Postharvest control of *Rhizopus stolonifer* on blackberry (*Rubus fruticosus*) by blackberry native crop bacteria

Control poscosecha de *Rhizopus stolonifer* en zarzamora (*Rubus fruticosus*) por bacterias nativas de zarzamora

Ismael F. Chavez-Diaz [1], Hortencia G. Mena-Violante [1], Ana N. Hernandez-Lauzardo [2], Guadalupe Oyoque-Salcedo [1], Ernesto Oregel-Zamudio [1], M. Valentina Angoa-Perez [1]

Originales: *Recepción:* 14/03/2018 - *Aceptación:* 25/10/2018

Abstract

The potential of four native bacterial strains of blackberries cv. Brazos (*Rubus fruticosus*): *Bacillus subtilis (BSS)*, *Bacillus subtilis (BSL), Bacillus licheniformis (BLI)* and *Leifsonia aquatica (LAQ)*, was evaluated for the postharvest control of soft rot caused by *Rhizopus stolonifer* in blackberry fruits. The fruits were treated with cell suspensions (CS) and cell-free supernatants (CFE) from each bacterial strain and were infected with two strains of *R. stolonifer (RSA and RSC)*. The severity and inhibition percentage of the disease were determined. Additionally, the inhibition by siderophores and the inhibition percentage of *R. stolonifer* spore germination were analyzed as possible control mechanisms. The CS of BSS inhibited RSA by 45.8%, followed by CFE of LAQ which controlled the same strain by 39.7%. The CS of BLI inhibited RSC by 37.7%, whereas the CFE of BSS and LAQ controlled it by 47.7 and 41.8%, respectively. All bacterial strains inhibited RSA and RSC by siderophores production (38.7 to 48.6 %) and the inhibition of spore germination of RSC was higher than 93% after 48 h. This work is one of the first to report *R. stolonifer* control by native bacteria CS and CFE, particularly LAQ in postharvested blackberry fruits. These results show the combination of mechanisms used by bacteria to control both *R. stolonifer* strains.

Keywords
Postharvest berries • Biocontrol • Cell-free Extract • *Leifsonia* • *Bacillus* • *Rhizopus stolonifer* • Soft rot

1 Instituto Politécnico Nacional. CIIDIR IPN Unidad Michoacán. Justo Sierra 28. Centro. C.P. 59510. Jiquilpan. Michoacán. México. vangoa@ipn.mx
2 Instituto Politécnico Nacional. CEPROBI. Departamento de Interacciones Planta-Insecto Carretera Yautepec-Jojutla. Km.6. Calle CEPROBI No. 8. Col. San Isidro. C.P. 62731. Apartado Postal 24. Yautepec. Morelos. Mexico.

Resumen

Fue evaluado el potencial de cuatro cepas bacterianas nativas de zarzamora var. Brazos (*Rubus fruticosus*): *Bacillus subtilis* (BSS), *Bacillus subtilis* (BSL), *Bacillus licheniformis* (BLI) y *Leifsonia aquatica* (LAQ), para el control en poscosecha de la podredumbre blanda causada por *Rhizopus stolonifer* en frutos de zarzamora. Los frutos fueron tratados con suspensiones celulares (CS) y extractos libres de células (CFE) de cada cepa bacteriana y fueron infectados con dos cepas de *R. stolonifer* (RSA y RSC). Se determinó el porcentaje de severidad e inhibición de la enfermedad. Además, fueron analizados como posibles mecanismos de control, la inhibición por sideróforos y el porcentaje de inhibición de la germinación de esporas de *R. stolonifer*. La CS de BSS inhibió un 45,8% a RSA, seguido por CFE de LAQ que controló la misma cepa en un 39,7%. El CS de BLI inhibió a RSC un 37,7%, mientras que el CFE de BSS y LAQ lo controlaron un 47,7 y 41,8%, respectivamente. Todas las cepas bacterianas inhibieron RSA y RSC por producción de sideróforos (38,7 a 48,6%) y la inhibición de la germinación de RSC por esporas fue mayor que 93% después de 48 h. Este trabajo es uno de los primeros en informar el control de *R. stolonifer* por CS y CFE de bacterias nativas, particularmente LAQ en poscosecha de frutos de zarzamora. Estos resultados muestran la combinación de mecanismos utilizados por las bacterias para controlar ambas cepas de *R. stolonifer*.

Palabras clave

poscosecha de frutillas • Biocontrol • Extracto libre de células • *Leifsonia, Bacillus* • *Rhizopus stolonifer* • Podredumbre blanda

Introduction

Global consumers' growing interest in including nutraceutical compounds in their diet has caused an increase in the market of fresh products rich in these compounds, such as beetroot and berries (1, 5); among them, blackberries, whose phytochemical and antioxidant content, in addition to their sweet flavor and smell, have contributed to make them a well-renowned and widely consumed product (12, 29). One of the most frequent production problems is the high susceptibility to mechanical damage during postharvest manipulation, which in turn contributes to the development of fungal diseases (8), such as soft rot, caused by *Rhizopus stolonifer* (Ehrenb.:Fr.) Vuill, thereby causing great economic losses in this crop. *R. stolonifer* control has been primarily performed through the application of synthetic fungicides (4, 16, 34). However, various collateral effects, such as the development of fungal resistance, environmental and human health damage, have been attributed to those products (2, 11). Postharvest disease control exacerbates the problem as producers face the dilemma of offering damage-free products with good external quality and innocuous at the same time. Biological control with the use of antagonistic microorganisms has showed positive results in the control of different fruit pathogens, and it is considered a viable alternative to chemical control since it is also environmentally friendly and low-cost (3, 6, 13, 22, 31, 36). An example of the above are the works of Arrebola, *et al.* (2010) and Govender *et al.* (2005), where *Bacillus* species strains were used to control postharvest diseases in citrus fruits and mango, respectively.

Furthermore, it has been posed that the use of cell-free supernatants, produced by phytopathogen antagonistic bacteria, is less risky than the use of bacterial cells; in this context, some works have reported that cell-free supernatants of *Pseudomonas* and *Bacillus* reduced the occurrence of phytopathogenic fungi in apple and tangerine fruits (17, 19, 21). For the control of *Rhizopus stolonifer,* it has been proposed to apply yeast (35) and bacteria (7, 34) in tangerines, peaches, apricots and grapes in postharvest; however, this is, as far as you know, the first work exploring its control in blackberry fruits. Due to the above, the objective of this work was the evaluation of the *Rubus fruticosus* native bacteria's potential, as well as its cell-free supernatants on the control of soft rot in blackberry fruits (*Rubus fruticosus*). Moreover, tests were conducted to explore possible mechanisms to control *Rhizopus stolonifer* used by the bacterial strains analyzed.

Materials and methods

Biological material

Fungal strains

Two strains of *Rhizopus stolonifer*, RSA and RSC, were used, which were selected because they presented different degrees of pathogenicity. Both were isolated from Brazos variety blackberries collected in Los Reyes, Michoacán, Mexico. The fruits were placed in a humid chamber and incubated in a growing room at 25°C during 5 d until the characteristic mycelium appeared; then, 5-mm-diameter sections were taken from the fruits covered with mycelium, and they were placed in Petri dishes with Potato Dextrose Agar (PDA) medium and were incubated at 25°C. Once the mycelium sporulated, serial dilutions were performed to obtain individual spores, which were placed in tubes with Potato Dextrose Broth (PDB) to obtain pure cultures (16). The isolates were identified by observation in an inverted microscope at 40X (1X71S8F-3, Olympus America Inc., Center Valley, PA, USA) and a stereoscope at 10X (SMZ-1500, Nikon Incorporated, Melville, NY, USA) using the keys reported by Schipper (1984). The strains identified were inoculated again in Petri dishes with PDA and incubated at 25°C during 5 d, at the end of which they were used to follow the Koch's postulates to confirm their pathogenicity. In both strains of *R. stolonifer*, blackberry fruits with export quality, uniform size, shape, weight and color, and free of physical damage, were used. To disinfect them, they were submerged in a sodium hypochlorite solution at 1.5% (vv^{-1}) during 5 min and were rinsed with sterile distilled water. The fruits were placed in humid chambers and inoculated with 50 µL of a spore suspension of the corresponding strain of *R. stolonifer* ($1x10^5$ spores mL^{-1} grown in PDB) and incubated in a digital incubator (M-815, Thermo Electron Corp., Marietta, OH, USA) at 25°C during 72 h. Fifteen fruits were evaluated per treatment with three replicates each.

Bacterial strains

Four bacterial strains isolated from a commercial crop of Brazos variety blackberries, located in Los Reyes, Michoacán, Mexico, were used. The antagonist activity against *R. stolonifer* was verified in a previous study (10): *Bacillus subtilis* isolated from soil (BSS), *Bacillus subtilis* (BSL), *Bacillus licheniformis* (BLI) and *Leifsonia aquatica* (LAQ) isolated from leaves, which were identified at a molecular level through the extraction of chromosome DNA in accordance with Harwood and Cutting (1990), amplification of 16S rDNA region, with the universal oligos: 27F TACGGYTACCTTGTTACGACTT and 2RAGAGTTTGATCMTGGCTCAG; the

amplified products were identified by the company Macrogen Corp. USA.

The comparison of the amplified products from 16S rDNA with the database (BLAST, nr/nt) revealed that *B. subtilis S* (BSS) (1403 pb), *B. subtilis* L (BSL) (1401pb), *B. licheniformis* (BLI) (1402pb) and *L. aquatica* (LAQ) (1393pb) corresponded to the species previously identified with the biochemical tests. The strains were preserved in Petri dishes with nutrient agar (Bioxon and Becton Dickinson, Mexico City, Mexico) at 4°C and were subcultured in the same growth medium 24 h before their use in the bioassays.

Antagonism by reduced siderophore production

The siderophore production by the bacterial strains was confirmed by growth in PDA medium with chrome azurol. To relate the production of siderophores to the inhibition of growth of both *R. stolonifer* (RSA and RSC), an antagonism test in iron-rich medium was performed under the supposition that, upon this element's high bioavailability, the microorganisms would not be forced to compete for it. PDA added with 100 mg of $FeCl_3$ (Baker Analyzed Reagent, J. T. Baker, Phillipsburg, NJ, USA) per liter of culture media (Rachid and Ahmed, 2005) was used. At four equidistant points of a 98 mm diameter Petri dish, the bacterial strains were inoculated by means of a roast, spreading them in an area of 1 cm in diameter at each point. Agar discs with mycelium were placed with 5-mm-diameter of each strain of the pathogen (RSA or RSC) in the center of the Petri dishes and were incubated in a digital incubator (M-815, Thermo Electron Corp., Marietta, OH, USA) at 28°C during 72 h; the growth diameter of the discs with mycelium was measured with a digital Vernier every 24 h. As control, iron-free PDA plates were used inoculating the pathogen as described above. The test ended when the mycelium fully covered the dishes of the control treatment. The formula used to calculate the antifungal index was Antifungal Index (%) = $(1 - Da\ Db^{-1})\times100$; where: Da is the diameter of the growth zone of the disc (cm) in the specimen dish, and Db is the diameter of the growth zone of the disc (cm) in the control dish (32). Three plates were used as an experimental unit and each treatment had 3 replicates.

Inhibition test of *R. stolonifer* spore germination

This test was performed in accordance with Bryk *et al.* (1998) with modifications. Bacterial suspensions of all the strains were prepared by inoculations thereof in 50 ml of PDB, they were incubated at 25°C during 24 h under constant stirring and adjusted to a $1X10^6$ cfu mL^{-1} density. Suspensions of RSA and RSC fungal strains were prepared by inoculations in PDB at 25 °C for 72 h, to obtain a final concentration of $1x10^5$ spores mL^{-1}. The suspensions of each bacterial strain (BSS, BSL, BLI and LAQ) were mixed with the RSA or RSC spore suspension in test tubes in a 1:2 proportion (v:v bacteria: fungi, respectively). The tubes were incubated at 25°C for 12, 24 and 48 h. As control, the spore suspension of the corresponding strain mixed with PDB in the same proportion as the remaining treatments was used. After each incubation time, an aliquot of each treatment was taken to be observed under the microscope. Three replicates per treatment were used. Ten spores in 10 fields were examined in each sampling time with a compound microscope at 40X (Axiolab-450907, Carl Zeiss Inc., Thornwood, NY, USA).

The amount of germinated and non-germinated spores was recorded and expressed as germination percentages of *R. stolonifer* present in the different strains.

Antagonism test (*in vivo*)

Suspensions of each bacterial strain were prepared in 150 ml of nutrient broth (Baker Analyzed Reagent, J. T. Baker, Phillipsburg, NJ, USA) incubating at 25°C for 24 h under constant stirring. Cell-free supernatants (CFE) were prepared to know the strains' capacity to produce water-soluble extracellular compounds with antifungal activity. These were obtained through CS centrifugation (RMC-2, Wheaton Science Products, Millville, NJ, USA) at 10 000 rpm per 10 min, the supernatant was filtered through a 0.22 µm nylon membrane (Millipore Corporation, Bedford, MA, USA).

Brazos variety blackberry fruits with export quality, uniform size, shape, weight and color, and free of physical damage, were used, which were disinfected as previously described and submerged in the corresponding bacterial suspension (CS) or cell-free extract (CFE) during 2 h, sprinkled with 10 ml of a spore suspension ($1x10^5$ spores mL^{-1}) of RSA or RSC strains, placed in humid chambers and incubated at 25 °C during 72 h. No damage was inflicted to the fruits prior to inoculation of the *R. stolonifer* strains. Fifteen fruits per treatment were evaluated with three replicates each. The treatments analyzed were: Fruits inoculated with the RSA or RSC strain (control with pathogen); fruits inoculated with PDB only (control without pathogen); fruits inoculated with cell suspensions (CS) of the 4 respective bacteria compared with the RSA or RSC strain; fruits inoculated with cell-free supernatants (CFE) of the 4 respective bacteria compared with RSA or RSC.

The severity of the infection was determined every 24 h during 5 d using the scale previously described in the pathogenicity test. The severity index was calculated with the formula reported by Pérez, *et al.* (1995) and the pathogen inhibition percentage with the same formula, but expressing the severity levels as percentages.

Statistical analysis

All of the experiments were performed with a completely random design. The fungal inhibition percentage data were transformed with the Arcsine of the square root. The data were processed by means of an analysis of variance and a Tukey means comparison using SAS® System for Windows 9.0 version (SAS Institute, Cary, NC, USA). The differences between the means were considered significant with a $p \leq 0.05$.

Results

***R. stolonifer* strains pathogenicity**

The identity of the *R. stolonifer* (RSA and RSC) strains obtained from the blackberry plants was confirmed by the Schipper (1984) keys. The fruits inoculated with both strains developed mycelium after 24 h, covered the fruit and generated exudates, the infection and softening appeared after 48 h, and after 72 h the fruits were degraded and with signs of fermentation. The infection percentage was 90% for RSA and 100% for RSC.

Antagonism by reduced siderophore production

The results showed that LAQ had a controlling effect on the RSA strain significantly higher than the remaining treatments for it presented an antifungal index

0.8 times higher than BSS and 0.7 times higher than BSL; followed by the BLI strain, which exceeded 0.9 and 0.8 times the inhibitory power of BSS and BSH, respectively. The RSC strain was controlled by BLI, which showed the higher antifungal index exceeding 0.8 times BSS and 0.85 times BSH, followed by LAQ whose antifungal index exceeded 0.9 and 0.8 times that presented by BSS and BSH, respectively (table 1). It is worth mentioning that BLI and LAQ, grown in an enriched medium, presented inhibition percentages on the growth of both *R. stolonifer* strains evaluated higher than those obtained in medium without $FeCl_3$.

Inhibition test of *R. stolonifer* spore germination

The results obtained showed that BSS and BLI allowed the germination of 6.10 and 18.7% of the RSC spores after 48 h, whereas, in the control, there was observed the germination of 90% of the spores after the same time (table 2, page 312).

In BSL and LAQ treatments, the amount of RSC spores reduced by 96% and 91% respectively after 12 h; this was due to their lysis, since fragments thereof were observed in all of the fields analyzed. In that time, no spore germination was found in said treatments; however, the remaining spores germinated after 48 h (3.5 and 5.2%, respectively) (table 2, page 312). Furthermore, the treatments applied had no effect on the germination of RSA spores, since they reached similar germination percentages to those of control (87-90%) after 48 h (Data not shown).

Antagonism test (*in vivo*)

The four native bacterial strains of blackberry plants showed positive effects to prevent soft rot caused by the RSA and RSC fungal strains.

Table 1. Antagonism of native bacterial strains of blackberry (*Rubus fruticosus* cv. Brazos) against R. *stolonifer* in medium enriched in $FeCl_3$.

Tabla 1. Antagonismo de cepas bacterianas nativas de zarzamora (*Rubus fruticosus* var. Brazos) contra *R. stolonifer* en medio enriquecido en $FeCl_3$.

Antagonist Agent	Antifungal Index (%)			
	With $FeCl_3$		Without $FeCl_3$	
	RSA	RSC	RSA	RSC
BLI	46.1±0.5^{c}	48.3±0.7^{b}	41.4±2.3^{d}	42.7±2.4^{d}
BSS	42.7±0.6^{d}	43.1±0.4^{d}	40.0±1.3^{d}	41.6±2.1^{d}
LAQ	48.6±0.3^{b}	47.0±0.3^{c}	36.4±2.0^{e}	41.9±2.9^{d}
BSL	38.7±0.5^{e}	41.2±1.2^{e}	37.6±1.1^{e}	36.2±1.1^{e}
Control (-)	0.00±0.0^{f}	0.00±0.0^{f}	0.00±0.0^{f}	0.00±0.0^{f}
Control (+)	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}

Means ± standard deviation are presented. The different superscript letters in the same column indicate significant differences according to the Tukey test ($p \leq 0.05$, n=3). RSA: *Rhizopus stolonifer* A strain; RSC: *Rhizopus stolonifer* C strain. BLI: *Bacillus licheniformis*; BSS*: Bacillus subtilis S*; LAQ: *Leifsonia aquatica*; *BSL: Bacillus subtilis* L; Control (-): PDB medium; Control (+) *Rhizopus stolonifer* A or C strains, respectively.

Se presentan las medias ± desviación estándar. Letras superíndices diferentes en la misma columna indican diferencias significativas de acuerdo a la prueba de Tukey ($p \leq 0,05$, n=3). RSA: *Rhizopus stolonifer* cepa A; RSC: *Rhizopus stolonifera cepa* C. BLI: *Bacillus licheniformis*; BSS*: Bacillus subtilis S*; LAQ: *Leifsonia aquatica*; *BSL: Bacillus subtilis* L; Control (-): medio PDB; Control (+) *Rhizopus stolonifer* cepas A ó C respectivamente.

Table 2. Inhibition of germination of spores of *R. stolonifer* (RSC) by the native bacterial strains of Blackberry (*Rubus fruticosus* cv. Brazos).

Tabla 2. Inhibición de la germinación de esporas de *R. stolonifer* (RSC) por cepas bacterianas nativas de Zarzamora (*Rubus fruticosus* var. Brazos)

Treatments	Spore germination percentage of RSC (%)		
	12 h	24 h	48 h
BLI+RSC	3.8±0.7[b]	4.7±0.8[c]	6.1±0.1[c]
BSS+RSC	9.2±0.5[b]	15.8±0.3[b]	18.7±0.4[b]
LAQ+RSC	0.0±0.0[c]	1.8±0.5[d]	5.2±0.7[d]
BSL+RSC	0.0±0.0[c]	1.1±0.7[d]	3.5±0.2[d]
RSC	81.3±6.7[a]	89.0±2.6[a]	89.9±1.1[a]

Means ± standard deviation are presented. The different superscript letters in the same column indicate significant differences according to the Tukey test ($p \leq 0.05$, n=3). RSC: *Rhizopus stolonifer* strain C; BLI: *Bacillus licheniformis*, BSS*: Bacillus subtilis S*, LAQ: *Leifsonia aquatica, BSL: Bacillus subtilis* L.

Se presentan las medias ± desviación estándar. Letras superíndices diferentes en la misma columna indican diferencias significativas de acuerdo a la prueba de Tukey ($p \leq 0,05$, n=3). RSC: *Rhizopus stolonifer* cepa C; BLI: *Bacillus licheniformis*, BSS*: Bacillus subtilis S*, LAQ: *Leifsonia aquatica, BSL: Bacillus subtilis* L.

The fruits treated with CS of BSS showed a significant inhibition (45.8%) on the growth of the RSA strain, which was 1.6, 1.6 and 2.5 times higher than that presented by BLI, LAQ and BSL treatments, respectively. This severity level was 1.8 times lower than that showed by the RSA control (4.25). Furthermore, the treatment with CFE of LAQ inhibited 39.7% the production of mycelium, which was 2.1, 1.9 and 1.7 higher than the inhibition percentage presented by CFE of BLI, BSS and BSL respectively; showing a severity level of 2.5, which was 1.7 times lower than that presented by the RSA control ($p \leq 0.05$). The fruits of the treatment with CFE of BSS showed an inhibition on the growth of RSC strain of 47.7%, with a severity level of 2.5, which was 1.8 and 1.6 times higher than that of CFE treatments of BLI and BSL, respectively, and 1.7 times lower than that presented by the RSC control. Similarly, the treatment with CFE of LAQ permitted the inhibition of fungal growth by 48.8%, which was 6 and 1.4 times higher than that of CFE treatments of BLI and BSL, with a severity level of 2.8, which was 1.5 lower than that of the RSC control (table 3, page 313).

Discussion

In the last years, postharvest pathogen biocontrol strategies have been developed for a wide range of fruits, including compact fruits with thick and waxy cuticle, such as: mangoes (*Manguifera indica*); citrus fruits such as oranges (*Citrus sinensis*) and tangerines (*Citrus reticulata*); tomatoes (*Solanum lycopersicum*), papaya (*Carica papaya*) and peaches (*Prunus persica*), among others (3, 6, 28). In the aforementioned fruits, it is necessary to make a small cut on the epidermal cuticle so that the pathogens enter successfully and cause an infection (18). However, berries, such as blackberries, are highly vulnerable to the attack of postharvest pathogens, even without any injuries, due to their soft and thin cuticle.

Table 3. Control of *R. stolonifer* with bacterial suspensions (CS) and cell-free extracts (CFE) of native bacterial strains of blackberry (*Rubus fruticosus* cv. Brazos).

Tabla 3. Control de *R. stolonifer* con suspensiones bacterianas (CS) y extractos libres de células (CFE) de cepas bacterianas nativas de zarzamora (*Rubus fruticosus* var. Brazos).

Treatments	Antagonist Agent	Severity Index *		Inhibition (%)**	
		RSA	RSC	RSA	RSC
Cell Suspension (CS)	*BLI*	3.0±0.1^{c}	3.05±0.05^{cd}	28.2±0.1^{c}	37.7±0.05^{cd}
	BSS	2.3±0.1^{b}	3.4±0.05^{de}	45.8±0.1^{b}	30.6±0.05^{de}
	LAQ	3.1±0.1^{cd}	3.2±0.05^{cde}	27.06±0.1^{cd}	33.6±0.05^{cde}
	BSL	3.3±0.1^{cd}	3.2±0.05^{cde}	22.3±0.1^{cd}	33.6±0.05^{cde}
Cell-Free Extract (CFE)	*BLI*	3.4±0.05^{d}	3.6±0.05^{e}	18.8±0.05^{d}	25.5±0.05^{e}
	BSS	3.4±0.3^{dc}	2.5±0.2^{b}	20.0±0.3^{cd}	47.7±0.2^{b}
	LAQ	2.5±0.1^{b}	2.8±0.1^{bc}	39.7±0.1^{b}	41.8±0.1^{bc}
	BSL	3.3±0.1^{cd}	3.4±0.3^{de}	22.3±0.1^{cd}	29.5±0.3^{de}
Control	Control (-)	1.0±0.0^{a}	1.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}
	Control (+)	4.2±0.05^{e}	4.9±0.1^{f}	0.0±0.0 ^{e}	0.0±0.0^{f}

Means ± standard deviation are presented. The different superscript letters in the same column indicate significant differences according to the Tukey test ($p \leq 0.05$, n=3). *Expressed as severity level based on a scale. **Calculated based on severity levels. RSA: *Rhizopus stolonifer* A strain; RSC: *Rhizopus stolonifer* C strain. BLI: *Bacillus licheniformis*; BSS*: Bacillus subtilis S*; LAQ: *Leifsonia aquatica; BSL: Bacillus subtilis* L; Control (-): PDB medium; Control (+) *Rhizopus stolonifer* A or C strains, respectively.

Se presentan las medias ± desviación estándar. Letras superíndices diferentes en la misma columna indican diferencias significativas de acuerdo a la prueba de Tukey ($p \leq 0,05$, n=3). * Nivel de severidad basado en una escala. **Calculado con base en los niveles de severidad. RSA: *Rhizopus stolonifer* cepa A; RSC: *Rhizopus stolonifera cepa* C. BLI: *Bacillus licheniformis*; BSS*: Bacillus subtilis S*; LAQ: *Leifsonia aquatica*; *BSL: Bacillus subtilis* L; Control (-): medio PDB; Control (+) *Rhizopus stolonifer* cepas A ó C respectivamente.

In this study, the *R. stolonifer* strains isolated from blackberries were capable of causing soft rot in the fruits even if they did not have any mechanical damage, for they are polydrupes, and this type of aggregates makes them susceptible to damages and hinders their treatment before selection for trade. Therefore, these berries are often packed without ensuring their preservation from pathogen attacks (20).

In this work, the effect of native bacteria of blackberry plants was evaluated, as well as their cell-free supernatants (CFE) in the control of soft rot of Brazos variety blackberry fruits (*Rubus fruticosus*). Interestingly, the growth of RSA was significantly reduced upon applying the CS of BSS, showing an inhibition percentage of 45.8%. These results coincide with those reported by Wang, *et al.* (2013) who used the *B. subtilis* SM21 strain to reduce by 37.2% the rot caused by *R. stolonifer* in peaches. Bonaterra *et al.* (2003) also reported the control of *R. stolonifer* in fruits damaged and subsequently treated by the *Pantoea agglomerans* EPS125 strain in different cultivars, such as nectarines (80-100%), apricots (60-97%) and peaches (76-86%).

Moreover, the use of cell-free supernatants (CFE) of the 4 bacterial strains tested in this study against *R. stolonifer*, allowed to confirm that they may be effective to control the pathogen, thus reducing the potential risks of applying bacterial directly cells to the fruits. This coincides with the proposal of Janisiewicz, W. and Roitman, J. (1988), who have proposed that the use of cell-free supernatants poses a lower risk

to human health than the application of living bacterial cells. Said authors applied *B. subtilis* 155 *in vitro* cell-free supernatants to inhibit the growth of gray mold caused by *Botrytis cinerea* with positive results. In contrast to the aforementioned study, in this work the evaluation was *in vivo*; however, the effect of CFE of BSS on postharvest disease control was shown with a 47.7% RSC development inhibition. In addition, the controlling capacity of CFE of LAQ was observed with a 39.7% RSA growth inhibition and a 41.8% of RSC and, as far as you know, this is the first report of the antagonistic effect of this bacterium and its CFE against soft rot caused by *R. stolonifer* in blackberries. Similar results were reported by using CFE of *Bacillus licheniformis* (EN74-1) to control gray mold in apple fruits, caused by *Botrytis mali,* accomplishing 58.8% of the pathogen growth (17). The CFE of *Pseudomonas syringae* showed antifungal activity against *Penicillium digitatum*, although this was lower than that obtained when the bacterial cells were applied (22).

Biocontrol may be understood as a dynamic process where interactions among fruit-pathogen-antagonist play a decisive role to preserve the system's integrity. Therefore, the biocontrolling activity of the four bacterial strains used in this study may have been the result of a combination of different mechanisms. In this regard, it has been proven that the strains pertaining to the *Bacillus* species, including *B. subtilis* and *B. licheniformis*, are capable of producing surfactants, such as: surfactin, iturin A and amicoumacin, with antagonistic activity capable of restricting pathogenic action (14, 25). This does not exclude the controlling capacity of *B. subtilis* through the production of antibiotics, siderophores and certain volatile compounds (more than 21 different volatile compounds with biocontrol activity have been reported) (3). Regarding the test with $FeCl_3$-enriched medium, an increase in the inhibition percentages was observed in two of the four bacterial strains used in comparison with the iron-free medium tests. LAQ was capable of inhibiting RSA growth with an antimycotic index of 48.6% *versus* a 36.4% of the iron-free medium and the RSC with an antifungal index of 47% of the $FeCl_3$ medium *versus* a 41.9% of the iron-free medium, which suggests that one of the mechanisms used by both strains to accomplish the control of the pathogen was the production of siderophores.

On the other hand, the results obtained in the inhibition of spore germination of the RSC strain showed that the four strains studied produce substances capable of inhibiting their germination; BLI and BSS strains were able to obtain high spore germination inhibition percentages (93.9% and 81.2%, respectively) after 48 h. Furthermore, BSL and LAQ treatments were able to lyse the RSC spores after 12 h (96 and 91%, respectively). These results coincide with the observations by Bryk *et al.* (1998), who reported that this mechanism was used by *Erwinia herbicola* to control *B. cinerea* and *P. expansum*.

Other authors attribute similar effects to the production of lytic enzymes, such as chitinases and glucanases by bacteria such as *B. subtilis*, which act on the cell wall of the pathogen compromising its integrity (21). Thus, the lysis of the spores is a mechanism that may have contributed to the inhibition of the pathogen germination observed. This early lysis capacity of the pathogen's spores may be fundamental to provide blackberry fruits with protection against postharvest diseases, given that the antagonist must act during the first stages of the infection of the fruit.

These results coincide with those reported by Panebianco *et al.* (2015), who observed that the *P. digitatum* spore germination inhibition by a *P. syringae* strain in liquid medium after 24 h ranged between 70-100%. Similar results were also observed by Ghosh *et al.* (2015), who reported *R. stolonifer* VBAM1 spore inhibition germination by the CFE of *Burkholderia cenocepacia* VBC7, *Pseudomonas poae* VBK1 (95%) and three lactic acid bacteria strains (97%). Even though the role of the *Leifsonia* species in the blackberry agroecosystem has not been yet elucidated, it is well known that this bacterial species are capable of producing biofilms involved in protective and food competition effects (27). It has been reported that oligopeptides, surfactants, and siderophores produced by some bacterial strains are capable of acting as inducing molecules that increase enzymes' activity, such as phenylalanine ammonia-lyase, polyphenol oxidase and superoxide dismutase, which are related to fruit defense mechanisms; as well as catalase and peroxidase which act as postharvest detoxifying agents in the fruit, as it was observed with the application of a cell suspension of *Pseudomonas putida* in papaya (31, 37). In this work, the four strains used were capable of producing siderophores with an important RSA and RSC growth inhibition activity by BLI and LAQ, which may have induced the production of some of the abovementioned enzymes, thereby causing the control effect observed.

Although the *Bacillus subtilis* is a well-known biological control agent, whose efficacy in postharvest control has been reported in many papers and from which the secondary metabolites that it actively produces have been isolated and characterized (23, 33), it is worth mentioning that there is no evidence of any work focused on postharvest control of fungal diseases in blackberry fruits. Additionally, *Leifsonia aquatica* has not been reported as an antagonist, producer of antifungals. Further research is required to characterize the action mechanisms involved in the fungicide effect of the bacteria related to blackberries in order to develop biotechnologies to control soft rot.

This study reports the effect of biocontrol of native bacterial strains associated with (*Rubus fruticosus*) Brazos variety, and their cell-free extracts, to control soft rot in the fruit, highlighting the potential of innovative biological agents such as *Leifsonia aquatica* and its metabolites, which represent an effective alternative for postharvest soft rot control.

References

1. Ahmad, M.; Masood, S.; Sutana, S.; Ben Hadda, T.; Bader, A.; Zafar, M. 2015. Antioxidant and nutraceutical value of wild medicinal Rubus berries. Pakistan Journal of Pharmaceutical Sciences. 28(1): 241-247.
2. Andrade-Bustamante, G.; Manelik García-López, A.; Cervantes-Díaz, L.; Aíl-Catzim, C. E.; Borboa-Flores, J.; Rueda-Puente, E. O. 2017. Estudio del potencial biocontrolador de las plantas autóctonas de la zona árida del noroeste de México: control de fitopatógenos. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 49(1): 127-142.
3. Arrebola, E.; Sivakumar, D.; Korsten, L. 2010. Effect of volatile compounds produced by Bacillus strains on postharvest decay in citrus. Biological control. 53(1): 122-128.

4. Baggio, J. S.; Goncalves, F. P.; Lourenco, S. A.; Tanaka, F. A. O.; Pascholati, S. F.; Amorim, L. 2016. Direct penetration of *Rhizopus stolonifer* into stone fruits causing rhizopus rot. Plant Pathology. 65(4): 633-642.
5. Ejaz, S.; Jezik, K. M.; Anjum, M. A.; Gosch, C.; Halbwirth, H.; Stich, K. 2017. Post-harvest nutritional and antioxidant profile of *Beta vulgaris* L. grown in low emission soilless microgarden system with organic and inorganic nutriments. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 49(2): 19-33.
6. Bonaterra, A.; Badosa, E.; Cabrefiga, J.; Francés, J.; Montesinos, E. 2012. Prospects and limitations of microbial pesticides for control of bacterial and fungal pomefruit tree diseases. Trees. 26(1): 215-226.
7. Bonaterra, A.; Mari, M.; Casalini, L.; Montesinos, E. 2003. Biological control of *Monilinia laxa* and *Rhizopus stolonifer* in postharvest of stone fruit by *Pantoea agglomerans* EPS125 and putative mechanisms of antagonism. International journal of food microbiology. 84(1): 93-104.
8. Boyzo-Marín, J.; Silva-Rojas, H.; Rebollar-Alviter, A. 2015. Biorational treatments to manage dryberry of blackberry caused by *Peronospora sparsa*. Crop Protection. 76: 121-126.
9. Bryk, H.; Sobiczewski, P.; Dyki, B. 1998. Antagonistic effect of *Erwinia herbicola* on *in vitro* spore germination and germ tube elongation of *Botrytis cinerea* and *Penicillium expansum*. Biocontrol. 43(1): 97-106.
10. Chávez-Díaz, I. F.; Angoa-Pérez, V.; López-Díaz, S.; Velázquez-del Valle, M. G.; Hernández-Lauzardo, A. N. 2014. Antagonistic bacteria with potential for biocontrol on *Rhizopus stolonifer* obtained from blackberry fruits. Fruits. 69(1): 41-46.
11. Enserink, M. 2009. Infectious Diseases Farm Fungicides Linked to Resistance in a Human Pathogen. Science. 326(5957): 1173-1173.
12. Gacasan, S. B.; Mwebi, N. 2011. Effects of storage on the anthocyanin, Vitamin C and antioxidant potential of raspberries and blackberries. Abstracts of Papers of the American Chemical Society. 241.
13. Ghosh, R.; Barman, S.; Mukhopadhyay, A.; Mandal, N. C. 2015. Biological control of fruit-rot of jackfruit by rhizobacteria and food grade lactic acid bacteria. Biological Control. 83: 29-36.
14. Govender, V.; Korsten, L.; Sivakumar, D. 2005. Semi-commercial evaluation of *Bacillus licheniformis* to control mango postharvest diseases in South Africa. Postharvest Biology and Technology. 38(1): 57-65.
15. Harwood, C. R.; Cutting, S. M. 1990. Molecular biological methods for Bacillus. Wiley. Chichester. New York.
16. Hernández-Lauzardo, A. N.; Velázquez-del Valle, M. G; Veranza-Castelán, L.; Melo-Giorgana, G. E.; Guerra-Sánchez, M. G. 2010. Effect of chitosan on three isolates of *Rhizopus stolonifer* obtained from peach, papaya and tomato. Fruits. 65(4): 245-253.
17. Jamalizadeh, M.; Etebarian, H.; Alizadeh, A.; Aminian, H. 2008. Biological control of gray mold on apple fruits by *Bacillus licheniformis* (EN74-1). Phytoparasitica. 36(1): 23-29.
18. Janisiewicz, W. J. 2009. *Quo vadis* of biological control of postharvest diseases. Postharvest Pathology. Springer. 137-148.
19. Janisiewicz, W.; Roitman, J. 1988. Biological control of blue mold and gray mold on apple and pear with *Pseudomonas cepacia*. Phytopathology. 78(12): 1697-1700.
20. Joo, M.; Lewandowski, N.; Auras, R.; Harte, J.; Almenar, E. 2011. Comparative shelf life study of blackberry fruit in bio-based and petroleum-based containers under retail storage conditions. Food Chemistry. 126(4): 1734-1740.
21. Leelasuphakul, W.; Hemmanee, P.; Chuenchitt, S. 2008. Growth inhibitory properties of *Bacillus subtilis* strains and their metabolites against the green mold pathogen (*Penicillium digitatum Sacc.*) of citrus fruit. Postharvest Biology and Technology. 48(1): 113-121.
22. Panebianco, S.; Vitale, A.; Polizzi, G.; Scala, F.; Cirvilleri, G. 2015. Enhanced control of postharvest citrus fruit decay by means of the combined use of compatible biocontrol agents. Biological Control. 84: 19-27.

23. Pereg, L.; McMillan, M. 2015. Scoping the potential uses of beneficial microorganisms for increasing productivity in cotton cropping systems. Soil Biology and Biochemistry. 80: 349-358.
24. Pérez, M.; Flores, P.; García, V.; Lozano, V. 1995. Factores genéticos y ambientales relacionados con la dinámica temporal y efecto de las enfermedades en fríjol (*Phaseolus vulgaris* L.) en Marín, Nuevo León, México. Revista Mexicana de Fitopatología. 13(1): 1-9.
25. Pinchuk, I. V.; Bressollier, P.; Sorokulova, I. B.; Verneuil, B.; Urdaci, M. C. 2002. Amicoumacin antibiotic production and genetic diversity of *Bacillus subtilis* strains isolated from different habitats. Research in Microbiology. 153(5): 269-276.
26. Rachid, D.; Ahmed, B. 2005. Effect of iron and growth inhibitors on siderophores production by *Pseudomonas fluorescens*. African Journal of Biotechnology. 4(7): 697-702.
27. Rickard, A.; McBain, A.; Ledder, R.; Handley, P.; Gilbert, P. 2003. Coaggregation between freshwater bacteria within biofilm and planktonic communities. FEMS Microbiology Letters. 220(1): 133-140.
28. Robl, D.; Thimoteo, S. S.; de Souza, G. C. C. F; Beux, M. R.; Dalzoto, P. R.; Pinheiro, R. L.; Pimentel, I. C. 2014. Occurrence of *Candida orthopsilosis* in Brazilian tomato fruits (*Lycopersicum esculentum* Mill.). Brazilian Journal of Microbiology. 45(1): 105-109.
29. Sanchez, L. C. A.; Real, C. P. V.; Perez, Y. B. 2014. Effect of an edible crosslinked coating and two types of packaging on antioxidant capacity of castilla blackberries. Food Science and Technology. 34(2): 281-286.
30. Schipper, M. A. 1984. Revision of the genus Rhizopus. Stud. Mycol. 25: 1-34.
31. Shi, J.; Liu, A.; Li, X.; Feng, S.; Chen, W. 2011. Inhibitory mechanisms induced by the endophytic bacterium MGY2 in controlling anthracnose of papaya. Biological Control. 56(1): 2-8.
32. Su, Y. C.; Ho, C. L.; Wang, E.; Chang, S. T. 2006. Antifungal activities and chemical compositions of essential oils from leaves of four eucalypts. Taiwan J For Sci. 21(1): 49-61.
33. Vlamakis, H.; Chai, Y.; Beauregard, P.; Losick, R.; Kolter, R. 2013. Sticking together: building a biofilm the *Bacillus subtilis* way. Nature Reviews Microbiology. 11(3): 157-168.
34. Wang, X.; Xu, F.; Wang, J.; Jin, P.; Zheng, Y. 2013. *Bacillus cereus* AR156 induces resistance against Rhizopus rot through priming of defense responses in peach fruit. Food chemistry. 136(2): 400-406.
35. Zahavi, T.; Cohen, L.; Weiss, B.; Schena, L.; Daus, A.; Kaplunov, K.; Zutkhi, J.; Ben-Arie, R.; Droby, S. 2000. Biological control of Botrytis, Aspergillus and Rhizopus rots on table and wine grapes in Israel. Postharvest Biology and Technology. 20(2): 115-124.
36. Zhang, D.; Fu, C. H.; Zheng, X.; Xi, Y.; Jiang, W.; Wang, Y. 2010. Efficacy of the antagonist *Aureobasidium pullulans* PL5 against postharvest pathogens of peach, apple and plum and its modes of action. Biological Control. 54(3): 172-180.
37. Zhao, Y.; Tu, K.; Shao, X.; Jing, W.; Su, Z. 2008. Effects of the yeast *Pichia guilliermondii* against *Rhizopus nigricans* on tomato fruit. Postharvest Biology and Technology. 49(1): 113-120.