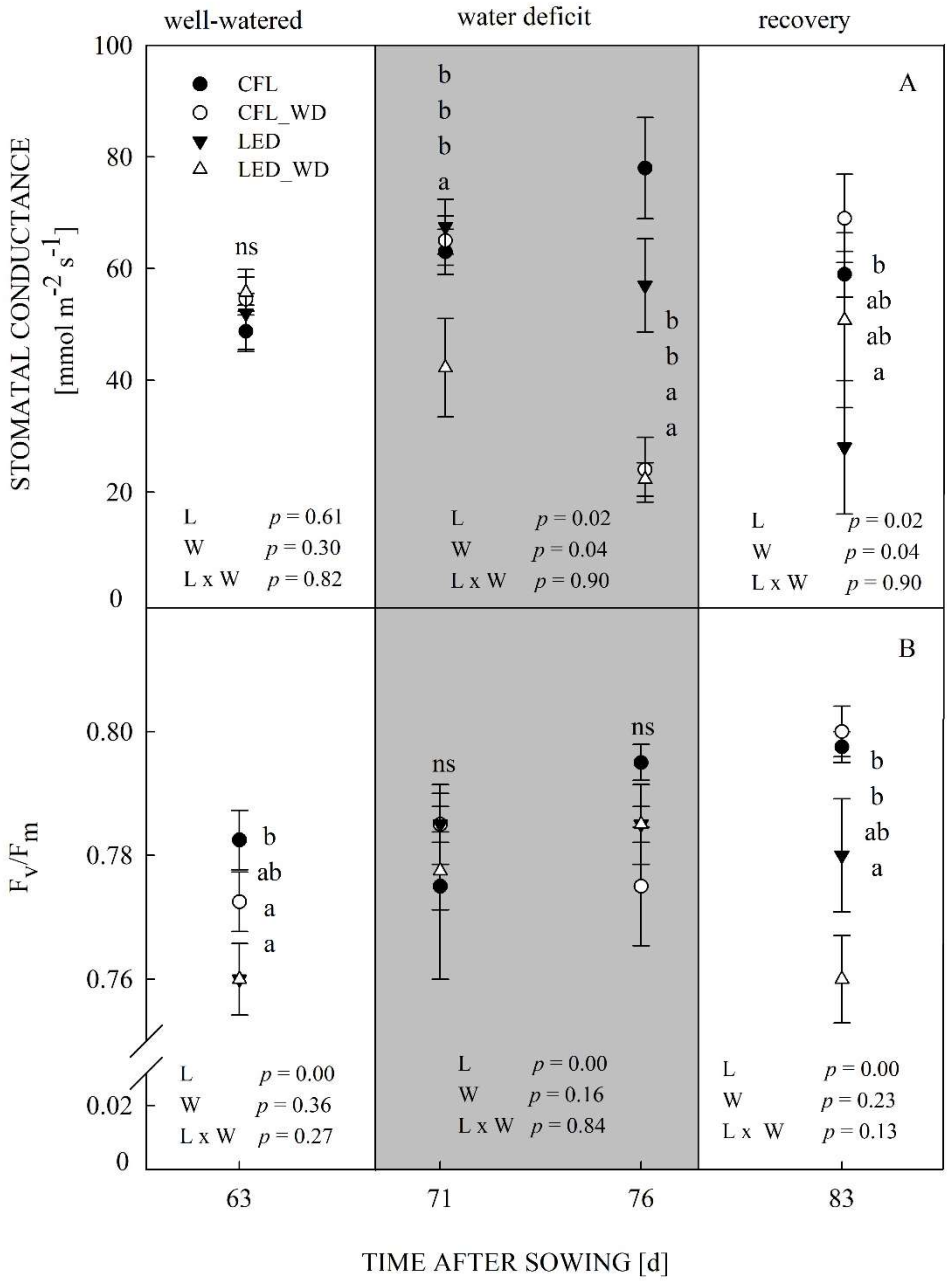

Fig. 5 A) Stomatal conductance B) and maximal photochemical efficiency quantum yield of PSII (F_v/F_m) of apple seedlings cultivated in a climate chamber under different light qualities (CFL – compact fluorescence lamps, and LED – light emitting diodes), during three watering phases: well-watered, water deficit and recovery. Treatments: CFL – control plants grown under CFL, CFL_WD – water deficit plants grown under CFL, LED – control plants grown under LED, and LED_WD – water deficit plants grown under LED. Data are means of 15 replicates, different letters indicate significant differences between treatments on each measurement day. Statistical analysis by two-way ANOVA, $p \leq 0.05$, n=60, mean values ± SE. L – Lighting. W – Watering. L*W – Interaction Lighting and Watering.

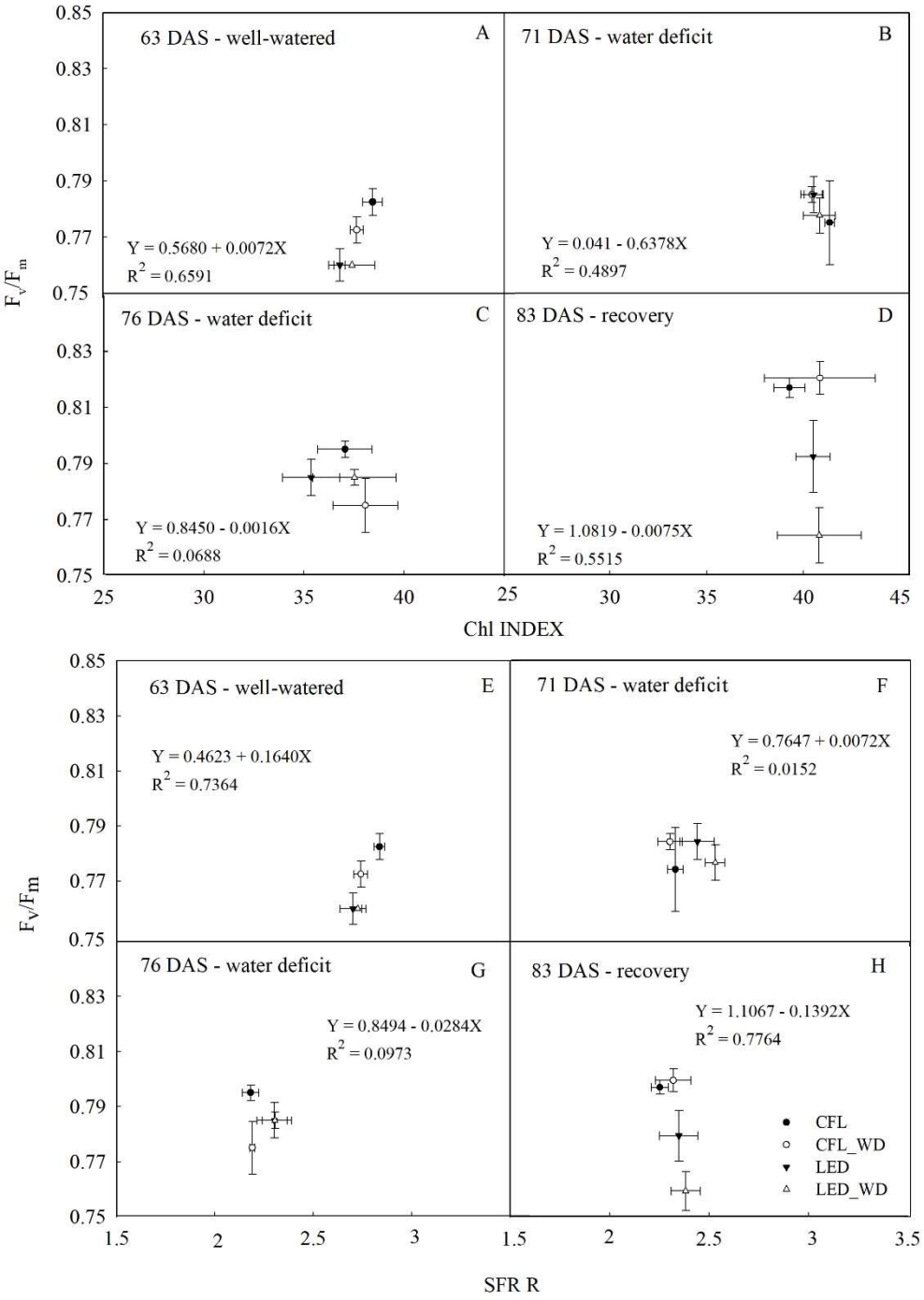

Fig. 6 A – H) Comparison between maximal photochemical efficiency (F_v/F_m) and A – D) Chl_Index and E – H) SFR_R of apple seedlings cultivated in a climate chamber under different light qualities (CFL – compact fluorescence lamps; LED – light emitting diodes) during three watering phases: well-watered, water deficit and recovery. Treatments: CFL – control plants grown under CFL, CFL_WD – water deficit plants grown under CFL, LED – control plants grown under LED, and LED_WD – water deficit plants grown under LED, n=16.Vertical and horizontal bars represent SE.

4 Discussion

In this study, physiological and biochemical responses of apple seedlings grown under different light sources and watering regimes were determined by wet chemical methods, as well as by non-destructive fluorescence sensors. The hypothesis of this study was that specific light conditions might mediate acclimation of apple seedlings to reduce their vulnerability to water deficit.

Light quality and watering regimes influenced physiological and biochemical indicators to different extents (Fig. 1). Relative water content is generally viewed as a good index for leaf water status, especially for turgor potential (Bolat et al., 2014). In this study, RWC decreased during advanced water deficit (76 DAS) and increased during re-watering (83 DAS), independently of the light source. However, LEDs affected chlorophyll and proline content, showing, lower and higher values during the advanced water deficit period, respectively. Wang et al. (2015), working with *Houttuynia cordata* seedlings, observed higher RWC and chlorophyll content in plants grown under fluorescence lamps and red LEDs. In general, proline accumulates to high levels in plants at low water potential caused by drought, describing a suitable biochemical indicator of water stress in plants (Hayat et al., 2012). However, blue- and red-light sources were also found to regulate proline accumulation, especially when combined with low water potential conditions, e. g. drought and salinity (Kovács et al., 2019). This might account for the faint, but significant proline accumulation in water restricted LED plants in the advanced water deficit phase, though this pattern was not followed in the early water deficit and recovery phases, so that further studies with longer water deficit periods are necessary for corroboration.

F_v/F_m reflects the maximal photochemical efficiency of the active centre of PSII in the dark (Krause and Weis, 1991; Kalaji and Guo, 2008). In this study, slightly higher photosynthetic efficiency was seen in CFL plants during the well-watered and recovery phases (Fig. 5B). Wang et al. (2015) verified higher F_v/F_m for plants grown under CFL and blue light modules, supporting higher light absorption in treatments with blue than with red LEDs. This might explain the lower performance of plants under LED in the present study, since blue light, according to the proportion R:B (2:1), was provided to a lesser extent. Wang et al. (2015) assume that the different absorption might influence CO_2 assimilation rates of plants grown under light with distinct wavelengths.

Additionally, light quality and watering regime also impacted stomatal conductance (G_s) (Fig. 5A). It is known that drought stress impairs stomatal conductance and ultimately results in stomatal closure to avoid water loss and limit gas exchange (Jaleel et al., 2009). Blue light has been found to be more effective than red light in causing stomatal opening or preventing stomatal closure (Farquhar and Sharkey, 1982; Shimazaki et al., 2007). This could explain the lower efficiency of stomatal conductance of water restricted plants under LED during the early water deficit regime, though it apparently did not directly affect maximal photochemical efficiency.

Indices obtained by portable fluorescence sensors related to chlorophyll estimation (Chl INDEX and SFR R) expressed increasing values throughout the experimental period (Fig. 2). A light quality effect was observed for LED treatments from the final period of water deficit onwards. The increase of Chl INDEX and SFR R in plants grown under LED radiation might have occurred due to blue and red light being absorbed by the chlorophyll molecules, as well as due to the incidence of blue light, which promotes more dark green coloured leaves and secondary metabolic accumulation (Runkle, 2015). However, no corroboration of chlorophyll fluorescence indices was given with chlorophyll concentration. -Compared with F_v/F_m, both Chl INDEX and SFR R revealed an opposite disposition (Fig. 6), since the Imaging-PAM parameter reproduced the tendency of the wet-chemically assessed chlorophyll content.

However, clear differences were verified regarding the nitrogen balance indices NBI and NBI R (Fig. 3). Both nitrogen indices showed higher mean values for plants grown under CFL lamps, similarly to the pattern of leaf chlorophyll content. Nitrogen is part of the chlorophyll molecule (Tremblay et al., 2012; Taiz and Zeiger, 2006) and therefore has a direct relation with the amount of chlorophyll accumulated in the mesophyll. Cerovic et al. (2012) described a direct relation between nitrogen and chlorophyll by analysing the efficiency of chlorophyll fluorescence to estimate the nitrogen status in crops by using the same leaf clip used in the present work. Moreover, Ben Abdallah et al. (2016) reported the practical usage of fluorescence indices, indicating the ratio chlorophyll content and phenolic compounds (Chl/Flav) as an initial basis to estimate leaf nitrogen balance.

Increased mean values of Flav Dx and Flav Mx were recorded in plants grown under LED light from 66 DAS onwards (Fig. 4). These indices are related to the accumulation of leaf epidermis phenolic compounds, including flavonoids and flavonols. The latter increased with higher sunlight intensity in *Gingko biloba* seedlings leaves (Xu et

al., 2014). This could be UV or, as in the present study, LED light, which has a higher red and blue light intensity than CFL lamps. Bantis et al. (2016) showed a significant increase in total phenolic content in *Ocimum basilicum* cultivated under the combination of high blue and green, high red:far-red (R/FR) and 1% ultraviolet illumination. It is known that the accumulation of flavonols and further secondary metabolites in plant cells is triggered in order to overcome stressful conditions. In addition, their synthesis can change due to environmental, physiological and genetic factors (Zhao et al., 2005), with light quality being one of the most influential factors (Kopsell et al., 2004; Kopsell and Sams, 2013).

5 Conclusions

Here, we show that LEDs with their specific spectral regions (blue and red) were not able to efficiently shield plants from water stress. In contrast, plants grown under a wider spectral range (CFL) performed better during and after a short period of water withdrawal, corroborated by fluorescence indices related to the nitrogen balance (NBI and NBI R) and the chlorophyll content. Phenolic compounds accumulation in leaf epidermis could also be well reproduced by flavonol indices (Flav Dx and Flav Mx), showing higher concentrations both in well-watered and water restricted plants set under LED. Hence, fluorescence indices related to nitrogen balance and flavonol accumulation promise to be a useful non-destructive tool to estimate physiological status of apple seedlings under different light sources and watering regimes. However, further studies should focus on different light intensities and wavelengths of LED lamps, and whether set up changes during the early cultivation phase of tree species would maximise a possible protective LED effect against water restrictions.

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C Monitoring physiological and biochemical responses of two apple cultivars to water supply regimes with non-destructive fluorescence sensors [2]

1 Introduction

Understanding physiological changes in plant tissues is one of the starting points for the development of non-destructive and non-time-consuming tools to assess current metabolic activities in response to environmental constraints. The fluorescence technique has been researched for several years to address the needs of farmers and producers on timely detection of plant stresses during different growth stages in the field (Gorbe and Calatayud, 2012; Tremblay et al., 2012; Bürling et al., 2013). Promising results have been reported on annual cultures for different abiotic factors, such as nitrogen fertilisation and water deficit (Shangguan et al., 2000; Bürling et al., 2011). At the orchard level, the effectiveness of any remedial measures also depends on the early detection and identification of the cause of stress (Kim et al., 2011). Chlorophyll fluorometers based on chlorophyll fluorescence kinetics techniques – such as Imaging-PAM (Heinz Walz GmbH , Effeltrich, Germany) and CF 1000 (P.K. Morgan Instruments, Andover, MA, USA) - are highly sensitive research instruments, which give quantitative information on the quantum yield of photosynthetic energy conversion, and are therefore extensively used in studies assessing responses to stress conditions in orchards plants, such as apple (*Malus domestica* Borkh.) trees (Fernandez et al., 1997). However, the necessity of dark-adaptation of leaves before measurements and the time-consuming nature during recording of fluorescence signals from the samples are just some of the drawbacks that lead, in many respects, to impracticability for plant stress assessments *in situ*.

Instead, portable fluorescence sensors with light emitting diodes would encompass a feasible technology in orchards to timely assess plant disorders and deliver a set of qualitative information about the plant physiological status. This technology has already

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been applied during crop production to evaluate performance and productivity of cultures growing under different stress conditions (Leufen et al., 2014a; Leufen et al., 2013; Peteinatos et al., 2016). Nevertheless, there are very few studies about the employment of portable fluorescence sensors to detect effects of water limitation on apple trees. In this culture, water is one of the most common limiting factors to reach productivity all over the world. Consequently, drought stress is a situation which apple trees have to deal with frequently. The increasing worldwide shortage of water emphasises the need to develop sparing irrigation systems. In recent years, there has been a wide range of proposed novel approaches to schedule irrigation which are based on sensing the plant response to water deficit directly, as opposed to sensing the soil moisture status (Alizadeh et al., 2011; Šircelj et al., 2007). Fluorescence sensors might also cover these requirements.

Plant responses to water limitation are usually monitored with traditional physiological parameters which have been proven to be good indicators of drought. The majority of studies on drought responses of apple trees investigated mainly plant water status (water potential, relative water content) and selected physiological responses such as stomatal reactions, photosynthesis, or osmotic adjustment. These studies showed that the physiological and biochemical reactions of apple trees to water stress are quite variable. This variability is associated with cultivar, time of year, previous water stress level, intensity of stress, and environmental conditions (Šircelj et al., 2007). However, based on the previous study of Fernandez et al. (1997), it seems likely that non-destructive fluorescence-based sensors are suitable to assess possible effects of water restriction on young trees. This present study focused on the behaviour of two different apple cultivars under different watering levels with non-destructive fluorescence sensors. In detail, we aimed to understand if and how water deficit leads to physiological responses in apple trees and if the cultivars ‘Gala Galaxy’ and ‘Pinova 10’ respond differently to the exposed stress conditions.

2 Material and Methods

2.1 Plant material and experimental design

The trial was run from mid of April to beginning of June 2017 at the Horticultural Science Department of the Institute of Plant Sciences and Resource Conservation, University of Bonn, Bonn, Germany. For this experiment, two-year-old healthy and vigorous apple trees (*Malus domestica* Borkh.) of two different cultivars ('Gala Galaxy' and 'Pinova 10') were grown in 27 cm Ø plastic pots filled with a mixed substrate composed of black soil, sand and perlite (4:2:1). Plants stood outside during winter and were pruned in March 2017. In April, trees were moved to tunnel-shaped greenhouses covered by transparent polyethylene foils and equipped with an automatic drip irrigation system. The rootstock used for both cultivars was M9. At the first measuring event - eight days after treatment (DAT) application, i. e. start of irrigation controlling in the acclimation phase - leaves were approximately one month old, with a mean size of 15.62 and 21.43 cm^2 for 'Pinova 10' and 'Gala Galaxy', respectively.

All plants, i.e. 32 unities, received a complete water supply so that the substrate reached its water holding field capacity (according to a pre-determined mean value). For this, pots were watered until the substrate was completely saturated and then left to drain for 24 h. The excess water was subtracted from the supplied water amount, resulting in the mean value of 1 L day^{-1} per pot. To determine for how long the irrigation had to be switched on, drippers attached to the automatic irrigation were placed in empty pots and the time it took to fill them up until 1 L was noted (20 min). Watering was performed daily in the early morning (7:00 a.m.).

Four different *phases* of water supply were adopted in the course of the experiment: *Phase I*) acclimation – for thirteen days, DAT 0 – 13 – *Phase II*) reduction of water supply – for fourteen days, DAT 13 – 27 - *Phase III*) introduction of complete watering withholding (water deficit – WD) – for fourteen days, DAT 27 – 41 - and *Phase IV*) recovery – for four days, DAT 41 - 45, totalising 45 days of experimental conduction. During acclimation (Phase I), the calculated water holding field capacity was supplied to all plants, corresponding to the variable 'Watering 100%' (W100). For phase II (reduction of water supply), a second watering level was added to the trial, i. e. 1 L day^{-1} per pot was

reduced to half the amount, in which 8 plants per cultivar received either the initial watering supply (Watering 100%) or the reduced variable ('Watering 50%' – W50). During Phase III (water deficit), both watering levels (W100 and W50) were subdivided into two parts: well-watered (WW), in which the testing plants continued receiving their respective water supply from the previous phase, and water deficit (WD), where these plants were submitted to a complete watering withholding. Finally, during the recovery period (Phase IV), all 32 trees were re-watered to the initially determined field capacity (Watering 100 %).

The experimental set-up was partially randomised, i.e. irrigation levels (W100 and W50) were conducted in two different green houses with both cultivars allocated in them in a randomised pattern. In Phase I, an initial measurement on DAT 8 was done, when all plants of both greenhouses were fully watered (W100). Table 1 summarises the plant watering and analysed parameters as well as the applied watering levels and phases.

2.2 Meteorological data

Temperature and relative air humidity values were recorded daily every hour during the course of the experiment by means of an indoor datalog recorder, the TinyTag Ultra 2® (Gemini Datalogers, Chichester, West Sussex, UK). Only data (daily mean value) from days when fluorescence recordings took place are presented in this study.

2.3 Physiological parameters

Physiological and biochemical indicators of drought stress in the apple trees were determined to compare with and validate the parameters assessed by the non-destructive fluorescence-based parameters. The so-called reference parameters were assessed in a destructive way, where leaves were harvested and sampled to undergo the laboratorial analysis. The evaluated parameters comprise leaf water potential, chlorophyll and proline concentrations, which were all measured on DAT 8, 17, 21, 24, 28, 31, 35, 38, 42, and 45. In addition, the leaf relative water content was determined on DAT 25, 30, 38 and 45, covering Phase II, III and IV.

Table 1 Experimental design and measured parameters. Two-year-old apple tree cultivars 'Pinova 10' and 'Gala Galaxy' tested under two watering regimes Watering 100 % and Watering 50 % and four watering phases: Phase I: acclimation, phase II: water restriction, phase III: cancelation of water supply, phase IV: recovery.

DAT	Phase	Watering levels				Measured Parameters	
8	I	Acclimation		W100		F. I.	P. P.
14	II	Water Reduction	W100	W50		F. I.	P. P.
17			W100	W50		F. I.	P. P.
21			W100	W50		F. I.	P. P.
24			W100	W50		F. I.	P. P.
25			W100	W50			P. P *
28	III	Water deficit	W100	W50		F. I.	P. P.
			WW	WD	WW	WD	
30			W100	W50			P. P.*
			WW	WD	WW	WD	
31			W100	W50		F. I.	P. P.
			WW	WD	WW	WD	
35			W100	W50		F. I.	P. P.
			WW	WD	WW	WD	
38			W100	W50		F. I.	P. P.**
			WW	WD	WW	WD	
42	IV	Recovery		W100		F. I.	P. P.
45				W100		F. I.	P. P.**

W100 = Watering 100%, W50 = Watering 50%, WW = Well Watered, WD = Water Deficit, F.I. = Fluorescence Indices, P. P. = Physiological Parameters (proline and chlorophyll concentration, relative water content and leaf water potential), * = only Leaf Relative Water Content (RWC), ** = all P.P., including RWC

2.3.1 Leaf water potential (Ψ_{leaf})

Leaf water potential (Ψ_{leaf}) was determined according to the Scholander et al. (1965) method, for which the sap pressure is measured by using a pressure chamber. The pressure chamber comprises an aluminium, steel or stainless steel pressure vessel that can withstand pressures up to 10 MPa and is connected to a pressurised supply of inert gas (generally nitrogen at 20 ± 5 bar or compressed air at 100 ± 5 bar) (Turner 1988). Per tree, one fully expanded leaf was cut off the stem close to the branch, placed in a plastic bag and immediately inserted in the pressure chamber (Soil Moisture Equipment Corp., Model 3000F01, Santa Barbara, CA, USA). Measurements were done between 9:00 and 11:30 am.

2.3.2 Relative water content (RWC)

Leaf relative water content, i.e. a measurement of leaf hydration status (actual water content) relative to its maximal water holding capacity at full turgidity (Mullan and Pietragalla, 2012), was measured according to the method described by Barr and Weatherley (1962) with few adaptions. One disc of 2.10 cm^2 was cut out from a detached leaf with a sharp cork borer. The samples were taken from the most expanded leaves in the middle part of the canopy. Sample sizes were large enough to avoid leaf veins. Discs were then weighed to obtain leaf fresh weight (W), after which the samples were immediately hydrated to full turgidity in petri dishes overnight under laboratory room light and temperature. After hydration, the samples were taken out of water, quickly dried of any surface moisture with filter/tissue paper and immediately weighed to obtain fully turgid weight (TW). Samples were then oven dried at 80 °C overnight and weighed again to determine dry weight (DW). The obtained weight for each sample was placed into the following calculation:

RWC (%) = [(W-DW) / (TW-DW)] x 100,

Where,

W – Sample fresh weight (g)

TW – Sample turgid weight (g)

DW – Sample dry weight (g).

2.3.3 Leaf chlorophyll concentration

Three fully expanded leaves of each tree on each measurement day were collected and cold-transported to the lab, immediately frozen (-20 °C ± 2), freeze-dried, ground for 1 minute using a ball mill (MM 2000, Retsch GmbH, Haan, Germany) and stored in the dark at room temperature (20 °C ± 5) for approximately one week, with silica gel to reduce air humidity and prevent chlorophyll degradation. Chlorophyll concentration was determined colorimetrically according to the method of Holden (1976) as well as Strobl and Türk (1990). Briefly, 5 ml of methanol were added to 50 mg of the dried and ground sample, mixed and centrifuged at 4,000 rpm for 15 min (Varifuge 3 OR, Heraeus Sepatech GmbH, Hanau, Germany). The supernatant was then decanted into 50 ml flasks and the pellet was extracted three more times until the extract was colourless. The collected supernatant was filled up to 50 ml with methanol. Absorbance of the extracts was measured with a UV-VIS spectrophotometer (Perkin-Elmer, Lambda 35, Waltham, MA, USA) at 650 nm and 665 nm. The following equations were used to calculate the concentrations of chlorophyll a (Chl a), chlorophyll b (Chl b) and total chlorophyll (Chl a + b):

Chl a = [(16,5 × A665) − (8.3 × A650)] × Vol / DM of sample material

Chl b = [(33,8 × A650) − (12.5 × A665)] × Vol / DM of sample material

Chl a+b= [(25,5 × A650) + (4 × A665)] × Vol / DM of sample material

2.3.4 Leaf proline concentration

Leaf proline concentration in the samples was determined colorimetrically according to the method described by Bates et al. (1973) and Dolatabadian et al. (2008) with slight modifications. Briefly, 3 ml sulfosalicylic acid (3 % w/v) were added to 0.1 g dried

and ground leaf material, and the mixture was homogenised and centrifuged at 4,000 rpm for 15 min (Varifuge 3.0R, Heraeus Sepatech GmbH, Hanau, Germany). Next, 0.2 ml of the supernatant were added to 1.8 ml sulfosalicylic acid, 2 ml glacial acetic acid and 2 ml ninhydrine acid and incubated in a hot water bath (100 °C) for 1 h. After cooling to 20 °C, 4 ml toluene were added and mixed. The absorbance of the supernatant was measured at 520 nm with a UV-spectrophotometer (Lambda 35 UV/VIS Spectrophotometer, PerkinElmer, Waltham, USA). Proline concentrations were calculated from a standard curve.

2.4 Fluorescence parameters

2.4.1 Fluorescence sensors

Two portable fluorescence sensors, Dualex® and Multiplex® (Force-A, Orsay, France), were adopted to record plant physiological conditions during the experiment. These devices can be used for instant evaluation of the plant status both under natural light irradiation in the field or with artificial lightning in greenhouses. Three different leaves per plant distributed in the lower, middle and upper level of the tree canopy were selected for fluorescence measurements. The measurements by Dualex® and Multiplex® were taken at DAT 8, 14, 17, 21, 24, 28, 31, 35, 38, 42, and 45 (Table 1).

The handheld sensor Dualex®4 scientific combines the use of fluorescence and light transmission of a leaf (Cerovic et al., 2012). It determines the optical absorbance of the leaf epidermis in the ultraviolet (UV) optical range through the differential measurement of the chlorophyll fluorescence and can also estimate the chlorophyll content of the leaf using different wavelengths in the red (R) and in the far-red (FR) region. Chlorophyll index (Chl_Index), flavonol index (Flav_Dx), and nitrogen balance index (NBI) are the three fluorescence indices measured by this instrument. The calculation details of these parameters can be found in the literature (Cerovic et al., 2012; Tremblay et al., 2012), their formulas are listed in Table 2.

Briefly, the Multiplex® sensor uses light emitting diodes (LED) which excite the plant material at three high energy excitation channels, i. e. at 375 nm (UV), 518 nm (green) and 630 nm (red), while the plant fluorescence was detected in the red (RF: 680–690 nm) and far-red (FRF: 720–755 nm) spectral regions. A disc with an aperture of 4

cm Ø was used in front of the optical unit to enable the illumination and measurement of an area of approximately 12.5 cm^2 by maintaining a constant distance of 10 cm between the light source in the device and the measured leaf surface. The portable multi-parametric fluorescence sensor Multiplex®3 (Force-A, Orsay, France) used in this study has been described in previous studies from our working group (Leufen et al. 2014b; Leufen et al. 2013; Bürling et al. 2011). The analysed indices obtained by Multiplex®3 were SFR_R, NBI_R, and Flav_Mx. The description of these parameters and their calculations can be found in Zhang et al. (2012). Table 2 also shows the listed formulas of Multiplex®-indices.

2.5 Statistical analyses

Three well expanded leaves were analysed on each measuring date. Data was statistically analysed with (SPSS) statistic software (PASW statistics version 23.0, SPSS Inc., Chicago, IL, USA). Independent-samples T test, one-way ANOVA and a *Duncan* post-hoc analysis were used to compare the mean values. The results are expressed as mean value ± standard error (SE) with the level of statistical significance of differences set to $p \leq 0.05$. Graphs were plotted with the software SigmaPlot®10 (Systat Software Inc., Chicago, IL, USA).

3 Results

3.1 Meteorological data

Temperature and relative air humidity varied greatly during the experimental course (Fig. 1A and B). Lowest temperatures were recorded on 14 DAT (11.18 °C, Phase II) and 31 (15.54 °C, Phase III). Similarly, DAT 14 and DAT 31 also represented days with highest relative humidity (87.93 % and 85.06 %, respectively). Relative air humidity was directly inversed compared to daily temperature values with two peaks on DAT 14 and DAT 31 (Phase II and III, respectively).

Table 2 Fluorescence indices recorded by fluorescence-based portable sensors used for non-destructive assessment of plant physiological status of two-years-old apple tress cultivated under different watering regimes.

Index	Sensor	Description	Formula
Chl Index	Dualex®	Chlorophyll content estimation	$FRT^{a} - RT^{b}$/ RT
SFR_R	Multiplex®	Simple Fluorescence Ratio (red light excitation)	FRF_R^{c}/RF_R^{d}
NBI	Dualex®	Nitrogen Balance Index	Chl Index/Flav_Dx
NBI_R	Multiplex®	Nitrogen Balance Index (red light excitation)	FRF_UV^{e}/RF_R
Flav_Dx	Dualex®	Epidermal flavonol content	Log FRF_R/FRF_UV
Flav_Mx	Multiplex®	Epidermal flavonol content	Log (FER_UV^{f})

a. FRT = far-red transmission

b. RT = red transmission

c: FRF_R = far-red fluorescence with red excitation light

d: RF_R = red fluorescence with red excitation light

e: FRF_UV = far-red fluorescence with UV excitation light

f: FER_UV = fluorescence excitation ratio with red and UV excitation lights

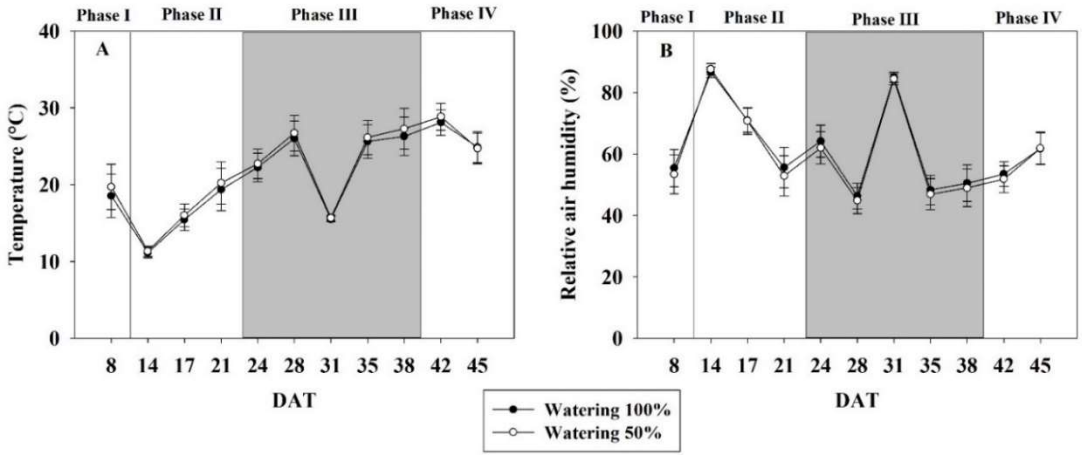

Fig.1 A) Daily mean values of air temperature in °C, and B) relative humidity in %, in the greenhouses with treatments Watering 100% (black circle) and Watering 50% (white circle).

3.2 Physiological parameters

3.2.1 Leaf water potential (Ψ_{leaf})

Leaf water potential (Ψ_{leaf}) did not significantly vary between the treatments for both cultivars under 100 % watering apart from DAT 38 (Phase III), when both water deficit regimes had significantly lower leaf water potentials than their well-watered controls (Fig. 2A). During the recovery period, Ψ_{leaf} was similar in all treatments. In addition, severe water deficit reduced Ψ_{leaf} of WD-treatments of both cultivars during phase III, irrespectively of the watering amount received beforehand (i.e. 100 or 50 %). On DAT 42, the first measurement day of the recovery period, Ψ_{leaf} was significantly lowest for 'Pinova 10' plants; however, at the end of the treatment, all plants showed similar values (Fig. 2B).

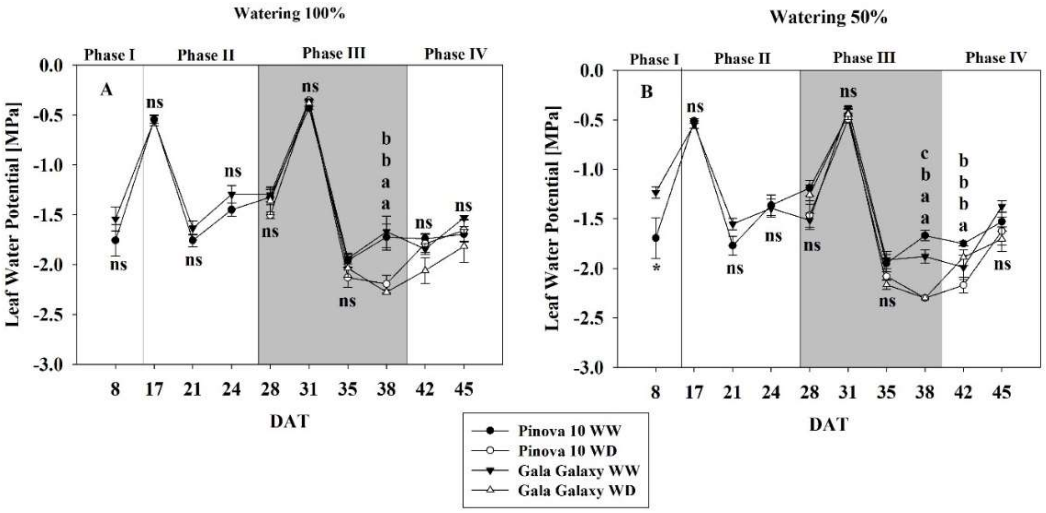

Fig. 2 Leaf water potential (MPa) of two different apple cultivars 'Pinova 10' and 'Gala Galaxy', cultivated under two irrigation regimes (field capacity – Watering 100 %, half field capacity – Watering 50 %) and four watering levels (Phase I: acclimation, phase II: water restriction, phase III: cancelation of water supply, phase IV: recovery). A) 100 % watering of the determined field capacity; B) 50 % watering of the determined field capacity. Statistical analysis by T-test and One-way ANOVA. Groups with the same letter do not show significant statistical differences by the complementary Duncan-test, $p \leq 0.05$. ns = no significance between all treatments levels. * = significance at T-Test.

3.2.2 Leaf relative water content (RWC)

Without any significant differences, leaf relative water content (RWC) of the apple trees which were fully watered from the start on was not affected by withholding of water supply during later phases in both cultivars (Fig. 3A). However, when plants only received 50 % watering from Phase II onwards, 'Pinova 10' WW had lowest RWC on DAT 30 (Phase II) and DAT 45 (Phase IV) and highest RWC was verified on 'Gala Galaxy' WW on the same dates (Fig. 3B).

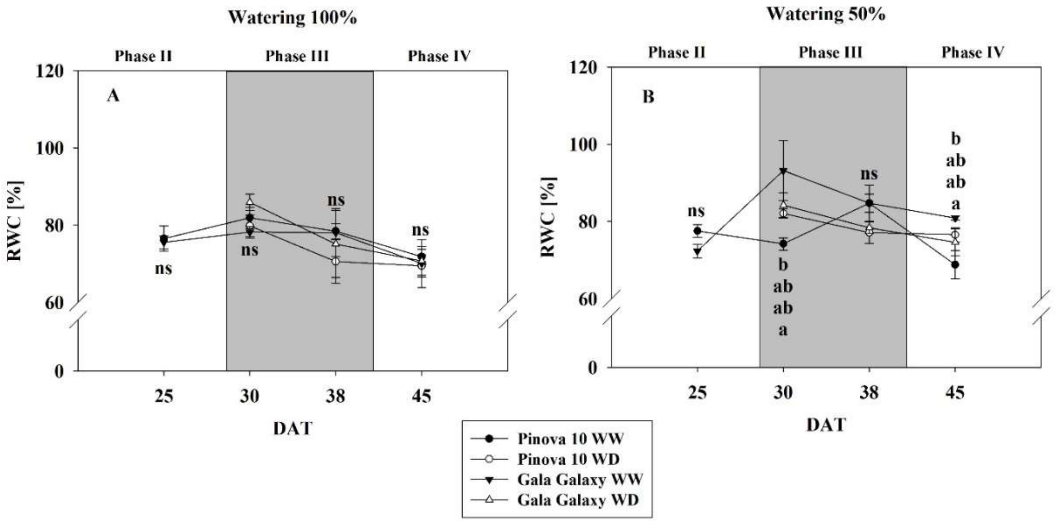

Fig. 3 Relative Water Content (RWC) (%) of two different apple cultivars 'Pinova 10' and 'Gala Galaxy', cultivated under two irrigation regimes (field capacity – Watering 100 %, half field capacity – Watering 50 %) and four watering levels (Phase II: moderate water deficit, phase III: severe water deficit, phase IV: recovery). A) 100 % watering of the determined field capacity; B) 50 % watering of the determined field capacity. Statistical analysis by T-test and One-way ANOVA. Groups with the same letter do not show significant statistical differences by the complementary Duncan-test, $p \leq 0.05$. ns = no significance between all treatments levels. * = significance at T-Test.

3.2.3 Leaf chlorophyll concentration

Figure 4 displays chlorophyll concentration analysed wet-chemically, revealing significant differences between the cultivars during Phase I and II, with 'Gala Galaxy' having highest leaf chlorophyll concentrations (Fig. 4A and B). Lowest chlorophyll concentration was recorded for 'Pinova 10' water-stressed plants during Phase III, even though statistical analysis did not reveal significant differences on most days. Both well-watered and water-stressed 'Gala Galaxy' plants had higher chlorophyll concentration during the recovery phase when compared to 'Pinova 10'.

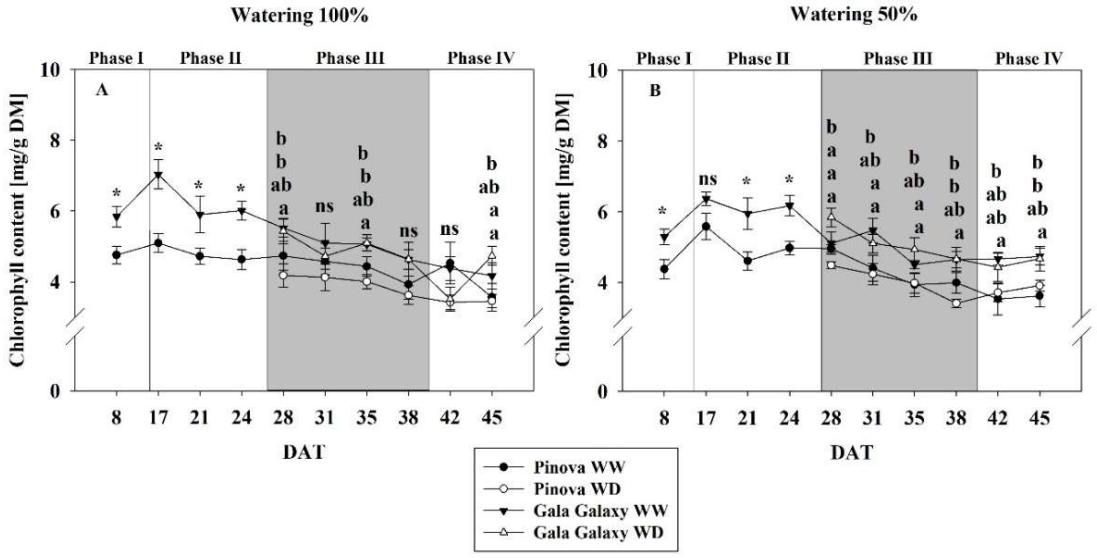

Fig. 4 Chlorophyll content (mg g^{-1} of dry mass) of two different apple cultivars 'Pinova 10' and 'Gala Galaxy', cultivated under two irrigations (field capacity – Watering 100 %, half field capacity – Watering 50 %) and four watering levels (Phase I: acclimation, phase II: water restriction, phase III: cancelation of water supply, phase IV: recovery). A) 100 % watering of the determined field capacity; B) 50 % watering of the determined field capacity. Statistical analysis by T-test and One-way ANOVA. Groups with the same letter do not show significant statistical differences by the complementary Duncan-test, $p \leq 0.05$. ns = no significance between all treatments levels. * = significance at T-Test.

3.2.4 Leaf proline concentration

There were no significant differences between treatments and watering regimes in proline concentration, apart from DAT 17, with 'Pinova 10' showing higher proline concentration when only watered with 50 % (Fig. 5B), and DAT 31 (Phase III), when highest values were analysed in well-watered 'Pinova 10' trees (Fig. 5A). When plants were water-stressed from Phase II onwards, highest proline concentrations were seen in 'Pinova 10' WD plants on DAT 35 and 38, even though there were no significant differences during the recovery period (Fig. 5B).

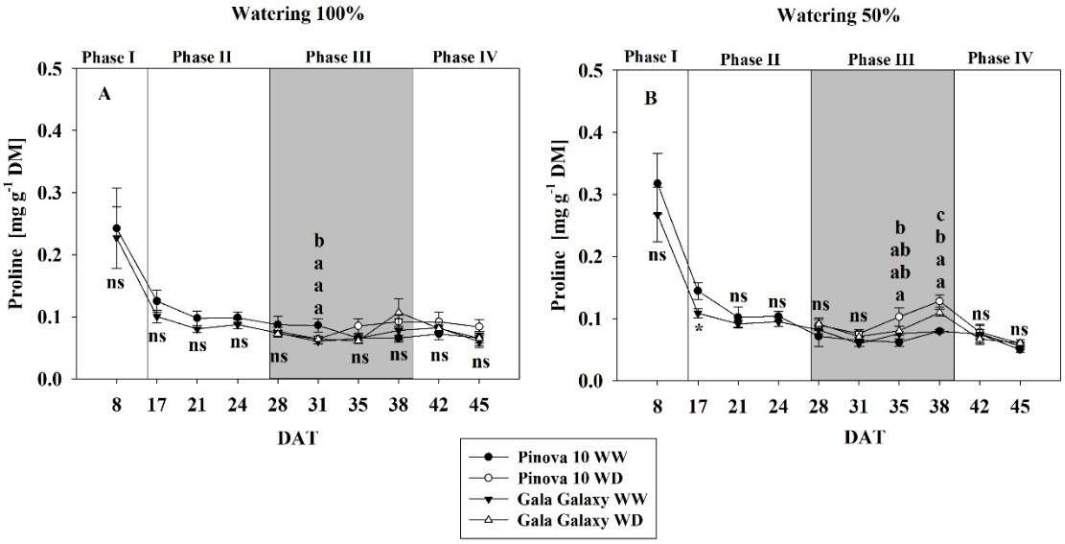

Fig. 5 Proline concentration (mg g^{-1} of dry mass) of two different apple cultivars 'Pinova 10' and 'Gala Galaxy', cultivated under two irrigation regimes (field capacity – Watering 100 %, half field capacity – Watering 50 %) and four watering levels (Phase I: acclimation, phase II: water restriction, phase III: cancelation of water supply, phase IV: recovery). A) 100 % watering of the determined field capacity; B) 50 % watering of the determined field capacity. Statistical analysis by T-test and One-way ANOVA. Groups with the same letter do not show significant statistical differences by the complementary Duncan-test, $p \leq 0.05$. ns = no significance between all treatments levels. * = significance at T-Test

3.3 Fluorescence indices

3.3.1 Chlorophyll content indices

Values of fluorescence indices estimating chlorophyll concentration - Chl_Index and SFR_R - during the experimental period are represented in Figure 6. Significantly higher Chl_Index values were recorded for 'Gala Galaxy' during Phase I and II, when plants were watered to their field and half field capacities, compared to 'Pinova 10' (Fig. 6A). For Phase III (watering withholding), lowest values were recorded for 'Pinova 10' WD. In contrast, during this phase, there were no significant differences between well-watered and water deficit 'Gala Galaxy' plants. During Phase IV, when plants were fully watered again, 'Pinova 10' WD (previously water stressed) did not recover. For plants that only received 50 % watering from Phase II onwards (Watering 50%), a similar observation in Chl_Index values was made during Phase I and II. Alternatively, from DAT 35 onwards, there were no significant differences between all four treatments (Fig. 6B).

SFR_R mean values followed a similar pattern for all treatments during Phase I and II. During Phase II, again lowest values were recorded for 'Pinova 10' WD, even though the differences were not as pronounced when compared with the Chl_Index and values also did not change during the recovery phase (Fig. 6C). Highest index expressions could be seen for 'Gala Galaxy' well-watered plants from DAT 38 onwards (Fig. 6D).

3.3.2 Nitrogen balance indices

NBI - representing an estimation of nitrogen status - was not significantly different during Phase I for all treatments (Fig. 7A and B). During Phase II, significantly highest values were recorded for 'Gala Galaxy' trees, independently of the watering regime. When water was withheld (Phase III), lowest NBI was measured for 'Pinova 10' plants (significantly different on DAT 28, 31 and 38). Even though those values did not recover to those from well-watered plants during Phase IV, there was no significant difference between all treatments on DAT 42 and 45 (Fig. 7A). For plants which were water-stressed from Phase II onwards, no significant difference could be observed throughout Phase III and IV (Fig. 7B).

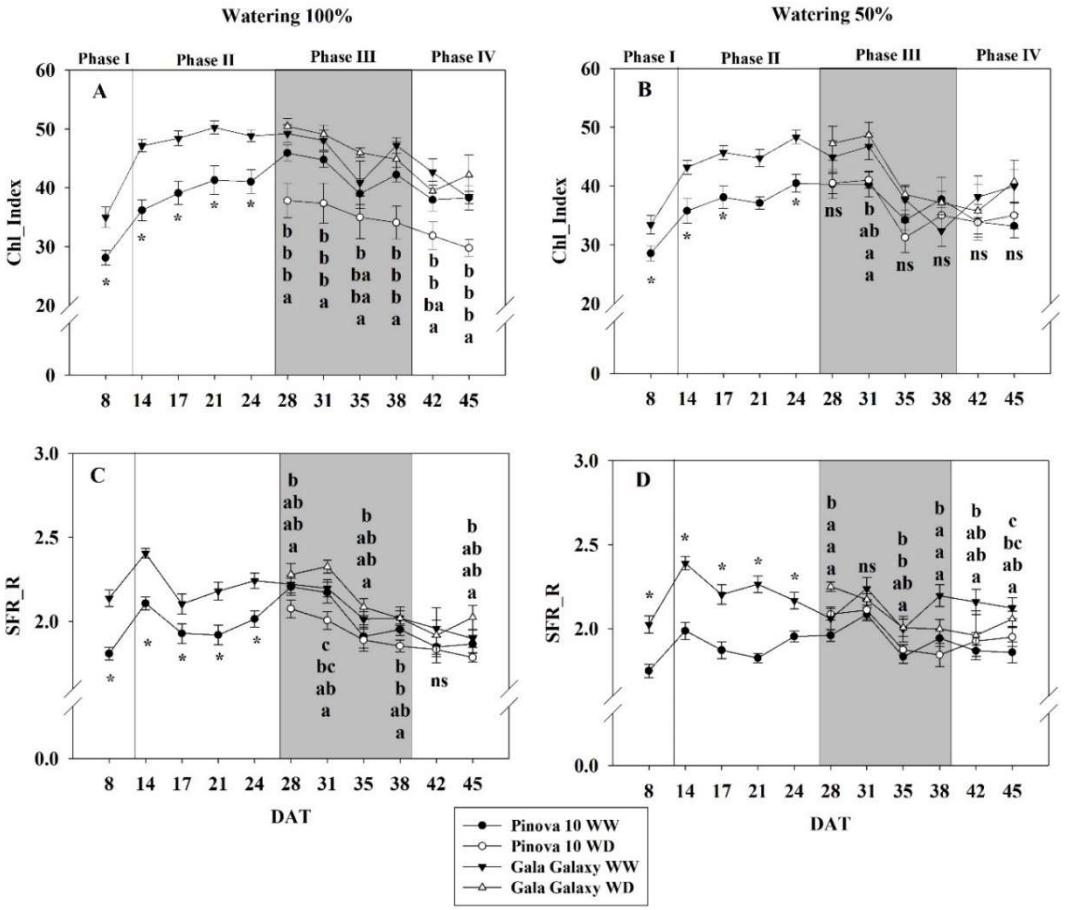

Fig. 6 Chlorophyll-fluorescence indices Chl_Index and SFR_R assessed, respectively, by Dualex® and Multiplex® on two different apple cultivars, 'Pinova 10' and 'Gala Galaxy', cultivated under two irrigation regimes (field capacity – Watering 100 %, half field capacity – Watering 50 %) and four watering levels (Phase I: acclimation, phase II: water restriction, phase III: cancelation of water supply, phase IV: recovery). A) Chlorophyll index (Chl_Index) with 100 % watering; B) Chlorophyll index (Chl_Index) with 50 % watering; C) Simple fluorescence ratio with red light excitation (SFR_R) with 100 % watering and D) Simple fluorescence ratio with red light excitation (SFR_R) with 50 % watering. Statistical analysis by One-way ANOVA. Groups with the same letter do not show significant statistical differences by the complementary Duncan-test, $p \leq 0.05$. ns = no significance between all treatments levels,* = significance at T-Test.

The NBI_R index did not record any significant difference between cultivars during Phase I and II (Fig. 7C and D). Even during Phase II and IV, when irrigation was restricted, no significant treatment effect was seen, apart from DAT 42, when 'Gala Galaxy' WD showed significantly lower NBI_R values compared to 'Gala Galaxy' well-watered.

3.3.3 Flavonol indices

The Flav_Dx index did either not at all (Fig. 8B) or only on DAT 31 (Fig. 8A) significantly vary between treatments and cultivars. The Flav_Mx index revealed signif-

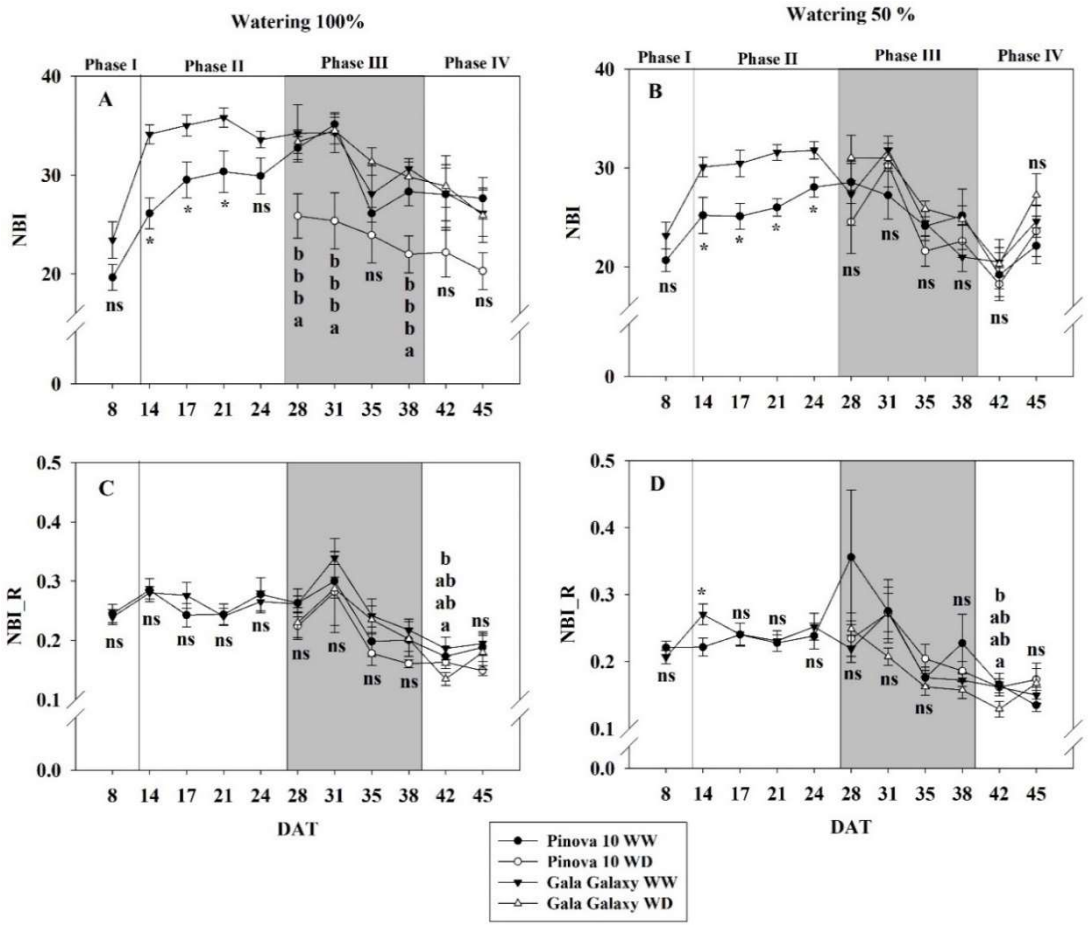

Fig.7 Nitrogen balance indices NBI and NBI_R assessed, respectively, by Dualex® and Multiplex® on two different apple cultivars 'Pinova 10' and 'Gala Galaxy', cultivated under two irrigation regimes (field capacity – Watering 100 %, half field capacity – Watering 50 %) and four watering levels (Phase I: acclimation, phase II: water restriction, phase III: cancelation of water supply, phase IV: recovery). A) Nitrogen balance index (NBI) with 100 % watering; B) Nitrogen balance index (NBI) with 50 % watering; C) Nitrogen balance index with red light excitation (NBI_R) with 100 % watering and D) Nitrogen balance index with red light excitation (NBI_R) with 50 % watering. Statistical analysis by One-way ANOVA. Groups with the same letter do not show significant statistical differences by the complementary Duncan-test, $p \leq 0.05$. ns = no significance between all treatments levels,* = significance at T-Test.

icant differences between the cultivars on DAT 8 (Fig. 8C and D). When plants received a water restriction (Watering 50%), 'Gala Galaxy' WD showed highest Flav_Mx values on DAT 21 (Phase II, Fig. 8D). There were no significant differences between the treatments during Phase II. Highest values on DAT 42 (Phase IV) were recorded for 'Gala Galaxy', which did not receive any water during Phase III.

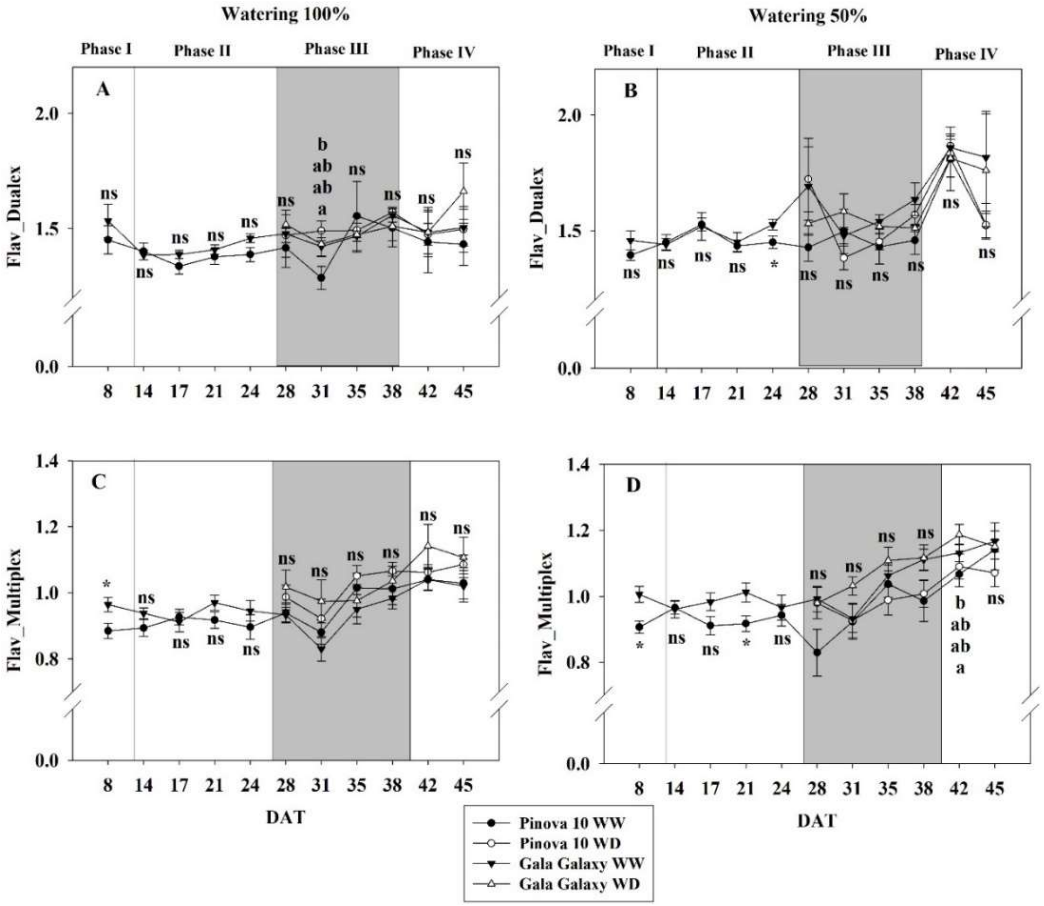

Fig. 8 Flavonol indices, Flav_Dualex and Flav_Multiplex. assessed, respectively, by Dualex® and Multiplex® on two different apple cultivars 'Pinova 10' and 'Gala Galaxy', cultivated under two irrigation regimes (field capacity – Watering 100 %, half field capacity – Watering 50 %) and four watering levels (Phase I: acclimation, phase II: water restriction, phase III: cancelation of water supply, phase IV: recovery). A) Flav_Dualex with 100 % watering; B) Flav_Dualex with 50 % watering; C) Flav_Multiplex with 100 % watering and D) Flav_Multiplex with 50 % watering. Statistical analysis by One-way ANOVA. Groups with the same letter do not show significant statistical differences by the complementary Duncan-test, $p \leq 0.05$. ns = no significance between all treatments levels, * = significance at T-Test.

4 Discussion

The aim of this study was to investigate the responses of two apple cultivars, 'Pinova 10' and 'Gala Galaxy', to water restriction regimes via physiological related parameters, measured both destructively and by non-destructive fluorescence signals.

Leaf water potential (Ψ_{leaf}) represents a parameter to assess the evaporation of water from leaf cells by transpiration. Coupled with the resistances to flow of water from soil to the leaf, a negative hydrostatic pressure builds up in the xylem (Scholander et al., 1965). This negative hydrostatic pressure is responsible for higher negative pressure values once plants are affected by water shortage and therefore, Ψ_{leaf} is commonly used as a physiological reference parameter to evaluate water stress (Parr et al., 1981). In our study, we could not verify significant differences between the cultivars and watering levels, except on DAT 38 during Phase III, when a complete watering withholding was applied (Fig. 2). It is well known that, apart from the watering regime, many other factors influence the momentary Ψ_{leaf}, especially meteorological conditions such as temperature or humidity. Naor et al. (1995) describe that plant water status is a function of soil water availability, hydraulic resistance along the flow path, plant water capacitance, and meteorological conditions (air temperature and humidity) that determine atmospheric evaporative demand. In our experiment, both varied greatly throughout the experimental period (Fig. 1.). Comparing these with Ψ_{leaf}, higher temperatures and lower air relative humidity led to decreases in Ψ_{leaf} mean values. Thus, it is very likely that the weather conditions overshadowed possible effects of the different watering regimes on Ψ_{leaf}.

Effects of Ψ_{leaf}, as well as further osmotic adjustments on plant cellular hydration can also be estimated through other plant water stress indicators, such as the leaf relative water content (RWC) (Gindaba, 1993). RWC is based on the mass of water held in relation to the mass that can be held at full turgor. This parameter is considered an important criterion of plant water status, where a decrease of RWC values is expected with increasing levels of water stress (Bolat et al. 2014). However, measurements of this parameter are not only destructive, they may also be time-consuming for day-to-day monitoring of plant water status in orchard conditions (Gindaba, 1993), as a regular frequency is often required to point out the effect of water stress in leaves. Barrs and Weatherley (1962) showed that in practice, the use of final dry weight to estimate initial water content was

found to be inaccurate, since a significant decrease in dry weight occurred during the 24 h period of floating, adopted to permit the tissue to become fully turgid, and this led to the initial water content being overestimated. Instead, they suggest collecting duplicate samples of tissue to be oven dried immediately to give the initial dry weight needed to accurately determine initial water content. This could explain why neither significant differences nor clear treatment influences on both cultivars were found in our study (Fig. 3), where we suggest that RWC could not perform as a reliable parameter to detect water stress in apple plants.

Biochemical parameters can also be important indicators of water stress in plants. Hayat et al. (2012) reviewed that proline plays a highly beneficial role in plants exposed to various stress conditions. Besides acting as an excellent osmolyte, this amino acid plays major roles during stress, i.e., as a metal chelator, an antioxidative defense molecule and a signaling molecule. The phenomenon of proline accumulation is known to occur for example under water deficit, salinity, low temperature, heavy metal exposure and UV radiation (Hayat et al., 2012) Proline also contributes to stabilising subcellular structures (e.g. membranes and proteins), scavenging free radicals and buffering cellular redox potential under stress conditions (Hayat et al., 2012). During the course of the present study, only minor differences in proline concentrations were seen, with higher concentrations in 'Pinova 10' leaves. According to Bertolli et al. (2013), it is always difficult to identify a single indicator of plant water stress because of the complexity of plant responses to water deficit. Our results suggest that proline concentration is not suitable *per se* for indicating water stress in apple trees.

Reports have explained that drought stress significantly decreases the chlorophyll content in crops such as chickpeas (*Cicer arietinum* L) (Mafakheri et al., 2010) or maize (*Zea mays* L.) (Gholamin and Khatnezhad, 2011). The amount of leaf chlorophyll *a* and *b* is frequently indicated as a reliable indicator of plant physiological status (Gitelson et al., 2003), because it can directly determine photosynthetic potential (pigment content per unity of leaf area) and primary production (Curran et al., 1990). In addition, chlorophyll is closely related to plant stress and senescence (Hendry et al., 1987; Merzlyak and Gitelson, 1995). Unfortunately, in this study it was not possible to validate foliar chlorophyll content as a reliable indicator of water stress indicator in apple trees. Similarly, Hailemichael et. (2016), investigated the relationships between water status, leaf chlorophyll content and photosynthetic performance in vineyards and pointed out that chlorophyll content

could not be used as a good indicator of water status, possibly due to the existence of narrow ranges of variation in leaf water potential (slight to moderate stress). In our experiment, we also observed a marked decrease in chlorophyll after DAT 17 for all treatments. Cran and Possingham (1973) indicated that chlorophyll loss and chloroplast degeneration in mature discs of cultured spinach (*Spinacia oleracea*) are accelerated by both increasing temperature and by increasing leaf and cell age. In our study, we could not verify any reliable relationship between water status and chlorophyll content (Fig. 2 and 4), even on DAT 38 (complete watering withholding – Phase III), when WD significantly affected leaf water potential, with lower Ψ_{leaf} mean values for both cultivars, whilst leaf chlorophyll concentration was not impaired by cessation of watering.

The non-destructive assessment of chlorophyll fluorescence techniques revealed interesting results concerning chlorophyll, nitrogen and flavonol content estimation. It is known that chlorophyll is degraded and phenolic compounds are accumulated in the leaf epidermis during ripening and stress conditions, suggesting that the Chl/Flav ratio should also change during the ripening process (Stewart et al., 2001; Junker and Ensminger, 2016). Therefore, increased flavonol levels were expected under a reduced water supply. This hypothesis could not be confirmed in our results, where no significant differences between the treatments and cultivars could be observed in the flavonol indices (Flav_Mx and Flav_Dx) measured with either sensor (Fig. 8).

The indices Chl_Index and SFR_R, estimating chlorophyll concentration, showed significant differences between both cultivars when water deficit was applied (Fig. 6). The two cultivars used in this study showed differences in both Chl_Index and SFR_R, with cultivar ‘Gala Galaxy’ having higher indices values throughout the experimental course. Fluorescence indices have been used to interpret plant physiological status in response to different stress factors, such as water restriction (Leufen et al., 2013; Leufen et al., 2014; Hoffmann et al., 2015; Bürling et al., 2013). Leufen et al. (2013) showed that parameters of multispectral fluorescence signature based on the far-red chlorophyll fluorescence are reliable indicators for sensing a temporary water deficiency stress in sugar beet (*Beta vulgaris* L.). Bürling et al. (2013) also demonstrated reliable results on wheat (*Triticum aestivum*) by assessing the reduction of chlorophyll content on water deficit plants by Chl_Index. In respect of the studied culture, Fernandez et al. (1997) conducted one of the first experiments on detection of drought stress in young apple trees through chlorophyll fluorescence. The authors determined the chlorophyll *a* fluorescence of PS II

using a chlorophyll fluorescence measurement system (CF 1000), evaluating variable and maximal chlorophyll fluorescence and fluorescence quenching. However, this technique was not sensitive enough to detect water stress. In contrast, our study suggests reliable usage of fluorescence sensors related to the assessment of chlorophyll status, based on the indices Chl_Index and SFR_R.

Drought stress can impair plant development due to changes in nutritional assimilation. Therefore, we evaluated the indices NBI and NBI_R, which serve as an estimation of nitrogen concentration (Fig. 7). Nitrogen is assimilated by plants and is part of the chlorophyll molecule, thereby strongly linking leaf chlorophyll content to plant nitrogen status (Goffart et al., 2013). In our study, chlorophyll and nitrogen balances indices plotted similar expression curves. For both indices, 'Pinova 10' showed a more sensitive response to water deficit, as those plants were not able to recover their values even after rewatering to field capacity. Interestingly though, the NBI_R was not able to distinguish differences between the two cultivars as much as the NBI.

In this study, different irrigation levels were used to impose water stress conditions in two different apple cultivars. 'Gala Galaxy' demonstrated a better performance than 'Pinova 10' during water stress conditions, indicated especially by increased chlorophyll indices measured non-destructively on certain measurement days throughout the experimental course. It is well known that cultivars can react differently to water stress. Sun et al. (2013) presented similar results, comparing apple scions from the cultivars 'Pink Lady' and 'Quinguan' which were affected differently by drought stress, indicating 'Pink Lady' as more sensitive to drought than 'Quinguan'. Tóth (2011) reported about suppressed yield of 'Pinova' if cultivation conditions are characterised by insufficient water supply or dry and warm habitats. In addition, 'Gala Galaxy' apple trees have lower water requirements, and, as long as they are planted in suitable soil and location, water should not be an issue in keeping trees healthy (Heath, 2017). However, this information is rather wary and therefore, our study suggests for the first-time actual differences in plant performance during water stress with a direct comparison of 'Gala Galaxy' and 'Pinova 10'. In addition, fluorescence-based indices, related to chlorophyll content and nitrogen balance, promise to be a useful non-destructive tool to estimate physiological status of young apple trees submitted to water restriction regimes.

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D Impact of nitrogen fertilisation on chlorophyll content and yield of barley cultivars assessed by fluorescence-based approaches [3]

1 Introduction

Nitrogen (N) is one of the most important chemical elements in plant nutrition, playing a major role in achieving higher crop productivity and yield (Tremblay et al., 2012; Sonnewald, 2014). Biochemically, it can be combined as amine ($-NH_2$) with other elements, i.e. carboxyl (-COOH) functional groups and a specific side chain (R group), to be assimilated into essential amino acids (Lam et al., 1996), which are necessary for forming proteins to trigger plant's growth and development (Silva and Uchida, 2000). Moreover, in chloroplasts, N atoms bind to the atom of magnesium (Mg), constituting a structural integrant of the chlorophyll molecule, which is the vital pigment for plants to absorb light energy and set off light-dependent reactions during photosynthesis (Taiz and Zeiger, 2006).

Nitrogen is considered a key plant fertiliser extensively applied in crop systems. However, its shortage can lead to impairment of leaf elongation by lowering turgor and/or cell wall extensibility and ultimately greatly reduce yield and quality of crops (Zhao et al., 2005). In contrast, N excess stimulates growth of foliage, surpassing flowering, fruiting and/or formation of storage organs such as tubers and roots (Radin et al., 1982; Palmer et al., 1996; Dodd et al., 2002). Excessive nitrogen fertilisation often leads to a reduction in net returns and groundwater contamination through nitrate (NO_3–N) leaching, denitrification, and volatilisation, what is not in lines with sustainable agricultural practices. Because of its mobility in the soil system, it can be found in many forms and it can easily change from one to the other (Leary et al., 2014). Due to this feature, it is well known that this nutrient needs to be replaced whenever it is removed (for example, when the crop is harvested), in order that soil's nutritional balance can be maintained (Goulding et al.,

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2008). Therefore, a balance between supply and utilisation of nitrogen is necessary, not only to optimise crop growth and economic returns, but also to minimise negative environmental impacts.

When important decisions on crop nutritional management are needed, non-destructive methods to assess crop nitrogen status qualitatively appear as a convenient and practical way to evaluate both plant's physiological condition and nutritional requirements in vegetative and pre-generative stages. Amongst several procedures offered currently in precision farming, the employment of portable sensors based on chlorophyll fluorescence (ChlF) seems to be a promising technique to be applied in crop fields. The principle of ChlF is based on the fluorescence emission of the chlorophyll *a* molecule, detected in the red (R) (F680) and far-red (FR) (F730) spectral regions (Briantais et al., 1986; Krause and Weis, 1991). In commercial sensors, fluorescence emissions are induced by the incidence of shorter wavelength electromagnetic signals emitted in the light spectrum, comprehending the region between ultraviolet (UV) and R (Schächtl et al., 2005; Ben Ghozlen et al., 2010; Cerovic et al., 2012). The red fluorescence light is selectively reabsorbed by chlorophyll, whereas the near-infrared fluorescence light is only slightly affected (Agati et al., 1993). The degree of reabsorption is strongly dependent on the chlorophyll concentration (Lichtenthaler and Rinderle, 1988), hence measuring the intensity of R and FR fluorescence light provides information about the chlorophyll content (Schächtl et al., 2005).

In addition, the natural occurrence of phenolic compounds in the leaf epidermis, especially flavonols (Flav), can be detected in the UV-A spectral region. Furthermore, the qualitative ratio of chlorophyll to flavonols (Chl/Flav) can serve as basis to estimate the balance of nitrogen level in leaves, resulting in a new index called nitrogen balance index (NBI) (Buschmann et al., 2000; Campbell et al., 2007; Cerovic et al., 2012). Unlike the traditional methods, such as transmission and reflectance measurements, chlorophyll fluorescence, although having a relatively weaker detected signal, has the advantage of being plant specific by characterising photosynthetic activity (Buschmann, 2007) and exhibiting greater sensitivity, since fluorescence signals only come from the plant (Schmidhalter et al., 2008).

Considering the cultivation of barley (*Hordeum vulgare* L.), fluorescence sensor techniques have been employed to some extent to understand the impact of constraining

growing conditions on the photosynthetic apparatus. Yu et al., (2013) performed the detection of fluorescence by using a portable sensor with light emitting diodes, demonstrating that non-invasive spectral measurements are able to detect mild disease symptoms before significant losses in leaf chlorophyll content appeared in field conditions. In turn, Kalaji & Guo (2008) applied variable ChlF, also called Kautsky kinetics technique (Krause and Weis, 1991), verifying that different ChlF parameters can be used as trustworthy indicators during selection processes of stress tolerant genotypes. In addition, imaging of fast chlorophyll fluorescence induction curve (OIJP) parameters proved to be suitable in screening and phenotyping studies on barley genotypic characterisation (Jedmowski and Brüggemann, 2015). Yet, there is only limited information concerning the employment of fluorescence-based sensors without leaf dark adaption to assess N fertilisation impacts under field conditions. In light of this, the purpose of this study was to verify whether fluorescence sensors can be a helpful non-destructive methodology to early estimate chlorophyll content and the final yield of four barley cultivars subjected to different nitrogen fertilisation levels under field conditions. This study also compares indices from three different sensors to understand whether they display similar trends.

2 Materials and methods

2.1 Plant material, growth conditions and experimental set-up

The experiment was conducted from May to July 2014 at the experimental fields of the Agricultural Faculty at the South-Westphalia University of Applied Sciences, Iserlohn, Germany (51°22′5.52″N, 7°41′12.48″E). The predominant soil in this region is stagnosol with the main compound being mid clay silt (80-85 %) and a humus content below 2.0 %. During the vegetation period, daily temperatures varied from 11.5 to 22 °C and relative air humidity from 52 to 96 %. The research area (approximately 0.2 ha) consisted of 96 randomised plots, each of 8.75 m² in size. Before the experiment was performed, maize (*Zea mays* L.) was grown and the residues were incorporated into the soil after corn harvest. Soil analysis taking subsamples was performed for the whole area. Determined mean values for NPK, Mg and pH were as follows: N_{min} = 52 kg/ha, P_2O_2 = 19 mg/100 g

K_2O = 13 mg/100 g, MgO = 6 mg/100 g, pH = 6.3.

For the field experiment, two factors were chosen:

1. Amount of nitrogen application (0, 40 and 80 kg/ha)
2. Barley cultivar ('Beatrix', 'Eunova', 'Sebastiana' and 'Victoriana')

Barley seeds, obtained from CropSense.net* seedbank, were sown into completely randomised plots with eight replicates for each treatment. The crop was cultivated according to the common practice; however, the nitrogen application was altered in the following way: Nitrogen fertilisation was given in the form of calcium ammonium nitrate, with 27 % N in its formulation, in three different levels (0, 40 and 80 kg/ha). Nitrogen was applied according to BBCH-scale (from German "*Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie*"; Witzenberger et al., 1989; Lancashire et al., 1991) between the stage 14 (leaf development) and 21 (beginning of tillering), while the 80 kg/ha treatment group received the remaining portion in a second application between 39 - 49 (booting stage). Plots without N-supply (0 kg/ha) were used as control. Grain harvest was undertaken on August 1st, (142 DAS), which corresponded to the stage BBCH 99 (the harvested product).

2.2 Fluorescence sensors and indices

Fluorescence recordings were taken at 65, 75, 85 and 110 DAS, corresponding to the BBCH stages in between 37 - 39, 49 - 51, 51 - 59 and 59 - 61, respectively. Three different non-sample destructive and non-sample dark-adapted sensors for instant fluorescence assessment were used for the measurements, as presented and described as follows.

The handheld sensor Dualex®4 scientific (Force-A, Orsay, France) combines the use of fluorescence and light transmission of a leaf to evaluate physiological and biochemical parameters (Cerovic et al., 2012). It determines the optical absorbance of the leaf epidermis in the ultraviolet (UV) range through the differential measurement of the chlorophyll fluorescence and can also estimate the chlorophyll content of the leaf using different wavelengths in the R and in the FR region. For each plot and evaluation date, 10 flag leaves were measured and averaged and the following indices reported: Chl (called 'Chl_Index' in this study to avoid misunderstanding with the quantitative leaf

chlorophyll content), NBI and Flav (here called Flav_Dx to avoid misunderstanding wit Flav_Mx from Multiplex®).

The portable multiparametric fluorescence sensor Multiplex®3 (Force-A, Orsay, France) used in this work has been described in previous studies (Leufen et al., 2014*b*; Leufen et al., 2013; Bürling et al., 2011). Briefly, light emitting diodes (LED) excite the fluorescence at three high energy excitation channels, i. e. at 375 nm (UV), 518 nm (green) and 630 nm (red), while the plant emitting fluorescence was detected in the red (RF: 680–690 nm) and far-red (FRF: 720–755 nm) spectral regions. A grid was used in front of the optical unit to enable the illumination of an area of approximately 5.10-3 m^2 by maintaining a constant distance of 0.10 m between the equipment and the leaf surface. The measurements were taken in each plot, with one measurement in 'one-shot-mode' per plot, and the indices SFR_R, NBI_R, and Flav_Mx were selected to be reported.

The fluorescence sensor MiniVeg-N (Fritzmeier Umwelttechnik GmbH, Großhelddendorf, Germany) is an active optical sensor using the measuring principle of laser-induced chlorophyll fluorescence (LICF). Core of the device is an internal laser diode (red light laser - R), inducing the chlorophyll molecules in plant cells to emit fluorescence light. The intensity of fluorescence light is detected with highly sensitive optical components at the wavelengths of 690 nm (red - R; F690) and 730 nm (far-red - FR; F730) and the vegetation index ratio is calculated (F690/F730) (Schmidhalter et al., 2008). In this study, the vegetation index ratio was inverted, i.e. F730/F690, to be directly correlated with Chl_Index and SFR_R. In the field, the equipment was mounted onto a pushcart, the way that its two measuring heads, one left- and the other right-sided, attached to a rod, could be driven along the rows between the plots while the fluorescence signals emitted by the leaves could be recorded in continuous mode.

Further information about the indices and their corresponding formula analysed in this study can be found in Table 1.

2.3 Leaf chlorophyll concentration

Chlorophyll concentration was determined wet-chemically at 110 DAS. For this purpose, 20 flag leaves of each plot were collected and cold-transported to the laboratory, immediately frozen (-20 °C ± 2), freeze-dried, ground and stored in the dark at room

Table 1 Fluorescence indices recorded by hand-held and implement-mounted sensors used for screening plant physiological status

Index	Sensor	Description	Formula
Chl Index	Dualex®	Chlorophyll content estimation	$FRT_1 - RT_2$/ RT
SFR_R	Multiplex®	Simple Fluorescence Ratio (red light excitation)	FRF_R_3/RF_R_4
F730/F690	MiniVegN	Chlorophyll estimation ratio	FRF_R/RF_R
NBI	Dualex®	Nitrogen Balance Index	Chl Index/Flav_Dx
NBI_R	Multiplex®	Nitrogen Balance Index (red light excitation)	FRF_UV_5/RF_R
Flav_Dx	Dualex®	Epidermal flavonol content	Log FRF_R/FRF_UV
Flav_Mx	Multiplex®	Epidermal flavonol content	Log (FER_UV_6)

[1]: FRT = far-red transmission

[2]: RT = red transmission

[3]: FRF_R = far-red fluorescence with red excitation light

[4]: RF_R = red fluorescence with red excitation light

[5]: FRF_UV = far-red fluorescence with UV excitation light

[6]:FER_UV = fluorescence excitation ratio with red and UV excitation lights

temperature (20 °C ± 5). Chlorophyll concentration was determined colorimetrically according to the method of Holden (1976) as well as Strobl and Türk (1990). Briefly, 5 ml of methanol was added to 50 mg of the dried and ground sample, mixed and centrifuged at 4.000 rpm for 15 min (Varifuge 3 OR, Heraeus Sepatech GmbH, Hanau, Germany). The supernatant was then decanted into 50 ml flasks and the pellet was extracted three more times until the extract was colourless. The collected supernatant was filled up 50 ml with methanol. Absorbance of the extracts was measured with a UV-VIS spectrophotometer (Perkin-Elmer, Lambda 35, Waltham, MA, USA) at 650 nm and 665 nm. The following equations were used to calculate the concentrations of chlorophyll a (Chl *a*), chlorophyll b (Chl *b*) and total chlorophyll (Chl *a* + *b*):

Chl *a* = [(16.5 × A665) − (8.3 × A650)] × Vol/ DM of sample material

Chl *b* = [(33.8 × A650) − (12.5 × A665)] × Vol / DM of sample material

Chl *a* + *b*= [(25.5 × A650) + (4 × A665)] × Vol / DM of sample material

Where:

A650 = absorbance at a wavelength of 650 nm

A665 = absorbance at a wavelength of 665 nm

Vol = volume of the filtrated material

DM = dry mass of the sample material

For data evaluation and statistical proceeds, we focused on the total leaf chlorophyll concentration, indicated in mg/g of the sampled DM.

2.4 Statistical Analysis

Data was statistically analysed with (SPSS) statistic software (PASW statistics version 23.0, SPSS Inc., Chicago, IL, USA). One-way ANOVA and independent-samples-T-Test for nitrogen fertilisation on 65 DAS, as well as a complimentary Duncan post-hoc analysis was used to compare the mean values. The results are expressed as mean ± standard error (SE), and the level of statistical significance of differences is $p \leq 0.05$. For regression analysis, mean values (of each treatment and fertilisation level) of yield and measured fluorescence indices and leaf chlorophyll content were correlated and the coefficient of determination (R^2), $p \leq 0.05$, is given.

3 Results

3.1 Grain Yield

In all cultivars, the highest nitrogen level (80 kg/ha) resulted in the highest yield, though no statistically significant difference was seen between the two applied doses (40 and 80 kg/ha), apart from ‘Eunova’, where 80 kg/ha resulted in the significantly highest yield (Fig. 1). The cultivar ‘Beatrix’ showed highest yield for all fertilisation levels, with 76.4, 73.1, and 62.3 dt/ha for 80, 40 and 0 kg/ha, respectively. In contrast, ‘Sebastiana’ had the lowest yield, with 70.7, 65.8, and 53.7 dt/ha respectively.

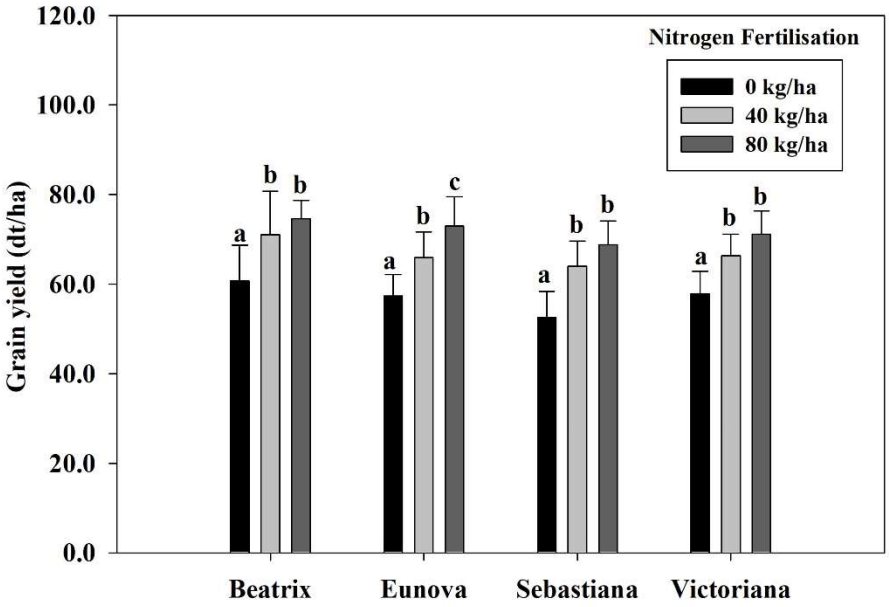

Fig. 1 Grain yield (dt/ha) of summer barley cultivar Beatrix, Eunova, Sebastiana and Victoriana fertilised with different nitrogen levels (0 kg/ha = black; 40 kg/ha = grey; 80 kg/ha = dark grey vertical bar). Means ± SE (n = 8 repetitions) of the same cultivar followed by different letters indicate significant differences between.

3.2 Chlorophyll-content fluorescence indices

All chlorophyll-content-linked fluorescence indices (Chl Index, SFR_R and F730/F690) showed similar tendencies for all cultivars in response to the different N fertilisation levels (Fig. 2). There was no significant difference between the 0 and 40 kg/ha nitrogen treatments on 65 DAS (first measurement day), except for 'Beatrix', where the plants with an applied dose of 40 kg/ha displayed a higher SFR_R expression (Fig. 2). From 75 DAS to 110 DAS, the highest N fertilisation treatment yielded highest indices for all cultivars and evaluated chlorophyll parameters. In general, all four cultivars displayed a decreased tendency for all indices recorded between the end of earing and beginning of blooming (110 DAS) (Fig. 2).

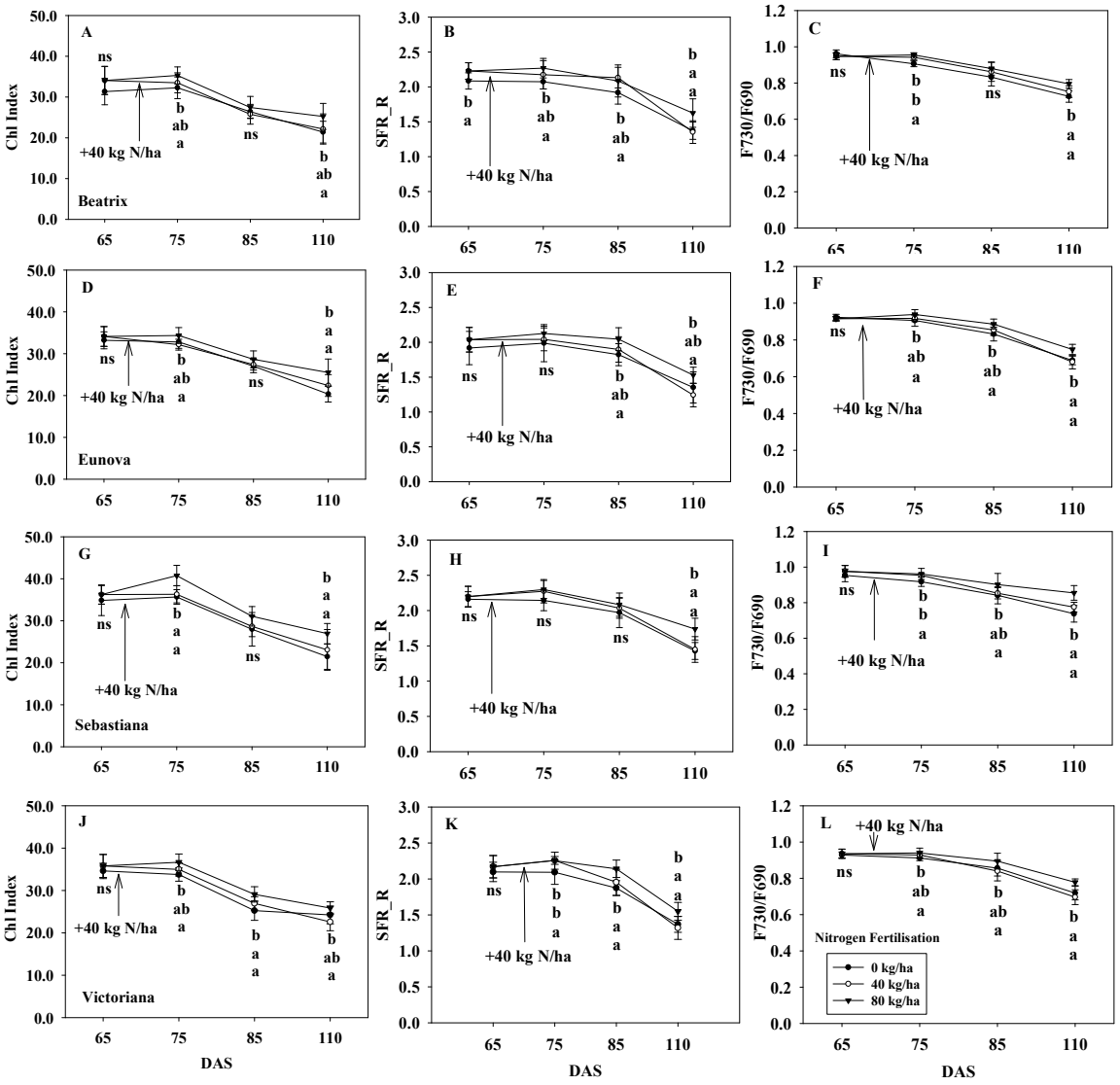

Fig. 2 Chlorophyll indices on four evaluation dates (DAS 65, 75, 85 and 110) recorded via Dualex® (Chl Index), Multiplex® (SFR_R) and MiniVeg-N (F730/F690) of the barley cultivars Beatrix (A, B, C), Eunova, (D, E, F), Sebastiana (G, H, I) and Victoriana (J, K, L) fertilised with different nitrogen levels (0 kg/ha = closed circle; 40 kg/ha = open circle; 80 kg/ha = closed triangle). Means ± SE (n = 8 repetitions) followed by different letters indicate significant differences between treatments, ns indicates no significance (t-test and ANOVA, $p \leq 0.05$) by Duncan Test.

3.3 Nitrogen balance indices

Figure 3 displays the effect of N fertilisation on the nitrogen balance indices (NBI and NBI_R). Both parameters showed decreased values subsequent to 75 DAS for all cultivars and fertilisation levels, though plots that received 80 kg/ha of N fertiliser continued to express slightly higher values than those that received the other doses. Similar to the

chlorophyll indices, nitrogen balance indices were lowest for all cultivars on 110 DAS. Interestingly, the NBI_R showed a sharp decrease from 85 DAS to 110 DAS for all cultivars and also resulted in no significant differences between the three nitrogen treatments at the last measuring date.

3.4 Flavonol indices

Figure 4 shows the evolution of fluorescence signals detected by Dualex® and Multiplex® from epidermal secondary metabolites belonging to the flavonoid-group, the flavonols. Flav_Dx did not reveal any significant differences between the fertilisation treatments for the cultivar 'Sebastiana' on all days measured and 'Victoriana' on 65, 85 and 110 DAS. Even though the other two cultivars showed varying Flav_Dx indices during the recording period depending on the nitrogen regime, there was no significant difference on 110 DAS. Flav_Mx values showed similar time-curves; however, the values between the different nitrogen regimes were more distinguishable.

3.5 Fluorescence Indices x Cultivars

In a similar way to the fluorescence signals evaluated for the nitrogen levels, the four cultivars displayed distinct patterns on the evolution of chlorophyll, nitrogen and flavonol fluorescence indices. Figure 5 presents a decreasing curve for F730/F690, Chl_Index, SFR_R, NBI and NBI_R whilst crop development advances. Although a tendency could not be assumed in all growing phases, the cultivar 'Sebastiana' expressed higher chlorophyll content and nitrogen balance indices (Fig. 5A-E) and lower Flav_Dx on 85 and 110 DAS (Fig. 5F). Together with 'Victoriana', 'Sebastiana' showed lower flavonol fluorescence also on 85 and 100 DAS for Flav_Mx (Fig. 5G). On the other hand, 'Beatrix' and 'Eunova' contrasted with a higher expression of the phenolic index on both evaluated parameters (Fig. 5G-H).

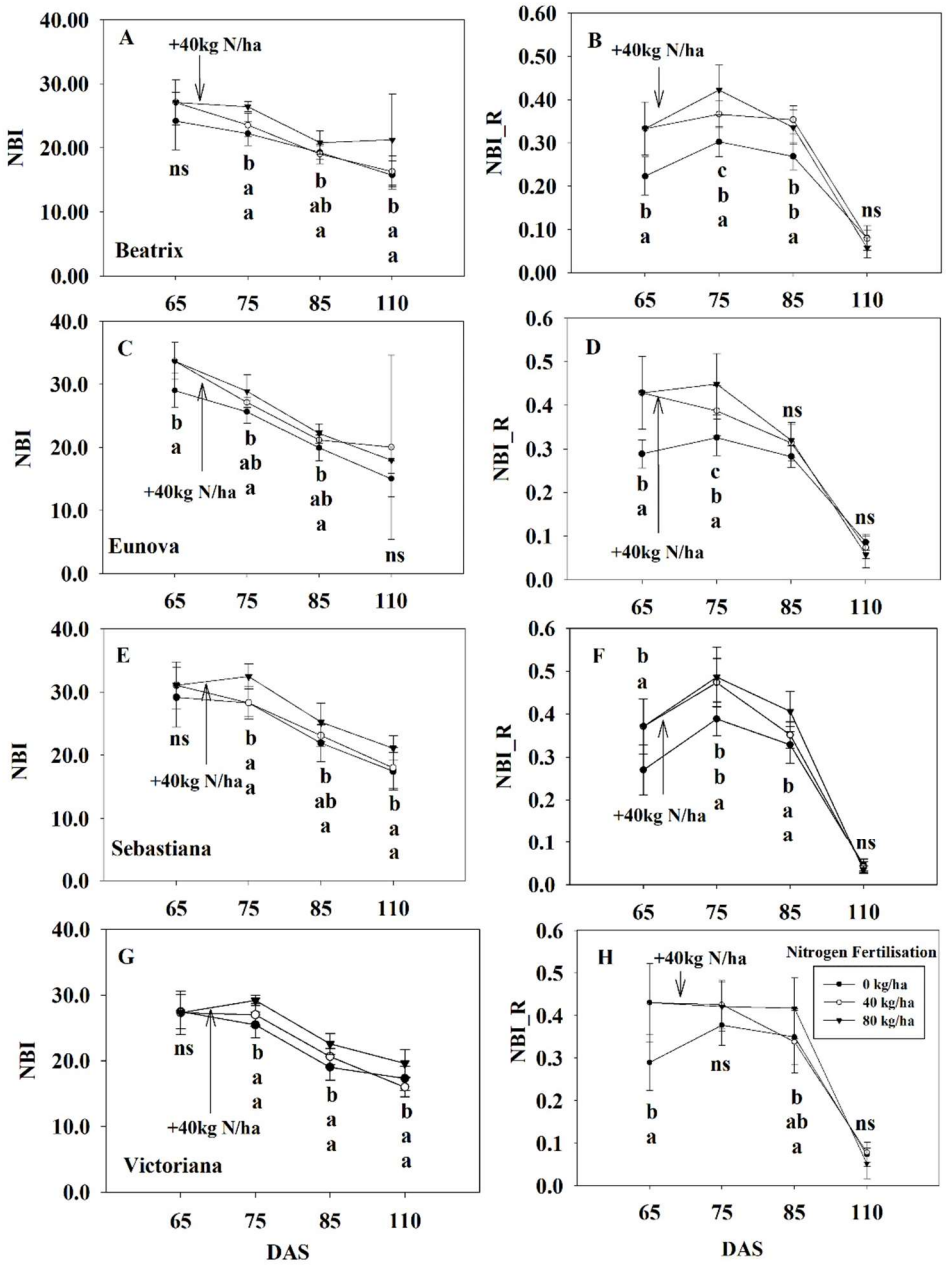

Fig. 3 Nitrogen Balance Indices on four evaluation dates (DAS 65, 75, 85 and 110) recorded via Dualex® (NBI) and Multiplex® (NBI_R) of the barley cultivars Beatrix (A, B), Eunova, (C, D), Sebastiana (E, F) and Victoriana (G, H) fertilised with different nitrogen levels (0 kg/ha = closed circle; 40 kg/ha = open circle; 80 kg/ha = closed triangle). Means ± SE (n = 8 repetitions) followed by different letters indicate significant differences between treatments, ns indicates no significance (t-test and ANOVA, $p \leq 0.05$) by Duncan Test

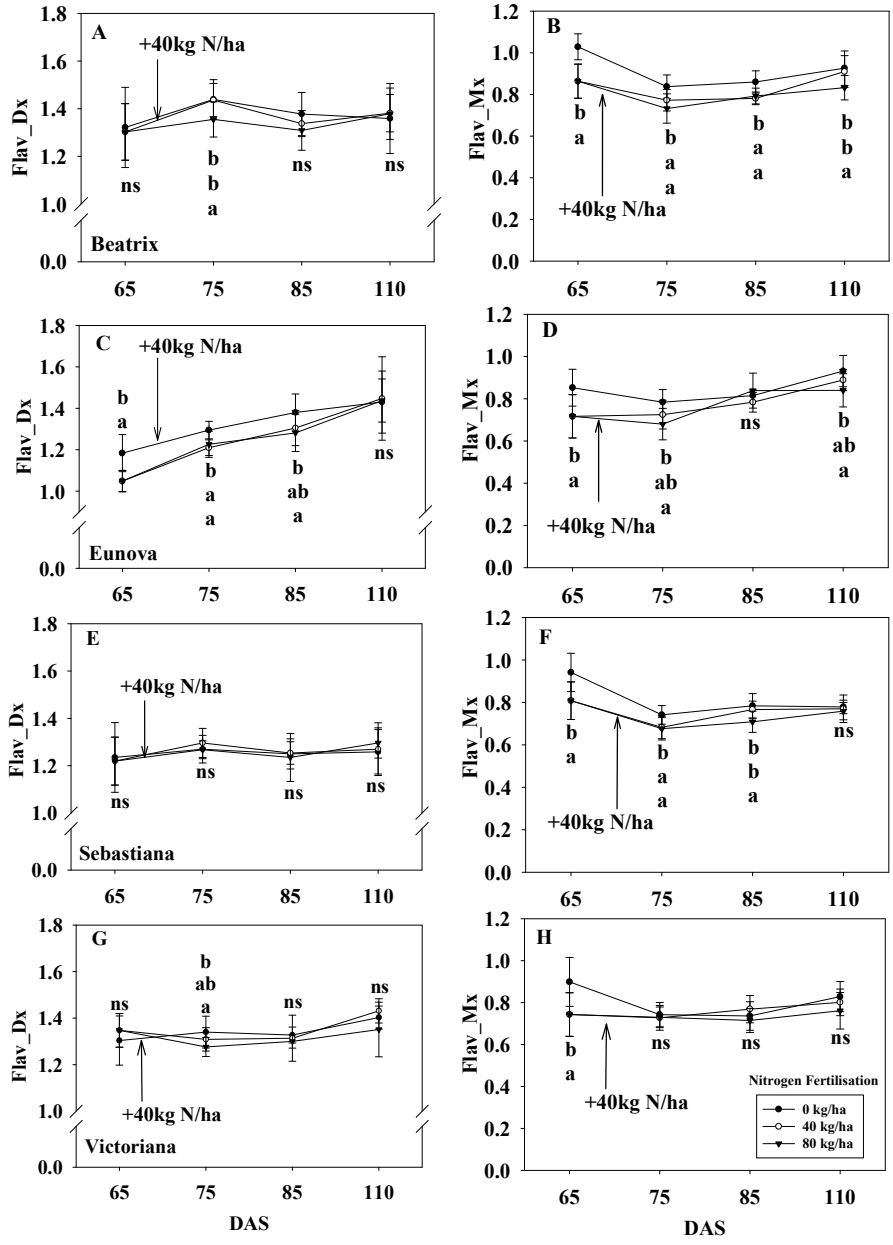

Fig. 4 Flavonol indices recorded with the Dualex® (Flav_Dx and Multiplex®Flav_Mx) devices of the barley cultivars Beatrix (A, B), Eunova, (C; D), Sebastiana (E; F) and Victoriana (G; H) on four evaluation dates (DAS 65, 75, 85 and 110) and influenced by nitrogen fertilisation levels (0 kg/ha = closed circle; 40 kg/ha = open circle; 80 kg/ha = closed triangle). Means ± SE (n = 8 repetitions) followed by different letters indicate significant differences between treatments, ns indicates no significance (t-test and ANOVA, $p \leq 0.05$) by Duncan Test.

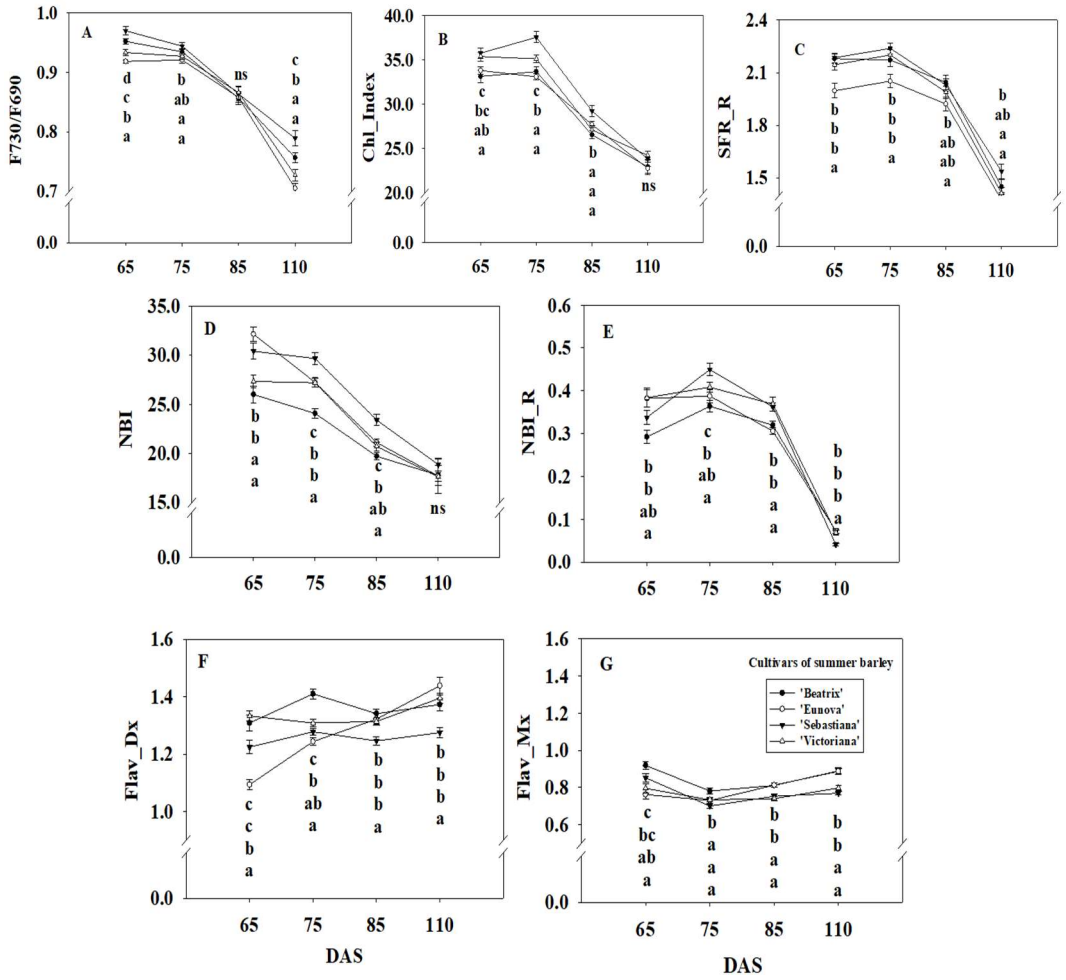

Fig. 5 Chlorophyll, nitrogen and flavonol indices on four evaluation dates (DAS 65, 75, 85 and 110) recorded via MiniVeg-N (A = F730/F690), Dualex® (B = Chl Index, D = NBI and F= Flav_Dx) and Multiplex® (C= SFR_R and E = NBI_R) of the barley cultivars Beatrix = close circle, Eunova = open circle, Sebastiana = close triangle, and Victoriana = open triangle. Means ± SE (n = 8 repetitions) followed by different letters indicate significant differences between treatments, ns indicates no significance (ANOVA, $p \leq 0.05$) by Duncan Test.

3.6 Leaf chlorophyll concentration

The laboratory analysis of chlorophyll content on 110 DAS showed significant differences between N fertilisation treatments, with higher concentrations of the photosynthetic pigment for the highest applied dose (80 kg/ha), even though no statistically significance was portrayed between the two applied doses, apart from ‘Eunova’, where

40 kg/ha did not induce higher chlorophyll accumulation (Fig. 6). There was no significant difference between the evaluated cultivars.

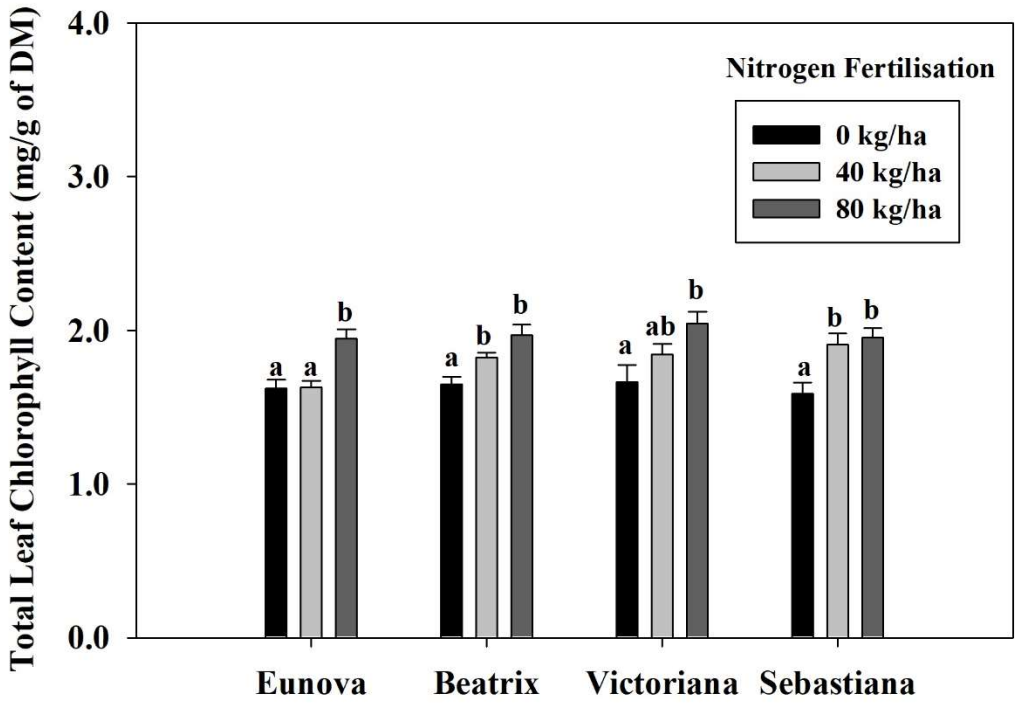

Fig. 6 Chlorophyll content (mg/g of DM) of leaf samples at 110 DAS of the barley cultivars Beatrix, Eunova, Sebastiana and Victoriana fertilised with different nitrogen levels (0 kg/ha = black vertical bar; 40 kg/ha = gray vertical bar; 80 kg/ha = dark gray vertical bar). Means ± SE (n = 8 repetitions) followed by different letters indicate significant differences between treatments within the cultivar (ANOVA, $p \leq 0.05$) by Duncan Test.

3.7 Linear model between chlorophyll content and grain yield

Final grain yield (142 DAS) and leaf chlorophyll content (110 DAS) strongly correlated (R^2 = 0.7) (Fig. 7). In contrast, only low correlations were seen between chlorophyll indices and grain yield for different measuring days: F730/F690 on 75 and 85 DAS (R^2 = 0.53), SFR_R on 85 DAS (R^2 = 0.55), and Chl Index on 110 DAS (R^2 = 0.43). In addition, neither flavonol indices (Flav_Dx and Flav_Mx) nor nitrogen balance indices (NBI and NBI_R) from any measuring day showed significant correlation with the final

grain yield (Table 2).

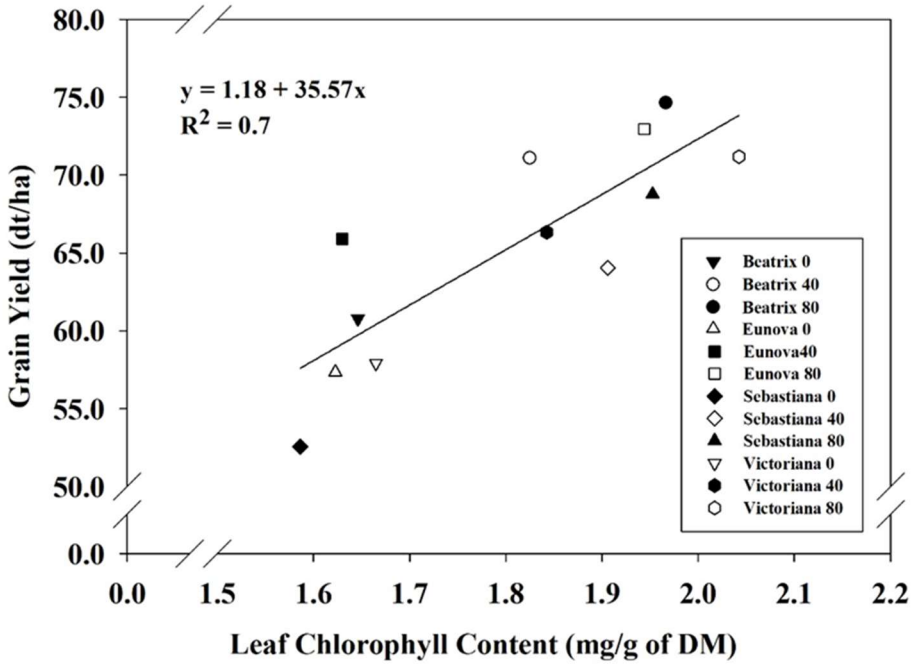

Fig. 7 Coefficient of determination between chlorophyll content (mg/g of DM) at 110 DAS (days after sowing) (BBCH 59 – 61) and grain yield (dt/ha) at 142 DAS (BBCH 99) of four barley cultivars fertilised with different nitrogen (N) doses. Data are means ± SE of 8 repetitions. Regression line obtained from coefficient of determination ($p \leq 0.05$).

4 Discussion

In this study, the effect of different nitrogen fertilisation levels was reflected in leaf chlorophyll content and in grain yield values, as dosage increments were responsible for higher chlorophyll content and productivity. This response of crop production to N supply is in accordance with other studies, furthermore proving the importance of the nutrient as a major constituent of the Chl molecule, whose supply can trigger an accumulation of the photosynthetic pigment in plants, thereby influencing yield (Bredemeier and Schmidhalter, 2002; Güler, 2009; Alazmani, 2015; Ben Abdallah et al., 2016).

Table 2 Coefficient of determination (R^2) of chlorophyll, nitrogen, and flavonol indices (75, 85 and 110 DAS), leaf chlorophyll content (mg/g DM) (110 DAS) and final grain yield (dt/ha) of barley (142 DAS). Data recorded by fluorescence sensors on four barley cultivars ('Beatrix', 'Eunova', 'Sebastiana', and 'Victoriana') fertilised with three nitrogen (N) doses (0, 40 and 80 kg/ha).

R^2		Days after seeding (DAS)	
Parameters	75	85	110
Fluorescence indices		Grain yield (dt/ha)	
Chlorophyll			
Chl Index	0.04	0.09	0.43*
SFR_R	0.28	0.55*	0.21
F730/F690	0.53*	0.53*	0.23
Nitrogen			
NBI	0.05	0.07	0.28
NBI_R	0.24	0.18	0.03
Flavonol			
FlavDx	0.00	0.02	0.07
FlavMx	0.15	0.01	0.01
Chlorophyll content (mg/g of DM)			0.70*

* Significant correlation at level 0.05

Chl_Index, SFR_R and F730/F690 are chlorophyll fluorescence indices considered as indicators of leaf chlorophyll content status. The recorded chlorophyll fluorescence signals expressed by these indices are all induced by incidence of light excitation in the R spectral region (Gitelson et al., 1999; Ghozlen et al., 2010; Cerovic et al., 2012; Schmidhalter et al., 2008). This incident light is transmitted to the mesophyll of the leaf, where the chlorophyll molecules are present at most (Tremblay et al., 2012). Nitrogen is assimilated by plants and integrates the chlorophyll molecules, so that the content of this leaf pigment is strongly related to plant nitrogen status. In this study, all evaluated FRF/RF indices yielded higher values with increased N dosages. Comparing these results with the total chlorophyll content at 110 DAS, those indices seem to provide a valid non-

destructive prediction of plant chlorophyll status based on the N fertilisation levels already established on 75 DAS. However, the differentiation between the barley cultivars, possibly due their similar growth and developing habit. On the other hand, this feature can also be positively scored, since these indices may be a useful instrument to estimate leaf chlorophyll status on any barley cultivar. A similar tendency was also found by Mauromicale et al. (2006), who studied chlorophyll fluorescence and chlorophyll content in field grown potato (*Solanum tuberosum* L.) as affected by nitrogen supply, genotype- and plant age, establishing a positive linear correlation between nitrogen supply, chlorophyll fluorescence signals and chlorophyll content. Schächtl et al. (2005), employing a laser induced chlorophyll fluorescence sensor for detecting N status on wheat canopies, were also able to show a clear differentiation between N treatments as early as the beginning of stem elongation (BBCH30).

Additionally, flavonol indices were tested as indicators of plant nitrogen balance. Flavonoids are a large family of polyphenolic compounds found in plant tissues, the main group being the flavonols, that can increase in leaves in response to nitrogen stress (Stewart et al., 2001). Although Flav_Dx and Flav_Mx increased during the course of the vegetative and pre-generative stages of barley, no significant differences between different fertilisation levels were seen. This was further proven by a weak coefficient of determination between those indices and final yield, suggesting that they are not completely suitable to be used as springboard to take decisions on crop nitrogen fertilisation.

Ben Abdallah et al. (2016) reviewed the utilisation of ChlF for crop nitrogen status, whereby they compared variable chlorophyll fluorescence (Pulse-Amplitude-Modulation technique – PAM) and chlorophyll fluorescence sensing methods (the same as employed in the present study) for the estimation of concentrations of leaf metabolite compounds in plants, i.e. leaf chlorophyll and flavonoids. The authors emphasised that fluorescence ratios (RF/FRF and FRF_UV/FRF_R), estimating the content of chlorophyll and flavonoids, can also be an indicator of plant N status (NBI). In our study, nitrogen balance indices (NBI and NBI_R) displayed similar tendencies compared to Chl indices, where plots without receiving any nitrogen fertilisation expressed lowest indices values for plant nitrogen balance.

5 Conclusion

Similar curve tendencies were observed for all evaluated fluorescence indices irrespectively of the employed sensor. Fluorescence-based approaches could substantially screen the effect of nitrogen fertilisation reflected by higher chlorophyll (F730/F690, Chl_Index and SFR_R) and nitrogen balance (NBI and NBI_R) indices at plots that received the higher fertiliser dosage - a pattern, subsequently, displayed on quantitative chlorophyll content and final grain yield. Thus, this assertion was discernible for those fluorescence indices, though performing low to moderate final yield predictions in some specific measuring events. Flavonol indices, in turn, were less precise in this prediction model, though a tendency could be observed in some stages, when higher Flav_Dx and Flav_Mx values figured on non-fertilised plots. In a similar way, the four cultivars did not ascertain a reliable prediction model, once leaf chlorophyll content and grain yield were not statistically distinct between cultivars, differently from the fluorescence indices. A predominately non-significant interaction between both treatments during the evaluation stages could explain the lower cultivar effect on the final outcomes. In addition, leaf chlorophyll content presented a strong correlation with the final grain yield. However, fluorescence-based indices presented here are initially rather wary and they should in principle be considered in studies to distinguish the effect of nitrogen fertilisation levels. Moreover, further studies might concentrate on more cultivars, field conditions, cultivation years, as well as fluorescence models, where other adapted indices should also be taken into account.

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E Summary

This study surveyed the potential and limitations of the use of fluorescence sensors to detect physiological adaptations to external factors in one perennial (*Malus domestica* Borkh.) and one annual (*Hordeum vulgare L.)* crop. Thereby, responses to abiotic factors such as light quality, water supply and nitrogen fertilisation were studied. The results of the single chapters can be summarised as follows:

1. Considering the influence of water shortage on plants exposed to different light quality – provided by light emitting diodes (LED) and compact fluorescence lamps (CFL) - on the physiological and biochemical status of apple seedlings: Results demonstrate that watering regimes had higher impact on biochemical indicators than light sources. Lower Chl concentration was determined on plants growing under LED, both on control and water deficit stressed plants. Considering fluorescence indicators, NBI and NBI_R showed similar pattern to leaf Chl in relation to light source, with higher efficiency of CFL. Flav was higher in plants cultivated under LED. Stomatal conductance (Gs) and maximal photochemical efficiency (Fv/Fm) revealed similar tendencies, demonstrating the effect of illumination with higher responsiveness of LED plants. Particular attention should be given to fluorescence indices related to nitrogen status and flavonol content as promising parameters to sense physiological impairments under the given conditions.
2. On young apple threes, ‘Gala Galaxy’ showed higher tolerance to water deficit stress than ‘Pinova 10’, expressed especially by increased chlorophyll fluorescence indices. Fluorescence indices related to chlorophyll content (Chl_Index and SFR_R) and nitrogen balance (NBI and NBI_R) showed similar curves. However, leaf chlorophyll analysis performed wet-chemically was not a reliable indicator of water stress in apple trees. Leaf water potential was affected during complete watering withholding, without significant differences between the cultivars.
3. Investigations on the impact of nitrogen fertilisation on chlorophyll content and yield of barley cultivars indicate that highest chlorophyll content at 110 DAS (days after sowing) as well as highest yield at 142 DAS were observed in all cultivars with 80 kg ha^{-1} nitrogen fertilisation. The final yield correlated strongly with leaf chlorophyll concentration (R^2=0.7), whereas yield and chlorophyll estimated

via fluorescence had a slight to medium correlation. Irrespectively of cultivars, fluorescence indices estimating chlorophyll content (Chl_Index, SFR_R and F730/F690) could serve as an early prediction of final yield, whereas indices estimating flavonol and nitrogen status (Flav_Dx, Flav_Mx, NBI and NBI_R) were less precise.

In summary, the results obtained in this study sustain the potential of the multiparametric fluorescence indices for an instant qualitative *in situ* detection of abiotic stresses at leaf level in apple, i.e. seedlings and two-years-old plants, cultivated, respectively, in climate chamber and greenhouse, and summer barley, cultivated in the field. Among the variety of detected and calculated fluorescence indices, we were able to select specific parameters, that more suitably reflected plant responses to light sources, restriction of water supply and nitrogen fertilisation according to the purposes of each specific trial, with emphasis on indices related to chlorophyll content, nitrogen balance and phenolic compounds.

However, based on the analysis and interpretation of data, some accuracy limitations could occasionally be verified when early symptoms of abiotic stresses were already visible on plants, but not reliable reproduced by the indices. Alterations on fluorescence indices related to chlorophyll content could quite often only be verified on young apple trees when plants presented lower water potential and visual symptoms of wilting. Furthermore, although acquisition costs still represent a substantial accessibility limitation for many growers, increasing investments in research and development of more accurate devices and launch of novel prototypes, have continuously been triggering new demands and consequently leading to a decline on commercial prices, which in turn indicates a forthcoming higher diffusion and acceptance of portable fluorescence sensors, together with the necessity of more environmentally friendly and precise practices in the agriculture worldwide.

On that note, prospective studies should also turn efforts into the creation of a wide-ranging database to continuously promote and improve the differentiation between cultivars with different characteristics of susceptibility and tolerance to abiotic stress factors. Optimally, such a system would provide accurate information on plant physiology already on early stress stages and without the need to measure control plants as a reference. In addition, changes in the composition pattern of biochemical compounds in the affected leaves should correlate with indices based on non-destructive fluorescence,

which is essential for the interpretation of the changes in the indices. Support vector machines, further developments in sensor technology and their combination could also lead to a fast and stable determination of complex fluorescence parameters, which are to be included in high-performance screening systems.

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