Application of chlorine dioxide (ClO₂) and marine yeasts to control postharvest anthracnose disease in mango (Mangifera indica L.)

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Abstract

J.J. Reyes-Perez, S. Vero, E. Diaz-Rivera, L. Lara-Capistran, J.C. Noa-Carrazana, and L.G. Hernandez-Montiel. 2019. Application of chlorine dioxide (ClO₂) and marine yeasts to control postharvest anthracnose disease in mango (Mangifera indica L.). Cien. Inv. Agr. 46(3): 266-275. Postharvest diseases in fruits cause serious losses of fresh produce worldwide. The application of synthetic fungicides for the control of postharvest diseases such as anthracnose in mangoes can cause adverse effects on the environment and on human and animal health and has generated phytopathogen resistance. Biological control with the application of marine yeasts and chlorine dioxide (ClO₂) to reduce the use of synthetic fungicides can be an alternative to prevent anthracnose in Ataulfo mango fruits. The results showed that different doses of ClO₂ inhibited the mycelium growth and spore germination of Colletotrichum gloeosporioides in vitro. When ClO₂ and the marine yeasts Debaryomyces hansenii and Rhodotorula minuta were applied to mango fruits, no signs of anthracnose disease incidence and lesion diameter were observed (LSD, p<0.05). Therefore, the application of ClO₂ plus antagonist yeasts provides excellent control of anthracnose disease in Ataulfo mango fruits.

Key words: Colletotrichum gloeosporioides, Debaryomyces hansenii, disinfectant, mango fruit, Rhodotorula minuta.

Introduction

The diseases caused by fungi in postharvest fruit cause economic losses during harvest, transport and commercialization (Mahunu et al., 2016; Tian et al., 2018). Mango (Mangifera indica L.) is an example of an agricultural product whose postharvest life is limited by the damage caused by plant pathogens, of which Colletotrichum gloeosporioides, the causal agent of anthracnose, is one of the main pathogens affecting production worldwide (Snowdon, 2010; Zhang et al., 2013). Losses are directly caused by this pathogen in the field and during processing, reducing quality and
commercialization (de Oliveira et al., 2017). The use of synthetic fungicides is among the main methods for controlling plant diseases, but their application has generated concerns about toxicity problems and environmental damage (Mailly et al., 2017). Moreover, there have been reports indicating that the control efficacy of fungicides has dwindled due to the appearance of resistant phytopathogen strains (Onyeani and Osunlaja, 2012; Esteriol et al., 2017).

Among the different methods that have been used as alternatives to decrease synthetic fungicide applications, the use of antagonist microorganisms and the application of disinfectants such as chloride dioxide (ClO₂) stand out, as they have demonstrated a potential for plant disease control (Liu et al., 2013; Lee et al., 2019).

Different microbial antagonists, including some yeast species isolated from fruits, plants or soil surfaces and/or lesions, have decreased several postharvest diseases (Sharma et al., 2009). Although the yeasts isolated from different land ecosystems have shown a capacity for phytopathogen control, the search for biological control agents continues. Different yeasts isolated from the marine ecosystem have been shown to have a high potential for phytopathogen antagonism (Rivas-Garcia et al., 2019). The application of several marine yeast species has reduced disease presence in different crops (Di Francesco et al., 2016; Hernandez-Montiel et al., 2017).

On the other hand, postharvest fruit disinfection with sanitizing agents plays an important role in conservation and shelf life. Traditionally, sodium hypochlorite (NaClO) has been applied as a fruit treatment; nonetheless, different studies have shown that trihalomethanes (THMs), which have carcinogenic effects, may be generated from the use of NaClO (Villanueva et al., 2003; Villanueva et al., 2017). Among the disinfectants that do not generate harmful subproducts, ClO₂ whose residual properties are limited, seems to be an alternative to NaClO (Arango et al., 2016) due to its oxidation capacity and wide antimicrobial spectrum (Wang et al., 2014; Sang-Hyun and Dong-Hyun, 2015). ClO₂ is capable of reducing phytopathogen populations that compromise food safety because of its high efficiency against the fungi and bacteria generally found on crops, such as pepper, tomato, grape, strawberry, among others (Trinetta et al., 2013; Sun et al., 2017a). Likewise, ClO₂ is a reliable and safe disinfectant for human and animal health (Calvo et al., 2019).

The application of antagonistic microorganisms with other management strategies could be an alternative to achieve improved control of the diseases caused by fungi in fruits, minimizing the application of synthetic fungicides. Thus, the objective of this study was to determine the postharvest application efficiency of chlorine dioxide (ClO₂) and marine yeasts on Ataulfo mango fruits protection against anthracnose caused by C. gloeosporioides.

Materials and methods

C. gloeosporioides

The phytopathogenic strain used in this study belongs to the fungal culture collection of the Laboratory of Phytopathology of Centro de Investigaciones Biológicas del Noroeste (CIBNOR). This strain was previously isolated from Ataulfo mango fruits with anthracnose symptoms from a commercial orchard located in El Carrizal, Baja California Sur, México, and its pathogenicity was determined (Hernandez-Montiel et al., 2017). The phytopathogenic fungus was activated in potato dextrose agar (PDA) and incubated at 28 °C for 7 days. Conidial suspensions were prepared from those cultures, and the concentration was adjusted to $1 \times 10^5$ conidia mL⁻¹ using a hemocytometer.

Marine yeasts

The marine yeasts were obtained from the CIBNOR collection of microbial antagonists. Two
yeast strains identified as *Debaryomyces hansenii* (1R11CB) and *Rhodotorula minuta* (1R4CF) and evaluated as agents of biocontrol were used (Hernandez-Montiel *et al.*, 2017; Rivas-Garcia *et al.*, 2019). Yeast inocula were prepared from a one-day-old potato dextrose broth (PDB) culture that was in an orbital shaker at 28 °C at 130 rpm. The yeast concentration of each inoculum was adjusted to $1 \times 10^4$, $1 \times 10^6$ and $1 \times 10^8$ cells mL$^{-1}$ with a hemocytometer.

**Chlorine dioxide (ClO$_2$)**

A biocide solution of the commercial product Dioxival® (manufactured by Suministros AZ, La Paz, Baja California Sur, México) that reports a concentration of 231.66 mg L$^{-1}$ of ClO$_2$ was prepared following the manufacturer’s instructions in concentrations of 1 mg L$^{-1}$, 3 mg L$^{-1}$ and 5 mg L$^{-1}$ with sterile distilled water.

**In vitro inhibition of the mycelial growth of C. gloeosporioides by ClO$_2$**

An aliquot (100 μL) from the solution of *C. gloeosporioides* was grown in Petri dishes containing PDA. Immediately afterwards, sterilized Whatman filter paper No. 1 discs (8 mm diameter) in which 30 μL of different ClO$_2$ solutions (1 mg L$^{-1}$, 3 mg L$^{-1}$ and 5 mg L$^{-1}$) were previously absorbed were placed in the center of each Petri dish. The control was prepared in the same way, but with discs containing sterile distilled water (SDW). An additional treatment using discs inoculated with 30 μL of 2% NaClO was also included in the study. Petri dishes were incubated at 28 °C for 7 days. At the end of the experiment, *C. gloeosporioides* mycelium growth diameter was quantified (mm), and the reduction was calculated with the following equation (Soylu *et al.*, 2006): 

\[ I(\%) = \frac{DC - DT}{DC} \times 100, \]

where $I(\%)$ = mycelium growth inhibition in percentage, $DC =$ mycelium measured in the SDW treatment and $DT =$ mycelium diameter in the presence of disinfectant. Fifteen replicates were performed per treatment, and the experiment was carried out twice.

**Application of ClO$_2$ and marine yeasts for the control of mango anthracnose**

Mango fruits were washed with sterile distilled water. Three equidistant 3 mm in width wounds were established with a sterile scalpel in the equator zone of each fruit and were disinfected with 3 mg L$^{-1}$ of ClO$_2$ (limit allowed by Food and Drug Administration, 2017). The fruits disinfected with ClO$_2$ were dried for 2 h, and each wound was inoculated with 15 μL of each marine yeast suspension at three different concentrations ($1 \times 10^4$, $1 \times 10^6$ and $1 \times 10^8$ cells mL$^{-1}$) and subsequently with 20 μL of a suspension of *C. gloeosporioides* adjusted to $1 \times 10^5$ spores mL$^{-1}$. One batch of fruit was treated with a solution of ClO$_2$ (3 mg L$^{-1}$) or with NaClO (2%) and then inoculated with the phytopathogen as described above. Another batch was used as a control and was only inoculated with *C. gloeosporioides*. The
fruit was stored in sterile plastic containers at 28 °C for 7 days. Disease incidence (%) and lesion diameter (cm) were quantified. Ten replicates were performed with three mangoes per repetition, and the experiment was performed twice.

**Wound site colonization**

At the end of the experiment, tissue samples containing the whole wound on fruits were removed with a sterile scalpel and placed in falcon tubes with 5 mL of phosphate buffer and 0.06% (v/v) of Tween 20. The samples were agitated at 200 rpm for 20 min. Subsequently, serial dilutions were performed with 0.85% saline solution, and 1 mL of each dilution was inoculated in PDA medium containing 100 ppm chloramphenicol, 50 ppm ampicillin and 2 ppm fluconazole (Benbow and Sugar, 1999; Parafati et al., 2015). Petri dishes were incubated at 28 °C for 48 h. The quantified yeast population was expressed in colony forming units (CFU/per wound); ten repetitions per treatment were performed.

**Scanning electron microscopy**

Samples from wounds of mango treated with ClO₂, marine yeasts and *C. gloeosporioides* were collected and fixed by immersion in 2.5% glutaraldehyde dissolved in 0.1 M of phosphate buffer at pH 7.0 for 24 h. Afterwards, the samples were partially dehydrated by means of an ethanol gradient (30, 50, 70, 80, 95 and 100%) for 20 min. They were dried at a critical point with CO₂ and later coated with gold using a coating bath (Bozzola and Russell, 1999). The samples were examined with scanning electron microscope (Hitachi®, S-3000N).

**Statistical analysis**

Data were processed by a one-way variance analysis (ANOVA) using the statistical package STADISTICA (StatSoft, Tulsa, OK). Prior to ANOVA, percentages were arcsine–square–root transformed. For separation of means, Fisher’s least significant difference (LSD) test was used with a significance level of 5% (*P* < 0.05).

**Results and discussion**

*In vitro inhibition of C. gloeosporioides by ClO₂*

The mycelium growth of *C. gloeosporioides* in PDA medium decreased significantly as ClO₂ doses increased in the *in vitro* assay. With 3 mg L⁻¹ and 5 mg L⁻¹ ClO₂, the phytopathogenic fungus was inhibited by 68% and 77%, respectively, and NaClO induced 78% inhibition (Fig. 1). When spores of the phytopathogenic fungus were suspended in water, 38% of them germinated after 6 h, while 87% germination was achieved after 10 h. In the presence of ClO₂ at all tested concentrations (1 mg L⁻¹, 3 mg L⁻¹ and 5 mg L⁻¹), no germinated spores were observed at any time (2, 4, 6, 8 and 10 h). The results showed that *in vitro* ClO₂ reduced mycelium growth and inhibited the spore germination of *C. gloeosporioides*.

Our results align with many previous *in vitro* studies that have shown that ClO₂ decreases the growth rate and spore germination of fungi, such as *Alternaria alternata*, *Fusarium oxysporum*, *Dothiorella gregaria*, *Fusarium tricinctum*, *Phytophthora cinnamomi*, *Pythium aphanidermatum*, and *Fusarium sulphureum*, among others (Scarlett et al., 2016; Mei et al., 2017; Sun et al., 2017b). ClO₂ directly alters the cellular membrane of fungi, releasing sugar, proteins, DNA, and ions and causing irreversible damage to the fungal cellular membrane (Sun et al., 2017a; Wen et al., 2017). ClO₂ also behaves as a free radical that eliminates the hydrogen atom of the lipid molecules contained within the fungal cellular membrane (Sharma et al., 2012), increasing the damage to the membrane (Zhu et al., 2013).
Disinfection and bioprotection of Ataulfo mango fruits

The incidence of anthracnose caused by *C. gloeosporioides* in mango fruits decreased significantly as the cellular dose of each of the yeasts applied to fruit increased (Fig. 2). The treatments where the disinfectant ClO₂ was applied with a dose of $1 \times 10^6$ and $1 \times 10^8$ cells mL⁻¹ of both yeasts (*D. hansenii*, strain 1R11CB, and *R. minuta*, strain 1R4CF) did not show disease incidence. Compared with the control condition, fruit disinfection with ClO₂ or NaClO significantly decreased the presence of anthracnose.

The application of marine yeasts after treatment with ClO₂ significantly reduced the presence of anthracnose on mangoes (Fig. 3). A large number of *D. hansenii* and *R. minuta* cells were observed to adhere to the mycelium of *C. gloeosporioides*, limiting their growth and decreasing anthracnose incidence and lesion diameter in Ataulfo mango fruits.

In the trial on mango fruits, the addition of marine yeasts after treatment with ClO₂ improved phytopathogen control principally through the biocide activity of ClO₂ (Meireles *et al.*, 2017) and the diverse antagonist mechanisms exerted.
by yeasts against phytopathogens such as space
and nutrient competence, lytic enzymes, parasit-
ism, resistance induction, killer toxins, volatile
organic compounds (VOCs), and biofilms (Di
Francesco et al., 2016; Arrarte et al., 2017; Chen
Marine yeasts have shown resistance to disin-
fecting agents, such as ClO₂, due to their ability
to grow under conditions of abiotic stress (i.e.,
Nisic, oxygen limitation, and
high saline concentration, among others) (Ochoa
et al., 1995; Ramirez-Orozco et al., 2001). This
study is the first report on the efficiency of the
application of ClO₂ and marine yeasts D. hansenii
and R. minuta on Ataulfo mango fruits to control
the anthracnose caused by C. gloeosporioides.

On the other hand, ClO₂ and NaClO are widely
used to disinfect equipment, tools and surfaces
in packing plants, as well as for the postharvest
disinfection of fruits and vegetables (Shinde
et al., 2017; Calvo et al., 2019). In this study,
Ataulfo mango fruits disinfected with ClO₂ or
NaClO developed anthracnose caused by C.
gloeosporioides. Kreske et al. (2006) and Zou
and Wang (2017) reported that the greatest an-
timicrobial effect of chemical disinfectants is
at the beginning of disinfection because they
lose effectiveness due to the type of product and
chemical composition, type of pathogen present
in the host, type of cell, location of pathogens
on the surface, among others.

Colonization of mango fruit
The data describing the colonization of D.
hansenii and R. minuta in wounds on mangoes
showed that the populations of both marine
yeasts increased inside the fruit wounds (Fig. 4).
The final colonization of R. minuta was signifi-
cantly increased when inoculated initially with
1 × 10⁶ and 1 × 10⁸ cells mL⁻¹ on fruit wounds.
The results showed that both marine yeasts ef-
ciently colonized host wounds. In this study, a
positive relationship was observed between the
application dose of each of the yeasts and the
colonization of the host wounds and the antagonist
activities of D. hansenii and R. minuta against
C. gloeosporioides in mango fruit.
In our current study, a positive relationship between the concentration of the inoculum added to wound and antagonistic activity towards \textit{C. gloeosporioides} was observed for the \textit{in vivo} trials with marine yeasts. Previous studies have reported the existence of a positive relationship between high population density of antagonist microorganisms on fruit wounds and the efficiency of the biocontrol activity of microorganisms in postharvest diseases (Grzegorczyk \textit{et al}., 2017). Moreover, a larger number of cells in the wound benefits antagonists when they increase their capacity to compete for space and nutrients, produce lytic enzymes, and inhibit other cells by VOCs and biofilms (Klein and Kupper, 2018).

The application dose of any antagonist against fruit phytopathogens could play an important role in disease development; therefore, knowing the minimum dose of an antagonist to protect the host would allow for improved control, efficiency and phytopathogen management (Mahunu \textit{et al}., 2016).

**Conclusions**

Our study shows that the application of chlorine dioxide (ClO$_2$) and the marine yeasts \textit{D. hansenii} and \textit{R. minuta} on Ataulfo mango fruits enhanced the control of anthracnose caused by \textit{C. gloeosporioides}. The mycelium growth of \textit{C. gloeosporioides} was inhibited by ClO$_2$ treatment in the \textit{in vitro} assay. Treatment with ClO$_2$ and medium- and high-density inocula of marine yeast significantly reduced the disease incidence and lesion diameter on mangoes fruits. Future studies will address the application of marine yeasts on Ataulfo mango fruits for the biocontrol of \textit{C. gloeosporioides} in storage. The combination of ClO$_2$ and marine antagonists could be a viable alternative to the synthetic fungicides used for the postharvest management of anthracnose disease in Ataulfo mango fruits.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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![Figure 4. Colonization of Debaryomyces hansenii and Rhodotorula minuta yeasts on the wounds of Ataulfo mango fruits. Different letters indicate significant differences detected by ANOVA followed by the LSD Fisher post hoc test (p<0.05).](Image)
Resumen

J.J. Reyes-Perez, S. Vero, E. Diaz-Rivera, L. Lara-Capistran, J.C. Noa-Carrazana, y L.G. Hernandez-Montiel. 2019. Aplicación de dióxido de cloro (ClO₂) y levaduras marinas para el control poscosecha de la antracnosis en mango (Mangifera indica L.). Cien. Inv. Agr. 46(3): 266-275. Las enfermedades poscosecha de las frutas causan pérdidas económicas en todo el mundo. La aplicación de fungicidas sintéticos para el control de las enfermedades en poscosecha como la antracnosis en mango puede causar efectos adversos al medio ambiente, la salud humana y animal, además de generar resistencia en los fitopatógenos. El control biológico a través de levaduras marinas y la aplicación del dióxido de cloro (ClO₂) pueden ser una alternativa para el control de la antracnosis en frutos de mango cv. Ataulfo y, en la reducción de la aplicación de fungicidas sintéticos. Los resultados indican que diferentes dosis de ClO₂ inhibieron in vitro el crecimiento del micelio y la germinación de esporas de Cladosporium gloeosporioides. Cuando se aplicó ClO₂ y las levaduras marinas Debaryomyces hansenii y Rhodotorula minuta sobre frutos de mango, no se observaron signos de incidencia y lesión de antracnosis (LSD, p<0.05). Por lo tanto, la aplicación de levaduras antagonistas más ClO₂ proporciona un control eficiente sobre la antracnosis en frutos de mango cv. Ataulfo.

Palabras clave: Colletotrichum gloeosporioides, Debaryomyces hansenii, desinfectante, mango, Rhodotorula minuta.

References


