ARTIGOS

Efficiency of a yeast-based formulation for the biocontrol of postharvest anthracnose of papayas

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ABSTRACT

Lima, J.R.; Viana, F.M.P.; Lima, F.A.; Pieniz, V.; Gonçalves, L.R.B. **Efficiency of a yeast-based formulation for the biocontrol of postharvest anthracnose of papayas.** ***Summa Phytopathologica,*** v.40, n.3, p.203-211, 2014.

To identify formulations of biological agents that enable survival, stability and a good surface distribution of the antagonistic agent, studies that test different application vehicles are necessary. The efficiency of two killer yeasts, *Wickerhamomyces anomalus* (strain 422) and *Meyerozyma guilliermondii* (strain 443), associated with five different application vehicles, was assessed for the protection of postharvest papayas. In this study, after 90 days of incubation at 4°C, *W. anomalus* (strain 422) and *M. guilliermondii* (strain 443) were viable with all application vehicles tested. Fruits treated with different formulations (yeasts + application vehicles) had a decreased severity of disease (by at least 30%) compared with untreated fruits. The treatment with *W. anomalus* (strain 422) + 2% starch lowered disease occurrence by 48.3%. *The most efficient treatments using M. guilliermondii (strain 443) were those with* 2% *gelatin* or 2% liquid carnauba wax, both of which reduced anthracnose by 50% in postharvest papayas. Electron micrographs of the surface tissues of the treated fruits showed that all application vehicles provided excellent adhesion of the yeast to the surface. Formulations based on starch (2%), gelatin (2%) and carnauba wax (2%) were the most efficient at controlling fungal diseases in postharvest papayas.

Additional keywords: Application vehicles, Carnauba wax, Gelatin, Killer yeasts, *Colletotrichum gloeosporioides*, biological control.

RESUMO

Lima, J.R.; Viana, F.M.P.; Lima, F.A.; Pieniz, V.; Gonçalves, L.R.B. **Eficiência de formulações à base de leveduras para biocontrole da antracnose pós-colheita do mamão.** ***Summa Phytopathologica,*** v.40, n.3, p.203-211, 2014.

A pesquisa de formulações com agentes biológicos exige estudos com diferentes veículos de aplicação para a seleção dos que permitam a sobrevivência, estabilidade e uma boa distribuição do agente antagônico na superfície a ser tratada. A eficiência de duas leveduras killer, *Wickerhamomyces anomalus* (cepa 422) e *Meyerozyma guilliermondii* (cepa 443), associadas com cinco diferentes veículos de aplicação, foi avaliada quanto à proteção do mamão na pós-colheita. Neste trabalho, com 90 dias de incubação a 4 °C, *W. anomalus* (cepa 422) e *M. guilliermondii* (cepa 443) mantiveram-se viáveis em todos os veículos de aplicação testados. Frutos tratados com diferentes formulações (leveduras + veículos de aplicação) tiveram menor incidência de doença (pelo menos 30%) quando comparados a frutos não tratados. No tratamento com *W. anomalus* (cepa 422) + amido 2% a doença foi 48,3% *menor. No*s tratamentos que empregaram a *M. guilliermondii (cepa 443), os mais eficientes foram aqueles que utilizaram a g*elatina 2% e a cera líquida de carnaúba 2%; ambos reduziram em 50% a antracnose pós-colheita dos mamões. Eletromicrografias do tecido da superfície dos frutos tratados revelaram que todos os veículos de aplicação proporcionaram excelente adesão das leveduras à superfície. As formulações baseadas em amido de milho (2%), gelatina (2%) e cera-de-carnaúba (2%) foram as mais eficientes no controle de doenças fúngicas em pós-colheita de mamão.

Palavras-chave adicionais: Veículos de aplicação, Cera-de-carnaúba, Gelatina, Leveduras killer *Colletotrichum gloeosporioides*, controle biológico.

The papaya tree (*Carica papaya* L.) is cultivated throughout most of the Brazilian territory, excluding some regions that have severe rainfall. The southeast and northeast regions of the country are responsible for over 90% of the national production (12). According to data from the Food and Agriculture Organization (FAO) of the United Nations, Brazilian production of papayas increased from 1.79 million tons in 2009 to 1.87 million tons in 2010, which maintained Brazil's position as the second largest producer of this fruit in the world (7).

Phytopathogens are responsible for significant losses, both during production and postharvest, of plant products in the tropics and may

reach figures of up to 60% of the total yield (24). For the papaya crop, a variety of pathogens can attack different parts of the plant, including the leaves and the fruit itself, and stem-end rot, anthracnose, black-spot disease and viruses are among the major diseases affecting the crop (28).

Papaya diseases are commonly controlled pre- and postharvest with agrochemicals. In recent years, however, the emergence of phytopathogens that are resistant to the main active ingredients and the associated risks of human and environmental contamination have generated a demand for new, alternative methods of control (16, 17, 25, 36).

In this context, the use of biological control agents constitutes an important tool in meeting the growing demand for products that are less toxic to humans, animals and the environment (14). Products that use microorganisms as an active ingredient to control postharvest pathogens are already registered and available in countries including South Africa, Spain, Israel, Canada, Germany and the United States (20, 30). Among these products are Candifruit®, Aspire®, Sporodex® and YieldPlus®, which have as active ingredients, respectively, *Candida sake* CPA-1, *Candida oleophila* I-182, *Pseudomonas flocculosa* PF-A22 and *Cryptococcus albidus*.

Among the microorganisms that could potentially be used for biological control, yeasts are noteworthy as they bring together the most desirable characteristics in a biocontrol agent, such as safety, ease of handling and application, low nutritional requirement and lack of production of toxic compounds (4, 6, 26, 27, 32). In addition, some yeast species are capable of producing a glycoprotein toxin, the killer toxin, which can have a direct effect on phytopathogens (5, 10, 11).

Various substances added to a microbial formulation can further improve cell survival by providing a protective milieu, including non-reducing disaccharides or certain amino acids (21).

This study evaluated the efficiency of formulations based on the killer yeasts *Wickerhamomyces anomalus* (strain 422) and *Meyerozyma guilliermondii* (strain 443) at protecting papayas postharvest. Their survival in different vehicles (Tween 80, sodium alginate, 2% gelatin, 2% liquid carnauba wax or 2% starch) and the ability of these formulations to adhere to the surface of the fruit were also tested.

MATERIALS AND METHODS

Killer yeast

Two killer yeasts that were previously isolated from papaya and identified as *Wickerhamomyces anomalus* (strain 422) and *Meyerozyma guilliermondii* (strain 443) were used. They are efficient in the *in vitro* control of *Colletotrichum gloeosporioides*, which is a major phytopathogen that affects postharvest papayas (18). The yeasts were identified by sequencing the D1/D2 region of rRNA, and the sequences were deposited into GenBank as JN627213 and JN627210 (18). The yeasts were kept at 4°C in potato dextrose agar (PDA; Difco™) until use.

Production of yeast biomass

The yeasts were cultured in five Erlenmeyer flasks containing 100 mL of yeast extract/peptone-dextrose (YEPD) broth (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose). The cultures were incubated for 24 hours at 28°C, and a medium-free cell suspension was obtained by centrifugation at 1,511 x g for 10 min at room temperature; the suspension was then washed twice with sterile distilled water for complete removal of the culture broth. The pellets were resuspended in sterile distilled water to a concentration of 1.0 x 10^8 cells/mL. The concentration was adjusted by using a Neubauer chamber under a microscope (35).

Formulation preparation

The yeasts were cultured in YEPD broth (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose), incubated for 24 hours at 28°C, as outlined above; then, they were harvested and centrifuged at 1,511 x g for 10 min and resuspended in the vehicles.

The cell pellets at the desired concentration were resuspended in (T1) Tween 80 (Vetec), (T2) 2% (w/v) sodium alginate (Sigma®), (T3) 2% (w/v) gelatin (Sigma®), (T4) 2% (v/v) liquid carnauba wax, and (T5) 2% (w/v) starch (Vetec). The final concentration of yeast in all vehicles was 1.0 x 108 colony-forming units (CFU)/mL.

Determination of yeast viability and stability in the application vehicles

The viability and the stability of *W. anomalus* (strain 422) and *M. guilliermondii* (strain 443) in each of the tested vehicles were assessed after incubation for 0, 10, 20, 30, 60 and 90 days at 4°C. Therefore, each tested formulation (yeast + vehicle) was subjected to serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}), and the cell concentration was determined by viable cell count performed according to the spread plate method (34).

Efficiency of yeast-based formulations of *W. anomalus* (strain 422) and *M. guilliermondii* (strain 443) in the control of papaya anthracnose.

Crops of healthy golden papayas (*Carica papaya* L.) showing uniform size and ripeness and no damage or dents were collected at Cacimbão Farm, in the municipality of Paraibapa, state of Ceará, Brazil, a region where papaya anthracnose is prevalent. The fruits were transported to the Postharvest Pathology Laboratory at the Embrapa Tropical Agroindustry, where they were initially washed in running water, surface sterilized with sodium hypochlorite at 2.0% for three minutes and then left to dry naturally. Later, they were distributed into groups to establish a randomized experiment with 4 replicates of 5 fruits used for each treatment (n = 20). The treatments were performed by using the formulations described in item 2.3 maintained at 28°C. The biological control agents were sprayed on the fruits, forming one cover layer; after that, the fruits were incubated at 28°C and 95% relative humidity (RH), conditions that are favorable to the development of disease (24).

The evolution of injuries was daily assessed based on a rating scale ranging from 0 to 6, where 0 – undamaged fruit; 1 – fruit with few spots and up to 3% of the surface affected; 2 – fruit with up to 6% of surface spots or small fungal colonies (<0.1 cm to 0.25 cm), 3 – fruit with up to 10% of the surface affected or with several fungal colonies from 0.26 to 1.0 cm; 4 – fruit with few medium to large spots (> 1.0 cm) affecting up to 25% of the surface; 5 – fruit with several small and/or large spots affecting 25-50% of the surface; 6 – fruits with more than 50% of the area occupied by spots and/or fungal colonies.

Sterile distilled water was used as a negative control, and a thiabendazole-containing fungicide was the positive control. The assay was completed when the negative control group, fruits, reached a score of 6.

Adherence of the formulations to the papaya surface.

Twenty-four hours after the application of each of the formulations to the fruit surface, samples of ± 1 cm of the surface (peel) were extracted by using a scalpel, washed twice in distilled water and processed for scanning electron microscopy (SEM) according to

Bozzola and Russell (2). The samples were immersed in modified Karnovsky's fixative for 48 hours and post-fixed in 1% osmium tetroxide in sodium cacodylate buffer (0.1 M, pH 7.2) for one hour. The samples were then dehydrated by immersion in solutions of increasing concentrations of ethanol (30%, 50%, 70% and 90%) for ten minutes each. The samples were subsequently immersed in three baths of ten minutes each of pure ethanol and taken to the critical point dryer (Emitech K850), yielding a gold sputter coating (Emitech K550) and then examined under a scanning electron microscope (Zeiss DSM 940 A) at an accelerating voltage of 15 kV.

Statistical analysis

The data were subjected to analysis of variance (ANOVA) [SISVAR software, package (version 5.3; Federal University of Lavras)] (8) and statistical significance was set to the level P≤0.05. Replicate averages with similar results were analyzed together. Averages and standard were significantly different, a comparison was performed by using Tukey's test (P ≤ 0.05).

RESULTS

Determination of yeast viability and stability in the application vehicles

After 90 days of incubation at 4°C, *W. anomalus* (strain 422) and *M. guilliermondii* (strain 443) remained viable in all tested application vehicles (Figures 1 and 2). For both organisms, the highest survival rates were obtained in 2% starch. Under refrigeration for the same period, formulations with 2% starch had a population reduction of only 1 log cycle for the *W. anomalus* population, and for *M. guilliermondii,* not only maintenance but also an initial increase in the yeast population

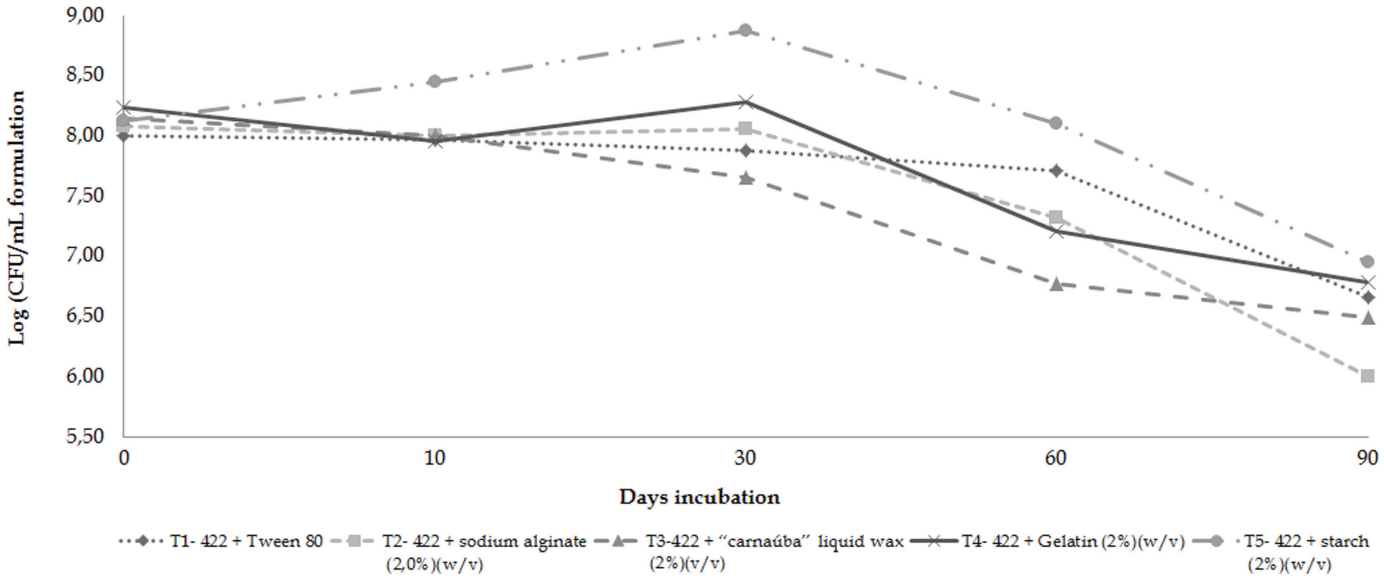

Figure 1. Survival of the killer yeast *W. anomalus* (strain 422) in different vehicles for the formulation of a biofungicide: A: Tween 80, B: Sodium alginate (2%), C: Gelatin (2%), D: Liquid carnauba wax (2%), and E: Starch (2%).

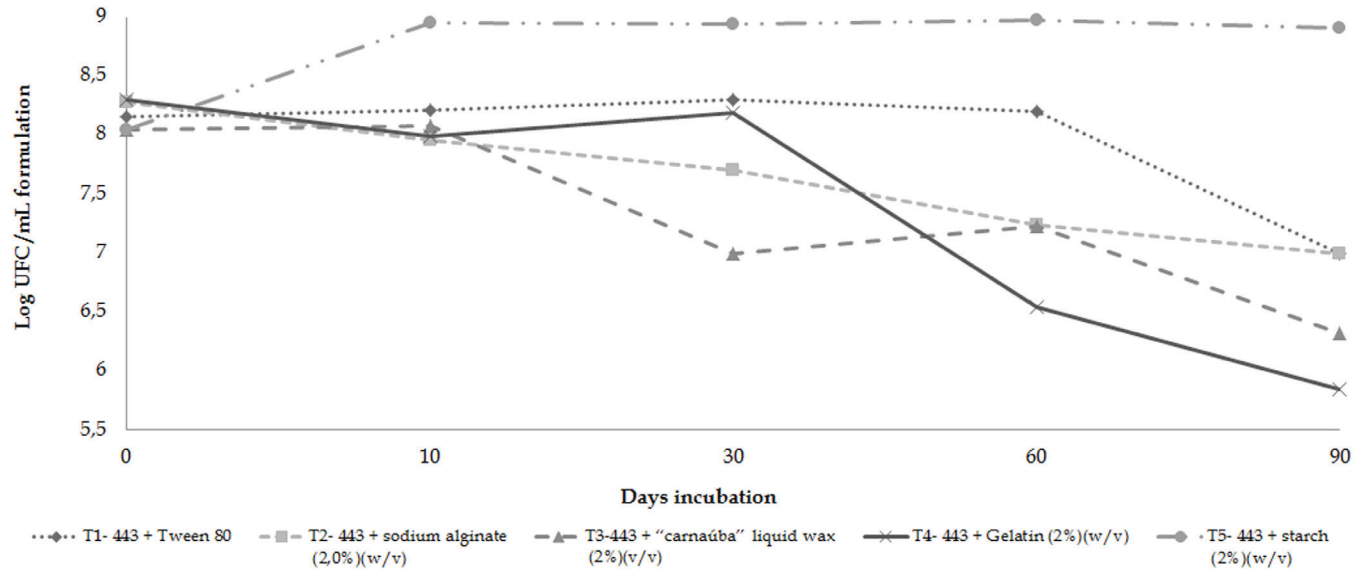

Figure 2. Survival of the killer yeast *M. guilliermondii* (strain 443) in different vehicles for the formulation of a biofungicide: A: Tween 80, B: Sodium alginate (2%), C: Gelatin (2%), D: Liquid carnauba wax (2%), and E: Starch (2%).

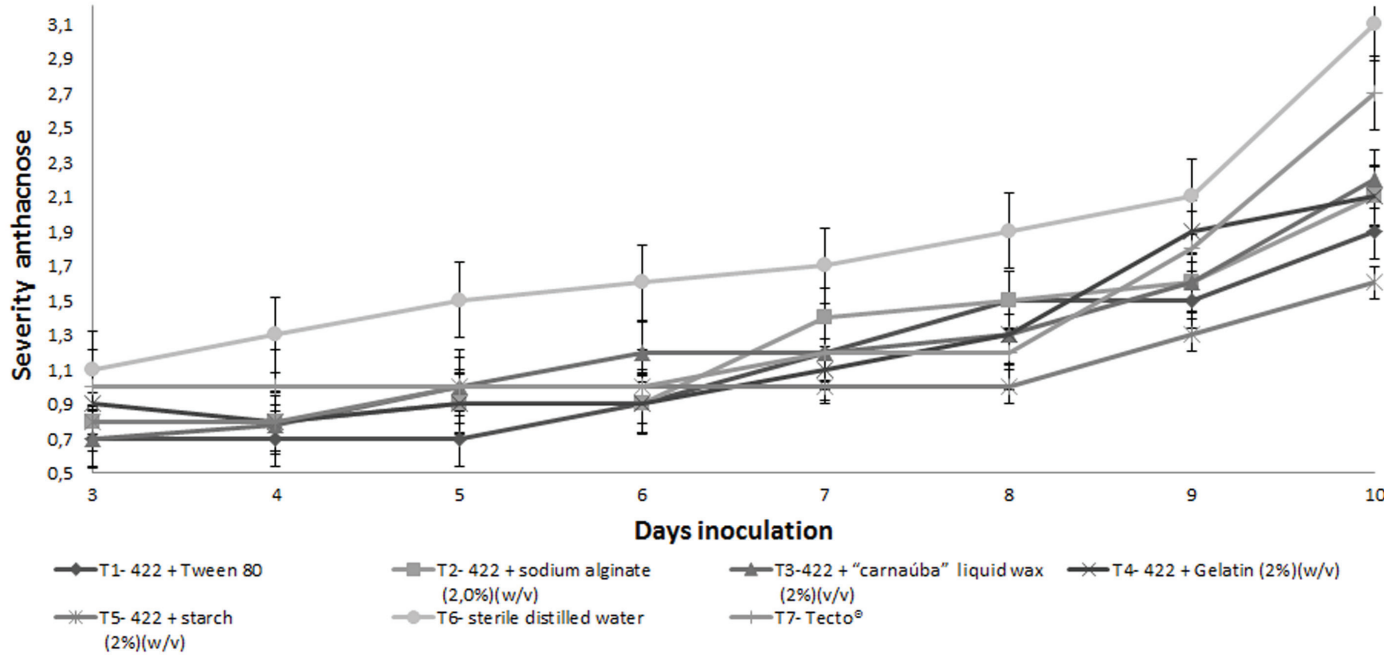

Figure 3. Effect of treatment of papayas with formulations based on the yeast *W. anomalus* (strain 422) with different vehicles during 10 days of incubation at 28°C and 95% RH. Vertical bars represent the standard error of means (0.05).

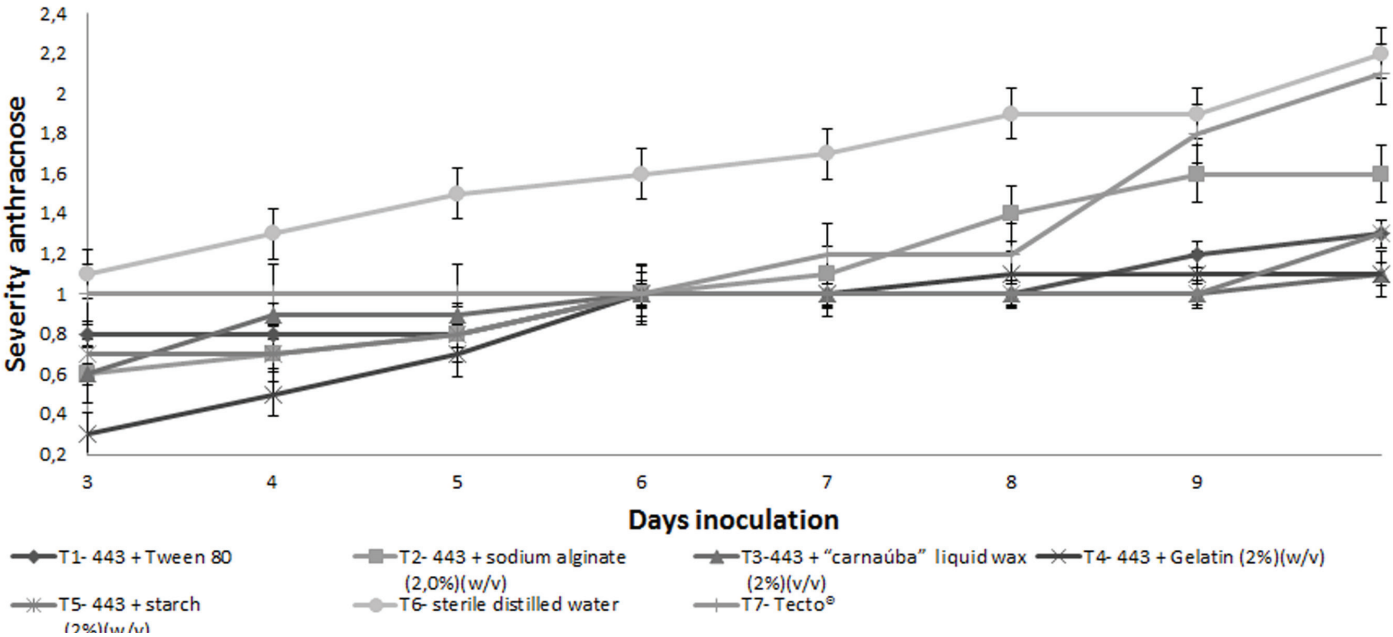

Figure 4. Effect of treatment of papayas with formulations based on the yeast *M. guilliermondii* (strain 443) with different vehicles during 10 days of incubation at 28°C and 95% RH. Vertical bars represent the standard error of means (0.05).

was observed (Figures 1 and 2). Tween 80 also ensured good population stability, with a decrease of only one log cycle for the two tested yeasts (Figures 1 and 2) after 90 days of incubation.

Liquid carnauba wax (2%) caused the largest reduction in the population of the two yeasts, which was evidenced by a reduction of 1.5 log cycles in the population of *W. anomalus* and 2 log cycles in the population of *M. guilliermondii* after 90 days (Figures 1 and 2). Formulations using sodium alginate (2%) also yielded reductions in population of 1.5 and 0.8 cycles for *W. anomalus* and *M. guilliermondii*, respectively, after 90 days.

Efficiency of killer yeast-based formulations of *Wickerhamomyces anomalus* (strain 422) and *Meyerozyma guilliermondii* (strain 443) in the control of papaya anthracnose.

The formulations of biofungicides reduced the severity of disease throughout the incubation period, and the results were statistically similar to those of the fungicide thiabendazole. Notably, fruits treated with this chemical product reached a maximum impairment of 2.7 at the end of the experiment, and fruits treated with the yeast-based formulations had a maximum impairment of merely 2.2 in the same period.

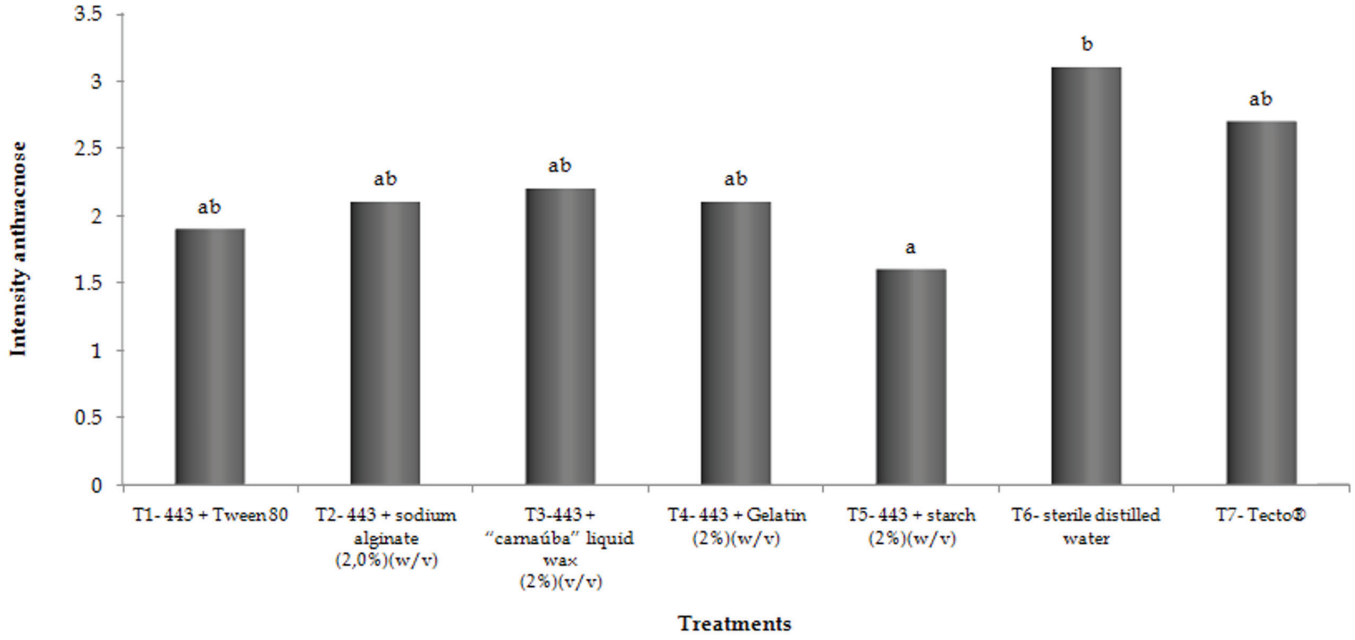

Figure 5. Effect of treatment of papayas with formulations based on *W. anomalus* (strain 422) with different application vehicles on the evolution of papaya postharvest injuries caused by *Colletotrichum*. Means above the bars followed by the same letter are statistically similar (Tukey $P \leq 0.05$).

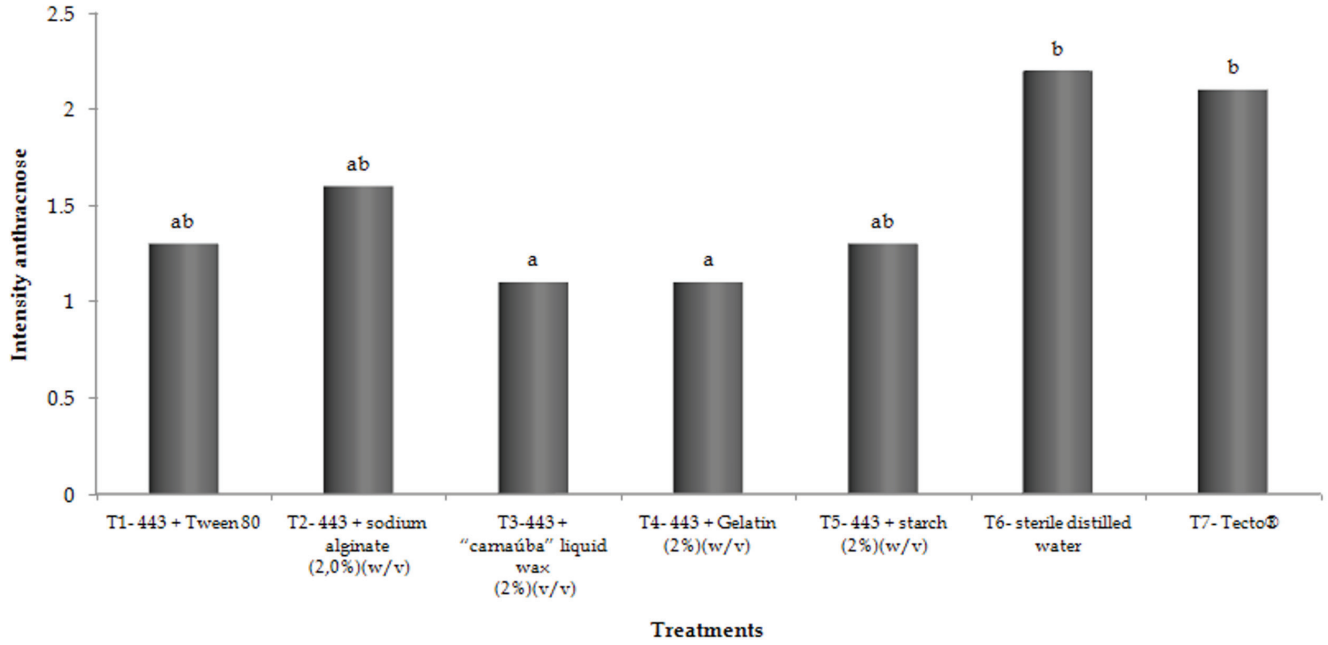

Figure 6. Effect of treatment of papayas with formulations based on *M. guilliermondii* (strain 443) with different application vehicles on the evolution of papaya postharvest injuries caused by *Colletotrichum*. Means above the bars followed by the same letter are statistically similar (Tukey $P \leq 0.05$).

The formulation composed of *W. anomalus* (strain 422) and 2% starch reduced the severity of anthracnose throughout the incubation period by at least 30% compared with untreated fruits, thus reaching a reduction of 48.3% ($P \leq 0.05$), compared with untreated fruits at the end of the incubation period (Figure 5).

Of the formulations based on the yeast *M. guilliermondii (strain 443), the most efficient treatments at controlling the disease were those with gelatin* (2%) and liquid carnauba wax (2%). These treatments significantly reduced ($P \leq 0.05$) the severity of papaya anthracnose in the postharvest during a storage period of 10 days compared with untreated fruits, promoting a reduction in the disease severity of at least 37% throughout this period and reaching a reduction of up to 50% at the end of incubation (Figure 6).

Ability of the formulations to adhere to the papaya surface.

Electron micrographs indicated that for all evaluated application vehicles, the yeasts remained firmly adhered to the surface of the fruit (Figure 7). Mycoparasitized hyphae and conidia of *C. gloeosporioides* on the surface of papayas were also observed (Figure 8).

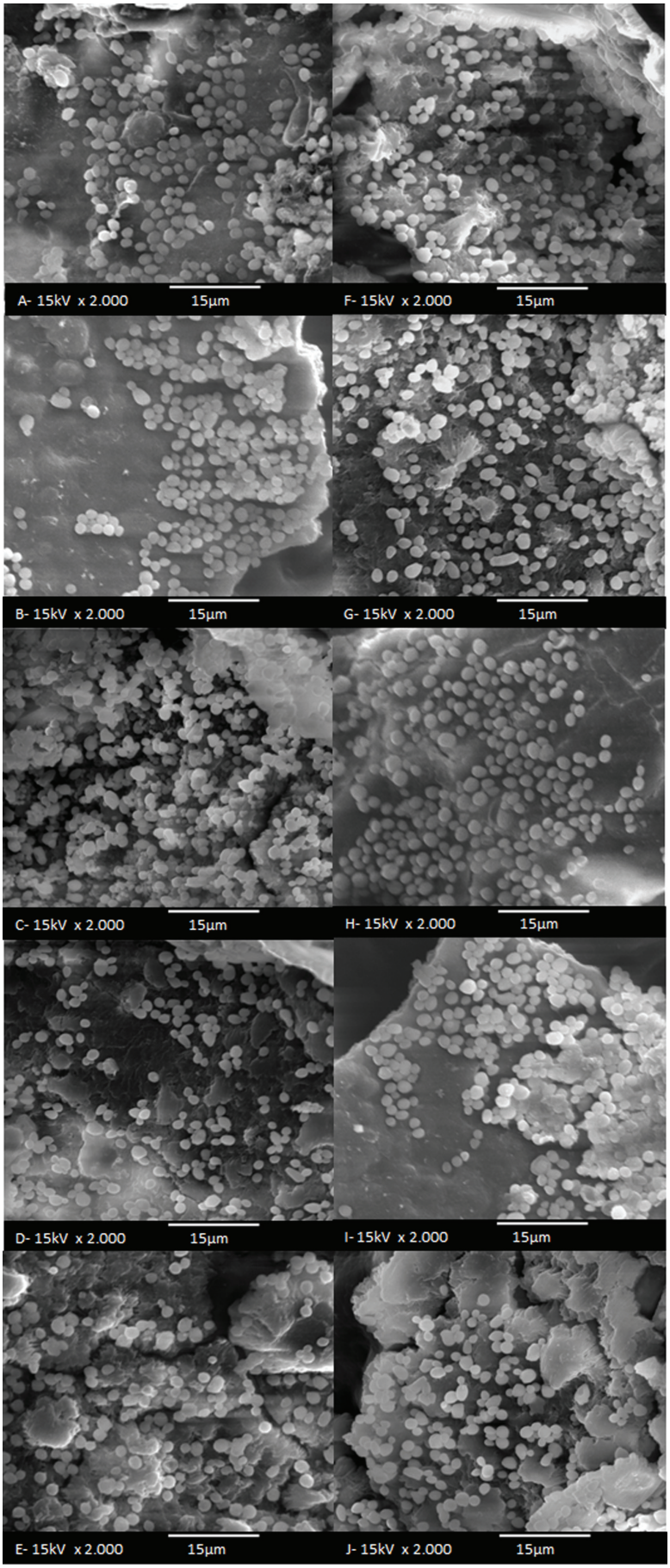

Figure 7. Electron micrographs of the surface of papayas treated with formulations based on *Wickerhamomyces anomalus* (strain 422) (A, B, C, D and E) and *Meyerozyma guilliermondii* (strain 443) (E, F, G H, I and J) associated with different application vehicles (Tween 80, 2% sodium alginate, gelatin, 2% liquid carnauba wax and 2% starch).

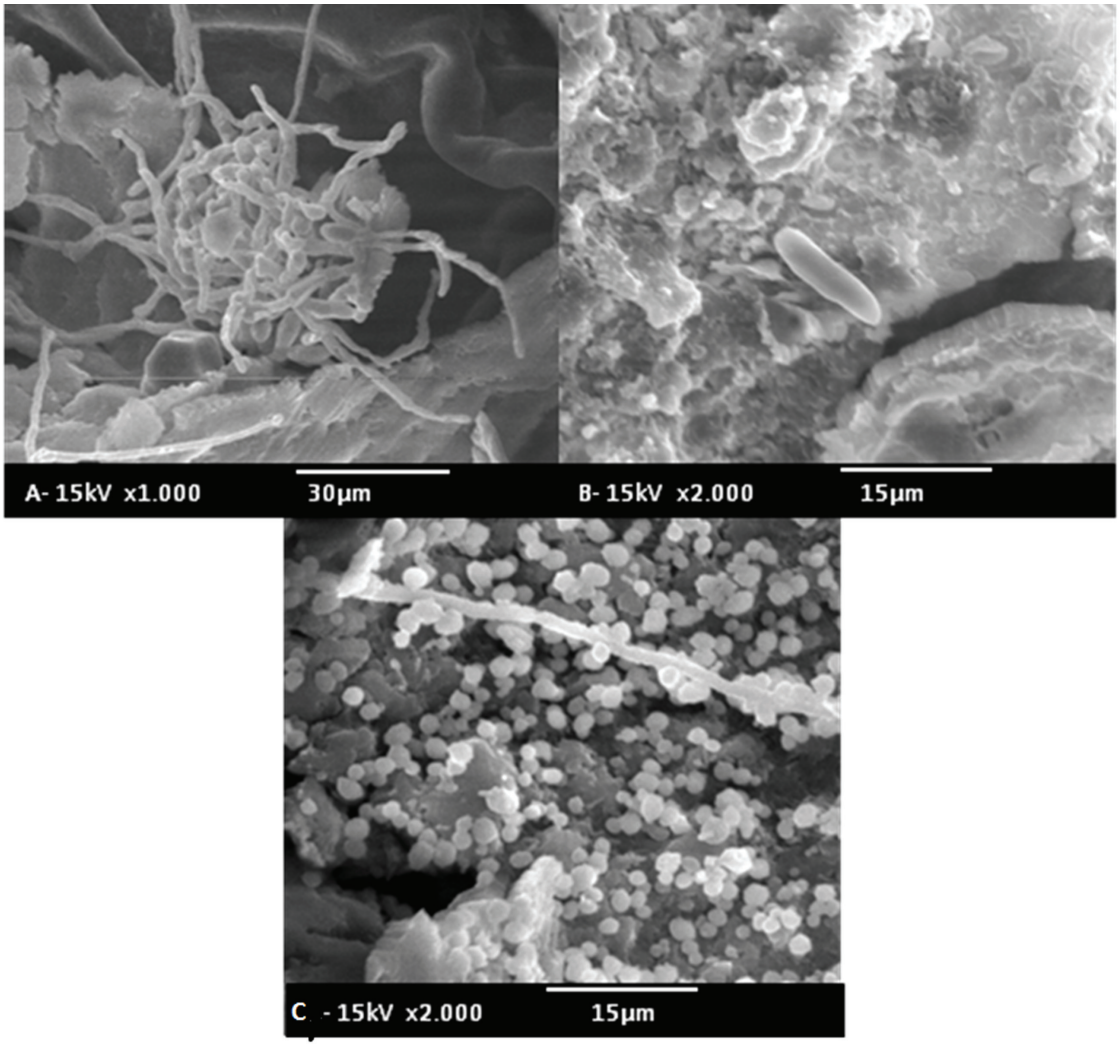

Figure 8. Electron micrographs of hyphae agglomerate (A), intact conidia (B) and hyphae of *C. gloeosporioides* (C) parasitized by the yeast *Meyerozyma guilliermondii* (strain 443) on the papaya surface.

DISCUSSION

The selection of application vehicles requires caution because these vehicles are fundamental to the survival and stability of the agent, the lifetime of the formulation and the efficiency of the product in commercial terms (22). All of the tested vehicles ensured the survival of the assessed yeasts for up to 90 days of incubation at 4°C. These findings suggest that the tested vehicles are promising substances because they were innocuous to yeasts and did not inhibit their growth. Similar results were described by Chakravarty and Kalita (3), who assessed the effect of organic-based formulations on *Pseudomonas fluorescens* for the biocontrol of bacterial wilt in eggplants (*Solanum melongena* L) and found that the survival period of the control agent was 120 days when incubated at 4°C or at room temperature. Likewise, Mounir (23) assessed the efficiency of a formulation based on a fungus, *Aureobasidium pullulans*, against *Penicillium expansum* in apples and reported that, in addition to the high efficiency of control on a pilot scale, the fungal control agent survived on the surface of the fruit.

When selecting an application vehicle, it is important to consider its availability and cost in addition to its toxicity. Therefore, the present study evaluated low-cost vehicles broadly used in the food industry (9, 13, 23, 29).

A reduction in the yeast population in the tested vehicles was only observed after 90 days of incubation. This result was likely due to intraspecific competition, especially for nutrients and space, with a consequent reduction in the population density.

Furthermore, in addition to maintaining the population stability, an increase in the initial yeast population was observed in the formulation of *M. guilliermondii* strain 443 and starch (2%). This finding is important because the concentration of the agent is directly related to its efficiency (33). It is also noteworthy that in addition to ensuring the survival of the biocontrol agent, the application vehicle must not be a food source for the organism, which could lead to increased population. A population increase could consequently cause a population decline due to the reduction of available nutrients and the accumulation of toxins produced in the substrate, thus resulting in reduced efficiency of the agent.

After 10 days of incubation under conditions favorable to disease development (28°C and 95% relative humidity - RH), fruits treated with the yeast-based formulations showed lower rates of disease compared with untreated fruits. The best results were obtained with a mixture of *W. anomalus* (strain 422) and starch (2%), a formulation that reduced the disease severity by approximately 48.3%; other functional formulations contained *M. guilliermondii* (strain 443) in liquid carnauba wax (2%)

and in gelatin (2%), which reduced the postharvest disease severity in papayas by 50%. Additionally, these formulations led to higher rates of protection than the fungicides such as thiabendazole that are currently used for postharvest papayas (31). These findings corroborate the results obtained from previous studies *in vitro* by Lima (18) and *in vivo* by Lima (19), who verified the antagonistic efficiency of these two strains of killer yeast against *C. gloeosporioides*.

The electron micrographs showed that all application vehicles provided the necessary adhesion of yeasts to the fruit surface, which is a key feature to ensure disease control, as competition for space and nutrients are the main mechanisms involved in biological control (15, 26, 32). The adherence of yeasts to the fruit surface was indispensable to ensure occupation of the site and delayed growth of the phytopathogen.

Although biological control agents are important alternatives to replace chemical agents (1), studies of the development of formulations that enhance defense against microbial agents, thus increasing their efficiency, are critical. The present study represents the first effort focused on the development of killer yeast-based formulations for the biocontrol of fungal diseases in postharvest papayas and shows that the killer yeasts *W. anomalus* (strain 422) and *M. guilliermondii* (strain 443) significantly reduced the severity of disease in papayas during the postharvest phase when used with vehicle substances, especially formulations containing starch (2%), liquid carnauba wax (2%) and gelatin.

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