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Study of the Action Mode of *Wickerhamomyces anomalus* against *Colletotrichum gloeosporioides*

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Abstract: *Colletotrichum gloeosporioides* is the causal agent of anthracnose disease in fruits and vegetables, representing a global problem. The use of biocontrol agents has proved effective against fungal diseases in a wide variety of products. In this work, the antifungal activity of *Wickerhamomyces anomalus* against *C. gloeosporioides* isolated from contaminated avocados was evaluated. The antagonism and volatile compound inhibition were measured on Petri dishes. In the mixed cultures, the mycelia damage was observed by scanning electron microscope (SEM). Chitinase and glucanase production by the antagonism was quantified by the reducing sugars method, and biofilm formation was evaluated with 1% crystal violet. The yeast *W. anomalus* could reduce the growth of *C. gloeosporioides* up to 65% by direct antagonism and 10% by volatile compounds. The antagonist did not allow the conidia germination and mycelia growth in any of the tested formulations. SEM showed mycelial damage caused by *W. anomalus*. The antagonist showed adhesion to the mycelium by a polysaccharide biofilm. The presence of mycelium stimulated the hydrolytic enzyme production with the maximal activity of 21.4 U/mg for chitinases at 24 h and 10 U/mg for glucanases at 60 h. These results showed that *W. anomalus* used together different mechanisms to express its antifungal activity against *C. gloeosporioides*. This study might be the first report for this phytopathogen isolated from avocado fruits, which could represent an opportunity to establish biocontrol of diseases for this agricultural product.

Key words: *Colletotrichum gloeosporioides*, *Wickerhamomyces anomalus*, biocontrol, avocado.

1. Introduction

Phytopathogenic microorganisms that cause decay in fruit and vegetables with commercial value represent a global problem, leading to a products loss percentages up to 20%-35% [1]. The anthracnose disease caused by *Colletotrichum gloeosporioides* not just affects fruits and vegetables, but also legumes and ornamental plants [2]. The infection on fruits can occur when the products are injured during transportation and management process, or when the fruits natural inhibitors start to decrease due to the maturation process, so the phytopathogen conidia present on the product skin initiate an accelerated reproduction, which leads to a subsequent invasion to the pulp. The anthracnose disease can be identified by the appearance of circular dark colored patches [3]. These fungal diseases are usually controlled by chemical fungicides [3, 4], and its application directly into food products is considered dangerous to human health and environment. These encouraged the search for safer and environmentally friendly alternatives to stop or reduce postharvest decay [2, 5]. One of the effective alternatives is the use of biocontrol agents, which involve the participation of antagonistic microorganisms applied directly to the fruits or vegetables. It can compete directly with the phytopathogenic microorganisms to prevent growth [6].

While the competition for space and nutrients is one of the principal mechanisms used by the biocontrol agents, they also make use of a wide variety of

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different mechanism to suppress the phytopathogen activities. Other mechanisms, like the capacity to adhere to specific sites or wounds of the host, exudation of different enzymes and secretion of antimicrobial substances or active metabolites like volatile compounds, are also an important part in the biocontrol activity [7, 8]. Yeast of *Wickerhamomyces anomalus* (*Pichia anomala*) has a wide history of biocontrol in various fruits due to its antagonistic effect against different fungal pathogens and their broad history of use for biotechnology process [6]. The goal of the present study was to conduct a study on the biocontrol strategy used by *W. anomalus* against *C. gloeosporioides* in the context of future research towards an efficient biocontrol using a formulation containing adequate conditions for the antagonist.

2. Materials and Methods

2.1 Biological Material

The isolate of *C. gloeosporioides* was isolated from field of infected avocados. *W. anomalus* strain KP238319 was provided by the Laboratory of Phytopathology, Centro de Desarrollo de Productos Bióticos-Instituto Politécnico Nacional (CEPROBI-IPN), Carretera Yautepec-Jojutla, Yautepec, Morelos, México.

2.2 Antagonistic Capacity

The antagonism *W. anomalus* was evidenced on Petri dishes, on two different solid media—yeast-malt agar (YMA) and potato dextrose agar (PDA). A dual culture was made placing a 6 mm disc of *C. gloeosporioides* at 1.5 cm of the dish end and streaking a 1.5 cm wide streak of *W. anomalus* 6 cm away from the disc (Fig. 1). The dishes were incubated at 28 °C for 15 d and then phytopathogen radius was measured. Dishes containing only the phytopathogen were used as controls.

2.3 Inhibition by Volatile Compounds

The assay was performed according to the methodology described by Vero et al. [9], making a double chamber with two Petri dishes containing PDA medium. On the upper side of the chamber, a 6 mm disc of *C. gloeosporioides* was placed and 100 μL of 5 × 10^6 cells of *W. anomalus* were placed on the bottom side. The chambers were sealed with parafilm and incubated for 15 d at 28 °C. Phytopathogen growth radius was measured and chambers in absence of antagonist were used as control.

2.4 Inhibition of Conidial Germination of C. gloeosporioides by W. anomalus

Flasks with potato dextrose broth (PDB) medium were inoculated with different cell numbers of *W. anomalus* starting with 1 × 10^6 cells and making serial dilutions (1:10). On the same flasks, conidia of *C. gloeosporioides* was inoculated using relations of dilution of 1:1, 10:1, 100:1 and vice versa. The cultures were maintained under stirring for 72 h at 28 °C. Prior the incubation time, aliquots were taken and observed under microscope for monitoring the presence of mycelia.

2.5 Interaction of W. anomalus with C. gloeosporioides by Scanning Electron Microscopy (SEM)

Flasks containing PDB medium were inoculated with 5 × 10^5 conidia from *C. gloeosporioides* during 72 h at 28 °C under stirring to allow mycelia growth. The mixed cultures were performed by adding 1 × 10^6 cells of *W. anomalus* to the flasks, containing the mycelia and incubating them under the same conditions. Samples were taken at 0, 24, 48 and 72 h of

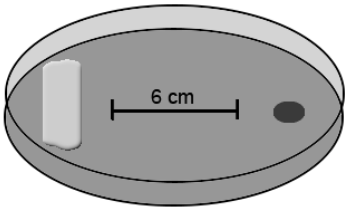

Fig. 1 Diagram of the dual culture *in vitro* test for the antagonism of *W. anomalus* (left) against *C. gloeosporioides* (right).

the interaction, and processed to be observed under SEM.

2.6 Evaluation of Chitinase and Glucanase Production of W. anomalus on the Mixed Cultures

Flasks containing PDB medium were inoculated with 1×10^6 cells of *W. anomalus* and either 5×10^5 conidia or the mycelia previously growth as mentioned above. Determinations were performed in the supernatant by the 3,5-dinitrosalicylic acid (DNS) method according to the methodology described by Tayel et al. [10]. The exo-β-1,3-glucanase and exo-chitinase activities were measured using laminarin and colloidal chitin as substrates, respectively. As control, flasks with 1×10^6 cells of *W. anomalus* were used.

For chitinase activity, 1 U of activity was defined as the amount of enzyme responsible for liberating 1 μmol of N-acetyl-β-D-glucosamine per minute per mL, using 1% solution of colloidal chitin as a substrate. And 1 U of β-1,3-glucanase activity was defined as the amount of enzyme, which required for liberating 1 μg of glucose equivalent per minute per mL of enzyme solution.

2.7 Determination of Biofilm Formation by W. anomalus

The determination was performed according to the methodology described by Vero et al. [9], using a 96-well plate containing 200 μL of PDB medium and inoculating 50 μL of a 1×10^6 cell suspension of *W. anomalus*. For the control wells, just the medium was used. The plate was incubated during 48 h at 72 °C, subsequently the wells were emptied, rinsed with distilled water and dried at room temperature. The biofilm present on the wells were stained with a 1% solution of crystal violet, rinsed and dried again. The stained biofilm was diluted with ethanol and measured at 600 nm using ELISA microplate reader. The biofilm formation was considered positive, if the absorbance of a well was greater than the mean of the negative controls plus three standard deviations.

3. Results and Discussion

3.1 Antagonistic Capacity

The antagonism on the PDA plates showed a 65% reduction in average growth radius of the pathogen, while no significant reduction was seen on the YMA plates (Table 1). In this case, both mediums contained glucose as carbon source, but had differences in the nutrients given by the potato dextrose or malt extract, showing involvement in the antagonistic activity. Jijakli and Lepoivre [11] described that the biocontrol exercised by *W. anomalus* is subject to the type of nutrients. The medium containing PDA is more efficient for *W. anomalus* than the ones containing malt on its composition. So the substrate specificity and selectivity are suggested towards certain types of fruits. These results were the base for just using PDA and PDB mediums and will be used to continue the experiments.

3.2 Inhibition by Volatile Compounds

The inhibition effect of volatile compounds showed 10% reduction in growth comparing with control (Table 2). However, the identity of the volatiles that inhibited *C. gloeosporioides* growth was not yet determined. Inhibition by volatile compounds has been demonstrated previously in different reports [9, 12]. Coda et al. [13] identified some volatile compounds produced by *W. anomalus*, such as ethyl acetate and ethanol, which caused inhibition for the pathogens. Also, the compound 2-phenylethanol produced by *W. anomalus* was attributed to have biocontrol properties that affect the conidia germination,

Table 1 Antagonism of *W. anomalus*a gainst *C. gloeosporioides* on PDA media.

Treatment	Growth in cm (± SD)
Control	5.2 ± 0.1128
W. anomalus	3.4 ± 0.2238

Control represents the growth of *C. gloeosporioides* in the absence of *W. anomalus*. Each value is the average of 10 repetitions. Values are significantly different ($P < 0.05$).

Table 2 Growth reduction of *C. gloeosporioides* by volatile compounds produced by *W. anomalus* on PDA media.

Treatment	Growth in cm (± SD)
Control	7.4 ± 0.1187
W. anomalus	6.7 ± 0.2415

Each value is the average of 10 repetitions. Values are significantly different ($P < 0.05$).

growth, toxin production and genes expression of *Aspergillus flavus* [14].

3.3 Inhibition of Conidial Germination of C. gloeosporioides by W. anomalus

In dual cultures, *W. anomalus* did not allow the conidia germination of *C. gloeosporioides* at any of the tested relationships, while conidial germination and mycelial growth in controls did it. *W. anomalus* showed antagonistic action against filamentous phytopathogens that affects fruits and vegetables, such as *Penicillium expansum*, *Botrytis cinerea* and *Aspergillus flavus* by inhibiting conidial germination and mycelial growth [11]. In these studies, the interaction of both microorganisms on a dual cell culture gives the data for the subsequent analysis of the hydrolytic enzymes activity.

3.4 Interaction of W. anomalus with C. gloeosporioides by SEM

Damage to the phytopathogen hyphae and its colonization by *W. anomalus* was observed by optical microscopy and SEM (Fig. 2). The damage by *W. anomalus* was seen on ratios of 10:1 cells or more against the phytopathogen by optical microscopy. On SEM, structural damage of mycelium was showed after 48 h, and presented cell wall degradation (Fig. 2c), compared with the control hyphae (Fig. 2a) and the beginning of the interaction (Fig. 2b), possibly due to the hydrolytic enzymes activity. *W. anomalus* is directly adherent to the hyphae of the pathogen, suggesting that adhesion mechanism is present (Fig. 2d), such as the production of an exo-polysaccharide might be contributes to adhesion. This adhesion had been reported as part of the mycoparasitism mechanism, and applied when the hyphae of a living fungus are utilized as a nutrient source for another microorganism. Tayel et al. [10] reported damage to the hyphae, mycoparasitism and finally a complete lysis of *Aspergillus flavus* hyphae caused by *W. anomalus*. This effect may vary depending

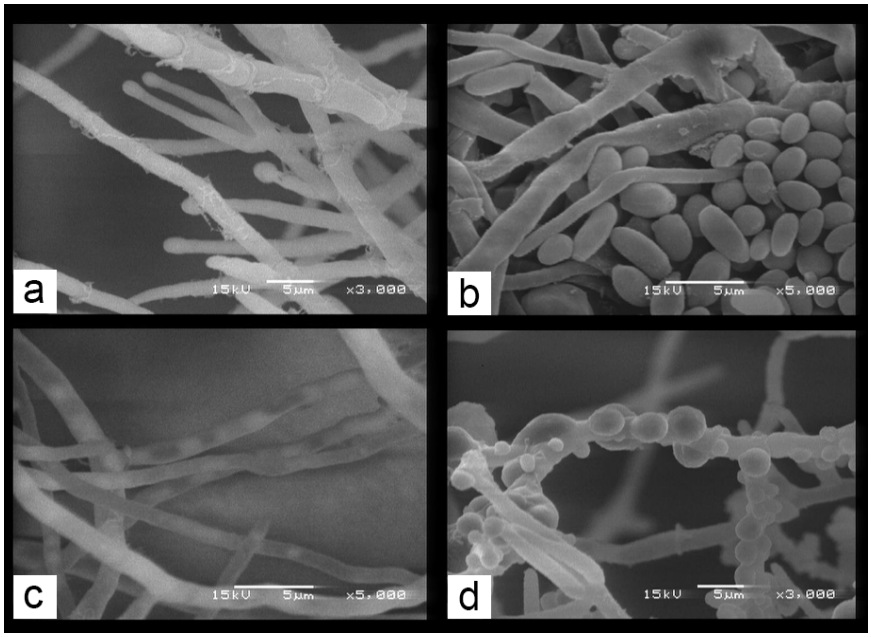

Fig. 2 Interaction of *W. anomalus* and *C. gloeosporioides* by SEM.
(a) control hyphae; (b) at the beginning of interaction of both microorganisms; (c) hyphae damage; (d) mycoparasitism of *W. anomalus* over *C. gloeosporioides* hyphae.

on which plant pathogen the yeast is facing. The antagonist yeasts effect over the phytopathogen suggests that their hyphae are more resistant to the attack made by the antagonist, mostly because they were not completed lysed. However, typical signs of these kinds of interaction can be seen, for example, deformations and the darkest zones with "sinkings" on the hyphae. Similar effects had been reported by other studies, where *W. anomalus* also adhered to the hyphae of *Monilinia fructicola*, *Penicillium expansum*, *Rhizopus stolonifer* and *Cryptococcus albidus* [11, 15]. The deformation effect was also attributed to the enzymatic action, mostly glucanases, which damage and cause degradation on the cell wall, moreover, making perforations or weakening of hyphae. These results correspond with the description of Mohamed and Saad [16], who reported these effect by *W. anomalus* joining together with the hyphae of *Lasiodiplodia theobromae* after 96 h of incubation. The presence of deformations or damage was also reported by Chan and Tian [17] using *W. anomalus* and its interaction against *Penicillium expansum*.

3.5 Evaluation of Chitinase and Glucanase Production of W. anomalus on the Mixed Cultures

To better analyze the times on which the different growth phases are presented and compare them to the enzyme production, a growth curve was made, showing a specific growth velocity of $\mu = 0.135/h$ (Fig. 3). As shown in Fig. 4, the chitinase activity produced by *W. anomalus* in the mixed cultures reached its maximum activity after 24 h in presence of *C. gloeosporioides* hyphae, with an activity of 21.4 U/mg, while the minimum at 48 h with near 0.4 U/mg. The presence of the conidia did not stimulate the chitinase production, and the enzyme activity was similar to that provided in absence of the phytopathogen. This suggests that chitinases are induced by the presence of chitin from the hyphae, where its amount is greater than in the conidia.

The glucanase activity presents two peaks of activities in presence of the fungal hyphae, at 30 h of 6.5 U/mg and 10.8 U/mg at 60 h, respectively (Fig. 5). Glucanase activity was lower in presence of conidia (6.4 U/mg). Unlike chitinases, the antagonist produces glucanases in absence of the phytopathogen. These results show that the presence of phytopathogen hyphae increases the production of both enzymes. While chitinases are produced during the exponential phase with activity almost double as glucanases which get its maximum activity on stationary phase. The glucanase production was also continuous from the start, but in low concentrations. Similar results were found by Jijakli and Lepoivre [11], who reported an

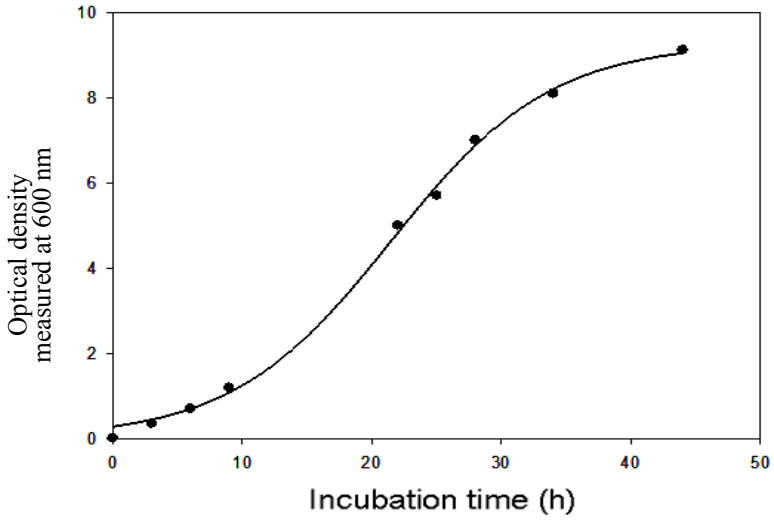

Fig. 3 Growth curve of *W. anomalus* (●) on PDB medium.

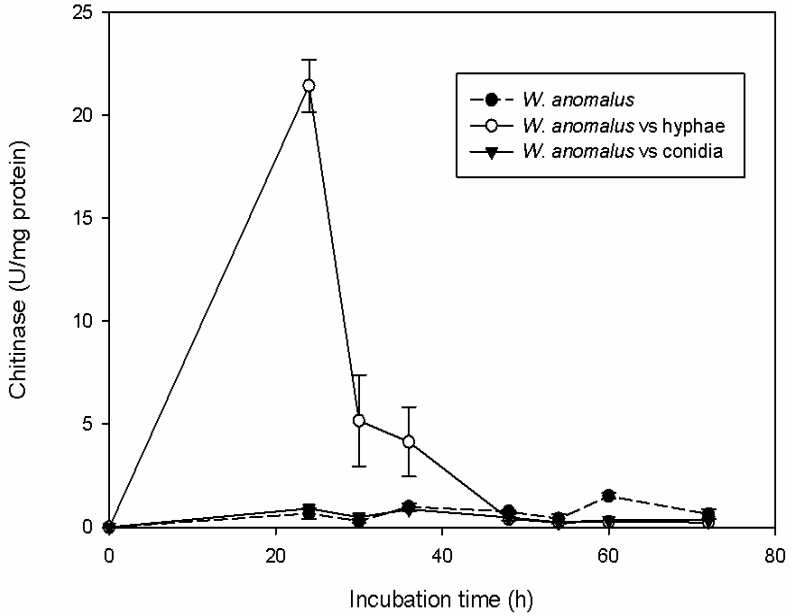

Fig. 4 Extracellular chitinase activity of *W. anomalus* (●) and its activity in presence of *C. gloeosporioides* conidia (▼) and hyphae (○) on PDB medium.

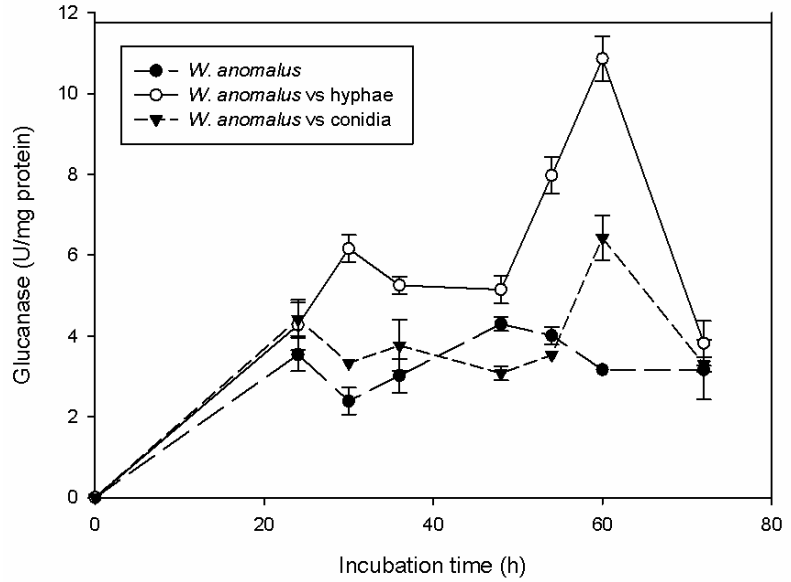

Fig. 5 Extracellular glucanase activity of *W. anomalus* (●) and its activity in presence of *C. gloeosporioides* conidia (▼) and hyphae (○) on PDB medium.

increase on the glucanase activity from *W. anomalus* in presence of cell wall fragments of *Botrytis cinerea*. Moreover, Tayel et al. [10] reported that glucanases activity production by *W. anomalus* was gradually increased on its stationary phase, and the production was decreased when the incubation times continued. Different studies reported higher activities of *Trichoderma harzianum*, *Pichia guilliermondii* and *Serratia marcescens* when the culture media was supplemented with the cell wall of a phytopathogenic cell walls in mediums that contain other substrates [18-21].

The production rate of these enzymes may vary depending on which phytopathogen *W. anomalus* is facing, as manifested by Tayel et al. [10], who found chitinase and glucanase activities around 2 U/mg and

3.5 U/mg, respectively, at 72 h when *W. anomalus* was on axenic culture. Jijakli and Lepoivre [11] reported glucanase activity between 60 h and 72 h period. The results in this study suggest that the hydrolytic enzymes produced by *W. anomalus* at different times allows a hydrolysis over *C. gloeosporioides* hyphae almost constantly. It has been mentioned that the hydrolytic enzymes are indeed a factor of biocontrol, working together with other mechanisms on degradation and lysis process. These results indicate that the interaction of both microorganisms produce the higher induction at different times for each enzyme. However, other studies about the production of these hydrolytic enzymes give more importance to the glucanases, although both enzymes are participants for these degradation processes and biocontrol.

3.6 Determination of Biofilm Formation by W. anomalus

The biofilm formation was measured at 600 nm, having an average value of an optical density (OD) of 0.3025 ± 0.0988, while the controls showed OD value of 0.0979 ± 0.0292 (Table 3). Biofilm formation is considered as an important attribute for antagonist yeasts, as it helps to successfully colonize and protect both the wound site and the intact fruit [22]. These biofilms are also a mechanism that gives the ability to withstand stress and improve their endurance [9]. The biofilm formation capacity of *W. anomalus* in absence of the phytopathogen could be tested and the adhesion to the hyphae during the interaction of both microorganisms was corroborated by SEM.

Biocontrol agents and their action mechanism have awakened great interest in the last 20 years, preventing postharvest diseases by pathogenic fungi. The present study focused on the mechanisms presented by the antagonist yeast *W. anomalus* against the phytopathogen *C. Gloeosporioides* causative of the anthracnose, isolated from avocados. Microorganisms as *Streptomyces* sp. have been shown antifungal

Table 3 Biofilm formation capacity of *W. anomalus* measured by the mean absorbance value (OD units) at 600 nm of the crystal violet stained samples.

Treatment	A_{600} (mean ± SD)
Control	0.0979 ± 0.0292
W. anomalus	0.3025 ± 0.0988

SD: standard deviation; A: absorbance at 600 nm.
A value higher than the mean plus three standard deviations its considered positive for biofilm formation.

activity against *C. gloeosporioides*, *Penicillium italicus* and *Fusarium oxysporum* [23]. *Trichoderma* species also exhibit antagonism toward these phytopathogens [24]. However, antagonistic yeasts are of the most promising, because they do not produce harmful molecules that could affect the stored products. The presence of the phytopathogen stimulates the production of hydrolytic enzymes and a biofilm formation that allows the development of the biocontrol effect. The hydrolytic enzymes glucanases and chitinases were reported in this study to inhibit the germination of conidia. And their production is an important factor for causing fungal mycelium damage, while the biofilm allows the invasion and parasitism. Directly, yeast as antagonist uses its ability to produce volatile compounds against mycelial growth of the pathogen. The mechanisms of antifungal compounds and/or enzymes production by *W. anomalus* work together synergistically and complement each other to achieve the biocontrol activity against *C. gloeosporioides*. Finally, this study might be the first report for this phytopathogen isolated from avocado fruits, which could represent an opportunity to establish biocontrol of diseases for this crop.

4. Conclusions

Regarding the results obtained, it can be concluded that *W. anomalus* is an efficient antagonist to inhibit growth of *C. gloeosporioides* isolated from avocados, using different mechanisms. The phytopathogen presence stimulates the production of hydrolytic enzymes glucanases and chitinases that are an important factor to inhibit the conidia germination and

cause structural damage on already developed hyphae. The biofilm allows *W. anomalus* to invade and parasite the hyphae, and indirectly, it can produce volatile compounds to also inhibit mycelia growth. The mechanisms used by the antagonist work together and complement each other to achieve the biocontrol activity over *C. gloeosporioides*.

Acknowledgments

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References

[1] Abano, E. E., and Sam-Amoah, L. K. 2011. "Effects of Different Pretreatments on Drying Characteristics of Banana Slices." *J. Eng. Appl. Sci.* 6 (3): 121-9.

[2] Ashwini, N., and Srividya, S. 2014. "Potentiality of *Bacillus subtilis* as Biocontrol Agent for Management of Anthracnose Disease of Chilli Caused by *Colletotrichum gloeosporioides* OGC1." *Biotech.* 4 (2): 127-36.

[3] Garbanzo, S. 2011. *Avocado Manual: Good Practices of Hass Variety Cultivation,* 2nd ed.. Costa Rica: Ministry of Agriculture and Livestock. (in Spanish)

[4] Nelson, S. 2008. "Anthracnose of Avocado." Plant Disease PD-58, College of Tropical Agriculture and Human Resources, University of Hawaii (UH–CTAHR). Accessed November, 2008. https://www.ctahr.hawaii.edu/oc/freepubs/pdf/PD-58.pdf.

[5] Kwasiborski, A., Bajji, M., Renaut, J., Delaplace, P., and Jijakli, M. H. 2014. "Identification of Metabolic Pathways Expressed by *Pichia anomala* Kh6 in the Presence of the Pathogen *Botrytis cinerea* on Apple: New Possible Targets for Biocontrol Improvement." *PLoS ONE* 9 (3): e91434.

[6] Haïssam, J. M. 2011. "*Pichia anomala* in Biocontrol for Apples: 20 Years of Fundamental Research and Practical Applications." *Antonie van Leeuwenhoek* 99 (1): 93-105.

[7] Castoria, R., Caputo, L., De Curtis, F., and De Cicco, V. 2003. "Resistance of Postharvest Biocontrol Yeast to Oxidative Stress: A Possible New Mechanism of Action." *Phytopathology* 93 (5): 564-72.

[8] Sharma, R. R., Singhb, D., and Singhc, R. 2009. "Biological Control of Postharvest Diseases of Fruits and Vegetables by Microbial Antagonists: A Review." *Biological Control* 50 (3): 205-21.

[9] Vero, S., Garmendia, G., González, M. B., Bentancur, O., and Wisniewski, M. 2013. "Evaluation of Yeasts Obtained from Antarctic Soil Samples as Biocontrol Agents for the Management of Postharvest Diseases of Apple (*Malus domestica*)." *FEMS Yeast Res.* 13 (2): 189-99.

[10] Tayel, A. A., El-Tras, W. F., Moussa, S. H., and El-Agamy, M. A. 2013. "Antifungal Action of *Pichia anomala* against Aflatoxigenic *Aspergillus flavus* and Its Application as a Feed Supplement." *J. Sci. Food Agri.* 93 (13): 3259-63.

[11] Jijakli, M. H., and Lepoivre, P. 1998. "Characterization of an Exo-β-1,3-Glucanase Produced by *Pichia anomala* Strain K, Antagonist of *Botrytis cinerea* on Apples." *Phytopathology* 88 (4): 335-43.

[12] Druvefors, U. Ä., Passoth, V., and Schnürer, J. 2005. "Nutrient Effects on Biocontrol of *Penicillium roqueforti* by *Pichia anomala* J121 during Airtight Storage of Wheat." *Appl. Environ. Microbiol.* 71 (4): 1865-9.

[13] Coda, R., Cassone, A., Rizzello, C. G., Nionelli, L., Cardinali, G., and Gobbetti, M. 2011. "Antifungal Activity of *Wickerhamomyces anomalus* and *Lactobacillus plantarum* during Sourdough Fermentation: Identification of Novel Compounds and Long-Term Effect during Storage of Wheat Bread." *Appl. Environ. Microbiol.* 77 (10): 3484-92.

[14] Hua, S. S., Hernlem, B. J., Yokoyama, W., and Sarreal, S. B. 2015. "Intracellular Trehalose and Sorbitol Synergistically Promoting Cell Viability of a Biocontrol Yeast, *Pichia anomala*, for Aflatoxin Reduction." *World J. Microbiol. Biotechnol.* 31 (5): 729-34.

[15] Masih, E. I., Slezack-Deschaumes, S., Marmaras, I., Barka, E. A., Vernet, G., Charpentier, C., and Paul, B. 2001. "Characterization of the Yeast *Pichia membranifaciens* and Its Possible Use in the Biological Control of *Botrytis cinerea*, Causing the Grey Mould Disease of Grapevine." *FEMS Microbiol. Lett.* 202 (2): 227-32.

[16] Mohamed, H., and Saad, A. 2009. "The Biocontrol of Postharvest Disease (*Botryodiplodia theobromae*) of Guava (*Psidium guajava* L.) by the Application of Yeast Strains." *Postharvest Biology and Technology* 53 (3): 123-30.

[17] Chan, Z. L., and Tian, S. P. 2005. "Interaction of Antagonistic Yeasts against Postharvest Pathogens of Apple Fruit and Possible Mode of Action." *Postharvest Biology and Technology* 36 (2): 215-23.

[18] De la Cruz, J., Pintor-Toro, J. A., Benitez, T., Llobell, A., and Romero, L. C. 1995. "A Novel Endo-β-1,3-Glucanase, BGN13.1, Involved in the

Mycoparasitism of *Trichoderma harzianum*." *J. Bacteriol.* 177 (23): 6937-45.

[19] Elad, Y., Chet, I., and Henis, Y. 1982. "Degradation of Plant Pathogenic Fungi by *Trichoderma harzianum*." *Canadian J. Microbiol.* 28 (7): 719-25.

[20] Ordentlich, A., Elad, Y., and Chet, I. 1988. "The Role of Chitinase of *Serratia marcescens* in Biocontrol of *Sclerotium rolfsii*." *Phytopathology* 78 (1): 84-8.

[21] Wisniewski, M., Biles, C., Droby, S., McLaughlin, R., Wilson, C., and Chalutz, E. 1991. "Mode of Action of the Postharvest Biocontrol Yeast, *Pichia guilliermondii*: Part I, Characterization of Attachment to *Botrytis cinerea*." *Physiological and Molecular Plant Pathology* 39 (4): 245-58.

[22] Droby, S., Wisniewski, M., Macarisin, D., and Wilson, C. 2009. "Twenty Years of Postharvest Biocontrol Research: Is It Time for a New Paradigm?" *Postharvest Biology and Technology* 52 (2): 137-45.

[23] Chen, H., Ma, Y., Zhang, W. F., Ma, T., and Wu, H. X. 2015. "Molecular Phylogeny of *Colletotrichum* (Sordariomycetes: Glomerellaceae) Inferred from Multiple Gene Sequences." *Genet. Mol. Res.* 14 (4): 13649-62.

[24] Kumar, K., Amaresan, N., Bhagat, S., Madhuri, K., and Srivastava, R. C. 2012. "Isolation and Characterization of *Trichoderma* spp. for Antagonistic Activity against Root Rot and Foliar Pathogens." *Indian J. Microbiol.* 52 (2): 137-44.