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Induced Defense in Avocado Fruits Mediated by Secondary Metabolites Produced by *Bacillus atrophaeus* B5

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Abstract: Biocontrol agents are an environmentally friendly alternative to chemical pesticides against phytopathogens. The effectiveness of metabolites produced by *Bacillus atrophaeus* B5 for controlling anthracnose produced by *Colletotrichum gloeosporioides* in harvested fruit and the possible action mechanisms have been studied. Avocado fruit treated with metabolites reduced 54% of anthracnose incidence and nearly 70% of the lesion than untreated fruit. The treatment enhanced the activities of peroxidase, polyphenol oxidase, and phenylalanine ammonia-lyase. Additionally, the transcription of *PAL* and *EIN3*-like genes related to defense were increased. These results suggest that metabolites produced by *B. atrophaeus* B5 enhance disease resistance against *C. gloeosporioides* in avocado fruit. In the fruit, the induced resistance is related to the *priming* of defense responses. Metabolites in the cell-free supernatant could also be a potential biological preservative for avocado protection.

Keywords: bacterial metabolites; gene expression; resistance induction; *priming*

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1. Introduction

Avocado (*Persea americana*) production has increased over the years. Avocado fruit is a food of functional and high nutritional value. In Mexico, this crop has a great economic impact [1]. Nevertheless, postharvest losses mainly caused by the incidence of fungal diseases reduce its consumption quality and shelf life. Several pathogens, including fungi such as *Colletotrichum* spp., attack the avocado crop. The phytopathogen *C. gloeosporioides* causes anthracnose in avocado fruit; this disease manifests during all stages of fruit development, causing economic losses of up to 20% of production [2]. As fungi control strategies, fungicides have been implemented because they are effective; nevertheless, they cause toxic effects in humans, animals, and the environment. For this reason, reducing their use and developing new innocuous strategies are essential [3].

Using non-pathogenic beneficial bacteria, such as plant-growth-promoting bacteria (PGPB), is considered an alternative or complement to pesticides for fungal plant disease management [4]. PGPB can promote plant growth, the induction of disease resistance, and abiotic stress tolerance, among others [5]. The rhizobacterium *Bacillus atrophaeus* has emerged as a new biocontrol agent against phytopathogenic fungi. Several *B. atrophaeus* strains have been applied to suppress the diseases of plants produced by *Colletotrichum* spp., *Botrytis cinerea*, *Fusarium* spp., and *Alternaria alternata* [6–10].

Bacillus atrophaeus strain B5 induces disease resistance and inhibits the anthracnose and Alternaria rot disease caused by *C. gloeosporioides* and *A. alternata*, respectively. *B. atrophaeus* B5 produces antifungal metabolites, which are contained in a cell-free supernatant (CFS) obtained from culture. The CFS applied as a preventive treatment on harvested soursop, avocado, and tomato fruit effectively suppress fungal infections [8,9]. According to this, it was suggested that disease resistance is associated with an enhanced capacity to trigger

defense responses in fruit. However, more information is needed about how *B. atrophaeus* B5 controls the disease in postharvest fruits.

This study aims to determine whether the disease resistance against anthracnose, mediated by *B. atrophaeus* B5 metabolites, is associated with *priming* defense responses in harvested avocado fruit. Additionally, we assessed the effects of the CFS on the quality and storability of avocado fruits. Metabolites synthesized by bacteria are gaining interest because of their potential to provide quality and safety benefits. The metabolites produced by *B. atrophaeus* B5 induce resistance against anthracnose disease in avocado fruit and are linked with *priming* defense responses. *B. atrophaeus* B5 metabolites showed potential in the safety of avocado management.

2. Materials and Methods

2.1. Microorganisms and Fruit Material

Bacillus atrophaeus B5 and the virulent strain *Colletotrichum gloeosporioides* used in this study were provided by the Biotechnology Laboratory of the Technological Institute of Tepic, Tepic, Mexico. Strain B5 was cultured on King agar B (KAB) at 25 °C for 24 h. *C. gloeosporioides* was grown on potato dextrose agar (PDA) at 28 °C for 7 days. Avocado fruits (*Persea americana*) were hand-harvested in a physiologically ripe state from orchards in Santa Maria del Oro, Nayarit, Mexico. Fruits of a similar size and lacking fungal diseases or mechanical injuries were selected.

2.2. Preparation of Suspension of Fungal-Spore-Cell-Free Supernatant

Colletotrichum gloeosporioides was cultured to prepare the fungal spore suspensions on a PDA plate for 7 days at 28 °C. After that, Petri plates were rinsed with sterile water, and the surface was scraped with a sterile glass rod. The liquid was filtered to remove the mycelia with degreasing cotton. The fungal spore suspension was adjusted to 1×10^6 spores/mL by microscopic counting in a Neubauer hemocytometer (Marienfeld, Lauda-Königshofen, Germany).

The bacterial cells were grown in King broth (KB) medium at 25 °C and shaken at 200 rpm for 24 h. The CFS was obtained by centrifugation of bacterial culture at 5000× *g* for 20 min at 20 °C, followed by filtering with a sterilized 0.22 µm pore size filter (Millipore Corp., Bedford, MA, USA).

2.3. Effect of CFS on Mycelial Growth

Assays for mycelial growth inhibition were made. The PDA medium containing CFS, a concentration of 20% (*v*/*v*), was inoculated with 7-day-old mycelia disks (5 mm diameter) at the center and was incubated at 28 °C. As a control, plates containing PDA without CFS were included. After incubation, the radial mycelial growth of *C. gloeosporioides* (*T*) was measured using a Truper Vernier caliper until the fungal growth in the control dishes (*C*) was almost complete. The inhibition of the radial growth of the fungal colony was evaluated daily, and the percentage of growth inhibition (*I*) was calculated using the following formula: $I\% = [(C-T/C)] \times 100$. Tests were carried out in triplicate.

2.4. Efficacy of CFS for Control of Avocado Anthracnose

The surface of the fruit was disinfected by immersion for 1 min in 1% NaOCl, rinsed with distilled water, and allowed to air-dry at 25 °C. The avocado fruit was soaked in the CFS for 1 min to induce disease resistance, taken out, and placed in trays at 25 °C for 2 h.

As controls, fruits were soaked in KB medium or distilled water. Later, the treated fruits were separated into six groups. All groups were wounded nine times at three sides of each avocado fruit with a cylindrical punch of 3 mm in diameter and 3 mm deep. The first, second, and third groups, treated with CFS, water or KB medium, respectively, were not inoculated with the spore suspension. The fourth, fifth, and sixth groups, treated with CFS, water, or KB medium, respectively, were inoculated with 15 µL of the spore suspension (1×10^6 spores/mL) using a sterile needle. The fruits were then incubated at 25 °C for 9 days.

The experiment was conducted twice, and each treatment comprised three replicates of ten fruits. The lesion diameter on each fruit wound was recorded at 3-day intervals during incubation. Additionally, the disease incidence (%) in each treatment was measured by the ratio between the number of symptomatic injuries and total injuries in the fruit.

In each replicate, fruit pulp tissue at 10 mm below the surface and 10 mm from the edge of the inoculated lesion were collected from five fruits. Tissue samples were mixed and immediately stored at −80 °C until used for protein measurements, enzyme assays, and transcriptional analysis.

2.5. Assay of Enzyme Activity

Enzyme extractions were made, as previously reported by Wang et al. [11]. Sample tissues were ground with 10 mL of ice-cold extraction buffer and 0.2 g polyvinylpolypyrrolidone with a mortar. For peroxidase, (POD, EC 1.11.1.7) and polyphenol oxidase (PPO, EC 1.10.3.1) assays, an extraction buffer (100 mM sodium acetate buffer; pH 6.4) was used. For phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), an extraction buffer (10 mM borax-borate, 5 mM β-mercaptoethanol, 2 mM EDTA; pH 8.8) was used. The supernatants were obtained by centrifugation of the homogenates at 11,000× *g* for 15 min at 4 °C, and then were used for enzyme assays. The determination of the protein content in the enzyme extracts was made by the Bradford method [12]. As standard, bovine serum albumin was used.

POD activity was measured at 30 °C and determined by monitoring the increased absorbance rate at 470 nm according to the methodology described by Tian et al. [12]. One unit of POD activity is defined as the amount of enzyme required to cause an increase in absorbance of 0.01 at 470 nm per minute.

PPO activity was evaluated as previously described by Tian et al. [12]. The quantity of enzyme required to raise the absorbance 0.01 at 420 nm per minute defines one unit of PPO activity.

PAL activity was determined according to the methodology described by Sellamuthu et al. [13]. One unit of PAL activity is defined as the amount of enzyme required to rise in absorbance of 0.01 at 290 nm in 1 h.

2.6. Analysis of the Expression of Defense-Related Genes

To investigate if *B. atrophaeus* B5-induced disease resistance against anthracnose is associated with the *priming* of defense responses in avocado fruit, tissue samples were collected from five fruits. Fruit tissue treated with water (control) or the CFS, uninoculated or inoculated with *C. gloeosporioides*, was used to evaluate the transcription profiling of genes related to defense in avocado fruit. Samples were collected before pathogen inoculation at 1 and 2 h after infection. Three defense genes were selected: the non-expression of pathogenesis-related genes 1 (*NPR1*), phenylalanine ammonia-lyase (*PAL*), and ethylene-insensitive 3 (*EIN3*-like). The housekeeping *ACT* gene, which codes for actin, was used as a reference.

Total RNA was extracted from avocado fruit as previously described by Healey et al. [14], with some adjustments. RT-PCR was achieved using 100 ng of DNA-free RNA and SuperScriptTM III Reverse Transcriptase (Invitrogen®). To design gene-specific primer pairs, Primer 5.0 software was employed. Nucleotide sequences were obtained from the GENBANK database, namely *NPR1*-like (GenBank ID: KR056089.1), *PAL* (GenBank ID: U16130.1), *EIN3*-like (GenBank ID: EU586509.1), and Actin (GeneBank ID: GU272027.1). The primer sequences used for RT-PCR analysis are depicted in Table 1.

The controls used for each RT-PCR reaction were as follows: (i) to verify the absence of DNA, PCR without the reverse transcription step was made; (ii) RT-PCR was performed without RNA templates to detect any contaminating DNA/RNA; (iii) to ensure primer fidelity, PCR performed using chromosomal DNA as a template was achieved; and (iv) amplification of a portion of the actin gene as an internal control was performed.

The RT reactions were conducted at 50 °C for 40 min; after that, PCR amplification was made at 94 °C for 5 min for 1 cycle; 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min

30 s for 30 cycles; and 72 °C for 10 min for 1 cycle. Each amplification reaction (10 µL) was analyzed by electrophoresis using 1.0% agarose gel, GelRed™ staining, and ultraviolet visualization.

Table 1. Primer sequences used for transcriptional analysis.

Primer Name	Primer Sequence	Gene
Npr1 Fw Npr1 Rv	GTGGAAAGGTGAGACAGCCGCCTG CGACATCATCAGAATCAAGAGCCCTG	*NPR1*
Pal Fw Pal Rv	CCTTGCACTTGTCAACGGCACTG GCTCCCTTGAACCCATAATCCAAGC	*PAL*
Ein3 Fw Ein3 Rv	GATTATGCAGCACTGTGACCCACCG GCCTTGGCTGTCATTTTGTCTTGG	*EIN3*
Act Fw Act Rv	CCAAAAGCCAACAGGGAGAAGATGAC ATCAGCAATGCCTGGGAACATGG	*ACT*

2.7. Semiquantitative Analysis of Gene Expression

Semiquantitative analyses measured the integrated density values (pixels) from the PCR products deposited on the gel after electrophoresis. The integrated density values of PCR products in the electrophoresis gel pictured were measured using ImageJ software 1.54d [15]. *ACT* gene-density quantification was used to normalize the intensity of the bands of the genes of interest. The expression levels of *NPR1*, *PAL*, and *EIN3*-like in inoculated and uninoculated avocado samples were calculated, a comparison was made between the control samples and those treated with CFS, and the relative accumulation of transcripts was determined by calculating the percentage difference in the normalized band intensity between the control.

2.8. Effects of Metabolites on Storage Quality of Avocado Fruit

An assessment of quality attributes, such as color, physiological weight loss, firmness, titratable acid, pH, and total soluble solids, was carried out. For each determination, avocado fruits were separated into two groups comprising fifteen fruits. As described above, one group was induced with CFS, and the other group was treated with water as a control. All treated fruits were air-dried at room temperature (25 °C). Later, they were kept at 25 °C and 50–65% relative humidity. Measurements at 1, 3, 6, and 9 days were made to assess the effects of *B. atrophaeus* B5 CFS on the fruit quality of avocados in a normal shelf-life condition. The evaluation was made twice with three replicates (three fruits for one replicate).

The color of the internal mesocarp (pulp color) was assessed at two different points of the fruit. A digital colorimeter NH300 was used, and the fruit color was recorded as Hue angle (°Hue).

Physiological weight loss was assessed according to the technique reported by Kassim and Workneh [16]. Measurements were achieved at 0, 1, 3, 6, and 9 days of storage, and four fruits were used per treatment. The difference between the initial weight and the weight at the measurement time was recorded. The percentage of physiological loss in weight was calculated using the following formula: % Weight loss = (initial weight − final weight)/initial weight × 100.

Flesh firmness was analyzed in the middle part of the fruit using a manual penetrometer (Fruit Test, Model FT, Wagner Instrument, Greenwich, CT, USA) and applying the two-point penetration test. The maximum force (Kgf) for penetration into the fruit determined the firmness of the fruit. Three readings were taken to measure the firmness of each fruit.

pH was determined as previously proposed [16]. To this end, 10 g of homogenized avocado pulp was diluted in 90 mL of distilled water, and a pH meter was used.

Titratable acid (TA) was assayed. As an indicator, three drops of phenolphthalein were added to 35 mL of the mixture (10 g of the avocado puree diluted with 90 mL of distilled water). Later, it was titrated with 0.1 N sodium hydroxide (NaOH) until a pink color was shaped and persevered. TA was expressed as a percentage of total acidity.

The content of total soluble solids (TSSs) on fruit flesh was evaluated using a refractometer (Atago, Master-53M, Tokyo, Japan) and expressed as °Brix.

2.9. Statistical Analysis

The results are shown as mean values ± standard errors. In each assay, n = 10 for each group. The means and standard deviations were calculated. They were statistically analyzed by ANOVA and Tukey's test for least significant differences. Results showing differences at $p < 0.05$ were considered statistically significant. Data were processed using Statistica software 12.0 (Statistica, Tulsa, OK, USA, SAD).

3. Results

3.1. Suppression of Mycelial Growth by CFS

Test inhibition assays were conducted to analyze the antifungal activity of extracellular bacterial metabolites contained in the CFS. The medium PDA supplemented with 20% CFS could inhibit 30.61% of mycelia growth (Figure 1a). These results revealed that *B. atrophaeus* B5 synthesizes antifungal metabolites secreted to the supernatant, which controlled the growth of *C. gloeosporioides* in vitro.

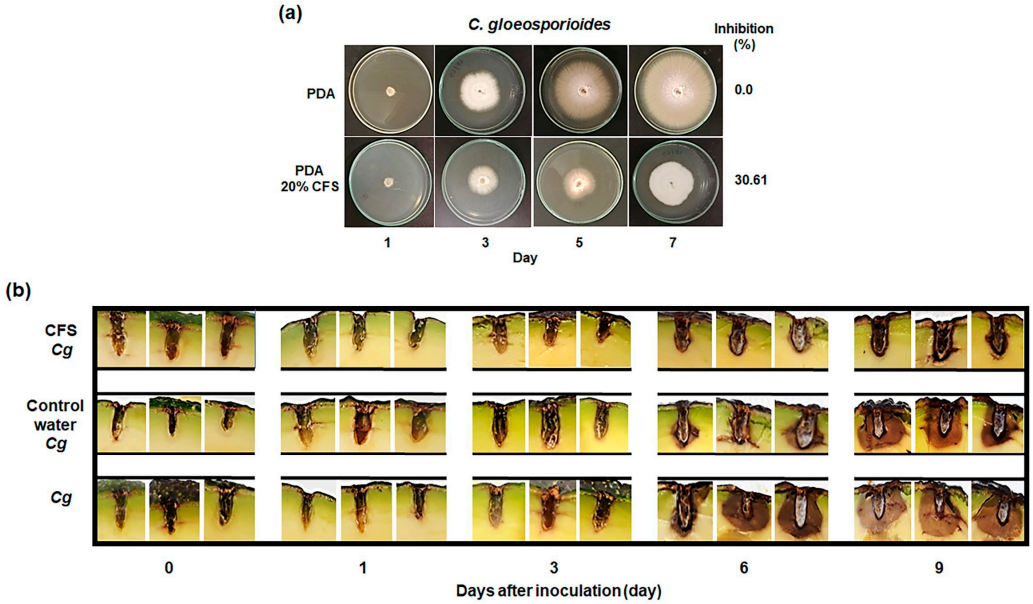

Figure 1. Effect of metabolites contained in the CFS against *C. gloeosporioides*. (**a**) Effect of CFS on mycelial growth. Inhibition (%) is indicated at the right of Figure (**b**), which shows CFS treatment against anthracnose disease in avocados. Treatments with *C. gloeosporioides* (Cg) inoculation are shown.

3.2. Biocontrol Effects of CFS from B. atrophaeus B5 on Anthracnose in Avocado Fruit

The efficacy of the metabolites in the CFS of *B. atrophaeus* B5 was confirmed by the diminution of anthracnose disease in avocado fruits. As controls, treatments with KB or distilled water were made; no significant difference between KB or water was observed. Therefore, water treatment was selected as a control in all assays. In untreated avocado fruits and those treated with water, pathogen hyphae caused dark-colored rot, distinctive of anthracnose. In contrast, CFS treatment restricted the development of *C. gloeosporioides* in vivo; consequently, the disease was reduced (Figure 1b).

This effect was more evident when the diameter of the lesion was assessed. At 9 days, the mean lesion diameter was 3.3 cm for the control and 1.02 cm for fruits treated with

CFS, representing nearly 70% of the lesion reduction (Figure 2a). While all the inoculated wounds in both the control and CFS-treated fruit developed anthracnose symptoms after 6 days of infection, and the diameter of the lesion in all CFS-treated fruit was consistently lower than in control (Figures 1b and 2a). Disease incidence was also evaluated, and we observed that CFS treatment reduced 54% of anthracnose incidence at 9 days post-inoculation (Figure 2b). These results indicate that *B. atrophaeus* B5 produces metabolites that restrict pathogen development and reduce the symptoms of anthracnose disease in avocado fruit.

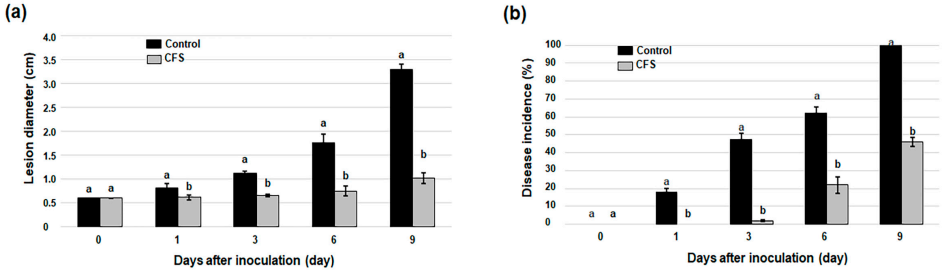

Figure 2. Effect of CFS in avocado fruit infected with *C. gloeosporioides*. (**a**) Lesion diameter; (**b**) disease incidence. $n = 10$ for each group. Means in each bar followed by equal letters are not significantly different consistent with Tukey's test ($p < 0.05$).

3.3. Effects of CFS Treatment on PPO, POD, and PAL Activities

The activity of defense-related enzymes (PPO, POD, and PAL) was assessed on fruit treated with CFS. The enzyme activity was evaluated post-treatment with CFS (IT: induction treatment) at 1, 3, 6, and 9 days post-*C. gloeosporioides* inoculation (days PI). The enzyme activities of PAL, PPO, and POD were evaluated in fruit treated with CFS before *C. gloeosporioides* inoculation (2 h-IT). Interestingly, CFS treatment increased the activities of PAL and POD compared to the uninoculated control (Figure 3). When *C. gloeosporioides* was inoculated on fruit treated with CFS, a rise in PAL, PPO, and POD activities was observed (Figure 3). The highest activity for POD and PPO was at 1 day PI, and for PAL, it was at 3 day PI. In all cases, this increased activity decreased during incubation; however, a higher level was always maintained compared to the control. These results demonstrate that *B. atrophaeus* B5 synthesizes metabolites that trigger the activities of defense-related enzymes.

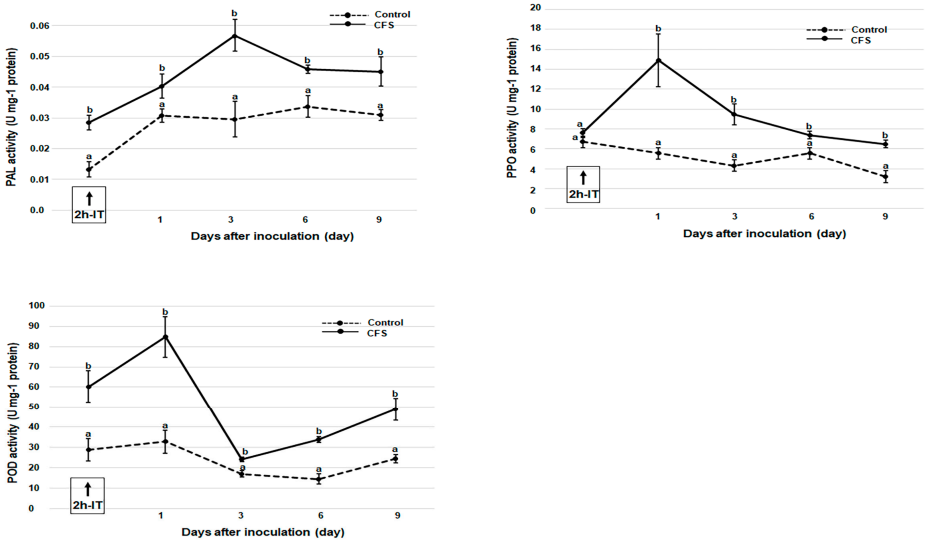

Figure 3. Effect of CFS on PAL, PPO, and POD enzyme activities. Equal letters are not significantly different consistent with Tukey's test ($p < 0.05$). Data are presented as the mean values of three repetitions and the standard error.

3.4. In Fruit, CFS Treatment Induced Higher Expression of Genes Related to Defense

The expression of defense-related genes induced by metabolites contained in the CFS was evaluated. The timeline representation of the sampling is depicted in Figure 4a. The control of the expression assay (*ACT* gene) showed its constitutive transcription level. The transcription pattern was assessed at 2 h post-treatment with CFS (2 h IT) and 1 and 2 h post-*C. gloeosporioides* inoculation (1 h PI; 2 h PI). In the uninoculated control, *NPR1*, *PAL*, and *EIN3*-like gene transcripts were undetected at 2 h post-water treatment, as well as at 1 or 2 h PI. Interestingly, *B. atrophaeus* B5 metabolites contained in the CFS induced the transcription of genes *PAL* and *EIN3*-like at 2 h IT in avocado fruit uninoculated, and this expression was detected at 1 h PI and 2 h PI (Figure 4b).

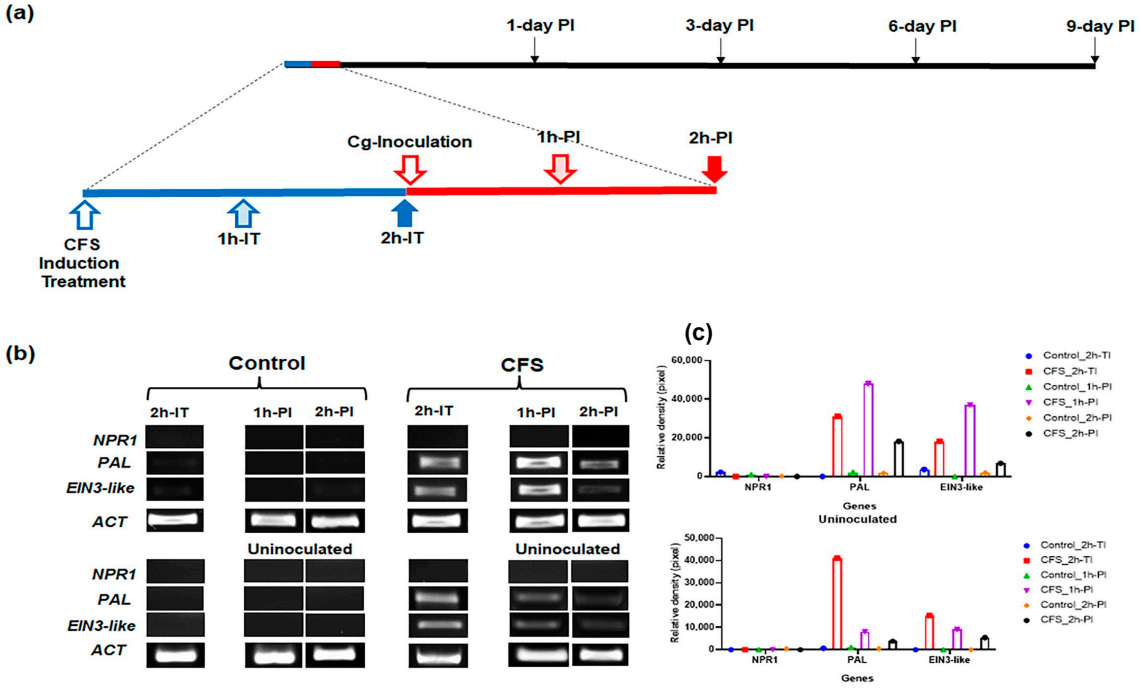

Figure 4. Expression pattern of representative genes related to defense in avocado fruit. (**a**) Timeline representation of the sampling; (**b**) expression pattern of *NPR1*, *PAL*, and *EIN3*-like genes in avocado fruit treated with CFS and control. h IT: hour post-treatment with CFS; h PI, post-*C. gloeosporioides* inoculation. Expression of the *ACT* gene was used as an internal transcription reference; (**c**) integrated density values of PCR products in the electrophoresis gel pictured with ImageJ software. The relative accumulation of transcripts was determined by calculating the percentage difference in the normalized band intensities.

When fruit treated with CFS was challenged with C. *gloeosporioides*, an apparent increase in the transcription of *PAL* and *EIN3*-like genes was detected at 1 h PI, compared to fruit only treated with CFS. This expression level decayed at 2 h PI. *NPR1* transcription was not detected in fruit treated with CFS or those inoculated with the pathogen (Figure 4b). Additionally, in this assay, we observed that C. *gloeosporioides* inoculation per se could not induce a clear expression of *NPR1*, *PAL*, or *EIN3*-like genes during the period evaluated.

ImageJ software 1.54d was used to determine the integrated density values from the PCR products in the electrophoresis gel pictured. The intensity of the bands was normalized using the *ACT* gene-density measurement. We observed that the inoculated and uninoculated controls had no detectable density of the *NPR1*, *PAL*, and *EIN3*-like genes. However, an increased relative density of the *PAL* and *EIN3*-like genes was observed in samples treated with CFS (Figure 4c). These results are consistent with those obtained in the expression analyses. According to this, metabolites synthesized by *B. atrophaeus* B5 enhance the transcription of *PAL* and *EIN3*-like genes in avocado fruit.

3.5. Effects of CFS on Storage Quality of Avocado Fruit

Six criteria were tested to assess the impact of CFS from *B. atrophaeus* B5 on avocado postharvest quality: pulp color, weight loss, firmness, titratable acidity, pH, and total soluble solids along storage days (Table 2). The results demonstrated that *B. atrophaeus* B5 metabolites do not affect the fruit-quality parameters evaluated, except for firmness, which was preserved in fruits treated with CFS at 9 days (Table 2).

Table 2. Effect of CFS treatment on fruit-quality parameters during storage.

Day	Pulp Color (°Hue)		Weight Loss (%)		Firmness (kgf)	
	Control	CFS	Control	CFS	Control	CFS
0	95.56 ± 4.30a	93.56 ± 4.30a	-	-	9.33 ± 0.28a	9.66 ± 0.76a
1	95.48 ± 3.25a	95.85 ± 2.13a	0.85 ± 0.65a	0.74 ± 0.60a	9.53 ± 0.15a	9.63 ± 0.15a
3	94.92 ± 2.77a	99.45 ± 5.46a	2.77 ± 0.38a	2.50 ± 0.92a	6.93 ± 0.40a	7.43 ± 0.15a
6	96.94 ± 3.70a	95.59 ± 3.78a	4.86 ± 0.73a	4.61 ± 1.16a	6.16 ± 0.92a	6.90 ± 0.18a
9	94.66 ± 1.46a	96.91 ± 1.63a	7.19 ± 0.97a	6.78 ± 1.76a	-	4.10 ± 0.33b
Day	**Titratable Acidity (%)**		**pH**		**TSS (°Brix)**	
	Control	CFS	Control	CFS	Control	CFS
0	0.04 ± 0.10a	0.04 ± 0.10a	6.96 ± 0.17a	6.96 ± 0.17a	8.00 ± 0.44a	8.00 ± 0.44a
1	0.04 ± 0.01a	0.04 ± 0.00a	7.42 ± 0.19a	7.24 ± 0.15a	6.66 ± 0.60a	7.25 ± 0.98a
3	0.07 ± 0.01a	0.09 ± 0.03a	7.08 ± 0.17a	6.86 ± 0.13a	8.05 ± 0.77a	7.75 ± 0.68a
6	0.10 ± 0.03a	0.09 ± 0.03a	6.94 ± 0.10a	6.83 ± 0.18a	8.50 ± 0.77a	8.50 ± 1.34a
9	0.11 ± 0.01a	0.10 ± 0.01a	6.89 ± 0.14a	6.82 ± 0.03a	8.00 ± 1.00a	7.58 ± 0.73a

Results were expressed as mean ± standard deviation. n = 15 for each group. Different letters represent significantly different scores ($p < 0.05$, Tukey's test). A dash (-) indicates not determinable.

4. Discussion

Chemical treatments used to control disease in the fruit provided good results; however, the residue of synthetic pesticides on fruit is one of the main worries of consumers and regulatory agencies. According to this, biocontrol strategies have arisen to reduce the use of chemicals, and now, they represent a suitable alternative to synthetic fungicides. Diverse approaches have been developed to offer new options to control phytopathogens. For example, plant metabolites have shown good antimicrobial activity [17,18]. Additionally, metabolites produced by antagonistic bacteria attracted particular interest because they can provide quality and safety benefits. Both strategies could be considered to achieve an integrated management program for sustainable disease control.

This study demonstrates that *Bacillus atrophaeus* B5 synthesizes metabolites that reduce mycelial growth in vitro and suppress *C. gloeosporioides* development in avocado fruit during postharvest. Furthermore, metabolites reduced the severity and incidence of anthracnose disease. In a previous study, preventive treatment with CFS from *B. atrophaeus* B5 suppressed anthracnose and Alternaria rot in soursop and tomato fruit [8,9]. Therefore, it has been proposed that the metabolites produced by the B5 strain induce the defense system of the fruits; however, the mechanisms by which this bacterial antagonist control postharvest diseases have yet to be completely elucidated.

The induction of disease resistance is proposed as a new strategy that could successfully prevent postharvest diseases by triggering the immune skill in fruits. Consequently, resistance against phytopathogens is enhanced [19]. Numerous biocontrol agents induce defense-related enzymes in harvested fruit, and this effect is correlated to disease resistance [20]. Several studies have shown that enzymes involved in the oxidation of phenols, such as POD and PPO, are the main enzymes related to defense reactions against pathogen infections [21]. POD contributes to the oxidation of phenols and cell-wall lignification of host-plant cells throughout the disease defense against pathogens. PPO catalyzes the oxidation of polyphenols into quinones and cell lignification in the plant through pathogen incursion. Furthermore, the PAL enzyme is key in the biosynthesis of numerous secondary compounds related to defense, such as lignin, phenols, phytoalexins, and several

metabolites associated with disease resistance in plants. PAL is the main enzyme in the phenylpropanoid pathway and catalyzes the synthesis of trans-cinnamic acid from the conversion of L-phenylalanine [21,22].

When *C. gloeosporioides* was inoculated on fruit treated with CFS, a rise in PAL, PPO, and POD activities was observed. These enzymes, as well as other defense-related enzymes, are frequently analyzed in postharvest biocontrol research because they are linked to induced resistance and the defense of plant hosts against fungal pathogens. PPO and POD are considered crucial enzymes linked to defense reactions against pathogen infections; both are involved in the lignification of host-plant cells [23], while PAL is related to localized disease resistance in plants because it is involved in the biosynthesis of lignin, phenolics, phytoalexins, and several compounds [24]. The induction of PAL, PPO, and POD mediated by biocontrol agents has been detected in harvested loquat, peach fruit and Chinese bayberry. It is associated with increased disease resistance and a reduction in disease severity [11,25–27]. In this study, we determined that metabolites contained in the CFS induced PAL, PPO, and POD activities and suppressed the anthracnose disease in avocado fruit challenged with *C. gloeosporioides* (Figures 1 and 3). These results suggest that postharvest treatment with CFS enhances resistance against anthracnose in avocado fruit.

It has been proposed that protection against diseases is based on the activation of defenses. Increasing evidence indicates that a common feature among different types of induced resistance in plants and fruit during the postharvest stage was observed, and this feature is named *priming* [20]. We analyzed the expression patterns of defense-related genes to understand if CFS-induced resistance against anthracnose is related to the *priming* of defense responses in avocado fruit. Three defense genes, namely the *NPR1*, *PAL*, and *EIN3*-like genes, were chosen because NPR1 is a central regulator that induces the expression of PR genes and increases disease resistance [28]; PAL is a key enzyme involved in the secondary production metabolites related to plant defense, including several phenolic compounds [29]; and *EIN3*-like is a transcriptional factor that participates in the defense signaling pathway dependent on ethylene [30].

It is interesting to note that preventive treatment with CFS induced the transcription of *EIN3*-like and *PAL* genes in avocado fruit but not *NPR1*. When CFS-treated fruit was confronted with *C. gloeosporioides*, the expression of *EIN3*-like and *PAL* genes increased, compared with fruit treated with CFS only. Similar results were obtained for studies in loquat fruit, where the treatment with *B. cereus* AR156 and inoculation with *C. acutatum* induced higher expression of genes related to defense [31]. In loquat fruit, the *NPR1* expression was observed until 6 h post-inoculation but not at 3 h, like our results. The *priming* strategy could be a common feature of the resistance responses triggered by *Bacillus* metabolites. For example, *B. cereus* AR156 induces resistance by *priming* against Rhizopus rot in peach fruit [27]. *B. subtilis* UMAF6639 activates JA- and SA-dependent defense responses, promoting melon resistance to powdery mildew [32]. Volatile compounds produced by *B. subtilis* KA9 induce the expression of defense-related genes against *R. solanacearum* in chili [33]. In addition, *B. subtilis* ABS-S14 synthesizes cyclic lipopeptides, which primed the transcription of defense-related genes in citrus fruit by the ISR, enhancing the induced resistance against the disease caused by *Penicillium digitatum* [34]. In all cases, resistance induction is attributed to metabolites produced by these strains. According to our results, *B. atrophaeus* B5 also synthesizes metabolites that induce disease resistance against anthracnose disease in avocado fruit. This process is related to the *priming* of defense responses in avocado fruit during the postharvest stage.

Diverse studies suggest that antifungal compounds' production by bacteria antagonists induce the defense mechanisms and biologically control fruit diseases. Metabolites synthesized by *B. subtilis* trigger the mechanisms of ISR by producing iturin and fengycin [35]. Other species of *Bacillus*, such as *B. cereus* strain AR156, reduced the development of *Rhizopus stolonifer* and *C. acutatum* in harvested peach fruit and loquat, respectively [27,31]. Likewise, *B. atrophaeus* TE7, XW2, and JZB120050 strains effectively controlled *Cladosporium cladosporioides*, *C. gloeosporioides*, and *Botrytis cinerea* respectively [7,36,37]. Re-

cently, it was reported that iturin A could delay the deterioration and improve the quality of cherry-tomato fruit by inhibiting the growth of *Rhizopus stolonifer* and inducing disease resistance [38]. The genes *srfAA*, *bmyB*, and *ituC* are coded into the chromosome of *B. atrophaeus* B5, suggesting that this strain produces surfactin, bacillomycin, and iturin [8]. It is plausible that these metabolites could trigger the mechanisms of ISR in avocado fruit during postharvest; however, further investigations are required to describe the mechanisms underlying the *priming* of defense responses mediated by *B. atrophaeus* B5.

The CFS obtained from *B. atrophaeus* B5 culture can be used to prevent anthracnose in harvested fruit. Fruit quality after treatments determines if antagonistic bacteria have the potential for application. No matter how effective the antifungal ability is, the quality of the fruits is reduced and the storage period is shortened after CFS application. This study demonstrated that CFS treatment did not affect fruit qualities such as pulp color, weight loss, titratable acidity, pH, and total soluble solids. The firmness was preserved throughout the storage period. According to previous reports, *B. atrophaeus* TE7 suspension applied in mangoes had better maintenance of firmness, peel color, TSS, and titratable acidity [37]. Firmness is one of the most important physical characteristics to evaluate the progress of fruit softening; for this reason, metabolites contained in CFS induce disease resistance and could contribute to preserving harvested avocado quality.

5. Conclusions

Our results demonstrate that *B. atrophaeus* B5 synthesizes metabolites that suppress anthracnose caused by *C. gloeosporioides* in harvested avocado fruit. The mechanism involved in induced disease resistance is associated with *priming*, in which triggering the immune skill in fruits could successfully prevent postharvest diseases. Furthermore, CFS, per se, can be applied as a preventive treatment to avocado fruit to reduce the severity and incidence of fungal diseases, showing their potential in the safety management of the avocado industry.

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