

Hexanal-containing dip treatment enhances kiwifruit resistance to *Botrytis cinerea*

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ABSTRACT

This study evaluated the effectiveness of a hexanal-based enhanced freshness formulation (EFF), containing antioxidants such as geraniol, α -tocopherol and ascorbic acid, to inhibit the fungal decay caused by *Botrytis cinerea* in 'Y368' kiwifruit. *In vitro* and *in vivo* experiments using three treatments: control (untreated), 0.03 and 0.06 % (v/v) EFF were set up. In the *in vitro* experiment, mycelial growth and spore germination of *Botrytis cinerea* exposed to EFF were examined. In the *in vivo* experiment, disease incidence, lesion diameter, activity of defense-related enzymes (Phenylalanine Ammonia Lyase, Guaiacol Peroxidase, β -1,3-glucanase and Chitinase), total phenolics and flavonoids were evaluated. The results demonstrated that 0.03 and 0.06 % EFF treatments reduced mycelial growth of *Botrytis cinerea* by 41 and 89 %, respectively. The lesion diameter of the 0.03 and 0.06 % EFF-treated fruit was 28 and 76 % smaller than the control. Furthermore, EFF-treated fruit exhibited significantly higher activities of defense-related enzymes compared to the control, with total phenolic content increasing by 1.2 and 1.3-fold for the 0.03 and 0.06 % treatments, respectively. These findings provide compelling evidence regarding the efficacy of EFF in suppressing *Botrytis cinerea* and highlight the role of defense-related enzymes in enhancing the fruit's resistance to decay. Overall, the study underscores the potential of EFF as a valuable and practical method for controlling fungal decay in kiwifruit during postharvest storage.

1. Introduction

Grey mold caused by *Botrytis cinerea* (*B. cinerea*) is a necrotrophic pathogen that results in severe deterioration of postharvest fruit quality in kiwifruit [1]. Postharvest losses attributed to *B. cinerea* are estimated between 20 and 30 % of the total kiwifruit produce [2]. Furthermore, economic losses incurred by the fruit industry due to *B. cinerea* are valued at approximately 10–100 million United States dollars annually [3]. The traditional management of postharvest diseases in kiwifruit relies on the use of synthetic fungicides [4,5]. However, growing concerns regarding fungicide residues and resistant strains have spurred researchers and growers to explore safe and environmentally friendly alternatives for pathogen management during postharvest storage [6,7].

Maximum residue limit (MRL) regulations and thresholds exert considerable pressure on growers and exporters to develop alternative disinfectants. When the chemical residues on a fresh produce surpass the

MRLs of the importing country, it can lead to the costly recalls and/or destruction of the consignment, often borne by the grower or marketer, resulting in significant financial losses and reputational damage. Hence, it is imperative to investigate alternative strategies to control fungal decay during storage, with the goal of reducing reliance on fungicides. Among the alternative control methods investigated are the use of certain strains of biocontrol agents, such as antagonistic bacteria and yeasts [8–10]. However, the commercial adoption of biocontrol agents has been hindered by their inconsistent and unreliable performance, biosafety concerns, and their limited applicability in real-world field or supply chain settings [5,8,11].

Other alternative treatments, such as edible coatings [12], ultraviolet C irradiation [13], ozone treatment [14], heat treatment [15] and combination treatments, such as salicylic acid combined with cinnamon plant extract [16] and methyl jasmonate combined with *Bacillus altitudinis* strains [17], have been evaluated as substitutes for fungicides.

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However, the majority of these control strategies remain in the experimental phase or not yet adaptable to commercial applications [18]. The high costs of implementing alternative control strategies have posed challenges for their adoption within the kiwifruit industry [19], leading to the continued reliance on more cost-effective and easy-to-apply synthetic fungicides. This dependence can adversely affect producers exporting to international markets with stringent food safety standards.

Hexanal is a promising alternative to fungicides, effective in suppressing fungal pathogens by inducing defense responses in plants [20–22]. Hexanal is a naturally occurring C6 aldehyde volatile compound, formed from linoleic acid via the lipoxygenase pathway during lipid peroxidation in plants [23]. It has been approved by the United States Food and Drug Administration (FDA) as a generally-regarded-as-safe (GRAS) food additive, with an oral mammalian lethal dose (ORL-MAM LD₅₀) of 3700 mg kg⁻¹ [24]. Prior studies have demonstrated the capacity of hexanal to suppress *B. cinerea* [25,26]. However, these studies only focused on investigating the effect of hexanal as a vapor treatment. The main drawback of implementing vapor treatments is the requirement of specialized equipment and facilities, which involves high capital investment. This constraint makes it less feasible for packhouses with limited resources to implement this treatment approach.

One advantage of hexanal over other vapor treatment methods is its versatility, as it can also be applied as a dip treatment. This is because hexanal as a dip treatment is straightforward, as it does not require specialized equipment or controlled environments. However, it is worth noting that there is a scarcity of studies assessing the efficacy of hexanal as a dip treatment for inhibiting fungal growth and *B. cinerea*, specifically. Furthermore, in our extensive review of the literature, we have not found studies evaluating the efficacy of hexanal dip treatment in controlling fungal growth of *B. cinerea* in kiwifruit.

Given the growing interest in developing novel alternative preservation strategies that are cost-effective and environmentally friendly [6], alongside the pressing challenge of *B. cinerea*, this study investigates the efficacy of an aqueous hexanal-based enhanced freshness formulation (EFF) as a dip treatment for controlling fungal growth in kiwifruit. We hypothesize that this treatment will reduce fungal growth in inoculated fruit by enhancing resistance to *B. cinerea*, thereby supporting sustainable practices in the fruit industry.

2. Materials and methods

2.1. Fruit source and pathogen

Kiwifruit (*Actinidia chinensis*) cv. 'Y368' were harvested from Roselands farm, a commercial farm located in the Richmond area (Latitude: 29.9033°S, Longitude: 30.2397°E), KwaZulu-Natal Province, South Africa. Kiwifruits were harvested when the total soluble solid content was greater than 6.2 % [27]. Harvested fruit were immediately transported in a ventilated vehicle to the Postharvest Laboratory of the University of KwaZulu-Natal (UKZN), where fruit without blemishes, decay or physical damage were graded and selected for uniformity in size, then assigned to the respective postharvest treatments. Thereafter, *in vitro* and *in vivo* experiments were conducted. Isolates of *B. cinerea* from a pure culture prepared by single colony isolation, were obtained from the Plant Pathology Laboratory of UKZN. All chemicals used to conduct the experiments in this study were purchased from Sigma-Aldrich, South Africa.

2.2. Pathogen identification

The pure cultures were identified by molecular methods at Inqaba Biotechnical Industries (Pty) Ltd. Deoxyribonucleic acid (DNA) extraction was performed using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005). The Internal Transcribed Spacer (ITS) target regions were amplified using primers ITS1 (5'-

TCCGTAGTGTAACCTGCGG-3') and ITS4 (5'- TCCTCCGCTTATGATATGC-3'). The DNA extracted from the cultures was used for Polymerase chain reaction (PCR). Briefly, the PCR protocol was denaturation at 94 °C for 5 min, Annealing at 94 °C for 30 s, Elongation at 50 °C for 30 s, 68 °C for 1 min and 68 °C for 10 min. There were 35 cycles performed and held at 4 °C. The PCR Amplicon fragments were enzymically purified using the ExoSAP procedure (NEB M0293L; NEB M0371). The amplicons were then purified for sequencing using the Zymo Research ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050, and sequenced in the forward and reverse direction (Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) using the ABI 3730xl Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific). Molecular identification ascertained the pathogen as *B. cinerea*.

2.3. Preparation of hexanal stock formulations

The hexanal-based enhanced freshness formulation (EFF) included ingredients such as hexanal (10 mL), geraniol (10 mL), α -tocopherol (10 g), ascorbic acid (10 g) and Tween 20 (100 mL), dissolved in 10 % (v/v) ethanol. The stock solution was prepared according to the method described by Paliyath and Murr [28] and was used consistently for both *in vitro* and *in vivo* experiments. Ethanol was first mixed with Tween 20, followed by the sequential addition of hexanal, geraniol, α -tocopherol, and ascorbic acid. The mixture was stirred until a homogeneous solution was obtained.

2.4. In vitro experiment

2.4.1. Mycelial growth

The effect of EFF on the mycelial growth of *B. cinerea* was evaluated at two concentrations, 0.03 and 0.06 % (v/v) using the agar dilution method [29]. The EFF stock solution was added to warm potato dextrose agar (PDA) medium to obtain final hexanal concentrations of 0.03 and 0.06 % (v/v). Each concentration of the amended medium was dispensed into 90 mm petri dishes, with each dish containing 20 mL, and then left to solidify. Mycelia of the pathogen were cut from actively growing culture and placed at the center of the new petri plates and incubated at 23 °C. Each treatment consisted of three replications, with three petri dishes per replication. The mycelial growth was recorded after 3, 5 and 7 days. The efficacy of EFF was expressed as percent inhibition in mycelial growth over control as demonstrated in Eq. (1).

$$\text{Per cent inhibition over control} = C - T \frac{x 100}{C} \quad 1$$

Where C = mycelial growth in control plate (mm) and T = mycelial growth in EFF amended plate (mm). The experiment was repeated three times.

2.4.2. Spore germination

Spores were collected from the cultures of *B. cinerea* grown in PDA medium. Spore suspensions were prepared by carefully adding the spores in each of the two EFF solutions. Similarly, spores were suspended in sterile water for use as control. A 100 μ L aliquot of the spore suspension was placed on sterile slides and incubated in petri dishes containing water agar in order to maintain the required humidity for germination. The spores were incubated for 24 h at 23 °C. After the incubation period, conidial germination was observed under a microscope (Zeiss AX10, ZEISS Group, Oberkochen, Germany) at 40-times magnification. Thereafter, the germination count was recorded as spore germination (%). Inhibition rate of spore germination was calculated using Eq. 2

$$\text{Inhibition rate of spore germination (\%)} = [(T - G) / T] \times 100 \quad 2$$

where T and G are the total number of spores observed and the number of germinating spores, respectively.

2.4.3. Morphological changes in mycelium and spores induced by enhanced freshness formulation

The effect of EFF on the mycelial morphology and spores of *B. cinerea* (7 day old fungi) was observed under a scanning electron microscope (SEM) (Zeiss EVO LS 15, ZEISS Group, Oberkochen, Germany). The segments (2 × 2 mm) were cut from the PDA plates and immersed for 3 h in 3 % (v/v) glutaraldehyde for primary fixation. Thereafter, the segments were subjected to dehydration in a series of ethanol solutions, namely, 10, 30, 50, 70 and 90 % for 10 min, respectively, followed by further dehydration in 100 % ethanol for 10 min (repeated three times). Thereafter, the samples underwent critical point drying, which involved heating and pressurizing liquid carbon dioxide to its critical point using a critical point dryer (Quorum K850, Quorum Technologies, United Kingdom). Afterward, the dried samples were mounted on SEM stubs and transferred to a rotary pumped coater (Quorum Q150R ES, Quorum Technologies, United Kingdom). In the sputter coater, the samples were subjected to gold sputter coating to make the samples conductive to the electron beam. The specimens were viewed under a SEM.

2.5. In vivo experiment

2.5.1. Postharvest treatment and inoculation

The EFF stock solution was diluted with distilled water at ratios of 1:3330 and 1:1660 to provide final hexanal concentrations of 0.03 and 0.06 % (v/v), respectively. Fruit disinfection and inoculation were done according to Hyun et al. [30]. The inoculated fruit were divided at random into three groups for the following treatments: control (untreated) and immersion in an aqueous solution of 0.03 and 0.06 % (v/v) EFF for 5 min. For inoculation, kiwifruits were punctured (depth 3 mm) at the equator with an autoclaved straight dissecting needle, and 15 µL of *B. cinerea* spore suspension (1×10^5 spores mL⁻¹) was inoculated into each wound. All the fruit were air-dried and then stored for 7 days in polyethylene plastic bags at 23 °C, to maintain a high relative humidity of approximately 85–90 % [15]. The tissue surrounding the wound of inoculated fruit was collected from 27 fruits from each treatment group at 3, 5 and 7 days post inoculation (dpi) and analyzed for total phenolic and flavonoid compounds, as well as defense-related enzyme activities. Each treatment consisted of three replicates (n = 3); each replicate had three fruit per sampling interval and experiments were repeated three times.

2.5.2. Lesion diameter, disease incidence and index

Lesion diameter was measured by crossing method using only infected wounds. Disease incidence was calculated as the percentage of the fruit with apparent disease symptoms using Eq. (3). According to the area of rot on the fruit surface, the extent of disease severity was scored using the following scale: 0, no disease; 1, decay area <20 %; 2, decay area of 20 %–40 %; 3, decay area of 40 %–60 %; 4, decay area >60 %, and disease index was calculated using Eq. (4) as previously described by Ge et al. [15].

$$\text{Disease incidence} = \frac{\text{number of symptomatic fruit}}{\text{total number of fruit}} \times 100 \quad 3$$

$$\text{Disease index} = \sum \frac{\text{disease scale} \times \text{number of fruit at that scale}}{\text{total number of fruit} \times 4} \quad 4$$

2.5.3. Total phenolic and flavonoid compounds

The concentration of total phenolics was determined using a method described by Singleton et al. [31], where 1 g of tissue sample was homogenized with 5 mL of 80 % (v/v) methanol and centrifuged at 13 000 × g for 15 min at 4 °C. Thereafter, 2.6 mL of distilled water and 200 µL of Folin-Ciocalteu's phenol reagent was added to 200 µL of the methanolic extracts. After a 6-min incubation, 2 mL of 7 % (w/v) sodium carbonate was introduced to the reaction mixture. The absorbance was

measured at 750 nm after 90 min using a Shimadzu UV spectrophotometer (Model UV-1800 240V, Kyoto, Japan). The total phenolic content (TPC) was determined using gallic acid as a standard and expressed on a fresh weight basis as gallic acid equivalents (GAE) g kg⁻¹.

The concentration of total flavonoids was determined by adding 3.2 mL of distilled water and 150 µL of 5 % (w/v) sodium nitrite to 500 µL of methanolic extract. After 5 min, 150 µL of 10 % (w/v) aluminium chloride was added. After 6 min, 1 mL of 1 M sodium hydroxide was added, and the absorbance recorded at 510 nm. The total flavonoid content (TFC) was determined using catechin as a standard and expressed on a fresh weight basis as catechin equivalents (CE) g kg⁻¹.

2.5.4. Defence-related enzyme activities

2.5.4.1. Phenylalanine ammonia lyase (PAL, EC 4.3.1.5). PAL enzyme activity was assessed using a protocol previously described by Khan et al. [32]. One gram of fruit tissue was homogenized in 10 mL of ice-cooled Tris-HCl buffer (50 mM, pH 8.5) containing 5 % (w/v) polyvinylpyrrolidone (PVPP) and 14.4 mM β-mercaptoethanol. After centrifugation for 20 min at 10 000 × g at 4 °C, the supernatant was recovered. PAL activity was quantified using a reaction mixture comprising of 800 µL of Tris-HCl buffer (0.5 mM, pH 8.0), 100 µL 6 µM of L-phenylalanine, and 100 µL of enzyme extract. After incubating the mixture at 40 °C for 1 h, the reaction was stopped by adding 100 µL of 5 N HCl. The absorbance was measured at 290 nm using trans-cinnamic acid as a standard to calculate enzyme activity, with results expressed as U kg⁻¹ of protein.

2.5.4.2. Guaiacol peroxidase (POD, EC. 1.11.1.7). POD enzyme was extracted by homogenizing fruit tissue in 7 mL of 100 mM potassium phosphate buffer (pH 7.0) containing 1.0 mM EDTA, 5 % (w/v) polyvinyl pyrrolidone, and 1 % (v/v) Triton X-100 at 4 °C. The homogenate was centrifuged at 13 000 × g for 20 min at 4 °C. POD enzyme activity was assessed using a protocol previously described by Sripong et al. [13]. The reaction mixture consisted of 100 µL enzyme solution, 2.82 mL of 0.1 M sodium phosphate buffer (pH 7.0), 30 µL of 12.3 mM H₂O₂ and 50 µL of 20 mM guaiacol. The increase in absorbance at 470 nm due to the guaiacol oxidation was recorded for 2 min. One unit of enzymatic activity was defined as the amount of the enzyme that caused a change of 0.001 in absorbance per minute. The POD activity was determined using Beer Lambert's law: $A = \epsilon bC$ and expressed as U kg⁻¹ of protein, using 25 mM⁻¹ cm⁻¹ as the extinction coefficient, A = absorbance, ϵ = molar absorptivity, b = path length, C = concentration.

2.5.4.3. Chitinase (CHI, EC 3.2.1.14). Chitinase enzyme activity was assessed using a protocol previously described by Pombo et al. [33]. Briefly, 1 g of fruit tissue sample was homogenized in 3 mL solution consisting of 10 mmol L⁻¹ sodium acetate buffer and 2 % (w/v) polyvinylpyrrolidone, adjusted to pH 5. The homogenate was stirred for 3 h at 4 °C, thereafter, centrifuged at 12 000 × g for 30 min. The supernatant was recovered and used to analyze chitinase activity. The reaction mixture consisted of 739 µL of 0.2 % chitin azure (Sigma) and 2.21 mL of extract. After incubating the reaction mixture at 37 °C for 1 h, 178 µL of 2 N HCl was added and immersed in ice to stop the reaction. Chitinase activity was measured at 575 nm using N-acetylglucosamine as a standard and expressed as U kg⁻¹ of protein.

2.5.4.4. β-1,3-glucanase (GLU, EC 3.2.1.39). GLU enzyme activity was assessed using a protocol previously described by Zheng et al. [34]. One gram of fruit tissue was homogenized with 3 mL of 50 mM sodium acetate buffer (pH 5) and centrifuged at 17 000 × g for 30 min at 4 °C. The reaction mixture comprised of 100 µL of enzyme extract, and 50 µL of 0.4 % laminarin and incubated at 37 °C for an hour. Thereafter, 200 µL of 0.63 % 3,5-dinitrosalicylic acid was added and the reaction mixture was boiled for 5 min. The reaction was cooled, and the absorbance was

measured at 500 nm. β -1,3-glucanase activity was determined as the amount of reducing glucose released from laminarin using glucose as the standard. Enzyme activity was expressed as U kg^{-1} of protein, where a unit is defined as 1 mg of glucose released per minute under these assay conditions.

2.6. Protein concentration

The Bradford assay [35] was used to determine the protein concentration using bovine serum albumin as a standard and used to calculate the specific activity of defense-related enzymes.

2.7. Statistical analysis

All experiments were performed using a completely randomized factorial design, with two factors: treatments and storage period. Statistical analysis and principal component analysis (PCA) were performed in R software version 4.3.1 [36]. The data were subjected to analysis of variance (ANOVA) and mean values were compared using Duncan's multiple range test at 5 % level of significance. Results were expressed as mean $\pm$ standard error (SE) values. For PCA, factor scores and loadings were calculated to determine the contributions of each variable to the principal components. Eigenvalues were examined to assess the amount of variance explained, and components with eigenvalues greater than 1 were retained.

3. Results

3.1. Morphological changes in mycelium and spores induced by enhanced freshness formulation

The SEM images showed that the EFF treatment had an adverse effect on the morphology of mycelia and spores of *B. cinerea* (Fig. 1). The mycelia exposed to the 0.03 % EFF treatment exhibited altered hyphae, that were shriveled and twisted, with a damaged surface (Fig. 1 B). Moreover, the spores of *B. cinerea* exposed to the 0.03 % EFF treatment were collapsed and distorted (Fig. 1 D). Similar effects were observed at

the 0.06 % EFF treatment level (Supplementary material: Fig. 1C–F).

3.2. Mycelial growth and spore germination

The results showed that EFF effectively reduced the mycelial growth of *B. cinerea* (Table 1), with the 0.03 and 0.06 % treatment level exhibiting an inhibition rate of 41 and 89 %, respectively. Similarly, the EFF treatments proved effective at reducing spore germination, which was 1.6 and 3.7-folds lower for the 0.03 and 0.06 % treatment level, respectively. The 0.03 and 0.06 % EFF treatment level inhibited spore germination by 34 and 70 %, respectively.

3.3. Lesion diameter, disease incidence and index

The lesion diameter (Fig. 2 A) increased for all treatments; however, EFF substantially reduced the size of the lesion (Fig. 3A and B). At 7 dpi, the lesion diameter of treated fruit was approximately 28 and 76 % smaller for the 0.03 and 0.06 % EFF treatment level, respectively, compared to the control. Similarly, the disease incidence (Fig. 2 B) also increased as the storage time progressed. At 7 dpi, the disease incidence of 0.03 % treated fruit was equal to that of control fruit. The 0.06 % treatment level significantly lowered the disease incidence, recording a value of 42 % at 7 dpi. The disease index (Fig. 2C) was registered at 91,

Table 1

Influence of enhanced freshness formulation on the inhibition of mycelial growth and spore germination of *Botrytis cinerea*.

Treatment	Inhibition (%)	Spore germination (%)	Inhibition rate of spore germination (%)
Control	0 $\pm$ 0c	91 $\pm$ 1.6a	0 $\pm$ 0c
0.03 % EFF	41 $\pm$ 0.6b	58 $\pm$ 1.4b	34 $\pm$ 1.2b
0.06 % EFF	89 $\pm$ 0.7a	27 $\pm$ 0.4c	70 $\pm$ 0.8a
P value	<0.001	<0.001	<0.001

Values are the means $\pm$ SE (n = 3). Means with different letters indicate a statistically significant difference according to Duncan's multiple range test ($P < 0.05$).

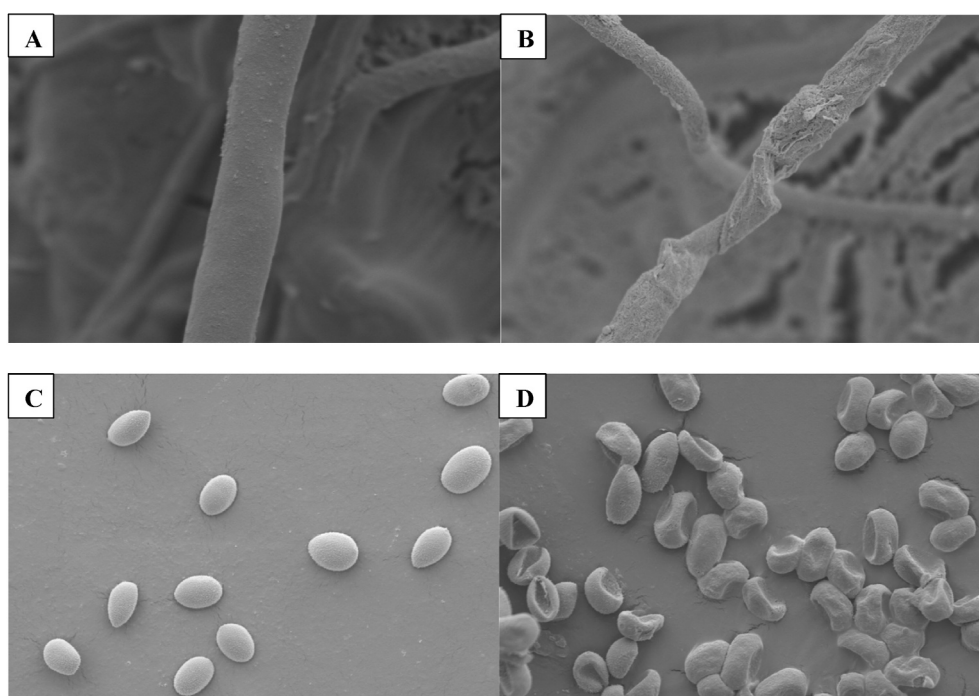


Fig. 1. Scanning electron microscope (SEM) images displaying the impact of enhanced freshness formulation on the mycelia and spore morphology of *Botrytis cinerea*. Control mycelia and spores (A, C); mycelia and spores treated with 0.03 % EFF (B, D). The SEM images are of 7 d old fungi.

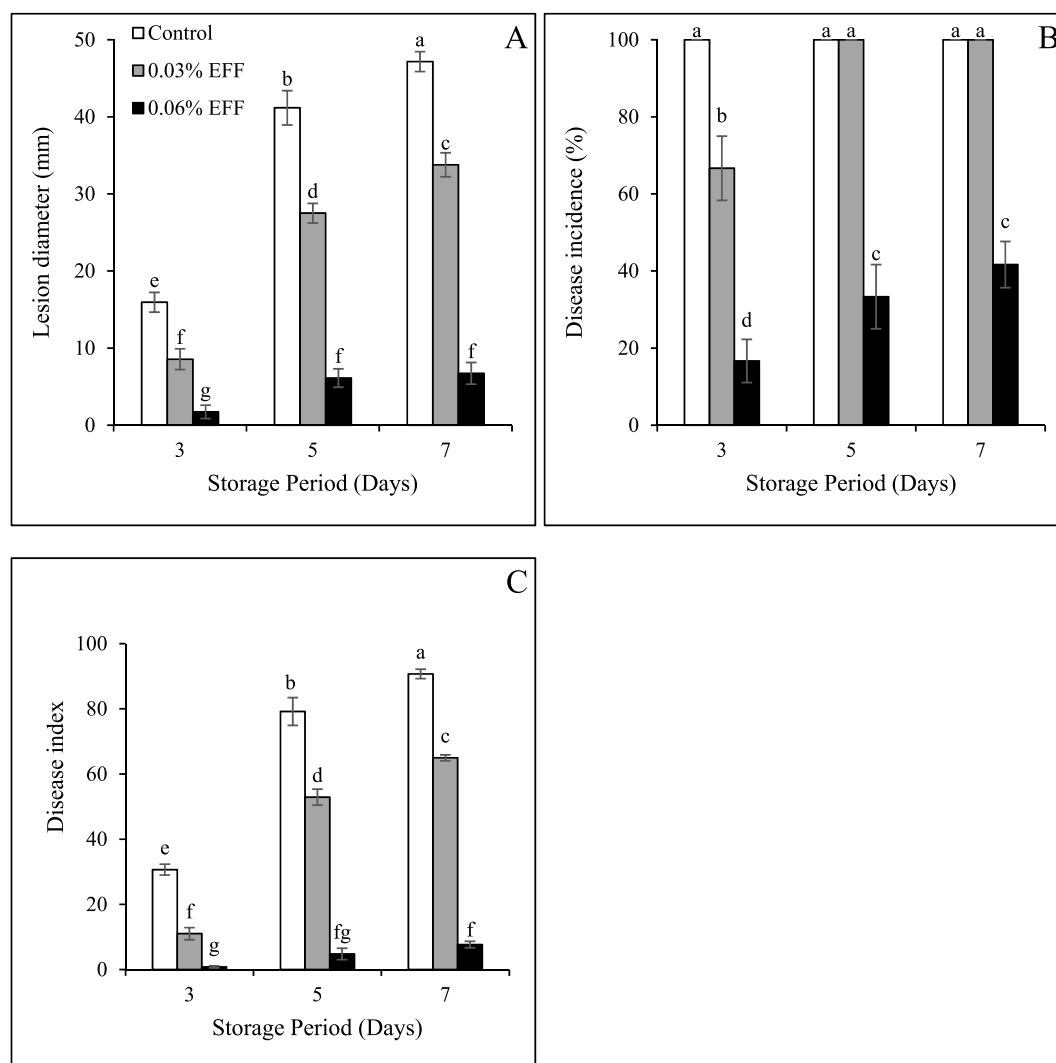


Fig. 2. Influence of enhanced freshness formulation on lesion diameter (A), disease incidence (B) and disease index (C) of 'Y368' kiwifruit after inoculation with *Botrytis cinerea*. Values are the means $\pm$ SE (n = 3). Means with different letters indicate a statistically significant difference according to Duncan's multiple range test ($P < 0.05$).



Fig. 3. Images displaying the influence of enhanced freshness formulation on kiwifruit inoculated with *Botrytis cinerea*. Unpeeled kiwifruit (A) and peeled kiwifruit (B). The photograph was taken 7 days after treatment.

65 and 7 for the control, 0.03 and 0.06 % EFF treatment level, respectively at 7 dpi.

3.4. Defense-related enzyme activities

The enzyme activity of PAL (Fig. 4 A) increased during storage, with treated fruit exhibiting significantly higher enzyme activity relative to

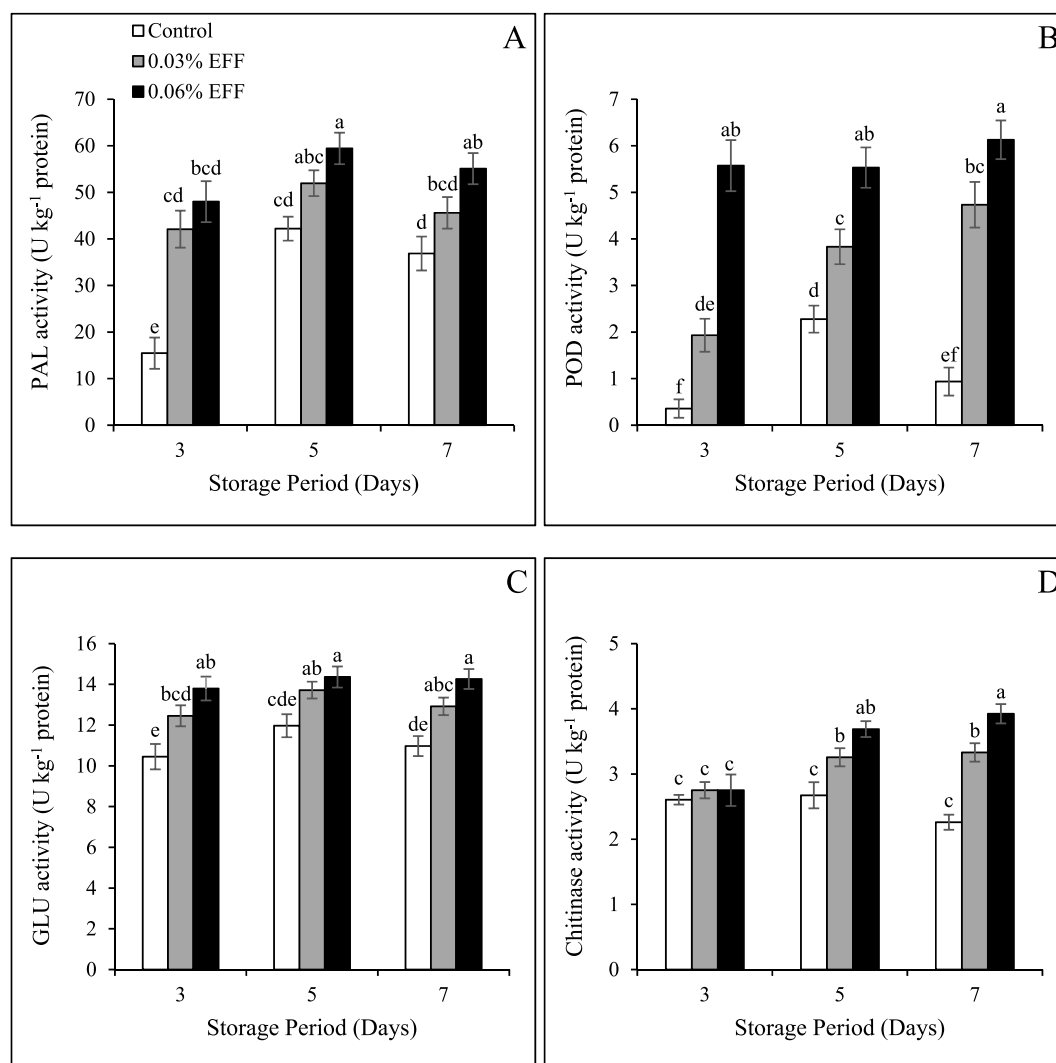


Fig. 4. Influence of enhanced freshness formulation on phenylalanine ammonia lyase (PAL) (A), guaiacol peroxidase (POD) (B), β -1,3-glucanase (GLU) (C) and Chitinase (D) of 'Y368' kiwifruit after inoculation with *Botrytis cinerea*. Values are the means $\pm$ SE (n = 3). Means with different letters indicate a statistically significant difference according to Duncan's multiple range test ($P < 0.05$).

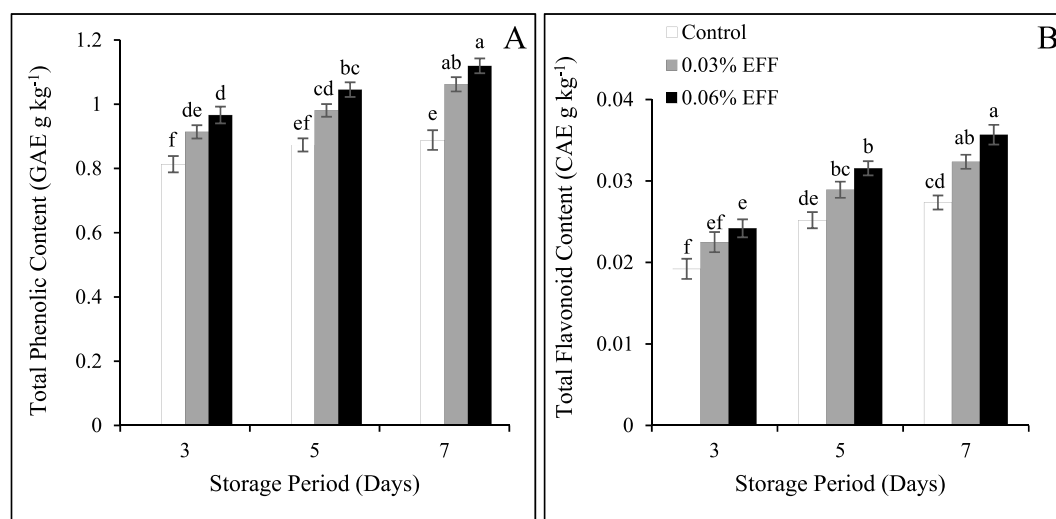


Fig. 5. Influence of enhanced freshness formulation on total phenolics (A) and total flavonoids (B) of 'Y368' kiwifruit after inoculation with *Botrytis cinerea*. Values are the means $\pm$ SE (n = 3). Means with different letters indicate a statistically significant difference according to Duncan's multiple range test ($P < 0.05$).

the control. At 7 dpi, PAL enzyme activity of 0.03 and 0.06 % EFF-treated fruit was approximately 20 and 33 % greater than the control, respectively. The enzymatic activity of POD (Fig. 4 B) increased significantly with a pronounced effect at the 0.06 % treatment level, followed by the 0.03 % and control treatment. The POD activity of control fruit declined after day 5, whereas treated fruit continued to exhibit an increase in enzyme activity. At 7 dpi, the POD activity of the 0.03 and 0.06 % treatment level was 5 and 6.5-folds higher than the control, respectively. The GLU enzyme activity (Fig. 4C) increased for all treatments, then declined on day 7. GLU enzymatic activity was registered at 11, 13 and 14 U kg⁻¹ protein for control, 0.03 and 0.06 % EFF at 7 dpi, respectively. The chitinase enzyme activity (Fig. 4 D) of treated fruit increased as the storage period progressed and remained substantially higher than the control. At 7 dpi, chitinase activity of treated fruit was 32 and 42 % higher than the control, respectively.

3.5. Total phenolic and flavonoid content

The treatments had a significant influence on total phenolic content (TPC) (Fig. 5 A), with EFF-treated fruit exhibiting higher TPC. At 7 dpi, the TPC of the 0.03 and 0.06 % EFF-treated fruit was 19 and 26 % higher than that of the control, respectively. Similar to TPC, the treatments had a substantial effect on the total flavonoid content (TFC) (Fig. 5 B), with treated fruit exhibiting greater TFC. At 7 dpi, the TFC of the treated fruit remained substantially higher than the control.

3.6. Principal component analysis

Principal component analysis (PCA) (Fig. 6) was performed to examine the relationship among the measured parameters and their response to the treatments over time. PCA was produced for the first two dimensions, where Dim1 and Dim2 accounted for 66.2 and 24.5 % of the total variance, respectively. A scatter distribution of the data showed that the interaction between treatments and storage time distinguished the inoculated fruit. The PCA biplot showed that the control group had greater fungal growth (lesion diameter, disease incidence and index)

compared to the EFF-treated samples, which were characterized by higher activities of defense-related enzymes (PAL, POD, GLU and CHI) and greater TPC and TFC.

4. Discussion

The ability of EFF to suppress the growth of *B. cinerea* (Table 1) may be attributed to its capacity to distort fungal hyphae by twisting, breaking and damaging the membrane (Fig. 1 B). Furthermore, suppression of spore germination (Table 1) may result from the capacity of EFF to induce the malformation of fungal spores, as illustrated by SEM images (Fig. 1 D). The results in this study are comparable to those reported by Anusha et al. [37], who demonstrated that hexanal treatment distorted fungal hyphae and spores of *Colletotrichum gloeosporioides* and *Lasiodiplodia theobromae*. Collectively, these findings suggest that EFF may be an effective antifungal treatment for controlling *B. cinerea*, potentially reducing postharvest losses in kiwifruit.

The defense-related enzymes involved in plant defense are PAL, POD, GLU and CHI. PAL is an enzyme that is part of the phenylpropanoid pathway, involved in the synthesis of disease-resistant substances such as phenolic compounds [38]. Phenolic compounds play a vital role in disease resistance by acting as toxins to the invading pathogen [39]. POD is an antioxidant enzyme that is involved in the synthesis of lignin and protects against pathogens by strengthening the cell wall [40]. GLU and CHI enzyme inhibit fungal growth by degrading β -1,3-glucan and chitin (key constituents of the pathogen cell wall), respectively [15].

The results of the current study suggest that the suppression of fungal growth (Fig. 2) in response to EFF treatment may be attributed to the enhancement of defense-related enzymes (Fig. 4) which confer disease resistance. These findings are similar to those reported by Seethapathy et al. [41], who demonstrated that dip treatment with hexanal-containing formulations induced disease resistance against *Lasiodiplodia theobromae* by enhancing the activities of PAL and POD in mango fruit. This reinforces the idea that hexanal-containing dip formulations have the potential to enhance disease resistance to fungi across different fruit types.

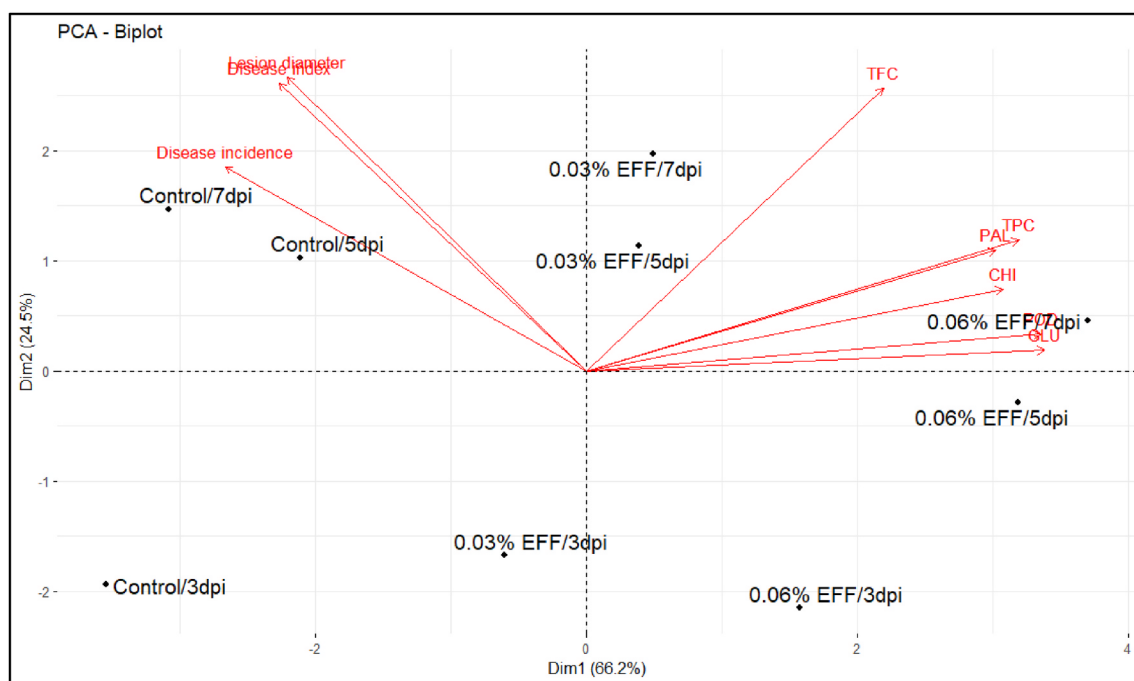


Fig. 6. Principal component analysis biplot showing the correlations between the measured variables and factors (treatment and storage time). Dimension one (Dim1), Dimension two (Dim2), Enhanced freshness formulation (EFF), days post inoculation (dpi), total phenolic content (TPC), total flavonoid content (TFC), Phenylalanine ammonia-lyase (PAL), Guaiacol peroxidase (POD), β -1,3-glucanase (GLU) and Chitinase (CHI).

The elevated activities of defense enzymes in response to EFF treatment may be attributed to the capacity of hexanal to upregulate the expression of defense-related genes such as PAL, GLU and CHI [42]. Additionally, EFF-treated fruit exhibited significantly greater TPC and TFC (Fig. 5), and lower fungal growth (Fig. 2) compared to the control fruit. This observation suggests that the higher levels of total phenolic and flavonoid contents enhanced the fruit's resistance to *B. cinerea*. As previously indicated, PAL is involved in the synthesis of phenolic compounds. Hence, it can be deduced that the greater TPC and TFC in treated fruit may be attributed to the capacity of EFF to enhance the enzyme activity of PAL (Fig. 4 A).

The results presented in the PCA biplot (Fig. 6), show that EFF-treated fruit were characterized by higher measurements of defense-related enzymes (PAL, POD, CHI and GLU) and disease-resistant substances (TPC and TFC), whereas control fruit exhibited greater fungal growth (lesion diameter, disease incidence and index). These observations suggest that EFF induced disease resistance in treated fruit by enhancing the activities of defense-related enzymes (Fig. 4) and the accumulation of TPC and TFC (Fig. 5). Therefore, the outcomes of the current study indicate that EFF dip treatment can effectively control the growth of *B. cinerea* in kiwifruit. Given that the ingredients of EFF (hexanal, geraniol, α -tocopherol and ascorbic acid) are food additives approved by the FDA with GRAS status, this treatment has the potential to serve as a safe and environmentally friendly alternative to synthetic fungicides. Therefore, the adoption of this treatment is worthy of consideration, as it has the potential to reduce the reliance on synthetic fungicides and alleviate the pressure on kiwifruit producers facing stringent MRL policies.

5. Conclusion

The outcomes of this study showed that EFF dip treatment effectively suppressed the growth of *B. cinerea* in kiwifruit through induced resistance, characterized by enhanced activities of defense-related enzymes (PAL, POD, CHI, GLU) and elevated levels of disease-resistant compounds (TPC and TFC). The distinct clustering in the PCA biplot further substantiates these findings, highlighting the substantial impact of EFF on the biochemical responses of kiwifruit and its role in enhancing resistance to *B. cinerea*. These findings suggest that EFF is a promising, practical alternative to traditional fungicides, offering a cost-effective and environmentally friendly method to manage *B. cinerea* in kiwifruit. This study contributes to the development of novel control strategies aimed at reducing reliance on synthetic fungicides, which is crucial for market access and building consumer confidence amid growing food safety and environmental health concerns. Future research should explore the molecular mechanisms by which EFF activates plant defense responses and investigate strategies for greater inhibition of *B. cinerea*, such as curative post-inoculation treatment, higher concentrations, or combinations with other treatments.

CRediT authorship contribution statement

Sisanda Sibusiso Mthembu: Writing – review & editing, Writing – original draft, Validation, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Samson Zeray Tesfay:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Asanda Mditshwa:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Lembe Samukelo Magwaza:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.pmpp.2024.102484>.

Data availability

Data will be made available on request.

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Hexanal-containing dip treatment enhances kiwifruit resistance to *Botrytis cinerea*

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