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# Suitability of non-destructive sensors for monitoring physiological and biochemical responses of tomato leaves and fruits to abiotic stresses

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**M.Sc. Tanja Groher**
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**Referent:** **Herr PD Dr. Mauricio Hunsche**

**Korreferent:** **Herr Prof. Dr. Uwe Rascher**

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# Suitability of non-destructive sensors for monitoring physiological and biochemical responses of tomato leaves and fruits to abiotic stresses

The major aim of the present work was to proof the suitability of chlorophyll fluorescence-based indices to non-destructively monitor and predict changes in the content of plant compounds in response to abiotic stresses. Simultaneously, another objective was to generate knowledge about the potential of tomato leaves from commercial production systems for the extraction of industrially relevant secondary metabolites, particularly rutin and solanesol, which are known to accumulate under abiotic stress conditions. For this purpose, tomato plants were grown under moderate abiotic stress treatments to induce the accumulation of the compounds of interest without lowering fruit yield as primary aim of commercial production. Plants' stress responses were monitored non-destructively with a hand-held multiparametric fluorescence sensor. First, a nitrogen and a general nutrient deficiency was applied to achieve changes in leaf compounds of different development stages. To prove the suitability of the fluorescence-based sensor to track changes in leaf compounds, physiological responses, which were recorded non-destructively, were compared to the biochemical parameters determined by means of HPLC analyses. Second, we hypothesized that supplementary light-emitting-diodes (LED) in the blue and red regions promote the accumulation of rutin in tomato leaves. Here again, fluorescence-based recordings and laboratory HPLC analyses were undertaken on young and mature leaves and the relationships between both were evaluated. Third and last, we examined the suitability of the multiparametric sensors to monitor and estimate concentrations of tomato fruit maturity compounds during ripening under a mild nitrogen and water deficiency in the greenhouse. The fluorescence-based indices were compared to the well-established reflection-based ripening index a*/b*. In summary, the following results were achieved in each chapter of this thesis:

1. The fluorescence-based indices SFR_R, NBI_G, FLAV and ANTH_RG were suitable to monitor differences in young leaves between plants grown under control conditions (full standard nutrient solution), nitrogen deficiency (standard nutrient solution without N-containing compounds) and a general nutrient deficiency (tap water). However, no differences were observed in mature leaves. The FLAV index, representing a parameter for polyphenol assessment, was not reliable to estimate rutin or solanesol concentrations non-destructively in tomato leaves. Nitrogen deficiency as well as a general nutrient deficiency led to an accumulation of rutin in young leaves, whereas solanesol concentration was higher in fully developed mature leaves. Fruits showing symptoms of blossom-end-rot were seen more frequently in plants exposed to a general nutrient deficiency.

2. Tomato plants exposed to supplementary LED light (red and blue in a ratio of 4:1) showed stress-related differences for the chlorophyll fluorescence-based indices SFR_R and FLAV in leaves of all investigated development stages, while the indices NBI_G and ANTH_RG were limited to more mature leaves. Supplementary LED light induced higher rutin concentrations mainly in young leaves and partly in mature leaves. Correlation analyses between FLAV index and rutin concentrations could not show a precise relationship between both parameters but clusters according to the leaf age and the time point of harvest.

3. Fluorescence-based indices and the reflection index a*/b* are suitable to monitor an acceleration of tomato fruit ripening for fruits grown under water deficit. Further, chlorophyll concentration was estimated non-destructively since the coefficient of determination between the SFR_R and the chlorophyll concentration determined analytically was very high with $r^2 = 0.84$. Moreover, the single signal FRF_G and the lycopene concentration showed a coefficient of determination of $r^2 = 0.81$ even if a precise differentiation between the maturity stages two (breaker) to four (pink) was not observed. Compared with fluorescence-based indices, the relation between the reflection index and pigment concentration was lower for chlorophyll ($r^2 = 0.74$) and higher for lycopene ($r^2 = 0.94$). The chlorophyll decrease was the driving force affecting all fluorescence signals. In consequence, an estimation of other maturity/quality compounds with chlorophyll fluorescence-based recordings, such as flavonoids with FLAV or FLAV_UV, is not appropriate.

# Eignung von nicht-destruktiven Sensoren zur Erfassung von physiologischen und biochemischen Reaktionen von Tomatenblättern und –früchten auf abiotischen Stress

Das Hauptziel der vorliegenden Arbeit war es, die Eignung chlorophyllfluoreszenz-basierter Indizes zur Erfassung und Abschätzung von Veränderungen in Blatt- und Fruchtinhaltsstoffen an Tomaten unter abiotischem Stress zu prüfen. Darüber hinaus war es ein weiteres Ziel, das Potential von Tomatenblättern aus der kommerziellen Gewächshausproduktion zur Extraktion von sekundären Inhaltsstoffen (Rutin und Solanesol) für die industrielle Nutzung zu evaluieren, da bekannt ist, dass diese durch abiotischen Stress gesteigert werden können. Zu diesem Zweck wurden Tomatenpflanzen moderaten Stressbedingungen ausgesetzt, um die Akkumulation der relevanten Stoffe zu induzieren ohne den Fruchtertrag, als primäres Ziel der Tomatenproduktion, zu beeinträchtigen. Physiologische Pflanzenreaktionen wurden nicht-destruktiv mittels eines multiparametrischen Fluoreszenzsensors gemessen. Zum einen wurden die Pflanzen einem Stickstoff- und generellem Nährstoffmangel ausgesetzt, um Veränderungen in den Gehalten von Rutin und Solanesol in Blättern verschiedener Entwicklungsstufen zu erreichen. Nicht-destruktiv erfasste physiologische Unterschiede wurden mit biochemischen Parametern verglichen, um die Eignung des multiparametrischen Fluoreszenzsensors für die Erfassung von Inhaltsstoffveränderungen zu prüfen. Zum anderen wurde angenommen, dass der Einsatz von zusätzlicher Beleuchtung mit lichtemittierenden Dioden (LED) im roten und blauen Lichtspektrum die Akkumulation von Rutin in Tomatenblättern fördert. Auch hier wurden chlorophyllfluoreszenz-basierte Messungen und nass-chemische Analysen an jungen und voll entwickelten Blättern durchgeführt und der Zusammenhang der Resultate untersucht. Darüber hinaus wurde in einem dritten Versuch die Eignung des multiparametrischen Sensors für die Erfassung und Abschätzung von Fruchtreifeparametern während der Tomatenreife unter einem milden Stickstoff- und Wasserdefizit im Gewächshaus getestet. Die fluoreszenz-basierten Messungen wurden mit dem bewährten reflektions-basierten Reifeindex a*/b* verglichen. Im Folgenden sind die Ergebnisse der einzelnen Kapitel der Arbeit zusammengefasst:

1. Die chlorophyllfluoreszenz-basierten Indizes SFR_R, NBI_G, FLAV und ANTH_RG zeigten Unterschiede in jungen Blättern zwischen Pflanzen, die unter Kontrollbedingungen (Standardnährlösung), Stickstoffmangel (Standardnährlösung ohne N-haltige Verbindungen) und einem generellen Nährstoffmangel (Leitungswasser) kultiviert wurden, nicht jedoch in alten Blättern. Der FLAV Index, als Parameter zur Polyphenolabschätzung, erwies sich als nicht geeignet für eine präzise Abschätzung des Rutin- oder Solanesolgehaltes in Tomatenblättern. Ein Stickstoffmangel sowie ein genereller Nährstoffmangel führte zu einer Akkumulation von Rutin in jungen Tomatenblättern, wohingegen höhere Solanesolkonzentrationen in voll entwickelten Blättern erreicht wurden. Früchte mit Blütenendfäule waren häufiger an Pflanzen zu finden, die einem generellen Nährstoffmangel ausgesetzt waren.

2. Tomatenpflanzen, die zusätzlichem LED Licht (rote und blaue LEDs im Verhältnis von 4:1) ausgesetzt waren, zeigten stressinduzierte Veränderungen in den fluoreszenz-basierten Indizes SFR_R und FLAV in allen untersuchten Blattaltern, während die Indizes NBI_G und ANTH_RG auf ältere Blätter begrenzt waren. Zusätzliche LED Beleuchtung erhöhte die Rutinkonzentration hauptsächlich in jungen Tomatenblättern und nur teilweise in alten Blättern. Die Korrelationsanalysen zwischen der Rutinkonzentration und dem FLAV Index zeigten keinen präzisen Zusammenhang beider Parameter, jedoch konnten deutliche Muster in Abhängigkeit von Blattalter und Erntezeitpunkt festgestellt werden.

3. Chlorophyllfluoreszenz-basierte Indizes konnten zuverlässig eine beschleunigte Reife von Tomatenfrüchten, die einem Wasserdefizit ausgesetzt waren, abbilden. Die Chlorophyllkonzentration konnte nicht-destruktiv mittels des SFR_R Index abgeschätzt werden, da ein Zusammenhang mit der nass-chemischen Referenzanalyse von Chlorophyll festgestellt werden konnte ($r^2 = 0.84$). Darüber hinaus zeigte sich ein Zusammenhang zwischen dem FRF_G Signal und dem Lykopingehalt ($r^2 = 0.81$), auch wenn eine exakte Differenzierung zwischen den Reifestadien „breaker“ bis „pink“ nicht sichtbar war. Im Vergleich zu fluoreszenz-basierten Indizes zeigte der Reflektionsindex a*/b* eine geringere Korrelation mit dem nass-chemisch analysierten Chlorophyllgehalt ($r^2 = 0.74$) und eine höhere Korrelation mit dem analysierten Lykopingehalt der Früchte ($r^2 = 0.94$). Der Abbau von Chlorophyll während der Tomatenreife beeinflusste alle Fluoreszenzsignale, sodass eine Abschätzung anderer Qualitäts- bzw. Reifestoffe, wie Flavonoide mittels FLAV oder FLAV_UV, nicht möglich war.

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# List of abbreviations

| | |
|---|---|
| % | percent |
| °C | degree Celsius |
| a* | red to green |
| ANTH_RG | anthocyanin index |
| b* | blue to yellow |
| BER | blossom end rot |
| BFRR_UV | ultraviolet excitation ratio of blue-green and far-red chlorophyll fluorescence |
| ca. | circa, around, about |
| cv. | cultivar |
| DaH | days after first harvest |
| DAT | days after treatment initiation |
| DW | dry weight |
| e.g. | exempli gratia, for example |
| EC | electrical conductivity |
| et al. | et alii (m.), et aliae (f.), and others |
| FLAV | flavonol index, decadic logarithm of the red to ultraviolet excitation ratio of far-red chlorophyll fluorescence |
| FLAV_UV | flavonol index without FRF_R as reference signal |
| FRF_G | far-red fluorescence excited with green light |
| FW | fresh weight |
| g | gram |
| h | hours |
| HPLC | high performance liquid chromatography |
| kg | kilogram |

| | |
|---|---|
| klx | kilolux |
| L | liter |
| L* | lightness |
| LED | light-emitting-diode |
| m | meter |
| mg | miligram |
| min | minutes |
| Mio. | Millions |
| mL | mililiter |
| n | number of replications |
| n.s. | not significant |
| NBI_G | nitrogen balance index excited with green light |
| NBI_R | nitrogen balance index excited with red light |
| nm | nanometer |
| p | probability of error |
| PAM | pulse amplitude modulated chlorophyll fluorescence |
| pH | pondus Hydrogenii |
| PPFD | photosynthetic photon flux density |
| $r^2$ | coefficient of determination |
| rpm | rounds per minute |
| SE | standard error |
| SFR_R | simple fluorescence ratio excited with red light |
| SM | secondary metabolites |
| UV | ultraviolet |

# A Introduction

## 1. Sustainable utilization of green biomass by-products in agriculture

Agricultural biomass ranks among the cheapest and most abundant renewable resources worldwide and represents a natural source of high-value secondary metabolites (SM) (1). These metabolites help the plant to adapt to environmental conditions, and also have beneficial effects on human health, making them a promising source of bioactive pharmaceuticals (2). The utilization of by-products from agricultural and horticultural cultivation for e.g. the extraction of SM would contribute to a more sustainable and ecologically friendly agriculture. Currently, green biomass is often considered as waste product in commercial greenhouse and fruit production systems and is mainly used for composting or as heating material.

Land availability and water saving production systems are strong determinants for modern agriculture. By using waste and by-products from food production for e.g. the extraction of SM, less agricultural land has to be occupied for the cultivation of medicinal plants and energy-consuming chemical synthesis of the desired substances would be reduced or avoided. In the case of tomato cultivation, greenhouse production can contribute to save water resources as it is known that water requirements for producing 1 kg fresh tomatoes in greenhouses is only a quarter of the water volume needed for field production (3). Besides ecological reasons, the utilization of green biomass from agricultural and horticultural cultivation would lead to an additional value for the farmers. Even though the utilization of agricultural by-products has been the focus of several studies (4–7), there is still a gap of knowledge regarding the real potential of SM from natural resources accruing from commercial production forms.

## 2. The potential of tomato as green-biomass utility

The Solanaceae family includes many economically important plant species such as potato, eggplant, tobacco, petunia, pepper and tomato (8). Tomato belongs to the

*Solanum* genus, is native in South America and was domesticated in Mexico for the first time. In the mid of the 16th century, it was introduced in Europe (9). Botanically, tomato is a fruit berry which is usually referred to as vegetable. Nowadays, tomato (*Solanum lycopersicum* L.) is one of the most important vegetable worldwide, consumed unprocessed or as soup, paste or concentrate (8). In 2016, about 177 Mio. tons tomatoes were produced worldwide with 85,287 tons in Germany (10). Under protective cultivation, the area of tomato production in Germany comprised about 332 ha in 2013, representing only 2% of the world-wide tomato production. (11).

**Figure 1.** Tomato plants grown in a commercial-like greenhouse. The oldest leaves were pruned to allow higher light exposition of the maturating fruit trusses in the lower part of the canopy.

In countries such as Holland, Belgium and Germany, under highly-intensified protected greenhouse cultivation, plants might reach 15 or more meters of stem length during a growing period that lasts from winter-to-autumn or winter-to-winter. In these high-input systems, large amounts of biomass arise in form of discarded fruits, pruned leaves during the growing period, and stems at the end of the cultivation. Especially the leaves of tomatoes contain several secondary metabolites which are so far commercially unused (12).

The focus of green biomass utilization for SM extraction is the major objective of the BioSC project "InducTomE" (Induction of secondary metabolites in tomato by-products for extraction and economic evaluation of the model process) (13). The project aims at the utilization of the secondary metabolites rutin and solanesol extracted from tomato leaves to achieve an added value for tomato producers and industrial purposes. The interdisciplinary project considers the most important steps for commercial implementation. This includes the identification of key genes involved in the biosynthetic pathway, the phenotyping and non-destructive assessment of suitable stress treatments for the accumulation of SM, the examination of practical greenhouse applicability, the extraction process as well as the evaluation of the market potential. However, for an economically viable application in commercial-like greenhouses, a precise knowledge of determinants influencing the biosynthesis of secondary metabolites in tomato leaves is necessary to achieve consistently high concentrations. This includes (optimum) abiotic conditions, time point of harvest, physiological age of plants as well as the variability within the leaf position.

# 3. Valuable secondary metabolites in plants

Secondary metabolites have quite a large scope of applications including pharmaceutical use, as food ingredients or in cosmetics (6, 14). A well-known example from the pharmaceutical sector is salicylate acid, a precursor molecule and the basis for the industrial production of acetylsalicylate, popularily known as aspirin (2). In recent years, research in evaluating, recovering and reusing natural components of by-products from agricultural production has gained increased interest (1, 14, 15). At least in Europe, this activity will become even more important in the future in the scope of the broad strategies to improve the Circular Economy.

Secondary metabolites can be distinguished in different groups according to their chemistry and biosynthetic pathways. The three major groups include phenolics, alkaloids and isoprenoids (the latter also known as terpenoids) which, in turn, are further divided to several subgroups (16). Phenolics are abundant in all higher plants because of their involvement in lignin synthesis, whereas other metabolites such as alkaloids are more limited to specific plant species (2). Biosynthesis of these compounds is a complex

process even if the precursors derive from the basic metabolic pathways as the shikimate pathway, the Calvin cycle and the glycolysis (17).

In the present study, the flavonoids as well as the group of isoprenoids are the main groups of interest. Flavonoids, omnipresent in the plant kingdom, are phenolic compounds and one of the major plant-based dietary constituents (18). Flavonoids are characterized by their general chemical structure (C6-C3-C6 backbones) including two C6 units with a phenolic form. According to Tsao (2010), they can be classified further into sub-classes according to the variation in the heterocyclic C ring. Anthocyanins, flavonols, flavones, flavanones and flavonols are the most common subgroups but isoflavones, neoflavonoids and chalcones are members of the flavonoid family as well (see Tsao 2010 for a detailed classification overview (19)).
Similarly, plant-based isoprenoids occur in a wide variation and structure. They are classified according to the number of presented isoprenoid (C5) units (Monoterpenoids, Sesquiterpenoids, Diterpenoids, Sesterterpenoids, Triterterpenoids, Tetraterpenoids and Polyprenols) which are further sub-classified. For example, monoterpenoids represent the simplest group of isoprenoids with 2 isoprene units (C10), whereas polyprenols consist of 40 or more carbon atoms (16).

## 4. Influence of abiotic stress on secondary metabolites in leaves

The composition of SM in plants depends on various factors such as the tissue and organ, the developmental stage of the plants and organs and also on individuals and populations. The function of SM in plants, although not fully understood, comprises an involvement in the adaption to adverse environmental conditions, the protection against pathogens as well as indirect influences on pollination due to the attraction of animals with signal colors. The latter help the plant to cope with their environment and enemies (20). For example, colored molecules are being used to attract animals for pollination and, at the same time, compounds might act as allelochemical protection against herbivores in unripe fruits (2, 20).

Further, secondary metabolites are involved in the plants' adaption to changes in their abiotic environment and, as consequence, their synthesis and accumulation often increase in response to abiotic stresses (20). For example, it is known that the concentrations of

SM are increasing under unfavorable environmental conditions such as intensive light, nutrient deficiency or salt stress (21). The present study is focusing on the influence of abiotic stress on the two components rutin, a flavonol, and solanesol which belongs to the group of isoprenoids.

## 4.1 Rutin

Chemically, rutin is a flavonol glycoside which is also known as vitamin P. The rutin molecule consists of quercetin and the disaccharide rutinose (rhaminose and glucose) (Fig. 2). When plants are exposed to stress, peroxidases (e.g. rutin glucosidase) might be activated, resulting in the separation of quercetin and rutinose. Quercetin is further used as a substrate for guaiacol peroxidases while rutinose is used for respiration as carbohydrate source. Hence, plants are protected against oxidation by increasing respiration rate (18, 22).

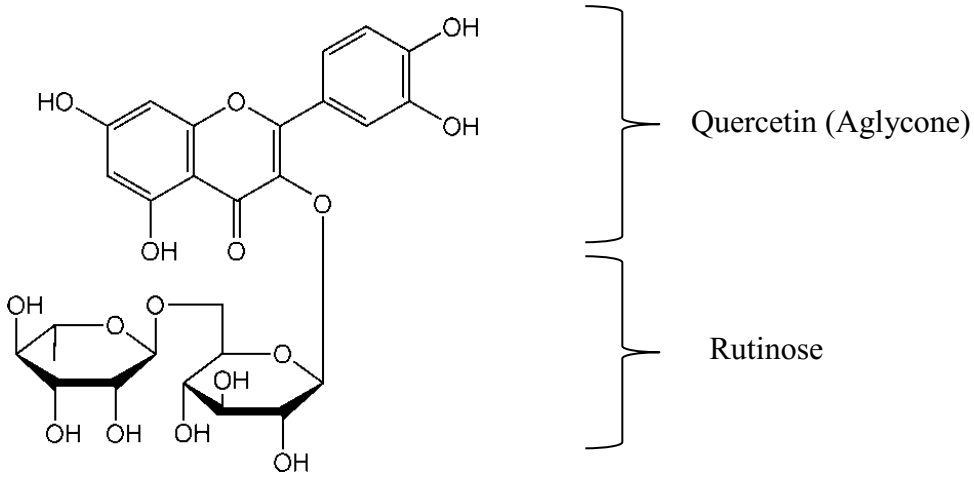


**Figure 2.** Rutin molecule (quercetin-3-O-rutinoside) (18).

From different perspectives, rutin has been in the focus of research. In this context, several studies investigated its activities as free radical scavenger and coupled with this, anti-bacterial, anti-inflammatory characteristics or its role in tumor defense (23, 24). Furthermore, the antioxidant capacity of rutin contributes to its application in the pharmaceutical and cosmeceutical sectors as natural stabilizer, colorant and preservative (25). Thus, the positive properties of rutin have resulted in a great interest in its natural production. Besides the fact that rutin was first detected in *Ruta graveolens* (26), it is

present in a wide range of plants, quantitative mainly in buckwheat but also in tobacco (27) and tomato (28). Nowadays, buckwheat is one of the most important sources of rutin although its concentration depends on several factors (29) including environmental conditions and varietal differences (30). In addition, the cultivar (31) and the plant organ (32) are determinants for the natural concentration of rutin. Suzuki et al. (2005) (33) showed that also solar radiation and day length as well as UV-B light, temperature and desiccation stress influence the rutin concentration in buckwheat. Furthermore, it has been widely shown that phenolic compounds such as flavonoids absorb UV-light, resulting in a higher tolerance against UV-light in plants which are able to synthesize these compounds (34, 35). In addition, Peng et al. (2008) (36) assumed that the flavonoid pathway had an impact on tolerance to nitrogen depletion in *Arabidopsis thaliana* even if the whole process is not fully understood. In a study with nine different tomato cultivars, a 2.5-fold increase in the content of leaf flavonoids was observed under reduced nitrogen supply in a glasshouse (37). Also, in young tomato plants grown in a controlled climate chamber, a combination of nitrogen deficiency, intensive light and low temperatures led to highest quercetin derivative concentrations in leaves whereby as single treatment, light radiation had the strongest impact on the concentration of quercetin derivates (28).

Especially the presence of single-spectra light in form of light-emitting-diodes, e.g. in the red or blue spectrum and the combination of both, led to an accumulation of secondary metabolites such as polyphenols and flavonoids (38). Spectral light properties can be adapted to the plant photoreceptors since most of the commonly used supplemental lighting sources apply a continuous range from 350 to 750 nm; this includes also wavelengths that are considered to be inefficient or less used by plants (39, 40). Although the impact of LED on horticultural plant development and metabolism under different light combinations was highlighted for some plant species (40), studies on the simultaneous effect of LED on the content of secondary metabolites in leaves are still missing.

## 4.2 Solanesol

Solanesol is a secondary metabolite belonging to the sub-group of isoprenoids. The molecule is a non-cycle terpene alcohol which consists of nine isoprene units (Fig. 3) (41). The chemical synthesis of solanesol is difficult, thus natural material of the

Solanaceous crops, particularly tobacco, is mainly used for extraction (7). Solanesol has its main application in the pharmaceutical and cosmetic sector. It is an intermediate for the synthesis of ubiquinone-based drugs and supplements for example in the anti-cancer synergizer N-solanesyl-N,N0-bis(3,4-dimethoxybenzyl) ethylenediamine (SDB), in coenzyme Q10 or as analogous of vitamin K (41, 42).

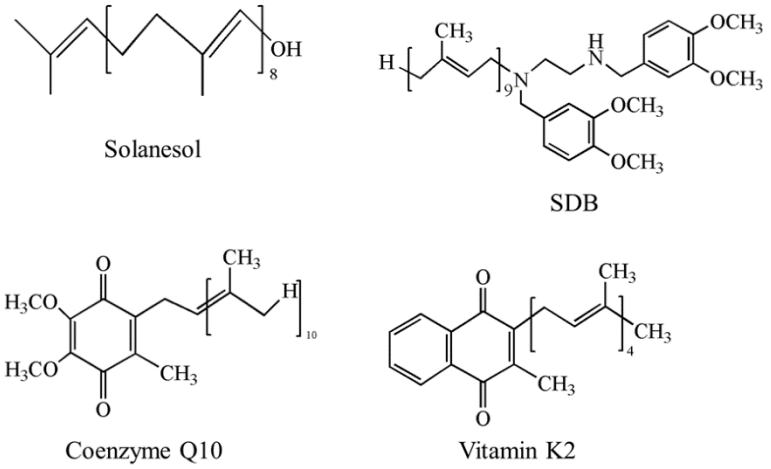


**Figure 3.** Solanesol and some of its derivatives (41).

Several plant species produce numerous highly-specific isoprenoids that play important roles in plant–environment interactions. Species containing significant concentrations of solanesol include potatoes, eggplants, peppers, tomatoes and tobacco, whereby the latter has the highest concentrations (41). As known from tobacco, the solanesol concentration is dependent on diverse factors such as the plant organ (43), the genotype and leaf age as well as environmental conditions such as drought, nitrogen fertilization, and irradiation (44). To exemplify, Burton et al. (1989) (44) found a 5-fold difference in solanesol concentration depending on the genetic line and a 4-fold increase in response to lower soil-moisture. However, previous studies on the impact of leaf age are controversial; while Burten et al. (1989) (44) showed highest solanesol concentrations in old leaves, Zhao et al. (2007) (43) found decreasing concentrations with decreasing leaf age in tobacco. In potato, the temperature is another important factor affecting solanesol concentration; Campbell et al. (2016) (4) observed a more than 6-fold increase in response to higher temperature (30°C day/20°C night) as compared to lower temperature (22°C day/16°C night). Besides the environmental and physiological parameters

influencing solanesol concentrations in the tissues, the extraction method after harvesting also affects the purity and concentration of solanesol (7).

## 5. Effect of abiotic stress on fruits

Abiotic stresses, depending on the type and intensity, can result in either increase or decrease of yield and/or fruit quality. In the literature, there are several studies demonstrating the impact of abiotic stress on yield and quality of crops. For example, Wang and Frei (2011) (45) reviewed the impact of abiotic environmental stresses on harvested food products and summarized negative effects on crop quality such as lower starch and lipid concentration, deterioration of the feed value and physical and sensory traits on the one hand. On the other hand, a stimulation of protein and antioxidant concentration resulting in an improvement of quality were identified as positive effects of environmental stresses (45). Also, Hodges and Toiven (2008) (46) documented among others the effects of abiotic stress in the preharvest production of fruits and vegetables. Examples of how abiotic stresses can influence product quality are the targeted irrigation deficit in peaches which resulted in a higher content of soluble solids and coloration (47) or the increased content of abscisic acid, proline, sugar and anthocyanin concentrations in Cabernet Sauvignon grapes in responses to water deficit (48).

In the case of tomatoes, ß-carotene and lycopene are the main antioxidants which contribute to fruit quality and the intensive red color (49). During the ripening process of tomatoes, the surface area undergoes a significant color transformation from green to red, whereby the final red color is commonly associated with taste, health value and overall quality (50, 51). When exposed to stress conditions, plants produce antioxidants to protect themselves from free radicals and as consequence in tomatoes, the ripening process can be accelerated. This aspect was shown in a study of Hoffmann et al. (2016) (52) where a water restriction led to accelerated ripening of tomatoes receiving only 50% of water as compared to control fruits (52). On the contrary, irrigation with saline water up to 0.25% improved carotenoid content and antioxidative activity in tomatoes but decreased the red surface color, fruit size and water content (53). Another targeted abiotic stress is the limitation of nitrogen supply which resulted in a higher sugar content and partly phenolic compounds such as rutin, whereby tomato yield was only slightly decreased (54).

The application of artificial radiation is another tool to influence yield and quality of tomatoes. White and red LEDs enhanced fruit yield of a single-truss tomato production system by 12 and 14%, respectively (55). Jiang et al. (2017) (56) have shown that LED modules from underneath or within the inner canopy enhanced fruit yield, whereby the radiation within the inner canopy also promoted the content of soluble solids in tomato fruits (56). Additionally, the application of red light and a combination of red light with UV in the postharvest storage of tomatoes increased the concentrations of fruit maturity compounds such as lycopene, ß-carotene, flavonoids and phenolics (57).
These studies suggested, that the balance between positive effects and negative effects on fruit yield has to be considered when manipulating growth conditions and applying abiotic stress in horticultural production.

## 6. Non-destructive sensors for horticultural purposes

Research on non-destructive sensors received a strong interest in the field of food science and technology (58). The use of such non-destructive sensors has advantages since it is based on fast and easy-to-repeat techniques which can be used directly in the greenhouse or field. For research purposes, the use of sensors represents a time- and cost-saving alternative to laboratory analysis of plant constituents. Simultaneously, a large amount of data can be recorded during the measurements of single leaves or the whole canopy as well as monitoring one and the same sample (e.g. leaf, fruit) during the whole season (59). So far, non-destructive sensors enable proper information about plant stress responses and estimation of tissue components in leaves and fruits (52, 59, 60).
A well-known technique to monitor plant stress status is the determination of chlorophyll fluorescence. Devices such as the PAM fluorometer provide information on the photosynthetic apparatus, especially on the functionality of the photosystem 2 (61). Another approach to obtain stress-related differences in plants is the adoption of multiparametric chlorophyll fluorescence which provides less details on photosynthetic activity but allows estimation of plant chlorophyll and polyphenol contents. For example, multiparametric fluorescence-based parameters were established in viticulture to estimate the accumulation of phenolic compounds in the berry skin, allowing to optimize fertilization practice and to determine the best harvest date to reach highest quality (59, 62). In the experiments presented here, the applicability of this multiparametric

fluorescence-based sensor was examined for monitoring biochemical changes of tomato leaves and fruits.

## 6.1 Multiparametric chlorophyll fluorescence

The multiparametric fluorescence sensor used in this study (commercial name Multiplex®, FORCE-A, Orsay, France) records the fluorescence signal emitted by chlorophyll molecules after excitation with radiation of defined wavelengths. When light reaches a chlorophyll molecule, a part of the light is emitted at longer wavelengths which is further called chlorophyll fluorescence (60). The light sources of the sensor might irradiate the sample with either UV-A (375 nm), Blue (425 nm), Green (530 nm) and Red (630 nm) light. A filtered photodiode detects the fluorescence intensity in the Blue (425-475 nm), Red (680-690 nm) and Far-Red (720-755 nm) spectral regions. The chlorophyll content is estimated from the chlorophyll fluorescence in the far-red and red spectral region. The parameter Simple Fluorescence Ratio (SFR) is directly related to the chlorophyll concentration of the sample and considers the reabsorption of red light by the chlorophyll molecules.

Besides chlorophyll, polyphenols such as flavonols and anthocyanins can be assessed due to their screening effect of light: A reference excitation light, which is not absorbed by the polyphenols, generates far-red fluorescence which is compared to the chlorophyll fluorescence excited with light specific to the type of polyphenols. Polyphenols located in the epidermis, morphologically above the chlorophyll, absorb the excitation light (green light for anthocyanins (ANTH_RG) and UV-light for flavonols (FLAV)) and only a part of the light reaches the chlorophyll molecules and generates fluorescence (62), leading to a comparatively lower chlorophyll fluorescence. The Nitrogen Balance Index (NBI) is defined by the chlorophyll-to-flavonol ratio, calculated as the ratio of far-red-fluorescence excited with UV-light and far-red fluorescence excited with red light (60).

Several signals can be detected and used as basis for the calculation of specific parameters, and can be further improved or used for the development of different parameters. An overview of parameters used in this study is summarized in table 1.

**Table 1.** Description of the fluorescence-based parameter used in this study.

| Index | Calculation | Description |
|---|---|---|
| SFR_R | (FRF_R/ RF_R) | Simple Fluorescence Ratio excited with Red light |
| FLAV | log(FRF_R/FRF_UV) | Flavonol index |
| ANTH_RG | log(FRF_R/ FRF_G) | Anthocyanin index |
| NBI_G | (FRF_UV/RF_G) | Nitrogen Balance Index excited with Green light |
| FLAV_UV | Log(1/FRF_UV) | Flavonol Index without FRF_R as reference signal |
| FRF_G | FRF_G | Far-red fluorescence excited with Green light |

One example of a newly developed index is the FLAV_UV. The FLAV_UV relies on the inverse signal of far-red fluorescence excited with UV-light and was first introduced by Ferrandino et al. (63). To eliminate the interference of anthocyanins on the FLAV index, which absorb in vivo at 630 nm and affect the FRF_R reference signal, the single signal (FRF_UV) is used in this calculation. Another important parameter in our study is the FRF_G signal (far-red fluorescence after excitation with green light) representing an indicator for compounds absorbed in this spectral region.

### 6.2 Fluorescence indices for monitoring biochemical changes in leaves and fruits

When plants are exposed to stress conditions, there is a shift in the proportions of the absorbed light energy used for photosynthesis and the energy emitted as heat or fluorescence (60). Also, dynamic changes in pigment compositions strongly influence optical parameters such as fluorescence. For example, during tomato ripening, the fruit color changes from green to red because chlorophyll is degraded and carotenoids such as lycopene are synthesized and accumulate in the tissues (64).

At leaf scale, various studies validated the applicability of fluorescence-based indices for abiotic stress detection in tomatoes. For example, in a study evaluating the impact of salinity and water deficit, Kautz et al. (2014) (65) observed significant changes for the FLAV and BFRR_UV (ratio of blue fluorescence to far-red fluorescence after excitation with UV-light) recorded on tomato leaves. In another study, the authors could track salinity-induced stress responses of tomato leaves with the SFR_G, NBI_G and BFRR_UV (66). The NBI is based on the balance between primary metabolism (chlorophyll) and secondary metabolism (flavonols): When plants are exposed to stresses

such as limited nitrogen supply, chlorophyll content decreases whereas flavonol content increases, resulting in a decrease of the NBI, a parameter that can be used as indicator for nitrogen fertilization (60). The usefulness of this index was confirmed in tomato plants exposed to water deficiency where the NBI_R allowed a fast detection of stress reactions with decreasing values for the drought-exposed plants. Additionally, the BFRR_UV and the FLAV significantly increased during water deficiency (67). The RG_G light was also suitable for monitoring temporary water deficiency in sugar beet genotypes (68). Besides observation of stress responses, fluorescence-based indices have been used for tomato and sugar beet genotypes screening (65–68).

Concerning alterations on berries and fruits, several studies have been conducted with winegrapes (59, 62, 6, 70). The potential to determine maturity components *in situ* was proposed as the ANTH_RG showed a good correlation with the wet chemistry analysis of anthocyanins in two different wine cultivars. Furthermore, the SFR_R showed a linear correlation with the concentration of sugar often used as technological maturity parameter Brix° of total soluble solids (59, 63). Additionally, Ferrandino et al. (2017) (63) showed that the FLAV_UV can be adopted for flavonol estimation in colored grapes, whereas in white cultivars the use of both FLAV and FLAV_UV were feasible.

Multiparametric fluorescence recordings were further done on apples showing linear regressions between the ANTH, FLAV and CHL indices and the corresponding laboratory reference analysis (71). In shaded and sun-exposed kiwifruits, a coefficient of determination of $r^2 = 0.88$ was observed between the FLAV index and chromatographically determined flavonol concentration (72). Some parameter recorded by the sensor are also applicable for the determination of the optimal harvest time of oil palm bunches. The BFRR_UV was able to distinguish between different ripening stages such as under ripe, ripe and over ripe fruits which varied in their oil extraction rate (73). The ripening development of tomato fruits was successfully monitored with the fluorescence-based indices NBI, FLAV and ANTH_RG in the pre- and postharvest phase and also for detecting time-shifts in maturity development due to water deficiency in the greenhouse (52). However, a precise relationship between fluorescence-based indices and the corresponding reference analysis has not been exploited in recent literature.

# 7. Objectives of the study

The major aim of the present work was to evaluate the suitability of chlorophyll fluorescence-based indices to non-destructively monitor and predict changes in the content of plant constituents in response to abiotic stresses. Relating thereto, another objective was to generate knowledge about the potential of tomato leaves from commercial production systems for the extraction of industrially relevant secondary metabolites (rutin and solanesol) which are known to accumulate under abiotic stress conditions.

Naturally, tomato leaves contain several secondary metabolites including the two compounds of interest in our research, rutin and solanesol. Although some positive indications have been made to observe and induce their accumulation in young tomato plants under fully-controlled conditions (5, 28), the real potential for using green biomass from commercial production as source of these compounds is still unknown. Up to now, there is a missing characterization of the origin material and a lack of knowledge on how different plant tissues respond to target applied stresses during different physiological developmental stages aiming at higher synthesis and enhanced accumulation of the metabolites of interest. Thus, a precise knowledge about factors influencing the natural concentration of both compounds is necessary, particularly the impact of leaf age, growing conditions and the time point of harvest during the year.

The available literature provides the evidence of the potential of non-destructive sensors for monitoring alterations in leaf constituents such as chlorophyll and secondary metabolites, e.g. polyphenols, in response to abiotic stresses. Furthermore, the potential to estimate fruit quality/maturity in order to gather information about ripening development, maturity classification and determination of the optimal time point of harvest of berries and fruits. In order to obtain and extend knowledge in this field, we conducted multiple experiments with tomato plants grown either under nitrogen/nutrient deficiency, supplementary LED light and water deficit to induce the accumulation of SMs in tomato leaves, observe the effect of targeted stress on tomato fruit ripening and prove the opportunity of non-destructive estimation of plants responses.

In summary, the suitability of fluorescence-based indices should be evaluated to monitor non-destructively changes in tomatoes leaf constituents as well as changes due to tomato fruit ripening. Moreover, the relation between fluorescence-based indices and

analytically determined biochemical parameters of leaves and fruits should be elaborated.

The present thesis is divided into three separated experimental chapters with the respective hypotheses, as follows:

1. Mineral deficiency is a tool to induce the accumulation of rutin and solanesol in tomato leaves grown under commercial-like greenhouse conditions. Non-destructive fluorescence parameters are able to monitor plant stress responses and to track alterations in the content of leaf constituents.

2. Light-emitting-diodes providing supplementary light support enhanced accumulation of rutin in tomato leaves. Physiological responses and stress-related changes in the concentration of rutin can be monitored with non-destructive fluorescence indices.

3. Mild nitrogen deficiency and reduced water supply affect the ripening of tomatoes. Fluorescence-based indices can be used for the non-destructive estimation of maturity compounds in tomatoes of different ripening stages.

# 8. References

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**B Limitation of mineral supply as tool for the induction of secondary metabolites accumulation in tomato leaves**[1]

# 1. Introduction

A wide range of molecules occurring in cultivated crops derive from the secondary metabolism (SM) of plants. These compounds have a quite large scope of application e.g. for pharmaceutical use, cosmetics or food ingredients (1). In plants, secondary metabolites are synthesized in different pathways mainly as response of the plants to their interaction with insects, pathogens, and adverse environmental factors (2, 3). In recent years, the potential of renewable resources as natural material containing SM is in the focus of research (1, 4-7).

Polyphenols and terpenoids (the latter also known as isoprenoids) represent two of the three main groups of secondary metabolites (8), both occurring in Solanaceae plants. As shown, abiotic factors, such as temperature (9), light intensity and quality (10), nitrogen availability (11) and combinations of different factors might promote the accumulation of polyphenols such as flavonoids in tomato leaves (12). Especially a decrease in nitrogen can lead to higher amounts of the flavonol glycosid rutin in tomato leaves (3, 11, 12). In this context, Løvdal et al. (2010) reported that N depletion influences the accumulation of flavonoids such as rutin more severe than light and temperature, as explained by the expression of related genes involved in flavonoid metabolism (12). In contrast, only a few studies deal with the relevance of the isoprenoid solanesol from tomato plants (1). As known from tobacco plants, its concentration is dependent on the plant organ (13), the variety and leaf age, whereas no effect could be found in solanesol concentrations depending on nitrogen fertilization (14). Furthermore, the extraction methods influence the solanesol yield after plant harvest (15). Solanesol has its main application in the pharmaceutical and cosmetical sector, since it is an intermediate for the synthesis of coenzyme Q10 or analogous of vitamin K (16). A recovery of solanesol from tomato/solanaceae residues for further utilization was proposed (1), but, to our knowledge, no study investigated the real potential of the commercial tomato production.

[1] Groher, T., Schmittgen, S., Noga, G., Hunsche, M., 2018. Limitation of mineral supply as tool for the induction of secondary metabolites accumulation in tomato leaves. Plant Physiology and Biochemistry 130, 105-111.

Tomato is one of the most important vegetables in the world. Under North-European intensive greenhouse cultivation, plants might reach 15 or more meters of stem length during a growing period that goes from winter-to-autumn or winter-to-winter. In these high-input systems, huge amounts of biomass occur in tomato production (17). Leaves and stems of tomato plants contain several so far unused SM in different concentrations (18). One key element is the steering of the plant metabolism to reach the compounds of interest. Although some positive indications have been observed with small plants under fully-controlled conditions (11, 12), the real potential for using these valuable compounds for industrial applications is still unknown. One of the reasons is a missing characterization of the origin material, and also a better understanding how plant tissues at different physiological developmental stages respond to approaches aiming higher synthesis and accumulation of selected metabolites. Another open question is whether optical sensors can support the monitoring of changes in the concentrations of the secondary metabolites in tomato leaves.

So far, non-invasive recordings provide proper information on plants stress response and different techniques based on chlorophyll fluorescence have been evaluated to monitor plants nitrogen status (19). With multiparametric sensors, fluorescence signals can be recorded after excitation with different light sources, whereby these signals are used to calculate specific fluorescence indices to track plant responses to environmental factors; in specific cases, such indices might also be related to the concentration of plants constituents. For example, the indices SFR_R and BFRR_UV could be used for rapid sensing of salinity induced stress in tomato leaves (20), while the SFR_R and FLAV are suitable for abiotic stress detection at leaf and fruit scale (21). Furthermore, the NBI_R ratio allows a fast detection of water deficiency in tomato plants (22) and the RG_G light is suitable for detection of a temporary water deficiency in sugar beet genotypes (23). Additionally, correlations between the non-destructive indices detected by the sensor and analytically determined plant ingredients such as pigments (24), anthocyanins, flavonoids (25) as well as sugar and acids (26) were found. In *Centella asiatica* (L.) Urb. leaf flavonoids (flavonol and anthocyanins) were estimated with the non-destructive indices FLAV and ANTH_RG (27).

Based on the current state of knowledge, the major objective of the present work was to evaluate the mineral deficiency as potential tool for inducing the accumulation of rutin and solanesol in tomato leaves in a commercial-like greenhouse. The second objective

was to evaluate the suitability of selected non-destructive fluorescence parameters to monitor plant stress responses and to track changes in the concentration of leaf ingredients. For this purpose, tomato plants were grown temporarily under three different nutrient solutions with different contents of mineral elements. Since fruits are the primary aim of commercial-like tomato production, fruits with blossom end rot (BER) were counted before and after stress application. The guiding hypotheses were that a modified nutrient solution leads to an accumulation of SM in tomato leaves without changing fruit yield, and that changes in leaf compounds can be recorded non-destructively with multiparametric fluorescence-based parameters.

## 2. Materials and methods

### Experimental setup

The experiment was conducted from January 2017 to May 2017 in a commercial-like greenhouse at the Research Station Campus Klein-Altendorf (University of Bonn, Germany). Seeds of the truss tomato (*Solanum lycopersicum*) F1 hybrid Lyterno (RijkZwaan, Netherlands) were sown in January 2017 into rockwool cubes (Grodan delta, Netherlands) and transferred into rockwool slabs (Grotop Expert, Netherlands) after one month. Tomato plants were cultivated as one-shoot system with two plants per rockwool slab (1m). The plants were cultivated under natural day length without artificial light supplementation and at day and night temperatures of about 20.7 ± 1.9 and 18.1 ± 0.4 °C on average. In the month prior to treatment initiation, drip irrigation was computer-controlled depending on time (4.0 min $h^{-1}$ on average) and irradiation sum (additional watering started at 15-20 kilolux). A total of 30 plants (10 plants per treatment group) were selected randomized in two greenhouse rows. In April, following nutrient regimes were applied: (1) control: full standard nutrient solution, (2) nitrogen deficiency: standard nutrient solution without nitrogen containing compounds, (3) nutrient deficiency: tap water without additional fertilizers. The control plants received all nutrients necessary for optimal growth mixed 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 all micronutrients: 0.0096 mM manganese, 0.0078 mM zinc, 0.0290 mM boron, 0.0008 mM copper, 0.0005 mM molybdenum, 0.000 mM 5 sodium) while the nitrogen deficient plants received a modified solution without the

nitrogen containing compounds $(NH_4)(NO_3)$, $KNO_3$ and $Ca(NO_3)_2$. Calcium chloride and potassium chloride were added (8.58 mM chloride in total) as substitutions while all other compounds remained unchanged. The tap water was provided from a rainwater tank (pH 7.5 ± 0.2, EC 0.6 ± 0.05, ppt (parts per thousand) 0.3 ± 0.03). To ensure equal starting conditions, each rockwool slab was washed out with tap water (60 to 70 liters) to remove all accumulated nutrients in the substrate before the different nutrient regimes were applied.

**Table 1.** Mineral concentrations of the water samples [mg $g^{-1}$ DW] collected from the rockwool slabs at -1 and 21 DAT.

| Mineral [mg $l^{-1}$] | -1 DAT (Before washing out) | -1 DAT (After washing out) | 21 DAT (After stress period) | | |
|---|---|---|---|---|---|
| | | | Control | Mineral deficiency | Nitrogen deficiency |
| **Calcium** | 492.04±12.00[1] | 66.00±2.75[1] | 423.28±32.9[3] | 85.50±10.0[3] | 358.88±38.3[3] |
| **Magnesium** | 253.54±41.51[1] | 20.44±0.97[1] | 122.67±14.80[3] | 18.41±1.73[3] | 104.68±17.65[3] |
| **Potassium** | 468.04±23.27[1] | 11.04±3.01[1] | 263.57±50.51[3] | 9.52±5.82[3] | 319.72±33.80[3] |
| **Sodium** | 152.00±7.95[1] | 133.39±8.35[1] | 135.87±50.40[3] | 109.05±45.14[3] | 124.11±54.99[3] |
| **Nitrate** | 419.26±11.14[2] | 7.97±0.37[2] | 262.19±16.73[4] | 0.69±0.13[4] | 2.23±0.28[4] |
| **Ammonium** | 0.49±0.06[2] | 0.52±0.03[2] | 0.51±0.07[4] | 0.46±0.11[4] | 0.51±0.13[4] |

1 = MW±SE of 15 rockwool slabs.
2 = MW±SE of 5 rockwool slabs.
3 = MW±SE of 5 rockwool slabs per treatment.
4 = MW±SE of 3 rockwool slabs per treatment.

Samples of the water were taken before and after the treatment application to determine the amount of nutrients in each rockwool slab (Table 1). Mineral concentrations (Mg2+, $Na^+$, $K^+$, $Ca^{2+}$) were determined with the AAS (Atomic Absorption Spectrometry, Perkin Elmer, Analyst 300) and the continuous flow analyzer ($NO_3^-$, $NH_4^+$) (Seal Analytical, QuATro39). The plants were supplied with the nutrient solutions for 3 weeks (2600 mL per day and plant) using an artificial watering system consisting of a Gardena Comfort 9000 (GARDENA Manufacturing GmbH, Ulm, Germany).

Sampling and preparation

Young and mature leaf samples were taken one day before, and 21 days after treatment induction (DAT) to assess the impact of the nutrient supply on the concentration of the

secondary metabolites rutin and solanesol. The samples were cooled directly after harvest, freeze-dried and then stored at -20 °C until further processing.

Determination of rutin and solanesol

Ground leaf samples were used for HPLC determination of rutin and solanesol (Agilent 1260 Infinity HPLC System Agilent Technology Deutschland GmbH, Ratingen, Germany). An amount of 0.12 g was extracted with methanol for 10 min in an ultrasonic bath, centrifuged for 10 min at 4 °C with 16100 g (Centrifuge 5415R, Eppendorf AG, Hamburg, Deutschland) and the supernatant was added in a volumetric flask. The procedure was repeated four times, and the supernatants were collected and filled up to 10 mL with methanol. After extraction samples were stored at -20 °C until HPLC analysis.

The samples were filtrated through a membrane filter (Phenomenex, Aschaffenburg, Germany) prior to injection whereby for rutin determination a mixture of 0.5 mL of the supernatant was diluted with 0.75 mL distilled water and centrifuged once more before 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 x 150 mm, 3 µm, Macherey-Nagel, GmbH & Co. KG, Düren, Germany) was eluted isocratically with a binary mixture of water and methanol (60:40) adjusted with phosphoric acid to a pH of 2.8. The flow rate was 0.15 mL $min^{-1}$, 10 µL samples were injected on the column equilibrated at 25 °C, and detection was made at 210 nm wavelength. Rutin peak was detected at 10.8 min, solanesol at 15.8 min. Both calibration curves were obtained from diluted series of standards provided by TCI Deutschland GmbH (Eschborn, Germany).

Non-destructive recordings

Non-destructive recordings were conducted two times per week from April 14th until May 4th on one young and one mature leaf per plant. Two measurements per plant were taken at the adaxial lamina of the tomato leaf using the multiparametric fluorescence excitation system Multiplex® (Multiplex®, Force-A, Orsay, France). All recordings

were done at a constant distance of 0.10 m to the leaf surface and a frontal cover plate with an aperture of 6 cm in diameter. The technical principle is based on chlorophyll fluorescence excited with different light sources. The sensor has light-emitting-diodes with different emission spectra (UV-A: 375 nm, Blue: 450 nm, Green: 530 nm, Red: 630 nm) and a filtered photodiode detection (in the blue: 425-475 nm, red: 680-690 nm and far-red: 720-755 nm). Based on the different signals, the system calculates different indices depending on the excitation light. The detailed working principle is extensively descried elsewhere (23, 25).

In our study, the following indices were selected for non-destructive stress detection:

(A) Simple Fluorescence Ratio (SFR_R): FRF_R/RF_R

(B) Nitrogen Balance Index (NBI_G): FRF_UV/RF_G

(C) Flavonol Index (FLAV): Log (FRF_R/FRF_UV)

(D) Anthocyanin Index (ANTH_RG): Log (FRF_R/FRF_G)

The selected indices were previously validated for abiotic stress detection in tomatoes (19, 20, 22). The Simple Fluorescence Ratio (SFR_R) is linked to the chlorophyll content of the sample. It is the ratio of chlorophyll fluorescence generated in the far red and red after excitation with red light (A). The Nitrogen Balance Index (B) combines the signals of far red fluorescence excited with UV and red fluorescence excited with green and is used to monitor the nitrogen status of the plant. The measurement principle for polyphenols can be explained by the screening effect of substances located morphologically above the chlorophyll. Flavonoids absorb UV (C), anthocyanins green light (D) while red light is not absorbed by polyphenols and used as reference signal. The higher the amount of polyphenols above the chlorophyll molecules the less chlorophyll fluorescence is generated because only a fraction of the excitation light reaches the chlorophyll molecules in the mesophyll tissue.

Fruit yield and quality

To assess the impact of the nutrient regime on fruit yield and quality, fruits with blossom end rot were counted before and at the end of the study for each plant. Additionally, the

primary trusses (from the bottom) were harvested at 21 DAT to estimate the fresh mass of fruits.

Statistical analysis

Differences between the experimental treatments (control, nitrogen deficiency, mineral deficiency) were tested using the Tukey HSD test and considered significant at $p \leq 0.05$ with SPSS statistical software (PASW statistics version 20.0, SPSS Inc., Chicago, USA). Graphs were done with SigmaPlot for Windows version 11.0 (Systat Software, Inc.).

# 3. Results and discussion

Effects of stress application on the accumulation of rutin

The rutin concentration of tomato leaves varied depending on leaf age and supplied nutrient solution (Fig. 1).

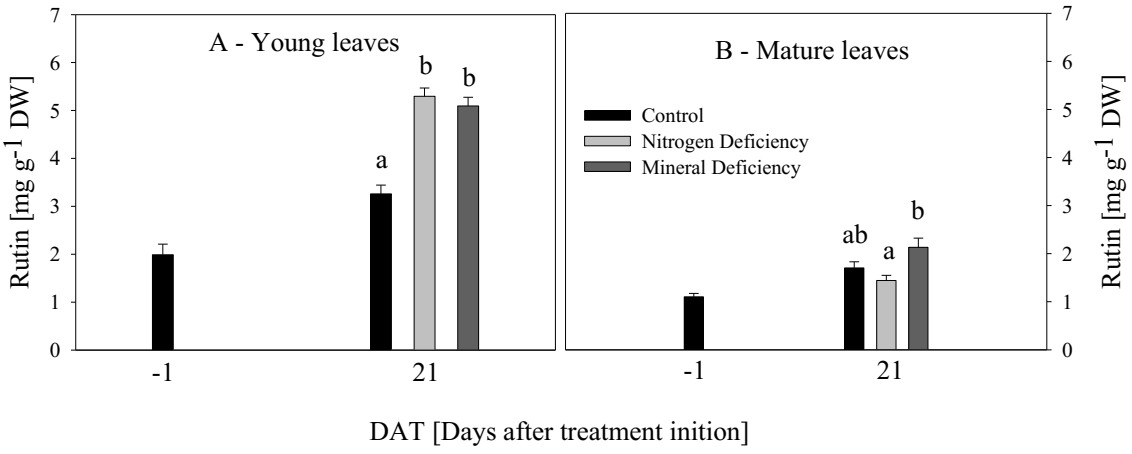


**Figure 1.** Rutin concentrations in young (A) and mature (B) tomato leaves at -1 and 21 DAT. Means ± SE (n = 10 leaves per treatment). Different letters indicate statistically significant differences at DAT 21 according to Tukey HSD test ($p \leq 0.05$).

In general, young leaves showed higher rutin concentrations compared to mature leaves while the concentration increased during the investigated three weeks for both leaf ages irrespective of nitrogen or general mineral limitation. After 21 days, young control leaves

revealed significantly lower concentrations with 3.26 mg $g^{-1}$ DW compared to the nitrogen deficient (5.3 mg $g^{-1}$ DW) and mineral deficient (5.09 mg $g^{-1}$ DW) leaves. However, no differences were found between the different stress treatments in young leaves. In mature leaves, the rutin concentration in stressed plants did not differ significantly from the concentration in control plants while significant differences were detected between both stress treatments. Leaves of nitrogen deficient plants had the lowest values followed by control plants and plants grown under a general mineral deficiency.

Our results in a commercial-like production greenhouse are in line with other studies which investigated the effect of nitrogen limitations on phenolic compounds such as rutin in young tomato plants (8, 11, 12). One reason for the accumulation in young leaves might be the protective function of rutin against UV-light (28). Light intensity increased from April to May and is more abundant in the upper leaves than in mature, shaded leaves. Moreover, young leaves might respond stronger to cultivation and environmental changes in terms of adjustment of metabolism and accumulation of secondary compounds, as demonstrated with our results. The time point of harvest during plant vegetation seemed to influence the accumulation of rutin as shown by the doubled amount in young leaves of control plants at DAT 21 compared to the first day of harvest.

Rutin is associated with several human health promoting functions thus resulting in a great interest in its natural production. A lot of research on potential rutin accumulation for industrial production was done with buckwheat (10, 29, 30). In buckwheat, the concentrations in leaves harvested 28 days after germination was less than 50 mg $g^{-1}$ DW in mature leaves and increased up to 200 mg $g^{-1}$ DW in young expanding leaves (30). Another study investigated the effect of different combinations and irradiation cycles of light-emitting-diodes on rutin concentration in buckwheat sprouts and found smaller amounts between 12.65 mg $g^{-1}$ DW to 13.13 mg $g^{-1}$ DW depending on light regime (10). Even if the amounts in tomato leaves are considerably lower as compared to buckwheat leaves, the recovery from tomato by-products e.g. from pruned leaves or whole tomato plants at the end of production would contribute to an added value and a resource saving production.

Effects of stress application on the accumulation of solanesol

The concentration of the isoprenoid solanesol was clearly higher in mature leaves as compared to young leaves regardless of the growing conditions (Fig. 2). Beside the leaf position, the solanesol concentration depended on the harvest time during the growing season. Whereas the solanesol concentration was stable in young control leaves (0.12 mg $g^{-1}$ DW) at -1 and 21 DAT, the solanesol concentration doubled from 1.07 mg $g^{-1}$ DW to 2.19 mg $g^{-1}$ DW in mature control leaves. After 21 days of treatment induction, both stress treatments promoted the accumulation of solanesol in young leaves without statistically differences between both treatments. The highest solanesol concentration in young leaves of up to 0.64 mg $g^{-1}$ DW was achieved with a mineral deficiency. Significant differences in the solanesol concentration of mature leaves could only be found between the control and nitrogen deficient plants. Mature leaves from plants of mineral deficiency contained 2.5 mg $g^{-1}$ DW and leaves from the nitrogen deficient plants 3.2 mg $g^{-1}$ DW, so that a 3-fold increase occurred due to nitrogen limitation after 21 DAT.

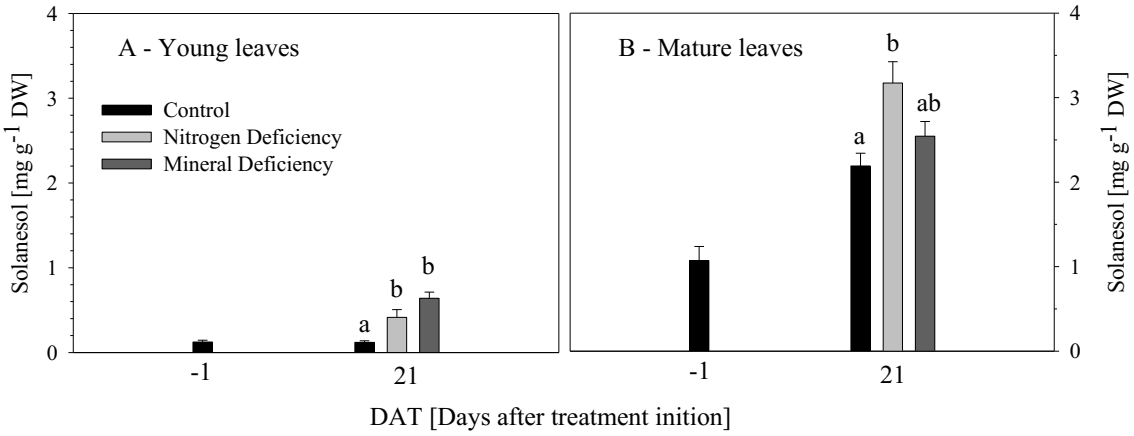


**Figure 2.** Solanesol concentrations in young (A) and mature (B) tomato leaves at -1 and 21 DAT. Means ± SE (n =10 leaves per treatment). Different letters indicate statistically significant differences at 21 DAT according to Tukey HSD test ($p \leq 0.05$).

The main source of solanesol for industrial use are crops belonging to the solanaceous family since the chemical synthesis is difficult. It is present in potato, eggplant, pepper, tomato, and mainly in tobacco (1). Up to now, there is a lack of information regarding the concentration and environmental influences on solanesol concentration in other plant than tobacco. Our experiment with tomato showed that the solanesol concentration

strongly depends on the leaf age. This is in line with the tobacco study of Burten et al. (1989) where the highest solanesol concentrations were found in old leaves (14). On the contrary, other studies showed opposite results with decreasing solanesol concentration with decreasing leaf age in tobacco (13). According to Zhao et al. (2007), leaves are the plant organs containing the highest amount of solanesol in tobacco plants with up to 6.12 mg $g^{-1}$ DW compared to stalks, flowers, seeds, fruits and roots. Besides the plant organ, the variety and the stage of growth have an influence on solanesol concentration in tobacco (14). The precise function of solanesol in plants is still not clear. Some studies suggested that solanesol has a protective function against reactive oxygen and is involved in biotic stress defense (1, 31). But also environmental conditions may affect the solanesol concentration. In tobacco, environmental factors such as drought, nitrogen fertilization, and irradiation could influence the concentration of solanesol (14, 32). We showed for the first time that a limitation in nitrogen and a general mineral deficiency lead to an accumulation of solanesol in tomato leaves.

Impact of nutrient supply on fluorescence signals

Fluorescence signal of young leaves showed the influence of the treatments at 11 DAT (Fig. 3). The SFR_R, estimating chlorophyll content of the leaves, slightly decreased from -1 to 8 DAT. From then, the index increased followed by a decrease at the last recording day. Significant differences between the control and the stressed leaves were found at 11 DAT. Here, the control leaves had the highest values followed by the nitrogen deficient leaves and leaves of the plants supplied with tap water. From 15 DAT, all three treatments differed significantly in the same order (Fig. 3A). NBI values did not differ significantly among the treatments from -1 to 8 DAT. First significant responses were observed at 11 DAT between all three treatments. At 15 DAT, control leaves had a marked increase as compared to both stress treatments. At the last day of recordings, the NBI index of all treatments differed significantly from each other (Fig. 3B). The Nitrogen Balance Index decreased under nitrogen limitation as consequence of its calculation method, which is the ratio of chlorophyll index and pidermal flavonol index. During abiotic stress conditions, such as nitrogen limitation, flavonol content increased while chlorophyll content stayed constant or decreased, resulting in a higher NBI index as compared to plants grown under control conditions. Leaves of plants grown under

mineral shortage had significantly higher FLAV values than control leaves from 11 to 21 DAT. Nitrogen deficient leaves and control leaves differed at DAT 15 and 21 significantly. The values for control leaves decreased during the experiment while the index increased for stressed leaves (Fig. 3C).

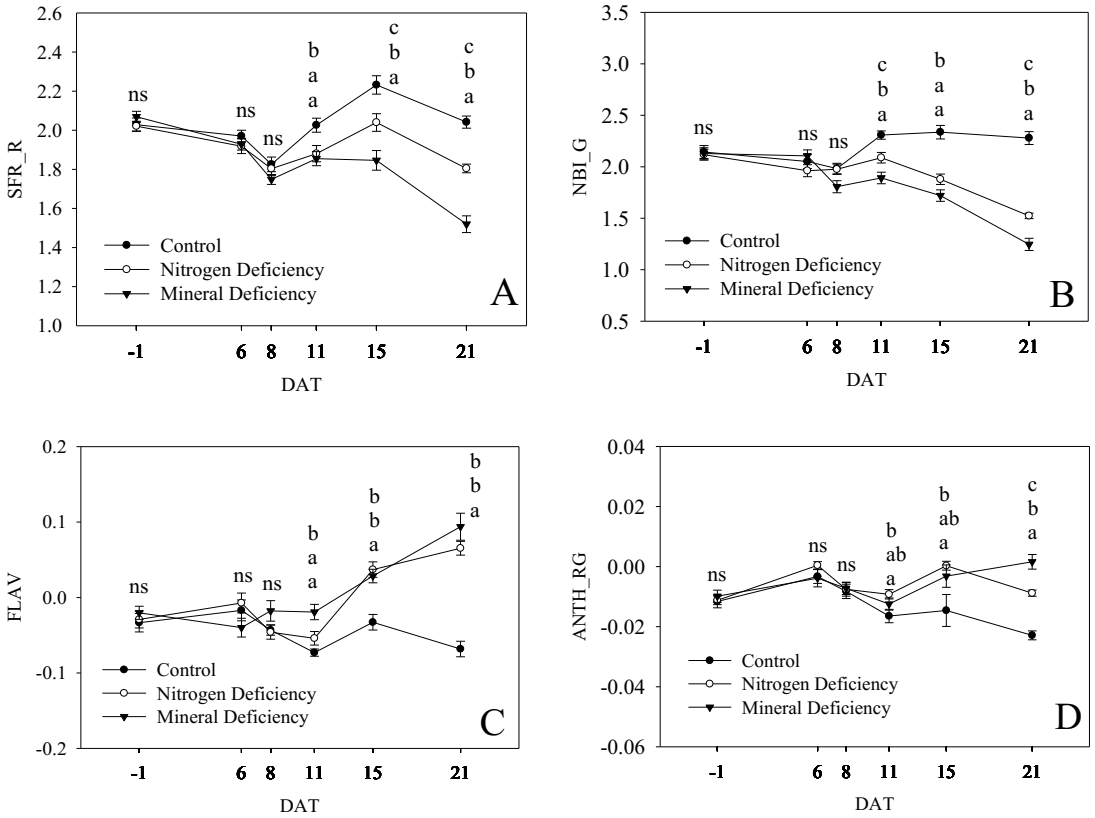


**Figure 3**. Simple Fluorescence Ratio (SFR_R), Nitrogen Balance Index (NBI_G), Flavonol Index (FLAV) and Anthocyanin Index (ANTH_RG) recorded from young leaves during the time course of the experiment. Means ± SE (n = 10). Different letters indicate statistically significant differences according to Tukey HSD test ($p \leq 0.05$).

A decrease in general minerals induced significantly higher ANTH_RG values at 11 and 15 DAT as compared to the control treatment. At the last day of recordings, all treatments differed significantly from each other with highest values for control leaves followed by the nitrogen and mineral deficiency (Fig. 3D). Anthocyanins represent a sub-group of phenolic compounds which are, similar to flavonoids, involved in plants UV protection. Larbat et al. (2014) showed that anthocyanin concentrations increased under low nitrogen supply in tomato leaves and stems (8). This is in line with our results obtained with non-destructive approaches in young tomato leaves exposed to nitrogen and general mineral

deficiency stress conditions. These findings are also confirmed by lower FLAV values for plants supplied with the full standard solution than those of the deficient plants. In general, SFR_R and FLAV values were lower in mature leaves compared to young leaves and higher for the ANTH_RG and NBI_G. However, no significant differences were found for non-invasive recordings in mature leaves (Appendix A).

Extensive correlation analysis indicates, however, that the fluorescence indices are not a reliable tool to estimate the concentration of rutin and solanesol in young or mature tomato leaves (Fig. 4A+B).

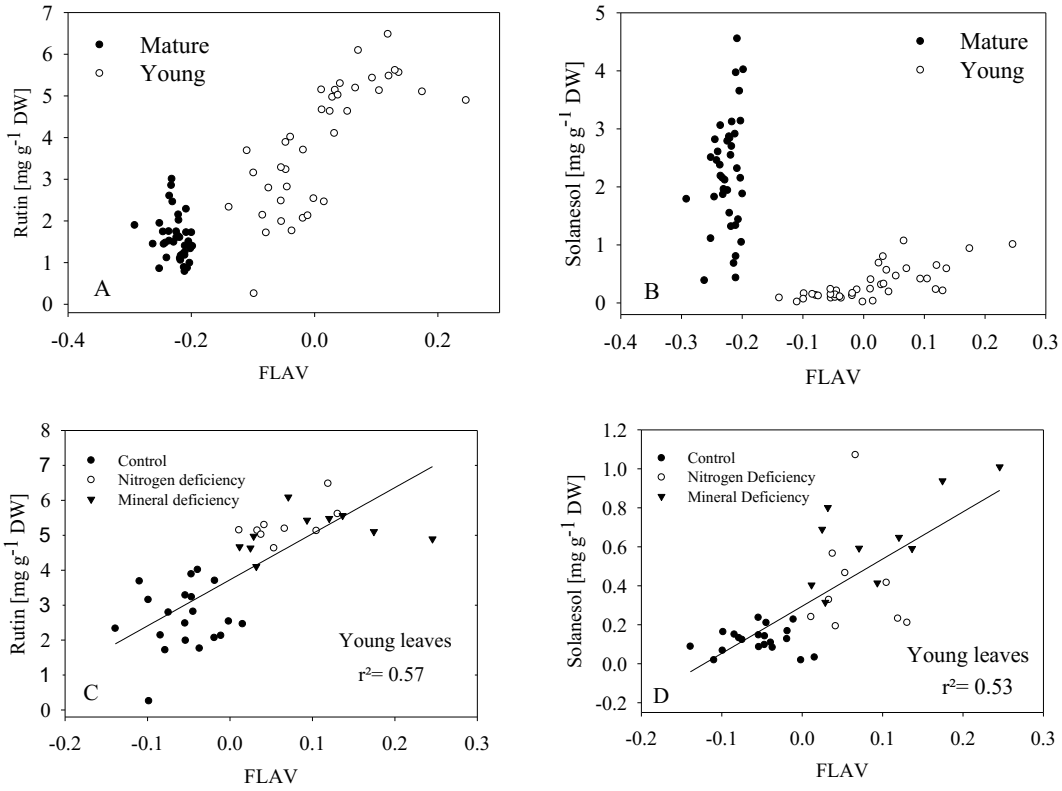


**Figure 4.** Correlation analysis between the FLAV index (Multiplex®) and the rutin (A) and solanesol (B) concentrations determined via HPLC. $R^2$ is given for linear regression for rutin (C) and solanesol (D) in young leaves.

In particular, the relationship between the FLAV (as indicator for the accumulation of SM) and rutin or solanesol shows a cluster for young and mature leaves but an estimation of rutin or solanesol is not possible. Especially the distribution of solanesol shows that the FLAV index gives values about -0.3 to -0.2 for a wide rutin range from 0.4 to 4.5 mg $g^{-1}$ DW. The correlation was stronger in younger leaves with $r^2 = 0.57$ for rutin (Fig. 4C) and $r^2 = 0.53$ for solanesol (Fig. 4D). These results might be related to the measuring

principle of the sensor, in which light is emitted into the tissue while the light penetration into comparatively old and firm tissue is lower as compared to young soft plant tissue. Irrespective of that, the correlation coefficients below 0.60 are too low to get reliable estimations of the concentration of solanesol and rutin in the young leaves.

Impact of nutrient supply on fruit quality

Changes in the nutrient solution for 21 days showed little effect on the fresh matter of the primary tomato trusses (Tab. 2) and confirm a previous study dealing with tomato fruit quality under a low nitrogen supply (33). Plants with fruits showing symptoms of blossom end rot were found most often on those exposed to general mineral deficiency with 80% of plants showed symptoms of blossom end rot. In comparison to only 20% of nitrogen deficient plants and 30% of plants from the control treatment. Only plants grown under mineral deficiency showed more than one truss with blossom end rot on a single tomato plant with 10 trusses out of eight plants (Tab. 2).

**Table 2.** Evaluation of fruits with blossom end rot (n = 10 plants) and the fresh matter of the primary truss at the end of the experiment (Means ± SE, n = 6 fruits of 10 plants per treatment).

| Parameter | Control | Nitrogen Deficiency | Mineral Deficiency |
|---|---|---|---|
| Plants with blossom end rot | 3 | 2 | 8 |
| Trusses with blossom end rot | 3 | 2 | 10 |
| FM (g) | 580.3±15.3a | 522.1±27.9a | 562.9±15.6a |

Different letters for FM indicate statistically significant differences according to Tukey HSD test ($p \leq 0.05$).

Interestingly, all symptoms of blossom end rot occurred at trusses eight or younger, confirming that young/developing fruits are more sensitive to unfavorable conditions compared to almost fully developed fruits. Blossom end rot of tomato fruits is a physiological disorder which becomes visible as lesions and nearly black tissue at or near the blossom end of the fruit creating high losses in tomato yield. The specific reasons for the occurrence of BER are still not fully understood. A calcium deficiency of tomato fruits or rather an abnormal calcium partitioning and distribution within the cells, is

considered as main cause of blossom end rot (34). However, it is still unclear how external factors affect the calcium concentration of the fruits (35). As shown in table 1, the concentrations of almost all mineral elements, including $Ca^{2+}$ have strongly declined after flushing the rockwool slabs. Some studies assume that a calcium deficiency causes an increase in ion permeability and a disintegration of the fruits cell membranes (36, 37). However, others focusing on blossom end rot of tomato conclude, that no significant differences in the calcium concentration of affected fruits could be found (38, 39). In the reviews of Saure (2001) and Taylor and Locascio (2004) symptom development, calcium availability and the influence of environmental factors on BER are comprehensively considered and summarized (35, 40).

# 4. Conclusion

In the present study, we proved for the first time that adjustments in the nutrient solution lead to the accumulation of SM in leaves of tomatoes grown under commercial-like conditions. Our results showed that the leaf age, the time of harvest in the vegetation cycle and the supplied nutrient solution affected rutin and solanesol concentration in tomato leaves. The highest rutin concentration was found in young leaves while higher amounts of solanesol were accumulated in mature leaves, both grown under nitrogen deficiency. The yield of marketable fruit decreased under a general mineral deficiency mainly because of quality losses due to higher incidence of fruits with blossom end rot. Thus, regarding the practical application, a modification of the nutrient solution in the last weeks of production should be consider to avoid economic losses due to more fruits with BER. Non-destructive fluorescence recordings successfully indicated the stress situation of the plants even though these parameters were not suitable for a reliable determination of the concentration of SM in the tissues. Before upscaling and commercial implementation, further work is needed to evaluate the potential of by-products from different tomato production systems (greenhouse or field), varieties, the time of harvest, and the influence of abiotic factors on the concentration of secondary metabolites.

## 5. Acknowledgments

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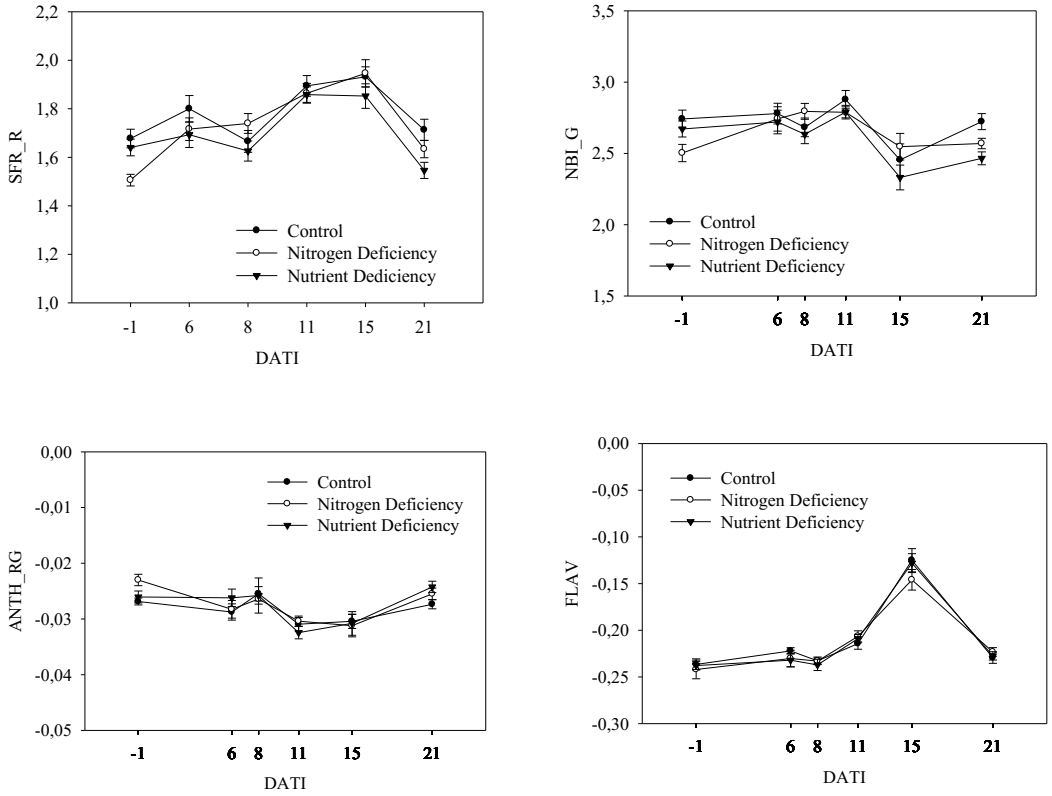
# 7. Appendix

A:




A. Fluorescence indices during the time course of the experiment measured at mature leaves. Means ± SE (n = 10). No differences between the treatments were found according to Tukey HSD test ($p \leq 0.05$).

**C Influence of supplementary LED lighting on physiological and biochemical parameters of tomato (*Solanum lycopersicum* L.) leaves[2]**

# 1. Introduction

Agricultural biomass ranks among the cheapest and most abundant renewable resources worldwide with a wide range of applications (1). In tomato cultivation, large amounts of biomass arise as hardly used by-product during and at the end of the cultivation cycle. So far, this green plant biomass accrues as waste product without specific consideration of its natural source of secondary metabolites. Flavonoids are omnipresent compounds in plant-based foods associated with many positive health-related functions in humans such as anti-inflammatory, anti-diabetic or anti-adipogenic (2, 3). In particular, Rutin is a flavonol glycoside also known as vitamin P. The hydrolysis of rutin produces quercetin, which commonly coexists with rutin itself. Peroxidases like rutin glucosidase will be activated when the plant is exposed to stress conditions to protect the plant against oxidation (4, 5). This aspect has been shown in buckwheat (*Fagopyrum esculentum* Moench) in which different plant organs contain high amounts of rutin (6, 7) whereas its concentration is influenced by environmental factors (8) like solar radiation and day length, as well as UV-B radiation, temperature and desiccation stress (9). So far, buckwheat is one of the major dietary source of rutin with its highest importance in Japan (10). In line with the raising interest of consumers for regional and sustainable forms of production, along with strategies of the circular economy, the accumulation and extraction of rutin from tomato leaves grown under LED could create added value to tomato production.

The anatomy, morphology and physiology of plants strongly depend on the spectral properties of light (11, 12), and also the concentration of flavonols may vary depending on the light source (13). In a recent review, Bantis and colleagues summarized the most important studies dealing with the impact of LED on physiological and biochemical parameters in tomato plants (14). In a recent experimental study, Jiang et al. (2017) observed a positive effect of supplemental LED light from underneath and within the inner canopy on growth and development of tomato plants as indicated by higher fresh

[2] Groher, T., Röhlen-Schmittgen, S., Fiebig, A., Noga, G., Hunsche, M., 2019. Influence of supplementary LED lighting on physiological and biochemical parameters of tomato (*Solanum lycopersicum* L.) leaves. Scientia Horticulturae 250, 154-158.

and dry weight, stem diameter and specific leaf area (15). Moreover, a combination of red and blue LEDs at a PPFD (photosynthetic photon flux density) of 300 µmol $m^{-2}$ $s^{-1}$ improved photosynthetic rate, increased leaf thickness and raised stomata density (16). Further, the fruit yield and the content of soluble solids and ascorbic acids was enhanced due to LED light (15). Although the innovative character, the recent research did not consider the potential effects of lighting on specific leaf compounds. In a study under controlled conditions in a growth chamber, Løvdal et al. (17) demonstrated higher content of rutin in tomato leaves grown under a combination of high light, low temperatures and nitrogen deficiency in growth chambers. These promising results, however, did not consider the applicability of the system under practice-related conditions, e.g. at production scale in a greenhouse. To close this information gap and extend the existing knowledge, the present study aimed at quantifying changes in rutin content in tomato leaves of different physiological ages due to blue and red LED intra-canopy lighting in commercial-like greenhouse systems. In addition, fluorescence-based indices were recorded to monitor non-destructively the physiological responses of plants with supplemental LED light, and to evaluate the applicability of the indices to estimate rutin content with a sensor-based system.

## 2. Materials and methods

### Plant material and growth conditions

The experiment was performed in a commercial-like greenhouse at the research station of the Chamber of Agriculture of the State North Rhine-Westphalia (Straelen, Germany), from October to December 2016. Tomato seedlings (*Solanum lycopersicum* L., cv. Lyterno grafted on Maxifort) were grown under optimal environmental conditions according to the common practice (temperature, humidity, fertilization, light), selected for uniformity and transplanted into rock wool slabs (Grotop Master, Grodan, The Netherlands) 43 days after sowing. The plants were cultivated as two-shoot V-system (plant density of 2.5 plants $m^{-2}$) fertilized with a commercial-like nutrient solution using an automatic drip irrigation steered by time and irradiation sum. Day and night average temperatures were 19.9±1.1 and 17.4±1.1 °C, respectively. For our study, we two rows in the middle of the greenhouse with a total of 20 plants per treatment group were used for non-invasive recordings from which 10 plants were harvested for laboratory analyses.

Lighting treatments

Two fully separated lighting treatments (control/LED) were installed in one greenhouse section. Two rows of control plants were grown under natural light conditions for 30 days, while two further rows were exposed to supplementary LED lighting during the same period. For further 15 days both the control and the LED treatment received LED lighting. Thus, LED plants received red and blue LED light during the time-course of the experiment starting after the first recordings (days after treatment initiation, DAT) for 45 days in total (LED plants from 0 to 45 DAT, Control plants from 30 to 45 DAT). Bi-directional LED lamps of 220 µmol $m^{-2}$ $s^{-1}$ light intensity (115 W per module, GreenPower LED Interlighting module DR/B, PHILIPS) were used for additional lighting with eight modules per double row (Fig. 1). Each module consisted of two light panels with 40 blue (20%) and 160 red (80%) lamps horizontally arranged in the plant canopy in addition to the natural day light from the top. The spectral distribution of the light in the greenhouse was determined with a handheld non-imaging spectroradiometer (ASD FieldSpec Pro FR spectrometer, Analytic Spectral Devices, Boulder, USA). LED lamps showed pronounced peaks in the blue (452 nm) and in the red (661 nm) spectral positions (Appendix A).

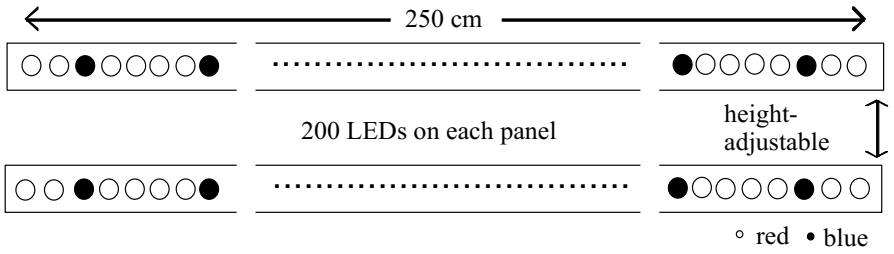



**Figure 1.** Schematic arrangement of a LED module with two light panels. The distance between the units as well as the distance from the ground are height-adjustable.

Sample preparation and laboratory analysis

On each measurement day, 10 leaves per treatment and leaf age were harvested after the non-destructive recordings: one young leaf (3rd to 4th from the apical meristem) and one mature leaf (5th to 6th, up to 13th to 15th fully developed leaf, according to the development stage during the vegetation period). Leaves were cooled down and then stored at -20 °C. Freeze-dried samples were ground for 1 min using a ball mill (MM 2000, Retsch GmbH,

Haan, Germany). Powdered plant material (0.12 g) was used for rutin extraction with methanol for 10 min in an ultrasonic bath. After centrifugation (4 °C/ 13000 rpm) for 10 min (Centrifuge 5415R, Eppendorf AG, Hamburg, Germany), the supernatant was filled in a volumetric flask, while the procedure was repeated four times. The supernatant phases were combined with methanol to 10 mL and stored at -20 °C until analyses. Prior to injection, 0.5 mL of the centrifuged supernatant was mixed with 0.75 mL distilled water, centrifuged and passed through a 0.2 µm membrane filter (Phenomenex, Aschaffenburg, Germany). Rutin content was analyzed using an Agilent 1260 Infinity HPLC System (Agilent Technology Deutschland GmbH, Ratingen, Germany) coupled with a quaternary solvent delivery system, an auto-sampler and a diode array UV–Vis detector. The column (Nocleodur C18, 3 x 150 mm, 3 µm, Macherey-Nagel, GmbH & Co. KG, Düren, Germany) was eluted isocratically with a binary mixture of water and methanol (volume ratio 60:40) adjusted to pH 2.8 with phosphoric acid. The flow rate was kept at 0.15 mL $min^{-1}$, and the detector was set at 210 nm wavelength for maximum absorption. Aliquots of 10 µL were injected onto the column equilibrated at 25 °C. Rutin peak was detected at 10.8 min. Finally, the content of rutin was calculated using the average area ratio obtained for each sample.

Non-destructive recordings

For each experimental treatment, a total of twenty plants were randomly selected for non-destructive recordings. At each measurement day (0, 16, 30 and 45 DAT), recordings were done *in situ* on leaves at different physiological ages as described above (section 2.3). Recordings were performed at the adaxial leaf lamina with a hand-held multiparametric sensor (Multiplex®, Force A, Orsay, France).

**Table 1.** Description of the fluorescence-based indices used in this study.

| Index | Calculation | Description |
|---|---|---|
| SFR_R | (FRF_R/ RF_R) | Simple Fluorescence Ratio excited with Red light |
| FLAV | log(FRF_R/FRF_UV) | Flavonol Index |
| NBI_G | (FRF_UV/RF_G) | Nitrogen Balance Index excited with Green light |
| ANTH_RG | log(FRF_R/ FRF_G) | Anthocyanin Index |

The LEDs were switched off during the recordings. The Multiplex® calculates different indices based on the chlorophyll fluorescence excited with different light sources (UV: 375 nm, blue: 450 nm, green: 518 and red: 630 nm). The sensor and its working principle are described in detail elsewhere (18–20). The selected indices used in our study are summarized in Table 1.

### Statistical analyses

Differences between treatments (control/LED) were statistically analyzed using the t-test ($p \leq 0.05$) with SPSS statistical software (PASW statistics version 20.0, SPSS Inc., Chicago, USA). Statistical analyses were done separately for each evaluation day because different leaves were recorded over the experimental period. All measurements are given as mean ± standard error. Graphs and curve fittings (linear regression) were done with SigmaPlot for Windows version 11.0 (Systat Software, Inc.).

## 3. Results

### Effect of supplementary lighting on rutin content in tomato leaves

Leaf rutin concentration did not significantly differ between the treatments on the first measurement date (DAT 0) as shown in Figure 2. However, the average value of the young leaves was clearly higher (1.2 mg $g^{-1}$ dry matter, DM) as compared to the amount in mature leaves (0.4 mg $g^{-1}$ DM). In young leaves, the rutin content slightly decreased from day 0 to 30 DAT and showed a strong increase until 45 DAT. First significant differences between the young leaves of control and LED plants were observed at 16 DAT and continued to the end of the experiment. At 45 DAT, when all plants received LED light, the rutin concentration strongly increased in both treatments to 1.6 and 2.5 mg $g^{-1}$ DM for control and LED plants, respectively (Fig. 2A).

In mature leaves, the concentration of rutin remained almost unchanged from 0 to 30 DAT, irrespective of the light conditions. At 45 DAT, a significantly higher concentration of rutin was detected in LED plants (4.1 mg $g^{-1}$ DM) as compared to the control plants (3.3 mg $g^{-1}$ DM, Fig. 2B).

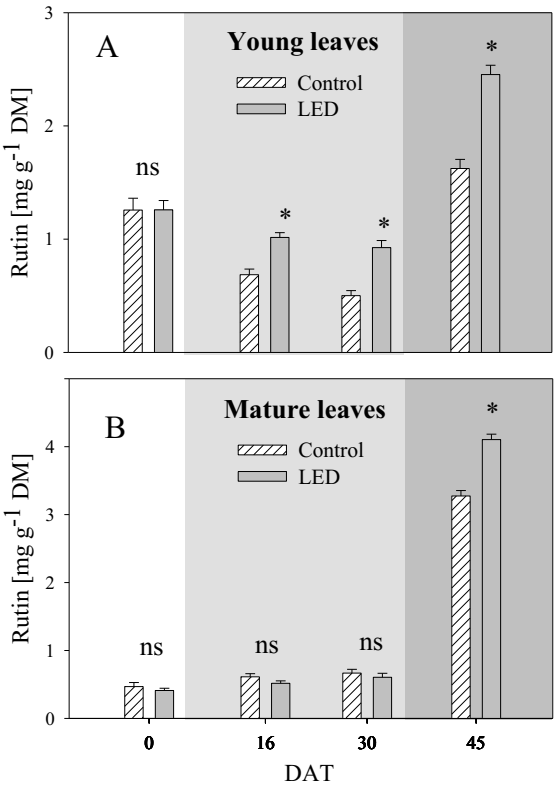



**Figure 2.** Rutin concentration [mg $g^{-1}$ DM] in young (A) and mature (B) tomato leaves. The area highlighted in gray represents the time period of supplementary LED lighting for half of the plants and the area highlighted in dark-gray the supplementary lighting for all plants. Mean±SE, n = 10, asterisks [*] indicate significant differences between the treatments, $p \leq 0.05$. ns = not significant.

Suitability of fluorescence indices

The Simple Fluorescence Ratio recorded under Red light (SFR_R) excitation, an indicator of chlorophyll content, was significantly higher in young leaves grown with supplemental LEDs as compared to control plants at 16 and 30 DAT (Fig. 3A). In mature leaves, the SFR_R decreased from 0 to 30 DAT, and rose to 45 DAT, with significantly higher values on leaves with LED, from 16 to 45 DAT (Fig. 3B).

In young leaves, the Flavonol Index (FLAV) was higher from 16 to 45 DAT when plants were grown with supplemental LEDs as compared to control plants (Fig. 3C), irrespective of the decrease during the time-course of the experiment in both experimental groups. Unexpectedly, the mature leaves of the control plants had higher FLAV values at 16 DAT and 30 DAT. On the contrary, at 45 DAT the same index was higher for leaves exposed to LEDs (Fig. 3D).

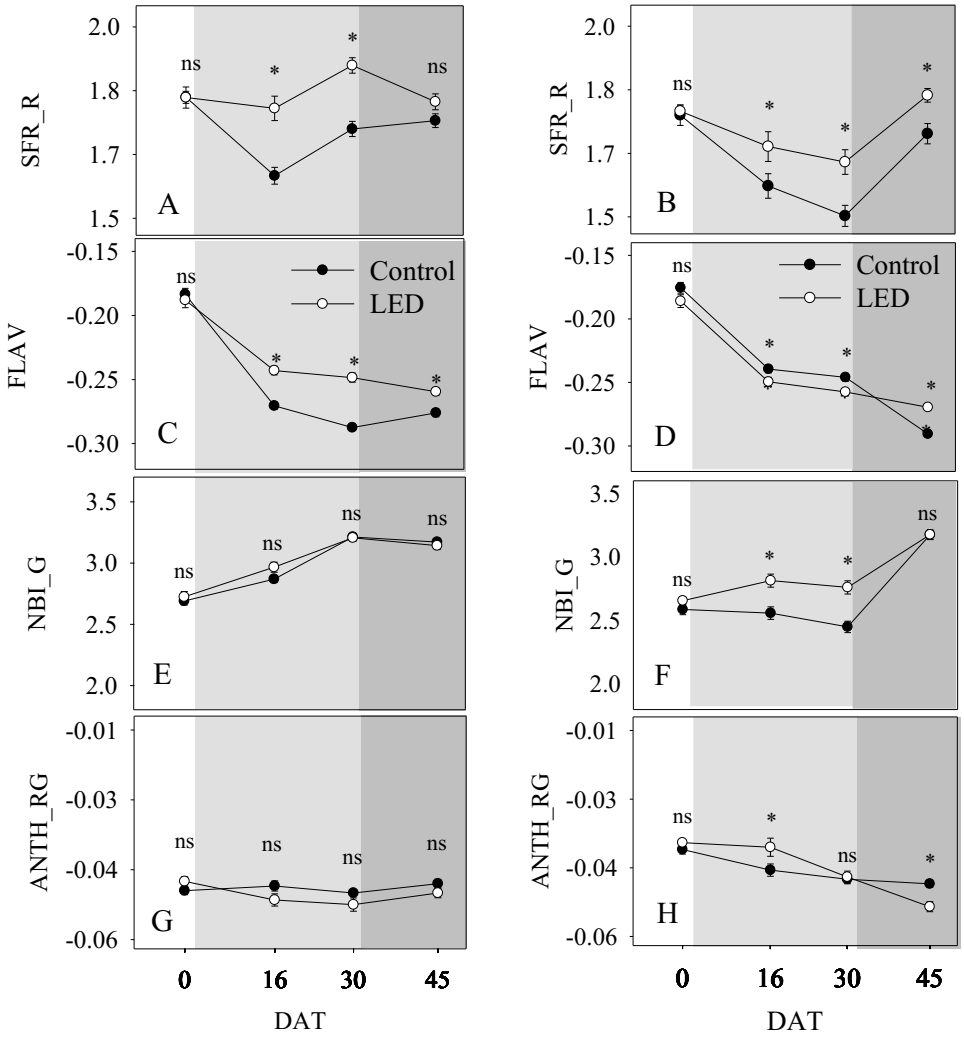



**Figure 3.** SFR_R (A, B), FLAV (C, D), NBI_G (E, F) and ANTH_RG (G, H) of young and mature tomato leaves as influenced by lighting regime. The area highlighted in gray represent the time period of supplementary LED lighting for half of the plants and the area highlighted in dark-gray the supplementary lighting for all plants (control/LED). Mean±SE, n = 20 asterisks [*] indicate significant differences between the treatments, $p \leq 0.05$, ns = not significant.

The Nitrogen Balance Index (NBI_G), in its calculation, includes the chlorophyll fluorescence after excitation with UV and green light, thus it represents a combination of Chlorophyll and Flavonol Index. In our experiment, the NBI_G as well as the Anthocyanin Index excited with red and green light (ANTH_RG) indicated no significant

differences in young leaves when comparing the control and LED treatment groups (Fig. 3E+G). In mature leaves, the NBI_G was significantly higher at 16 and 30 DAT for plants exposed to LEDs (Fig. 3F). In mature leaves, the ANTH_RG slightly decreased during the evaluation period and showed differences between the treatments at DAT 16 (higher value for plants with LEDs) and 45 DAT (higher values for leaves of control plants, Fig. 3H).

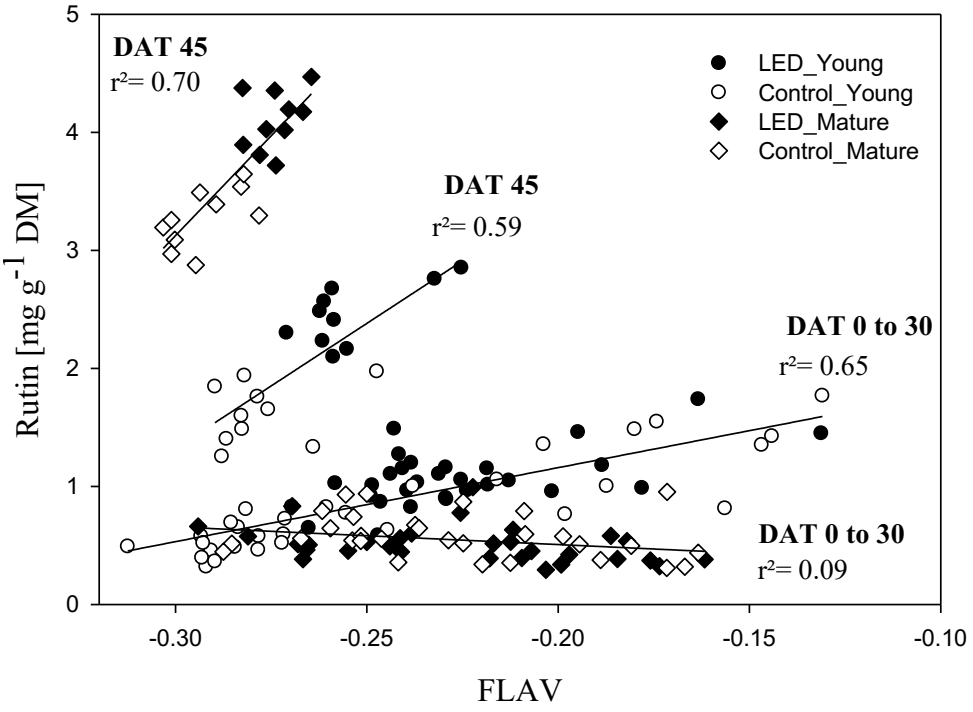



**Figure 4**. Correlation of the FLAV index with the rutin concentration according to the evaluation period and leaf age.

As shown in figure 4, clear correlation clusters between rutin and FLAV could be identified. However, these relationships depended on the leaf age, light treatment and time of exposure. The best linear correlation was found at DAT 45 for mature tomato leaves ($r^2 = 0.7$) followed by young leaves at DAT 45 ($r^2 = 0.59$). The data from 0, 16 and 30 DAT showed a better fitting between the FLAV index and the rutin content in young leaves ($r^2 = 0.65$) and nearly no correlation in mature leaves ($r^2 = 0.09$).

# 4. Discussion

In the present work, the accumulation of rutin due to supplemental LED light within commercial-like tomato cultivation was observed. Young leaves contained more rutin than mature leaves; this might be attributed to the fact that the tomato plants were rather small at the beginning of the experiment (Appendix B) so that more additional light reached the upper part of the plants. During vegetation, the mature leaves localized at the lower leaf levels grew closer to the LED modules resulting in higher amounts of rutin at 45 DAT. The effect of irradiation also explains why the rutin amount of young leaves decreased from the first to the second day of harvest (Fig. 2A) as plants were provided with supplementary lighting during the pre-cultivation period to maintain optimal seedling development. When plants were transferred to the greenhouse, the light conditions were weaker as compared to initial cultivation phase. In addition, the light distribution within the vertical layers of the tomato canopy influences plant growth since the mature, lower leaves were often shaded (21). This problem was recently discussed in a study by Jiang et al. (2017) where it was indicated that supplemental LED from underneath and within the tomato canopy positively induces plant growth, development and fruit yield (15). In this context, the optimal positioning of LEDs in the canopy can positively affect vegetative performance and fruit yield, as well as raise the content of secondary metabolites in the leaves. Another feasible explanation for the higher rutin concentrations in young leaves might be provided by Suzuki et al. (2005). The authors attribute this fact to the UV screening function of rutin since rutin absorbs UV-light in the same range as lignin and wax. Young leaves are softer with less amount of lignin and wax, thus, rutin is more suitable as a light screening compound. Mature leaves are often harder compared to young leaves indicating the production of lignin and wax instead of e.g. rutin. This explanation is also transferable to tomato plants (9).

The SFR_R index as an indicator of chlorophyll content was higher in LED exposed leaves, which was in accordance with other studies describing an influence of red and blue LEDs on different anatomical and morphological leaf parameters, as well as the enhancement of chlorophyll containing chloroplasts (11). Our results indicate that leaves radiated with LED light had a higher chlorophyll concentration leading to higher photosynthesis rate and a general better plant performance/health. In contrast, ANTH_RG did not provide any consistent result in our experiment.

The FLAV index and the rutin content showed a similar development in young and mature leaves. While the values were higher for young leaves exposed to LEDs from 30 to 45 DAT, the mature leaves of control plants showed marginally higher rutin and FLAV values at 30 and 45 DAT. At the last day of recording, both FLAV and rutin values were higher in mature leaves exposed to LEDs, suggesting a relationship between both parameters. Even though there was no consistent correlation for the whole dataset, specific data clusters were identified. Hence, when interpreting FLAV values of tomato leaves to estimate rutin content, information on leaf age and duration of LED lighting was crucial.

# 5. Conclusion

With the present study, we demonstrate for the first time under production conditions in a greenhouse the added value that can be exploited from the tomato biomass residues when using supplemental radiation with LEDs during plant cultivation. In particular, we found a significant increase in rutin content, which might be extracted and used as a natural source for different industrial applications in the pharmacological or food industry. Thereby, rutin content was influenced by leaf age as well as the light conditions since supplemental LEDs placed within the canopy led to an accumulation of rutin in young and mature tomato leaves. The non-destructive SRF_R index revealed differences between the treatment groups in both young and mature leaves, while the suitability of the FLAV index was limited to young leaves. A precise relation between the FLAV index and the real rutin content could not be established. However, clear data clusters as influenced by leaf age and sampling day were revealed. Further experiments and optimization work should focus on additional variables such as duration of LED light exposure, potential seasonal fluctuations, impact of varieties and other cultivation practices.

# 6. Acknowledgements

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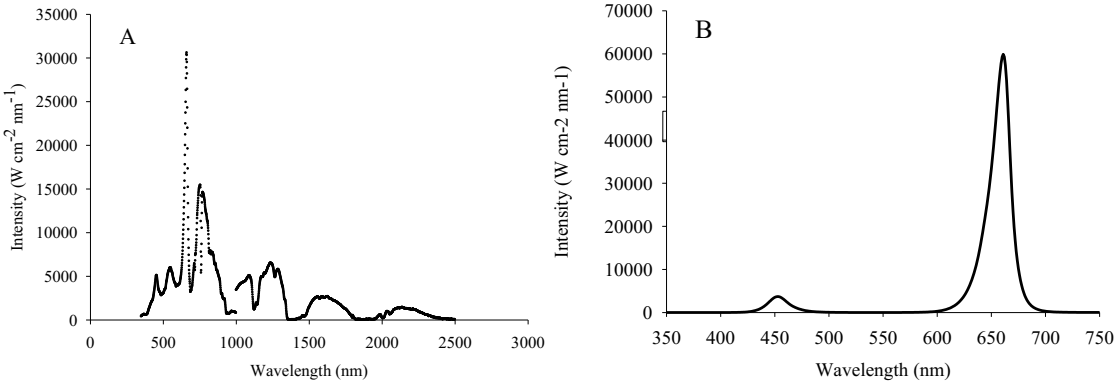
# 8. Appendix

A:




A. Spectral composition of the light in the greenhouse section measured at noon (A). The graph at the right side demonstrates the blue and red light peaks of the LEDs (B).

B:

B. View in the greenhouse section at 0, 16, 32 and 45 days after treatment (from top left to bottom right). The LED panels are placed above (0 DAT) and later (16 to 45 DAT) within the plant stock.

**D Suitability of fluorescence indices for the estimation of fruit maturity compounds in tomato fruits[3]**

# 1. Introduction

During the ripening process, the surface of tomatoes undergoes a significant color transformation, where the consumer commonly associates the final color with taste, health value and overall quality. Color categories - from green to tannish-yellow, pink or red up to fully red - have been established and standardized as maturity stages to allow classifications of fruit maturity according to the percentage of colored surface (1). For the consumers' perception, color is one of the most important external quality characteristic of fruits and vegetable (2, 3). Moreover, quality aspects are becoming more and more important worldwide, and play a crucial role in human nutrition concepts such as 'functional food', or vegetarian and vegan nutrition (4-6). In particular, tomatoes are a rich source of polyphenolic compounds such as flavonoids which are known to contribute to a healthy diet (6).

Different approaches have been proposed for non-destructive evaluation of food quality (7-9). In particular, chlorophyll fluorescence represents a tool to assess fruit quality attributes as it is a fast and easy-to-repeat technique, and suitable for use directly in the greenhouse. During the ripening process of tomatoes, a breakdown of chlorophyll takes place. Carotenoids, such as ß-carotene and lycopene, accumulate in the plastids as chloroplasts are converted to chromoplasts. Thus, while green tomato fruits contain mainly chlorophyll, no chlorophyll is present in fruits at harvest maturity (10, 11). This dynamic change in pigment composition and concentration strongly influences optical parameters such as the reflection and fluorescence signals (8).

Quality determinations based on the color reflection readings L*(lightness), a*(red to green) and b* (blue to yellow) are well established to evaluate tomato ripening. In particular, the a*/b* color index has been proposed to predict lycopene content at different maturity stages (12-14). Recently, Vazquez et al. (15) could not confirm the suitability of this ratio as appropriate color index, but integrated the readings in

[3] Groher, T., Schmittgen, S., Fiebig, A., Noga, G., Hunsche, M., 2018. Suitability of fluorescence indices for the estimation of fruit maturity compounds in tomato fruits. Journal of the Science of Food and Agriculture 98, 5656-5665.

regression models and artificial neural networks to estimate carotene and lycopene in tomato fruits. Lai et al. (16) showed that laser-induced fluorescence allows monitoring of pigments and detection of damage to the tomato fruit surface. Multi-parametric fluorescence recordings have been used to evaluate ripening and quality attributes of apples (4), classify maturity of oil palm bunches (17) and for monitoring of maturation (18-20) and anthocyanin content in grapes (21). In our group, Hoffmann et al. (22) proposed for the first time the use of fluorescence-based recordings to assess ripening of tomatoes in the pre- and postharvest phases without exploring the precise relationship between the recordings and the biochemical quality attributes. Beside the time-course of the indices depending on the different growing conditions and maturity stages, the focus of the current study was to explore the relationship between the compound concentrations and fluorescence and reflectance recordings. Therefore, indices were chosen which are directly related to biochemical compounds in the fruit.

As a promising parameter to estimate the content of chlorophyll, we selected the Simple Fluorescence Ratio excited with red light (SFR_R) which comprises the re-absorption of red light by chlorophyll. So far, this index has been used for leaf and fruit measurements, being directly related to the concentration of chlorophyll in the sample (4, 17, 23). The second chosen parameter to predict the content of flavonoids is the FLAV index. Flavonoids, which are mainly localized in the tomato skin (24), absorb UV-A light, whereby the FLAV index refers to wavelengths at around 360 nm. The amount of polyphenols, such as flavonoids, in the samples can be estimated because of their screening effect on chlorophyll excitation, depending on the wavelength of the excited light and the localization of the light absorbing compounds in the profile of the tissue. The more light is absorbed by epidermal compounds, the less light reaches the chlorophyll molecules in the mesophyll to generate fluorescence in the red and far-red regions. A few studies have demonstrated the relationship of the non-destructive FLAV index and flavonoid content, e.g. for grapes (20), kiwi exocarp (25) and apples (4). Additionally, we selected the FLAV_UV index for the quality determinations. This index was proposed for the first time by Ferrandino et al. (26) to estimate the flavonol concentration in colored berries relaying on an inverted, single signal of far-red fluorescence excited with UV to eliminate the interference of anthocyanins on the FLAV index. Anthocyanins in vivo can absorb at the 630 nm excitation band provided by the Multiplex®, affecting then the FRF_R signal used as reference for FLAV. As a last

parameter for this experiment, the single signal FRF_G was chosen as it uses only green light excitation and the resulting emission of far-red fluorescence.

So far, the relationship of chlorophyll fluorescence parameters recorded with a multi-parametric sensor and the effective pigment concentration in tomato fruit has not been the primary focus of current studies. One objective of this study is to characterize the changes in the selected parameters during the maturation of tomato fruits in the pre-harvest phase. Additionally, the relationship between non-destructive indices based on chlorophyll fluorescence and analytically determined maturity compounds was investigated and compared to the well-established reflectance sensor for tomato maturity. For this purpose, tomato plants were cultivated under different conditions (control, water deficiency and modified nitrogen supply) in a long-term greenhouse experiment to induce fruits of different ripening speed and behavior. The hypothesis guiding this work is that multiple fluorescence indices provide useful results to draw conclusions on biochemical maturity across different growing conditions. Classification of tomato maturity was always based on the percentage of colored surface. The suitability of the proposed sensors and parameters has to be proven under real conditions, since so far, many of these parameters were not validated under practical conditions.

## 2. Materials and methods

### Plant cultivation and growth conditions

The study was carried out in commercial-like greenhouses at the research station Campus Klein-Altendorf (University of Bonn, Germany). Tomato seeds (Lycopersicon esculentum, F1 hybrid Lyterno, Rijk Zwaan Distribution B.V., The Netherlands) were sown in January 2016 into rock wool cubes (Grodan delta, Grodan, The Netherlands) and grown under supplemental assimilation light. After four weeks, seedlings were transplanted into 1 m long rock wool slabs (Grotop Expert, Grodan, The Netherlands) with two plants per meter and 70 plants per row (distance between rows: 1 m). Plants were cultivated as one-shoot systems and fruit trusses were thinned out to six fruits per truss. Automatic drip irrigation was used for fertigation providing a full standard nutrient solution mixed from two stock solutions. The irrigation setup was controlled by time (on average 3.5 min $h^{-1}$) and additionally adjusted according to the plant needs as estimated by the sum of daily irradiation (additional irrigation starting at 15-20 kilolux). In one of

the greenhouses, all nitrogen compounds of the nutrient solution were decreased by 30% compared to the control plants. To maintain the pH value of the solution, potassium chloride was added while the amounts of other nutrients were not changed. In the other greenhouse, a moderate water deficit was implemented by decreasing the amount of nutrient solution up to 50% of the control treatment. The plants were cultivated during the time course of the experiment under natural day length and light intensity conditions with average day and night temperatures until the last harvest of 20.4±2.5 and 16.2±1.3 °C for greenhouse section 1 (c1, normal N supply/-N, modified nitrogen supply) and 20.7±2.3 and 16.0±1.1 °C for greenhouse section 2 (c2, normal water supply/wd, water reduction).

Non-destructive recordings

For each treatment, two fruits per truss (in a defined fruit position) from 50 plants were labeled for posterior in situ fluorescence readings. At the start of the recordings, all fruits had the same development stage (hazelnut-size, first to second truss from the apical meristem). The external fruit color was assessed on each measuring day according to the Standards for Grade of Fresh Tomatoes. Non-destructive optical recordings were performed one to three times a week from September to October 2016 for all selected plants. Recordings were taken at the equatorial zone of the light exposed fruit side. Reflectance determinations of tomato surface color were done with a portable spectrophotometer (CM-503d, Konica Minolta Inc., Tokyo, Japan). A sensing area of 7 mm² was used to read the L, a* and b* values of the CIELAB model. For fluorescence determinations, a hand-held multi-parametric sensor (Multiplex®, Force A, Orsay, France) with a mask of 2 cm diameter and a constant distance of 0.10 m to the fruit surface was used. The sensor, its working principle and the selected parameters are extensively described in the literature (17, 18, 25, 26, 27). For the current work, we selected the fluorescence indices SFR_R, FLAV, FLAV_UV and FRF_G to assess fruit maturity status.

The selected parameters are calculated as follows:

(1) SFR_R = (FRFred/ RFred)

(2) FLAV = log (FRFred/ FRFUV)

(3) FLAV_UV = log (1/FRFUV)

(4) FRF_G = FRFgreen

During fruit ripening from the green stage to the stage of harvest maturity, chlorophyll is degraded, whereas carotenoids, such as ß-carotene and lycopene, are synthesized (11). Chlorophyll molecules re-absorb red fluorescence and as a consequence the SFR_R increases with rising chlorophyll content and vice versa (Equation 1). The FLAV index is calculated from the ratio between far-red fluorescence excited with red light, which is not absorbed by flavonols, and the far-red fluorescence excited with UV-A, which is partially absorbed by flavonols (Equation 2). The log transformed FLAV_UV (Equation 3) is based on an inverted, single signal without using FRF_R as reference signal. The last chosen parameter was the FRF_G which is the far-red fluorescence excited with green light since lycopene absorbs in this spectral region (13).

The a*/b* reflectance index is well-established for fruit color readings and constitutes the ratio of green to blue-yellow compounds in the fruit.

Fruit harvest and sample preparation

Prior to harvest, tomato fruits were marked and classified into one of the six maturity stages (‘green’, ‘breaker’, ‘turning’, ‘pink’, ‘light red’ and ‘red’) according to their external fruit color (1). At the first harvest date, five fruits of the ‘green’ stage were sampled for each treatment group. Furthermore, tomato samples were taken at stages ‘breaker’, ‘turning’, ‘pink’, ‘light red’ and ‘red’, resulting in a constant decrease of fruits for the concomitant non-invasive recordings throughout fruit ripening. Due to the influence of the nitrogen and water supply, fruit maturation differed considerably between treatments, so that the harvest date of each treatment was postponed over time. Immediately after non-destructive recordings, selected fruits (five fruits per treatment and day) were harvested and cold transported to the laboratory, washed with distilled water, frozen and then freeze-dried for posterior sample preparation and analysis. Dried samples were ground for 1 min using a ball mill (MM 2000, Retsch GmbH, Haan, Germany).

Pigment extraction and determination

For each harvested fruit, chlorophyll a and b, lycopene and ß-carotene content were determined according to the method described by Nagata and Yamashit (28). Freeze-dried and ground samples (0.1 g DW) were homogenized and extracted with a 2 ml acetone-hexane mixture (4:6). The absorbance of the extracts was measured at 453, 505, 645 and 663 nm with an UV/VIS spectrophotometer (Lambda 35 UV/VIS, spectrophotometer, Perkin Elmer®, USA). Concentrations of pigments were calculated according to the following equations:

(5) Chlorophyll a (mg $100ml^{-1}$): 0.999A663 – 0.0989A645

(6) Chlorophyll b (mg $100ml^{-1}$): -0,328A663 + 1.77A645

(7) Lycopene (mg $100ml^{-1}$): -0.0458A663 + 0.204A645 + 0.372A505 – 0.0806A453

(8) ß-Carotene (mg $100ml^{-1}$): 0.216A663 – 0.304A505 + 0.452A453

(9) Total chlorophyll (mg $100ml^{-1}$): (5) + (6)

Flavonoid extraction and determination

The total flavonoid content was determined according to the methodology described by Hamrouni-Sellami et al. (29) with slight modifications. Briefly, a methanolic extract (80% methanol + 1.0% hydrochloric acid) was prepared from 0.05 g freeze-dried and powdered fruit sample. From an aliquot of the methanolic extract, 1 ml was mixed with 0.1 ml of 0.72 mol $l^{-1}$ $NaNO_2$ solution, 0.1 ml of 0.75 mol $l^{-1}$ $AlCl_3$ solution and 0.1 ml of 1 mol $l^{-1}$ NaOH at defined time intervals. After vortexing for 10 s, 1.7 ml water was added to adjust the sample to a final volume of 3.0 ml. After 30 minutes of incubation, absorbance was measured at 510 nm using a UV/VIS spectrophotometer (Lambda 35 UV/VIS, spectrophotometer, Perkin Elmer®, USA). Quercetin was used as the standard for a linear curve between 0.1 and 1.0 mg $ml^{-1}$, and the results were expressed as milligram of quercetin equivalents in dry mass. Analyses were performed in triplicate and the following equation was used to calculate flavonoid content:

(10) $Y = 0.9865x + 0.00113$

Statistical analysis

Statistical differences between control fruits (c1, normal N supply) and fruits grown under reduced nitrogen supply (-N) as well as control fruits (c2, sufficient supply of water) and fruits grown under water deficit (wd) were determined using the t-test ($p \leq 0.05$) with SPSS statistical software (IBM statistics version 22.0, Armond NY, USA). Graphs and curve fittings were done with SigmaPlot for Windows version 10.0 (Systat Software, Inc.).

# 3. Results and discussion

Non-destructive recordings

The mean values (±SE, standard error) of the selected indices SFR_R, FLAV, FLAV_UV, FRF_G and a*/b* of fruits harvested from plants grown under different cultivation conditions are shown in Figures 1 and 2. Fruits grown under normal N supply and fruits grown under nitrogen deficiency showed similar fluorescence (Fig. 1A, C, E, G) and reflection signals (Fig. 2A), whereby the only significant differences were found for the FLAV index which was significantly higher in the control fruits on the first measurement day. However, this difference faded from the second to the last recording day. The mild nitrogen deficiency had only slight effects on fruit development, resulting in a similar maturation pattern for samples of normal N supply and nitrogen deficiency (–N) samples. Only at 23 DaH (days after first harvest), fruits in the –N treatment were already in maturity stages six while fruits in the control needed three more days to reach the stage of harvest maturity. Fruit from plants cultivated under water defiency (wd-fruits) matured faster compared to their control fruits, reaching the ‘breaker’ stage four days earlier than control fruits. This finding suggests an accelerated maturation and confirms earlier studies from our group targeting the accelerated process of tomato ripening due to a lower water supply monitored with the multi-parametric fluorescence sensor (22). For the non-destructive SFR_R index, wd-fruits presented significantly higher values for day 0, 17 and 20 as compared to their control fruits (Fig. 1B). The SFR_R is sensitive to the chlorophyll content of the samples due to the re-absorption of red fluorescence. Therefore, the index decreased from unripe to mature red fruits. The specific pattern of the SFR_R is explained by the accelerated maturation of the wd-fruits

compared to control samples. Control fruits were in the `pink` stage at 17 DaH and `light-red` at 20 DaH, while wd samples were already more mature with maturity stage `light red` at 17 DaH and `red` at 20 DaH. The later maturity stages had less or almost no chlorophyll but mainly carotenoids while earlier stages contained more chlorophyll and fewer carotenoids resulting in the different fluorescence patterns. If the content of chlorophyll in the sample decreases, less red-light will be absorbed, resulting in a lower amount of far-red fluorescence detected by the sensor.

The FLAV index started with values at about zero and increased gradually, peaking between 0.3 to 0.4 units at the 'light-red' maturity stage, followed by a decrease at the last evaluation date in the 'red' stage (Fig. 1D). A similar development for this index was found in a pre-harvest monitoring of tomato fruit ripening (22). Again, fruits from the wd-treatment revealed an earlier maximum than control fruits.

Technically, the calculation of the FLAV index includes two signals, the FRF_R and the FRF_UV, both based on chlorophyll excitation with light (in the red or UV region) resulting in the far-red-fluorescence signal (FRF). The FRF_R serves as a reference for chlorophyll content in samples, while the FRF signal after UV excitation (FRF_UV) is affected by both UV-light-absorbing flavonoids located morphologically above the chlorophyll in the leaf or fruit shell and the decreasing level of chlorophyll, both reducing the far-red fluorescence signal. Thus, the FRF_UV is affected by the absorption of flavonoids of the energy-rich UV-light shielding chlorophyll molecules and therefore, reducing the far-red fluorescence signal. In the case of tomato fruits, the FRF_R cannot serve as a reference signal because the FRF_R signal strongly decreases during the chlorophyll degradation of fruit maturation process. Besides chlorophyll breakdown (reduced FRF_R signal), the maturity-related accumulation of UV-absorbing flavonoids affects the FLAV index as well. Based on these altering FRF_R and FRF_UV signals, it cannot be clearly stated whether changes in FLAV were related to chlorophyll reduction or flavonoid accumulation. Hence, the FLAV index cannot be used to monitor fruit maturity and to estimate the content of flavonoids in tomato fruits of different ripening stages. Even though the FRF_UV is affected by changes in the flavonoid content, the effect of FRF_R signal reduction superimposes FLAV index based estimations of the flavonoid content in fruits.

Due to this technical issue of two affected signals, the single signal-based FLAV_UV index was tested for the potential estimation of flavonoid accumulation, that refers solely to the FRF_UV signal. The FLAV_UV index increased during the time-course of the experiment showing significant differences between c2 and wd-fruits only at 17 DaH (Fig. 1F).

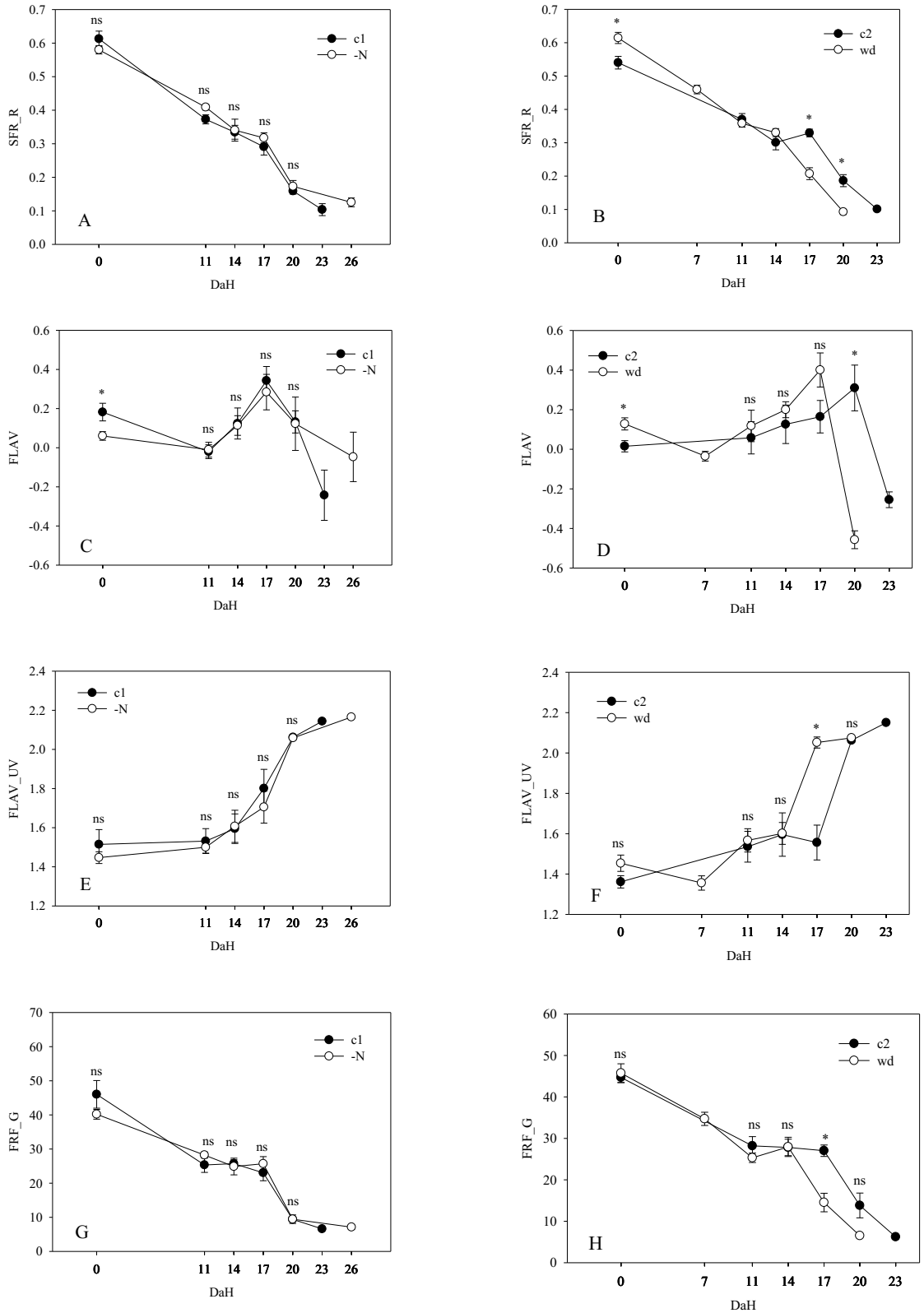



**Figure 1.** Fluorescence-based indices SFR_R [A+B], FLAV [C+D], FLAV_UV [E+F] and FRF_G [G+H] as influenced by nitrogen (c1: Control, -N: Nitrogen deficiency) and water supply (c2: Control, wd: Water deficiency). Means ± SE (n = 5 replicates per treatment and day) were compared for each harvesting date (DaH: Day after first harvest) with the t-test ($p \leq 0.05$); asterisk indicates significant differences between the treatments; ns: non-significant.

Thus, this index seemed to be more suitable for monitoring flavonoid accumulation during fruit ripening, even though the single fluorescence signal is also dependent on chlorophyll breakdown. However, the FRF_UV signal is only indirectly affected by flavonoid absorption (shielding of chlorophyll molecules), and mainly provided by fluorescence signals that decrease during the ripening-related chlorophyll reduction. So, at later maturity stages corresponding with low chlorophyll concentrations, the single signal FRF_UV index becomes inaccurate itself; however, the comparison of plants under control and stress conditions allows to distinguish between stress-induced alterations.

As last index, the single FRF_G signal was selected due to the green light excitation with the aim to evaluate lycopene concentration based on its absorption in the green spectral range (13). The FRF_G signal decreased from the green to the mature red stage of maturity showing a significantly higher value at 17 DaH for c2 fruits (Fig. 1H). The wd-fruits showed lower values for the FRF_G at 17 and 20 DaH due to lower chlorophyll content of the fruits on those days. The measuring principle is equal to the FLAV_UV calculation. The more compounds absorbing green light in the layers above the chlorophyll molecules, less light reaches the chlorophyll molecules. In general, the chlorophyll content is crucial for the measurement principle of chlorophyll fluorescence, regardless of the energy level of excitation signals. Hence, maturity related chlorophyll breakdown in tomato fruits affects all used fluorescence indices. Nevertheless, SFR_R, FLAV and single signal indices (FLAV_UV and FRF_R) showed maturity-related alterations in our study.

As an additional measurement of surface reflection, we considered including the a*/b* reflectance index that showed an increasing development for all applied treatments (Fig. 2). While no difference was found between nitrogen limitation and control conditions, reduced water supply resulted in significantly higher values of the a*/b* index 11 days after the first harvest compared with the control (Fig. 2B). This parameter seemed to be more sensitive to the biochemical modifications in fruit maturation than fluorescence-based indices, even though the differences faded by day 14.

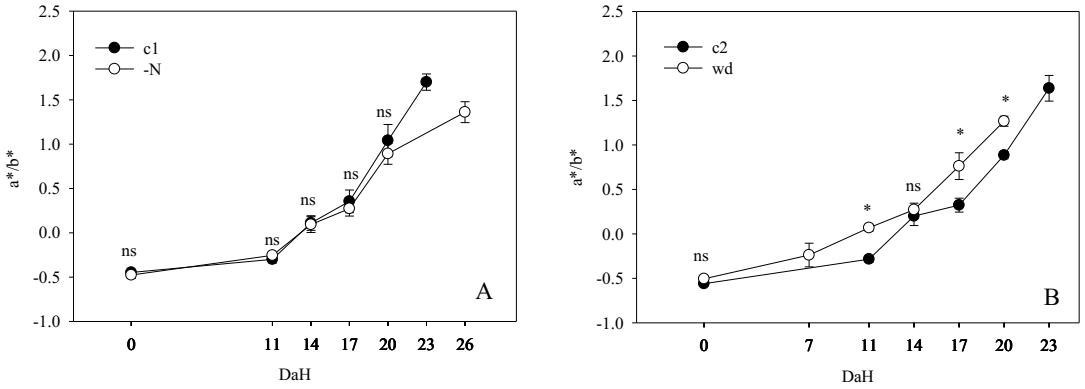



**Figure 2.** Reflection index a*/b* as influenced by nitrogen (c1: Control, -N: Nitrogen deficiency) and water supply (c2: Control, wd: Water deficiency). Means ± SE (n = 5 replicates per treatment and day) were compared for each harvesting date (DaH: Day after first harvest) with the t-test ($p \leq 0.05$); asterisk indicates significant differences between the treatments; ns: non-significant.

Lab analysis

The effective pigment concentrations determined destructively are shown in Figure 3. The mean values presented here are in line with those of other publications dealing with the ripening development from green to red in tomato fruits (30, 31). Regardless of the nutrient supply of the plants, chlorophyll content steadily declined while lycopene content increased with progressing maturity stage (Fig. 3A-D). The content of ß-carotene first increased, followed by a slight decrease (Fig. 3E+F).

In general, during tomato ripening, changes in pigment composition start when a fruit has reached its final size at the end of development even though the chlorophyll concentration peaks much earlier at the beginning of fruit growth (11).

The mean chlorophyll, lycopene, and ß-carotene and flavonoid values did not significantly differ from each other for c1 and -N fruits. Two exceptions were noted at day 0 (first harvest date), where the control fruits showed significantly higher values for the chlorophyll content, and at day 17, where the control fruits also showed higher amounts of ß-carotene (Fig. 3A+E). A trend of increasing flavonoids was observed for fruits at 11 DaH until the last harvest date although these were not significantly different between the cultivation conditions (Fig. 3G+H).

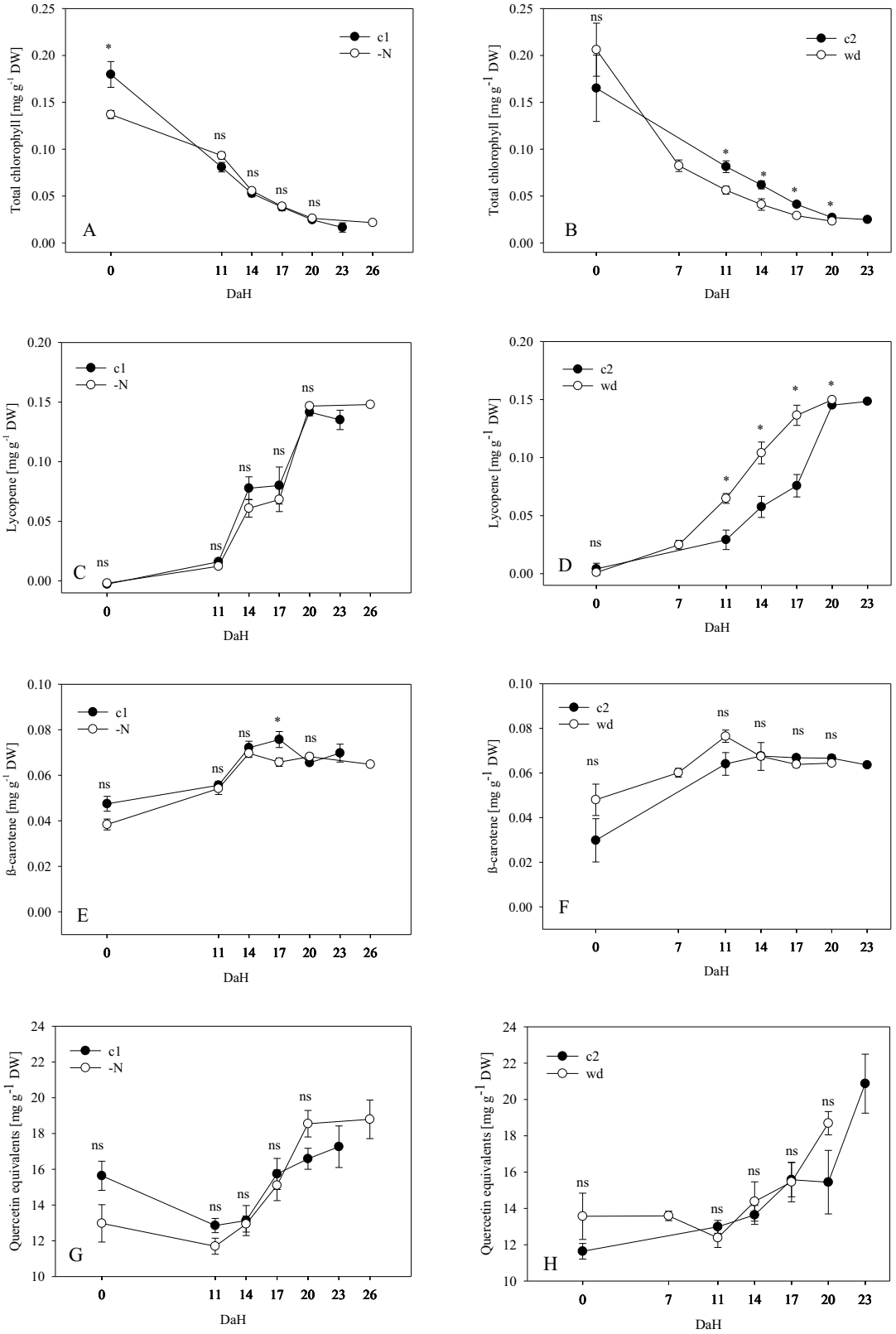



**Figure 3.** Total chlorophyll [A,B], lycopene [C,D], ß-carotene [E,F] and flavonoid content [G,H] as influenced by nitrogen (c1: Control, -N: Nitrogen deficiency) and water supply (c2: Control, wd: Water deficiency). Means ± SE (n= 5 replicates per treatment and day) were compared for each harvesting date (DaH: Day after first harvest) with the t-test ($p \leq 0.05$); asterisk indicates significant differences between the treatments; ns: non-significant.

Usually, fertilization with nitrogen is associated with increased carotene concentrations in plants (32), but there is little information on specific data in the literature (33). Bénard et al. (34) observed in a practical tomato greenhouse experiment that different nitrogen regimes only had a poor significant effect on carotene and phenol levels during the vegetation period. From this, they resumed that a conservation of nitrate at about 75% would not change fruit yield and external quality. Moreover, our results are in line with various studies testing the effects of a modified nitrogen supply (0 to 250 kg N $ha^{-1}$) on fruit quality attributes. No significant differences were found for color, weight, firmness and total solids (35) and the ratio of total solids/ titratable acidity (36) in these field studies.

Due to the lower water supply, fruit ripening accelerated. This result is reflected in the chlorophyll and lycopene content from day 11 to 20 showing significant differences between c2 and wd-fruits (Fig. 3B+D). The decreased water supply did not affect the concentrations of ß-carotene and flavonoids in the tomato fruits (Fig. 3 F+H). Various environmental stresses, such as water deficit in our study, stimulate the ethylene formation of fruits which is an important factor for the initiation and continuation of maturation, not only in tomatoes but in all climacteric fruits (11).

Correlation between non-destructive indices and effective pigment concentration

One advantage of using non-destructive sensors for quality evaluations is the large amount of data which can be recorded in a very short time on the same individual fruit without reducing the number of samples in time-series evaluations. The fluorescence-based sensor used in this study provides several fluorescence signals and parameters resulting in a big dataset at the end of the experiment. However, not all of these indices are suitable for monitoring maturity/quality attributes of tomato fruits. Therefore, we chose the most promising indices and investigated whether the biochemical analyses of maturity compounds and the multiple fluorescence indices provide similar results.

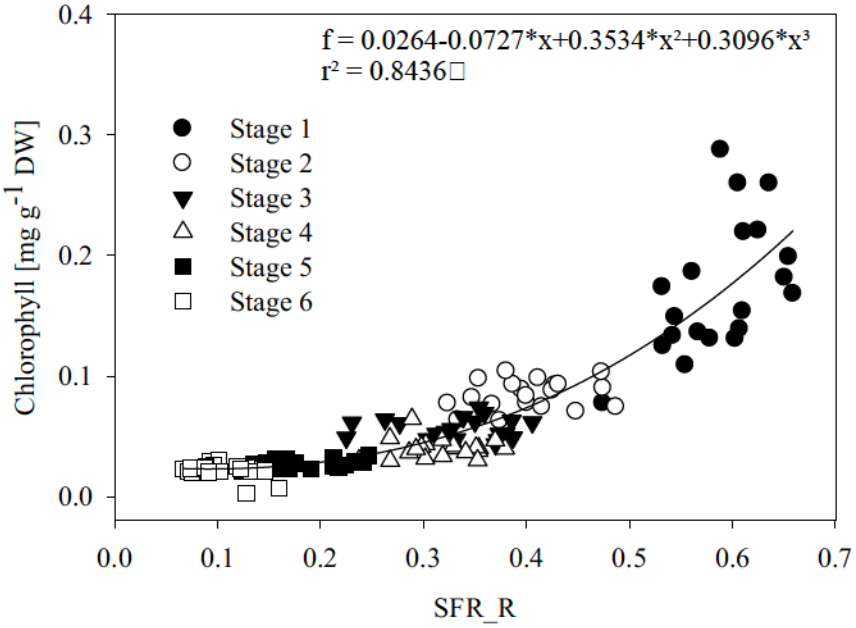



**Figure 4.** Correlation between the optical SFR_R index (Multiplex®) and the destructively measured total chlorophyll content in tomato fruits at different maturity stages. The solid line indicates curve fitting. Equation and $r^2$ for the regression line (all data) are given.

Correlation analysis between the non-destructive parameters and the concentration of the pigments in the tissue were done using the whole dataset of each experiment, from the first until the last evaluation date. In each diagram (Fig. 4-7), the values are presented pairwise for the corresponding parameters. Figure 4 indicates the relationship between the non-destructive SFR_R index and the destructively determined chlorophyll content of 120 fruits across the six maturity stages. The values of the SFR_R decreased alongside the decreasing chlorophyll content of the samples. The fitted exponential model based on the SFR_R index explains 84% of the variance in chlorophyll content. By using this index in tomato fruits, the chlorophyll content can be estimated non-invasively. Similar results were obtained in apple fruits, where the SFR_R and the corresponding chlorophyll values in the skin showed a good positive correlation ($r^2 = 0.872$) (4). In addition, we fitted an exponential function ($r^2 = 0.8143$) for the relationship of the FRF_G signal with the spectrophotometrically determined lycopene content (Fig. 5).

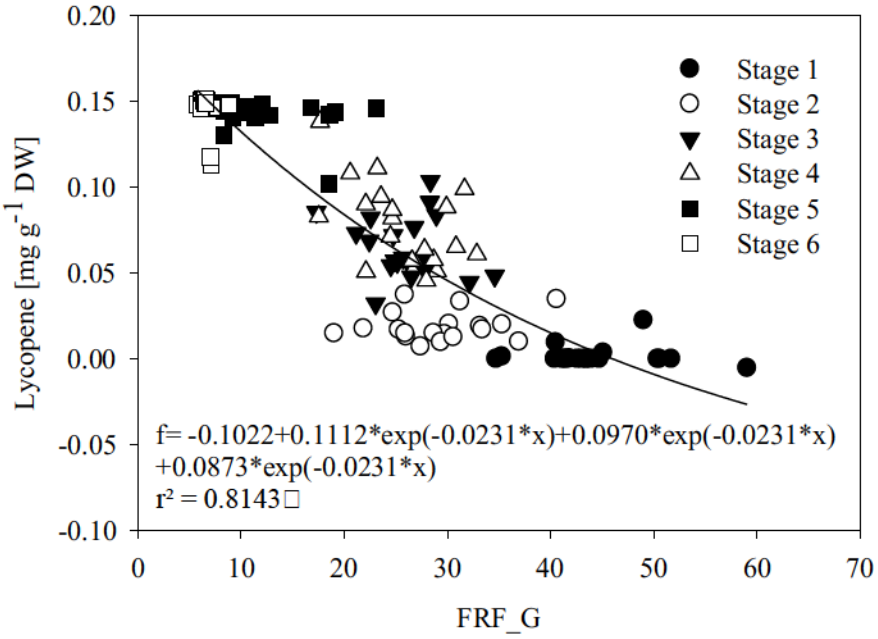



**Figure 5.** Correlation between the optical FRF_G signal (Multiplex®) and the destructively measured lycopene content in tomato fruits at different maturity stages. The solid line indicates curve fitting. Equation and $r^2$ for the regression line (all data) are given.

The FRF_G signal strongly decreased for rising maturity stages while analytically determined lycopene concentration increased. The correlation showed low FRF_G values for high lycopene concentrations and vice versa, but within the different maturity stages, there was a wide variation with values between 18 to 40 units for maturity stages two to four. The high correlation could be due to the fact that lycopene absorbed the green excitation light and, as is always the driving force, that the chlorophyll content in general strongly decreased, thus less fluorescence was detected by the sensor. Regarding the practical point-of-view, a differentiation between unripe fruits and fruits with harvest maturity is clearly indicated by the relationship of both parameters. However, realistically, an exact prediction of lycopene concentration based on the FRF_G signal only is not possible.

Correlation analyses for the a*/b* reflectance index and lycopene as well as chlorophyll concentrations are shown in Figure 6.

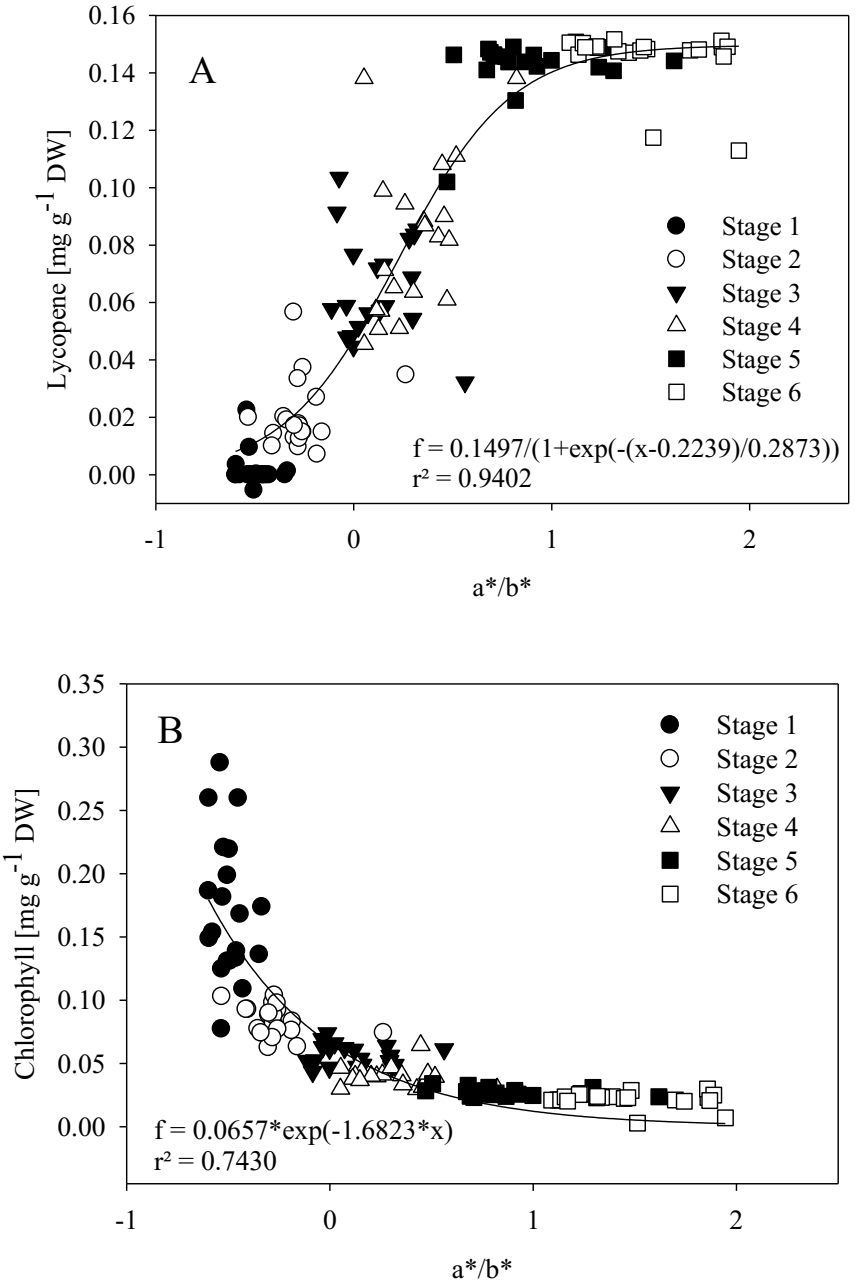



**Figure 6.** Correlation between the optical a*/b* index (Minolta®) and the destructively measured lycopene [A] and total chlorophyll [B] content in the fruits depending on maturity stage. The solid line indicates curve fitting. Equations and $r^2$ for the regression lines (all data) are given.

While a steep slope for the a*/b* reflectance index and lycopene concentration increased for a*/b* values from about -0.5 to 2 (Fig. 6A), the correlation of the index with chlorophyll concentration was contrary, e.g. a higher chlorophyll concentration at the beginning (maturity stage 1) and a continuous decrease to nearly zero for the sixth maturity stage (Fig. 6B). The coefficient of determination for the a*/b* reflectance index

and the effective lycopene content was more precise ($r^2 = 0.9402$) than the FRF_G signal based on chlorophyll fluorescence. Comparing the relationship between chlorophyll concentrations and reflection or fluorescence-based indices, the SFR_R provided better results ($r^2 = 0.8436$) than the a*/b* reflectance index ($r^2 = 0.7430$). It has been published before that lycopene content and color readings in tomato fruits correlate well (13) as also proved in the current greenhouse experiment.

The relationship between FLAV index, which is based on the ratio of the chlorophyll fluorescence excited with red and UV-A light, and the effective flavonoid content determined destructively, is shown in Figure 7A. The correlation between both variables ($r^2 = 0.0696$) indicates that the FLAV index is not a reliable parameter to predict flavonoid content in tomato fruits since both fluorescence signals were affected by chlorophyll breakdown. As reported for apple fruits, Hagen et al. (37) found a positive correlation ($r^2 = 0.899$) between chlorophyll fluorescence parameters and lab analyses in a postharvest apple study, similar to the results of Betemps et al. (4) where they compared the FLAV index with flavonol concentrations in apple peel extracts ($r^2 = 0.85$). In kiwi fruits, a correlation between the fluorescence-based index and the flavonol concentration of the exocarp could be established ($r^2 = 0.83$) (25), whereas in grapes, a relationship for both parameters was also shown depending on the cultivar (Vermentino with $r^2 = 0.766$ (20), Nascetta with $r^2 = 0.8056$ and Chardonnay $r^2 = 0.83$ (26)).

However, when correlating the single signal FLAV_UV and the corresponding flavonoid content of whole fruits (Fig. 7B), a higher correlation ($r^2 = 0.4536$) compared with the FLAV index was found, but a precise prediction of flavonoid content in tomato fruits was not possible in our case. In other studies, the calculated FLAV_UV index showed a high correlation with flavonol concentration in the colored grape Barbera with $r^2 = 0.74$, whereas the FLAV index was not suitable for this cultivar with $r^2 = 0.04$ (26). In our study with tomato fruits, this result could be partially confirmed. In addition, no significant correlations could be established for the ß-carotene content or any of the indices provided by the employed measurement devices.

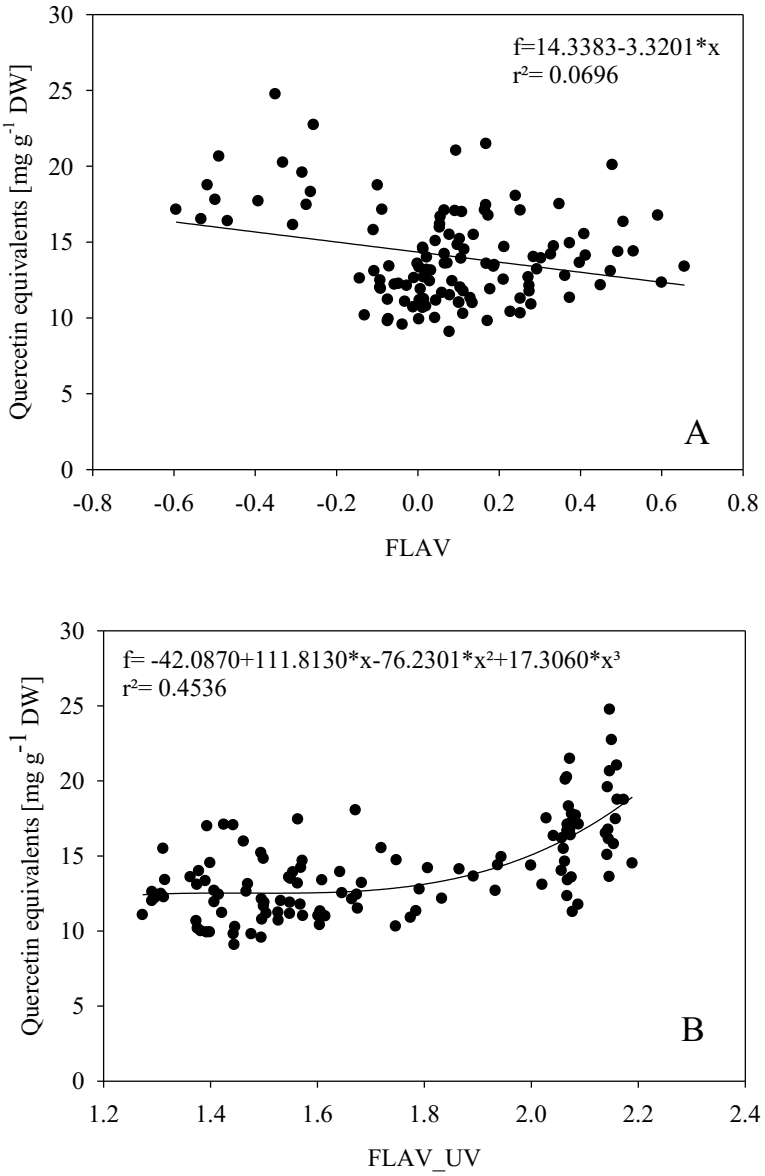



**Figure 7.** Correlation between the FLAV index provided by the Multiplex® [A] and the calculated FLAV_UV index [B] with the destructively measured flavonoid content in tomato fruits. The solid line indicates curve fitting. Equations and $r^2$ for the regression line (all data) are given.

In summary, the weak correlation between the FLAV index and FLAV_UV with the corresponding flavonoid concentrations may arise from the evaluation methods. Firstly, the fluorescence signals depend on chlorophyll content that decreases during maturation and therefore, allows no conclusion about whether chlorophyll decreases or flavonoids accumulate. Secondly, the used analytical method for the flavonoid determination was not specific for those flavonols, which are the class of flavonoids with the absorption spectrum shifted to longer wavelengths and closer to the excitation wavelength of the sensor (375 nm).

# 4. Conclusion

For the first time, we have shown that fluorescence indices correlate well with the content of chlorophyll in tomato fruits. The SFR_R index provides proper information on the chlorophyll content of fruits at different maturation stages while alterations in the lycopene content can be estimated indirectly using the FRF_G. An application of the FLAV and FLAV_UV index to predict changes in flavonoid content in ripening tomatoes could not be found. Decreases in chlorophyll during tomato ripening are the driving force affecting all fluorescence signals. The knowledge generated in this study might be used to estimate the content of chlorophyll in tomato fruits non-destructively during ripening at different pre-harvest or postharvest phases.

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# E Summary and conclusions

The first main objective of this study was to track plants' physiological and biochemical changes in response to abiotic stresses with a multiparametric chlorophyll fluorescence-based sensor to prove the suitability of non-destructive estimations of leaf and fruit constituents. A second objective was to generate knowledge about the potential of tomato leaves from commercial production systems for the extraction of industrially relevant secondary metabolites, particular rutin and solanesol, which are known to increase due to abiotic stress. For this purpose, tomato plants were grown in commercial-like greenhouses and exposed to moderate abiotic stress conditions to induce physiological and biochemical changes in leaves without lowering fruit yield, the primary aim of commercial production. First, either nitrogen or a general nutrient deficiency was applied to enhance rutin and solanesol concentrations in leaves of different physiological ages. Physiological changes were recorded with a multiparametric sensor and selected indices were compared to biochemical parameters determined by HPLC analyses to prove the applicability of the provided indices to track changes in leaf compounds due to altered nutrient supply. Second, supplementary intracanopy blue and red light-emitting-diodes (LED) were used to promote the accumulation of rutin in young and mature tomato leaves. Here again, the relationship between fluorescence-based recordings and laboratory HPLC analyses were evaluated. Third, the suitability of two devices - the multiparametric fluorescence sensor and a reflection-based spectrophotometer – was evaluated to track changes and estimate concentrations of tomato maturity compounds during ripening under mild nitrogen and water deficiency cultivation conditions.

The following main results were achieved in the single chapters:

1. The chlorophyll fluorescence-based indices SFR_R, NBI_G, FLAV and ANTH_RG were able to monitor differences between the treatments in young leaves, but no differences were found in mature leaves. None of the evaluated indices were suitable for a reliable estimation of rutin or solanesol concentrations in tomato leaves. Nitrogen deficiency (fertigation with standard nutrient solution without N-containing compounds) as well as a general nutrient deficiency (fertigation with tap water) led to an accumulation of rutin in young tomato leaves, whereas solanesol concentrations were higher in fully-developed mature leaves. The quantity of fruit showing symptoms of blossom-end-rot as a

parameter to assess the impact of altered nutrient supply on fruit quality and marketable yield was higher in plants grown under a general nutrient deficiency.

2. The SFR_R and FLAV showed stress-induced differences in leaves of all investigated development stages while the usefulness of the indices NBI_G and ANTH_RG was limited to mature leaves. Supplementary intracanopy light with red and blue light-emitting-diodes (ratio 4:1) induced rutin concentrations mainly in young tomato leaves and partly in mature leaves. Correlation analyses between rutin concentrations and the FLAV index displayed clusters according to the leaf age and time point of harvest. However, a precise rutin determination or even estimation could not be reached with these methods.

3. Fluorescence-based indices were suitable to track the accelerated tomato ripening of fruits grown under water deficit. The coefficient of determination between the SFR_R and the chlorophyll concentration determined analytically was very high with $r^2 = 0.84$. Moreover, the single signal FRF_G and the lycopene concentration showed a coefficient of determination of $r^2 = 0.81$ even if a precise differentiation between the maturity stages two (breaker) to four (pink) was not observed. Compared with fluorescence-based indices, the relation between the reflection index a*/b* and pigment concentration was lower for chlorophyll ($r^2 = 0.74$) and higher for lycopene ($r^2 = 0.94$). In particular, the decrease in chlorophyll content was the driving force affecting all fluorescence signals. In consequence, an estimation of other maturity/quality compounds with chlorophyll fluorescence-based recordings, such as flavonoids with FLAV or FLAV_UV, is not appropriate.

Specific parameters recorded with the multiparametric chlorophyll fluorescence-based sensor were suited to monitor stress-related differences in tomato leaves and fruits and to estimate the chlorophyll content of tomato fruits, although an estimation of rutin or solanesol in tomato leaves was not feasible. As expected for this type of sensor, the content of chlorophyll in the sample was always the driving force affecting all fluorescence signals. Thus, as a consequence of the decrease of chlorophyll content during tomato ripening, the fluorescence signals changed accordingly.

Based on the findings of this study, the potential of different growing conditions and varieties on the accumulation of rutin and solanesol are encouraged. The potential of other crops containing high amounts of secondary metabolites in their green biomass should be investigated to evaluate the potential of other well-established fruit production systems to achieve a second gain of production from available by-products. It was shown here that abiotic factors such as mineral deficiencies or supplemental LED illumination enhance the content of secondary metabolites in tomato leaves. This enhancement in the content of SM is further influenced by other factors such as seasonal variation and optimal environmental conditions. Finally, the concentrations vary in different plant organs and probably in different production systems. In particular, the adoption of light-emitting-diodes with different light spectra offers several possibilities for further research since it has a positive impact on both yield and fruit quality, as well as SM content in leaves. In order to avoid any negative impairment on yield related to the applied nutrient deficiencies, an implementation in commercial growing systems is most suitable at the end or in the last weeks of fruit production when the whole plant remains in the greenhouse.

Our results indicate a potential of tomato leaves for SM extraction; nevertheless, comprehensive analyses of the entire value chain are necessary to estimate a real applicability for commercial production. In particular, besides considering other components beyond rutin and solanesol, inconvenient aspects need to be carefully evaluated. For farmers, an additional workload occurs due to collection and storage of tomato by-products. Furthermore, using e.g. tomato leaves from commercial production, a contamination with pesticides or fungal infestation under wet weather conditions cannot be ruled out. A further aspect is the seasonal variation in the amount of SM which negatively impacted SM yield compared to chemically synthesised constituents. Therefore, the technical feasibility on the spot should be evaluated more detailed.

The applicability of the multiparametric fluorescence-based sensor for the real-time estimation of tomato fruit and leave constituents was limited mainly to chlorophyll. For further analyses of the relation between non-destructive indices and secondary metabolites, other evaluation methods as well as the suitability of other sensors based on e.g. reflection including a wider range of wavelength should be considered. This also offers the opportunity to choose specific wavelengths and calculate new indices which can be correlated with the content of secondary metabolites.

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