Original article

Antagonistic bacteria with potential for biocontrol on *Rhizopus stolonifer* obtained from blackberry fruits

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Antagonistic bacteria with potential for biocontrol on *Rhizopus stolonifer* obtained from blackberry fruits.

Abstract – Introduction. México is one of the most important producers and exporters of blackberries in the world. During postharvest handling, blackberry fruits are exposed to the attack of phytopathogenic fungi. **Materials and methods**. To obtain *Rhizopus stolonifer* isolates, samples of leaves and rhizospheric soils were placed on Petri plates containing Potato Dextrose Agar (PDA). In addition, fruits were also placed in humidity chambers at 25 °C. To describe the infection process on blackberry fruits by *Rhizopus stolonifer*, samples were taken at different intervals for a period of 72 h. All fungal isolates obtained were maintained on PDA. The bacterial colonies were isolated and purified by streaking on PDA. The antagonistic activity of the bacteria was assessed against *R. stolonifer* by dual culture technique on PDA. To detect the production of siderophores, the chrome azurol S assay was carried out. **Results**. The infection process of *R. stolonifer* on blackberry fruits was described for the first time in this work. Eighty-six bacterial isolates from different parts of the plant and rhizospheric soil were obtained. Bacterial isolates with antagonistic activity were identified and the production of siderophores was measured. Four isolates showed antagonistic activity against *R. stolonifer*. *Bacillus subtilis* obtained from soil was the most effective isolate. **Conclusion**. In this study we isolated and identified antagonistic bacteria with potential for biocontrol on *Rhizopus stolonifer* obtained from blackberry fruits. To our knowledge this is the first report regarding this topic.

México / *Rubus fructicosus* / fruits / postharvest diseases / *Rhizopus stolonifer* / biological control / antagonistic bacteria

Bactéries antagonistes ayant un potentiel de lutte biologique contre *Rhizopus stolonifer* obtenu à partir des fruits du mûrier.

Résumé – Introduction. Le Mexique est l'un des plus importants producteurs et exportateurs de mûres dans le monde. Au cours de la manutention post-récolte, ces fruits sont exposés à l'attaque de champignons phytopathogènes. **Matériel et méthodes**. Pour obtenir des isolats de *Rhizopus stolonifer*, des échantillons de feuilles et de rhizosphère ont été placés en boîtes de Pétri contenant un milieu PDA (Potato Dextrose Agar). En outre, des fruits ont également été placés dans des chambres humides à 25 °C. Pour décrire le processus d'infection des mûres par *R. stolonifer*, des échantillons ont été prélevés à des intervalles différents pendant une période de 72 h. Tous les isolats fongiques obtenus ont été maintenus sur milieu PDA. D'autre part, les colonies bactériennes ont été isolées et purifiées par stries sur le milieu PDA. L'activité antagoniste des bactéries contre *R. stolonifer* a été évaluée par la technique de double culture sur PDA. Pour détecter la production de sidérophores, un dosage à l'azurol de chrome a été effectué. **Résultats**. Le processus d'infection des mûres par *R. stolonifer* a été décrit pour la première fois dans ce travail. Quatre-vingt-seize isolats bactériens provenant de différentes parties de la plante et de la rhizosphère du sol ont été obtenus. Les isolats bactériens ayant une activité antagoniste ont été identifiés et la production de sidérophores a été mesurée. Quatre isolats ont montré une activité antagoniste contre *R. stolonifer*. *Bacillus subtilis* isolé à partir du sol a été l'isolat le plus efficace. **Conclusion**. Dans notre étude, nous avons isolé et identifié des bactéries antagonistes ayant un potentiel de lutte biologique contre *R. stolonifer* obtenu à partir mûres. À notre connaissance, il s'agit du premier rapport sur le sujet.

Mexique / *Rubus fructicosus* / fruits / maladie de post-récolte / *Rhizopus stolonifer* / lutte biologique / bactérie antagoniste

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1. Introduction

Blackberry fruit (*Rubus fructicosus*), like other berries, is a highly nutritious fruit. It is an important dietary source of fiber, vitamins and minerals, in addition to phytochemical compounds which are essential for human health [1, 2]. Among other countries, México is one of the most important producers and exporters of blackberries in the world [3]. However, during the postharvest handling of fruits, they are exposed to diseases and insect pests [4].

Rhizopus stolonifer (Ehrenb.: Fr.) Vuill. is the causal agent of *Rhizopus* rot disease in various fruits and vegetables [5]. For many years, this pathogen has been controlled by using synthetic fungicides. However, it has been suggested that such chemical fungicides represent a potential risk to the environment and to human health [6].

Antagonistic bacteria have been used to control postharvest pathogens and their positive effects have been demonstrated on different fruits [7]. For many years, postharvest biocontrol research has seen tremendous advances and the creation of several products [8]. Nonetheless, there are no bacterial biocontrol agents reported against postharvest fungal diseases of blackberries. Therefore, the aim of our work was to isolate and identify antagonistic bacteria with potential for biocontrol on *R. stolonifer* obtained from blackberry fruits.

2. Materials and methods

2.1. *Rhizopus stolonifer* isolation and infection process

Blackberry plants (var. 'Brazos') with rhizospheric soil were collected in Los Reyes, Michoacán, México. To obtain *R. stolonifer* isolates, samples of leaves and rhizospheric soils were placed on Petri plates containing Potato Dextrose Agar (PDA). In addition, fruits were also placed in humidity chambers at 25 °C. All fungal isolates obtained were maintained on PDA. Serial dilutions were carried out on pure cultures and individual spores were collected and grown on PDA for 96 h. In order to fulfill Koch's postulates, the isolates were re-inoculated on fruits and placed in humidity chambers at 25 °C until symptoms appeared. For their identification, morphological characterization was carried out taking into account particular features of the mycelium and rhizoids [9]. To describe the infection process on blackberry fruits by *R. stolonifer*, samples were taken at different intervals for a period of 72 h at 25 °C. Random samples were processed by longitudinal and transversal cuts. Images (10 ×) of different parts of the fruits were obtained using a stereo microscope (Nikon, SMZ1500).

2.2. Isolation and antagonistic bacterial activity *in vitro* assays

Bacterial samples were obtained as described previously; fruits and rhizospheric soil samples were placed on PDA. The bacterial colonies were isolated and purified by streaking on PDA. The antagonistic activity of the bacteria was assessed against *R. stolonifer* by the dual culture technique on PDA. A 5-mm mycelial disc of *R. stolonifer* was placed on the center of the Petri plates. PDA plates inoculated with the pathogen alone were maintained as control. The plates were incubated at 28 °C in a regulated incubator for 72 h and the inhibition zone was measured from the edge of the mycelium to the bacterial streak, when the control plates showed full growth. Mycelial growth was measured with a digital vernier at 24 h and expressed as average diameter (mm). The antagonistic activity was calculated according to previous studies [10].

2.3. Identification of the antagonistic bacteria

The isolates with antagonistic activity were identified using the biochemical test of BBL CRYSTAL™ (Identification System Rapid Gram-positive ID Kit, BD Company, Becton, Dickinson and Company).

2.4. CAS assay for analysis of siderophores produced by bacteria

To detect the production of siderophores, the universal CAS (chrome azurol S) assay was carried out [11]. Colonies were plated in the center of CAS Petri plates and incubated for 24 h at 28 °C. The production of an orange halo was considered as positive for the production of siderophores. To measure the production of siderophores, the halo diameter was assessed in mm.

2.5. Data analysis

Experiments were repeated three times with three replicates per treatment. The data was analyzed by ANOVA (SigmaPlot 11.0, USA).

3. Results and discussion

3.1. Description of the infection process of *R. stolonifer* on blackberry fruits

In our study, two isolates showed the distinctive characteristics of *R. stolonifer* such as aerial mycelium and complex rhizoids, well defined according to Schipper's identification [9]. Additionally, the formation of sporangiophores, sporangia and sporangiospores (globose, ellipsoidal and angular) typical of *R. stolonifer* were observed as previously described [12]. These isolates were identified as *R. stolonifer* 1 (from leaves) and *R. stolonifer* 2 (from fruits). The infection process of *R. stolonifer* on blackberry fruits was similar in the two isolates. Germination of spores on blackberry fruits was observed 12 h after the inoculation (*figure 1A*). Later, the mycelium was observed between the fruits (*figure 1B*). The formation of erected sporangiophores anchored to the substrate by complex rhizoids, well defined (48 h), was observed (*figure 1C*). Dark sporangia were observed on the tips of the sporangiophores (*figure 1D*). This indicates that the spores are ripe. Macerated tissues were observed by longitudinal and transversal cuts (*figure 1E, 1F*). Mycelia inside the fruits after 72 h of inoculation were not observed. The infection process of *R. stolonifer* on blackberry fruits was described for the first time in this work. We agree with other authors who suggested that the macerating process on the fruit tissue is carried out by the excretion of different enzymes, such as polygalacturonase and pectin methylesterase, among other factors [13, 14].

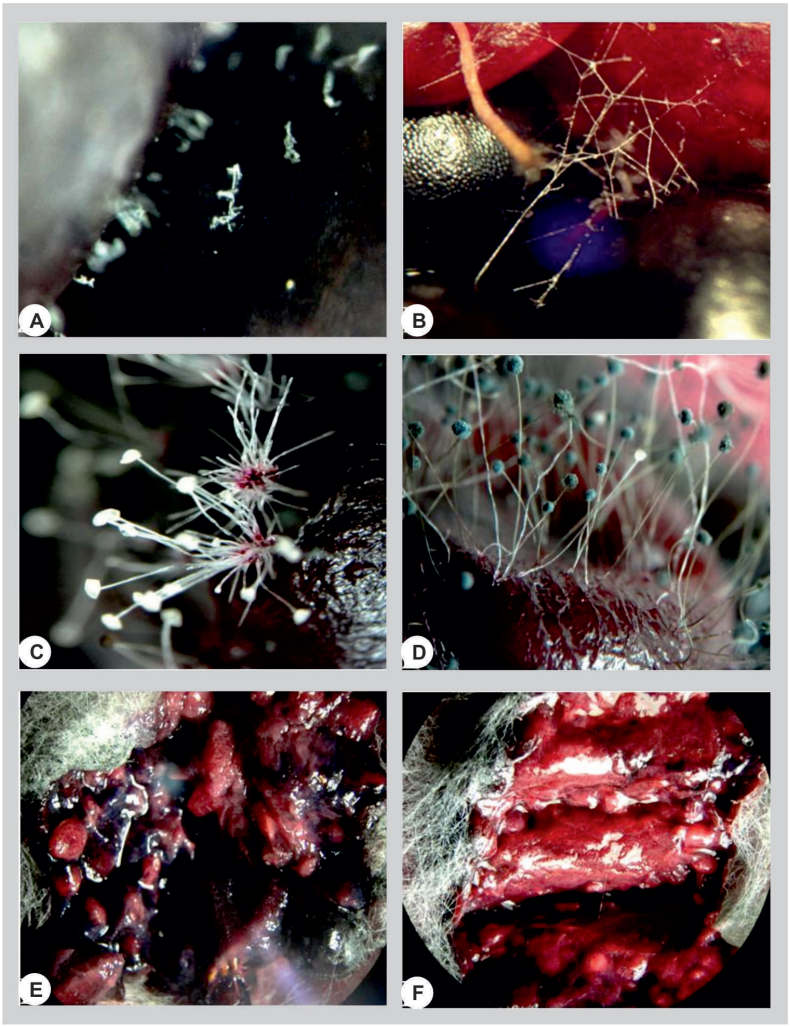

Figure 1.
Infection process of *Rhizopus stolonifer* on blackberry fruits in humidity chambers for 72 h at 25 °C. Images were taken using a stereo microscope (Nikon, SMZ1500). Magnifications of the images were 10×: A) and B) at 12 h; C) at 24 h; D) at 48 h; E) and F) at 72 h.

3.2. Effect of antagonistic bacteria towards *R. stolonifer* and their identification

Eighty-six bacterial isolates were obtained from different parts of the plant and

Table I.
Mycelial growth inhibition in a dual culture assay (72 h at 28 °C) of *Rhizopus stonolifer* and identification of the antagonistic bacterial isolates by BBL CRYSTAL™.

Antifungal index (%) (Zone of mycelial growth inhibition)		Sample	Identification by BBL CRYSTAL™
Rhizopus stolonifer (1)	*Rhizopus stolonifer* (2)		
41.42 a	42.78 a	Leaves	*Bacillus licheniformis*
40.04 a	41.46 a	Rhizospheric soil	*Bacillus subtilis* (S)
36.64 b	41.87 a	Leaves	*Leifsonia aquatica*
37.56 b	36.18 b	Leaves	*Bacillus subtilis* (L)
0.0 c	0.0 c	–	Control

Means with the same letter within columns are not significantly different according to ANOVA, Tukey´s test $p < 0.05$.

Table II.
Chrome Azurol S (CAS) assay for analysis of siderophores produced by bacteria on PDA medium (24 h at 28 °C) and the antifungal index.

Isolate	CAS-blue agar (color change)	Measurement of color change zone (mm)
Bacillus licheniformis	Orange	18.00 a
Bacillus subtilis (S)	Orange	21.60 a
Leifsonia aquatica	Orange	17.80 a
Bacillus subtilis (L)	Orange	20.20 a
Control	–	0.00 b

Means with the same letter are not significantly different according to ANOVA, Tukey´s test $p < 0.05$.

rhizospheric soil. The results of the antagonistic activity of the bacteria showed that four isolates were effective against *R. stolonifer*. These isolates were identified as *Bacillus licheniformis* (from leaves), *Bacillus subtilis* [from rhizospheric soil (S) and leaves (L)] and *Leifsonia aquatica* (from leaves). *Bacillus licheniformis* (41.42% and 42.78%) and *Bacillus subtilis* (S) (40.04% and 41.46%) showed the highest antifungal index against two isolates of *R. stolonifer* (*table I*). *Bacillus subtilis* obtained from soil was the most effective isolate.

3.3. Siderophore production

The color of the CAS (chrome azurol S) agar changed from blue to orange in all bacteria tested. This indicates that the bacteria could produce siderophores. The values were between 17.8 mm and 21.6 mm (*table II*). In general, *Bacillus subtilis* has been reported as a biocontrol agent because it produces several antifungal metabolites that contribute to inhibition of mycelial growth [15]. Among others, the production of siderophores has been reported in this species [16]. *Bacillus subtilis* might be a promising biocontrol agent against postharvest fungal disease of blackberries. Therefore, studies like this have an important role in contributing to the potential use of Gram-positive bacteria. To our knowledge, this is the first report of isolation of antagonistic bacteria with potential for biocontrol of *R. stolonifer* obtained from blackberry fruits.

4. Conclusion

In our study, we isolated and identified antagonistic bacteria with potential for biocontrol of *Rhizopus stolonifer* obtained from blackberry fruits.

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Bacterias antagonistas con potencial de biocontrol contra *Rhizopus stolonifer* obtenido de frutos de zarzamora.

Resumen – **Introducción**. México es uno de los productores y exportadores más importantes de zarzamoras del mundo. Durante el manejo postcosecha, los frutos de zarzamora están expuestos al ataque de hongos fitopatógenos. **Material y métodos**. Para obtener aislados de *Rhizopus stolonifer*, muestras de hojas y de suelo rizosférico fueron colocados en cajas Petri conteniendo Papa Dextrosa Agar (PDA). Además, los frutos también fueron colocados en cámaras húmedas a 25 °C. Para describir el proceso de infección de *Rhizopus stolonifer* en frutos de zarzamora, se tomaron muestras a diferentes intervalos por un periodo de 72 h. Todos los aislados fúngicos obtenidos fueron mantenidos en PDA. Por otra parte, las colonias bacterianas se aislaron y purificaron estriando en PDA. La actividad antagonista de las bacterias contra *R. stolonifer* fue evaluada mediante la técnica de cultivo dual en PDA. Para detectar la producción de sideróforos, se realizó el ensayo de cromo azurol. **Resultados**. El proceso de infección de *R. stolonifer* en frutos de zarzamora fue descrito por primera vez en este trabajo. Se obtuvieron 86 aislamientos bacterianos de diferentes partes de la planta y de suelo rizosférico. Los aislados bacterianos con actividad antagonista fueron identificados y la producción de sideróforos fue medida. Cuatro aislados mostraron actividad antagonista contra *R. stolonifer*. *Bacillus subtilis* obtenido de suelo fue el aislado más efectivo. **Conclusión**. En este estudio aislamos e identificamos bacterias antagonistas con potencial de biocontrol sobre *Rhizopus stolonifer* obtenido de frutos de zarzamora. Para nuestro conocimiento este es el primer reporte en este tema.

México / *Rubus fructicosus* / frutas / enfermedad de postcosecha / *Rhizopus stolonifer* / control biológico / bacterias antagonistas