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D-limonene in the postharvest of 'THB' papaya

Flavia Figueira de Souza Patrício[1], Cátia Aparecida Simon[2], Wilgner Fernandes Paradizo[3], Luciano Rastoldo Sigismondi[4], Leandro Demetrio Becattini Pereira[5], Edilson Romais Schmildt[1], Marcelo Barreto da Silva[1], Karin Tesch Kuhlcamp[3], Enilton Nascimento de Santana[3] and Sara Dousseau-Arantes[3] ✉

[1] Universidade Federal do Espírito Santo, Centro Universitário Norte do Espírito Santo, Departamento de Ciências Agrárias e Biológicas, Rodovia Governador Mário Covas, Km 60, Bairro Litorâneo, CEP 29932-540 São Mateus, ES, Brazil. E-mail: flaviafspatricio@gmail.com, edilson.schmildt@ufes.br, marcelo.silva@ufes.br

[2] Universidade de São Paulo, Escola Superior de Agricultura Luiz de Queiroz, Avenida Pádua Dias, nº 235, CEP 13418-900 Piracicaba, SP, Brazil. E-mail: catiasimonsimon@gmail.com

[3] Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural, Rodovia BR 101, Km 151, CEP 29900-000 Linhares, ES, Brazil. E-mail: wilgnerparadizo@gmail.com, karin.agronomia@gmail.com, enilton@incaper.es.gov.br, saradousseau@gmail.com

[4] Universidade Federal do Espírito Santo, Campus de Alegre, Alto Universitário, s/ nº, Campus de Alegre, Guararema, CEP 29500-000 Alegre, ES, Brazil. E-mail: luciano.rastoldo@lithoplant.com.br

[5] Faculdade Pitágoras, Avenida São Mateus, nº 1.458, Bloco 1, Sala 3, Bairro Araçá, CEP Linhares, ES, Brazil. E-mail: leandrobecattini@hotmail.com

✉ Corresponding author

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Abstract – The objective of this work was to verify if D-limonene in vitro presents inhibitory action on the mycelial growth of *Colletotrichum* spp. and antimicrobial activity against *Enterococcus faecium* and *Escherichia coli*, as well as to evaluate the effect of its concentrations on papaya disinfestation in the post-harvest process. For the in vitro tests, fungi were isolated from papaya fruit infected with anthracnose. For this, fruit fragments were disinfected and transferred to Petri dishes. Another test was carried out in vitro, to evaluate bactericidal activity, by for adding D-limonene to the suspension of the tested bacteria. Fruit physicochemical quality and disease incidence were evaluated. The application of D-limonene inhibited the mycelial growth of *Colletotrichum* spp. in vitro, with an increasing effect at increasing concentrations and a decreasing effect with time of product application. D-limonene was effective in inhibiting *E. faecium* and *E. coli*. There was no significant interaction between D-limonene rates and storage time for fruit quality or for disease incidence in ex vitro studies. D-limonene can be applied to papaya at postharvest, as it does not affect fruit quality and controls the growth of phytopathogens in vitro.

Index terms: *Carica papaya*, firmness, fungal pathogen, plant health.

D-limoneno na pós-colheita do mamoeiro 'THB'

Resumo – O objetivo deste trabalho foi verificar se o D-limoneno apresenta ação inibitória sobre o crescimento micelial de *Colletotrichum* spp. e atividade antimicrobiana contra *Enterococcus faecium* e *Escherichia coli*, bem como avaliar o efeito de suas concentrações na desinfestação do mamão no processo de pós-colheita. Para os testes in vitro, os fungos foram isolados de mamões infectados com antracnose. Para tanto, pedaços dos frutos foram desinfestados e transferidos para placas de Petri. Outro teste foi realizado in vitro para avaliação da atividade bactericida, tendo-se adicionado D-limoneno à suspensão de bactérias em teste. Foram avaliadas a qualidade físico-química dos frutos e a incidência de doenças. A aplicação de D-limoneno inibiu o crescimento micelial de *Colletotrichum* spp. in vitro, com efeito crescente com o aumento da concentração aplicada e efeito decrescente com o tempo da aplicação do produto. O D-limoneno foi eficaz na inibição de *E. faecium* e *E. coli*. Não houve interação significativa entre as doses de D-limoneno e o tempo de armazenamento quanto à qualidade dos frutos e à incidência de doenças nos estudos ex vitro. O D-limoneno pode ser utilizado na aplicação pós-colheita do mamão, por não afetar a qualidade dos frutos e por controlar o crescimento de fitopatógenos in vitro.

Termos para indexação: *Carica papaya*, firmeza, patógeno fúngico, fitossanidade.

Introduction

Papaya (*Carica papaya* L.) ranks as the third most cultivated tropical fruit globally, encompassing regions with tropical and subtropical climates (Chávez-Pesqueira & Núñez-Farfán, 2017). Brazil stands out as the second-largest producer and third-largest exporter of papaya, with 1,256,703 tonnes average annual production (FAO, 2021). Production is primarily concentrated in the states of Espírito Santo and Bahia, accounting for about 67% of the national production, and Espírito Santo yields three times more than Bahia (IBGE, 2021).

Despite its economic significance, papaya cultivation faces substantial challenges related to postharvest losses, due to its physiological characteristics and improper handling after harvesting. Papaya, being a climacteric and highly perishable fruit, exhibits a relatively short shelf life (Chitarra & Chitarra, 2005). Its relatively thin skin over a thick pulp makes it particularly susceptible to mechanical damage and pathogen attacks that accelerate the deterioration process (Tan et al., 2022).

To preserve fruit quality and mitigate postharvest losses, various technologies have been developed, including physical, chemical, and biological methods. Among these approaches, the heat treatment combined with fungicides and followed by refrigeration, during storage and transportation, has been commonly used to delay the development of phytopathogens (Rodrigues et al., 2021). However, extensive use of fungicides may result in adverse effects on the environment and public health, while also hindering exports, particularly to markets such as Europe (Murakami et al., 2020).

In this context, essential oils extracted from medicinal plants have garnered attention, due to their multifunctional properties, including antibacterial, antioxidant, antifungal, insecticidal, as well as cosmetic and medicinal applications (Bolouri et al., 2022). Among these oils, those derived from citrus fruit peels have gained prominence, for being obtained from fruit processing residue and for their antioxidative, anti-inflammatory, analgesic, antimicrobial, and anticancer properties (Haokip et al., 2023). Citrus peels constitute half the fruit weight, and the essential oil yield can reach up to 5% of the fresh peel weight, depending on the species and extraction method (Singh et al., 2021). The substantial generation of peel by orange juice industries makes the extraction of these oils advantageous (Mohsin et al., 2022).

The predominant component in citrus essential oils is D-limonene (4-isopropenyl-1-methylcyclohexene), representing approximately 91.4% of the oil (Ferronatto & Rossi, 2018). Studies, such as those by Cerna-Chávez et al. (2019), highlight the fungicidal action of D-limonene against various phytopathogens. Previous research has proven that the inclusion of citrus essential oils or D-limonene in coatings shows promising results for the controlling of phytopathogens and extending the shelf life of various fruits, such as strawberries (Shehata et al., 2020), bananas (Hou et al., 2022), and papayas (Yu et al., 2023).

Given the antifungal potential of D-limonene, this study's hypothesis is that its application on papaya at postharvest may disinfect the fruit, extending their shelf life and preserving their quality.

The objective of this work was to verify if D-limonene in vitro presents inhibitory action on the mycelial growth of *Colletotrichum* spp. and antimicrobial activity against *Enterococcus faecium* and *Escherichia coli*, as well as to evaluate the effect of its concentrations on papaya disinfestation in the post-harvest process.

Materials and Methods

The source of D-limonene used in the present work came from a commercial product composed of 53% D-limonene, 1,2,3 propanetriol, glycerol, emulsifier, and surfactant used to stabilize the product. The Clean Fruit is a sanitizer registered by the national health surveillance agency (Agência Nacional de Vigilância Sanitária - Anvisa), under process number 5351.318401/2022-88.

Fungi were acquired in a store located in the city of São Mateus, in the state of Espírito Santo, isolated from papaya fruit with symptoms of anthracnose (*Colletotrichum* spp.).

The fruit were taken to the phytopathology laboratory of the Universidade Federal do Espírito Santo, where they were washed in running water, and incisions were made in the transition region, between the lesions and healthy tissues, to remove the fragments. Fragments were disinfected in sodium hypochlorite solution (1:3), 70% alcohol, and distilled water and, then, dried on sterile filter paper, transferred to Petri dishes containing potato-dextrose-agar (PDA) culture medium, and incubated in BOD-type chambers, at 25±2ºC and 12-hour photoperiod.

The treatments were conducted in triplicate, and the radial growth of the fungus was evaluated in BOD at 25°C, after 72, 96, 120, 144, and 168 hours. Five concentrations of the D-limonene emulsion (1,000, 2,000, 3,000, 4,000, and 5,000 μL L^{-1}) were used in PDA culture medium containing fruit inocula. The control consisted of the fungi disk, without the addition of product.

Mycelial growth was determined by measuring the diameter of the colonies, in two diametrically opposite directions, using a caliper. The percentage of growth inhibition was calculated using the following formula: Growth (%) = (1 - Da/Db) × 100, where: Da is the diameter of the growth zone on the test plates; and Db is the diameter of the growth zone on the control plate.

The assay was conducted in a completely randomized design and subjected to a regression analysis. Polynomial fit was verified.

For the evaluation of the bactericidal activity of antiseptics and chemical disinfectants based on D-limonene (containing orange essential oil and citrus terpene) for *Escherichia coli* – ATCC 11229 and *Enterococcus faecium* – ATCC 6569, the European Standard Norme methodology was used (German Institute for Standardization, 2006).

For both microorganisms tested, an aliquot of the suspension tested for two microorganisms tested, containing approximately 1.5×10^8 CFU mL^{-1}, was added to D-limonene. I use the dilutions of D-limonene in a concentration of 8, 60, and 210 mL per liter of sterile purified water. The mixture was maintained at 20±1°C for 15 minutes. At the end of the contact period, the aliquots were taken, and the bactericidal activity in this portion was immediately suppressed by the dilution/neutralization method. The controls are also carried out in parallel with the study to validate the test. All plates were incubated at 36°C for 48 hours. The colony-forming units were counted and recorded and the calculations of the averages and reductions were done.

To verify the effect for disinfestation and fruit quality, different concentrations of the commercial D-limonene solution were evaluated in a papaya post-harvest processing packing house. Fruit were harvested from an area of commercial papaya cultivation belonging to the group Solo 'THB', located in the municipality of Sooretama, in the state of Espírito Santo, Brazil. Harvesting was carried out when fruit were at stage 1, that is, up to 15% of the peel surface was yellow (Brasil, 2010; Reis et al., 2018). Fruit were sent to the washing tank of the packing house of Caliman Agrícola S.A., located in Sooretama, where the treatments were carried out by washing on an automatic conveyor belt with brushes, with capacity of 2,200 L of water. Subsequently, fruit were taken to the laboratory of plant physiology / post-harvest and phytopathology, located at the experimental farm of the Instituto Capixaba de Pesquisa e Assistência Técnica e Extensão Rural (Incaper), in the municipality of Linhares, in the state of Espírito Santo.

The experiment was set in a 5x2 factorial arrangement – five doses of D-limonene (commercial product), and two storage periods – with a chlorine-based (20 ppm of chlorine) treatment as control, in a randomized complete block design with four replicates of five fruit.

After harvest, fruit were washed according to the described treatments and conditioned at two different temperatures, according to the performed evaluation. To evaluate the physicochemical characteristics, fruit were stored at 16±2°C, and they were evaluated at 8 and 11 days after storage. Fruit maturation stage was evaluated (Brasil, 2010), for which the visual scales correspond to: 0, grown and developed fruit, 100% green; 1, fruit ripening, 15% yellow; 2, ¼ ripe fruit, with 15 to 25% yellowish skin surface; 3, ½ ripe fruit, with 25 to 50% yellowish skin surface; 4, ¾ ripe fruit, with more than 50 to 75% yellowish skin surface; and, 5, ripe fruit, with 75 to 100% yellowish peel surface.

The following characteristics were evaluated: loss of fresh mass, determined by the difference between the initial mass of the fruit and the mass obtained at the end of each storage time, with the aid of a 0.01 g semi-analytical electronic Marconi precision scale, model AS5500C; fruit firmness, evaluated with the aid of a penetrometer, with a tip measuring 8 mm diameter by 20 mm length, at four opposite points in the equatorial region of each fruit, model IP-90DI, Impac; titratable acidity (TA), evaluated in the pulp juice, obtained from the midsection of the fruit by manual pressure and determined by the NaOH method at 0.1 mol L^{-1}, in the Titrino Plus Metrohn/848 automatic titrator, obtaining the result in percentage of citric acid; hydrogen ionic potential (pH), also evaluated in the pulp juice and determined in a digital potentiometer, pH lab Metrohm/827; total soluble solids (TSS), also evaluated in the pulp juice, using mL aliquot of juice,

with the aid of a Schmidt Haensch ATR-BR benchtop digital refractometer, with a variation from 0 to 100 °Brix, and TSS/TA ratio, obtained by the ratio between total soluble solids and titratable acidity.

To evaluate the incidence of diseases, fruit were stored at an ambient temperature of about 27.7°C for 7 days, when they reached maturation stage 5. Fruit were visually and individually examined, for the identification of the presence or absence of fungal diseases.

Fungal diseases were evaluated through the observation of symptoms characterized by lesions on the surface with a rounded shape, and necrotic, black, or grayish white spots, with depressed tissue centers, where brown or orange conidia masses are produced. The following diseases were evaluated: anthracnose (*Colletotrichum gloeosporioides*), early blight (*Alternaria solani)*, chocolate spot (*Glomerella cingulata*), stem-end-rot (*Lasiodiplodia theobromae, Phomopsis caricae-papayae*, *Colletotrichum gloeosporioides*, *Botryodiplodia theobromae*, *Fusarium* spp., *Alternaria alternata*, *Stemphylium lycopersici*, *Mycosphaerella* sp.), corynespora spot (*Corynespora cassiicola*), papaya mosaic (*Papaya sticky disease virus* – PSDV), and black rot (*Phoma caricae-papayae).*

To evaluate the physicochemical characteristics of the fruit and the incidence of diseases on them, data were subjected to the normality check using the Shapiro-Wilk's test, to the homoscedasticity check using the Bartlett's test, and to the check for independence of errors using residual plot. The assumptions were met. Data were subjected to the analysis of variance using the F test (α=0.05). When significant, the qualitative variables were subjected to Dunnett's test, to compare the doses of D-limonene with the standard treatment. The quantitative variables were subjected to regression analysis, to adjust the curves depending on the doses of D-limonene. All analyses were carried out using the software R version 4.2.2 (R Core Team, 2022), using the Tratamentos.ad package version 0.2.4 (Azevedo, 2022).

Results and Discussion

The D-limonene emulsion inhibited the mycelial growth of *Colletotrichum* spp. in vitro, and the outcome varied depending on the compound concentration and application time (Figure 1). Inhibition increased proportionally with concentration and decreased over the application time. The decline of *Colletotrichum* spp. inhibition over the application time was lower, when using 4,000 and 5,000 µL L^{-1} of the D-limonene emulsion (Figure 1 A). The mycelial growth inhibition

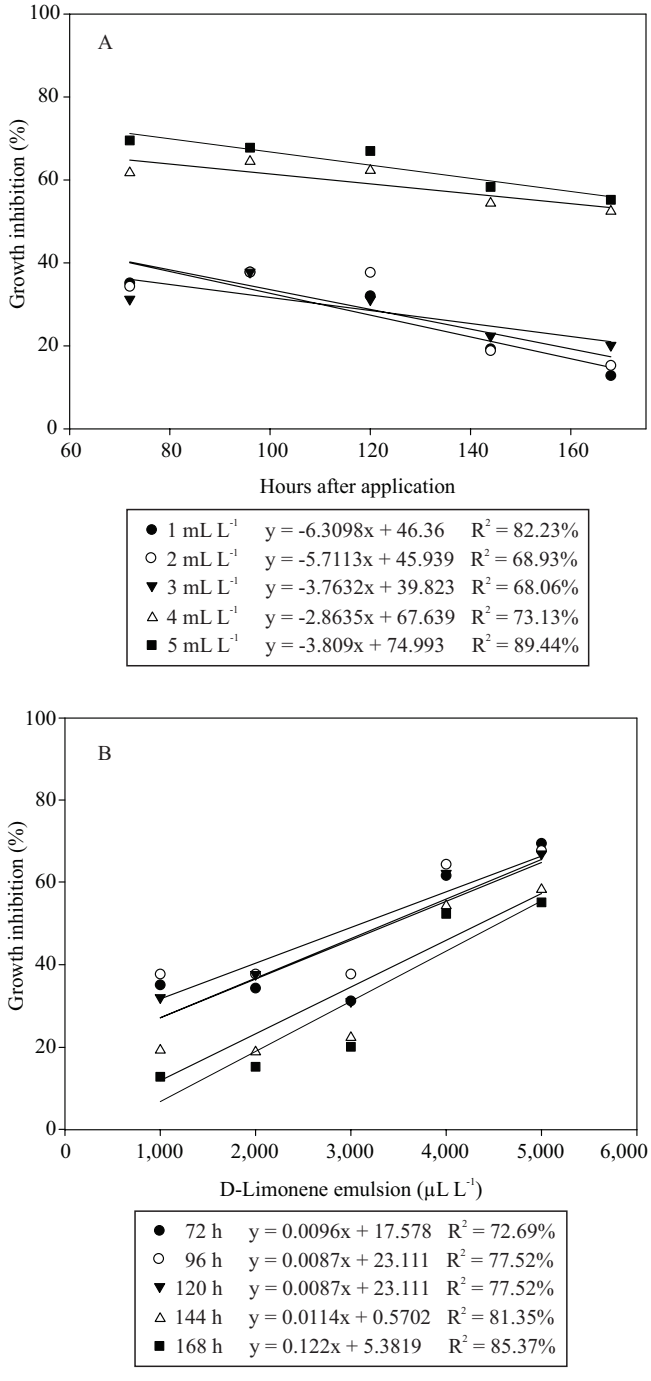

Figure 1. Inhibition percentage of *Colletotrichum* spp. isolated from papaya (*Carica papaya* 'THB') fruit, as a function of five concentrations of D-limonene emulsion evaluated after 72, 96, 120, 144, and 168 hours of application.

was more pronounced up to 96 hours after the D-limonene application, and the effect increased with concentration (Figure 1 B).

Considering that the emulsion contains 53% of D-limonene, applying 5,000 µL of the emulsion amounted to 2,650 µL L^{-1} of D-limonene, resulting in an observed average mycelial growth inhibition of approximately 70%, after 72 hours of inoculation (Figure 1 A). In a study conducted by Samithri et al. (2020), the addition of 1,000 µL L^{-1} of citrus essential oil provided only 2% to 13% inhibition of *Colletotrichum* sp. of mycelial growth, 48 hours after inoculation. These results indicate that the D-limonene emulsion is more efficient than pure essential oil, which is similar to the observations in banana post-harvest by Hou et al. (2022).

The D-limonene emulsion inhibited the growth of the microorganisms *Enterococcus faecium* and *Escherichia coli*, with a response varying with the concentration used (Table 1). The highest inhibition percentage (97%) was observed for *E. faecium* and *E. coli* at the D-limonene emulsion concentrations of 60 and 210 µL mL^{-1}, respectively. The present study showed a greater effectiveness of D-limonene against *E. faecium* (Gram-positive) than that against *E. coli* (Gram-negative). Gupta et al. (2021b) suggest that the relatively impermeable outer membrane surrounding Gram-negative bacteria might reduce the effectiveness of D-limonene.

The findings of the current study is consistent with well-documented reports on the antibacterial property of D-limonene (Gupta et al., 2021a). D-limonene causes membrane rupture, cell leakage, and cell death in *E. coli* cells. However, with increased contact time from 15 min to 2 hours, the reduction of the population ranges from 7% to 100% (Gupta et al., 2021b). As the objective of this research was to evaluate the emulsion potential as a disinfectant, the bactericidal action was assessed after 15-min contact. Further studies are recommended to test for longer contact times with the emulsion.

The response to concentrations was consistent with the studies of Gupta et al. (2021b), showing a greater effect at higher concentrations. For instance, observable damage to *E. coli* was evident through scanning electron microscopy at 16 µL mL^{-1} of D-limonene, while an increase to 128 µL mL^{-1} resulted in chemical alterations suggestive of membrane breakdown.

The physicochemical characteristics of the fruit were not affected by the interaction between D-limonene emulsion

Table 1. Results obtained for surviving cell counts, after a 15-minute contact with D-limonene emulsion, and calculated reductions in relation to the added inoculum.

Concentration (µL mL^{-1})	Culture	Result after 48-hour incubation at 36°C[(1)]		
		RVC (CFU mL^{-1})	Reduction (%)	REL
8	*E. faecium*	2.97	66.330	0.47
	E. coli	4.19	76.145	0.62
60	*E. faecium*	4.67	78.588	0.67
	E. coli	3.06 x 101	96.736	1.49
210	*E. faecium*	3.25 x 101	96.927	1.51
	E. coli	2.82	64.548	0.45

[(1)]CFU, colony forming units; RVC, reduction of the viable cells; and REL, reduction expressed in logarithm.

Table 2. Means of six physicochemical characteristics of papaya (*Carica papaya* 'THB') fruit subjected to different doses of D-limonene and control treatment with chlorine after 8 and 11 days of storage[(1)].

D-Limonene (ppm)	Mass loss (g)		Fruit firmness (N)		Total soluble solids (TSS, ° Brix)		pH		Titratable acidity (TA)		TSS/TA ratio	
	8 days	11 days	8 days	11 days	8 days	11 days	8 days	11 days	8 days	11 days	8 days	11 days
0	18.21a	32.23A	21.13a	19.26A	10.81a	10.68A	5.55a	5.55A	0.12a	0.10A	91.23a	107.15A
3,000	17.61a	30.69A	21.90a	19.10A	10.75a	10.34A	5.60a	5.64A	0.11a	0.10A	99.68a	103.31A
4,000	19.63a	34.86A	19.48a	16.85A	10.85a	10.66A	5.61a	5.63A	0.10a	0.08A	110.70a	130.62A
5,000	17.29a	27.80A	21.61a	19.24A	10.78a	10.42A	5.52a	5.62A	0.12a	0.08A	101.05a	132.91A
6,000	20.98a	29.45A	18.54a	15.94A	10.81a	10.55A	5.56a	5.61A	0.10a	0.09A	104.84a	121.46A
Chlorine	19.30a	33.51A	24.21a	19.81A	10.26a	10.12A	5.40a	5.53A	0.11a	0.08A	92.90a	126.92A
CV (%)	18.58	8.33	21.42	9.35	3,21	4.50	1.86	1.80	17.82	11,76	20.52	11.87

[(1)]Means followed by equal letters of each characteristic for each dose of D-limonene, lowercase at eight days and uppercase at 11 days, do not differ from the control (chlorine) by Dunnett's test at 5% of probability.

concentrations and storage time, nor in the comparison of doses with the standard treatment (Table 2).

Therefore, these results indicate that the physicochemical characteristics of 'THB' papaya fruit were not affected by treatments with D-limonene, which is assumed to be favorable. In the comparison of, an increase of weight loss with longer storage was evident, from 18.74 g, after 8 days, to 31 g after 11 days of storage, regardless of the D-limonene doses applied. Fruit weight loss is associated with the rate of respiration and evaporation of moisture through the epidermis or water loss (Dhital et al., 2018). The results of the present work corroborate those obtained by Lan et al. (2020), as they evaluated the effect of D-limonene on post-harvest mangoes, with greater weight loss values after 10 days of storage.

Fruit firmness decreased with increasing days of storage, dropping from 20.93 N, after 8 days, and decreasing to 18.04 N after 11 days of storage, regardless of the D-limonene treatment. Changes of fruit firmness have been correlated with ripening processes. As the fruit ripens, ethylene activates pectinolytic enzymes, when a substantial portion of the cell wall pectins are converted to a water-soluble form, affecting texture (Yahia, 2019).

Fruit showed little variation for total soluble solids, regardless of the days of storage, with a slight decrease from 8 to 11 days. Although papaya is a climacteric fruit, the increase of sugar content is observed only while fruit are attached to the plant (Ruggiero et al., 2011). The total soluble solids content varies minimally in post-harvest, as these fruit have a low starch content to hydrolyze (Dantas et al., 2018).

No significant difference was observed for the disease incidence results in papaya fruit subjected to different D-limonene rates compared to chlorine (control) (Table 3). Similarly, no significant statistical difference was found when comparing chlorine with dose 0 (water).

The regression analysis did not fit the curve. Hence, there was no significant difference between treatments for which the disease incidence percentages in relation to the average doses and water control were equal (Table 3). This fact suggests that washing with water and chlorine proved to be ineffective methods for controlling the pathogenic fungi.

The findings of this study are relevant for the evaluation of the effect of different D-Limonene concentrations, as the trial was conducted on-site, at a packing house; besides, the post-harvest papaya disinfection could not be verified. These results have generated new hypotheses. The first is that pathogen concentrations on fruit were low, suggesting the need for further basic research studies with inoculum control, to confirm the sanitizing effect on papaya fruit. The second hypothesis is that greater D-limonene effectiveness is achieved with longer contact time. Thus, only disinfection may not efficiently extend fruit longevity. This second hypothesis is supported by a recent study of Yu et al. (2023), who found that including 5,000 ppm of D-limonene in edible coatings can delay fruit ripening, reduce microbial infection and, consequently, extending their storage life.

Table 3. Mean incidence of diseases in papaya (*Carica papaya* 'THB') fruit, after seven days of storage, subjected to different doses of D-limonene and control treatment with chlorine[(1)].

D-Limonene (ppm)	Anthracnose	Chocolate spot	Stem-end-rot	Corynespora spot
0	22.50a	5.00a	21.88a	95.00a
3,000	10.00a	2.50a	17.50a	80.00a
4,000	30.00a	2.50a	22.50a	80.00a
5,000	2.78a	15.28a	20.55a	86.95a
6,000	17.50a	5.00a	30.00a	100.00a
Chlorine	13.06a	2.50a	20.88a	81.95a
CV (%)	72.53	125.32	54.58	17.71

[(1)]Means of each dose of D-limonene followed by equal letter, does not differ from the control (chlorine), by Dunnett's test at 5% of probability.

Conclusions

1. D-limonene emulsion effectively inhibits fungal growth and suppresses bacterial proliferation in vitro.

2. D-limonene does not affect the physicochemical quality or shelf life of 'THB' papaya (*Carica papaya*) fruit.

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