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Original article

Biological control for basal rot in yellow pitahaya fruits (*Selenicereus megalanthus*): Ex vivo trials

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ABSTRACT

Endophytic microbiome for biocontrol of plant pathogens has been the subject of recent research. *Fusarium oxysporum* (F54) and *Fusarium fujikuroi* (F17) are fungi associated with basal rot in yellow pitahaya fruits (*Selenicereus megalanthus*), which limits the possibilities of fruit commercialization.
Objective: In this study, the ability of *Lactobacillus plantarum* (endophytic bacterium), *Weissella cibaria* (non-endophytic), and their metabolites to control the basal rot of yellow pitahaya (caused by fungi F54 and F17) was evaluated.
Methods: Healthy yellow pitahaya fruit was sprayed with lactic acid bacteria or their metabolites, and one hour later, spore solutions of F54 and F17 were individually injected into the fruit peduncle. The affected area of the peduncle was measured at 15 days. The percentage of the area of infection was measured using image evaluation software. The percentage reduction of infection was also calculated.
Results: When healthy fruits were inoculated with pathogens (positive control), these developed a severe disease (more than 14 cm^2 of infection). Healthy fruits sprayed with *W. cibaria* and *L. plantarum* or their metabolites (negative control without pathogens) remained healthy for more than 15 days. Treatments without lactic acid bacteria began with symptoms of senescence on day 9. *L. plantarum* (and its metabolites) reduced the manifestations of basal rot in yellow pitahaya fruits by up to 99.99%.
Conclusions: Specific microbiome of yellow pitahaya as *L. plantarum* is an effective biological control agent against *Fusarium* associated with basal rot. *L. plantarum* and its metabolites could reduce the environmental impact caused by synthetic fungicides used to control basal rot of yellow pitahaya. Finally, the results of this research suggest that a fungal consortium may not be necessary to develop the basal rot disease.

1. Introduction

In Colombia, the yellow pitahaya (*Selenicereus megalanthus* [K. Schum. ex Vaupel] Moran) has been considered a promising exportable exotic fruit with high commercial value. Colombia was the first country to cultivate yellow pitahaya for export to Japan, and currently, Ecuador and Israel ship yellow pitahaya to Europe and the US (ProColombia, 2019). There is an ever-increasing demand for pitahaya from the US and European import markets due to this fruit has medicinal and industrial applications (Vilaplana et al., 2018). The export of this fruit has great remains. Seventeen genera and 25 species of plant pathogens can infect pitahaya fruits. The majority (21 fungal species) cause fungal diseases in the stem, fruits, and flowers. Diseases of pitahaya crop such as armillaria root, gray mold, the roya disease, Fusarium wilt, bacterial spot, necrotic, crown gall, reddish-brown spot disease, stem canker and root, stem and fruit basal rot have been reported (Mohd Hafifi et al., 2019). Fruit and stem rot, canker, anthracnose, and viral disease are the most frequently reported diseases (Balendres and Bengoa, 2019). The first demonstrated significant impact of disease in yellow pitahaya was reported in the 1990s in Colombia (Balendres and Bengoa, 2019; Caetano et al., 2011).

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The basal rot of yellow pitahaya fruits is characterized by presenting small yellow to brown patches, resulting in soft rot. This disease begins at the base of the peduncle, at more advanced stages, the affected area undergoes necrosis (Balendres and Bengoa, 2019; Salazar-González et al., 2016).

Many microorganisms have been associated with basal fruit rot of yellow pitahaya, but research has shown that different species of *Fusarium* are the main causes of this disease (Salazar-González et al., 2016). *Fusarium* species are capable of colonizing the soil and the whole plant. Warm temperatures favor their growth, and they have resistance structures that allow them to survive in the soil for up to six years (Verma et al., 2016a; Xu et al., 2020). The management of the disease is therefore complicated.

Temperature management, hot water treatment, and gamma irradiation have been reported to reduce disease incidence and severity of pitahaya fruit diseases (Balendres and Bengoa, 2019). Usually, the control of pitahaya postharvest diseases is carried out by applying synthetic fungicides imazalil or thiabendazole (Vilaplana et al., 2020). However, agrochemicals to control this pathogen has caused soil problems and resistance to these compounds. The prevention of acquired fungicide resistance in target pathogens has promoted the development of biological control agents as an effective alternative to chemical products.

The use of endophytic lactic acid bacteria (LAB) to control fungal diseases could be a helpful option partially or totally to overcome the problems caused by the use of agrochemicals (Daranas et al., 2018; Verma et al., 2016b). The effect as a biocontrol agent of LAB had been shown in the prevention of diseases in fruits such as apples, grapes, kiwi, prune, strawberries and tomatoes (Daranas et al., 2018; Gajbhiye and Kapadnis, 2016; Konappa et al., 2016). In addition, Shehata et al. (2019) demonstrated LAB's potential for the control of fungal infections in cereals. The antifungal compounds produced by LAB include organic acids, short-chain fatty acids, hydrogen peroxide, reuterin, diacetyl and bacteriocins, and bacteriocin-like inhibitory substances (Shehata et al., 2019).

Several studies on the topic, including *in vitro* studies revealing the fungistatic activity of a bacterium isolated from an animal niche (*W. cibaria*) against *F. oxysporum* and *F. fujikuroi* (Valencia-Hernández et al., 2016). Similarly, *In-vitro* assessment for the control of the same *Fusarium* species using a lactic acid bacterium isolated from yellow pitahaya (Valencia-Hernández et al., 2020). *F. oxysporum* and *F. fujikuroi* have been associated with basal rot of yellow pitahaya (Salazar-González et al., 2016). Finally, Munir et al. (2020), indicate that biological control of most pathogens mediated by native endophytes is considered one of the crucial determinants of plant health.

This research aimed to evaluate the capacity of a endophytic bacterium (*Lactobacillus plantarum*), *Weissella cibaria,* and their metabolites to control the basal rot of yellow pitahaya fruits caused by *Fusarium oxysporum* and *Fusarium fujikuroi* using Ex-vivo assessment.

2. Materials and methods

2.1. Microorganisms

Two lactic acid bacteria (*W. cibaria* IBUN-090-03684 and *L. plantarum* IBUN-090-03774) or their metabolites were used as fungistatic agents. *W. cibaria* was isolated from an animal source (Bovine Rumen) (Serna Cock and Hernández, 2010a) and *L. plantarum* was isolated from yellow pitahaya fruit crops (Valencia-Hernández et al., 2020). Two pathogenic strains, *F. oxysporum* (F54) and *F. fujikuroi* (F17), associated with basal yellow pitahaya rot were used (Salazar-González et al., 2016).

2.2. Reproduction of lactic acid bacteria and separation of their metabolites (fungistatic agents)

W. cibaria and *L. plantarum* were grown separately by six batch fermentations (37 °C, 10% inoculum in relation to the volume of the substrate, 100 rpm) according to the method reported by (Valencia-Hernández et al., 2016, 2020). Commercial substrate (MRS broth) was complemented with glucose (20 g) (SC) (De Man et al., 1960). Fermentations were maintained with orbital shaking (8 h for *W. cibaria* and 48 h for *L. plantarum*) (VWR Incubating Orbital Shaker VWR model 5000I, USA). Erlenmeyer of 1000 ml with working volumes of 500 ml was used for each fermentation. The fermentations were adjusted to pH 6.0 using 1 M NaOH. The separation of bacterial biomass and metabolites was made according to the procedure reported by Valencia-Hernández et al. (2020) with some modifications. The fermentations were divided into two volumes. The biomass was separated from its metabolites in volume one by centrifuging of fermented (4472 g, 15 min) (Eppendorf AG, Germany). The supernatants were decanted into 50 ml of Eppendorf tubes and named metabolites (ML) or (MW) depending on whether the fermentations were from *L. plantarum* or *W. cibaria*. The biomass was washed with 5 ml of 0.9% NaCl, again centrifuged (4472 g, 15 min), and subsequently, the biomass was decanted. The biomass was rewashed with 10 ml of sterile distilled water, centrifuged, and decanted. This biomass was designated as (W) or (L) depending on both strains *W. cibaria* or *L. plantarum*, respectively (Table 1). Volume two (containing a mixture of biomass and metabolites) was labeled as (W + M_W) or (L + M_L) depending on whether it came from *W. cibaria* or *L. plantarum*, respectively. See the description of the treatment in Table 1.

2.3. Culture of pathogens

F. oxysporum and *F. fujikuroi* have been cultured on potato dextrose agar (PDA) at 26 °C for 8 d until sporulation occurred. Spores were subsequently collected in test tubes, and 0.002 kg L^{-1} peptone water was added, then tubes were vigorously shaken (Magnusson et al., 2003) (Suspensions were adjusted into 10^5 spores mL^{-1} through the microscopic determination of the cell number using

Table 1
Experimental treatments to evaluate the capacity of *Lactobacillus plantarum*, *Weissella cibaria,* and their respective metabolites in the control of fungi associated with basal rot of yellow pitahaya fruits.

Treatment	Fungistatic agent	Pathogen
T1	(L + M_L)	*F. fujikuroi*
T2	(M_L)	*F. fujikuroi*
T3	(L)	*F. fujikuroi*
T4	(L + M_L)	*F. oxysporum*
T5	(M_L)	*F. oxysporum*
T6	(L)	*F. oxysporum*
T7	(W + M_W)	*F. fujikuroi*
T8	(M_W)	*F. fujikuroi*
T9	(W)	*F. fujikuroi*
T10	(W + M_W)	*F. oxysporum*
T11	(M_W)	*F. oxysporum*
T12	(W)	*F. oxysporum*
C1	Without fungistatic	Without fungus
C2	Without fungistatic	*F. fujikuroi*
C3	Without fungistatic	*F. oxysporum*
C4	L + M_L	Without fungus
C5	M_L	Without fungus
C6	L	Without fungus
C7	W + M_W	Without fungus
C8	M_W	Without fungus
C9	W	Without fungus

L. plantarum (L), *W. cibaria* (W), metabolites of *L. plantarum* (M_L), metabolites of *W. cibaria* (M_W). C1–C9 positive and negative controls.

a Neubauer Chamber (Brand, Germany). These pathogen suspensions were referred to as (F54) and (F17).

2.4. Applications of fungistatic agent and infection of yellow pitahaya fruits

Thirty-three kilograms of yellow pitahaya fruit (*Selenicereus megalanthus* [K. Schum. ex Vaupel] Moran) were harvested in crops of the farm "Las Camelias," located in the municipality the Trujillo (Valle del Cauca, Colombia). The place is 1630 masl. The fruits were collected healthy, with an average weight of 230 g per fruit and in ripening status three according to Colombian technical standard NTC 3554 (ICONTEC, 1996). The fruits were disinfected by immersion in a chlorine solution (5%v/v); subsequently, the fruits were immersed in alcohol (70% v/v) for 5 min. Finally, the fruits were washed with distilled water (Crowley et al., 2013a). The fruits were treated with antifungals the same day they were harvested.

The applications of fungistatic agents and pathogens were made using modifications to the methods proposed by Hernández-Rodríguez et al. (2008). Initially, each healthy fruit was sprayed with 50 μL^{-1} of each fungistatic agent described in Table 1 using a plastic manual spray pump. One hour after the use of fungistatic agents, 50 µL of (F54) and (F17) were individually injected into the fruit peduncle (Dimkić et al., 2013). Fruits were stored in an environmental chamber (DIES, Colombia) with 24 °C and 65% relative humidity.

2.5. Infection measurement

The fruit's infection area (cm^2) was measured at 15 days (after inoculation with F54 and F17). Measurement of the area of infection (basal fruit rot) was calculated using image assessment software (ImagenJ software) (Rasband, 1997). The percentage of infection reduction (*% R*) was calculated using Eq. (1).

$$\%R = (CHC - CH)/CHC \times 100 \quad (1)$$

where CHC is the average area (m^2) of the tissue affected by basal rot in control treatments (C2 and C3), while CH is the average area (m^2) of the tissue affected by basal rot in each of the treatments.

The appearance of visible orange color on the fruits' surface was associated with the beginning of the browning of the fruit. In addition, a subjective scale assessed the loss of firmness of the fruits, classifying as firm those fruits that did not present deformation before the pressure on the surface of the fruit with the thumb and index fingers and cataloging as loss of firmness those fruits that showed some deformation before the same subjective test.

2.6. Experimental design

A randomized 7*3 factorial design with three replicates was used. The results were analyzed using Statistical Analysis System (SAS). Treatment means were compared using the Tukey test with a $p < 0.05$ probability.

3. Results

Fruits corresponding to treatment C1 (No fungistatic and no pathogens) did not develop basal rot as expected and showed the first symptoms of senescence (browning and loss of firmness) as of day 9 (Fig. 1). When F54 and F17 were embedded in healthy fruits (Fig. 1: C2 and C3), severe basal rot disease developed, and more than 40% of the tissue at the base of the fruit was affected, and soft, depressed watery rot was found in the base segment. The fungus was seen in the peduncle and bracts on day four after inoculation of F54 and F17, and browning lesions appeared. On day 6, the lesions became dark brown. After another six days, rot appeared with browning and softening the basal fruit. In addition, the fruits were necrotic and brown, and the firmness was lost.

Applying lactic acid bacteria or their metabolites in healthy fruits (C4 to C9) caused the fruit to remain fresh, healthy, firm, with yellow bracts and peduncles in good condition. Fruits corresponding to C4 to C9 began senescence from day 18 (24 °C and 65 % RH). This time was considerably longer than the time required for that without treatment fruit to start the senescence process. According to Estrella et al. (2005), the shelf life of yellow pitahaya without refrigeration (24 °C and 85% RH) at physiological maturity can be summarized as follows: after six days, it develops sensory maturity, presenting optimum sensory color, texture, taste, and aroma. The fruits had browning reactions after the sixth day. The senescence stage begins from day 9, when enzymes such as polyphenol oxidases and peroxidases act on phenols that cause condensation and polymerization and result in fruit browning. The shelf life of the yellow pitahaya is estimated to be 15 days on average.

ANOVA has shown a statistically significant effect on the interaction between fungistatic agents and pathogens. Comparisons between the means showed a difference betweenC2 and C3 (except for T12). The percentage reduction in disease showed statistically significant differences between treatments (Table 2). The mean comparison showed that the fungistatic activity of *W. cibaria* and its metabolites against *F. oxysporum* and *F. fujikuroi* was different, while *L. plantarum* and its metabolites had a similar fungistatic effect on both pathogens.

L. plantarum and its metabolites showed greater fungistatic activity against *F. fujikuroi*, reducing the infection by up to 99% in yellow pitahaya fruits infected with this fungus. At the same time, *W. cibaria* and its metabolites reduced the *F. fijikoroi* infection by up to 95%. *L. plantarum* and its metabolites reduced *F. oxysporum* infection by 86%, while *W. cibaria* metabolites reduced only 57% of the infection in yellow pitahaya fruits infected with this fungus.

T1, T2, and T3 treatments remained healthy until the 9th day. On the 10th day, the disease began in a small area of the fruit basal, with the appearance of a reddish color at the tips of the bracts. In T7, T8, and T9, basal rot symptoms developed in the peduncle and bracts; however, this condition was less than C2 (Control). The fruits of T12 lost firmness in the bracts, and the area of treatment for tissue injury was of the same magnitude as C3 (control) treatment (Fig. 1).

4. Discussion

In this investigation, statistical differences were found in the area of infection, and the percent reduction of basal rot of yellow pitahaya fruits treated with two lactic acid bacteria (*L. plantarum* and *W. cibaria*. These fruits were infected with the pathogens *F. oxysporum* and *F. fujikuroi*. The difference in the fungistatic activity between *L plantarum* y *W. cibaria* can be explained by their isolation source. *W. cibaria* was isolated from the bovine rumen (Serna Cock and Hernández, 2010b), whereas *L. plantarum* was a bacterium-specific niche directly derived from healthy yellow pitahaya crops. *L. plantarum,* as endophytic bacteria of yellow pitahaya, can trigger an increase in the metabolic response against plant pathogens. Authors suggest that endophytic bacteria, can increase the production of amino acids such as lysine and tyrosine that are associated with the antimicrobial response of plant tissue (Munir et al., 2020). However, the specific metabolic response of pitahaya fruit needs to be investigated. Also, two microorganisms belonging to the same environmental niche can cause competition for nutrients and space. In addition, the difference can be explained by the Quorum Sensing (*QS*) Mechanism. Bacteria use this system to communicate through autoinducers, inducing a resistance response of

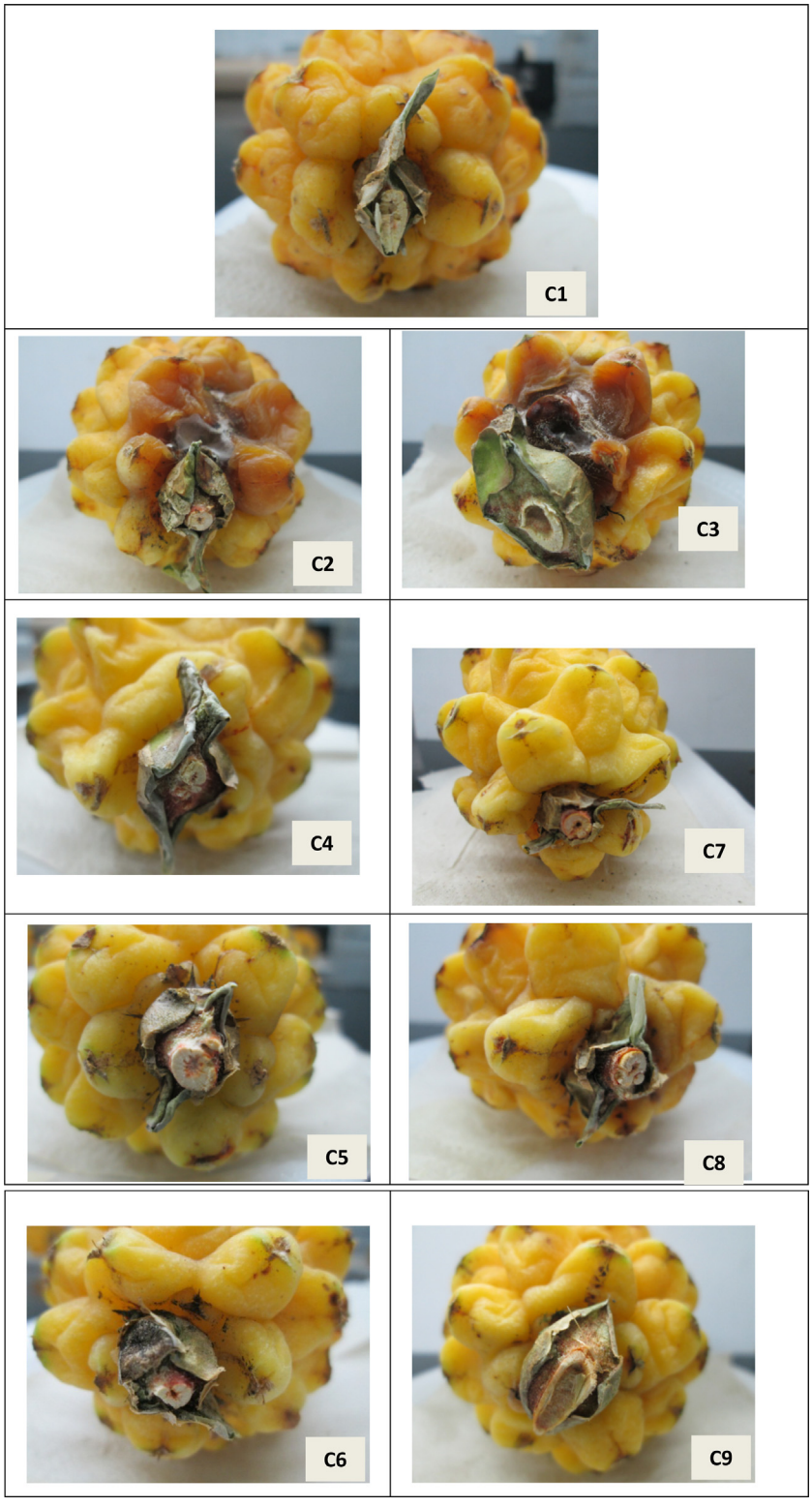

Fig. 1. Photographs of yellow pitahaya fruits (*Selenicereus megalanthus* [K. Schum. ex Vaupel] Moran) on the 15th day of storage (24 °C, 65% RH). C1: negative control (without fungistatic, without fungus). C2: positive control (without a fungistatic agent, with *F. fujikuroi)*. C3: positive controls (without a fungistatic agent, with *F. oxysporum*). C4: with a fungistatic agent (*L. plantarum* + metabolites). C5: with a fungistatic agent (metabolites of *L. plantarum*), C6: with fungistatic agent (*L. plantarum*). C7: with a fungistatic agent (*W. cibaria* + metabolites). C8: with a fungistatic agent (Metabolites of *W. cibaria*). C9: with a fungistatic agent (*W. cibaria*).

Table 2
Infection area (cm^2) and percent reduction of basal rot (%R) of yellow pitahaya fruits treated with *L. plantarum, W. cibaria,* and its metabolites and inoculated in the peduncle with *F. oxysporum* and *F. fujikuroi*. See treatment nomenclature in Table 1.

Treatments	Infection Area Average (cm^2)	Treatments	Infection Reduction %R
C2	14,98 ± 0,92^{a}	T2	99,99 ± 0a
C3	14,33 ± 2,04^{a}	T1	99,99 ± 0a
T12	13,38 ± 2,14^{a}	T3	98,47 ± 0,23^{ab}
T10	8,64 ± 1,16^{b}	T9	95,22 ± 0,51^{abc}
T11	6,05 ± 0,61^{c}	T7	94,71 ± 3,14^{abc}
T5	5,93 ± 0,11^{c}	T4	86,37 ± 0,51^{abc}
T6	2,47 ± 0,40^{d}	T8	84,14 ± 0,39^{bc}
T8	2,38 ± 0,06^{d}	T6	82,79 ± 2,77^{c}
T4	1,95 ± 0,07^{de}	T5	58,64 ± 0,77^{d}
T7	0,79 ± 0,47^{de}	T11	57,80 ± 4,25^{d}
T9	0,72 ± 0,08^{de}	T10	39,68 ± 8,06^{e}
T3	0,23 ± 0,04^{de}	T12	6,61 ± 14,94^{f}
T1	0,001 ± 0^{e}		
T2	0,001 ± 0^{e}		
C1	0 ± 0^{e}		
C5	0 ± 0^{e}		
C6	0 ± 0^{e}		
C4	0 ± 0^{e}		
C7	0 ± 0^{e}		
C8	0 ± 0^{e}		
C9	0 ± 0^{e}		

Different letters for each column indicate a significant difference ($p \leq 0.05$).

the fruit to infection by Fusarium species (Papenfort and Bassler, 2016). Although *L. plantarum* and *W. cibaria* use *QS*, their antifungal activity is different because they use signals of different chemical nature.

The use of *L. plantarum* or its metabolites as fungistatic biocontrol agents significantly reduced the manifestations of basal rot in yellow pitahaya. *Therefore, L. plantarum and its metabolites can* be used as effective biocontrol measures against *F. oxysporum* and *F. fujikuroi*. This lactic acid bacterium could be used as a biocontrol agent to manage fungal plant diseases.

Identifying metabolites responsible for the fungistatic activity was not included in this investigation. However, this fungistatic activity of lactic acid bacteria can be explained by the fact that lactic acid bacteria produce organic acids such as lactic acid and acetic acid as well as low molecular weight compounds (Niku-Paavola et al., 1999), peptides (Okkers et al., 1999) and proteins (Magnusson and Schnürer, 2001). Mauch et al. (2010) suggest that organic acids are involved in antifungal activity against *Fusarium* species at low pH levels. Ghanbari et al. (2013) argued that acetic acid restricts the growth of bacteria and fungi and produces synergistic effects with lactic acid. Crowley et al. (2013a) suggested that lactic acid bacteria restrict the growth of pathogenic bacteria and fungi caused by the production of weak organic acids such as lactic acid, acetic acid, and propionic acid are final products of carbohydrate metabolism in acidic media. Gajbhiye and Kapadnis (2016) reported that the antifungal effect of lactic acid bacteria is attributed to a mixture of more than one compound, including organic and fatty acids and proteinaceous compounds cyclic dipeptides, phenolic compounds, and H_2O_2. Fungistatic metabolites penetrate the hydrophobic membranes of pathogens and reduce cell pH. It is possible that the spraying of these metabolic compounds on yellow pitahaya fruits acts as a layer that protects the fruits against enzymatic, microbiological, and physical deterioration, and this would explain the longer shelf life of the fruit corresponding to C4 to C9 treatments. Organic acids, such as lactic acids and acetic acids obtained from *W. cibaria* would be the compounds responsible for antifungal activity (Baek et al., 2012). *L. plantarum* with *in vitro* inhibitory potential against pathogenic fungi was reported by several authors (Arasu et al., 2014; Sangmanee and Hongpattarakere, 2014; Ström et al., 2002). However, there are very few reports on antifungal LAB as a biocontrol agent in rot fruit. Lan et al. (2012) found that the application of *W. cibaria* could effectively inhibit the growth of *P. oxalicum* for six days on grape surfaces. *Ped. pentosaceus* has been used to delay the rotting caused by *P. expansum* in pear fruits (Crowley et al., 2013a,b). Daranas et al. (2018) reported the used of LAB to control Pseudomonas in kiwi fruits, *Xanthomonas arboricola* pv. pruni in prunes and *Xanthomonas fragariae* in strawberries. Konappa et al. (2016) reported the treatment of tomato seeds with *Ralstonia solanacearum* to promote the production of defense-related enzymes and thus impart resistance to tomato crops.

Finally, the literature reports that basal rot disease in yellow pitahaya is caused by a fungal complex that includes several species of *Fusarium* (Caetano et al., 2011; Salazar-González et al., 2016). However, in the present investigation, when individually inoculating *F. oxysorum* and *F. fujikuroi* (isolated strains of yellow pitahaya fruits affected with basal rot obtained from previous research by Salazar-González et al. (2016) in Peduncles of healthy yellow pitahaya fruits; we observed that the fruits developed the disease. Therefore, this research confirms that a fungal consortium may not be necessary to develop the basal rot disease.

5. Conclusions

In this research, through *ex-Vivo* tests, a specific niche lactic acid bacterium's potential for controlling pathogens associated with basal rot of yellow pitahaya fruits was demonstrated. *L. plantarum* (an endophytic bacterium isolated from healthy yellow pitahaya fruits) and its metabolites efficiently reduce up to 99.9% of the basal rot of yellow pitahaya fruits caused by *F. fujikuroi* and *F. oxysporum*. In addition, the microbial compounds delay both the appearance of browning and the softening of these fruits. *W. cibaria* and its metabolites are not recommended to develop antifungal agents because their fungistatic action is limited. The results suggest that a fungal consortium may not be necessary to develop the basal rot disease.

L. plantarum and its metabolites can be used as a biological control agent in yellow pitahaya crops, reducing the environmental impact caused by agrochemicals. However, new research is necessary to elucidate the mechanism of biological control.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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