

Article

Grapevine Response to Pyroligneous Acid: Antifungal, Physiological, and Biochemical Impacts

Efoo Bawa Nutsukpo , Peter Amoako Ofori, Raphael Ofoe , Anagha Pradeep Kumar , Samuel K. Asiedu, Chijioke Emenike  and Lord Abbey 

Department of Plant, Food, and Environmental Sciences, Faculty of Agriculture, Dalhousie University, 50 Pictou Road, Truro, NS B2N 5E3, Canada

* Correspondence: bw761972@dal.ca (E.B.N.); loab07@gmail.com (L.A.)

Abstract: *Botrytis cinerea* is a major fungal pathogen causing significant economic losses in grapevines worldwide. To address the environmental concerns associated with overreliance on synthetic fungicides, this study investigated the antifungal efficacy of varying concentrations of pyroligneous acid (PA) (0, 2, and 4%) compared to a commercial fungicide (Switch[®]) against *B. cinerea* in grapevines (*Vitis vinifera* ‘Himrod’), as well as its physiological and biochemical responses. Our preliminary in vitro assays using the poisoned food method showed that PA significantly ($p < 0.05$) inhibited *B. cinerea* mycelial growth by approximately 0.70-, and 1-fold, respectively, compared to the 0% PA during the three weeks of observation. The results also demonstrated that the 2% PA and 4% PA treatments, as well as the Switch[®] application, significantly ($p < 0.05$) reduced average lesion length by 0.19-, 0.52-, and 0.85-fold, respectively, compared to the untreated plants with Botrytis alone. Both the 4% PA and Switch[®] significantly ($p < 0.05$) increased the maximum quantum efficiency of photosystems II (F_v/F_m) and potential photosynthetic capacity (F_v/F_o) by approximately 0.02-fold and 0.1-fold, respectively, compared to the untreated plants with Botrytis alone. The 2 and 4% PA treatments also increased total carotenoids and flavonoids. Further molecular studies are recommended to elucidate the mechanisms underlying the observed physiological and biochemical changes.



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Keywords: wood vinegar; *Botrytis cinerea*; necrotic symptoms; lipid peroxidation; hydrogen peroxide

1. Introduction

A wide array of pathogenic organisms can inflict devastating diseases on crops, resulting in severe economic losses [1] estimated at 20 to 40% of crop production. These crop losses can reach approximately USD 40 billion per year worldwide [2]. Among these pathogens, fungi represent some of the greatest biotic challenges facing global agriculture [3]. Among these pathogens, *Botrytis cinerea* is one of the most notable fungi pathogens causing severe economic losses due to its ability to infect and colonize a diverse range of crops [4,5]. The economic impact of *B. cinerea* is staggering, with estimated annual losses ranging from USD 10 to 100 billion worldwide [6,7]. This pathogen poses a serious threat to the health and productivity of over 1400 plant species, causing a devastating mold disease [8,9]. The pathogen’s host range is extensive, affecting both ornamental and food crops. High-value ornamental flowers such as roses (*Rosa rubiginosa*) and gerbera (*Gerbera jamesonii*), as well as economically important fruit and vegetable crops such as tomatoes (*Solanum lycopersicum*), grapevines, and strawberries (*Fragaria × ananassa*), are

particularly susceptible [10]. *B. cinerea*'s infection strategy is versatile. It can directly penetrate intact host leaf surfaces like the cuticle or enter through natural openings such as stomata, wounds, and previously infected sites [11]. The fungus thrives in wet and damp conditions, which are necessary for its conidia to germinate and initiate infection [12]. Once established in agricultural fields, *B. cinerea* becomes particularly challenging to control due to its ability to produce long-lasting sclerotia, which serve as survival structures in adverse conditions [13]. While fungicide application has traditionally been an effective control method, it has raised significant concerns. The environmental impact of these chemicals and the increasing development of fungicide-resistant strains have prompted a search for alternative control strategies [13]. Researchers are exploring various alternatives to reduce or eliminate the use of synthetic chemicals in controlling *B. cinerea*. One of the promising approaches involves the use of pyroligneous acid (PA), a natural compound with antifungal properties [14].

Pyroligneous acid (PA), also known as wood vinegar, is a crude reddish-brown liquid by-product of pyrolysis of plant biomass under high temperatures and limited oxygen supply [15,16]. During pyrolysis, the organic material is converted into biochar, charcoal, and organic vapor (smoke), which is then condensed to form PA as a by-product [15]. Chemical analysis has shown that PA consists primarily of 80 to 90% of water and more than 200 organic compounds comprising 10 to 20% organic acids, ester, alcohol, phenolic, and alkene [15,17]. Among these organic compounds, acetic acid represents about 1.5% of PA [18], which contributes to its low pH of less than 3, while phenolic compounds contribute to its smoky odor [15,18,19]. The nature and the chemical composition of PA depend on the feedstock, particle size, residence time, temperature, and heating rate [15].

Several studies have demonstrated that PA induces beneficial antimicrobial effects on various plant diseases. For instance, PA has been shown to inhibit the growth of numerous strains of fungi, including *Fomitopsis palustris*, *Trametes versicolor* [20], *Alternaria mali*, *Rhizoctonia solani*, *Sclerotium oryzae*, *Helminthosporium maydis*, *Pythium* sp., *Colletotrichum gloeosporioides*, and *Choanephora cucurbitarum* [15,21,22]. Specifically, PA has potential in controlling *B. cinerea* [14]. It has been studied in grape fruits [23] and apple fruits [24], with these studies mainly reporting PA inhibition of mycelium growth of *B. cinerea* using plate assays. However, there is limited research on its application to grapevines specifically for controlling *B. cinerea* strains in growing leaf tissues. Transitioning from plate-based assays to the use of living plant tissues is indispensable, as it can provide more accurate and ecologically relevant results, especially for understanding how *B. cinerea* behaves in a more natural environment. This study aims to address this knowledge gap by investigating the effects of PA on *B. cinerea* in grapevine plants. Furthermore, the research determines appropriate PA application rates and examines the physiological and biochemical responses of grapevines to both *B. cinerea* infection and PA treatment. By exploring these aspects, this study provides valuable insights into using PA as a potential method for managing *B. cinerea* infections in grapevine cultivation, contributing to the development of more sustainable disease management strategies in viticulture.

2. Materials and Methods

2.1. Study Area

The experiment was conducted in the Department of Plant, Food, and Environmental Sciences, Faculty of Agriculture, Dalhousie University, Truro, Canada, between February 2023 and June 2024.

2.2. Pyroligneous Acid (PA)

The PA used in this study was produced from white pine (*Pinus strobus*) biomass using fast pyrolysis technology developed by Proton Power Inc. (Lenoir City, TN, USA). The detailed description of the process and composition of the PA were described well by Ofoe et al. [25]. In brief, the chemical profile of the PA was 2.3 pH, 1.03 g/L salt content, 1.42 g/L total dissolved solid content, 2.05 mS/cm electrical conductivity, and 9.35 °Brix. Chemical analysis revealed high concentrations of bioactive compounds, including 95.81 mg Gallic Acid Equivalent (GAE)/mL of total phenolic, 49.46 µg quercetin/mL of total flavonoids, and a 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of 78.5%. Metabolite profiling revealed 156 distinct compounds categorized into organic acids, hexose, carnitine, and derivatives of phospholipids. Additionally, the PA was found to contain 33 different mineral elements [25].

2.3. Origin and Maintenance of Fungal Strain

The *B. cinerea* isolate DAOMC155189 was obtained from Canadian Collection of Fungal Cultures. The isolate was sub-cultured onto potato dextrose agar (PDA) and incubated at 27 °C for 14 days. The PDA medium was prepared by combining 24g/L potato dextrose broth and 15g/L agar (both from BioShop®, Burlington, ON, Canada) in 1 L distilled water and autoclaved.

2.4. Plant Materials

Thirty-two-week-old grape (*Vitis vinifera* ‘Himrod’) seedlings were produced from stem cuttings of mother plants obtained from Gray’s Greenhouse in Antigonish, Nova Scotia, Canada. The seedlings were planted in 2.84 L pots filled with Pro-Mix® BX (Premier Tech Horticulture, Québec City, QC, Canada) soilless growing media.

These seedlings were raised under greenhouse conditions with day/night temperatures of 22 °C/15 °C and 70% relative humidity. A 16 h photoperiod was maintained and supplemented by a 600 W HS2000 high-pressure sodium lamp with NAH600.579 ballast (P.L. Light Systems, Beamsville, ON, Canada). Fertigation was performed using compound fertilizer nitrogen–phosphorus–potassium (NPK 20-20-20) Miracle-Gro® (Scotts Canada Ltd., Mississauga, ON, Canada.) and applied following the manufacturer’s recommended application rate of 6 mL/L at 14-day intervals. All plants were watered regularly to field capacity until they were ready for use in the study.

2.5. In Vivo Antifungal Activity Assay

The grape seedlings were treated with PA concentrations of 0%, 2%, and 4%, as well as a commercial fungicide (Switch®) (Syngenta Canada Inc, Guelph, Ontario, Canada) at the manufacturer’s recommended rate. A PDA plug assay was performed following the method of Kirkby et al. [26]. In brief, the treatments were PDA plugs alone (T1), untreated plants with Botrytis alone (T2), 2% PA-treated plants with Botrytis (T3), 4% PA-treated plants with Botrytis (T4), and Switch®-treated plants with Botrytis (T5). Each treatment, including various PA concentrations, the recommended fungicide rate (positive control), and water (negative control), was applied to the plants using a pressure sprayer (China made). Approximately 500 mL of each treatment was sprayed on the leaves from a distance of about 20 to 30 cm while the researchers wore personal protective equipment. The plants were then left for 24 h before inoculation.

The experimental setup included three plants for each treatment. Five leaves per plant were inoculated using either 7 mm PDA plugs (for control) or *B. cinerea* fungal plugs for the fungal inoculation. The plugs were held in place using sterilized pins (Figure 1A). The

entire experiment was conducted in a tent chamber (Figure 1B) under controlled conditions with a 16/8 h photoperiod at 15° to 20 °C.

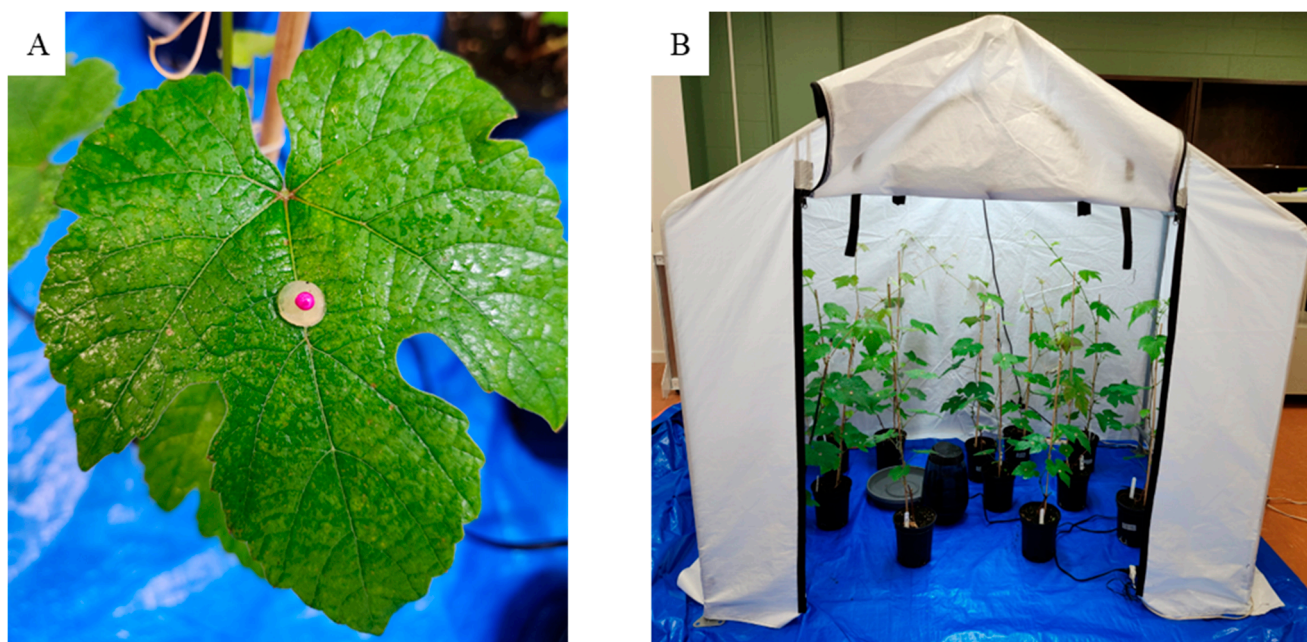


Figure 1. Inoculation of attached leaves on grape seedlings. (A) Inoculated attached leaf with *Botrytis* fungal plug secured using a sterilized pin. (B) Setup placed in a tent chamber.

After 4 days, the progression of the disease was assessed by measuring the length of diseased lesions that had spread beyond the mycelial plugs with a digital Vernier caliper (Mastercraft, ON, Canada). This study was repeated twice.

2.6. Plant Physiological Analysis

Physiological parameters were examined seven days post-inoculation. Leaf greenness was used to estimate chlorophyll content on the five inoculated leaves per plant using a SPAD 502-plus chlorophyll meter (Spectrum Technologies Inc., Aurora, IL, USA). Chlorophyll fluorescence parameters i.e., maximum quantum efficiency (F_v/F_m) and potential photosynthetic capacity of photosystems II (F_v/F_o), were assessed on the same leaves with a Chlorophyll fluorometer (Opti-Sciences, Inc., Hudson, NY, USA). In addition, photosynthetic parameters, including net photosynthetic rate ($\mu\text{mol m}^{-2} \text{s}^{-1}$), transpiration rate ($\text{mol m}^{-2} \text{s}^{-1}$), intracellular carbon dioxide concentration ($\mu\text{mol mol}^{-1}$), and stomatal conductance ($\text{mol m}^{-2} \text{s}^{-1}$), were assessed from the same five leaves using an LCI portable photosynthesis system (ADC Bioscientific Ltd., Hoddesdon, UK), following the manufacturer's protocol.

2.7. Biochemical Analysis

On the seventh day post-inoculation, the inoculated leaves were sampled and immediately frozen in liquid nitrogen. The frozen samples were ground into fine powder and stored in a -80°C freezer for further biochemical analysis.

2.7.1. Chlorophylls and Carotenoids

Chlorophylls (*a* and *b*) and carotenoids were extracted from 0.2 g of leaf tissue using 80% acetone a method described by Linchtenthaler [27] and adopted by Nutsukpo et al. [28], with some modifications. The mixture was vortexed and centrifuged, and the supernatant's absorbance was measured at 646.8 nm, 663.2 nm, and 470 nm. Chlorophyll concentrations

were calculated using specific formulas, while carotenoid content was determined using absorbance at 470 nm. The results were expressed as $\mu\text{g/g}$ fresh weight (FW).

2.7.2. Total Phenolics

Total phenolic content (TPC) was assessed using the Folin–Ciocalteu method described by Ainsworth and Gillespie [29] and adopted by Ofori [30], with slight modifications. Leaf tissue (0.2 g) was homogenized in 95% methanol, incubated for 48 h, and centrifuged. The supernatant was mixed with the Folin–Ciocalteu reagent and sodium carbonate, and absorbance was measured at 765 nm. TPC was quantified using a gallic acid standard curve and expressed as mg gallic acid equivalents (GAE)/g FW.

2.7.3. Total Flavonoid

Flavonoid content was determined by homogenizing 0.2 g of leaf tissue in 95% methanol, followed by centrifugation, a method described by Chang et al. [31] and adopted by Nutsukpo et al. [32], with some modifications. The supernatant was mixed with aluminum chloride, potassium acetate, and distilled water, and absorbance was measured at 415 nm. Flavonoid levels were calculated using a quercetin standard curve and expressed as μg quercetin/g FW.

2.7.4. Total Sugar

Total sugar was quantified using the phenol-sulfuric acid procedure, as described by Dubois et al. [33] and adopted by Negassa et al. [34], with small modifications. Leaf tissue (0.2 g) was extracted with 90% ethanol, incubated at 60 °C, and centrifuged. The supernatant was mixed with phenol and sulfuric acid, and absorbance was measured at 490 nm. Sugar content was determined using a glucose standard curve and expressed as μg glucose/g FW.

2.7.5. Lipid Peroxidation and Hydrogen Peroxide

Lipid peroxidation was measured by quantifying malondialdehyde (MDA) using thiobarbituric acid (TBA). Leaf tissue (0.2 g) was homogenized in trichloroacetic acid (TCA), centrifuged, and mixed with TBA. Absorbance was measured at 532 nm and 600 nm, and MDA concentration was calculated. Hydrogen peroxide (H_2O_2) levels were determined by homogenizing leaf tissue in TCA, centrifuging, and reacting the supernatant with potassium iodide. Absorbance was measured at 390 nm, and H_2O_2 levels were quantified using a standard curve [35,36].

2.7.6. Total Protein and Peroxidase Enzyme Activity

Total protein was extracted from 0.2 g of leaf tissue using a phosphate buffer, centrifuged, and mixed with a Bradford reagent. Absorbance was measured at 595 nm, and protein content was calculated using a bovine serum albumin (BSA) standard curve. Peroxidase (POD) activity was determined by reacting crude enzyme extract with pyrogallol and H_2O_2 . Absorbance was measured at 420 nm, and activity was expressed in units based on purpurogallin formation [37,38].

2.8. Data Analysis

Data were analyzed using one-way ANOVA, Tukey's post hoc test, bivariate correlation, and principal component analysis (PCA) with Minitab version 21 (Minitab, Inc., State College, PA, USA), XLSTAT version 2022.3 (Addinsoft, New York, NY, USA), GraphPad Prism 9.5.0 (GraphPad Software, San Diego, CA, USA), and Microsoft Excel. Significance was set at $p \leq 0.05$.

3. Results

3.1. *Botrytis cinerea* Necrosis

The leaves of the grape plants did not show necrotic symptoms for the first three days post *B. cinerea* inoculation. On the fourth day post inoculation (dpi), initial dark-brownish necroses were observed, which increased in size at 5 to 7 dpi (Figure 2).

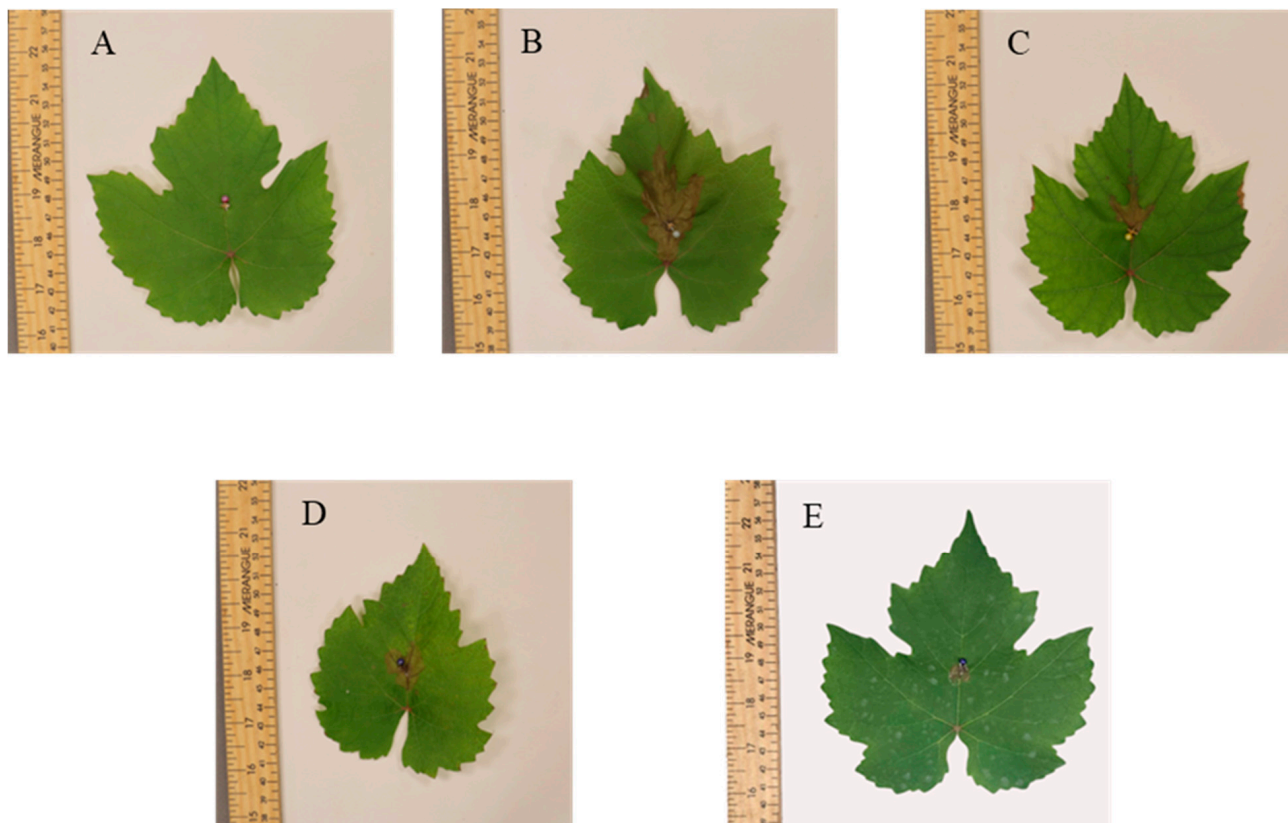


Figure 2. Inhibition of *B. cinerea* growth as affected by different concentrations of pyroligneous acid using the in vivo antifungal activity method. (A) Untreated plants with PDA plug alone; (B) untreated plants with Botrytis alone; (C) 2% PA-treated plants with Botrytis; (D) 4% PA-treated plants with Botrytis; and (E) Switch[®]-treated plants with Botrytis.

It was observed that the PDA plugs alone developed no necrotic symptoms (Figure 2A). The 2% and 4% PA-treated plants developed necrotic symptoms, which continued to grow with time (Figure 2C,D), but the symptoms were more severe in Botrytis alone (Figure 2B). The Switch[®]-treated plants developed very small lesions (Figure 2E).

Figure 3 showed the trend of *B. cinerea* lesion progression according to the various treatments over time. Here, the impact of PA against the lesion development of *B. cinerea* compared to the controls was checked on a daily basis.

Botrytis alone showed significantly ($p < 0.05$) higher average daily lesion length throughout the study, followed by 2% PA, 4% PA, and the Switch[®]-treated plants. The PDA plugs alone had no lesions throughout the study (Figure 3).

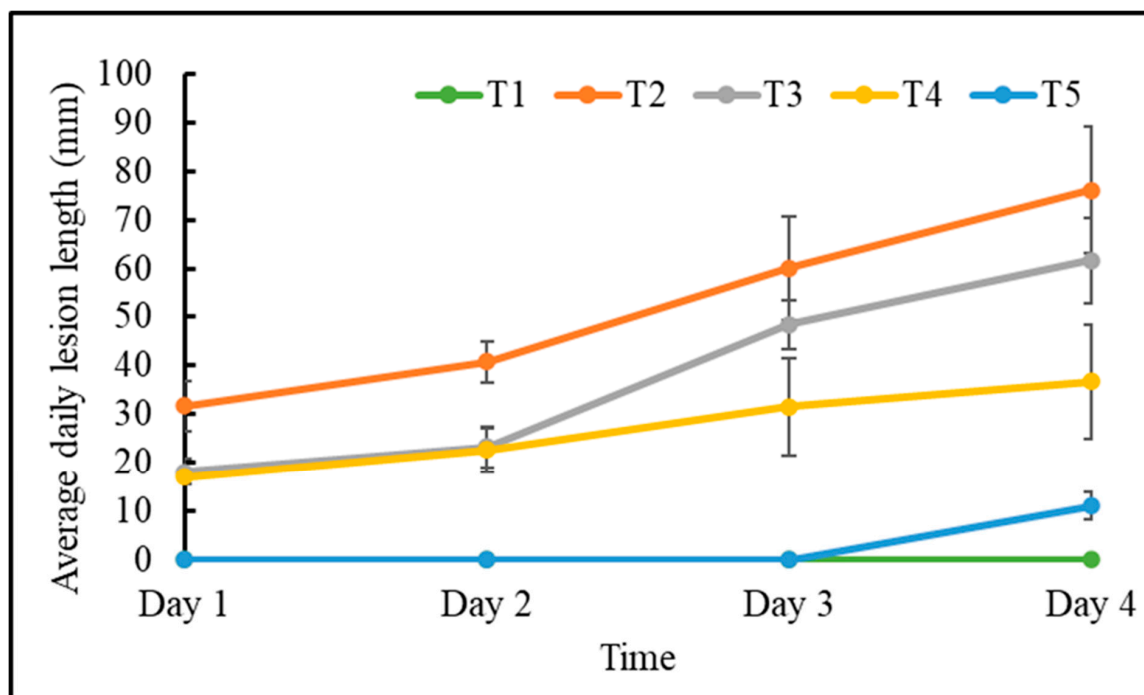


Figure 3. Average daily lesion length. PDA plugs alone (T1), untreated plants with Botrytis alone (T2), 2% PA-treated plants with Botrytis (T3), 4% PA-treated plants with Botrytis (T4), and Switch[®] treated plants with Botrytis (T5). Error bars represent standard deviation.

3.2. Physiological Response of Grape Plants to PA Application and *B. cinerea* Inoculation

To further confirm the response of the grape seedlings to the application of PA and *B. cinerea* inoculation, we measured the plant physiological parameters. In general, the PDA plugs alone had significantly ($p < 0.05$) the highest maximum quantum efficiency of photosystems II (PSII) (F_v/F_m), potential photosynthetic capacity (F_v/F_o), photosynthetic rate, transpiration rate, and stomatal conductance (Table 1). It was observed that the 4% PA and Switch[®] with Botrytis showed a non-significant ($p > 0.05$) increase in chlorophyll content, while the 2% PA with Botrytis showed a non-significant ($p > 0.05$) reduction in chlorophyll content of the leaves compared to Botrytis alone (Table 1).

Table 1. Grape seedlings leaf chlorophyll content and photosynthetic parameters as affected by PA application and *B. cinerea* infection.

Treatments	SPAD	F_v/F_m	F_v/F_o	A ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	E ($\text{mol m}^{-2} \text{s}^{-1}$)	C_i ($\mu\text{mol mol}^{-1}$)	g_s ($\text{mol m}^{-2} \text{s}^{-1}$)
T1	35.20 $\pm$ 3.54a	0.798 $\pm$ 0.00a	3.97 $\pm$ 0.06a	2.75 $\pm$ 1.13a	1.90 $\pm$ 1.33a	395.20 $\pm$ 34.50a	0.17 $\pm$ 0.13a
T2	34.23 $\pm$ 4.25a	0.778 $\pm$ 0.01b	3.52 $\pm$ 0.16b	1.80 $\pm$ 0.87a	1.08 $\pm$ 0.10a	392.60 $\pm$ 20.23a	0.07 $\pm$ 0.01ab
T3	32.51 $\pm$ 2.05a	0.787 $\pm$ 0.00ab	3.71 $\pm$ 0.08ab	2.12 $\pm$ 0.64a	1.01 $\pm$ 0.35a	363.80 $\pm$ 28.60a	0.06 $\pm$ 0.02ab
T4	37.47 $\pm$ 3.16a	0.794 $\pm$ 0.00a	3.87 $\pm$ 0.11a	1.42 $\pm$ 0.27 a	0.96 $\pm$ 0.19a	378.40 $\pm$ 18.80a	0.05 $\pm$ 0.01b
T5	34.54 $\pm$ 0.23a	0.797 $\pm$ 0.00a	3.95 $\pm$ 0.29a	1.92 $\pm$ 0.99a	1.42 $\pm$ 0.34a	397.80 $\pm$ 41.80a	0.08 $\pm$ 0.02ab
<i>p</i> -value	0.168	0.001	0.001	0.187	0.153	0.370	0.037

F_v/F_m , maximum quantum efficiency of PSII; F_v/F_o , potential photosynthetic capacity; A, photosynthetic rate; E, transpiration rate; C_i , Sub-stomatal CO_2 ; g_s , stomatal conductance. Values are means $\pm$ standard deviation of four replicates, and different letters indicate significant ($p < 0.05$) differences according to Tukey's honestly significant difference post-test at $\alpha = 0.05$. PDA plugs alone (T1), untreated plants with Botrytis alone (T2), 2% PA-treated plants with Botrytis (T3), 4% PA-treated plants with Botrytis (T4), and Switch[®]-treated plants with Botrytis (T5).

The results also showed that treatments 4% PA and Switch[®] with Botrytis significantly ($p < 0.05$) improved maximum quantum efficiency (F_v/F_m) by approximately 0.02-folds each compared to Botrytis alone. The 4% PA and Switch[®] with Botrytis significantly ($p < 0.05$) increased potential photosynthetic capacity (F_v/F_o) by approximately 0.09- and 0.12-folds compared to Botrytis alone.

3.3. Changes in the Chemical Composition of Grape Plants in Response to Treatments

The application of PA, Switch[®], and *B. cinerea* inoculation caused significant ($p < 0.05$) changes in the chemical composition of the grape plants (Figures 4–8).

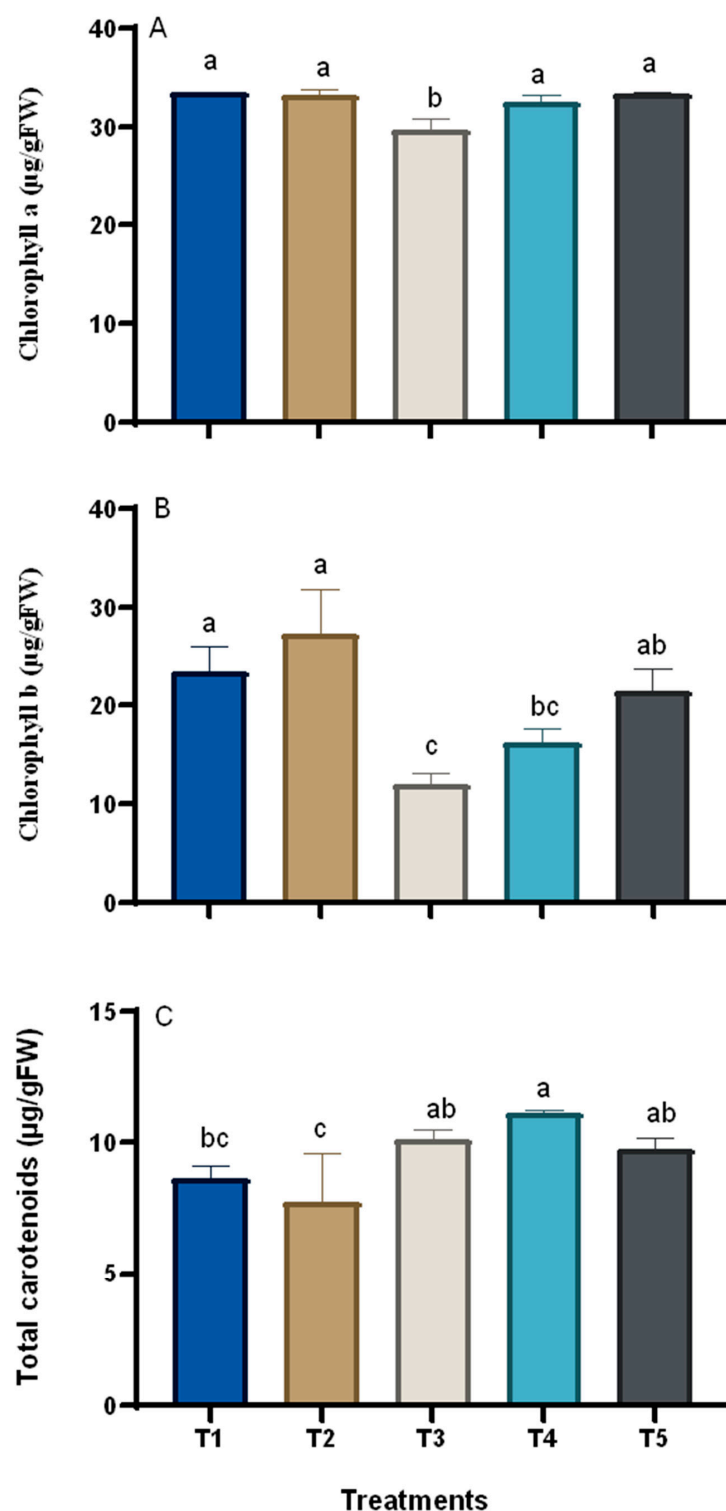


Figure 4. Application of PA, fungicide, and *B. cinerea* inoculation altered photosynthetic pigments accumulation in the grapevines. (A) chlorophyll a, (B) chlorophyll b, and (C) total carotenoids. PDA plugs alone (T1), untreated plants with Botrytis alone (T2), 2% PA-treated plants with Botrytis (T3), 4% PA-treated plants with Botrytis (T4), and Switch[®]-treated plants with Botrytis (T5). Values are the means of four replicates. Different letters indicate significant differences ($p < 0.05$) according to Tukey's test at $\alpha = 0.05$. Error bars represent standard deviation.

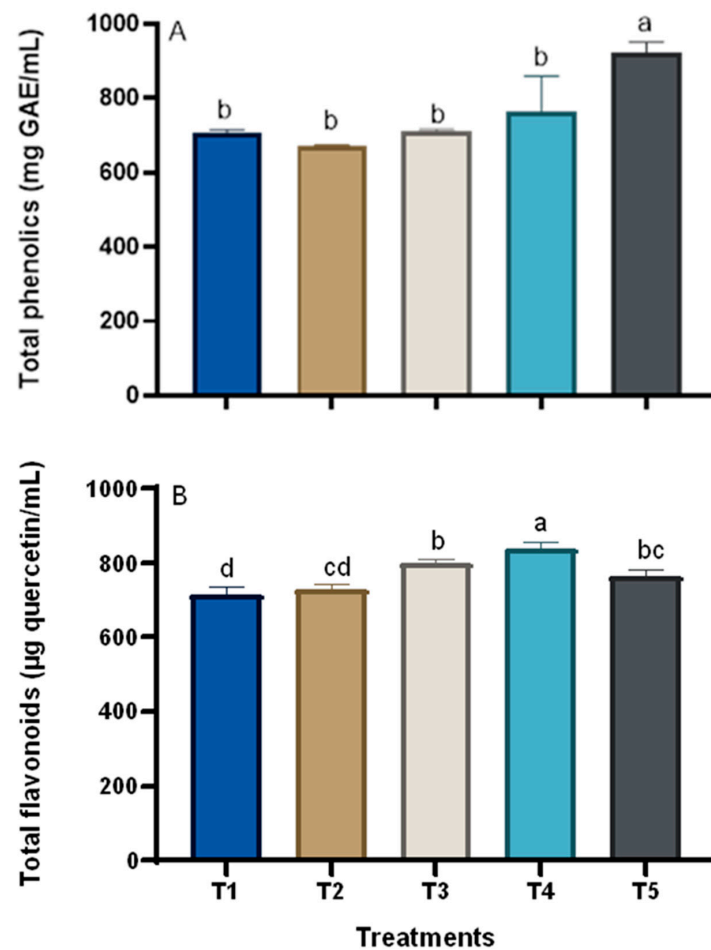


Figure 5. Application of PA, fungicide, and *B. cinerea* inoculation altered antioxidant accumulation in the grapevines. (A) total phenolics and (B) total flavonoids. PDA plugs alone (T1), untreated plants with Botrytis alone (T2), 2% PA-treated plants with Botrytis (T3), 4% PA-treated plants with Botrytis (T4), and Switch[®]-treated plants with Botrytis (T5). Values are the means of four replicates. Different letters indicate significant differences ($p < 0.05$) according to Tukey's test at $\alpha = 0.05$. Error bars represent standard deviation.

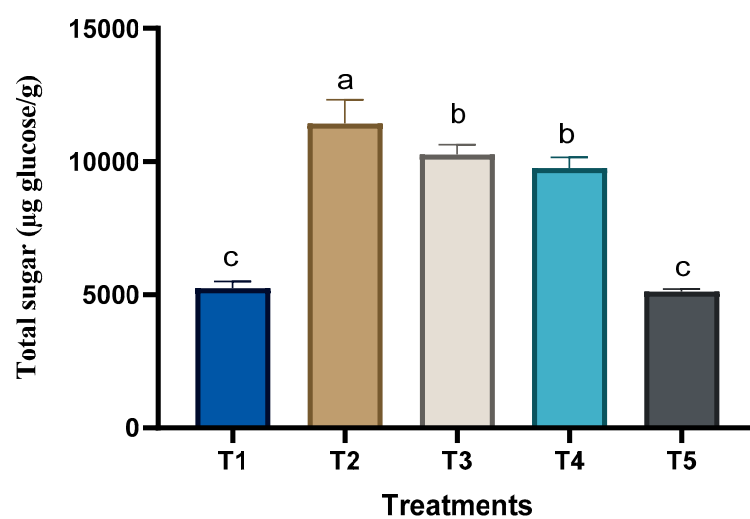


Figure 6. Application of PA, fungicide, and *B. cinerea* inoculation altered osmolyte (sugar) accumulation in the grapevines. PDA plugs alone (T1), untreated plants with Botrytis alone (T2), 2% PA-treated plants with Botrytis (T3), 4% PA-treated plants with Botrytis (T4), and Switch[®]-treated plants with Botrytis (T5). Values are the means of four replicates. Different letters indicate significant differences ($p < 0.05$) according to Tukey's test at $\alpha = 0.05$. Error bars represent standard deviation.

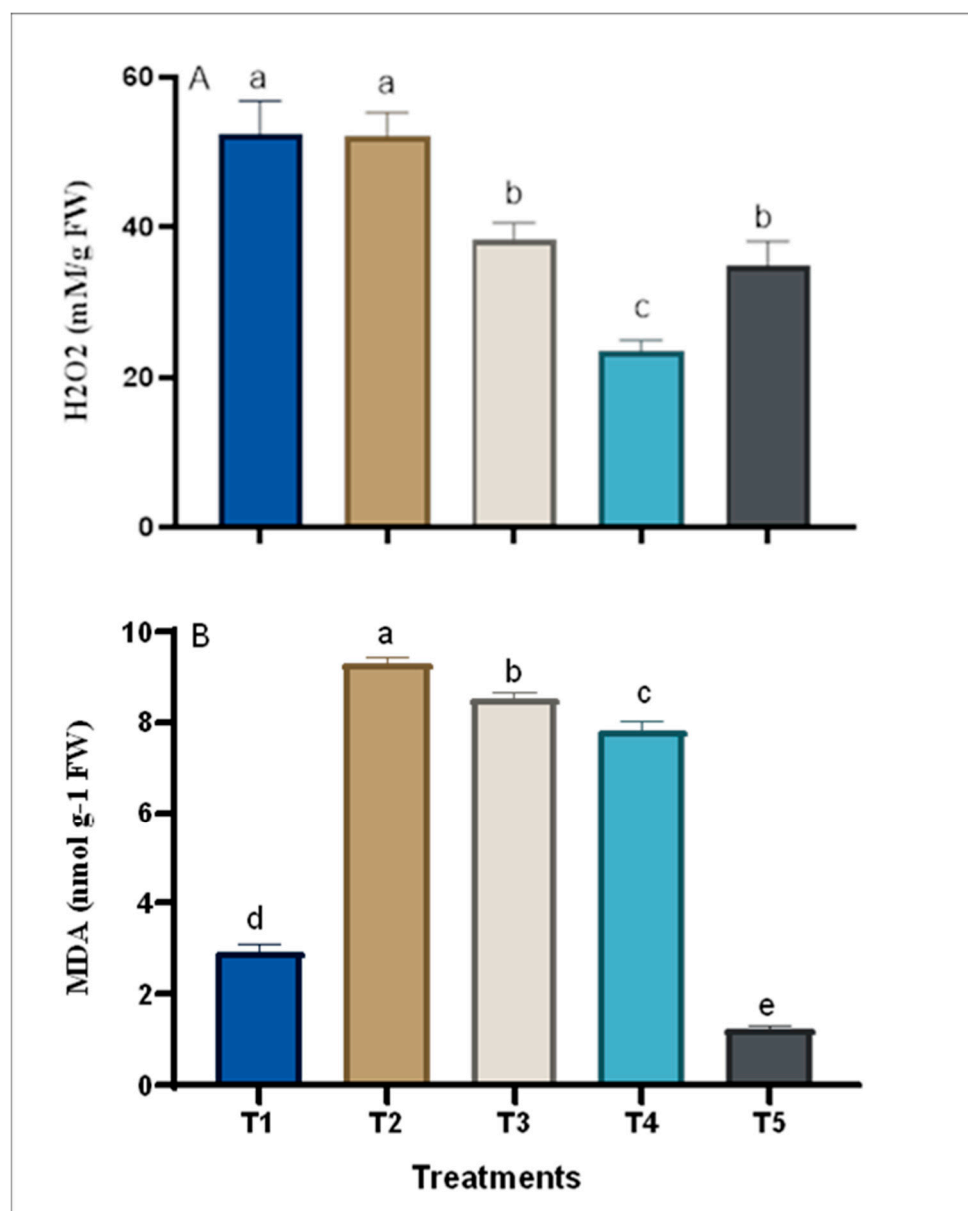


Figure 7. Application of PA, fungicide, and *B. cinerea* inoculation altered (A) H₂O₂ content and (B) MDA content in the grapevines. PDA plugs alone (T1), untreated plants with Botrytis alone (T2), 2% PA-treated plants with Botrytis (T3), 4% PA-treated plants with Botrytis (T4), and Switch[®]-treated plants with Botrytis (T5). Values are the means of four replicates. Different letters indicate significant differences ($p < 0.05$) according to Tukey's test at $\alpha = 0.05$. Error bars represent standard deviation.

The 2% PA plants with Botrytis showed significant ($p < 0.05$) reduction in chl *a* by approximately 0.11-fold compared to Botrytis alone. Similarly, the 4% PA plants with Botrytis showed a non-significant ($p > 0.05$) reduction in chl *a* compared to Botrytis alone. The Switch[®]-treated plants with Botrytis showed a non-significant ($p > 0.05$) increase in chl *a* compared to Botrytis alone (Figure 4A). The 2 and 4% PA plants with Botrytis showed significant ($p < 0.05$) decreases in chl *b* by approximately 0.56- and 0.41-folds, respectively, compared to Botrytis alone. Switch[®]-treated plants with Botrytis showed a non-significant ($p > 0.05$) decrease in chl *b* compared to Botrytis alone (Figure 4B). The 2% PA-, 4% PA-, and Switch[®]-treated plants with Botrytis showed significant ($p < 0.05$) increases in total carotenoids by approximately 0.31-, 0.44-, and 0.26-folds, respectively, compared to Botrytis alone (Figure 4C).

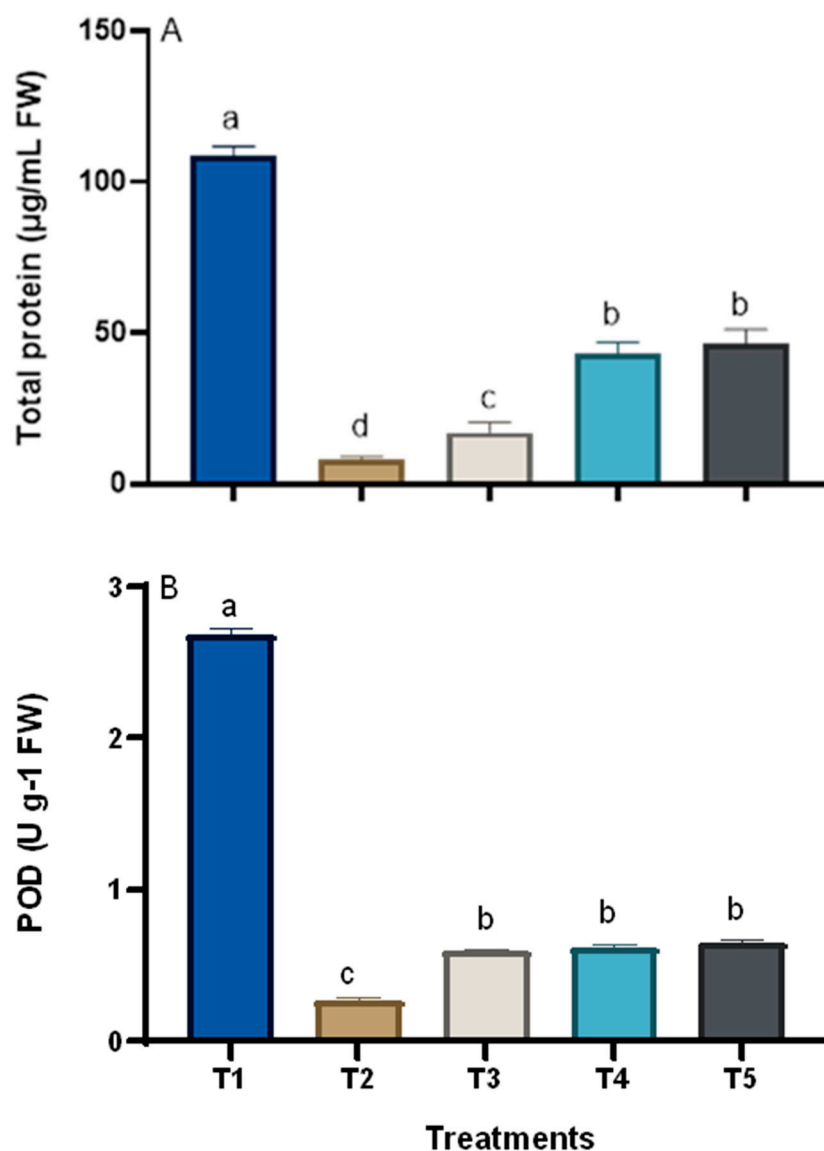


Figure 8. Application of PA, fungicide, and *B. cinerea* inoculation altered (A) (I) total protein content and (B) POD content in the grapevines. PDA plugs alone (T1), untreated plants with Botrytis alone (T2), 2% PA-treated plants with Botrytis (T3), 4% PA-treated plants with Botrytis (T4), and Switch[®]-treated plants with Botrytis (T5). Values are the means of four replicates. Different letters indicate significant differences ($p < 0.05$) according to Tukey's test at $\alpha = 0.05$. Error bars represent standard deviation.

The 2% and 4% PA plants with Botrytis had non-significant ($p > 0.05$) increases in total phenolics compared to Botrytis alone, while the Switch[®]-treated plants with Botrytis showed significant ($p < 0.05$) increases in total phenolics by approximately 0.38-fold compared to Botrytis alone (Figure 5A). Total flavonoids were significantly ($p < 0.05$) increased by the 2% PA and 4% PA with Botrytis by approximately 0.09- and 0.15-folds, respectively, compared to Botrytis alone. Switch[®]-treated plants with Botrytis showed a non-significant ($p > 0.05$) increase in total flavonoids compared to Botrytis alone (Figure 5B).

Total sugar was significantly ($p < 0.05$) decreased by 2% PA, 4% PA, and Switch[®] with Botrytis by approximately 0.10-, 0.15-, and 0.55-folds, respectively, compared to Botrytis alone (Figure 6).

The 2% PA with Botrytis showed significant ($p < 0.05$) decreases in MDA content and hydrogen peroxide by approximately 0.08- and 0.26-folds, respectively, compared to Botrytis alone. The 4% PA plants with Botrytis had significant ($p < 0.05$) decreases in

MDA content and hydrogen peroxide by approximately 0.16- and 0.55-folds, respectively, compared to Botrytis alone. The Switch[®] plants with Botrytis showed significant ($p < 0.05$) decreases in MDA content and hydrogen peroxide by approximately 0.87- and 0.33-folds, respectively, compared to Botrytis alone (Figure 7).

The 2% PA plants with Botrytis showed significant ($p < 0.05$) increases in total protein and peroxidase enzyme activity by approximately 1.16- and 1.25-folds, respectively, compared to Botrytis alone. The 4% PA plants with Botrytis showed significant ($p < 0.05$) increases in total protein and peroxidase enzyme activity by approximately 4.49- and 1.35-folds, respectively, compared to Botrytis alone. The Switch[®]-treated plants with Botrytis showed significant ($p < 0.05$) increases in total protein and peroxidase enzyme activity by approximately 4.93- and 1.46-folds, respectively, compared to Botrytis alone (Figure 8).

The heatmap in Figure 9 shows strong positive correlations between the zone of lesion and total sugar ($r = 0.95$) and MDA content ($r = 0.91$). The zone of lesion has weak positive correlations with total flavonoid ($r = 0.21$) and hydrogen peroxide ($r = 0.07$). The zone of lesion also has strong negative correlations with maximum quantum efficiency (F_v/F_m) of PS II ($r = -0.95$), potential photosynthetic capacity (F_v/F_o) ($r = -0.96$), and total protein ($r = -0.89$) (Figure 9).

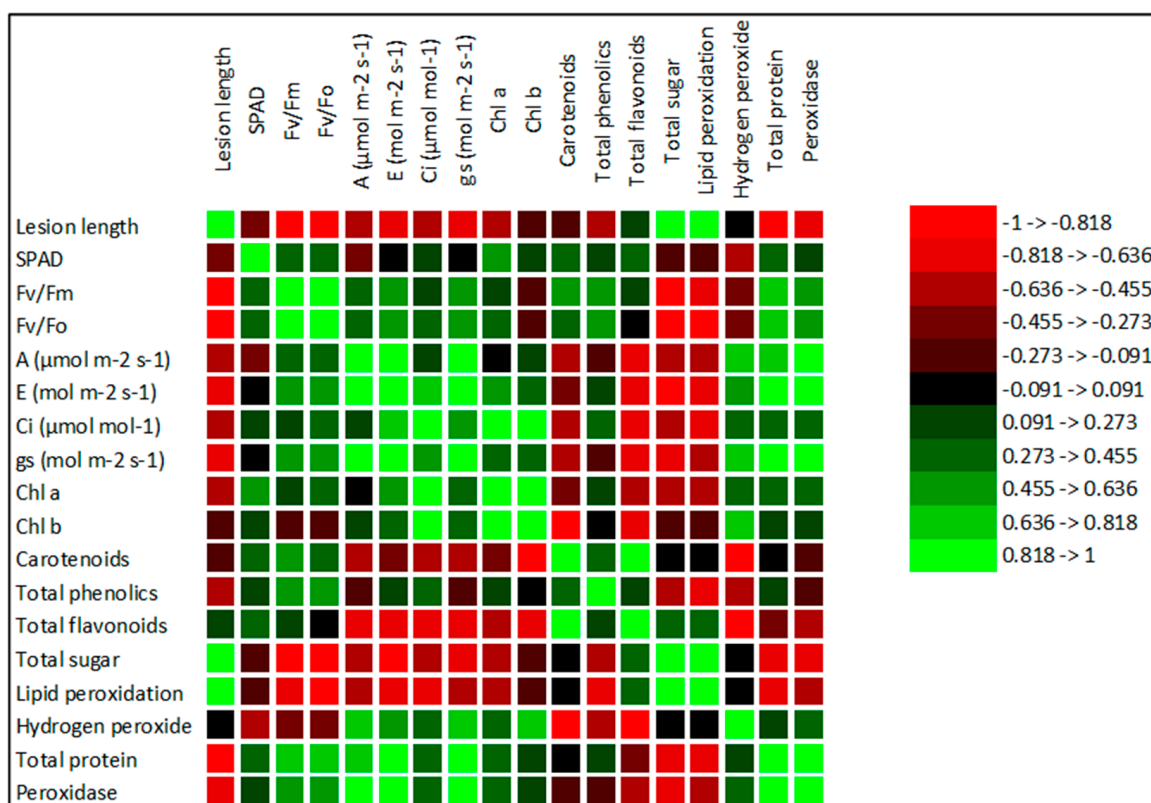


Figure 9. A heatmap showing the response of grape plants to application of PA, fungicide, and *B. cinerea* inoculation in a correlation matrix of the individual traits observed PDA plugs alone (T1), untreated plants with Botrytis alone (T2), 2% PA-treated plants with Botrytis (T3), 4% PA-treated plants with Botrytis (T4), and Switch[®]-treated plants with Botrytis (T5). The red color represents a strong negative association, and the green color represents a strong positive association.

To further assess the relationship between the treatments and the physiological and chemical parameters, a principal component analysis (PCA) was performed (Figure 10). The first and second principal components (PC1 and PC2) accounted for approximately 50% and 28%, respectively, explaining approximately 79% of the total variations in the dataset.

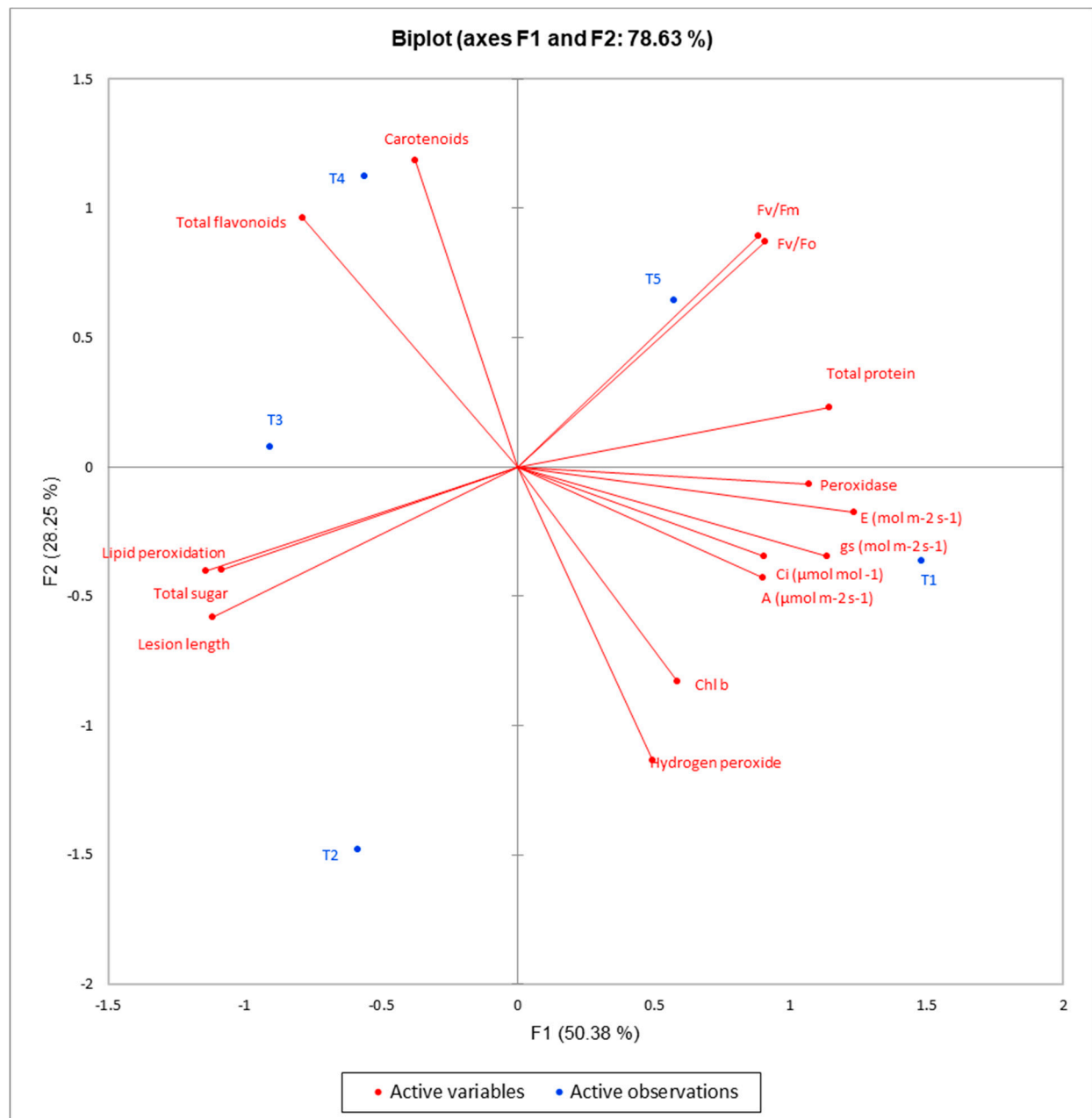


Figure 10. A two-dimensional (2-D) principal component analysis (PCA) showing relationships among the applications of PA, fungicide, and *B. cinerea* inoculations to grape plants. F_v/F_m , maximum quantum efficiency; F_v/F_o , potential photosynthetic capacity; A, photosynthetic rate; E, transpiration rate; C_i , Sub-stomatal CO_2 ; g_s , stomatal conductance; chl *a*, *b*, chlorophylls *a* and *b*. PDA plugs alone (T1), untreated plants with Botrytis alone (T2), 2% PA-treated plants with Botrytis (T3), 4% PA-treated plants with Botrytis (T4), and Switch[®]-treated plants with Botrytis (T5).

The parameters that were clustered together suggested positive relationships, while parameters that separated suggested a negative relationship. Parameters located at the right-angle suggested no relationship. Parameters assembled with a treatment indicated that the treatment influenced them, while parameters that were distant from a treatment indicated that the treatment did not influence them. The PCA biplot revealed that PDA plugs alone had an influence on photosynthetic rate, transpiration rate, sub-stomatal CO_2 , stomatal conductance, hydrogen peroxide, total protein, chl *b*, and peroxidase enzyme activity. Switch[®]-treated plants with Botrytis influenced F_v/F_m and F_v/F_o . The PA treatments

influenced total carotenoids and total flavonoids. In addition, Botrytis alone showed an influence on total sugar, lipid peroxidation, and lesion length (Figure 10).

4. Discussion

The study effectively demonstrates that pyroligneous acid (PA) inhibits *Botrytis cinerea* growth and reduces its impact on grapevines. It also provides valuable insights into the plant's physiological and biochemical responses to both the pathogen and PA treatment. This research contributes to our understanding of potential natural fungicides and plant defense mechanisms against *B. cinerea*, a significant pathogen in viticulture.

Many researchers have identified PA as antibacterial and antifungal agent due to its chemical composition. For instance, Wei et al. [39] tested PA from walnut branches against various plant pathogens, such as *Valsa mali*, *Colletotrichum orbiculare*, *Phytophthora infestans*, *Phytophthora capsica*, *Cochliobolus sativus*, and *Helminthosporium sativum*. They showed that PA inhibited these pathogens. Chen et al. [40] tested PA from *Eucommia ulmoides* against *B. cinerea* using the plate method and table grape fruits, and found that PA at dilutions lower than 1:200 can inhibit fungal mycelium growth. Pertile and Frac [14] used the plate method and found that 1:1600 dilution of PA affected the metabolic profiles of *B. cinerea*. Liu et al. [24] also used the plate method to examine the antifungal effect of PA from bamboo against *B. cinerea* in apple fruits. In the present study, PA from white pine inhibited *B. cinerea* mycelium growth at 2% concentration, as in a preliminary study. These different outcomes can be attributed to the quantity and type of molecules characterizing the PAs used due to the origin of the feedstocks and, possibly, manufacturing conditions such as temperature [15,18]. This preliminary study using the plate method has been reported by a few researchers; hence, our main focus was to investigate the effects of PA on *B. cinerea* in grapevines.

In the present study, a known fungicide (Switch®) and varying concentrations of PA were tested for efficacy against *B. cinerea* lesion development in grape seedlings. The most consistent reduction in lesion growth was obtained from Switch®. Switch® is a synthetic fungicide known to control Botrytis [41]. Pyroligneous acid is organic and environmentally friendly, with the ability to control Botrytis, and may be preferable to synthetic fungicides for reducing chemical residues in the environment [26].

This result is particularly promising, as it indicates that pyroligneous acid's antifungal activity observed in vitro could be translated effectively to real-world applications in living plant tissues. Thus, the differences obtained from the efficacy test present a great opportunity to further examine the physiological and biochemical responses of the grape seedlings challenged with *B. cinerea*. Plants depend on their internal immune systems to recognize and respond to biotic stimuli [42]. During plants' disease defenses, the physiological and biochemical conditions of the plants change accordingly [43,44]. In the present study, the stomatal conductance significantly ($p < 0.05$) influenced CO₂ assimilation and transpiration rates. Thus, the lower the CO₂ assimilation and transpiration rates, the more the stomatal conductance decreases in response to the treatment. Changes in stomatal conductance can affect the intercellular CO₂ concentration, which, in turn, impacts the carboxylation efficiency and overall photosynthetic rate [45], which agrees with our findings in this study. In addition, the reduction in the stomatal conductance may be triggered as stress response in the grape seedlings, leading to stomatal closure as a protective mechanism [46]. F_v/F_m ratios decreased, suggesting damage to the photosynthetic apparatus under stress. This reduction in photosynthetic efficiency is often preceded by a decline in chlorophyll content, especially of PSII [47]. This matches what we found in our study, except that the 4% PA with Botrytis non-significantly ($p > 0.05$) increased the chlorophyll content of the treated plants. The increase in chlorophyll production by the 4% PA with Botrytis may be a mechanism

for protective response. PA may stimulate chlorophyll biosynthesis or protect existing chlorophyll from degradation under stress conditions.

Plants have evolved complex defense mechanisms to respond to pathogen attacks, which often involve alterations in the levels of various metabolites including chlorophylls, carotenoids, phenolics, flavonoids, and sugars [48,49]. In this study, Botrytis alone produced the highest chl *a*, *b*, and soluble sugar compared to the other treatments. The reduction in the chlorophylls with the PA and fungicide treatments could be attributed to the ability of these treatments to degrade the chlorophylls in the leaves or inhibiting chlorophyll biosynthesis [50–52]. The upregulation of the soluble sugar could be attributed to *B. cinerea* impairing carbohydrate metabolism [53]. These treatments, on the other hand, significantly ($p < 0.05$) increased the total carotenoids, phenolics, and flavonoids. These antioxidants helped to scavenge ROS produced during the infection process [48,54].

The change in the MDA amount was consistent with the degree of damage caused by the pathogen in the grape leaves. Based on the results, the lower lipid peroxidation observed in the Switch[®]-treated plants with *B. cinerea* fungal plugs was most effective. Malondialdehyde (MDA) is a key byproduct of lipid peroxidation and serves as a widely used biomarker to assess the extent of lipid peroxidation and, by extension, the degree of membrane damage [55]. In this study, the amount of hydrogen peroxide was higher in untreated plants with PDA plugs alone and Botrytis alone. Hydrogen peroxide is, indeed, a potent signaling molecule in plants that plays a crucial role in regulating growth, metabolism, and stress responses [56]. It is produced routinely in both stressed and non-stressed conditions through various mechanisms, primarily via the dismutation of superoxide radicals by superoxide dismutase (SOD) during electron transport in different cellular compartments [57–59]. Nurnaeimah et al. [60] reported that hydrogen peroxide can also act as a potent signaling molecule that mediates various physiological and biochemical processes in plants. Hydrogen peroxide in this study had positive correlations with photosynthetic rate, transpiration rate, sub-stomatal CO₂, stomatal conductance, chl *a* and *b*, total protein, and peroxidase enzyme activity. The total protein and peroxidase enzyme activity were high with untreated plants with PDA plugs alone and were reduced per the level of leaf damage [61]. These biochemical responses could serve as useful markers for the development of *B. cinerea*-resistant grapevines. The effective application of key metabolite-based markers can facilitate the identification of candidate genes associated with disease resistance. The study provides valuable insights into the biochemical response mechanisms used by grapevines to combat pathogens such as *B. cinerea*, emphasizing the integration of genomics and metabolomics for the sustainable development of pathogen-resistant cultivars [48,49].

5. Conclusions

The in vitro assays in our preliminary study demonstrated that PA concentrations ranging from 0.25% to 4% effectively inhibited the growth of *B. cinerea*, with higher concentrations of >2% exhibiting enhanced antifungal activity. These findings were further supported by in vivo assessments, which also revealed that both 2% and 4% PA treatments remarkably decreased the average lesion length on grapevines, with a clear trend showing that greater PA concentrations resulted in shorter lesions. The findings of this study have significant implications for sustainable viticulture practices. The ability of PA to control *B. cinerea* infection while simultaneously improving plant physiological parameters suggests that it could be an effective tool in integrated pest management strategies. Its natural origin and potential for sustainable production make it an attractive alternative to synthetic fungicides. It addresses concerns about environmental impact and development of resistance against synthetic chemical fungicides under the current dispensation of climate change. Further molecular studies are recommended to elucidate the mechanisms underlying the

observed physiological and biochemical changes. The downregulation of chlorophyll content caused by PA also requires further studies to evaluate the impact of PA on *Vitis* crops in the field.

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