Antifungal activity of *Zingiber officinale* Roscoe (ginger) oil and extracts, associated with carnauba wax nanoemulsions, on fungal control of harvest papaya

M. Miranda[1,a], X. Sun[2,b], O.B.G. Assis[3,c], C. Ference[2,d], M.D. Ferreira[3,e] and E.A. Baldwin[2,f]

[1]São Paulo State University (UNESP), School of Pharmaceutical Sciences, Araraquara, SP, Brazil; [2]United States Department of Agriculture (USDA) - ARS Horticultural Research Laboratory, Ft. Pierce, FL, USA; [3]Brazilian Agricultural Research Corporation, Embrapa Instrumentação, São Carlos, Brazil.

Abstract

Essential oils and plant extracts can be safe alternatives for reducing post-harvest decay in foods compared to synthetic preservatives. Ginger oil (GO) and ethanolic extracts (GE) has been in vitro studied. Antifungal activity associated with fruit coatings on papaya has not been exhaustively investigated until now. In this study, the antifungal activity of GOs and GEs to control *Colletotrichum gloeosporioides* was investigated. In vitro results showed that GO has higher activity compared to GE, significantly reducing mycelial growth. The measured minimum inhibitory concentration (MIC) of GOs and GE were 0.1 to 0.8% (v/v) and 2.5 to 5%, respectively. Petri dishes inoculated with *C. gloeosporioides* were coated with carnauba wax nanoemulsion (CWN), GO nanoemulsion (at 3 and 6%), and their combination. Results showed that after 24h plates treated with 3 or 6% of GO, and CWN exhibited significant inhibition of the mycelial zone (MZI). The combination of CWN coating and GO was more effective than GO alone. CWN coatings resulted in significantly higher MZI alone or when associated with GO, compared to GO itself or control-water. After 7 days, plates treated with GO resulted in the same MZI as control, and CWN demonstrated inhibition against *C. gloeosporioides.* In vivo tests on 'Redland' papayas coated with CWN showed effective control upon fungal growth. The lowest values of fungal disease severity occurred when CWN was associated with GO (at 3 and 6% v/v). By comparing the CWN and GO alone activities, CWN resulted in higher decay inhibition. At a concentration of 6% GO, the diseases severity were superior than that found in uncoated fruits, suggesting GO phytotoxicity at that level.

Keywords: *Colletotrichum gloeosporioides,* papaya decay, ethanolic extracts, edible coatings, carnauba wax nanoemulsion

INTRODUCTION

Considerable losses of fruits and vegetables are caused by plant pathogens, mainly by fungal-induced post-harvest spoilage and deterioration (Palou et al., 2016). Papaya (*Carica papaya* L.), a native fruit of tropical America, is particularly susceptible to postharvest decay as a consequence of fungal and bacterial infestation that reduce shelf-life, and consequently fruit marketability (Singh, 2010). To tackle this problem, essential oils and plant extracts were explored as an eco-friendly alternative for reducing microorganisms compared to commercial, chemical fungicides.

Potential control of phytopathogens using essential oils (EOs) has been intensified in the last years and showed the promising application in the post-harvest field (Palou et al.,

[a]E-mail: mmiranda.bio@gmail.com
[b]E-mail: XiuXiu.Sun@ars.usda.gov
[c]E-mail: odilio.assis@embrapa.br
[d]E-mail: Chris.Ference@ars.usda.gov
[e]E-mail: marcos.david@embrapa.br
[f]E-mail: Liz.Baldwin@ars.usda.gov

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2016; Singh, 2010). The development of low cost, low tech postharvest techniques can represent a significant advance for post-harvest product conservation.

Several essential oils have been tested against post-harvest diseases of papaya fruit, including ginger oil (*Zingiber officinale*), which has active phenolic compounds (Singh et al., 2008) and the capacity for reducing common microorganisms in food (Sa-Nguanpuag et al., 2011) with potential use associated with edible coatings (Atarés et al., 2010). Ginger extracts have potential to be used as a natural antimicrobial agent, replacing artificial preservatives and chemical fungicides. Additionally, combining essential oils (EO) and plant extracts with coatings can be a suitable alternative to overcome problems related to EO in vivo application, such as undesirable flavor and odor, phytotoxicity, and efficacy in vivo. By combining EO in a coating matrix, volatile release of antimicrobial volatiles could be controlled and phytotoxicity reduced (Oliveira et al., 2019).

The aim of the present study was to evaluate the antifungal activity of ginger oil (GO) and ginger extracts (GE) for control of fungal decay on papaya fruit, and also to explore the potential of their incorporation into a postharvest coating consisting of carnauba wax nanoemulsion for reduction of postharvest decay.

MATERIALS AND METHODS

Extraction of ginger extracts (GE) and essential oils (GO)

Ginger rhizome (*Zingiber officinale*), 'Gigante' was purchased from Ceasa-SP (Brazil). Samples were washed, rinsed, sanitized and cut in small pieces (0.5 cm). Then, the material was ground and divided into three parts: One portion was frozen; another submitted to oven air at 40°C and the other freeze-dried until 3 to 9% final moisture. After drying, the material was packed in low-density polyethylene packages and stored in a dry environment at room temperature (~25°C).

GE was obtained by mixing each plant (fresh or dried) with absolute ethanol (ethanolic extract - GEE) or 70% ethanol (hydroethanolic extract - GHE), according to Breda et al. (2016). Each extraction was carried out in triplicate and then combined.

GOs were obtained by hydro-distillation (steam distillation) using a Clevenger's type apparatus according to Natta et al. (2008) procedure with slight modifications. In short, fresh ginger (600 g), freeze-dried (50 g) and oven air-dried ginger (50 g) were mixed with distilled water (1 L), and heated under soft boil over 24 h. The oil was collected and dried over anhydrous sodium sulfate. Extractions were conducted in triplicate and stored at 4±2°C in a dark container for further use. Commercial ginger oil (GOCom), food grade, CAS Number: 8007-08-7, was purchased from Sigma-Aldrich (China) and used as a reference.

In vitro antifungal activity evaluation – microorganism maintenance and inoculum preparation

The plant extracts and essential oils were evaluated for activity against *Colletotrichum gloeosporioides* provided by Horticultural Research Laboratory (USHRL) in Ft. Pierce, FL, US-Agricultural Research Service (USDA-ARS). The microorganisms were grown in potato-dextrose-agar (PDA). After inoculation, the cultures were incubated at 25°C until sporulation (between 7 to 10 days).

Petri dishes containing the sporulating fungal cultures were covered with 1 mL of a steril solution of Tween 20 at 0.5% (v/v) and gently homogenized with a Drigalski spatel. An aliquot of spore suspension was analyzed in Neubauer chamber and adjusted with 0.85% sodium chloride solution to approximately 0.4×10^6 CFU mL^{-1} to determine MIC (Minimum Inhibitory Concentration), MFC (Minimum Fungal Concentration), and to inoculate Petri dishes with postharvest coatings.

Dilution of GEs and EOs for the direct contact method

Crude extracts were dissolved in 0.016 $g\times mL^{-1}$ ethanolic solution of polyvinylpyrrolidone at 2:1 (v/v), according to El-Arini and Leuenberger (1996). Essential oils were dissolved in dimethyl sulfoxide 10% at a proportion of 2:1 (v/v).

The direct contact method in broth

The MIC test was performed using a sterile test tube containing 500 µL of potato-dextrose (PD) broth with the sample, and 500 µL of a standardized inoculum of *C. gloeosporioides* was added to all test tubes, which were incubated at 25°C for 7 days at 125 rpm. The observations were carried out after 24, 48, 72, 96 and 168 h. The concentration ranges for extracts were 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0 and 6.0% (v/v) and for essential oil were 1.6, 0.8, 0.4, 0.2, 0.1, 0.5, 0.025, 0.0125, 0.0062, 0.0031 and 0.0015% (v/v). The diluted sample was added to broth in an amount calculated to obtain the final desirable concentration. Cycloheximide and broth were used as positive and negative controls, respectively. The MIC was defined as the lowest concentration of extract or ginger essential oil that inhibited visible microorganism growth. In order to determine the MFC, an aliquot of 10 µL of each incubated test tube (7d) of MIC and the higher concentrations were sub-cultured on PDA Petri dishes and incubated at 25°C for 5 days (Donlan and Costerton, 2002). The MFC was defined as the lowest concentration of extract that prevented visible growth. The results are expressed as the average of three repetitions.

Coatings preparations

Carnauba wax nanoemulsion (CWN) coating, was prepared with the oil phase (O) composed for carnauba wax type 1 (8 to 18% wt/v), and oleic acid (2.6 to 6% wt/v), from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The aqueous phase (W) was composed of ammonium hydroxide (1 to 3% wt/v), and dimethylpolysiloxane (0.02 to 0.1% v/v) (from Sigma-Aldrich) and deionized water (71 to 89% wt/v). The nanoemulsion was accomplished by the inversion phase of W/O to O/W system, using the morpholine-free method as proposed by Hagenmaier and Baker (1997) with modifications. The carnauba wax nanoemulsion with average diameter size of 44.1±7.6 nm was generated, with a narrow polydispersion index (0.28) and zeta potential -43.8 mV, according to measurements previously carried out by Zetasizer Nano ZS (Malvern Instruments Inc., Westborough, MA, USA), (Miranda, 2015). Oil-in-water (O/W) GO nanoemulsions (with 3 and 6% v/v) were prepared following Otoni et al. (2014). Commercial ginger oil was used considering the low yield attained by steam distillation extraction in laboratorial scale. Higher concentrations of GO (3 and 6%) were chosen due to preliminary in vivo experiments which showed interactions among the EO components and the fruit matrix, what reduces the antimicrobial EO efficacy. Carnauba wax nanoemulsion containing GO was prepared with continuous and gradually additions of Tween 80 at 6% (v/v), into previous prepared CWN, followed by mixing using an Ultra-Turrax at 16,000 rpm for 4 min to obtain the final coating.

Inoculated Petri dishes coated with postharvest coatings

The amount of 10 µL of *C. gloeosporioides* spore suspension 10^6 CFU mL^{-1} was placed on solid PDA. After 24 h incubation, aliquots of 1 mL of each coating formulation (filtered through sterile 0.2 µm pore size membrane) were cast on the inoculated Petri dishes and gently spread with Drigalski spatel and incubated at 25°C for 7 days. A control was similarly prepared by replacing coating with sterile deionized water. Three Petri dishes per treatment were assayed. The coatings treatments were: CWN at 18%; CWN at 18% with commercial GO at 3 and 6%, and GO at 3 and 6%. Percentage of mycelial inhibition (%MZI) was estimated after 2 and 7 days and expressed as the mean value of three repetitions per treatment. Radial growth reduction was calculated in relation to fungal growth as measured in the control, as follows: (C-T/C)×100 = % MZI, where C is the radial growth measurement in control and T is radial growth of the pathogen in the presence coating formulations (Sivakumar et al., 2002).

Coated papaya fruits natural decay

'Redland' papaya (*Carica papaya* L.) were harvested at the first maturity stage, with 10% yellow peel color, from a commercial farm in Homestead-FL. The fruit was selected, washed and sanitized by immersion in 200 mg L^{-1} peroxy acetic acid during 3 min, air-dried and stored at room temperature with 80% relative humidity (RH). When fruits achieved 40% yellow peel color, they were coated with 2 mL of the different formulations. The coatings

compositions were the same six that were applied to the Petri dishes. Coated fruit was air-dried at room temperature and stored for 6 days at 22°C. Experiments were conducted in a completely randomized factorial design. The incidence and severity of natural diseases were determined based on the correlation between frut surface and the percentage of affected area. For samples from each treatment during the storage period were assessed. Each fruit was scored using a 6-point category scale. The scores were: 1 (0% of affected area), 2 (1 to 20% of affected area), 3 (21 to 40%), 4 (41 to 60%), 5 (61 to 80%) and 6 (81 to 100%) of affection (Romanazzi et al., 2013).

Statistical analysis

Statistical analysis for percentage of mycelial zone inhibition for *C. gloeosporioides*-inoculated Petri dishes coated with postharvest coatings was performed using univariate parametric analysis of variance (ANOVA) and multiple comparisons Tukey test. The comparison between disease severity scores was performed using non-parametric ANOVA and Kruskal-Wallis multiple comparisons due to the ordinal level of the variable and independent samples assumed in each experiment. The significance level for all analyzes was 5% ($p<0.05$). Statistical software R (Core Team, 2016) was used.

RESULTS AND DISCUSSION

Table 1 shows the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of *C. gloeosporioides* after exposure by direct contact in broth medium for ginger extracts (GE) and ginger oils (GO).

Table 1. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) for *Colletotrichum gloeosporioides* after exposure by contact to different concentrations of ginger extracts and essential oils incorporated into the medium.

Class	Samples	MIC (% v/v)	MFC (% v/v)
Ginger extracts (GE)	GEE-fresh ginger	3.0<MIC ≤ 4.0[a]	6.0
	GEE-freeze-dried ginger	2.0<MIC ≤ 2.5[a]	5.0
	GEE-air oven ginger	2.0<MIC ≤ 2.5[a]	5.0
	Ethanol 96% - control	3.0<MIC ≤ 4.0[a]	5.0
	GHE-fresh ginger	4.0<MIC ≤ 5.0[a]	>6.0[b]
	GHE-freeze- dried ginger	2.5<MIC ≤ 3.0[a]	4.0
	GHE-air oven ginger	2.0<MIC ≤ 2.5[a]	4.0
	Ethanol 70% - control	4.0<MIC ≤ 5.0[a]	6.0
Ginger essential oils (GO)	GO-fresh ginger	0.2<MIC ≤ 0.4[a]	0.4
	GO-freeze-dried ginger	0.05<MIC ≤ 0.1[a]	0.2
	GO-air oven ginger	0.4<MIC ≤ 0.8[a]	1.6
	GO-commercial	0.8<MIC ≤ 1.6[a]	>1.6 [b]

GEE=ethanolic ginger extract, GHE=hydroethanolic extract, GO=ginger oil. *n*=3 for each sample tested. Ginger extracts concentrations tested: 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0% (v/v) and ginger oil: 0.0015, 0.0031, 0.0062, 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6% (v/v).

[a]Lowest concentration of GEE, GHE or GO that inhibited microorganism visible growth in broth medium.

[b]Total inhibition not observed at the highest concentration tested.

Oven air and freeze-dried ginger extracts resulted in lower MIC compared to fresh ginger extracts, indicating that the drying process may influence the activity of the extracts. GEs from fresh ginger showed a MIC equal to the alcoholic controls (ethanol 96 and 70%), showing that the ethanolic portion might takes place in the antifungal activity of these extracts. Additionally, alcoholic controls showed MFC at 5 and 6% (v/v) concentrations, corroborating the participation of ethanol in the extract activity. GEs exhibited MFC against *C. gloeosporioides* between 4 and 6% (v/v), except GHE-fresh ginger which did not show any fungicidal activity at the highest concentration tested.

GOs performed better for MIC and MFC assays compared to GEs. GO from freeze-dried ginger was the most effective at low concentration in inhibiting growth and fungal activity, with 0.1 and 0.2% (v/v) for MIC and MFC, respectively. Commercial ginger oil required a higher concentration (1.6%) when compared to extracted GOs. However, because the low yield of oil extraction processing, the use of commercial ginger oils for postharvest decay control was also adopted. Isolation of antifungal compounds from ginger could be another alternative to reduce concentration, for example [6], [8] and [10]-gingerols and [6]-gingerdiol metabolites. The antifungal mechanism of these compounds has been associated with the fungal cell wall and intracellular organelles, provoking morphological alterations in pathogen's hyphae, according to a study presented by Oliveira et al. (2019) .

Table 2 shows the percentage of mycelial zone inhibition (% MZI) of *C. gloeosporioides* after exposure by contact with the coatings in Petri dishes. After 2 days of inoculation, CWN coating showed a significantly higher percentage of fungal inhibition, alone or with addition of GO, as compared to GO itself and the control. Similar MZI (21.0 and 22.5%) were attained by GO nanoemulsion for both evaluated concentrations: 3 and 6% respectively. However, after 7 h no significant mycelial inhibition was observed in Petri dishes treated with GO nanoemulsion compared to control plates and to treatments containing CWN, which resulted in satisifatory inhibition of *C. gloeosporioides.*

Table 2. Percentage of mycelial zone inhibition (% MZI) of *Colletotrichum gloeosporioides* after contact with coated Petri-dishes with different coatings (mean values ± SD, *n*=3).

Coatings	% MZI	
	After 2 days	After 7 days
Water - control	00.0±0.0 [c]	00.0±0.0 [b]
GO nanoemulsion at 3%	21.9±5.5 [b]	0.2±0.7 [b]
GO nanoemulsion at 6%	22.5±2.6 [b]	2.5±1.6 [b]
CWN coating	50.6±3.0 [a]	45.0±2.6 [a]
CWN containing GO at 3%	42.0±7.6 [a]	44.4±1.9 [a]
CWN contating GO at 6%	48.2±8.0 [a]	47.8±2.8 [a]

CWN=carnauba wax nanoemulsion, GO=ginger oil nanoemulsion made from commercial oil. Means followed by a different letter in the same column are significantly different by Tukey test ($p<0.05$).

Antimicrobial activity of essential oils (EOs) and extracts from spices and herbs are attributed to phenolic compounds and there are considerable differences in chemical structures and amount of phenolics present in GO and oleoresin extracts (Singh et al., 2008). The hypothesis for EO activity is based in the hydrophilic character of its components. Oil compounds can cross the lipid layers of fungal cell membranes causing detachment and disruption, impairing membrane integrity and structure. There is an imbalance of hydrogenic and potassium cation exchange, which may lead to cell death (Beckman, 2000). GO also can induce high levels of cytoplasmic vacuolation, vacuole fusion and detachment of the plasma membrane from the cell wall, making it thinner causing loss of integrity (Da Cruz Cabral et al., 2013).

After 7 days (Table 2), % MZI for Petri dishes treated with GO nanoemulsion coating at both concentrations were not significantly different compared to the untreated control. Coatings containing CWN resulted in the highest percentage of mycelial growth inhibition (Table 2). It is known that carnauba wax is an inert substance; however, for the experimental conditions adopted in this work, the carnauba wax nanoemulsion coatings have showed protection against fungal colonization and invasive infection. The potential antimicrobial activity of carnauba can be attributed mainly to its hydrophobic nature. Carnauba wax provides a water-repellent coating which difficults microorganisms attachment and fruit penetration, reducing feed on living cells what have strong influence on pathogen survival. Additionally, other compounds found in wax composition have been suggested as having antifungal ativity, as reported by Cruz et al. (2002), that showed that defense active proteins,

such as chitinase and a β-1,3-glucanase, in carnauba wax presents in vitro action of inhibiting *Colletotrichum lindemuthianum*, *Colletotrichum musae*, and *Fusarium oxysporum* growth in their early stage. Gonçalves et al. (2010) also reported antifungal activity of carnauba wax, under in vitro and in vivo conditions (as coating on nectarines and plums), against the development of *Monilinia fructicola* and *Rhizopus stolonifera* infection.

Figure 1 shows the performance of GO, CWN coating and their combination when in vivo experiment with papaya. CWN coatings containing GO (3 or 6%) exhibited the smallest decay severity, however statistically not different from CWN neat formulation (without GO). The nanoemulsion coating containing 3% GO resulted similarly to the control, however, not different if compared to 6% GO, which have shown some peel phytotoxicity. By considering that, the results attained by CWN formulation was not different from that of 3% GO addition and of untreated control. Although it is not possible to confirm the joint effect of both compounds, a probable synergic action between coating and GO was reported by Ali. et al. (2016). In evaluating coating combinations of 2% GO and 10% gum Arabic (GA) applyied on 'Eksotika-II' papaya fruits along 28 days storage at 12±1°C it was shown that the mixture of GO and GA provided to be most effective in reducing anthracnose development than the individual application of GO or GA.

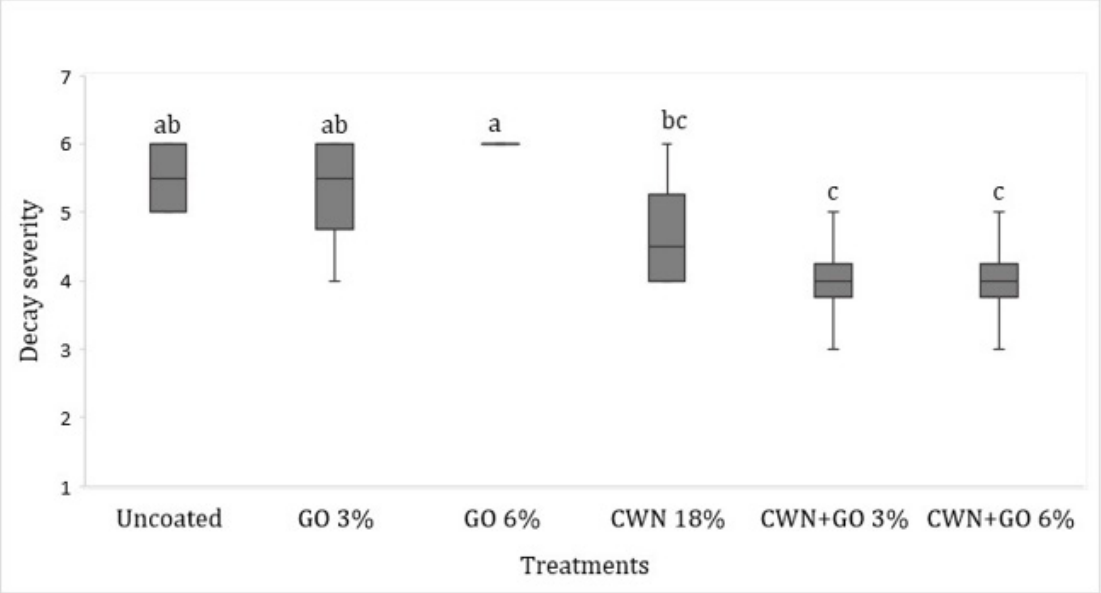

Figure 1. A) Median of the score for natural decay severity of 'Redland' papaya fruit coated with different coatings. Fruits were storage for 6 days at 80% RH. CWN=carnauba wax nanoemulsion and GO=ginger oil nanoemulsion made up with commercial oil. Bars with different letter are significantly different by Kruskal-Wallis test ($p<0.05$).

It is worth noticing that carnauba wax nanoemulsion itself showed some antifungal control, despite being an inert coating. In addition to the previously commented features of carnuba active compouds, there is the possibility that some ammonia residues may remain in coating. A ammonium is used as emusificate during the preparation of wax nanoemulsions. When the coating is drying, is expected that both ammonium and water evaporate and a thin carnauba film is formed. However, some ammonia residues may remain, what can provide certain action toward reducing fungal severity, when compared to uncoated fruits. Nevertheless, Gonçalves et al. (2010), have pointed out three possible antifungal mechanisms acting, either conjunctly or separately, in neat carnauba wax coatings: i) the formation of a steady physical barrier that prevents pathogen passage through the film; ii) alteration of the fruit internal atmosphere and, iii) a presumed direct action of the wax against the pathogens.

The incorporation of essential oils can interfere in the water vapor diffusion of the coatings but allow the delivery of active compounds to fruit's surface, where contamination occurs (Sanchez-Gonzalez et al., 2011). For an effective inhibition of fungal growth, a high concentration of phenolic compounds could be required, particularly when the attack takes place in a microorganism favorable environment, such as a the food matrix (Ali et al., 2016). However, the use of high concentrations of GO should be carefully considered due its phytotoxicity, as here observed when coating fruits with a 6% GO nanoemulsion. Normally for in vivo analizes of the antifungal activity, the concentration of active oil required is higher than the amount tested in in vitro conditions. This is because several interactions occur between oil phenolic compounds and the food matrix, decreasing overall activity (Feng and Zheng, 2007). These complex interactions may justify why less inhibition of *C. gloeosporioides* fungus is observed on the surface of the fruits (Ali et al., 2016).

CONCLUSIONS

GOs and GEs showed activity against *C. gloeosporioides,* and GOs required lower concentrations compared to alcoholic GE. Extracts from fresh material displayed less activity than oven air and freeze-dried ginger roots. Petri dishes inoculated with CWN coatings showed mycelial inhibition (after 48 h and 7 days), in neat condition or associated with commercial GO. On the other hand, GO at 3, or 6% reduced fungal growth after 48 h compared to uncoated plates. However, after 7 days, only GO was not efficient to further reduction of fungus spreading. The in vivo assays pointed to the complexity of the processes involving the volatile compound release, interactions with fruit matrix, and action on fungal development, when EOs are incorporated. To control disease severity in papayas, CWN containing GO showed the highest activity, with superior performance than GO alone, followed by the CWN neat coating. GO at 6% exhibited some peel phytotoxicity and consequent elevated fungal severity (probably due to peel injuries resulting from the GO totoxicity). In summary, CWN coating alone or associated with EOs might be a potential alternative to chemical fungicides as a natural and safe product to reduce papaya fungal decay.

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