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Multiple, simultaneous abiotic stresses differentially impact downy mildew infection in grapevine.

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KEYWORDS

(1) leaf gas exchange, (2) heat stress, (3) hydrogen peroxide (H₂O₂), (4) phenols, (5) pathogens (*Plasmopara viticola*), (6) water stress.

RUNNING HEADER

Impacts of combined stresses on downy mildew pathogenesis.

ABSTRACT

Grapevine health and resilience under climate change demand an understanding of how multiple stress interact. Climate change has led to more frequent and intense droughts and heat waves, which often occur together and may affect disease pressure. Here, we explored a complex multi-stress framework by combining drought and heat stress with downy mildew infection in grapevine, determining their possible interactions (synergistic or antagonistic) on pathogenesis, and detailing their effects on grapevine leaf physiology. We subjected grapevines to drought, heat stress, or a combination of both, subsequently inoculating leaves with *Plasmopara viticola*. In these same leaves we quantified their physiological responses and changes in defense-related metabolite (H_2O_2 and phenols) concentrations. Our results revealed common and distinct responses to the multi-stress interactions. Drought and drought-heat treatments led to low stomatal conductance (a reduction in stomatal aperture), which acted as a biophysical mechanism restricting *P. viticola* entry and resulting in a lower *P. viticola* sporulation rate. Defense-related metabolites were differentially regulated according to the stress combination and suggesting a role in the biochemical mechanism against the *P. viticola* infection. Stilbene concentration increased by 36, 57 and 113% in response to drought, heat, and drought-heat, respectively. Flavonoids and phenolic acids exhibited distinct patterns in response to different stress treatments. These results shed light on the mechanisms that drive complex interactions between abiotic and biotic stressors, enhancing our understanding of multi-stress resilience and allowing us to better predict how grapevines will respond to the changing climate.

INTRODUCTION

Cultivating optimum plant health demands an understanding of the mechanisms that drive complex interactions between abiotic and biotic stressors. Although the consequences of the many possible biotic-abiotic interactions are largely unexplored, two theoretical outcomes can be assumed: synergy or antagonism. Synergistic effects, when the sum of the overall effects is greater than any individual effect, could occur if an abiotic stress had previously depleted plant resources to withstand a disease. In contrast, antagonistic effects, when one stressor reduces the impact of another, could occur if an abiotic stress triggered plant defenses, delaying or even preventing an infection. Comprehensive pathoecophysiology studies within multi-stress frameworks are needed to determine which outcome is most likely.

Grapevine downy mildew (DM), caused by the obligate biotrophic oomycete *Plasmopara viticola* (Berk. & Curt. Ex de Bary), infects young organs including shoots, leaves, and berries. Recognized as one of the most destructive foliar diseases of the genus *Vitis*, DM

results in significant losses of yield and wine quality (Gessier et al., 2011; Koledenkova et al., 2022) and requires the use of fungicides that potentially have negative consequences on human and environmental health. *P. viticola* infection has three distinct phases: (1) the attachment and entry of zoospores through the stomata, (2) the colonization of the intercellular space of the leaf mesophyll and haustoria growth into the cells, and, finally, (3) the production and release of sporangiophores through the stomata (Kiefer et al., 2002; Gessier et al., 2011; Koledenkova et al., 2022).

Although quantitative trait loci for *P. viticola* resistance have been identified (Moreira et al., 2011; Sargolzaei et al., 2020), the mechanisms by which grapevines respond to reduce *P. viticola* infection are complex and not fully understood. Grapevines can potentially respond at any or all of the three stages of the infection. Stomatal regulation may provide a physical barrier that may restrict the *P. viticola* at the point of entry for zoospores and the point of release for sporangiophores. For example, laboratory treatment with abscisic acid (ABA) resulted in stomatal closure and reduced the number of infection sites following *P. viticola* inoculation (Allègre et al., 2009). The presence of *P. viticola* mycelium within the leaf mesophyll triggers a combination of related defense responses involving various biochemical and molecular mechanisms (Adrian et al., 2012; Adrian et al., 2024). These include the accumulation of reactive oxygen species (ROS; Kortekamp and Zyprian, 2003) and antimicrobial phenols, such as caffeic acid (Figueiredo et al., 2008) and stilbenes, which are grapevine phytoalexins, a class of specialized antimicrobial metabolites (i.e., viniferins and piceid; Pezet et al., 2004; Malacarne et al., 2011; Jeandet et al., 2021).

DM pathogenesis is a dynamic process in which the plant and the pathogen are intimately related to, and mutually influenced by, the environment. In the classical disease-triangle model (for a review see Scholthof, 2007), the role of the environment in a disease outcome is fundamental. The current changing climate is increasing the intensity, frequency, and duration of droughts and heat waves, which have a high probability of occurring together (IPCC, 2023). These combined abiotic stresses will most certainly impact disease patterns and pressures and will likely have an influence across scales and in a host-pathogen specific manner (Garrett et al., 2006; Singh et al., 2023).

To endure and adjust to water deficits, plants regulate water loss by decreasing stomatal conductance (Jones, 1998). However, leaf transpiration, evaporative cooling, and carbon dioxide (CO₂) uptake for photosynthesis are critical processes and are primarily controlled by the stomata (Jones, 1998; Flexas and Medrano, 2002; Medrano et al., 2002). Despite extensive studies on water deficits and their negative impacts on grapevine yield and physiology (Gambetta, 2016; Gambetta et al., 2020), studies on the combination of water deficits with other abiotic and biotic stresses are limited.

During heat waves, the ambient temperature rises above plant tolerance thresholds and, depending on the duration and intensity, may cause irreversible damage. Temperatures of 40 °C and above can impair carbon assimilation in grapevine leaves (Greer and Weston, 2010; Greer and Weedon, 2012) and lead to dysfunctions of the photosystem machinery such as inhibition of photosystem II (PSII; Luo et al., 2011), as well as, low F_v/F_m ratio (Zhang et al., 2018). The heat stress responses mentioned above lead to secondary physiological responses, such as oxidative stress and initiation of scavenging activities (Foyer and Noctor, 2005; Carvalho et al., 2015b; Czarnocka and Karpiński, 2018); although, little is known about it within a multi-stress framework. Previous studies focused on berry and wine quality have employed different systems and methods to simulate heat waves, ranging from sophisticated devices to heat individual grapevine organs (Tarara et al., 2008), to closed chambers to heat the whole canopy in the vineyard (Soar et al., 2009). Here, we developed and used a simple, reliable, low-cost method using commercially available radiant heaters to simulate a heat wave on whole-potted-plants, aiming to better understand physiological responses to combined stresses.

In this study, we aimed to explore an extremely complex multi-stress framework, combining different abiotic stresses (drought and heat) with a biotic stress, DM. Our overall goal was to understand the effects of drought, heat, and their combination on the *P. viticola* infection phases, and more particularly, determine if these abiotic stresses have a synergistic or antagonistic impact. We further explored whether these stresses, alone or in combination, provoke changes in the grapevine defense responses. Specifically, hydrogen peroxide (H_2O_2) and phenols accumulation in leaves, which are metabolites that play a role in antagonizing *P. viticola* infection (Pezet et al., 2004; Kim Khiook et al., 2013; Adrian et al., 2024).

We hypothesized that drought would have an antagonistic effect on *P. viticola*, as the stomatal aperture will be reduced (i.e. low stomatal conductance), restricting *P. viticola* entry. Conversely, we hypothesized grapevines would upregulate stomatal conductance to promote evaporative cooling in response to heat stress alone, which could have a synergistic effect favoring *P. viticola* entry. Finally, we hypothesized that the production of defense-related metabolites in response to these abiotic stresses could antagonize *P. viticola* infection.

MATERIAL AND METHODS

1) Plant material and experimental design

Vitis vinifera cv. Cabernet Sauvignon was planted from self-rooted cuttings in 12 L plastic pots filled with peat-clay growing medium (Horticultural Soil TH210, Dumona, France) mixed with sand in a 2:1 (v:v) ratio and slow-release fertilizer (Osmocote Scott, 19:4:5). Growth was maintained under greenhouse conditions, with daily irrigation to soil capacity. Plants were 1-year old and were trained to have two vegetative shoots (not bearing fruit).

We used a factorial experimental design with four abiotic treatments (control, drought, heat, and the combination of drought-heat) and two biotic treatments (inoculation with *P. viticola* and water; as control). Grapevine pots were arranged in two rows of three plants each (2x3, n= 6), repeating this design twice per treatment (n = 12 per stress treatment). The time frame of the experiment consisted of exposing grapevine plants first to continuous water stress (days 1 to 23), then exposing them to heat stress (days 20 and 21), followed by two days of temperature recovery interval (days 22 and 23). Finally, on day 24, leaves were harvested for *P. viticola* inoculation and disease assessment (described below). During the experiment, the day/night temperatures were 26.5 ± 4.3 / 20.4 ± 1.7 °C (mean $\pm$ SD), excluding heating day temperatures (described below).

To induce water stress, irrigation was stopped on day 1 (June 15, 2023), and water stress levels were monitored by measuring leaf gas exchange (g_s) and leaf water potential (Ψ_{leaf}). Plants in the drought and drought-heat treatments were not irrigated throughout the experiment (days 1 to 23), and the remaining plants (control and heat treatments) were irrigated daily (600 mL), keeping soil moist. Prior to the start of the experiment (day 0), all plants were watered to soil saturation and then left to drain the excess water. All pots were covered with aluminum foil to reduce soil water evaporation of the water stressed treatments and to ensure the same conditions between treatments.

To manipulate temperature, we used commercially available radiator heaters designed for <10 m² (QH-1000D, Cixi Crown Electric Appliance Co. Ltd.) to elevate the temperature to about +5 °C around the plants that received the heat stress (heat and drought-heat treatments). Heaters were turned on/off at 9:00 and 21:00, respectively. Two heaters were placed on each side of the row, on a 40 cm high platform, and 50 cm away from the space between two potted plants to avoid direct heat to a plant. Lastly, each plant had a thermocouple (TinyTag, Talk2 TK-4023; Gemini Data Loggers) attached to the grapevine training stake at a height of 70 cm, which measured and recorded the temperature hourly throughout the period of the experiment. This number of thermocouples allowed us to capture all individual plant canopy temperatures across the heat treatments (heat and drought-heat).

2) Assessments of leaf physiological traits under abiotic stress treatments

In order to target a moderate water stress, a leaf porometer/fluorometer (600 Li-Cor, Lincoln, NE, USA) was used to measure daily leaf stomatal conductance (g_s) and quantum yield of PSII, calculated from fluorescence (Φ_{PSII}); we measured six leaves per plant (n= 72 leaves per treatment). Because leaf water potential (Ψ_{leaf}) is a destructive method, we collected leaves on specific dates: on day 1 as a baseline, on days 6, 13, and 19 as the g_s declined and remained low in the water stressed treatments, on day 21 during the heat stress,

and on day 23 after the heat stress (n= 6 leaves per treatment). Both measurements were taken between, approximately, 11:00 and 13:00.

3) *Plasmopara viticola* inoculation and sporulation assessment

The third and the fourth leaves (counted from the apex; n= 4 leaves per plant) were collected at the end of the greenhouse experiment (day 24), placed on pre-wetted paper towels, enclosed in a plastic resealable bag, and then executing the inoculation assay. The *P. viticola* isolate ANN-01 was kindly provided by Dr. Corio-Costet (INRAE Bordeaux, France). Adapting from the method of Corio-Costet et al. (2011), the leaves were placed abaxial side up over a pre-wet paper filter (3 mL), and six 15 μ L droplets (inoculation sites) containing 5,000 sporangia mL⁻¹ or distilled water were placed on the leaf surface (n= 4 leaves per biotic stress treatment, total 192 leaves). Leaves were in a dark room for 24 h, and any remaining inoculum were removed by aspiration with a water pump. The Petri dishes were sealed, and leaves were incubated at 25 °C with a 16 h photoperiod for 2 to 8 days post inoculation (dpi).

To evaluate the effect of different abiotic stress treatments on *P. viticola* infection, we monitored the incidence of sporulation (emergence of sporangiophores) at each of the six inoculation sites daily (2-8 dpi). We then calculated the incidence of sporulation (%) by dividing the number of sites with sporulation by the total number of inoculation sites on each leaf. At 8 dpi, we visually scored disease severity as the percentage of the leaf area showing sporulation (sporulation severity, %) by comparing the spread and density of sporulation on all leaves. Spread indicates the extent of sporulation on the leaf surface and density indicates the concentration ranging from sparse to intense. This score was based on the International Organization of Vine and Wine Descriptor 452-1 (OIV, 2009) and expressed on a scale of 0-100%, where 0% indicates no sporulation and 100% indicates the greatest amount of sporulation among the leaves. Leaves were then frozen with liquid nitrogen and kept at -80 °C for further analysis.

4) Analyses of metabolites linked to stress responses

Prior to biochemical analysis, lyophilized leaves (n=2 leaves per inoculation treatment) were ground under liquid nitrogen using a ball mill (MM 400, Retch, Germany) at 30 Hz for 15 seconds.

To quantify hydrogen peroxide (H₂O₂) concentrations, we followed the colorimetric protocol of Junglee et al. (2014), based on potassium iodide (KI) oxidation. Briefly, 20 mg of ground leaves were treated with a stock solution containing 1 M KI, trichloroacetic acid (0.1% w/v), 0.01 M potassium phosphate buffer, and 50mM tricine buffer (pH 8). They were thoroughly mixed using a mill grinder (MM200, Retch, Germany) for 1 min at 30 Hz. Simultaneously, for every sample, a control sample was prepared with the same stock

solution, but without KI to capture any tissue background coloration. Next, samples were kept at 4°C for 10 min, protected from light, for extraction and colorimetric reaction combined. Then, samples were centrifuged in a microcentrifuge at 12,000 rpm for 15 min at 4 °C, and 200 µL of the supernatant was transferred to three microplate wells where the optical density (OD 340-350 nm) was recorded using a spectrophotometer (MP96, SAFAS, France). Finally, H₂O₂ concentrations were calculated from a standard curve and expressed as µmol of H₂O₂ per g of fresh leaf tissue (µmol of H₂O₂ g⁻¹ FW).

To quantify the concentrations of 19 phenols within the classes of stilbenes, phenolic acids, and flavonoids (Table 1) in the leaf tissue, we first did an extraction by adding 0.5 mL of methanol (100 %; v/v, Fisher Chemical, U.K.) to lyophilized leaf powder (50 mg) and placed in an ultrasonic water bath (FB 15054, Fisher Scientific) at 20-25 °C for 15 minutes. Next, samples were centrifuged in a microcentrifuge at 14,000 rpm for 5 min at 7 °C, and the supernatant was removed and transferred to another Eppendorf tube. Then, we did a second extraction with 0.5 mL methanol (70 %; v/v) and repeated steps aforesaid. Both supernatants were combined. An aliquot of 4 µL was injected into a column Zorbax SB-C18 (2.1 × 100 mm, 1.8 µm) of a high-performance liquid chromatography (HPLC, 1260 Infinity, Agilent Technologies) that was coupled to a 6430 triple quadrupole mass spectrometer and two solvents: solvent A (water/formic acid 99.9:0.1, v/v) and solvent B (acetonitrile/formic acid 99.9:0.1, v/v). The elution gradient was for solvent B: 5–18% (0–5 min), 43% (15 min), 95% (17–20 min), and 5% (22–25 min), with a flow rate of 0.3 mL/min. Data were processed using the MassHunter software against a calibration curve with pure standards in the range of 0.05 to 16 mg L⁻¹. All compounds were quantified with the corresponding standard curve and expressed as mg of phenols per kg of fresh leaf tissue (mg Kg⁻¹ FW).

5) Data analyses

We used one-way ANOVAs to determine whether (a) the physiological traits during the abiotic stress treatments were different at each time point, (b) the incidence of sporulation differed among the abiotic stress treatments at each time point, and (c) the sporulation severity differed among the abiotic stress treatments at 8 dpi. Next, we used two-way ANOVAs to compare the changes on defense metabolites post-inoculation among the four abiotic stress treatments and the two biotic stress treatments. We also used a repeated-measurements ANOVA to evaluate the incidence of *P. viticola* sporulation since we examined the same leaf over seven days (2-8 dpi). After each analysis of variance, a Tukey's HSD post-hoc test was done, if traits were significantly different ($p < 0.05$).

We ran a machine learning-based classification generalized linear model (GLM) to predict whether the defense metabolites were linked to abiotic or biotic stress treatments. The classification models considered H₂O₂ and each phenol as input variables using CARET and

GLMNET packages in R (V 4.3.0) to construct lasso and elastic net models in order to predict experimental treatments (presence/absence of stress). The dataset was randomly divided into two parts, 75% for training and 25% for testing, using stratified sampling for each predicted trait. During the training step, both regularization and penalization hyperparameters were optimized using a grid search over 40 values. To account for variability in data partitioning and parameter optimization, 100 different models were constructed, and the accuracy of these predictions was evaluated by comparing real and predicted data. The exact same approach was used for regression model to predict the relationship between defense metabolite concentrations and various traits associated with abiotic and biotic stress treatments, such as stomatal conductance (g_s), temperature, maximum temperature, sporulation incidence (from 5 to 8 dpi), and sporulation severity. The regression results were represented as a distribution of R^2 scores between the real and predicted data (R^2 values closer to 1 indicate better-performing models).

RESULTS

1) Microclimate and grapevine leaf physiological responses

The mean temperature during the heat stress period (days 20 and 21, from 9:00 to 21:00) was similar between the heat and drought-heat treatments (31.3 ± 0.2 and 30.8 ± 0.2 °C, respectively), and ~5 °C (17%) higher than the control and drought treatments on average (25.8 ± 0.1 and 25.8 ± 0.1 °C, respectively) (Fig. 1, Table S1; available as Supplementary Data at Tree Physiology Online). The use of radiator heaters generated non-homogeneous heating between individual plant canopies within the heat and drought-heat treatments. During the heat stress period, the variance was 9.1 and 11.7 °C for the heat and drought-heat treatments, respectively; accordingly, the mean temperatures ranged from 29.1 to 36.2 °C and 27.1 to 36.5 °C for the heat and drought-heat treatments, respectively (Fig. S1; available as Supplementary Data at Tree Physiology Online). Likewise, the drought treatment had a temperature variance of 5.3 °C, and mean temperatures ranged from 24.8 to 27.3 °C. Lastly, the control treatment exhibited the lowest variance of 4.0 °C, and mean temperatures ranged from 25.3 to 26.5 °C (Fig. S1).

We targeted a mild to moderate water stress on the grapevines (Gambetta et al., 2020), in which the leaf water potential (Ψ_{leaf}) for drought and drought-heat treatments decreased from -0.71 ± 0.03 to -1.43 ± 0.03 MPa and -0.64 ± 0.02 to -1.50 ± 0.09 MPa, respectively, during the experiment (day 1 to 23) (Fig. 2A, Table S2). In contrast, Ψ_{leaf} means for control (-0.73 ± 0.03 MPa) and heat (-0.73 ± 0.03 MPa) treatments remained at similar values throughout the experiment (Fig. 2A, Table S2). The mean stomatal conductance (g_s) for the control treatment persisted with little change throughout the experiment, its mean was 0.21 ± 0.002 mol-H₂O m⁻²s⁻¹ and variance was 0.008 (Fig. 2B, Table S3). In contrast, drought

and drought-heat treatments resulted in a significant drop compared to the control, in which g_s persisted at low levels for 13 days (days 11-23) for both the drought ($0.008 \pm 0.002 \text{ mol-H}_2\text{O m}^{-2}\text{s}^{-1}$) and drought-heat treatments ($0.01 \pm 0.02 \text{ mol-H}_2\text{O m}^{-2}\text{s}^{-1}$). The g_s responded to the heat treatment alone by decreasing 76% (from 0.22 ± 0.06 to $0.05 \pm 0.03 \text{ mol-H}_2\text{O m}^{-2}\text{s}^{-1}$) and did not fully recover ($0.13 \pm 0.07 \text{ mol-H}_2\text{O m}^{-2}\text{s}^{-1}$) after the abiotic stress experiment. In addition, on days 6 and 7, mean g_s decreased for all treatments due to heavy precipitation on those days (53 mm and mean ambient temperature of 21.9°C ; INRAE Climatic Database) although all plants were in the greenhouse (closed space), the g_s responded to the external weather event. The following day (day 8), the g_s recovered in the control and heat treatments ($0.22 \pm 0.007 \text{ mol-H}_2\text{O m}^{-2}\text{s}^{-1}$ and $0.23 \pm 0.006 \text{ mol-H}_2\text{O m}^{-2}\text{s}^{-1}$, respectively) and slightly recovered to $0.10 \pm 0.005 \text{ mol-H}_2\text{O m}^{-2}\text{s}^{-1}$ in both drought and drought-heat treatments, indicating the onset of water stress (Fig. 2B, Table S3).

The mean Φ_{PSII} was significantly lower in leaves subjected to drought and drought-heat treatments compared to leaves in the heat and control treatments. Before the heat stress (days 15-19), the mean Φ_{PSII} had already decreased in the drought and drought-heat leaves (0.70 ± 0.008 and 0.70 ± 0.006 , respectively) compared to control (0.75 ± 0.002) and heat treatments (0.74 ± 0.002) (Fig. 2C, Table S4). During the heat stress period (days 20-21), heat treatment alone had no effect on Φ_{PSII} (0.75 ± 0.001), but it had a synergistic effect with the water stress in the drought-heat treatment, and the mean Φ_{PSII} decreased to 0.63 ± 0.005 . At the end of the experiment, the mean Φ_{PSII} was the lowest for the drought and drought-heat treatments (0.67 ± 0.009 and 0.67 ± 0.012 , respectively), which were not statistically different from each other, however, they were statistically different from the heat (0.72 ± 0.010) and control (0.74 ± 0.003) treatments (Fig. 2C, Table S4).

2) *Plasmopara viticola* inoculation and sporulation assessment

Control leaves that received only water (water inoculated) never showed signs of *P. viticola* development at any time point. *P. viticola* inoculated leaves began to show sporangiophore formation at 5 dpi (Fig. 3A, Table S5). Between 5-8 dpi, the increase in the incidence of sporulation was 31% in the control (from 71.5 ± 7.5 to 93.7 ± 3.4 %), 65% in the heat (from 49.3 ± 7.8 to 81.2 ± 7.0 %), 212% in the drought-heat (from 21.5 ± 6.8 to 67.0 ± 8.5 %), and 648% in the drought treatments (from 9.0 ± 4.7 % to 67.4 ± 7.7 %) (Fig. 3A, Table S5). The mean incidence of sporulation in the drought and drought-heat treatments lagged behind the heat treatment from 5 to 7 dpi. On 8 dpi, although the stress treatments were not statistically different from each other, they were still statistically different from the control (Fig. 3A, Table S5). Lastly, we found a positive correlation between the mean incidence of *P. viticola* sporulation at 5 dpi and mean leaf g_s at day 23 ($R^2 = 0.3$, $p < 0.0001$; Fig. 3B), where both

drought and heat-drought stressed leaves had low g_s corresponding low incidence of sporulation.

The outcomes of individual abiotic stresses, drought and heat, on the sporulation severity of *P. viticola* were opposite. Compared to the control treatment, drought stress treatment reduced sporulation severity by 50% (14.7 ± 1.8), meaning the sporangioophores were sparse and less widespread. In contrast, although not statistically significant heat stress treatment increased sporulation severity by 12% (33.2 ± 3.0), meaning the sporangioophores were denser and extensive. In the drought-heat treatment, the mean sporulation severity was similar to that of the control treatment at 29.7 ± 3.1 and $29.6 \pm 2.6\%$, respectively (Table 1, Fig. 3C).

3) Defense metabolism analyses

We assessed the concentration of H_2O_2 and phenols in the leaf tissue at 8 dpi. The mean concentration of H_2O_2 varied significantly between the biotic stress treatments. *P. viticola* inoculated leaves had ~20% less H_2O_2 than those water inoculated, except in the heat stress treatment, in which *P. viticola* and water inoculations had a similar concentration of H_2O_2 (Fig. 4A, Table S6). The concentration of H_2O_2 in water inoculated leaves was statistically different across the abiotic treatments. The concentration of H_2O_2 in the drought stress treatment increased significantly by 39% compared to the control treatment ($1.62 \pm 0.3 \mu\text{mol } H_2O_2 \text{ g}^{-1}$), by 74% compared to the heat stress treatment ($1.32 \pm 0.2 \mu\text{mol } H_2O_2 \text{ g}^{-1}$), and by 14% compared to the drought-heat treatment ($1.97 \pm 0.2 \mu\text{mol } H_2O_2 \text{ g}^{-1}$). However, this last difference was not statistically significant. (Fig. 4A, Table S6).

Compared to their corresponding water inoculated controls, stilbene concentrations in *P. viticola* inoculated leaves increased by 113% in drought-heat ($7.58 \pm 1.2 \text{ mg kg}^{-1}$), 57% in heat ($8.42 \pm 1.9 \text{ mg kg}^{-1}$), 36% in drought stress ($5.74 \pm 0.7 \text{ mg kg}^{-1}$), and 2% in control ($4.47 \pm 0.6 \text{ mg kg}^{-1}$) treatments (Fig. 4B, Table S6). Among the analyzed stilbenes, the viniferins were below the limit of quantification (LOQ) of the assay (data is not shown in Table 1).

The concentrations of both, phenolic acids and flavonoids, in the *P. viticola* inoculated leaves showed a contrasting pattern depending on the abiotic stress to which they were subjected to (Table 1). Although not statistically significant, compared to their corresponding water inoculated controls, phenolic acids concentrations in the *P. viticola* inoculated leaves were 118% and 101% higher, respectively, in drought and drought-heat treatments ($1,590.8 \pm 220$ and $1,837.5 \pm 198 \text{ mg kg}^{-1}$, respectively), in contrast, it was 12% lower in the heat ($1,537.4 \pm 194 \text{ mg kg}^{-1}$) and 19% lower in the control ($1,827.3 \pm 209 \text{ mg kg}^{-1}$) treatments (Fig. 4C, Table S6). The abiotic stress treatment led to an overall decline in the phenolic acids concentration in the leaves. Leaves subjected to drought and drought-heat treatments had significantly lower concentrations of phenolics compared to the control treatment. Although phenolic

concentrations were 23% less in the leaves exposed to heat stress treatment than in the control leaves, the difference was statistically significant (Fig. 4C, Table S6). Flavonoids concentrations in the *P. viticola* inoculated leaves were statistically significantly lower compared to the water inoculated controls (Fig. 4D, Table S6). The abiotic stress treatment led to an overall decline in the flavonoids concentration in the leaves however, only the leaves subjected to heat stress treatment had significantly lower concentrations of flavonoids compared to the control treatment.

Our GLM-based modeling significantly outperformed the no-information rate - corresponding to a naive model that always predicts the most frequent class. Defense metabolites (H_2O_2 and phenols) were the best predictors of g_s as an indicator of drought ($R^2 = 0.44$), followed by earlier infection progression as demonstrated by 5 and 6 dpi from the onset of sporulation ($R^2 = 0.24$ and 0.26 , respectively; Fig. 5). In contrast, defense metabolites were weakly predictive of later infection progression, as demonstrated by 7 and 8 dpi from incidence of sporulation ($R^2 = 0.15$ and 0.10 , respectively, Fig. 5). Lastly, defense metabolites appeared unrelated to mean and maximum temperatures ($R^2 = 0.02$ and 0.02 , respectively, Fig. 5).

DISCUSSION

This study explored a complex, multi-stress framework combining drought and heat stress treatments with biotic stress imposed by DM to study their interaction (synergistic or antagonistic) and the consequences on grapevine leaf physiology. Our data show that drought and heat treatments alone had different outcomes on *P. viticola* infection. Drought stress hindered its sporulation, while in contrast, heat stress increased its sporulation severity. These differences corresponded to differences in the biophysical and biochemical mechanisms presented in each abiotic-biotic stress combination. Drought and drought-heat stress treatments led to a reduction in the stomatal aperture (due to low g_s , Fig. 3B) suggesting a biophysical mechanism that physically limited *P. viticola* entrance through the stomata and, consequently, delayed the incidence of sporulation (Fig. 4A). Defense-related metabolites (H_2O_2 and phenols) were differentially regulated according to the abiotic stress treatment combination playing a unique role in the biochemical mechanism protection against the *P. viticola* infection. Although the concentration of H_2O_2 decreased in the abiotic-biotic stress treatments, drought stress leaves had the highest H_2O_2 levels under both water and *P. viticola* inoculations, which could have contributed to a biochemical mechanism. While stilbene concentration increased in response to the *P. viticola* (biotic stress treatment), flavonoids and phenolic acids had different patterns depending on the abiotic-biotic stress combination to which they were subjected. The differences among the concentrations of defense metabolites induced by each abiotic stress likely contributed to the specific disease responses. These results shed light on the mechanisms that underlie complex interactions between abiotic and

biotic stressors, indicating a combination of biophysical and biochemical mechanisms, particularly in the context of stress combinations.

1) Simulating heat waves and its effects, alone and in combination

Our heat stress treatments yielded realistic (+5 °C; Fig. 1), but not extreme, maximum temperatures. While temperatures of 40 °C and above impair carbon assimilation (Greer and Weston, 2010; Greer and Weedon, 2012), partially due to the inhibition of PSII (Luo et al., 2011) and a low F_v/F_m ratio (Zhang et al., 2018). The low g_s , which is a rapid response to heat stress (Fig. 3B), suggests that stomatal regulation contributes to this mechanism. Heat stress treatment of 31 °C (Fig. 1) decreased g_s but did not impact the Φ_{PSII} , however, it led to a shift in the concentration of phenols in the leaves, such as a reduction of phenolic acids and flavonoids (Fig 4B-D). The same reduction of flavonoids concentration was found in *Salix* spp. leaves (40 °C, Austen et al., 2019) and grape berries (42°C, Pastore et al., 2017) subjected to heat stress.

Heat stress may induce oxidative stress through the production of reactive oxygen species (ROS), as shown in assessments of H_2O_2 concentration, which increased immediately after a heat exposure (35 °C for 1 h; Carvalho et al., 2015a). Here, we found that two-days of heat stress period led to a reduction of H_2O_2 concentration in grapevine leaves, when assessed after the stress (Fig. 4A), probably due to the activation of scavenging activities (Foyer and Noctor, 2005; Carvalho et al., 2015b; Czarnocka and Karpiński, 2018). However, heat stress had a synergistic effect with the water stress, and H_2O_2 concentration increased by 21% in the drought-heat treatment (Fig. 4A), which may be probably because ROS are also involved in signaling pathways and systemic responses to stress (Foyer and Noctor, 2005; Carvalho et al., 2015b; Czarnocka and Karpiński, 2018). This difference in H_2O_2 concentration could infer different plant defense outcomes depending on the type of stressor.

2) Antagonistic and synergistic interactions between abiotic and biotic stress

Our data reveal contrasting effects of drought, heat stress, and their combination on the *P. viticola* infection. Drought stress treatment had an antagonistic effect that may be attributed to both biophysical, stomatal closure, and biochemical, an increase in defense related metabolites, mechanisms. In contrast, heat stress treatment had a synergistic effect, leading to the greatest sporulation severity. While the biophysical mechanisms may have initially restricted *P. viticola* entry, the incidence of sporulation was still higher compared to other abiotic stress treatments. In addition, the g_s at the end of the greenhouse experiment (day 23) was also greater. The biochemical profile changes, including a decrease in the concentration of phenolic acids and flavonoids, could have played a larger role in the increase of greatest sporulation severity. Like drought alone, combined drought-heat stress resulted in

an antagonistic effect, likely resulting from stomatal closure restricting *P. viticola* entry, although it lagged behind the drought stress treatment. At the same time however, the inoculated area showed denser and more widespread sporangiophores as assessed by sporulation severity and resulted in ~50% greater severity than the drought stress treatment. Although the defense metabolite profile was similar between drought and drought-heat treatments, their concentrations were different which could have influenced these different outcomes (Fig. 4, Table S6). Finally, other metabolites may explain these findings as well. Further studies are necessary to more robustly and comprehensively identify and determine the metabolites involved.

The GLM models predicted that g_s , as an indicator of drought, and earlier infection progression (5 and 6 dpi) were best predicted by the defense metabolites (Fig. 5). These results suggest the presence of biophysical mechanisms within this pathosystem. This hypothesis may be extended to be tested in other pathosystems. Stress-induced stomatal closure likely produced a physical barrier at the first phase and point of entry of zoospores. As an oomycete, *P. viticola* does not produce fusicochin, a toxin that permits fungi to lock wide open the stomata and enter in the stomatal cavity (Evangelisti and Govers, 2024), and thus they are contingent on the leaf stomatal aperture to enter into the leaf mesophyll. The drought and drought-heat treatments had $g_s \leq 0.01 \text{ mol-H}_2\text{O m}^{-2}\text{s}^{-1}$ for 13 days, and to a lesser extent, g_s also responded to the heat stress treatment with a 76% decrease (Fig. 2B and Fig. 3B). Dark-measurements of g_s of non-stressed leaves inoculated with *P. viticola* revealed a continuous g_s rise from 6-8 dpi, simultaneously with an increase in stomatal aperture and oil spot symptom development (Allègre et al., 2007). Although we did not measure g_s during disease development (to maintain microenvironmental humidity conditions), the g_s relief and, mainly, water stress recovery are not immediate, and it can take days (Tombesi et al., 2015; Morabito et al., 2022).

Moreover, to tolerate and adjust to water deficits, plants regulate water loss by decreasing stomatal conductance (Jones, 1998; Medrano et al., 2002), which leads to an increase of photorespiration and subsequent H_2O_2 accumulation (Voss et al., 2013). Production of H_2O_2 , as a reactive oxygen species (ROS), is rapidly triggered in response to stress, and although their accumulation in the cells is harmful, ROS are also signaling molecules for local and systemic responses to stress, with constant regulation by the activation of scavenging activities (Foyer and Noctor, 2005; Carvalho et al., 2015b; Czarnocka and Karpiński, 2018). Drought stress treatment resulted in the highest concentrations of H_2O_2 , regardless of whether the leaves were inoculated or not (Fig. 4A). Since, signaling by H_2O_2 acts through different pathways depending on the environmental stimulus, here, it may have acted as a long-term signaling under prolonged water stress. Further time-series experiments are necessary to identify and validate long-term signaling.

There are very few patho-ecophysiology studies within a multiple-stress framework in grapevine. For example, the foliar symptoms of the wood disease ESCA were inhibited under drought, exhibiting an antagonist interaction between water stress and pathogenicity (Bortolami et al., 2021). In another study, grapevines infected with the vascular bacteria *Xylella fastidiosa* (agent of Pierce disease) had a reduction of g_s and leaf hydraulic conductive accelerating leaf symptom development; a synergistic interaction (Choat et al., 2009). Lastly, the presence of the fungus *Erysiphe necator* (agent of powdery mildew) in the leaf mesophyll reduced g_s in symptomatic and asymptomatic leaf areas (Moriondo et al., 2005), suggesting a possible synergistic outcome to water stress. Collectively, it is clear that the consequences of multiple stresses on different pathosystems can vary greatly and cannot be presumed from single-stress studies.

3) The putative role of phenols

The presence of *P. viticola* mycelium in the apoplast of the leaf mesophyll triggers a series of resistance responses that serve as basal defenses, in which phenols are part of the biochemical mechanism in grapevines. The cultivar Cabernet Sauvignon and all *Vitis vinifera* species are susceptible to DM, as they lack effector-triggered immunity (for a review, see Jones and Dangl, 2006). In the absence of plant resistance genes against *P. viticola*, the best outcome is attenuating DM symptoms through the biochemical mechanisms associated with basal defenses (i.e., PAMP-triggered immunity, Jones and Dangl, 2006).

Grapevines were subjected to abiotic stress or treated with various substances prior to being inoculated with *P. viticola*, a process that could potentially create a primed state. Under this state, plants can respond faster by activation of defense responses when subsequently challenged by pathogens or herbivorous insects, it depends, however, the intensity and duration of the priming stimuli (Conrath, 2009; Nair et al., 2022). In this study, abiotic stress treatments yielded higher stilbene concentrations in all *P. viticola* inoculated leaves. Drought and drought-heat stress treatments also presented higher concentrations of phenolic acids and flavonoids in the *P. viticola* inoculated leaves (Fig. 4B-C), suggesting that phenol production could be induced under abiotic stress against further biotic attack. Further studies should investigate the accumulation of phenols in time-series experiments, especially in the earlier stages of the *P. viticola* infection process, in conjunction with omics approaches. This will facilitate a deeper understanding of this antagonistic relationship and the manner in which drought could act as a priming stimulus.

Stilbenes, especially ϵ - and δ -viniferins, are phenols that accumulate in leaves in response to *P. viticola* infection, particularly in naturally DM-resistant *Vitis* genotypes (Langcake, 1981; Pezet et al., 2004) and in previously elicited grapevines, in which decreased the sporangioophores emergence at 8 dpi (Aziz et al., 2003; Godard et al., 2009). Stilbenes

compounds, however, are not equally toxic to *P. viticola*, for example, resveratrol and piceid have low toxicity, whereas ϵ - and δ -viniferins have high toxicity (Langcake, 1981; Pezet et al., 2004; Adrian et al., 2012; Adrian et al., 2024). In this study, viniferins were below the limit of quantification (LOQ), which is likely associated with little reduction of DM disease symptoms at 8 dpi. Moreover, although we waited for *P. viticola* to complete its life cycle (8 dpi), the GLM-model predicted that earlier infection progression (5 and 6 dpi) was best predicted using defense metabolites (Fig. 5). Similarly, stilbenes have been identified at 2-4 dpi in DM-resistant *Vitis* genotypes (Langcake, 1981; Pezet et al., 2004); future multiple stress studies should focus on the earlier infection process to have a better action profile of this phytoalexin.

To conclude, under natural conditions, grapevines are impacted by a combination of stresses (abiotic and biotic), triggering synergistic or antagonistic physiological responses unique to each combination. These complex interactions cannot be revealed by single-stress studies, highlighting the need for more multi-stress framework studies. Resilience to multiple stressors, from the perspective of viticulture and other perennial crops, is required to not only to maintain productivity within the current season but also to avoid negative carry-over effects across future seasons. It is still unclear how these complex and sometimes contradictory short-term interactions would manifest over the long-term, where combined abiotic stress and disease could act synergistically to contribute to a global plant decline. Under the current climatic scenario, knowledge of plant resilience and defense under combined abiotic and biotic stresses are critical for predicting plant survival and the broader consequences for agricultural and forest ecosystem functions.

AUTHORS CONTRIBUTIONS

ACF, EG, GG, SC planned the project. ACF and MS conducted the sampling and did statistical data analyses. ACF and JVF did the metabolomics assessment. SP did the GLM-based model. ACF took the lead in writing the manuscript, with feedback and approval from co-authors.

TABLES

Table 1. List of phenolics compounds analyzed and their mean $\pm$ SD concentration (mg Kg⁻¹ FW). The limit of quantification threshold (LOQ) was 0.0340 mg L⁻¹ adjusted by leaf weight ($\pm$ 0.50 g).

SUPPLEMENTAL MATERIAL

Supplemental Figure 1. Hourly temperature means for individual plants (n= 12) during the two days of heat stress. Grey-lines indicate the time when radiator heaters were turned on/off. Points represent the mean $\pm$ SE.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Both the manuscript and the supplementary data contain all of the information from this study. The raw data that support the findings of this study are available from the corresponding author upon reasonable request.

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FIGURES LEGEND

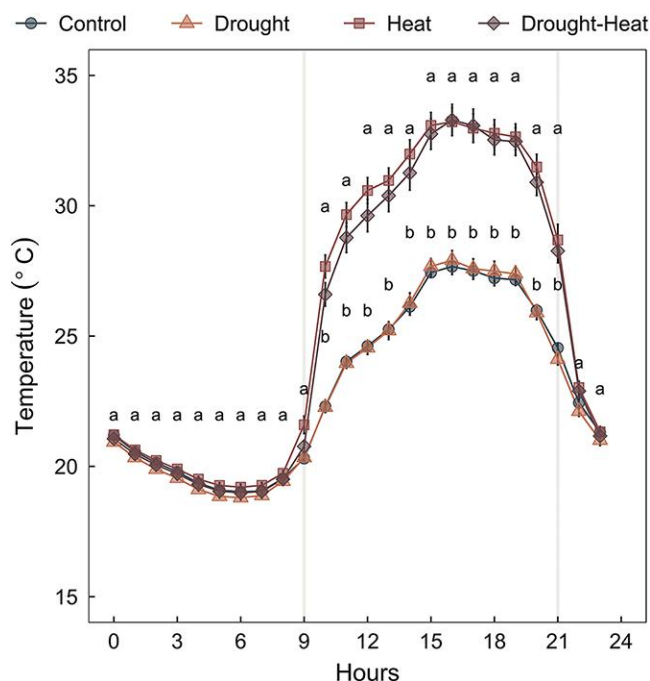


Figure 1. Hourly temperatures recorded for the heat stress treatment on days 20 and 21. Data points show the means $\pm$ SE for the four abiotic stress treatments: control, drought, heat, and drought-heat. Radiator heaters were turned on at 9h00 and off at 21h00 as indicated by the gray vertical lines. Lowercase letters indicate significant differences among abiotic stress treatments according to the Tukey's HSD test ($n=12$ plants with individual thermocouples).

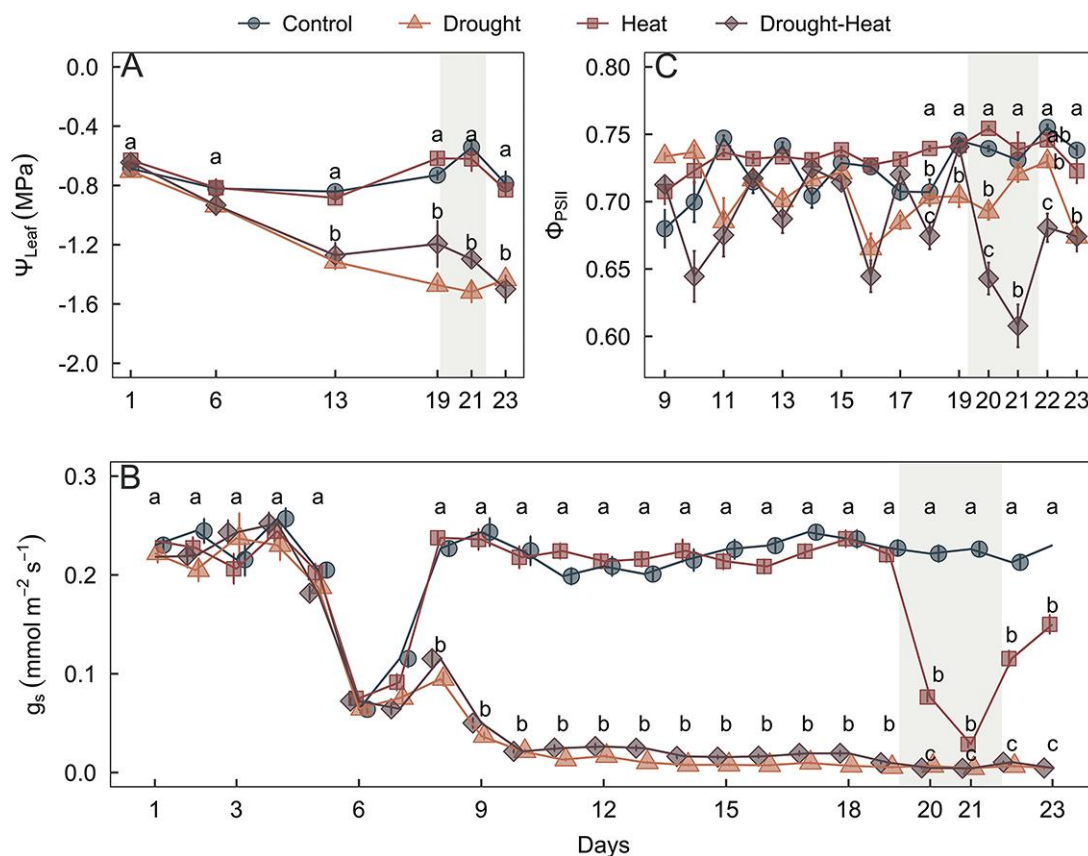


Figure 2. Leaf physiological traits monitored throughout the 23 days of experiment to impose the four abiotic stress treatments: control, drought, heat, and drought-heat. (A) Leaf water potential (Ψ_{leaf}) was measured at midday and collected on selected dates ($n=6$ leaves per abiotic stress treatment per time point). (B) Leaf gas exchange (g_s) was measured daily at midday to track water stress severity. (C) Quantum yield of photosystem II (Φ_{PSII}) was calculated from daily midday fluorescence measurements as an indicator of how the abiotic stress treatments affect photosynthetic performance; these data are presented from the onset of water stress (day 9). (B and C) $n=72$ leaves per abiotic stress treatment per time point. Grey lines indicate heat stress treatment period on days 20 and 21 ($\sim +5^\circ\text{C}$). Data points represent the mean $\pm$ SE. Lowercase letters indicate significant differences among abiotic stress treatments according to Tukey's HSD test.

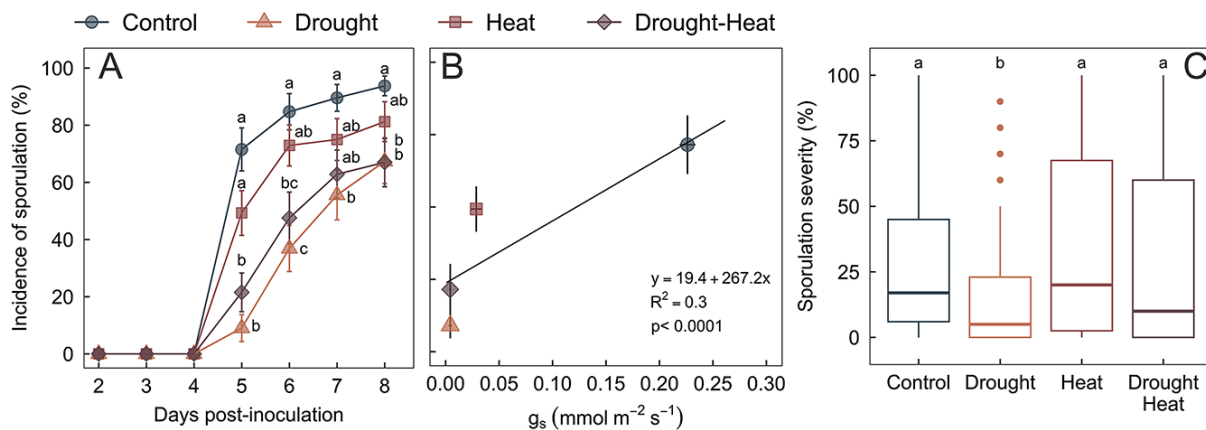


Figure 3. Visual assessment of *P. viticola* sporulation on the third and fourth leaves. (A) *P. viticola* incidence of sporulation, during the disease cycle (2 to 8 dpi), was calculated as the percentage of inoculation sites showing sporulation relative to the total number of sites on each leaf (n= 96 leaves per biotic stress treatment per time point). (B) Correlation between mean incidence of *P. viticola* sporulation on 5 dpi and mean leaf g_s on day 23 represents how the abiotic stress treatments affect sporulation incidence. (C) Sporulation severity of *P. viticola* at the 8 dpi, estimated as spread and density of the sporulation site. Spread refers to the extent of sporulation on the leaf surface, and density indicates the concentration of sporulation, ranging from sparse to intense. This score was based on OIV descriptor 452-1 and expressed on a scale of 0-100% (n= 24 leaves per abiotic stress treatment). Data points represent the mean $\pm$ SE and boxplots represent data distribution. Lowercase letters indicate significant differences among abiotic stress treatments according to Tukey's HSD test.

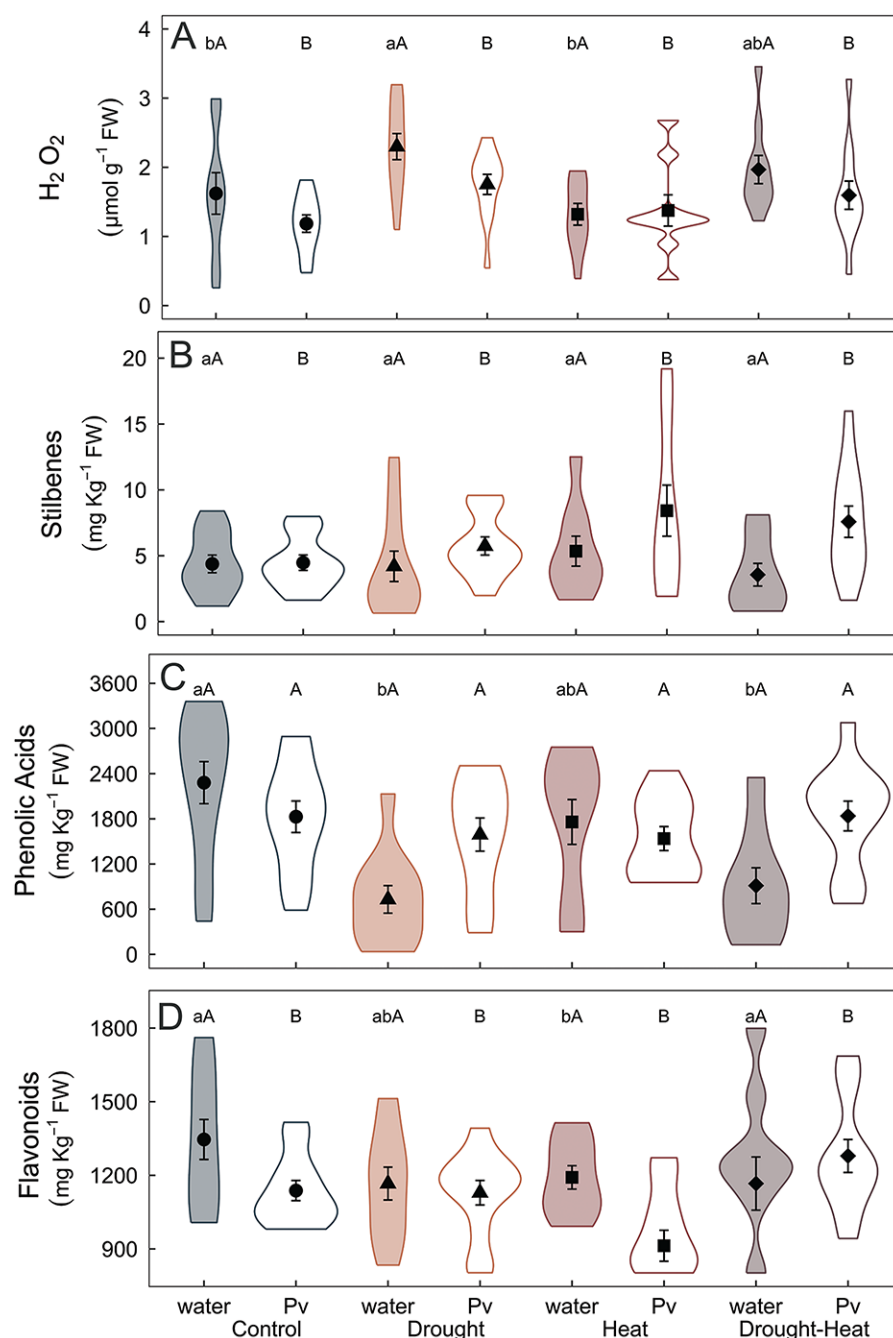


Figure 4. Concentration of defense-related metabolites in the leaves assessed on 8 dpi from control (water) and *P. viticola* (Pv) inoculations (biotic stress) across four abiotic stress treatments: control, drought, heat, and drought-heat. (A) hydrogen peroxide (H₂O₂), (B) stilbenes, (C) phenolic acids, and (D) flavonoids. Data points represent mean $\pm$ SE, and the shades of the violin plots indicate the distribution of the data. Lowercase letters denote different abiotic stress treatments, and uppercase letters denote different biotic stress treatments (inoculation assay). Differences indicate significant differences between treatments according to Tukey's HSD test. (n = 4 leaves per plant; total = 96).

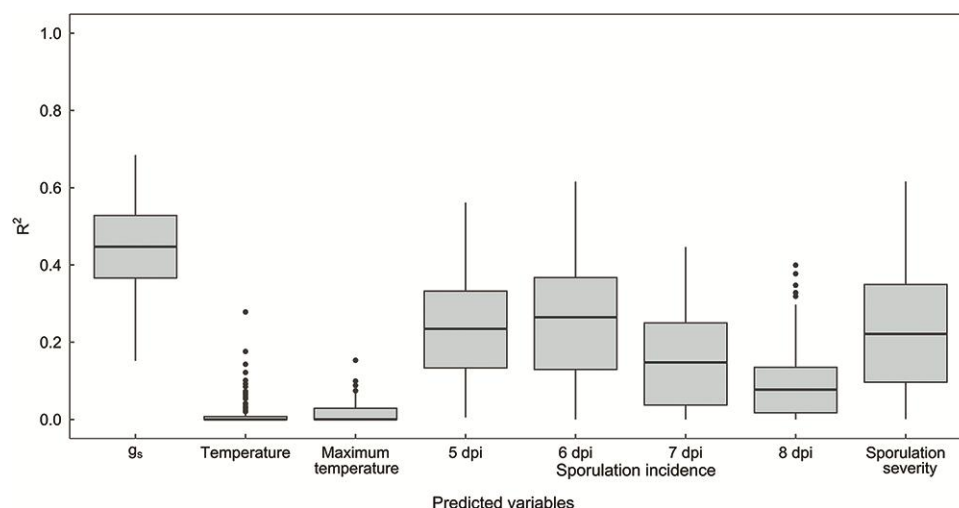


Figure 5. Result of the GLM-model to predict the relationship between defense metabolite concentrations and various traits associated with abiotic and biotic stress treatments. The traits analyzed were stomatal conductance (g_s), temperature, maximum temperature, sporulation incidence (from 5 to 8 dpi), and sporulation severity. They were predicted from the defense metabolites (H_2O_2 and each phenols) concentration. The model yielded a distribution of R^2 scores (obtained by comparing real and predicted values over 100 repeats), in which higher R^2 scores indicate stronger correlation between the defense metabolites and the predicted traits.