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Type of Stress Induces Differential Responses in *Acer rubrum* (Red Maple), but Induced Responses Have No Effect on Herbivorous Pests

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Abstract: Plants thrive in dynamic environments requiring adaptive strategies in response to environmental stressors. Furthermore, insect herbivores may be attracted or deterred by the expression of these traits. This study examines growth, physiological, and phytochemical adaptations of maple trees in response to stressors and how these stressors effect herbivore feeding behavior within an agricultural production system. Agricultural systems are unique because plants experience environmental stressors unique to production such as herbicide sprays and girdling. Using four environmental stressors commonly observed in agricultural production (control, mechanical defoliation, chemical defoliation, and girdling), applied to two cultivars of red maple (*Acer rubrum*, 'Brandywine' and 'Franksred'), this study analyzed differentiation of expressed traits in a production system. Responses varied depending on cultivar and stress treatment but had no effect on insect herbivore behavior. Understanding the ecological interactions within these systems will provide information for better plant production and pest management recommendations.

1. Introduction

Environmental stressors are defined as any environmental conditions (biotic and abiotic) that cause a sharp reduction in fitness [1–3]. For plants, environmental stressors may be climatic [4,5], chemical [6,7], physical [8], biological [9,10] or a combination of stress types [11] that reduce plant fitness (i.e., growth and productivity). To minimize reductions of overall fitness, plants may utilize *constitutively* expressed traits (traits that are always present), or *induced* traits (responses that are "immune-like") to counter environmental stressors [10,12,13]. Induced responses are often energetically costly compared to constitutive traits [9,14,15]. To reduce energetic costs, optimization of multiple metabolic pathways may be utilized to induce responses [16,17]. For example, the growth-differentiation balance hypothesis predicts that plants should re-allocate resources away from primary metabolic functions (i.e., growth) to differentiated process (i.e., defense production) [18–20]. Alternatively, plants may change resource acquisition strategies, such as increasing photosynthetic capacity, to meet the additional nutrients requirements of induced responses [21–23]. The balancing act of plants to minimize the negative effects of induced responses and maximize overall fitness often leads to the expression of a broad arsenal of induced traits [24,25]. To fully understand an induced response, analyses should include plant nutritional quality (e.g., non-structural carbohydrates), physical characteristics (e.g., leaf morphology, trichome density), regrowth capacity, direct chemical defenses (e.g., polyphenols and tannins), and indirect chemical defenses (e.g., volatile organic compounds). Thus, it is more informative to consider induced responses as a suite of traits with synergistic interactions [24–26].

1.1. Effects of Induced Responses on Herbivorous Insects

Induced plant-responses transcend all ecological interactions in ecosystems and strongly influence herbivore behavior [27,28]. The plasticity of induced responses changes the magnitude of expressed traits, thereby altering the nutritional quality of plant tissues [29]. Nutritional characteristics of plant tissues, including primary metabolites such as carbohydrates, may positively influence the growth and development of herbivores [30,31]. Contrastingly, anti-nutritional compounds, including secondary metabolites such as polyphenols and tannins (a subset of polyphenols), and induced volatile organic compounds (VOCs) may negatively influence herbivore growth, development, and behavior [32–34]. Recent advances in VOC analyses suggest that VOCs emitted in response to stress play a role in host-locating behavior of herbivores [35–37]. More specifically, green leaf volatiles [GLV, various C_6 compounds such as limonene, (Z)-3-hexene-1-ol, (Z)-3-hexenyl acetate, and DMNT] are commonly emitted compounds by stressed plants [36,38–40] that often act as olfactory cues for insect herbivores [28,39,41].

1.2. Targeting Trait Complexes to Identify Stress Signals and Insect Herbivore Responses in an Agro-Ecosystem

We wanted to identify changes in red maple (*Acer rubrum*) growth, physiology, and phytochemistry in response to common types of injury-induced stress in nursery production. We hypothesized that stressed trees would have increased production of chemical defenses [42,43]. Additionally, to reduce the energetic costs associated with defense production, stressed trees should express changes to physiological processes (i.e., increased photosynthetic capacity and water potential) and physical characteristics (i.e., reduced leaf aspect ratio, leaf area, specific leaf area, trichome density, and growth rates) as resources are allocated to stress responses and away from primary metabolic functions [18,20]. Because pests are commonly attracted to induced stress responses [36,37], we hypothesized that stressed trees would have increased pest pressure (i.e., spider mites, broad mites, aphids and plant leafhoppers). Finally, we hypothesized that stressed trees should also express greater quantities of GLV [i.e., limonene, (Z)-3-hexene-1-ol, (Z)-3-hexenyl acetate, and DMNT] [44,45].

2. Materials and Methods

2.1. Red Maple Propagation and Treatment Applications

We used two sites in middle Tennessee, United States that have similar environmental conditions for replication of this experiment. One site was located at the Tennessee State University Otis L. Floyd Nursery Research Center, McMinnville, Tennessee, United States (NRC, 35.708867° N, −85.743953° W) (Warren Co.), using the 'Brandywine' cultivar. The second site was at the Moore Nursery in Irving College, Tennessee, United States (MN, 35.583889° N, 85.713056° W) (Warren Co.), using the 'Franksred' cultivar. Red maple cultivars have known lineages, which allow us to control for genotypical differences of expressed phenotypes [46]. Each site had a different cultivar to reproduce common nursery production methods. Mean height of trees at planting was 194.03 ± 45.71 cm, and a mean stem diameter of 23.01 ± 5.07 mm. We analyzed four stress treatments at both sites in a randomized complete block design. The stress treatments were applied in early spring, immediately after tree canopies were completely flushed. The treatments and date applied were as follows: (1) no stress (*control*; Figure 1a), (2) mechanical defoliation by removing all leaves (*defoliated*; NRC defoliated on 5 May 2021, MN defoliated 12 May 2021, Figure 1b), (3) chemical defoliation (*herbicided*; Scythe (Gowan, Yuma, AZ, USA) application at both sites on 11 May, 2021 with a concentration of 266 mL/3.78 L applied to runoff over the entire canopy, Figure 1c), and (4) girdling stress (*girdled*; a 10 cm portion (approximately 2.5 cm wide) of bark and cambium was removed 7.5 cm above the root crown; NRC girdled on 5 May 2021, MN girdled on 12 May 2021, Figure 1d). Treatments were replicated 20 times for a total of 80 trees at each site.

Figure 1. Stress treatments applied to red maple (*Acer rubrum)* cultivars. Photographs are of 'Frank's red' cultivars at the Moore Nursery in Irvine College, TN, USA, but 'Brandywine' cultivars at the Otis Floyd Nursery Research Center, McMinnville, TN, USA received the same treatments. (**a**) control treatment with no stress application. (**b**) complete mechanical defoliation of tree canopy. (**c**) complete chemical defoliation with a foliar contact herbicide. (**d**) girdling of the sapling trunk.

2.2. Analysis of Growth, Canopy, and Leaf Traits

We measured height (of apical meristem) and stem diameter growth as relative growth rate (RGR; final measurement -initial measurement/# of days) as described by [47]. This calculation avoids bias caused by initial plant size by subtracting the initial size from the final sampling size and dividing by the total number of days that elapsed (190 days in this experiment). Initial plant height (cm) and stem diameter (mm) were recorded on the date of treatment application previously mentioned and final height and stem diameter were recorded at the end of the season (18 November 2021). Height of canopy crown (cm; distance between ground and bottom of canopy) and mean canopy diameter (cm; calculated as the mean length of the widest part of the canopy and length perpendicular to the widest length) were measured on 15 September 2021, so canopies were fully flushed, but fall senescence had not begun.

After re-flush of leaves from defoliated and herbicided treatments (14 June 2021), leaf morphology was assessed for each tree. Five matured leaves from different dominant lateral branch tips of each sapling were subsampled, scanned, and analyzed using WinFO-

LIA™ (Regent Instruments Inc., Quebec City, Quebec, CA, USA) computer image analysis software. Key leaf morphological parameters (described in [48]) were determined as the mean measurement of the five subsamples. Parameters included total leaf area (cm^2), leaf perimeter (cm), vertical length (cm), horizontal length (cm), and leaf aspect ratio (maximum leaf breadth/maximum leaf width). Specific leaf area (SLA) was calculated by dividing the total leaf area by the leaf's dry weight (cm^2/g). Trichome density was determined by using a 7 mm hole punch to remove 3 disks from each of the five leaves collected per tree. The trichomes were counted on each disk, then disks were dried and weighed. The mean number of trichomes per dry mass (g) of leaf was recorded as the trichome density (# of trichomes/g) for each sapling.

2.3. Physiological Trait Measurements

Tree physiological characteristics were also measured after re-flush of leaves. All physiological measurements were performed on four randomly selected replications of each treatment (a total of 16 saplings) from each site. For chlorophyll a, chlorophyll b, and total carotenoid (xanthophylls and carotenes) analyses, we collected three mature leaves from each replication. Three 6 mm leaf disks were punched from deveined tissues of each leaf and placed into vials with 50 µL of 2.0 mm beads into 1.5 mL locking Eppendorf tubes and 1 mL of methanol. We bullet blended the leaf tissues into a slurry consistency. Vials were then incubated at 4 °C for 24 h to extract pigment compounds. After extraction, vials were centrifuged at $4000\times g$ rpm for 15 min then 200 µL from each sample were pipetted into a 96 well microplate. Total carotenoid (λ = 470 nm), chlorophyll a (λ = 665.2 nm), and chlorophyll b (λ = 652.4 nm) were determined using the UV-VIS Epoch microplate spectrophotometer (BioTek, Winooski, VT, USA) and conversion equation according to the Lichtenthaler and Buschmann [49] method. From the same saplings, we used a LI-6400XT Portable Photosynthesis System (LI-COR Biosciences, Lincoln, NE, USA) to measure photosynthetic rate ($\mu mol\ m^{-2}\ s^{-1}$) and stomatal conductance ($mol^{-2}\ s^{-1}$). The measurements were analyzed under ambient conditions (20–25 °C) with 400 µmol $CO_2\ mol^{-1}\ CO_2$ concentration, a fixed flow rate (set at 300 µmol s^{-1}) and ambient PAR with an average 1000 $m^{-2}\ s^{-1}$ PPFD. For consistency of environmental variables, we conducted gas exchange measurements between 0800 and 1200 over a period of two days (14 and 15 June 2021). We used a Scholander water pressure chamber (Model 600 Classic Pressure Chamber 40 Bar, PMS Instrument, Albany, OR, USA) to measure water potential. Plant water potential is dynamic throughout the day [50,51]. Measurements were performed pre-dawn as a more reliable indicator of plant water status because the plant is assumed to be in equilibrium with soil water potential at this time [51,52] and mid-day to detect mid-day water stress [50].

2.4. Plant Phytochemistry

Plant foliar phytochemistry was analyzed by collecting five mature leaves from each sapling. We collected leaf samples after reflush (14 June 2021) and at the end of the season (30 September 2021) to observe immediate and delayed induced responses. For both collection dates, leaves were immediately oven-dried at 65 °C for 48 h, ground using a 2 mm mesh on a Wiley® Mill (product no. 3375P76, Thomas Scientific, Swedesboro, NJ, USA) and homogenized for each tree. Certain phenolic compounds are depleted using this method [52–54]. However, we were interested in relative concentrations and not absolute concentrations [55,56]. Polyphenols and tannins were extracted from the dried tissues using a 70% acetone solvent [57,58]. Polyphenol concentrations were analyzed using the 96-well modified Prussian blue assay for total phenols [57]. Total tannin concentrations were quantified using the radial diffusion assay [57]. There are no unique concentrations for polyphenols or tannins, so we standardized against gallic acid (gallic acid equivalents, G.A.E.) and tannic acid (tannic acid equivalents, T.A.E.), respectively [57]. Non-structural carbohydrates from foliar tissues were analyzed using the Fournier [59] method with

a modification by Tomlinson et al. [60] that uses a phenol-sulfuric acid solvent for a colorimetric reaction with sugars and starches.

Green leaf volatiles were collected from 4 replicates of each treatment for each cultivar ('Brandywine' on 14 June 2021 and 'Franksred' 15 June 2021). A 15 cm branch tip was clipped from each tree. The clipped ends of the samples were placed in a 30 mL centrifuge tube filled with water to keep branch tips from desiccating during volatile collections. The branch tips with centrifuge tubes were each set in their own "collecting chamber". The collection chambers consisted of cylindrical glass jars (custom made by Sigma Scientific, LLC, Micanopy, FL, USA) that were 31.5 cm tall with a 10 cm diameter. Each chamber had an opening at the top where air could flow in through a carbon filter to prevent external volatile contamination, and an opening at the bottom with a SuperQ filter which absorbed emitted volatiles. Volatile organic compound profiles were collected for three hours/branch sample using a pull rate of 0.5 ± 0.05 L/min. After collection of VOCs, leaves were counted from each sample, dried, weighed, and averaged to normalize emission rates per sample. The filters containing the VOCs were eluted with 250 μL of pentane into vial inserts that were placed in 2 mL glass vials. Elution vials were sealed with Teflon tape and stored in a -18 °C freezer until analyses could be performed. Eluted samples were analyzed by gas chromatography using a Shimadzu GCMS-QP2010 gas chromatograph mass spectrometer (Shimadzu Corp, Kyoto, Kyoto, Japan). One microliter of the extracts was injected into a splitless injector at 220 °C. The DB-1 column (30 m × 0.25 mm i.d. × 0.1 μm film thickness; Agilent Technologies, Santa Clara, CA, USA) was kept at 50 °C for 5 min, followed by a temperature ramp of 15 °C/min to 180 °C a and a final temperature ramp of 20 °C/min to 280 °C. Helium carrier gas flow rate was 1 mL/min (constant flow) and the transfer line temperature was 260 °C. The EI mode ion source temperature was 230 °C and 70 eV. Major plant volatiles were identified by matching compound mass spectra to the reference spectra library (NIST08) and compared to analytical standards. Green leaf volatiles were compared between samples by using the area % of peaks of each compound (i.e., limonene, (Z)-3-hexene-1-ol, (Z)-3-hexenyl acetate, and DMNT).

2.5. Quantification of Common Nursery Pests

We collected two 30.0 cm branch tips from each tree, once a month in June, July, and August. Branch tips were placed in a zip lock bag and stored at 4 °C until insect counts could be conducted. For each tree, we totaled the number of adult spider mites (Trombidiformes: Tetranychidae), broad mites (Trombidiformes: Tarsonemidae), aphids (Hemiptera: Aphididae), and thrips (Thysanoptera) counted and summed from the two branch tips collected each month. We measured plant leaf hopper damage (Hemiptera: Cicadellidae) in August as the percentage of branch tips with characteristic 'Hopperburn' (i.e., browning and curling of leaf tips, [61]).

2.6. Statistical Analysis

To analyze induced responses of maples to stress treatments, we used multivariate analysis of variance (MANOVA) for multiple dependent variables to minimize type I statistical error with the *dplyr* package [62] in R analytical software [63]. Transformations were made as necessary to meet all assumptions of normality and heteroscedasticity [64]. Due to differences in sampling techniques, we created several different models. The first model included variables growth (height and stem diameter), canopy height, canopy width, and leaf morphological traits (total leaf area, leaf perimeter, vertical length, horizontal length, leaf aspect ratio, and SLA) as the dependent variables, treatment (control, defoliated, girdled, or herbicided) and cultivar ('Brandywine' or 'Franksred') as the independent variables. We did not include site (MN or NRC) because site was confounded with cultivar type. The second model included variables that were subsampled: physiological changes (total carotenoid, chlorophyll a, and chlorophyll b concentrations, photosynthetic rate, stomatal conductance, and pre-dawn/mid-day water potential measurements) with the same independent variables as the first model. The third model included phytochemical traits (June

and September measurements of polyphenol, tannin, and non-structural carbohydrate concentrations) as the dependent variables and with the same independent variables as the first model. Graphs were made using the *ggplot2* package [65]. The final model included the GLV area % of peaks for each compound [limonene, (Z)-3-hexene-1-ol, (Z)-3-hexenyl acetate, and DMNT] as the dependent variables and the same independent variables as the first model.

Pearson correlations were performed between leaf morphological and phytochemical traits (using June measurements as this is when pest populations start to grow) and count data (spider mites, broad mites, and aphids) and % damage (leaf hopper burn). Phytochemical traits were categorized as nutritional (i.e., non-structural carbohydrates) and antinutritional (i.e., polyphenols and tannins).

3. Results

3.1. Growth, Canopy and Leaf Traits

Growth, canopy characteristics, and leaf morphological traits were significantly different among treatments (MANOVA: Wilk's $\lambda = 0.56$, $F_{(12, 2307)} = 13.66$, $p < 0.001$, Table 1) and between cultivars (MANOVA: Wilk's $\lambda = 0.36$, $F_{(4, 767)} = 109.87$, $p < 0.001$, Table 1). There was an interactive effect of treatment and cultivar on response variables (MANOVA: Wilk's $\lambda = 0.97$, $F_{(12, 2307)} = 1.11$, $p = 0.049$). Overall, 'Brandywine' cultivars grew much faster and had larger canopies than 'Franksred' cultivars (ANOVA: height RGR $F = 50.94$, $p < 0.001$, canopy height: $F = 192.85$, $p < 0.001$, canopy width: $F = 12.95$, $p = 0.001$, Table 2, Figure 2). RGR was reduced in defoliated treatments compared to controls in 'Brandywine' cultivars (*post hoc* TukeyHSD: 'Brandywine' $p < 0.001$, Figure 2a). However, 'Brandywine' treatments grew significantly faster than 'Franksred" regardless of stress (ANOVA: $F = 5.37$, $p = 0.002$, Table 2, Figure 2a,b). Trichome density was greater in defoliated and herbicided treatments for both cultivars (*post hoc* TukeyHSD: 'Brandywine' $p = 0.04$, $p = 0.021$, 'Franksred' $p = 0.031$, $p = 0.012$), but was not different between cultivars (ANOVA: $F = 0.38$, $p = 0.673$, Table 2). 'Brandywine' cultivars had a larger leaf area than 'Franksred' cultivars (ANOVA: $F = 440.28$, $p < 0.001$, Table 2). However, control treatments had greater leaf area than defoliated treatments in both cultivars (*post hoc* TukeyHSD: 'Brandywine' $p < 0.001$, 'Franksred' $p = 0.013$). 'Franksred' cultivars had a greater specific leaf area in control treatments compared to defoliated and herbicided treatments (*post hoc* TukeyHSD: $p < 0.001$, $p = 0.026$, respectively).

3.2. Physiological Traits

Physiological characteristics were affected by the stress treatments (MANOVA: Wilk's $\lambda = 0.70$, $F_{(15, 114)} = 2.29$, $p = 0.007$), cultivar type (MANOVA: Wilk's $\lambda = 0.62$, $F_{(5, 36)} = 11.81$, $p < 0.001$), and the interaction between treatment and cultivar (MANOVA: Wilk's $\lambda = 0.66$, $F_{(15, 114)} = 2.56$, $p = 0.012$). Chlorophyll a decreased in herbicided treatments of the 'Brandywine' cultivars (ANOVA: $F = 8.61$, $p < 0.001$ Table 2, Figure 3a), but there were no differences between treatments in 'Franksred' cultivars (*post hoc* TukeyHSD $p > 0.05$, Table 2, Figure 3b) or in other pigments (*post hoc* TukeyHSD chlorophyll b: $p > 0.05$ or carotenoids: $p > 0.05$, Table 2). There were no changes to photosynthetic rates or stomatal conductance in response to stress treatments (ANOVA: $F = 1.50$, $p = 0.230$ and $F = 0.1.28$, $p = 0.0.290$, respectively, Table 2), but 'Franksred' cultivars did express a higher conductance rate compared to 'Brandywine' cultivars (ANOVA: $F = 19.34$, $p < 0.001$, Table 2, Figure 3a,b). Trees were not water-stressed pre-dawn (ANOVA: $F = 0.072$, $p = 0.974$), but became more water-stressed throughout the day (ANOVA: $F = 31.64$, $p < 0.001$, Table 2, Figure 4). Stressed trees became even more water-stressed midday compared to controls (*post hoc* TukeyHSD: all $p < 0.05$, Figure 4b,d).

Table 1. MANOVA results testing the effect of treatment (control, defoliated, herbicided, and girdled) on responses of two red maple (*Acer rubrum*) cultivars ('Brandywine' and Franksred'). Significant values are in **bold**.

	Treatment				Cultivar				Treatment × Cultivar			
	λ	df	$F_{(12, 2307)}$	*p*	λ	df	$F_{(4, 767)}$	*p*	λ	df	$F_{(12, 2307)}$	*p*
Growth, canopy, and leaf traits	0.06	3	3.66	**<0.001**	0.36	1	109.87	**<0.001**	0.02	3	1.11	0.049
Physiological traits	0.70	3	2.29	**0.007**	0.62	1	11.81	**<0.001**	0.66	3	2.56	**0.012**
Phytochemical Constituents	1.10	3	13.1	**<0.001**	0.45	1	18.31	**<0.001**	0.33	3	2.82	**<0.001**

Table 2. ANOVA results testing the effect of treatment (control, defoliated, herbicided, and girdled) on responses of two red maple (*Acer rubrum*) cultivars ('Brandywine' and Franksred'). Significant values are in **bold**.

| Response Variable	Treatment			Cultivar			Treatment × Cultivar		
	df	*F*	*p*	*df*	*F*	*p*	*df*	*F*	*p*
Growth, canopy, and leaf traits									
RGR height	3	50.94	**<0.001**	1	191.96	**<0.001**	3	5.37	**0.002**
RGR stem diameter	3	73.53	**<0.001**	1	27.13	**<0.001**	3	5.77	**<0.001**
Canopy height	3	1.23	0.30	1	192.85	**<0.001**	3	0.14	0.940
Canopy diameter	3	20.04	**<0.001**	1	13.03	**<0.001**	3	2.39	0.071
Trichome density	3	2.76	**0.041**	1	0.38	0.536	3	0.51	0.673
Leaf area	3	10.75	**<0.001**	1	440.28	**<0.001**	3	2.33	0.073
Specific leaf area	3	7.09	**<0.001**	1	210.57	**<0.001**	3	1.51	0.209
Dry leaf mass	3	0.34	0.799	1	0.37	0.544	3	0.21	0.889
Physiological traits									
Chlorophyll a	3	8.61	**<0.001**	1	4.30	**0.040**	3	1.68	0.190
Chlorophyll b	3	5.58	0.300	1	2.07	0.158	3	3.40	**0.027**
Total carotenoids	3	0.68	0.570	1	0.47	0.50	3	0.59	0.632
Photosynthetic rate	3	1.50	0.230	1	0.57	0.454	3	0.56	0.646
Stomatal conductance	3	1.28	0.293	1	19.34	**<0.001**	3	4.22	**0.011**
Water potential (pre-dawn)	3	0.072	0.974	1	86.02	**<0.001**	3	0.13	0.943
Water potential (mid-day)	3	31.64	**<0.001**	1	3.30	0.082	3	0.96	0.427
Phytochemical constituents									
June polyphenols	3	1.70	0.171	1	0.01	0.989	3	0.61	0.607
June tannins	3	81.79	**<0.001**	1	20.62	**<0.001**	3	7.49	**<0.001**
June TNC	3	202.60	**<0.001**	1	0.075	0.785	3	0.839	0.476
September polyphenols	3	1.02	0.384	1	3.58	0.061	3	0.96	0.412
September tannins	3	210.38	**<0.001**	1	80.35	**<0.001**	3	9.14	**<0.001**
September TNC	3	0.85	0.471	1	0.48	0.488	3	0.80	0.494
Limonene	3	0.448	0.721	1	26.97	**<0.001**	3	2.14	0.687
(Z)-3-hexene-1-ol	3	-	-	1	-	**-**	3	-	-
(Z)-3-hexenyl acetate	3	0.303	0.823	1	67.22	**<0.001**	3	0.79	0.445
DMNT	3	2.421	0.104	1	29.03	**<0.001**	3	1.67	0.385
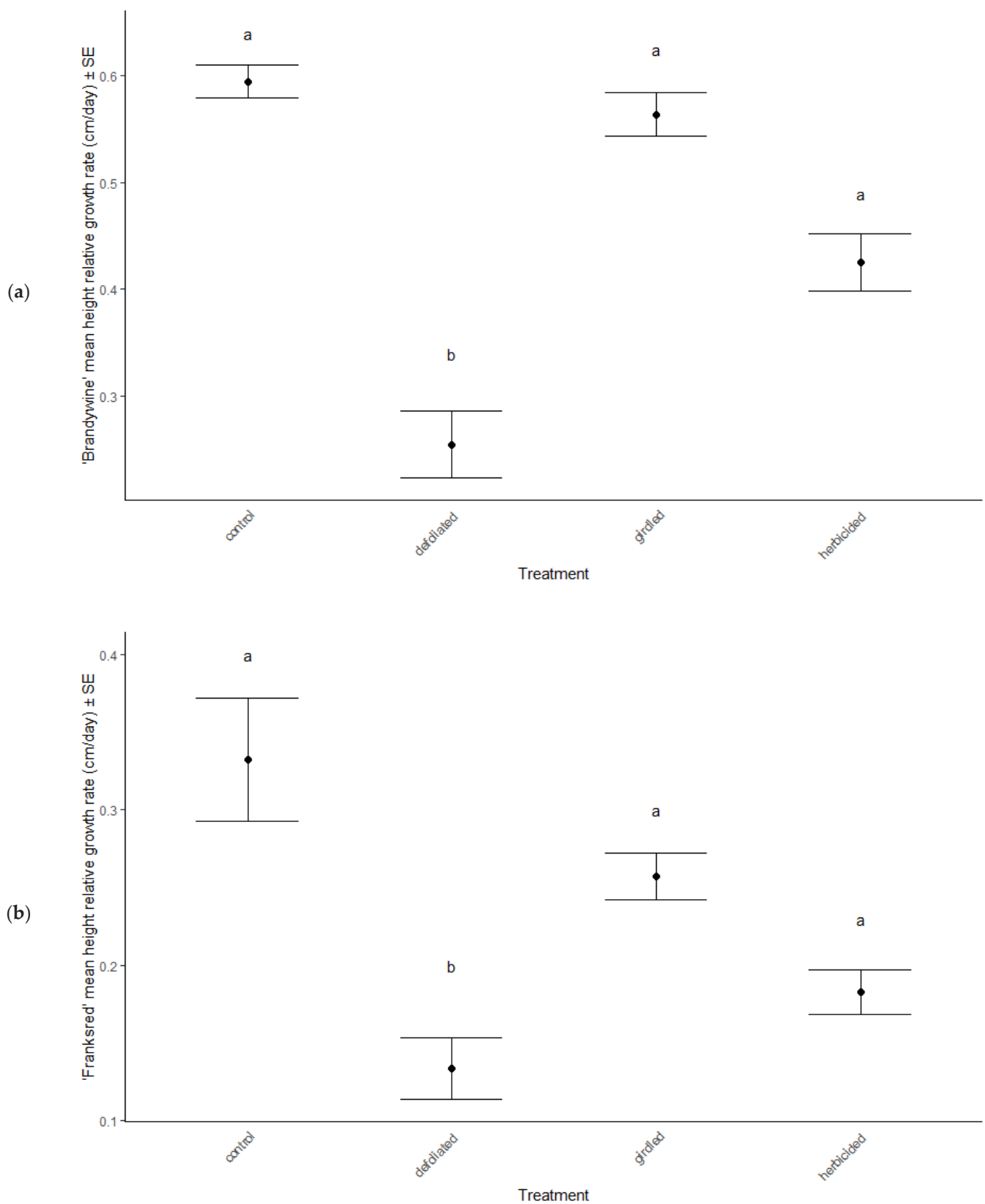
Figure 2. Relative growth rate (RGR in cm/day) of red maple (*Acer* rubrum) cultivars (**a**) 'Brandywine' and (**b**) 'Franksred' in response to stress treatments. Different letters represent statistical differences between treatments according to a *post hoc* Tukey HSD analysis (*p* < 0.05).
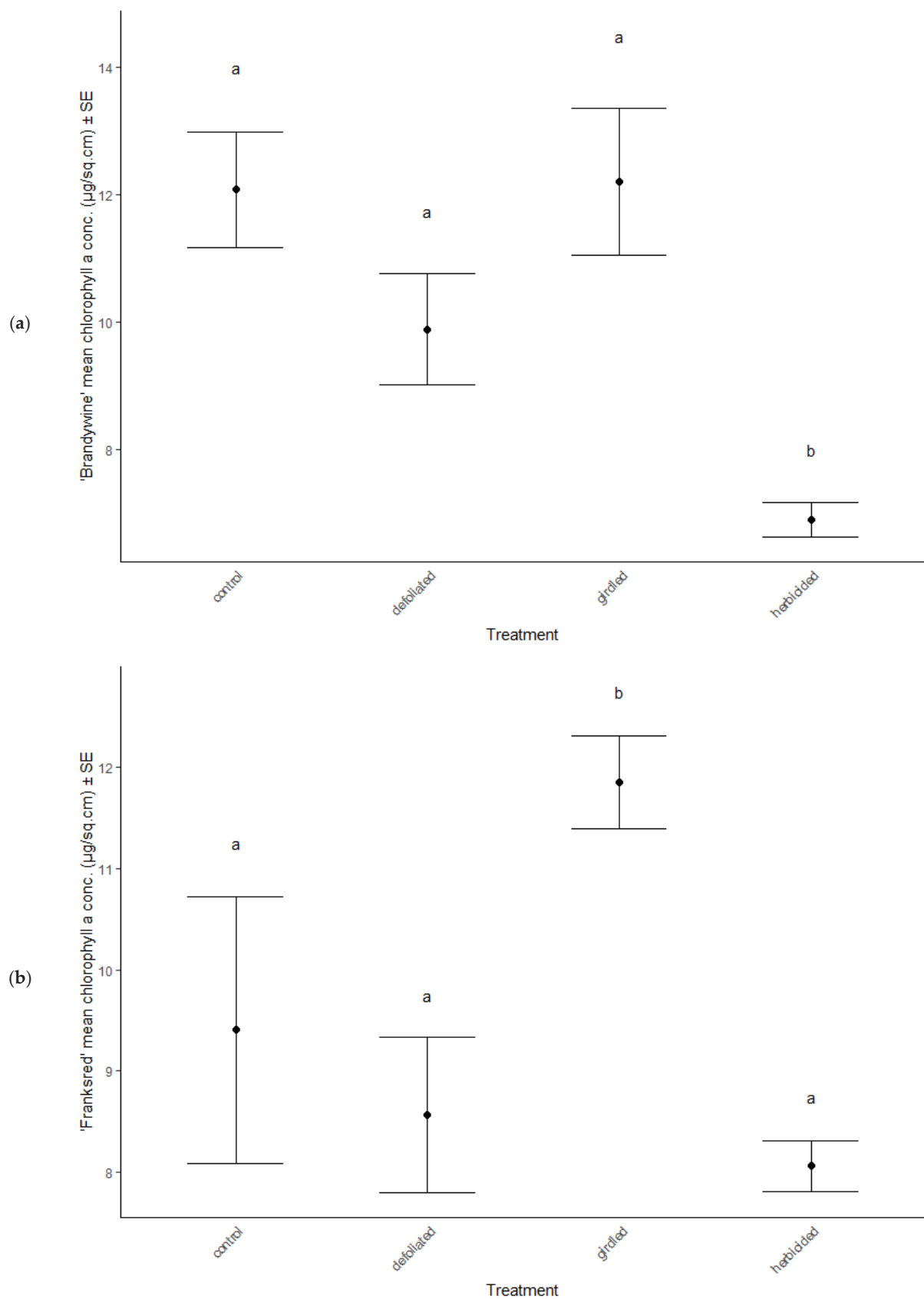
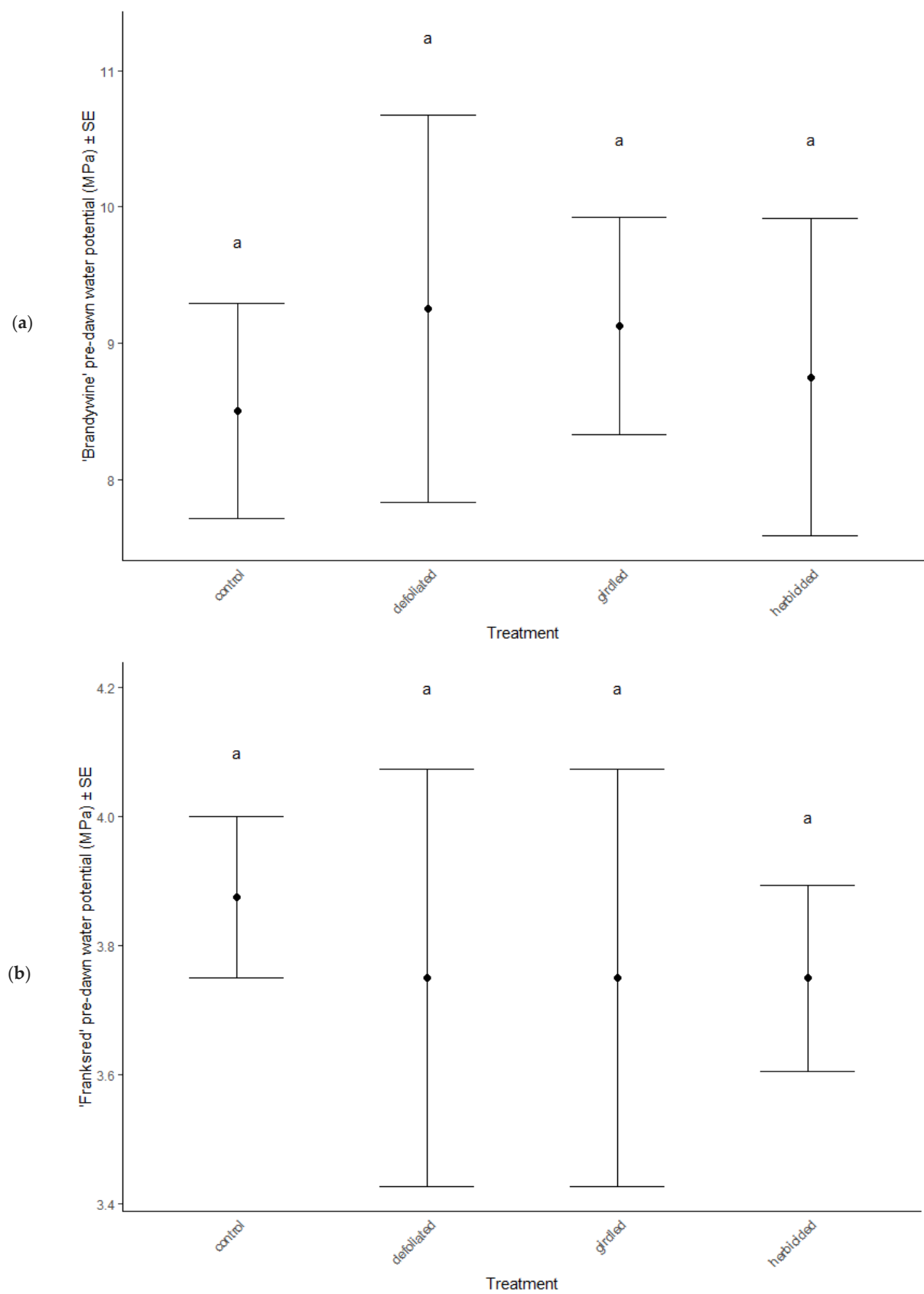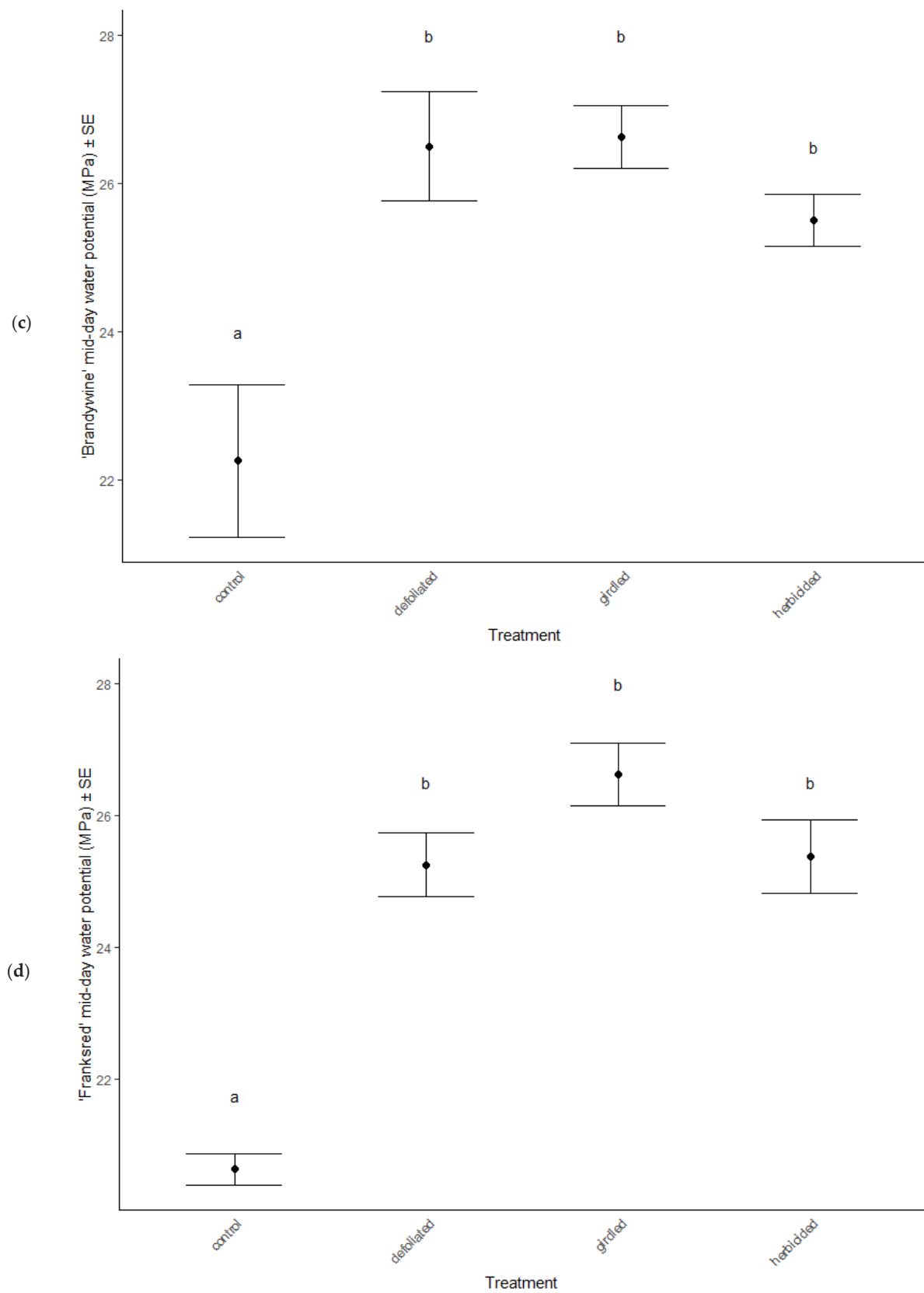**Figure 3.** Changes in chlorophyll a concentrations in response to stress treatments in red maple (*Acer rubrum*) cultivars (**a**) 'Brandywine' and (**b**) 'Franksred'. Different letters represent statistical differences between treatments according to a *post hoc* Tukey HSD analysis.

Figure 4. *Cont.*

Figure 4. Water potential (MPa) of control and stressed treatments of 'Brandywine' (**a**) pre-dawn and (**b**) mid-day, and 'Franksred' (**c**) pre-dawn and (**d**) mid-day cultivars of red maples (*Acer rubrum*). Different letters represent statistical differences between treatments according to a *post hoc* Tukey HSD analysis.

3.3. Plant Phytochemistry

Phytochemistry was significantly different among the stress treatments (MANOVA: Wilk's λ = 1.10, $F_{(13, 405)}$ = 13.10, $p < 0.001$), between cultivars (MANOVA: Wilk's λ = 0.45, $F_{(6, 133)}$ = 18.31, $p < 0.001$) and in the interaction between treatment and cultivar (MANOVA: Wilk's λ = 0.33, $F_{(18, 405)}$ = 2.82, $p < 0.001$). In June, total polyphenol concentrations remained consistent across treatments (ANOVA: F = 1.70, p = 0.170) and cultivars (ANOVA: F = 0.01, p = 0.989), but total tannin concentrations significantly decreased in defoliated and herbicided treatments compared to controls of 'Brandywine' cultivars (*post hoc* TukeyHSD: p = 0.032 and $p < 0.001$, respectively, Figure 5a). September polyphenol concentrations also remained the same across treatments (ANOVA: F = 1.23, p = 0.384, Table 2) and cultivars (ANOVA: F = 3.58, p = 0.061, Table 2), but total tannin concentrations were elevated in defoliated and herbicided treatments compared to controls of 'Brandywine' cultivars (*post hoc* TukeyHSD: $p < 0.001$, $p < 0.001$, respectively, Figure 5b).

In June, total non-structural carbohydrate concentrations were elevated in defoliated and herbicided treatments compared to controls of both cultivars (*post hoc* TukeyHSD: all $p < 0.001$, Figure 6a,b), but all treatments had similar concentrations of TNC by the end of the season, in September (ANOVA: F = 0.85, p = 0.471, Table 2).

Emissions of selected GLV were not affected by stress treatments (F and p values range by compound, see Table 2), but were affected by cultivar type (F and p values range by compound, see Table 2). Interestingly, (Z)-3-hexene-1-ol was not detected in 'Brandywine' cultivars.
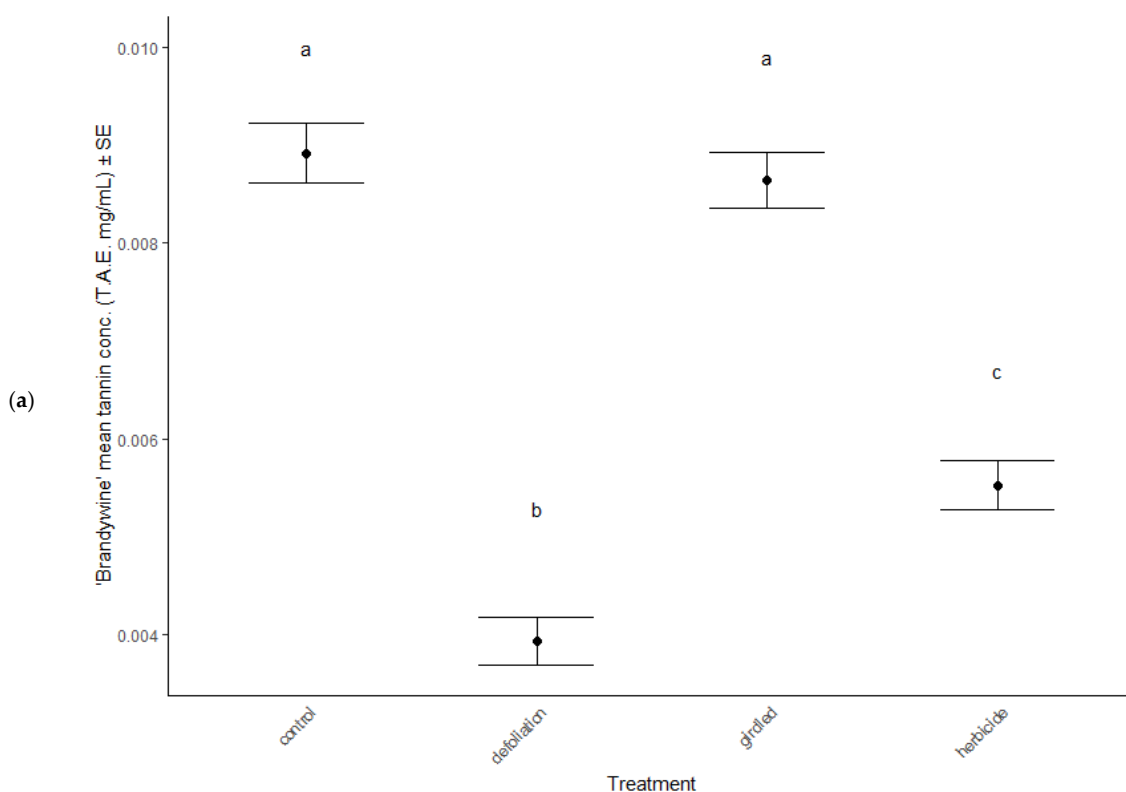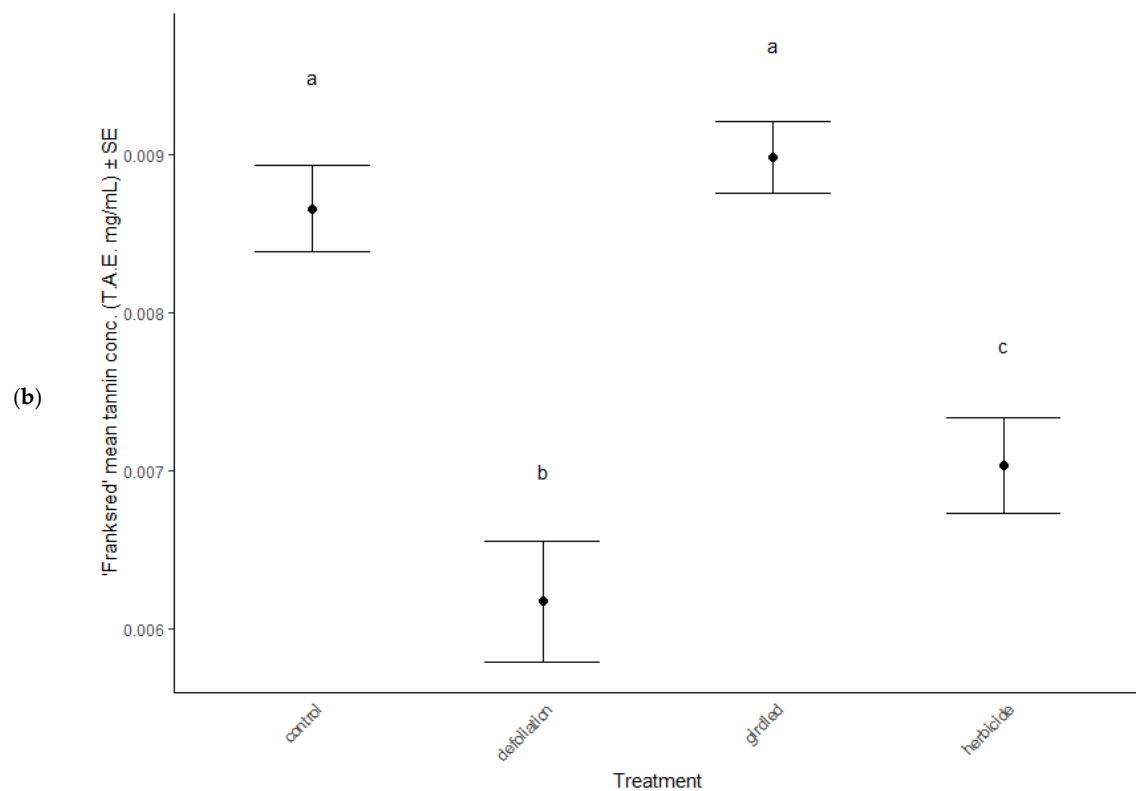
(a)

Figure 5. *Cont.*

Figure 5. Total tannin concentrations in foliage of stress treatments in June, after reflush of defoliated and herbicided treatments. (**a**) 'Brandywine' and (**b**) 'Franksred' cultivars of red maples (*Acer rubrum*). T.A.E. = tannic acid equivalents (see methods section for details). Different letters represent statistical differences between treatments according to a *post hoc* Tukey HSD analysis.
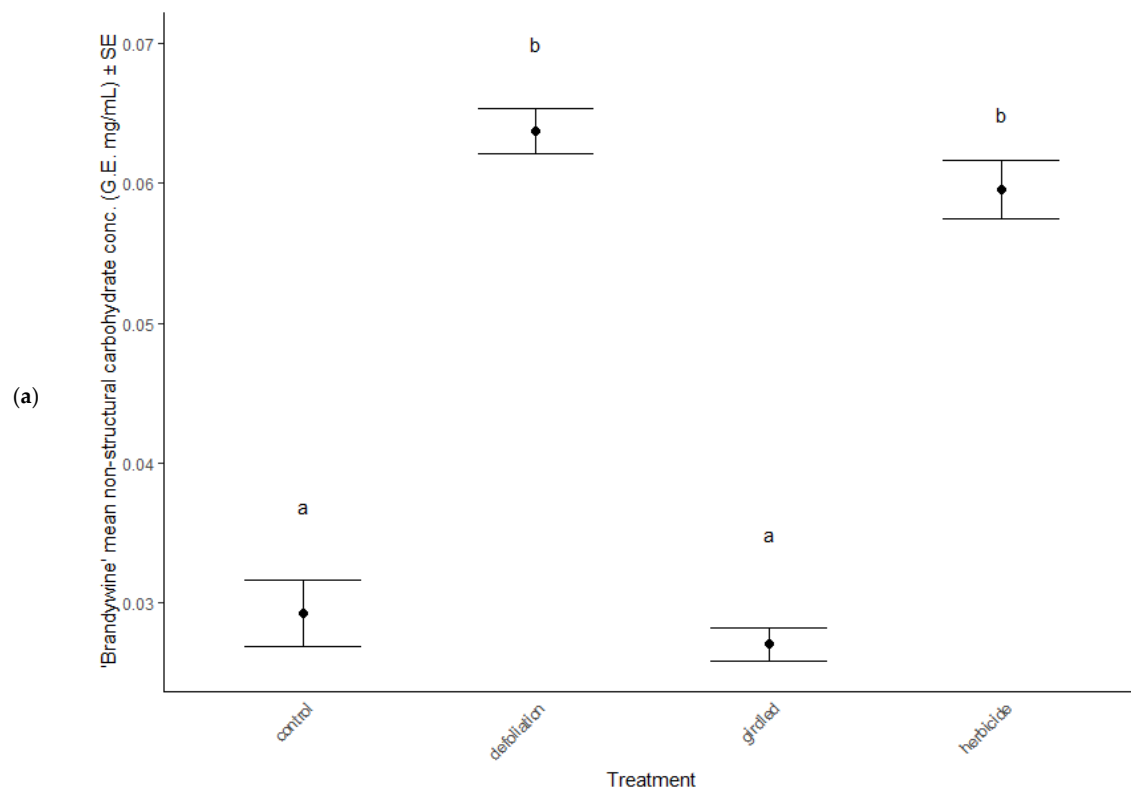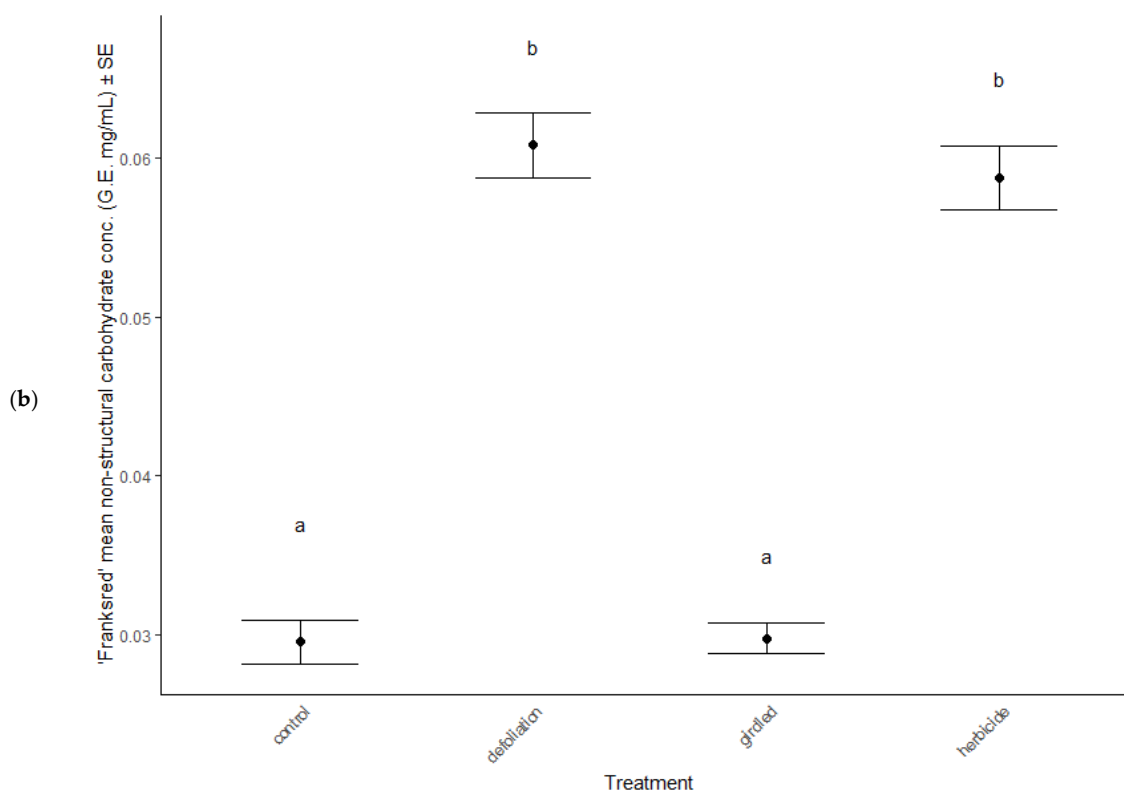

Figure 6. *Cont.*

Figure 6. Total non-structural carbohydrates (combined sugars and starches) in foliage of stress treatments in June, after reflush of defoliated and herbicided treatments. (**a**) 'Brandywine' and (**b**) 'Franksred' cultivars of red maples (*Acer rubrum*). G.E. = glucose equivalents (see methods for details). Different letters represent statistical differences between treatments according to a *post hoc* Tukey HSD analysis.

3.4. Correlations between Common Nursery Pests and induced Responses

Pest populations were not influenced by changes to foliar nutritional and antinutritional constituents due to stress treatments (see Table 3). However, aphid populations were smaller on leaves with greater trichome density ($r = -0.57$, $p = 0.039$, Table 3).

Table 3. Pearson correlations (r) testing for relationships between leaf morphological and phytochemical traits. Significant values are in **bold**.

Correlative Trait	Spider Mites		Broad Mites		Aphids		Leaf Hoppers	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Leaf morphological traits								
Trichome density	0.32	0.051	0.17	0.416	−0.57	**0.039**	0.34	0.266
Leaf area	0.22	0.319	0.04	0.698	−0.07	0.211	−0.12	0.116
Specific leaf area	0.22	0.513	−0.18	0.117	−0.21	0.338	−0.08	0.242
Dry leaf mass	0.09	0.661	−0.19	0.215	0.16	0.053	0.16	0.333
Nutritional compounds								
Non-structural carbohydrates	−0.07	0.513	0.10	0.403	−0.07	0.568	−0.01	0.298
Anti-nutritional compounds								
Polyphenols	−0.05	0.676	−0.09	0.444	−0.02	0.836	−0.02	0.162
Tannins	−0.15	0.221	−0.29	0.126	−0.06	0.641	−0.25	0.359

4. Discussion

Environmental stress influences induced responses of plants, including changes in developmental, physiological processes, and biochemical traits [66,67]. We found signifi-

cant changes in red maple responses to different types of stress. A potential mechanism that is driving these induced responses is the expression of multiple phenotypes from a single genotype—or phenotypic plasticity [68,69]. Alternatively, environment × genotype interactions (adaptive phenotypic plasticity) may also result in multiple phenotypic expressions from a single genotype (*sensu* [70,71]). We used two different cultivars of red maple to explore possible genotypic limitations. However, the cultivar type was confounded by possible changes in environment between the two sites. Site limitations restrains our ability to make determinate conclusions about differences in cultivar response, but there are some overall recognizable patterns that we will discuss for further exploration.

4.1. Phenotypic Plasticity in Expression of Stress-Induced Responses

Research interests have successfully illustrated phenotypic plasticity using the concept of a reaction norm, which is a representation of trait expression in response to different environments or environmental pressures [72,73]. Different genotypes may express variation in reaction norms in response to the same environmental stress [74]. We found that water stress increased in response to our stress treatments in both cultivars. Pre-dawn measurements of water potential showed no sign of stress (Figure 4a,b). This is when plants should be experiencing the least amount of water stress because the soil and plant water potential are at equilibrium [50,51]. As temperatures warm throughout the day, we anticipate additional water stress. Interestingly, we found that the mid-day water potential measurements in treatments that had been defoliated, girdled, or herbicided suffered greater water stress than the control treatments for both sites. This suggests a confounding effect of daily water stress and additional environmental stressors [75]. Many other plant species have adapted physiological mechanisms to tolerate water stress such as reducing stomatal conductance, which lowers CO_2 availability, resulting in reduced photosynthetic capabilities [75,76]. However, we did not observe any changes in stomatal conductance or photosynthetic capacity in response to stress treatment in either cultivar (Table 2). Similarly, Li et al. [76] found that several species of trees in different environmental conditions showed high plasticity in response to stress, but that stomatal conductance showed little variation. Despite the additional stress treatments we applied, both cultivars were able to compensate for changes in water potential without compromising photosynthetic capacity at two separate sites. This suggests that the adaptation to tolerate additional water stress expressed by both cultivars may result from phenotypic plasticity and not necessarily adaptive phenotypic plasticity to specific environments [71,77,78].

Further evidence of plastic traits expressed in response to stress are the non-structural carbohydrate (TNC) patterns observed (Figure 6a,b). Defoliated and herbicided treatments from both cultivars responded with elevated TNC concentrations. TNC concentrations in trees are commonly found to display high environmental and genetic plasticity [79]. Either both sites have very similar environmental conditions, or the elevated TNC concentrations in defoliated and herbicided treatments must be a plastic inducible trait.

Contrastingly, maple cultivars showed differentiated responses in growth and tannin production. Differentiation of expressed traits, combined with selective pressures dictate the evolutionary trajectory of induced responses, and may result in increased plasticity for a specific trait [80]. 'Brandywine' cultivar responses to growth and tannin production support the growth-differentiation balance hypothesis. In defoliated and herbicided treatments, 'Brandywine' individuals expressed an increase in tannin production, but an overall reduction in growth rate. According to the growth-differentiation balance hypothesis, plants should express a trade-off between growth and differentiated functions such as tannin production [18,20]. Perkovich and Ward [20] suggested that induced expression of growth-defense trade-offs is likely a result of adaptations to herbivory. However, we found differential expression between the two cultivars of maple in this experiment, and trade-offs are often scale-dependent [25]. Alternatively, differences in environmental conditions may result in differential resource acquisition and allocation which masks the presence of a trade-off [21,81]. It may be that the MN ('Franksred' cultivars) environment allows

for greater resource acquisition, therefore no trade-off between growth and defense is observed. Unfortunately, we are unable to disentangle differences between site and cultivar in our study.

Many studies have suggested changes in the specific GLV compounds we analyzed (e.g., [39,40,82]), but we did not detect any differences between the treatments. A meta-analysis by Ameye et al. [39] found that wounding and stress increased the concentrations of GLV emitted in wooded plants. The Ameye et al. [39] meta-analysis also showed that herbivory increased GLV emissions. The interaction between stress treatment and the intensity of pest damage could mask changes in GLV emissions. That is, in our study, we only evaluated population densities and not feeding intensity. Insect pests often alter consumption rates depending on plant-host quality [31,83,84]. Increases in plant defenses and decreases in nutritional constituents of stressed plants could have reduced feeding so that feeding intensity was greater in control treatments, increasing GLV emissions at the same rate as stress in the in the treatments. The lack of changes to GLV emissions is an anomaly and more research is needed to disentangle the effects of stress, herbivory, and the interaction between stress and herbivory on GLV emissions.

4.2. Pest Correlations with Maple Characteristics (or Lack of)

Interestingly, we observed few correlations between leaf morphological or phytochemical characteristics. The only observed correlation was a negative association between aphid populations and leaf trichome density. Trichome density has been shown to be an inducible defense in several plant species [85,86], including maples [87]. We did not identify the species of aphids, but many aphids suffer negative consequences associated with trichomes [88,89]. The relationship between trichomes as a defense mechanism and their effectiveness on paid attacks is still fully understood [89]. However, we did not find an increase in trichome density in response to stress treatments, suggesting that trichome density is most likely a constitutively expressed trait, and does not necessarily act as a defense or stress response in maples. In regard to pest population correlations with defensive chemical constituents, several studies suggest that plant express high genetic variation for constitutive and induced resistance that is heterogenous across individuals [90]. The heterogeneity of defenses may have been lost when we homogenized samples and pests may be responding in spatially distributed patterns which we did not analyze [91,92].

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