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Alessandra; COELHO, Alexandre Rodrigo
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In vitro and *in vivo* antifungal activity of natural inhibitors against *Penicillium expansum*

Inibidores naturais no controle in vitro e in vivo de Penicillium expansum

Claudia FIEIRA¹, Francieli OLIVEIRA², Rubens Perez CALEGARI³,
Alessandra MACHADO¹, Alexandre Rodrigo COELHO^{1*}

Abstract

Penicillium expansum is the causative agent of apple blue mold. The inhibitory effects of the capsaicin derived from *Capsicum* spp. fruits and yeast *Hansenula wingei* against *P. expansum* were evaluated in an *in vitro* and in *in vivo* assay using Fuji apples. The minimum inhibitory concentration of capsaicin determined using the broth micro-dilution method was 122.16 µg mL⁻¹. Capsaicin did not reduce blue mold incidence in apples. However, it was able to delay fungal growth in the first 14 days of the *in vivo* assay. The *in vivo* effect of the yeast *Hansenula wingei* AM2(-2), alone and combined with thiabendazole at low dosage (40 µg mL⁻¹), on the incidence of apple diseases caused by *P. expansum* was also described. *H. wingei* AM2(-2) combined with a low fungicide dosage (10% of the dosage recommended by the manufacturer) showed the best efficacy (100%) up to 7 days of storage at 21 °C, later showing a non-statistically different decrease ($p > 0.05$) after 14 (80.45%) and 21 days (72.13%), respectively. These results contribute providing new options for using antifungal agents against *Penicillium expansum*.

Keywords: capsaicin; *Hansenula wingei*; blue mold.

Resumo

Penicillium expansum é o agente causador da doença em maçã conhecida como mofo azul. O efeito inibitório da capsaicina derivada dos frutos *Capsicum* spp. e da levedura *Hansenula wingei* foi avaliado através de ensaios *in vitro* e *in vivo* em maçã. A concentração inibitória mínima da capsaicina de 122,16 µg mL⁻¹ foi determinada usando microdiluição. A capsaicina não mostrou capacidade em reduzir a incidência do mofo azul na maçã. Entretanto, um retardo no crescimento do fungo foi observado nos 14 primeiros dias dos ensaios *in vivo*. Também descrevemos o efeito da levedura *Hansenula wingei* AM2(-2) isolada e em combinação com tiabendazol em baixa dosagem (40 µg mL⁻¹) no controle da doença de maçãs por *P. expansum*. *Hansenula wingei* AM2(-2), em combinação com baixa dosagem de tiabendazol (10% da recomendada pelo fabricante), apresentou 100% de eficácia após 7 dias de estocagem a 21 °C, seguido de 80,45% e 72,13% de eficácia após 14 e 21 dias, respectivamente ($p > 0,05$). Estes resultados contribuem com as novas opções de emprego de agentes antifúngicos contra *Penicillium expansum*.

Palavras-chave: capsaicina; *Hansenula wingei*; mofo azul.

1 Introduction

Blue mold disease is one of the most important causes of postharvest losses in apple production in Brazil (COELHO et al., 2007; BRASIL, 2005). *Penicillium expansum* is responsible for fruit infection and patulin production (COELHO et al., 2011; LIMA et al., 2011; PITT, 1997), a mycotoxin that causes genotoxicity in mammalian cells (MOSS; LONG, 2002). This fungus has proven able to proliferate in bags, wooden boxes, and storage chambers causing losses of up to 30% during storage and transportation of apples in Brazil (CANAVAR; PIERO, 2011).

Several methods have been used to solve post-harvest losses, such as fungicide treatment and modified controlled atmosphere (MONTERO et al., 2010; ROMANAZZI et al., 2012). Postharvest fungicide treatment is a primary method of control of *Penicillium* infections. Thiabendazole is one of the most commonly used chemical fungicides in the control of postharvest fungal apple decay, but it has led to the emergence of

resistant strains (SPOTTIS; CERVANTES, 1986; BARALDI et al., 2003).

Public concern regarding eating food grown with the addition of synthetic fungicides and the demand for more natural foods, free of chemical additives, has resulted in the need to find new strategies to develop fungicides to control fruit postharvest diseases. In fact, new approaches have been studied, such as the use of biological control agents, sanitizing products, and naturally-occurring compounds (MARI; BERTOLINI; PRATELLA, 2003; MARTÍNEZ-ROMERO, 2007; ROBIGLIO et al., 2011).

In terms of biological control, yeasts used in fruits are safe antagonists, do not pose risk and with low ecological impact, whose mechanism of antagonistic action is based on competition over space and nutrients (JANISIEWICZ; KORSTEN, 2002). Additionally, some species produce extracellular substances

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¹ Graduate School of Food Technology, Federal University of Technology-Parana – UTFPR, Av. dos Pioneiros, 3131, CEP 86036-370, Londrina, PR, Brazil, e-mail: arcoelho@utfpr.edu.br

² School of Food Technology, Federal University of Technology-Parana – UTFPR, Linha Santa Barbara, s/n, CP 135, CEP 85601-970, Francisco Beltrão, PR, Brazil

³ School of Environmental Engineering, Federal University of Technology-Parana – UTFPR, Linha Santa Barbara, s/n, CP 135, CEP 85601-970, Francisco Beltrão, PR, Brazil

*Corresponding author

which act against a variety of filamentous fungi in treated fruits, such as killer toxins, β -1,3-glucanase enzymes, and chitinase (CASSONE et al., 1997; IPPOLITO et al., 2000; BALLESTER et al., 2010). *Hansenula* sp is one of the most often yeast genera described as killer toxin producing yeast (GOLUBEV, 1998) including HK toxin of *Hansenula mrakii* that are targeted to β -1,3-D-glucan skeleton in the cell wall (THEISEN; MOLKENAU; SCHMITT, 2000). In a preliminary study, a killer positive strain of *H. wingei* AM2-2 showed stable in vitro antagonism against *Fusarium verticillioides* 103F for more than 30 days (GASPERINI et al., 2011).

Lima et al. (2011) showed a reduction of *Penicillium* rot in apple fruit by 35 and 52% when low dosages of *Rhodosporidium kratochvilovae* strain Ls11 and *Cryptococcus laurentii* strain LS28, respectively, were used.

In addition, naturally occurring antimicrobial substances have been found to be an alternative for the control of postharvest pathogens (VASANTHA RUPASINGHE et al., 2006). Phytoalexin, phenolic, and essential oils have been shown to have antifungal activity against *Penicillium expansum*; *Botrytis cinerea*, *Aspergillus flavus*, and *Fusarium* ssp. (PEDRAS; PEARSON, 2005; AHUJA; KISSEN; BONES, 2012; SINDHU et al., 2011; NERI et al., 2006; MARTÍNEZ-ROMERO et al., 2007). Capsaicin is an alkaloid responsible for the pungent flavor of hot pepper, and it is known for its high biological activity (LUO; PENG; LI, 2011). Many studies have shown that capsaicin and its analogues exhibit antimicrobial activity against *Bacillus subtilis* (MOLINA-TORRES; GARCIA-CHÁVEZ; RAMÍREZ-CHÁVEZ, 1999), *Helicobacter pylori* (ZEYREK; OGUZ, 2005), *Streptococcus mutans* (SANTOS et al., 2012), *Escherichia coli* (LIU et al., 2012), and *Colletotrichum capsici* (KRAIKRUAN; SANGCHOTE; SUKPRAKARN, 2008). Moreover, capsaicin micro-capsules were able to inhibit the growth of *Botrytis cinerea* and *Aspergillus niger* (XING; CHENG; YI, 2006).

In the present study, the antifungal activity of capsaicin was measured and whether this naturally occurring compound is efficient in controlling *P. expansum* on apples was investigated. Additionally, the *in vivo* antifungal activity of antagonistic yeast *Hansenula wingei* against *P. expansum* on apples was tested.

2 Materials and methods

2.1 Fruits and chemicals

Fuji apples were selected according to homogeneous size, maturity, color, and absence of injuries from a local market in Francisco Beltrão (Brazil).

Capsaicin from *Capsicum* ssp. was acquired from Sigma [$\geq 50\%$ capsaicin (HPLC) and $\sim 35\%$ dihydrocapsaicin, Sigma-Aldrich, Schnelldorf, Germany] and dissolved in Dimethyl Sulfoxide (DMSO) ($\geq 99.9\%$, Sigma, Saint Louis, USA) to obtain a stock solution of 2.44 mg.mL^{-1} . Thiabendazole was kindly provided by Syngenta (Chemical group benzimidazole; trade formulate Tecto®, 48.5% w/v a.i., São Paulo, Brazil). All other chemicals used were of analytical grade and were obtained from Vetec (Rio de Janeiro, Brazil).

2.2 Biological control Agent (BCA)

The antagonistic killer yeast *Hansenula wingei* strain AM2(-2), previously isolated from corn at the Laboratory of Food Microbiology of Federal University of Technology - Parana, was the BCA used in this study. The yeast was kept on Potato Dextrose Agar [(PDA) HIMEDIA, Mumbai, India] at 4°C for subsequent assay; *H. wingei* strain AM2(-2) was activated in Yeast Medium Agar [(YM agar) 2.0% glucose, 0.5% yeast extract; 0.23% NaH_2PO_4 ; 1.0% NaCl; 0.5% $(\text{NH}_4)_2\text{SO}_4$; 1.8% agar] at 25°C overnight, followed by cell suspension in distilled water at $10^8 \text{ cells.mL}^{-1}$ (Mac Farland n.3 scale).

2.3 Pathogen

Single-spored *Penicillium expansum* no. 2 was isolated from naturally decaying apple (NELSON; TOUSSON; MARASAS, 1983). The culture stored in PDA at 4°C was grown on PDA slants at 21°C for 120 hours before use. A spore suspension was prepared by inoculating the culture into 3 mL sterile YM broth, and the cell number was adjusted to $1 \times 10^5 \text{ spores.mL}^{-1}$ (Newbauer chamber).

2.4 In vitro antifungal activity of capsaicin against *P. expansum*

The antifungal activity was evaluated using a liquid growth inhibition assay, as described by Fehlbauer et al. (1994), with some modifications. Briefly, different volumes of the capsaicin stock solution (0.63, 1.25, 2.5, 5, 10 μL) were added to 80 μL of fungus ($1 \times 10^5 \text{ spores.mL}^{-1}$) in YM broth and diluted in sterile distilled water to a final volume of 100 μL . The capsaicin concentrations tested were 15.25, 30.54, 61.08, 122.16, and $244.30 \mu\text{g mL}^{-1}$, and the corresponding DMSO concentrations achieved were 0.63, 1.25, 2.5, 5, and 10%, respectively. Thiabendazole was used as positive control at concentrations of 16.10, 8.05, 4.02, 2.01, and $1.00 \mu\text{g mL}^{-1}$. For the negative control preparation, capsaicin or thiabendazole were replaced with sterile water. Fungal growth was measured by monitoring the OD increase at 630 nm using a TP Reader NM microplate (Thermoplate, Curitiba, Brazil). The minimal inhibitory concentration (MIC) was determined following incubation at 25°C for 48 hours. The MIC is the lowest concentration of capsaicin preventing visible antifungal growth, and the determinations were carried out independently 3 times, using triplicate samples each time.

2.5 Ability of *H. wingei* and capsaicin in controlling *P. expansum* in vivo

The *in vivo* assay was based on the methods described by Lima et al. (2006, 2011). For the antifungal assay with *H. wingei* AM2(-2), 45 cv Fuji apples were superficially disinfected by dipping the fruits in a sodium hypochlorite solution (2.0% active chlorine) for 15 minutes, rinsing with sterile distilled water, and drying them at room temperature. Four wounds (3 mm wide and 3 mm deep) were made on each fruit. Two treatments were performed: BCA treatment (yeast cell suspension alone); and BCA + thiabendazole at low dosage ($40 \mu\text{g mL}^{-1}$, 10% of the full suggested dosage). For the BCA treatment, 30 μL of yeast cell

suspension in distilled water (3.0×10^8 cells.mL⁻¹) (Mac Farland n.3 scale) were placed in each wound, while for the combined treatment (BCA + thiabendazole at low dosage), the same yeast cell suspension was placed in each wound, followed by 30 µL of fungicide at low dosage. In each experiment, the controls were represented by the apple fruits in which the wounds were treated with 30 µL of sterile distilled water alone (negative control), 30 µL of thiabendazole at full dosage, as recommended by the manufacturer (400 µg mL⁻¹), or 30 µL of thiabendazole at low dosage (40 µg mL⁻¹), as positive controls.

After two hours at room temperature, each wound was inoculated with 10^5 conidia of *P. expansum* n. 2. The fruits were packed in polypropylene plastic bags (previously disinfected with 70% ethanol) and incubated at 21 °C; the number of wounds showing rot symptoms was assessed after 7, 14, and 21 days. Each treatment included three replicates, and each consisted of 15 fruits (three fruits per treatment).

One treatment was performed for the capsaicin assay, and in addition to the capsaicin solution, it consisted of four wounds in each fruit tested. Twenty-seven apples were used to carry out the capsaicin experiment. The treatments were: A) 30 µL of sterile distilled water (positive control); B) 30 µL of capsaicin stock solution 2.44 mg.mL⁻¹; and C) 30 µL of fungicide 400 µg mL⁻¹ (negative control). The number of wounds showing rot symptoms was assessed at 7, 14, and 21 days, and photographic records were performed.

To compare the integrated treatments with those applied separately, the percentage values of infected wounds were transformed into percentages of protection efficacy (PE), as follows: $PE = [(C - T)/C] \times 100$, where C is the number of infected wounds in the control (water + pathogen), and T is the number of infected wounds in the examined treatment (BCA alone or BCA + fungicide). Values of PE ranged from 0 (no PE) to 100 (maximum PE) (LIMA et al., 2011).

2.6 Statistical analyses

The mean values for fungal growth obtained from the *in vitro* antifungal activity assay with capsaicin, represented as the absorbance by ELISA assay, were evaluated by the Tukey test ($p < 0.05$) using the Anova/Manova software (Statistica version 7.0, Inc. Tulska OK, USA, 2005). For the *in vivo* assays, the mean values obtained from the three replicates during the entire experiment (PE values) were evaluated by the Tukey test ($p < 0.05$) using the Anova/Manova software (Statistica version 7.0, Inc. Tulska OK, USA, 2005).

3 Results and discussion

3.1 In vitro antifungal activity of capsaicin against *P. expansum*

Growth-inhibiting effect of MIC found in the experiments of capsaicin against *Penicillium expansum* was described in Table 1. The maximum inhibitory effect of capsaicin was found at a concentration of 112.16 µg mL⁻¹, and it was statically independent of the DMSO concentration (Figure 1).

Table 1. Minimal inhibitory concentration (MIC) of capsaicin and controls - *in vitro* growth of *P. expansum*.

Thiabendazole Conc. (µg mL ⁻¹)	DMSO Conc. (%)	Capsaicin Conc. (µg mL ⁻¹)
16.10 (-)	10 (-)	244.30 (-)
8.05 (-)	5 (+)	122.16 (-)
4.02 (+)	2.5 (+)	61.08 (+)
2.01 (+)	1.25 (+)	30.54 (+)
1.00 (+)	0.63 (+)	15.25 (+)

(+): Presence of fungal growth; (-) absence of fungal growth; positive control: thiabendazole; negative control: YM agar + water; DMSO: capsaicin solution control. Measurements were analyzed using Tukey's test ($P < 0.05$). Value in bold is the MIC.

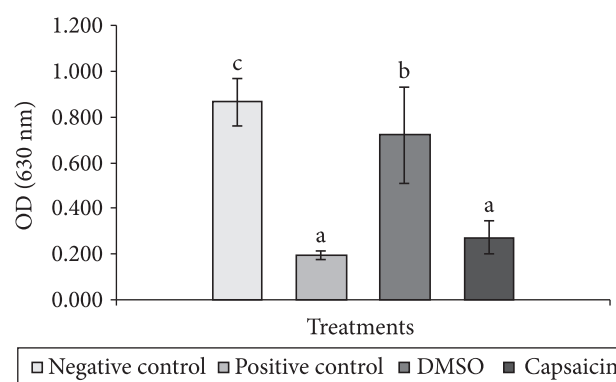


Figure 1. Comparison of the optical density of antifungal assay after 48 hours using: negative control (YM agar + water); positive control [thiabendazole (8.05 µg mL⁻¹)]; DMSO (5%); capsaicin (122.16 µg mL⁻¹). Three separate experiments were performed, and in each assay three replicates per treatment were used. Measurements marked by the same letter do not differ statistically according to the Tukey test ($p > 0.05$).

This finding is in accordance with the National Committee for Clinical Laboratory Standards (1997), which states that dilution of insoluble compounds should be done with DMSO, and its concentration must not exceed 10% of the medium. However, incubation with DMSO alone affected the growth of the fungus, but it was not responsible for antifungal activity of capsaicin at its MIC value. Compared with the positive control, capsaicin was 15-fold less active against the microorganism strains studied. On the other hand, Xing, Cheng and Yi (2006) demonstrated that capsaicin microcapsules exhibited antifungal activity against filamentous fungi, *B. cinerea* and *A. niger* at a concentration of 590 µg mL⁻¹. Interestingly, the authors believe that the inhibitory effect of the system originated from synergistic actions of capsaicin and tannins. According to the measuring fluorescence polarization of liposomes, capsaicin's changed membrane fluidity could be a result of disruption of the microorganisms' membrane (TSUCHIYA, 2001). Further, DNA microarray analysis suggests that the antimicrobial mechanism of capsaicin is related to the induction of an osmotic stress and key genes for membrane biosynthesis (KURITA et al., 2002).

3.2 Activity of *H. wingei* and effects of capsaicin against *P. expansum* *in vivo*

The results of the experiments carried out on wounded apples with capsaicin did not show protective efficacy after 21 days. Its visual protective efficacy on 7, 14, and 21 days is shown in Figure 2. As can be seen, capsaicin presented low efficacy; the diameters of the infected wounds were lower than those observed in the control, indicating low activity against the fungus. According to Vasantha Rupasinghe et al. (2006) the differences in the *in vivo* and *in vitro* treatments can be related to apple tissue features, such as pH, levels of essential nutrients (vitamins, minerals, and nitrogen-containing compounds) or natural phenolic compounds. Furthermore, Salas et al. (2011) reported that *in vivo* antifungal activity depends on the nature of the fungi and compound used.

The protective efficacy (PE) of *H. wingei* AM2 (-2) alone and in combination with thiabendazole against *P. expansum* on apples is shown in Figure 3. The visual protective efficacy of the biological control agent – BCA *Hansenula wingei* AM2 (-2) alone and in combination with a low dosage of thiabendazole ($40 \mu\text{g mL}^{-1}$) against *Penicillium expansum* no.2 on apples stored at 21°C for 7 days is shown in Figure 4.

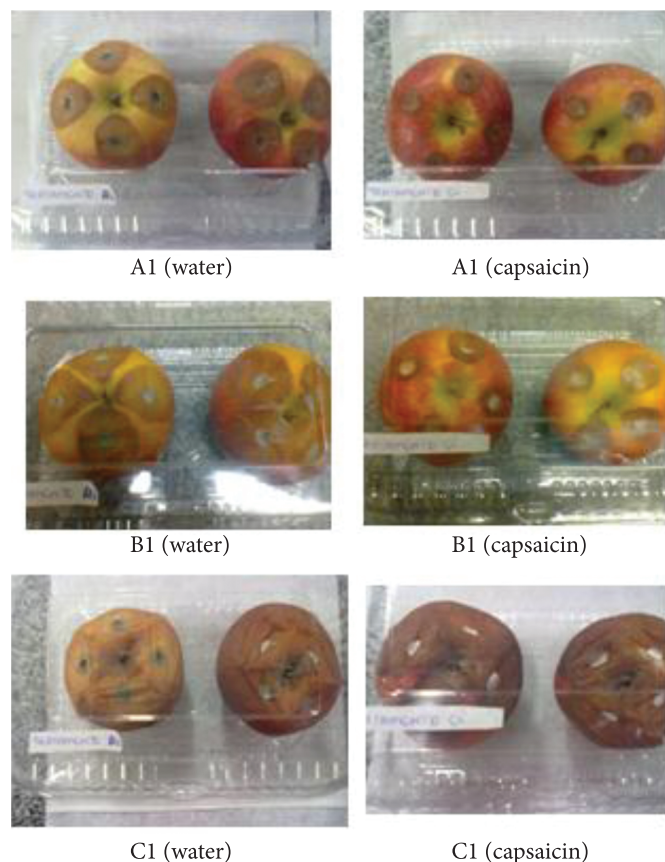


Figure 2. *In vivo* antifungal assay of capsaicin (2.44 mg mL^{-1}) against *Penicillium expansum* n. 2 on apples stored at 21°C . a) 7 days; b) 14 days; c) 21 days.

After 7 days of storage at 21°C , the control apples (water + *P. expansum*) showed no protective efficacy (zero PE), i.e., 100% percentage of disease incidence (Figure 3). Although the treatment with *H. wingei* alone exhibited no efficacy either (zero PE), as shown in Figure 3, the diameter of the infected wounds (represented as brown rot circumferences) in Figure 4b were smaller in size than those observed in the control (Figure 4a), indicating a slight inhibition of the fungus. In this case, the high initial amount of yeast inoculum used in the experiment ($3.0 \times 10^8 \text{ cells mL}^{-1}$) contributed to some fruit decay by nutrient competition since yeasts are also regarded as deteriorating agents when in contact with the inside of the fruit. In studies carried out by other researchers, including a 10^6 - $10^8 \text{ cells mL}^{-1}$ initial dosage of antagonistic yeasts, it was reported low efficacy in controlling disease incidence up to 6 or 7 day storage (GENG et al., 2011; LIMA et al., 2011). Another explanation for the *in vivo* ineffectiveness of the yeast alone treatment could be the difficulty of the killer toxin in diffusing into the fruit, consequently, affecting performance; the killer toxin is an extracellular substance that inhibits the development of other microorganisms (WALKER; McLEOD; HODGSON, 1995). In a previous study, *H. wingei* AM2 (-2) showed a positive killer factor against standard yeast isolates used as reference (GASPERINI et al., 2011).

In our preliminary *in vitro* study, 100% of conidia germination of *P. expansum* no. 2 ($10^5 \text{ conidia mL}^{-1}$) was inhibited by cell-free culture supernatant of *H. wingei* AM2 (-2) in a broth medium (data not shown). Thus, it is suggested that the antagonistic activity of *H. wingei* AM2 (-2) is essentially associated with the production of an extracellular substance, not yet reported in the literature for this species.

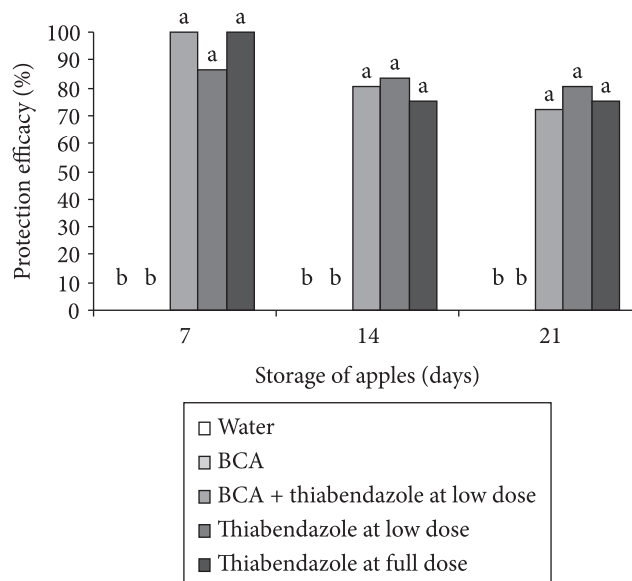


Figure 3. Activity of the biological control agent – BCA *Hansenula wingei* AM2(-2) alone and in combination with a low dosage of thiabendazole ($40 \mu\text{g mL}^{-1}$) against *Penicillium expansum* n. 2 on apples stored at 21°C for 7, 14 and 21 days. Values marked by the same letter do not differ statistically according to the Tukey test ($p > 0.05$). BCA: biological control agent.

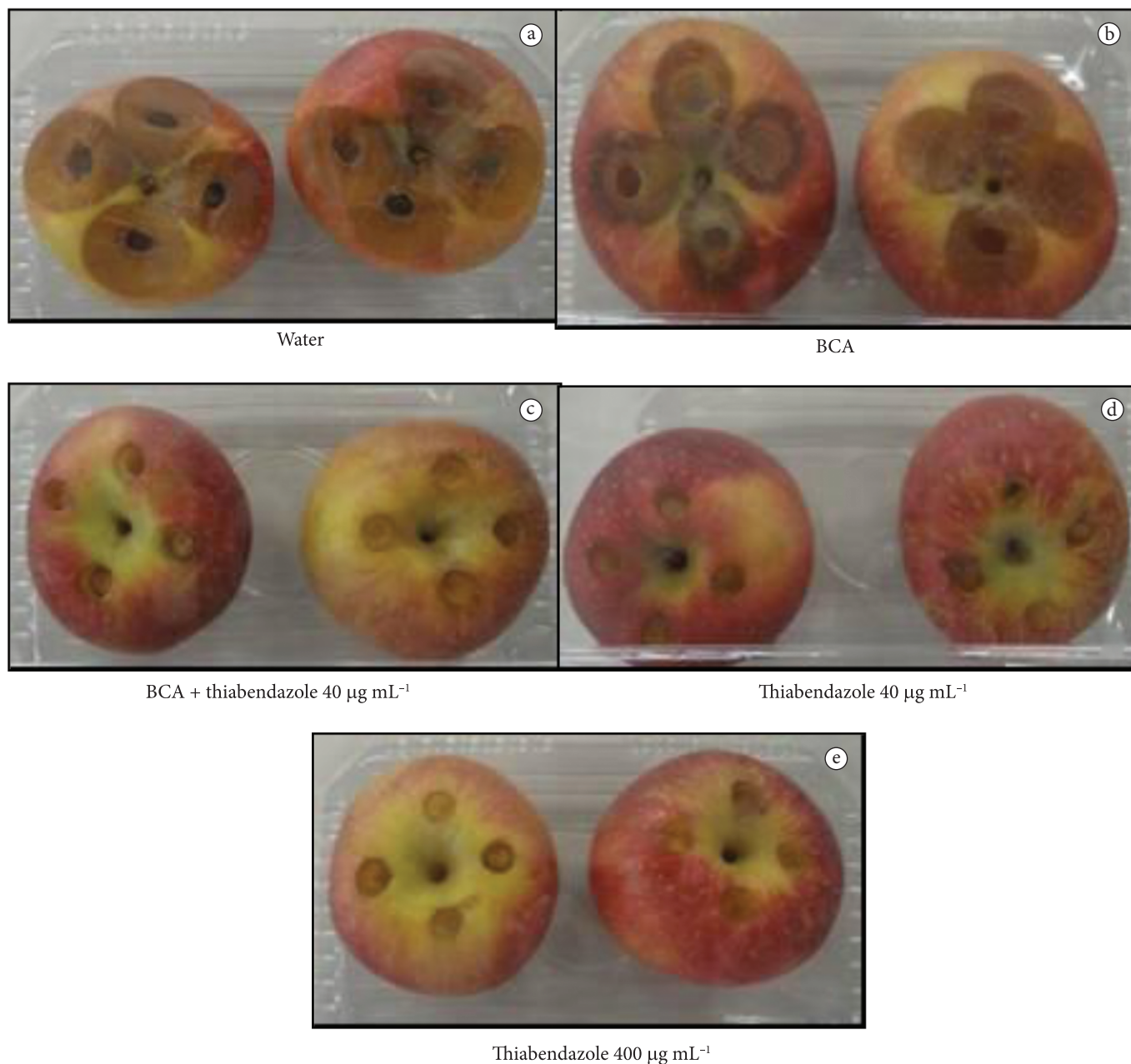


Figure 4. Visual protective efficacy of the biological control agent – BCA *Hansenula wingei* AM2(-2) alone and in combination with a low dosage of thiabendazole (40 $\mu\text{g mL}^{-1}$) against *Penicillium expansum* n. 2 on apples storage at 21 °C for 7 days. a) water; b) *H. wingei* AM2(-2) alone; c) *H. wingei* AM2(-2) in combination with thiabendazole (40 $\mu\text{g mL}^{-1}$); d) thiabendazole alone at low dose (40 $\mu\text{g mL}^{-1}$); E: thiabendazole alone at full dose recommended by manufacturer (400 $\mu\text{g mL}^{-1}$).

In addition, PE in the treatments BCA with thiabendazole at 40 $\mu\text{g mL}^{-1}$, thiabendazole at 40 $\mu\text{g mL}^{-1}$ alone, and thiabendazole at 400 $\mu\text{g mL}^{-1}$ alone were significantly effective against the fungus with PE higher than 85% (Figure 3). Figure 4c-e showed no incidence of disease in the treatments BCA in combination with thiabendazole at 40 $\mu\text{g mL}^{-1}$, thiabendazole at 40 $\mu\text{g mL}^{-1}$ alone, and thiabendazole at 400 $\mu\text{g mL}^{-1}$ alone, respectively. On the other hand, the incidence of disease was clearly visible

in Figure 4a (positive control, water), represented as a brown rot circumference. Comparing the protection efficacy between BCA + thiabendazole at a low dosage and thiabendazole at a low dosage alone, the former showed the maximum effect (100%), while the fungicide alone, in this study, controlled 86.15%, showing antagonistic activity of yeast against fruit decay (Figure 3). It is important to mention that the optimization of biological control efficacy also depends on the survival

and colonization of biological agents in wounded fruits in the presence of low amounts of fungicides. Lima et al. (2011) reported a significant rot reduction of 98% after 7 days of storage by using an integrated treatment based on antagonistic yeasts with chemical fungicides, such as boscalid or cyprodinil, both at low dosage. The combination of *Kluyveromyces marxianus* with sodium bicarbonate was as effective as the imazalil treatment in natural infection trials, which gave about 90% control of green mold (GENG et al., 2011).

After 14 and 21 days of storage, BCA + thiabendazole at low dosage and thiabendazole at low dosage alone showed no significant PE ($p > 0.05$), 84.45-72.13% and 83.3-80.50%, respectively, while the fungicide at full dosage maintained 75% PE. In this study, the application of yeast in combination with a low dosage of thiabendazole was as effective in the control of *P. expansum* rot after 21 days of storage as the chemical fungicide applied separately, at low and full dosage (Figure 3, $p > 0.05$).

Although thiabendazole is one of the most important synthetic fungicides still allowed for postharvest treatment of fruit in many countries, the low efficacy or inefficacy against *P. expansum* reported by Lima et al. (2011) could be related to the widespread diffusion of fungal pathogen isolates that have become resistant to fungicides used for a long time in packing houses.

According to the results obtained, the combination of BCA with thiabendazole at a low dosage showed the best efficacy (100%) up to 7 days of storage at 21 °C, followed by a non- statistically significant decrease after 14 (80.45%) and 21 days (72.13%). Several studies on storage of fruits at 20-25 °C have reported efficacy by combining antagonistic yeasts with chemical substances up to 7 days (LIMA et al., 2006, 2011; GENG et al., 2011). However, Fazio, Gonçalves and Hoffmann (2012) reported a PE of 87.5% for the control of *P. expansum* on apples stored at 21 °C for 21 days when a combination of *Dekkera bruxellensis* and thiabendazole 40 µg mL⁻¹ was used.

Therefore, the application of antagonistic yeasts in combination with fungicides at low dosage (10% of that recommended by the manufacturer) becomes a very promising alternative to control postharvest spoilage fungi, reducing a large amount of toxic waste in the treated fruits.

4 Conclusion

In conclusion, the present study describes, for the first time, the antifungal effect exhibited by capsaicin against *P. expansum*. This study expands the existing knowledge of antimicrobial activity of natural compounds suggesting that it is likely that hot pepper could be used to control blue mold disease. In *in vivo* experiments, the yeast combined with fungicide at low dosage showed satisfactory efficacy in the control of fruit decay up to 14 days, becoming a control strategy from an economic and technical point of view, such as better control of fruit rot diseases caused by prolonged fruit storage. Future investigations will focus on the synergistic effect of *Hansenula wingei* with other naturally occurring antimicrobial substances on the growth of filamentous fungi.

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