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Inhibitory effect of coatings with different polymeric bases on germination and in *vitro* growth of anthracnose fungus

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Abstract

Colletotrichum theobromicola is one of the fungus species that causes anthracnose in the papaya fruit. This study aimed to evaluate the effect of three polymeric bases in their usual concentrations – starch (6% m/v), carboxymethylcellulose (CMC, 1% m/v), and chitosan (2% m/v) – on the *in vitro* growth parameters of *C. theobromicola*: germination, conidia count, and membrane permeabilization, to identify a more suitable polymeric base to be applied in the development of an active coating for the postharvest conservation of papaya. *In vitro* growth was determined in Petri dishes for 7 days, while germination and membrane permeabilization were assessed after 24 h of incubation at 25 ± 3°C. It was found that the different polymeric bases interfere with the germination and mycelial growth of *C. theobromicola*. The chitosan coating completely inhibited germination and *in vitro* growth of the fungus. Germination and *in vitro* growth were more easily achieved with the 6% starch coating. The 1% CMC also showed a high proportion of conidia germination but with a lower proportion of mycelial growth. Thus, based on the high cost of chitosan, CMC can be considered a more suitable polymeric base for the formulation of active coatings.

Keywords: starch; carboxymethylcellulose; chitosan; *C. theobromicola*.

Practical Application: This work evaluates how the different coatings used to preserve the postharvest quality of papaya can affect the growth of anthracnose fungus.

1 INTRODUCTION

Some species of *Colletotrichum* spp. cause anthracnose disease in papaya fruit. Ten species have been identified in papaya crops in Brazil: *C. truncatum*, *C. okinawense*, *C. gloeosporioides*, *C. karsti*, *C. siamense*, *C. fructicola*, *C. plurivorum*, *C. theobromicola*, *C. chrysophilum*, and *C. queenslandicum* (Santos Vieira et al., 2022).

Anthracnose is characterized by rounded orange and dark lesions on the fruit's skin. Around 30% of papaya production is lost due to anthracnose. Treatments with synthetic fungicides are used to combat anthracnose, but their prolonged use can lead to pathogen resistance and environmental contamination and pose a health risk to consumers (Ali et al., 2015).

Active coatings have proved to be an interesting technology to control fungi in the postharvest phase of fruits as they are low cost, widely available in nature, nontoxic, and biodegradable (Passos Braga et al., 2019). Active coatings extend the shelf life of fruits by forming a semipermeable barrier to gases, reducing water loss and respiration rates (Dotto et al., 2015). Polysaccharides, proteins, and lipids are the main components used to prepare active coatings (Chen et al., 2019).

Cassava starch is a polysaccharide widely used in active coatings due to its hydrophilic characteristics, low cost, and biodegradability. The starch coating can delay fruit ripening and reduce product mass loss (Costa et al., 2022).

Carboxymethylcellulose (CMC) is a polymer derived from cellulose; it is highly soluble in water, has a low cost, has a transparent coating, and has good oxygen barrier properties, resulting in delayed fruit ripening and improved commercial appearance (Dong & Wang, 2017).

On the contrary, chitosan is a commercially expensive polysaccharide derived from chitin extracted from the wastes of mollusks and crustaceans. It is used in active coatings due to its antimicrobial action and barrier properties,

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as noted by Pavinatto et al. (2020) in studies with fresh strawberries.

Research usually evaluates the action of starch, carboxymethylcellulose (CMC), and chitosan coatings incorporated with oils and plant extracts, against anthracnose (Trigo et al., 2012). However, all the works do not consider the influence of different polymeric bases on fungal growth. This study evaluates the effect of three polymeric bases (starch, CMC, and chitosan), at the usual concentrations used in the fruit coatings, on the parameters of in vitro fungal growth, germination, and conidia count. The objective is to identify a more suitable polymeric base for developing active coatings to papaya fruits.

2 Materials and methods

2.1 Materials

The soluble starch p.a. and CMC were purchased from Dinâmica Química Contemporânea Ltd and Chitosan from Sigma Aldrich.

2.2 Isolation and characterization of the fungus

Colletotrichum spp. was isolated directly from papaya fruits (*Carica papaya* L.) purchased in a market at the Campos dos Goytacazes, RJ, Brazil. Part of the anthracnose lesion was placed on a Petri dish containing 20 mL of Potato Dextrose Agar (PDA) culture medium (KASVI). The plates were then incubated in an incubator chamber at 28°C for 5 days to observe the fungal growth.

The morphological characterization of the fungus was carried out according to the methodology described by Chaudhary et al. (2020), observing the color of the culture, the mycelial and morphological growth pattern, and spore characteristics, such as length and diameter, conducted with the aid of a microscope.

2.3 Genetic identification

The identification of fungi was conducted through the identification service provided by the Micoteca URM Culture Collection of UFPE (WFCC 604) (UFPE, 2024), using a standardized DNA extraction protocol and polymerase chain reactions (PCRs). In short, for DNA extraction, the Promega Genomic DNA Purification Kit was used (Wizard Genomic DNA Purification Kit), following the methodology recommended by the manufacturer. After obtaining DNA, PCRs were performed to amplify genes or regions more appropriate, as described in the current literature (Santos Vieira et al., 2020). The following sequence was identified for *Colletotrichum theobromicola:*

Gene glyceraldehyde 3-phosphate dehydrogenase

CCGTCAACGACCCCTTCATTGAGACCAAGTACGCTGTGAGTATCACCCCACTTACCCCTCCAAACTCGCCACTACTTCACACCCGCCACGGCCGCTGCTGTCGTTCACACCTTCCCGCCTGTATTTGGTAGACTACAAGGCCAACGTGAATTGATGCCAATTGATACCATGGCTCGGCACGGCCGGACACAGCTATCACTCATCTCAGCCCCATCTGTCACATGTACTGACTCGCACTTCACAGGCCTACATGCTCAAGTACGACTCCACC.

2.4 Preparing the coatings

2.4.1 Starch

The starch was previously dried in an oven (Quimis) at 105°C for 24 h, weighed on an analytical balance (GEHAKA AG 200), and transferred to a 250-mL Erlenmeyer flask where it was dissolved in distilled water at a concentration of 6% (w/v). After being dissolved, the coating was heated in a water bath on a hotplate (FISATOM) to a temperature of 70°C, remaining at this temperature for 15 min with constant magnetic stirring until the starch granules had completely gelatinized. The coating was then cooled in an ice bath until it reached room temperature and had its pH corrected to 5.6 (Silva et al., 2019).

2.4.2 Carboxymethylcellulose

The CMC coating was prepared at a concentration of 1% (w/v) and followed the same preparation pattern described for the starch. The CMC was dissolved in distilled water (1% w/v), and the coating was heated in a water bath on a hotplate to a temperature of 70°C, remaining at this temperature for 15 min with constant magnetic stirring until complete gelatinization occurred. Then, the coating was cooled in an ice bath until it reached room temperature and had its pH corrected to 5.6 (Santos et al., 2021).

2.4.3 Chitosan

Chitosan at a concentration of 2% (m/v) was dissolved in an aqueous solution (1% v/v) of acetic acid, stirring constantly on a hotplate for 6 h at a temperature of 40°C. The pH of the solution was corrected to 5.6 to ensure that the acetic acid did not interfere with the antifungal action. The coating was then cooled to room temperature (Granja et al., 2021).

2.5 Preparing the Petri dishes

Petri dishes 9 cm in diameter were prepared with 20 mL of PDA culture medium (KASVI), duly sterilized in an autoclave at 121°C and 1 kgf/cm^2 for 15 min. Once the PDA medium had solidified, 10 mL of active coating was added to the dish, which was placed in the fridge for 24 h to allow the coating solidification.

The fungus was inoculated under a laminar flow chamber by making a well in the center of each Petri dish using a copper punch and adding 100 µL of *C. theobromicola* spore solution containing 65.5×10^5 cells/mL. The plates were sealed with PVC film and kept in an incubator chamber at 25°C for 7 days with a 12-h photoperiod.

2.6 Analysis of mycelial growth

Mycelial growth was assessed daily by measuring the colonies' diameter in four diametrically opposite directions, obtaining the average diameter of the developing mycelia. After 7 days, the fungal growth curve was constructed, and the area of mycelial growth was calculated, considering the area (circle) of the mycelium on the plate (cm^2).

2.7 Conidia count

From each Petri dish containing the fungus with 7 days of growth, 10 disks of 5 mm were removed, using a copper punch, in two regions of the growth halo: in the central region around the inoculation point and at the edge of the growth region. The disks were transferred to individual Falcon tubes containing 10 mL of a mixture (1:1) of saline solution (0.85% v/v NaCl) and Tween 20 solution (0.1% v/v). After shaking in a Vortex type tube shaker (QL-901) for 30 s, an aliquot of 0.5 mL of the solution was placed in the Neubauer chamber, and the conidia were counted in quadrant C at points A, B, C, D, and E using an optical microscope (Nikon eclipse E200) at 40 x magnification. The results were expressed as the number of conidia per mL (Guerra et al., 2015).

2.8 Germination of conidia

Aliquots of 1 mL of *C. theobromicola* conidia suspension (10^5 conidia/mL) were placed in Falcon tubes containing 10 mL of coating, shaken manually, and kept in an incubator chamber at 25°C for 24 h. The lacto phenol blue dye was then added to stop germination, and the mixture was shaken in a Vortex tube shaker (QL-901) for 30 s. An aliquot of 0.5 mL was then placed in a Neubauer chamber to count the germinated conidia using an optical microscope (Nikon Eclipse E200) at 40× magnification, considering the germination tube as 50% of the conidium size (Guerra et al., 2015). The effectiveness of the inhibition of conidia germination was assessed by comparing the percentage of germinated conidia in the medium containing dispersions of the active coatings concerning the control test with PDA medium.

2.9 Scanning electron microscope of mycelial growth

From each Petri dish containing the fungus with 7 days of growth, a 5-mm disc was removed from each treatment, with the aid of a copper awl, in the central region surrounding the inoculation point. Subsequently, the samples were fixed in a 2.5% glutaraldehyde solution, left for 1 h at room temperature, and then stored at 4°C for 24 h. The samples were then dehydrated in an acetone solution (from 30 to 100%), dried using the CO_2 critical point, and mounted on an aluminum support (covered with a layer of aluminum foil and fixed with carbon tape). Samples were coated with gold (20–30 nm) in an evaporator for observation in a ZEISS Scanning Electron Microscope EVO: 40 (Portes et al., 2015).

2.10 Permeabilization of the plasma membrane

The membrane permeabilization of conidia was assessed by fluorescence microscopy using the Sytox Green probe. After the germination inhibition test, 100 µL of cells subjected to the different coating conditions were incubated with 0.2 µL of Sytox Green, under protection from light, for 15 min. Then, the cells were centrifuged at 800 g and analyzed by differential interference contrast (DIC) microscopy using an optical microscope (Axioplan.A2, Zeiss) equipped with a set of fluorescence filters for detecting fluorescein (excitation wavelengths 450–490 nm; emission 500 nm). Cells incubated only with Sytox Green were used as a negative control. The percentage of permeabilized cells was calculated based on the total number of cells in the DIC and fluorescent images of 10 random microscopic fields for each sample, assuming the total number of cells in the clear field of each sample to be 100% (Taveira et al., 2022).

2.11 Statistical analysis

The mycelial growth test was conducted in a completely randomized design using 3 coating types (starch, CMC, and chitosan) with 10 replicates. Conidia germination was analyzed using 5 replicates, while the number of conidia produced in each coating treatment was counted using 10 replicates. The data was evaluated using ANOVA, and the means were compared by the Tukey test at $p \leq 0.05$ using the R *studio* computer software.

3 RESULTS AND DISCUSSION

3.1 Mycelial growth

The starch and CMC polymeric bases provided the growth of *C. theobromicola*, while the chitosan inhibited its growth (Table 1). After 7 days of incubation, the largest growth area occurred in the control treatment, followed by the starch coating and the CMC coating.

Mycelial growth is characterized as a filamentous mass formed from the germination of conidia that form germ tubes, which are long filaments that branch out; its simple filament is called a hypha (Putzke & Putzke, 1998). For their development, the fungus secretes exoenzymes that degrade compounds to obtain carbon, nitrogen, sulfur, and other nutrients (Donini et al., 2005).

In Figure 1, the microscopic images of fungal samples that were taken on the surface of the Petri dishes containing the mycelium after 7 days of incubation in different coatings are presented. Notable similarities can be observed between hypha structures growing in 6% starch coating (Figure 1B) and 1% CMC coating (Figure 1C), which seem like a smooth intricate and regular tissue as compared to the agglomerated structure

Table 1. Areas of *in vitro* mycelial growth (cm^2) of *Colletotrichum theobromicol,* in coatings of starch (6%), CMC (1%), chitosan (1%), and control (PDA medium), over 7 days of incubation at 25 ± 3°C. The data represent the mean values from 10 replicates*.

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Control	0.4 ± 0.1 gA	2.9 ± 0.4 fA	7.3 ± 0.3 eA	14.8 ± 1.4 dA	23.2 ± 1.4 cA	34.8 ± 2.1 bA	46.5 ± 3 aA
CMC	0.4 ± 0.03 gA	2.2 ± 0.4 fB	6.5 ± 0.5 eB	9.7 ± 0.9 dB	11.8 ± 1.2 cC	14.8 ± 1.3 bC	23.6 ± 2.5 aC
Starch	0.4 ± 0.03 gA	2.2 ± 0.3 fB	5.5 ± 0.5 eC	10.6 ± 0.6 dB	15.5 ± 1.4 cB	22.4 ± 2.2 bB	28.9 ± 2.1 aB
Chitosan	0 ± 0 aB	0 ± 0 aC	0 ± 0 aD	0 ± 0 aC	0 ± 0 aD	0 ± 0 aD	0 ± 0 aD

*Means followed by different uppercase letters in the column and lowercase letters in the line differ by the Tukey test at 5% significance.

observed in the control treatment with pure PDA (Figure 1A), showing overlays of dense hypha structures forming valleys and hills over the plate.

The starch coating is a polymeric base widely used on fruits precisely due to its barrier properties and its ability to serve as a vehicle for incorporating active agents such as oils and extracts that have antifungal action against *C. gloeosporioides* (Serpa et al., 2014). Costa et al. (2022) evaluated the usefulness of cassava starch coatings to preserve the quality of fruits.

The 1% CMC coating showed a smaller growth area of *C. theobromicola* than the starch coating, with a 49% reduction in growth area compared to the control treatment at the end of 7 days of incubation. CMC has been used as an active coating on various fruits such as strawberries (Shahbazi, 2018), minimally processed papaya (Trigo et al., 2012), and mangoes (Amariz et al., 2010).

The only coating with no mycelial growth of *C. theobromicola* was the treatment with 2% chitosan. Liu et al. (2007) found total inhibition of the mycelial growth of *B. cinerea* using chitosan at 5%. Camili et al. (2007) also verified the total inhibition of *B. cinerea* in 5 days of incubation at concentrations of 0.5–2% chitosan.

Chitosan is a potent antimicrobial agent, and its action is due to its surface charge, which becomes positive when it comes into contact with physiological fluids, helping the protonated amino groups of chitosan to bind to the anionic groups of the cell walls of microorganisms inhibiting their growth (Kong et al., 2010).

Maia et al. (2010) found that the treatment with 0.016% chitosan reduced mycelial growth of *Elsione ampelina* (causal agent of anthracnose in grapevines) by 57%. Botelho et al. (2010) also found that 0.016% chitosan reduced the mycelial growth of *Penicillium* sp. by 34.2%. According to Gonzáles et al. (2009), chitosan (0.1%) had a fungistatic effect on *Alternaria padwickii* and *Bipolaris oryzae*, which cause Alternaria leaf spot and brown spot in rice.

Considering the commercial costs for the polymeric bases (US$1 = R$ 5.40, IPEA, 2024): chitosan (R$ 17.98/g), CMC (R$ 0.21/g), and starch (R$ 0.05/g), the cost of the raw material to prepare 100 mL of coatings is equivalent to R$ 35.96 for 2% chitosan, R$ 0.21 for 1% CMC, and R$ 0.30 for 6% starch, indicating that the costs of CMC coating is 30% lower than the starch coating.

3.2 Conidia count

The conidia count is shown in Table 2. The central region of the mycelium has higher conidia quantities than at the edge region due to the intense formation of conidiophores that produce conidia (Putzke & Putzke, 1998). In the edge of the mycelium, there are fewer vegetative hyphae, resulting in a lower number of conidia.

The coating type does not affect the conidia count, except for chitosan coating, which shows a very low conidia production (Table 2). Debnath et al. (2022) observed that chitosan is toxic to the fungal cell and reduces sporulation.

3.3 Germination of conidia

Conidia need heat, nutrients, and moisture to germinate and form one or more thin filaments known as germ tubes (Putzke & Putzke, 1998). Figure 2 shows germinated *C. theobromicola* conidia (A), with the germ tube visible, and non-germinated *C. theobromicola* conidia (B).

The conidia germination trials were tested on starch coatings with lower concentrations to check the effect of substrate concentration on conidia germination. The Tween20 and sterile water used to prepare the chitosan coating did not affect conidia germination when evaluating the total inhibition of conidia germination caused by chitosan (Table 3).

Table 2. Conidia count (conidia/mL) of *Colletotrichum theobromicola* after 7 days of incubation at 25 ± 3°C on different polymeric bases, at the central region and the outer edge of the mycelium. The data represent the mean values of 10 replicates*.

Treatments	Central region	Outer-edge region
Control	46.0 ± 5.8 aA	32.3 ± 5.8 bA
CMC 1%	31.1 ± 2.9 aA	22.1 ± 2,2 bA
Starch 6%	32.6 ± 5.7 aA	25.9 ± 5.7 bA
Chitosan 2%	2.4 ± 0.7 aB	1.0 ± 0.4 aB

*Means with standard errors followed by different uppercase letters in the same column and lowercase letters in the same line differ by the Tukey test at 5% significance.

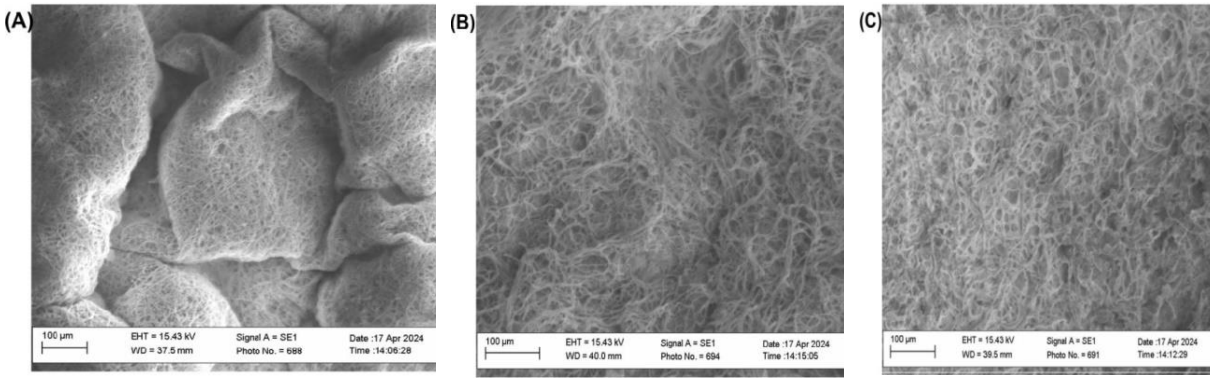

Figure 1. Microscopic images of the mycelia growth of *C. theobromicola* after 7 days of incubation at 25 ± 3°C in different coatings: (A) Control treatment with pure PDA, (B) 6% starch coating, and (C) 1% CMC coating.

The control treatment (3.9% PDA solution) provided 100% germination of *C. theobromicola*. On the contrary, the treatments with starch in different concentrations and the 1% CMC treatment promoted partial germination of the conidia (Table 3). The 6% starch concentration was most suitable for coating papaya in preliminary laboratory tests. However, conidia germination reached 80.5% at these concentrations, achieving a germinating proportion similar to that obtained with the 1% CMC coating, which is a limit value for achieving a higher consistency for immersing the fruit. In this case, specific enzymes are required to degrade cellulose into glucose: endo-acting cellulase (endoglucanase), exo-acting cellulase (cellobiohydrolase), and cellobiase (glycosidases) (Eriksson et al., 1990).

The tests on the 2% chitosan coating showed total inhibition of conidia germination (Table 3). Tests using concentrations of 0.5 and 1.0% chitosan were conducted in the laboratory, but they also caused total inhibition of conidia germination (data not shown). The chitosan effects on the germination and morphology of the fungal hyphae that grow on fruit were described by Verlee et al. (2017). The fungicidal activity of chitosan was verified by Lopez-Moya et al. (2015). According to Kong et al. (2010), the amine group becomes positively charged ($-NH_3^+$) at slightly acidic pH, making chitosan more soluble and having better antimicrobial activity than chitin.

3.4 Permeabilization of the plasma membrane

The membrane permeabilization tests were accompanied by microscopic image analyses of the conidia (Figure 3). After 24 h of incubation, only the treatment using 2% chitosan could cause permeabilization of the plasma membrane of *C. theobromicola* conidia, as indicated by an intense fluorescence signal, denoting that the chitosan facilitated the entry and subsequent marking of the probe, thus suggesting the effective permeabilization of this membrane (Table 4).

The fungal cell wall is composed of chitin, a polysaccharide that in some phytopathogenic fungi present in papaya occurs in the composition of 13–37%. The chitinase enzyme in chitosan can degrade the β 1.4 bonds of chitin, contributing to damage to the cell wall (Lucas-Bautista et al., 2019; Muzzarelli, 2011).

Chitosan hinders the growth of different phytopathogens and fungi, including *Alternaria* spp., *Colletotrichium* spp., or *Trichoderma* spp. In addition, it permeabilizes the plasma membrane, triggering the intracellular production of reactive oxygen species (Lopez-Moya et al., 2015).

Different mechanisms of action have been proposed for chitosan, the most widely accepted of which are alteration of the permeability of the microorganism membrane or cell wall, causing it to rupture; interaction of chitosan with the microorganism DNA, affecting protein synthesis due to mRNA inhibition; chelation of nutrients (metal ions) by chitosan, which are essential for microbial growth; and formation of a thick polymeric film on the cell surface of the microorganism, preventing gas and nutrient exchange with the external environment, leading to cell death (Lee & Je, 2013; Li et al., 2010; Park et al., 2011).

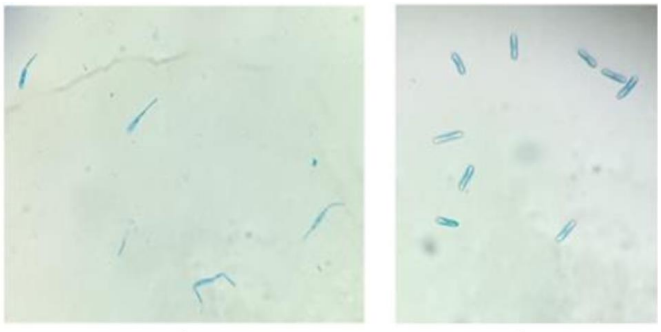

Figure 2. Microscopic images (40 X) of *C. theobromicola* conidia with germ tubes (A) and non-germinated *C. gloeosporioides* conidia (B).

4 CONCLUSIONS

The different polymeric bases interfere with the germination and mycelial growth of the fungus *C. theobromicola*. The chitosan coating caused total inhibition of germination and *in vitro* growth of the fungus, with a fungicidal effect. Part of the mechanism of conidial death caused by chitosan can be explained by the permeabilization of the plasma membrane in 100% of the conidia.

Germination and *in vitro* growth of the fungus were facilitated in starch coating. There was an increase in germination and in vitro growth with increased starch concentration up to 6%, reaching values lower than the control treatment (3.9% PDA). The 1% CMC coating also showed a high proportion of conidia germination but a lower proportion of mycelial growth than the 6% starch coating.

Table 3. Conidia count (conidia/mL) of germinated and non-germinated *Colletotrichum theobromicola* on different polymeric bases after 24 h of incubation at 25 ± 3°C. The data represent the mean values of five repetitions*.

Treatments	Germinated conidia	Ungerminated conidia	Proportion of germinated conidia (%)
Sol. PDA 3.9%	15.3 ± 0.8 aA	0 ± 0 bC	100 A
Sterile water	0 ± 0 bD	10.6 ± 0.7 aA	0 E
Tween 20 1%	0 ± 0 bD	12.1 ± 0.3 aA	0 E
Starch 1.5%	0.4 ± 0.2 bD	13.7 ± 0.9 aA	2.9 D
Starch 3 %	5.4 ± 0.7 bC	9.1 ± 0.8 aAB	37.5 C
Starch 6%	12.5 ± 0.7 aAB	3.0 ± 0.4 bBC	80.5 B
CMC 1%	9.9 ± 0.6 aB	3.6 ± 0.5 bB	73.4 B
Chitosan 2%	0 ± 0 bD	13.5 ± 0.9 aA	0 E

*Means followed by different uppercase letters in the same column and lowercase letters in the same line differ by the Tukey test at 5% significance.

Considering the high cost of chitosan, CMC can be considered a more suitable polymeric base for the formulation of active coatings applied to the postharvest conservation of papaya.

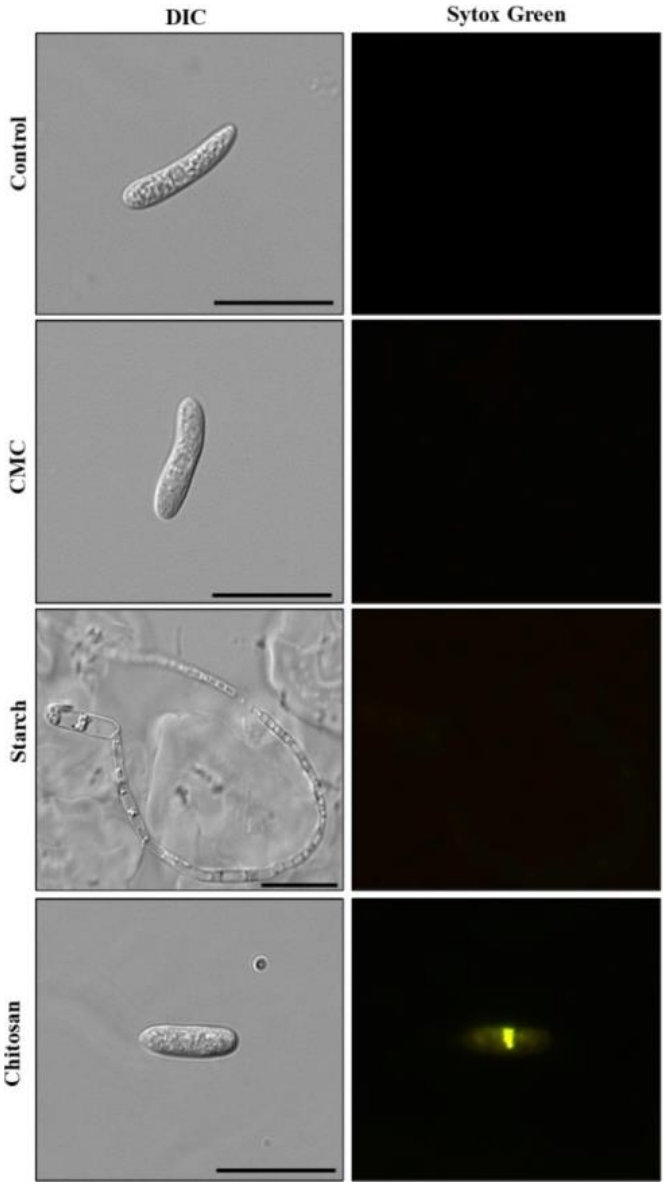

Figure 3. Fluorescence microscopic images of the conidia, highlighting the images taken in brightfield, using a differential interference contrast (DIC), and in darkfield, using the fluorescent probe Sytox green. The conidia were evaluated after 24 h of incubation at 25 ± 3°C on different polymeric bases. Bars of 20 µm.

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Table 4. Number of spores (conidia/100 µL) under brightfield (DIC) and darkfield (Sytox green) and the proportion of membrane permeabilization after 24 h of incubation at 25 ± 3°C in different polymeric bases*.

Treatments	Conidia in the DIC	Conidia in Sytox green	Proportion of permeabilized conidia (%)
Control	11.0 ± 0.3 Aa	0 ± 0 Bb	0 B
Starch 6%	11.0 ± 0.3 Aa	0 ± 0 Bb	0 B
CMC 1%	10.0 ± 0.0 Ba	0 ± 0 Bb	0 B
Chitosan 2%	11.0 ± 0.3 Aa	11.0 ± 0.3 Aa	100 A

*Means followed by different uppercase letters in the same column and lowercase letters in the same line differ by the Tukey test at 5% significance.

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