Effect of UV Radiation and Salt Stress on the Accumulation of Economically Relevant Secondary Metabolites in Bell Pepper Plants

Authors

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Key words

Capsicum annuum, flavonoids, fluorescence monitoring, bio-waste utilization

Abstract

The green biomass of horticultural plants contains valuable secondary metabolites (SM) which can potentially be extracted and sold. When exposed to stress, plants accumulate higher amounts of these SMs, making the extraction and commercialization even more attractive. We evaluated the potential for accumulating of the flavones cynaroside and graveobioside A in leaves of two bell pepper cultivars (Mavras and Stayer) when exposed to salt stress (100 mM NaCl), UVA/B excitation (UVA 4-5 W/m²; UVB 10-14 W/m² for 3 hours per day) or a combination of both stressors. HPLC analyses proved the enhanced accumulation of both metabolites under stress conditions. Cynaroside accumulation is effectively triggered by high-UV stress, whereas graveobioside A contents increase under salt stress. Highest contents were observed in plants exposed to combined stress. Effects of stress on overall plant performance differed significantly between treatments, with least negative impact on aboveground biomass found for high-UV stressed plants. The usage of two non-destructive instruments (Dualex and Multiplex) allowed us to gain insights in ontogenetical effects at the leaf level and temporal development of SM contents over time. Indices provided by those

devices correlate fairly with amounts detected via HPLC (Cynaroside: R^2 = 0.46 – 0.66; Graveobioside A: R^2 = 0.51 – 0.71). The concentrations of both metabolites tend to decrease at leaf level during the ontogenetical development even under stress conditions. High-UV stress is a promising tool for enriching plant leaves with valuable SM without major effects on plant biomass. All data is available online [1].

Abbreviations

DATI – days after treatment inception

HPLC – high performance liquid chromatography

ROS – reactive oxygen species

SM – secondary metabolite

Introduction

Green biomass as a source of valuable chemicals

Commercial vegetable production is accompanied by large quantities of so far under-utilized green biomass in all stages of production and especially after harvest [2]. While the use of biomass for the purpose of energy production is becoming a standard procedure in northern Europe in recent years [3], the extraction and the use of high-value SMs from vegetable plant leaves are just being developed. Research strategies and legacy in Europe are heading towards cascade use of agricultural byproducts and pave the way for extracting and using “valuable substances or molecules before ultimately discarding the left-overs” [4]. The pharmaceutical industry – as an example – is highly dependent on plant SMs, since approximately 60% of anticancer compounds and 75 % of drugs for infectious diseases are derived from plants [5]. In this frame, research on targeted enrichment of valuable substances in plant biomass is gaining in importance [6].

Plant stress as a measure to increase leaf secondary metabolite content

The biochemical background of enhanced accumulation of SMs in plant leaves as a measure to cope with stress is a well described phenomenon [3,7,8]. In short, the cultivation of plants under suboptimal conditions leads to an increased amount of reactive oxygen species (ROS) in plant tissues. Accumulation of SMs is a plant strategy to avoid oxidative damage caused by reactive oxygen species. In theory, both biotic and abiotic stressors could lead to higher amounts of valuable SMs in plants, but while biotic stressors such as fungi and insects are hard to control and may cause major phytosanitary problems, abiotic stressors are easier to manage and applicable by practitioners. The results of several studies in recent years indicate that abiotic stressors are a useful tool for SM accumulation in leaves of horticultural plants. SMs in *Centella asiatica* leaves increase under enhanced UV-B light, especially in the epidermis [9]. In bell pepper, increased flavonoid contents can be found in leaves exposed to elevated UV [10]. The promoting effect of UV-B radiation on flavonoid accumulation in plant leaves has recently been reviewed [11]. The effects of salt stress on the antioxidant machinery may be adverse and depends on the plant's tolerance. While tolerant plants increase leaf SM contents to cope with salt stress, sensitive plants do not have this mechanism and induce senescence instead, finally dying off if the stressor is persistent [12]. Studies directly comparing effects of salt and UV stress on leaf SMs are rare. One study shows both stressors to similarly affect leaf contents of the flavonoids quercetin and luteolin in *Ligustrum vulgare* [13].

Abiotic stressors such as drought and salt stress are easily applicable in commercial greenhouse production in soilless systems, which are the predominant systems in many parts of the world, including Europe [14].

Non-invasive monitoring of secondary metabolites in plant leaves

Quantification of secondary metabolites including flavonoids with portable optical devices is well established in plant sciences [15]. The use of non-invasive optical sensors to investigate plant leaf components has several advantages over laboratory analyses: Data acquisition is faster and more cost effective than laboratory analyses [16]. Moreover, considerate handling

of leaves allows for several measurements of the same leaf, enabling to gain insights in temporal developments. Several studies demonstrated the viability of optical devices to access secondary metabolites in plant leaves: A multiparametric fluorescence sensor was used to evaluate the influence of nutrient deficiency on the chemical properties of tomato leaves and to quantify the content of the flavonoids rutin and solanesol [17,18]. In bell pepper, a fluorescence sensor was used to evaluate the impact of priming plants with high-light conditions on leave flavonoid content [10].

Cynaroside and Graveobioside A

The vast diversity and chemical complexity of plant SMs often prohibit an economically feasible chemical synthesis. Therefore, extraction either from wild or cultivated plants often represents the best source of supply [2].

Cynaroside (Luteolin-7-glucoside) potentially has a range of medicinal applications: Both its capability to prevent ROS-induced apoptosis in heart cells [19] and its positive effect on kidney injury as a side effect of cancer treatments with the chemotherapeutic drug cisplatin. A potential medicinal use of graveobioside A (Luteolin-7-apiosyl-glucoside) is proven by a patent on its application in preparation of drugs for preventing hyperuricemia and gout [20]. Graveobioside A was shown to be contained in several plants, such as celery seeds, parsley and bell pepper [21,22].

Several SMs in Solanaceae leaves have the potential to biologically control insects [23]. Graveobioside A is such a potential natural insecticide, since oviposition of the American serpentine leafminer fly (*Liriomyza trifolii*) was shown to drop in kidney bean leaves treated with a graveobioside A containing solution [22]. It is expected that the demand for natural insecticides will increase across the EU due to more rigid legislation [24].

We hypothesize that cynaroside and graveobioside A contents in bell pepper leaves can be enhanced by abiotic stressors that are potentially applicable by practitioners in the future. Another aim is to check weather non-invasive devices can be used for assessments of cynaroside and graveobioside A in bell pepper leaves. We furthermore attempt to get insights

in interactions between different stressors and differences in stress response between two bell pepper cultivars.

Material and Methods

Plant material and growth conditions

Sweet pepper plants (*Capsicum annuum*) cultivar 'Stayer' (Rijk Zwaan, The Netherands), and 'Mavras' (Enza Zaden, The Netherlands) were grown in rock wool (Grotop Master, Grodan, The Netherlands). All plants received all nutrients mandatory for optimal growth prepared from two stock solutions (17.2 mM nitrogen, 5.4 mM calcium, 4.7 mM potassium, 0.4 mM phosphorous, 5.4 mM sulfur, 2.4 mM magnesium, 0.01 mM iron and all micronutrients; electrical conductivity 2.5 mS cm^{-1}, pH 5.5). Plants were cultivated at the greenhouse facility in Bonn-Endenich (University of Bonn, Germany) at day/night temperatures of 24.5 °C ± 5.4 and supplemental light intensity of 203-540 µm $m^{-2}s^{-1}$ provided by sodium vapor lamps (Philips Lighting GmbH, Hamburg, Germany).

To apply salt stress, a salt concentration of 100 mM NaCl for a period of 14 days was added to the standard nutrient solution. To apply UV stress, plants were exposed to UV light (UVA 4-5 W m^{-2}; UVB 10-14 W m^{-2}) for 3 hours per day (Philips Lighting GmbH, Hamburg, Germany). In addition, some plants were exposed to combined salt and UV stress. A total of 5 plants per treatment (control, salt stress, UV stress, combined stress) were randomized in the growth chamber.

Non-destructive recordings

Non-destructive measurements were performed on all leaves per plant, from mature to young. Measurements were taken using the multiparametric fluorescence excitation system Multiplex® (Multiplex®, Force-A, Orsay, France), as described in previous studies [25]. 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 4 cm in diameter opening to assess the index of epidermal flavonols (FLAV index): $\log \frac{FRF\_R}{FRF\_UV}$

Plant harvest

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

Leaf sample preparation and laboratory analysis

Samples were taken at the harvesting at 16 DATI, of the mature leaf 4 and the young leaves 10 and 12, to assess the impact of stress application on the amount of the two luteolins graveobioside A and cynaroside. The samples were freeze-dried and then stored at - 20 °C until further processing. Ground leaf samples were prepared for HPLC determination (Agilent 1260 Infinity HPLC System Agilent Technology Deutschland GmbH, Ratingen, Germany). An amount of 0.3 g was extracted with water-diluted methanol (60:40, v/v) for 10 min in an ultrasonic bath, centrifuged for 10 min at 4 °C with 13000 rpm (Centrifuge 5415R, Eppendorf AG, Hamburg, Deutschland) repeated four times. The supernatants were collected and stored at - 20 °C until HPLC analysis. The samples were filtrated through a membrane filter (Phenomenex, Aschaffenburg, Germany) prior to injection. The HPLC system consisted of an autosampler, a diode array UV-Vis detector and was coupled with a quaternary solvent delivery system. The column (Nocleodur C18, 3 x 150 mm, 3 µm, Macherey-Nagel, GmbH & Co. KG, Düren, Germany) was isocratically eluted with a binary mixture of water and methanol (60:40) adjusted to pH 2.8 with phosphoric acid. The flow rate was 0.3 mL min^{-1}, 10 µL samples were injected onto the column equilibrated at 25 °C (detection at 355 nm). Graveobioside A peak was detected at 14.1 min, cynaroside at 15.6 min. Both calibration curves were obtained from diluted series of standards provided by PhytoLab (Vestenbergsgreuth, Germany).

Statistics

Data analysis was performed with R [26] in RStudio [27]. According to the data structure, e.g. balanced or imbalanced, type I or type III ANOVA were used to compare group means. Applied post-hoc test was Tukey's HSD. Figures were created in RStudio, with the package ggplot2 [28].

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Results

Stress-related effect varies among secondary metabolites and cultivars

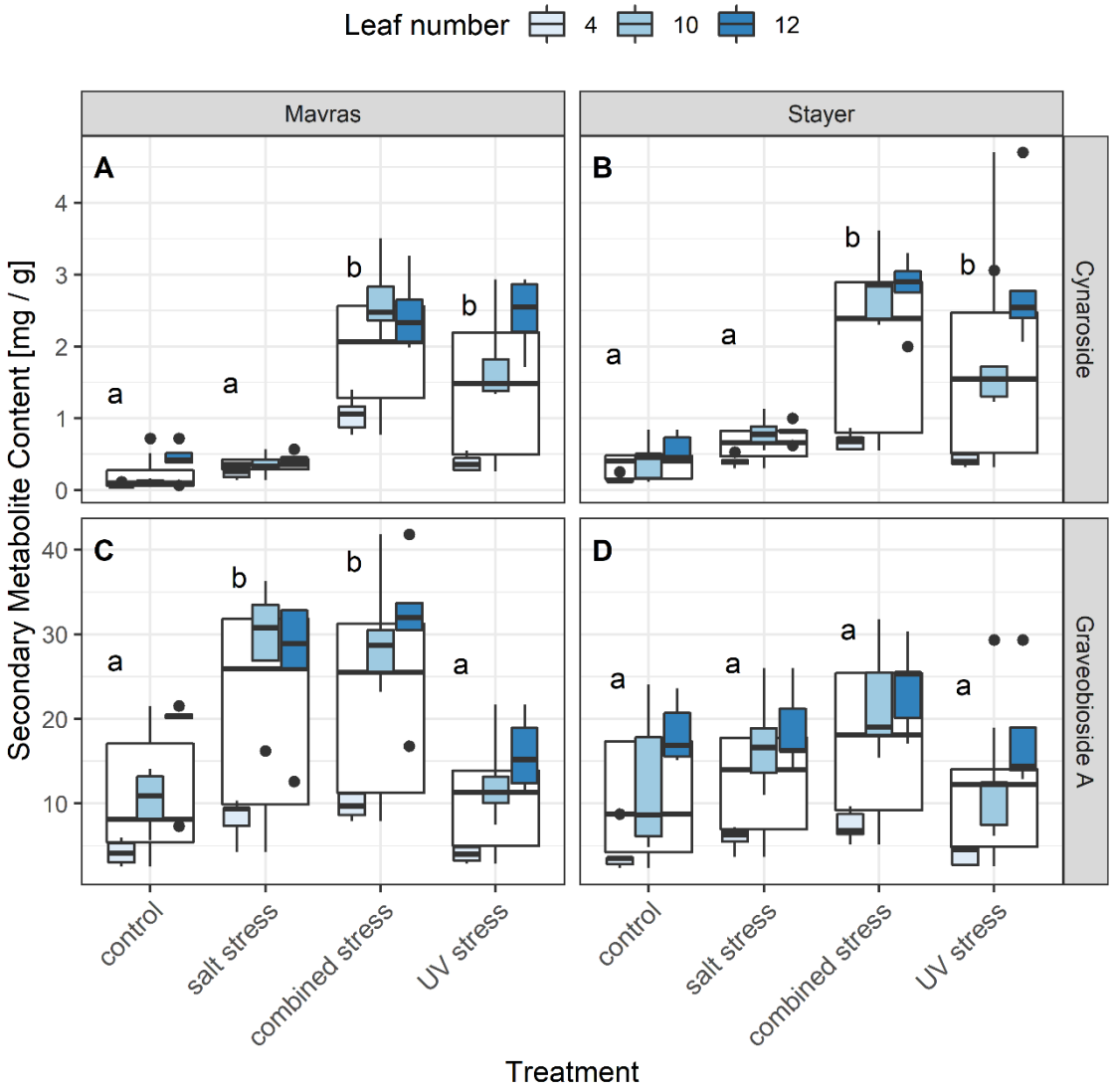

Figure 1: HPLC-determined leaf cynaroside (A, B) and graveobioside A (C, D) contents, for bell pepper cultivars 'Mavras' (A, C) and 'Stayer' (B, D) under different growth conditions, 15 days after treatment inception (n = 5). Transparent boxplots show pooled data from all leaves (n = 15). Letters (a,b) indicate differences within each cultivar * secondary metabolite – combination (Tukey HSD, $p < 0.05$).

A treatment effect was observed on contents of both cynaroside and graveobioside A, while no significant effect of the variable cultivar on either metabolite content was found. There was a strong tendency for higher graveobioside A in 'Mavras' as compared to Stayer ($p = 0.055$). No interactions between cultivar and treatment were observed (Tab. 1). Both combined

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stressed plants and plants under UV-exposure accumulated significantly higher amounts of cynaroside in their leaves than control and salt stressed plants (Fig. 1, A+B). Plants of the cultivar 'Mavras' accumulated significantly higher graveobioside A amounts in salt-stressed and combined-stressed plants than in control and UV-stressed plants (Fig. 1, C). No significant treatment effect on graveobioside A content in plants of the cultivar Stayer was found (Fig. 1, D). Levels of SM in leaves of different ontogenetical stages are shown as an illustration of uneven distribution within the plants. SM contents decrease with leaf ontogenetical stage.

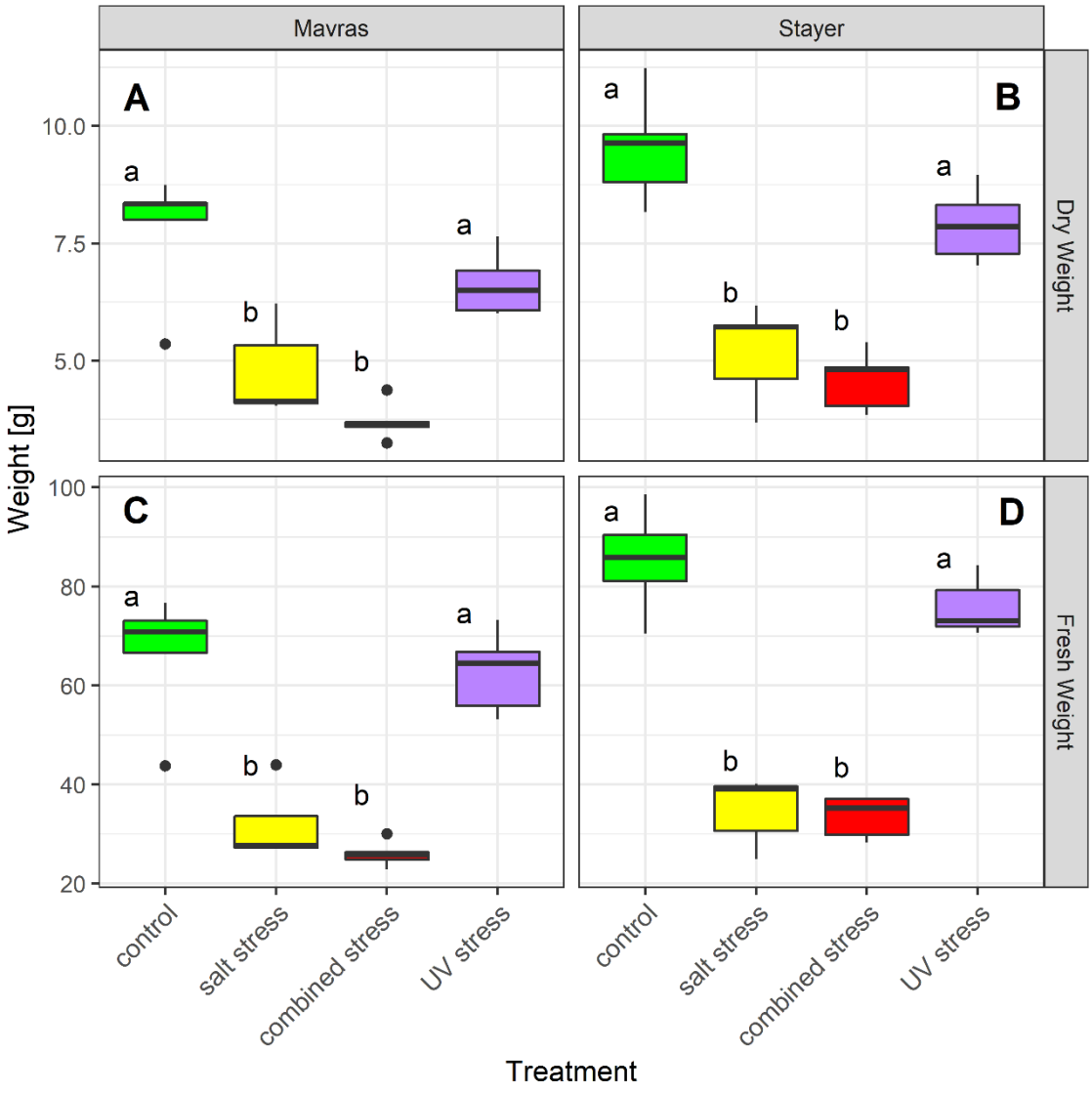

Figure 2: Aboveground biomass of bell pepper cultivars 'Mavras' and 'Stayer' under different growth conditions, 15 days after treatment induction (n = 5). Letters (a,b) indicate differences within each cultivar * dry/fresh weight – combination (Tukey HSD, $p < 0.05$).

Both fresh and dry weight of bell pepper plants differed significantly depending on the cultivar, with Stayer attaining higher weights than Mavras. Treatment had a significant effect on both

fresh and dry weight. There was no interaction between the treatment and cultivar regarding plant fresh or dry weight. Dry weight of plants of the cultivar Mavras was significantly higher in control plants than in any other treatment (Tab. 1). UV stressed plants of the cultivar Mavras exhibited higher dry weights than plants under combined stress conditions. The mean dry weight of salt stressed plants tended to be higher than under combined stressed, but lower than those of salt stressed plants, without statistical significance (Fig. 2, A). Dry weight of Stayer was higher in control and UV-stress plants than in plants exposed to salt and combined stress (Fig. 2, B). The same applies to plant fresh weights, for both cultivars respectively (Fig. 2, C+D).

Table 1: Interaction and main effect for treatments (control, salt-stress, combined stress, UVstress) and cultivars (Mavras and Stayer), calculated with a type I two-way ANOVA. Greyed area indicates significant effect ($p \leq 0.001$).

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

Non-invasive monitoring of secondary metabolites

Figure 3 shows exponential regressions between three indices (Multiplex indices FLAV and NBI_R; Fig. 3 A - D and Dualex index Flav; Fig. 3 E + F) and leaf contents of the SMs cynaroside (Fig. 3 A, C, E) and graveobioside A (Fig. 3 B, D, F), respectively. Predictions of graveobioside A contents based on the indices are better than predictions of cynaroside contents. Multiplex indices are more accurate predictors than the Dualex index, as outlined by correlation coefficients (r^2). Index values level off at cynaroside contents above 1.5 mg g^{-1}. The connection between graveobioside A and the indices is more linear, but still levelling off at graveobioside A contents above approximately 25 mg g^{-1}.

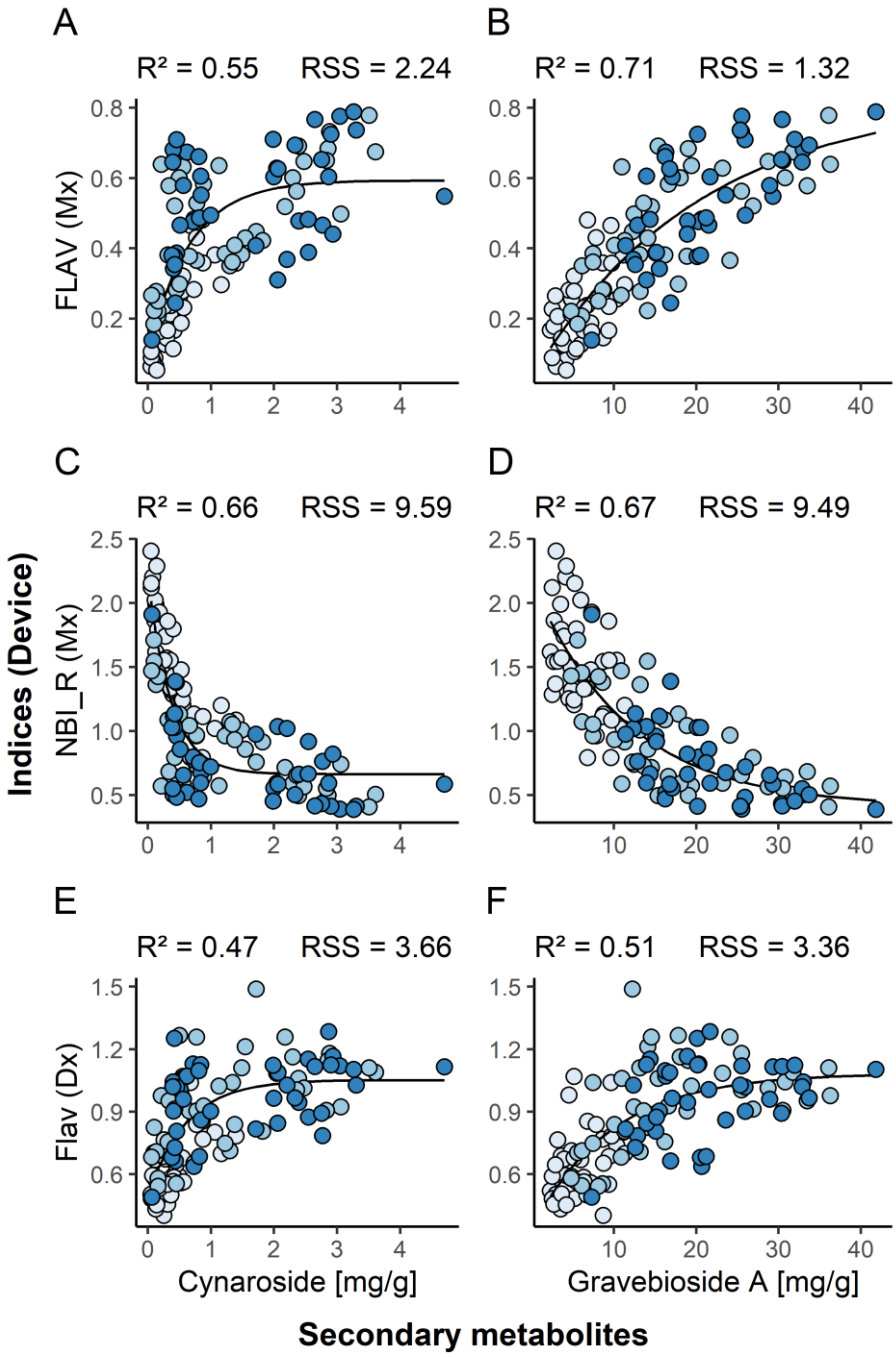

Figure 3: Exponential regression between indices of non-invasive devices and leaf secondary metabolite concentrations in bell pepper leaves, determined via HPLC. Color of points represents leaf age, with darkest colors for youngest leaves. Lines indicate exponential regressions (n = 60). RSS = residual sum of squares.

Spatial and temporal development of secondary metabolite contents

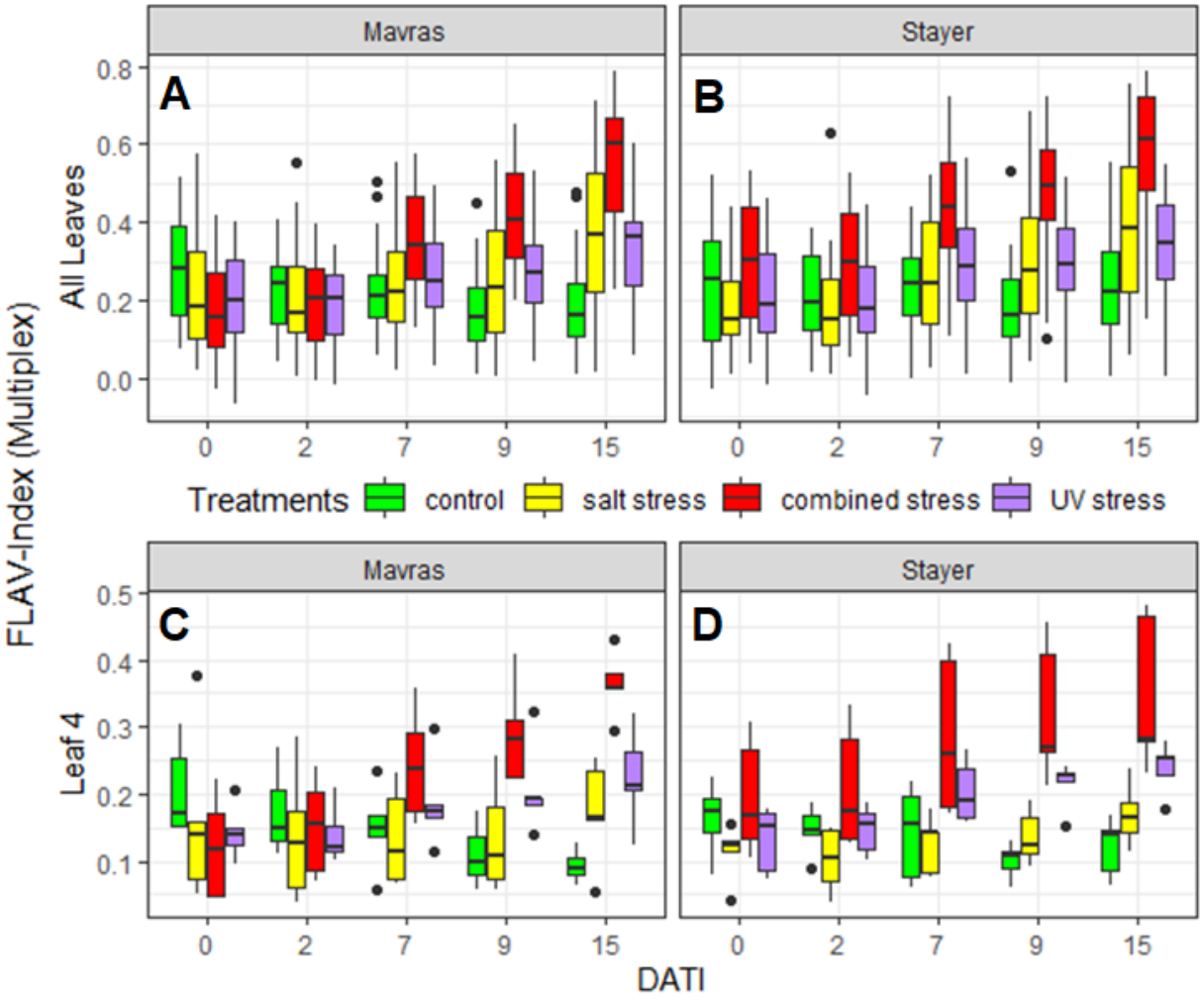

Figure 4: Temporal development of secondary metabolites in leaves of bell pepper cultivars 'Mavras' and 'Stayer', expressed with the FLAV-index (Multiplex) (C, D: n = 5; A, B: n = 5 – 50). DATI = Day after treatment initiation.

The only significant changes in FLAV values within cultivar * treatment groups were seen among the fourth leaves of combined stressed Mavras plants at days 0 vs. 9 and 0 vs. 15 respectively (Fig. 4, C). A clear trend was observed for the fourth leaves of combined stressed Stayer plants at days 0 vs. 15 (TukeyHSD, $p = 0.053$) (Fig. 4, D). Generally, FLAV values for stressed plants tend to increase, while the values for control leaves tend to decrease.

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

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

Discussion

We are among the first groups accessing the amount of graveobioside A in pepper leaves [5]. For cynaroside, the range of values detected corresponds to the results of other studies [29,30].

Stress-related effect varies according to secondary metabolites and cultivars

Since cynaroside contents under single UV-stress and combined UV- and salt-stress are not significantly different (Fig. 1, A+B), cynaroside accumulation appears to be triggered mainly by high radiation conditions. Interestingly, and in contrast to cynaroside, graveobioside A accumulation is triggered more effectively by salt stress than by UV-stress, especially in the cultivar Mavras (Fig. 1, C). Thit is a surprising result, since biosynthesis of flavonoids is said to be enhanced similarly by UV radiation and salinity [13,31]. On the other hand, some authors report that the regulation of SM production in response to salt stress differs between salt sensitive (upregulation) and salt tolerant (downregulation) plants [12]. However, differences in salt stress tolerance between the cultivars used in this study are not supported by differing plant biomasses (Fig. 2). The chemical group of flavonoids is highly diverse, and metabolic pathways are not entirely understood to date. At this point, it remains unclear how exactly upregulation of cynaroside synthesis under UV stress and upregulation of graveobioside A synthesis under salt stress occures.

Our results indicate – as expected – that salt stressed plants acquire a significantly lower biomass than both control plants and UV-stressed plants. Stunted growth is a well described symptom of salt stress in plants [12,32], whereas plants reaction to UV-B exposure varies from growth reduction to enhancement, dependent on species, cultivar and stress level [11,33]. Since the overall aim of the stress application is the accumulation of higher amounts of secondary metabolites in the plant´s green biomass, it is necessary to consider not only the share of desired metabolite in the plant´s biomass, but also the biomass reduction caused by the treatment. Considering this background, we can state that stressors with minor negative effects on plant biomass accumulation, but major positive effects on contents of desired metabolite in the plant tissues, are necessary to achieve these aims. In our specific setup with two single stressors and one combined stress, the single UV stress is most promising, whereas salt stress, although promoting the accumulation of graveobioside A, is less promising as a tool to enhance whole plant SM amounts, due to the decrease in total biomass. Effects on plants grown over a whole season are a matter of ongoing research.

Non-invasive monitoring

The indices provided by both optical devices deliver better estimates for leaf graveobioside A contents than for leaf cynaroside contents. That is an expected result, since the amount of graveobioside A as determined via HPLC is up to ten-fold higher than the amount of cynaroside (0 – 4 mg g^{-1} vs. 2 – 40 mg g^{-1}) and both secondary metabolites share similar optical properties. Any estimate of concentrations based on non-invasive, optical devices will be best for the predominant fraction of a group of metabolites with similar optical properties. By the same token, signals of metabolites that occur in small quantities are more likely to be superimposed by other signals and therefore difficult to quantify. Additional factors known to influence non-invasive assessment of leaf compounds include the concentration of other pigments potentially influencing the measurement [34], leaf thickness [35] and the device used [36].

In our study, the FLAV-index of the Multiplex shows an almost linear response to changes in leaf graveobioside A content (Fig. 2, B). The same applies for the NBI_R index, which

correlates negatively with the actual graveobioside A content. Both indices use the far-red fluorescence of leaves excited with UV-light and normalize that signal for the red fluorescence emitted after excitation with red light [37]. As an enhanced graveobioside A content leads to a stronger absorption of UV light in the leaf epidermis, less radiation penetrates into the mesophyll, which in turn leads to a lower chlorophyll fluorescence. We have to highlight the broad distribution of fluorescence values, though, which prohibits a precise prediction of actual graveobioside A levels on the individual leaf level. The Flav-index of the Dualex is almost indifferent to changes at graveobioside A levels above 25 mg g^{-1}.

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

Insights in spatial and temporal accumulation of secondary metabolites

The usage of non-invasive phenotyping tools such as the Multiplex and Dualex devices allows to analyze leaf constituents during ontogenesis. The observed drop of the flavonol content in leaves of unstressed plants during ontogenesis (Fig. 4, C+D) is in line with the theories that (a) the production of phenolics, such as flavonols, is mainly caused by photodamage [38] and (b) that ontogenetically young leaves are in general more prone to be affected by high light stress than older leaves, since their photosynthetic apparatus is not yet well developed [39] and the photoprotective cuticula is thinner compared with older leaves [40]. Therefore, young leaves show stress-related reactions in conditions that are neither stressful for older leaves nor for the entire plant. However, the described ontogenetic effects, tend to be overcompensated by stress related effects in all three stress treatments (Fig. 4, C+D). Thus, flavonol contents of the fourth leaf as measured with the FLAV (Mx) index slightly increased in

plants experiencing single stresses, while plants exposed to combined stress showed major increases in leaf flavonol contents (Fig. 4, C+D).

Implications and future challenges

The present study proves that abiotic stresses, in particular salt stress and UV stress, can enhance the amount of economically valuable SMs, namely cynaroside and graveobioside A, in bell pepper leaves. The main objective of growing bell pepper plants, however, is the production of fruits of adequate quantity and quality for human nutrition. Considering the decline in plant biomass in response to stress conditions, it is very likely that the stressors applied would also lead to a reduction in fruit production. Salt stress in particular is known to be an important factor limiting crop productivity [41]. We have shown that the type of stressor has magnificent effects on both plant biomass and leaf secondary metabolite content. Other studies have proven that this also applies for different levels of abiotic stress [42]. The search for the best stressors and stress levels for the accumulation of secondary metabolites in plant leaves with negligible effects on fruit yield is a major future challenge for research in stress physiology. Several authors reported neutral or positive responses of product quality to mild stress [42]. Low UV radiation reduces the antioxidative capacity and therefore the fruit quality of bell pepper fruits [43]. Additional UV radiation may help to overcome this problem and at the same time induce the production of valuable SM in the leaves. Cultivation of plants under mild water stress conditions can also enhance water use efficiency. To avoid any competition with food production, post-harvest treatment of leaves could be an appropriate measure to achieve high contents of promising metabolites [44,45]. These effects should also be taken into account when evaluating the value of production systems that are based on commercialization of both fruits and SMs in leaves of horticultural plants.

To enhance precision of non-invasive estimation of SMs in pepper leaves, future studies should consider hyperspectral sensors as well as chlorophyll fluorescence-based sensors, ideally a combination of both. Sensors covering the UV range are just entering the market and

appear as a promising tool to access SMs in plants, as they cover absorption bands of flavones and other phenolic leaf compounds [46].

Conclusions

Both additional UV light and salt stress can enhance concentrations of the two SMs graveobioside A and cynaroside in bell pepper leaves. Highest concentrations were reached by combining both treatments. Stressed bell pepper leaves contain up to 30 mg graveobioside A and abou 2 mg cynaroside per gram dry weight. While salt stress has a major negative impact on plant vegetative growth, UV stress has no significant impact on the fresh mass of the plants. The tendency of decreasing SM contents in leaves during ontogenesis is outweighed by the stress treatments. Graveobioside A contents can be assessed with the multiparametric fluorescence sensor Multiplex. Reliable quantification of cynaroside is not possible with the non-invasive sensors used. If future experiments exclude major negative impacts on fruit quality, UV stress can be recommended as one tool to enhance valuable SMs in bell pepper leaves and potentially in vegetable leaves in general.

Author contributions

Conceptualization, S.R.-S.; data analysis, J.E., N.S., P.K., J.B.S.L, D.P., M.R., L.S.; methodology, S.R.-S.; writing – original draft preparation, J.E.; writing – review and editing, J.E., S.R-S., N.S., P.K., J.B.S.L, D.P., M.R., L.S.

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Conflict of interest

The authors declare no conflict of interest.

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