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Bacteria associated with soft rot of *Agave tequilana* Weber var. Azul in Nayarit

Bacterias asociadas a la pudrición blanda del cogollo del *Agave tequilana* Weber var. Azul en Nayarit

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ABSTRACT

The blue agave is a species of significant economic, social, and cultural importance to Mexico. Unfortunately, it is susceptible to serious diseases, such as soft rot. Approximately 11 different bacterial species have been reported as causal agents, and it is believed to be a syndrome. In the Nayarit state, no previous records exist regarding the specific microorganism responsible for this condition. In the present study, samples were collected from two municipalities in Nayarit with confirmed disease incidence, yielding a total of 35 bacterial isolates. Three pathogenicity tests were performed: *in vitro* on potato slices, *in vitro* on agave leaf fragments, and *in vivo* on agave plants. Bacterial isolates that induced disease symptoms in at least one of the assays were subjected to molecular analysis of the 16S rRNA gene. Results identified *Pectobacterium aroidearum* as the causative agent in all three tests. Other identified bacteria included *Enterobacter hormaechei* and *Pseudomonas mendocina*. This is the first study to report *Pectobacterium aroidearum* as a causal agent of soft rot in agave in Mexico.

KEY WORDS: Pectobacterium aroidearum, 16 rDNA gene, bacteria, identification.

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RESUMEN

El agave tequilero es una especie de gran importancia económica, social y cultural para el país, desafortunadamente, se enfrenta a importantes enfermedades, como la pudrición blanda del cogollo. Se reportan alrededor de 11 especies diferentes de bacterias como agente causal, y se cree que se trata de un síndrome. En Nayarit no existen reportes sobre el microorganismo causante de la enfermedad. En el presente trabajo se recolectaron muestras de dos municipios de Nayarit con incidencia de dicha enfermedad, de las cuales se obtuvieron 35 aislados. Se realizaron tres pruebas de patogenicidad: in vitro en papa, in vitro en fragmentos de hoja de agave, e in vivo en plantas de agave. A las bacterias que provocaron los signos de la enfermedad al menos en una de las pruebas, se les realizó análisis molecular del gen 16S, los resultados identificaron a *Pectobacterium aroidearum*, que causó enfermedad en las tres pruebas realizadas; las otras bacterias identificadas fueron *Enterobacter hormaechei* y *Pseudomona mendocina*. Este sería el primer trabajo que reporta a *Pectobacterium aroidearum* como causante de la pudrición blanda del cogollo del agave en México.

PALABRAS CLAVE: Pectobacterium aroidearum, 16S rDNA gene, bacteria, identificación.

Introduction

The blue agave (*Agave tequilana* Weber var. Azul) is a Mexican species cultivated in the region protected under the Tequila Denomination of Origin, which includes the state of Jalisco and selected municipalities in Nayarit, Guanajuato, Michoacán, and Tamaulipas (CRT, 2025). Blue agave is the primary raw material used in tequila production (SE, 2006) and holds substantial economic, social, and cultural value for the country (González *et al.,* 2007), largely due to its role in generating employment in both agriculture and the industry (Ángeles-Espino *et al.,* 2013). Additionally, it is a significant source of foreign revenue, as tequila ranks second among Mexican agri-food products in export value, generating $4.085 million of US dollars in 2023 (SIAP, 2023).

Since the agave stores high concentrations of sugars in the stem and aggregate of leaf bases, termed `piña', it provides favorable conditions for microbial proliferation. In *A. tequilana*, the endophytic bacterial population in leaf tissue has been estimated at three million colony forming unit per gram (CFU g^{-1}) of fresh plant tissue. 16S rDNA-based identification revealed 99-100 % sequence homology with *Acinetobacter sp*., *A. baumannii*, *A. bereziniae*, *Cronobacter sakazakii*, *Enterobacter hormaechei*, *Bacillus sp*., *Klebsiella oxytoca*, *Pseudomonas sp*., *Enterococcus casseliflavus*, *Leuconostoc mesenteroides subsp. mesenteroides*, and *Gluconobacter oxydans*.

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These microorganisms were considered potential plant growth-promoting bacteria (Martínez-Rodríguez *et al.,* 2014).

Thus, the internal environment of the plant favors the proliferation of various microorganisms, including pathogens (Fucikovsky, 2004), which are among the main factors negatively impacting agave productivity. The principal pathogens affecting this crop include *Fusarium oxysporum* and *F. moniliforme*, which colonize and obstruct the plant's vascular tissues, leading to leaf wilting, chlorosis, and eventually plant death, a condition known as agave wilt; *Cercospora agavicola*, a fungus that spreads on the leaves causing gray leaf spot; and the bacterium *Pectobacterium carotovorum*, considered highly aggressive and responsible for the soft rot, which leads to leaf death. Due to their impact, these diseases are regulated by Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria (SENASICA), which aims to reduce infection levels in states within the Tequila Denomination of Origin Zone (SENASICA, 2020).

Characteristic symptoms of soft rot include necrotic and water-soaked lesions, often beginning at the apical or lateral spines and progressing toward the shoot base and into the leaf. The shoot takes on a dark purple hue and may detach easily (Valenzuela, 2003). The disease spreads downward, reaching the 'piña' and producing a foul-smelling internal rot that eventually leads to tissue disintegration and plant death (Rubio-Cortés, 2007).

In the case of this disease, SENASICA (2020) reported the isolation of several microorganisms as potential causal agents. Three separate studies initially identified the same microorganism: *Erwinia spp*., followed by *Pectobacterium (Erwinia) sp*. of the *carotovora* group, and later, *Pectobacterium carotovorum*. In 2004, Jiménez-Hidalgo and colleagues isolated *Erwinia cacticida*, *Pantoea agglomerans*, and *Pseudomonas sp*., proposing that soft rot symptoms may be caused by different microorganisms. In 2011, *Erwinia sp*. was again reported as the causal agent (Martínez-Ramírez, 2011). In 2012, *Bacillus pumilus* was identified, and in 2014, four additional microorganisms, *Pantoea sp.*, *Bacillus sp*., *Arthrobacter sp*., and *Streptomyces sp*., were reported in association with the disease. In 2017, *Pectobacterium carotovorum subsp. atroseptica* was once again identified as a causative agent. However, the Centro Nacional de Referencia Fitosanitaria (CNRF, 2017) confirmed that more than one pathogen may be involved and that these microorganisms might interact to produce the syndrome known as soft rot of agave.

Therefore, the objective of this study was to identify the microorganism responsible for soft rot from isolates obtained in Nayarit, Mexico.

Material and Methods

Plant material

Leaves were collected from agave plants aged between two and four years that exhibited characteristic signs and symptoms of soft rot. Four samples were collected from two localities:

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Miguel Hidalgo, in the municipality of Santa María del Oro, and Lázaro Cárdenas, in Ixtlán del Río.

Isolation and preservation

From each leaf, three discs of 1.0 cm in diameter were extracted, containing both symptomatic and healthy tissue. Samples were rinsed with running water, then surface-disinfected first with 2 % sodium hypochlorite, followed by three rinses with distilled water. Subsequently, samples were disinfected with 70 % ethanol and rinsed again three times with sterile distilled water. The discs were placed on Luria-Bertani (LB) agar plates and incubated at 28 °C for 24 hours. All resulting colonies were re-streaked on LB agar until axenic cultures were obtained. In total, 35 isolates were recovered and preserved in 80 % glycerol solution at -80 °C.

Pathogenicity assessment of the isolates

Suspensions were prepared from the 35 axenic cultures. A pre-inoculum was cultivated in LB broth and incubated at 28 °C until reaching an optical density (OD) at 500 nm, determined from individual growth curves. Serial dilutions were prepared in 0.85 % sterile saline solution to a final concentration of 1.5 x 10^8 CFU mL^{-1} (Jiménez-Hidalgo *et al.,* 2004).

Pathogenicity tests

For all three pathogenicity assays, a bacterial suspension of 1.5 x 10^8 CFU mL^{-1} was used (Jiménez-Hidalgo *et al.,* 2004), with three replicates per isolate.

In vitro pathogenicity test on potato

Following the methodology proposed by Duarte *et al.* (2004), potato tubers were surface-sterilized by immersion in 1 % sodium hypochlorite solution (NaClO) for one minute, rinsed three times with distilled water, followed by immersion in 70 % ethanol, and a final rinse with sterile distilled water. Slices of 0.5 cm thickness were cut and placed in Petri dishes on filter paper moistened with sterile distilled water. Three wounds were made on each slice, rectangular incisions 5 mm deep and 10 mm long, into which the inoculum was applied. Sterile saline solution was used as a negative control. Tubers were evaluated 24, 48, and 72 hours post-inoculation (Corzo-López & Quiñones-Pantoja, 2017; Jiménez-Hidalgo *et al.,* 2004).

In vitro pathogenicity test on agave leaf fragments

A modified version of the method by Duarte *et al.* (2004) was used. Healthy agave leaves were disinfected by immersion in 1 % sodium hypochlorite solution (NaClO) for one minute, rinsed with sterile distilled water, then immersed in 70 % ethanol and again rinsed with sterile distilled water. Leaf fragments measuring 1 cm x 5 cm were cut, and three segments were placed in each Petri dish on a cotton bed moistened with sterile distilled water. Inoculation was performed with the bacterial isolates, and the fragments were evaluated at 24, 48, and 72 hours post-inoculation (Corzo-López & Quiñones-Pantoja, 2017; Jiménez-Hidalgo *et al.,* 2004).

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In vivo pathogenicity test on agave plants

This test was conducted only with isolates that caused disease in either the potato or agave *in vitro* pathogenicity tests. Healthy two-year-old blue agave plants were grown in 40 x 40 cm bags filled with locally sourced soil disinfected with quaternary ammonium salts (Mezo, 2018). Each treatment included three replicates, consisting of three plants per isolate and three plants as negative controls, which were inoculated with sterile saline solution. Inoculation was carried out by subcutaneous injection, the injection site was first disinfected with 70 % ethanol, and 1 mL of bacterial suspension was injected per plant using a hypodermic syringe (Mezo, 2018; Navarro, 2018). To induce stress conditions, plants were subjected to waterlogging by positioning them over a plastic sheet, on which a 10 cm water layer was maintained.

Biochemical and physiological identification

For isolates that induced disease in the *in vitro* potato test, the following assays were performed: growth on casein peptone-glucose agar, Gram staining, growth in 5 % NaCl, growth at 37 °C, anaerobic growth, growth in 10 % glucose, and hydrogen sulfide (H_2S) production (Schaad *et al.,* 2001).

Molecular identification of bacterial isolates

Molecular identification was performed only on those isolates that produced characteristic symptoms of soft rot in any of the pathogenicity tests. DNA extraction followed the protocol from ZYMO Research (Quick-DNA Fungal/Bacterial Miniprep Kit). DNA concentration was quantified using a NanoDrop microvolume spectrophotometer (Thermo Scientific). PCR amplification of the V3-V4 region of the 16S rRNA gene was carried out using universal primers for enterobacteria (Widmer *et al.,* 1998): fD1 (AGAGTTTGATCCTGGCTCAG) and rD1 (AAGGAGGTGATCCAGCC) (Bastos-Silva *et al.,* 2009). The expected product length ranged between 500-1500 bp (Apolinario, 2018).

PCR reactions were prepared according to Bastos-Silva *et al.* (2009), using a 50 µL master mix containing 0.2 mM of each dNTP, 10 pmol/µL of each primer, 1 U of Taq DNA polymerase (Jena Bioscience), and 50 ng of bacterial DNA. Thermal cycling conditions included one cycle at 94 °C for 3 minutes; 30 cycles of denaturation at 92 °C for 50 s, annealing at 57 °C for 50 s, and extension at 72 °C for 1 minute; followed by a final extension at 72 °C for 7 minutes. PCR reactions were run on a Biorad 100 gradient thermocycler. Products were visualized on 1 % agarose gel electrophoresis using 1x TBE buffer and ethidium bromide, under a UV transilluminator (Thermo Scientific).

PCR products were submitted for sequencing to the Molecular Biology Unit at UNAM. Sequence similarity was assessed using the BLAST database (National Center for Biotechnology Information, NCBI).

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Koch's postulates

Based on the *in vitro* pathogenicity test on agave fragments, the tissue affected by isolate three, which consistently caused disease in the three pathogenicity tests, was re-isolated. It was preserved with 80 % glycerol and placed in ultra-freezing at -80 °C.

The procedure described above was also performed for sequencing. This isolate was named K3.

Results and Discussion

A total of 35 isolates were obtained from the field samples; however, only those that produced disease symptoms in at least one pathogenicity test were considered for molecular identification. The geographical location of the isolates is shown in Table 1.

Table 1. Geographic location of isolates obtained from plants exhibiting symptoms of soft rot.

			Cordinates	
Isolate	Location	Municipality	Latitude N	Longitude W
2	Sta. María del Oro	Miguel Hidalgo	21°28'22.9"	104°40'15.3"
3	Sta. María del Oro	Miguel Hidalgo	21°28'36.4"	104°39'41.1"
4	Ixtlán del Río	Lázaro Cárdenas	21°02'04.3"	104°20'19.3"
8	Ixtlán del Río	Mexpan	21°02'09.3"	104°25'45.9"
9	Ixtlán del Río	Mexpan	21°02'09.3"	104°25'45.9"
10	Sta. María del Oro	Miguel Hidalgo	21°28'36.4"	104°39'41.1"

Pathogenicity assessment of isolates

In the *in vitro* pathogenicity assay using potato, six isolates 2, 3, 8, 9, and 10, produced disease signs and symptoms (Table 2).

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Table 2. Results of the pathogenicity tests of the bacterial isolates conducted in vitro on potato slices and agave leaf segments, and *in vivo* on field-grown agave plants.

	Phytopathogenicity tests		
Isolate	***In vitro* in potato**	***In vitro* in agave**	***In vivo***
2	+	-	-
3	+	+	+
4	-	+	-
8	+	-	-
9	+	+	+
10	+	-	-

+ Showed signs and symptoms; - Did not show signs or symptoms.

Isolates 2, 3, and 8 began inducing symptoms 48 hours post-inoculation, while isolates 9 and 10 showed visible symptoms after 72 hours. In general, the inoculated areas on the potato slices exhibited translucent exudates, darkened edges, and water-soaked rot, in contrast to the control, where wounds healed without any signs or symptoms (Figure 1).

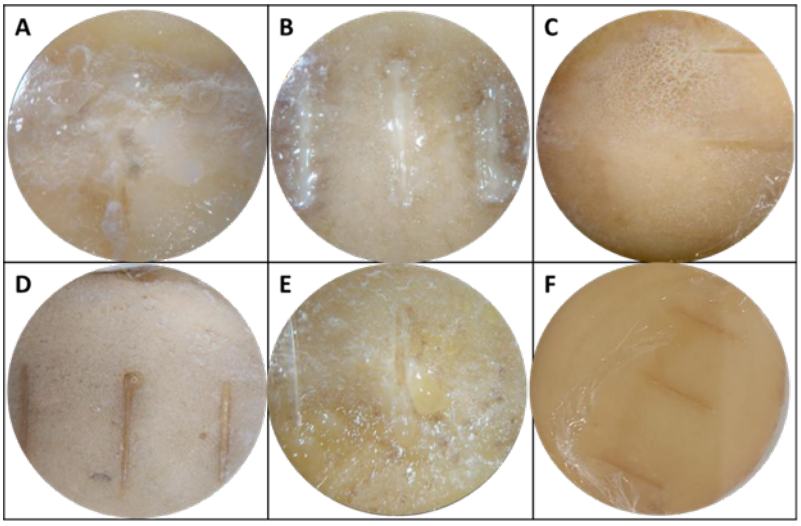

Figure 1. Signs and symptoms produced by the isolates in the *in vitro* pathogenicity test on potato slices.

A) Isolate 2, B) Isolate 3, C) Isolate 8, D) Isolate 9, E) Isolate 10, and F) Control.

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In contrast, when testing *in vitro* on agave leaf segments, only three isolates, 3, 4, and 9, elicited a symptomatic response (Table 2). Isolates 3 and 4 began causing symptoms within 24 hours post-inoculation, observed as localized darkening around the wound margins. After 48 hours, more pronounced signs appeared, such as exudates, water-soaked lesions, and dark spots (Figure 2).

Figure 2. Signs and symptoms caused by isolates in the *in vitro* pathogenicity test on agave leaf fragments.

A) Isolate 3, B) Isolate 4, and C) Control.

In the *in vivo* pathogenicity test, only agave plants inoculated with isolates 3 and 9 developed signs and symptoms (Table 2). Isolate 3 began to cause darkening around the injection site 29 days after inoculation, whereas isolate 9 induced symptoms starting on day 34. Both isolates resulted in water-soaked rot and exudate formation (Figure 2). In contrast, control plants healed without developing any symptoms, as shown in Figure 2.

Biochemical and physiological identification

Characterization was performed only on isolates 2, 3, 4, 8, 9, and 10, as these caused disease symptoms in at least one of the three pathogenicity tests.

Biochemical and physiological assays were conducted in conjunction with molecular identification to account for metabolic variability among individuals of the same species (Fonseca, 2014). The results of the biochemical tests used for isolates identification are presented in Table 3.

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Table 3. Results of the biochemical and physiological tests performed on the isolates that caused symptoms in at least one pathogenicity assay.

			Growth				H_2S production
Isolate	pH	Gram stain	NaCl 5 %	at 37 °C	Anaerobic	Glucose 10 %	
2	M	-	+	-	+	+	+
3	A	-	+	+	-	-	-
4	A	-	+	+	+	+	+
8	M	-	+	+	+	+	-
9	A	-	+	+	+	+	-
10	M	-	-	-	-	-	-

pH: M = pH ≥ 7.4, A = pH ≤ 5.5.

Molecular identification of bacterial isolates

The universal primers rD1 and fD1 successfully amplified DNA from all bacterial isolates, except for the negative control, in which no DNA was used. All isolates produced amplicons with an approximate molecular weight of 1000-1200 bp, as shown in Figure 3.

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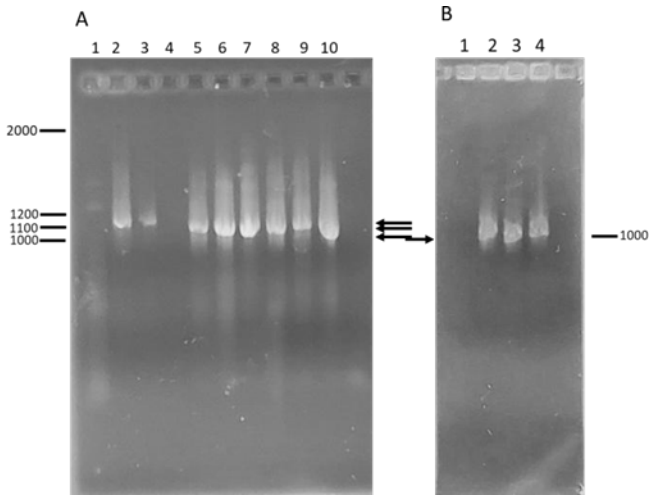

Figure 3. PCR amplification of bacterial isolates.

A) Lane 1, molecular marker (Sigma, 50 bp); lane 2, isolate 2; lane 5, isolate 8; lane 6, isolate 10 (positive control); lane 8, isolate 9; lane 11, negative control. B) Lane 1, molecular marker; lane 2, isolate 10; lane 3, isolate 4; lane 4, isolate 3. Arrows indicate the fragment sizes.

The BLAST analysis results of the 16S rRNA gene sequences from the evaluated isolates are shown in Table 4.

Table 4. BLAST analysis results for 16S rRNA sequences obtained from bacterial isolates.

Isolate	Microorganism	Percentage of similarity
2	*Enterobacter asburiae*	99.90 %
3	*Pectobacterium aroidearum*	99.71 %
4	*Enterobacter hormaechei*	99.00 %
8	*Enterobacter hormaechei*	99.61 %
9	*Pectobacterium aroidearum*	99.62 %
10	*Pseudomona mendocina*	99.90 %

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Isolates 3 and 9 produced signs and symptoms in all pathogenicity assays: *in vitro* in potato, *in vitro* in agave leaf fragments, and *in vivo* in agave plants (Table 2). However, isolate 3 exhibited symptoms 24 hours earlier than isolate 9 in the *in vitro* tests, and five days earlier in the *in vivo* test.

Based on the ribosomal DNA sequence, these isolates showed 99.71 % and 99.62 % similarity with *Pectobacterium aroidearum*. This species comprises non-gas-producing bacteria (Nabhan *et al.,* 2013). Likewise, in the biochemical and physiological assays, these isolates did not produce H_2S.

In the first studies on soft rot in *Agave tequilana* var. azul, which did not involve molecular analyses, *Erwinia* and *Pectobacterium carotovorum* or *P. carotovorum subsp. carotovorum* were indicated as the causal agents of the disease (SENASICA, 2020). This may be related to the fact that, until a few years ago, *P. carotovorum subsp. carotovorum* was used to group isolates that differed from other *Pectobacterium* species. It was not until 2013 that molecular phylogenetic analyses led to the proposal of *Pectobacterium aroidearum* as a new species grouping pathogens responsible for soft rot (Nabhan *et al.,* 2013). Since then, it has been reported causing soft rot in zucchini (*Cucurbita pepo*) (Moraes *et al.,* 2016), in chili pepper fruits (*Capsicum annuum*) in Brazil (Moraes *et al.,* 2020), in calla lily (Zantedeschia spp.) (Li *et al.,* 2022), and in Olecranon Honey peach (*Prunus persica*) in China (Liang *et al.,* 2022), as well as in *Alocasia amazonica* in Poland (Mikiciński *et al.,* 2023). It is generally considered a species with a preference for monocots, but it is also capable of causing soft rot in dicots.

The 16S rRNA gene sequence of isolate 2 showed 99.9 % similarity with *Enterobacter asburiae*. In the *in vitro* tests, this isolates only produced symptoms in the potato. However, in biochemical tests, it showed growth under anaerobic conditions and produced hydrogen sulfide (H_2S). The genus Enterobacter is facultatively anaerobic and generally does not produce H_2S (Jha *et al.,* 2011). Nonetheless, atypical strains of *Escherichia coli* have been reported that produce H_2S and cause a wide range of diseases in humans and animals (Mazumder *et al.,* 2023). Reports of *E. asburiae* as a plant pathogen are scarce. The first report came in 2020, identifying it as the causal agent of ginger (*Zingiber officinale* Roscoe) tuber rot in China (Zhang *et al.,* 2020); in 2021, it was reported as the causal agent of bacterial blight in rice, occasionally in co-infection with *Pantoea ananatis* in China (Xue *et al*., 2021); and in 2023, as the cause of root rot in radish (*Raphanus sativus* L.) (Wang *et al.,* 2023).

In contrast, *Enterobacter asburiae* strain RS83 has been studied for its plant growth-promoting capabilities (Jetiyanon, 2015). Furthermore, *the Enterobacter asburiae* strain JAS5 has shown potential for bioremediating pesticide-contaminated agricultural soils (Abraham and Silambarasan, 2015).

Isolates 4 and 8 showed 99.00 % and 99.61 % similarity with *Enterobacter hormaechei*, respectively. In biochemical tests, isolate 4 showed growth at 37 °C, under anaerobic conditions, and produced hydrogen sulfide. Although the optimal growth temperature for Enterobacter is 30 °C, most clinical strains grow at 37 °C (Grimont & Grimont, 2015).

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Isolate 8 shares most of these characteristics, except for H_2S production. There were also differences in the pathogenicity test responses: isolate 8 produced symptoms only in potato, while isolate 4 showed symptoms in agave leaves, although not in the *in vivo* test (Table 2). Susceptibility to pectolytic bacteria depends on several factors, such as inoculum concentration, temperature, and crop variety, among others (Franco *et al.,* 2007); in this case, one or more of these factors may not have been adequate for symptom development. Microorganisms can also colonize plants asymptomatically and act as pathogen reservoirs throughout the plant's development cycle (Marín-Ortiz *et al.,* 2018). However, further research is required. Martínez-Rodríguez *et al.* (2014) isolated this bacterium from *Agave tequilana* leaves and suggested that it could be a plant growth-promoting bacterium due to its nitrogen-fixing properties, indole acetic acid production, and phosphate solubilization. Although *Enterobacter hormaechei* belongs to the "*Enterobacter cloacae"* complex, which includes six species: *E. asburiae, E. cloacae, E. hormaechei, E. kobei, E. ludwigii,* and *E. nimipressuralis*, that are usually isolated from hospital infections (O'Hara *et al.,* 1989), the first report of *E. hormaechei* as a plant pathogen was in Malaysia in 2019, as the causal agent of stem rot in dragon fruit (*Hylocereus costaricensis*), and in the same year in Costa Rica (Retana *et al.,* 2019). Notably, both agave and dragon fruit are succulent plants.

Isolate 10 showed 99.90 % similarity with *Pseudomonas mendocina*. Jiménez-Hidalgo *et al.* (2004) conducted a study where isolates were characterized using molecular, biochemical, and fatty acid analyses. The molecular tests identified *Pseudomonas sp*., with 97 % similarity to *P. mendocina* and *P. alcalophila*. The microorganisms identified in the present study include *E. asburiae, P. aroidearum, E. hormaechei,* and *P. mendocina*. Among the isolates evaluated, *E. asburiae* was the only one that consistently reproduced the disease in all three tests, making it the most virulent.

These results partially agree with the findings of Jiménez-Hidalgo *et al.* (2004), who identified *Erwinia cacticida, Pantoea agglomerans*, and *Pseudomonas sp.* associated with soft rot in agave and suggested that more than one bacterial species may cause the disease. However, the species identified in the present study differ from those previously reported.

Conclusions

Based on the bacterial diversity identified and the results of the pathogenicity tests, it is concluded that soft rot of the agave is associated with a complex of pathogens. The bacteria detected were *Enterobacter asburiae, Pectobacterium aroidearum, Enterobacter hormaechei*, and *Pseudomonas mendocina*. This study represents the first report identifying these species as causal agents of soft core rot in isolates obtained in Nayarit, Mexico.

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