JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Article

Impact of Moderate Cold and Salt Stress on the Accumulation of Antioxidant Flavonoids in the Leaves of Two *Capsicum* Cultivars

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Cite This: *J. Agric. Food Chem.* 2021, 69, 6431−6443

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ABSTRACT: The horticultural production of bell peppers generates large quantities of residual biomass. Abiotic stress stimulates the production of protective flavonoids, so the deliberate application of stress to the plants after fruit harvest could provide a strategy to valorize horticultural residuals by increasing flavonoid concentrations, facilitating their industrial extraction. Here we exposed two *Capsicum* cultivars, a chilli and a bell pepper, to cold and salt stress and combinations thereof to determine their valorization potential. Noninvasive image-based phenotyping and multiparametric fluorescence measurements indicated that all stress treatments inhibited plant growth and reduced the leaf chlorophyll fluorescence index, with the chilli cultivar showing greater sensitivity. The fluorescence-based FLAV index allowed the noninvasive assessment of foliar luteolin glycosides. High-performance liquid chromatography−mass spectrometry (HPLC-MS) analysis showed that moderate cold increased the levels of two foliar antioxidant luteolin glycosides in both cultivars, with bell pepper containing the highest amounts (induced to maximum 5.5 mg g^{-1} DW cynaroside and 37.0 mg g^{-1} DW graveobioside A) after combined stress treatment. These data confirm the potential of abiotic stress for the valorization of residual leaf biomass to enhance the industrial extraction of antioxidant and bioactive flavonoids.

KEYWORDS: *abiotic stress, graveobioside A, cynaroside, apiin, apigetrin, antioxidants, chilli, bell pepper*

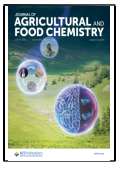
INTRODUCTION

The increasing human need for plants and plant-derived compounds has encouraged the intensified agricultural production of food, feed, and biomass crops, as well as medicinal and aroma plants. The agriculture, horticulture, and food/feed industries therefore generate large amounts of waste biomass.[1] The sustainable use of such biomass residuals adds industrial value and contributes to the efficient utilization of resources. Currently, most waste biomass is converted into energy or biogas,[2] but there is increasing interest in the use of residual green biomass as a source of high-value secondary metabolites.[3,4] Bell pepper (*Capsicum annuum* L.) is a crop plant grown for its fruits, which provide a good source of vitamins, carotenoids, and phenylpropanoids.[5] However, the leaves of pepper plants also contain valuable bioactive compounds, such as the antioxidant flavonoids cynaroside (luteolin-7-glucoside), graveobioside A (luteolin 7-*O*-(2-apiosyl)glucoside), apigetrin (apigenin 7-glucoside), and apiin (apigenin-7-apiosylglucoside).[4,6−8] Cynaroside possesses antibacterial, antiviral, and anticancer properties,[9−11] whereas graveobioside A can inhibit oviposition in the leafminer *Liriomyza trifolii* and is therefore a candidate for the development of natural insecticides.[6] Furthermore, apigetrin can suppress neuro-inflammation by inhibiting microglial activity,[12] and apiin has been used for the synthesis of antimicrobial nanoparticles.[13] The postharvest extraction of such valuable flavonoids from bell pepper leaves would utilize the residual green biomass and thus valorize the horticultural production cycle. The economic feasibility of industrial extraction would be improved by increasing the content of valuable secondary metabolites.

When plants experience biotic and abiotic stress, their growth and yield are negatively affected.[14,15] Plants have evolved various morphological and physiological stress responses for protection against unfavorable environmental conditions, including the accumulation of secondary metabolites.[16−18] The composition and abundance of secondary metabolites in plants is dependent on the species, developmental stage, tissue, and environmental conditions.[19,20] Strongly genotype-dependent variation has been observed in the flavonoid content of the genus *Capsicum*.[5] Furthermore, *Capsicum* cultivars differ widely in pungency based on the content of alkaloid compounds known as capsaicinoids, which are derived from the phenylpropanoid pathway.[5,21] Therefore, pungency may interfere with flavonoid biosynthesis.[22]

Higher concentrations of phenylpropanoids, such as flavonoids and phenolic acids, may confer greater tolerance toward abiotic stress conditions.[23] High salinity and low temperatures tend to increase the content of phenolic compounds in *C. annuum*.[24,25] Generally, the stress response is enhanced by combinations of stress factors.[26] Flavonoids are known for their antioxidant,[22] UV-protective,[27] and anti-

Received: February 12, 2021
Revised: May 18, 2021
Accepted: May 18, 2021
Published: June 3, 2021



https://doi.org/10.1021/acs.jafc.1c00908
J. Agric. Food Chem. 2021, 69, 6431−6443

bacterial properties,[28] thus playing a key role in the protection of plants against abiotic and biotic stresses. Flavonoids and related secondary metabolites therefore are considered important components of a healthy diet and are valued by the food, pharmaceutical, and cosmetics industries.[1,29,30]

The flavonoid content of bell pepper or chilli plant residuals could be increased by applying deliberate stress treatments to induce their synthesis. Similar valorization methods have been proposed for the utilization of tomato biomass.[3,31] Commercial valorization procedures for bell pepper plants must be reproducible and controlled, which requires the analysis of plant stress responses to determine the correlation between stress intensity and the content of target metabolites. Image-based methods allow the nondestructive monitoring of dynamic stress responses in plants based on parameters such as growth[32] and changes in the relative abundance of photosynthetic pigments, which provide an indication of photosynthetic capacity.[19,33] The noninvasive analysis of changes in foliar pigment concentrations has been achieved by using color quantification[34] as well as fluorescence-based and reflectance-based methods.[4,35−37] Such noninvasive methods allow individual plants to be followed throughout stress treatments to estimate changes in the concentration of foliar compounds.[34]

Here we evaluated the ability of different abiotic stress treatments to maximize the content of valuable flavonoids in the leaves of two *Capsicum* cultivars, a pungent chilli cultivar and a nonpungent bell pepper. We hypothesized that moderate cold and salt stress (and combinations thereof) might induce the accumulation of flavonoids such as cynaroside, graveobioside A, apigetrin, and apiin in the leaves of both cultivars. We also compared the two cultivars to measure differences in the accumulation of flavonoids, reflecting cultivar-specific metabolic adjustments. Genetic variations in flavonoid induction will determine how residual biomass can be utilized in different crops. We integrated nondestructive imaging and fluorescence-based methods to monitor the response of *Capsicum* plants during 14 days of moderate cold and salt stress, allowing the quantification of stress responses and the dynamic profiling of flavonoid levels in the foliar tissues.

MATERIALS AND METHODS

Plant Material and Growth Conditions. We compared two *Capsicum* cultivars: (1) the bell pepper *Capsicum annuum* cv. Mazurka (Rijk Zwaan, De Lier, Netherlands) and (2) the chilli pepper *C.* sp. CAP1035 (Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany). Seeds of both cultivars were sown on Rockwool cubes (Grodan, 2 × 2 × 4 cm), watered with quarter-strength Hoagland solution[38] (pH 5.6) until germination (~2 weeks), and then watered with half-strength Hoagland solution for another 2 weeks. Plants were then placed in big Rockwool cubes (Grodan, 7.5 × 7.5 × 6.5 cm) and watered with full-strength Hoagland solution. The plants were cultivated in growth chambers (Hühren Kälte-Klima-Elektrotechnik, Erkelenz, Germany) under the following conditions: 24 °C/18 °C day/night temperature, 10 h photoperiod, 300 μmol m^{-2} s^{-1} light provided by metal halide lamps, and 55% relative humidity. Two months after sowing, stress treatments were applied for 14 days. This plant stage was chosen because rather mature leaves were already present, and the size of the plants was still appropriate for the image-based phenotyping device. The plants were randomly distributed within the growth chambers, comprising four plants per treatment, cultivar (chilli and bell pepper), and harvest day (0, 7, and 14 days after start of treatment). The treatments consisted of control, moderate cold, 200 mM salt, 400 mM salt, moderate cold combined with 200 mM salt, and moderate cold combined with 400 mM salt (Table 1). Control plants were watered with full-strength Hoagland solution, and the salt treatment was applied by adding NaCl to a final concentration of 200 or 400 mM. For the moderate cold treatment, plants were transferred to an identical growth chamber with a day/night temperature regime of 18 °C/12 °C.

Table 1. Cultivation Temperature of the Individual Stress Treatments of Chilli and Bell Pepper during Stress Application

condition	temperature (°C day/night temperature)	salt (mM NaCl)
control	24/18	0
cold	18/12	0
S200	24/18	200
S400	24/18	400
cold + S200	18/12	200
cold + S400	18/12	400

Image-Based Assessment of Phenotypic Parameters. The image-based phenotyping setup consisted of a modular aluminum frame (1.3 × 0.6 × 0.8 m) enclosed within a lightproof textile cover. Inside the frame, a MANTA G-235C camera (Allied Vision, Stadtroda, Germany) and two Mosaic2 light panels (Limelite, Bowens, U.K.) were aimed at a turntable for the plants, which could be accessed via a curtain. A customized LabVIEW system design software (National Instruments Corporation, Austin, TX, U.S.A.) was used to automatically take four pictures per plant at 90° rotations. Pictures of plants were taken twice each week starting 1 day before the start of the stress treatments and always 1 day before leaf sampling. Pictures were saved as tagged image file format (TIFF) files and were processed using a support vector machine (SVM)-based image segmentation approach to separate plants from the background.[39,40] The resulting masks were used to determine the projected leaf area (PLA) and plant width in pixels, and the average color was calculated as the average of the red, green, and blue (RGB) values for the whole plant. The relative growth rate (RGR_{PLA}) was calculated for all plants as shown in eq 1,

$$RGR_{PLA}\ (\%/\text{day}) = \left(\frac{\ln PLA2 - \ln PLA1}{t}\right) \times 100 \quad (1)$$

where t denotes the time (in days) between measurements.

Likewise, the relative change of plant width was calculated as shown in eq 2:

$$\text{relative change of plant width}\ (\%/\text{day}) = \left(\frac{\ln \text{width2} - \ln \text{width1}}{t}\right) \times 100 \quad (2)$$

Tolerance efficiency[17] was calculated for the RGR_{PLA} and the relative change of plant width in order to determine the effect of the stress treatment on plant growth, as shown in eq 3:

$$\text{tolerance efficiency}\ (\%) = \left(\frac{\text{stressed}}{\text{control}}\right) \times 100 \quad (3)$$

To quantify leaf color, the color index excess greenness (ExG)[41] was selected because it represents stress-induced changes in leaf color. ExG was calculated as shown in eq 4:

$$ExG = 2G - (R + B) \quad (4)$$

The ExG was calculated from images of whole plants (ExG_{plant}) captured using the phenotyping instrument under controlled illumination. To investigate the potential of smartphones for quick and simple phenotyping, images of the sample leaf were captured in the growth chamber using an iPhone 6S smartphone from a distance of 30 cm. Mean RGB values were extracted using the software ImageJ and used to calculate the ExG_{leaf} as previously described.[3]

https://doi.org/10.1021/acs.jafc.1c00908
J. Agric. Food Chem. 2021, 69, 6431−6443

Fluorescence-Based Analysis. A portable Multiplex 3 fluorometer (Force A, Orsay, France) was used for the noninvasive estimation of epidermal pigments.[42] The adaxial surface of the leaf beneath the first branching of each plant was assessed on each measurement day with five technical replicates. The index of flavonols (FLAV), simple fluorescence ratio (SFR_G) linked to the chlorophyll concentration, and anthocyanin index (ANTH_RG) were calculated using eqs 5−7, where FRF_red = chlorophyll fluorescence emitted in response to far-red excitation at 730−780 nm, FRF_UV = chlorophyll fluorescence emitted in response to UV excitation, and RF_green = chlorophyll fluorescence emitted in response to green excitation at 680−690 nm:

$$\mathrm{FLAV} = \log\left(\frac{\mathrm{FRF\_red}}{\mathrm{FRF\_UV}}\right) \quad (5)$$

$$\mathrm{SFR_G} = \frac{\mathrm{FRF\_green}}{\mathrm{RF\_green}} \quad (6)$$

$$\mathrm{ANTH_{RG}} = \log\left(\frac{\mathrm{FRF\_red}}{\mathrm{FRF\_green}}\right) \quad (7)$$

Biomass Harvesting and Extraction of Phenolic Compounds. Plants were harvested directly before stress treatment and after 7 or 14 days of treatment. The fresh weight was directly recorded, and the leaf beneath the first branching was immediately frozen in liquid nitrogen and stored at −80 °C. Leaf material was ground in liquid nitrogen using an MM400 mixer mill in a 25 mL grinding beaker with two 15 mm diameter grinding balls (Retsch, Haan, Germany) for 30 s at a frequency of 30 s^{-1}. Phenolic compounds were extracted according to the modified method described by León-Chan et al.[8] by immersing 20 mg of ground material in 2 mL of cold 40:60 (v/v) methanol/water (VWR International, Langenfeld, Germany) and shaking at 900 rpm for 30 min at 4 °C. The mixture was centrifuged (20 800*g*, 5 min, 4 °C) and the supernatant was used for the quantification of total phenolics and flavonoids and for the HPLC-MS analysis of flavonoids. To assess the dry/fresh weight ratio, the leaves beneath and above the harvest leaf were dried for 7 days at 60 °C. Pretests with *Capsicum* plants showed that the averaged dry mass fraction of leaves above and below the sampled leaf represent its dry mass fraction well.

The effect of the applied stress treatments on foliar phenolic compounds was assessed using spectrophotometric assays and HPLC-MS measurements. Although spectrophotometric methods do have their limitations regarding specificity,[43] they represent good methods for a fast identification of compound classes that are affected by, e.g., certain stress treatments and can be targeted in subsequent chromatographic analyses. The Folin−Ciocalteu assay does not only measure the phenolic contents.[44] However, it seems to be suitable for the quantification of phenolics in leaves of *Capsicum* because the content of interfering ascorbic acid is rather low, while the content of phenolics is high.[45]

Quantification of Total Phenolic Content. The total phenolic content was determined using the Folin−Ciocalteu method.[44] An aliquot of the sample supernatant (100 μL) was mixed with 200 μL of 10% Folin−Ciocalteu reagent (Sigma-Aldrich, Darmstadt, Germany) and 800 μL of 700 mM sodium carbonate. The reaction was incubated for 2 h at room temperature in the dark before centrifugation (20 800*g*, 1 min, room temperature). The absorbance of the supernatant was measured at 765 nm using a UV/vis SPECORD 200 PLUS (Analytik Jena, Jena, Germany). The total phenolic content was quantified based on a gallic acid (Sigma-Aldrich) calibration curve and was expressed in gallic acid equivalents in mg g^{-1} dry weight.

Quantification of Flavonoids. Flavonoids were quantified using the modified aluminum chloride method.[8] An aliquot of the sample supernatant (75 μL) was mixed with 560 μL of distilled water, 300 μL of 40:60 (v/v) methanol/water, and 40 μL of 5% aluminum chloride (Sigma-Aldrich). The blank contained distilled water instead of aluminum chloride. After incubation for 30 min at room temperature in the dark, the absorbance was measured at 405 nm using the UV/vis SPECORD 200 PLUS. Flavonoid levels were quantified based on a luteolin (Sigma-Aldrich) calibration curve and were expressed as luteolin equivalents in mg g^{-1} dry weight.

HPLC-MS Analysis of Flavonoids. Four flavonoids (two glycosides of luteolin and two glycosides of apigenin) were quantified by HPLC-MS. The sample supernatant was diluted 10-fold with 40:60 (v/v) methanol/water and filtered through a 0.2 μm polytetrafluoroethylene (PTFE) membrane (VWR) into an HPLC vial (CS-Chromatographie Service, Langerwehe, Germany). Target flavonoids were detected using an Agilent 1260 Infinity HPLC device with a binary pump, thermostated column compartment, and autosampler (Agilent Technologies Germany, Waldbronn, Germany) linked to a Triple Quad 6420 MS (Agilent Technologies Germany). Extracts were separated by injecting 10 μL samples onto a Nucleodur C18 ec column (3 μm, 250 × 3 mm) protected by a Nucleodur 300-5 C18 ec 4 × 3 mm precolumn (both supplied by Macherey-Nagel, Düren, Germany) at 35 °C. Fractions were eluted in gradient mode at a flow rate of 0.4 L min^{-1} eluent A (HiPerSolv CHROMANORM water, VWR) containing 0.5% formic acid and in eluent B (100% methanol, VWR). The gradient was established by increasing the proportion of eluent B from 50% to 90% over 20 min. The electrospray ionization (ESI) interface was driven in positive mode. The capillary voltage was set to 5500 V, and the fragmentation voltage was set to 150 V. The nitrogen gas temperature was 300 °C, and the flow rate was 6 L min^{-1}. The nebulizer pressure was set to 60 psi. Multiple reaction monitoring (MRM) mode was applied for the quantification of flavonoid glycosides. The graveobioside A (luteolin 7-*O*-(2-apiosyl)-glucoside) peak was detected at 5.6 min, cynaroside (luteolin-7-glucoside) was at 6.2 min, apiin (apigenin-7-apiosylglucoside) was at 7.1 min, and apigetrin (apigenin 7-glucoside) was at 7.9 min (Table S1 and Figure S1). Flavonoids were identified based on retention time and cochromatography with the following reference substances: graveobioside A, $[M + H]^+$ 581.100; cynaroside, $[M + H]^+$ 449.100; apiin, $[M + H]^+$ 565.100; and apigetrin, $[M + H]^+$ 433.100 (Table S1). The reference substances graveobioside A (purity >99%) and cynaroside (purity ≥99.87%, PhytoLab, Vestenbergsgreuth, Germany), apiin (purity ≥97%), and apigetrin (purity ≥97%, Sigma-Aldrich) were used to generate external calibration curves for quantification without correction for differences in the purity. For each of the four biological replicates (four plants per treatment, cultivar, and time point), the leaf beneath the first branching was sampled and HPLC-MS measurements were done in technical duplicates. Data were analyzed using Mass Hunter software vB05.00 (Agilent Technologies, Germany) and were presented as mg g^{-1} dry weight.

Data Analysis. All data were analyzed using SigmaStat in Sigma Plot 13 (Systat Software, San Jose, CA, U.S.A.). Dependent on the outcome of tests for homogeneity of variance (Brown−Forsythe test) and normal distribution (Shapiro−Wilk test), the data were subjected to one-way or two-way analysis of variance (ANOVA) or Kruskal−Wallis one-way ANOVA on ranks, followed by Tukey's HSD post hoc test or Dunn's test for the comparison of means, respectively.

RESULTS

The impact of moderate cold and salt stress on the performance and flavonoid content of two *Capsicum* cultivars was studied over the course of 14 days after treatment initiation. Image-based phenotyping revealed a significantly lower RGR_{PLA} in both cultivars after treatment with 400 mM salt alone or in combination with cold (Figure 1). In contrast, the cold and 200 mM salt treatments (alone and combined) significantly reduced the RGR_{PLA} of the chilli cultivar but not that of the bell pepper (Figure 1). A lower RGR_{PLA} on day 13 compared to day 6 was observed for treatments with 200 mM salt, 400 mM salt, and the combination of cold plus 400 mM salt for both cultivars. In the bell pepper, this was also true for the cold plus 200 mM salt treatment (Figure 1). The PLA was

https://doi.org/10.1021/acs.jafc.1c00908
J. Agric. Food Chem. 2021, 69, 6431−6443

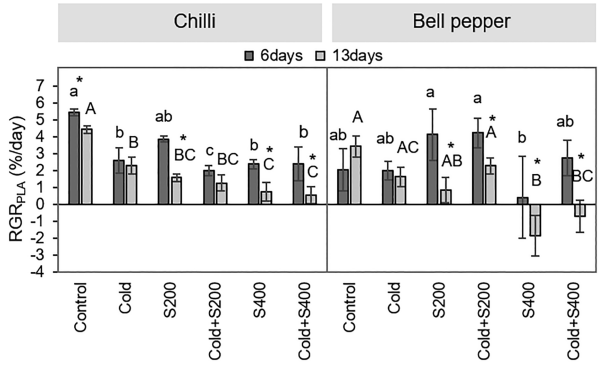


Figure 1. Relative growth rate based on projected leaf area (RGR_{PLA}, in %/day) of chilli and bell pepper plants shown for control, cold treatment (cold), 200 mM salt (S200), 400 mM salt (S400), cold plus 200 mM salt (cold + S200), and cold plus 400 mM salt (cold + S400) after 6 and 13 days. Data are means ± standard deviations (n = 4 plants). Different letters (lowercase letters for day 6, uppercase letters for day 13) indicate significant differences between treatments per cultivar within each time point ($p \leq 0.05$). Asterisks indicate significant differences between time points ($p \leq 0.05$).

a good measure of plant biomass (Table S2) as shown by the strong correlation between PLA and dry weight (chilli R^2 = 0.97, bell pepper R^2 = 0.84; Table S3).

Wilting reduced the width of plants in both cultivars (Figure S2), especially in response to 400 mM salt alone and in combination with cold (Figure S3). In contrast to the chilli cultivar, the bell pepper showed a significantly reduced relative width after 6 days of treatment. A further reduction in relative width in response to the salt treatments on day 13 compared to day 6 was observed for the chilli cultivar, leading to a significant decrease for the combination of cold plus 200 mM salt (Figure S3). For the bell pepper, the relative width of salt-treated plants was higher after 13 days than after 6 days, and this difference was significant for the treatment with 400 mM salt.

Both cultivars generally showed lower tolerance efficiencies in response to salt than to cold, except for the tolerance efficiency of plant width in the chilli cultivar, which was lower for cold than for 200 mM salt (Table 2). The salt tolerance efficiency of the bell pepper was much lower than that of the chilli cultivar, and this was more pronounced for the tolerance efficiency of the plant width compared to the tolerance efficiency of RGR_{PLA} (Table 2). For the bell pepper, tolerance efficiencies were higher for the combined treatment compared to the treatment with salt alone, whereas the chilli cultivar showed lower tolerance efficiencies for the combined versus single treatments, except for the tolerance efficiency of the plant width following treatment with cold plus 400 mM salt (Table 2).

Table 2. Tolerance Efficiency Calculated for Relative Growth Rate (RGR_{PLA}) and Relative Change of Plant Width for the Cold, 200 mM Salt (S200), 400 mM Salt (S400), Cold Plus 200 mM Salt (Cold + S200), and Cold Plus 400 mM Salt (Cold + S400) Treatments of Chilli and Bell Pepper Plants after 13 Days

	treatment	chilli	bell pepper
tolerance efficiency RGR_{PLA} (%)	cold	52.04	47.77
	S200	36.04	25.15
	cold + S200	29.04	66.69
	S400	17.58	−53.23
	cold + S400	13.22	−20.42
tolerance efficiency plant width (%)	cold	−8.63	34.67
	S200	9.28	−129.69
	cold + S200	−41.04	−94.66
	S400	−74.26	−289.87
	cold + S400	−59.72	−212.65

The color index (ExG_{plant}) increased gradually with time under stress conditions, whereas a slight decrease was observed for the control plants (Figure 2A and B). Cold alone increased the ExG_{plant} of both cultivars, with the chilli cultivar showing an earlier significant response than the bell pepper. The 200 mM salt treatment significantly increased the ExG_{plant} of bell pepper plants after 6 days, whereas a significant effect was only seen after 13 days in the chilli plants (Figure 2A and B). For both cultivars, a significantly higher ExG_{plant} was observed for the combined treatments at both salt concentrations starting after 6 days (Figure 2A and B). The single treatment with 400 mM salt led to the highest ExG_{plant} in both cultivars.

The applied stress treatments significantly affected indices obtained from multiparametric fluorescence measurements depending on the cultivar, time point, and stress treatment (Figure 3). The SFR_G index, which represents chlorophyll, tended to increase during the control conditions in both cultivars, whereas no change or a slight decrease was observed in the stress-treated plants (Figure 3A and B). Cold treatment reduced the SFR_G in both cultivars, with chilli showing an earlier significant response (after 9 days) compared to bell pepper (Figure 3A and B). Although treatment with 200 mM salt also reduced the SFR_G, this was not significant in either cultivar. The combination of cold plus 200 mM salt significantly reduced the SFR_G in both cultivars after 14 days. The treatment with 400 mM salt significantly reduced the SFR_G of chilli leaves starting after 9 days, whereas the reduction was nonsignificant in the bell pepper leaves. The combination of cold and 400 mM salt significantly reduced the SFR_G in both cultivars, with chilli showing an earlier significant reduction after 9 days (Figure 3 A and B).

Cold treatment caused an increase in the ANTH_RG index, representing epidermal anthocyanins, in both cultivars, with bell pepper showing an earlier significant response after 9 days (Figure 3C and D). The single treatments with salt had no significant effect on ANTH_RG in either cultivar. The combination of cold plus 200 mM salt increased ANTH_RG in the chilli but not the bell pepper cultivar after 9 days (Figure 3C and D). The combination of cold plus 400 mM salt increased ANTH_RG in both cultivars, with the bell pepper already showing a significant increase after 9 days (Figure 3C and D).

The FLAV index, representing epidermal flavonoids, did not change over time in control plants but significantly increased over 14 days in both cultivars exposed to the cold treatment alone, starting after 7 days (Figure 3E and F). In contrast, treatment with 200 mM salt had no significant effect on the FLAV index of either cultivar. The FLAV index increased significantly after 14 days in bell pepper plants treated with the combination of cold plus 200 mM salt, whereas there was no significant increase in the chilli plants (Figure 3E and F). Treatment with 400 mM salt caused the FLAV index to decline significantly in bell pepper plants after 14 days but had no significant effect in chilli plants. The combination of cold plus

https://doi.org/10.1021/acs.jafc.1c00908
J. Agric. Food Chem. 2021, 69, 6431−6443

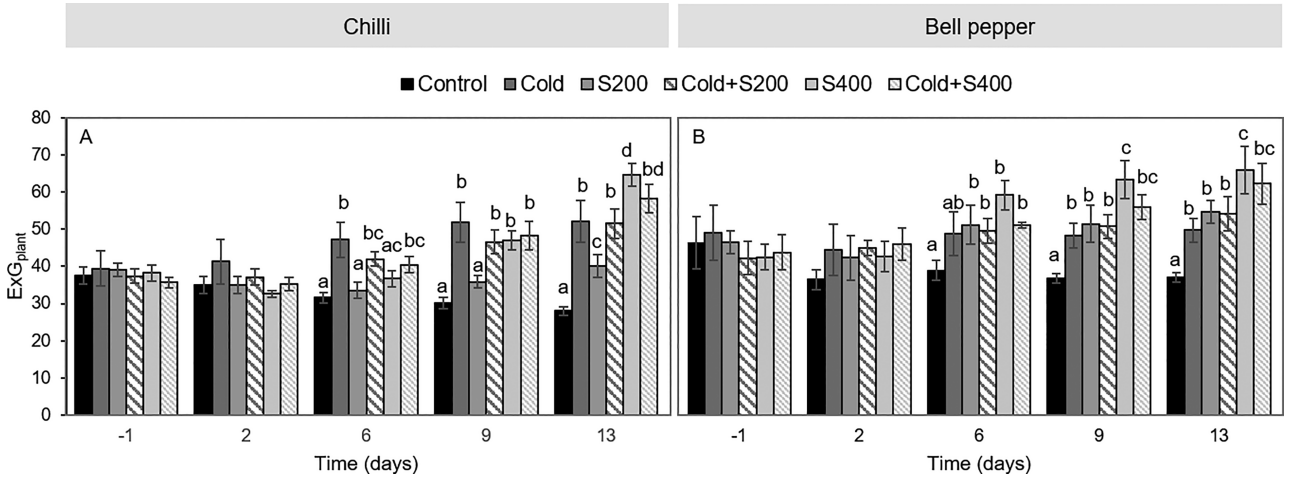


Figure 2. Changes in plant color based on the color index (ExG_{plant}) of chilli (A) and bell pepper (B) plants shown for control, cold, 200 mM salt (S200), 400 mM salt (S400), cold plus 200 mM salt (cold + S200), and cold plus 400 mM salt (cold + S400) at time points −1, 2, 6, 9, and 13 days. Data are means ± standard deviations ($n = 4$). Different letters (a, b, c, d) indicate significant differences between treatments within each time point ($p \leq 0.05$).

400 mM salt caused the FLAV index to increase, but not significantly, in both cultivars (Figure 3E and F).

The stress treatments significantly affected the content of total phenolics and flavonoids in the leaves of both cultivars after 14 days, but the outcome differed according to the cultivar and specific treatment (Figure 4). Combined cold and salt stress significantly increased the total phenolic content in both cultivars after 14 days, whereas the individual treatments with cold or 200 mM salt had no significant effect (Figure 4A). In response to 400 mM salt, the total phenolic content significantly increased in the chilli cultivar but not in the bell pepper cultivar after 14 days (Figure 4A). The flavonoid content increased significantly in the bell pepper after 14 days of cold or cold combined with salt, but there was no corresponding response in the chilli cultivar (Figure 4B).

The cold treatment alone or in combination with salt generally increased the content of the four target flavonoids (graveobioside A, cynaroside, apiin, and apigetrin) in both cultivars, with lower concentrations in chilli than in bell pepper for most treatments (Figure 5). Graveobioside A and cynaroside also accumulated in response to individual treatments with salt in both cultivars but to a much lower extent than with the cold treatment. Indeed, cold treatment caused a significant increase in the concentration of graveobioside A in both cultivars after 14 days (Figure 5A and B), representing a 3.4-fold change in chilli and a 2.8-fold change in bell pepper relative to the control (Figure 5I). The cynaroside content increased significantly after 7 days of cold treatment in both cultivars (Figure 5C and D), leading to a 52.8-fold change in chilli and a 13.6-fold change in bell pepper after 14 days (Figure 5I). In contrast, the apigetrin content was not significantly affected by the cold treatment (Figure 5G and H). The single treatment with 200 mM salt significantly increased the cynaroside concentration after 14 days in both cultivars (Figure 5C and D), but there was no significant effect on graveobioside A, apiin, or apigetrin (Figure 5A, B, E, F, G, and H). The single treatment with 400 mM salt only significantly increased the content of cynaroside in chilli after 14 days (Figure 5C). The combined treatment of cold plus salt (200 or 400 mM) significantly increased the content of cynaroside in both cultivars, with bell pepper already showing a significant increase after 7 days (Figure 5C and D). Moreover, the graveobioside A content of bell pepper leaves increased significantly after 14 days (Figure 5B), reaching a maximum 5.5-fold increase in response to cold + 200 mM salt (Figure 5I) as well as significantly higher levels of apigetrin after 7 and 14 days (Figure 5H), reaching a maximum of 7.9-fold higher (Figure 5E). The combined cold plus salt treatment did not significantly enhance the levels of graveobioside A or apigetrin in chilli plants, although the cold plus 200 mM salt treatment exerted a slightly stronger effect than cold plus 400 mM salt (Figure 5A and G). The apiin content was not significantly affected by either of the treatments in either cultivar (Figure 5E and F).

Significant correlations were observed between the luteolin glycosides graveobioside A and cynaroside, between the apigenin glycosides apiin and apigetrin, and also between the luteolin and apigenin glycosides in both cultivars, with higher correlation coefficients in bell pepper than in chilli (Table S4). In both cultivars, significant positive correlations were observed between the total phenolic content and graveobioside A (chilli $R^2 = 0.52$, bell pepper $R^2 = 0.64$) as well as between the total phenolic content and apiin (chilli $R^2 = 0.53$, bell pepper $R^2 = 0.49$). The correlation between total and individual flavonoids was weaker and was only significant in bell pepper for graveobioside A ($R^2 = 0.40$) and cynaroside ($R^2 = 0.41$). In both cultivars, the FLAV index showed a good correlation with graveobioside A (chilli $R^2 = 0.65$, bell pepper $R^2 = 0.70$) and cynaroside (chilli $R^2 = 0.70$, bell pepper $R^2 = 0.72$) but a weaker correlation with apiin (chilli $R^2 = 0.32$, bell pepper $R^2 = 0.49$) and apigetrin (chilli $R^2 = 0.51$, bell pepper $R^2 = 0.46$) (Table S4). A significant correlation between the FLAV index and the total phenolic content or total flavonoids was only observed in bell pepper. Furthermore, the anthocyanin index ANTH_RG showed a good correlation with the cynaroside and apigetrin content of bell pepper, whereas the correlation in the chilli cultivar was much weaker (Table S4). Similarly, the SFR_G index showed a low significant correlation with graveobioside A and cynaroside in bell pepper but no significant correlation in the chilli cultivar. The SFR_G index showed a strong negative correlation with the ExG_{plant} ($R^2 = -0.82$) and ExG_{leaf} ($R^2 =$

https://doi.org/10.1021/acs.jafc.1c00908
J. Agric. Food Chem. 2021, 69, 6431−6443

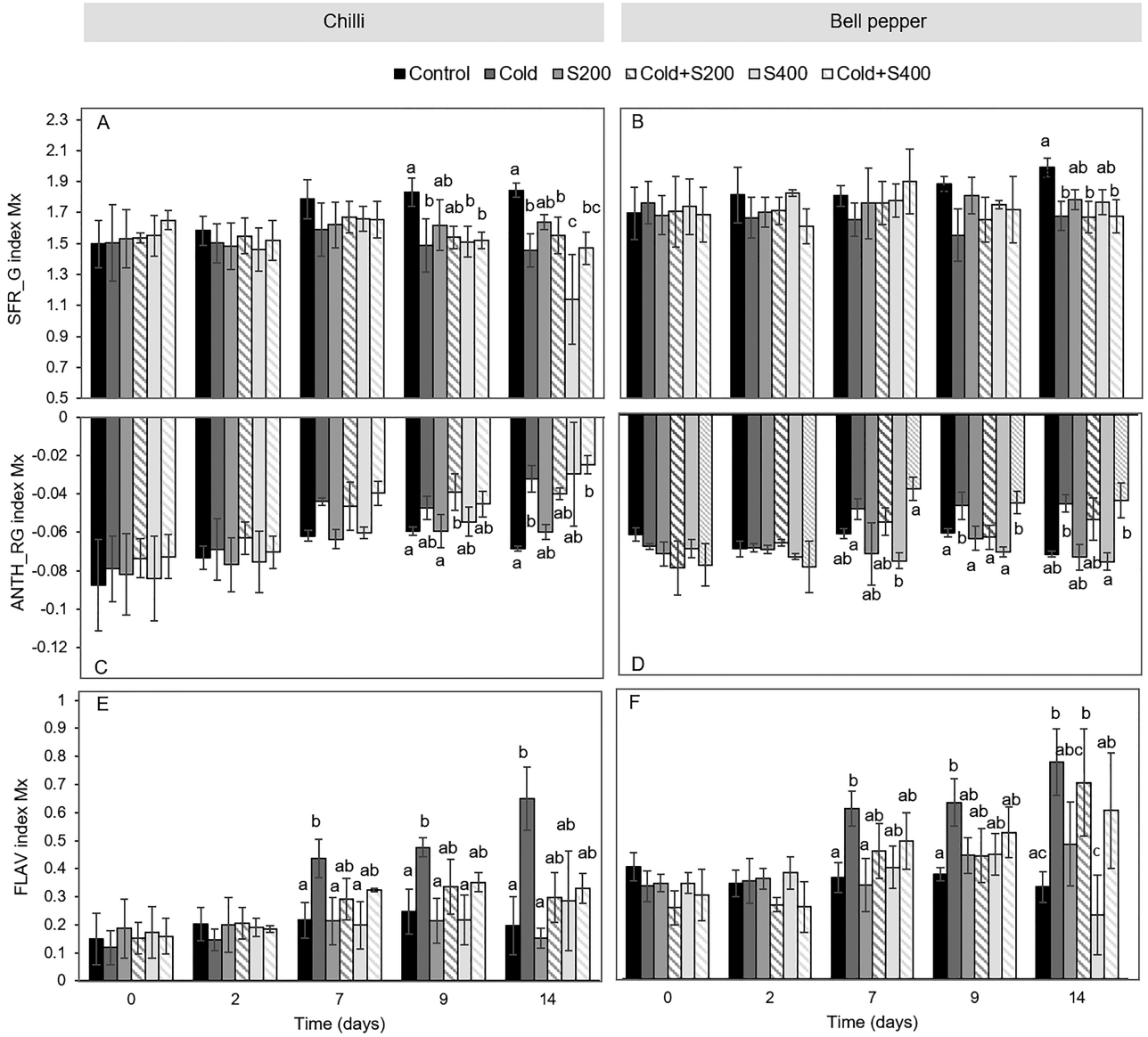


Figure 3. Temporal changes in the SFR_G index Mx (A, B), ANTH_RG index Mx (C, D), and FLAV index Mx (E, F) of chilli (A, C, E) and bell pepper (B, D, F) plants under control conditions and after treatment with cold, 200 mM salt (S200), 400 mM salt (S400), cold plus 200 mM salt (cold + S200), and cold plus 400 mM salt (cold + S400) after 0, 2, 7, 9, and 14 days. Data are means ± standard deviations (n = 4). Different letters (a, b, c) indicate significant differences between treatments within each time point ($p \leq 0.05$).

−0.86) in chilli plants, but the negative correlation was weaker in bell pepper (ExG_{plant}, R^2 = −0.58; ExG_{leaf}, R^2 = −0.50).

DISCUSSION

In this study, the impact of moderate cold stress and high salt stress, applied alone and in combination, on plant growth and the accumulation of phenolic compounds in the leaves of a pungent chilli and a nonpungent bell pepper cultivar was investigated. We specifically focused on the accumulation of the flavonoids graveobioside A, cynaroside, apiin, and apigetrin, which have previously been quantified in the leaves of *C. annuum* plants.[4,7,8] The accumulation of these flavonoids in response to stress could be exploited to valorize *Capsicum* plants after the fruit production cycle.[3,31,46] The ability of phenolic compounds to confer tolerance against abiotic stress has been described in numerous studies.[14,16,23] The potential contributions of the quantity of phenolic compounds and of individual flavonoids to stress tolerance in each of the cultivars are discussed.

Sensitivity to Cold and Salt Stress in a Chilli and Bell Pepper Cultivar. Moderate cold and salt stress reduced the relative growth rate (RGR_{PLA}; Figure 1) and caused wilting (Figure S2) in both cultivars, in agreement with previous studies.[47−49] Although the cold treatment was moderate,[47,49] the RGR_{PLA} of the chilli cultivar was significantly reduced, whereas the change was a nonsignificant trend in the bell pepper (Figure 1). Cold-induced wilting was much stronger in the chilli cultivar, indicating lower cold tolerance than in the bell pepper cultivar. Cold stress has been shown to reduce chlorophyll levels in chilli-type pepper plants.[24,47] The earlier significant cold-induced reduction of the chlorophyll index SFR_G in the chilli cultivar (Figure 3) provides further evidence that it is less tolerant to low temperatures than the bell pepper.

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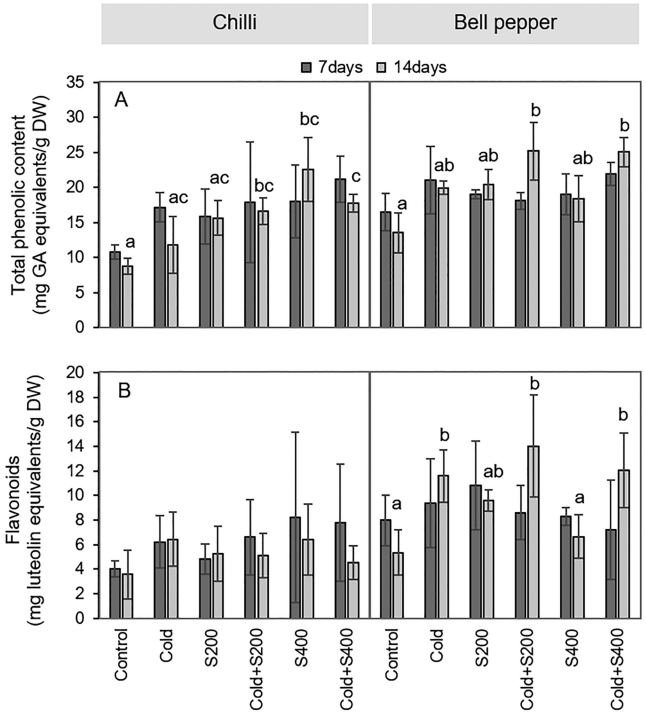

Figure 4. Stress-induced increases in total phenolics and flavonoids. (A) Total phenolic content in gallic acid (GA) equivalents (mg g^{-1} dry weight). (B) Total flavonoid content in luteolin equivalents (mg g^{-1} dry weight). Each panel shows chilli and bell pepper leaves under control conditions, cold, 200 mM salt (S200), 400 mM salt (S400), cold plus 200 mM salt (cold + S200), and cold plus 400 mM salt (cold + S400) after 7 and 14 days. Data are means ± standard deviations (n = 4). Different letters (a, b, c) indicate significant differences between treatments within each cultivar and time point ($p \leq 0.05$).

The genotype-dependent differences observed in growth inhibition induced by salt stress have been reported in previous studies of *Capsicum* cultivars.[25,50] In this study, individual treatments with 400 mM salt significantly reduced the RGR_{PLA} in both cultivars, whereas only chilli was affected in the presence of 200 mM salt (Figure 1). The chilli cultivar therefore appears more sensitive to salt stress. Previous studies have shown the inhibition of plant biomass accumulation even at much lower salt concentrations.[4,25] Pepper plants show a substantial genetic variation in salt tolerance, from highly sensitive to tolerant.[51] Also, 400 mM salt had a significant negative effect on the chlorophyll index SFR_G, specifically in the chilli cultivar (Figure 3A and B), agreeing with earlier reports that chlorophyll loss is more severe in sensitive compared to salt-tolerant cultivars.[25] The reduction of width as a wilting indicator responded very strongly and early to the 200 mM salt treatment in the bell pepper (Figure S3) without affecting the RGR_{PLA}. Similarly, 150 mM salt triggered a strong wilting response in habanero pepper plants without a significant effect on plant growth.[50] The treatment with 400 mM salt had a more severe effect on the RGR_{PLA} and width of bell pepper plants compared to the chilli cultivar, indicating that the bell pepper has lower tolerance to the higher salt concentration. The severe reduction in RGR_{PLA} with negative growth rates reflects the combination of wilting and leaf rolling (Figure S2), which was not observed for the chilli cultivar. The projected leaf area used to estimate plant growth is affected by morphological changes due to turgor loss, which is more severe in bell pepper than in chilli, indicating that the two cultivars have different morphological responses to salt stress.

Combinations of stresses generally have more severe effects than individual stresses.[26] Furthermore, combined stress treatments increase flavonoid levels to a greater extent than single treatments.[4] However, combinations of stresses cannot be interpreted simply as the sum of individual stresses because stress combinations also trigger genes that do not respond to the individual stresses.[52] Interestingly, the combination of salt and cold stress did not result in an additive effect on growth or wilting in the more cold-tolerant bell pepper, and the additional cold did not increase the negative impact of salt stress. Instead, more severe growth reduction was observed in the chilli plants exposed to the combined stress treatment (Figure 1). Accordingly, tolerance efficiencies were lower for the combined stress compared to salt stress alone in the chilli cultivar, but the opposite was observed in the bell pepper cultivar (Table 2). However, the combined stress treatment and individual cold treatment reduced the chlorophyll index SFR_G to a comparable extent in both cultivars (Figure 3). Low-temperature stress and salt stress reduced the photosynthetic capacity and thus promoted the generation of reactive oxygen species (ROS).[49,53] Oxidative stress has been shown to reduce chlorophyll biosynthesis.[54] However, the combined stress treatment did not cause any further reduction of chlorophyll levels.

Noninvasive Quantification of Stress Responses and Changes in the Foliar Flavonoid Content. The application of stress treatments at bell pepper/chilli production sites would benefit from the reliable noninvasive monitoring of stress responses and flavonoid accumulation. Accordingly, the use of noninvasive phenotyping technologies such as the quantitative analysis of plant and leaf color changes and the multiparametric fluorescence responses of leaves was tested to identify indices correlating with the accumulation of specific foliar flavonoids or indices allowing the estimation of stress intensity.

Among various color indices, the ExG was selected because it repeatedly showed a strong response to the applied stress treatments, with increasing significance in plants exposed to cold and/or salt stress (Figure 2). This color index is often calculated from aerial or satellite images to monitor forest canopies and has been used to assess the health of pine trees, focusing on biotic stress factors.[55] Here, the ExG was calculated from individual plant images to monitor abiotic stress responses under controlled conditions in a growth chamber. Stress-induced changes in the ExG are not directly related to changes in plant morphology because there was a significant correlation between the ExG_{leaf} (calculated from images of the sample leaf) and the ExG_{plant} (calculated from images of the whole plant; Table S4). The significant correlation between the chlorophyll index SFR_G, the ExG_{leaf}, and the ExG_{plant} indicates that this color index may provide a useful indicator of the dynamic foliar chlorophyll content influenced by abiotic stress. Color indices like the ExG can be calculated from images taken with a smartphone, which could reduce the complexity and cost of the method compared to multiparametric fluorescence measurements.[56]

Noninvasive multiparametric fluorescence measurements allowed us to estimate stress-induced changes in the concentration of individual foliar flavonoids. In agreement with the destructive analysis of flavonoids, the FLAV index in chilli increased sharply following the individual cold treatment,

https://doi.org/10.1021/acs.jafc.1c00908
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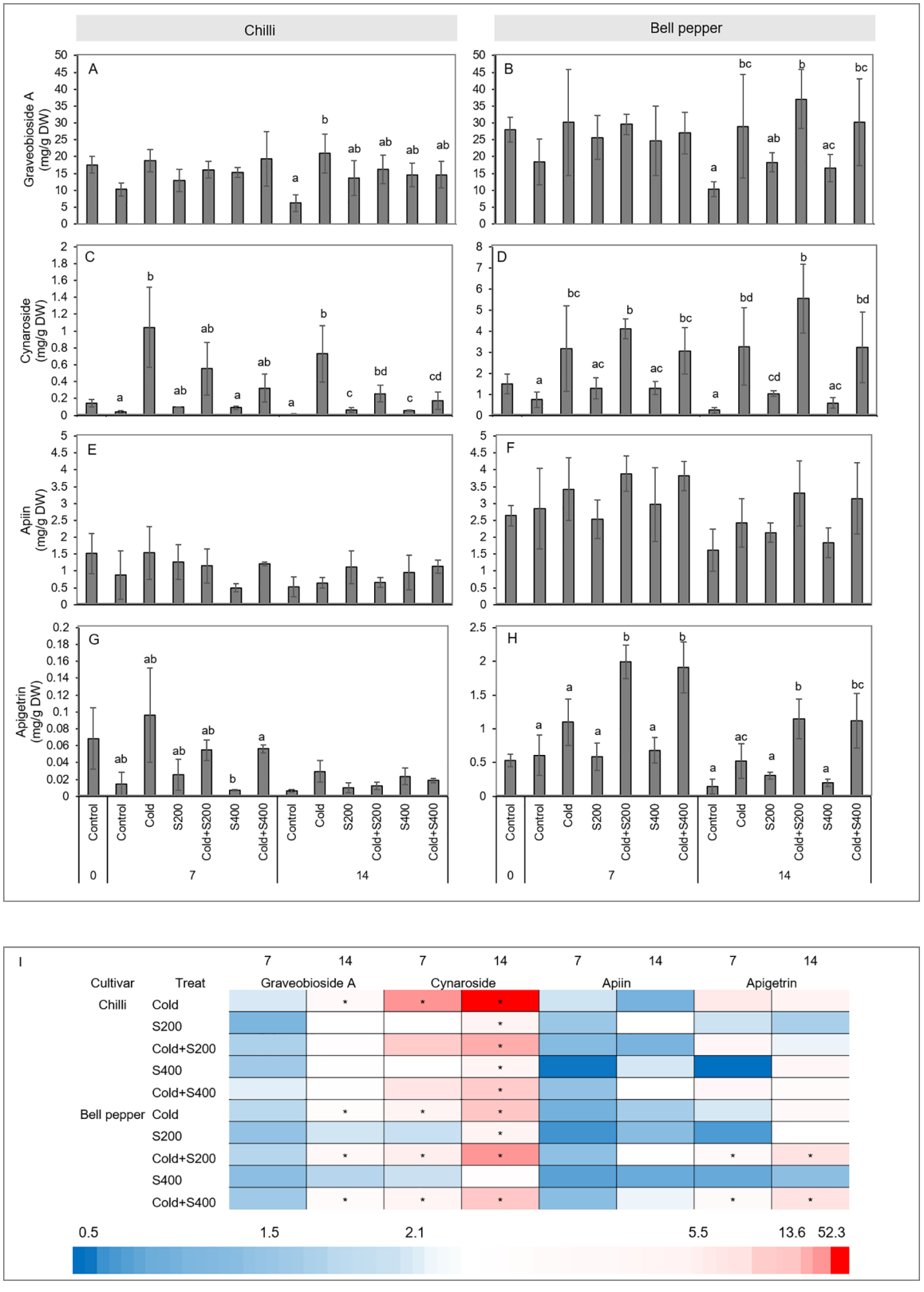

Figure 5. Contents and fold changes (relative to the control, I) of graveobioside A (A, B), cynaroside (C, D), apiin (E, F), and apigetrin (G, H) in chilli and bell pepper leaves under control conditions, cold, 200 mM salt (S200), 400 mM salt (S400), cold plus 200 mM salt (cold + S200), and cold plus 400 mM salt (cold + S400) after 0, 7, and 14 days. Data are means ± standard deviations (n = 4). Significant differences between treatments within each time point ($p \leq 0.05$) are indicated by different letters (a, b, c, d). Fold changes are color coded from blue (low) to red (high) with significant differences from the control indicated by asterisks ($p \leq 0.05$).

but in bell pepper the highest increase was observed in response to cold alone and cold plus salt (Figure 3). In agreement with earlier reports,[4] a good correlation between the FLAV index and graveobioside A and cynaroside levels was

https://doi.org/10.1021/acs.jafc.1c00908
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observed (Table S4). Significant correlations were also observed for apiin and apigetrin, but with lower correlation coefficients (Table S3). The aglycon of graveobioside A and cynaroside is luteolin, which has a dihydroxy B-ring (catechol structure), whereas the aglycon of apiin and apigetrin is apigenin, and this has a single hydroxyl group in the B-ring. In *Ligustrum vulgare*, there was good agreement between the FLAV index and the content of flavonoids containing a dihydroxy B-ring, but no correlation with the apigenin 7-*O*-glycoside content.[57] In this study, the content of total phenolics and flavonoids was correlated with the FLAV index in the bell pepper cultivar but not in the chilli. The FLAV index mainly represents the epidermal flavonoids, whereas destructive analysis measures the content of phenolic compounds throughout the leaf, explaining this lack of correlation. The biosynthesis of dihydroxy B-ring flavonoids increased under intense illumination, suggesting that they may be highly concentrated in the epidermis.[57] This would explain the high correlation between the dihydroxy B-ring flavonoids graveobioside A and cynaroside and the FLAV index in the present study. In addition to the FLAV index, the ANTH_RG index, which has been used to estimate anthocyanin levels,[58] increased significantly in response to cold or cold plus 400 mM salt (Figure 3). In agreement with this, cold was shown to induce the accumulation of anthocyanins in purple-leaved pepper,[59] and salt stress had a similar effect in various *Capsicum* varieties.[16,60,61] However, salt stress has also been shown to decrease the anthocyanin content in salt-sensitive species.[62] No significant changes in the ANTH_RG index were observed in response to individual salt treatments (Figure 3). Indeed a foliar anthocyanin was described in one hot pepper cultivar,[7] but the amount of anthocyanins was not quantified here, so results must be interpreted carefully.

Moderate Cold Stress That Strongly Induced the Accumulation of Flavonoids with High Antioxidant Activity. The accumulation of total phenolics under stress conditions confers enhanced tolerance to abiotic stress.[16] In the present study, cold treatment tended to increase the content of flavonoids after 7 days in both cultivars (Figure 4), as shown also at lower temperatures.[8] The moderate cold conditions selected in this study could be applied in production greenhouses at the end of fruit production in autumn, when the heating is reduced. Prolonging the cold treatment up to 14 days increased the total flavonoid content significantly in the bell pepper but not in the chilli cultivar. The total phenolic content was shown to increase more in salt-tolerant compared to salt-sensitive *C. annuum* cultivars.[25] A significant increase in the total phenolic content was observed in response to salt treatment alone, but only at the high concentration of 400 mM in the chilli cultivar (Figure 4). However, in both cultivars the total phenolic content increased significantly when the cold treatment was combined with 200 mM salt, whereas the individual treatments had no significant effects (Figure 4). The combined treatment also significantly increased the total flavonoid content in bell pepper and slightly enhanced their accumulation compared to the individual stress treatments. Similarly, a combination of cold and exposure to UV-B increased the total flavonoid content of *C. annuum* to a greater extent than each individual treatment.[8] Genes involved in the regulation of secondary metabolism were also differentially expressed when comparing single and combined stress treatments.[52] The biosynthesis of individual flavonoids may be affected differently under specific stress conditions as shown in spinach (*Spinacia oleracea* L.), where cold stress increased the abundance of antioxidant flavonoids while other flavonoids were unchanged or depleted.[63] We therefore investigated four individual flavonoids, previously identified among the antioxidant compounds of *C. annuum* leaves.[7]

Cynaroside showed the strongest response to individual and combined stress treatments in both cultivars, with maximum increases of 52-fold in chilli (maximum average of 1.04 mg g^{-1} DW) and 23-fold in bell pepper (maximum average of 5.54 mg g^{-1} DW, Figure 5C, D, and I). The accumulation of cynaroside in bell pepper achieved concentrations ~3 times higher than those reported in the leaves of *Lonicera macranthoides*, a common source of this flavonoid.[64] Cold and combined stress treatments increased the levels of graveobioside A up to 3 times higher (maximum average of 20.9 mg g^{-1} DW in chilli) or 6 times higher (maximum average of 37.0 mg g^{-1} DW in bell pepper) than reported in the seeds of *Apium graveolens*,[65] which is often utilized for the extraction of graveobioside A. Although apigetrin levels increased significantly under the combined stress treatment in bell pepper (Figure 5G–I), the concentration was lower than that reported in the apigetrin-rich henna tree, *Lawsonia inermis*.[66] To our knowledge, apiin has previously been detected in the leaves of chilli cultivars but not in bell pepper leaves. Apiin was unaffected by the stress treatments in either cultivar (Figure 5E, F, I). Taking the stress-induced reduction of plant biomass accumulation into account, the yields of graveobioside A, cynaroside, and apigetrin were still higher following the individual cold treatment in bell pepper but not in chilli. Given the stronger inhibition of growth by salt stress, the yield of these flavonoids did not increase following the individual salt-stress treatments in either cultivar. Cold plus salt stress increased the total yield of graveobioside A and apigetrin, but this increase was only observed in bell pepper, as well as in the case of graveobioside A only for the combination of cold and the lower salt concentration of 200 mM. Therefore, the bell pepper cultivar seems better suited than the chilli cultivar for the targeted enrichment of flavonoids via deliberate stress treatment.

Flavonoids, which are widely distributed in the plant kingdom, form a major group of secondary metabolites including chalcones, flavones, flavonols, anthocyanins, and proanthocyanidins.[67] Recent studies indicate that flavones are the major group of flavonoids present in the leaves of *Capsicum*.[7,8,68] Furthermore, in the leaves of three hot pepper cultivars, the four flavones investigated in this study represented 67–88% of the extracted phenolic compounds.[7] Flavonoids show various physiological activities, but their role during plant stress responses is not fully understood.[69] Cold and salt stress are known to induce the generation of ROS.[26,70] The accumulation of ROS in cells leads to the oxidation of proteins, nucleic acids, and lipids and ultimately causes cell death.[26,71] Chilling tolerance conferred by the treatment of pepper plants with exogenous abscisic acid (ABA) is thought to reflect protection against ROS-induced chilling injury by enhancing the activity of antioxidant enzymes.[49] Graveobioside A and cynaroside are more efficient free radical scavengers than apiin and apigetrin,[7] potentially due to their catechol structure.[29] The strong accumulation of graveobioside A and cynaroside during cold treatment (Figure 5A–D) may indicate that these two flavonoids are more relevant than apiin and apigetrin for antioxidant stress responses in the cultivars tested in this study. The accumulation of flavonoids containing a catechol structure in response to low temperature has also

https://doi.org/10.1021/acs.jafc.1c00908
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been described in spinach.[63] Apigetrin was induced by combined cold and salt stress in bell pepper but not by the individual stress treatments (Figure 5G and H). These results differ from a previous study in which cold treatment significantly increased the content of apigetrin in *C. annuum* but only slightly increased the levels of cynaroside.[8] The single salt treatment induced the accumulation of graveobioside A and cynaroside in both cultivars, albeit to a much weaker extent than cold, in agreement with a recent study investigating the effect of salt and UV stress.[4] A rather low inducing effect of single salt treatment on the concentration of foliar flavonoids was also observed in tomato.[72] Nevertheless, salt as well as low-temperature stress have been shown to increase the enzymatic activity of phenylalanine ammonia lyase, an important enzyme of the phenylpropanoid/flavonoid pathway.[72,73] However, the regulation of specific phenolic compounds seems to be differently affected by certain stress factors, as has been shown in tomato where salt treatment increased the concentration of caffeoylquinic acids, while heat stress led to an increase of flavonols.[72] The comparison of genetic diversity among different crop species, cultivars, and wild relatives helps to identify stress-tolerance traits for future breeding programs.[74] There is a broad range of salt tolerance within the genus *Capsicum*,[25,50] but few studies have considered tolerance of low temperatures.[24,75] Moreover, the mechanisms underlying salt and cold tolerance in this genus are poorly understood.[49,50] In this study, the cold treatment, alone or in combination with salt, enhanced the accumulation of graveobioside A, cynaroside, and apigetrin to a greater extent than salt alone in both cultivars (Figure 5A−D, G, and H), suggesting that these flavonoids may play an important role in cold stress adaption. Even though graveobioside A and cynaroside were more strongly induced by cold in the chilli cultivar, they reached much lower concentrations compared to the bell pepper cultivar, potentially explaining the difference in cold tolerance. Other flavonoids may also play a role in the bell pepper cultivar because the total flavonoid content increased in response to cold, but this is not the case in the chilli cultivar (Figure 4B). In the chilli cultivar, the accumulation of graveobioside A and cynaroside during cold treatment was limited in the presence of salt, whereas salt increased this response to cold stress in the bell pepper cultivar. This correlated with the slightly reduced tolerance efficiency of growth (RGR_{PLA}) during the combined stress treatment in the chilli cultivar (Table 2), indicating that luteolin glycosides are important components of the tolerance against cold and salt stress combinations. Although both luteolin glycosides were also strongly induced in the chilli cultivar, bell pepper leaves contained higher amounts of these flavonoids and may therefore be more suitable for industrial extraction. Variations in the total content of phenolics and flavonoids in different *Capsicum* cultivars, as well as individual antioxidant flavonoids, have been reported in previous studies.[4,7,25] The selection of cultivars with higher concentrations of specific target flavonoids would therefore support the sustainable use of bell pepper side streams.

In this study, the chilli cultivar was less tolerant to the applied stresses than the bell pepper cultivar, which showed less growth reduction under low temperature and salt stress. Plants that showed a higher tolerance performed better and with less growth reduction under stressful conditions and often contained higher amounts of, e.g., phenolic compounds.[16] A higher content of antioxidant flavonoids, as observed for graveobioside A, cynaroside, apiin, and apigetrin in the bell pepper cultivar under control as well as cold and salt stress conditions, likely contributed to the higher tolerance. These results also indicate that the bell pepper cultivar is more suitable for an industrial extraction of these flavonoids from residual foliar biomass than the chilli cultivar.

In summary, differences in cold tolerance between two *Capsicum* cultivars were observed based on noninvasive phenotyping. These differences reflected differences in the content and inducibility of antioxidant flavonoids, which were slightly more abundant in the bell pepper than in the less cold-tolerant chilli. Thus, antioxidant flavonoids seem to contribute to cold tolerance in *Capsicum*. Noninvasive analysis of leaf color and multiparametric fluorescence measurements provided good estimates of stress intensity and flavonoid accumulation, especially the foliar levels of graveobioside A and cynaroside, which correlated well with the FLAV index. Moreover, the enrichment of flavonoids in leaves (specifically graveobioside A and cynaroside) for extraction could be achieved by cold treatment in two different *Capsicum* cultivars. In contrast, salt stress was less effective for the accumulation of these flavonoids and even reduced their abundance when combined with cold stress in the chilli cultivar. The induced accumulation of cynaroside and graveobioside A could enhance the value of bell pepper residual plants for industrial extraction and utilization.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.1c00908.

Information on MRM quantification of flavonoids; fresh and dry weights of plants at harvest; chromatograms of reference substances and bell pepper extracts; untreated and treated plants; relative change of plant width; Pearson correlation coefficients of the width, projected leaf area, and plant biomass; and Pearson correlation coefficients of the contents of total phenolics, total flavonoids, individual flavonoids, and fluorescence-based and image-based color indices (PDF)

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Funding

This research was supported by the German Federal Ministry of Education and Research (Grant no. 031B0361B). Construction and development of the image-based phenotyping instrument was funded by the Ministry of Innovation, Science and Research of the German State of North Rhine-Westphalia in the framework of the Bioeconomy Science Center (BioSC, NRW Strategie Projekt no. 313/323-400-00213).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank E. Berg and J. von Ameln for support with plant phenotyping and leaf sampling, as well as B. Uhlig, K. Wolter-Heinen, and M. Heinen for support in the greenhouse. The authors also thank Dr. Richard M. Twyman for editing the manuscript.

■ ABBREVIATIONS

DW, dry weight; ExG, excess greenness; FW, fresh weight; PLA, projected leaf area; RGR, relative growth rate; ROS, reactive oxygen species; S200, 200 mM salt; S400, 400 mM salt

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