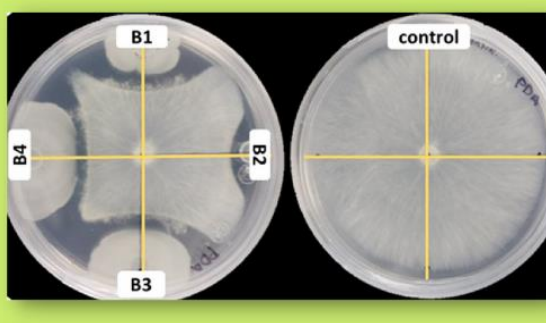
Investigaciones Biológicas, Agrícolas y Ambientales de México

Leandris Argentel Martínez
Ofelda Peñuelas Rubio
Organizadores



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Rua Abaete, 83, Sala B, Centro. CEP: 78690-000.
Nova Xavantina – Mato Grosso – Brasil.
Telefone (66) 99682-4165 (Whatsapp).
https://www.editorapantanal.com.br
contato@editorapantanal.com.br

Prólogo

Investigaciones Biológicas, Agrícolas y Ambientales de México es un libro electrónico científico, basado en estudios experimentales desarrollados por un colectivo de prestigiosos investigadores de México y de otros países que, en colaboración, aportan respuestas a problemáticas existentes en dichas ramas del saber. Estos trabajos aparecen divididos en capítulos donde se ofrece información actualizada sobre los avances más recientes en dichas áreas, con un estilo de artículo científico y con referencias bibliográficas de gran nivel de actualización científica.

El proceso de revisión de los capítulos fue desarrollado, bajo la modalidad a doble ciegas, por varios investigadores que participan en el comité editorial de PANTANAL EDITORA. Se agradece a los autores de los respectivos capítulos por la dedicación al atender las sugerencias y comentarios realizados por los revisores, optimizando el tiempo de los procesos de revisión y aceptación.

Los autores

Sumário

Capítulo 7

Isolation and characterization of endophytic bacteria from maize and giant reed with biotechnological and biocontrol potential against *Rhizoctonia zeae*

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Estefanía Morales-Ruiz[1,4]
Gloria M. Zamudio-Aguilasocho[2]
Carmen Martínez-Valenzuela[3]
Ignacio E. Maldonado-Mendoza[1]
Jesús Damián Cordero-Ramírez[2*]

ABSTRACT

Bacterial endophytes provide beneficial effects such as growth promotion and protection against phytopathogens to their host plants. The objective of this study was to isolate endophytic bacteria from maize (*Zea mays* L.) and giant reed (*Arundo donax* L.) to evaluate both their plant growth promotion traits and antagonistic potential against *Rhizoctonia zeae*, an emergent maize pathogen. In total, 133 bacteria from the leaves, stems and roots of giant reed and maize were isolated, and the 10 strains that had the highest percentage of growth inhibition of the phytopathogenic fungus *R. zeae* were selected for further characterization. The strains were identified by 16S rDNA sequencing as belonging to the genera *Rhizobium*, *Staphylococcus*, *Bacillus*, *Acinetobacter*, and *Pseudomonas*. Phenotypic characterization showed that six strains produced endoglucanases, proteases, and lipases; one produced chitinase; five produced siderophores; seven produced IAA; and five produced solubilized phosphate, all of which are important plat-growth promotion and antagonistic traits. In conclusion, this suggests the potential biotechnological use of naturally occurring bacterial endophytes obtained from wild plants for use in agronomically important crops. Additionally, our findings highlight the importance of working with native organisms to

[1] Departamento de Biotecnología Agrícola, CIIDIR Unidad Sinaloa, Instituto Politécnico Nacional, Boulevard Juan de Dios Bátiz Paredes 250, 81101, Col. San Joachin Guasave, Sinaloa, México.
[2] Departamento de Ciencias Naturales y Exactas, Universidad Autónoma de Occidente, Unidad Regional Guasave. Guasave, Sinaloa, México. Avenida Universidad S/N Colonia Villa Universidad, Guasave, Sinaloa, CP 81048
[3] Unidad de investigación en Ambiente y Salud. Boulevard Macario Gaxiola y Carretera Internacional S/N Col. Conrado Espinosa. CP. 81273, Los Mochis Sinaloa México.
[4] Present address: Unidad Académica de Sistemas Arrecifales, Instituto de Ciencias del Mar y Limnología, Universidad Nacional Autónoma de México, Puerto Morelos, México.
* Corresponding author e-mail: elcofra@yahoo.com.mx

fight local fungal infections and reaffirm the need to protect ecological preservation zones as reservoirs of bacterial biodiversity.

INTRODUCTION

One of the most important agricultural crops worldwide, maize (*Zea mays*), is used to feed both humans and livestock and is used in diverse industrial applications, such as biofuel production. In terms of global maize production, Mexico, at 28 million tons, is ranked just behind the USA, China, Brazil, Argentina, Ukraine, and India (FAOSTAT, 2017). Maize is certainly one of the most profitable crops and has experienced increased demand over the past decade (Shiferaw et al., 2011). This has favored its monoculture but has also led to the appearance and increase in fungal diseases such as those caused by *Rhizoctonia* and *Fusarium* (Castro del Ángel et al., 2017).

The genus Rhizoctonia comprises phytopathogenic fungi that infect the roots and leaves of many host plants, including various important agricultural crops such as maize, tomato, rice, and common bean (Devi and Thakur, 2018). It includes a heterogeneous group of filamentous saprophytes and necrotrophic fungi that share several characteristics: they do not form spores but grow by hyphae, some of which specialize into monilioid cells that fuse together to form hard resistant sclerotia that persist in the soil until the conditions are favorable for colonizing a host plant (González García et al., 2006). *R. solani* is considered the most important pathogen in this group and has been isolated from over 200 hosts ranging from ornate plants to agricultural crops. Importantly, in recent decades, *R. solani* and other species, such as *R. zeae* (teleomorph *Waitea circinata*), have been more frequently isolated from agricultural crops with which they do not typically associate (Blanco et al., 2018). This phenomenon has occurred despite agricultural management practices such as crop rotation (i.e., soy-maize, potato-maize, or rice–maize), suggesting that what were once considered to be host-specific strains can actually adapt to new hosts (González-Vera et al., 2010; Ajayi-Oyetunde and Bradley, 2017) with severe consequences for plants that have not evolved mechanisms to contend with this specific fungus (Anderson et al., 2016).

In Mexico, *Rhizoctonia* research has focused mainly on potatoes since this crop suffers the greatest economic damage from this pathogen. *Rhizoctonia*-infected potatoes were previously identified in the northern region of the Mexican state of Sinaloa (Fernández-López, 2011). In recent growing seasons, growers and field advisors have observed the increased incidence and severity of *Rhizoctonia* stem canker and black scurf in potato; dry sunken lesions on stems and roots near the soil line, such as those caused by *Rhizoctonia,* have also been observed on common bean (Rábago-Zavala, 2017). Moreover, maize at the silking stage has been seen to develop blight on the first and second leaf sheaths that spreads and causes nodal cankers in the first and second basal leaves. As stated above, the incidence and severity of these

diseases are more relevant in commercial fields subject to crop rotation, such as potato, common bean, and maize.

Rábago-Zavala (2017) identified 14 *Rhizoctonia* spp. isolates from maize plants in northern Sinaloa, in which ten were identified as *R. zeae* and three as *R. solani*. One of these isolates from *R. zeae* and another from *R. solani* were applied to maize seeds in an earlier study, resulting in 100% and 37% disease severity, respectively, five days after germination; furthermore, the endophytic bacterium *Bacillus cereus sensu lato B*25 inhibited the growth of both fungi in vitro by 35.71% (*R. zeae*) and 43.75% (*R. solani*) (Carreño-Chávez, 2014).

The biological control of *Rhizoctonia* (and phytopathogenic fungi in general) is an attractive, environmentally friendly approach, and several studies have reported the success of endophytic bacteria as biocontrol agents (Rahman et al., 2018; De Silva et al., 2019; White et al., 2019). Endophytic bacteria are a subclass of rhizospheric plant-associated bacteria that live inside plant tissues without causing any damage to the host. Aside from acting as biocontrol agents, some of these bacteria can promote host plant growth and are therefore characterized as plant growth-promoting rhizobacteria (PGPR) (De Silva et al., 2019). Endophytic bacteria provide beneficial effects to plants by directly obtaining nutrients and modulating growth-related hormones, while they indirectly affect the growth of pathogens that produce lytic enzymes or scavenge nutrients (Afzal et al., 2019).

The use of endophytic microorganisms to specifically control *Rhizoctonia* infections has been demonstrated broadly. For example, *Trichoderma harzianium* inhibits mycelial growth and sclerotia formation of *R. solani* f. sp. *sasakii* during maize infection (Devi and Thakur, 2018); *T. harzanium* inoculated with *B. megaterium* increases maize yield in the presence of *R. solani* (Kubheka et al., 2018); and *T. atroviride*, *Phomopsis* sp., *Epicoccum nigrium* and *Alternaria longipes* have shown antagonistic activity in greenhouse experiments against *R. solani* in potato (Lahlali and Hijri, 2010).

The efficacy of endophytes depends greatly on host specificity. Indeed, endophytic colonization of plants is determined by a set of plant and bacterial traits in a complex process that requires very strict communication between the two partners. The endophytic diversity present in a plant depends on several factors, including the species of the host plant. Evidence suggests that endophytes are only capable of plant growth promotion in plants closely related to their natural host (Long et al., 2008). In contrast, other reports have shown that endophytes can promote growth in diverse plant hosts (Compant et al., 2005; Sessitsch et al., 2005; Ma et al., 2011). Thus, it is important to test the host range of endophytes to learn about their potential as growth-promoting or biocontrol agents.

It has been observed that different plant species growing in the same soil can have very different endophytic communities (Afzal et al., 2019). This highlights the importance of isolating and using native – rather than exotic or foreign – microorganisms to fight local infections (Long et al., 2008). Indeed, in

Sinaloa, Mexico, the native endophytic bacterium *Bacillus cereus B*25 has been applied to biologically control maize ear and root rot caused by the fungus *Fusarium verticillioides* (Lizárraga-Sánchez et al., 2015).

Thus, in the present work, we aimed to isolate endophytic bacteria from maize native to the state of Sinaloa and from wild-growing giant reed (*Arundo donax* L.) – a close relative of maize – to test their biocontrol potential against *Rhizoctonia zeae* isolated from infected maize crops in Sinaloa. Those bacteria that inhibited the growth of the fungus in higher percentages were molecularly identified and phenotypically characterized according to their plant growth-promoting traits. Our observations open new possibilities for endophytes obtained from wild-growing plants and their application to become biotechnologically relevant in agriculturally important crops such as maize.

MATERIALS AND METHODS

Isolation of endophytic bacteria

Wild-growing giant reed plants (*Arundo donax* L.) were collected (February 2018) in the Ecological Preservation Zone known as "La Uba", located 26 km south of the municipality of Guasave in the state of Sinaloa, Mexico (25° 29' 42" north latitude, 108° 27' 12" west longitude). Maize (*Zea mays* L.) Voluntary plants (growing in between crops) at approximately 40 to 55 days post-emergence (from vegetative stage V4 up to tasseling stage VT) were harvested adjacent to La Uba. This preservation zone represents the last relict area of endemic natural vegetation of the region and was therefore selected for the isolation of endemic bacteria.

The leaves, stems and roots of five maize plants and five giant reed plants were superficially disinfected by washing them with fresh water and commercial soap for 5 min. Subsequently, tissue sections 1-2 cm in length were disinfected by soaking in 70% ethanol for 1 min and were then immersed in a 1% sodium hypochlorite solution for 10 min, washed with a 10% v/v Tween-20 solution (Sigma, Cat. P7949), rinsed three times with sterile distilled water, and dried over sterile filter paper (Abdallah et al., 2016).

Transverse and longitudinal cuts were made in the tissue fragments, which were then plated onto LB (Sigma, Cat. L3022), King B (Sigma, Cat. 6078) and actinomycete isolation agar (AIA) (Fluka, Cat. 17117) plates and incubated at 30°C for 2 days. Morphologically distinct colonies were replated until they were fully purified. All isolates were cryopreserved in 15% glycerol.

Fungal isolate

The *Rhizoctonia zeae* strain was kindly provided by Dr. Rubén Félix-Gastélum (Universidad Autónoma de Occidente; Los Mochis, Sinaloa, Mexico). The strain was isolated from maize crops, preserved on PDA (BD Bioxon, Cat. 211900) slant cultures that were incubated for five days at 25°C in

the dark and then covered with a 1-cm layer of mineral oil (Faga-Lab, Cat. 1300) (Montesinos-Matías et al., 2015) and kept at 4°C until use.

In vitro antifungal activity/antagonism assays

The interaction between the bacterial isolates and *R. zeae* was assayed using a dual culture technique (Prince et al., 2011). *R. zeae* was grown on PDA for seven days at 25°C in the dark. On day seven, 5-mm disks containing mycelia were extracted with a sterilized cork borer. Prior to assaying, the bacterial isolates were grown on LB broth for 24 h at 30°C with 200 rpm shaking. For the assay, a 5-mm PDA disk with *R. zeae* mycelia was placed at the center of a PDA agar plate, and 10 µL of a bacterial isolate was inoculated on opposite (yet equidistant) sides of the plate in the shape of a cross. One PDA plate could thus be used to assay four different bacterial strains. The plates were then incubated at 25°C for three days in the dark. As a control, a plate was inoculated with *R. zeae* alone in LB broth. Each assay was performed in triplicate. The percent inhibition was calculated using the formula:

$$\%\ inhibition = \frac{r - r_1}{r} \times 100$$

in which r = fungal growth measured in mm from the center of the colony radially toward the edge of the plate in the absence of the antagonist, and r_1 = fungal growth measured in mm from the center of the colony radially toward the antagonist.

Enzyme activity assays

All growth conditions were the same in the following assays, with single colonies grown in 5 mL of LB broth at 30°C and 200 rpm for 24 h. When needed, 1 mL of culture was centrifuged for 10 min at 16,000 x *g*, and the supernatant was recovered for use. All assays were performed in triplicate.

Total protease production was tested using skim milk agar plates (Jones et al., 2007). Fifty microliters of the supernatant was inoculated inside 6-mm diameter holes previously made in skim milk agar plates (LB agar with 10% w/v of a 10% w/v skim milk solution (Svelty, Nestle®) both previously sterilized at 121°C for 15 min and mixed while still warm) and incubated for 24 h at 30°C. *B. subtilis* (*Bs*TA16) was used as a positive control (Khalil et al., 2021). Protease activity was identified by the formation of a clear halo around the well.

Chitinase production was evaluated on colloidal chitin agar plates. Ten microliters of the culture was spotted onto colloidal chitin agar plates: $(NH_4)_2SO_4$, 0.5 g (Faga Lab, Cat. 2332); $K_2HPO_4$4, 0.1 g (Fermont, Cat. 35842); NaCl, 1 g (Faga Lab, Cat. 2153); $MgSO_4 \cdot 7H_2O$, 0.01 g (Faga Lab, Cat. 2344); yeast extract, 0.05 g (BD Bioxon, Cat. 230900); colloidal chitin, 0.5 g (Roberts and Selitrennikoff, 1988); and agar, 1.5 g (BD Bioxon, Cat. 215000) in 50 mL of distilled water and sterilized at 121°C for 20 min. *B.*

cereus sensu lato B25 was used as a positive control. Chitinase enzyme activity was identified by the formation of a clear zone around bacterial cells after 5 days of growth at 30°C (Figueroa-López et al., 2017).

β-1,4-endoglucanase activity was qualitatively evaluated using carboxy methyl cellulose (CMC) (Sigma Aldrich, Cat. 419273) as a substrate and 1% (w/v) CMC agar plates. For this test, 50 µL of each supernatant was inoculated into 6-mm diameter holes previously made in 1% CMC agar plates. Next, the plates were incubated for 24 h at 50°C (Ramírez and Coha, 2003). Cellulase enzyme from *Trichoderma reesei* (Sigma Aldrich, Cat. C8546) was used as a positive control. β-1,4-endoglucanase activity was revealed by adding 5 mL of 1% (w/v) Congo red dye (Sigma, Cat. C6767) for 15 min. The dye was subsequently removed, and the excess was washed with 5 mL of 2 M NaCl (Faga Lab Cat. 2153) for 5 min. Finally, glucanase activity was determined according to the presence of a clear halo around the well, measured in centimeters (Teather and Wood, 1982). Microorganisms were grouped according to the glucanase activity (hydrolysis halo size) criterion described in Ramírez and Coha (2003), with some modifications: absent, 0.6 – 0.79 cm; scarce, 0.80 – 0.99 cm; regular, 1.00 – 1.19 cm; good, 1.20 – 1.39; very good, ≥ 1.4.

Lipolytic activity was qualitatively evaluated on glyceryl tributyrate (Sigma Aldrich, Cat. T8626) (1% (w/v) tributyrin) agar plates (Stead, 1984). Fifty microliters of each culture supernatant was transferred and placed into 6-mm diameter holes previously made in the plates and incubated for 48 h at 30°C. Lipolytic activity was identified by the formation of a clear halo around the well. *Serratia liquefaciens* was used as a positive control.

Hemolysis tests were performed to rule out isolates with a potential pathogenic effect on humans, *i.e.*, bacteria that partially (α-hemolysis) or totally (β-hemolysis) lyse erythrocytes. For the assay, 50 µL of each supernatant was transferred and placed into 6-mm diameter holes previously made in blood agar plates (MCD LAB, Cat. 7504). LB broth was used as a negative control. The plates were then incubated at 37°C for 48 h. The strains were classified according to the observed lysis halos as follows: α (partial lysis), β (total lysis), or ɣ (absence of hemolysis) (Tille, 2013).

Indole acetic acid (IAA) production

IAA production was assayed colorimetrically, as described by Loper and Schroth (1986). Single colonies of each strain were grown on LB broth for 48 h at 30°C with 200 rpm shaking. After 10 min of centrifugation at 12,000 x *g*, the culture supernatants were recovered. One hundred microliters of each supernatant was incubated with 100 µL of Salkowski's reagent (0.2 g of ferric chloride (Faga Lab, Cat. 2143), 4.23 mL of sulfuric acid (Faga Lab, Cat. 2047) in a final volume of 10 mL) for 30 min in the dark, after which the absorbance was recorded at 530 nm. A calibration curve (R^2 = 0.9962) was performed with IAA (Sigma, Cat. 12886) concentrations ranging from 0 to 60 µmol l^{-1} in LB broth to extrapolate the obtained data. Each assay was performed in triplicate.

Phosphate solubilization

The phosphate solubilization ability of the strains was assayed on Pikovskaya agar plates (for 1 L: $Ca_3(PO_4)_2$, 2.5 g (Sigma, Cat. 693898); glucose, 13 g (Faga Lab, Cat. 2165); $(NH_4)_2SO_4$, 0.5 g (Faga Lab, Cat. 7738); NaCl, 0.2 g (Faga Lab, Cat. 2153); $MgSO_4 \cdot 7H_2O$, 0.1 g (Faga Lab, Cat. 2344); KCl, 0.2 g (Faga Lab, Cat. 1062); yeast extract, 0.5 g (Sigma, Cat. 8213); agar, 15 g (BD Bioxon, Cat. 215000); pH 7.2).

Single colonies of each strain were grown on LB broth for 24 h at 30°C with 200 rpm shaking. Next, 10 µL of each culture was spotted onto Pikovskaya agar plates in triplicate and incubated for 7 days at 30°C. A clear halo surrounding the colony indicates a positive reaction. The solubilization index (SI) was calculated using the following formula (Edi Premono et al., 1996):

$$SI = \frac{colony\ diameter + halo\ zone\ diameter}{colony\ diameter}$$

Siderophore production

Siderophore production was tested using the overlaid chrome azurol S (O-CAS) method (Pérez-Miranda et al., 2007) with slight modifications. First, all tested strains were grown in LB broth for 24 h at 30°C with 200 rpm shaking. Next, 10 µL of each culture was spotted onto LB agar plates supplemented with 200 µmol l^{-1} 2,2-bipyridyl (Sigma, Cat. D216305) to generate an iron-deprived state (Kalidasan et al., 2018). The agar plates were then incubated at 30°C for 24 h.

The CAS medium to be poured as an overlay was prepared as two different solutions: a PIPES solution (PIPES, 6.048 g (Sigma, Cat. P6757) in 180 mL of double-distilled water, pH 5.8) and a chrome azurol S (CAS) solution, which is a mix of three different solutions: solution A (chrome azurol S (Pfaltz and Bauer, Cat. C21430), 30 mg in 25 mL of ddH_2O); solution B ($FeCl_3$ (Faga Lab, Cat. 2143), 2.7 g in 10 mL of 10 µmol l^{-1} HCl (J. T. Baker, Cat. 9535-02)); and solution C (hexadecyltrimethylammonium bromide (HDTMA, Sigma, Cat. H6269), 36 mg in 40 ml of ddH_2O). The three solutions were mixed in the following proportions: 4.5 mL of solution B and 40 mL of solution C were added to 25 mL of solution A for a final volume of 69.5 mL. Both the PIPES and CAS solutions were autoclaved at 121°C for 15 min. Subsequently, 20 mL of the CAS solution was added to the PIPES solution. Finally, 1% (w/v) agarose (Nara Biotec, Cat. NR-90125) was added as a gelling agent and heated to dissolve it. Then, 10 or 30 mL (for 80- or 150-mm diameter petri dishes) was poured over the previously inoculated LB + bipyridyl agar plates. With this assay, after 25 min to a maximum of 24 h, a change in color from blue to purple or orange can be observed exclusively surrounding catechol- or hydroxamate-producing microorganisms, respectively. *Acinetobacter calcoaceticus* (AcDB3) was used as a positive control for siderophore production (Khalil et al., 2021), and LB broth was used as a negative control.

Molecular identification

Genomic DNA (gDNA) extraction was performed on the selected bacterial isolates using the DNeasy® Blood and Tissue kit (QIAGEN, Cat. 69506), according to the supplier's instructions. The gDNA concentration was quantified using a NanoDrop® spectrophotometer (Thermo Fisher, Cat. ND2000).

The gDNA was then used as a template for PCR amplification of the 16S region of the ribosomal DNA with the universal oligonucleotides F2C (5'-AGAGTTTGATCATGGCTC-3') and C (5'-ACGGGCGGTGTGTAC-3') (Shi et al., 1997) and the GoTaq® Flexi DNA Polymerase enzyme (Promega, Cat. M3005). The PCR conditions were as follows: one denaturing cycle at 95°C for 4 min; 33 cycles of denaturing at 95°C for 30 s, annealing at 60°C for 30 s, and polymerization at 72°C for 45 s; and a final extension cycle at 72°C for 7 min. The amplicons obtained were sequenced at the Laboratorio de servicios genómicos (Labsergen), LANGEBIO, CINVESTAV (Irapuato, Mexico), and a BLAST search of the sequences was performed using the NCBI database.

RESULTS

Isolation and selection of endophytic bacteria with antagonistic potential toward Rhizoctonia

A total of 133 endophytic bacteria were isolated from maize (43 isolates: 10 from leaves, 6 from stems, and 27 from roots) and giant reed (90 isolates: 23 from leaves, 17 from stems, and 50 from roots). To avoid the possibility of working with potential human pathogens, all α- (33) and β-hemolytic (18) strains were discarded, leaving 79 γ-hemolytic strains (6 from maize and 73 from giant reed).

The antifungal activity of the 79 γ-hemolytic strains was evaluated *in vitro* in PDA plates using a dual culture technique. Ten strains (4 from maize and 6 from giant reed) decreased the growth of *R. zeae* (Figure 1), with inhibition percentages ranging from 21 to 50% (Table 1).

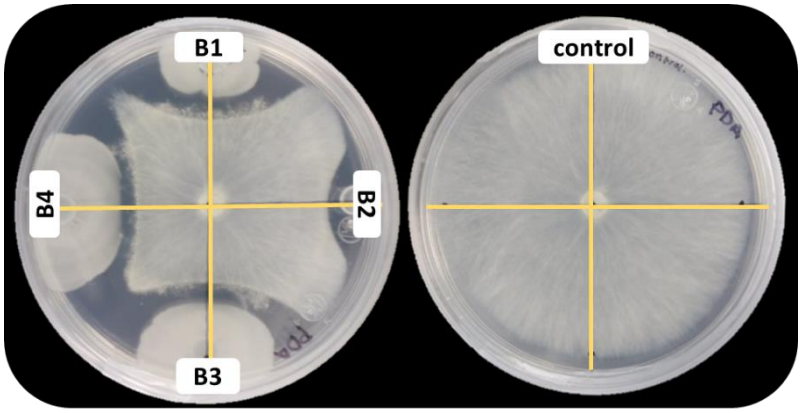

Figure 1. *Bacteria-fungus antagonism assays*. Dual culture assays were performed to determine if any of the isolated bacterial strains inhibited the growth of *Rhizoctonia zeae*. Left plate: *R. zeae* (center of the plate) and 4 different bacteria forming a cross (B1-B4) were grown on PDA plates. An inhibition halo is formed

around the colony if a bacterium inhibits fungal growth. Right plate: as a control, *R. zeae* was grown alone in LB broth without any bacterial inoculum.

Molecular identification of endophytic isolates

The 10 strains that inhibited the growth of *R. zeae* were identified by 16S rDNA amplification and sequencing. The sequences were submitted to BLAST searches using the NCBI database, and the results are summarized in Table 1. We found species belonging to *Rhizobium*, *Staphylococcus*, *Bacillus*, *Acinetobacter*, and *Pseudomonas*, with an identity percentage at the sequence level above 98%.

Table 1. BLAST results for the sequences from the 10 bacterial isolates that inhibited the growth of *Rhizoctonia zeae.*

Strain	Query Cover (%)	E value	Identity (%)	Accession no.
Rhizobium pakistanense	96	0	100	NR_145565.1
Staphylococcus warneri	100	0	100	NR_025922.1
Bacillus aryabhattai	100	0	99	NR_118442.1
Staphylococcus saccharolyticus	100	0	100	NR_113405.1
Acinetobacter radioresistens	98	0	100	NR_114074.1
Bacillus velezensis	100	0	100	NR_075005.2
Pseudomonas guariconensis	96	0	98	NR_135703.1
Bacillus velezensis	100	0	100	NR_075005.2
Pseudomonas plecoglossicida	94	0	100	NR_114226.1
Pseudomonas aeruginosa	98	0	99	NR_117678.1

Characterization of plant growth promotion and antagonism traits

Next, the ten strains were evaluated for their ability to produce protease, chitinase, endoglucanase, lipase, siderophores, and IAA and to solubilize phosphate. These traits are related to both plant growth-promoting and antagonistic activities, and their results are summarized in Table 2. Examples of the phenotypic characterization are shown in Figure 2.

Only *Staphylococcus warneri* produced chitinase on colloidal chitin agar. β-1,4-endoglucanase activity was observed in the maize strain *B. velezensis* and the giant reed strain *B. aryabhattai* with good activity and in four other strains with very good activity, including *S. warneri, S. saccharolyticus, B. velezensis* and *Rhizobium pakistanense.*

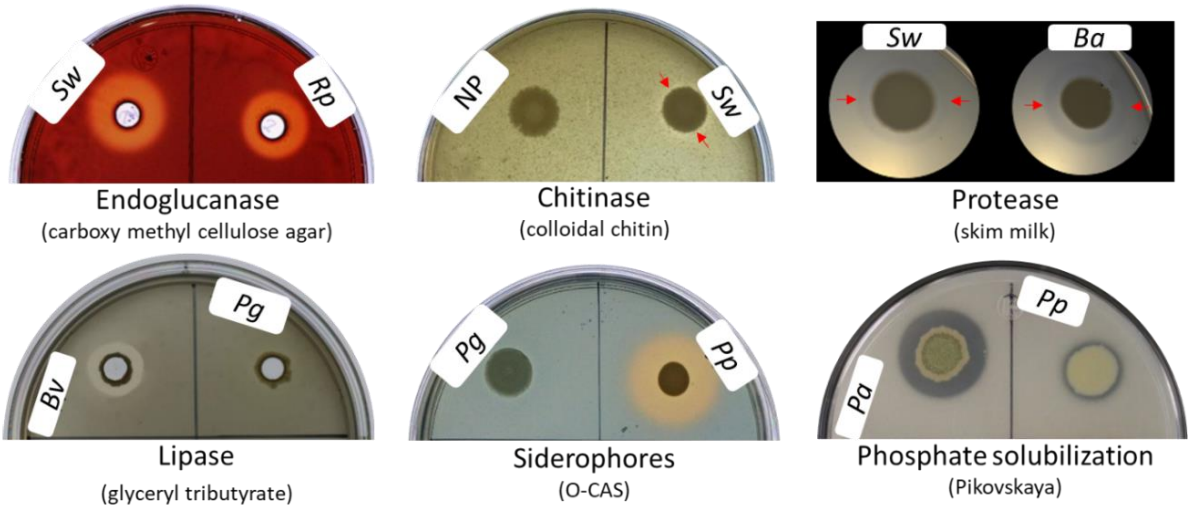

Figure 2. *Examples of the phenotypic characterization of bacteria that antagonize fungal growth*. Phosphate solubilization, protease production, lipase activity, endoglucanase activity, and chitinase production were all revealed by the formation of a clear halo around the colony on their respective plates. Siderophore production was revealed by the formation of an orange halo around the colony. Abbreviations: *Pa, Pseudomonas aeruginosa; Pp, P. plecoglossicida; Sw, Staphylococcus warneri; Ba, Bacillus aryabhattai; Bv, B. velezensis; Pg, P. guariconensis; Rp, Rhizobium pakistanense*; NP, nonproducer unidentified strain.

Protease activity was found in six out of the 10 strains, i.e., *S. saccharolyticus*, *S. warneri*, *B. aryabhattai*, *B. velezensis* (both strains isolated from maize and giant reed), and *P. aeruginosa*, of which *B. aryabhattai and P. aeruginosa* had the highest activity according to the production of clear halos (>4 mm) in the skim milk assay. Lipase activity was detected in the six strains *A. radioresistens*, *S. saccharolyticus*, *S, warneri*, *B. velezensis* (both strains isolated from maize and giant reed), and *P. aeruginosa*, which formed 2-4.5 mm hydrolysis halos on glycerol tributyrate agar plates, with *S. warneri* displaying the highest activity.

Regarding siderophore production, five strains, *A. radioresistens*, *S. warneri*, *P. guariconensis*, *P. plecoglossicida*, and *P. aeruginosa*, produced 3-6 mm orange halos, corresponding to hydroxamate siderophores in the O-CAS experiment. All the strains except *S. saccharolyticus*, *B. velezensis* and *R. pakistanense* produced IAA as determined by the Salkowski reaction, with the strain *P. aeruginosa* displaying the highest IAA production (9.17 μM).

Finally, the phosphate solubilization assay on Pikovskaya agar plates revealed that five strains, *S. saccharolyticus*, *B. velezensis*, *P. guariconensis*, *P. plecoglossicida*, and *P. aeruginosa*, were able to solubilize phosphate (solubilization index > 1), of which *P. aeruginosa* from maize and *B. velezensis* from giant reed had the highest SI values (1.83 and 1.47, respectively).

Table 1. Plant growth promotion and antagonistic traits of the bacterial isolates evaluated.

Strain	*R. zeae* % *inhibition**	*Endoglucanase*†	*Chitinase*‡	*Protease*‡	*Lipase*‡	*Siderophores*‡	*IAA (µM)*	*SI*
Giant reed								
Acinetobacter radioresistens	50.57±0.05	0.6	-	-	++	+++	0.075	-
Staphylococcus saccharolyticus	45.71±0.1	1.46	-	+	++	-	-	1.35
Staphylococcus warneri	45.71±0.1	1.8	+	++	+++	++	0.31	-
Bacillus aryabhattai	44±0.05	1.26	-	+++	-	-	4.3	-
Bacillus velezensis	44±0.05	1.93	-	+	+	-	-	1.47
Rhizobium pakistanense	21.14±0.05	1.46	-	-	-	-	-	-
Maize								
Pseudomonas aeruginosa	40±0.05	0.6	-	+++	+	+	9.17	1.83
Bacillus velezensis	34.28±0.05	1.26	-	++	++	-	3.49	-
Pseudomonas guariconensis	29.71±0.05	0.6	-	-	-	+++	6.2	1.08
Pseudomonas plecoglossicida	25.71±0.1	0.6	-	-	-	+++	5.3	1.08

*The values correspond to the mean ± SD. †Hydrolysis halo size in centimeters. Corresponding glucanase activity: absent, 0.6 – 0.79 cm; scarce, 0.80 – 0.99 cm; regular, 1.00 – 1.19 cm; good, 1.20 – 1.39; very good, ≥ 1.4. ‡The results are expressed as a function of the diameter of the halos formed around the colonies: +, <3 mm; ++, >3<4 mm; +++, >4 mm. IAA, indole acetic acid; SI, phosphate solubilization index.

DISCUSSION

One of the most important cereals grown around the world, maize, is susceptible to fungal infections that inflict important economic losses. *Fusarium verticillioides* is the most commonly found fungus to infect maize, and a number of biocontrol strategies have been successfully applied to control this pathogen (Lizárraga-Sánchez et al., 2015; Figueroa-López et al., 2016; Martínez-Álvarez et al., 2016). With this in mind, we aimed to find a native endophyte that could antagonize *Rhizoctonia zeae* in maize, as the incidence of this emerging pathogen has been increasing in maize fields in the Mexican state of Sinaloa (Rábago-Zavala, 2017). In the municipality of Guasave (Sinaloa), we collected giant reed plants (*Arundo donax* L.) from the ecological preservation zone known as La Uba, an area of endemic relictual vegetation, and voluntary maize plants were collected from an adjacent maize field. Giant reed is a promising grass

for use in the production of biofuel (Antal, 2018). Since both giant reed and maize are members of the family Poaceae, we isolated endophytes of giant reed to determine if they could also colonize maize and be used as a possible source of biocontrol agents, as well as contribute to our understanding of endophyte-plant host specificity.

We isolated a total of 133 endophytes from maize and giant reed. After discarding α- and β-hemolytic isolates, 79 γ-hemolytic strains were retained to evaluate their potential to antagonize the growth of *R. zeae*. Ten strains inhibited fungal growth by 21-50%, similar to the inhibition percentages reported for other maize endophytes used as biocontrol agents (Mousa et al., 2015; Figueroa-López et al., 2016; Marag and Suman, 2018). We amplified and sequenced the 16S rDNA region of these strains and found species belonging to *Rhizobium, Staphylococcus, Bacillus, Acinetobacter,* and *Pseudomonas*. Among these genera, all strains have been reported as endophytes, except *S. saccharolyticus*. For example, *Rhizobium pakistanense* was previously isolated from peanut (*Arachis hypogaea* L.) nodules (Khalid et al., 2015); *S. warneri* has been reported as an endophyte of maize (Bodhankar et al., 2017), rice (Chaudhry et al., 2017), and common bean (*Phaseolus vulgaris*) (Costa et al., 2012); *B. aryabhattai* has been isolated from sugarcane (*Saccharum officinarum* L.) (Kruasuwan and Thamchaipenet, 2016), maize (Marag and Suman, 2018), and tomato (*Solanum lycopersicum* cv. Jiabao) (Tian et al., 2017); *A. radioresistens* has been shown to be an endophyte of both tomato (Rashid et al., 2012) and maize (Thanh and Diep, 2014). *B. velezensis* is reported to be a maize endophyte with biocontrol activity against *Sclerotinia sclerotium* (Massawe et al., 2018) and has also been found in peanut (Chen et al., 2019). *P. guariconensis* is a sugarcane (Kruasuwan and Thamchaipenet, 2016) and tomato endophyte (Ramírez-Bahena et al., 2015). *P. plecoglossicida* improves banana yield and reduces the severity of *Mycospharella fijiensis* infections (Marcano et al., 2016) and has also been reported as a poplar tree (*Populus trichocarpa*) endophyte (Moore et al., 2006). Finally, *P. aeruginosa* has been reported as an endophyte in black pepper, where it controls the oomycete plant pathogen *Phytophtora capsici* (Kumar et al., 2013), and in chili (*Capsicum annum* L.), where it has shown antagonism against the fungus *Colletotrichum capsica* (Allu et al., 2014).

We then evaluated several traits related to plant growth promotion and antagonism. Only *S. warneri* produced chitinase on colloidal chitin agar. Chitinase, an enzyme that degrades the chitin found on the cell wall of fungi, is produced by several biocontrol bacteria and fungi (Banerjee and Mandal, 2019). The production of other hydrolytic enzymes with biocontrol activity, such as protease, glucanase and lipase, has also been described for several bacteria (Afzal et al., 2019). Accordingly, we found that several of our isolates produced some of these enzymes to some extent (Table 1).

Siderophore production is both an antagonistic and a plant growth-promoting trait, and we found that some of our isolates produce hydroxamate siderophores. IAA and phosphate solubilization traits were also present in some of our strains. None of the ten isolates produced every trait that we evaluated, not

even those isolates that had the highest percentages of *R. zeae* growth inhibition. Nevertheless, it has been reported that the antagonism displayed by biocontrol agents is the result of the synergistic mode of action of different mechanisms (White et al., 2019). In our group, we are currently working to develop biocontrol/biofertilizer agents based on native bacteria for commercial purposes.

CONCLUSION

This report highlights the importance of working with native organisms to resolve local problems caused by fungal pathogens. Our results also reaffirm the need to protect ecological preservation zones, which are an important reservoir of bacterial biodiversity that harbor biotechnological potential for use as biofertilizers in agronomically important crops.

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Ciencias Ambientales

Índice

Sobre los organizadores

Dr. Leandris Argentel Martínez

Profesor e Investigador Titular "C" del Tecnológico Nacional de México, Campus Valle del Yaqui (ITVY). Miembro del Sistema Nacional de Investigadores, Nivel 1. Profesor Perfil Deseable (PRODEP) de la Secretaría de Educación Pública de México, Líder del Cuerpo Académico ITVAYA-CA-3. Líneas de investigación: Fisiología Vegetal, Bioquímica, Biología Celular y Molecular en plantas y microorganismos. Doctorado en Ciencias Biotecnológicas. Desarrollo de investigaciones sobre mecanismos fisiológicos, rutas anapleróticas y mecanismos moleculares activados por los organismos durante su adaptación estreses abióticos. Uso de marcadores moleculares de tolerancia de los organismos al estrés abiótico (salinidad, sequía y calor). Manejo de técnicas de isótopos estables para el seguimiento de reacciones bioquímicas en células y tejidos. Aplicación de técnicas experimentales univariadas y multivariadas para el procesamiento de datos. Entre sus principales proyectos, se encuentra vigente en 2022 "Aplicaciones del microbioma y el metaboloma de la *Parkinsonia aculeata* L. Sp. Pl. para la mitigación de estreses biótico y abiótico en el semidesierto y en especies de interés agrícola en México" correo electrónico para contacto: oleinismora@gmail.com

Dra. Ofelda Peñuelas Rubio

Profesor e Investigador Titular "C" del Tecnológico Nacional de México, Campus Valle del Yaqui (ITVY). Miembro del Sistema Nacional de Investigadores, Nivel 1. Profesora con Perfil Deseable (PRODEP) de la Secretaría de Educación Pública de México, miembro del Cuerpo Académico ITVAYA-CA-3. México. Realizó dos estancias posdoctorales (Enero 2016 - Diciembre 2017) dentro del programa de Estancias Nacionales de CONACYT en el Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional unidad Sinaloa del Instituto Politécnico Nacional en el área de Ecología Molecular de la Rizósfera. Es Doctora en Ciencias especialidad en Biotecnología. Su quehacer científico lo desarrolla en el área agrícola, principalmente en el manejo sustentable de los recursos implicados en los agroecosistemas y el aprovechamiento de la microbiota del suelo. Ha participado en colaboración con distintos grupos de investigación lo que le ha permitido participar en proyectos multidisciplinarios y en publicaciones científicas. Email para contacto: ofeperub@gmail.com

Pantanal Editora
Rua Abaete, 83, Sala B, Centro. CEP: 78690-000
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