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Penicillium citrinum and *Penicillium mallochii*: New phytopathogens of orange fruit and their control using chitosan

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ABSTRACT

The antimicrobial action of chitosan against several phytopathogens in agriculture has been tested, including *Penicillium digitatum*, which is the major pathogen that causes postharvest decay of oranges. However, the biopolymer action has not been tested against other fungi that are capable of developing molds in orange fruit. This study have demonstrated that chitosan is able to inhibit the growth *in vitro* and *in vivo* of two *Penicillium* species, which were isolated from decay oranges fruit and identified as *Penicillium citrinum* and *Penicillium mallochii*, using molecular methods. This is the first report of *P. mallochii* acting as an orange phytopathogen. The commercial chitosan with higher molecular weight demonstrated a reduction in the disease incidence of 50–70 % for the inoculum *P. citrinum* and of 40 % for the inoculum *P. mallochii* for the *in vivo* experiments. The data obtained opens interesting alternative options to synthetic fungicide to prevent orange decay caused by the potential phytopathogenic species of *Penicillium* here identified.

1. Introduction

Citrus fruit is one of the most commonly produced and traded fruit crops, being cultivated in subtropical and tropical regions around the world. Brazil has led the world market of citrus fruit for many years, being oriented toward processing, followed by the United States and China (Ladaniya, 2008). The huge economic losses in worldwide citriculture are generally a reflection of the incidence of several diseases in post-harvest stages (Talibi, Boubaker, Boudyach, & Ben Aoumar, 2014). Diseases such as peduncle rot, anthracnose, black spot and melanose may be important under certain conditions (Kimati, Amorim, A., B. F., & Rezende, 1997), but the mold is considered the most recurrent postharvest disease in citrus production (Talibi et al., 2014). Fischer, Lourenco, and Amorim (2008)) have demonstrated that the mold reaches up to15 % of all post-harvest diseases.

The major fungal pathogen that causes mold in citrus fruit is *Penicillium digitatum*, which develops the green mold consisting of a white mycelium surrounding the area being sporulated, whilst the outer region of the lesion is composed of a softened rind (Snowdon, 2010; Timmer, Garnsey, & Graham, 2000). The development of green mold lesions is predominantly faster; but is common to find fruits that presents more than one type of mold caused by *Penicillium* spp., such as *P. italicum*, *P. fellutanum*, *P. expansum*, *P. crustosum*, *P. ulaiense* (Garcha & Singh, 1976; Louw & Korsten, 2015; Youssef et al., 2010). Al-Sheikh and Yehia (2016) investigated fungi causing mold in citrus fruit and they have found 39.5 % of incidence of *P. digitatum* and *P. italicum*, 2.5 % of *P. citrinum* and 0.91 % of *Fusarium solani*. Although the presence of *P. digitatum* is more relevant, the pathogenicity of different *Penicillium* species in citrus post-harvest causing molds should be more explored, since it could be a key issue in controlling molds in citrus fruit production.

Currently, the most employed treatment to control citrus postharvest disease are the chemical fungicides made from imazalil and thiabendazole groups (Dore, Molinu, Venditti, & D'hallewin, 2013; Sepulveda et al., 2015). However, they have several restrictions regarding their usage, such as high residual content and acute toxicity, long degradation time, possible side effects on human health and especially the development of resistant strains (Jimenez et al., 2015; Moretto, Cervantes, Batista, & Kupper, 2014). Especially in Brazil, where most of the citrus fruit produced are destined for exportation, the use of chemical fungicides may interfere the commercialization into countries with legislation that do not allow such substances (Nakano

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et al., 2016). All these restrictions and the demand for foods without chemical preservatives have encouraged the search for natural antimicrobial compounds (Talibi et al., 2014). Several approaches have been proposed as alternatives to synthetic fungicides for post-harvest control of citrus molds. These strategies include the use of food products (Youssef, Sanzani, Ligorio, Ippolito, & Terry, 2014), plant extracts (Simas et al., 2017), physical treatment (Terao et al., 2017), biological control (Zhu et al., 2013), being the treatments synergistic or not.

A promising strategy to control mold in citrus fruit is the use of chitosan, which is currently amongst the most assessed antifungal edible coating for postharvest preservation of fresh fruit (Cazon, Velazquez, Ramirez, & Vazquez, 2017; Fasihi, Fazilati, Hashemi, & Noshirvani, 2017; Li, Ye, Hou, & Zhang, 2018). Chitosan is a polysaccharide derived from chitin deacetylation, which is produced from crustacean processing waste, representing a renewable and biodegradable product (Rege & Block, 1999). Besides the ability to act as an antimicrobial agent against a variety of phytopathogens (Freddo, Mazaro, Brun, & Wagner, 2014; Mohammadi, Hashemi, & Hosseini, 2016; Oliveira, El Gueddari, Moerschbacher, & Franco, 2012), chitosan enhance fruit shelf-life by reducing respiration rate and water loss (Elsabee & Abdou, 2013; Verlee, Mincke, & Stevens, 2017). The effectiveness of chitosan as an antifungal agent against molds caused by *P. digitatum* and *P. italicum* in citrus fruit has been recently reported (Al-Sheikh & Yehia, 2016; El Guilli, Hamza, Clement, Ibriz, & Ait Barka, 2016; Lu et al., 2014; Tayel, Moussa, Salem, Mazrou, & El-Tras, 2016). Despite this advance, the investigation of different potential species causing mold in citrus, as well as the evaluation of chitosan effect against new molds, might ensure treatments more effective to control the disease.

Considering this context, the primary purpose of this work was to investigate by molecular analysis if molds developed on oranges exposed to the ambient for contamination were *P. digitatum* and/or *P. italicum*. In the case of new fungi being identified, their potential as phytopathogens in oranges fruit would be investigated by pathogenicity test, conducted according to the Koch's postulate. Then, the antifungal effect of chitosan *in vitro* and *in vivo* on the identified fungi would be evaluated, using commercial samples of chitosan with different molecular weight. The aim of the present work is to demonstrate that the chitosan biopolymer already consolidated in the literature as an antimicrobial agent, is able to inhibit the growth of new potential phytopathogens causing post-harvest disease in oranges.

2. Materials and methods

2.1. Materials

The chitosan samples used in this study (Chitoclear) were obtained from the shrimp *Pandalus Borealis* and provided by Primex from Island. The three administrations of chitosan were characterized by Fraguas et al. (2015) as molecular weights and acetylation degrees (Table 2).

2.2. Fungi isolation

Isolates of *Penicillium* spp. were obtained from citrus fruits (*Citrus sinensis* L.) which were collected from a local market and exposed to the ambient air for contamination (25 °C, 56 % relative humidity, with a photoperiod of 12 h for 20 days). The fungi were isolated from the collected fruit sample of traditional cultivars of Brazil, 'Serra d'água', 'Pêra Rio' and 'Bahia'. Portions of 0,5 cm from the symptomatic tissue were cut with a sterile scalpel and placed on Petri dishes containing agar Sabouraud medium at 25 °C for seven days. A pure culture was obtained by single spore isolation on Sabouraud medium. The fungal spores were collected in a sterile saline solution of 0.85 % NaCl and isolates were stored in 15 % glycerol in a refrigerator (−20 °C).

2.3. Molecular identification of fungal isolates

For fungal species identification, the samples from each isolates (S, P and B) were analyzed using the regions ITS and Beta-tubulin. Total DNA extraction from each sample was performed according to methodology described by Rosa, Vaz, Caligiorne, Campolina, and Rosa (2009). ITS1 and ITS4 primers were used for the amplification of the internal transcribed region ITS-5.8S of the rRNA gene region, as described by Innis, Geldand, Sninsky, & White (1989). The BT2a and BT2b primers were used for partial amplification of the β-tubulin gene as described by Glass and Donaldson (1995). The amplifications generated by the PCR reaction were purified by using 5 mM EDTA (Ethylenediamine tetraacetic acid). The product obtained was dosed into a NanoDrop ND 1000 (NanoDrop Technologies) in order to be used in the sequencing reactions. The sequencing reactions from the products obtained after amplifying the purification were performed through the use of the Big Dye version 3.1 kit combined with the ABI 3730 automated sequencing system.

The sequences obtained were analyzed with a SeqMan P and with Lasergene software and a consensus sequence was obtained by using Bioedit v. 7.0.5.3 software. Representative consensus sequences for fungal taxa were deposited into GenBank (Table 1). The DNA sequences were compared to the GenBank deposited type sequences by using BLASTn (Basic Local Alignment Serch Tool - version 2.215 of BLAST 2.0), in accordance to Altschul et al. (1997), available at NCBI (2015). Phylogenetic analysis was conducted through the use of the Mega Molecular Evolutionary Genetics Analysis (version 6) software. The maximum composite likelihood method was employed to estimate evolutionary distances with bootstrap values calculated from 1000 replicate runs. To complete the molecular identification, the sequences of known fungal strains or reference sequences obtained from fungal species deposited in the international culture collections, which are found within the GenBank [36], were added to improve the accuracy of the phylogenetic analysis. Information on the hierarchical levels used in fungal taxonomy may be obtained from MycoBank (IMA, 2017) and from the Fungorum Index (Fungorum, 2015).

Table 1
Molecular identification of fungi isolated from moldy oranges of the cultivars 'Bahia' (B), 'Pêra Rio' (P), and 'Serra d'Água' (S).

Isolated fungus	Results of Top BLAST (GenBank access nº)	Coverage (%)	Identity (%)	Number of base pairs sequenced and analyzed	Proposed taxa (GenBank access n°)
B	*P. mallochii* (NR111674) [1]	98	99	481	*P. mallochii* (MG591446) [3] (MG591449) [4]
	P. mallochii (JN625973) [2]	100	99	400	
P	*P. citrinum* (NR121224) [1]	100	100	439	*P. citrinum* (MG591447) [3] (MG591450) [4]
	P. citrinum (KP329975) [2]	100	100	439	
S	*P. citrinum* (NR121224) [1]	98	100	455	*P. citrinum* (MG591448) [3] (MG591451) [4]
	P. citrinum (KP329975) [2]	100	100	445	

[1]Identification performed by BLASTn using fragments of the ITS1-58S and ITS2 regions of the rRNA gene. [2] Identification performed by BLASTn using β-tubulin gene fragments. [3] ITS1-5.8S-ITS2 and [4] β-tubulin sequences deposited in GenBank.

Table 2
Chitosan MIC (μg × mL^{-1}) and growth reduction (GR in %) values for the fungi assessed, using the three chitosan samples with different molecular weights (Mw) and degrees of acetylation (DA).

Chitosan	Mw [1] (kDa)	DA [1] (%)		Fungus S *P. citrinum*	Fungus P *P. citrinum*	Fungus B *P. mallochii*
1	132 ± 1.0	5.9 a	MIC	100	100	80
			GR [3]	77.9 a B	86.4 a A	81.4 a B
2	228 ± 9.0	6.8 a	MIC	100	100	80
			GR [3]	78.4 a A	83.4 a AB	88.9 b B
3	245 ± 2.0	6.3 a	MIC	100	100	80
			GR [3]	78.6 a B	93.4 b A	98.4 c A

[1,2]Source for analysis: Fraguas et al. (2015). Means followed by the same letter do not differ statistically (Tukey's test; p ≤ 5 %).
[3]Means followed by the same letter do not differ statistically (Tukey's test; p ≤ 5 %); lower case (a) evaluates differences between the chitosans; upper case (A) evaluates differences between the P, B, and S fungi.

2.4. In vitro determination of antifungal activity

Antifungal activity of the three chitosan samples was evaluated by the cultivations of the *Penicillium* sp. strains in Sabouraud medium (composition in g × L^{-1}: 20 glucose, 5 yeast extract and 10 peptone). Spore concentrations were adjusted to 1×10^5 spores × mL^{-1} by counting spores in a Neubauer chamber. The solution of chitosan was prepared using acetic acid 0.15 mol × L^{-1} and the pH was adjusted to 5.5, then it was filtered through a membrane with 22 μm. Bioassays took place in polystyrene microtiter plates, as the following amounts of reagents were added in each well: i) 115 μL of sterile Sabourad medium; ii) Chitosan in μg × mL^{-1}: 0, 40–800 ; iii) 10 μL of spore suspension; iv) sterile distilled water to make up a final volume of 200 μL. The cultures were performed as triplicates and the microtiter plates were incubated at 25 °C under 180 rpm for 5 days. Microbial growth was monitored by optical density (OD) measured at 405 nm in microplate reader (MR-96 A, Mindray) in 24 h intervals, according to methodology suggested by Oliveira et al. (2012). The minimum inhibitory concentration (MIC) was determined by statistical Tukey test (P ≤ 5 %) applied to the data obtained at the end of cultivation stage (after 120 h). The percentage of growth reduction (GR) duo to chitosan action was calculated according to the Eq. 1:

$$GR(\%) = \frac{100 - (A_{MIC} \times 100)}{A_{control}} \quad (1)$$

where A_{MIC} refers to the area under the growth kinetics curve for the MIC and $A_{control}$ means the area under the growth kinetics curve for the control.

2.4.1. Type of inhibitory effect

In order to assess the fungistatic or fungicidal effect of chitosan, the most resistant isolated to the chitosan was chosen and then it was cultivated in Falcons of 20 mL in the presence of Chitosan 3 (that presents the higher molecular weight) with a concentration twice the MIC. After 5 days of cultivation at 25 °C the culture was diluted 10 times with Sabouraud medium, reaching a concentration of chitosan much lower than MIC. Growth was monitored by OD at 405 nm for 5 days. Tests were performed as triplicates, where growth would mean fungistatic effect and no growth would mean fungicidal effect.

2.5. Postharvest experiments

For the postharvest experiments 'Serra d`água' and 'Pêra Rio' oranges fruits (*Citrus sinensis* L.) were collected 8 months after flowering from a citrus plantation located in the city Cristiano Otoni – Brazil (20°47′19.7″S 43°48′39.5″W). The fruits had their surface disinfected by immersion in 1 g × 100 mL^{-1} sodium hypochlorite solution for 5 min. Then, they were washed with distilled water and the following post-harvest experiments were performed.

2.5.1. Pathogenicity test and in vivo evaluation of antifungal activity

The postharvest experiments to assess the pathogenicity of isolates and the *in vivo* effect of chitosan were conducted simultaneously, using oranges from the cv. 'Pêra Rio'. The experiment consisted of 8 treatments, with 10 oranges for each treatment: i) control without fungus and without chitosan; ii) control without fungus and with chitosan; iii) fungus B with chitosan; iv) fungus B without chitosan; v) fungus P with chitosan, vi) fungus P without chitosan; vii) fungus S with chitosan and viii) fungus S without chitosan. In the treatments (ii), (iii), (v) and (vii) the oranges were immersed in chitosan solution 1 g × 100 mL^{-1} with acetic acid 0.15 mol × L^{-1} and exposed to the environment for approximately 10 h for drying. In the treatments from (iii) to (viii) the oranges were wounded in two equatorial positions with the aid of a sterile needle at a depth of 1.5 mm and a width of 2.0 mm, and 10 μL of spore suspension (10^8 spores × mL^{-1}) were added over each wound. The fruits were kept at ambient temperature (21 °C) and ambient air (56 % relative humidity), with a photoperiod of 12 h for 10 days.

The treatments (i), (iv), (vi) and (viii) were analyzed separately from the other treatments representing the pathogenicity test, which consisted in reinoculate the isolates obtained from the moldy oranges in healthy oranges to assess whether the disease would develop (Jakobi, 2010). On the other hand, the treatments from (ii) to (viii) represented the experiment to assess the disease incidence (%) in the presence of chitosan. The percentage of disease incidence was calculated with the Eq. 2

$$disease\ incidence\ (\%) = \frac{number\ of\ rotting\ wounds}{total\ number\ of\ wounds} \text{x}\, 100 \quad (2)$$

2.5.2. Shelf-life of orange fruit treated with chitosan

The shelf-life experiments were conducted by analyzing the physicochemical parameters of oranges from cv. 'Serra d'água' treated with Chitosan 3 (presenting higher molecular weight). Every day, the pulps of three replicate of fruit coated with chitosan and uncoated (control) were collected. These pulp samples were filtered in order to eliminate interference and the following parameters were analyzed for 10 days. The total soluble solids (SST) was determined through readings in the refractometer (°Brix). The pH was measured by digital pH-meter. Acid concentration (fixed and volatile) was measured by titration: a predefined volume of 10 mL of orange juice titrated with sodium hydroxide 0.1 mol × L^{-1}, with alcoholic phenolphthalein solution 1 g × 100 mL^{-1} as the final indicator of titration. The results were expressed in g × L^{-1} and calculated in terms of citric acid. The vitamin C content was measured by the colorimetric method suggested by Kampfenkel, VAnmontagu, and Inze (1995)) and total vitamin C was expressed as mg of ascorbic acid × 100 mL^{-1} of fresh pulp. Differences between the averages were detected by the Tukey multiple comparison test.

3. Results and discussion

3.1. Fungal identification

The orange fruits (*Citrus sinensis* L.) from the cultivars 'Bahia', 'Serra d'água' and 'Pêra Rio' were exposed to the environment for contamination and then presented soft rot, covering the fruit with white mycelium and a large number of green spores, as can be seen in Fig. 1. The isolated fungi obtained from the cultivars 'Bahia', 'Serra d'água' and 'Pêra Rio' were named as fungus B, fungus S and fungus P, respectively. The colonies of the fungi P, B and S resemble *Penicillium* spp. (Fig. 1) as their agar plate culture present similar characteristics, with green conidia, granular shape, white mycelia and rough appearance (Frisvad & Samson, 2004; Kimati et al., 1997).

Due to macroscopic similarities between species of *Penicillium* and

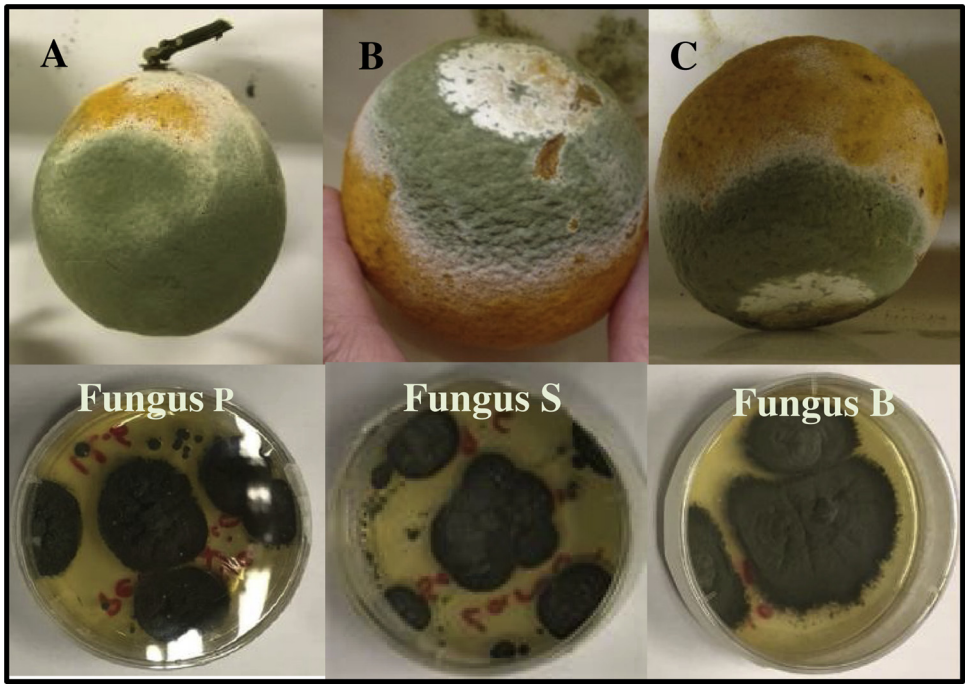

Fig. 1. Moldy orange fruit after exposure to contamination, and fungi isolated from them. Decayed oranges of cultivars (A) 'Pêra Rio', (B) 'Serra d'Água', and (C) 'Bahia' after 20 days exposure to ambient air for contamination. Plate cultures of fungal isolates P, S, and B on Sabouraud agar after 7 days at 25 °C.

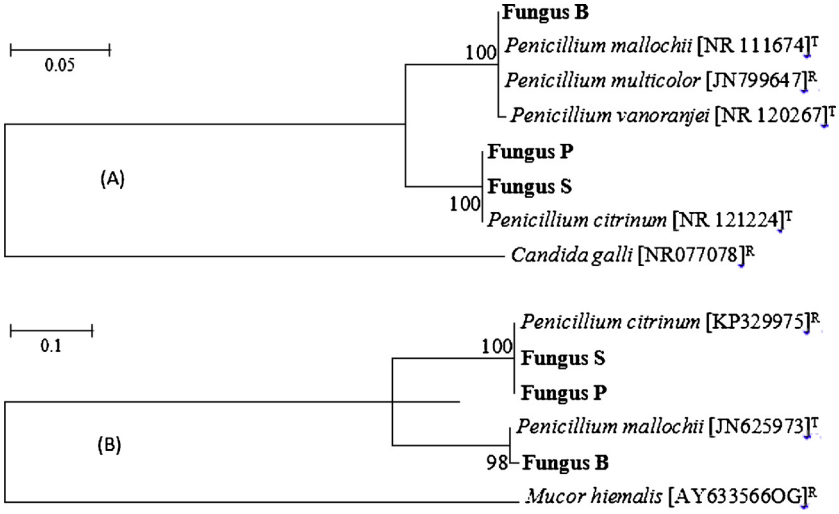

Fig. 2. Phylogenetic tree of fungi isolated from different orange cultivars: 'Bahia' 'Pêra Rio', and 'Serra d'Água'. The tree was constructed based on (A) fragments of the internal transcribed region ITS-5.8S of the rRNA gene, and (B) the partial sequencing of the β-tubulin gene, using the neighbor-joining method. [R] Sequence of reference. [T] Sequence type.

for the fact that *P. digitatum* is the most commonly fungus found in moldy citrus fruit, many studies have considered the isolated fungi from post-harvest citrus fruit as *P. digitatum*, based only on morphological characteristics, without any molecular analysis (El Guilli et al., 2016; Tayel et al., 2016). For these reasons, the molecular analysis of the isolates was done in order to obtain reliable information about the fungal species. The molecular analysis, through the sequencing of ITS and Beta-tubulin regions, identified the species *Penicillium mallochii* for fungus B and *Penicillium citrinum* for fungi P and S (Table 1). Both fungi have presented sequences with identity ≥ 99 % to the fungal sequences deposited in the GenBank (NCBI, 2015). The phylogenetic tree is shown in Fig. 2. Micrographs of fungi were obtained using an optical microscope and are illustrated in Supplementary Material.

This is the first report of *Penicillium mallochii* causing mold in orange fruit. *P. mallochii* is an unexplored specie that was reported by Rivera et al. (2012) as a host in ''cajá'' fruit (*Spondias mombin*) and recently it has been isolated from insets presents in strawberry crops by Poitevin (Poitevin, Porsani, Poltronieri, Zawadneak, & Pimentel, 2018). On the other hand, the *Penicillium citrinum* fungus has been isolated from different food sources including cereals, rice, wheat, corn, barley, peppers, coffee and grapes (Barkai-Golan & Paster, 2008). There are some reports of its isolation from citrus (Akhtar, Anjum, & Jabeen, 2013; Al-Sheikh & Yehia, 2016; Wyatt, Parish, WidmeR, & Kimbrough, 1995), although no studies have evaluated its pathogenicity. It is known that *P. citrinum* produces a mycotoxin citrinin with strong nephrotoxic action (Houbraken, Frisvad, & Samson, 2011; Wu, Yang, Yu, & Liu, 2012). In this way, more elaborate studies were necessary to investigate *P. citrinum* and *P. mallochii* aggressiveness and relevance as citrus fruit pathogens. With this purpose, the pathogenicity test of the identified fungus was performed according to the Koch's Postulate (Jakobi, 2010), which is better described in item 3.3.

3.2. Antifungal activity in vitro of chitosan

The *in vitro* growth of *P. citrinum* and *P. mallochii* in the presence of chitosan has demonstrated a positive correlation between polymer concentration over the development of these fungi. The minimum inhibitory concentration (MIC) represents the dose of the antimicrobial

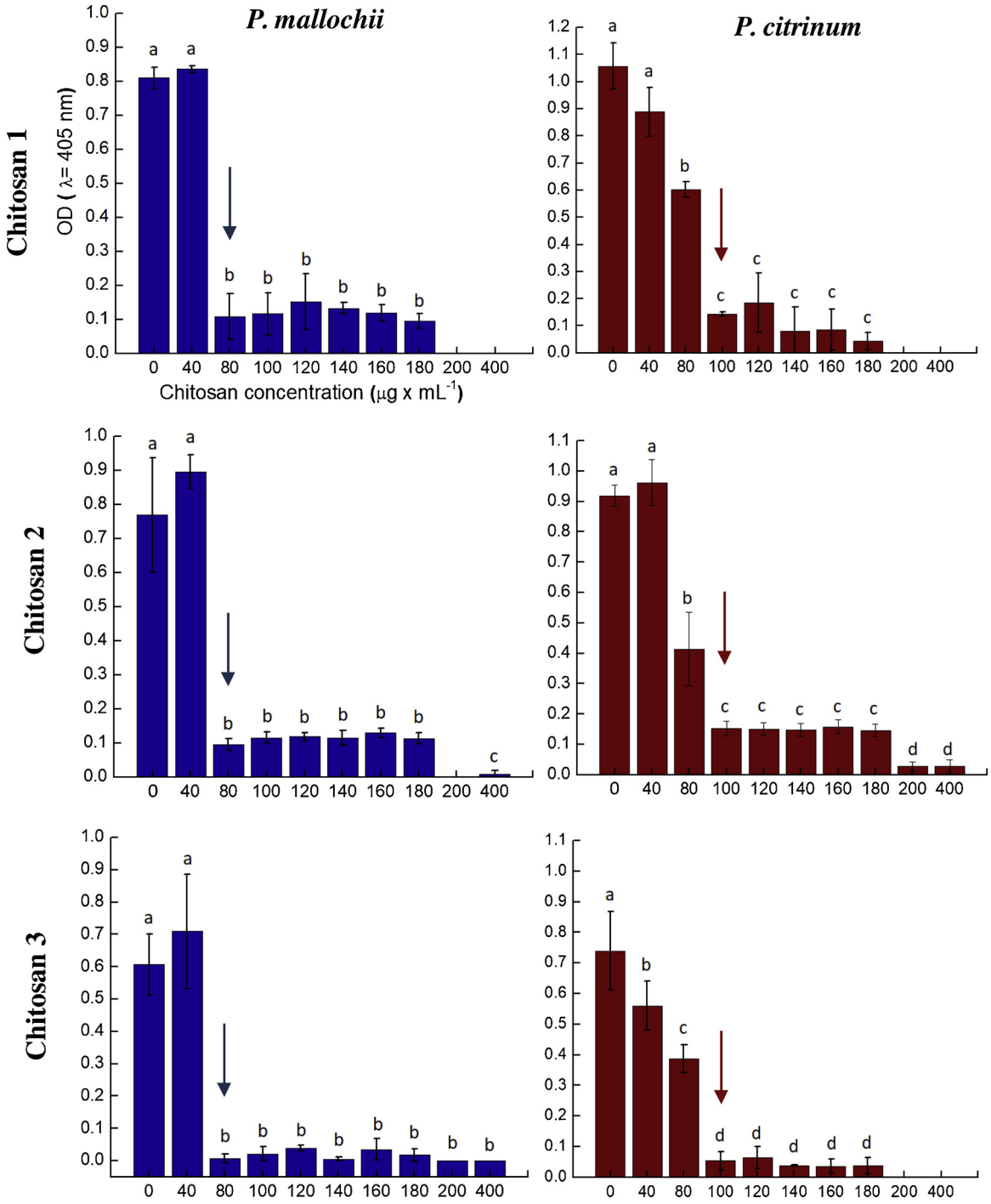

Fig. 3. Determination of antifungal activity. Results of the *in vitro* growth of *P. mallochii* (fungus B) and *P. citrinum* (fungus P) after 120 h, in the presence of chitosan. The OD values were obtained for different concentrations of chitosan. The MIC values are indicated by the arrows. The means of the triplicates followed by the same letter did not differ statistically (Tukey's test; $p \leq 5$ %).

agent that may completely inhibit microorganism growth until the end of the cultivation. Chitosan MIC values were 80 μg × mL^{-1} for *P. mallochii* and 100 μg × mL^{-1} for *P. citrinum* (Fig. 3). It is important to highlight that the most relevant citrus molds from the genus *Penicillium* (*P. digitatum* and *P. italicum*) have exhibited the same behavior in the presence of chitosan, *i.e.* a dose-related inhibitory effect, as demonstrated for by El Guilli et al. (2016); Shao et al. (2015) and Al-Sheikh and Yehia (2016). This indicates the potential of chitosan against new and already established phytopathogens that cause mold in oranges.

Chitosan MIC values can vary according to the polymer characteristics such as source, molecular weight and degree of acetylation. Chitosan with lower degree of acetylation, reflected by higher content of amino groups, may mean a more expressive antimicrobial effect (Oliveira et al., 2012). The results found in this work did not allow this verification, since chitosan samples used in this work were characterized by Fraguas et al. (2015) and showed very close degrees of acetylation and no statistical difference (Table 2). The MIC values for *P. citrinum* and *P. mallochii* was 80 and 100 μg × mL^{-1}, respectively, while Al-Sheikh and Yehia (2016) have found MIC of 4000 μg × mL^{-1} for *P. digitatum* when also using shrimp chitosan. The difference found between MIC values could have occurred due to the distinctive behavior of each fungus specie for the chitosan, that is, *P. citrinum* and *P.*

mallochii may be more resistant to the antifungal action of the biopolymer than *P. digitatum*. Another factor that may explain the difference in MIC values is the degree of acetylation of chitosan. Chitosan used by Al-Sheikh and Yehia (2016) had a higher degree of acetylation (10.4 %) than the chitosan used in this work (approximately 6 %).

The antimicrobial action of chitosan with lower degree of acetylation should be more pronounced than the antimicrobial action of chitosan with higher degree of acetylation, because it has more amino groups available to alter the permeability of cellular membrane of microorganism. The amino groups of chitosan have strong attraction to the negative groups of macromolecules exposed on the surface of the fungal cell wall. This attraction causes changes on the permeability of the cellular plasmatic membrane of the fungi leading to the release of proteins and other intracellular constituents, being this mechanism the most widely accepted to explain the chitosan antimicrobial activity (Benhamou, 1996).

The relationship between molecular weight and antimicrobial activity of chitosan is another parameter that has been used to explain the polymer inhibitory effect. Most of the studies classify the action of chitosan as molecular weight-dependent (Jeon, Park, & Kim, 2001). The growth reduction (GR) for the inoculum P (*P. citrinum*) and B (*P. mallochii*) was more pronounced when using Chitosan 3, which had the higher molecular weight (Table 2), suggesting that Chitosan 3 has a greater inhibitory effect for these fungi. When evaluating the mechanism of action by chitosan, it is possible to infer that molecules with higher molecular weight are theoretically bigger, and when they bind to the fungal cell wall they cause a more expressive steric hindrance. This steric hindrance makes it impossible for nutrients and metabolites to be exchanged by the cell, interfering in the development of the phytopathogens (Jeon et al., 2001).

The efficiency of chitosan as antimicrobian agent depends not only on the polymer characteristics, but also on the characteristics of the phytopatogen, such as the species and the host fruit in which it develops (Palou, Valencia-Chamorro, & Perez-Gago, 2015). Although both S and P fungi were identified as *P. citrinum* and showed the same MIC, they presented difference in the total growth reduction (Table 2). When comparing growth reduction data, *P. citrinum* isolated from 'Serra d'água' orange seems to be more resistant than *P. citrinum* isolated from 'Pêra Rio' orange fruit. Fischer et al. (2008) have found a higher incidence of green mold in 'Serra d'água' orange fruit (referred to as "Lima" orange in their study) than in 'Pêra Rio' cultivars. These results suggest that 'Serra d'água' orange strains may have developed more elaborate resistance mechanisms than 'Pêra Rio' orange strains.

In the experiment conducted to assess whether chitosan has fungistatic or fungicidal effect, it was chosen the isolates of ''Pêra Rio" orange fruit (fungus S, *P. citrinum,*) because they have shown to be the most resistant. The cultures which had previously their growth inhibited by chitosan with a dose of 200 and 400 μg × mL^{-1} (above the MIC), were then diluted 10 times for a dose of chitosan below the MIC, consequently, unable to inhibit fungal growth. The results have made evident the fungistatic effect of chitosan, once the spores restarted to growing (Fig. 4 and Fig. S6 of Supplementary Material). This behavior indicates that *P. citrinum* has germination inhibited by chitosan, but when reinserted into the medium containing new nutrients without chitosan or with very low doses of the polymer, it becomes feasible again. So far, no fungicidal or bacterial effect by chitosan has been reported against any microorganism (de Britto & Assis, 2012; Freddo et al., 2014; Oliveira et al., 2012){Freddo et al., 2014, Chitosan as fungistatic mycelial growth of Rhizoctonia solani Kuhn}. It has been evident that chitosan presents a protective effect, rather than a therapeutic effect, as it prevents the development of spores, but is unable to eliminate an established infection (Bautista-Banos, Hernandez-Lopez, Bosquez-Molina, & Wilson, 2003).

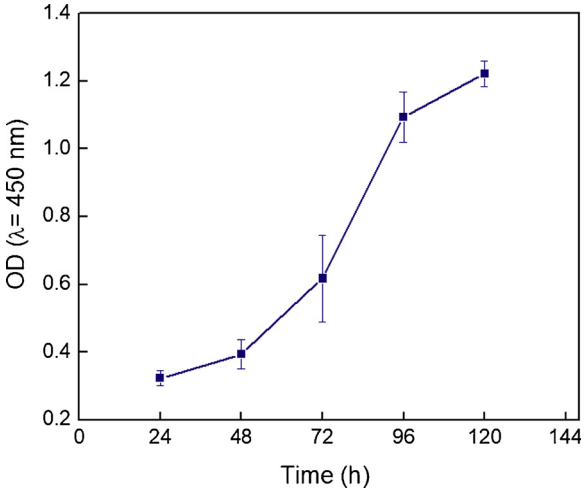

Fig. 4. Fungistatic effect of chitosan. OD data presented as means of triplicates, representing the growth kinetics of *P. citrinum* (fungus S) in the presence of a dose of chitosan much lower than the MIC (20 μg chitosan × mL^{-1}).

3.3. Post-harvest experiment: pathogenicity of isolates and in vivo effect of chitosan

The pathogenicity tests consisted on inoculating spores of the isolated fungi (fungus S, *P. citrinum*; fungus P, *P. citrinum* and fungus B, *P. mallochii*) into healthy oranges (from the cv. 'Pêra Rio'). The aim of the experiment was at verifying whether there was disease incidence after 10 days of inoculation and thus validate Koch's postulate (Jakobi, 2010). The Fig. 5 shows that after 10 days of inoculation, oranges showed 100 % disease incidence, with the development of soft rot in all the fruits, evidencing the pathogenicity of the three isolated.

In the third day after inoculation into the wounds, the oranges showed signs of internal infection; in the fifth day, oranges were covered with white mycelium and in the ninth day the peels were predomntly covered by spores of green coloration. A singularity observed at the end of the experiment was that 10 % of the oranges inoculated with *P. mallochii* presented only internal signs of infection, without the development of mold throughout the peel. In general, the results indicates that both *P. citrinum* (isolated from cv. 'Pera Rio' and from cv. 'Serra d'água') and *P. mallochii* (isolated from cv. 'Bahia') may not behave as saprophytes or opportunistic, but as potential phytopathogens of orange fruit. The pathogenicity test validate the Koch's postulate and suggest a similar aggressiveness between the new isolates.

In the post-harvest experiments to evaluate the *in vivo* antifungal effect of chitosan, the polymer with the highest molecular weight (chitosan 3) was chosen, taking into account that it was the most effective in the *in vitro* experiments. The course of the experiment is illustrated in Fig. S8-S11 of the Supplementary Material. All the oranges fruit from the controls group without fungus and without chitosan did not showed signs of disease (Fig. 5). The same behavior was observed for the controls group with chitosan and without fungus, indicating that there was no proliferation of other molds present naturally in the fruits, hence only the spores that were inoculated in the wounds would be able to cause any infection in the fruits.

Oranges untreated with chitosan presented complete development of the disease (after 10 days) by the three inoculums (fungus S, *P. citrinum*; fungus P, *P. citrinum* and fungus B, *P. mallochii*). On the other hand, the orange fruit that were treated with chitosan presented a reduction in the disease incidence of 40 % for *P. mallochii* B, 70 % for *P. citrinum* P and 50 % for *P. citrinum* S (Fig. 5). Regarding the *in vitro* experiments, chitosan 3 represented a growth reduction of fungi B, P and S of 98, 93 and 78 %, respectively. Although the parameters 'growth reduction' and 'disease incidence reduction' have different mathematical foundations, they can be compared once both indicate

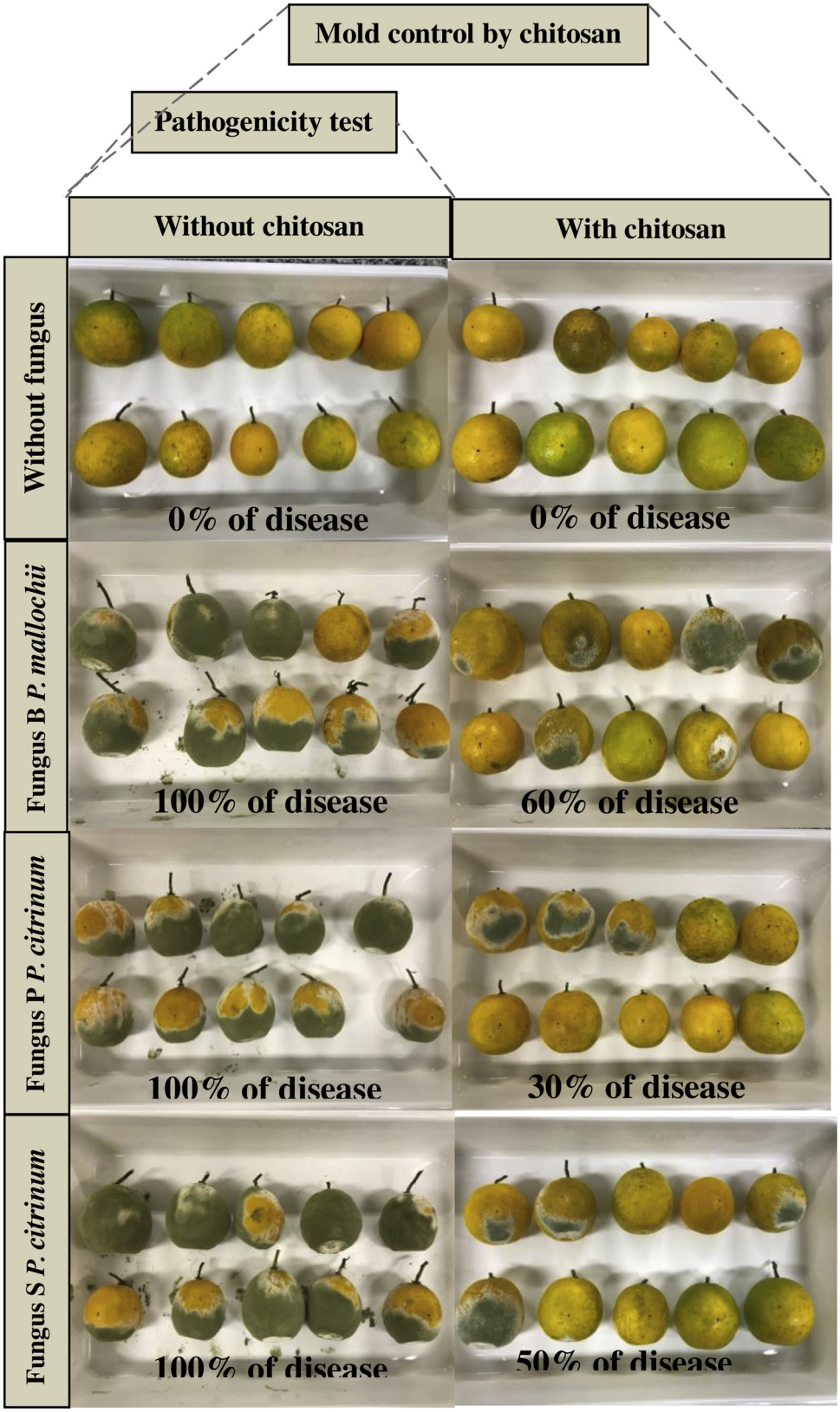

Fig. 5. Postharvest experiment performed using cv. 'Pêra Rio' oranges, after 10 days of storage. Oranges not treated with chitosan showed 100 % of disease incidence. *P. mallochii* (fungus B), *P. citrinum* (fungus P), and *P. citrinum* (fungus S) showed reductions of disease incidence of 40, 70, and 50, respectively, in the presence of chitosan.

the percentage of fungal growth reduction *in vitro* and *in vivo*, respectively. The *in vitro* results have shown to be better than *in vivo* results, probably due to the better operational conditions for antimicrobial activity of chitosan when it is soluble in the liquid medium and upon agitation, granting greater availability and homogeneity of polymer during the culture. Tayel et al. (2016) have also demonstrated greater growth inhibition of *Penicillium* spp. by chitosan in the *in vitro* experiments than in the *in vivo* experiments performed on lemons (*Citrus limon* L.). In plate cultures the inhibition zone was 100 %, whereas in lemons coated with chitosan there was a reduction in the disease incidence of 65.9 % for *P. digitatum* and 84.5 % for *P. italicum* after 14 days of stocking the fruits. Despite the discrepancies between the *in vitro* and post-harvest tests, it can be assumed that the polymer has potential application in reducing the incidence of the moldy disease in orange fruit.

Another interesting point that should be highlight is that in the oranges with absence of infection symptoms, it was observed healing symptoms of the wounds by chitosan, indicating that the polymer acted to combat the spores and also the wounds. An increase in the wound healing process generates by chitosan in citrus fruit was noted by Zhao et al. (2018), whose proved this fact by demonstrating lignin accumulation, increases in cell wall compounds (pectin, cellulose), wound tissue appearance changes and reduced activities of cell wall enzymes.

3.4. Post-harvest experiment: physico-chemical parameters of orange fruit

All the physicochemical parameters analyzed did not show changes for the fruit treated with chitosan, confirming that the polymer does not affect the quality of the fruit (cv. 'Serra d'água, randomly chosen). Table 3 presents the mean values for ºBrix of SST, pH, acidity, vitamin C content and *Ratio* (indicator used to determine the maturation stage of citrus fruit) after 10 days of storage. These parameters remained constant over the 10 day assayed (only data from the tenth day are shown) and the values were compatible with results from literature for the cv. 'Serra d'água' (Couto & Canniatti-Brazaca, 2010; Fischer et al., 2008), indicating that chitosan can be considered a safe product.

The ability of chitosan to form a semipermeable film and regulate gas exchange, reducing sweat losses, represents a prolongation to the shelf-life of fruit and vegetables (Cazon et al., 2017; Elsabee & Abdou, 2013). In order to evaluate if there was a prolongation to the shelf-life of orange fruit coated with chitosan, the experiment was conducted by measuring fruit mass daily which were stored for 10 days. The weight loss increased over the storage time, reaching almost 8 % (Fig. 6). Although the weight loss tended to be lower for the fruits coated with chitosan, the data between oranges treated and untreated with chitosan were not statistically different (Tukey's test data not shown), so it is not possible to affirm that the antifungal agent could extend the shelf-life of fruit during the 10 days of storage. (Gao et al., 2018) observed that coating oranges (cv. 'Nave') with cinnamaldehyde-chitosan significantly reduced the decay rate and weight loss of the fruits. The experiment was conducted for 120 days, suggesting that the benefits in terms of the shelf life of the fruit could be achieved in the case of long storage times.

Others surveys assessing the antifungal activity of chitosan films to prevent the green mold in oranges have been concluded (Al-Sheikh & Yehia, 2016; El Guilli et al., 2016; Tayel et al., 2016). However, these researches have concentrated on the susceptibility of *P. digitatum* and sometimes, on the susceptibility of *P. italicum*, because of the predominance of these phytopathogen on mold development. The purpose of this work was to show that others *Penicillium* spp. such as *P. citrinum* and *P. mallochii* may be potential phytopathogens in oranges, which can be efficiently controlled by coating with chitosan films.

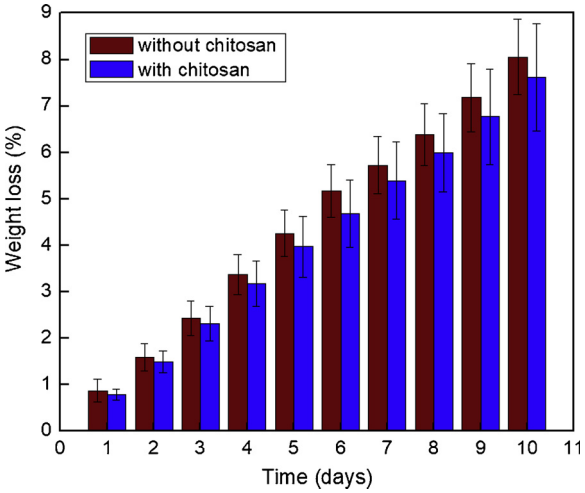

Fig. 6. Weight losses of oranges treated with chitosan. The oranges with and without (control) chitosan treatment were assessed for 10 days. The data are means of six independent experiments.

4. Conclusion

The current study has showed that chitosan can inhibit *in vitro* and *in vivo* growth of *P. citrinum* and *P. mallochii*, two fungi species identified as potential phytopathogens causing decay in orange fruit. This is the first report of *P. mallochii* being isolated from mold in orange fruit, and the first pathogenicity test reported of *P. mallochii* and *P. citrinum*. The chitosan action has demonstrated to be a concentration-dependent mode and to have a fungistatic effect against both fungi *in vitro*. Moreover, shelf-life tests have proved that this polymer is a safe product, once did not alter any physicochemical parameters of the fruit. Evidence suggests that chitosan may be a promising alternative to synthetic fungicide to prevent decay in oranges, since it is able to inhibit the newly identified phytopathogens species of *Penicillium* spp.

CRediT authorship contribution statement

Thamara Carvalho Coutinho: Conceptualization, Methodology, Writing - original draft. **Mariana Costa Ferreira:** Methodology. **Luiz Henrique Rosa:** Methodology. **Ana Maria de Oliveira:** Data curation, Validation, Visualization. **Enio Nazaré de Oliveira Júnior:** Writing - review & editing, Supervision, Funding acquisition.

Table 3
Means of physicochemical parameters analyzed during a 10-day period for 'Serra d'Água' oranges treated with chitosan.

'Serra d'Água' orange	SST (ºBrix)	pH	Acidity (g × L^{-1})	Vitamin C [2]	*Ratio* [3]
Control	8.7 a^1 ± 0.8	5.0 a^1 ± 0.1	2.5 a^1 ± 0.4	56.5 a^1 ± 5.8	35.7 a^1 ± 9.1
With chitosan	8.8 a ± 0.5	5.0 a ± 0.1	2.4 a ± 0.5	48.6 a ± 14.3	37.7 a ± 9.2
Literature [4]	9.1 ± 0.5	5.4 ± 0.4	2.3 ± 0.6	64.6 ± 0.4	38.8

[1]Means followed by the same letter do not differ statistically (Tukey's test; p ≤ 5 %).
[2]In terms of ascorbic acid (mg × 100 mL^{-1}). The results showed a high coefficient of variation (CV = 22.23 %).
[3]SST (ºBrix) / acidity (mg × 100 m L^{-1}).
[4]Data obtained by Couto and Brazaca [56].

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.carbpol.2020.115918.

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