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Induction of Time-Dependent Tolerance through Thermopriming in Tomatoes

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Abstract: The risk of potential yield losses due to heat waves and other challenging weather phenomena is globally increasing. So far, plant producers have only had limited possibilities to adapt their cultivation methods. Plants exposed to (any form of) stress naturally adapt to environmental changes by synthesizing and accumulating protective metabolites to become more tolerant to subsequent stress events. Priming, or thermopriming if induced by heat, relies on this concept to increase plant tolerance. However, it is still unclear how to apply heat stress under consideration of plant physiological costs and benefits in regard to the further development and growth of plants. In this study, 90 min thermopriming (at 40 °C) for seven consecutive days induced an accumulation of flavonols in leaves that were directly affected by the treatment and thus identified as suitable for inducing thermotolerance in tomato var. Adeleza seedlings. The initial costs on plant growth and development were compensated a few weeks after thermopriming and even benefited the plants later. Thereby, thermopriming may enable global plant production to cope with unpredictable and more frequently occurring environmental stress by a sustainable hardening method of seedlings that can be incorporated in the plant production cycle.

Keywords: thermomemory; abiotic stress; heat stress; tomato plants; solanum lycopersicum; climate change; plant growth; plant development; secondary metabolites

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1. Introduction

In global plant production, there is an emerging need for sustainable plant protection against abiotic and biotic stresses due to diverse challenges related to climate change [1]. Particularly horticultural crops are facing extreme environmental conditions in open-field production and even more in overheating greenhouses [2,3]. Greenhouses are supposed to be protective cultivation systems that allow for optimal plant production. However, more frequently occurring heatwaves combined with high light intensities challenge the technical capabilities of common greenhouses [1]. Simultaneously, it is not unusual that under extreme weather phenomena, plants experience multiple biotic and abiotic stresses, which can potentially interact and thereby amplify their harmful effects [4]. Therefore, it is not sufficient to protect crops from only one stress but to ensure a more sustainable (long-lasting) preventive measure against a set of potential risks without causing any collateral damage to surrounding organisms. At the same time, any new kind of plant treatment also needs to be cost-efficient to motivate plant producers to implement new treatments on their own initiative.

Treatments, such as priming, are becoming more and more relevant. Although current studies focus on various approaches, they have one aspect in common: they have shown a successful induction of cross-tolerances in plants by priming [4]. Priming (also referred to as hardening or conditioning) basically describes the controlled application of tolerable stress to plants or seeds to trigger their natural adaptive abilities and ultimately increase their resilience against subsequent stress events. Seed priming is already well-established in commercial seed production to primarily accomplish uniform germination, reduced

germination, and emergence times, but only secondarily to improve the stress tolerance in the offspring due to an inevitable trade-off in seed quality [5,6]. The latter is addressed by the priming of transplants. Martinez-Medina et al. [7] summarized the benefits in a more robust plant defense and low fitness costs in an overall positive cost–benefit balance on the condition that plants experience stress. There are plenty of studies on promising types of priming [4,8–11], but they concentrate on different organisms and apply stress stimuli under inconsistent conditions (e.g., plant age, plant developmental stage, type of stress, intensity, duration, etc.). In this context, thermopriming comprises the treatment of plants with a sublethal heat shock for an as yet undefined duration and intensity to trigger plant defense mechanisms and to obtain a stress memory [12–16]. The underlying molecular mechanisms of thermopriming and their effects on various types of crops in response to different subsequent stresses in varying plant developmental stages have been extensively reviewed by Khan et al. [17].

Based on the promising results of heat as a priming stimulus [9,10], thermopriming was chosen in our study. The prior research questions by Serano [9] and Röhlen-Schmittgen et al. [10] were extended here to examine how a reduced or prolonged thermopriming duration affects i. plant growth, ii. plant development (vegetative and generative), and iii. the accumulation of primary and secondary metabolites in leaves. The main aim of this study was to identify suitable thermopriming conditions, particularly treatment duration, for subsequent experiments on the yield performance of tomato crops.

Producers of transplants obtain the opportunity to adapt and implement this kind of hardening in their production systems [18]. However, the plant physiological benefit in increasing plant tolerance by thermopriming can result in higher initial financial costs for a more comprehensive and consequently more expensive transplant cultivation due to additional energy costs. In accordance with the sustainable development goals [19], the overall goal of all participants in the value chain is to protect food production from unpredictable risks and yield losses to ensure global food security and sustainable agriculture (Goal 2). This is investigated here in this study of thermopriming.

2. Materials and Methods

2.1. Experimental and Priming Conditions

Three sets of experimental replicates were performed in 2022 with the tomato (*Solanum lycopersicum* L.) var. Adeleza (Enza Zaden Deutschland GmbH & Co. KG, Dannstadt-Schauernheim, Germany) at Geisenheim University (Geisenheim, Germany) from 15 June to 26 October with a duration of seven weeks (first set) and five weeks (second and third set), respectively, after sowing. Thermopriming (at 40 °C, adapted from Serano [9]; abbreviated as "priming" in this paper because no other priming types were investigated) was applied 1 WAS (week after sowing) for seven consecutive days in climate chambers (Fitotron® HGC 0714, Weiss Technik GmbH, Reiskirchen, Germany) once per day starting at 11:30 a.m.—with a preceding and subsequent period of 30 min to increase and decrease the air temperature—with four durations: 0 min (non-primed control treatment), 45 min, 90 min, and 180 min, respectively.

In the climate chambers, environmental conditions were set to 22 °C (day)/20 °C (night) air temperature, 70% air humidity, and 200 µmol m^{-2} s^{-1} (metal halide lamps) for a 16 h photoperiod (8.56 mol m^{-2} d^{-1}). Plants were sown in multipot plates "HerkuPak D 77" (Herkuplast Kubern GmbH, Ering/Inn, Germany) in the peat substrate "Floradur A" (pH 5.6; Floragard Vertriebs-GmbH, Saterland, Germany) and transplants potted in "Floradur B" (pH 5.6; Floragard Vertriebs-GmbH, Saterland, Germany) in 10 cm-diameter pots. For the experiment, plants were further cultivated in the greenhouse (18 °C day and 16 °C night air temperature) in a completely randomized block design to consider local environmental conditions (n = 3–15 plants per treatment (n = 4) and block (n = 3); decreasing weekly due to sample taking until the end of experiment). However, the total number of potted plants per treatment (n = 45) after thermopriming was the same in all experimental sets (Table 1). After sowing, the plates and later pots were irrigated once per

day without fertilization, and fertigated after the emergence of seedlings with 0.5% "Ferty 2 mega" (Hauert HBG Dünger AG, Grossaffoltern, Switzerland).

Table 1. Summary of experimental settings.

	Experiments		
Replicate:	1	2	3
Months:	June–August	August–September	September–October
Timing of thermopriming (week after sowing):	2nd	2nd	2nd
Duration of experiment (weeks after thermopriming):	5	3	3
Duration of experiment (weeks after sowing):	7	5	5
Number of blocks:	3	3	3
Leaf parameters after thermopriming			
Sample size per treatment, block, and week:	3	5	5
Total sample size per treatment and week:	9	15	15
Total sample size per treatment:	45	45	45
Growth parameters			
Total sample size per treatment:	45	45	45
. . . decreasing after thermopriming each week by:	−9	−15	−15

2.2. Growth Parameters

In all experimental sets, plant height, relative growth rate (referred to height), number of leaves (principal growth stages defined by the BBCH-scale [20]), specific leaf area calculated from leaf area (LI-3100C, LI-COR Environmental, Lincoln, Nebraska, NE, USA) and the corresponding leaf dry matter, internode length, number of inflorescences (only available for the first set), as well as above-ground fresh (FM) and dry matter (DM; only assessed at the end of the experiment) were determined. The relative growth rate (RGR) was calculated as follows [21]:

$$RGR = (\ln H_2 - \ln H_1)/(t_2 - t_1),$$

where H_1 and H_2 are plant heights at times t_1 and t_2 (one week difference). The day after the end of thermopriming, plants were potted after measuring their height. Hence, one week after priming (WAP), RGR was not calculated due to incomparable and inconsistent height changes caused by potting.

Furthermore, because of the different experimental durations of 5 WAP (set 1) and 3 WAP (set 2 and 3), the number of observations per week and treatments are also varied (n = 9 in set 1 or n = 15 in set 2 and 3).

2.3. Leaf Compound Analysis

Total chlorophyll content (TCC), total carotenoid content (TCarC), total anthocyanin content (TAC; expressed as cyanidin-3,5-O-diglucosid equivalents, CyEs), total phenolic content (TPC; expressed as gallic acid equivalents, GAEs), and flavonoid contents (FCs) were colorimetrically measured weekly in young (1–2 leaves per sample depending on leaf size) and mature (only one leaf per sample) tomato leaves using an Infinite M200 microplate reader with Magellan 7.2 software (Tecan Group Ltd., Männedorf, Switzerland) according to Dörr et al. [22]. Hereby, FC was selectively determined by two procedures for i. flavanols and flavones luteolin ($FC_{Quercetin}$; expressed as quercetin equivalents, QEs) and ii. rutin, luteolin, and catechin ($FC_{Catechin}$; expressed as catechin equivalents, CEs) [23]. Three technical replicates were measured (undiluted) for each sample, from which the mean was calculated to minimize technical bias by the microplate reader.

Additionally, chlorophyll (Chl) and flavonoid (Flav) indices were non-invasively determined by a leaf transmittance (Dualex Chl index) and fluorescence (Dualex Flav index) measurement device (Dualex Scientific+™, Force-A, Orsay, France). Per leaf, three

abaxial spots—one at each side of the leaf and one at the leaf tip—were measured as technical measurement replicates and summarized as means.

As stated before, the sample size per week differed between the sets, but not in total for sampling as well as for Dualex measurements. For both methods, freshly formed and already fully unfolded true leaves were classified and measured as young and the oldest primary true leaves as mature leaves.

2.4. Data Analysis

All statistical analyses were carried out using R (version 4.2.2) with a linear mixed-effects model for ANOVA (type 2, $\alpha = 0.05$; car–package, version 3.1.1) and post-hoc analysis by estimated marginal means (EMMs, $\alpha = 0.05$, Tukey-adjusted; emmeans–package, version 1.8.4.1) combined with the cld–function (multcomp–package, version 1.4.23) for the display of compact letters indicating significant differences in pairwise comparisons ($\alpha = 0.05$). The lmer–models (lmerTest–package, version 3.1.3) were specified depending on measurement date and, respectively, the corresponding random effects by experimental set, completely randomized blocks, and repeated measurements. The multiple factor analysis (MFA) was computed with the factoextra–package (version 1.0.7) for the standardized quantitative variables determined by Dualex and colorimetric measurements with the active variable treatment and the supplementary variables: leaf age, time (WAS), and set [24]. In advance, the Dualex indices (used for ANOVA and MFA) and colorimetrically measured leaf compounds (used for MFA) were cleared of outliers that were detected using the interquartile range criterion to avoid data distortion by technical bias. Plots were created by ggplot2–package (version 3.4.1).

3. Results

3.1. Plant Height after 90 min Thermopriming Most Similar to Control

Thermopriming had a strong effect on the elongation of plants after the heat treatment was performed (Figure 1a). All primed groups demonstrated a significant increase in plant height compared to the control (45 min: +13%; 90 min: +21%; 180 min: +18%) consequently to the thermopriming treatment—two weeks after sowing. This effect was more pronounced in plants primed for 90 min and 180 min, respectively. One week after priming, the plant height of 90 min-primed plants was similar to the control but showed a longer internode length due to increased stem elongation (Figure A1a). At WAP 2, 90 min thermopriming resulted in a decreased height (Figure 1a). Additionally, at that time, 45 min and 90 min were similar, but higher than the 180 min-primed group.

In contrast to the initial elongation and the corresponding increase in internode length caused by thermopriming (Figure A1a), 180 min-primed plants had a decreased internode length at 2 WAP compared to the other thermoprimed groups and to the control group. After 3 WAP, no differences between height and stem elongation were found between all the primed groups as well as the control group. Thus, thermopriming initially increased plant height by elongation (based on an increased internode length), but in comparison to all primed groups, 90 min was eventually most similar to the control.

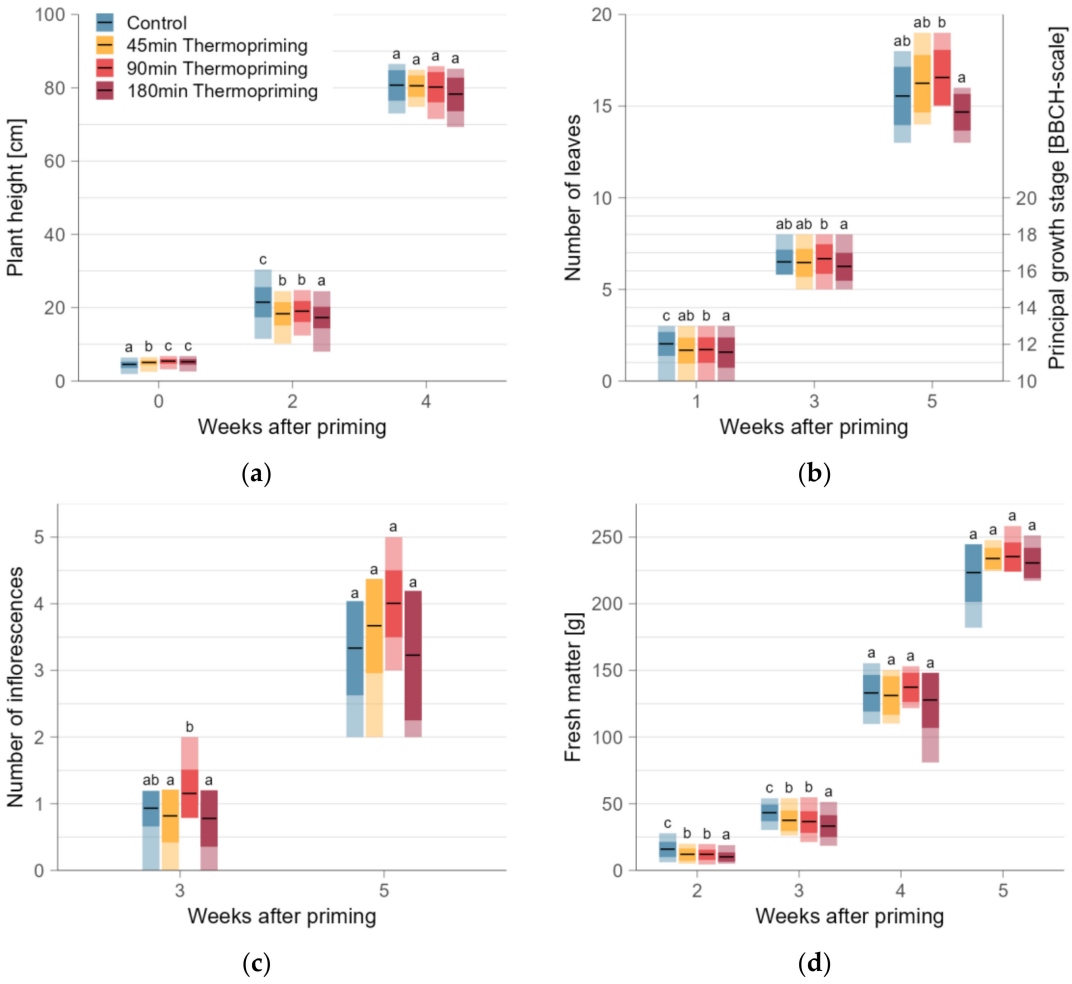


Figure 1. Growth parameters, displayed by mean (black horizonal line), standard deviation (intensely colored inner box), as well as maximum and minimum (lightly colored outer box) of tomato plants summarized for three experimental sets weeks after priming, differentiated for the four treatments: control (blue), 45 min (yellow), 90 min (red), and 180 min (dark red) thermopriming. (**a**) Plant height measured from soil to shoot apical; (**b**) number of leaves and growth stage; (**c**) number of inflorescences; (**d**) fresh matter. The different letters indicate significant differences (ANOVA and EMMs post-hoc; $\alpha = 0.05$) between groups at the same week after priming. The shown data of each timepoint were summarized for all three sets. In all sets, the initial sample size after priming was n = 45 plants per treatment. In the first set, n = 9 samples per treatment were taken each week for 5 weeks after priming, whereas in the second and third set, 15 samples per treatment were taken each week for 3 weeks after priming.

3.2. Initially Delayed, but Later Accelerated Development by 90 min Thermopriming

In regard to the vegetative plant development, the number of leaves (Figure 1b), which is linked to the principal growth stages, was similar after 45 min and 90 min thermopriming, whereas 90 min thermopriming showed a higher number of leaves and an increased growth stage in comparison to 180 min, respectively. In the first week after thermopriming, the 90 min-primed group had formed less leaves than the control group (−16%), like the 45 min (−18%), but still more than the 180 min-thermoprimed group (−23%). Although, at 3 WAP, the control did not differ from the 90 min-primed group anymore. All in all, 90 min thermopriming resulted in the smallest initial delay in plant development (−2%). However, the number of leaves at 5 WAP indicated that 90 min priming led to an accelerated plant development (+6%; not significant, but in trend) compared to the control.

The generative plant development was assessed by the total number of inflorescences per week (Figure 1c). For the entire duration of the experiment, the 90 min-thermoprimed group showed an increased number of inflorescences compared to the other priming treatments but did not differ from the control. In comparison to the control, plants treated for 45 min (−2%) and 180 min (−9%) had less inflorescences, whereas 90 min thermopriming displayed an accelerated flower development (+15% number of inflorescences compared to control), even though it was not significantly different.

3.3. Relative Growth Rates after 45 min Thermopriming Most Similar to Control

In general, plant growth after 45 min thermopriming was most similar to the control (Table A1). Directly after priming and two weeks after sowing, the RGR of the 90 min-primed plants did not differ from that of the control plants or the other thermoprimed groups (Figure A1b). Nevertheless, the other primed groups showed a higher RGR compared to the control (45 min: +31%; 180 min: +27%). At WAP 2, the control plants exhibited a higher RGR than the thermoprimed plants. Hereby, the RGR decreased less in the 45 min-primed plants compared to the RGR of the longer-primed plants. On the contrary, at WAP 3, the highest RGR was found in the 45 min- and 180 min-primed plants and the lowest RGR in the control. From 3 WAP on, there was no different RGR measured between the treatments. Altogether, the 45 min-thermoprimed plants showed not only the most similar pattern to the control, but (from all treatments) also the highest RGR (+9%) compared to the control.

3.4. Decreased Biomass by Thermopriming—Most Pronounced after 180 min

Thermopriming had a consistent effect on fresh matter (Figure 1d) as well as dry matter (Table A1). The plant biomass (FM and DM) was reduced as a result of priming at WAP 1 to 3. The strongest decrease in biomass was measured in the 180 min-primed group (FM: −22%; DM: −17%, referred to control), whereas the biomass of the 45 min- and 90 min-primed groups was less affected but still significantly reduced (FM: −14%; DM: −12%) in comparison to the non-primed control. At 5 WAP, all treatments showed a similar FM and DM.

3.5. Flavonols Increased after 90 min Thermopriming

Dualex flavonol index was significantly increased at 1 WAP in mature leaves in the 90 min thermopriming treatment (by +3%) compared to the control group (Figure 2a). At the same time, the shorter- and longer-primed groups followed the same trend as the 90 min group with significant differences to the control. After that, the flavonol accumulation in thermoprimed leaves was balanced out (2 WAP) and even decreased at 3 WAP in the most intensely treated group (180 min; −5% compared to the control). Hence, 90 min thermopriming temporarily resulted in an increased accumulation of flavonols in mature leaves.

However, the leaf flavonol content at 1 WAP in newly grown young leaves (Figure 2b), that were not exposed to heat stress, also did not change, such as it occurred in mature leaves after thermopriming. In the following weeks, the 180 min-primed group was characterized by a decreased flavonol content in young leaves, while groups that were 45 min- or 90 min-thermoprimed showed a similar Dualex Flav index to the control.

The leaf pigments and secondary metabolites, assessed each week after sowing, were affected by the treatment (Tables 2 and 3). As a result of the most intense thermopriming (with the longest duration of 180 min), the concentrations of chlorophylls, carotenoids, phenols, and flavonoids (such as rutin, luteolin, and catechin) decreased at 2 WAP in young leaves due to an increase in specific leaf area (Table A2), which led to a dilution of these leaf compounds in the corresponding area (related to a decreased accumulation in young leaves). This effect was neither found for lower thermopriming durations nor in mature leaves in all primed groups.

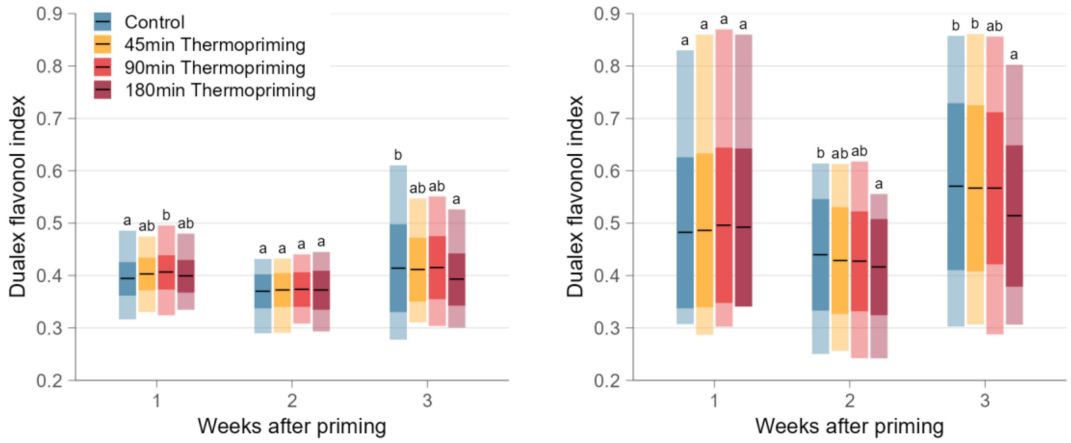


Figure 2. Dualex flavonol index, displayed by mean (black horizonal line), standard deviation (intensely colored inner box), as well as maximum and minimum (lightly colored outer box) of tomato plants summarized for three experimental sets weeks after priming in (**a**) mature and (**b**) young leaves differentiated for the four treatments: control (blue), 45 min (yellow), 90 min (red), and 180 min (dark red) thermopriming. The different letters indicate significant differences (ANOVA and EMMs post-hoc; $\alpha = 0.05$) between groups at the same week after priming. The shown data of each timepoint were summarized for all three sets. In all sets, the initial sample size after priming was n = 45 plants per treatment. In the first set, n = 9 samples per treatment were taken each week for 5 weeks after priming, whereas in the second and third set, 15 samples per treatment were taken each week for 3 weeks after priming.

Table 2. Primary and secondary metabolites in young and mature tomato leaves differentiated for the four priming durations.

Priming Duration [min]	Leaf Age	Developmental Stage	TCC [1] [µg mg^{-1} DM^{-1}]	TCarC [2] [µg mg^{-1} DM^{-1}]	TAC [3] [µg CyEs mg^{-1} DM^{-1}]	TPC [4] [µg GAEs mg^{-1} DM^{-1}]	$FC_{Catechin}$ [5] [µg CEs mg^{-1} DM^{-1}]	n
0	young	early [6]	3.3 ± 1.1 ^{a}	1.1 ± 0.4 ^{b}	3.7 ± 1.1 ^{a}	6.3 ± 2.3 ^{a}	23.0 ± 6.3 ^{a}	38–41
		late [7]	4.2 ± 0.6 ^{a}	1.8 ± 0.3 ^{b}	4.3 ± 1.0 ^{ab}	9.5 ± 2.8 ^{b}	26.1 ± 8.0 ^{a}	96
	mature	early	3.6 ± 0.6 ^{a}	1.0 ± 0.4 ^{a}	4.2 ± 0.6 ^{b}	4.6 ± 0.4 ^{a}	22.0 ± 3.9 ^{a}	30
		late	3.6 ± 0.7 ^{a}	1.4 ± 0.5 ^{c}	4.5 ± 0.8 ^{b}	6.0 ± 1.4 ^{b}	17.0 ± 4.9 ^{a}	86
45	young	early	3.3 ± 0.8 ^{a}	0.8 ± 0.8 ^{a}	3.5 ± 0.9 ^{a}	6.1 ± 1.8 ^{a}	22.2 ± 6.5 ^{a}	38–41
		late	4.1 ± 0.6 ^{a}	1.7 ± 0.3 ^{ab}	4.3 ± 0.8 ^{ab}	9.1 ± 2.5 ^{ab}	26.4 ± 8.7 ^{a}	96
	mature	early	3.5 ± 0.5 ^{a}	0.8 ± 0.6 ^{a}	3.8 ± 0.6 ^{ab}	4.7 ± 0.4 ^{a}	22.2 ± 4.1 ^{a}	29
		late	3.5 ± 0.5 ^{a}	1.2 ± 0.6 ^{b}	4.5 ± 0.7 ^{ab}	5.7 ± 1.4 ^{a}	17.0 ± 6.3 ^{a}	87
90	young	early	3.4 ± 0.9 ^{a}	0.8 ± 0.6 ^{a}	3.4 ± 1.1 ^{a}	6.1 ± 1.9 ^{a}	22.4 ± 6.2 ^{a}	37–40
		late	4.1 ± 0.6 ^{a}	1.7 ± 0.3 ^{ab}	4.1 ± 0.8 ^{a}	9.1 ± 2.6 ^{ab}	25.6 ± 8.1 ^{a}	96
	mature	early	3.6 ± 0.4 ^{a}	0.7 ± 0.8 ^{a}	3.5 ± 0.7 ^{a}	4.7 ± 0.5 ^{a}	23.0 ± 2.8 ^{a}	30
		late	3.6 ± 0.6 ^{a}	1.2 ± 0.6 ^{ab}	4.2 ± 0.6 ^{a}	5.8 ± 1.6 ^{ab}	16.4 ± 5.2 ^{a}	87
180	young	early	3.3 ± 0.9 ^{a}	0.6 ± 0.8 ^{a}	3.6 ± 1.0 ^{a}	6.2 ± 2.3 ^{a}	22.3 ± 7.0 ^{a}	37–40
		late	4.1 ± 0.6 ^{a}	1.6 ± 0.4 ^{a}	4.3 ± 0.8 ^{b}	8.7 ± 3.1 ^{a}	25.2 ± 8.0 ^{a}	96
	mature	early	3.6 ± 0.5 ^{a}	0.6 ± 0.9 ^{a}	3.7 ± 0.7 ^{a}	4.7 ± 0.7 ^{a}	22.7 ± 5.1 ^{a}	29
		late	3.6 ± 0.6 ^{a}	1.0 ± 0.9 ^{a}	4.3 ± 0.7 ^{ab}	5.7 ± 1.4 ^{ab}	17.5 ± 5.7 ^{a}	86

[1] TCC: total chlorophyll content; [2] TCarC: total carotenoid content; [3] TAC: total anthocyanin content (expressed as CyEs, cyanidin-3,5-O-diglucosid equivalents); [4] TPC: total phenolic content (expressed as GAEs, gallic acid equivalents); [5] FC: flavonoid content (expressed as CEs, catechin equivalents); [6] early: 1 week after priming (indicating the short-term effects of thermopriming); [7] late: 2–5 weeks after priming (further cultivation period). Notes: The different letters indicate significant differences (ANOVA and EMMs post-hoc; $\alpha = 0.05$) between groups with the same developmental stage and leaf age of the corresponding parameter. n corresponds to the sample size of the previous parameters.

Table 3. Primary and secondary metabolites in young and mature tomato leaves differentiated for the four priming durations.

Priming Duration [min]	Leaf Age	Developmental Stage	$FC_{Quercetin}$ [1] [µg QEs mg^{-1} DM^{-1}]	n	Dualex Chlorophyll Index	Dualex Flavonol Index	n
0	young	early [2]	13.3 ± 2.5 ^{ab}	38–41	28.06 ± 6.84 ^{a}	0.50 ± 0.15 ^{a}	315
		late [3]	15.2 ± 2.5 ^{a}	96	28.06 ± 6.84 ^{b}	0.50 ± 0.15 ^{b}	315
	mature	early	13.9 ± 2.2 ^{a}	30	25.78 ± 2.94 ^{a}	0.40 ± 0.06 ^{a}	234
		late	13.6 ± 2.4 ^{a}	86	25.78 ± 2.94 ^{a}	0.40 ± 0.06 ^{b}	234
45	young	early	13.1 ± 1.9 ^{ab}	38–41	28.26 ± 6.19 ^{b}	0.50 ± 0.15 ^{a}	315
		late	15.1 ± 2.5 ^{a}	96	28.26 ± 6.19 ^{a}	0.50 ± 0.15 ^{ab}	315
	mature	early	13.5 ± 1.4 ^{a}	29	27.47 ± 3.83 ^{b}	0.40 ± 0.04 ^{ab}	234
		late	13.5 ± 2.4 ^{a}	87	27.47 ± 3.83 ^{b}	0.40 ± 0.04 ^{ab}	234
90	young	early	13.9 ± 2.8 ^{b}	37–40	28.73 ± 6.11 ^{c}	0.50 ± 0.15 ^{a}	309
		late	14.9 ± 2.5 ^{a}	96	28.73 ± 6.11 ^{a}	0.50 ± 0.15 ^{a}	309
	mature	early	14.0 ± 2.0 ^{a}	30	27.73 ± 2.46 ^{c}	0.40 ± 0.05 ^{b}	229
		late	13.7 ± 2.4 ^{a}	87	27.73 ± 2.46 ^{b}	0.40 ± 0.05 ^{ab}	229
180	young	early	12.8 ± 1.5 ^{a}	37–40	28.13 ± 6.22 ^{bc}	0.49 ± 0.15 ^{a}	321
		late	15.0 ± 2.5 ^{a}	96	28.13 ± 6.22 ^{a}	0.49 ± 0.15 ^{a}	321
	mature	early	13.3 ± 1.0 ^{a}	29	27.89 ± 2.67 ^{c}	0.39 ± 0.04 ^{ab}	239
		late	13.6 ± 2.4 ^{a}	86	27.89 ± 2.67 ^{b}	0.39 ± 0.04 ^{a}	239

[1] FC: flavonoid content (expressed as QEs, quercetin equivalents); [2] early: 1 week after priming (indicating the short-term effects of thermopriming); [3] late: 2–5 weeks after priming (further cultivation period). Notes: The different letters indicate significant differences (ANOVA and EMMs post-hoc; $\alpha = 0.05$) between groups with the same developmental stage and leaf age of the corresponding parameter. n corresponds to the sample size of the previous parameters.

No clear differences, nor patterns between the treatments in the accumulation of secondary metabolites have been found. Therefore, multiple factor analysis was conducted to reduce the dimensionality and thus, increase the interpretability of the data.

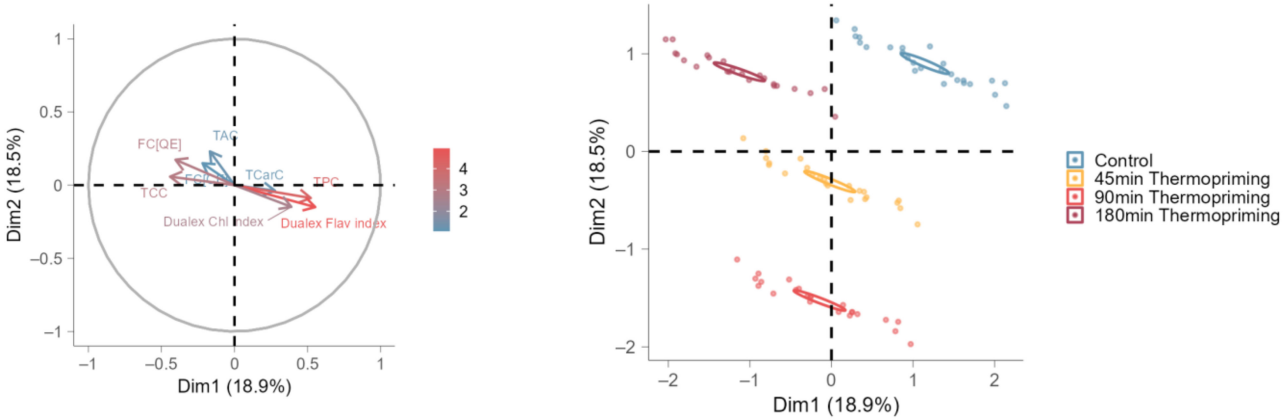
3.6. Multiple Factor Analysis

The multiple factor analysis (MFA) revealed priming effects on the contents of secondary metabolites (Figure 3). As visualized by arrows (Figure 3a), positively correlated variables were grouped together in the same quadrants. Hereby, the increasing distance between variables and the origin (displayed as arrow length) was interpreted as a good representation of the input parameters by the calculated dimensions (Dim). The total phenolic content and the Dualex flavonol index contributed the most to the two dimensions, indicating their high variance in the data. Moreover, TPC and Dualex flavonol content were evidently positively correlated (Figure 3a). Data points represent group means that were calculated for the active variable treatment (n = 4) and the supplementary variables: leaf age (n = 2), date (n = 7), and set (n = 3).

The clustering of the variable treatment clearly outlines treatment effects (Figure 3b). For all variables, the treatments contributed the most to the first and second dimensions. Ellipses visualize the confidence intervals ($\beta = 0.95$) for the four treatments—control, 45 min, 90 min, and 180 min thermopriming—to minimize the bias due to tested, yet undetected outliers. The 45 min- and 90 min-thermoprimed groups are positioned in the opposed quadrant of the control, which indicates a negative correlation between those groups in the accumulation of leaf compounds in response to the thermopriming treatment.

Leaf pigments and secondary metabolites such as chlorophylls (Dualex Chl index and TCC) and flavonoids (Dualex Flav index, FC_{QE} and FC_{CE}), measured by the non-invasive Dualex device as well as invasive colorimetric measurements in the laboratory, were identified by MFA as clearly negatively correlated to each other (Figure 3a). This partially explains the conflicting results in this study by the two used, supposedly complementary,

methods for evaluating the primary and secondary plant metabolism. However, in combination, the two procedures were used to find patterns of the active variable, the treatment, which was the aim of this study.



(**a**) (**b**)

Figure 3. Multiple factor analysis of standardized primary and secondary leaf compounds (TCC: total chlorophyll content; TCarC: total carotenoid content; TAC: total anthocyanin content; TPC: total phenolic content; FC: flavonoid content (expressed as CEs, catechin equivalents, or QEs, quercetin equivalents); Dualex chlorophyll (Chl) index; Dualex flavonol (Flav) index) for group means (specified by treatment, leaf age, date, and set; displayed as points in (**b**) and the four treatments (displayed as confidence ellipses in (**b**), β = 0.95): control (blue), 45 min (yellow), 90 min (red), and 180 min (dark red) thermopriming. Multiple factor analysis was performed on the active variable treatment as well as the supplementary variables: leaf age, date, and set. The color gradient in (**a**) indicates the contribution of the variables to the dimensions (Dim).

4. Discussion

Thermopriming was applied seven days after sowing when seedlings had already formed cotyledons. Cotyledons, as the main photosynthetic sources of fixed carbon in seedlings, provide energy for growth processes and the formation of the first true leaves [25]. Olas et al. [25] emphasized the importance of cotyledons for establishing the thermomemory in the shoot apical meristem and thus in the freshly developed true leaves by providing essential sugars. In this study, young tomato plants synthesized and accumulated flavonols for stress protection in leaves that were formed as the first true leaves just when thermopriming was performed [26]. Therefore, thermoprimed seedlings successfully obtained a memory (at least when they were thermoprimed for a duration of 90 min). Hence, we confirmed an enhanced tolerance (thermotolerance) by applying thermopriming during an early developmental stage [11]. However, it is still uncertain if the obtained memory will last "long enough" to protect the plants from subsequent stresses even after a couple of weeks or multiple months have passed. This is relevant for greenhouse crops, such as tomatoes, that are cultivated for long periods and thereby exposed to a wide range of environmental stresses throughout the year.

Furthermore, in accordance with other studies [11,25,27], thermoprimed plants did result in an initially delayed vegetative development and growth (decreased number of leaves and reduced biomass) that was even more pronounced in the more intensely thermoprimed plants. In contrast to elevated ambient temperatures, which can promote plant growth and induce earlier flowering, thermopriming showed in the early plant developmental phase a different effect on growth and vegetative developmental processes [25,28]. The ultimate aim of plants is reproduction and thereby propagation in order to survive. In response to delays in growth, the accelerated transition to flowering in thermoprimed plants is an adaption that reduces the risk of flowering under unfavorable conditions in upcoming warm periods to avoid potential losses of yield [25,29]. Despite the lack

of statistical significance, we displayed an enhanced number of inflorescences by up to 15% compared to the non-primed control and thus accelerated flower development due to adequate thermopriming (of 90 min duration). In contrast to Fan et al. [11], we did observe effects of thermopriming on the specific leaf area. In this study, we found an increased specific leaf area in the most intensely thermoprimed plants (by 180 min) which led to decreased concentrations of chlorophylls, carotenoids, phenols, and flavonoids in young leaves.

Nevertheless, the deficit in vegetative plant growth and development (regarding height and biomass), stated by Martinez-Medina et al. [7] as initial costs of stress-induced priming tolerance, was balanced out 4 weeks after priming. From this week on, thermoprimed plants did not differ in their growth stages from the control anymore. Moreover, plants did not only indicate an accelerated generative, but also vegetative plant development as the result of thermopriming at the end of the first experimental set (5 WAP). After all, it is necessary to verify the developmental prospects with long-term experiments that also cover yield performance.

We conclude that thermopriming with a daily duration of 90 min for seven days in early plant development on seedlings with established cotyledons is most qualified for inducing a stress memory by heat (thermomemory) in transplants. The physiological costs of stress adaptation by initially delayed growth and vegetative development were later balanced out. Beyond that, our experimental results even displayed an accelerated plant development. In comparison to the non-primed control, the 90 min-thermoprimed plants performed and recovered best after the heat shock. The potential benefit of thermomemory was indicated by the accumulation of protective flavonols in leaves that were formed under or just after thermopriming conditions. The effects of the acquired thermotolerance must next be verified on yield performance in greenhouse production over a longer period in interaction with (repeated) subsequent stress events. In the future, thermopriming could support sustainable and environmental-friendly plant protection in accordance with Goal 2 of the sustainable development goals [19].

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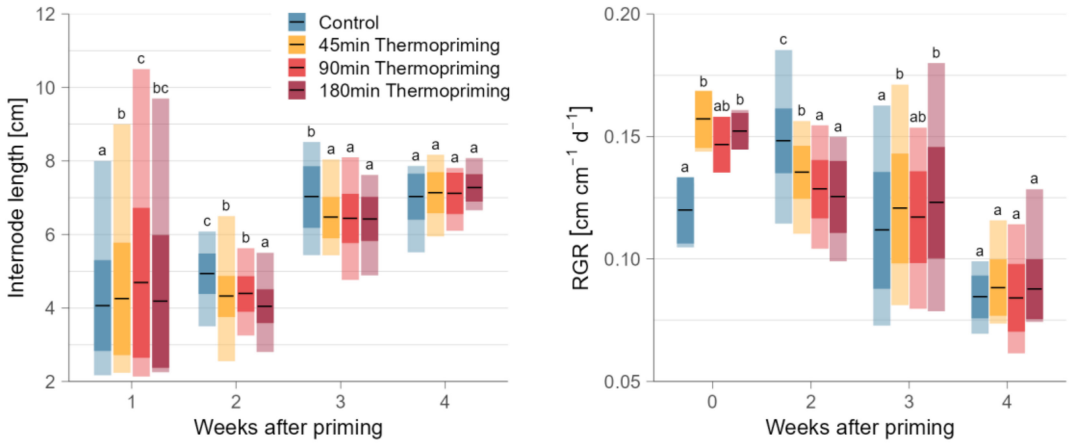
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Appendix A

Table A1. Growth parameters of tomato var. Adeleza weeks after sowing differentiated for the four priming durations.

Priming Duration [min]	WAS [1]	FM [2] [g]	n	DM [3] [g]	n	RGR [4] [cm cm^{-1} d^{-1}]	n	Number of Inflorescences	n
0	5	33.2 ± 8.2 ^{c}	39	2.1 ± 0.7 ^{c}	30	0.12 ± 0.03 ^{b}	183	0.8 ± 0.4 ^{ab}	27
	7	230.6 ± 11.4 ^{a}	9	25.9 ± 1.9 ^{a}	9			3.2 ± 1.0 ^{a}	9
45	5	37.2 ± 7.7 ^{b}	39	2.5 ± 0.8 ^{b}	30	0.12 ± 0.03 ^{b}	183	0.8 ± 0.4 ^{a}	27
	7	233.9 ± 8.0 ^{a}	9	25.3 ± 2.0 ^{a}	9			3.7 ± 0.7 ^{a}	9
90	5	36.2 ± 8.1 ^{b}	39	2.4 ± 0.7 ^{b}	30	0.12 ± 0.03 ^{a}	183	1.1 ± 0.4 ^{b}	27
	7	235.1 ± 10.9 ^{a}	9	26.4 ± 2.8 ^{a}	9			4.0 ± 0.5 ^{a}	9
180	5	43.1 ± 6.3 ^{a}	39	3.3 ± 1.1 ^{a}	30	0.12 ± 0.03 ^{a}	183	0.9 ± 0.3 ^{a}	27
	7	223.0 ± 21.6 ^{a}	9	25.3 ± 2.8 ^{a}	9			3.3 ± 0.7 ^{a}	9

[1] WAS: weeks after sowing; [2] FM: fresh matter; [3] DM: dry matter; [4] RGR: relative growth rate referred to plant height. Notes: The different letters indicate significant differences (ANOVA and EMMs post-hoc; $\alpha = 0.05$) between groups in the same week after sowing of the corresponding parameter. n corresponds to the sample size of the previous parameter.



(**a**) (**b**)

Figure A1. Growth parameters, displayed by mean (black horizonal line), standard deviation (intensely colored inner box), as well as maximum and minimum (lightly colored outer box) of tomato plants summarized for three experimental sets weeks after priming differentiated for the four treatments: control (blue), 45 min (yellow), 90 min (red), and 180 min (dark red) thermopriming. (**a**) Internode length; (**b**) RGR (relative growth rate). The different letters indicate significant differences (ANOVA and EMMs post-hoc; $\alpha = 0.05$) between groups at the same week after priming. The shown data of each timepoint were summarized for all three sets. In all sets, the initial sample size after priming was n = 45 plants per treatment. In the first set, n = 9 samples per treatment were taken each week for 5 weeks after priming, whereas in the second and third set, 15 samples per treatment were taken each week for 3 weeks after priming.

Table A2. Leaf compounds and specific leaf area of young tomato leaves two weeks after priming differentiated for the four priming durations.

Priming Duration	TCC [1]	TCarC [2]	TPC [3]	$FC_{Catechin}$ [4]	n	SLA [5]	n
[min]	[µg mg^{-1} DM^{-1}]		[µg GAEs mg^{-1} DM^{-1}]	[µg CEs mg^{-1} DM^{-1}]		[cm g^{-1} DM^{-1}]	
0	4.4 ± 0.6^{b}	1.8 ± 0.4^{b}	8.1 ± 1.6^{c}	27.6 ± 9.6^{b}	39	573.4 ± 170.6^{a}	38
45	4.3 ± 0.5^{ab}	1.8 ± 0.3^{b}	7.4 ± 1.6^{b}	28.5 ± 9.8^{b}	39	574.0 ± 166.5^{a}	38
90	4.4 ± 0.5^{ab}	1.8 ± 0.4^{b}	7.6 ± 1.4^{bc}	27.6 ± 8.9^{b}	39	574.9 ± 173.7^{a}	39
180	4.3 ± 0.6^{a}	1.6 ± 0.5^{a}	6.8 ± 1.4^{a}	23.8 ± 8.0^{a}	39	644.5 ± 219.4^{b}	39

[1] TCC: total chlorophyll content; [2] TCarC: total carotenoid content; [3] TPC: total phenolic content (expressed as GAEs, gallic acid equivalents); [4] FC: flavonoid content (expressed as CEs, catechin equivalents); [5] SLA: specific leaf area. Notes: The different letters indicate significant differences (ANOVA and EMMs post-hoc; $\alpha = 0.05$) between treatments (priming duration) of the corresponding parameter. n corresponds to the sample size of the previous parameters.

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