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Antagonistic microorganisms efficiency to suppress damage caused by *Colletotrichum gloeosporioides* in papaya crop: Perspectives and challenges

Eficiencia de los antagonistas microbianos para suprimir el daño ocasionado por *Colletotrichum gloeosporioides* en el cultivo de papaya: Perspectivas y desafíos

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Abstract

Papaya (*Carica papaya* L.) is one of the most valued tropical fruits worldwide due to its nutritional content, but its production is drastically affected by *Colletotrichum gloeosporioides*, one of the main pathogens responsible for anthracnose disease. Several techniques as an alternative of conventional chemical treatments for disease control have been studied. Among these techniques, the use of antagonist microorganism has emerged as a promising, eco-friendly alternative for postharvest disease control. This review is focused on the inhibition of *Colletotrichum gloeosporioides* in papaya applying microbial antagonists. The main purpose of this study, is to provide the results of *in vivo* and *in vitro* assays that addressed the use of microorganisms and their activity as biocontrol agents in papaya, considering its application to diminish crop losses and suggesting possible future researches applicable to their attractive usage. We believe that a specific compilation is helpful for groups that are in research of pre- and postharvest fruits management, providing useful information to create new perspectives and/or alternative in emerging technologies.

Keywords: microbial antagonists, biological control, postharvest disease, papaya, *Colletotrichum gloeosporioides*.

Resumen

La papaya (*Carica papaya* L.) es uno de los frutos tropicales más apreciados a nivel mundial debido a su contenido nutricional. Sin embargo, su producción es afectada drásticamente por *Colletotrichum gloeosporioides*, uno de los principales patógenos responsables de la antracnosis. Se han estudiado diversas estrategias alternativas al tratamiento químico convencional para el control de antracnosis. Entre éstas, el uso de microorganismos antagonistas ha surgido como una alternativa amigable con el medio ambiente y eficiente para el control de enfermedades poscosecha. Esta revisión está enfocada en la inhibición de *Colletotrichum gloeosporioides* en papaya aplicando microorganismos antagonistas, proveyendo los resultados más importantes de ensayos de inhibición *in vivo* e *in vitro*, considerando así su aplicación para disminuir las pérdidas en poscosecha, y sugiriendo los posibles trabajos de investigación que requieren realizarse para hacer más atractivo su uso. Creemos que esta compilación específica será útil a los grupos de investigación que trabajan en el manejo pre y poscosecha de frutas para crear nuevas perspectivas o alternativas en el uso de tecnologías emergentes.

Palabras clave: microorganismos antagonistas, control biológico, enfermedades poscosecha, papaya, *Colletotrichum gloeosporioides*.

1 Introduction

Papaya (*Carica papaya* L.) is one of the main tropical fruits produced in Mexico (Carballo-Sánchez *et al*., 2016), but it can be infected with, anthracnose, one of the main diseases that affects it. In recent years, anthracnose prevalence in crops and postharvest production of papaya and other tropical fruits, has grown in Mexico despite the treatments with fungicides, resulting in economic losses (Chávez-Magdaleno *et al*., 2018; Hernández-López *et al*., 2018, Ramos-Guerrero *et al*. 2020). More than 50% of waste of fresh fruits and vegetables are caused by *Colletotrichum* species (Paull *et al*., 1997; Awang *et al*., 2011; Chávez-Magdaleno *et al*., 2018). This disease is associated to the fungus *Colletotrichum gloeosporioides* that causes deep rounded stains, soaked with water in orange-pink zones formed due to the conidia mass that covers

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the center of the lesion and sometimes, a pattern of concentrically rings. Another species of this fungus is Colletotrichum truncatum, also known as Colletotrichum capsici; this fungus causes lesions with color from brown to black, with spore mass in gray color and plenty of concentrically rings (Tapia-Tussell *et al*., 2008; Torres-Calzada *et al*., 2013). Furthermore, it causes cell damage, and the production of acervuli is associated with dark sunken areas on the surface of fruit with diameter of about 4.2 cm. This pathogen infects the host fruit through a subcuticular intramural infection, causing anthracnose in 72 hours, and producing acervuli in 96 hours after inoculation, completing its life cycle (Rojo-Báez *et al*., 2016). Recently, Colletotrichum magnum was identified in papaya infected with anthracnose; colonies exhibiting white-orange color with acervuli, unicellular cylindrical conidia with rounded edges were isolated (Tapia-Tussell *et al*., 2016). Also, Colletotrichum fructicola has been first evidenced as a phytopathogen for papaya in Oaxaca, Mexico, with lesions characterized by sunken and irregular edges, presenting white to gray sporulation at the center. Even if these characteristics are similar to *C. gloeosporioides* species, identification through molecular techniques, revealed different species (Marquez-Zequera *et al*., 2018).

Biological control agents (BCA) emerged as an alternative for chemical fungicides, promoting a sustainable, eco-friendly treatment. In literature, the first report of BCA was Trichoderma spp. as an antagonist microorganism against the strawberry pathogen Botrytis rot (Tronsmo and Denis, 1977). Since then, numerous antagonists were isolated and identified as BCA. In this review, we collect information of BCA against the pathogen *Colletotrichum gloeosporioides*, the main pathogen responsible for papaya anthracnose.

2 *Colletotrichum gloeosporioides*

Colletotrichum gloeosporioides is a fungal pathogen that belongs to the order Melanconiales. It can infect different crops, from seeds to trees, and cause postharvest diseases, such as anthracnose. Optimal conditions for *C. gloeosporioides* growth are 25-28°C and pH 5.8-6.5. This pathogen grows when conditions of humidity are suitable, but in dry seasons, it does not develop. It has been isolated in various growing media for growth and sporulation, e.g. potato dextrose agar (PDA) or malt extract agar. Anthracnose caused by *C. gloeosporioides* infects fruits, like avocado, apple, mango, papaya, strawberry, and many others. Symptoms of infection by this pathogen are expressed as small, dark lesions that appear on leaves, fruits, and flowers, which produce concentric ring patterns, as depicted in Figure 1 (Sharma and Kulshrestha, 2015).

Fig. 1. Anthracnose symptoms in papaya (*Carica papaya* L.).

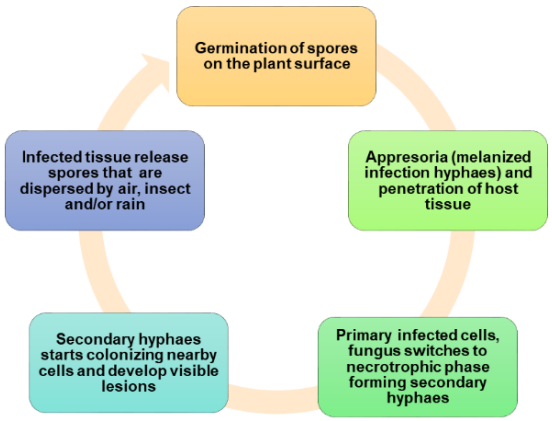

Fig. 2. Life cycle of *Colletotrichum gloeosporioides* (Sharma and Kulshrestha, 2015)

2.1 *Life cycle*

C. gloeosporioides follows two routes: pathogenic and saprophytic. Pathogenic germination takes place on plants or hydrophobic surfaces, with fast mitosis and development of a single germ tube. This process is initiated immediately and results in the formation of appressoria (Barhoom and Sharon, 2004). Figure 2 depicts this process.

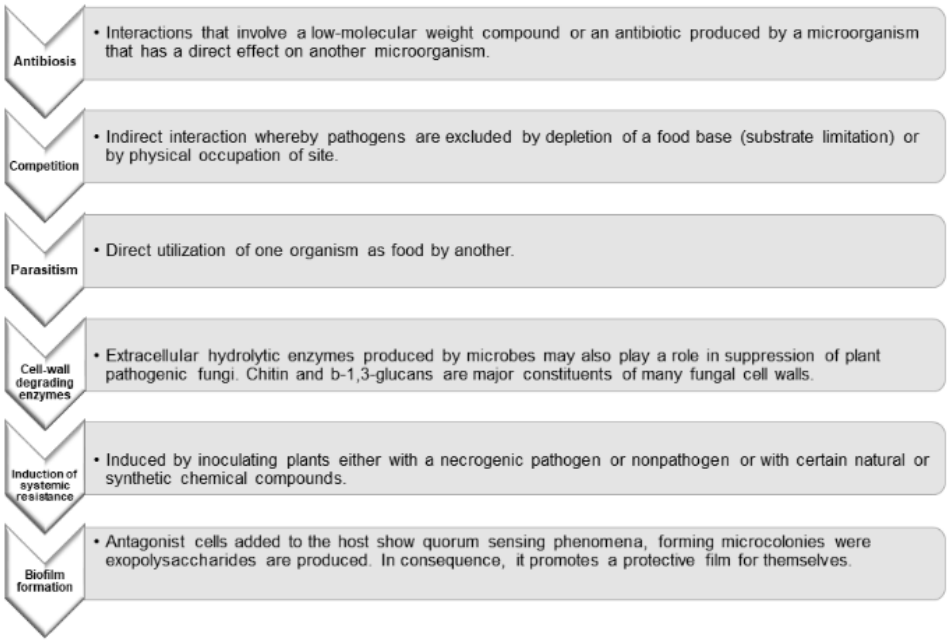

Fig. 3. Antagonist mechanisms of action.

2.2 Anthracnose disease spreading

Some fungi are capable to displace short distances on their own. Fungal spores spread by wind driven rain, irrigation water, insects, mammals, soil water, and humans as carriers (LeClair *et al*., 2015). When fungal spores are transferred to new host plants, they attach to them and begin the infection process one more time. To understand this, the spread of infectious material from one site to another is a control issue on crop losses, hence, elucidate its mechanism of dissemination is a complex of mixed genes involved, and their identification and characterization still generates challenges. On this topic, some of these identified genes and their function are listed below: i) Shpx2, Shpx5, Shpx6, Shpx12 and PepCYP (host defense); ii) Cap 20 and CgDN3 (host pathogenesis); iii) Chip 6 (pathogenesis, conidial germination and appressorium formation); iv) Pnl-1 and Pnl-2 (pathogenesis), v) Pel-B (degrade plant cell-wall); vi) CgDN24 and Pel-1,Pel-2 (pathogenesis and hyphal development); vii) CgCTR2 (putative copper transporter); viii) CgRac1 (morphogenesis, nuclear division and pathogenesis); ix) GDH2, GS1, GLT and MEP (induce ammonia accumulation and pathogenesis); and x) PacC (create alkaline environment and regulate activity of several genes) (Sharma and Kulshrestha, 2015). Polymerase chain reaction (PCR) still predominates on fungal species screening. Currently, the list of *Colletotrichum* names in use has a total of 66 species, and 20 were recently added but considered as doubtful. Thus, this demands the increasing reliance on molecular methods for species definition. Finally, because carbon and nitrogen are the most important and essential elements, besides others, for their sources and incorporation into infect ion, growth and reproduction, the strain identification vary by geographical and host sources due to substrate properties (Sangeetha and Rawal, 2008).

3 Antagonistic microorganisms on pathogens

3.1 Inhibition mechanisms

Several research groups have studied the possible mechanisms of antagonist inhibition in pathogens, which can be: antibiosis, competition, parasitism, enzymatic cell-wall degradation, induction of systemic resistance, and biofilm formation (Cook and Baker, 1983; Fravel, 1988; Handelsman and Parke, 1989; Adams, 1990; Bautista-Rosales *et al*., 2013; González-Estrada *et al*., 2017a). In Figure 3 the aforementioned mechanisms are briefly described.

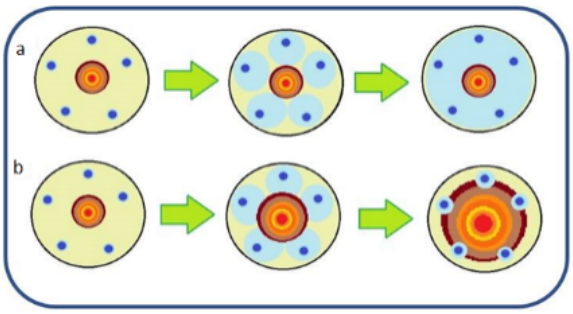

Fig. 4. *In vitro* assays, challenge of antagonists against *Colletotrichum gloeosporioides*, a) High inhibition of pathogen b) Poor inhibition of pathogen.

Fig. 5. *In vivo* assays, fruits are coated with a suspension of antagonists by immersion or aspersion.

3.1.1 In vitro assays of fungal inhibition

In vitro assays can be conducted as described by Hernández-Montiel *et al*. (2013), where a disc from a previously grown inoculum of around 5 mm diameter is placed in the center of a Petri dish with PDA by substitution. BCA are inoculated at a defined concentration on sterile filter paper and placed at equal distance from the center of the Petri dish. After incubation at specific conditions for the pathogen, diameters of inhibition are measured. Procedure is shown in Figure 4. Furthermore, spore germination test is needed to complete *in vitro* assays on the antagonistic activity (Rahman *et al*., 2007).

3.2 In vivo assays for antagonist-pathogen interaction

For *in vivo* assays, wounds are performed in papaya and *C. gloeosporioides* is inoculated. Antagonists are placed by submerging the fruit in a suspension with a defined concentration of them (Figure 5). After incubation at specific conditions for the pathogen, diameters of inhibition and disease severity are measured (Rahman *et al*., 2009).

3.3 Antagonistic activity of microorganisms against Colletotrichum gloeosporioides in papaya (Carica papaya L.)

Gamagae *et al*. (2003) evaluated the inhibitory activity of yeast Candida oleophila, individually and in combination with a suspension of sodium bicarbonate (2%), against *C. gloeosporioides* in papaya (*Carica papaya* L.) at storage conditions (13.5°C, 95% R.H., 10 days). They observed that the combination of both treatments reduced the population of the pathogen and anthracnose severity in papaya inoculated and naturally infected with *C. gloeosporioides*. Treatments by themselves were effective suppressing the infection, but combination of both proved to be better. Gamagae *et al*. (2004) evaluated the same treatments inside a wax coating, and results showed that the coating increased the inhibitory activity of yeast and sodium bicarbonate compared to the previous study. Wax coating created a modified atmosphere, where *C. oleophila* was able to adapt and survive while inhibiting the pathogen. This suggests that coating methods enhance microbial survival (Luján-Hidalgo *et al*., 2019) and could be used as an important strategy to extend viability of biological agents to control postharvest diseases (González-Estrada *et al*., 2017b).

Three strains of *Bacillus firmus* and four of *Pseudomonas fluorescens* were evaluated by Baños-Guevara *et al*. (2004) against anthracnose disease in Maradol red papaya (*Carica papaya* L). They found that in the *in vitro* assay, two strains of *B. firmus* (B10 and B3) reduced growth of *C. gloeosporioides* in 75.32 and 69.17% in 96 hours, respectively, while *P. fluorescens* strains, did not present antagonism against the pathogen. *B. firmus* strains were able to inhibit *C. gloeosporioides* in postharvest, this can be due to nutrient competition and space, or due to the production of antibiotics and other antimicrobial substances.

Epiphytic microorganisms from papaya leaves and fruit were isolated by de Capdeville *et al*. (2007a), 164 distinct microorganisms were found, from which 67 yeasts were identified. 30 strains were able to inhibit *C. gloeosporioides* growth *in vitro*, and 10 of these were evaluated *in vivo*. Ribosomal RNA

sequences for the yeast strain with better inhibitory activity resulted in *Cryptococcus magnus* MZKI K-479. *in vivo* tests were carried out in papaya infected with *C. gloeosporioides* and inoculation of *C. magnus* on different cell concentration at three different times (0, 24 and 48 hours) prior to pathogen inoculation. The results showed that high cell concentration of *C. magnus* (10^7 and 10^8 cell/mL) was effective, independently of the inoculation time. Later, electronic microscopy imaging of antagonist effect was observed. Fungal cells were able to colonize superficial lesions faster than the pathogen, competing for the space and probably the nutrients. This fungus produces a flocculent matrix that affects the integrity of hypha. Summarizing, this yeast can inhibit *C. gloeosporioides* growth by competition of space and nutrients, and also, by modifying integrity of hypha (de Capdeville *et al*., 2007b).

Burkholderia cepacia and *Pseudomonas aeruginosa* were studied by Rahman *et al*. (2007). Mycelial growth and spore germination of *C. gloeosporioides* in presence of *B. cepacia* were completely inhibited, while P. aeruginosa only had inhibitory activity over mycelial growth, but not on spore germination. The possible mechanism of inhibition is due to bacterial production of antibiotics.

Antifungal substances produced by *B. cepacia*, strain B23, were extracted by Kadir *et al*. (2008), and evaluated against *C. gloeosporioides*. Cultured in nutrient broth (NB), *B. cepacia* was able to produce higher amounts of antifungal substances. Dilutions of antifungal substances (1:8) in the supernatant of *B. cepacia* B23, inhibited mycelial growth and spore germination (41 and 100%, respectively) of *C. gloeosporioides*. Substances produced in NB medium inhibited mycelial growth in 82.67% at concentrations of 1:1, where pyrrolnitrin was detected among other compounds.

Rahman *et al*. (2009) combined *B. cepacia* B23 with chitosan and calcium chloride (0.75 and 3%, respectively), and found that anthracnose infection was effectively controlled in fruits inoculated with the pathogen during storage at 14°C and 95% R.H. for 18 days. This combination reduced disease severity in 99% by the 14th day and at 6 days of ripening at 28 ± 2°C.

Osman *et al*. (2010) evaluated the efficiency of *Bacillus amyloliquefaciens* PPCB004 in Solo papaya, previously treated with 1-methylcyclopropene (1-MCP) during storage, against anthracnose and rottenness. The combined treatment of 1-MCP and *B. amyloliquefaciens* reduced disease severity compared to singled or commercial treatments (NaOCl). Production of iturin A, fengycin and surfactin were shown on fruit individually treated with *B. amyloliquefaciens* or in combination with 1-MCP, suggesting that this bacterium can be used as a protectant of fruit when previously treated with 1-MCP.

Endophytic bacterial strain *Pseudomonas putida* MGY2, was isolated from papaya fruit by Shi *et al*. (2011). Papaya treated with *P. putida* MGY2, was challenged against *C. gloeosporioides* in postharvest, and the possible inhibition mechanisms were studied. They observed that fruit with treatment showed reduction in disease index, disease incidence and lesion diameter. Decrease in reduction of firmness, and production of ethylene delay in papaya harvest and storage at 25°C was also observed. Reduction of disease severity was up to 35% compared to the untreated control. Phenylalanine (PAL), catalase (CAT) and peroxidase (POD) activities were increased in the presence of *P. putida* and phenolic content rose. They suggested that papaya inoculated with *P. putida* MGY2, may activate defensive enzymes and genes that can induce resistance against pathogens disease.

Our group (Magallón-Andalón *et al*., 2012), observed parasitism mechanism of *Rodothorula mucilaginosa* 2 and *Candida famata* on *C. gloeosporioides* in papaya (Figure 6).

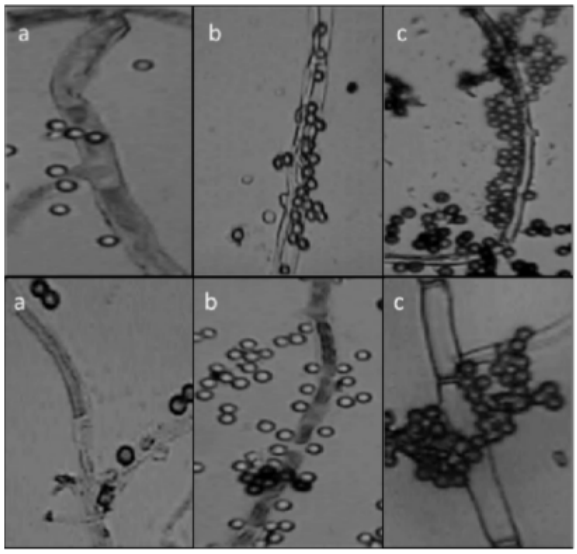

Fig. 6. *Candida famata* (above) and *Rhodotorula mucilaginosa* 2 (below) adhesion to the *Colletotrichum gloeosporioides* mycelium observed by optical microscopy (100x) after a) 12h, b) 24h and c) 48h (Magallón-Andalón *et al*., 2012).

Both yeasts were capable to attach to the pathogen, producing hydrolytic enzymes responsible for fungi cell wall degradation. The enzyme production in both strains was increased when yeast was in presence of sterile mycelium of *C. gloeosporioides*, confirming the usage of mycelium as a nutrient. When cultured in a medium enriched with nutrients, *C. famata* had a better inhibition of *C. gloeosporioides*, probably due to faster intake of nutrients.

Colletotrichum gloeosporioides is considered the most significant of fungal species that cause anthracnose in papaya in Brazil. To suppress it, two killer yeasts, *Wickerhamomyces anomalus* and *Meyerozyma guilliermondii*, were tested against *C. gloeosporioides*, and the influence of inoculation time was examined along with the occurrence of mycoparasitism. Assays of hydrolytic enzyme β-1-3-glucanase and chitinase were carried out. In papaya that was treated with *W. anomalus* 24 h before the inoculation of *C. gloeosporioides*, fruits showed lesions 31.35% smaller than those without the treatment, while on those inoculated 24 h after, lesions were 4% smaller. *M. guilliermondii* had a similar response, 24 h before inoculation of *C. gloeosporioides*, a lesion 41.17% smaller than control was observed, while 24 h after inoculation lesion was 10% smaller (Lima *et al*., 2013). *W. anomalus* reduced the lesion induced by *C. gloeosporioides* in 24.62%, and *M. guilliermondii* in 20.68% after 6 days of inoculation.

Colonization of killer yeast achieved maximum growth between the third and fourth day after inoculation, with a posterior stabilization of similar levels to the initial inoculum. The speed of colonization of the yeast was faster than the growth of the pathogen, providing protection to the fruits. This is related to the competition for nutrients and space used as a mechanism for biocontrol.

Killer yeast mycoparasitism was observed on *C. gloeosporioides*, causing physical damage, like turgidity loss, occurrence of concave areas and, in some cases, complete hypha rupture with yeast penetration into the pathogen hyphae, possibly due to production of hydrolytic enzymes. Activity of β(1-3)-glucanase was of 1.18 and 0.78 nkat/mg protein for *W. anomalus* and *M. guilliermondii*, respectively. 1 nkat is equivalent to 1.0 nmol glucose released/mL/s. This enzyme hydrolyzes glucans, an abundant component of the fungal cell wall, giving support to the use of this yeast against the anthracnose disease induced by *C. gloeosporioides* (Lima *et al*., 2013).

Another bacteria, *Streptomyces violascens* produces antibiotics and low molecular weigth siderophores; this bacteria was tested against various fungi responsible for rotting in fruits. *In vitro* assays demonstrated that *C. gloeosporioides* growth was inhibited in about 30 mm, also they correlated the production of antibiotics with the biomass formation; by the third day, production of antibiotics reached its maximum values, as well as the inhibition zone. Extraction of antifungal compounds was carried out with five organic solvents, with n-butanol being the best at percentage of extraction and antifungal activity. Further analysis demonstrated that this antifungal compounds are of polyene nature (Choudhary *et al*., 2015).

Landero-Valenzuela *et al*. (2016) compiled information with different methods for controlling *C. gloeosporioides* in different fruits. The most commonly used is the addition of chemical fungicides, although they represent a threat to consumers due to residues remaining in the fruits. Another alternative for controlling *C. gloeosporioides*, is the application of biorationals produced by different antagonist microorganisms, like essential oils, chitosan (Sotelo-Boyás *et al*., 2015), or glucosinolates. Molecular manipulations have been carried out to repress the expression of genes involved in ripening (production of ethylene). Although this is a promising technique, it requires major investments and results take longer than other methods.

Table 1 summarizes the studied microorganisms for *C. gloeosporioides* inhibition. Percentage of inhibition for both *in vivo* and *in vitro* tests, and antagonist mechanism are also shown.

3.4 Perspectives and challenges

Papaya is a valuable fruit due to its nutritional contribution and bioactive compounds (Vallejo-Castillo *et al*. 2020), but its shelf life in postharvest storage is short, with consequent economic losses. Anthracnose caused by *Colletotrichum gloeosporioides* is the main disease responsible for these losses. In order to control the disease, traditional chemical fungicides are still in use since alternative treatments, like antagonist microorganism studied in this review, have not proved to be sustainable and/or attractive for commercialization yet, due to various factors such as temperature and humidity conditions for pathogen growth. Biocontrol agents are a capable technique, despite that, control efficiency could vary from *in vitro* to *in vivo* conditions significantly; perhaps biocontrol efficacy is influenced by the

Table 1. *C. gloeosporioides* inhibition with biocontrol agents. DSR: Disease Severity Reduction, MG: Mycelial Growth, SG: Spore Germination, NR†: Not Reported. 1-MCP: 1-methyl-cyclopropene.

Treatment	*In vitro* inhibition (%)	*In vivo* inhibition (%) DSR	Antagonist mechanism	Reference
C. oleophila + $NaHCO_3$	NR†	NR†	NR†	Gamagae *et al.* (2003)
C. oleophila+ $NaHCO_3$ + wax coating	NR†	NR†	NR†	Gamagae *et al.* (2004)
B. firmus B10	75.32 MG	NR†	Competition	Baños- Guevara *et al.* (2004)
B. firmus B3	69.17 MG	NR†	Competition	Baños-Guevara *et al.* (2004)
C. magnus	NR†	NR†	Competition	de Capdeville *et al.* (2007)
B. cepacia	100 MG, 100 SG	NR†	Antibiosis	Rahman *et al.* (2007)
P. aeruginosa	100 MG, 3.7 SG	NR†	Antibiosis	Rahman *et al.* (2007)
B. cepacia	41 MG, 100 SG	NR†	Antibiosis	Kadir *et al.* (2008)
B. cepacia + chitosan + $CaCl_2$	NR†	98.2	Antibiosis	Rahman *et al.* (2009)
P. putida	NR†	35	Induction of systemic resistance	Shi *et al.* (2010)
B. amyloliquefaciens + 1-MCP	NR†	NR†	Antibiosis	Osman *et al.* (2010)
W. anomalus	NR†	24.62	Competition, parasitism, cell-wall degrading enzymes	Lima *et al.* (2013)
M. guilliermondii	NR†	20.68	Competition, parasitism, cell-wall degrading enzymes	Lima *et al.* (2013)

amount of pathogen present (Roberts, 1994; Siddiqui and Ali, 2014).

Although several antagonist have proved to be efficient in suppressing ancthranose caused by *C. gloeoesporioides*, several reports do not have tests simulating in situ conditions (Bautista-Baños *et al*., 2013), which makes this technique not sustainable yet. Antagonist mechanism of action requires more elucidation. Furthermore, several preharvest studies are needed because papaya can be infected with conidia from isolates of *Colletotrichum gloeosporioides*, regardless of the original host plant. For example, *Colletotrichum* isolates from papaya can cause infection in mango fruits (Freeman and Shabi, 1996). On this regard, genetic and geographical data provided, suggest that *C. gloeosporioides* was disseminated as an endophyte around the world (Akem, 2006). Thus, understanding the origins and diversity of *C. gloeosporioides* in papaya would have importance to future research on control techniques, according to specific locations (Siddiqui and Ali, 2014). Even to improve the preharvest studies, the existing pathogen could be identified, using infrared spectroscopy as a spectral marker, and in this way, it could provide quicker information in comparison with PCR; therefore, an early action could be established for the control of postharvest diseases (Salman *et al*., 2010). In addition, some authors have bet on combining the antagonists with different vehicles to improve disease management (Gamagae *et al*., 2004). Clearly, there is still much research needed in order to use antagonists. Future studies could contemplate nanotechnology with greener methods for encapsulation and for strengthening the antagonist capacity. These studies could also include the use of biofilms with antagonists in a bioactive matrix (González-Estrada *et al*., 2017b) and perhaps a new generation of hybrids that allows these microorganisms to be sustainable and attractive to producers and consumers.

Conclusions

In order to explore full potential of microbial antagonists, preliminary research goals should be focused on its inhibition mechanism and molecular analysis, microbial viability, market storage conditions, among others. Certainly, the above reviewed studies demonstrate that antagnostic microorganisms are able to suppress the disease. Hence, further studies should be conducted to escalate their activity at industrial levels as eco-friendly

technology.

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