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Fluorescence-based systems for detection of abiotic stresses on horticultural crops

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Fluorescence-based systems for detection of abiotic stresses on horticultural crops

The main objective of this thesis was to assess the impact of economically important abiotic stresses on the plant physiology using non-destructive fluorescence indices, and to evaluate the potential use of the sensor techniques as supporting tool for plant phenotyping in horticulture. The early detection of water deficiency and salinity was studied at leaf level in tomato (*Solanum lycopersicum* L.) plants by means of non-destructive fluorescence techniques. Evaluations comprised multiparametric fluorescence indices and pulse-amplitude modulated (PAM) chlorophyll fluorescence parameters for an effective and rapid sensing of water deficiency stress and stress recovery in three tomato genotypes. In addition, the impact of salinity on tomato genotypes was also studied using both methods. In the next step, the response of tomato genotypes exposed to simultaneous occurrence of salinity and water deficiency was examined by multiparametric fluorescence indices. An additional objective of the work was to investigate the suitability of chemically induced osmotic stress by polyethylene glycol (PEG) for drought stress experiments based on key physiological parameters of apple (*Malus domestica* Borkh.) leaves. The results of the single chapters can be summarized as follows:

1. Multiparametric fluorescence indices and PAM fluorescence imaging were adopted for an effective and rapid sensing of water deficiency stress and recovery capability in three tomato genotypes. The multiparametric fluorescence indices were selected for the evaluations since they enable faster sensing of water deficiency without the need of dark-adaption as required for the PAM recordings. The results of this study indicate that the multiparametric indices are one reliable tool for the early detection of drought impact on tomato plants. The combination with the obtained PAM parameters allows a better estimation of impairments in the primary and secondary plant metabolism.
2. Compared with the PAM method, multiparametric fluorescence indices provide an effective and timely technique for the *in situ* sensing of salt stress in plants. UV light-induced blue fluorescence to far-red fluorescence and green light-induced far-red fluorescence to red fluorescence were the most sensitive indices for the rapid sensing of salinity. Moreover, the temporal development of the indices was in accordance with the concentrations of Na, proline and chlorophyll in the leaves, parameters well-known for salt tolerance. The selected indices might be used as a tool to evaluate genotypes for salt tolerance.
3. Using multiparametric fluorescence indices allowed detecting the simultaneous occurrence of salinity and water deficiency in tomato plants within eight days after treatment induction. The modification pattern in the complex parameters was principally caused by differences in the chlorophyll concentration and the functionality of the electron flux and less by an accumulation of blue fluorescing pigments in the leaves.
4. As compared to drought, chemically-induced osmotic stress in hydroponic solutions with different PEG 6000 concentrations revealed similar impact on relative water content and chlorophyll content in leaves of apple seedlings. In contrast, strong discrepancies were observed between net photosynthetic rate, indices of the multiparametric fluorescence technique, proline concentration and the leaf thickness. Thus, when using PEG, the appropriate concentration of PEG as well as the target parameters should be tested and defined on basis of preliminary experiments. Due to mismatch in biochemical, physiological and morphological parameters caused by PEG in hydroculture and drought in soil cultivation, PEG might be used with care to induce drought-like physiological changes, but it cannot be considered as an unconditional equivalent for natural drought, particularly in long-term studies.

Fluoreszenzbasierende Systeme zur Erkennung von abiotischem Stress in gartenbaulichen Kulturen

Ziel dieser Arbeit war es, das Potential ausgewählter fluoreszenzbasierter Indizes für die Erfassung der Pflanzenreaktion auf ökonomisch bedeutende abiotische Stressfaktoren zu ermitteln. Zudem sollte evaluiert werden, inwiefern sich diese Technologie für die Pflanzenphänotypisierung im Gartenbau eignet. Dazu wurden nicht-destruktive Fluoreszenztechniken zur Früherkennung von Wassermangel und Salinität bei Tomatenpflanzen (*Solanum lycopersicum* L.) auf Blattebene getestet. Die Evaluierung umfasste multiparametrische Fluoreszenzindizes und Puls-Amplituden-modulierte (PAM) Chlorophyllfluoreszenzparameter, untersucht sowohl in der Wassermangelphase als auch in der darauf folgenden Erholungsphase an drei Tomatengenotypen. Diese Methoden wurden ebenfalls für die Untersuchung der Tomatengenotypen auf deren Salztoleranz verwendet. Darüber hinaus wurde ermittelt, wie sich das zeitgleiche Auftreten der Stressfaktoren Salinität und Wassermangel auf die Fluoreszenzindizes auswirkt. Als ein weiterer Aspekt der Studie wurde die Eignung von Polyethylenglycol (PEG) als osmotisch aktive Substanz zur Induktion von Trockenstress an Apfelblättern (*Malus domestica* Borkh.) evaluiert. Die Ergebnisse der einzelnen Kapitel werden nachfolgend zusammengefasst:

1. Die multiparametrischen Fluoreszenzindizes und die PAM-Fluoreszenz wurden sowohl für die Erkennung von Wassermangel als auch für die Ermittlung der Erholungsfähigkeit bei drei Tomatengenotypen getestet. Gegenüber den PAM-Messungen erwiesen sich die Indizes aufgrund der effizienteren und schnelleren Erfassung des Wassermangels als geeigneter. Es konnte bestätigt werden, dass ausgewählte Fluoreszenzindizes ein zuverlässiges Instrument für die schnelle Detektion von Wassermangel an Tomatenpflanzen sind. Die Kombination mit den PAM-Parametern ermöglichte allerdings eine bessere Bewertung der entstandenen Einschränkungen für den primären und sekundären Pflanzenmetabolismus.
2. Verglichen mit der PAM-Methode, stellen die multiparametrischen Fluoreszenzindizes eine effektive und auch zügig durchzuführende Technik zur *in situ* Erkennung von Salzstress in Pflanzen dar. Die sensitivsten Indizes für die frühe Erkennung von Salinität waren die durch UV-Licht induzierte blaue Fluoreszenz zu dunkelroter Fluoreszenz und mit grünem Licht induzierte dunkelrote Fluoreszenz zu roter Fluoreszenz. Die zeitliche Entwicklung der Indizes entsprach dabei den Konzentrationen von Na, Proline und Chlorophyll im Blatt, die als Parameter für Salztoleranz bekannt sind. Somit könnten die ausgewählten Indizes sich als ein hilfreiches Werkzeug zur Bewertung verschiedener Genotypen hinsichtlich Salztoleranz herausstellen.
3. Mit dem Einsatz von multiparametrischen Fluoreszenzindizes konnte das gleichzeitige Auftreten von Salinität und Wassermangel in Tomatenpflanzen innerhalb von acht Tagen nach Stressbeginn nachgewiesen werden. Änderungen dieser komplexen Parameter waren maßgeblich auf Veränderungen der Chlorophyllkonzentration und der Funktionsweise des Elektronenflusses zurückzuführen und weniger auf eine Akkumulation blau-fluoreszierender Pigmente in den Blättern.
4. PEG-induzierter Stress hatte ähnliche Auswirkungen beim relativen Wasser- und Chlorophyllgehalt der Blätter von Apfelsämlingen zur Folge wie natürliche Trockenheit. Große Unterschiede wurden hingegen bei der Nettophotosyntheserate, den multiparametrischen Fluoreszenzindizes, der Prolinkonzentration und der Blattdicke festgestellt. Aufgrund der Diskrepanzen bei den biochemischen, physiologischen und morphologischen Parametern zwischen PEG induziertem Stress und Trockenheit sollte PEG mit Bedacht verwendet werden, wenn es darum geht, physiologische Veränderungen hervorzurufen. Insbesondere bei Langzeitstudien kann PEG nicht als uneingeschränktes Äquivalent zur natürlichen Trockenheit angesehen werden.

Table of Contents

Inhalt

List of abbreviations

A	absorbance
ABA	abscisic acid
ANOVA	analysis of variance
AOI	area of interest
BF	blue fluorescence
BFRR_UV	blue-to-far-red fluorescence ratio
BGF	blue-green fluorescence
C	control plants
Ca^{2+}	calcium
CAT	catalase
CCD	charged-coupled-device
Chl	chlorophyll
ChlF	chlorophyll fluorescence
Cl^-	chloride
cm	centimeter
cm^2	square centimeter
CO_2	carbon dioxide
Cupido	*Solanum lycopersicum* L. F1 hybrid Cupido
cvs	cultivars
°C	degree Celsius
DAT	day(s) after treatment initiation
DM	dry matter
dm	decimeter
e.g.	exempli gratia/ for example
EC	electrical conductivity
ETR	electron transport rate
FAO	Food and Agriculture Organization of the United Nations
Fig.	figure
FLAV	Flavonol-Index, logarithm of the ratio of red-excited far-red fluorescence to the ultra-violet-excited far-red fluorescence
FM	fresh matter
Fm	maximum fluorescence from dark-adapted leaf
Fm'	maximum fluorescence from light-adapted leaf
Fo	round/minimal fluorescence from dark-adapted leaf
Fo'	ground/minimal fluorescence from light-adapted leaf
Fv	variable fluorescence from dark-adapted leaf
FRF	far-red fluorescence
g	force of gravity
g	gram
GF	green fluorescence
GmbH	Gesellschaft mit beschränkter Haftung
h	hour
H-2274	*Solanum lycopersicum* L. variety H-2274
H^+	hyrdogen
H_2O	water
H_2O_2	hydrogen peroxide
Harzfeuer	*Solanum lycopersicum* L. F1 hybrid Harzfeuer
i.e.	id est/ that is
Inc.	incorporated

K^+	potassium
kg	kilogram
l	liter
LED	light-emitting diode
m	meter
Mg^{2+}	magnesium
min.	minutes
ml	milliliter
mm	millimeter
mmol	millimole
MPa	megapascal
mS	millisiemens
µl	microliter
µm	micrometer
µmol	micromole
n	number of replications
N	nitrogen
Na^+	sodium
NaCl	sodium chloride
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	reduced form of nicotinamide adenine dinucleotide phosphate
NBI_G	ratio of UV-excited far-red fluorescence to the green-excited red fluorescence
NBI_R	ratio of UV-excited far-red fluorescence to the red-excited red fluorescence
nm	nanometer
NO_3^-	nitrate
NPQ	non-photochemical quenching
n.s.	not significant
%	percent
O_2	oxygen
$O_2^{\bullet-}$	superoxide radical
P	probability of error
p.	page
PAM	pulse-amplitude-modulated chlorophyll fluorescence
PAR	photosynthetically active radiation
PEG	polyethylene glycol
P_N	net photosynthetic rate
pp.	pages
PPFD	photosynthetically active photon flux density
ppm	parts per million
PSII	photosystem II
Q_A	primary quinone electron acceptor of photosystem II
qL	the coefficient of photochemical quenching based on a ‘lake model’
qP	coefficient of photochemical quenching based on a ‘puddle model’
RF	red fluorescence
Rio Grande	*Solanum lycopersicum* L. var. Rio Grande
ROS	reactive oxygen species
rpm	revolutions per minute
RWC	relative water content
s	second
S1	well-watered plants cultivated in substrate

S2	plants exposed to drought cultivated in substrate
SE	standard error
SFR_G	far-red fluorescence to red fluorescence ratio after green light excitation
SFR_R	far-red fluorescence to red fluorescence ratio after red light excitation
SOD	superoxide dismutase
T1	control plants
T2	well-watered plants with nutrient solution amended with sodium chloride
T3	water deficiency (50% of the nutrient solution)
T4	water deficiency (50% of the nutrient solution amended with sodium chloride)
TM	turgid mass
UV	ultra-violet
V	volume
var.	variety
vol. %	volume percent
WD	water deficit
Ψ	water potential
Ψ_π	osmotic potential

A Introduction

1 Abiotic constraints in horticultural production

During their lifetime, plants are frequently exposed to several adverse situations impairing growth and development, commonly expressed as stress factors. Depending on their origin, these stresses might be classified as biotic or abiotic stress factors. Examples of biotic stresses are insects and pathogens while abiotic stresses comprise cold, heat, salinity or water deficiency (Lichtenthaler 1996). Particularly water deficit and salinity affect plant physiology and agronomic performance in important horticultural regions worldwide. This situation becomes even more complex when considered that horticultural crops have high water consumption, and crops such as tomato (*Solanum lycopersicum* L.) and apple (*Malus domestica* Borkh.) are in general susceptible to abiotic stresses (Maas 1986).

Drought tolerance in tomatoes depends strongly on the species. According to the newest taxonomical classification, tomatoes consist of twelve wild species and the cultivated *Solanum lycopersicum* L. (Fischer et al. 2011). For example, *Solanum chilense*, a wild type growing in the Atacama Desert, is much better adapted to water deficit conditions than *Solanum lycopersicum* L. (Loyola et al. 2012). However, even within the species *Solanum lycopersicum* L. cultivars might be classified from susceptible to tolerant to water shortage (Achuo et al. 2006, Sánchez-Rodríguez et al. 2010, Sánchez-Rodríguez et al. 2011a, Sarlikioti et al. 2010). In many cases, also in tomato and perennial fruit crops such as apple, grafting is adopted to overcome specific stresses. In apple, rootstocks can significantly increase the tolerance to water deficit (Liu et al. 2012, Schwarz et al. 2010). For this reason, the rootstocks are more important for classification than the cultivar they are grafted onto. For example, the rootstock *Malus sieversii* was shown to be less drought-sensitive than *Malus hupehensis* (Liu et al. 2012).

Horticultural crops in most cases are glycophytes and might be classified into sensitive, moderately sensitive, moderately tolerant, or tolerant to salinity (Greenway and Munns 1980, Katerji et al. 2001, Maggio et al. 2004, Munns and Tester 2008). In comparative studies, depending on the experimental design including conditions and evaluated cultivars, tomato (*Solanum lycopersicum* L.) might be classified as sensitive (Dasgan et al. 2002), moderately sensitive (Jenks et al. 2007), or moderately tolerant (Ghanem et al. 2008) to salt stress (Hunsche et al. 2010). The final classification also depends on the target parameters since salt stress affects not only yield-related characters. Salinity impairs almost every aspect of the

plant's physiology and biochemistry. Because of this, it has to be taken into account how tolerance to salinity is defined (Cuartero et al. 2006).

In terms of economy and nutrition, tomato and apple are two of the most significant horticultural crops in the world. The strong increase in production of tomatoes (+39%) and apples (+37%) (FAO 2015) during the last decades underline their economic and social value. Particularly in the Mediterranean region, the performance of these two crops may be impaired by water deficiency and salinity. Due to this, one major goal for improving horticultural crop production is to select cultivars and varieties for better tolerance of these environmental constraints.

1.1 Water deficiency

Water deficiency occurs if the transpiration of aerial parts is higher than the water uptake by the roots. The magnitude and duration to which plants can prevent or buffer this negative impairment depends on the degree of resistance to water shortage (Sánchez-Rodríguez et al. 2010). Because of its essential role in metabolism, any decrease in water availability has an immediate effect (Pugnaire et al. 1999), leading to biochemical, physiological and morphological responses at cellular and whole-plant level (Yordanov et al. 2000).

In addition to the direct effects of water shortage, the stronger accumulation of reactive oxygen species (ROS), e.g. superoxide radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) impair functionality of membranes and metabolic processes. Drought induces a root-to-leaf signaling by abscisic acid (ABA), which is produced in the roots and transported to the aerial plant parts, resulting in stomatal closure to reduce water loss (Ajay et al. 2002). Stomatal closure decreases transpiration, but at the same time decreases internal CO_2 concentration, finally inhibiting the whole photosynthetic process (Biswal and Biswal 1999, Reddy et al. 2004). The accrued imbalance between the generation and the use of electrons leads to the overproduction and accumulation of ROS. Free ROS damage nucleic acids and membranes prompting the oxidation of amino acids and proteins, and attacks photosynthetic pigments such as chlorophylls, carotenoids and xanthophylls (Apel and Hirt 2004, Biswal and Biswal 1999, Liu et al. 2012, Smirnoff 1993).

These photosynthetic pigments serve as antenna (light harvesting complex) of the photosynthetic apparatus and are the primary initiators of energy transduction in the photosynthesis process by absorbing light and transferring its energy to the reaction centers (Krause and Weis 1991). Under optimal conditions, more than 90% of the absorbed light quanta are used in the photosynthetic light reaction and the associated electron transport to $NADP^+$ reduction and NADPH as well as ATP formation. These are required for further CO_2

assimilation in the Calvin cycle (Krause and Weis 1991, Lichtenthaler et al. 2005). In this context, the photosynthetic apparatus with its two major components (1) the lamellar network, collectively referred to as thylakoids, and (2) the stroma matrix with soluble enzymes of the Calvin cycle can be strongly affected by drought stress (e.g. ROS). To alleviate the oxidative damage, plants use complex defense mechanisms, like non-enzymatic and enzymatic antioxidants. Latter include amongst others superoxide dismutase (SOD) and catalase (CAT). SOD as a major scavenger of $O_2^{\bullet-}$, converts $O_2^{\bullet-}$ into O_2 and H_2O_2. Then, H_2O_2 is scavenged by CAT into H_2O and O_2 (Liu et al. 2012, Mittler 2002, Navari-Izzo and Rascio 1999).

In addition, alteration of water relationships within the plant changes content and quality of non-enzymatic antioxidants synthesized to protect cellular structures. Amongst others, osmotic active solutes of low molecular weight are produced to overcome water deficit since they are non-toxic and do not interfere with cellular metabolism (Bartels and Souer 2003). They include sugars (e.g. sucrose, mannitol), betaines (e.g. glycine betaine) and amino acids (e.g. proline) (Parry et al. 2005). These organic compounds preferentially accumulate in the epidermal layer of the leaves, and they frequently include nitrogen-based compounds (e.g. proline). Other solutes include ions such as Cl^-, K^+ and Na^+. They act as a mediator to maintain turgor during water deficiency, stabilize subcellular structures or buffer redox potential. Further, the solute accumulation decreases the cellular water potential (Ψ), which maintains temporarily the ability of plants to absorb water. Plants can absorb water as long as their Ψ is lower than that of the soil water (Sánchez-Rodríguez et al. 2010, Takagi 2008).

Other non-enzymatic antioxidants are phenols (e.g. flavonoids, anthocyanins, carotenoids), ascorbic acid and glutathione (Reddy et al. 2004). Phenolic compounds sequester ROS, such as the anion superoxide or the radicals hydroxyl and peroxyl (Sánchez-Rodríguez et al. 2010). Hernández et al. (2006) detected an increase of the oxidation products of flavan-3-ols in drought stressed tea leaves, de Abreu and Mazzafera (2005) observed increased levels of the flavonoids quercetin and rutin in the medicinal herb *Hypericum brasiliense* exposed to drought. As a consequence of this protective effect, plant tissues with a higher content of anthocyanins usually have a higher tolerance to water deficiency (Rodziewicz et al. 2014). For example, the purple cultivar of pepper is more tolerant to drought stress than the green cultivar (Bahler et al. 1991).

Drought-induced inhibition of the photosynthesis apparatus is caused both by damaged photosynthetic pigments or lower CO_2 assimilation, but irrespective of the type of constraint, it results in decreased plant growth. Plant growth is the result of cell division and enlargement, water deficit directly lowers growth by reducing photosynthetic activity and by

cell wall relaxation, which adversely affect turgor pressure, cell division and elongation (Pugnaire et al. 1999, Taiz and Zeiger). Consequently, water deficit stress also affects leaf area and yield (i.e., quality and quantity) negatively (Brix 1961, Chartzoulakis et al. 2002, Krasensky and Jonak 2012).

A different kind of water deficiency in plants occurs when soil salinity appears. It is well known that growth impairment is directly related to osmotic potential of soil water (Greenway and Munns 1980). Due to the relatively high solute concentration in the soil and the associated osmotic effect of the salt around the roots, water absorption is impaired (Demetriou et al. 2007, Munns and Tester 2008). The low water availability caused by salinity induces the same root-to-leaf signaling by ABA (Fricke et al. 2004) or increased formation of ROS, SOD and CAT as common with plants in drying soils (Apel and Hirt 2004, Davies et al. 2005, Foyer and Noctor 2005). Osmotic adjustment, at the physiological level, is an adaptive mechanism to maintain turgor under conditions of water deficit (Morgan 1984). Under saline soil conditions, osmotic adjustment is partially achieved by the absorption of inorganic ions from the soil, which could result in mineral toxicity or mineral imbalance. Contrariwise, under water deficit, osmotic adjustment is rather attained by synthesizing and accumulating compatible organic solutes (Alain et al. 2000). However, both drought and saline soil conditions lead to decreased photosynthesis (Chaves et al. 2009) with all its impairment of plant performance.

1.2 Salinity

Salinity is a status of the soil or growing medium characterized by a high concentration of soluble salts. Here, mainly chlorides of calcium, magnesium and sodium are the most important soluble salts. Latter is the most soluble and abundant salt released (Levitt 1972). Natural salinity is developed due to soil-forming processes. Another way to saline soils is the salinization caused by improper irrigation. About 50% of the existing irrigation systems of the world are under influence of secondary salinization. Saline soils are a consequence of high fertilizer input (i.e., many solved ions on the water) in arid and semiarid regions, where rainfall is insufficient to leach salts out of the rhizosphere and high evaporation rates leave ions behind (Pessarakli and Szabolcs 2011).

Soil salinity can affect plants in two ways: The osmotic stress affects the plant immediately. High concentrations of salts in the soil increase the osmotic potential in the soil and make it harder for the roots to absorb water (Demetriou et al. 2007, Munns and Tester 2008). A major difference between the low-water-potential environments caused by high salt contents in the soil versus soil desiccation is the total amount of water available. During

drought periods, a finite amount of water can be absorbed from the soil by the roots, causing ever-decreasing soil water potentials. In most saline soils, a large amount of water at a constant, low water potential is available. As stated above, the low availability of water due to saline soil conditions causes the same plant biochemical (i.e. synthesis of ROS, SOD, CAT and ABA) and physiological (e.g. reduced stomatal conductance) effects as under dry soil conditions (Apel and Hirt 2004, Fricke et al. 2004, Davies et al. 2005, Foyer and Noctor 2005).

Salinity also leads to osmotic adjustment due to vacuolar accumulation of compatible solutes and ions to increase the turgor pressure (Cayuela et al. 1996, Rivero et al. 2014). In leaves, amino acids such as proline (Aziz et al. 1999, Cayuela et al. 1996, Khatkar and Kuhad 2000, Lin et al. 2002, Singh et al. 2000), carbohydrates such as sugars (fructose, glucose, sucrose) (Gao et al. 1998, Khavarinejad and Mostofi 1998), and phenolic compounds (Juan et al. 2005, Parida et al. 2002), such as anthocyanins (Eryilmaz 2006, Ramakrishna and Ravishankar 2011) and flavonoids (Sánchez-Rodríguez et al. 2011b), accumulate in response to salinity. Phenolic compounds play an essential role in the detoxification of free radicals (Ksouri et al. 2007). *In vitro* studies have shown that flavonoids are able to scavenge molecular species of active oxygen (e.g. O_2 and H_2O_2) directly by donating electrons or hydrogen atoms (Arora et al. 1998, Inzé and Montagu 1995, Sakihama et al. 2000, Sakihama et al. 2002). Thus, salt-sensitive species tend to have a low anthocyanin level or their level decreases under strong salt impact (Daneshmand et al. 2010). Amino acids and carbohydrates mainly act as agents for osmoprotection, osmotic adjustment, carbon storage, and radical scavenging. These responses are related to the activity and concentration of enzymes such as sucrose phosphate synthase (Carvajal et al. 2000), sucrose synthetase (Rosales et al. 2007), and phosphoenolpyruvate carboxykinase (Saito et al. 2008).

In contrast to this, other studies have reported that in a number of species, including tomatoes, salt stress leads to a higher concentration of reducing sugars (glucose, fructose), sucrose and fructans in the leaves (Hunsche et al. 2010, Kerepesi and Galiba 2000, Khatkar and Kuhad 2000, Singh et al. 2000). These diverse responses are linked to the tomato genotypes, and their different susceptibility to salinity, used in these studies. The adjustment of water and osmotic potential, which usually occurs by the accumulation of high amounts of inorganic or organic solutes, are important aspects of salt-tolerance (Chen et al. 2009).

Under saline conditions, the increase in turgor potential is not always related with an increase in cell water content, because the size of the cell could be reduced under salinity, which limits the water uptake capacity. In addition to that, saline soil conditions could reduce

cell expansion in tomato plants, which is linked to a decrease in osmotic potential and water potential and to a rise in the turgor potential (Munns 1993, Rivero et al. 2014, Romero-Aranda et al. 2001). However, despite reduced cell expansion (i.e. smaller leaves) under salinity, leaf thickness could increase due to greater leaf succulence (mg H_2O cm^{-2}) in consequence of the accumulation of chloride (Longstreth and Nobel 1979, Kemp and Cunningham 1981).

Though, in salinity affected plants rates of photosynthesis per unit leaf area are often unchanged, even though stomatal conductance is decreased (James et al. 2002). The reason for this could be explained by the changes in cell anatomy, i.e. smaller but thicker leaves, resulting in a higher chloroplast density per unit leaf area. In case of expressing photosynthesis on unit chlorophyll, rather than on leaf area, a decrease due to salinity stress can usually be detected. However, reduced leaf area caused by saline soil conditions means that photosynthesis per plant is always reduced (Munns and Tester 2008). Additionally, the stomatal density of tomato leaves might decrease due to salinity (Romero-Aranda et al., 2001), which might result in reduced plant water uptake. These changes in leaf anatomy could also contribute to changes in photosynthesis performance.

Contrasting the direct effects of salinity, the ion-specific stress develops over time due to combination of ion accumulation in the plant cells, the inability to tolerate the ions that have been accumulated and nutritional constraints by decreasing uptake of essential ions such as calcium, nitrate and potassium.

One strategy by which plants protect actively growing and metabolizing cells is the regulation of ion movement into tissues (Hasegawa et al. 2000, Munns 1993). The accumulation of huge quantities of ions in mature and old leaves, which than dehisce, has been observed in plants affected by salinity (Hasegawa et al. 2000, Munns 1993). Here, old leaves are supposed to act as ion sinks to restrict ion deposition into meristematic and actively growing and photosynthesizing cells. Another explanation is that cellular ion discrimination is a natural consequence of transpirational and expansive growth fluxes, cell morphology and intercellular connection. Tissues like meristematic cells are not directly connected to the vasculature and less exposed to ions delivered through the transpiration stream, and their small vacuolar space is not conductive to ion storage (Hasegawa et al. 2000).

For example, Na^+ toxicity mainly occurs in the leaf blade, where Na^+ accumulates after being deposited in the transpiration stream, rather than in the roots (Evangelou and McDonald 1999, Munns 2002). External Na^+ negatively impacts intracellular K^+ influx. When Na^+ accumulation increases, Na^+ reduces the acquisition of K^+ by cells. The lack of this essential

nutrient and the abundant supply of Na^+ inhibit protein synthesis through competition for K^+-binding sites (Hasegawa et al. 2000, Wyn Jones and Pollard 1983). The altered ratio of Na^+/K^+ is only one consequence of high Na^+ and Cl^- concentrations in the soil solution. Further impacts are the depression of nutrient-ion activities and the production of other extreme ratios of Na^+/Ca^{2+}, Ca^{2+}/Mg^{2+}, and Cl^-/NO_3^-. Changes in the ratios result in susceptibility to osmotic and specific-ion injury as well as to nutritional disorders (Grattan and Grieve 1999). To avoid or to alleviate changes in ratios like Na^+/K^+, the main mechanism of ion homeostasis in plants for Na^+ extrusion is caused by the plasma membrane H^+-ATPase. Using the energy of ATP hydrolysis, H^+-ATPase pumps H^+ out of the cell, inducing an electrochemical H^+ gradient (Sussman 1994). Plasma membrane Na^+/H^+ antiporters couple the movement of H+ into the cell along the electrochemical gradient of H^+ to the extrusion of Na^+ against its electrochemical gradient.

In case of insufficient extrusion of Na^+, compartmentalisation of Na^+ into vacuoles averts the detrimental effects of Na^+ in the cytosol (Blumwald 2000, Parida and Das 2005). Further, salinity is often accompanied by a decrease of nitrogen (N) accumulation in plants (Kafkafi et al. 1982, Martinez and Cerdá 1989) since Cl^- is rather absorbed than NO_3^- as caused by the antagonism and preferential uptake (Bar et al. 1997, Feigin et al. 1987) as well as lower water uptake (Lea-Cox and Syvertsen 1993). Sodium-caused K^+ deficiency implies in growth and yield depression of tomato plants (Grattan and Grieve 1999, Song and Fujiyama 1996). K^+ and Na^+ compete for absorption by the plant, but at the same time that K^+ absorption is impaired by salinity, higher K^+ concentrations in tissues are required for growth. Although increases in leaf-Na^+ levels may help to maintain turgor during salinity, Na^+ is not able to substitute K^+ completely, which is required for enzyme activation and protein synthesis (Hasegawa et al. 2000, Wyn Jones and Pollard 1983). Another detrimental effect of lower K^+ concentrations, especially in the stroma, due to salt stress is the decreased photosynthetic capacity (Chow et al. 1990).

Increased salinity in the soil solution is accompanied by increased Ca^{2+} requirement of the plant (Bernstein 1975). At the same time, Ca^{2+} uptake is limited because of ion interactions (e.g. Na^+), increased ionic strength or precipitation. These influences are responsible for reduced Ca^{2+} activity in the root zone and consequently for lower Ca^{2+} availability to the plant (Cramer et al. 1986, Suarez and Grieve 1988, Grattan and Grieve 1999).

Calcium has major impact on processes that preserve the structural and functional integrity of plant membranes, stabilize cell wall structures, regulate ion transport, selectivity,

and ion-exchange performance or cell wall enzyme activities (Rengel 1992, Marschner 1995, Grattan and Grieve 1999). On the other hand, Ca^{2+} appears to be easily displaced from its membrane binding sites by other cations. The consequence is that these essential functions may become strongly impaired by decreased Ca^{2+} availability. Further, low to moderate levels of NaCl often raises the occurrence of blossom-end rot (Adams and Ho 1989), primary caused by the lower Ca^{2+} content in the fruit.

Na^{+} and Ca^{2+} are strongly competitive with Mg^{2+}, and the binding sites on the root plasma membrane appear to have less affinity for the highly hydrated Mg^{2+} than for Ca^{2+} (Marschner 1995), ending in reduced leaf Mg^{2+} concentration (Ruiz et al. 1997). Due to its essential role as central atom in the chlorophyll structure (Brace et al. 1978, Chow et al. 1975), Mg^{2+} deficiency is associated with decrease in chlorophyll synthesis.

2 Experimental methods for osmotic stress induction and stress evaluation

2.1 PEG as osmotic stress agent

Several methods might be used to induce water deficit and evaluate its impact on plants. Most commonly, plants are allowed to grow under soil desiccation accompanied by increasing stress intensity or provided with a specific amount of water to maintain steady-state stress conditions. Another method commonly used is to induce water shortage by changing the osmotic potential in the growth medium. Osmotic stress can be induced by various osmotically active substances such as sucrose (Cui et al. 2010), sorbitol (Al-Khayri and Al-Bahrany 2002, Wang et al. 1999), mannitol (Lawlor 1970) or inorganic salts (Termaat and Munns 1986) as well as by nonionic synthetic, long chain, inert polymers like polyethylene glycol (PEG) (Comeau et al. 2010, Nepomuceno et al. 1998). Shortcoming of these methods is that these substances might be absorbed by the plant roots from the growth medium and influence physiological processes such as blockage of the transpiration pathways (Komor 1977, Lawlor 1970).

Polyethylene glycols are highly water-soluble compounds available in different molecular weights (Lawlor 1970). As already shown, in order to use PEG as adequate method for water deficit induction, it should have a molecular mass of 6000 or above, otherwise the polymers are expected to penetrate intact plant tissues (Chazen et al. 1995, Fan and Blake 1997, Hohl and Schopfer 1990, Mexal et al. 1975). It has also to be considered that PEG not only lowers the surface tension and increases viscosity of the nutrient solution but also decreases the movement and supply of O_2 (Verslues et al. 1998). Hence, the entry of PEG into damaged

cells make PEG less suitable for the usage as an osmoticum (Lawlor 1970). Therefore, careful handling of the roots is the highest priority when working with PEG. Nevertheless, despite controversial discussions about the suitability of PEG as appropriate method to study the impact of water shortage in plants, PEG has been used for this purpose in several studies (Bressan et al. 1981, Comeau et al. 2010, Fan and Blake 1997, Pérez-Alfocea et al. 1993, Ranjbarfordoei et al. 2000, Türkan et al. 2005, Zhang et al. 2011).

As external osmotica, PEG reduces the osmotic potential (Ψ_π) of the nutrient solution, causing a decrease of the Ψ in the plant, and finally generates water deficiency in a similar manner as soil desiccation. Moreover, the general water balance of plants is affected, because root hydraulic conductance and transport into cells of leaves requires development of even lower Ψ to maintain a downgrade gradient of Ψ that facilitates water movement from the nutrient solution into the leaves (Sánchez-Rodríguez et al. 2010, Takagi 2008).

Advantages of using PEG for osmotic stress induction are the precise adjustment and maintenance of the stress level (i.e. Ψ_π) in the hydroponic solution. Furthermore, due to the fact that PEG with a molecular weight $\geq$ 6000 does not enter the apoplast, water is withdrawn not only from the cell but also from the cell wall. Nevertheless, PEG-induced stress means osmotic stress, and for this reason, results have to be taken with caution in terms of drought stress studies (Michel and Kaufmann 1973). Until now, studies using PEG were performed mainly with herbaceous species such as wheat (Shangguan et al. 2000) or barley (Bandurska 2001). Moreover, precise comparisons of the physiological responses to PEG or drought stress in model plants are missing, and examples of the use of PEG in perennial plants that have more lignified tissues such as apple leaves are rare.

3 Non-destructive fluorescence based sensors in horticultural crops

Most of the experimental studies dealing with the impact of water deficiency or salinity on the physiology of the plant focus either on traditional physiological parameters such as photosynthesis and plant growth, or on destructive analysis in the laboratory (Hunsche et al. 2010, Manaa et al. 2011, Sánchez-Rodríguez et al. 2010, Šircelj et al. 2007). Such studies are time consuming and often imply costly laboratory analyses. In contrast, for the effective evaluation of crop performance, rapid, non-invasive techniques are required (Baker and Rosenqvist 2004). Non-destructive fluorescence-based sensors allow measurements of the same plants over a long period, providing information with high temporal resolution while plant development is influenced by growing conditions. Additionally, non-destructive methods enable the timely evaluation of the physiological status of the plants and might

contribute to the precise selection of stress tolerant cultivars. In general, these technologies might add to the realisation of cost-effective, more environmentally friendly, sustainable horticulture (Chaerle and van der Straeten 2001). Some examples on sensing the impact of insufficient water supply and salinity on horticultural crops were already published (Bertolli et al. 2014, Leufen at al. 2013, Rivero et al. 2014). Nevertheless, the potential of the non-destructive optical sensors particularly in horticultural crops is far away from being completely exploited.

In order to get as much information as possible on morphological, biochemical and physiological adaptations of plants to environmental conditions, detection systems built up with different excitation light sources and detection systems might be used. Besides lasers, xenon lamps and LEDs are the common light sources for fluorescence excitation. Fluorescence decay curves, i.e. lifetime, can only be measured via short-pulsed LEDs or lasers. The general principles of fluorescence and its application in plant sciences are well described in books (Albani 2007, Papageorgiou and Govindjee 2004) and scientific articles (Baker 2008, Buschmann and Lichtenthaler 1998, Lichtenthaler and Rinderle 1988), thus we provide here a brief summary of the most important aspects.

Excitation of a leaf with blue or red light enables the recording of the chlorophyll fluorescence (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, Buschmann et al. 2008), as demonstrated in figure 1.

At present, the available technique which detects and calculates multiparametric fluorescence indices is not able to separate between BF and GF properly. For this reason a signal between the BF and the GF at 475 nm is used. More accurate information about the BF or GF can be obtained by detection of the fluorescence lifetime. Because of the phenolic origin of the blue-green fluorescence (BGF), the BGF emission, i.e. yield and spectral characteristics, strongly depends on the temperature and other environmental factors (e.g. pH, polarity, heavy metals, etc.), like any fluorophore *in vitro*, in contrast to ChlF emission, which is linked to proteins (Cerovic et al. 1999).

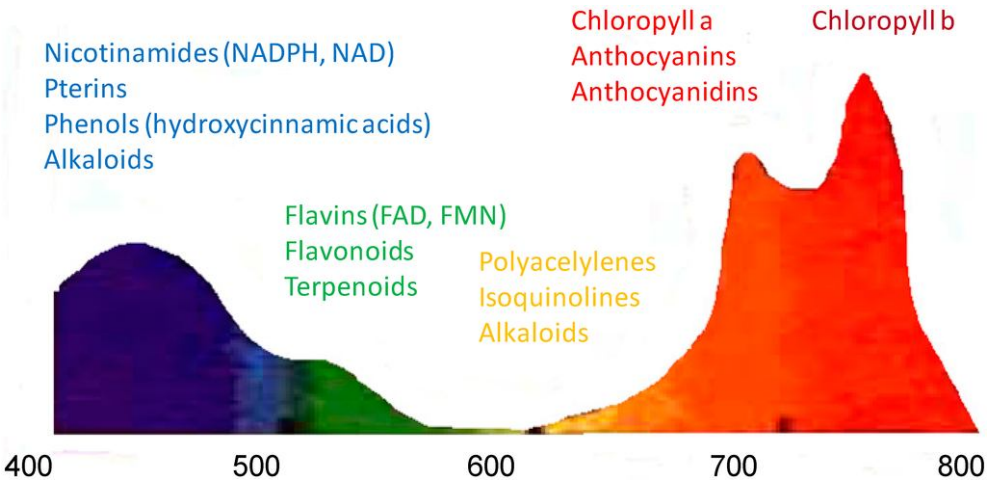

Fig. 1 Fluorescence emission spectrum of a typical green leaf under UV-radiation. Talamond et al. 2015.

3.1 Pulse-amplitude-modulated chlorophyll fluorescence (ChlF)

Chlorophyll fluorescence (ChlF) is a tool to determine in a fast way changes in the photosynthetic capacity of the tissues, thereby exploiting in detail the electron flow between the photosystems inside the chloroplasts. With the Pulse-Amplitude-Modulation (PAM) technique, the ChlF is usually recorded between 680 and 690 nm either as a spot or as spatially resolved information enabled by imaging instruments. Systems usually can record both the fast parameters and the kinetic fluorescence parameters. In the sum, the collected data allows the calculation of numerous complex parameters related to photosynthetic efficiency (Baker and Rosenqvist 2004), i.e. extensive information about the photosynthetic apparatus, or more precisely, about the photosystem II (PSII) and indirect information about the photosystem I, too (Belkhodja et al. 1994, Bilger et al. 1995).

The basic requirement in using the PAM technique to get the full information range is the dark-adaptation of plants and leaves. With this, the primary quinone acceptor of the PSII (Q_A) becomes maximally oxidized and the PSII reaction centers are open, i.e., capable of performing photochemical reduction of Q_A (Baker and Rosenqvist 2004). If dark-adapted leaves are exposed to a non-actinic, weak modulated measuring beam (photosynthetically active photon flux density (PPFD) of ca. 0.1 µmol m^{-2} s^{-1}) the minimal level of fluorescence (Fo) can be recorded (Fig. 2) (Baker 2008). After a short pulse at high PPFD of several hundred µmol m^{-2} s^{-1} and generally less than 1 s, the maximal level of fluorescence (Fm) is generated. As a consequence of this light pulse, Q_A becomes maximally reduced and the PSII reaction centers close, i.e., the capacity of PSII photochemistry drives almost to zero (Baker and Oxborough 2004). The calculation of the ratio (Fm – Fo = Fv)/Fm, estimates the

maximum efficiency of the PSII, i.e., the quantum efficiency if all PSII centers are open (Maxwell and Johnson 2000).

When plants are exposed to drought stress or salinity, Fv/Fm might increase (Li et al. 2010) or decrease (Mishra et al. 2012). As proposed, abiotic stress, such as water deficiency and salinity, do not affect rates of photosynthesis automatically, even though stomatal conductance is decreased (James et al. 2002). The reason for this could be the way how photosynthesis is expressed, on unit leaf area or on unit chlorophyll. Measuring the maximum efficiency of the PSII (Fv/Fm) with the PAM technique means, measuring the photosynthesis on unit leaf area. The increase of Fv/Fm in plants exposed to abiotic stress conditions might indicate higher efficacy of PSII, as part of an adaptation process in the plants. If Fv/Fm decreases (e.g. in tomato leaves or lemons), stress has affected the photosynthetic apparatus negatively and possibly also damaged (Mishra et al. 2012, Nedbal et al. 2000b).

Under continuous actinic light, the fluorescence level F' can be measured. This phenomenon (the Chl *a* fluorescence dark/light induction curve) has been observed first by Kautsky and Hirsch (1931). F' rises to the maximal fluorescence level (Fm') when the leaf is exposed to a brief saturating light pulse that maximally reduces Q_A. A prime notation (') is used after fluorescence parameter if the leaf is exposed to light that drives photosynthesis, i.e., actinic light (Baker 2008). The difference between Fm' and F' termed Fq' since this is the fluorescence that has been quenched from the maximal level. For healthy leaves operating at steady-state photosynthesis under moderate to high PPFDs, Fm' generated by the saturating light pulse will be considerably less than Fm generated from dark-adapted leaves by the same pulse (Fig. 2) (Baker and Rosenqvist 2004). Genty et al. (1989) demonstrated that the ratio Fq'/Fm' estimates the quantum yield of PSII photochemistry for a leaf at any given light condition. This led to the fact that Fq'/Fm' is being widely used to estimate the operating quantum efficiency of PSII electron transport (ETR) (Baker and Rosenqvist 2004). The quenching analysis describes the stable, i.e. the steady state photosynthetic activity. According to this, the ETR depends indirectly on the stomatal conductance, too. Under water deficit and stomatal closure, the lower CO_2 assimilation through stomata is co-responsible for the decrease of ETR (Zribi et al. 2009).

Additional parameters allow the estimation of the non-photochemical quenching (NPQ) that reflects heat dissipation of excitation energy in the antenna system (Bilger and Björkman 1991). When tomato plants are subjected to drought or salinity stress processes a higher regulated-energy dissipation (e.g. NPQ) might happen to protect the photosynthetic apparatus

(Sarlikioti et al. 2010, Zribi et al. 2009), whereas a decrease is expected when the stress impact becomes more severe or in times of recovery (Gorbe and Calatayud 2012).

The coefficient of photochemical quenching (qP) (fraction of open PSII reaction centres based on a 'puddle model' (Genty et al. 1989)), and the coefficient of photochemical quenching (qL) (fraction of PSII centres in the open state based on a 'lake model' for the PSII photosynthetic apparatus (Kramer et al. 2004)) are two other indicators detectable with the PAM technique (Baker 2008, Buschmann 1999, Sperdouli and Moustakas 2011). Both qL and qP give supplementary information about the fraction of Q_A in its oxidized state (Kramer et al. 2004). Depending on type, intensity and duration of the stress situation, qL and qP might lead to different results. Low qL or qP in tomato leaves of plants grown under drought and salinity stress conditions reveal a strong impact on the PSII, i.e. low fractions of open PSII reaction centers, commonly revealed as immediate effect (Haupt-Herting and Fock 2000, Krause and Jahns 2004). In contrast, adaptation of the plants over the time may increase the values for these parameters due to strong accumulation of non-reduced primary electron acceptors of PSII, ready to accept the excitation energy for passing it further towards other photochemical processes (Hura et al. 2007). However, although qP represents an approximate measure of the fraction of open PSII reaction centers, qP does not take into account the efficiency of the PSII reaction centers (Genty et al. 1989, Juneau et al. 2005). Further, it should be considered that qP might overestimate the fraction of open centres, except at the extreme boundary conditions, the differences are higher at low fractions of open centres (e.g. abiotic stress conditions) where photoprotective mechanisms break down (Calatayud et al. 2006, Kramer et al. 2004). Compared to this, recording steady state fluorescence signals is certainly easier than recording fluorescence kinetics with the need of dark adaption. On the other hand, steady state fluorescence signals strongly depend on highly variable prevailing daylight conditions, are lower than dark-adapted, and measurable changes might be less clearly detectable (Bauriegel and Herppich 2014).

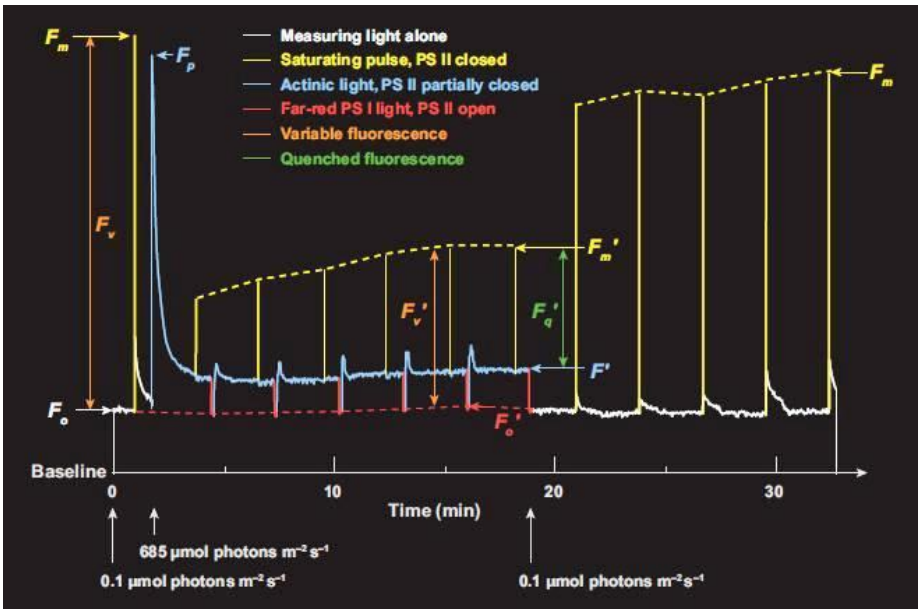

Fig. 2 Example of a typical fluorescence quenching analysis by the saturation pulse method. Source: Baker (2008).

3.2 Pulse-amplitude modulated chlorophyll fluorescence imaging

In addition to the averaged ChlF values over the entire measured object obtained by the punctual PAM technique, imaging fluorescence visualizes the fluorescence characteristics of an object (e.g. leaf or fruit) with spatial resolution. Generally, for imaging fluorescence signals sensitive high-resolution charged-coupled device (CCD) cameras are used, and each pixel can be understood as a separate measurement (Chaerle and van der Straeten 2000, Langsdorf et al. 2000). Current ChlF imaging techniques permit close pre-symptomatic non-invasive monitoring of even minor changes in the physiological state of plants at leaf and fruit level, e.g. due to the impact of abiotic stresses (Bauriegel and Herppich 2014, Chaerle and van der Straeten 2000, Martínez-Peñalver et al. 2011, Nedbal et al. 2000a, Oxborough 2004, Rolfe and Scholes 2002). Further, fluorescence imaging allows the simultaneous measurement of many samples, e.g. tomato leaves or apple seedlings (Bauriegel and Herppich 2014). The use of UV-laser-induced fluorescence imaging systems provides the simultaneous measurement of the fluorescence emission from the blue, green, red and far-red spectral band, and a visualization of these measurements (Buschmann et al. 2000).

3.3 Multi-indices fluorescence excitation

In general, the RF and FRF is only originated by chlorophylls (Buschmann et al. 2000, Lichtenthaler and Schweiger 1998), whereas the BF and GF emission of green leaves originate from several compounds, primarily from fluorophores produced in the shikimate pathway such as plant phenolics and polyphenols. Amongst others, hydroxycinnamic acids,

and in particular the ferulic acid as major substance, as well as chromones, phenolic acids, flavonols, flavones are responsible for this spectral component of the fluorescence (Fig. 1) (Buschmann and Lichtenthaler 1997, Buschmann et al. 2000, Langsdorf et al. 2000).

Based on the absolute fluorescence intensities at various excitation bands, fluorescence ratios (indices) can be calculated. Pulsed excitation light in different colors allied to the synchronized detection, allow this method to be used at ambient light and in the field. Thus, fluorescence indices determined *in situ* under light conditions provide fast information about the physiological status of the plant. Depending on the features of the measuring system, and the experimental conditions, absolute fluorescence intensities in spectral band as well as simple or complex fluorescence indices might be used to evaluate the impact of stress factors.

A common approach is to measure the fluorescence emission spectra and determine the ratio between two Chl maxima as indicator for Chl content (Lichtenthaler 1990). Typically for this method is to determine two peaks, in the red (685 – 690 nm) and far-red region (730 – 740 nm). Generally the ChlF ratio of RF to FRF decreases with increasing Chl concentration in a curvilinear relationship. This is due to the re-absorption of the light mainly of the red ChlF band emitted inside the leaf by the Chl absorption bands (Buschmann 2007, Gitelson et al. 1997). However, changes in cell anatomy, i.e. as a consequence of water deficiency, may result in a higher chloroplast density per unit leaf area with no significant decrease of the RF to FRF ratio (Buschmann et al. 2000, Lang et al. 1996).

The simple fluorescence ratio (SFR) depends on the pigment concentration and is based on the partial reabsorption of RF by the chlorophyll itself (Buschmann 2007), while the FRF band is not reabsorbed. The SFR (FRF to RF ratio) can be calculated after excitation with green (SFR_G) or red light (SFR_R). According to the fact that SFR is related to the Chl *a* + *b* concentration (Leufen et al. 2014), chlorophyll degradation caused by drought or salinity results in a decrease of SFR.

The nitrogen balance index (NBI) compares the FRF after UV-light excitation and the RF after green (NBI_G) or after red (NBI_R) light excitation. Both NBI indices were defined to be proportional to the chlorophyll to flavonol ratio. They are based on the balance between primary and secondary metabolism of the plants, where flavonol content increases, and chlorophyll content decreases, in plants grown under nitrogen deficiency (Agati et al. 2013). Irrespective of the original purpose of NBI, the optimization of the nitrogen fertilization in cereals, it might be used as indicator of other stress situations. In terms of salinity and drought, NBI is expected to decrease due to chlorophyll degradation. The decline could be strengthened or caused by itself by the accumulation of phenolic compounds (Juan et al.

2005) such as flavonoids (Sánchez-Rodríguez et al. 2011b) to detoxify free radicals (Ksouri et al. 2007).

A well-known sensitive indicator for detection of early water deficit impact is the ratio of BF to FRF after UV-light excitation (BFRR_UV) (Buschmann et al. 2000, Buschmann and Lichtenthaler 1998). The use of BF/FRF is preferred compared to BF/RF, because FRF at 735 nm is less affected by re-absorption of the red fluorescence by the photosynthetic pigments. As shown, BFRR_UV might increase fast due to shrinking cell volume that may result in a higher density of BF emitting fluorophores per unit leaf area caused by water shortage. Later, the increase in BFRR_UV is due to an accumulation of leaf secondary metabolites in the epidermal layer that can emit BF. Under water deficiency and salinity, chlorophyll content might decrease while BF emitting phenolic compounds such as flavonoids (quercetin 3-O-rutinoside and luteolin 7-O-glucoside) or hydroxycinnamates (echinacoside) might be strongly produced (Ksouri et al. 2007, Tattini et al. 2004). Further, the absorption of the UV-excitation light in the epidermis attenuate the UV-excitation of Chl molecules in the mesophyll cells, which consequently decreases ChlF and results in an increase of BF/FRF (Cerovic et al. 1999, Chaerle and van der Straeten 2000).

Another fluorescence index is also based on the filtering effect of UV-absorbing phenolic compounds present in leaf epidermises and fruit skins that are screening under-laying Chl, the ChlF screening method. The logarithm of the ratio of FRF after R light excitation to the FRF after excitation with UV-light (FLAV) represents this differential absorption measurement (according to the Beer-Lambert's law) that is proportional to the concentration of flavonols in the epidermal layer (Agati et al. 2011, Agati et al. 2013, Cerovic et al. 2008, Tremblay et al. 2012). Due to the fact that epidermal flavonoids are representative of the total leaf flavonoids (Agati et al. 2008), the ChlF screening method enables the detection of Chl content itself and the content of the epidermal flavonoids (Agati et al. 2013, Tremblay et al. 2012). FLAV is expected to increase under water deficiency due to changes in the synthesis and accumulation of blue fluorescing flavonoids, particularly the epidermal flavonols. The accumulation itself might also be driven by cell shrinking when leaves lost their turgor due to water deficit. Consequently, a size-decrease of the cells might result in higher BF per unit leaf area.

Besides the general understanding how stress parameters might affect fluorescence signals of leaves, only a few scientific studies have addressed the impact of more than one stress, and their consequences, on the fluorescence signature of different cultivars. Thus, information on multiple abiotic constraints that simultaneously affect horticultural crops remains scarce. With the demand to obtain robust data for efficient abiotic stress detection,

calculating fluorescence indices (e.g. SFR, NBI, BFRR_UV, FLAV) might provide valuable, much more stable information compared to the absolute fluorescence intensities. Fluorescence indices restrict the influence of external factors (e.g., equipment type, measurement setup), optical properties of the samples (leaf morphology) as well as environmental conditions and offer better conditions for comparisons of e.g. treatments or cultivars (Lichtenthaler 1996).

4 Limitations and potential of fluorescence-based systems in horticulture

Optical sensors based on fluorescence recordings are fast, reliable and non-destructive tools for physiological evaluations. However, it has to be considered that a fluorescence value by itself has no meaning (Kalaji et al. 2014). For an appropriate interpretation of the data, a well-defined reference state for the photosynthetic sample, in case of chlorophyll fluorescence, is needed. Further, several important factors have to be considered to detect and evaluate fluorescence signals properly. Standardized or at least well-defined measuring conditions are necessary to minimize disturbances in general. At first, the possibility of affecting molecules in the excited state increases with increasing ambient temperature, which could result in lower or higher fluorescence lifetime or intensity (Morales et al. 1996). Therefore, temperature-dependency could influence fluorescence measurements and restricts them in its opportunities. As consequence of the temperature-dependency, leaf BGF increased when leaf temperature decreased (5% of BGF change per degree Celsius) (Bongi et al. 1994, Tremblay et al. 2012). Consequently, when using BGF as a parameter for assessment of plant physiological characteristics, temperature should be constant over measuring time. Moreover, increasing distance of the light source and sensing optics from the sample causes a decrease of the intensity of the excitation radiation and consequently of the fluorescence emission; here, the distance *per se* also decreases the amount of light which can be detected by the equipment, irrespective of the intensity of the emitted fluorescence. With regard to all these demands, fluorescence measurements provide a tool with a lot of challenges in field operation.

Considering the PAM chlorophyll fluorescence technique, the fact that the determination of fluorescence quenching parameters takes about 3-5 minutes and requires a time-consuming dark-adaptation, this method is less suitable for field measurements or a high quantity of samples and does not offer images. In case of using leaf clips for dark adaption in the field, the leaf clips tend to be sensitive to smooth leaves (i.e. clips shift) if the leaf is not flat or some stray light may enter the leaf clip via the spaces left between the clip and the surface (Kalaji et al. 2014). In addition to that, this method offers only point measurements without any spatial resolution. On the other hand, the parameters of the fluorescence kinetics without

dark adaption (e.g. Fo', Fm') are rapidly detectable and thus offer higher potential for this purpose. As in the case of using leaf clips, a single detector with a measuring area of a few square millimeters is sufficient for spectral ChlF. The advantage of these point measurements is that they provide information on the whole fluorescence spectra including position and intensity of fluorescence maxima. On the other hand, neither local fluorescence differences nor fluorescence gradients over the whole leaf area can be detected, because one leaf part only yields one spectral information (Lang et al. 1996).

In addition to abiotic stress detection, the techniques might be also used to evaluate the impact of other factors. Nedbal et al. (2000b) investigated and predict post-harvest damage, such as mould-infected or damaged areas in lemons long before visible damage appears, by monitoring ChlF. Langsdorf et al. (2000) used fluorescence ratio imaging at leaf level as a non-destructive diagnostic tool for monitoring nitrogen supply to plants. Other studies revealed the potential of the ChlF ratio (Eullaffroy and Vernet 2003) as a tool for determination of herbicide toxicity or ChlF imaging for a rapid detection of herbicide resistance (Kaiser et al. 2013).

As a big challenge, the data obtained by multispectral or ChlF fluorescence imaging require a high level of know-how and still have to be calibrated against established, classical parameters, such as concentration of Chl or phenolic compounds, for an appropriate interpretation and decision-making (Chaerle and van der Straeten 2000). Furthermore, fluorescence imaging studies are mostly limited at the level of single leaves or the seedling level of model crops. Other limitations of fluorescence imaging in controlled environment are the challenge to analyze complicated whole-shoot species and the requirement of pre-acclimation conditions. Under field conditions, it is difficult to measure at the canopy scale, because of the small signal to noise ratio, though laser-induced fluorescence transients can extend the range available, while solar-induced fluorescence can be used remotely (Li et al. 2014).

To address the use of large scale phenotyping and to develop a standard procedure for fluorescence image processing robustness, reproducibility and data analysis software are needed. In addition to this, the power requirements of fluorescence imaging (for example, using short-wave laser stimulation) may be limiting for field phenotyping applications (Li et al. 2014). Nevertheless, the combination of ChlF imaging with other measurement techniques might provide a powerful tool. For example, the use of ChlF imaging combined with infra-red gas exchange technique enables the correlation of the PSII photosynthetic efficiency directly to the measured CO_2 assimilation rate by eliminating photorespiration as a result of the

reducing O_2 or increasing CO_2 within the chamber. Combining ChlF imaging with other imaging techniques, such as thermography, can also be an extremely strong tool. First studies demonstrate promising results showing the relation of photosynthetic rate to stomatal behavior (Chaerle et al. 2007) or imaging intrinsic water use efficiency (Lawson 2009). ChlF imaging in combination with hyperspectral imaging has the advantage of being able to distinguish between chlorophyll degradation and the impact of different diseases based on changes in photosynthetic efficiency and spectral signatures (Murchie and Lawson 2013). Considering the fact that PAM ChlF parameters are useful to indicate overall photosynthetic activity and reflect closely the status of the entire photosynthetic apparatus, the combination with hyperspectral imaging would include a much higher information density than multispectral or RGB images on its own (Bauriegel and Herppich 2014).

Another reason for the combination of UV-induced fluorescence and hyperspectral imaging technique is due to the fact that fluorescence parameters are not detectable from mobile or airborne platforms. First studies have already shown promising results of combining fluorescing and multispectral or hyperspectral remote stress detection (Chaerle et al. 2007, Lenk et al. 2007, Moshou et al. 2006), underlining that the approach of combined techniques for remote stress detection has to be pursued. In addition to that, another study has revealed that non-invasive spectral measurements have the potential to assist and complement disease scoring in breeding plot experiments. Nevertheless, established indices are not disease-specific, meaning that they can be used for quantifying an infestation or damage, i.e. they do not allow distinguishing between different types of disease (Jansen et al. 2014).

Despite all these promising applications of fluorescence-based sensors in field crops (Bürling et al. 2013, Leufen et al. 2013) and horticultural (Kautz et al. 2014, Müller et al. 2013) crops, there is still a high development demand before their practical use is ensured. In particular, fluorescence sensors might be used in different research fields of stress physiology and practical applications, including salinity-induced stress and water deficiency. In general, the optical sensors could contribute to optimize yield and product quality in intensive horticulture, and also to decrease the negative impacts of these intensive cultivation systems to the environment. In this context, breeding and crop production might be optimized through faster stress detection and stress differentiation, high-throughput whole-plant phenotyping, the selection of more stress tolerant genotypes or rootstocks, and improvements in fruit quality assessment.

5 Objectives of this study

Water deficiency as well as salinity are major limiting factors for horticultural crop production. In this context, breeding of cultivars that are more tolerant to drought and/or salinity stress is of great importance. For this purpose, early detection of the effects of abiotic stress on plants, the discrimination between the type of stresses, and the differentiation between tolerant and susceptible genotypes is required. To optimize and accelerate the process of evaluating the physiological status of plants, the use of non-destructive fluorescence-based sensors has been proposed. Nevertheless, existing techniques need to be adapted and improved, and their potential use should be further investigated and exploited. In the present study, pulse-amplitude-modulated (PAM) fluorescence imaging and multispectral fluorescence-based indices were used to evaluate the impact of abiotic stresses on the fluorescence signature of tomato (*Solanum lycopersicum* L.) leaves. Thereby, changes in the fluorescence signature as influenced by water deficiency and salinity were related to changes in photosynthesis and quantitative changes in secondary plant metabolism.

Additionally, the suitability of osmotic stress chemically-induced by polyethylene glycol has been examined in drought stress experiments by employing multispectral fluorescence-based indices and key physiological parameters of apple (*Malus domestica* Borkh.) leaves.

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 the plant much faster as compared to the PAM technique requiring dark adaption of the plants. In this context, we hypothesized that multiparametric fluorescence indices reveal the onset and intensity of long-term drought stress in tomato plants, as well as the effect of re-watering of the plants. On this basis, we also wanted to investigate if the multiparametric fluorescence indices are supportive for the fast screening of tomato genotypes regarding drought tolerance.

2. The aim was to identify appropriate indices of the multiparametric fluorescence technique to evaluate the response of tomato (*Solanum lycopersicum* L.) genotypes to salinity. In addition, we wanted to estimate the potential of multiparametric fluorescence indices as a tool to assess genotypes for salt tolerance. In this regard, we hypothesized that multispectral fluorescence based indices can be used to sense *in situ* the impact of salinity in three tomato genotypes.

3. In this chapter, the aim was to examine the influence of water deficit and salinity on plant physiology and in particular on specific parameters of the fluorescence signature of tomato (*Solanum lycopersicum* L.) leaves. Thereby, we hypothesised that multiparametric fluorescence indices support the monitoring of stress-specific physiological changes.

4. In general, the use of PEG is considered to be equivalent to physical water deficit. In this context, our objective was to examine physiological responses of apple (*Malus domestica* Borkh.) leaves to water deficit induced in nutrient solutions by PEG and in soil by interrupting irrigation. Here, we hypothesized that PEG-induced osmotic stress impacts plant physiology, morphology and biochemistry in a way similar to physical water deficit.

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B Controlled long-term water deficiency and its impact on the fluorescence emission of tomato leaves during stress and re-watering[1]

1 Introduction

The fluorescence emission of leaves has emerged as a fast and reliable approach to detect and evaluate the influence of stresses on plants, and might be used to amend or partially replace time consuming evaluations based on plant growth or lab analysis. Fluorescence datasets provide fast information about the plant's physiological status (Cerovic et al. 1999). In this context, the chlorophyll fluorescence (ChlF) has become a well-established tool in the last decades (Baker and Rosenqvist 2004). In particular, the chlorophyll (Chl) *a* fluorescence emission, recorded by pulse amplitude modulation (PAM) systems, provides extensive information about the photosystem II (PSII) (Bilger et al. 1995). On the other hand, the value of the blue fluorescence (BF) for a better understanding of plant physiological responses has received more attention (Morales et al. 1996).

Fluorescence can be excited by ultra-violet (UV)-radiation and by visible light from blue to orange-red. In order to generate fluorescence emissions carrying as much information as possible, the use of different excitation lights is indispensable. The UV-excitation (375 nm) mainly penetrates the upper cell layers of the tissue, being predominantly absorbed in the epidermis. Similar pattern has been observed with red light excitation (635 nm). In contrast, green light (510 nm) penetrates into deeper cell layers of the leaf (Buschmann et al. 2008). Thus, the combination of BF and ChlF, excited by more than one light source, seems to be a promising approach to get detailed information about genotype specific response in terms of water deficiency and during re-watering.

Absolute fluorescence intensities contain essential evidences about the plant physiology. However, they are susceptible to morphological and external factors such as leaf geometry and measurement settings, respectively. To achieve more reliable information, the calculation of ratios of the peaks from the absolute intensities represents a sensible solution for comparisons of treatments (Cerovic et al. 1999).

Recent investigations demonstrate the potential of the ChlF based detection of water deficiency in tomato plants (Mishra et al. 2012); although the potential of the multiparametric fluorescence technique particularly in horticultural crops remains widely underexplored.

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Particularly fluorescence ratios determined *in situ* under light conditions provide much faster information about the physiological status of the plant as the PAM technique requiring dark adaption of the plants. Therefore, the aim of the present study was to explore the influence of water deficiency and the re-watering on the fluorescence emission of leaves of adult tomato (*Solanum lycopersicum*) plants.

In this context, we hypothesized that the fluorescence ratios, the BF to far-red fluorescence (FRF) ratio after UV-light excitation (BFRR_UV), the logarithm of the ratio FRF after red light excitation to the FRF after excitation with UV-light (FLAV), and the ratio of FRF after UV-light excitation to red fluorescence after red light excitation (NBI) of the multiple fluorescence technique reveal the onset and intensity of stress in plants exposed to long-term water deficiency, as well as re-watering of the plants. As reference parameters of the PAM ChlF we choose the relative apparent electron transport rate (ETR) and the coefficient of photochemical quenching (qP). These records estimate the fraction of open centers of the PSII (Baker 2008).

2 Materials and methods

2.1 Plant material and growth conditions

Seeds of the *Solanum lycopersicum* L. cultivars Cupido, Harzfeuer (both Volmary GmbH, Münster, Germany) and Rio Grande (donation of Dr. Mustafa Demirkaya, Erciyes University, Turkey) were used in the present study.

Experiments were conducted under greenhouse conditions. Seeds were steeped in the dark (20 °C for three days). Germinated seeds were transferred into rock wool trays and cultivated until the third leaf stage. As next, plantlets were transplanted into 10 l pots filled with perlite (Perligran G, Knauf Perlite GmbH, Dortmund, Germany) and placed on two greenhouse tables. Fertigation was based on Kristallon^{TM} Blau (Yara GmbH & Co. KG, Dülmen, Germany) and amended with calcium nitrate tetrahydrate ($Ca(NO_3)_2$) (99% purity, AppliChem GmbH, Darmstadt, Germany). Excessive nutrient solution was drained off at the bottom of the pots after perlite saturation. Nutrient solution and its spillover were kept separated from each other and nutrient solution was not recycled. The average day/ night air temperature 40 cm above the tables was 28/ 18 °C with an air humidity of 50 – 70%.

2.2 Treatments and sampling

Plants of each cultivar were grown for about 5 weeks and then divided into two treatments (n = 10 plants per treatment): T1, well-watered control plants; T2, water deficiency

(20% of the volume of the control plants). Starting at plant age of 33 days water deficiency was induced and held for 46 days; thereafter all plants were fertigated in excess for 16 days enabling the plants to recover. Leaf samples were taken at day 46 and 62 after treatment induction. Freeze dried samples (Gamma 1-16 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) were ground for determinations of Chl and proline.

2.3 Analytical determinations

The relative water content (% RWC) of the leaves was calculated as:

$$\% \, RWC = \left(\frac{FM - DM}{TM - DM}\right) x \, 100$$

Leaf disks (13 mm diameter) were punched out and the fresh matter (FM) was determined. To determine the turgid mass (TM), samples were immersed in deionized water for 24 h in the dark. As next, samples were oven dried (80 °C/ 48 h) to obtain the dry matter (DM).

About 4 g of leaf FM were squeezed; 200 µl of the extract was centrifuged at 10,000 rpm for 10 min. at 4 °C. Osmotic potential (Ψ_π) of 15 µl supernatant was analyzed twice (Osmomat 030-D, Gonotec GmbH, Berlin, Germany). Means [osmol kg^{-1}] were multiplied by -2.437 (correction coefficient valid for 20 °C) to get Ψ_π in MPa (Taiz and Zeiger 2007).

To determine the proline concentration, 3 ml sulfosalicylic acid were added to 0.1 g ground DM and centrifuged at 4,200 rpm for 20 min. at 20 °C. Afterwards, 0.2 ml of the supernatant was filled up with 1.8 ml sulfosalicylic acid, 2 ml glacial acetic acid and 2 ml ninhydrine acid. The mixture was boiled at 100 °C for 1 h in a hot water bath. After the samples cooled down to 20 °C, 4 ml toluene was added to the mixture. The upper, organic part was collected for spectrophotometric measurements (Lambda 35 UV/VIS Spectrophotometer, PerkinElmer, USA). The absorbance of extracts was evaluated at 520 nm.

The concentration of Chl *a* and *b* was analyzed from dried and ground samples (0.05 g DM) after extraction with 5 ml methanol and centrifugation at 4,000 rpm for 15 min. at 4 °C. The supernatant was transferred to 50 ml volumetric flasks. The extraction procedure with methanol was repeated three times; thereafter, the flasks were filled up to 50 ml with methanol. The absorbance of the extracts was determined at 647 nm and 664 nm with an UV-VIS spectrophotometer (Lambda 35 UV/VIS Spectrophotometer, PerkinElmer, USA).

2.4 Fluorescence measurements

Fluorescence measurements were performed under laboratory conditions on detached leaves, either immediately after sampling or after dark-adaptation to the room conditions. In order to minimize possible modifications after the sampling, batches of three leaves each were

harvested for the sequential handling. Fluorescence determination in the time-course of the experiment was done on leaves of different physiological ages: the first measurements were conducted on the fourth leaf counted from bottom (cotyledons excluded), every 7-10 days a higher leaf level was selected according to the plant growth. Fluorescence readings were taken with two devices: the Imaging-PAM® (Heinz-Walz GmbH, Effeltrich, Germany) chlorophyll fluorometer and the multiparametric fluorescence excitation system Multiplex®3 (Force-A, Orsay Cedex, France), as described elsewhere (Leufen et al. 2013).

PAM ChlF parameters were recorded from the adaxial side of dark-adapted (30 min.) leaves. After determining ground (Fo) and maximum fluorescence (Fm), specific parameters as related to the kinetic curves were evaluated over a period of 300 seconds. Based on the literature (Kramer et al. 2004), we selected the coefficient of photochemical quenching (qP; Imaging PAM, calculated as (Fm' – F)/(Fm' – Fo')) and the relative apparent electron transport rate (ETR; Imaging PAM, calculated as 0.5 x Yield x PAR x 0.84 µequivalents m^{-2} s^{-1}) as meaningful parameters.

The light source (0.5 µmol m^{-2} s^{-1} PAR) used for fluorescence excitation and actinic illumination contains 96 blue light diodes emitting at 470 nm. Fluorescence images were recorded by a black and white CCD (8.458 mm chip with 640 x 480 pixels) camera operated in 10-bit-mode at 30 frames per second, as described elsewhere (Bürling et al. 2010). Data evaluation was based on the recorded pictures. In each single image, three areas of interest (AOI) were selected: leaf edge, apex and center. Afterwards the mean of the three AOIs was calculated before running the statistical analysis.

For multiple fluorescence excitation and fluorescence ratios, BF, RF and FRF spectral bands, excited with UV, green and red light, were recorded with a hand-held multiparametric fluorescence sensor at the adaxial lamina of detached leaves. These fluorescence recordings were done immediately after harvesting the leaves. Leaves were fixed horizontally on a sample holder at a defined distance (10.5 cm) to the sensor body. A frontal cover plate having an aperture (6 cm diameter) was used to standardize the area to be measured. As indicative parameters we selected three fluorescence ratios, the BF to FRF ratio after UV-light excitation (BFRR_UV), the FLAV Index (FLAV) as expressed by the logarithm of the ratio of FRF after red light excitation to the FRF after excitation with UV-light, and the Nitrogen Balance Index (NBI) given by the ratio of FRF after UV-light excitation to RF after red light excitation.

2.5 Data analysis

Statistical analysis was done with IBM SPSS Statistics 20.0 (IBM Corporation, New York, USA). Means were compared by t-test ($P \leq 0.05$) and graphs (mean ± SE) were drawn using SigmaPlot 11.0 (Systat Software Inc., San Jose, CA, USA).

3 Results

3.1 Relative water content, osmotic potential, proline and chlorophyll

RWC was significantly reduced after 46 days of water deficiency in comparison with leaves of the control treatment (Table 1). RWC was 20% ('Harzfeuer') to 27% ('Cupido') lower in those plants cultivated under water deficiency. The Ψπ of the leaves revealed a significant decrease in all three cultivars growing under water deficiency as compared to control plants and was about 40% lower than under well-watered conditions (Table 1). Proline contributed significantly to the adjustment of Ψπ in the leaves. Proline concentrations were significantly higher 46 days after treatment induction (DAT) as compared to the respective control treatment (Table 2). At the end of the water deficiency phase, 'Rio Grande' grown under water deficiency emerged followed by 'Harzfeuer' and 'Cupido'. After the re-watering period, plants of 'Harzfeuer' showed no significant differences between control and former water deficiency treatment. In contrast, proline concentrations in the previous water deficiency treatments from 'Cupido' and 'Rio Grande' were significantly lower compared to the respective control treatment. Finally, the concentration of Chl *a*, Chl *b* and Chl *a*+*b* in leaves of the water deficiency plants decreased significantly after 46 days compared with the control treatment (Table 2). During the re-watering period, the plants of the former water deficiency treatment revealed a significantly higher concentration of Chl *a*, Chl *b* and Chl *a*+*b* in comparison with the fulltime well-watered plants.

Table 1 Relative water content (% RWC), osmotic potential (Ψπ) and alteration in percent (%). Tomato leaves were harvested 46 days after inducing water deficit (WD). Control plants (C) served as control.

Cultivar	Treatments	RWC [%] [a]	%	Ψπ [a]	%
Rio Grande	C	83.34 ± 1.40*		-1.34 ± 0.02*	
	WD	64.66 ± 0.89	-22.25 ± 1.62	-1.91 ± 0.02	-42.03 ± 1.64
Harzfeuer	C	77.15 ± 0.89*		-1.39 ± 0.04*	
	WD	58.43 ± 0.40	-24.19 ± 0.83	-1.90 ± 0.01	-36.65 ± 1.31
Cupido	C	78.33 ± 1.03*		-1.47 ± 0.08*	
	WD	56.67 ± 0.40	-27.56 ± 0.86	-2.05 ± 0.02	-39.87 ± 3.14

[a] Values are mean ± SE of five replicates. * Significant difference between treatments (t-test, $P \leq 0.05$).

Table 2 Concentrations of proline, chlorophyll (Chl) *a*, Chl *b* and the total Chl (Chl *a*+*b*) of tomato leaves. The water deficit (WD) was conducted until 46 days of treatment (DAT); thereafter, plants were allowed to recover until 62 DAT when the final evaluation was done. Control plants (C) served as control.

			Concentration [mg g^{-1} DM][a]			
Cultivar	**Treatments**	**DAT**	**Proline**	**Chl *a***	**Chl *b***	**Chl a+*b***
Rio Grande	C	46	5.08 ± 0.22*	14.87 ± 0.58*	2.15 ± 0.09*	17.02 ± 0.66*
	WD	46	9.15 ± 0.36	10.92 ± 0.32	1.55 ± 0.05	12.47 ± 0.36
	C	62	5.88 ± 0.47*	13.68 ± 0.51*	1.35 ± 0.10	15.03 ± 0.60*
	WD	62	4.07 ± 0.28	16.15 ± 0.19	1.60 ± 0.09	17.75 ± 0.27
Harzfeuer	C	46	5.28 ± 0.11*	14.88 ± 0.26*	2.24 ± 0.07*	17.12 ± 0.32*
	WD	46	8.60 ± 0.49	11.36 ± 0.52	1.66 ± 0.12	13.02 ± 0.64
	C	62	3.61 ± 0.24	13.76 ± 0.48*	1.39 ± 0.09*	15.15 ± 0.57*
	WD	62	3.37 ± 0.15	16.16 ± 0.77	1.96 ± 0.12	18.12 ± 0.89
Cupido	C	46	5.86 ± 0.24*	14.18 ± 0.77*	1.96 ± 0.12*	16.14 ± 0.89*
	WD	46	7.35 ± 0.69	10.34 ± 0.57	1.53 ± 0.07	11.87 ± 0.64
	C	62	6.29 ± 0.26*	11.48 ± 0.59*	1.29 ± 0.06*	12.77 ± 0.65*
	WD	62	3.97 ± 0.19	14.42 ± 0.34	1.57 ± 0.05	15.99 ± 0.39

[a] Values are mean ± SE of five replicates. * Significant difference between treatments (t-test; $P \leq 0.05$) at each day after treatment.

3.2 PAM parameters

The time curves of the ETR were distinct in plants with water deficiency, evaluated 6 and 27 DAT, as compared to the well-watered control treatment (Fig. 1). The strongest influence of water shortage on the ETR was observed for 'Rio Grande'. Despite the distinct pattern of the curves, the biggest numerical difference between leaves of control and water deficiency was observed in the timeframe of 100 to 150 s after start of the measurement. After the re-watering phase, the ETR of the former water deficiency treatment was slightly higher than of the control plants irrespective of the cultivar (Fig. 1C, F, I).

The time course analysis of the qP demonstrated that qP was strongly affected by water deficiency 6 DAT (Fig. 2A, D, G). The most pronounced difference was noticed in 'Rio Grande', particularly on day 6 at 100 s after first illumination (Fig. 2A). Further, the PAM-images clearly demonstrate the spatial variability of the values over individual leaves after 80 and 300 s of first illumination (Fig. 3). During the re-watering phase, an approximation of the

curves between the former water deficiency and the respective control treatment was observed (Fig. 2C, F, I).

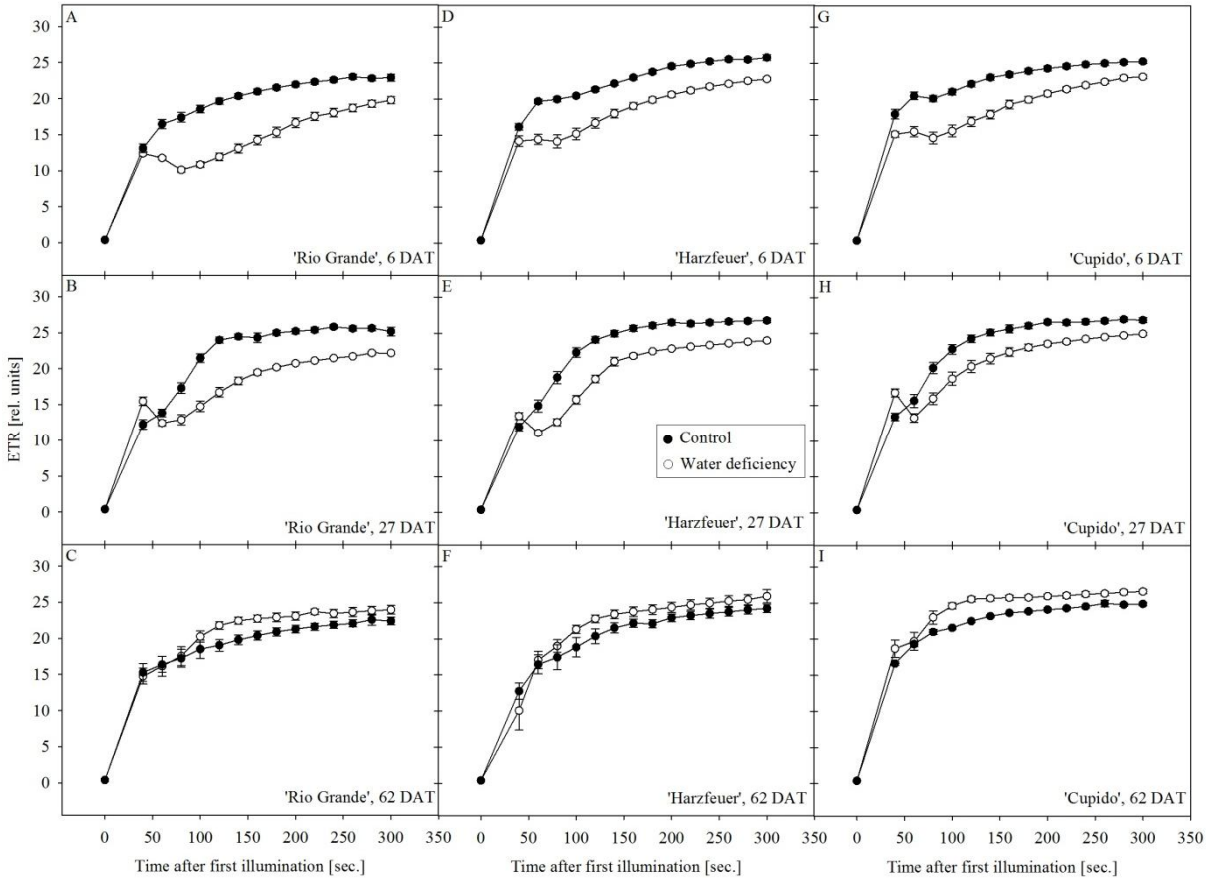

Fig. 1 Time curves of the relative apparent electron transport rate (ETR). ETR was measured on tomato leaves from the cultivars Rio Grande (A - C), Harzfeuer (D - F) and Cupido (G - I) on day 6 (A, D, G), 27 (B, E, H) and 62 (C, F, I) of the experiment. Values represent the mean ± SE (standard error) of ten (until day 44) or five (from day 48) samples.

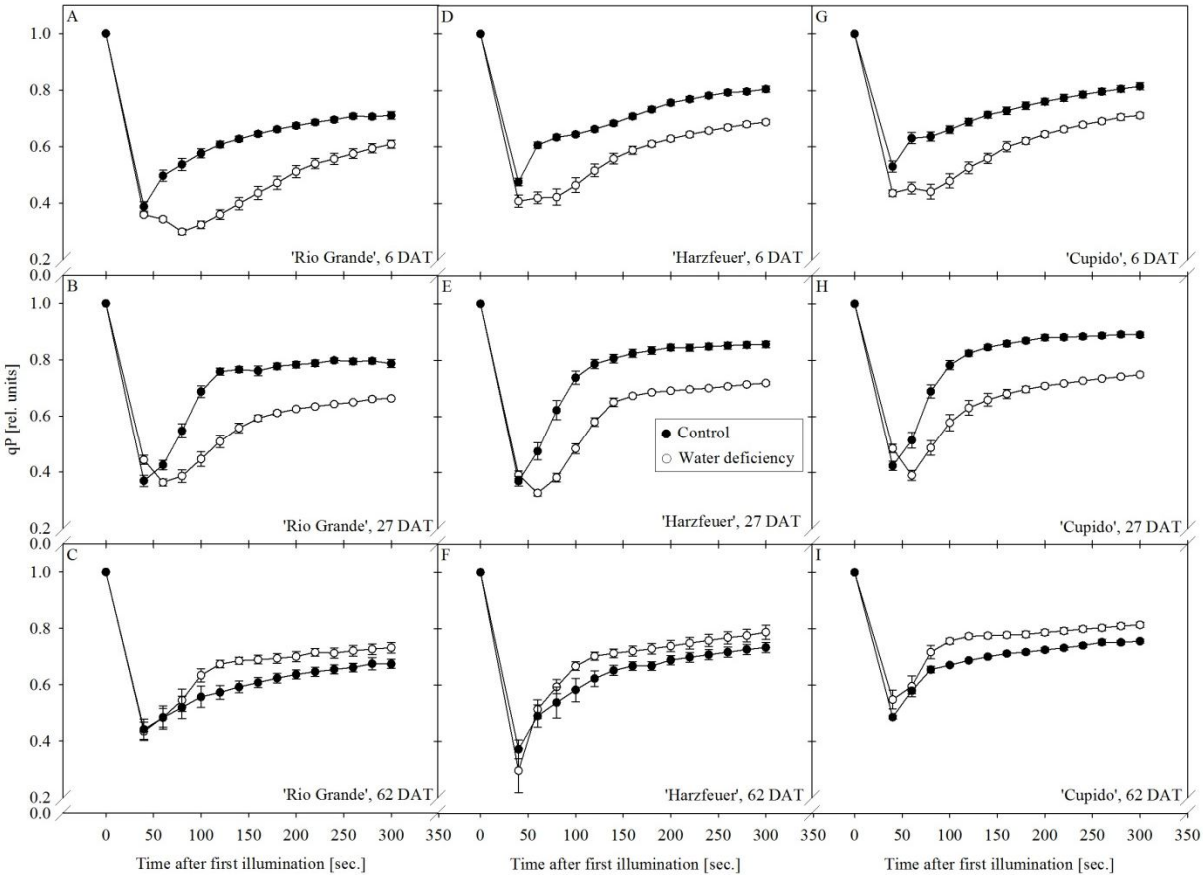

Fig. 2 Time curves of the coefficient of photochemical quenching (qP). The parameter was measured on tomato leaves from the cultivars Rio Grande (A - C), Harzfeuer (D - F) and Cupido (G - I) on day 6 (A, D, G), 27 (B, E, H) and 62 (C, F, I) of the experiment. Values represent the mean ± SE (standard error) of ten (until day 44) and five (from day 48) samples.

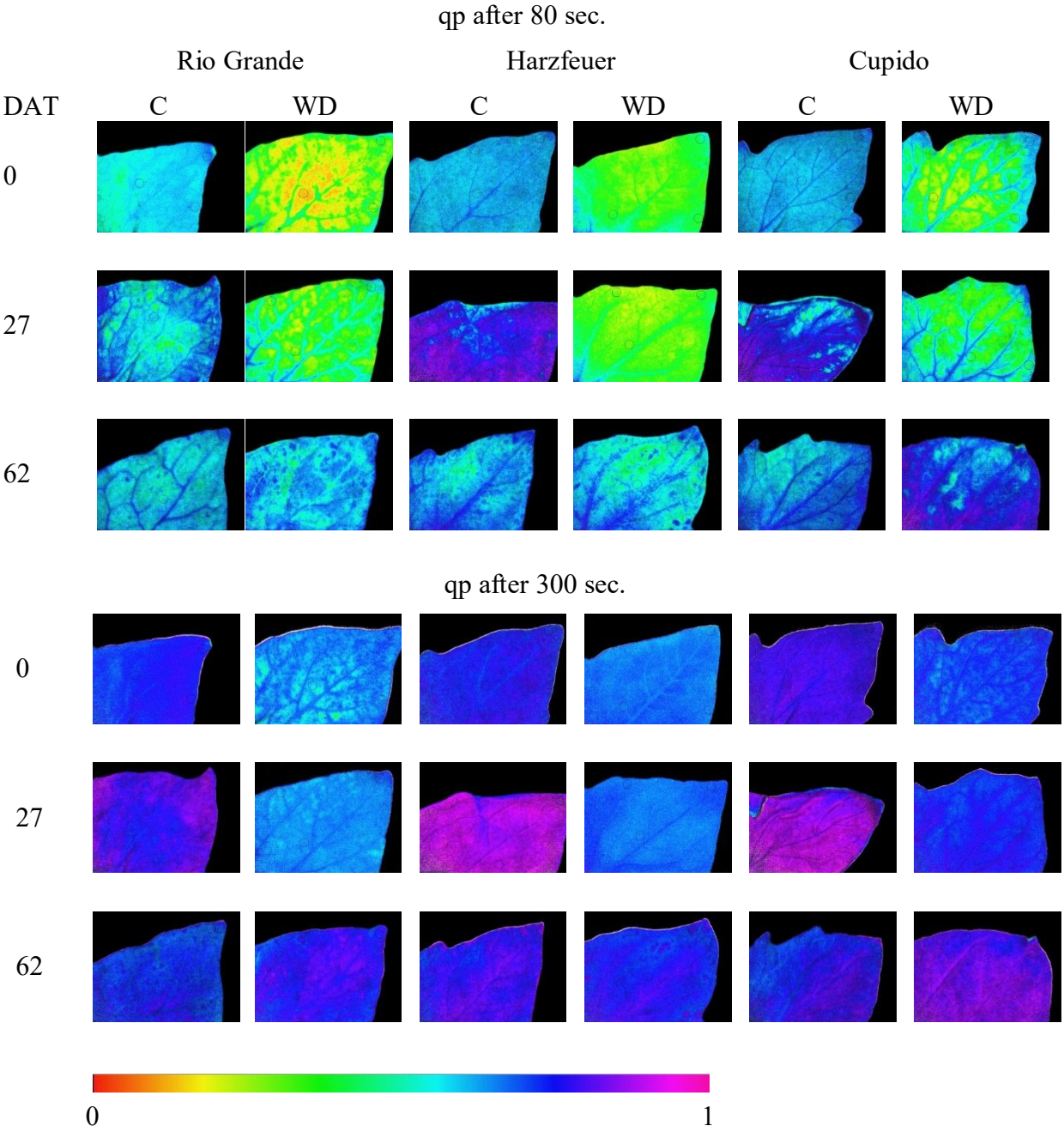

Fig. 3 Time course of the coefficient of photochemical quenching (qP). The images of the parameter were recorded 80 and 300 sec. after the first illumination of the tomato leaves from the cultivars Rio Grande, Harzfeuer and Cupido at day 6, 27 and 62 of the experiment (DAT). C = Control plants; WD = plants treated with water deficiency.

3.3 Multiparametric fluorescence ratios

The BFRR_UV revealed water deficiency induced changes in ‘Rio Grande’ and ‘Harzfeuer’ at 6 DAT. In general, this parameter was significantly higher in plants of the water deficiency treatment. The significant differences between control and water deficiency plants lasted until the DAT 46, when the re-watering phase was initiated. Then, the significant differences registered in the previous phase were not evident anymore in ‘Rio Grande’ and ‘Harzfeuer’ (Fig. 4A, B). In contrast, the BFRR_UV in ‘Cupido’ remained high (Fig. 4C).

The FLAV, which is related to the accumulation of epidermal flavonols in the leaves, indicates a clear impact of the water deficiency at 6 DAT (Fig. 4). Drought exposed plants had higher FLAV values than well-watered plants. ‘Rio Grande’ responded within 7 days to the re-watering phase (Fig. 4D); in ‘Harzfeuer’ the approaching of the FLAV values was observed after 11 days (Fig. 4E). Values of ‘Cupido’ decreased, too, but remained significantly higher than the control plants until the end of the experiment.

The NBI_R of the water deficiency exposed plants were lower than the values of the control treatment. However, this parameter responded with some delay to the stress situation. Reliable significant differences between the treatments were observed from 37 DAT in ‘Rio Grande’ and ‘Harzfeuer’. In ‘Cupido’, significant changes were observed starting at 23 DAT. By trend, the re-watering of the plants led the values to approach the normal values, but this was more evident for ‘Rio Grande’ and ‘Harzfeuer’ (Fig. 5A, B).

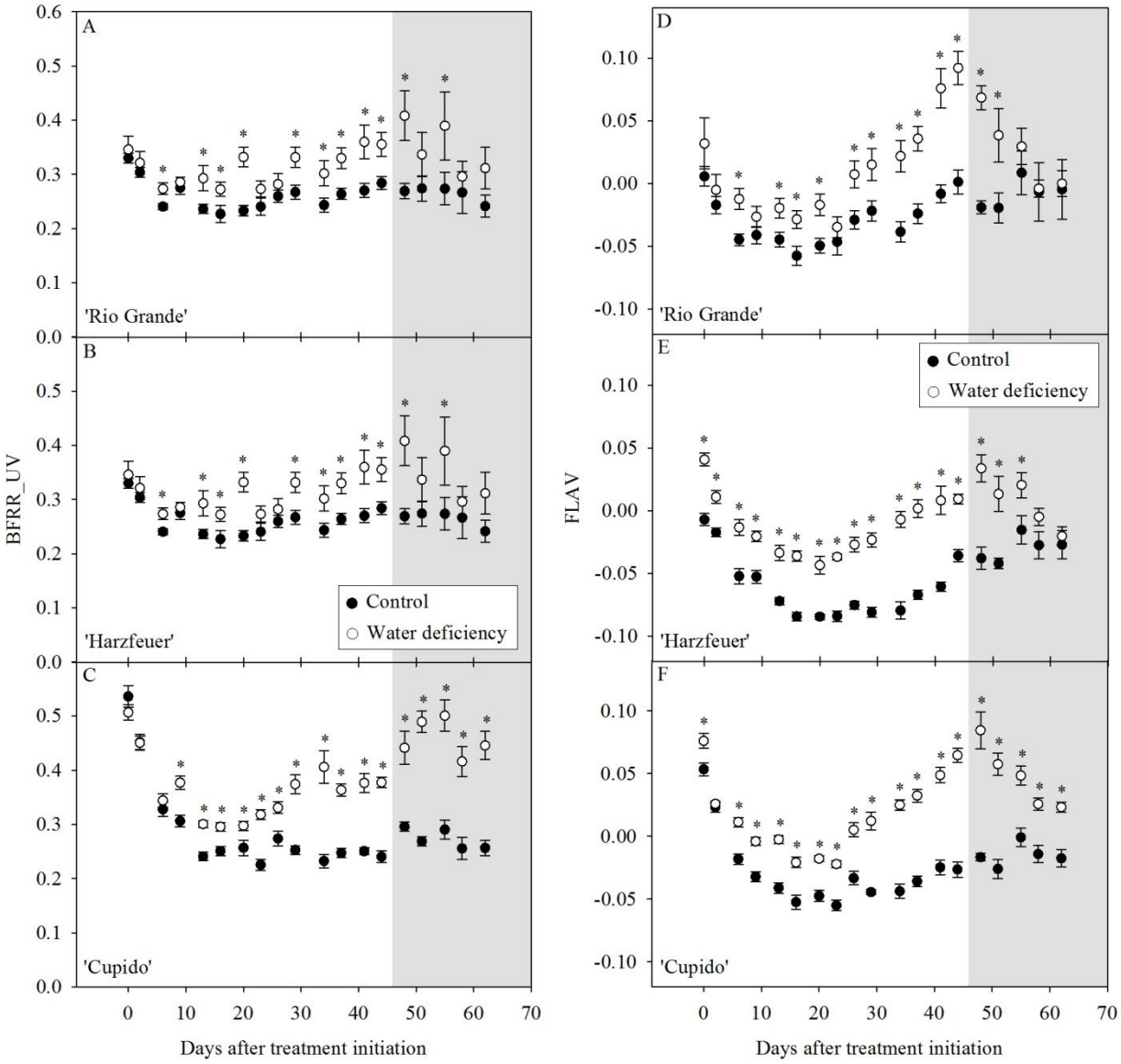

Fig. 4 Blue-to-far-red fluorescence ratio after excitation with UV-light (BFRR_UV) (A - C) and the FLAV-Index expressing the logarithm of the ratio of far-red fluorescence after red light excitation and the far-red fluorescence after excitation with UV-light (D - F). Values were recorded on tomato leaves from the cultivars Rio Grande (A, D), Harzfeuer (B, E) and Cupido (C, F). The grey regions represent the re-watering time without water deficiency. Values represent the means ± SE (standard error) of ten (until day 44) and five (from day 48) samples. * Significant differences ($P \leq 0.05$) between control and water deficiency treatment for each cultivar and measuring day assessed by t-test.

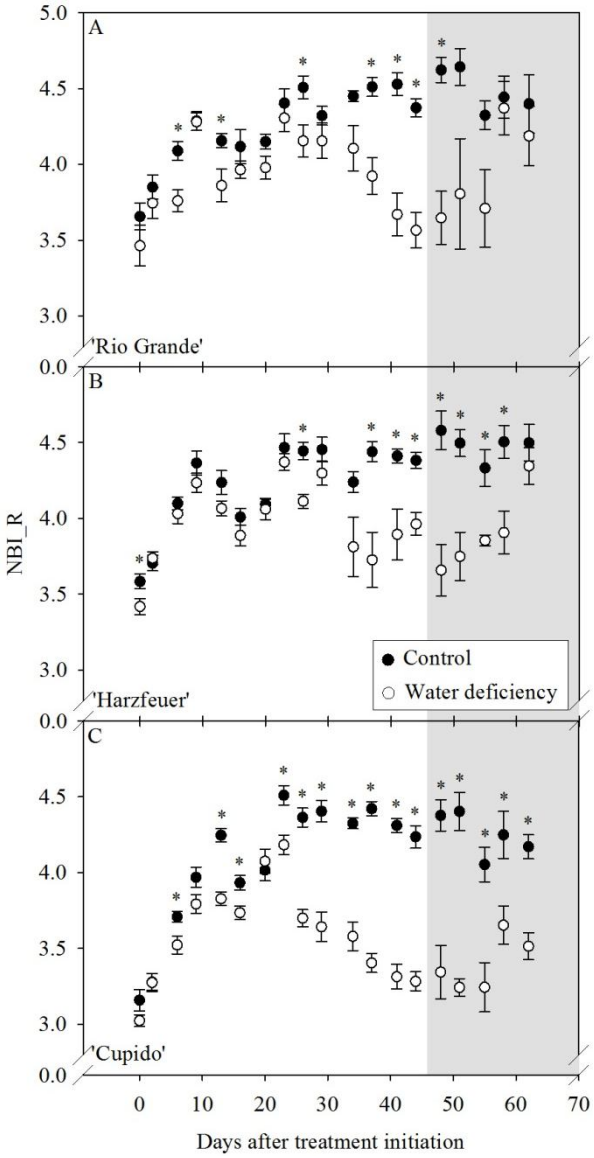

Fig. 5 Time course of the ratio of FRF after UV-light excitation to RF after red light excitation (NBI_R). NBI was recorded on the cultivars Rio Grande (A), Harzfeuer (B) and Cupido (C). The grey regions represent the re-watering time without water deficiency. Values represent the mean ± SE (standard error) of ten (until day 44) and five (from day 48) samples. * Significant differences ($P \leq 0.05$) between control and water deficiency treatment for each cultivar and measuring day assessed by t-test.

4 Discussion

Here, we proof the suitability of specific ratios of the multiparametric fluorescence technique to reveal the onset and intensity of stress in different tomato genotypes exposed to long-term water deficiency, as well as during re-watering. Thereby, we selected traditional parameters (e.g., RWC, Chl *a*, proline) for the monitoring of the plant's physiological status.

Plants growing under water deficiency can undergo several anatomical, morphological, physiological, biochemical and molecular adaptations in order to maintain a positive turgor. It is evident that stomata close progressively with increased drought stress, followed by reduced net photosynthesis rates. Additionally, it is well known that a good correlation between leaf water potential and stomatal conductance exists, even under water shortage (Reddy et al. 2004). In our trial, plants exposed to water deficiency had significantly lower RWC and Ψπ as well as higher proline concentration (Table 1). Previously, these parameters were highlighted as reliable indicators of drought stress and resistance, respectively (Parry et al. 2005). To prevent water loss due to drought stress, plants accumulate osmolites such as proline, amongst others to support the osmotic adjustment in the cells (Morant-Manceau et al. 2004) or act as stabilizer of subcellular structures (Sánchez-Rodríguez et al. 2010). Water deficiency induced a significant increase in proline concentration (Table 2), which is in line with other publications (Kishor and Sreenivasulu 2014).

Furthermore, changes in the Chl concentration are frequently used as stress indicator (Matile and Hörtensteiner 1999). In our experiment water deficiency significantly decreased the Chl *a*+*b* concentration in the three evaluated genotypes. The Chl degradation was accompanied by disfunction in the functionality of the photosynthetic apparatus (Tuba et al. 1996). This was also confirmed by us in the three genotypes at 6 and 27 DAT taking the ETR and qP as indicative parameters. Based on this results and findings of Tuba et al. (1996) the three used cultivars revealed a rather indicate to be poikilochlorophyllous behavior with significant decrease of chlorophyll content in the water deficiency phase followed by an increase in response to re-watering. In our study, the Chl *a*+*b* concentration was 23% (‘Harzfeuer’) to 26% (‘Rio Grande’ and ‘Cupido’) lower during water deficiency and about 18/ 19% (‘Rio Grande’/ ‘Harzfeuer’) to 25% (‘Cupido’) higher after re-watering than in fulltime well-watered plants. The fast increase of the Chl *a*+*b* concentration after re-watering provides another evidence that the three cultivars are better classified as poikilochlorophyllous.

Further support for this opinion is the ETR, which reflects the stomatal limitations imposed on photosynthesis and thus, the activity of CO_2 assimilation (Baker and Rosenqvist 2004). Consequently, the decrease of the ETR under water deficiency 6 as well as 27 DAT stands for a lower CO_2 assimilation activity. Complementary, the adjustment of the ETR shows an effect of re-watering (Fig. 1). Further, the qP gives supplementary information about the functionality of PSII concerning the photosynthetic quantum conversion and open PSII reaction centers or more specifically, the fraction of Q_A in its oxidized state (Kramer et

al. 2004). Briefly, qP = 1 stands for the probability by which excitons in the PSII antenna system will initiate a photochemical reaction, hence the probability is zero when q = 0 (Krause and Jahns 2004). Thus, low qP in the water deficiency plants reveals a strong impact on the PSII, i.e. a reduction of the open PSII reaction centers (Fig. 2). The immediate effect of water deficiency as well as the plant adaptation during the water deficiency and re-watering phases is highlighted also by the spatially resolved fluorescence pictures (Fig. 3). The approximation of the values after 16 days of normal water supply confirms those results observed for ETR measurements. Nevertheless, the changes in Chl *a*+*b* concentrations during the stress and re-watering phases might have influenced these results.

The BFRR_UV and the FLAV indices, recorded by the multiple fluorescence excitation technique, revealed early physiological changes in the water deficiency exposed plants (Fig. 4). Here, we observed a cultivar-dependent response to the water deficiency, which was more pronounced in 'Cupido' than in 'Rio Grande' and 'Harzfeuer'. Further, the BFRR_UV of 'Rio Grande' and 'Harzfeuer' showed significant effects of re-watering after a few days of full water supply as compared with 'Cupido'. Analysis of the raw data demonstrate that the increase of the BFRR_UV is explained by a strong ('Harzfeuer') and a slight ('Cupido', 'Rio Grande') increase of the absolute intensities of BF, and by a very strong ('Cupido') and a moderate ('Harzfeuer', 'Rio Grande') decrease of ChlF in the far-red band, respectively (*data not shown*). While the decrease of FRF might be associated with the reduction of the Chl content and the shielding of the excitation light by epidermal UV-absorbing compounds, the increase of the BF is directly related to the accumulation of blue-fluorescing compounds. It is well known that phenolic compounds are the major substances contributing for the BF (Lichtenthaler and Schweiger 1998). As shown in Fig. 4, a significant increase of phenols as well as of flavonoids due to drought stress might be expected in tomato leaves (Sánchez-Rodríguez et al. 2010). Furthermore, the decrease of the Chl *a*+*b* concentration and lower quantity of carotenoids, which normally re-absorb BF and green fluorescence emission, might intensify this effect (Szigeti 2008).

The accumulation of flavonols in the leaf epidermis can be monitored by the fluorescence screening technique (Bilger et al. 1997), in our study provided by the FLAV index. The FLAV recordings in the time-course enable to recognize a strong and early influence of water deficiency on 'Harzfeuer' and a delayed response of 'Rio Grande' and 'Cupido' (Fig. 4). The re-establishment of water supply revealed a fast response of 'Rio Grande' and 'Harzfeuer', but not of 'Cupido'. The pronounced FLAV increase during the water deficiency phase, and its decrease after re-watering, confirms previous observations that the FLAV Index is a

reliable indicator of drought-induced stress (Bürling et al. 2013). Alterations of the FLAV might arise due to changes in the synthesis and accumulation of flavonoids in the tissue, particularly the epidermal flavonols, as well as the 'apparent' concentration of these compounds per leaf area as driven by the water loss and the size-reduction of single cells. In a re-watering phase cells absorb huge amounts of water, inducing a 'dilution effect', as observed e.g., for the osmolites (Table 2) and also other cellular compounds. This explains the almost total approximation of the FLAV values, except for 'Cupido'.

The NBI_R, which depends on the content of Chl and epidermal phenolics, provides a promising ratio for fast and non-invasive sensing of changes of the Chl and flavonoid concentration (Tremblay et al. 2012). We show a significant decrease of the ChlF ratio during water deficiency e.g., at 6 DAT in 'Rio Grande' and 'Cupido'. In contrast, 'Harzfeuer' demonstrates a delayed response (Fig. 5). Further, the general course of adaptation of 'Rio Grande' and 'Harzfeuer' to re-watering was quite similar, while 'Rio Grande' responded significantly within 5 days, 'Harzfeuer' approached this level within 16 days. In 'Cupido' the values did not show any approximation after re-watering. Due to the fact that the NBI depends on both Chl and epidermal phenolics, and taking into account that the difference in the Chl concentration of control and stress did not differ appreciable between the genotypes, our trails indicate a stronger relevance of the synthesis of phenolic compounds in 'Cupido', also confirming the trend observed for FLAV. Irrespective of the similar response of the three cultivars to the PAM ChlF (Figs. 1-3), the fluorescence indices BFRR_UV, FLAV and NBI indicate a stronger drought-induced activation of the secondary metabolism in 'Cupido'.

5 Conclusions

We demonstrate that the ChlF provides reliable parameters for sensing water deficit and re-watering processes in adult tomato plants. Thereby, the BFRR_UV, FLAV and NBI_R ratios of the multiparametric fluorescence enable a more effective and faster sensing of water deficiency stress without the need of dark-adaption as required for the PAM recordings. Fluorescence emissions of 'Cupido' revealed the strongest changes to water deficiency and also the slowest approximation after re-watering. In contrast, 'Harzfeuer' and 'Rio Grande' showed less influence of water deficiency and faster response to re-watering. Our results indicate that the three cultivars were similarly impaired in their primary metabolism while 'Cupido' was comparatively stronger influenced in its secondary metabolism. On this basis, we also highlight the potential of the multiparametric fluorescence ratios for the fast screening of horticultural genotypes. Nevertheless, further studies are needed to analyze the

accumulation pattern of compounds at cell level leading to the alterations of the fluorescence signals.

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C Salinity-induced changes of multiparametric fluorescence indices of tomato leaves[2]

1 Introduction

The problem of salinity and its increasing relevance for horticultural crops is well described in the literature (Cuartero et al. 2006, Hunsche et al. 2010a, Hunsche et al. 2010b). Particularly for tomato, most of the salinity studies have evaluated morpho-physiological modifications such as vegetative development, fresh and dry matter and marketable yield (Cuartero et al. 2006, Gautier et al. 2010). In many cases, biochemical parameters, e.g., the concentrations of ions, sugars and secondary compounds, were also analyzed (Incerti et al. 2007). For example, it is well known that plants respond to salinity by accumulating specific compounds, such as proline, sugars, organic acids and flavonoids (Cayuela, et al. 1996) as key components in plant resistance. Phenolic compounds can also be accumulated as a stress response in susceptible cultivars (Juan et al. 2005). All these parameters are relevant for understanding how cultivars deal with adverse environmental conditions. However, the recording of these data is time consuming and often requires costly laboratory analysis after sampling. In contrast, rapid and non-destructive techniques offer a timely evaluation of the physiological status of the plants and might contribute to the precise selection of stress-tolerant genotypes.

As one of the most traditional non-destructive techniques, pulse-amplitude modulated (PAM) chlorophyll fluorescence (ChlF) recorded at 680–690 nm provides several parameters for sensing environment-triggered physiological changes at the leaf level (Baker and Rosenqvist 2004, Bilger et al. 1995, Lichtenthaler et al. 1996). The major limitation of this technique is that reliable recordings require a time-consuming dark-adaptation and measurements performed in the dark.

In contrast, the detection of fluorescence in the entire range of visible light (380–750 nm) provides information about the localization, type and concentration of specific fluorophores, including chlorophyll (Chl) molecules, in the plant tissue (Cerovic et al. 1999). Chl molecules emit their fluorescence in the red and far-red bands, whereas cinnamic acids and a small fraction of phenolics, covalently bound to the cell walls, are the principal emitters in the blue and green bands (Buschmann and Lichtenthaler 1998, Morales et al. 1996, Lichtenthaler et al. 1998). To overcome variations in intensity resulting from measuring conditions and leaf

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morphology, fluorescence ratios might be adopted to provide more reliable information for treatment comparisons (Cerovic et al. 1999). The suitability and the use of multispectral fluorescence based indices for stress detection is gaining importance for field (Bürling et al. 2013, Leufen et al. 2013) and horticultural (Kautz et al. 2014, Müller et al. 2013) crops. However, there is still a high demand for the further development and use of fluorescence sensors in different research fields of stress physiology and practical applications, including salinity-induced stress.

The objective of this work was to evaluate the suitability of multiparametric fluorescence indices for sensing salinity-induced stress in tomato (*Solanum lycopersicum*) plants *in situ* without dark-adaptation. For this purpose, we evaluated three tomato genotypes grown in standard or saline environments. As a reference, we recorded the traditional Fv/Fm ChlF parameter and analyzed sodium (Na), potassium (K), magnesium (Mg) and proline as well as chlorophyll (Chl) concentrations for a precise characterization of the salinity-triggered stress. In this context, we hypothesized that the fluorescence indices BFRR_UV (ratio of BF (blue fluorescence) to FRF (far-red fluorescence), both excited with UV (ultraviolet)-light), FLAV (logarithm of the ratio of red-excited FRF to UV-excited FRF), NBI (ratio of UV-excited FRF to green-excited red fluorescence) and SFR (ratio of FRF to RF after green-light excitation) of the multiple fluorescence technique, allow the identification of the impact of salinity on the leaves of the three tomato genotypes.

2 Materials and methods

2.1 Plant material and growth conditions

Experiments were conducted under greenhouse conditions from August to October. Seeds of *Solanum lycopersicum* L. F1 hybrid Harzfeuer (Volmary GmbH, Münster, Germany), *S. lycopersicum* var. H-2274 and var. Rio Grande (both donations from Mustafa Demirkaya, Erciyes University, Kayser, Turkey) were used in the present study. Tomato seeds were steeped in the dark at 20 °C for three days. The germinated seeds were transferred into rock wool trays and cultivated until the third leaf stage. The plantlets were transplanted into 10 L pots filled with perlite (Lerligran G, Knauf Perlite GmbH, Dortmund, Germany) and placed on two greenhouse tables. Fertigation was based on Kristallon™ Blau (Yara GmbH & Co. KG, Dülmen, Germany) and amended with calcium nitrate tetrahydrate ($Ca(NO_3)_2$) (99% purity, AppliChem GmbH, Darmstadt, Germany). Nutrient solution in excess was drained off at the bottom of the pots after perlite saturation. The nutrient solution and its spillover were isolated from each other, and the nutrient solution was not recycled.

2.2 Treatments

Plants of each genotype were separated into two treatments (n = 10 plants per treatment group): T1, control plants provided with standard nutrient solution (electrical conductivity, EC = 2 mS·cm^{-1}); T2, plants provided with nutrient solution amended with sodium chloride (NaCl) (99% purity, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) targeting an EC value of 12.4 mS cm^{-1}. To avoid osmotic shock in the NaCl-treated plants, the EC of the solution was increased in three steps starting at 7 mS cm^{-1} in the 1st week to EC = 9.6 mS cm^{-1} in the second week, reaching a final concentration of 12.4 mS cm^{-1} in the third week.

2.3 Analytical determinations

The mineral concentrations were analyzed at the end of the experiment from freeze-dried (Gamma 1-16 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) and ground samples of middle-aged leaves. After acid-digestion of 0.1 g ground dry matter (DM) in a microwave, the concentration of Mg, K and Na was determined by atomic absorption spectrometry (AAS, Perkin-Elmer, Analyst 300, Wellesley, MA, USA) as described by Hunsche et al. (2010).

Proline concentration in the leaves was determined as described by Bates et al. (1973). A mixture of 3 ml sulfosalicylic acid and 0.1 g DM grounded leaves was centrifuged at 4200 rpm for 20 min at 20 °C. A total of 1.8 ml sulfosalicylic acid, 2 ml glacial acetic acid and 2 ml ninhydrin acid was then added to 0.2 ml of the supernatant. The mixture was boiled at 100 °C for one hour in a hot water bath. After the sample cooled down to 20 °C, 4 ml toluene was added to the mixture. The upper, organic portion was collected for spectrophotometric measurements (Lambda 35 UV/VIS Spectrophotometer, PerkinElmer, Waltham, MA, USA). The absorbance of the extracts was measured at 520 nm.

The chlorophyll (Chl) concentration (Chl a + b) of the samples was determined from 0.05 g ground DM (Munné-Bosch and Alegre 2000); the material was mixed with 5 ml methanol and centrifugated at 4000 rpm for 15 min at 4 °C. The supernatant was transferred to 50 ml volumetric flasks. The extraction procedure with methanol was repeated three times; the flasks were then filled up to 50 ml with methanol. The absorbance of the extracts was determined at 647 nm (A_{647}) and 664 nm (A_{664}) with a UV-VIS spectrophotometer (Lambda 35 UV/VIS Spectrophotometer, PerkinElmer, Waltham, MA, USA).

2.4 Fluorescence measurements

Fluorescence measurements were performed on detached leaves under laboratory conditions, either immediately after sampling in the case of the multiparametric fluorescence excitation system (Multiplex®3, Force-A, Orsay, France) or after dark adaptation to the room conditions in the case of pulse-amplitude-modulated (PAM) chlorophyll fluorescence (Imaging-PAM®, Heinz-Walz GmbH, Effeltrich, Germany). Fluorescence determination over the time-course of the experiment was performed on leaves of different physiological ages: the first measurements were conducted on the fourth leaf level counted from the bottom (cotyledons excluded), and every 6–8 days the upper leaf level was selected, according to the growth of the plant.

ChlF parameters were recorded from the adaxial side of dark-adapted (30 min) leaves with our system as described elsewhere (Bürling et al. 2010). Briefly, the light source (0.5 µmol m^{-2} s^{-1} PAR) used for fluorescence excitation and actinic illumination at 470 nm contains 96 blue light diodes. Fluorescence images were recorded with a black and white CCD (8.458 mm chip with 640 × 480 pixels) camera operated in 10 bit mode at 30 frames per second. Determinations of the ground (Fo) and maximum fluorescence (Fm) were used to calculate the variable fluorescence Fv (Fv = Fm − Fo) and to estimate the maximum quantum efficiency of PSII photochemistry (Fv/Fm) (Baker and Rosenqvist 2004). Data evaluation was based on the recorded pictures. In each single image, three areas of interest (AOI), at the leaf edge, apex and center, were selected. The mean of the three AOIs was calculated before running the statistical analysis.

The fluorescence in the blue (BF), red (FR) and far-red (FRF) spectral bands, excited with UV and green (G) light, was recorded on detached leaves with a multiparametric hand-held fluorescence sensor (Leufen et al. 2013; Müller et al. 2013). The fluorescence recordings were performed immediately after harvesting the leaves. Leaves were fixed horizontally on a sample holder at a defined distance (10.5 cm) to the sensor. A frontal cover plate having an aperture of 6 cm in diameter was used to standardize the area to be measured. As indicative parameters, we selected four fluorescence ratios: the BF to FRF ratio after UV light excitation (BFRR_UV); the FLAV Index (FLAV), as expressed by the logarithm of the ratio of FRF after red light excitation to the FRF after excitation with UV light, the Nitrogen Balance Index (NBI), given by the ratio of FRF after UV light excitation to RF after G light excitation; and the FRF to RF ratio after G light excitation (SFR_G).

2.5 Data analysis

The statistical analysis was performed with IBM SPSS Statistics 20.0 (IBM Corporation, New York, NY, USA). Means were compared with a *t*-test ($p \leq 0.05$), and graphs (mean ± SE) were drawn using SigmaPlot 11.0 (Systat Software Inc., San Jose, CA, USA).

3 Results

3.1 Mineral, proline and chlorophyll concentrations

Leaves of the three tomato genotypes significantly accumulated Na over the 40 days of the experiment in salinity-grown plants compared with the control (Table 1); the concentration increase in the salinity-exposed plants ranged from 231% in "H-2274" to 525% in "Harzfeuer". In contrast, the K concentration in leaves decreased in a range of −8% ("H-2274") to −47% ("Harzfeuer"), whereas the Mg concentration was between −14% ("Rio Grande") and −33% ("Harzfeuer") lower in the salinity-grown plants.

As a biochemical indicator of plant stress, proline concentration increased more than 600% in all genotypes (Table 1). In addition, we observed significant differences among the genotypes; here, the proline concentration in "Harzfeuer" was significantly lower than in "Rio Grande" and "H-2274". Lastly, the Chl concentration decreased due to salinity in the leaves of "Harzfeuer" only, whereas it remained unaffected in the other genotypes.

3.2 Maximum quantum efficiency of photosystem II (Fv/Fm)

Fv/Fm values indicated no significant differences at 7 and 13 days after treatment initiation (DAT), irrespective of the genotype (Fig. 1). Significantly higher values in the NaCl treatment group were observed at 20 DAT in "Rio Grande" and "Harzfeuer" and at 26 DAT in "H-2274". More pronounced differences between salinity and control treatment were observed in "Rio Grande".

Table 1 Sodium, potassium, magnesium, proline and total Chl (Chl *a* + *b*) concentrations in tomato leaves. Samples were taken 40 days after initiation of the treatments.

Tomato Cultivar	**Treatments**	**Na [mg/g DM]**	**K [mg/g DM]**	**Mg [mg/g DM]**	**Proline [mg/g DM]**	**Chl *a* + *b* [mg/g DM]**
Rio Grande	Control	4.74 ± 0.20*	70.95 ± 1.44*	3.74 ± 0.14*	0.54 ± 0.04*	12.67 ± 0.26*$^{n.s.}$
	NaCl	20.95 ± 7.26	53.97 ± 2.35	3.20 ± 0.07	4.14 ± 0.18	13.69 ± 0.63
H-2274	Control	4.99 ± 0.21*	69.07 ± 1.84*	3.32 ± 0.12*	0.44 ± 0.07*	12.28 ± 0.28*$^{n.s.}$
	NaCl	16.56 ± 1.20	63.36 ± 1.97	2.87 ± 0.05	3.36 ± 0.24	12.40 ± 0.29
Harzfeuer	Control	5.40 ± 0.13*	73.04 ± 2.40*	2.47 ± 0.07*	0.25 ± 0.03*	14.32 ± 0.35*
	NaCl	33.79 ± 1.27	38.71 ± 1.87	1.83 ± 0.09	1.79 ± 0.21	12.54 ± 0.41

* Significant differences according to *t*-test ($p \leq 0.05$; $n = 10$) between control and NaCl treatment for each genotype and measuring day; all data were expressed as the mean ± SE; *$^{n.s}$ not significant.

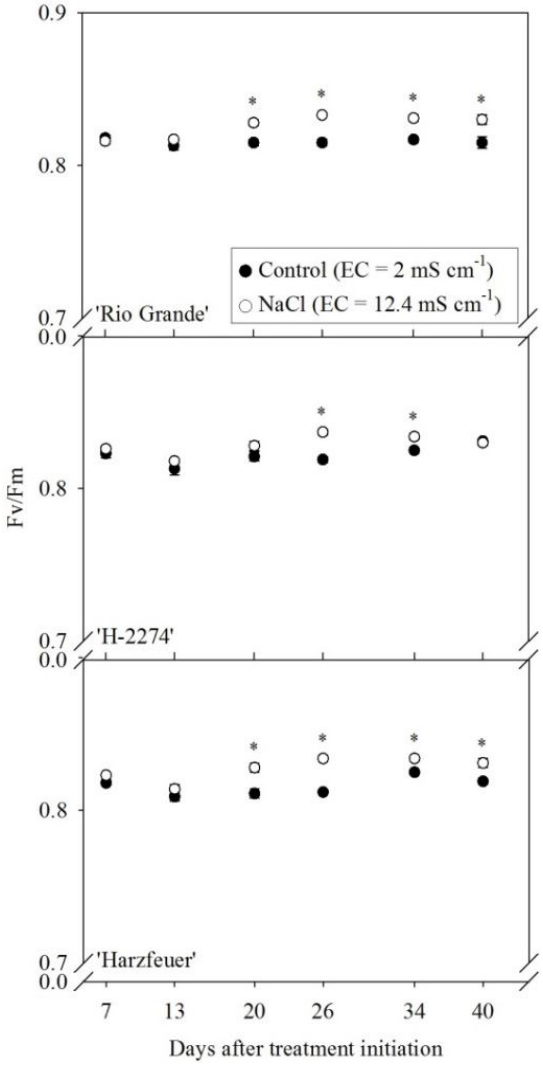

Fig. 1 Maximal photochemical efficiency (Fv/Fm) of tomato leaves. Measurements were taken from "Rio Grande", "H-2274" and "Harzfeuer" in the course of the experiment. * Significant differences according to *t*-test ($p \leq 0.05$; $n = 10$) between control and NaCl treatment for each genotype and measuring day; all data were expressed as the mean ± SE.

3.3 Blue-to-far-red fluorescence ratio (BFRR_UV)

In general, the BFRR_UV was significantly higher in salinity exposed plants. Already at 7 DAT, the BFRR_UV was significantly higher due to NaCl in "Rio Grande" and "H-2274" compared with the respective control plants (Fig. 2). "Harzfeuer" had a delayed response, showing significant differences between the experimental treatments at 20 DAT (Fig. 2). Unexpectedly, the values in the NaCl treatment of "H-2274" approached the control values at 26 DAT and thereafter. In contrast, the values for the NaCl-treated plants of "Rio Grande" and "Harzfeuer" remained higher than those for the control plants.

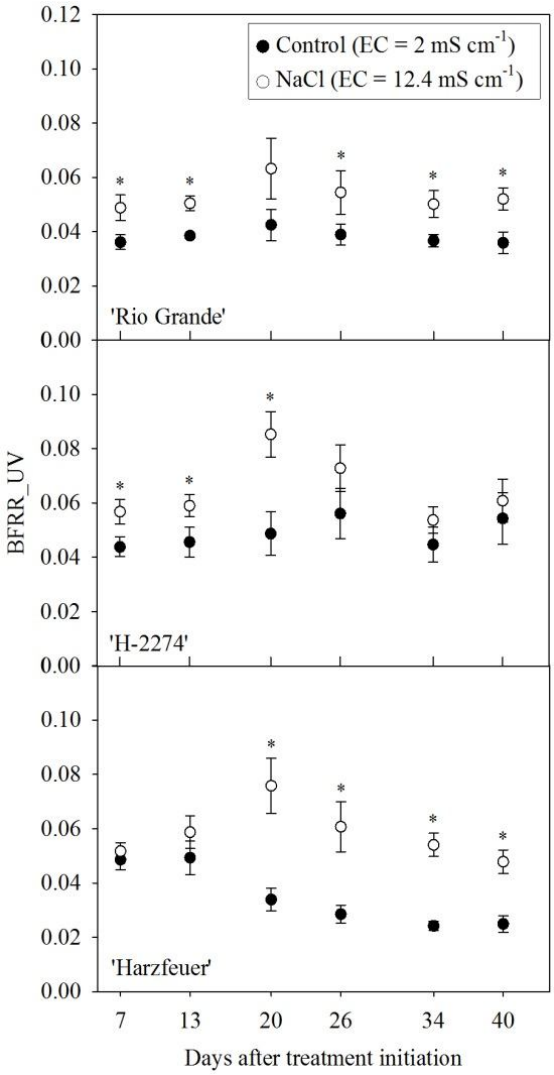

Fig. 2 Blue-to-far-red fluorescence ratio (BFRR_UV) after excitation with UV light displayed over the time course of the experiment. Readings were taken on tomato leaves from the genotypes Rio Grande, H-2274 and Harzfeuer. Values represent the mean ± SE (standard error, $n = 10$). * Significant differences ($p \leq 0.05$) between control and NaCl treatment for each genotype and measuring day, assessed by *t*-test.

3.4 Flavonol-index (FLAV)

The FLAV, which is related to the accumulation of flavonols in the leaf epidermis, showed a delayed response to salinity. The first significant responses were observed at 20 DAT (Fig. 3). The salt-exposed plants had higher FLAV values than the control plants. Generally, “Harzfeuer” responded with a stronger increase compared with the control treatment than “Rio Grande” and “H-2274”. However, no significant differences between the control and salt treatments could be measured at the end of the experiment.

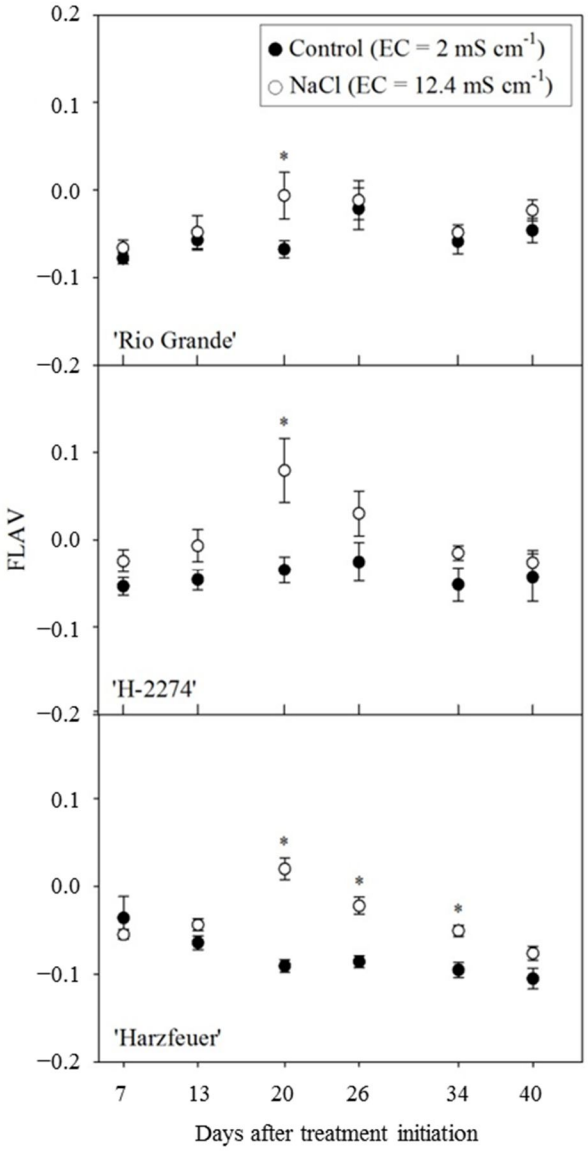

Fig. 3 The FLAV-Index expressing the logarithm of the ratio of far-red fluorescence after red light excitation to far-red fluorescence after excitation with UV-light displayed over the time course of the experiment. Readings were taken on tomato leaves from the genotypes Rio Grande, H-2274 and Harzfeuer. Values represent the mean ± SE (n = 10). *Significant differences ($p \leq 0.05$) between control and NaCl treatment for each genotype and measuring day, assessed by t-test.

3.5 Nitrogen balance index (NBI_G)

The NBI_G values of the salinity-exposed plants were higher than those of the control plants (Fig. 4). At 7 DAT, significant differences between the salinity and control treatments were observed in all genotypes. Subsequently, the three genotypes showed distinct courses of development over the time. In the case of “Harzfeuer”, significant differences in the salt-

treated plants were observed until the end of the experiment, whereas the values for “Rio Grande” and “H-2274” declined to the level of the control treatments.

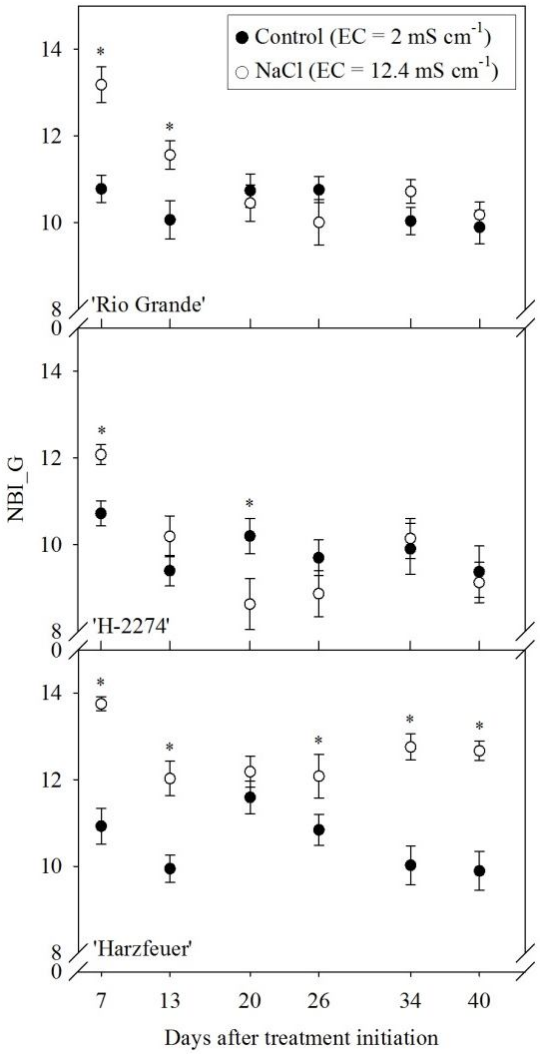

Fig. 4 The ratio of UV-excited far-red fluorescence to green-excited red fluorescence (NBI_G). Readings were taken on tomato leaves from “Rio Grande”, “H-2274” and “Harzfeuer”. Values represent the mean ± SE (n = 10). * Significant differences ($p \leq 0.05$) between control and NaCl treatment for each genotype and measuring day, assessed by t-test.

3.6 Simple fluorescence ratio (SFR_G)

Salinity-exposed plants had higher SFR_G values than control plants at 7 DAT (Fig. 5). The ratios for the salt treatment in “Rio Grande” and “H-2274” approached the control treatments over the course of the experiment. In contrast, SFR_G of the salt treated plants in “Harzfeuer” remained higher than the control treatments.

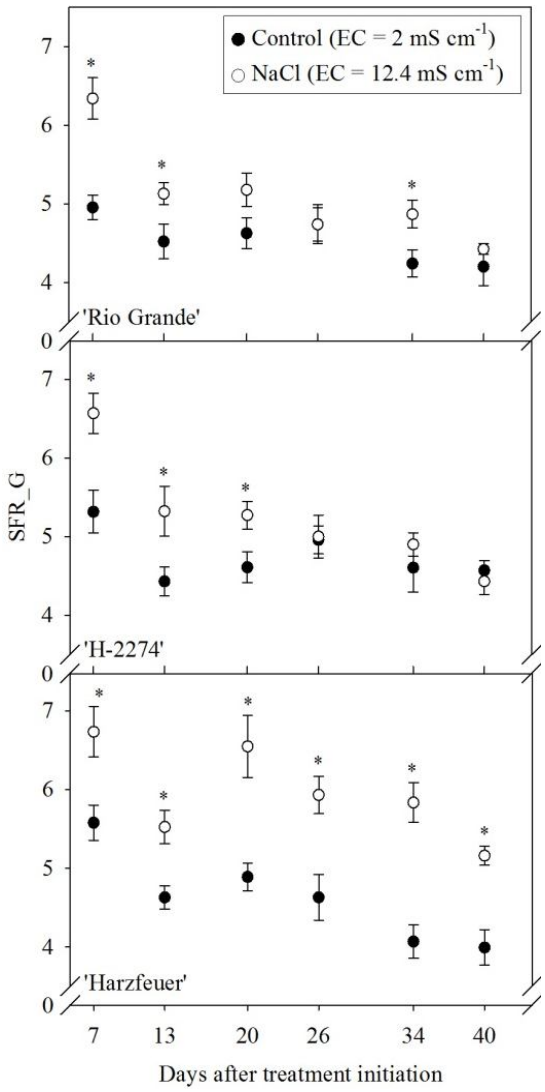

Fig. 5 The ratio of far-red fluorescence to red fluorescence after green light (SFR_G). Readings were taken on tomato leaves from "Rio Grande", "H-2274" and "Harzfeuer". Values represent the mean ± SE (n = 10). * Significant differences ($p \leq 0.05$) between control and NaCl treatment for each genotype and measuring day, assessed by t-test.

4 Discussion

In the present work, we demonstrate that selected indices of the multiparametric fluorescence technique reveal the impact of rootzone salinity on tomato leaves and plants. Biochemical parameters, such as the content of Na, K, Mg, proline and Chl a + b, as well as parameters recorded with the classical PAM chlorophyll fluorescence technique, served as a reference.

Salinity negatively affects plant growth and development (Hunsche et al. 2010). In addition to mineral imbalances at the root zone, causing a lower uptake of minerals such as K and Mg (Table 1), the high Na uptake and transport to the cells in the leaves alters specific biochemical and physiological processes. For example, cells might undergo a hyperosmotic shock by lowering the water potential, which causes the reduction of turgor (Kishor and Sreenivasulu 2014). As a protective measure, tissues accumulate proline (Table 1), a well-known indicator of drought and salinity-stress (Cayuela et al. 1996, Santa-Cruz et al. 1999). Proline supports intracellular osmotic adjustment (Botella et al. 2005, Chinnusamy and Zhu 2004). In certain organisms, it serves to scavenge reactive oxygen species as well as to stabilize membranes and proteins (Takagi 2008). As suggested, high intracellular proline concentrations contribute to improve the stability of chlorophyll molecules (Kumar et al. 2003), as demonstrated in our study by the not-significant difference between the Chl concentrations in “Rio Grande” and “H-2274”. Overall, the analytical results confirm that the experimental plants suffered from salinity-induced stress.

Similarly, the maximum photochemical efficiency of the photosystem II (Fv/Fm) indicated particular responses of the genotypes. Significant differences between the treatment groups, as indicated by the increase of Fv/Fm in the salt-stressed plants, were observed at 20 DAT and thereafter. This finding is in agreement with Li et al. (2010), who detected an increase in Fv/Fm as a consequence of salinity. In contrast, studies of other plant species did not show any significant impact of abiotic stress on photosystem II (Havaux 1992, LU et al. 2002). Given that Fv/Fm shows the maximum efficiency at which light absorbed by the light-harvesting antennae of PSII is converted to chemical energy (Baker and Rosenqvist 2004), plants exposed to salinity appear to have a more efficient PSII. Consequently, the long-term saline environment could produce an adaptation process in the plants.

To cite promising results of the study, several multiple fluorescence excitation indices, such as BFRR_UV (Fig. 2), NBI_G (Fig. 4) and SFR_G (Fig. 5), already showed physiological changes in the salt-exposed plants at 7 DAT. We observed different responses of the cultivars to salinity. Early responses, as indicated by BFRR_UV, were more pronounced and long-lasting in “Rio Grande” and “H-2274” than in “Harzfeuer”. The indices NBI_G and SFR_G also indicated cultivar-specific modification patterns.

The BFRR_UV, as a complex fluorescence index calculated from the blue fluorescence divided by far-red fluorescence after excitation with UV light, increased in those plants exposed to salinity (Fig. 2). This result was primarily driven by a significant (“Harzfeuer”), moderate (“Rio Grande”) and low (“H-2274”) decrease in the absolute intensities of ChlF in

the far-red spectral region, whereas changes in blue fluorescence were virtually absent. Lastly, these changes in the FRF might be associated with alterations in the amount of chlorophyll and the efficiency of light use in the photosystems as well as the shielding of the excitation light by epidermal UV-absorbing compounds (Buschmann et al. 2000).

The accumulation of epidermal flavonols in the leaves can be monitored by the fluorescence screening technique (Bilger et al. 1997), indicated by the fluorescence index FLAV (Fig. 3). Previous observations by our group indicate that the FLAV-Index might be adopted for use as a reliable indicator of drought stress in wheat (Bürling et al. 2013). In contrast, FLAV did not outperform the other indices in the present study of the impact of salinity on tomatoes (Fig. 2).

The NBI_G and SFR_G are two other complex excitation emission indices (Tremblay et al. 2012) that support the rapid and non-destructive detection of changes in the Chl *a* + *b* concentration (Gitelson et al. 1999) and epidermal phenolics. In our study, we observed a significant increase in NBI_G in salt-exposed tomato genotypes 7 DAT (Fig. 4), which was less accentuated in “H-2274”. Although these results are consistent with the trends indicated by SFR_G (Fig. 5), they do not agree with the chlorophyll content. Moreover, we observed lower absolute intensities of RF than of FRF emission, a result that is consistent with the findings of previous studies (Lichtenthaler 1996, Lichtenthaler and Rinderle 1998). However, we observed an increase in NBI_G and SFR_G due to the NaCl treatment, in contradiction to results in the literature (Buschmann 2007, Cerovic et al. 2009). In view of this finding, the weak differences between untreated and salt-affected plants, in, e.g., Chl *a* + *b* in “H-2274” and “Rio Grande”, are not consistent with the results of other studies (Bhivare and Nimbalkar 1984).

An explanation for these controversial data could be an increase in leaf thickness due to salinity (Bhivare and Nimbalkar 1984), changing the penetration of the excitation light and reducing the fluorescence emission. Another important reason might be the use of a higher leaf level for the sequential measurements. Young leaves tend to have lower leaf ion concentrations than mature leaves (Maggio et al. 2007). Accordingly, their physiology is less affected than that of old leaves. Moreover, compared with the older leaves at the bottom of the plant, the young leaves had less time for synthesizing fluorescing pigments and flavonols, and this observation would explain the minor differences between the control and salt-exposed plants. Lastly, plants might also adapt to the stressful environment, so that the stress factors did not affect plant physiology as strongly as initially expected.

5 Conclusions

Compared with the PAM method, multiparametric fluorescence ratios (BFRR_UV, NBI_G, SFR_G) provide an effective and timely technique for sensing salt stress without the need for dark adaptation. BFRR_UV and SFR_G were the most sensitive ratios for the rapid sensing of salinity. Of the evaluated genotypes, the fluorescence emissions in “Harzfeuer” revealed the strongest responses to salinity. Overall, the temporal development of NBI_G and SFR_G in “Rio Grande” and “H-2274”, allied to the low Na and the high proline concentrations as well as the unchanged chlorophyll content, provide evidence that these genotypes are more salt tolerant than “Harzfeuer”. Nevertheless, further in-depth physiological studies are required to analyze salinity-induced changes in the composition pattern of fluorophores. Lastly, additional validation studies with other genotypes and plant species are required.

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D Sensing drought- and salinity-imposed stresses on tomato leaves by means of fluorescence techniques[3]

1 Introduction

In ecophysiological studies, the chlorophyll *a* fluorescence (ChlF) measured with pulse-amplitude-modulated (PAM) fluorometers provides a fast and non-destructive tool to evaluate the physiological status of leaves (Lang et al. 1996). Thus, the method is widely used for screening the tolerance of plants to salinity (Belkhodja et al. 1994) and drought (Walter et al. 2011; Boureima et al. 2012). With the PAM technique, the ChlF is usually recorded between 680 and 690 nm as a spot or as spatially resolved information enabled by imaging systems. The collected information allows the calculation of numerous complex parameters related to plant energetic efficiency (Baker and Rosenqvist 2004). Compared to the classical photosynthesis analysis, kinetic chlorophyll fluorescence parameters enable more profound analysis of single processes responsible for changes in the energy conversion in response to environmental stresses.

Amongst other parameters of the chlorophyll fluorescence, the coefficient of photochemical quenching (qL) (Kramer et al. 2004) and the non-photochemical quenching (NPQ) (Maxwell and Johnson 2000; Schreiber 2004; Lichtenthaler et al. 2005) are highlighted as relevant indicative parameters. They estimate the fraction of open centres of the photosystem II and the apparent rate constant for non-radiative decay (heat loss) from photosystem II (PSII) and its antennae (Baker and Rosenqvist 2004). Impairments of the photosynthetic process lead to changes in the nominal values of qL and NPQ, as well as the shape of their curves during the measurement. In this context, qL and NPQ provide suitable information on the stress-induced changes in plant physiology.

Leaf pigments (e.g., chlorophylls, carotenoids) absorb light, which is used as energy for photosynthesis. Chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) emit fractions of the absorbed light energy as fluorescence light (Lichtenthaler et al. 1986). The ChlF that is emitted in the red (RF) and far-red (FRF) spectral bands can be detected not only by PAM equipment but also by other techniques such as laser-induced fluorescence and the multi-indices fluorescence excitation (Chappelle et al. 1984; Cerovic et al. 2008). The latter technique also enables recordings of the blue (BF) and green (GF) fluorescence (Cerovic et al. 2008). The fluorescence emission in the blue and green region is mainly caused by cinnamic acids

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(mostly ferulic acid) and phenolics, covalently bound to the cell walls (Morales et al. 1996; Lichtenthaler and Schweiger 1998). In contrast, the RF and FRF is mainly emitted by Chl *a* molecules in the antenna and reaction centre of the chloroplast PSII, located in the mesophyll cells (Buschmann and Lichtenthaler 1998; Cerovic et al. 1999; Buschmann 2007). Specific fluorescence ratios can be calculated based on the absolute fluorescence intensities (e.g., BF, GF, RF, FRF). These ratios offer better conditions for comparisons of treatments by reducing the relevance of external factors such as equipment type, measurement setup, optical properties of the samples and environmental conditions (Lichtenthaler 1996; Cerovic et al. 1999). The fluorescence intensity associated with the fluorescence ratios support precise conclusions about the physiological status of the plants (Lichtenthaler et al. 1997; Buschmann and Lichtenthaler 1998; Cerovic et al. 1999).

The fluorescence signals combined with a characteristic fluorescence signature provide valuable information about the type, localisation and concentration of specific fluorophores in the plant tissue (Cerovic et al. 1999). Changes in the fluorescence signature caused by alterations in the amount and composition of fluorescing pigments might be used as indicators for the impact of the growing environment on plant physiology (Lichtenthaler et al. 1998). This has been shown for different plant species including horticultural crops (Lichtenthaler and Babani 2000). Amongst other techniques, the multiparametric fluorescence technique has been adopted in ecophysiological studies in cereals (Bürling et al. 2013), sugar beets (Leufen et al. 2013) and medicinal plants (Müller et al. 2013).

In the past, many studies focused on traditional approaches to characterise the physiological response of tomato plants to abiotic stresses such as drought or salinity (Hunsche et al. 2010 and references therein). However, particularly in horticultural crops, optical techniques have the advantage of being rapid and non-invasive but are still underutilised. One reason for their underutilisation might be the higher complexity in long-term greenhouse experiments compared with studies conducted with seedlings in climate chambers. Two examples demonstrate the non-destructive sensing of stress induced by NaCl (Zribi et al. 2009) and drought (Haupt-Herting and Fock 2000) on tomato plants. However, only a few studies have addressed the impact of more than one stress and their consequences, on the fluorescence signature of different cultivars. Thus, information on multiple abiotic constraints that simultaneously affect horticultural crops remains scarce.

Tomato (*Solanum lycopersicum* L.) plants are one of the most important commercial vegetables in the world. Particularly in the Mediterranean region, tomato plants may be impaired simultaneously by water deficiency and salinity. A widely accepted opinion is that

drought and salinity have predominantly the same influence on plant physiology (Mahajan and Tuteja 2005). Salinity affects the cellular concentration of ions, and consequently the osmotic potential, which may worsen when the tomato plants are exposed to water deficit.

In this context, the aim of our study was to investigate the influence of water deficiency and salinity, applied separately or combined, on specific parameters of the fluorescence signature of tomato leaves. Thereby, we hypothesised that multiparametric fluorescence indices might support the monitoring of the physiological status of the plants without the need to dark-adapt the plants, a prerequisite for reliable PAM measurements. Our results provide evidence about the stress-specific changes in the fluorescence signature. With the identification of appropriate parameters, we contribute to the development of fast and non-destructive stress detection required to screen genotypes and optimise cultural practices in a more applied scope.

2 Materials and methods

2.1 Plant material and growth conditions

Experiments were conducted under greenhouse conditions from August to October 2011. Tomato seeds of the genotypes of *Solanum lycopersicum* L. F1 hybrid Harzfeuer (Volmary GmbH, Münster, Germany) and *S. lycopersicum* var. Rio Grande (donation of Dr. Mustafa Demirkaya, Erciyes University, Turkey) were steeped in the dark at 20 °C for three days. Germinated seeds were transferred into rock wool trays and cultivated until the third leaf stage. Then, plantlets were transplanted into 10 1itre pots filled with perlite (Perligran G, Knauf Perlite GmbH, Dortmund, Germany) and allocated randomly on two greenhouse tables. Fertigation was based on Kristallon™ Blau (Yara GmbH & Co. KG, Dülmen, Germany) amended with calcium nitrate tetrahydrate ($Ca(NO_3)_2$) (99% purity, AppliChem GmbH, Darmstadt, Germany). Excess nutrient solution was drained off at the bottom of the pots after perlite saturation. Nutrient solution and its spillover were isolated from each other, and the nutrient solution was not recycled in the experiment.

2.2 Treatments

The plants were assigned to four treatments (n = 5 plants): T1, control plants (well-watered + nutrient solution; electrical conductivity, EC = 2 mS cm^{-1}); T2, well-watered plants; EC = 12.5 mS cm^{-1} standard nutrient solution amended with sodium chloride (NaCl) (99% purity, Carl Roth GmbH & Co. KG, Karlsruhe, Germany); T3, water deficiency (50% of the nutrient solution compared to the control plants; EC = 2 mS cm^{-1}); T4, water deficiency

(50% of control; EC = 12.5 mS cm^{-1}). To avoid osmotic shock in the NaCl-treated plants, the EC value of the solution was increased in three steps starting at 2 mS cm^{-1} in the 1st week (36 days after sowing) to EC = 7 mS cm^{-1} in the 2nd week (42 days after sowing), reaching the final concentration of 12.5 mS cm^{-1} in the 3rd week (48 days after sowing). Plants in the NaCl treatment groups received the NaCl in each irrigation cycle.

2.3 Sampling and analytical determinations

Samples of middle-aged leaves were collected at the end of the experiment, freeze-dried (Gamma 1-16 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) and ground for processing. To quantify the sodium (Na) concentration, 0.1 g dry matter (DM) of the samples was acid-digested in a microwave and analysed by atomic absorption spectrometry (AAS, Perkin-Elmer, Analyst 300, Wellesley, USA) as described by Hunsche et al. (2010).

Chlorophyll (Chl) concentration (Chl *a*+*b*) of the samples was determined from 0.05 g ground DM (Munné-Bosch and Alegre 2000); the material was mixed with 5 ml methanol and centrifuged at 4,000 rpm for 15 min. at 4 °C. The supernatant was filled in a 50 ml volumetric flask. The procedure of mixing with 5 ml methanol and centrifugation was repeated three times; after this procedure, the flasks were filled up to 50 ml with methanol. The extract absorbance was recorded at 647 nm (A_{647}) and 664 nm (A_{664}) with a UV-VIS spectrophotometer (Lambda 35 UV/VIS Spectrophotometer, Perkin Elmer, USA).

Proline concentration in the leaves was determined as described by Bates et al. (1973). Here, a mixture of 3 ml sulfosalicylic acid and 0.1 g ground DM was centrifuged at 4200 rpm for 20 min. at 20 °C. After collecting 0.2 ml of the supernatant, 1.8 ml sulfosalicylic acid, 2 ml glacial acetic acid and 2 ml ninhydrin acid were added, and the mixture was boiled at 100 °C for 1 h in a water bath. After the sample cooled to 20 °C, 4 ml toluene was added to the mixture. The upper, organic part was collected for spectrophotometric measurements (Lambda 35 UV/VIS Spectrophotometer, Perkin Elmer, USA). The absorbance of extracts was evaluated at 520 nm.

Osmotic potential was determined from 4 g fresh matter (FM) of squeezed tomato leaves. From that, 200 µl of the extracted liquid was collected and centrifuged at 10,000 rpm for 10 min. at 4 °C. Osmotic potential (Ψ_π) of 15 µl supernatant was analysed twice (Osmomat 030-D (Gonotec GmbH, Berlin, Germany). The means of the results, given in [osmol kg^{-1}], were multiplied by -2.437 (correction coefficient valid for 20 °C) to obtain Ψ_π in MPa (Holbrook et al. 2007).

2.4 Fluorescence measurements

Fluorescence measurements were performed on detached leaves in a laboratory under dark conditions, a prerequisite for reliable PAM measurements. To minimise possible modifications after sampling, batches of three leaves each were harvested and transported to the measuring room. Following the plant growth, a higher leaf level was selected for the evaluations in the time-course of the experiment. Thus, the first measurements were performed 49 days after sowing on the fourth leaf counted from bottom (cotyledons excluded). Every 7-10 days, a higher leaf level was selected, ending at 77 days after sowing on the eighth leaf. Fluorescence readings were taken with the Imaging-PAM® (Heinz-Walz GmbH, Effeltrich Germany) chlorophyll fluorometer (Bürling et al. 2010) and the multiple excitation fluorescence recording system Multiplex®3 (Force-A, Orsay Cedex, France) as described elsewhere (Leufen et al. 2013; Müller et al. 2013).

PAM chlorophyll fluorescence was recorded from the adaxial leaf side of dark-adapted leaves (30 min.) over a period of 320 seconds. First, the ground (Fo) and maximum fluorescence (Fm) were recorded, followed by specific parameters as related to the kinetic curves. Thereby, we focused on the coefficient of photochemical quenching (qL; Imaging PAM), calculated as (Fm' – F)/(Fm' – Fo') (Fo'/F) and the non-photochemical quenching (NPQ, Imaging PAM), calculated as (Fm – Fm')/Fm', as reliable indicative parameters highlighted in the literature (Müller et al. 2001; Kramer et al. 2004).

The evaluation of data was performed on the recorded pictures. In each single image, three areas of interest (AOI) were set: the leaf edge, apex and centre. Afterwards, the mean of the three AOIs was calculated before running the statistical analysis.

The fluorescence in the blue (BF), red (RF) and far-red (FRF) spectral bands, excited with UV, green or red light, was recorded on detached leaves with a multiparametric hand-held sensor (Leufen et al. 2013; Müller et al. 2013). Fluorescence recordings were conducted immediately after detaching the leaves. For this purpose, leaves were fixed horizontally on a sample holder and the distance between sensor and leaf was kept constant at 10.5 cm. Further, we used a frontal cover plate with an aperture of 6 cm in diameter to standardise the measuring area. We selected two ratios from a number of parameters provided by the equipment: the BF to FRF ratio after UV-light excitation (BFRR_UV) and the logarithm of the ratio of FRF after red light excitation to the FRF after excitation with UV-light (FLAV). The former ratio is well known to be a sensitive indicator for drought impact (Buschmann and Lichtenthaler 1998); the latter is related to the concentration of flavonols in the epidermis (Cerovic et al. 2008).

2.5 Data analysis

Statistical analysis was performed with IBM SPSS Statistics 20.0 (IBM Corporation, New York, USA). Means were subjected to analysis of variance (ANOVA, $P \leq 0.05$; $n = 5$), and significant differences were compared using Duncan's multiple range test; each cultivar was analysed separately. Graphs were drawn using SigmaPlot 11.0 (Systat Software Inc., San Jose, CA, USA).

3 Results

3.1 Osmotic potential and concentration of sodium and proline

The Ψ_π of leaves in the two evaluated cultivars was significantly reduced by salinity and water deficit (Table 1). Salinity had a stronger effect in reducing the Ψ_π of the leaves (-41.54% 'Rio Grande; -30.06% 'Harzfeuer') compared to water deficit (-8.95% 'Rio Grande'; -17.67% 'Harzfeuer'). The combined stresses, salinity and water deficit, had a more pronounced effect (-55% 'Rio Grande'; -61% 'Harzfeuer') compared to the single stresses.

The reduction in Ψ_π followed the accumulation of sodium in the leaves. Well-watered plants of both cultivars, when treated with NaCl, displayed a significantly higher Na concentration compared to plants cultivated with the standard fertigation (Table 1). The combination of water deficiency and salinity (EC = 12.5 mS cm^{-1}) led to significantly higher Na concentration (+1100% 'Rio Grande'; +1860% 'Harzfeuer') than the well-watered plants with the same EC value (+712% 'Rio Grande'; +990% 'Harzfeuer'). Further, we show that 'Harzfeuer' accumulated significantly more Na in the leaves than 'Rio Grande'.

A significant contribution for the adjustment of the osmotic potential of the leaves was also given by proline. In both cultivars, proline concentrations increased significantly under water deficiency (+183% 'Rio Grande'; +814% 'Harzfeuer') and saline (+469% 'Rio Grande'; +987% 'Harzfeuer') conditions (Table 1). Thereby, salinity induced a stronger proline accumulation. The highest concentration of proline was observed in those plants exposed to water deficit and salinity (+620% 'Rio Grande'; +3653% 'Harzfeuer'). In general, the changes in the proline concentration were more pronounced in 'Harzfeuer'.

Table 1 Osmotic potential (Ψ_π), sodium and proline concentrations, and alteration in percent (%) in tomato leaves. Samples were collected from 'Rio Grande' and 'Harzfeuer' 42 days after initiation of the treatments.

	Treatments		Ψ_π		Na		Proline	
Tomato cultivar	**Water supply**	**EC value [mS cm^{-1}]**	**[MPa]**	**% changes**	**[mg g^{-1} DM]**	**% changes**	**[mg g^{-1} DM]**	**% changes**
Rio Grande	Well-watered	2.0	-1.22 ± 0.02 c	-	2.00 ± 0.13 a	-	1.59 ± 0.14 a	-
	Well-watered	12.5	-1.73 ± 0.08 b	-41.54 ± 6.09	15.50 ± 1.73 b	+712.73 ± 149.12	8.91 ± 0.56 c	+469.23 ± 35.04
	Water deficiency	2.0	-1.33 ± 0.04 c	-8.95 ± 4.00	2.41 ± 0.11 a	+23.44 ± 11.92	4.51 ± 0.71 b	+183.82 ± 35.89
	Water deficiency	12.5	-1.90 ± 0.01 a	-55.63 ± 2.08	23.92 ± 2.85 c	+1100.63 ± 124.24	11.12 ± 0.20 d	+620.55 ± 57.22
Harzfeuer	Well-watered	2.0	-1.20 ± 0.02 d	-	2.60 ± 0.15 a	-	0.59 ± 0.05 a	-
	Well-watered	12.5	-1.57 ± 0.01 b	-30.06 ± 1.80	27.76 ± 0.72 b	+990.21 ± 98.88	6.27 ± 0.15 c	+987.48 ± 84.88
	Water deficiency	2.0	-1.42 ± 0.03 c	-17.67 ± 2.38	3.42 ± 0.13 a	+34.37 ± 12.28	5.23 ± 0.14 b	+814.05 ± 94.17
	Water deficiency	12.5	-1.93 ± 0.04 a	-60.78 ± 4.69	50.04 ± 1.95 c	+1859.54 ± 165.58	21.74 ± 0.56 d	+3653.26 ± 219.90

The means ± SE in the columns followed by the same letter do not differ statistically according to Duncan's multiple range test ($p \leq 0.05$; $n = 5$). Statistics were performed separately for each cultivar.

3.2 Chlorophyll concentration

The total Chl concentration (Chl *a*+*b*) was stronger influenced in 'Rio Grande'. While water deficit caused only a slight reduction (-4.95% 'Rio Grande'), salinity led to a significant lower Chl concentration (-9.18%) (Table 2). Both combined stresses resulted in a significant decrease in the total Chl concentration (-22.83% 'Rio Grande'; -4.11% 'Harzfeuer'). In contrast to 'Rio Grande', 'Harzfeuer' responded with an increase in Chl *a*+*b* in the salinity treatment (+9.23%) when applied as a single stressor. Changes in total chlorophyll concentration were accompanied by alterations of the Chl *a*/*b* ratio.

In 'Rio Grande', Chl *a*/*b* increased significantly under saline conditions irrespective of the water supply (+43.01% T2; +34.43% T4) (Table 2), whereas the water deficit itself had no significant influence on the Chl *a*/*b*. In 'Harzfeuer', we observed a slightly different behaviour; the water deficit induced a significant rise of the Chl *a*/*b* (+19.28%). The highest increase in the Chl *a*/*b* was observed when salinity was imposed as a single stressor (+40.36%), whereas the combined salinity and water deficit had no significant changes in the Chl *a*/*b* ratio (+5.51%).

3.3 Coefficient of photochemical quenching (qL)

Typical curves of the photochemical quenching are presented in figure 1. In 'Rio Grande', T2, T3 and T4, an overall increase in the qL curve was observed 8 days after treatment induction (DAT). The most pronounced difference between the treatments was noticed at 80 s after first illumination (Fig. 1). Only a slight increase in the qL was recorded in T2 treated plants, whereas both water deficiency treatments (T3 and T4) lead to a strong increase in qL. The highest increase was measured on plants of the T3 group. Under appropriate water supply, salinity did not significantly affect qL kinetic curves of 'Harzfeuer'. The qL curve of water deficit plants was higher compared with well-watered plants, even if the effect was not as strong as that observed in 'Rio Grande'. The impact of salinity and water deficit on the qL was also noticed 36 days after treatment induction. At this time, the qL of both cultivars in water deficiency-exposed plants was significantly higher when compared to plants receiving a sufficient amount of water. Particularly in 'Rio Grande', NaCl did not significantly influence the qL, neither in plants of the T2 group nor in plants of the T4 group. A slight but not-significant increase in the qL curve was observed over the 300 s measurement period in 'Harzfeuer'.

Table 2 Total chlorophyll concentration (Chl *a*+*b*) and Chl *a* : Chl *b* (Chl *a*/*b*) ratio of tomato leaves as well as alteration in percent (%). Samples were collected from 'Rio Grande' and 'Harzfeuer' 42 days after initiation of the treatments.

	Treatments		**Chl *a*+*b***		**Chl *a*/*b***	
Tomato Cultivar	**Water supply**	**EC value [mS cm^{-1}]**	**[mg g^{-1} DM]**	**% changes**	**[rel. units]**	**% changes**
Rio Grande	Well-watered	2.0	18.13 ± 0.45 c	-	7.35 ± 0.38 a	-
	Well-watered	12.5	16.45 ± 0.49 b	-9.18 ± 2.38	10.28 ± 0.79 b	+43.01 ± 17.54
	Water deficiency	2.0	17.23 ± 0.52 bc	-4.95 ± 1.96	7.02 ± 0.38 a	-2.87 ± 8.83
	Water deficiency	12.5	13.95 ± 0.20 a	-22.83 ± 2.53	9.75 ± 0.61 b	+35.43 ± 14.65
Harzfeuer	Well-watered	2.0	15.81 ± 0.56 a	-	7.26 ± 0.15 a	-
	Well-watered	12.5	17.27 ± 0.49 b	+9.23 ± 6.34	10.19 ± 0.10 c	+40.36 ± 3.21
	Water deficiency	2.0	15.58 ± 0.41 a	-1.45 ± 2.00	8.66 ± 0.15 b	+19.28 ± 3.55
	Water deficiency	12.5	15.16 ± 0.24 a	-4.11 ± 3.76	7.66 ± 0.33 a	+5.51 ± 4.87

The means ± SE in the columns followed by the same letter do not differ statistically according to Duncan's multiple range test ($p \leq 0.05$; $n = 5$). Statistics were performed separately for each cultivar.

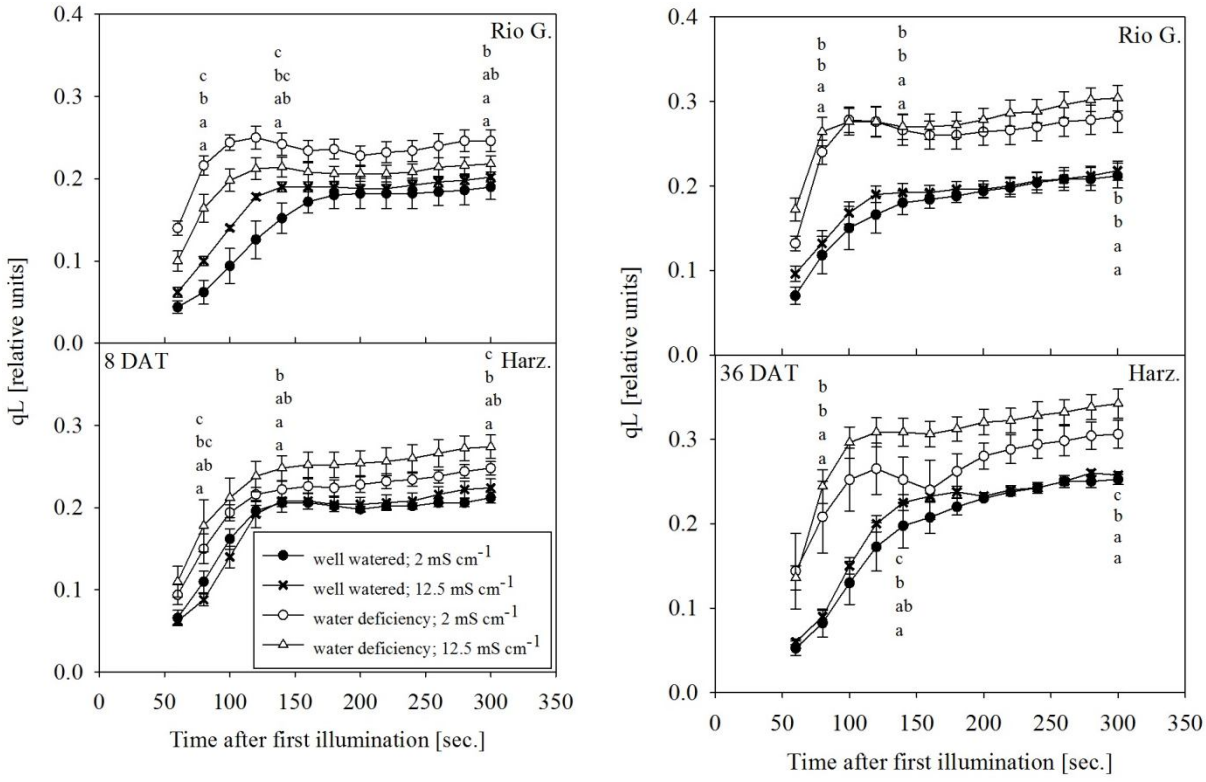

Fig. 1 Temporal development of the coefficient of photochemical quenching (qL) calculated as (Fm' – F)/(Fm' – Fo') (Fo'/F). Readings were taken on tomato leaves from 'Rio Grande' and 'Harzfeuer' at 8 (left) and 36 (right) days after initiation of the treatments. Mean ± SE (n = 5); letters above or below the lines (selected times only) indicate the separation of the means by Duncan's multiple range test ($p \leq 0.05$; n = 5).

3.4 Non-photochemical quenching (NPQ)

The development of the NPQ curves during the measuring period exhibited different patterns depending on the cultivar, experimental treatment and time after treatment initiation. The kinetic curves for 'Rio Grande' increased after 60 s in deficit-irrigated plants (Fig. 2). In general, salinity had no significant influence on NPQ during the 300 s measurement. Similarly, 'Harzfeuer' exhibited a reliable differentiation between the influence of water supply and salinity in the time frame from 100 to 160 s after first illumination. As indicated, saline conditions on well-watered and water deficit plants did not influence NPQ significantly. In both cultivars, multiple stresses, i.e., salinity and water deficiency, had no clear additive effect on NPQ.

Measurements at 36 days indicate that NPQ increased due to salinity (Fig. 2). The NPQ increased strongly when both stresses were applied simultaneously in contrast to the single stresses, particularly in 'Rio Grande'.

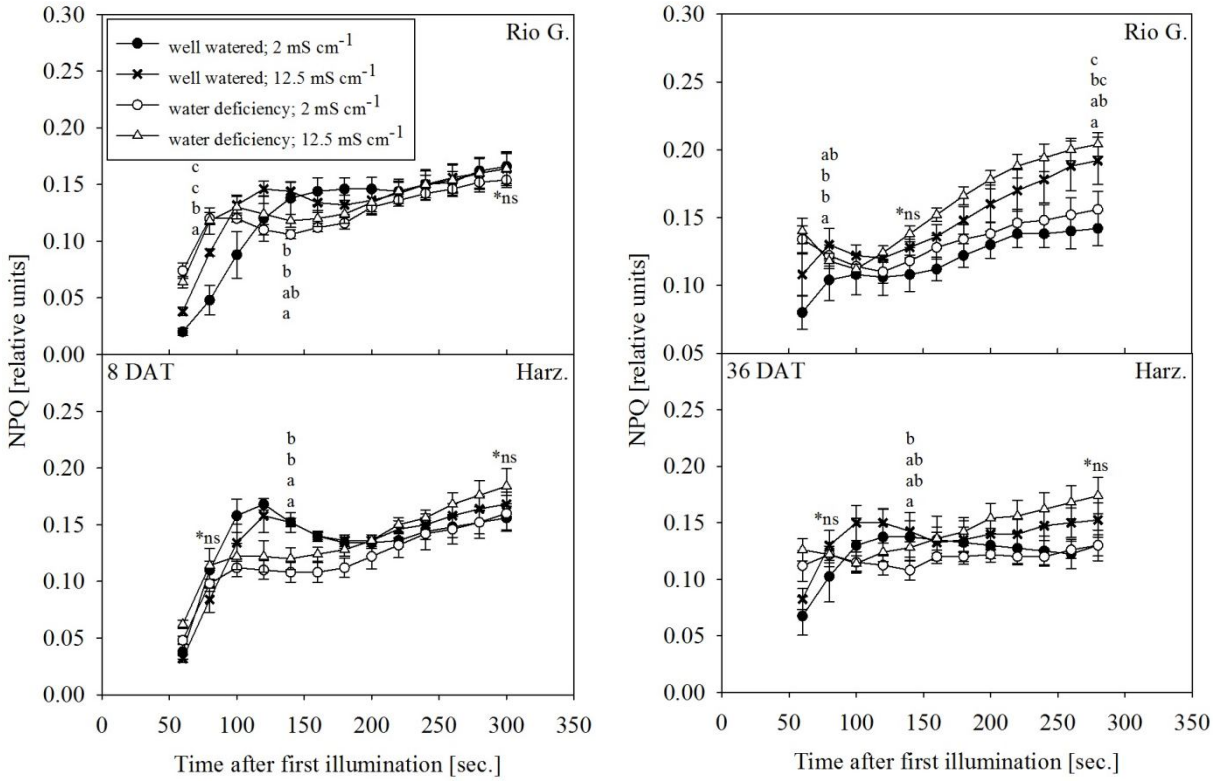

Fig. 2 Temporal development of the non-photochemical quenching (NPQ) calculated as (Fm – Fm')/Fm'. Readings were taken on tomato leaves from 'Rio Grande' and 'Harzfeuer' at 8 (left) and 36 (right) days after initiation of the treatments. Mean ± SE (n = 5); letters above or below the lines (selected times only) indicate the separation of the means by Duncan's multiple range test ($p \leq 0.05$; n = 5).

3.5 Blue to far-red fluorescence ratio (BFRR_UV) and flavonol-index (FLAV)

The BFRR_UV indicates stress-induced changes at 8 DAT in both cultivars (Fig. 3). In general, this parameter was significantly higher in those plants exposed to combined salinity and water deficiency. At 36 DAT, the increase in BFRR_UV in response to water deficit combined with NaCl was confirmed in both cultivars. Plants exposed to a single stress, water deficiency or salinity, revealed a distinct increase in BFRR_UV in 'Rio Grande'.

The FLAV index, which is related to the accumulation of epidermal flavonols, indicates clear trends of stress-induced changes at 8 DAT (Fig. 4). At this time, the drought- and/or salinity-exposed plants exhibited higher values than the well-watered control plants. We observed a pronounced impact of the water deficit, compared to the salinity, in 'Harzfeuer' at 36 DAT. Plants exposed to the combined salinity and water deficiency treatment showed statistically significant increases of FLAV, irrespective of the cultivar.

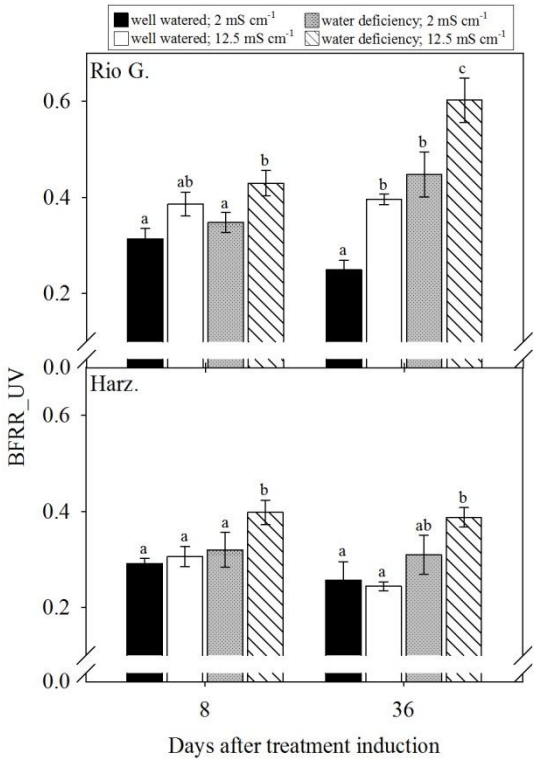

Fig. 3 Blue to far-red fluorescence ratio (BFRR_UV) of tomato leaves, cultivars 'Rio Grande' and 'Harzfeuer'. Data were recorded at 8 and 36 days after treatment induction. The vertical bars (mean ± SE) with different letters are significantly different from each other at ($p \leq 0.05$; $n = 5$) according to Duncan's multiple range test.

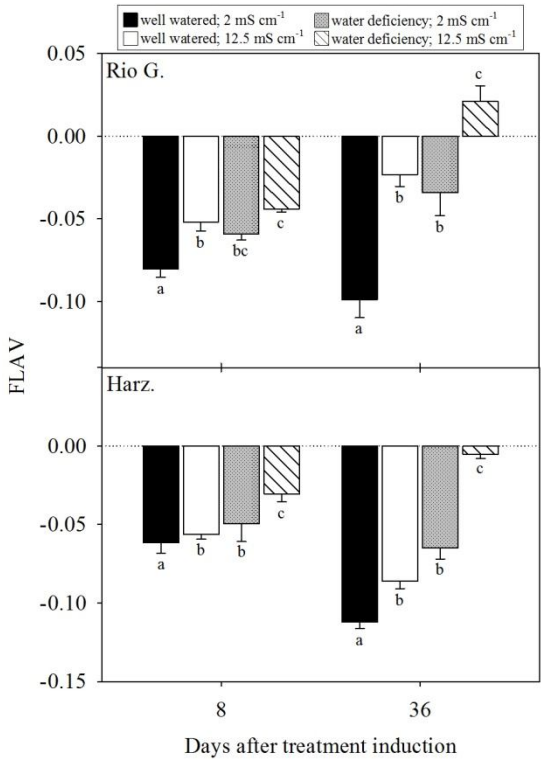

Fig. 4 FLAV representing the logarithm of the ratio of far-red fluorescence after red light excitation and the far-red fluorescence after excitation with UV-light. Data were recorded on 'Rio Grande' and 'Harzfeuer' tomato leaves at 8 and 36 days after treatment induction. The vertical bars (mean ± SE) with different letters are significantly different from each other at ($p \leq 0.05$; $n = 5$) according to Duncan's multiple range test.

4 Discussion

The aim of this long-term study was to examine the impact of water deficit and salinity as single and combined stresses on the fluorescence signature of tomato leaves. In this context, we hypothesised that multiparametric fluorescence indices enable the monitoring of the physiological status of plants without the need to dark-adapt the plants, as needed for reliable PAM measurements. In the example of selected fluorescence parameters, we accomplished the sensor-based monitoring of tomato plants to low water availability, salinity or the combination. Classical physiological parameters were referenced.

As a consequence of osmotic adjustment caused by the accumulation of ions and organic compounds in the cells (Morant-Manceau et al. 2004), stress treatments induced a significant decrease in the Ψ_π under water deficiency and particularly due to the impact of NaCl (Table 1). This is proven by the increase in Na and proline concentration and is in line with other publications (Cayuela et al. 2007, Hunsche et al. 2010). Further, proline concentration increased under both water deficiency and salt impact (Table 1) but remarkably increased as a

consequence of salinity (Cayuela et al. 1996, Juan et al. 2005, Zushi and Matsuzoe 2009). Plants growing under water shortage and/or salinity undergo several anatomical, morphological, physiological, biochemical and molecular adaptations to maintain a positive turgor and aid in detoxification of reactive oxygen species (Chaves et al. 2003; Chinnusamy and Zhu 2004; Claussen 2005). Additionally, the content of chlorophyll and the Chl a/b ratio was significantly influenced by the treatments. Thereby, the stronger decline in Chl *a*+*b* in 'Rio Grande' compared with 'Harzfeuer' was mainly due to a decrease in the Chl *b* concentration. This highlights the general assumption that Chl *a* is more tolerant to NaCl than Chl *b* (El-Meleigy et al. 2004; Santos 2004). Chl *b* has analogous functions to Chl *a* (Holbrook et al. 2007, Stoll 1936); thus, in many cases, the ratio Chl *a*/Chl *b* better indicates impairment of or damage to the photosynthetic apparatus.

Chl *a* fluorescence, which in many cases is used to replace the determination of the current photosynthetic rate, revealed a major impact of water deficit on the coefficient of photochemical quenching (qL) at 8 DAT. Salinity alone, or in combination with water deficit, had a less pronounced effect on qL. Besides the fact that our results are in agreement with those reported by Zribi et al. (2009), the first effects of salinity were recorded delayed (4 weeks after salt addition). Assuming a 'lake model' for PSII, in which all PSII reaction centres are considered to be embedded within one antenna matrix and capable of receiving excitation energy from antenna pigments throughout the matrix. The qL presents a more reliable fluorescence parameter than qP, which is based on a 'puddle model' where each PSII reaction centre and its associated antenna are not able to transfer excitation energy to antennae of the other PSII reaction centres (Baker 2008). Accordingly, the increase in qL indicates that the electron transport via PSII and the transmembrane thylakoid proton gradient was affected during dehydration. More precisely, an increase in qL caused by water deficit can be explained by the strong accumulation of non-reduced primary electron acceptors of PSII, ready to accept the excitation energy for passing it further towards other photochemical processes (Hura et al. 2007). Thus, stomatal closure in response to water shortage could be responsible for these observations, whereas salinity did not have a large effect on this process. With advanced stress duration, the differences became more evident, particularly in plants exposed to water deficiency. As demonstrated here, the qL determination between 60 and 100 s after first illumination better displays differences between treatments when evaluating the impact of environmental stresses, irrespective of cultivar and Chl concentration.

Contrary to the qL, NPQ curves did not show clear trends at 8 DAT. However, the longer exposure of plants to the treatments was followed by an increase in the NPQ in salt-treated

plants, as demonstrated at the end of the 300 s measurement 36 days after treatment induction. Compared to qL, which was more affected by water deficit, NPQ was more pronounced in salt-treated plants. The rise of energy dissipation in PSII antennae, as a consequence of low photosynthetic activity under stress conditions (Baker 2008), is represented in our study in the form of higher NPQ. Further, the increase might indicate a damage of PSII and light-harvesting complexes as well as the activity of electron transport rate (Lichtenthaler 1996; Buschmann and Lichtenthaler 1998). Salt impact and its possible toxic consequences due to high cellular concentrations of Na affected 'Rio Grande' more. In addition, we observed that the NPQ was closely related to the Chl *a*/*b* in 'Rio Grande' because plants with a higher Chl *a*/*b* showed higher NPQ curves. As suggested, this increase in the NPQ might also be an indicator of an increase of the steady state proton gradient over the thylakoid membrane in times of abiotic stress (Schmuck et al. 1992).

The BFRR_UV, a complex fluorescence index calculated from the BF divided by FRF after excitation with UV-light, increased particularly in 'Rio Grande' when plants were exposed for a longer period to the combined stresses. Analysis of the data reveals no alteration of the absolute fluorescence intensity in the blue band (*data not shown*), while changes in this parameter were mainly related to alterations of the far-red (chlorophyll) fluorescence. This effect is in line with previous studies (Buschmann et al. 2000), but it should be considered that different aspects might have contributed, e.g., changes in the Chl concentration, the electron transport in the photosystems and also the accumulation of epidermal flavonols due to abiotic stress, which might absorb the UV excitation light. In contrast, the decrease in the quantity of pigments, e.g., Chl or carotenoids, which normally re-absorb blue and green fluorescence emission, might intensify this effect (Szigeti 2008).

It is well proven that the biosynthesis of flavonoids is up regulated in response to abiotic stresses (Di Ferdinando et al. 2012 and references therein). One spectroscopic method to determine the accumulation of epidermal flavonols in the tissue is using the screening technique (Bilger et al. 1997), which in the case of the multiple-index fluorescence system, is given by the FLAV Index. The UV-screening effect depends on the amount of epidermal flavonols and the optical properties of the leaf (Cerovic et al. 2012). Our trials reveal a cultivar-specific response in the FLAV, which was maintained at 8 and 36 DAT. In 'Rio Grande', the salinity had a stronger effect on the biosynthesis of epidermal flavonols; in the case of 'Harzfeuer', the water deficit played the most pronounced role, especially on day 36. Nevertheless, the combined stresses in both cultivars led to the strongest increase in the FLAV index, not only confirming the effectiveness of this parameter in monocotyledons

(Bürling et al. 2013) but also identifying it as a robust indicator of abiotic stress in dicotyledons.

5 Conclusion

The multiparametric fluorescence indices BFRR_UV and FLAV are promising fluorescence parameters for the fast detection of abiotic stresses at the leaf level. The impact of combined stresses (salinity and water deficiency) was particularly sensed at 8 days after treatment induction. The simultaneous occurrence of salinity and water shortage caused significantly higher ratios compared with plants exposed to single stresses. The modification pattern in these complex parameters is principally explained by differences in the chlorophyll concentration and the functionality of the electron flux and less by an accumulation of blue fluorescing pigments in the leaves. In most of the evaluated parameters, 'Rio Grande' had a stronger response to the treatments than 'Harzfeuer'. These results highlight the potential of the fluorescence-based, non-invasive techniques for genotype screening.

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E PEG and drought cause distinct changes in biochemical, physiological and morphological parameters of apple seedlings[4]

1 Introduction

Worldwide, the impact of water deficit on plants is one of the most studied abiotic stress factors. Despite controversial and critical discussions on the use of polyethylene glycol (PEG) to manipulate and study water availability for plants (Comeau et al. 2010, Fan and Blake 1997), many studies on controlled water deficit stress rely on the use of PEG. One major advantage of using PEG is the precise adjustment of the stress level in the hydroponic solution. Nevertheless, PEG-induced stress means osmotic stress, and for this reason, results have to be taken with caution (Michel and Kaufmann 1973). While some studies with PEG have focused on destructive methods to identify the physiological response of plants to PEG (Türkan et al. 2005), others have analyzed the impact of PEG on the chlorophyll fluorescence (ChlF) (Kocheva et al. 2004, Shangguan et al. 2000) and photosystem I and II of monocotyledonous plants (Oukarroum et al. 2009).

ChlF is a widely used tool for the evaluation of the impact of adverse environmental conditions on plant physiology (Lichtenthaler and Babani 2000, Bürling et al. 2011). Chlorophyll (Chl) *a* and Chl *b* emit fractions of absorbed light energy as fluorescence light (Lichtenthaler et al. 1986). In addition to the traditional PAM method, the ChlF that is emitted in both the red and far-red spectral bands can be detected and used in the multi-indices fluorescence excitation technique (Cerovic et al. 2008). Changes in the fluorescence signature caused by alterations in the amount and composition of fluorescing pigments might be used as indicators for the impact of the growing environment on plant physiology (Lichtenthaler et al. 1998).

In general, the use of PEG is considered to be equivalent to physical water deficit. Until now, studies were performed mainly with herbaceous plants such as wheat (Shangguan et al. 2000) or barley (Bandurska 2001). Thus far, an extensive comparison of the physiological responses to PEG or drought stress in model plants is missing; moreover, there is no example of the use of PEG in plants that have more lignified tissues such as apple plantlets. In this context, our objective was to compare the defined physiological responses of apple (*Malus domestica* Borkh.) leaves to water deficit induced in nutrient solutions by PEG and in soil by

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interrupting irrigation. Thereby, we hypothesized that PEG-induced osmotic stress impacts plant physiology (relative water content, photosynthesis and chlorophyll fluorescence), morphology (leaf cross section) and biochemistry (proline and chlorophyll content) in a similar way to physical water deficit. The relative water content is well-proven indicator of the water status of plants grown under water deficit (Kautz et al. 2014a, Weatherley 1950) and proline is an intensively studied compatible amino acid, which works as indicator of drought (Chaves et al. 2003 and references therein). Net photosynthetic rate (P_N) allows the detection of the impact of water deficit on plants early and over the entire experimental period (Abrams et al. 1990, Pedrol et al. 2000). Changes in the chlorophyll concentration are frequently used as a stress indicator (Matile and Hörtensteiner 1999). Additionally, chlorophyll degradation is accompanied by dysfunction in the photosynthetic apparatus (Tuba et al. 1996). Fluorescence measurements from leaves are commonly used for monitoring photosynthetic events and physiological status of the plant (Kocheva et al. 2004).

2 Materials and methods

2.1 Plant material and growth conditions

Apple (*Malus domestica* Borkh., cv. Golden Delicious) seeds were stratified for 28 days at 4 °C in the dark. Subsequently, seeds were sown in sterilized substrate filled trays containing 60% commercial potting mixture, 20% sand and 20% perlite (Perligran G, Knauf Perlite GmbH, Dortmund, Germany). After germination, seedlings were transplanted to Teku-Pots (V = 0.23 l) and filled with the same substrate mixture indicated above. Seedlings were grown in the climate chamber (100 µmol m^{-2} s^{-1} light intensity, 14 h photoperiod, 20/12 ± 2 °C (day/night temperature), 60/70 ± 15% relative humidity). Plants received a nutrient solution based on Kristallon™ Blau (Yara GmbH & Co. KG, Dülmen, Germany) combined with the irrigation water three times a week. At the third leaf level, 30 uniform plants were selected and transferred to pots (V = 1 l), filled with 800 ml Hoagland's nutrient solution and provided with a continuous air supply to establish a pure hydroponic system. The weight of each single pot (+ 800 ml nutrient solution) was recorded daily and filled up with the Hoagland solution. To prevent fungal and bacterial growth, the nutrient solution was changed once a week. In parallel to the hydroponic system, 20 uniform apple seedlings were transplanted into pots (V = 1 l) filled with substrate and supplied with nutrient solution every second day. Excess nutrient solution was spilled out.

2.2 Treatments, sampling and evaluation

Plants in the hydroponic system were assigned to three treatments (n = 10 plants per treatment) with different concentrations of polyethylene glycol (PEG 6000, AppliChem GmbH, Darmstadt, Germany): 0 g dm^{-3}; 50 g dm^{-3} and 100 g dm^{-3}. The seedlings cultivated in substrate were assigned to two treatments (n = 10 plants): S1, well-watered plants; S2, plants exposed to drought. The experimental treatments were applied in two phases: the 1st period of 27 days (starting at 8 weeks after germination) was followed by a recovery phase of 21 days. Thereafter, the 2nd experimental period was conducted for 20 days with the same treatments as described above. The sixth to ninth leaves were collected at the end of the experiment, freeze dried (Gamma 1-16 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) and ground for the sequential determinations of proline and chlorophyll.

2.3 Osmotic potential

Osmotic potential of the hydroponic growing medium was recorded during the experiment. A 200 µl nutrient solution and nutrient solution supplemented with PEG were collected 27 and 63 days after PEG addition and centrifuged at 21689 *g* for 10 min. at 4 °C, respectively. Osmotic potential (Ψ_π) of 15 µl supernatant was analyzed twice (Osmomat 030-D, Gonotec GmbH, Berlin, Germany). The recorded values [osmol kg^{-1}] were multiplied by -2.437 (correction coefficient valid for 20 °C) to get Ψ_π in MPa (Holbrook et al. 2007). Values for 0, 50 and 100 g dm^{-3} were -0.12, -0.18 and -0.32 MPa, respectively, at the first sampling (27 days), and -0.09, -0.14 and -0.29 MPa at the second sampling (63 days).

2.4 Relative water content

The relative water content (RWC) of the leaves was calculated as developed by Weatherly (1950):

$$\% \, RWC = \left(\frac{FM - DM}{TM - DM}\right) x \, 100$$

Leaf disks (13 mm diameter) were punched out and their fresh matter (FM) was determined. To determine the turgid mass (TM), leaf disks were immersed in deionized water for 24 h in the dark. Afterwards, samples were oven dried at 80 °C for 48 h to obtain the dry matter (DM).

2.5 Proline and chlorophyll concentration

Proline concentration in the leaves was determined as described by Bates et al. (1973). A mixture of 3 ml sulfosalicylic acid and 0.1 g ground DM was centrifuged at 3826 *g* for 20 min. at 20 °C. After collecting 0.2 ml of the supernatant, 1.8 ml sulfosalicylic acid, 2 ml glacial acetic acid and 2 ml ninhydrin acid were added, and the mixture was boiled at 100 °C

for 1 h in a water bath. After the sample cooled to 20 °C, 4 ml toluene was added to the mixture. The upper, organic fraction was collected for spectrophotometric measurements (Lambda 35 UV/VIS Spectrophotometer, Perkin Elmer, USA). The absorbance of the extracts was evaluated at 520 nm.

Chlorophyll (Chl) *a* and *b* concentration were determined from 0.05 g ground DM (Munné-Bosch and Alegre 2000); the sample was mixed with 5 ml methanol and centrifuged at 3470 *g* for 15 min. at 4 °C. The supernatant was transferred to a 50 ml volumetric flask. The procedure of mixing with 5 ml methanol and centrifugation was repeated three times, and after this procedure, the flasks were filled up to 50 ml with methanol. The extract absorbance was recorded at 647 nm (A_{647}) and 664 nm (A_{664}) with a UV-VIS spectrophotometer (Lambda 35 UV/VIS Spectrophotometer, Perkin Elmer, USA).

2.6 Leaf cross section

Prior leaf cross section, samples (0.5 cm^2) were fixed in the AFE solution (5 vol. % acetic acid and 5 vol. % formaldehyde in 90 vol. % ethanol) and left for one week at room temperature. Fresh samples were dehydrated using an ethanol concentration series (75%, 96% and 100%; 1-2 h per step) at room temperature. After dehydration, samples were infiltrated for 2 h with a solution containing 50% Technovit 7100 (Heraeus Kulzer GmbH, Wehrheim, Germany) and 50% ethanol. Afterwards, the leaf segments were incubated overnight in a solution of 1 g Hardener I (Heraeus Kulzer GmbH, Wehrheim, Germany) in 100 ml Technovit 7100 (Heraeus Kulzer GmbH, Wehrheim, Germany). Polymerization was enabled by adding Hardener II. Sections of 1.5 µm thickness were cut using a rotary microtome (HM 360, Microm International GmbH, Walldorf, Germany). Leaf cross sections were analyzed using a light microscope (Axio Scope, Carl Zeiss AG, Oberkochen, Germany) with a mounted camera (AxioCam MRc 5, Carl Zeiss AG, Oberkochen, Germany) at magnification of 400x. The thicknesses of the epidermis and leaves were measured using AxioVision 4.8.2 (Carl Zeiss AG, Oberkochen, Germany).

2.7 Net photosynthetic rate

Photosynthesis (µmol CO_2 m^{-2} s^{-1}) was measured *in situ* on the sixth leaf counted from bottom using a portable CO_2/H_2O porometer type CIRAS-1 with a PLC-B Parkinson leaf chamber (PP-Systems, Hitchin Herts, UK). Flow rates into and out of the leaf chamber were controlled by two mass flowmeters and maintained at 200 ml min^{-1} and the boundary layer resistance of the chamber reduced to less than Rb = 0.27 m^2 s mol^{-1} by vigorous stirring. The

CO_2 concentration within the chamber was set to 440 ppm using CO_2 soda cartridges and soda lime absorber columns, and the humidity was retained at ambient concentration.

2.8 Multiparametric fluorescence excitation

The fluorescence in the red and far-red spectral bands, excited with red light, was recorded with the hand-held sensor Multiplex®4 (Force-A, Orsay, France), as described previously (Kautz et al. 2014b). To perform recordings, leaves were placed in front of a black plate to reduce influences from the environment. The distance between the sensor and the leaf was kept constant at 7.5 cm and a frontal cover plate with aperture of 4 cm in diameter was used to standardize the measuring area. As a representative parameter, we selected the simple fluorescence ratio (ratio of far-red fluorescence to red fluorescence) excited with red light (SFR_R).

3 Data analysis

Statistical analysis was accomplished with IBM SPSS Statistics 20.0 (IBM Corporation, New York, USA). Means were subjected to analysis of variance (ANOVA, $P \leq 0.05$), and in cases of significant differences, compared by Duncan's multiple range test. Therefore plants cultivated in the hydroponic system and substrate were analyzed separately if necessary, e.g., analysis of the fluorescence lifetimes. Graphs were drawn using SigmaPlot 11.0 (Systat Software Inc., San Jose, CA, USA).

4 Results and discussion

In the present study, we compared the impact of osmotic stress induced by PEG in a hydroponic system and the drought stress in substrate cultivation using apple seedlings as a non-herbaceous model plant. In this context, we hypothesized that PEG-induced osmotic stress influences the photosynthesis and chlorophyll fluorescence in a similar way such as withholding water from soil. As references, we selected established biochemical and morphological parameters.

Plants exposed to water deficit undergo several physiological, biochemical and molecular adaptations to maintain a positive turgor (Chaves et al. 2003). In our study, plants exposed to osmotic stress or water shortage had significantly lower RWC and higher proline concentration than the respective control treatments (Table 1). PEG treatments and drought caused similar decrease in leaf RWC and no significant difference was observed between both

control treatments. To support the osmotic adjustment in the cells and to prevent water loss from drought impact, plants accumulate osmolytes like proline (Alonso et al. 2001, Yamada et al. 2005). Proline also acts as stabilizer of subcellular structures (Sánchez-Rodríguez et al. 2010). Our results confirm previous reports in which proline concentration increased in plants growing under PEG (Bandurska 2000, Bandurska 2001, Türkan et al. 2005) and drought stress (Sánchez-Rodríguez et al. 2010). However, the proline concentration in soil-grown seedlings was significantly higher than in those grown in hydroponic solution containing PEG. Here, we observed a mismatch between the similar values of RWC in 100 g dm^{-3} PEG and drought soil (39.25% versus 37.83%) and the very distinct values of proline (0.85 versus 2.43 mg g^{-1}). While the impact of PEG on the plant occurs suddenly, and its effect remains relatively constant because of the constant $\Psi\pi$ in the nutrient solution, interruption of irrigation causes slow but continuous soil drying. This results in the stress situation, here indicated as the accumulation of proline, increasing in the time course of the experiment.

The lower water availability due to PEG or drought also affected the leaf thickness (Tab. 1, Fig. 1). In particular, the thinner upper epidermis significantly contributed to the differences in leaf thickness between stressed and non-stressed plants in the hydroponic system. In contrast, in soil-grown plants, a relevant contribution was made by the (thinner) abaxial epidermis. Because of the impact of drought in leaf anatomy, a general effect on CO_2 diffusion and consequently the photosynthetic activity (Chartzoulakis 2002) cannot be excluded. If existent, however, it might have affected all of the stress-exposed plants because we observed similar effects from PEG and drought on the leaf anatomy.

Water shortage in the tissue also affected the concentration of photosynthetic pigments in the leaves. Both osmotic and drought stress caused a significant decrease in the Chl *a* and *b* concentrations. Here, Chl *a* was more strongly affected than Chl *b*, confirming that Chl *a* is more sensitive to abiotic stresses (Zayed and Zeid 1997). However, for both chlorophyll components we observed that a similar trend was induced by either osmotic or drought stresses.

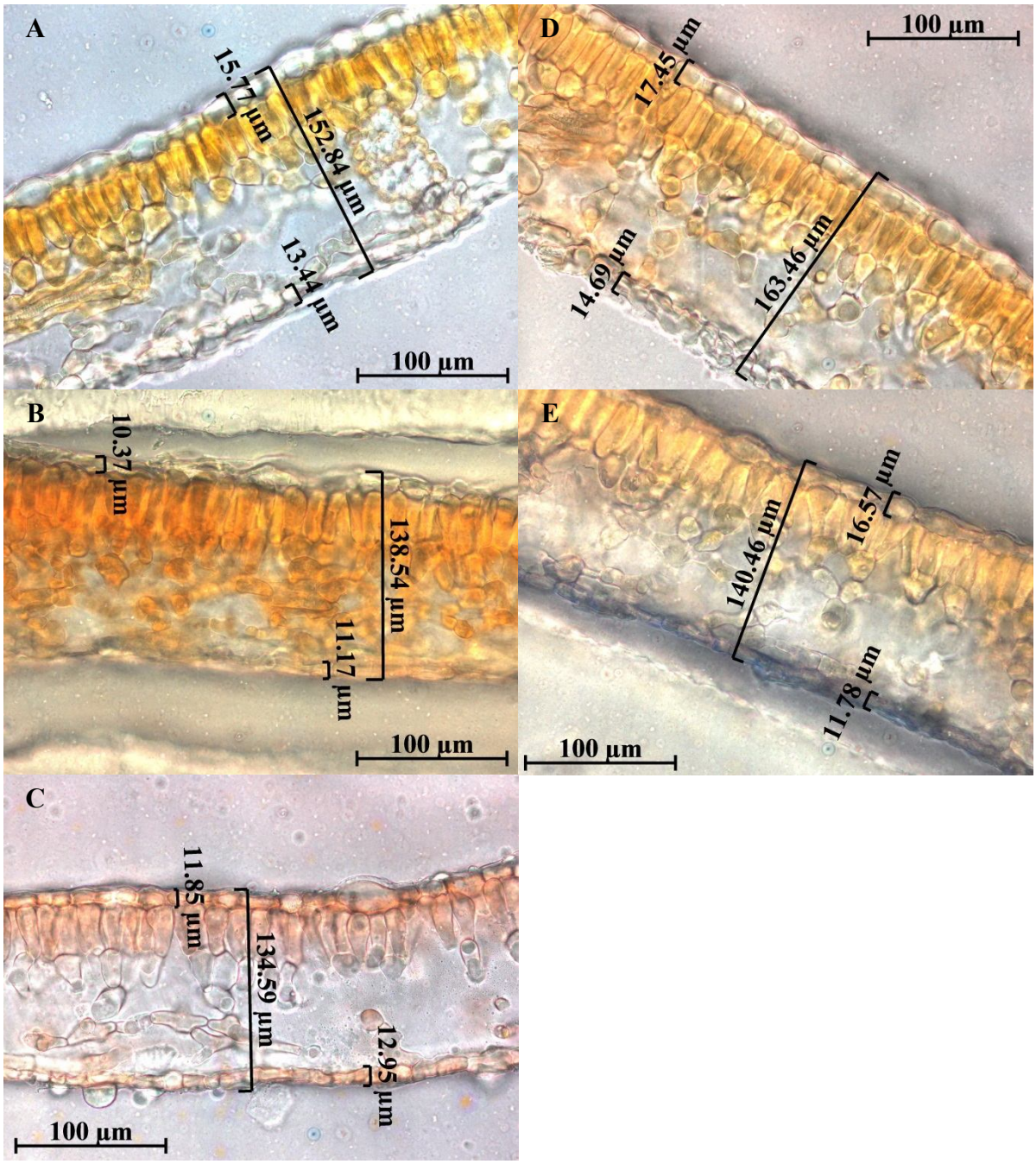

Fig. 1 Leaf cross sections of apple leaves after treated with 0 g PEG/ l (A), 50 g PEG/ l (B), 100 g PEG/ l (C) in hydroponic system as well as after sufficient (D) and deficit (E) water supply in soil. Samples were taken 70 days after the first treatment initiation. Magnification = x 400.

Table 1 The effect of PEG-induced osmotic stress and drought on relative water content (RWC), proline and chlorophyll concentration (Chl *a*+*b*) of apple leaves as well as the thickness of the leaves.

	Physiological parameters								Leaf thickness [µm]					
Treatment	**RWC [%]**		**Proline[1]**		**Chl *a*[1]**		**Chl *b*[1]**		**Leaf**		**Upper epidermis**		**Lower epidermis**	
0 g PEG dm^{-3}	79.96 ± 2.26	b	0.18 ± 0.05	a	6.52 ± 0.12	b	1.80 ± 0.03	b	149.1 ± 2.3	b	15.5 ± 0.6	b	13.5 ± 0.4	bc
50 g PEG dm^{-3}	42.70 ± 8.90	a	1.06 ± 0.29	b	4.74 ± 0.50	a	1.50 ± 0.13	a	138.9 ± 6.0	a	10.3 ± 0.9	a	11.3 ± 1.4	a
100 g PEG dm^{-3}	39.25 ± 6.02	a	0.85 ± 0.16	b	5.36 ± 0.31	a	1.57 ± 0.09	a	135.4 ± 3.7	a	11.5 ± 0.8	a	12.1 ± 0.5	abc
Watered soil	82.59 ± 2.90	b	0.08 ± 0.01	a	6.98 ± 0.17	b	1.86 ± 0.05	b	163.9 ± 3.5	c	17.3 ± 0.6	b	14.2 ± 0.7	c
Drought soil	37.83 ± 3.40	a	2.43 ± 0.43	c	5.33 ± 0.22	a	1.46 ± 0.07	a	142.4 ± 2.3	ab	16.0 ± 0.8	b	11.6 ± 0.8	ab

Mean ± SE in the columns followed by the same letter do not differ statistically according to the Duncan's multiple range test ($p \leq 0.05$; $n = 10$).

[1]Values given in [mg g^{-1} DM].

The net photosynthetic rates (P_N) of plants were monitored over time; under the comparatively low-light conditions of the climate chamber, the mean P_N of 0 g dm^{-3} PEG was 2.3 µmol CO_2 m^{-2} s^{-1}, and the mean P_N of well-watered plants reached 2.4 µmol CO_2 m^{-2} s^{-1}. For an easier comparison of the treatments, P_N is displayed as percent modification to the respective control groups (Fig. 2A). P_N was not significantly affected by 50 g dm^{-3} PEG in contrast to 100 g dm^{-3}. Analogous to that, drought strongly decreased the P_N, although if occurred after many days of delay (Fig. 2A). Here, we observed a pronounced stomatal closure influencing the P_N in stress-exposed plants (*data not shown*). As is well known, stomata closure in response to declining leaf turgor causes lower CO_2 diffusion through the leaf mesophyll and down-regulates photosynthesis (Chaves et al. 2009). The temporal changes in the P_N underline the differences of the treatments in their speed at inducing drought-like symptoms. Interestingly, in the second stress phase we did not observe any significant impact of the water supply on the P_N. Although unexpected, similar results were previously observed (Pedrol et al. 2000). One explanation might be related to the measuring technique; providing CO_2 to the leaf may compensate lower diffusion of CO_2 across the leaf mesophyll under PEG-induced osmotic or drought stress, leading to similar rates of photosynthesis in stressed and well-watered plants (Chaves et al. 2003).

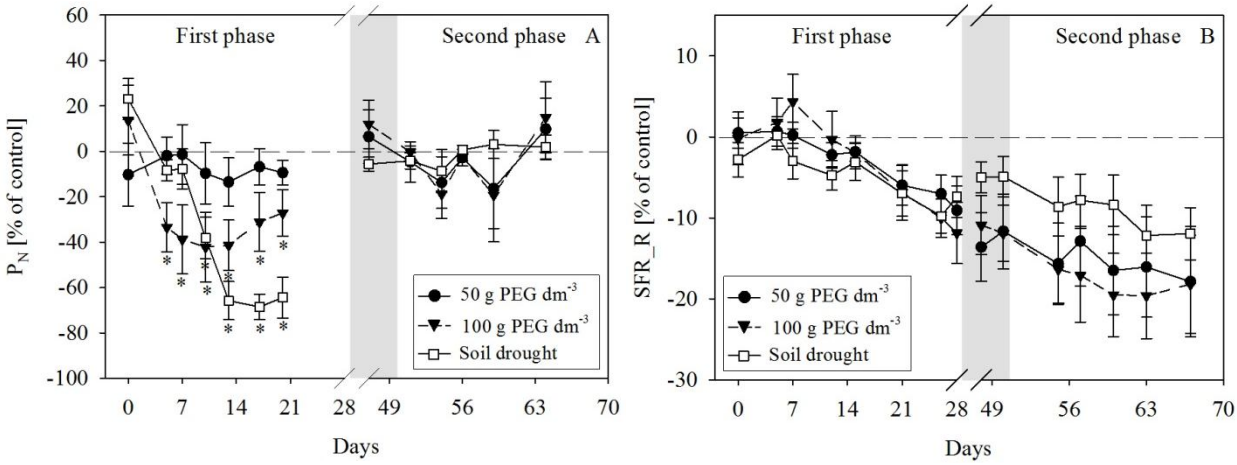

Fig. 1 The effect of PEG-induced osmotic stress and drought on A - net photosynthetic rate (P_N), B - simple fluorescence ratio (SFR_R) in apple leaves (% of control; Mean ± SE; *n* = 10). Gray zones in the graph indicate the recovery phase.

The simple fluorescence ratio (SFR), a fluorescence index that is related to both the chlorophyll content and the photosynthetic activity of the leaves, indicated a significant impact of the treatments with delay. Moreover, SFR did not show any significant differences between the stress-exposed groups. The strongest decrease of SFR was observed in the second stress phase; even if it was not statistically significant, drought in soil cultivated plants

affected the SFR less than in the osmotic stress induced by PEG (Fig. 2B). The stronger decrease in SFR was mainly because of a decline in the far-red fluorescence intensity (*data not shown*). This agrees with Lichtenthaler and Rinderle (1988) who report a proportionally stronger impact on far-red fluorescence under severe stress conditions than on red fluorescence. Here, again we observed relevant discrepancies between parameters; both P_N and SFR were affected by water withholding or PEG (particularly 100 g dm^{-3}), but there was a pronounced difference concerning time, speed and intensity in which these physiological indicators were affected.

In summary, in some cases PEG might induce drought-like symptoms in apple seedlings, as confirmed by changes in RWC, proline, chlorophyll, P_N and fluorescence parameters. The PEG concentrations (i.e., the different osmotic potentials of the hydroponic solutions) had a similar impact on the RWC and chlorophyll content compared with drought. On the other hand, the concentration of proline and the leaf thickness of the seedlings grown in hydroponics did not match the values of the plants exposed to drought in soil cultivation. Similarly, we observed strong discrepancies between P_N and the indices of the multiparametric fluorescence technique. Thus, when using PEG 6000, both the concentration of the chemical and the target parameters should be tested and defined on basis of preliminary experiments. Because of the mismatch concerning the biochemical, physiological and morphological parameters caused by PEG in hydroculture and drought in soil cultivation, we do not recommend the use of PEG to simulate drought stress in long-term studies.

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F Summary and conclusion

The main objective of this thesis was to assess the impact of economically important abiotic stresses on the plant physiology using non-destructive fluorescence indices, and evaluate the potential use of the sensor techniques as supporting tool for plant phenotyping in horticulture. The early detection of water deficiency and salinity was studied at leaf level in tomato (*Solanum lycopersicum* L.) plants by means of non-destructive fluorescence techniques. Evaluations comprised multiparametric fluorescence indices and pulse-amplitude modulated (PAM) chlorophyll fluorescence parameters for an effective and rapid sensing of water deficiency stress and stress recovery in three tomato genotypes. In addition, the impact of salinity on tomato genotypes was also studied using both methods. In the next step, the response of tomato genotypes exposed to simultaneous occurrence of salinity and water deficiency was examined by multiparametric fluorescence indices. An additional objective of the work was to investigate the suitability of chemically induced osmotic stress by polyethylene glycol (PEG) for drought stress experiments based on key physiological parameters of apple (*Malus domestica* Borkh.) leaves. The results of the single chapters can be summarized as follows:

1. Multiparametric fluorescence indices and PAM fluorescence imaging were adopted for an effective and fast sensing of water deficiency stress and recovery capability in three tomato (*Solanum lycopersicum* L.) genotypes. The multiparametric fluorescence indices were selected for the evaluations since they enable faster sensing of water deficiency without the need of dark-adaption as required for the PAM recordings. The results of this study indicate that the multiparametric indices are one reliable tool for the early detection of drought impact on tomato plants. The combination with the obtained PAM parameters allows a better estimation of impairments in the primary and secondary plant metabolism.

2. Compared with the PAM method, multiparametric fluorescence indices provide an effective and timely technique for the *in situ* sensing of salt stress in plants. UV light-induced BF/FRF and green light-induced FRF/RF were the most sensitive indices for the rapid sensing of salinity. Moreover, the temporal development of the indices was in accordance with the concentrations of Na, proline and chlorophyll, parameters well-known for salt tolerance. The selected indices might be used as a tool to evaluate genotypes for salt tolerance.

3. By use of multiparametric fluorescence indices it was possible to detect the simultaneous occurrence of salinity and water deficiency in tomato plants within eight days after treatment induction. The modification pattern in the complex parameters was principally caused by differences in the chlorophyll concentration and the functionality of the electron flux and less by an accumulation of blue fluorescing pigments in the leaves.

4. As compared to drought, chemically-induced osmotic stress in hydroponic solutions with different PEG 6000 concentrations had only similar impact on relative water content and chlorophyll content in leaves of apple (*Malus domestica* Borkh.) seedlings. In contrast, strong discrepancies were observed between net photosynthetic rate, indices of the multiparametric fluorescence technique, proline concentration and the leaf thickness. Thus, when using PEG, the appropriate concentration of PEG as well as the target parameters should be tested and defined on basis of preliminary experiments. Due to mismatch in biochemical, physiological and morphological parameters caused by PEG in hydroculture and drought in soil cultivation, PEG might be used with care to induce drought-like physiological changes, but it cannot be considered as an unconditional equivalent for natural drought, particularly in long-term studies.

In summary, the results obtained in these studies endorse the potential of the multiparametric fluorescence indices for the fast *in situ* detection of abiotic stresses at leaf level in tomato (*Solanum lycopersicum* L.) without the need of dark adaptation. Furthermore, our study indicates that the use of PEG is not recommendable to simulate drought stress in long-term studies. The discrepancy concerning the biochemical, physiological and morphological parameters in apple (*Malus domestica* Borkh.) seedlings caused by PEG in hydroculture and drought in soil cultivation reveals that PEG might only induce drought-like symptoms.

Specific multiparametric fluorescence indices were identified for effective and fast evaluations of the impact of water deficiency and salinity, indicating a potential use for classification of the tolerance degree of genotypes. In addition to that, multiparametric fluorescence indices enable the detection of the simultaneous occurrence of salinity and water shortage compared with plants exposed to single stresses. However, based on multiparametric fluorescence indices a stress differentiation between drought stress and salinity was not possible. To develop a reliable and precise assessment tool, further in-depth physiological studies as well as a larger quantity of cultivars and other plant species with distinct tolerance skills should be examined. Therefore, future works should focus on the development of a

broad database to support the distinction between cultivars with different qualities of susceptibility and tolerance. In the ideal case, such a system would allow a precise picture of the plant physiology without the need to measure non-stressed, control plants as reference. In addition, drought and salinity-induced changes in the composition pattern of biochemical compounds in affected leaves should be correlated with the non-destructive fluorescence-based indices. This would be helpful to interpret the alterations of the indices. With future perspectives, novel support vector machines, further developments in sensor technology and their combination could lead to rapid and stable determination of complex fluorescence parameters to be included in high performance screening systems.

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