Published by :
http://www.ijert.org

International Journal of Engineering Research & Technology (IJERT)
ISSN: 2278-0181
Vol. 14 Issue 06, June - 2025

Outcome of Polyphenolic Compound Application on Stem end Rot of Avocado Fruits

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Abstract: **Avocados have been identified to contain essential secondary metabolites, which are important to the fruit as a defense system and to humans as key nutrients in health management. The application of biological compounds has been proven to strengthen the fruit's defence approach against fungal invasion, with documented limitations. This study evaluated the impacts of applying selected polyphenolic compounds to promote antifungal properties and natural defence mechanisms in green-skinned avocado fruits during postharvest storage. The fruits were artificially inoculated and treated curatively and preventatively with vanillic acid (700 mg L^{-1}), (b) caffeic acid (700 mg L^{-1})], ethanol solution (0.05%), Prochloraz® (reference antifungal) and control (untreated), then stored for 28 days and placed on the shelf for another 5 days to simulate market shelf life. The treated fruits were evaluated for incidence, biochemical, and phytochemical activity. The results demonstrated that fruits treated with caffeic and vanillic acids significantly reduced stem-end rot incidence than prochloraz® and control fruits. Treatment with vanillic and caffeic acids at 700 mg L^{-1} induced the activities of phenylalanine ammonia-lyase, peroxidase, chitinase, and β-1, 3-glucanase and increased the exocarp epicatechin content. The effects of vanillic and caffeic acids enhanced the shelf life and quality of avocado fruits during storage, which is attributed to their antifungal activities and induction of biochemical defense responses in the fruit. The incorporation of caffeic acid as an alternative control of postharvest diseases of avocados could enhance the quality and shelf life of this cultivar. Due to the biological properties of caffeic acid, this compound is safe for application to fruits.**

Keywords: **Fruit, disease incidence, metabolites, postharvest, caffeic**

I. INTRODUCTION

Secondary metabolites in avocado fruit comprise beneficial antioxidants that are necessary for human health, which, when consumed, add functional elements that support human well-being [1]. In avocado fruits, metabolites such as phenolic compounds and their derivatives serve as key machinery that stimulates the production of other defence compounds to fight against invading phytopathogens that initiate enzyme-causing disease incidences in the fruit [2]. Avocados may be infected by *Lasiodiplodia theobromae*, which causes a pronounced symptomatic lesion at the stem end of the fruit, known as stem end rot disease [3]. This disease impacts the quality, shelf life, and marketability of green-skinned avocados, resulting in a loss of profits for growers, wholesalers, and retailers.

Generally, once the fruit is under attack by this fungal pathogen, the phenolic compounds that are generated through the phenylpropanoid pathway release enzymes that act as chemical barriers and induce other natural defense mechanisms to create resistance and protect the fruit cell wall [4]. However, these natural defense enzymes can become weakened due to their quantity or inactivation by fungal enzymes [5]. Application of external phenolic compounds can bolster the postharvest quality of fruits by decreasing the occurrence of diseases, preserving nutraceutical compounds, and retarding the onset of senescence [6]. When these compounds are introduced to the fruit's skin, they interact with the enzymes within the cell walls and membrane, thereby suppressing the function of defensive enzymes and disrupting the synthesis of ergosterol, a fundamental component of fungal cell membranes. The external elicitors also contribute to strengthening the cell walls of fruits by delaying disease incidence and maintaining the fruit quality over time. In packhouses, a commercial broad-spectrum imidazole has been applied to the fruit to extend its quality and shelf life. However, the current trend of healthy eating, primarily comprising the consumption of fruits and vegetables treated or exposed to non-cancerous substances, is becoming the norm [7]. There are studies to develop more eco-friendly and organic products to combat microbial-related incidents involving avocados. Some commercially available products, when applied to fruits in the form of a coating, biofumigant, or solutions, have been shown to enhance the fruit's secondary metabolites' ability to incapacitate pathogens by lysing fungal cell walls and inducing active defense-related enzymes [8,9]. Phenylalanine ammonia-lyase (PAL), PR-proteins, Superoxide dismutase (SOD), Catalase (CAT), and Peroxidase (POD) are some of the key defense-related enzymes present in the fruit which regulate the rate at which the disease develops.

A study by Nguyen [2] demonstrated that the combination of gallic acid, rutin, and chlorogenic acid extracted from four plant extracts reduced the Fusarium wilt of tomatoes by 26.7% and 28% after 28 days of the fungal infection. Another study demonstrated that the application of caffeic acid to Alternaria rot-infected pears enhanced the enzymatic mechanisms and gene transcription levels of PAL, polyphenol oxidase, and other related enzymes. Therefore, this study investigated the impact of selected phenolic compounds on the incidence and severity of stem-end rot, epicatechin content, and active defense response-related enzymes in the 'Fuerte' cultivar.

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II. MATERIALS AND METHODS

2.1 Fruits samples

Late season 'Fuerte' avocado fruits without defects were harvested at the commercially matured stage and purchased from Bassan Fruit Packers, Tzaneen, South Africa. The cultured fungal isolates were maintained on Potato Dextrose Agar (PDA) (Sigma-Aldrich Johannesburg, South Africa) at 25-30°C.

2.2. Fungal strain

L. theobromae strain was obtained from the culture collection of the Fruit and Vegetable Technology Laboratory of Tshwane University of Technology, Pretoria, South Africa.

2.3. Fruit inoculation

The stock spore suspension was prepared from a young viable culture of 4-5 days old, and the spore counts were adjusted (10^5 spores mL^{-1}) with a hemocytometer before inoculation on the fruits [10]. The healthy 'Fuerte' avocado fruits were sterilized with 0.05% sodium hypochlorite and air-drie. Then, a cut approximately 2 mm in diameter was made on each fruit at the stem end.

2.4. Reagents

The functional compounds: epicatechin standard (≥ 97.0% HPLC), vanillic acid, caffeic acid, ferulic acid, coumaric acid and gallic acid, and other reagents such as sodium phosphate buffer, sodium acetate trihydrate, glacial acetic acid, sodium hydroxide, borate buffer, β-mercaptoethanol and ethylenediaminetetraacetic acid, L-phenylalanine, hydrochloric acid, laminarin, 3,5- dinitrosalicyclic reagent, bovine serum albumin was purchased from Sigma-Aldrich (Johannesburg, South Africa). Prochloraz® (450 g L^{-1}; imidazole) was purchased from Adama SA (Pty) Ltd, South Africa.

2.5. Artificial infection of fruits

The surface-sterilized fruits were subjected to two treatment methods, and four punnets containing five fruits each were used for each functional compound. The treatment methods that were adopted for this study include: (i) The preventative method [infected fruits were stored at 6.5°C for 28 days and thereafter at 25°C for 5 days to simulate market shelf (28 + 5)], and (ii) The curative method, which involves storage of fruit at room temperature (25°C) for 7 days.

For the preventative treatment method, the fruits were dipped in functional compounds [vanillic acid (700 mg L^{-1}), (b) caffeic acid (700 mg L^{-1})], ethanol solution (0.05%) and untreated control for 5 min and allowed to air dry for 6 h before uniformly wounding with a sterile needle, thereafter inoculated with 20 µL of spore suspension (10^5 spores mL^{-1}) at the stem-end region.

For the curative treatment method, a similar method was adopted as abovementioned. However, the fruits were inoculated prior to exposure to functional compounds [vanillic acid (700 mg L^{-1}), (b) caffeic acid (700 mg L^{-1})], ethanol solution (0.05%) and untreated control.

At the end of the storage period, the percentage incidence was recorded, and the lesion diameter (mm) was measured to evaluate the severity of stem-end rot on the fruit.

2.6. Determination of active defense-related enzymes

In this study, the levels of PAL (phenylalanine ammonia-lyase), Chitinase and β-1, 3-glucanase in the artificially infected fruits subjected to functional compounds and prochloraz solution were evaluated. Frozen samples (0.5 g) of avocado tissue from five replicate fruits per treatment were homogenized in buffers to determine the enzyme activities mentioned above. The method adopted for this analysis was described by [11].

The PAL activity was determined according to the method described by Sellamuthu1[1] with slight modifications. The expression of the enzyme activity was as nmol cinnamic acid h/mg of protein.

POD activity was determined according to the method described by [11]. The expression of the specific activity of the enzyme was ΔA460 min^{-1} mg of $protein^{-1}$.

Chitinase activity was determined by adding 600 µL of the enzyme extract to the reaction mixture containing 125 µL of 2% (w/v) dye-labeled chitin azure in 50 mM sodium acetate buffer (pH 5.0) and incubated for 2 h at 40°C. The measurement of the supernatant was taken at an absorbance of 550 nm, with one unit defined as the amount of enzyme required to catalyze the rate of production of moles of product per mass of protein (1 nmol $product^{-1}$ h^{-1} mg of protein).

To determine β-1, 3-Glucanase activity, 100 µL of the enzyme extract was added to 100 µL of 2% (w/v) laminarin (Aldrich, Missouri, USA) and incubation was for 24 h at 40°C. The amount of reducing sugar was determined at an absorbance of 540 nm, and the enzyme activity was expressed in units with one unit defined as the amount of enzyme required to facilitate the formation of glucose equivalent production rate per product mass protein (1 µmol glucose $equiv^{-1}$ h^{-1} mg of protein). The protein estimate from the enzyme extract was determined using the method by [11]. All the enzyme assays for each treatment per sample were replicated three times.

2.7. Epicatechin content

The epicatechin content was determined according to the method described by [12, 13], with slight modifications. A fresh-frozen sample (0.5 g) of artificially inoculated avocado skin was weighed and homogenized in a 1:1 (v/v) methanol-water mixture by vortexing the mixture for 30 s. The homogenates were filtered through 0.45 µm membrane (Nylon syringe filter, PerkinElmer™, China) prior to injection into the HPLC system (PerkinElmer, USA). The expression of the data obtained was µg 100 g^{-1} of fresh weight.

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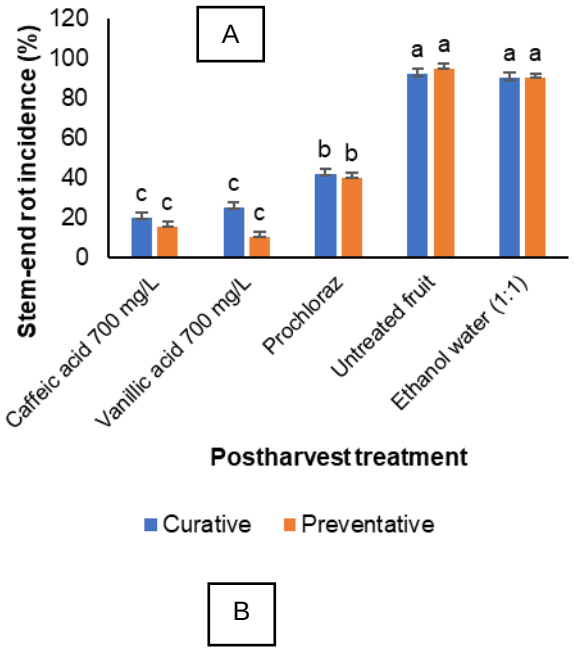
International Journal of Engineering Research & Technology (IJERT)
ISSN: 2278-0181
Vol. 14 Issue 06, June - 2025

2.8.Statistical analysis

The experiments were in a completely randomized design, with 60 fruit per treatment and repeated twice to confirm the preliminary observations. Each treatment, consisting of 20 fruit replicates, was subjected to analysis of variance using the GenStat for Windows (2004) statistical package. The Fishers' protected least significant difference (LSD) test was performed at a 5% level of significance.

III. RESULTS

Fruits subjected to caffeic, and vanillic acids showed an incidence of less than 26% and a severity of 7 mm for stem-end rot, respectively, for both curative and preventative treatments. In comparison, fruits exposed to Prochloraz showed a 42% incidence and 19 mm severity for curative treatments and 40% and 20 mm for preventative treatments. A higher incidence and severity of stem-end rot were recorded for the ethanol-water treatment and the untreated control, with ~ 100% and 40 mm, respectively (Fig. 1). The impact of caffeic and vanillic acid was evident in the enzyme activities of PAL (phenylalanine ammonia-lyase), β-1,3 glucanases, chitinase, and POD, as well as the epicatechin content, which showed higher activity levels of these biochemical mechanisms. The abovementioned enzymes (phenylalanine ammonia-lyase), β-1, 3 glucanases, chitinase, POD, and the epicatechin content) were relatively low in prochloraz, ethanol solution, and untreated control fruits (Figs 2 & 3).



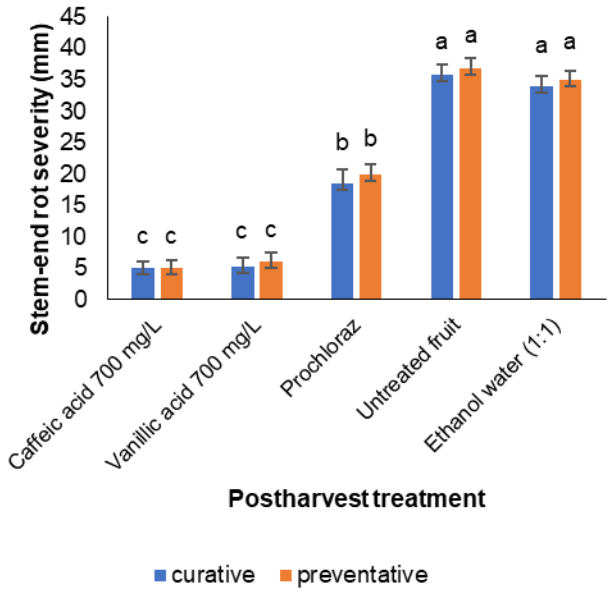
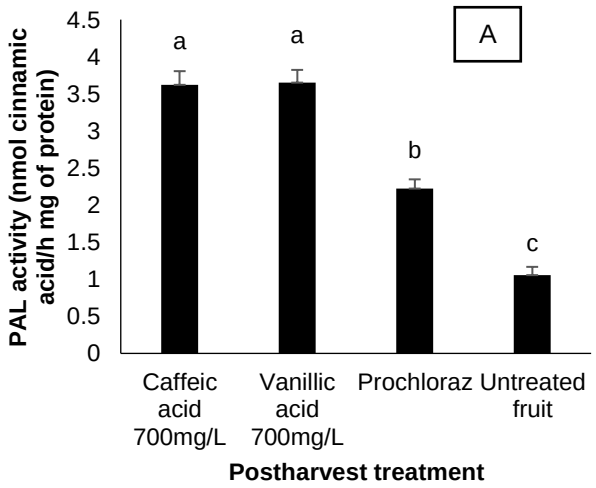


Fig1. Influence of phenolic compounds on the A) Incidence of stem-end rot and B) Severity of stem-end rot on curative and preventative treatment. Data presented are the means of five replicate punnets per treatment each punnet containing five fruits. Different alphabets indicate significant differences between treatment means at $P < 0.05$; vertical lines represent standard deviations.



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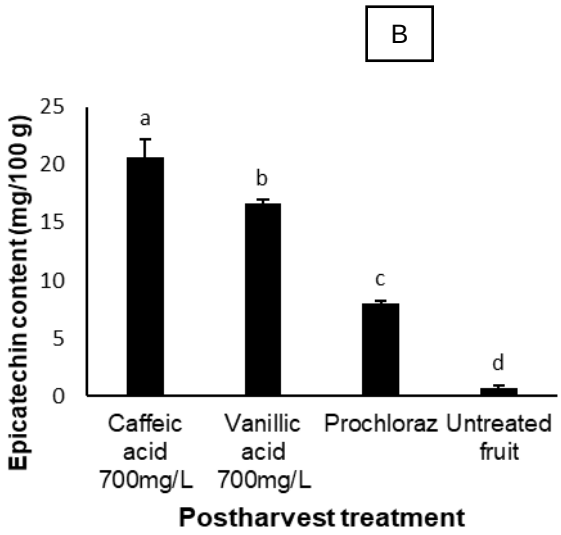


Fig 2. Influence of phenolic compounds on A) phenylalanine ammonia-lyase activity and B) exocarp epicatechin content, *on Lasiodiplodia theobromae* inoculated fruit. Data presented are the means of three replicate punnets. Different alphabets indicate significant differences between treatment means at $P < 0.05$; vertical lines represent standard deviations.

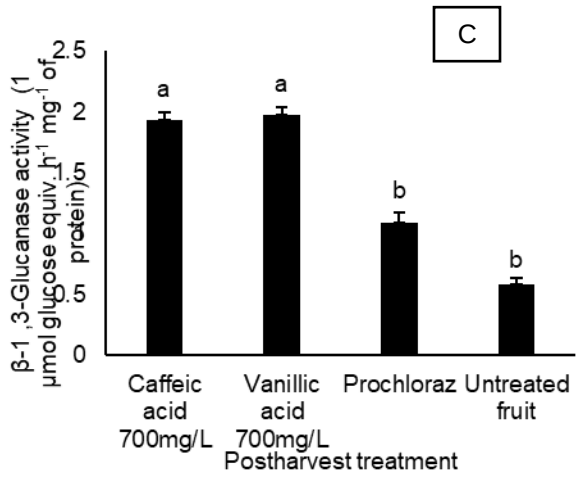
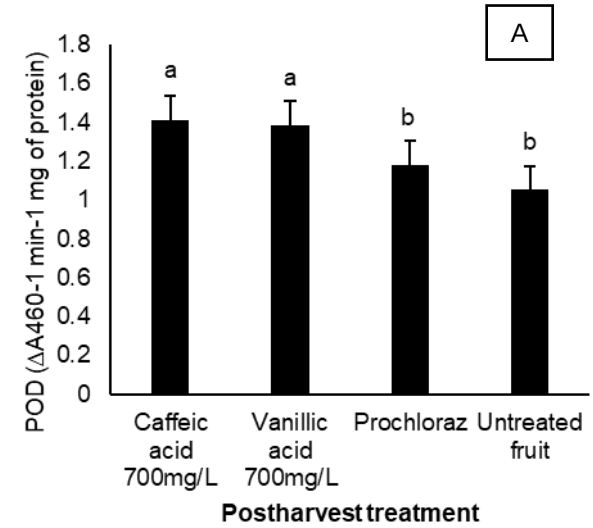
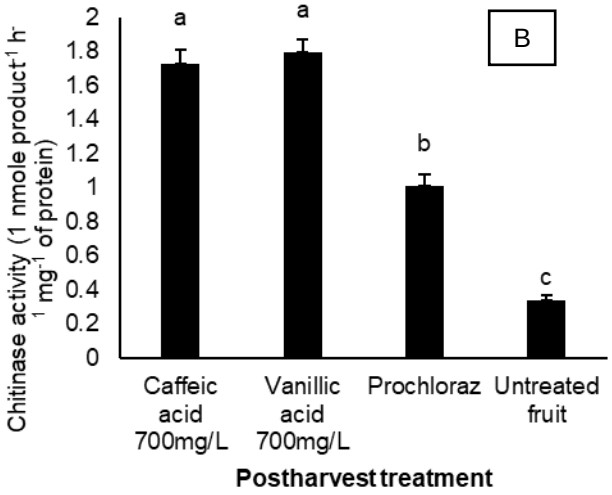


Fig 3. Influence of phenolic compounds on A) peroxidase (POD) B) chitinase activity and C) β-1,3 glucanase activity, on *Lasiodiplodia theobromae* inoculated fruit. Data presented are the means of three replicate punnets. Different alphabets indicate significant differences between treatment means at $P < 0.05$; vertical lines represent standard deviations.





IV. DISCUSSIONS

The safety and efficacy of the replacement fungicide in maintaining the freshness and quality of horticultural produce until it reaches the consumer's plate are critical factors in selecting a fungicide for controlling postharvest diseases. According to [14], phenolic compounds, which are plant secondary metabolites, are widely distributed in the tissues of resistant fruit varieties and have the potential to trigger defense mechanisms against fungi by forming a chemical barrier that restricts the growth of pathogens and increases plant resistance. In this study, it was evident that vanillic acid and caffeic acid at a concentration of 700 mg L^{-1} significantly reduced the occurrence of stem end rot in avocado fruit varieties "Fuerte" after storage. [15] found in their study that a positive correlation exists between the antifungal activity of phenols and their hydrophobicity, which could facilitate the diffusion of these compounds through the fungal membranes.

Phenolic compounds play a crucial role in combating pathogens and triggering defense mechanisms in plants [14]. PAL is a key enzyme that induces secondary metabolic pathways (phenylpropanoid) and increases the synthesis of phenolic compounds and pathogenesis-related proteins as a defense response to pathogen invasion [13]. However, the abundance of PR proteins in plants is important for defense responses against fungal pathogens. They degrade the cell wall of the fungus, which is composed of the main structural components of fungal cell walls, including chitin and β-1,3-glucan [16]. This mechanism was evident in the activity of the enzymes PAL, chitinase, and β-1,3-glucanase which were induced by caffeic and vanillic acids (700 mg L^{-1}), which resulted in a delay in the development of stem rot in the fruit. These current results suggest that caffeic and vanillic acids triggered the accumulation of epicatechin content in the exocarp of avocado fruits by maintaining the concentration in the infected fruits between 20.7 and 16.6 mg 100 g^{-1} [17]. In

International Journal of Engineering Research & Technology (IJERT)
ISSN: 2278-0181
Vol. 14 Issue 06, June - 2025

correlation with this, [18] suggested that the increase in phenolic compounds and PAL activity played a key role in the phenylpropanoid metabolic pathway in mangoes.

The content of epicatechin, a product of the phenylpropanoid pathway, in the fruit pericarp, regulates the degradation of the antifungal compound (1-acetoxy-2-hydroxy-4-oxoheneicosa 12,16-diene), which in unripe fruits is responsible for the resistance to *L. theobromae* infections [19]. The epicatechin content in avocado fruits has been shown to decrease with ripeness due to oxidative metabolism by the enzyme lipoxygenase, which facilitates infection by *L. theobromae* in ripe fruits [19]. A study by [20] highlighted that avocado cultivars are resistant to *C. gloeosporioides* decay, which is maintained by a higher epicatechin content, and that this content slowly decreases during ripening. Therefore, we hypothesized that the retention of epicatechin in the pericarp of "Fuerte" avocados is the primary mechanism of action that increases resistance to *L. theobromae* infection. The POD activity enhanced the oxidation of phenolics, as well as the suberization and lignification processes, during the defense effects against pathogens [21, 22]. From the current results, vanillin and caffeic acid at a concentration of 700 mg L^{-1} induced the activities of POD, PAL, β-1,3-glucanase, and chitinase, which maintained higher epicatechin content and played a crucial role in disease resistance to stem rot in avocado fruits. Finally, using vanillin and caffeic acid at a concentration of 700 mg L^{-1} can be recommended as a natural postharvest biocide for antifungal activity and induction of a defence mechanism in avocado fruits. However, the application of vanillic and caffeic acids at 700 mg L^{-1}, combined with edible coatings, to the fruit will provide more resistance to postharvest pathogens in the avocado industry.

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