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Sensor-Based Phenotyping of Plant's Physiological Responses to Abiotic Stress

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Contents

Summary

Plant phenotyping can be defined as the systematic recording of morphological, anatomical, physiological and chemical characteristics of plants, as well as their developments and responses to stimuli. The common use of sensors in crop phenotyping today has stretched the limits of what can be recorded as a phenotype:

Firstly, because sensors expand the perceptual horizons of human senses, and computer-assisted acquisition, storage and analysis of large amounts of data provides insights that would not be possible through purely human observation. Secondly, measurement standardized through sensor use potentially enables better comparability of phenotyping activities performed at different locations by different work-groups.

This thesis focuses on three challenges of modern sensor-based phenotyping of plants under abiotic stress:

1. The chapter "Phenotyping in Arabidopsis and Crops – Are We Addressing the Same Traits? A Case Study in Tomato" addresses the challenges that arise from comparing phenotypes of different plant species. Using Arabidopsis thaliana and tomato under drought stress as examples, causes of lack in comparability of phenotyping data generated by scientists from different disciplines with different goals and perspectives on plants are discussed. In addition, ways toward overcoming this problem are presented.
2. In the chapter "Factors Influencing Chlorophyll Meter Readings – Toward a Conceptual Framework", the influence of confounding variables on phenotypic measurements is analyzed using non-invasive chlorophyll measurements as an example. The chapter provides an overview of the functioning of noninvasive chlorophyll meters. In addition, a possible way to deal with confounding variables, namely explicit inclusion in the statistical model, is presented.
3. Finally, in the chapter "Effect of UV Radiation and Salt Stress on the Accumulation of Economically Relevant Secondary Metabolites in Bell Pepper Plants," the potential of sensor-based phenotyping to quantify economically relevant secondary metabolites in bell bell pepper leaves is discussed. The physiological basis of non-invasive detection is also explained.

The present work may be helpful to scientists in the field of phenotyping primarily in that it may provide new perspectives on phenotyping as a whole. Advanced phenotyping may in turn help both plant breeders and farmers and the society as a whole.

Zusammenfassung

Die Phänotypisierung von Pflanzen lässt sich definieren als die systematische Erfassung von morphologischen, anatomischen, physiologischen und chemischen Eigenschaften von Pflanzen, sowie deren Entwicklungen und Reaktionen auf Reize. Durch den heute üblichen Einsatz von Sensoren in der Phänotypisierung von Nutzpflanzen haben sich die Grenzen des erfassbaren Phänotyps verschoben:

Zum einen, weil Sensoren den Wahrnehmungshorizont menschlicher Sinne erweitern und das computergestützte Erfassen, Speichern und Auswerten großer Datenmengen Einsichten ermöglicht, die durch rein menschliche Beobachtung nicht möglich wären. Zum anderen ermöglicht die durch durch Sensoreinsatz standardisierte Messung potentiell eine bessere Vergleichbarkeit von Phänotypisierungsaktivitäten, die an unterschiedlichen Orten durch verschiedene Arbeitsgruppen durchgeführt werden.

Die vorliegende Arbeit befasst sich schwerpunktmäßig mit drei Herausforderungen moderner, sensorbasierter Phänotypisierung von Pflanzen unter abiotischem Stress:

1. Das Kapitel "*Phenotyping in Arabidopsis and Crops – Are We Addressing the Same Traits? A Case Study in Tomato*" befasst sich mit den Herausforderungen die sich aus dem Vergleich von Phänotypen unterschiedlicher Pflanzenarten ergeben. Am Beispiel von *Arabidopsis thaliana* und Tomaten unter Trockenstress werden Ursachen mangelnder Vergleichbarkeit von Phänotypisierungsdaten, die von Wissenschaftlern unterschiedlicher Fachbereiche mit unterschiedlichen Zielen und Sichtweisen auf Pflanzen generiert werden, erörtert. Außerdem werden Wege hin zur Überwindung dieser Problematik aufgezeigt.
2. Im Kapitel „*Factors Influencing Chlorophyll Meter Readings – Toward a Conceptual Framework*” wird am Beispiel von nichtinvasiven Chlorophyllmessungen der Einfluss von Störvariablen auf phänotypische Messungen analysiert. Das Kapitel liefert einen Überblick über die Funktionsweise nichtinvasiver Chlorophlyllmessgeräte. Außerdem wird ein möglicher Weg um mit Störvariablen umzugehen, nämlich die explizite Inklusion in das statistische Modell, aufgezeigt.
3. Im Kapitel „*Effect of UV Radiation and Salt Stress on the Accumulation of Economically Relevant Secondary Metabolites in Bell Pepper Plants*” wird schließlich das Potential sensorbasierter Phänotypisierung zur Quantifizierung von ökonomisch relevanten Sekundärmetaboliten in Paprikablättern erörtert. Dabei werden auch die physiologischen Grundlagen des nicht-invasiven Nachweises erläutert.

Die vorliegende Arbeit kann Wissenschaftlern im Bereich der Phänotypisierung vor allem insofern helfen, als dass sich eventuell neue Blickwinkel auf die Phänotypisierung insgesamt ergeben. Verbesserte Phänotypisierung kann sowohl Pflanzenzüchtern und Landwirten als auch ultimativ der Gesellschaft insgesamt von Nutzen sein.

Abbreviations and Units

2D	2 dimensional
3D	3 dimensional
A. thaliana	Arabidopsis thaliana
ABA	abscisic acid
ANOVA	analysis of variance
BRT	boosted regression tree
Chl	chlorophyll
Chl(Dualex)	Chlorophyll index of the Dualex device
Chl NDI	chlorophyll normalized difference index
cm	centimeter
CO_2	carbon dioxide
cv.	cultivated variety
DAG	directed acyclic graph
DATI	days after treatment inception
DW	dry weight
e.g.	example given
et al.	et alii
FAO	Food and Agriculture Organization of the United Nations
fig	figure
FLAV	Flavonol index of the Multiplex device
FRF_{UV}	far-red fluorescence when illuminated with ultra violet light
FRF_R	far-red fluorescence when illuminated with red light
g	gramm
GC-MS	gas chromatography – mass spectroscopy
GER	Germany

HCl	Hydrochloric acid
HPLC	high pressure liquid chromatography
HSD	honest significant difference
i.e.	id est
L	liter
LAD	leaf area density
LAI	leaf area index
LCM	leaf chlorophyll meter
LiDAR	light detection and ranging
m	meter
min	minutes
µmol	micromol
mL	milliliter
mm	millimeter
mM	millimol
mS	millisievert
n	sample size
na	not available
NBI_R	nitrogen balance index under red excitation light
NL	The Netherlands
nm	nanometer
no.	number
PEG	polyethylenglucol
PAM	pulse amplitude modulation
QTL	quantitative trait locus
RF_R	red fluorescence when illuminated with red light
RGB	red green blue

RMSE	root mean square error
ROS	reactive oxygen species
rpm	rounds per minute
RWC	relative water content
RSA	root system architecture
s	second
SEM	structural equation model
SFR_R	Simple Fluorescence Ratio under red excitation light
SM	secondary metabolite
ssp.	subspecies
Tab.	Table
UAV	unmanned aerial vehicle
UK	United Kingdom
USA	United States of America
UV	ultra violet
v	volume
Vol	volume
W	watt
Wi	*weighted-in portion*
WUE	water use efficiency

1. Introduction

Definitions, historic development and aims of plant phenotyping

Plant phenotyping is the systematic assessment of a plant's phenotype. But to the question of what exactly the phenotype of a plant is, different answers can be found in the literature:

Houle and colleagues defined phenotypes as "the characteristics of organisms that are of the most interest" [1], which highlights the subjective, human-centrist perspective. Other authors use more general definitions, e.g. Pieruschka and Poorter state, referring to the classical concept of Johanssen [2], that "the phenotype can be seen as the combination of all the morphological, physiological, anatomical, chemical, developmental and behavioural characteristics that, when put together, represent the individual organism" [3]. When taking the latter definition of plant phenotype, one could define plant phenotyping as the systematic assessment of a plant's morphological, physiological, anatomical, chemical, developmental and behavioral characteristics. The plant's phenotype could also be termed the physical footprint of the plant in the observable world. The anatomical and morphological properties of the plant, which can only be incompletely delineated from one another, can best be described technically as the three-dimensional structure of the plant and its parts in space. The theoretically possible precision of the detection of these properties is infinite, therefore it must be stated that a complete detection of these plant properties is impossible. The same applies to chemical and physiological properties of plants. Some physiological processes, such as wilting or the production of certain pigments, are quite easy to capture even by human senses alone, but capturing physiological responses especially at the molecular level, is only possible with considerable effort. Developmental and behavioral characteristics add yet another layer of complexity, as these processes have an inherent temporal component, and the observation requires the continuous recording of the plant in its entirety. Thus, it can be stated that plant phenotyping is a task that can never be fully accomplished, capturing a plant's phenotype completely is not possible. At the same time, for thousands of years, humans have been able to modify plants through selection (based on the phenotype of the plant) in a way that increases the plant's usability by humans, and the very development of mankind is closely related to its success in this selection process [4]. After all, crop species itself are the results of up to thousands of years of plant phenotyping.

It is difficult to trace when and where plant phenotyping was first done systematically – and difficult to decide what "systematic" can mean in this context. In any case, many authors refer to Fisher as the founder of the theoretical basis for the systematic recording and analysis of plant

phenotypes [5], while Fisher himself also acknowledges older works from Bayes [6] and Pearson [7] in this context. Fisher suggested the randomized screening of plants in order to exclude any bias based on wrong assumptions of the experimenter and in a way that “each variety has an equal chance of being tested on any particular plot of ground” [8]. Even today, randomized controlled trials are still widely used in crop phenotyping. The good control of environmental effects represents both a strength and a weakness: On the one hand, effects of those environmental parameters that are included in the analysis are actually well accounted for. On the other hand, the external validity is not always given, as, in order to control of environmental effects, trials have to be applied, which differ strongly from the real environment of crops in the field. Finally, it has to be considered that Fisher implicitly assumes that the experimenter knows all relevant factors influencing his measurements and randomizes according to them. This omniscient experimenter is a strong assumption. Therefore, in recent decades, new, partly “uncontrolled” experimental designs and statistical methods have become more and more common in plant phenotyping [9–11].

The focus has traditionally been on yield maximization and increasingly on product quality as phenotypic traits. With changing goals in plant breeding from maximum yield to product quality and most recently to resource use efficiency, the focus of phenotyping associated with breeding is also shifting. Plants respond with different mechanisms to abiotic stresses such as nutrient or water deficiency, and these responses provide clues to stress tolerance and resource use efficiency of plants. Since these responses can be partially detected by sensors, a new field of application of sensor-based phenotyping emerges. As one example here, consider the use of thermal cameras to detect drought stress tolerant plants (e.g. [12]). In addition to the importance of sensor-based plant phenotyping in the development of new varieties in plant breeding, another field of application emerged with the advent of precision agriculture: the management of plant populations. To stay with the example of drought stress, it is also possible to detect acute drought stress in a plant stand through thermal imaging and to counteract possible yield losses by adequate irrigation [13,14]. In conclusion, plant phenotyping today is an essential part of both plant breeding and plant management.

Sensor-based plant phenotyping: Beyond the obvious

Early plant phenotyping has probably been based on visual assessment of plants, fruits, *et cetera*. The use of primitive measuring instruments (e.g. folding rule, scales) has later allowed for a minimum level of reproduceability of phenotyping activities. The use of electronic sensors opened up new ways to systematically record – and thus improve – crop traits.

A sensor is broadly defined as “a device that discovers and reacts to changes in such things as movement, heat, and light” [15]. With regard to plant phenotyping in the broader sense as described above, various plant characteristics can be inferred from movement, heat, and light (reflection and absorption): Structural properties (e.g. anatomical, morphological and – when measured over a period of time – developmental and behavioral) can be inferred with appropriate algorithms based on absorption, reflection and scattering of electromagnetic radiation ("light") [16]. Different physiological reactions such as changes in stomatal conductivity or increases and decreases in the concentration of various secondary metabolites can also be assessed with the aid of sensors [17,18].

The systematic use of technical sensors has massively expanded the limits of what phenotyping can achieve. This has two main causes: first, sensors massively expand human perception. To stay with the example of electromagnetic radiation: While our eyes in interaction with our central nervous system can process radiation information in the wavelength range of 400-700 nm, technical sensors know almost no limits. The absorption of UV radiation in plant leaves can be detected [19], as can the root structure in the soil [20], both without touching the plant or affecting its development. On the other hand, the use of sensors makes it possible to record, store and finally process very large amounts of data. While data alone is not useful information, it does provide the basis for knowledge [21]. Finally, the precision of technical sensors is partly superior to human perception. For example, modern thermal cameras can reliably detect temperature differences in the range of 0.1 Kelvin. As of today, sensors are used to gain information on all three pillars of the phenotype – genotype – environment concept.

Showing differences in stressed plants

By definition, the phenotype of a stressed plant is different from the phenotype of a non-stressed plant. If the phenotype does not differ, there is no stress situation.

Both to identify stress-resistant and stress-tolerant lines in plant breeding and for optimal resource management in crop production, the identification of plant stress is necessary. In order to understand which phenotypic differences can be expected under which stress and how these can be detected, it is necessary to understand the mechanisms of plant stress physiology. A detailed account of plant responses to a wide range of stressors is beyond the scope of this dissertation. Various textbooks and reviews shed light on this topic [22–24]. Using drought stress as an example, the response of different plants and various phenotyping options will also be discussed in detail in the chapter “Phenotyping in Arabidopsis and Crops – Are We Addressing the Same Traits? A Case Study in Tomato”.

Breakthroughs in plant biology lead to new challenges

Beginning with the sequencing of the Arabidopsis thaliana genome in 2000 [25], more and more information about the genotype of plants, including in particular important crops, has been generated in recent decades. Through this development in the field of biology, the focus of phenotyping has also partially shifted: Henceforth, it has been of great interest to understand which parts of the plant genome provide which phenotypic traits – initially without these phenotypes necessarily providing direct agronomic added value. The identification of quantitative trait loci (QTLs) related to yield and the identification of resistance genes to various plant diseases also drove the rise of marker-assisted breeding. Genetic sequencing has become faster, more accurate and less expensive over the years. As a result, more and more genotypic information became available, which had to be matched with phenotypic information to gain an understanding of the relationships between genome and phenotype. Starting from around 2005, the term "phenotyping bottleneck" was coined to express that the lack of phenotype information was holding back progress in understanding plants and ultimately in plant breeding [11,26–28], and more and more efforts were made to overcome this shortage of phenotypic information.

Plant phenotyping today – relevance and challenges

Plant phenotyping today takes place on many different levels. This applies not only to spatial scales from the molecular to the acre scale, but also to the way phenotyping experiments are designed and performed. On the one hand, there are still single experiments under very controlled environmental conditions to answer a specific question, such as the change in expression of a particular gene under different light conditions. On the other hand, there are large phenotyping consortia and private-sector breeding companies that conduct phenotyping experiments under a wide variety of environmental conditions, some of which are internationally coordinated and standardized (for an overview, see e.g. [29]). From a scientific point of view, both approaches have their justification, although in the case of the former, small-scale experiments, caution is required when interpreting the results with regard to general validity.

There are global inequalities in phenotyping capabilities: Most high-throughput phenotyping platforms are based in the northern hemisphere and in moderate to Mediterranean climate. Also, much work is done for model organisms like *Arabidopsis* or model crops such as rice and tomato, while rather few studies focus on tuber crops like yams (*Dioscorea*) or cassava (*Manihot esculenta*), despite their outstanding importance for the cheap nutrition of millions of people [30].

With regard to the traits of interest, it can be seen that the focus is shifting in some cases. Yield and product quality – although still of great importance – are no longer the only decisive factors, but process quality is also increasingly being considered. Among other things, water and nutrient use efficiency, and in the future probably increasingly CO_2 footprints of different varieties, are moving into focus. These issues are *inter alia* addressed in our conference paper "Eco-friendly tomatoes: saving water and nutrient resources?" [31]. In addition, with the emergence of bio-economy, some entirely new demands on crops are emerging. Co-products are becoming more important in a society increasingly steeped in zero-waste thinking. Examples of quantifying novel co-products in vegetable crop leaves are included in the chapter "Effect of UV Radiation and Salt Stress on the Accumulation of Economically Relevant Secondary Metabolites in Bell Pepper Plants", as well as another publication by Röhlen-Schmittgen et al. [32], further discussed in the section "Topic-related collaborations". In the future, the use of sensor-based phenotyping could determine optimal harvesting times and identify suitable crop management measures in order to optimally exploit the bio-economic potential.

There have been several breakthroughs on the technological side, improving both throughput and quality of phenotypic data in recent decades [33]. However, phenotyping even when using modern sensors, is still often done methodically in the same way as suggested by Fisher of more than 100 years ago. But there are some steps towards new methodological milestones.

Concepts of phenotype – genotype – environment interactions

The relationship between genotype and phenotype has already been mentioned, the following section now provides an overview of the different concepts and modeling approaches used to understand the interplay between genetics, environment and phenotype.

The first concept including a distinction between an organisms genotype and phenotype is often attributed to Johannsen [34], e.g. [35]. However, Garrod [36] already argued that the physiological reaction to a drug is not only a result of the genes, but that the environment (e.g. diet) can modify the phenotypic reaction. The simplest mathematical expressions of the relationship between genotype, phenotype and environment is

$$Phenotype = Genotype \times Environment$$

If one takes this formula seriously, then the exact determination of each of the three parameters should be possible if the other two are known. In reality, however, neither the phenotype, nor the genotype, nor the environmental conditions are factors that can be unambiguously determined by a numerical value. Moreover, the real relationships are more complex than the

above equation suggests. A concept still in use today in university teaching is the "phenotype – genotype – environment – triangle" (Fig. 1). The origin of this schematic representation cannot be clearly determined. There are also different variations: In part, the relationships between genotype and phenotype as well as between environment and phenotype are represented bidirectionally, unlike in the representation chosen here. Although there is certainly an influence of the phenotype of plants on their environment – think, for example, of root exudates and the associated modification of physical soil properties – the majority of the interaction here is a directional one, as shown in figure 1. The relationship between genotype and phenotype is of a similar nature.

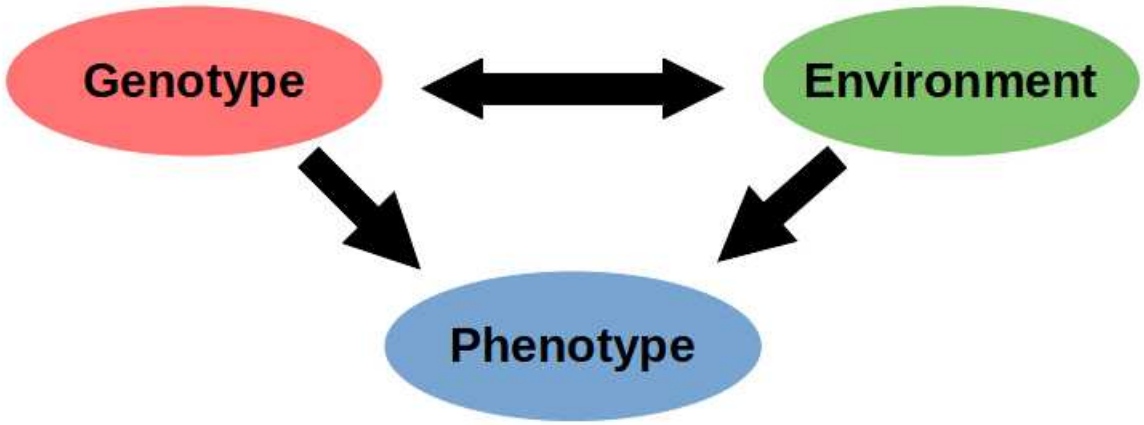

Figure 1: Simple schematic representation of the interactions between genotype, environment and phenotype.

The simple triangle is nevertheless a highly simplified representation of the real relationships. Taking into account some of the parameters that are hidden behind the large generic terms, it quickly becomes clear how confusing modeling approaches can become in the area where genotypes, phenotypes and the environment are in tensions (Fig. 2). For reasons of clarity, the drawing of correlations and dependencies between individual factors was omitted.

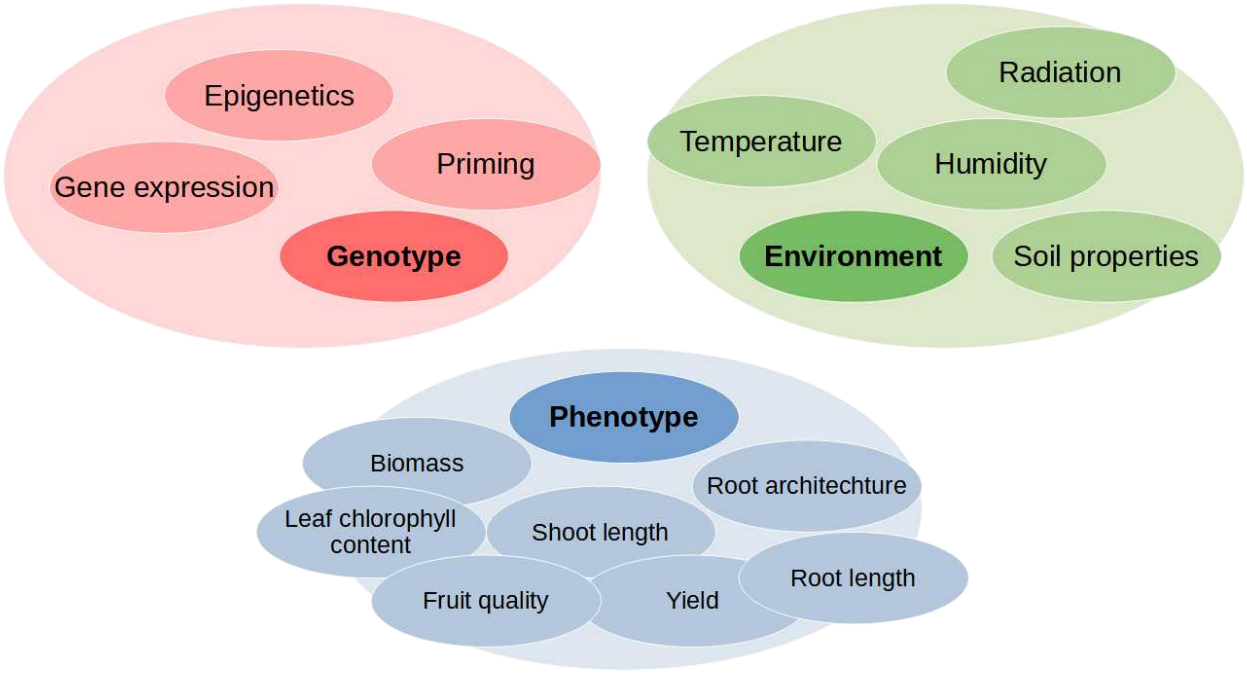

Figure 2: Several elements of the broad generic terms "genotype", "environment," and "phenotype." The manifold interrelationships between the individual elements are not shown here.

Given the complexity indicated in figure 2, it is questionable whether randomized controlled trials and simple statistical methods are sufficient to analyze the system as comprehensively as possible. One of the prerequisites for the use of linear models and, for example, analyses of variance for these models is that the "independent variables" are not correlated with one another. This central assumption is violated in many cases when relationships between genotype, environment and phenotype are observed. In fact, there are numerous and strong correlations, for example within environmental factors such as radiation, temperature and humidity, but also between environmental factors and epigenetics. In some cases, the dependencies are bidirectional, and thus potentially representable by systems of equations. For example, the relationship between root system and soil moisture influences each other reciprocally. In other cases, however, the direction of influence is clear: air temperature affects the rate of growth, and thus the biomass production, of a plant at a point in time. Conversely, however, the influence of biomass on temperature is very limited. Whether systems of equations and linear models are always the best mathematical representation of reality must be doubted.

Neural networks offer a way to model the complex relationships described above more realistically than is possible with classical linear models. Nichol and colleagues [35] proposed a neural network's structure after Gerlee and Anderson [37], that can in be used to predict phenotypes based on environments as input variables and genotypic effects as modulation of

the effect transmission between individual nodes of the neural network (Fig. 3).

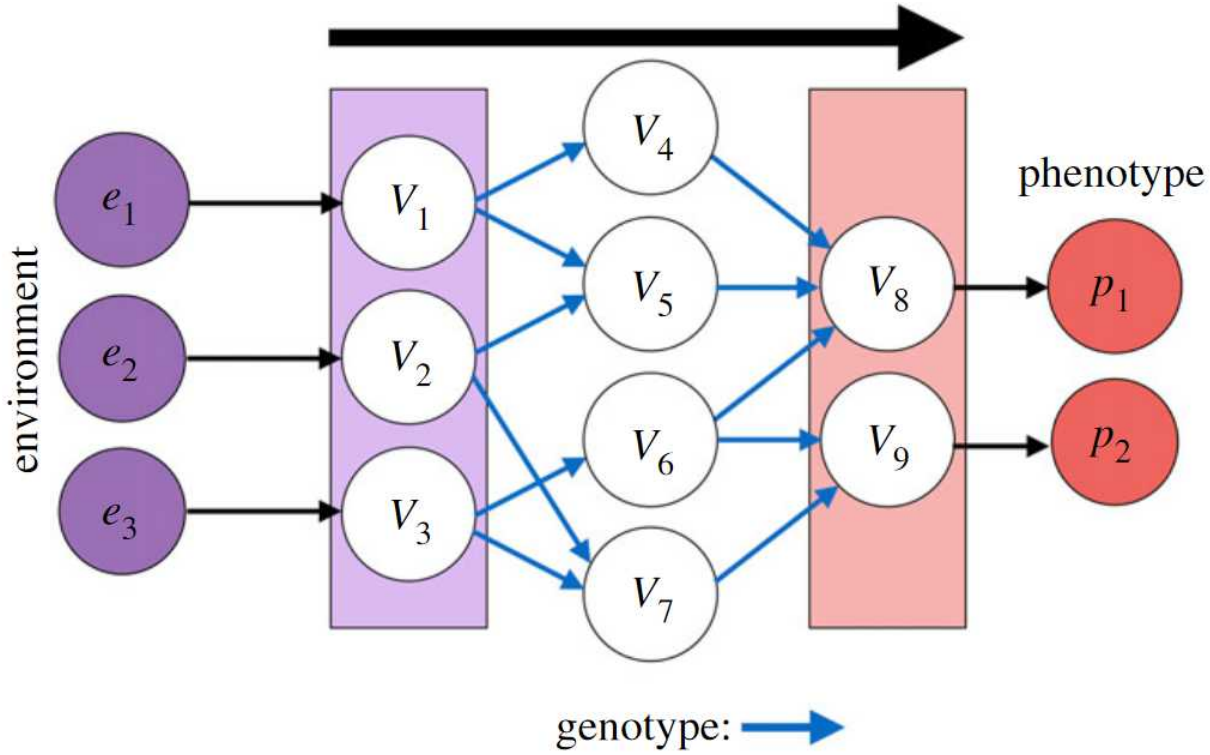

Figure 3: Structure of a “feed-forward” neural network. The genotype determines the internal weights of the neural network. Slightly modified from [35].

Note that the neural network shown in figure 3 only ever receives inputs from exactly one other layer. Among the different structures of neural networks, directed acyclic graphs (DAGs) in particular offer an even higher degree of structural complexity. Individual layers of these models can process inputs from multiple layers and project outputs to multiple layers, thereby representing natural relationships more realistically. DAGs play an increasing role in modeling genotype-environment interactions for human disease studies [38,39], but not yet in plant phenotyping. DAGs might also enable the incorporation of confounders of phenotypic measurements, captured alongside the phenotypic data. This possibility is further discussed in the chapter “Factors Influencing Chlorophyll Meter Readings – Toward a Conceptual Framework”.

Overall phenotyping benchmarks

The development of landraces well adapted to local conditions independently around the world has probably often been achieved without any sensor support and perhaps even without strictly systematic phenotyping. One should be aware of this: modern, systematic, sensor-based

phenotyping, which is the subject of the rest of this dissertation, undoubtedly offers various advantages. However, from an agronomical point of view, the breeding progress compared to landraces must outweigh all the additional effort compared to simple phenotyping as it has been done for centuries.

Overview of topics addressed in this dissertation

The following main body of the dissertation is divided into three parts, each addressing a current challenge in plant phenotyping. A brief introduction to the problem addressed is followed by a manuscript addressing the problem at hand. Finally, the findings are summarized in a general discussion.

First challenge

The available amount of phenotypic data of the biological model organism *Arabidopsis thaliana* is considerably larger than the amount of phenotypic data of any crop plant. At the same time, it is cost and labor intensive to perform detailed phenotyping studies also on crop plants. Therefore, plant scientists try to transfer the phenotypic knowledge from the model organism to crop plants. For example, if a mutation in *A. thaliana* leads to increased drought stress tolerance in the plant, it is reasonable to assume that this could also be the case for crop plants. Numerous QTLs in *A. thaliana* can also be found in crop plants. Indeed, much of the work on A. thaliana is justified on the basis that the knowledge gained is transferable to crop plants. However, this transfer is not trivial, in part because the phenotyping methods used in molecular biological studies on *A. thaliana* are often massively different from those used in crop plants. For example, "drought stress tolerance" of *A. thaliana* is often equated with survival rate under desiccation, or even with the ability to grow on a medium with low osmotic potential. Such "drought stress tolerance" is useless from an agronomic point of view, because the simple survival of a plant in drought has no value *per se*, but only if this ability is not unduly detrimental to yield.

The first chapter of this thesis, "Phenotyping in Arabidopsis and Crops – Are We Addressing the Same Traits? A Case Study in Tomato", addresses in detail the problems of "translational phenotyping", or the transfer of phenotypic knowledge between species, using *A. thaliana* and tomato as examples. In addition, this chapter provides a detailed overview of state of the art methods for sensor-based plant phenotyping, and therefore represents an ideal extended introduction to this dissertation.

This chapter of the dissertation was also published as a review paper in Genes [40].

2. Phenotyping in Arabidopsis and Crops – Are We Addressing the Same Traits? A Case Study in Tomato[1]

Paolo Korwin Krukowski *, Jan Ellenberger *, Simone Röhlen-Schmittgen, Andrea Schubert and Francesca Cardinale

* These authors contributed equally to this work.

Abstract

The convenient model *Arabidopsis thaliana* has allowed tremendous advances in plant genetics and physiology, in spite of only being a weed. It has also unveiled the main molecular networks governing, among others, abiotic stress responses. Through the use of the latest genomic tools, *Arabidopsis* research is nowadays being translated to agronomically interesting crop models such as tomato, but at a lagging pace. Knowledge transfer has been hindered by invariable differences in plant architecture and behavior, as well as the divergent direct objectives of research in *Arabidopsis* vs. crops compromise transferability. In this sense, phenotype translation is still a very complex matter. Here, we point out the challenges of "translational phenotyping" in the case study of drought stress phenotyping in *Arabidopsis* and tomato. After briefly defining and describing drought stress and survival strategies, we compare drought stress protocols and phenotyping techniques most commonly used in the two species, and discuss their potential to gain insights, which are truly transferable between species. This review is intended to be a starting point for discussion about translational phenotyping approaches among plant scientists, and provides a useful compendium of methods and techniques used in modernphenotyping for this specific plant pair as a case study.

1 Krukowski, P.K.; Ellenberger, J.; Röhlen-Schmittgen, S.; Schubert, A.; Cardinale, F. Phenotyping in Arabidopsis and Crops—Are We Addressing the Same Traits? A Case Study in Tomato. *Genes* **2020**, *11*, 1011, doi:10.3390/genes11091011.

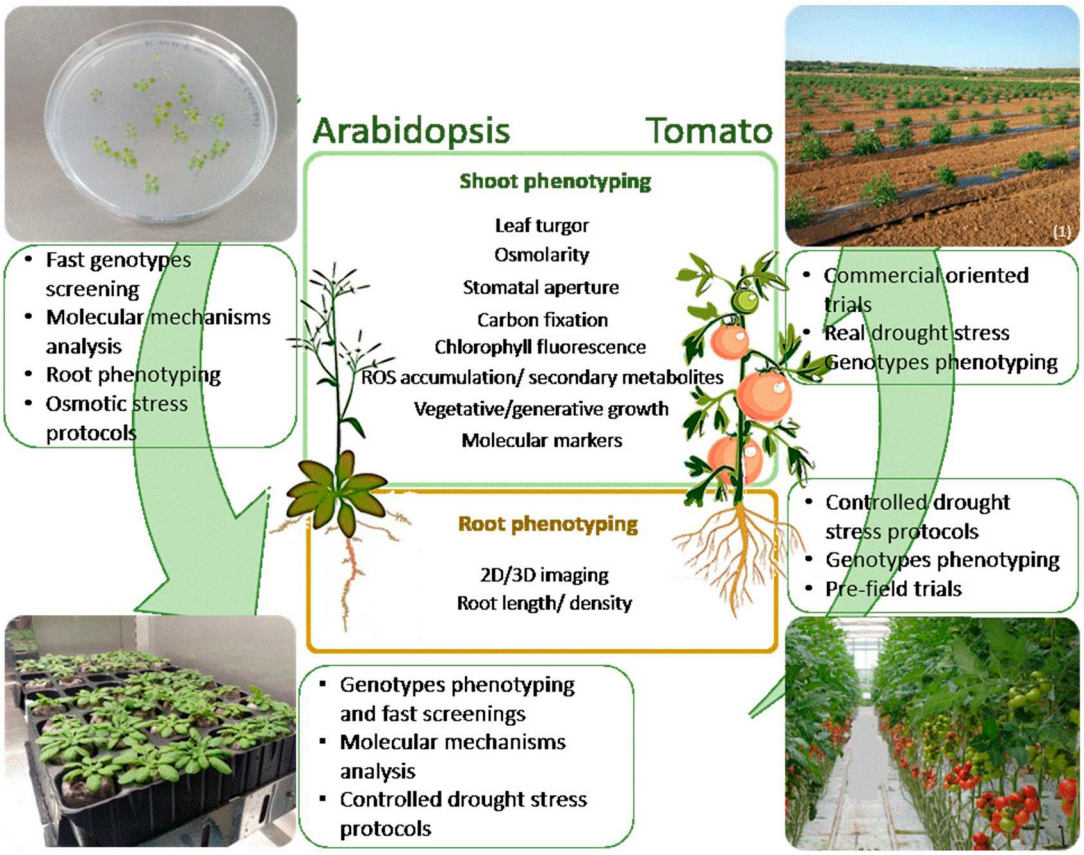

Figure 4: Grafical abstract. Phenotyping Arabidopsis and tomato with similar technology in the conflict of different phenotyping objectives and stress protocols.

Introduction

The quest for drought resistant genotypes has been, for a long time now, one of the principal challenges in plant sciences: Drought stress can seriously hamper crop development leading to a decrease in yield, with serious socioeconomic consequences [41]. Historically, a decrease in crop yield has always resulted in social disorders, for example, in Egypt when the Nile flooded under emperor Claudius govern [42]; in Ireland, during the potato blight famine [43], and now seen in the effects of climate change on agriculture, including drought have been recognized, among other interconnected social, political and economic factors, as a concurring cause of the current African migration [44].

Climate change influence on temperature and rainfall occurrence and intensity is rapidly mutating the water balance of ecosystems, resulting, amidst other extreme climatic phenomena, in unusually extended drought periods in temperate countries [45]. Consequently, unless serious countermeasures are adopted, these countries may face a tremendous water shortage

affecting both water and food security. According to a recent Food and Agriculture Organization (FAO) report [46], agriculture accounts nowadays for 70 per cent of water usage worldwide. It is clear that reducing its consumption in this sector could be very proficient. Such a complex task must be addressed through the combination of several integrated solutions among which the development of water-use efficient crops may hold a position of high relevance.

In the beginning, new drought resilient plants were obtained by conventional breeding among promising genotypes, exploiting the genetic pools offered by natural variation [47]. Following the advances of genetics, new methods were developed to overcome the limits of traditional breeding, attaining the possibility of gene editing at single-base definition [48].

No matter the techniques used, modified plants need to be phenotyped. Following the classical equation, where “phenotype = genotype × environment”, the mutation of a single gene can have various effects on plant phenotype [49]. *Arabidopsis thaliana* (*Arabidopsis*) has been for decades the most important model for genetics and molecular biology of angiosperms due to numerous characteristics that made it very convenient for research [50]. A short life cycle, compact dimensions, high number of seeds and a very small, sequenced and well-annotated genome. All these advantages, however, do not really overcome the fact that there is no commercial use for this weed. Consequently, *Arabidopsis* research is only a first step towards the characterization of a gene that can be useful for crop improvement. The results must be translated into more economically interesting models, such as a tomato. *Solanum lycopersicum L.* is a convenient crop model; popular for its taste and nutritional value of its fruits, it is one of the most economically important crops around the globe [51] and a high quality sequence of its genome is available [52]. Tomato is a good model for molecular, physiological and agronomical studies, and a perfect endpoint for translational biology. As an example, many tomato genes that strongly influence yield, a trait that is often overlooked in *Arabidopsis* research, are homologs of *Arabidopsis* genes involved in flowering, seed production or other reproductive processes [53]. In general, translational biology is currently undertaking the quest for adapting Arabidopsis molecular models to more agronomically interesting crop models, especially through the use of “omic” techniques and data mining [54]. While, possibilities and issues of *Arabidopsis*-to-crop genomic translation have been discussed elsewhere [54–57], the problematics of translating phenotyping studies have not been addressed until now. Despite both being widely used models in physiology, the different nature of *Arabidopsis* and crops prohibits an absolute equalizing of phenotyping methods and leads to different endpoints. Additionally, certain physiological variables and fruit-related traits are easier to quantify in tomato. This leads to the paradox that physiological phenotypes, described in model crops,

would profit from the molecular underpinnings being investigated in *Arabidopsis*. While, meaningful physiological phenotyping of the latter plant, which is needed to correctly identify mutants in a forward genetic approach, can be a bottleneck. We believe that a careful assessment of available techniques in either plant species may help the homogenization of phenotyping methods and protocols where possible, and ease the tricky task of comparing them meaningfully. This review is a first attempt to describe the difficulties of translational phenotyping. Such a complex topic is too broad to be dissected in a single paper. Here, we will focus on translating drought stress studies from *Arabidopsis* to tomato as a case study. Drought is one of the most detrimental stressors in crop production and, as a consequence, resistance is one of the most studied traits in crop science. However, there is not a unique definition of drought and different ways to impose drought are used in experimental procedures. When comparing *Arabidopsis* and tomato studies, it is therefore important to understand the nature of drought. For instance, the drought stress that occurs during a field study in tomato differs dramatically from an osmotic stress often imposed *in vitro* in *Arabidopsis*.

The Multiple Facets of Drought

Drought is generally defined as a prolonged period of water shortage, resulting in an insufficient supply for the environment. However, drought stress and its precise definition, heavily rely on a number of environmental variables, as previously discussed [58], including the severity and duration of water deprivation, seasonal variations as well as the dynamics of drought occurrence, such as slightly reduced, merely sub-optimal water availability or a more serious and persistent water shortage that may reveal lethal.

In plant physiology more specifically, drought is a form of stress, i.e. an external factor that seriously affects plant growth, productivity, reproductive capacity or survival [23]. As a consequence of stress, plants acclimate through a complex set of physiological, molecular, biochemical and developmental mechanisms to create a new homeostatic equilibrium. Therefore, drought can be described as water deficiency imposed (in various forms e.g., pulsed or persistent drought periods) to induce, identify and understand morphological, physiological and molecular mechanisms of acclimation [59]. Similarly, in agronomical sciences, drought is also defined in function of the studied trait. However, due to the different nature of agronomy itself, other socioeconomic and environmental factors are taken into account as well. Indeed, the points of view of researchers in different scientific disciplines interested in the topic often differ noticeably. While, a molecular scientist may design a very controlled osmotic stress, *in vitro*, to follow the precise expression kinetic of a gene set, an agronomist may be more

interested in running a field experiment to quantify whole crop stands' yield of two genotypes, in order to identify the more tolerant one. Phenotyping performed by the two researchers will, thus, address very different traits. The type and intensity of drought stress imposed cannot be the same in both trials. Actually, the nature of the experiments the two scientists are designing and conducting will differ greatly, but plant science as a whole should still seek for ways to integrate results of both trials.

A crucial step towards understanding drought impacts across species and environments is to understand adaptation and acclimation mechanisms, and to incorporate them into experimental design.

How Do Plants Cope with Drought? A Trait-Oriented Perspective

When a drought spell occurs, plants react to raise their survival chances. There is no unique response for all plants, even when limiting the case study to *Arabidopsis spp*., responses may change dramatically among ecotypes [60]. Therefore, comparing drought stress coping strategies among different species is a complex, but a necessary task. In fact, drought acclimation strategies should be the main drivers of drought stress experiments [61].

The classical definition divides survival mechanisms in three broad categories: Drought escape, avoidance and tolerance [59]. In case of water scarcity, escaping plants will try to complete their life cycle before stress becomes too severe to manage (i.e., by early flowering or early maturity). In contrast, avoiding drought involves the ability of plants to maintain a stable water status despite a water shortage in soil. This is usually achieved through root architecture and water use optimization. Finally, tolerant plants will acclimate to the new environmental equilibrium and spend resources to; (a) maintain turgor in unfriendly conditions through osmotic adjustments; and (b) produce antioxidants to avoid oxidative damage caused by the generation of reactive oxygen species (ROS) as a consequence of stress. However, no plant applies only one of the three strategies. In fact, each species adopts its own combination of some drought avoidance, tolerance and escape mechanisms. This is a critical concept when comparing two different species like tomato and Arabidopsis.

Recently, Gilbert and Medina [61] proposed a new set of four terms linking increasing drought severity to distinct physiological mechanisms underlying the acclimation: Soil water deficit avoidance (e.g., by better soil exploration, water conservation), stress avoidance (e.g., by osmotic adjustments, optimization root-soil interactions), damage avoidance (e.g., by optimized leaf orientation, increased evaporative cooling, more favorable root-to-shoot ratio) and damage tolerance (e.g., by night-time recovery, or molecular protection conferred by heat shock

proteins). Since these definitions point to the combination of specific traits and stress severity levels, they can be monitored by precise molecular and morpho-physiological markers and thus make it easier to design experiments to study preferred traits.

While tomato and Arabidopsis do not react in the exact same way to the same stress, they share molecular and physiological responses that are activated in response to stresses. As a consequence, we propose that in order to generate comparable datasets across species under drought, ensuring that a specific reaction of interest – be it molecular or morpho-physiological – is present at a similar level in the two species under even dissimilar environments may be more useful operationally than struggling to precisely impose the same stress to the two species. For example, in order to build a deficit irrigation protocol for tomato and potato, Jensen and colleagues [62] decided to use ABA xylem concentration to observe and synchronize stress among different species. In this way, they developed two slightly divergent watering regimes that yielded similar responses in the two *Solanaceae*. In this sense, drought stress protocols are in function of the studied traits, rather than the opposite: A similar approach is advisable when translating from *Arabidopsis* to crop and vice versa.

Drought Stress Protocols

When trying to study a drought response, scientists have to design a stress protocol suitable to follow that specific response or trait. Gilbert and Medina [61] previously discussed general experimental procedures to study different categories of responses. Instead of repeating their excellent work, we will describe which stress application methods are commonly used in both, or either plant species, discussing advantages, pitfalls and suitability for cross-species phenotyping. These protocols are often the result of a compromise between field and experimental conditions and range from very artificial *in vitro* setups, commonly used for molecular studies because of the absence of contamination and ease of standardization, to open-field trials suitable for applied agricultural research (summarized in Table 1). As a general rule, the more a protocol is close to field conditions, the less its results are predictable and reproducible. When precise kinetics are to be followed (e.g., ABA accumulation in tissues, metabolite or protein accumulation, gene expression), artificial setups under very controlled conditions are more convenient.

Table 1. *Drought stress protocols commonly used in Arabidopsis and/or tomato. The table discriminates protocols based on the stress application method; for each protocol, growth substrates, advantages and disadvantages and phenotyping suitability is listed. When possible, an example for both plants is given.*

Stress Application Method	Growth Substrate	Advantages (+)/Disadvantages (−)	Phenotyping Suitability	Arabidopsis	Tomato
Water withholding	Soil (open or protected field)	(+) realistic drought conditions (+) best method for market-oriented phenotyping (−) other stresses such as salinity and heat can co-occur (−) not used/useful for Arabidopsis (−) strongly affected by weather conditions	All traits can be phenotyped, but root phenotyping can be unfeasible	NA	Landi et al., 2017 [63]
	Soil (pot)	(+) quite close to commercial conditions (+) suitable for every growth stage (−) influenced by environmental conditions (−) can be laborious (−) stress can be slow to occur	All phenotyping methods here described can be used, but root phenotyping needs appropriate apparatus (e.g., rhizotrons, x-ray tomography)	Vello et al., 2015 [64]	Visentin et al., 2016 [65] Halperin et al., 2017 [66] Galdon-Armero et al., 2018 [67]
	Soil (pellet)	(+) as for pot protocols, but the limited size of pellets speeds up drought stress occurrence (−) not used for tomato	All phenotyping methods described here can be used	Vello et al., 2015 [64]	NA
	Inert substrate e.g., sand, vermiculite (pot)	(+) stress is reached faster than in soil-based protocols (+) easier to uproot plants (−) nutrient stress occurs	All phenotyping techniques described here can be carried out	Santaniello et al., 2017 [68]	Takayama et al., 2011 [69]

		together with water withholding, as plants are fertigated (−) more artificial than soil-based protocols			
Transfer to stressing substrate	Agar with low osmotic potential	(+) very reproducible (+) a wide range of stress intensities can be achieved (+) fast (+) sterile (−) far from naturally occurring conditions (−) depending on osmolyte nature, off-target effects can be a concern (−) suitable only for small/young plants (−) stomata dynamics hard to assess in very young plants	Phenotyping, especially for tomato, is limited to the first stages of plant growth (seedling stage). Very convenient for early screenings	Frolov et al., 2017 [70]	Aazami et al., 2010 [71]
	Hydroponics-Osmotic stress	(+) very reproducible (+) fast (+) a wide range of stress intensities can be achieved by gradually increasing osmolyte concentration (−) artificial (−) depending on solute nature, off-target effects can be a concern (−) root growth is altered (−) need for a hydroponic apparatus	All phenotyping techniques described here can be carried out. Very suitable for the description of precise kinetics. Absence of soil makes root phenotyping not always feasible	Nieves-Cordones et al., 2012 [72]	Ali et al., 2018 [73] Amitai-Ziegerson et al., 1995 [74]
	Inert substrates-	(+) reproducible (+) fast	All phenotyping techniques described here can be carried	NA	Jin et al., 2000 [75]

	Osmotic stress	(+) a wide range of stress intensities can be achieved by gradually increasing osmolyte concentration (+) cost-effective (−) artificial (−) depending on solute nature, off-target effects can be a concern	out. Very good if precise kinetics are analyzed		
Transfer to dry substrate	Inert substrate	(+) very fast (+) reproducible (−) very artificial (−) severe stress only (−) only early responses can be analyzed	Due to very fast stress, only early responses can be studied. Root phenotyping is not convenient	NA	Visentin et al., 2020 [76]
Uproot and let dehydrate	Inert substrate to no substrate	(+) very fast (+) reproducible (−) very artificial (−) severe stress only (−) only early responses can be analyzed	Due to very fast stress, only early responses can be studied. Root phenotyping is not convenient	Virlouvet et al., 2014 [77]	NA

Soil-based protocols, ranging from pot-grown plants in growth chambers or greenhouses [66] to field studies [63], are the most used when phenotyping drought stress in tomato. Their similarity to real conditions makes them perfect for applied research. Similarly, Arabidopsis is often grown in soil in small pots or pellets [78], while usually there is no point in studying it in the field. Drought occurs from water withdrawal in test plants, while controls are watered regularly to prevent stress responses. In general, the most obvious procedure to monitor and control stress levels is to weigh pots daily and to add different water volumes to each pot, in order to reach the same soil water content for all replicates [79]. Nonetheless, with a large experimental population such apparently trivial steps can become very time- and labor-consuming, unless a complex (and costly) automated irrigation system is available. As a consequence, do-it-yourself devices based on open source technologies, such as Arduino chip-sets and/or single-board computers, are gaining interest thanks to their high versatility and cost effectiveness [80,81].

Almost all phenotyping methods discussed in this review can fit in soil-based protocols, but sometimes soil is not the recommended substrate. For example, soil dehydration is achieved through water evaporation and plant transpiration, two factors only partially controlled by the operators. Soil dehydration rates can be different among genetically identical biological replicates under identical environmental conditions, thus, reproducibility and predictability of these experiments are not always guaranteed [82]. The fact that synchronizing stress among individuals can be tricky adds complexity to this picture, especially when comparing mutants featuring differences in biomass, leaf area and/or stomatal density/width. A common, elegant solution used to minimize the latter problem is to grow mutants and wild type *Arabidopsis* plants in the same wide pot, to expose different genotypes to the same environment, better synchronizing stress appearance across individuals [83]. However, this approach may fail in comparing individuals with very different developmental features (e.g., very different root length/structure, growth rate or exudates production) and is possible only on small plants. For bigger plants phenotyping, an easy and cheap method was adopted by Marchin and colleagues [12] through a very simple hydraulic setup. The authors were able to equalise soil moisture among individuals of different species. Another concern relates to stress duration, and depending on environmental conditions, it may be controlled, only in part. Soil drying rates can be either too fast or too slow to phenotype a specific trait optimally. For example, a stress occurring too quickly can be an issue when studying late responses, such as the accumulation of osmolytes or cell wall hardening [84], or when very detailed time-courses of stress responses are to be compared between genotypes with subtle phenotypic differences. A solution can be too air-tight and cover the soil surface to lower evaporation rates. By contrast, a stress too slow

to occur be concerning when very fast stress is needed to highlight differences in genotype performances, or (for example) when repeated stress is under study. In these cases, fast stress can be achieved by limiting the size of pots. In Arabidopsis studies, the use of peat pellets allows to achieve faster soil dehydration than in soil-filled pots, with very comparable results [64,78,85]. Surely, this is not always possible in plants, such as tomato. In this case, inert materials, such as perlite, vermiculite or rockwool are worth considering as growth substrates. These protocols are based on hydroponic-like systems where plants are grown in an inert substrate and a nutrient solution is supplied periodically [68,76]. Stress can be imposed by water withdrawal faster than soil based protocols and, if a very fast stress is needed, plants can be easily uprooted and dehydrated in air or transferred to a dry substrate [75,76]. However, care should be taken when designing fast, severe stress quickly followed by re-watering, since late responses may not have the time to be activated. Moreover, these artificial substrates lack nutrients and, consequently, nutrient stress could occur coupled with dehydration.

Sometimes, the need for a fast, precise and reproducible stress pushes researchers away from field-like conditions. While sacrificing stress authenticity, an induced physiological drought represents a good proxy of drought stress effects and allows fast and easy screening procedures; of course, it must be noted that osmotic stress slightly differs from drought stress both, at the molecular and physiological level, so care should be taken when interpreting results. Osmotic stress can be obtained supplementing growth media with osmolytes causing a decrease in the water potential of the substrate, to the point that water absorption by the plant is impaired [73,82,84,86]. While, in the past a wide range of solutes has been used, it turned out that most of them are able to penetrate plant cells resulting in a range of off-target effects dependent on the solute nature [87,88]. Therefore, the use of high molecular weight, bio-inactive compounds, such as PEG-8000 is now the standard for these experiments. Stress can be imposed to a severe degree immediately, or by gradually increasing the supplemented osmolytes and better mimicking, this way, real-world drought occurrence [82].

Systems based on PEG-infused agar are very interesting for Arabidopsis drought stress screenings; practically, plants can be germinated directly in PEG-infused agar or transferred at a later stage. The main reason to adopt such methods relies on their simplicity. With few manipulations, it is possible to achieve a wide range of water potentials avoiding most of the problems related to the lack of full control on environmental conditions or soil drying rates [86]. However, the same simplicity sets these models far apart from field experiments and, while it is possible, though uncommon, to adapt protocols to every stage of *Arabidopsis* growth [70], the same cannot be said of bigger plants [84]. Indeed, this approach is rarely reported on tomato,

with very few examples [71]. In contrast, hydroponic systems can be easily applied to both *Arabidopsis* and tomato [72–74], but with potential pitfalls, for example, PEG solutions are highly viscous and can hamper aeration of the root apparatus [82]. If side effects are not a concern, other solutes, such as sorbitol or mannitol can be used. Alternatively, osmotic stress protocols can be applied to plants grown in inert substrates, obtaining a hydroponic-like system without the need for a complex apparatus [75].

When obtaining field-like conditions is not necessary, and a very fast, cost-effective and easy to handle stress is needed, dehydration can be achieved through air drying. Uprooted plants can quickly reach a severe level of stress (usually in 60–120 min), maintaining easiness of handling and independence from environmental conditions; if plants must recover from drought, it is sufficient to immerse roots in water or nutrient solution [89–91]. However, there are clear drawbacks: these protocols are far from field conditions and make many relevant physiological measurements difficult to carry out. Still, they can be very interesting if correctly used, as done by Fromm and colleagues when studying stomatal responses to recurring drought spells [77,92,93]. These experiments were translated to corn and rice using the same air-drying protocol [93,94], but never in tomato.

Drought Stress Phenotyping

Plant phenotyping is an incredibly broad and fast evolving research field in the plant sciences (for a recent systematic review on past development and upcoming trends in the research area, see [95]). Many excellent reviews address certain areas of plant phenotyping, ranging from the phenotyping of submicroscopic features in specific plant organs by electron microscopy, to whole plant or field of plants in agronomic contexts by UAVs (unmanned aerial vehicles) [96] and satellites. Phenotyping is often performed in specific phenotyping platforms that allow the analysis of multiple plant features at once [29] (e.g., hyperspectral reflectance, thermal signature and chlorophyll fluorescence). These platforms are particularly useful in drought stress phenotyping, as the plant environment can be precisely monitored and potentially manipulated [97]. The large costs involved in building and maintaining such platforms [98] is one limitation, along with the need for specialized personnel. To address the challenges in translational phenotyping, we present a selection of standard drought stress phenotyping approaches in Arabidopsis and tomato, summarized in Table 2, and highlight similarities and differences between those approaches when applied to either species. As there are no studies directly comparing the phenotypes of Arabidopsis and tomato lines, there is no literature available to directly compare threshold values for single traits/quantifiable variables. Some

parameters like plant height are inevitably different across species, but this does not necessarily apply to properties of the photosynthetic apparatus, or stomatal regulation. The absence of universal drought stress and phenotyping protocols, to date, still limits easy comparisons of obtained phenotypic results across species. Some examples for specific phenotyping techniques are given in the respective paragraphs.

Table 2. *An overview of common phenotyping targets in Arabidopsis and tomato under drought. Referenced publications contain detailed information on the methods applied.*

Physiological Reaction Monitored	Accessible Traits	Arabidopsis	Tomato
Leaf turgor drop	- Direct assessment (high-precision pressure probe) - Wilting (RGB-imaging) - Drop in projected leaf area - Lower specific leaf area - Relative water content	Direct assessment: Ache et al., 2021 [99] Wilting (RGB-imaging): Bouzid et al., 2019 [60] Projected leaf area: de Ollas et al., 2019 [85]	Direct assessment: Lee et al., 2012 [100] Plant architecture (Light Detection and Ranging—LiDAR): Rose et al., 2015 [101]
Osmolarity increase	- proline quantification - osmolarity quantification	Proline: Li et al., 2019 [102] Zhang et al., 2013 [103] Osmolarity: Frolov et al., 2017 [70] Verslues & Bray, 2004 [104]	Proline: Aghaie et al., 2018 [105] Osmolarity: Rodríguez-Ortega et al., 2019 [106]
Stomata closure	- Leaf temperature (by infrared thermography) - Direct stomata aperture measurements (by microscopy; destructive) - Stomatal conductance (by porometer)	Infrared thermography: Li et al., 2017 [83] Merlot et al., 2002 [107] Kuromori et al., 2011 [108] Microscopy: Virlouvet & Fromm, 2014 [93]	Infrared thermography: Leinonen & Jones, 2004 [109] Porometer: Visentin et al., 2020 [76] Caird et al., 2007 [110] Microscopy: Galdon-Armero et al., 2018 [67]
Lower carbon fixation	- Leaf gas exchange	Harb et al., 2010 [78]	Galdon-Armero et al., 2018 [67]
Enhanced chlorophyll fluorescence	- Hand-held devices to assess chlorophyll fluorescence - Fluorescence imaging (e.g., PAM imaging)	Hand-held device: Jung, 2004 [111] PAM imaging: Yao et al., 2018 [112]	Imaging system (within crop stand): Takayama et al., 2011 [69] Imaging system (FluorCamFC1000-H): Mishra et al., 2012 [113]
Higher concentrations of Reactive Oxygen Species (ROS) in the leaf	- Chemical staining and imaging: destructive or non destructive	Non-destructive chemical imaging: Fichman et al., 2019 [114] Destructive chemical imaging: Lee et al., 2012 [100]	Destructive chemical imaging: Ijaz et al., 2017 [115]

Higher concentrations of ROS-scavenging secondary metabolites (e.g., flavonoids, anthocyanins, carotenoids)	- Hand-held devices for accessing specific leaf compounds (e.g., Dualex, Multiplex, FieldSpec) - Hyperspectral - Full metabolic profiling (destructive) imaging	Hyperspectral imaging: Mishra et al., 2019 [116] Matsuda et al., 2012 [117] Metabolomics: Nakabayashi et al., 2014 [118]	Hyperspectral imaging: Susic et al., 2018 [119] Metabolomics: Ali et al., 2018 [73]
Changes in vegetative growth	- RGB-Imaging: lower projected leaf area, compact habitus - Lower fresh and dry mass - Lower specific leaf area - Slowed longitudinal growth of individual leaves - Senescence	RGB-Imaging: Ollas et al., 2019 [85] Senescence: Jin et al., 2018 [120]	LiDAR: Hosoi et al., 2011 [121] 3D point clouds: Paulus et al., 2014 [122] Trichomes: Galdon-Armero et al., 2018 [67]
Changes in root growth	- 2D features - 3D features	Xu et al., 2013 [123] Mathieu et al., 2015 [124]	Alaguero-Cordovilla et al., 2018 [125] Mairhofer et al., 2012 [126]
Changes in generative growth	- Earlier fruit set - Lower fruit weight - Higher number of non-marketable fruits - Lower overall yield	Seed mass and yield: Jofuku et al., 2005 [127]	Flowering and yield: Sivakumar et al., 2016 [128]
Molecular markers	*- 9-Cis-Epoxycarotenoid Dioxygenase* *- NCED* *- Responsive to dehydration 29 (RD29)* *- Homeobox protein 6 (HB6)* *- Dehydration-responsive Element-Binding protein 2 (DREB2)*	*AtNCED3* Hao et al., 2009 [129] Sussmilch et al., 2017 [130] *AtRD29B* Ma et al., 2019 [131] Virlouvet et al., 2014 [77] *HB 6* Ding et al., 2013 [132] Harb et al., 2010 [78] *AtDREB2A* Ma et al., 2019 [131] Harb et al., 2010 [78]	*SlNCED1, SlNCED2* Yu et al., 2019 [133] Munoz-Espinoza et al., 2015 [134] *SlRD29* Gao et al., 2020 [135] Iovieno et al., 2016 [136] NA *SlDREB2* Gao et al., 2020 [135] Hichri et al., 2016 [137]

Leaf Turgor Drop

Reduced leaf turgor pressure and subsequent wilting are among the first signs of drought stress, and therefore, assessed in numerous studies in both, Arabidopsis and tomato. In Arabidopsis, wilting is often not assessed as a quantitative but rather as a qualitative trait, and scientists categorize a plant as either wilted or not wilted based on visual assessment (e.g., [60]). In crops, Red Green Blue (RGB) cameras are often used to quantify projected leaf areas (reviewed e.g., in [138]), and the ratio of projected leaf area and actual leaf area can be used as an indicator of wilting. In tomato, a portable Light Detection and Ranging (LiDAR) system has been used to detect leaf angles, among other parameters [121]. Such a system, combined with powerful algorithms, can be a more useful tool than RGB images only, as more traits that are relevant for plant breeding (e.g., the dynamics of light harvesting as a function of plant architecture and daily growth rates) can be extracted from the generated point-clouds [122]. In theory, the same phenotypic methods could be used to analyze both Arabidopsis and tomato, as the systems are precise enough to detect changes in relatively small Arabidopsis leaves [139].

Whether the more detailed and more complicated phenotyping approach, described above, will replace the common practice of visual binary categorization of Arabidopsis in “wilted” and “non-wilted” plants is hard to tell.

Leaf turgor can also be used to monitor plant recovery from drought stress, since during this phase, leaf water potential rises to pre-stress levels; this parameter, measured with the Scholander pressure bomb, was successfully used to monitor stress in tomato plants [76]. In Arabidopsis studies, the Scholander pressure bomb is rarely, used mostly due to the small dimension of the leaves, and therefore, the destructive measure of leaf Relative Water Content (% RWC) is used instead. This procedure can also monitor recovery in Arabidopsis, since recovered leaves have similar % RWC levels compared to pre-stress values [93,132]. Another approach to address leaf turgor is via high-precision pressure probes [100]. These systems are capable of non-destructively monitoring leaf turgor, and thereby allow insights in its temporal development under drought and during recovery. The system was, e.g., used in Arabidopsis, to study leaf turgor responses to several abiotic stressors, in wild-type and different mutants [99], and can replace destructive methods involving the Scholander pressure bomb.

Osmolarity

A key plant strategy to avoid physiological drought is to increase osmolarity within cells, leading to a more negative water potential, and therefore, an influx of water from the surrounding

substrate into the plant. A standard method of destructive phenotyping is to measure the overall osmolarity of cell sap with osmometers, as done in Arabidopsis [70,104] and tomato [106].

Among the several classes of osmolytes (i.e., osmoprotective compounds, including sugars and amino acids), proline is the metabolite that is most commonly quantified in drought stress studies [102,105,140,141]. A recent study in tomato has suggested that the ratio of proline content in stressed and non-stressed plants can serve as an indicator for drought stress tolerance in a given genotype, with a high ratio (e.g., 1.86-fold increase in stress compared to the control) associated with the most tolerant [105]. An earlier study suggested the opposite [141], a cultivar labelled as drought stress tolerant showed no differences in leaf proline content between “stressed” and non-stressed plants. However, the reported leaf relative water content of this cultivar did not differ between treatments, suggesting that no physiological drought stress had occurred after all for otherwise undefined reasons. In *Arabidopsis*, a study highlights that proline plays a key role in the ROS scavenging system of the plant, and at the same time, acts as an osmolyte [142].

Polyamines also play a protective role against drought stress consequences, as shown in several studies in Arabidopsis [143,144] and tomato [145,146], at least partially by reducing ROS in the plant tissues.

The published methods to quantify leaf proline and polyamine contents are similar for *Arabidopsis* and tomato, and in theory, the same (destructive) protocols could be used. If similar drought stress protocols are applied, it may be feasible to transfer knowledge on drought resistance from Arabidopsis to tomato, based on osmolyte accumulation patterns as a readout.

Water Loss at the Leaf Level

Both direct and indirect analyses of stomatal dynamics can be conducted in *Arabidopsis* and tomato in similar ways. For the rather direct analysis via (microscopic) images of the leaves, a fixation of the tissue is performed, which can be done by creating a die with nail polish [147] or by fixating leaves using the chemical glutaraldehyde [93]. Stomata can subsequently be counted and measured under an optical or confocal microscope. For more sophisticated analyses, variable pressure scanning electron microscopes are used to address stomata features [67]. Using this method, a fixation of leaf material is not necessary and damage through fixation can be avoided. Recent advancements in automated image analysis will probably pave the way to an automated analysis of relevant stomatal features like density, length, width and guard cell size from microscopic images [148].

The analysis of trichomes in drought studies is common, as these specialized epidermal cells manipulate the microclimate of the thin air layer surrounding the leaf, and can thereby, prevent unproductive water losses. Enhanced trichome density in drought tolerant genotypes is found in tomato [67] and Arabidopsis [149], and can be assessed via light microscopy or scanning electron microscopy.

A common, non-invasive, although indirect, method in addressing transpiration is thermal imaging. This technique has been used to identify *Arabidopsis* mutants defective in stomatal regulation already in 2002 [107]. The combination of thermal and visible images was later used to remotely access drought stress in crops under greenhouse and field conditions. Sunlit and shaded leaves were separated using RGB-image data and the corrected thermal information correlated fairly well with measured stomatal conductance [109].

Stomatal conductance—and thereby transpiration through stomata—can also be assessed using a Porometer, as previously described in *Arabidopsis* [108] and tomato [76,110]. Devices measuring carbon assimilation can also provide information on leaf transpiration, with more precision than the latter instrument but with longer measurement times.

Whole-plant transpiration dynamics are observed with gravimetric systems. In short, potted plants are placed on wages and the growth substrate is covered by water-impermeable materials to avoid evaporation. This also allows for a calculation of water use efficiency (WUE) in its agronomic sense as either biomass or yield produced per unit of transpired water. Efforts are being made to combine 3D imaging systems (capable of estimating biomass) with gravimetric transpiration control, allowing dynamic phenotyping over time [150]. A commercially available gravimetric system has been used in tomato already, addressing drought stress tolerance of an introgression population [66].

Stomatal water loss is also used to analyze recovery when a plant is re-watered after stress, stomata start reopening and gas exchange rates reach values very close to pre-stress ones. However, it is important to note that stomatal conductance does not fully recover immediately after stress, as it does not depend only on hydraulic signals. Therefore, even when leaf water potential or % RWC are back to the levels of irrigated plants, stomatal conductance will lag behind (hysteresis of stomata closure). This phenomenon, often called “after effect” of drought, is well documented both in Arabidopsis and tomato [76,93,151] and it is by all means a reflection of drought stress memory at the stomatal level [151].

Gas Exchange

Gas exchange and carbon assimilation measurements are straightforward ways to assess the photosynthetic efficiency of a plant in a given environment. A drop in gas exchange can be a sign of a range of different plant stresses, including drought. In *Arabidopsis*, LI-COR gas exchange systems were used in several studies to assess leaf gas exchange under drought [152,153]. In tomato, carbon assimilation under drought stress is studied across different scales and levels of environmental control, from chambers with artificially elevated CO_2 [136] to greenhouse and field [63,64]. As carbon assimilation is highly influenced by irradiation and temperature, studies in greenhouses and in the field should be conducted in reproducible weather conditions, ideally during sunny days and virtually at the same time. For studies in the field, hand-held devices are the most practical choice. Good care has to be taken when comparing leaf gas exchange values across studies: a study on tomato [64] reports 0.15–0.25 µmol H_2O m^{-2} s^{-1}, with slight differences between control and drought, while a study on *Arabidopsis* [131] reports a more than four-fold increase during drought stress, but still lower absolute values of stomatal conductance than any tested tomato (0.02–0.09 µmol H_2O m^{-2} s^{-1}). As drought stress protocols, instrument settings (e.g., photon flux density) and growth systems are inconsistent across studies, the comparison of absolute carbon assimilation rates across studies (and species) is inappropriate.

Carbon fluxes inside the plant can be studied in even more detail by using $^{13}CO_2$ and mass spectrometry [135].

Enhanced Chlorophyll Fluorescence

As drought stress impairs photosynthetic activity and enhanced chlorophyll fluorescence is a direct result of this impairment [154], the quantification of chlorophyll fluorescence is a standard procedure in stress phenotyping both in *Arabidopsis* and horticultural crops [154,155]. In general, a plant that maintains high photochemical quenching, and therefore relatively low non-photochemical quenching and associated variable chlorophyll fluorescence under stress conditions, is described as tolerant against this stressor. In tomato, imaging systems are mainly used in molecular studies on plants in early growth stages and in artificial environments like growth chambers (e.g., [113]), while at later growth stages, and/or in less artificial environments like greenhouses, leaf clip-based systems are more commonly used (e.g., [156]). However, it is possible to apply fluorescence imaging in commercial-like greenhouses [69]. Many chlorophyll fluorescence measurement systems require a dark adaptation of measured leaves; a prerequisite that may be hard to fulfil, depending on the growth system.

ROS and Leaf Secondary Metabolite Contents

The formation of ROS is a hallmark of cellular stress also upon drought; it can be observed in vivo, based on the oxidation of fluorescence probes like H2DCFDA, as shown in *Arabidopsis* [114]. In the presence of ROS, this chemical starts to emit fluorescence signals that can be observed with hyperspectral cameras. While destructive assessment of ROS is carried out in tomato (e.g., [115,157]), the recently introduced method of non-destructive, whole-plant ROS imaging is to our knowledge not yet applied in tomato, despite the potential for knowledge transfer on ROS production and scavenging mechanisms.

A common measure to address persistent stress is the quantification of secondary metabolites (SM) with the capability to reflect or absorb excessive amounts of sunlight, thus, mitigating the risk of excessive ROS production, and also to scavenge ROS directly [158,159]. SMs such as flavonoids or anthocyanins can be quantified destructively, as done in *Arabidopsis* [160] and tomato [161]. Identification and quantification of SMs can be achieved photometrically (e.g., [162]), via High Performance Liquid Chromatography (HPLC) (e.g., [163]) or via Gas Chromatography-Mass spectrometry (GC-MS) (e.g., [152]). The latter allows a more precise analysis of chemical subgroups of metabolites, potentially offering detailed insights in their metabolism ("metabolomics"). When the researcher is interested in the spatial or temporal development of SM contents, the use of either imaging [117,119] or non-imaging [146,147] remote sensors should be considered to avoid destructive measurements. Several non-imaging sensors rely on leaf clipping, and therefore, require a minimum leaf size, which can be a limiting factor especially in *Arabidopsis*. For reviews on available devices, see [139,148]. Many hyperspectral imaging systems can be used not only under lab conditions, but are also extensively used in the field, as they are, either hand-held [149] or can be mounted on UAVs for rapid phenotyping of large numbers of plants [96]. Factors like leaf age and morphology may have a large impact on SMs estimation based on non-destructive methods [150], and therefore must be taken into account.

Root Structure

Roots can either be phenotyped two-dimensionally, by using a normal camera and plants grown either hydroponically or in agar (e.g., [123,124]); or three-dimensionally for plants grown in systems closer to actual crop production systems (e.g., [126]). While the former are quick, easy and cheap, the latter allows more sophisticated analyses of complex traits like three-dimensional (3D) root system architecture (RSA).

RSA phenotyping allows dynamic interactions between roots and their surrounding substrate to

be understood by evaluating, e.g., fine root diameters, specific root length, root angles and root length density (reviewed by [164]). Understanding genotypic differences in RSA responses to abiotic stressors, like drought has the potential to improve the breeding of resilient cultivars [20,165]. In order to analyze dynamic rhizosphere interactions and spatial alterations, recommended detection methods do not interfere with the 'natural' habitat of roots [166]. Particular approaches mostly refer to plants grown artificially in hydroponics, paper pouches, gel and in appropriate soil types, *inter alia* in soil-filled rhizotrons (up to a volume of ~18 L), which limits phenotyping to young or small plants [165]. Growth media limitations do also apply for 3D methods, like magnetic resonance imaging [20] and X-ray [167], visualizing the 'natural' growth and architecture, as well as the impacts of biotic and abiotic stresses. In order to bridge the gap between phenotype and genotype, recent studies revealed insight into intertwined genetic factors of root and shoot development, in both, *Arabidopsis* and *Solanum* [125,168]. However, plants are often analyzed during their early growth and transferability to mature plants may be limited [169].

Changes in Vegetative Growth

Leaf area densities and related source-sink relationships are known to be important for final yield in horticultural crops [162] and grains. These traits are therefore studied extensively in crops, but the *Arabidopsis* model is due to its compact habitus unsuitable for translation of most information in this respect. The differences in growth habitus between *Arabidopsis* and tomato indeed complicate a homogenization of phenotyping methods regarding vegetative growth. While the rosette-like structure of *Arabidopsis* allows relatively straightforward analyses, the three-dimensional structure of tomato is more difficult to parameterize. For tomato indeed, not only leaf area index (LAI), but also leaf area density (LAD) in several horizontal layers within a high-wire-system tomato canopy have been analyzed with the LiDAR-based system described above [97]. In *Arabidopsis* instead, 3D plant architecture analyses are not common, as its rosette-like structure is rather plain. So, the additional information on the third dimension does not seem to justify the effort needed to capture it, and stress effects can be detected as projected leaf area observed non-destructively via RGB cameras located above the plants [29].

Changes in Generative Growth

Early fruit set is also part of the drought escape strategy and therefore a symptom of drought stress both in *Arabidopsis* [170] and tomato [136]. Many genes that apparently control yield in tomato, especially through the regulation of auxin contents, are homologs of genes found in Arabidopsis [53]. However, there are major differences in generative growth of the two model

plants. Tomato is a plant insensitive to day length, e.g., the fruit set is not influenced by season [171], whereas *Arabidopsis* flowers earlier under long-day conditions [172]. Thus, researchers interested in drought-induced early flowering in *Arabidopsis* and tomato have to take day length (in-) sensitivity of the respective plant into account, either through appropriate experimental design and/or through statistical models.

Fruit yield is a highly integrative phenotypic trait, and genetically controlled by at least 28 QTLs in tomato [173]. Operationally, the temporal development of generative growth can be assessed quite easily, as flowers and fruit setting are directly visible in both *Arabidopsis* [174] and tomato. Direct yield quantification in tomato is common, although quite labor intensive, as fruits must be harvested once a week over a period of several weeks to obtain robust results. Also, to obtain meaningful results, plants must be grown in commercial-like systems, an often challenging task for molecular biology groups.

Another important difference in reproductive physiology of *Arabidopsis* and tomato that has to be considered is that the short life cycle in the former ends with fruit production, whereas constant fruit production over months and theoretically over years is possible with indeterminate tomato varieties.

Observing Stress through Marker Genes

After sensing drought, plants start activating a complex network of gene-expression changes affecting plant behavior. While some of these may vary among plant species, others are pretty well-conserved, thus, representing a signature of drought stress. Transcripts of such marker genes are often quantified in physiological studies and can be used to monitor stress response intensities.

Describing the specific intricacies of molecular responses during drought stress, a complex and still partially elusive network, is far from the purposes of this review; among the impressive body of literature on the topic, the reader is referred to two up to date and influential reviews [175,176]. Here, we will quickly suggest some useful stress marker genes that are shared (or not) between the two species.

Some of the most prominent molecular responses to drought stress are governed by the stress hormone ABA (abscisic acid). Firstly, ABA biosynthesis is augmented during stress through the transcriptional induction of the genes encoding its biosynthetic enzymes. Among these, the NCED (9-Cis-Epoxycarotenoid Deoxygenase) genes, which catalyze one of the last steps of ABA biosynthesis, can be used to monitor plant sensing of drought stress in tomato and

Arabidopsis. AtNCED3 is expressed quickly during drought stress [129] as soon as Arabidopsis leaves lose turgor [130]. In tomato, the two genes SlNCED1 and SlNCED2 seem to play similar roles [133,134]. ABA-responsive genes can be used as stress markers, too: the transcript of the dehydrin-encoding gene AtRD29B (Responsive to Dehydration 29 B) is typically profiled in drought stress experiments [77,131] and possesses a similarly behaving orthologue in tomato: SlRD29 [136].

Another commonly used drought stress marker gene in Arabidopsis is Homeobox Protein 6 (HB6), an ABA-activated gene in drought stress that encodes a transcription factor governing several stress responses [78,132]; however, no obvious tomato homologue has been characterized until now. Similarly, the tomato ABA-dependent, dehydrin-encoding Solyc02g084850 is a good drought marker (our unpublished data) still not characterized in *Arabidopsis*.

In some cases, such as the study of genotypes with disturbed ABA sensing/biosynthesis, the use of ABA-dependent stress markers may not be appropriate. In this case, ABA-independent, drought-activated genes can be used instead; one of these is DREB2 (Dehydration-responsive Element-Binding protein 2). Both AtDREB2A and SlDREB2 expression is induced in either plant species by drought stress [78,131,135,137], and they encode for ABA-independent transcription factors, involved in drought stress responses; signaling genes downstream of DREB2 are, consequently, good putative stress markers as well.

Conclusions

Nowadays, more than 200 angiosperm species have been sequenced, and this number is predicted to increase rapidly [177]. Together with the levels reached by our understanding of genetics, this is raising consistently the possibility of developing new marketable crop genotypes suitable for future agricultural challenges. However, until these new genotypes are characterized, they remain just a possibility: the need for precise phenotyping is stronger than ever. In spite of the difficulties outlined in the introduction, some efforts in adjusting drought stress and phenotyping protocols across species have already been made, and technological advances in plant phenotyping offer further potential for translational phenotyping. Therefore, we hope that future research efforts will account for the need of comparable phenotyping in *Arabidopsis* and crops.

As technology evolves, phenotyping facilities addressing multiple traits simultaneously are becoming the new standard in plant phenotyping [155,178]. The combination of several of the techniques mentioned above allows integrated phenotyping to a detail level never matched

before, and that could never be reached by single-sensor approaches. As the often mentioned phenotyping bottleneck [27] is gradually being overcome, the scientific focus will have to shift towards developing universal phenotyping approaches which integrate results of phenotypic observations across scales, environments, and even across species. In this sense, the advent of phenomics [179] coupled with the newest bioinformatic approaches such as machine learning [180] will probably play a major role in this transition. Still, more traditional phenotyping approaches will always be necessary to some extent.

The knowledge gathered on the *Arabidopsis* model is more valuable than ever, especially if the scientific community manages to translate it to crop models from which we can obtain a real advantage, including in food, fodder or fiber. We are convinced that knowledge can be better translated between species in relation to mechanisms involved in tolerance against abiotic stresses like drought, as well as on many other plant traits, such as fruit development, light response, or resistance against pests and diseases. At present, the transferability of knowledge is still limited, as stress protocols, as well as phenotyping protocols (if at all existent) are often incoherent among different species. Researchers interested in translating the vast knowledge gained on Arabidopsis to crops and vice versa must carefully design their studies and ideally build interdisciplinary teams to gather knowledge on genetic background, expected and desired phenotypes and on the agricultural production systems the crops are grown in.

While the idea of modeling the performance of plants with virtual allele combinations under a range of environments is not new [181], it seems that its potential has still not been realized, to date. Some of the existing molecular and physiological plant models of water status and drought stress in tomato (e.g., [182,183]) and Arabidopsis (e.g., [184]) may be connected to improve our understanding of drought and plant responses to it. Moreover, new modeling approaches, including the causal inference approaches by Pearl and colleagues, which provide mathematical tools to describe causal relations, rather than correlation, and explicitly include the scientist's causal knowledge in the design of a statistical model. These methods, until now widely overlooked in the plant sciences, have the potential to allow insights in systems hardly comparable by classic statistical approaches [185], and may thereby help to lift translational phenotyping to the next level.

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Second challenge

Because of their ease of use, and because leaf chlorophyll content is an important parameter for estimating the physiological status of crops, leaf chlorophyll meters are a class of sensors commonly used for phenotyping. However, the leaf chlorophyll contents indicated by the sensors vary not only with different chlorophyll contents of the measured leaves, but also depending on a number of other factors, such as leaf age, leaf thickness, and the species under consideration. The second chapter of this thesis "Factors Influencing Chlorophyll Meter Readings – Toward a Conceptual Framework" deals with the influence of these and other confounders on measurements of different Leaf Chlorophyll Meters. In this context, the modes of operation of the different sensors are discussed in detail. Furthermore, a way to quantify the influence of confounders is described, since the strength of influence determines whether the confounder is relevant enough to justify the effort to capture it. Finally, a conceptual model of chlorophyll measurements with Leaf Chlorophyll Meter under eplicit inclusion of various confounders is proposed. This model can help to obtain more realistic estimates of chlorophyll content of plant leaves in the future. Some of the lessons learned in this context can be applied to a broader range of problems in plant phenotyping, since phenotyping as a whole often takes place in confusing cause-and-effect contexts, and building conceptual models can be an important step in identifying the relationships that are truly involved. This chapter of the dissertation is submitted to Precision Agriculture as a research manuscript.

3. Factors Influencing Chlorophyll Meter Readings - Toward a Conceptual Framework[2]

Authors

Jan Ellenberger, Katja Schiffers, Luca Marie Erbe, Tanja Groher and Simone Röhlen-Schmittgen

Abstract

Portable chlorophyll meters are commonly used for fast and non-invasive assessment of leaf chlorophyll content. Although several physiological and environmental factors are known to affect chlorophyll meter readings, the vast majority of studies ignore this bias and treat the values as accurate representations of leaves' chlorophyll content. In this study, we designed an experiment to quantify the impact of drought, nutrient deficiency, leaf age and leaf thickness, of two plant species (maize and tomato) on the chlorophyll predictions obtained with four commonly used portable chlorophyll meters. Results indicate that leaf age, leaf thickness and drought have major impacts on readings of most chlorophyll meters. Major differences were observed in the predictive capability of the devices. Based on our findings and previously published studies, we formulate a comprehensive conceptual model of the relationship between portable chlorophyll meter readings and leaf chlorophyll content, including physiological and environmental factors. We recommend this model to be further developed and to replace the extremely simplistic prevailing perception, which implicitly assumes that chlorophyll meter readings depend solely on the chlorophyll content of measured leaves and ignores all confounders.

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Introduction

Leaf Chlorophyll Meters (LCMs) are widely used non-invasive tools for the *in vivo* assessment of leaf chlorophyll content. Since the introduction of SPAD-502 (Konica-Minolta, Tokyo, Japan) [186], a wide range of LCMs entered the market in the subsequent decades. While measurement principles of the devices differ, their common aim is to give a quick and accurate insight in the leaf chlorophyll content, to evaluate plant performance without harming the leaf at the point of measurement. Several areas of application for LCMs can be defined: Plant growers can use the devices to identify the best time and amount for fertilizer applications in a wide range of crops [187,188], for optimizing crop yield [189]. Also, plant breeders can use the devices to identify lines tolerant to certain biotic or abiotic stressors, as changing leaf chlorophyll contents are an early indicator of plant stress [190]. Such a systematic selection process can prevent major financial losses and the loss of potentially promising genetic material, when based on reliable estimates. Hence, important decisions with major economic and ecological consequences are informed by LCM measurements. Although few decision makers in the situations described above will make their decision solely based on LCM measurements, inaccuracies in LCM readings will lower the quality of the decisions.

Quality of chlorophyll meter data

A raising number of studies, however, identified deviations in chlorophyll measurements of the very same leaves among different devices (e.g. [191]), and there is growing evidence that the relationship between LCM readings and leaf chlorophyll content is in fact very complex: A wide range of factors have impacts on LCM measurements, and indications by different devices are impacted to different degrees. Plant-related factors that are known or suspected confounders for LCM projections include leaf age [163], leaf thickness [192], heterogeneous chlorophyll distribution within the leaf [191], as well as physiological alterations due to e.g. plant malnutrition [193] and drought [194]. Already more than 30 years ago, Campbell and colleagues described differences in the relationship between SPAD-502 values and leaf chlorophyll content for apple trees grown in the greenhouse and on the field, potentially related to physiological alterations resulting from differences in radiation and the micro-climate throughout the plants life (e.g. radiation, wind, temperature) [195]. The effect of different radiation regimes on SPAD-502 measurement was later analyzed in detail, revealing major differences of LCM readings as a result of radiation-depended chloroplast movement, rather than actual changes in leaf chlorophyll content [196]. It was shown that this leads to relatively lower LCM readings at noon as compared to measurements around dusk or dawn [197]. An entire research branch now deals with leaf chlorophyll fluorescence alterations caused by plant pathogens (e.g. [198]). As

some LCMs use chlorophyll fluorescence emission at different wavelengths as a proxy for leaf chlorophyll content, biological stressors can be seen as another potential cause of anomalies in LCM measurements. However, only few of the aforementioned studies actually quantified the impact of the potential confounders on the LCM reading.

We suggest a classification of the impacts of disturbing factors in two categories: Firstly confounders can change (mostly increase) the heterogeneity of phenotypic data (e.g. the effect of heterogeneous chlorophyll distribution in plant leaves on the assessment of leaf chlorophyll content with chlorophyll meters). This leads to less precise, but not necessarily less accurate data. Secondly confounders can have a directed effect on phenotypic data, resulting in inaccurate data. Especially if confounding effects on the phenotypic measurement are directed (e.g. chlorophyll measurements are biased towards higher leaf content), not accessing those factors and mathematically correcting for the bias they induce, will produce systematically wrong phenotypic data. Relying on such wrong data may cause unfavorable decisions in plant management (e.g. application of inadequate fertilizer quantities) and plant breeding (e.g. accidentally excluding a promising line from a breeding program).

As some LCMs are based on entirely different principles of measurement (e.g. transmitted vs. reflected radiation) and some differ only slightly (e.g. minimal divergence in observed wavelengths), it is plausible that potential confounding effects affect the devices to different degrees. Moreover, some factors that influence measurements might not be independent from each other, but highly correlated, such as leaf age and leaf thickness, but also drought stress and leaf thickness, making it difficult to disentangle their effects on LCM measurements.

In this study, we address this difficulty with a boosted-regression tree approach that allows us to reflect and quantify the intricate relationships between chlorophyll content, potential confounders and the readings of different LCMs. Only if the effect caused by individual confounders is known, scientists may decide whether or not collecting information on that confounder and mathematically correcting for the error in future studies is worth the effort.

We designed an experiment to test the effect of drought, nutrient deficiency, leaf thickness, leaf age, on leaf chlorophyll measurements, carried out with four different LCMs on maize and tomato leaves. Results of this experiment and the literature described above were used to formulate a conceptual framework of the complex interactions between leaf chlorophyll content, leaf characteristics, the plants environment and chlorophyll meter measurements. This conceptual framework can be improved in future and used as a starting point for advanced structural equation models, gaining even more insights in the complex environment-plant-sensor-interactions.

Material and Methods

The following paragraphs provide a comprehensive overview over the functioning of the devices used in this study. Information on experimental setup and procedures follow subsequently.

Operating principles of leaf chlorophyll meters

Table 3 provides an overview of the four devices compared in this study. Positioning of sensor and light source relative to one another and the leaf is important, as sensors that are based on reflected light (sensor and light source on the same side of the leaf) are more sensitive to changes in chlorophyll content of the upper parts of the leaves (e.g. the palisade parenchyma in dicotyledons). The inclusion of fluorescence effects in leaf pigment analysis may increase the accuracy of chlorophyll content projections [199], but chlorophyll fluorescence is not only dependent on the leaf chlorophyll content, but also on the plant health status [200]. Plants exposed to different stressors use a lower proportion of irradiated light for photosynthesis and release a higher proportion of the irradiated energy as heat or chlorophyll fluorescence [201,202].

Table 3: *Sensor-specifications of four leaf chlorophyll meters.*

Device	Position of sensor relative to light source and leaf	Fluorescence-based chlorophyll assessment	Considered wavelengths [nm]	Distance to probe [mm]	Measured area [mm²]
SPAD-502	0	0	0	0	6
Dualex	0	0	0	0	20
Multiplex	0	0	0	0	314
FieldSpec	0	0	0	contact (Leaf clip)	314

SPAD

SPAD-502 (Konica Minolta, Japan) was one of the first chlorophyll meters commercially available and still is one of the most common devices today. Leaf chlorophyll content is approximated by comparing absorption (here defined as the difference between incident radiation at one side of the leaf and detected radiation at the opposite side) of electromagnetic radiation at one wavelength sensitive to chlorophyll content (650 nm) and one wavelength indifferent to chlorophyll content, but sensitive to leaf structure (940 nm). Transmitted radiation without a plant leaf attached between light source and sensor is used as calibration [203].

$$SPAD = \log\left[\frac{I\,940}{I_0(940)}\right] - \log\left[\frac{I\,650}{I_0(650)}\right] \quad (1)$$

with *I 940* and *I 650* as electromagnetic radiation of the respective wavelength transmitted through the plant leaf and I_0 *(940)* and I_0 *(650)* as detected radiation without an attached leaf.

Dualex

Dualex 4 (Force-A, France) can be described as a more advanced LCM as compared to SPAD-502, as its measurement capabilities are not limited to a chlorophyll indication. Additional measurement of ultraviolet absorption of the leaf allows for indications on the leaf flavonoid content and a nitrogen index is calculated based on assessed chlorophyll and flavonoid concentrations. With regard to chlorophyll measurements, however, Dualex is very similar to SPAD-502, the measurement principle is the same as described above. Main differences in chlorophyll measurement are the formula and the wavelengths used, as Dualex uses the red-edge region, which is very sensitive to changes in leaf chlorophyll contents: As the red-edge shift from high to low absorption at wavelengths above the absorbance maximum of chlorophyll at around 685 nm is pushed toward longer wavelengths for leaves with higher chlorophyll content, indices including the red-edge are long known to be accurate chlorophyll predictors in plant leaves [204].

$$Chl(Dualex) = \frac{I\,820 - I\,705}{I\,705} \quad (2)$$

with *I 820* and *I 705* as electromagnetic radiation of the respective wavelength transmitted through the plant leaf [205].

Multiplex

Multiplex (Force-A, France) is a device originally tested for the assessment of flavonols and anthocyanins in grapes [206], but was also used as leaf chlorophyll meter (see e.g. [207]). A

major difference to the other devices used in this study is the distance between sensor surface and leaf. While SPAD-502, Dualex and FieldSpec were used as contact-based devices, with a clip placed right at the leaf, Multiplex measurements are always conducted from a distance of 10 cm. Another difference is the usage of chlorophyll fluorescence rather than the absorption of radiation only to determine the leaf chlorophyll content. Chlorophyll fluorescence occurs both in the red and near-infrared (here: “far-red”) region. As chlorophyll re-absorbs red fluorescence but does not absorb near infrared fluorescence, the quotient of near-infrared fluorescence and red fluorescence is correlated with the leaf chlorophyll content. This quotient is called Simple Fluorescence Ratio under red excitation light (SFR_R):

$$SFR_R = \frac{FRF_R}{RF_R} \quad (3)$$

With FRF_R and RF_R representing the far-red and red fluorescence of the plant leaf respectively, when illuminated with red light. Although information on the exact wavelength of radiation used by the Multiplex is not publicly available, it is likely that wavelength around the chlorophyll absorption maximum at around 685 nm and maximum chlorophyll fluorescence at around 735 nm are used.

FieldSpec

The ASD FieldSpec 4 StandardRes (Palvern Analytical, UK) is a spectroradio-meter, capable to detect radiation in the visible, near infrared and short-wave infrared region of the electromagnetic spectrum (350 – 2500 nm). Equipped with a leaf clip, the device can be used to analyze the spectral pattern of plant leaves. Both light source and sensor are placed on the same side of the leaf (e.g. adaxial side in our experiment), resulting in the detection of the fraction of radiation reflected directly from the leaf surface, or reflected back after interaction with pigments and structures inside the leaf. Given the vast number of chlorophyll-related indices for hyperspectral data described in the literature [208], we selected the one with the closest correlation to chlorophyll contents detected in the lab. The ChlNDI Index we used in this study is calculated from relative reflectance from the leaf surface in the chlorophyll-sensitive red-edge region around 700 nm (here: 705 nm; same as for Dualex) and the chlorophyll insensitive near-infrared region (here: 750 nm). Radiation in the near-infrared region is used to normalize for the influence of leaf structure, but the used wavelength differs notably from the wavelengths used by SPAD-502 and Dualex. The Chl NDI is calculated as follows:

$$Chl\ NDI = \frac{R750 - R705}{R750 + R705} \quad (4)$$

With *R705* and *R750* representing the share of reflected radiation from the leaf at wavelength 705 and 750 nm respectively.

Experimental setup

In short, maize (*Zea mays*) and tomato (*Solanum lycopersicum*) plants were grown in a greenhouse under three fertigation regimes (full nutrition = control; drought; nutrient deficiency). LCMs were used to assess leaf chlorophyll contents non-invasively and a range of possible confounders was assessed alongside the LCM measurements. Chlorophyll in plant leaves was subsequently extracted with an organic solvent and quantified photometrically. We used boosted regression trees to disentangle and quantify the confounding effects on LCM measurements and correlation coefficients and root mean square errors of the boosted trees to compare the predictive performance of different LCMs under the given conditions.

Plant material and growth conditions

Seeds of tomato (*Solanum lycopersicum*, cv. 'Lyterno'; Rijk Zwaan, NL) and maize (Zea *mays*, cv. 'Mallory'; Limagrain Europe, GER) were sown into rockwool cubes (10×10×8 cm; Grodan delta, NL) in late August and early September respectively, and grown under controlled conditions in a greenhouse. Temperature and relative humidity were adjusted to 24.8 ± 0.9 °C and 41.0 ± 8.2 % during the day and 19.5 ± 0.5 °C and 61.2 ± 3.9 % during the night. A total of 60 plants (30 per species) was randomly selected and assigned to treatment groups (n = 10 plants per treatment: control, nutrient deficiency, drought) at the 3 leaf stage (tomato: 28 days after seeding, maize: 14 days after seeding). Rockwool cubes of control plant were kept at a relative humidity of 80 % throughout the experiment, to ensure optimal growth conditions. Drought stressed plant received half the amount of fertigation as compared to control plants. The fertigation was prepared according to a protocol for commercial-like tomato growth from two stock solutions (17.2 mM nitrogen, 5.4 mM calcium, 4.7 mM potassium, 0.4 mM phosphorous, 5.4 mM sulfur, 2.4 mM magnesium, 0.01 mM iron and micronutrients; electrical conductivity 2.5 mS cm^{-1}; pH 5.5). Nutrient deficit was induced be watering the plants with deionized water instead of nutrient solutions. All data presented are results from measurements conducted at 21 days after treatment induction, resulting in a plant age of 49 and 35 days for tomato and maize respectively.

Chlorophyll meter measurements

Chlorophyll meter measurements with all four LCMs (SPAD, Dualex, FieldSpec and Multiplex) were subsequently performed at the same leaf spot. For tomato leaves, the front leaflet of the respective leaf was used, while for maize leaves a region 10 cm distal from the main shoot was

marked and used for measurement. Due to differences in measurement area of the devices user, for SPAD and Dualex two measurements per leaf were taken and averaged, while for Multiplex and FieldSpec one measurement per leaf was conducted.

Confounder assessment

The thickness of plant leaves at the very point of chlorophyll-meter measurements was assessed with a digital thickness gauge (Mitutoyo 547-300S; Mitutoyo, Japan) right before chlorophyll-meter measurements. The device has an accuracy of 0.01 mm. Additional confounders like species, relative leaf age (derived from leaf number) and treatment were noted by the experimenters.

Laboratory analysis

Pigment extraction and quantification was slightly modified according to the method described in [209].

Leaf samples were harvested right after the above mentioned measurements, immediately cooled and later freeze-dried. Dried leaf samples were ground in ball mills and prepared for spectrophotometric analyses (Lambda 35 UV/VIS spectrophotometer, Perkin Elmer, USA). Additionally to 0.03 g leaf powder, each sample contained 0.1 g MgO to ensure complete extraction of pigments. Folch mixture (chloroform : methanol (2:1)) was added to the sample, followed by centriefuging (4000 rotations per minute, 10 minutes, 10 °C; Heraeus Multifuge X3 FR Centrifuge, Thermo Scientific, Germany). Supernatants were collected and 1.2 ml distilled water added prior to another round of centriefuging. The remaining pellet was put aside in order to dry until further processing. After centriefuging of the supernatants, the upper phase (Methanol-water phase) was separated from the lower chloroform phase, photometrically measured at 750 nm and 360 nm and put back to the centrifuge tube. The latter was left in the dark until measurements took place. Next, the water-methanol phase was acidified with two drops of HCl (37%) and measured photometrically at 750 nm and 530 nm. The chloroform phase was filled up with Folch mixture to a total of 50 ml for tomato samples and 25 ml for maize samples. The solutions were measured photometrically at 750 nm, 665.6 nm, 647.6 nm and 480 nm. After the pellet had been dried, 4 ml of a methanol-HCl solution (100:1) were added to the samples, which were centrifuged afterwards (4000 rotations per minute, 10 minutes, 10°C). Supernatants were collected and the last steps repeated, so the supernatants could be united and again centrifuged. The samples were measured at 750 nm and 360 nm, then acidified with two drops of HCl and measured at 750 nm and 530 nm. Photometric data was gathered with the UVWinlab software (Perkin Elmer, USA).

$$Chlorophyll\,a = \frac{A \times Vol\left(CHCl_3\right) \times 10^3}{M\left(Chl\,a\right) \times Wi} \quad (5)$$

$$A = 11.47 \times \left(I\,665.6 - I\,750\right) - 2 \times \left(I\,647.6 - I\,750\right) \quad (6)$$

$$Chlorophyll\,b = \frac{\left(B \times Vol\left(CHCl_3\right) \times 10^3\right)}{M\left(Chl\,b\right) \times Wi} \quad (7)$$

$$B = 21.85 \times \left(I\,647.6 - I\,750\right) - 4.53 \times \left(I\,665.6 - I\,750\right) \quad (8)$$

$$Chlorophyll = Chlorophyll\,a + Chlorphyll\,b \quad (9)$$

With *A* and *B* representing the *chlorophyll a* and *chlorophyll b* concentrations in µm/ml, *Vol(CHCl₃)* is the volume of the chloroform-phase in ml, *Wi* is the weighed-in portion of the sample in g and *M* is the molar mass of *chlorophyll a* and *b* respectively, defined as (a) 893.49 g/mol and (b) 907.47 g/mol. *I [number]* represents the absorption of electromagnetic radiation at the respective wavelengths in nm, as detected photometrically.

Data analysis

While the handling of SPAD, Dualex and Multiplex data is quite straightforward, FieldSpec data, like most hyperspectral data, has to be rehashed before it can be analyzed. A guide for ASD FieldSpec 4 data preparation is available online [210]. Data analysis was performed using the R programming language [211]. Figures were created with the R package ggplot2 [212].

Quantifying the impact of leaf chlorophyll content and a range of possible confounders on LCM readings requires the simultaneous analysis of categorical and metric data in one statistical model. For the analysis of the 120 data points (2 species * 2 leaf ages * 3 treatments * 10 biological repetitions), we chose to use the boosted regression tree (BRT) approach. BRTs are well suited, because predictor variables can be of any type (numeric, binary, categorical, etc.) [213] and trees are rather insensitive to outliers. Also, one main disadvantage of most tree-based models, the limited capacity to model smooth functions, is avoided in BRTs, through averaging of many tree-based models. To avoid over-fitting, we performed 5-fold cross validation by randomly splitting the data set in training and test data (90:30). Variable importance in the projection was calculated following the methods proposed by Friedman [214], implemented in the gbm-package [215] in R. Data is available online: https://github.com/JanEllenberger/Factors-Influencing-Chlorophyll-Meter-Readings/blob/main/data_to_publish.csv

Results

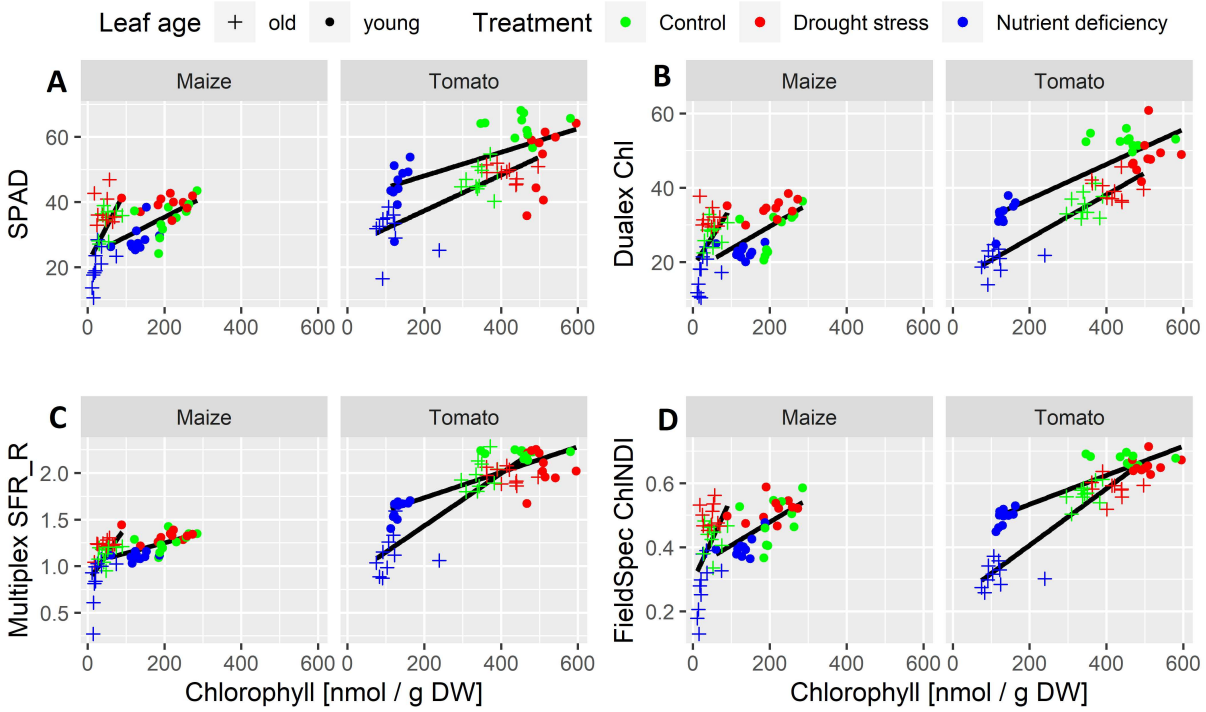

Figure 5: Correlations of maize and tomato leaf chlorophyll content obtained by wet-chemical photometric measurement and chlorophyll indices of 4 portable chlorophyll meters. Clusters depending on plant nutrition (colors) and leaf age (shapes) can be observed.

In general, there was a high degree of error in the LCM measurements in relation to the chlorophyll content quantified by the lab analysis (Fig. 5). Linear models for explaining the chlorophyll content determined in the laboratory by measured values of the four LCMs without including further factors such as leaf age or species show highly significant correlations for all four models ($p < 0.0001$). The measured values from all four PCMs show a positive linear correlation to chlorophyll contents determined in the laboratory. However, examination of the correlation coefficients reveals differences in the strength of the relationship. For SPAD ($r^2 = 0.60$), Dualex ($r^2 = 0.66$) and FieldSpec ($r^2 = 0.63$) the considered correlations are similarly strong, while the Multiplex-index shows a closer correlation ($r^2 = 0.76$). Measurements for maize and tomato leaves show different patters, with maize leaf chlorophyll contents not exceeding 300 nmol * gDW^{-1}, while tomato leaves contain up to 600 nmol * gDW^{-1}. Also, plant nutrition- and leaf age-related clusters can be seen. For some subsets of plant leaves, LCM readings are extremely weak predictors for lab-based chlorophyll content, e.g. a drought stressed (blue) tomato leaf may have the same SPAD value as a nutrient deficient tomato leaf that actually contains twice the amount of chlorophyll (Fig. 5, A). Low laboratory values for chlorophyll

content, especially in maize leaves, were not consistently detected by the LCMs. This is especially observed in young leaves with optimal nutrient supply, as well as drought-stressed young leaves.

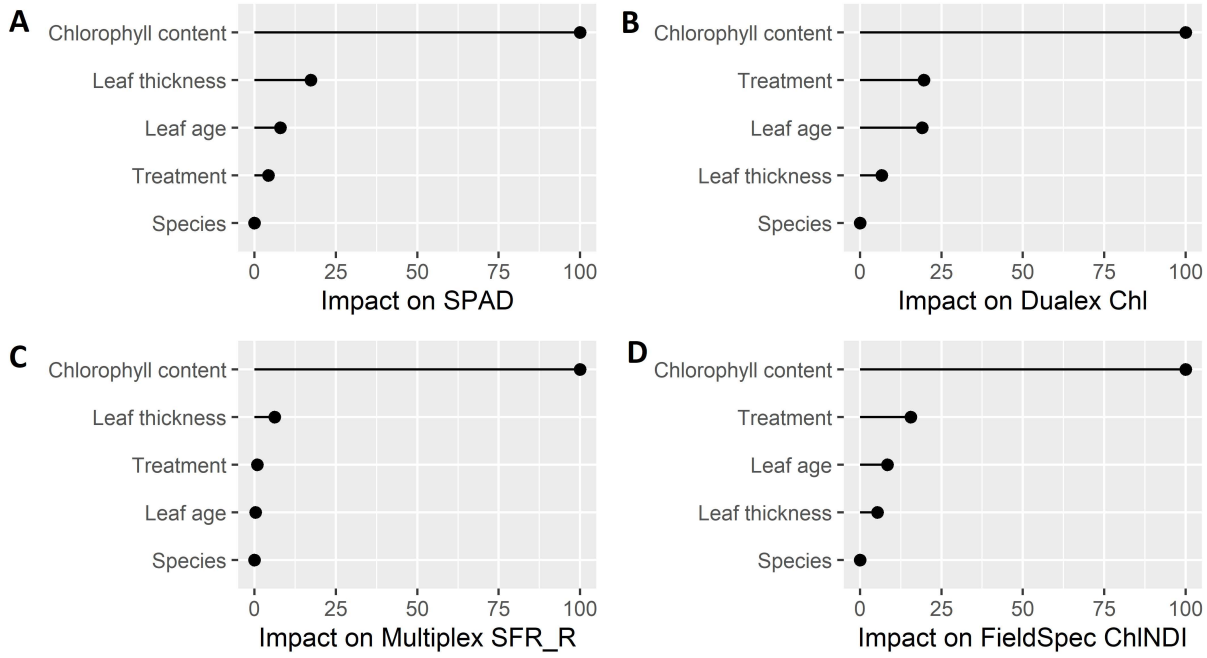

Figure 6: Relative impact of considered explanatory variables on four chlorophyll meter readings as identified through variable importance of boosted regression tree models. Strongest impact for each index is scaled to 100.

Figure 6 shows the relative impacts of different explanatory variables on PCM readings, with the strongest impact scaled to 100. Leaf chlorophyll content as quantified through wet-chemical methods explains most heterogeneity in readings of all four leaf chlorophyll meters (Fig. 6). For Multiplex (Fig 6. C), the share of information carried in the explanatory variables ‘Leaf thickness’, ‘Treatment’, ‘Leaf age’ and ‘Species’ is negligible. For SPAD, the impact of leaf thickness is about one fifth the impact of chlorophyll content and also both leaf age and treatment contribute to the devices’ measurement (Fig. 6, A). Dualex readings are affected by leaf age and treatment as well, while leaf thickness plays a minor role (Fig. 6, B). FieldSpec’s ChlNDI index is affected by treatment and leaf age, with leaf thickness again paying a minor role (Fig. 6, C). Interestingly, there was no information identified that was solely carried by the factor ‘Species’. This information is apparently completely mediated through other factors included in the analysis.

Table 4: *Performance of boosted regression tree models to explain index values based on leaf chlorophyll content and considered confounders.*

	SPAD	**Dualex Chl**	**Multiplex SFR_R**	**FieldSpec ChlNDI**
r^2	0.70	0.84	0.87	0.84
RMSE	8.03	5.20	0.20	0.06

Accuracy metrics for the four models including described confounders and the leaf chlorophyll content as detected by photometric measurements described above are presented in Table 4. These accuracy metrics relate to the overall performance of the models for which the importance of individual explanatory variables is shown in figure 6. Higher r^2 indicate a better model fit and therefore a successful identification and inclusion of most factors influencing the respective chlorophyll meter reading. Root mean square error (RMSE) is given in the artificial units of the respective index and is therefore not suitable for comparisons between devices. While the chosen model performed well for Dualex, Multiplex and FielsSpec values (r^2 = 0.84 – 0.87), SPAD values were rather poorly explained (r^2 = 0.70) (Table 4).

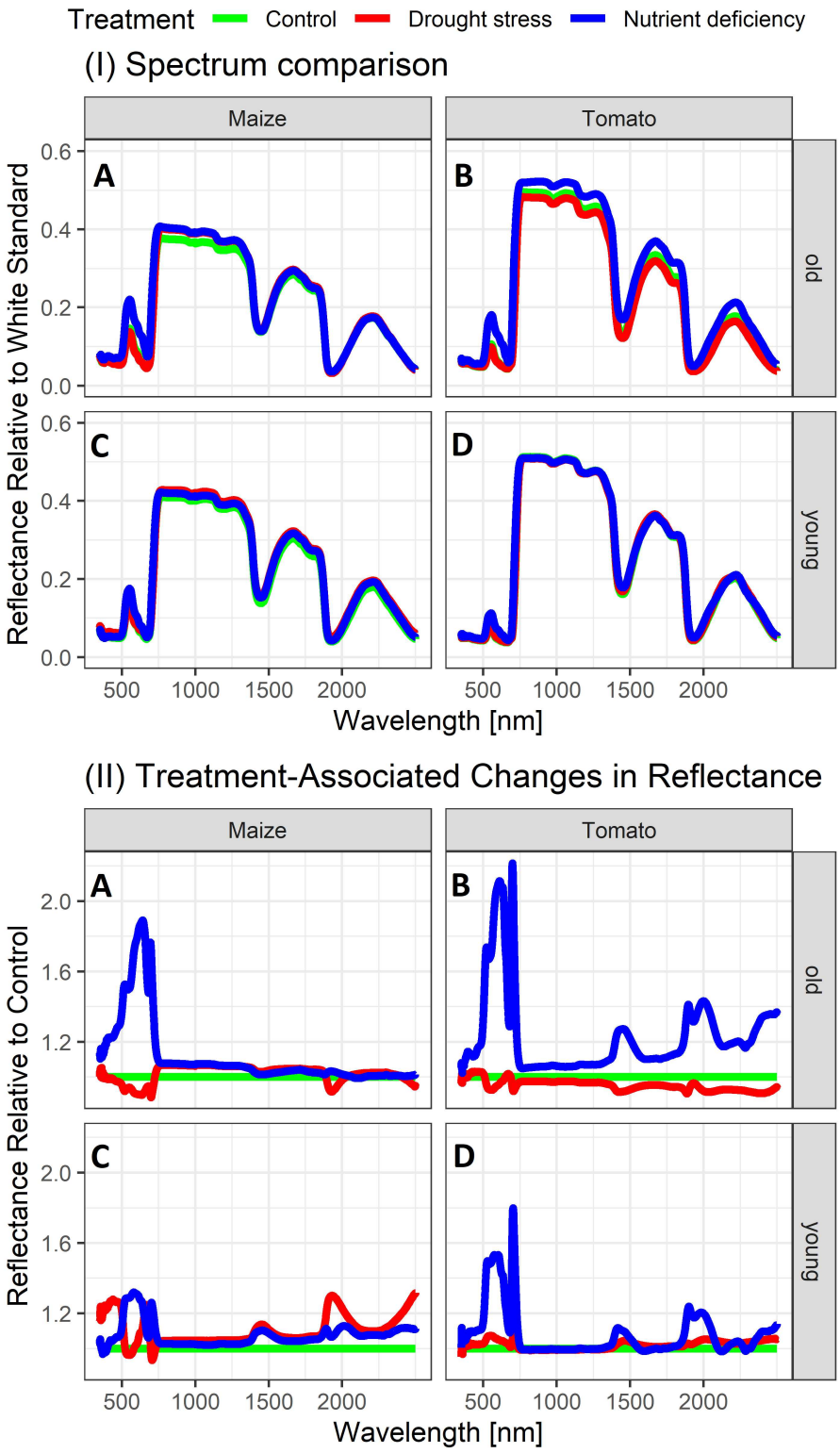

Figure 7: Differences in hyperspectral reflectance of maize and tomato leaves. Spectra are mean values. N = 10 per species * leaf age * treatment – combination (120 spectra in total).

Figure 7 shows FieldSpec data: reflectance patterns of measured plants leaves, averaged by leaf age, species and treatment. In figure 7 I, reflectance is shown relative to “absolute reflectance” of a barium sulfate white standard. While relative reflectance of young leaves shows only minor differences between treatments, differences in old leaves are more prominent, with nutrient deficient leaves reflecting more (= absorbing less) radiation that control and drought stressed leaves (Fig. 7 I). Tomato leaves generally reflect more light than maize leaves, while relative changes in reflectance at different wavelengths remain similar for both species. Figure 7 II shows reflectance of drought stressed and nutrient deficient young and old leaves of maize and tomato plants relative to the reflectance of leaves from optimally supplied plants. Leaves of nutrient deficient plants stick out, in the red region (600 – 700 nm) up to twice as much radiation is reflected, as compared to optimally nourished plant leaves. Looking at the spectral reflectance pattern, leaves from drought stressed plants differ much less from leaves of optimally supplied plants, than from those of plants suffering from nutrient deficits.

Discussion

In the following paragraph, we discuss the results presented above with emphasis on the different devices used and the plants nutritional status. In subsequent paragraphs, available models representing the interactions between leaf chlorophyll content, leaf characteristics, the plants environment and chlorophyll meter measurements are reviewed, and a new conceptual model is developed and presented.

Choice of Portable Chlorophyll Meter matters

If and to what degree a non-invasive chlorophyll measurement is biased through several confounders is largely dependent on the LCM chosen for the measurement. In order to understand why the various influencing variables have such different effects on the various measuring instruments, we need to build a bridge back to the way the instruments work. SPAD and Dualex have a similar mode of operation: both instruments are leaf-clip based, and both use very similar wavelengths for chlorophyll estimation. This functional similarity is reflected in a comparably strong dependence of the measurements of both instruments by the external disturbance variables considered. The rather bad performance may also be related to the small measurement area as compared to FieldSpec and Multiplex (Tab. 1). The FieldSpec hyperspectral instrument is also similar in function to the instruments described above. Consequently, the influence of all relevant disturbance variables (treatment, leaf age, leaf thickness) on the measurements of the FieldSpec was comparable than on the measurements of the other leaf-clip based instruments. Great care has to be taken when analyzing the results of the chosen FieldSpec index, as it was manually selected: while SPAD and Dualex do not provide a choice, but each device provides only one chlorophyll index, many different indices can be calculated based on FieldSpec data. By selecting the index that best matches the chlorophyll contents detected in the laboratory (ChlNDI), we have probably at the same time selected the index that is least influenced by other factors. In a sense, one can speak of "overfitting". However, the FieldSpec radiometer provides a lot more information than SPAD and Dualex, as depicted in figure 6. Other authors have shown that both categorization of plants into stressed and unstressed and regressions for estimation of leaf chlorophyll content based on analyses (e.g. partial least-squares regression or Support vector machines) of total hyperspectral reflectance are possible [216,217]. We deliberately did not use this approach here to ensure some comparability between the data from the PCMs used. Another difference compared to SPAD and Dualex is the design of the FieldSpec instrument: light source and detector are located on the same side of the leaf. Thus, since light does not shine through the leaf to the detector, the thickness of the sheet is of secondary importance to the detected light

than with transmission-based instruments. This effect also exists with Multiplex measurements. In addition, the Multiplex relies on chlorophyll fluorescence to quantify chlorophyll. Chlorophyll molecules in the leaf are excited by pulses of red light, resulting in chlorophyll fluorescence of different wavelengths, which is absorbed to a greater or lesser extent depending on the chlorophyll content of the leaf. Chlorophyll fluorescence-based measurements are known to be less susceptible to external influences than measurements based on reflection and absorption of irradiated light alone [200], but at the same time chlorophyll fluorescence also varies with plant physiological status, and can therefore be used as an indicator of plant stress [218,219]. In our experiment, Multiplex was the device least susceptible to the analyzed confounders. Chlorophyll predictions of Multiplex are least influenced by leaf thickness, leaf age or nutritional status of the observed plant.

Insights from full-spectra analyses

Nutrient deficiency causes characteristic changes in the spectral reflectance pattern of both tomato and maize leaves. Although these changes differ in strength between leaves of different age, with younger leaves showing a stronger reaction, the characteristic pattern remains the same across leaf age and even species (Fig 7 B). Discrimination of drought-stressed and optimally supplied plants based on chlorophyll meter readings is much harder, as the interaction of radiation with drought stressed and optimally supplied leaves shows only minor differences, as indicated by the reflectance patters (Fig 7 B). This result is contrasting a body of literature claiming that drought detection *inter alia* with chlorophyll meters is possible [220]. The acute drought stress applied in our experimental approach probably resulted in reduced cell elongation and simultaneous chlorophyll degradation in the observed drought stressed leaves. These two effects have adverse effects on leaf-radiation interactions and may have eliminated one another, as PCMs address chlorophyll content per unit leaf area.

Available models for remote sensing of foliar pigment contents

The general trends for modeling complex phenotypes with explicit inclusion of environmental as well as genetic effects are summarized here [221]. In this section, we restrict ourselves to models specifically developed for leaf pigments and their detection.

An overview of early efforts to quantify leaf pigment contents is provided in the review from Ustin and colleagues [222]. In short, the first mathematical models were used to quantify contents of a single pigment (e.g. chlorophyll), usually using reflectance of light at different wavelengths as input parameters. An early example is the work of Gitelson and Merzlyak, using light in the red and near-infrared region of the electromagnetic spectrum to predict leaf

chlorophyll content per area [223]. A downside of early, simple models of that kind is the need of re-calibration for new datasets, especially if the models are transferred across species or environmental conditions [222]. Moreover, the exact wavelengths used for the different indices varies from study to study, because wavelengths are slightly adopted to the properties of the dataset, choosing the wavelengths that explain most variation in the data. This artificial selection may cause overfitting and limited transferability of the resulting model. Data dependent re-calibration is also done for multivariate statistical models like partial least squares regression, or different data-driven machine learning approaches [224], at least, if these models rely on hyperspectral data only.

Aiming at the development of „generic algorithms“, a next generation of models was developed with multi-species datasets. Gitelson and Solovchenko used several hyperspectral datasets from overall 45 different species and used cross-validation within the same dataset to evaluate the models predictive performance regarding leaf chlorophyll, carotenoid and anthocyanin contents [224].

More recently, radiative transfer models like the PROSPECT-D model [225] were used and constantly improved to project leaf chlorophyll, carotenoid and anthocyanin contents from spectral reflectance. These models require assessment and incorporation of some confounding factors (e.g. water content and leaf density) in the model. A general overview on different early PROSPECT models (introduced already in 1990 [226]) was reviewed by Jacquemoud and colleagues [227]. These approaches might pave the way to better leaf pigment estimations based on remote sensing data on large scales, where manual assessment of most confounders is hard or impossible. When using handheld devices, however, capturing more additional data e.g. on leaf age and leaf thickness is not an unreasonably large effort, given the accuracy benefit an incorporation of such factors may have on chlorophyll prediction. Most recent versions of PROSPECT also include leaf protein content and carbon-based constituents [228]; parameters that are well known to be correlated with plant nutritional status.

In order to generate a truly generic model for leaf chlorophyll prediction, the incorporation of more information than plain hyperspectral data seems unavoidable. This is supported by our results presented in figure 6 of this work, as well as the fact that the most advanced PROSPECT model is being developed in that direction. After more than 30 years of development, the PROSPECT models are well proven to be reliable tools. However, despite the introduction of new model parameters over the years, the model structure remained untouched – it still is a linear model after all. This simplistic structure does not correspond to the complexity of leaf pigment measurements: The interactions between the plants’ environment, the physical

properties of the leaf itself, and the different non-invasive sensors.

Toward a better understanding of chlorophyll measurements

The results obtained in our experiment stimulate the re-thinking of leaf chlorophyll measurements. The simplest models implicitly underlying chlorophyll measurements with LCMs are a poor representation of reality. They are based on reflection of light of a few wavelengths without incorporation of any information on leaf characteristics, plant nutrition or even the plant species. We argue that this simplistic concept is insufficient for reliable phenotyping and should be extended as indicated in figure 8.

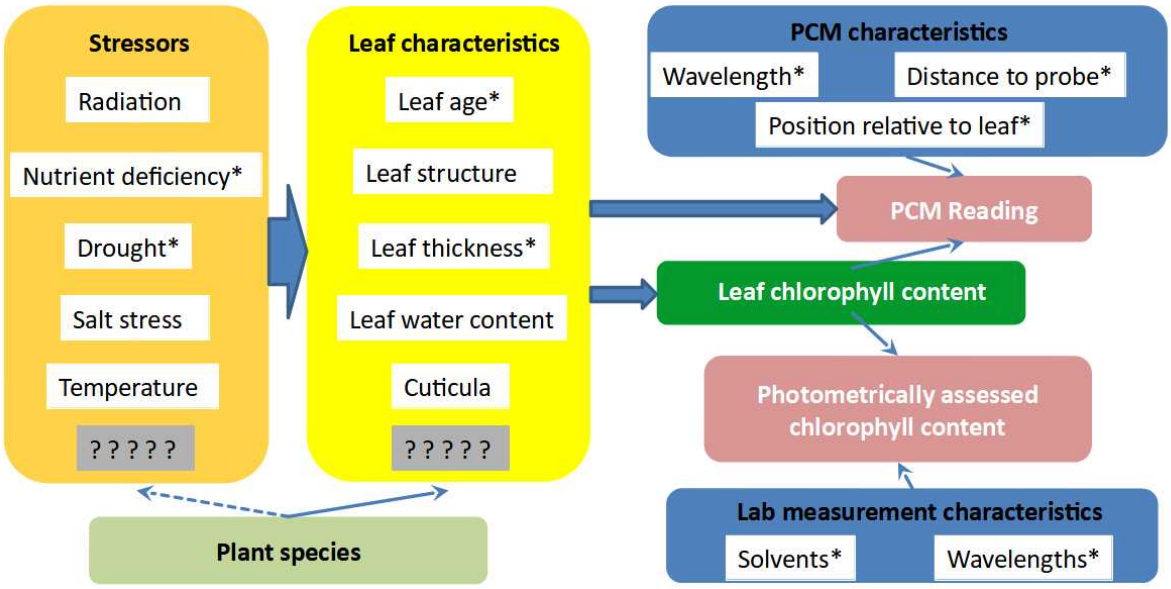

Figure 8: Conceptual framework of leaf chlorophyll content assessment with non-invasive devices. Text boxes marked with * contain factors that were considered in our experiment. Grey boxes represent unknown factors.

While some stressors like nutrient deficiencies may have a direct effect on leaf chlorophyll content, many other stressors act to some degree indirect via changes in leaf characteristics such as leaf water content and leaf thickness. LCM readings are in fact not solely informed by the actual leaf chlorophyll content, but also sensitive to leaf characteristics which in turn are manipulated by the environment (and stressors present therein). As different LCMs indicate different chlorophyll contents for the very same leaf, it cannot be denied that the instruments characteristics influence the measurement as well. Finally, looking at different plant species changes both leaf characteristics and the definition of “stressor”, as different plants tolerate different amounts of radiation, fertilizer, *et cetera*. Temporal stress development adds yet another layer of complexity we do not cover in this work. We hope that a better understanding of the complex interactions leading to the plain numeric output of LCMs leads to a more cautious

use of the results obtained with the devices. Finally, it is important to note that chlorophyll determinations in the laboratory, either via a photometer or more sophisticated via mass spectrometry, are not an unbiased representation of the “real” chlorophyll content either. Values obtained in the laboratory are mainly informed by the actual leaf chlorophyll content, but also vary with the solvents used for extraction and the exact wavelengths used for photometric analysis, probably among other factors. Although only few available studies compare several laboratory methods of chlorophyll extraction in plant leaves, many phenotyping studies (including our present study) refer to laboratory measurements as a gold standard, implicitly neglecting any imprecision on that end.

Next steps: From a conceptual framework to parameterized models

The conceptual framework presented in figure 8 is not complete and should possibly include more or different potential confounders. However, even if that framework was complete, how could we turn it into a working model? As some effects depicted in the conceptual model (Fig 8) are directed, e.g. plant species has an impact on leaf structure, but not vice versa, the mathematical model cannot consist of simple equations, as the latter are non-directed.

The interwoven concepts of directed acyclic graphs (DAGs) and structural equation models (SEMs) could provide a solution. Originally described by Wright 100 years ago [229] and later rediscovered and developed by Pearl and others (e.g. [185,230]), these methods deliver a framework for incorporation of both known or suspected causal interaction between factors in a dataset, and observational data.

More recently, Grace and Irvine published an article in order to widen the adoption of causal diagrams and SEMs in the field of ecology [231]. SEMs have also been used for phenotyping purposes, for example in rice, to distinguish direct genetic effects on rice water use from those effects that come to stand indirectly through a change in projected shoot area [232]. There is reason to believe that several questions that arise in the analysis of the data set presented here can be well answered with similar models: To what degree does the measured chlorophyll content of the leaf depend on the actual chlorophyll content? To what extent does drought stress influence the measurement result (a) by changing the chlorophyll content of the leaf, and to what degree is the changed measurement (b) the result of a drought stress-induced change in leaf structure? While the boosted regression trees used in our work must detect patterns in data based on the data alone, without a logical structure provided by the scientist, SEMs can use both existing knowledge of causal relationships and data collected in the experiment to answer such questions and are therefore presumably even better suited.

In summary, we suggest that, in order to generate reliable phenotypic data, confounders of phenotypic measurements have to be analyzed systematically. The potential error of known or suspected confounders should be quantified, to provide the best possible phenotypic data for most efficient plant breeding and crop management. Many previous approaches to modeling the relationship between chlorophyll meter measurements and leaf chlorophyll contents are inadequate and should be abandoned in favor of the approach outlined above.

Acknowledgements

Luca Marie Erbe passed away completely unexpectedly. We are grateful not only for her contributions to this work, but for everything she did as a person to enrich our lives.

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Third challenge

Demands on crops are subject to constant change. As a result, new challenges are also constantly arising for crop phenotyping. In recent years, the bio-economy has become increasingly important: Products that are traditionally produced petrochemically are to be more and more derived from renewable resources. In this context, secondary metabolites from vegetable plant leaves are playing an increasingly important role. Therefore, in the third chapter, "Effect of UV Radiation and Salt Stress on the Accumulation of Economically Relevant Secondary Metabolites in Bell Pepper Plants", we highlight the potential of sensor-based phenotyping to quantify selected secondary metabolites in bell pepper plant leaves. For the detection of specific secondary metabolites, a detailed knowledge of both the physical properties of these metabolites and the metabolic pathways and environmental properties under which these substances are formed is required.

In the following chapter, we will discuss the stress metabolism of crops and the resulting possibilities for sensor-based phenotyping.

The chapter is also published as a research manuscript in Agronomy [163].

4. Effect of UV Radiation and Salt Stress on the Accumulation of Economically Relevant Secondary Metabolites in Bell Pepper Plants[3]

Authors

Jan Ellenberger, Nils Siefen, Priska Krefting, Jan-Bernd Schulze Lutum, Daniel Pfarr, Maja Remmel, Lukas Schröder and Simone Röhlen-Schmittgen

Abstract

The green biomass of horticultural plants contains valuable secondary metabolites (SM), which can potentially be extracted and sold. When exposed to stress, plants accumulate higher amounts of these SMs, making the extraction and commercialization even more attractive. We evaluated the potential for accumulating the flavones cynaroside and graveobioside A in leaves of two bell pepper cultivars (Mavras and Stayer) when exposed to salt stress (100 mM NaCl), UVA/B excitation (UVA 4–5 W/m^2; UVB 10–14 W/m^2 for 3 h per day), or a combination of both stressors. Plant age during the trials was 32–48 days. HPLC analyses proved the enhanced accumulation of both metabolites under stress conditions. Cynaroside accumulation is effectively triggered by high-UV stress, whereas graveobioside A contents increase under salt stress. Highest contents of secondary metabolites were observed in plants exposed to combined stress. Effects of stress on overall plant performance differed significantly between treatments, with least negative impact on above ground biomass found for high-UV stressed plants. The usage of two non-destructive instruments (Dualex and Multiplex) allowed us to gain insights into the ontogenetic effects at the leaf level and temporal development of SM contents. Indices provided by those devices correlate fairly with amounts detected via HPLC (Cynaroside: r^2 = 0.46–0.66; Graveobioside A: r^2 = 0.51–0.71). The concentrations of both metabolites tend to decrease at leaf level during the ontogenetic development even under stress conditions. High-UV stress should be considered as a tool for enriching plant leaves with valuable SM. Effects on the performance of plants throughout a complete production cycle should be evaluated in future trials. All data is available online.

3 Ellenberger, J.; Siefen, N.; Krefting, P.; Schulze Lutum, J.-B.; Pfarr, D.; Remmel, M.; Schröder, L.; Röhlen-Schmittgen, S. Effect of UV Radiation and Salt Stress on the Accumulation of Economically Relevant Secondary Metabolites in Bell Pepper Plants. *Agronomy* **2020**, *10*, 142, doi:10.3390/agronomy10010142.

Introduction

Green Biomass as a Source of Valuable Chemicals

Commercial vegetable production is accompanied by large quantities of so far under-utilized green biomass in all stages of production and especially after harvest [233]. While the use of biomass

for the purpose of energy production is becoming a standard procedure in northern Europe in recent years [161], the extraction and the use of high-value secondary metabolites (SMs) from vegetable plant leaves are just being developed. Research strategies in Europe are heading toward a cascade use of agricultural byproducts and pave the way for extracting and using "valuable substances or molecules before ultimately discarding the left-overs" [234]. The pharmaceutical industry – as an example – is highly dependent on plant SMs, since approximately 60% of anticancer compounds and 75% of drugs for infectious diseases are derived from plants [235]. In this frame, research on targeted enrichment of valuable substances in plant biomass is gaining importance [236].

Plant Stress as a Measure to Increase Leaf Secondary Metabolite Content

The biochemical background of enhanced accumulation of SMs in plant leaves as a measure to cope with stress is a well-described phenomenon [161,237,238]. In short, the cultivation of plants under suboptimal conditions leads to an increased amount of reactive oxygen species (ROS) in plant tissues. Accumulation of SMs is a plant strategy to avoid oxidative damage caused by reactive oxygen species [239]. In theory, both biotic and abiotic stressors could lead to higher amounts of valuable SMs in plants. While biotic stressors such as fungi and insects are hard to control and may cause major phytosanitary problems, abiotic stressors are easier to manage and applicable by practitioners. The results of several studies in recent years indicate that abiotic stressors are a useful tool for SM accumulation in leaves of horticultural plants. Secondary metabolites in *Centella asiatica* leaves increase under enhanced UV-B light, especially in the epidermis [240]. In bell pepper, increased flavonoid contents can be found in leaves exposed to elevated UV [241]. The promoting effect of UV-B radiation on flavonoid accumulation in plant leaves has recently been reviewed [242]. The effects of salt stress on the antioxidant machinery may be adverse and depend on the plant's tolerance [243] and salt concentration in the rootzone [244]. Another extensive study on leaf metabolism in bell pepper under different levels of salt stress revealed an increasing reduction in growth with increasing NaCl contents in the rootzone [245]. While tolerant plants increase leaf SM contents to cope with salt stress, sensitive plants do not have this mechanism and senesce, finally dying off if the

stressor is persistent [243]. Studies directly comparing effects of salt and UV stress on leaf SMs are rare. One study shows both stressors to similarly affect leaf contents of the flavonoids quercetin and luteolin in *Ligustrum vulgare* [246]. Abiotic stressors such as drought and salt stress are easily applicable in commercial greenhouse production in soilless systems, which are the predominant systems in many parts of the world, including Europe [247].

Non-Invasive Monitoring of Secondary Metabolites in Plant Leaves

Quantification of secondary metabolites including flavonoids with portable optical devices is well established in plant sciences [248]. The use of non-invasive optical sensors to investigate plant

leaf components has several advantages over laboratory analyses: data acquisition is faster and more cost effective than laboratory analyses [249]. Moreover, considerate handling of leaves allows for several measurements of the same leaf, enabling to gain insights in temporal developments. Several studies demonstrated the viability of optical devices to access secondary metabolites in plant leaves: a multiparametric fluorescence sensor was used to evaluate the influence of nutrient deficiency on the chemical properties of tomato leaves and to quantify the content of the flavonoids rutin and solanesol [250,251]. In bell pepper, a fluorescence sensor was used to evaluate the impact of priming plants with high light conditions on leaf flavonoid content [241].

Cynaroside and Graveobioside A

The vast diversity and chemical complexity of plant SMs often prohibit an economically feasible chemical synthesis. Therefore, extraction either from wild or cultivated plants often represents the best source of supply [233].

Cynaroside (Luteolin-7-glucoside) potentially has a range of medicinal applications: it has the capability to prevent ROS-induced apoptosis in heart cells [252]. Cynaroside furthermore diminishes kidney injury as a side effect of cancer treatments with the chemotherapeutic drug cisplatin. A potential medicinal use of graveobioside A (Luteolin-7-apiosyl-glucoside) is proven by a patent on its application in preparation of drugs for preventing hyperuricemia and gout [253]. Graveobioside A was shown to be contained in several plants, such as celery seeds, parsley, and bell pepper [254,255].

Several SMs in Solanaceae leaves have the potential to biologically control insects [256]. Graveobioside A is such a potential natural insecticide, since oviposition of the American serpentine leafminer fly (*Liriomyza trifolii*) was shown to drop in kidney bean leaves treated with

a graveobioside A containing solution [255]. It is expected that the demand for natural insecticides will increase across the EU due to more rigid legislation [257].

We hypothesize that cynaroside and graveobioside A contents in bell pepper leaves can be enhanced by abiotic stressors that are potentially applicable by practitioners in the future. Another aim is to check whether non-invasive devices can be used for assessments of cynaroside and graveobioside A in bell pepper leaves. Furthermore, we attempt to get insights in interactions between different stressors and differences in stress response between two bell pepper cultivars.

Material and Methods

Plant Material and growth conditions

Seeds of sweet pepper plants (*Capsicum annuum*) cultivar 'Stayer' (Rijk Zwaan, The Netherlands) and 'Mavras' (Enza Zaden, The Netherlands) were sown in soil under greenhouse conditions. Fourteen-days old pepper plants were transplanted into small rockwool cubes (3 × 3 × 5 cm) and one further week later into larger cubes (10 × 10 × 7.5 cm) (Grotop Master, Grodan, The Netherlands). On day 32 after seeding, plants were transferred to a grow chamber to ensure a stable environment. From that day on, stress was applied for 16 days, resulting in a plant age of 48 days at the end of the trial. A longer trial was not feasible due to limitations of the chosen facility. All plants received all nutrients mandatory for optimal growth prepared from two stock solutions (17.2 mM nitrogen, 5.4 mM calcium, 4.7 mM potassium, 0.4 mM phosphorous, 5.4 mM sulfur, 2.4 mM magnesium, 0.01 mM iron and micronutrients; electrical conductivity 2.5 mS cm^{-1}; pH 5.5). Plants were cultivated at the greenhouse facility in Bonn-Endenich (University of Bonn, Germany) at day/night temperatures of 24.5 °C ±5.4 and supplemental light intensity of 203–540 µm m^{-2} s^{-1} provided by sodium vapor lamps (Philips Lighting GmbH, Germany).

To apply salt stress, a salt concentration of 100 mM NaCl for a period of 16 days was added to the standard nutrient solution, since that concentration was shown to trigger a higher total phenolic content in leaves of bell pepper seedlings in a previous study [245]. To apply UV stress, plants were exposed to UV light (UVA 4–5 W m^{-2}; UVB 10–14 W m^{-2}) for 3 h per day (Philips Lighting GmbH, Hamburg, Germany) over a 16-day period. In addition, some plants were exposed to combined salt and UV stress. Plant age at stress onset was 32 days. A total of 5 plants per treatment (control, salt stress, UV stress, combined stress) were randomized in the growth chamber.

Non-Destructive Readings

Non-destructive measurements were performed on all leaves per plant, from mature to young. Measurements were conducted using two well-established devices in stress physiology monitoring. First device is the multiparametric fluorescence excitation system Multiplex® (Multiplex®, Force-A, France), described in previous studies [258]. All recordings with this device were done at a constant distance of 0.10 m to the leaf surface and a frontal cover plate with an aperture of 4 cm in diameter opening to assess the index of epidermal flavonols (FLAV) and the nitrogen balance index under red excitation light (NBI_R):

$$FLAV = \log \frac{FRF_R}{FRF_{UV}} \quad (10)$$

$$NBI_R = \frac{FRF_{UV}}{RF_R} \quad (11)$$

with FRF_R and FRF_{UV} representing the far-red fluorescence of the leaf, when illuminated with red or UV-light respectively and RF_R representing the red fluorescence when illuminated with red light.

Secondly, the transmittance-based fluorescence measurements were conducted with the Dualex sensor (Force-A, France). The Dualex is a device with a leaf-clip; measurements were taken with virtually no distance to the leaf surface. The device is extensively described in the literature [205,206].

Plant Harvest

Plants were harvested 16 days after treatment inception (DATI) at a plant age of 48 days. The total fresh weight of shoots was determined immediately. Leaves were dried for 7 days at 50 °C (drying oven) to collect dry weights.

Leaf Sample Preparation and Laboratory Analysis

Samples were taken at the harvesting at 16 DATI, of the mature leaf 4 and the young leaves 10 and 12, to assess the impact of stress application on the amount of the two luteolins, graveobioside A and cynaroside. All leaf numbers are given as the number of true leaves, counted from the base of the plant. The samples were freeze-dried and then stored at −20 ◦C until further processing. Ground leaf samples were prepared for HPLC determination (Agilent 1260 Infinity HPLC System Agilent Technology Deutschland GmbH, Germany). An amount of 0.3 g was extracted with water-diluted methanol (60:40, v/v) for 10 min in an ultrasonic bath, centrifuged for 10 min at 4 ◦C with 13,000 rpm (Centrifuge 5415R, Eppendorf AG, Germany)

repeated four times. The supernatants were collected and stored at −20 ◦C until HPLC analysis. The samples were filtrated through a membrane filter (Phenomenex, Germany) prior to injection. The HPLC system consisted of an autosampler, a diode array UV–Vis detector and was coupled with a quaternary solvent delivery system. The column (Nocleodur C18, 3 ×150 mm, 3 µm, Macherey-Nagel, GmbH & Co. KG, Germany) was isocratically eluded with a binary mixture of water and methanol (60:40) adjusted to pH 2.8 with phosphoric acid. The flow rate was 0.3 mL min−1; 10 µL samples were injected onto the column equilibrated at 25 ◦C (detection at 355 nm). Graveobioside A peak was detected at 14.1 min, and cynaroside at 15.6 min. Both calibration curves were obtained from diluted series of standards provided by PhytoLab (Germany).

Data Analysis and Statistics

All data is available online [259]. Data analysis was performed with R [260] in RStudio [261]. According to the data structure, e.g., balanced or imbalanced, type I or type III ANOVA were used to compare group means. Applied post-hoc test was Tukey's HSD. Figures were created in RStudio, with the package ggplot2 [212].

Results

Stress-Related Effect Varies Among Secondary Metabolites and Cultivars

A treatment effect was observed on contents of both cynaroside and graveobioside A, while no significant effect of the variable cultivar on either metabolite content was found. There was a strong tendency for higher graveobioside A in Mavras as compared to Stayer ($p = 0.055$). No interactions between cultivar and treatment were observed (Table 5). Both combined-stressed plants and plants under UV-exposure accumulated significantly higher amounts of cynaroside in their leaves than control and salt-stressed plants (Fig. 9, A + B). Plants of the cultivar 'Mavras' accumulated significantly higher graveobioside A amounts in salt-stressed and combined-stressed plants than in control and UV-stressed plants (Figure 9 C). No significant treatment effect on graveobioside A content in plants of the cultivar Stayer was found (Figure 9 D). Levels of SM in leaves of different ontogenetic stages are shown as an illustration of uneven distribution within the plants. SM contents decrease with leaf ontogenetic stage (Fig. 9).

Table 5. *Interaction and main effect for treatments (control, salt-stress, combined-stress, UV-stress) and cultivars (Mavras and Stayer), calculated with a type I two-way ANOVA. Grayed area indicates significant effect ($p \leq 0.001$).*

Factor	Cultivar	Treatment	Cultivar × Treatment
Cynaroside	0.179	$< 2 \times 10^{-16}$	0.917
Graveobioside A	0.055	1.25×10^{-5}	0.141
Dry Weight	0.00082	3.8×10^{-12}	0.426
Fresh Weight	0.00017	1.15×10^{-15}	0.146

Both fresh and dry weight of bell pepper plants differed significantly depending on the cultivar, with Stayer attaining higher weights than Mavras. Treatment had a significant effect on both fresh and dry weight. There was no interaction between the treatment and cultivar regarding plant's fresh or dry weight. Dry weight of plants of the cultivar Mavras was significantly higher in control plants than in any other treatment (Table 5). UV-stressed plants of both tested cultivars exhibited higher fresh and dry weights than plants under salt-stress and combined-stress conditions (Fig. 10). Observed mean fresh weight decreased in salt-stress and combined-stress plants compared to control and UV stress, which were in the magnitude of 50% (Fig. 10 C, D). The mean dry weight tended to be higher for salt-stressed plants as compared to plants under combined stress, but lower than the dry weights of plants experiencing UV stress or control conditions (Fig. 10 A, B).

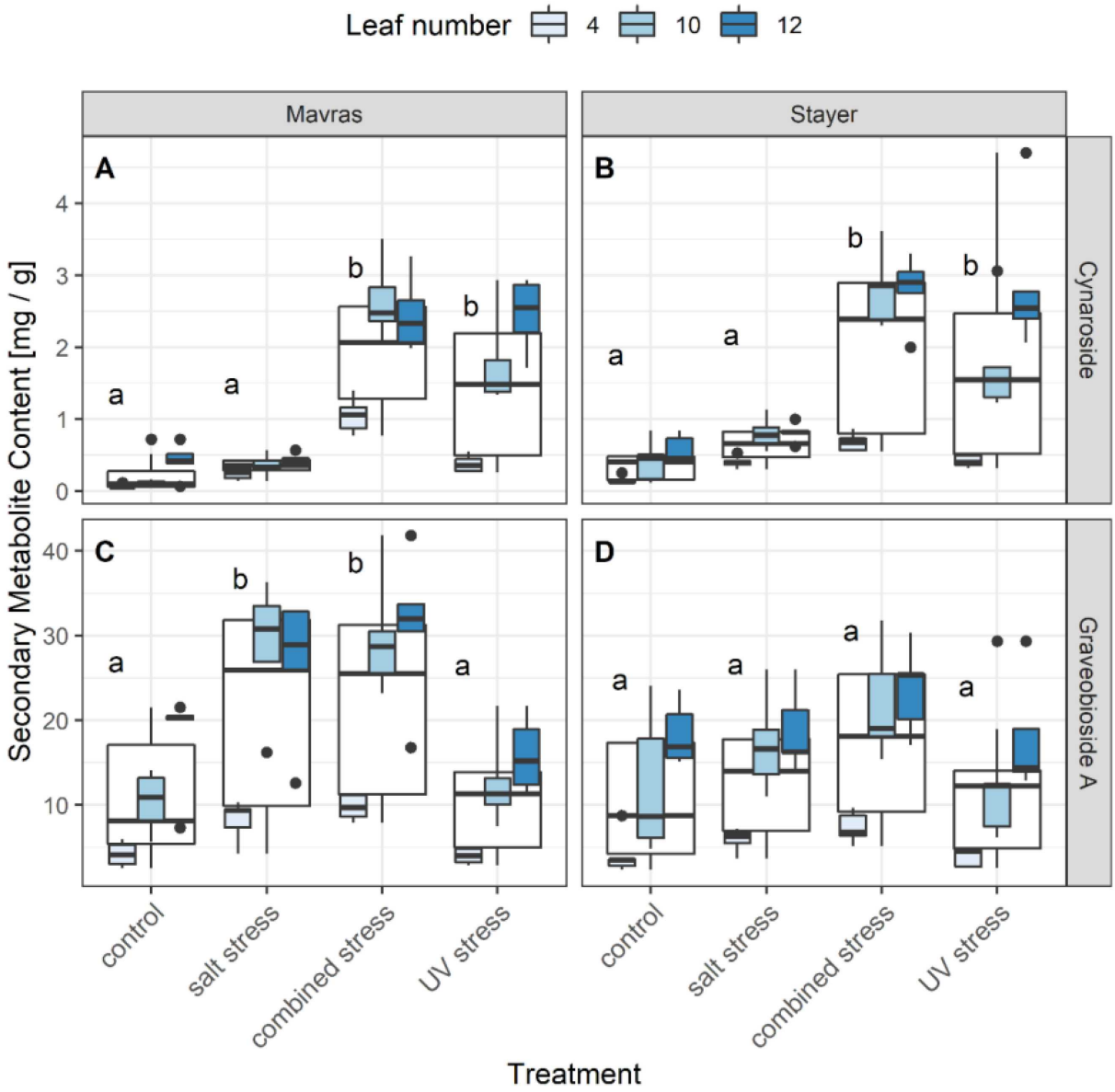

Figure 9: HPLC-determined leaf cynaroside (A, B) and graveobioside A (C, D) contents, for bellpepper cultivars ‘Mavras’ (A, C) and ‘Stayer’ (B, D) under different growth conditions, 15 days after treatment inception (n = 5). Transparent boxplots show pooled data from all leaves (n = 15). Colored boxplots represent leaf age – subgroups (Leaf 4, 10, and 12 as counted from the base, with darkest colors for youngest leaves). Letters (a, b) indicate differences within each cultivar × secondary metabolite – combination (Tukey HSD, $p < 0.05$).

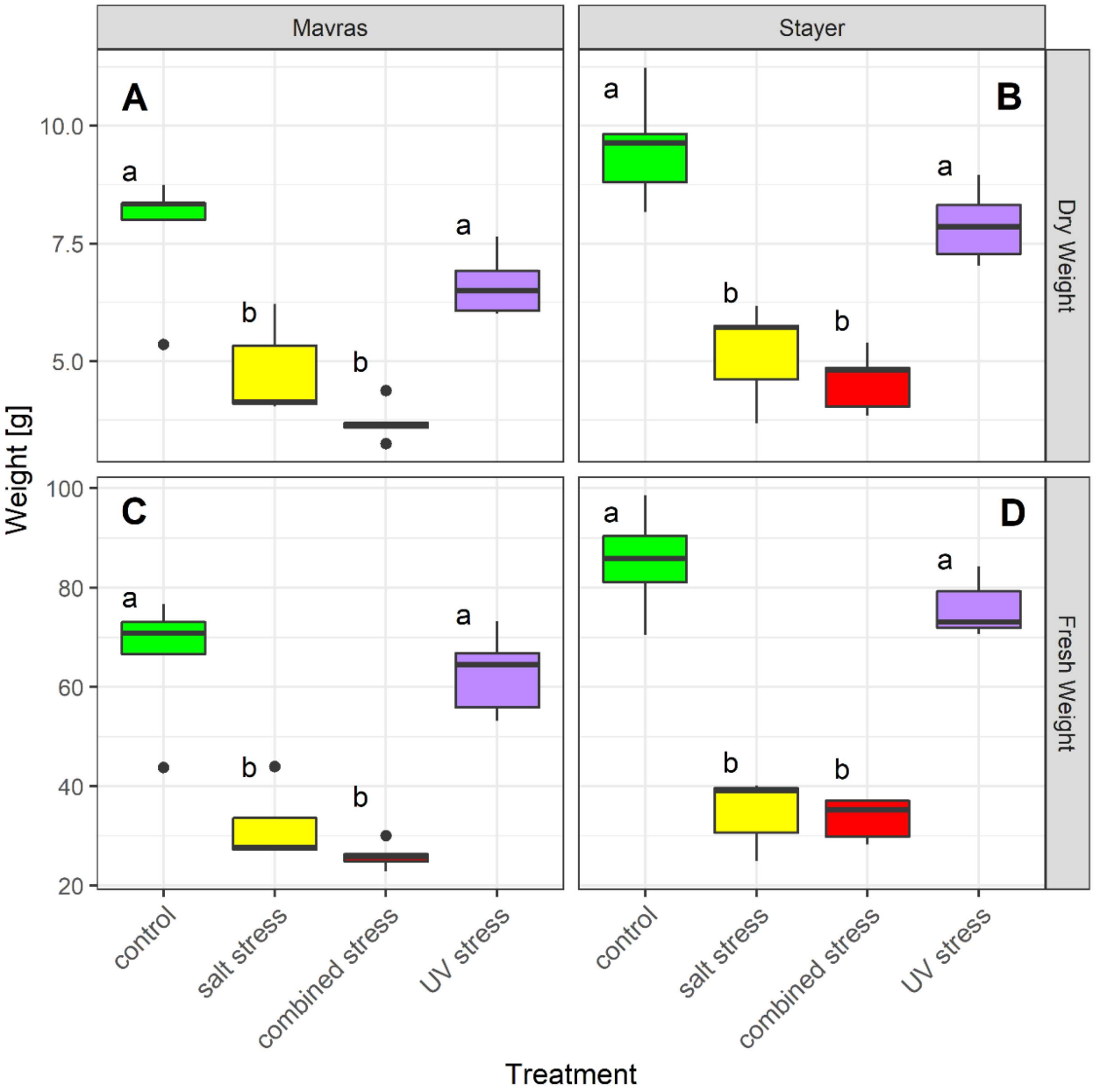

Figure 10: Aboveground biomass (dry weight: (A), (B); fresh weight: (C), (D)) of bell pepper cultivars "Mavras" (A), (C) and "Stayer" (B), (D) under different growth conditions, 15 days after treatment induction (n = 5). Letters (a, b) indicate differences within each cultivar × dry/fresh weight – combination (Tukey HSD, $p < 0.05$).

Non-Invasive Monitoring of Secondary Metabolites

Figure 11 shows exponential regressions between three indices (Multiplex indices FLAV and NBI_R; Fig. 11 A–D and Dualex index Flav; Fig. 11 E, F) and leaf contents of the SMs cynaroside (Fig. 11 A, C, E) and graveobioside A (Fig. 11 B, D, F), respectively. Predictions of graveobioside A contents based on the indices are better than predictions of cynaroside contents. Multiplex indices are more accurate predictors than the Dualex index, as outlined by

correlation coefficients (r^2). Index values level off at cynaroside contents above 1.5 mg g^{-1}. The connection between graveobioside A and the indices is more linear, but still leveling off at graveobioside A contents above approximately 25 mg g^{-1}.

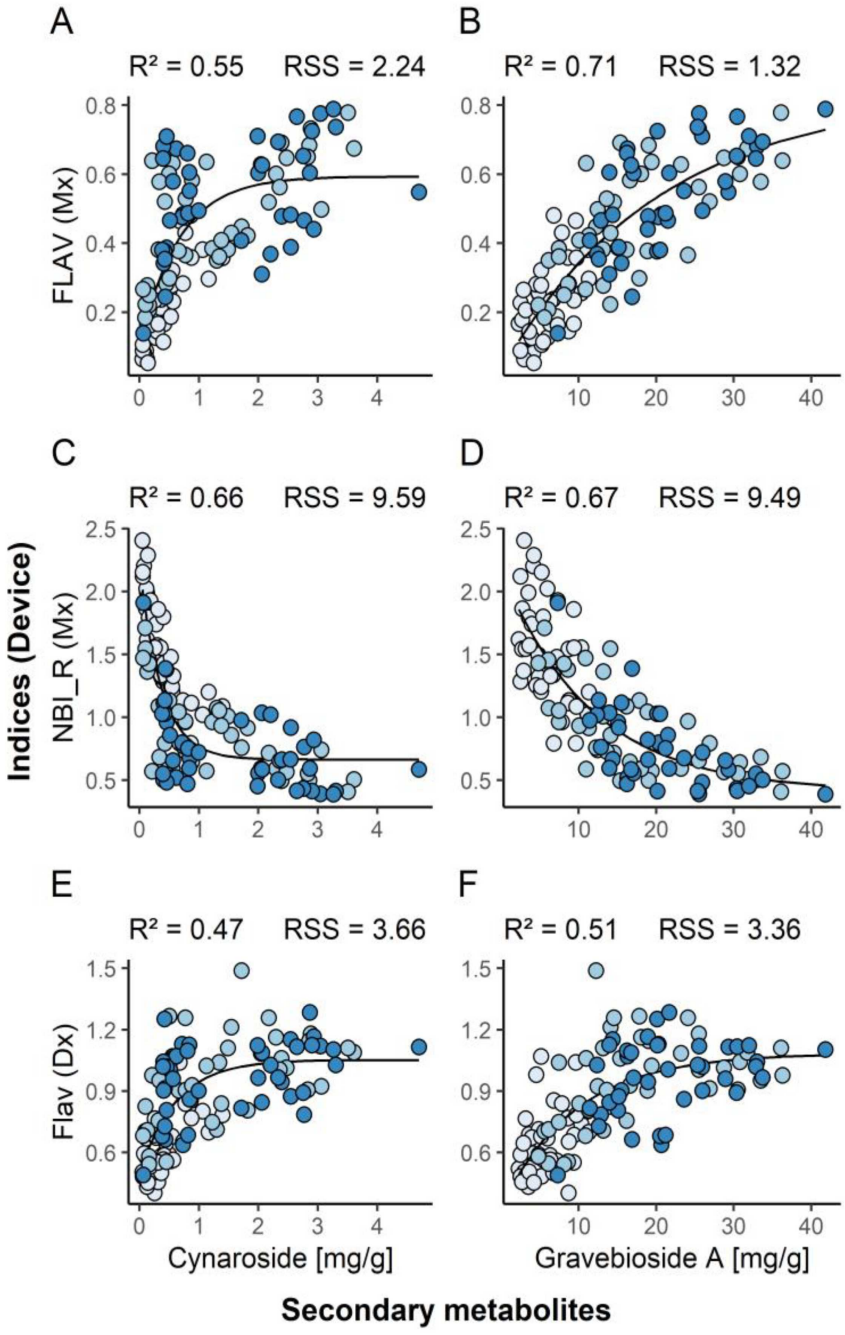

Figure 11: Exponential regression between indices of non-invasive devices and leaf secondary metabolite concentrations in bell pepper leaves, determined via HPLC. Contents of cynaroside and graveobioside A correlated with FLAV (Mx) (A), (B), NBI_R (Mx) (C), (D), and Flav (Dx) (E), (F). Color of points represents leaf age (Leaf 4, 10, and 12 as counted from the base, with darkest colors for youngest leaves). Lines indicate exponential regressions (n = 60). RSS, residual sum of squares.

Spatial and Temporal Development of Secondary Metabolite Contents

The only significant changes in FLAV values within cultivar × treatment groups were seen among the fourth leaves of combined-stressed Mavras plants at days 0 versus 9 and 0 versus 15, respectively (Fig. 12 C). A clear trend was observed for the fourth leaves of combined-stressed Stayer plants at days 0 versus 15 (TukeyHSD, p = 0.053) (Fig. 12 D). Generally, FLAV values for stressed plants tend to increase, while the values for control leaves tend to decrease. A comprehensive overview of associated main effects is given in Table 6.

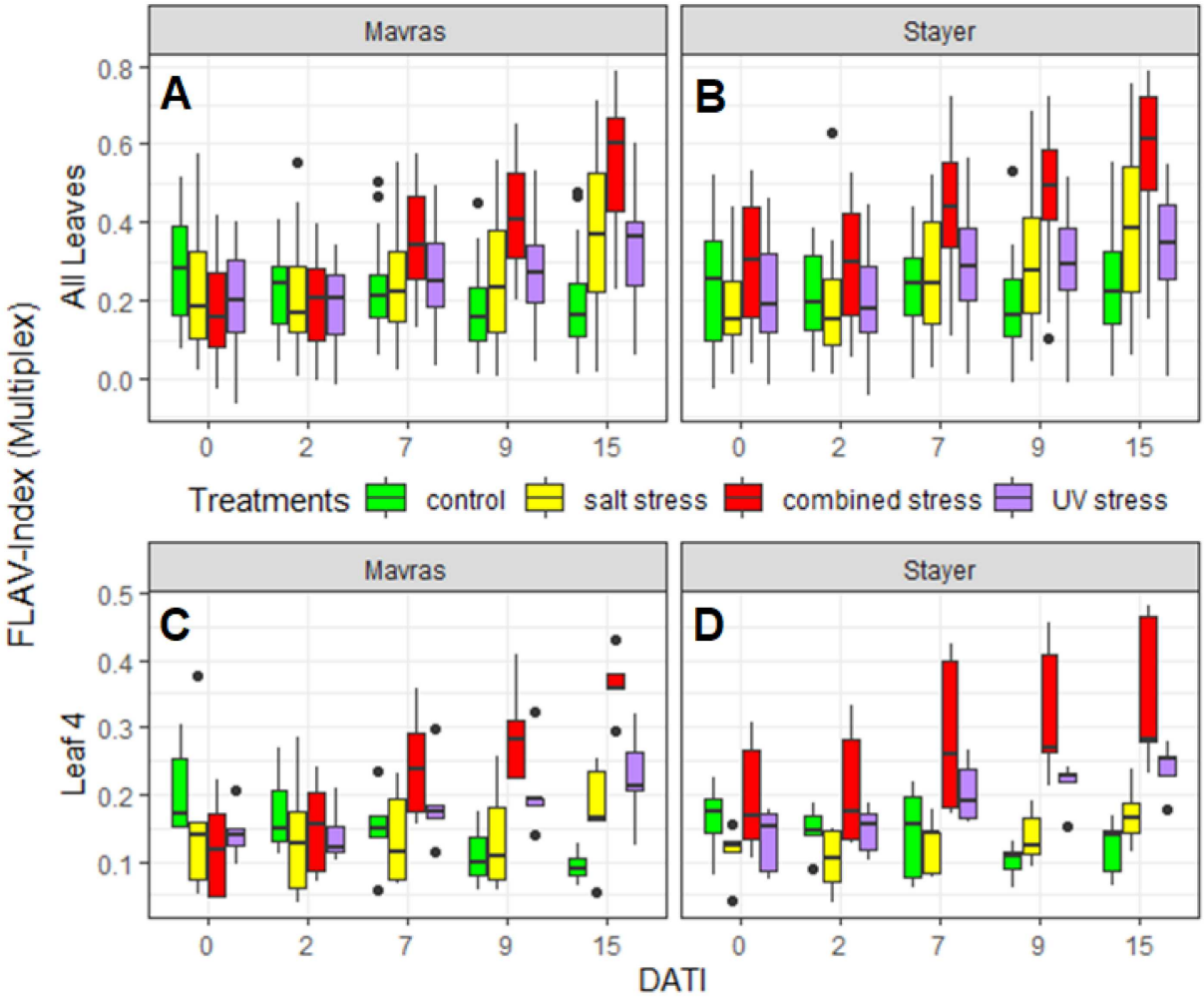

Figure 12: Temporal development of secondary metabolites in leaves of bell pepper cultivars “Mavras” and “Stayer”, expressed with the FLAV-index (Multiplex). (C), (D), n = 5; (A), (B), n = 5 – 50; DATI, day after treatment initiation.

Table 6. *Interaction and main effect for treatments (control, salt-stress, combined-stress, UV-stress) and DATI (0, 2, 7, 9, 15). To account for the unbalanced design (unequal numbers of observations within each level of DATI), type III ANOVA was selected to compare differences between factor means for FLAV values of "All leaves". Grayed area indicates significant effect at p ≤0.05 (light), p ≤0.01 (medium), and p ≤0.001 (dark).*

Leaf	Cultivar	Treatment	DATI	Treatment × DATI
All leaves	Mavras	0.085	0.027	$< 2 \times 10^{-16}$
	Stayer	0.079	0.509	2.17×10^{-6}
Leaf 4	Mavras	0.00011	0.055	0.00027
	Stayer	8.37×10^{-12}	0.00484	0.081

Discussion

We are among the first groups accessing the amount of graveobioside A in pepper leaves [235]. For cynaroside, the range of values detected corresponds to the results of other studies [262,263].

Stress-Related Effect Varies According to Secondary Metabolites and Cultivars

Since cynaroside contents under single UV-stress and combined UV- and salt-stress are not significantly different (Fig. 9 A, B), cynaroside accumulation appears to be triggered mainly by high radiation conditions. Interestingly, and in contrast to cynaroside, graveobioside A accumulation is triggered more effectively by salt stress than by UV-stress, especially in the cultivar Mavras (Fig. 9 C). This is a surprising result, since biosynthesis of flavonoids is said to be enhanced similarly by UV radiation and salinity [246,264]. On the other hand, some authors report that the regulation of SM production in response to salt stress differs between salt-sensitive (upregulation) and salt-tolerant (downregulation) plants [243]. However, differences in salt-stress tolerance between the cultivars used in this study are not supported by differing plant biomasses (Fig. 10). The chemical group of flavonoids is highly diverse, and metabolic pathways are not entirely understood to date. At this point, it remains unclear how exactly upregulation of cynaroside synthesis under UV stress and upregulation of graveobioside A synthesis under salt stress occurs.

Our results indicate – as expected – that salt-stressed plants acquire a significantly lower biomass than both control plants and UV-stressed plants. Stunted growth is a well-described symptom of severe salt stress in plants [243,265]. If the applied salt concentration would have been lower, negative effects could probably have been avoided to a certain extent, as recently

discussed in a review on the potential of seawater use in soilless culture [244]. Reaction of plants to UV-B exposure varies from growth reduction to enhancement, depending on species, cultivar, and stress level [242,266]. Since the overall aim of the stress application is the accumulation of higher amounts of secondary metabolites in the plant's green biomass, it is necessary to consider not only the share of desired metabolite in the plant´s biomass, but also the biomass reduction caused by the treatment. Considering this background, we can state that stressors with minor negative effects on plant biomass accumulation, but major positive effects on contents of desired metabolites in the plant tissues, are necessary to achieve these aims. Finding the perfect trade-off between biomass and fruit yield loss, on the one hand, and SM increase, on the other hand, will be crucial to improve the production system. In our specific setup with two single stressors and one combined stress, with respective levels of stress described above, the single UV stress is most promising, whereas salt stress (100 mM NaCl), although promoting the accumulation of graveobioside A, is less promising as a tool to enhance whole plant SM amounts, due to the decrease in total biomass. Effects on plants grown over a whole season are a matter of ongoing research.

Non-Invasive Monitoring

The indices provided by both optical devices deliver better estimates for leaf graveobioside A contents than for leaf cynaroside contents. That is an expected result, since the amount of graveobioside A as determined via HPLC is up to ten-fold higher than the amount of cynaroside (0–4 versus 2–40 mg g^{-1}) and both secondary metabolites share similar optical properties. Any estimate of concentrations based on non-invasive, optical devices will be best for the predominant fraction of a group of metabolites with similar optical properties. By the same token, signals of metabolites that occur in small quantities are more likely to be superimposed by other signals and therefore difficult to quantify. Additional factors known to influence non-invasive assessment of leaf compounds include the concentration of other pigments potentially influencing the measurement [267], leaf thickness [192], and the device used [268]. In our study, the FLAV-index of the Multiplex shows an almost linear response to changes in leaf graveobioside A content (Fig. 11 B). The same applies for the NBI_R index, which correlates negatively with the actual graveobioside A content. Both indices use the far-red fluorescence of leaves excited with UV-light and normalize that signal for the red fluorescence emitted after excitation with red light [206]. As an enhanced graveobioside A content leads to a stronger absorption of UV light in the leaf epidermis, less radiation penetrates into the mesophyll, which in turn leads to a lower chlorophyll fluorescence. We have to highlight the broad distribution of fluorescence values, though, which prohibits a precise prediction of actual graveobioside A

levels on the individual leaf level. The Flav-index of the Dualex is almost indifferent to changes at graveobioside A levels above 25 mg g^{-1}. None of the indices is strongly related to the leaf cynaroside contents quantified by HPLC. Neither the Dualex nor the Multiplex provide any indices that allow to quantify cynaroside contents higher than approximately 1 mg g^{-1} dry weight. An exact evaluation of high levels of this specific SM in bell pepper leaves is therefore not possible with the tested devices. However, the correlations we have identified between the FLAV index and HPLC measurements still allow us to analyze the gradual changes in SM contents as they occur during the prolonged period of stress.

Insights in Spatial and Temporal Accumulation of Secondary Metabolites

The usage of non-invasive phenotyping tools such as the Multiplex and Dualex devices allows to analyze leaf constituents during ontogenesis. The observed drop of the flavonol content in leaves of unstressed plants during ontogenesis (Fig. 12 C, D) is in line with the theories that (a) the production of phenolics, such as flavonols, is mainly caused by photodamage [269] and (b) that ontogenetically young leaves are, in general, more prone to be affected by high light stress than older leaves, since their photosynthetic apparatus is not yet well developed [270] and the photoprotective cuticula is thinner compared with older leaves [271]. Therefore, young leaves show stress-related reactions in conditions that are neither stressful for older leaves nor for the entire plant. However, the described ontogenetic effects tend to be overcompensated by stress-related effects in all three stress treatments (Fig. 12 C, D). Thus, flavonol contents of the fourth leaf as measured with the FLAV (Mx) index slightly increased in plants experiencing single stresses, while plants exposed to combined stress showed major increases in leaf flavonol contents (Fig. 12 C, D).

Implications and Future Challenges

The present study proves that abiotic stresses, in particular, salt stress and UV stress, can enhance the amount of economically valuable SMs, namely cynaroside and graveobioside A, in bell pepper leaves. The main objective of growing bell pepper plants, however, is the production of fruits of adequate quantity and quality for human nutrition. Considering the decline in plant biomass in response to stress conditions, it is very likely that the stressors applied would also lead to a reduction in fruit production. Severe salt stress, in particular, is known to be an important factor limiting crop productivity [272]. We have shown that the type of stressor has magnificent effects on both plant biomass and leaf secondary metabolite content. Other studies have proven that this also applies for different levels of abiotic stress [245,273]. The search for the best stressors and stress levels for the accumulation of secondary metabolites in plant

leaves with negligible effects on fruit yield is a major future challenge for research in stress physiology. Several authors reported neutral or positive responses of product quality to mild stress [273]. For salt stress, several studies in the model-crop tomato reveal positive impacts of mild stress on fruit quality (e.g., antioxidant capacity and nutritional value) [274,275]. Low UV radiation reduces the antioxidative capacity and, therefore, the fruit quality of bell pepper fruits [276]. Additional UV radiation may help to overcome this problem and, at the same time, induce the production of valuable SM in the leaves. Cultivation of plants under mild water stress conditions can also enhance water use efficiency. To avoid any competition with food production, post-harvest treatment of leaves could be an appropriate measure to achieve high contents of promising metabolites [277,278]. These effects should also be taken into account when evaluating the value of production systems that are based on commercialization of both fruits and SMs in leaves of horticultural plants. To enhance precision of non-invasive estimation of SMs in pepper leaves, future studies should consider hyperspectral sensors as well as chlorophyll fluorescence-based sensors, ideally a combination of both. Sensors covering the UV range are just entering the market and appear as a promising tool to access SMs in plants, as they cover absorption bands of flavones and other phenolic leaf compounds [19].

Conclusions

Both additional UV light and salt stress can enhance concentrations of the two SMs graveobioside A and cynaroside in bell pepper leaves. Highest concentrations were reached by combining both treatments. Stressed bell pepper leaves contain up to 30 mg graveobioside A and about 2 mg cynaroside per gram dry weight. While salt stress (100 mM NaCl) has a major negative impact on plant vegetative growth, UV stress (UVA 4–5 W m−2; UVB 10–14 W m−2; 3 h per day) has no significant impact on the fresh mass of the plants. The tendency of decreasing SM contents in leaves during ontogenesis is outweighed by the stress treatments. Graveobioside A contents can be assessed with the multiparametric fluorescence sensor Multiplex. Reliable quantification of cynaroside is not possible with the non-invasive sensors used. If future experiments exclude major negative impacts on fruit quality, UV stress can be recommended as one tool to enhance valuable SMs in bell pepper leaves and potentially in vegetable leaves in general. A less-intense salt stress should also be considered in future experiments.

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5. Topic-related Collaborations

During my research in the last years I had the opportunity to work in different constellations with various scientists and to contribute to their works. In addition, we have presented part of my work not in scientific journals, but in the context of conferences. This chapter is dedicated to a part of these works, and contains besides this short introduction the abstracts of the different papers.

The first work presented here, “Eco-friendly tomatoes: saving water and nutrient resources?”, is a conference paper in which some work within the EU-project TOMRES, of which I was a part during my PhD studies, is presented. Using the tomato as a scientific model organism and economically important crop in one, the project investigated how water and nutrient consumption in European agriculture can be reduced. Our work in the project context consisted primarily of sensor-based phenotyping of young tomato plants in the vegetative stage. Our goal was to identify, as early as possible, promising genotypes that would thrive under reduced water and nutrient inputs.

Another topic-related work I was privileged to contribute to, "Boosting leaf contents of rutin and solanesol in bio-waste of *Solanum lycopersicum*", led by Simone Röhlen-Schmittgen, is a paper with a bio-economic focus. Similar work to that done on bell pepper plants in the fourth chapter of this thesis is described here with a focus on tomato plants under commercial conditions. Plants were also subjected to abiotic stress (in this case, including nutrient deficiency and salt stress), and the effect on plant physiology was investigated using non-invasive sensors, among other techniques. Parallel laboratory studies also allowed quantification of two plant secondary metabolites relevant in human medicine.

Finally, the last work I would like to present here shows once again what a broad field phenotyping is. In the paper "Effect of postharvest irradiation with red light on epidermal color and carotenoid concentration in different parts of tomatoes", led by Lachinee Panjai, non-invasive sensors were used to document the effect of red light on the ripening process of tomato fruits after harvest. In fact, the detection is possible with the Multiplex, a device that we have seen in the course of this dissertation can also be used to determine the chlorophyll content in plant leaves quite confidently. And even when observing the ripening process of tomato fruits, it is ultimately the chlorophyll degradation in the fruit that provides the highest information content.

Eco-Friendly Tomatoes: Saving Water and Nutrient Resources?[4]

Authors

Jan Ellenberger, Simone Schmittgen, Hannah Jaenicke and Georg Noga

Abstract

Nutrient and water availability as well as sustainable use of resources are of high importance for modern horticulture aiming toward optimal and sustainable food production, particularly in arid and nutrient-poor areas. This also applies to the production of tomato, one of the world's most favored fruit crops, for which water and nutrient availability will be restricted due to climate change effects and increasing intensification of agricultural production. In the EU funded project TOMRES (www.tomres.eu), 25 partners from 10 countries work together on the optimization of tomato plants coping well with reduced nitrogen, phosphorus and water availability to ensure sustainable resource utilization at minimal yield and fruit quality loss. A collection of tomato lines is being screened to select most promising genotypes for more detailed analyses. Plant performance is being evaluated both under optimal and stress conditions, from the beginning of active vegetative growth to flowering and fruit production, also considering shoot to root zone interactions. Management strategies and decision-making tools will be established to support farmers, inform customers and demonstrate innovative scientific approaches in horticultural research. In the context of our work package, we screened tomato genotypes at the vegetative stage of growth to identify effects of 50% reduced nitrogen, phosphorus and water supply on plant metabolism as well as on biomass accumulation. We found that first effects due to stress application were assessable by thermal detection, showing higher leaf canopy temperature in stressed and less well performing plants. Differences in composition of constituents were detected at later developmental stages by evaluation of hyperspectral leaf reflectance. Overall, the selection of promising genotypes as well as more resource-efficient management strategies can effectively contribute to improve sustainability of modern production processes.

4 Ellenberger, J.; Schmittgen, S.; Jaenicke, H.; Noga, G. Eco-Friendly Tomatoes: Saving Water and Nutrient Resources? In Proceedings of the Acta Horticulturae; International Society for Horticultural Science (ISHS), Leuven, Belgium, December 3 2020; pp. 273–280, doi: 10.17660/ActaHortic.2020.1297.37

Boosting Leaf Contents of Rutin and Solanesol in Bio-Waste of *Solanum lycopersicum*[5]

Authors

Simone Röhlen-Schmittgen, Jan Ellenberger, Tanja Groher and Mauricio Hunsche

Abstract

In tomato production, the accruing green biomass shows promising potential as source of health-promoting compounds, such as rutin and solanesol, that are of high interest due to their medicinal properties. Naturally, they accumulate in plants growing in suboptimal growing conditions, e.g. influenced by biotic and abiotic stressors. With the aim to evaluate the potential use of tomato residues as source, we analyzed both leaf metabolites during a complete cultivation cycle, while applying single and combined stresses practically realized in greenhouse production.

In the late season, contents of both metabolites were significantly enhanced by nutrient deficit in combination with 2 °C colder nights for 4 weeks and prolonged for in total 9 weeks. Particularly, higher solanesol contents were achieved by salt stress and elevated temperature after one week, even stronger when combined with drought. At harvest, stressed plants consist of less green biomass reducing the overall economic potential. However, practicable abiotic stresses should be considered as potential tool to induce the accumulation of beneficial compounds. Extracting profitable metabolites from the green biomass of the model crop tomato supports the overall goal to promote sustainable approaches in horticultural production.

5 Röhlen-Schmittgen, S.; Ellenberger, J.; Groher, T.; Hunsche, M. Boosting Leaf Contents of Rutin and Solanesol in Bio-Waste of Solanum Lycopersicum. *Plant Physiol. Biochem.* **2020**, *155*, 888–897, doi:10.1016/j.plaphy.2020.08.035.

Effect of Postharvest Irradiation with Red Light on Epidermal Color and Carotenoid Concentration in Different Parts of Tomatoes[6]

Authors

Lachinee Panjai, Simone Röhlen-Schmittgen, Jan Ellenberger, Georg Noga, Mauricio Hunsche and Antje Fiebig

Abstract

The aim of this study was to investigate the effect of red light irradiation during postharvest ripening with focus on the outer (epicarp and mesocarp) and inner (endocarp and seed) parts of tomatoes by evaluating concomitant alterations in bioactive compounds, such as lycopene, β-carotene, total phenolic and total flavonoid concentrations, external fruit color and spectral reflectance pattern, and the Simple Chlorophyll Fluorescence ratio. As promising measure, deriving from previous studies, green stage-1 tomatoes were harvested and treated daily with red light for 12 h per day, for 15 days (followed by storage in darkness for additional 6 days) or continuously radiated with red light for 21 days. Control untreated tomatoes were kept in the dark for the same period. Application of continous red light strongly accelerated changes in the outer layer of fruit, for example visible in color parameters. Significant differences between treatments were analyzed for major secondary metabolite compounds such as lycopene, β-carotene, total phenolic and total flavonoid in both outer and inner fruit layers. Continuous red light treatment led to the highest concentration of secondary metabolite compounds in all parameters. Therefore, it can be concluded that continuous red light radiation is the most effective treatment to accelerate the color development and ripening of the outer layer of the epicarp. Furthermore, it plays a role in stimulating the inner layer of the endocarp to provide beneficial secondary metabolite compounds.

6 Panjai, L.; Röhlen-Schmittgen, S.; Ellenberger, J.; Noga, G.; Hunsche, M.; Fiebig, A. Effect of Postharvest Irradiation with Red Light on Epidermal Color and Carotenoid Concentration in Different Parts of Tomatoes. *J. Food Meas. Charact.* **2021**, doi:10.1007/s11694-020-00770-0.

6. Conclusions

The following sections revisit the three challenges in plant phenotyping identified in the introduction, focusing on the degree to which the challenges have already been overcome and what is needed in the future to further improve plant phenotyping.

The challenge discussed last seems to be the easiest to overcome: To identify and reliably and non-invasively quantify various metabolites in plant leaves, it is necessary to use data that are based on the properties of the respective compound. We could also give indications that more precise results can be obtained when confounders are included in the measurement. Whether the additional work to increase precision is worthwhile, in the sense of economically rewarding, is not something we have addressed in this paper, although it should certainly be part of any future investigation on this topic. In addition, if the metabolite of interest is known to be part of the plants metabolic response to a specific stress, there is the possibility to quantify metabolites generated in other simultaneously triggered metabolic pathways known to be part of the plant's response to this specific stress. This indirect quantification can be particularly useful when the metabolite of interest is present only at low concentrations in the leaf and has no characteristic property (e.g. absorption of electromagnetic radiation of a particular wavelength) that sets it apart from the bulk of the other substances in the leaf. Although some of the sensors used were not designed for the purpose of quantifying secondary metabolites in plant leaves, and non of the sensors was designed to quantify the two very metabolites of interest, we were able to show that they are also suitable for this purpose. The presented study thus represents an example of a phenomenon that can be observed again and again in the phenotyping of plants: The fewest technologies used were originally developed for the purpose of plant phenotyping. Rather, devices and technologies are used for this purpose that were originally used in other contexts. A similar approach was used in the study led by Panjai mentioned above, where fruit ripening in tomato was accessed via chlorophyll breakdown, rather than the synthesis of ripeness-related metabolites. What is not necessary at this point, however, is a complete rethinking of the scientific methodological approach to quantifying a target metabolite. On the contrary, this approach is by and large the same, regardless of whether it is classically about the determination of chlorophyll contents or, as often seen in the context of bio-economy, about secondary metabolites of economic importance.

However, a fundamental rethinking of the methods usually applied may be part of the answer to the second challenge addressed, the influence of confounders on phenotypic data. Using chlorophyll data from non-invasive measurements as an example, we have seen that the measured values do not depend exclusively on the chlorophyll content of the leaves considered,

but on numerous other factors, some of which may even be unknown. A conceptual model for this specific context was designed in the corresponding chapter, but parameterizing and validating will be part of future work and include directed acyclic graphs. The approach used, namely building a conceptual model based on existing knowledge and own investigations, could be, and increasingly already is, a role-model for complex phenotype-genotype-environment interactions.

At the same time, as sensors, experimental methods, and mathematical models become more sophisticated, it is important not to neglect economics. Insights gained with more complex methods often come at the price of higher investments. Especially where sensor-based phenotyping is not used to gain scientific knowledge but, for example, to increase the operating result in agriculture, horticulture and viticulture, the inclusion of the economic component is indispensable. The science-driven question "How accurately can we measure something?" is being replaced in more applied areas by the question "How accurately do we need to know something?".

Finally, a short review of the challenge discussed in the third chapter. How quickly and how well the scientific community will succeed in transferring current and future phenotypic knowledge gained from *Arabidopsis* to crop plants will probably be determined mainly by two factors: One is, how much emphasis will be placed on making phenotyping protocols compatible between species. Interdisciplinary teams of agronomists, biologists, engineers and data scientists should work together to design phenotyping efforts in a way that maximizes value for all scientific disciplines, but ultimately and most importantly agricultural production. First steps in that direction are being made, interdisciplinary research teams to tackle challenges in plant phenotyping are formed and the way these teams are managed will determine how useful their outputs are going to be. At this stage, data management is still a major challenge, as it does not always meet the FAIR (Findable, Accessible, Interoperable, and Reusable) [279] requirements.

Acknowledgments

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