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ORIGINAL ARTICLE

The Mexican giant maize of Jala landrace harbour plant-growth-promoting rhizospheric and endophytic bacteria

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Abstract

The giant landrace of maize Jala is a native crop cultured in Nayarit and Jalisco States in the occident of México. In this study, after screening 374 rhizospheric and endophytic bacteria isolated from rhizospheric soil, root, and seed tissues of maize Jala, a total of 16 bacterial strains were selected for their plant-growth-promoting potential and identified by 16S rRNA phylogenetic analysis. The isolates exhibited different combinations of phenotypic traits, including solubilisation of phosphate from hydroxyapatite, production of a broad spectrum of siderophores such as cobalt, iron, molybdenum, vanadium, or zinc (Co^{2+}, Fe^{3+}, Mo^{2+}, V^{5+}, Zn^{2+}), and nitrogen fixation capabilities, which were detected in both rhizospheric and endophytic strains. Additional traits such as production of 1-aminocyclopropane-1-carboxylate deaminase and a high-rate production of Indoleacetic Acid were exclusively detected on endophytic isolates. Among the selected strains, the rhizospheric *Burkholderia* sp., and *Klebsiella variicola*, and the endophytic *Pseudomonas protegens* significantly improved the growth of maize plants in greenhouse assays and controlled the infection against *Fusarium* sp. 50 on fresh maize cobs. These results present the first deep approach on handling autochthonous microorganisms from native maize with a potential biotechnological application in sustainable agriculture as biofertilizers or biopesticides.

Keywords Indoleacetic acid · Maize · Nitrogen fixation · Phosphate solubilisation · Plant-growth-promoting bacteria (PGPB) · Siderophore

Introduction

Jala maize (*Zea mays*) is a Mexican giant maize landrace cultured in the fertile volcanic valley of Jala in the State of Nayarit, Mexico (Kempton 1924). Its farming demands waterlogged soils, producing plants up to 5 m long and cobs between 45 and 60 cm long after a life cycle of 7–8 months (Rice 2007; Prasanna 2012). Outside the Jala Valley, the growth of Jala maize do not reach its normal size (Aguilar-Castillo et al. 2006). Extensive agriculture, economic pressures, and market demands have significantly reduced the number of landraces cultivated in the country and have displaced many native varieties, including Jala (Rice 2007). Moreover, intensive cropping homogenizes bacterial diversity and reduces the complexity of bacterial networks in soils (Figuerola et al. 2015; Karimi et al. 2019; Wang et al. 2020). Geographical, climatic, and edaphic differences between crop soils can be used to predict the high and specific microbial diversity associated with each native landrace,

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soil type, and agricultural practice (Aira et al. 2010; Bouffaud et al. 2012; Zhou et al. 2017). The conservation of the current diversity of maize landraces and their germplasm represents a valuable cultural heritage and a profitable biological resource for the development of improved varieties with suitable phenotypes. Research on maize ecology should include the study of its own germplasm and microbiome as a holobiont.

Plant-growth-promoting bacteria (PGPB) colonize plant tissues without causing any symptom of disease or competition for nutrients. PGPB facilitate nutrient uptake, participate in rhizoremediation, modulate plant stress control, reduce disease damage, antagonize soil-borne pathogens, and induce systemic resistance, among other activities (Saleem et al. 2007; Beneduzi et al. 2012; Weber et al. 2018). The culturable fraction of autochthonous PGPB associated with maize has an important biotechnological application in sustainable agriculture as biofertilizers and/or biopesticides.

Several rhizospheric and endophytic bacteria have been isolated from maize plants with potential plant-growth-promoting (PGP) characteristics such as biological nitrogen fixation, phosphate solubilization, siderophore secretion, heavy metal stress tolerance, and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase production. Among these maize-associated bacteria, *Azospirillum*, *Enterobacter*, *Brevundimonas*, *Pseudomonas*, *Pantoea*, *Arthrobacter*, and *Achromobacter* are frequently described as PGPB; *Bacillus* and *Pseudomonas* are highlighted as antagonists of *Fusarium* species; and *Klebsiella*, *Herbaspirillum*, *Burkholderia*, and *Rhanella* are known nitrogen-fixing bacteria on grass-like plants including maize (Reis et al. 2004; Montañez et al. 2009; Pereira and Castro 2014; Douriet-Gámez et al. 2018). Due to the rhizospheric effect, the largest population of these bacteria has been found to be associated to the root, which is an environment of high metabolic activity between the plant and microorganisms (Bulgarelli et al. 2013; Gaiero et al. 2013).

A recent report on microbial endophytes from ten different varieties (landraces) of maize from Canada, United States of America, and Mexico, described an isolate of *Pantoea ananatis* from Jala seeds that produces auxins (Johnston-Monje and Raizada 2011). Other works have reported the biofertilization potential of a mixture of *Sphingomonas* sp., *Azospirillum brasiliense*, and *Acinetobacter* sp. when applied to an autochthonous blue maize from the state of Tlaxcala, Mexico (non-specified landrace), which improves plant growth under desiccation stress (Molina-Romero et al. 2017).

Although information about the endophytic bacteria of maize is extensive, few works address the bacterial diversity within native maize of Mexico, which is the origin centre, domestication, and diversification of this grass (Buckler and Stevens 2006). The purpose of this study was to evaluate the biotechnological potential of a collection of PGPB isolated from the rhizosphere and maize tissues of the native landrace Jala. This collection of culturable PGPB might harbour an important potential for species-specific biofertilization and biopesticide application on maize.

Materials and methods

Sample collection and soil characterization

Soil and maize plants were collected from the Jala municipality (14° 25.2′ 24″ N; 21° 05.7′ 6″ E) in the state of Nayarit, Mexico. Plants were carefully removed from soil, collected in sterile plastic bags, and stored at 4 °C for the isolation of PGPB. Soil was collected from five equidistant points within the field, using a soil profile sampler at 20–30 cm depth. Soil samples were mixed before storage at 4 °C to be later analysed. The soil had a neutral pH in water (6.97), a loamy texture, and was composed of 39.32% sand, 40.36% silt, 20.32% clay, 3.08% organic matter, 12 ppm inorganic N, 120 ppm Bray P, 478 ppm K, and 94.1 ppm Fe.

Bacterial isolation and growth conditions

Root and grain samples were disinfected for 5 min with a 2% sodium hypochlorite solution containing 100 µL of Tween-20 followed by a 3-min rinse with 70% ethanol. Samples were washed five times with sterile distilled water and aseptically macerated in a mortar. One gram of rhizospheric soil attached to the roots was washed out and processed. Direct isolation and enrichment cultivation were performed in solid and semi-solid nitrogen-free media, including Winogradsky, Bridges, Haahtela with 1% of glucose and Haahtela with 1% of mannitol and sucrose as carbon sources (Bridges 1981; Haahtela et al. 1983; Atlas 2010). Direct isolation of bacteria was performed using the serial dilution method. The enrichment cultures were obtained after inoculating 1 g of the samples into nitrogen free semi-solid agar. They were incubated at 28 °C and transferred to fresh media every 15 days for 60 days. Pure cultures were obtained from both direct isolation and enrichment cultivation and preserved at − 70 °C in a solution of 25% glycerol (*v*/*v*). All cultures were incubated under aerobic conditions at 28 °C.

DNA extraction and PCR amplification of 16S rRNA

DNA extraction was performed as described by Hoffman and Winston (1987) DNA was diluted in 20 µL of water and quantified by measuring the absorbance at 260 nm.

Bacterial isolates were identified by the analysis of the 16S rRNA sequence using the primers and the conditions reported by Relman (1993) using 100 ng of template DNA,

1 × Green GoTaq Flexi Buffer, 10 pmol L^{-1} of a dNTP mixture, 1 × GoTaq Flexi DNA, 10 pmol L^{-1} of each primer, and 3.5 mmol L^{-1} of $MgCl_2$ in a final volume of 25 µL. PCR products (1.4 kb) were purified using the ZymocleanTM Gel DNA Recovery Kit (ZYMO RESEARCH, USA) and sequenced through a DNA sequencing service (Macrogen Inc., Korea).

Sequence analysis

The resultant nucleotide sequences were compared using the BLAST tool (Altschul et al. 1997) and aligned with the most similar sequences and with sequences from related species collected from the non-redundant GenBank database of 16S ribosomal RNA from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov).

All the sequences were aligned and manually edited with SEAVIEW 3.2 software (Galtier et al. 1996). The most appropriate model of nucleotide substitution was selected using the software jModelTest 2.0 (Darriba et al. 2012). The similarity percentage between sequence pairs was calculated using MatGAT software (Campanella et al. 2003). All sequences were submitted to the GenBank database.

Characterization of isolates as PGPB

Phosphate solubilization

The phosphate solubilization capacities of the strains were tested on the National Botanical Research Institute Phosphate (NBRIP) agar medium, which uses $Ca_3(PO_4)_2$ as a sole source of insoluble phosphate (Mehta and Nautiyal 2001). The agar plates were inoculated with 5 µL of bacterial solution adjusted to an optical density at 600 nm (OD600) of 0.8. After incubation at 28 °C for 3 days, the P-solubilizing halo zone was measured, and the phosphate solubilization index (PSI) was calculated as the ratio between the diameter of the halo and the diameter of the colony.

The quantitative phosphate solubilization test was performed by the modified molybdenum blue method (He and Honeycutt 2005; Bashan et al. 2013) with hydroxyapatite as the sole source of insoluble phosphorus.

Siderophore production

Siderophore production was detected following the universal chrome azurol S (CAS) agar plate assay (Schwyn and Neilands 1987) supplemented with five different ions from the inorganic salts: ferric sulfate $Fe_2(SO_4)_3$, copper sulfate $CuSO_4$, sodium molybdate Na_2MoO_4, sodium metavanadate $NaVO_3$ and zinc sulfate $ZnSO_4$. CAS-blue agar plates were inoculated with a spot of 5 µL of a bacterial suspension adjusted to 0.8 (OD600) and incubated at 28 °C for 48 h. The positive production of siderophore was detected by the production of a yellow halo around the bacterial growth. The ion capture index was calculated as the PSI.

Quantification of indoleacetic acid (IAA)

Bacterial indole-3-acetic acid production was estimated by a colorimetric assay using Salkowski's reagent (35% $HClO_4$, 0.01 mol L^{-1} $FeCl_3$), which is a common method for indole-type molecule estimation, including auxins (Patten and Glick 1996). The isolates were grown in Tryptic Soy Broth (TSB) and TSB supplemented with L-tryptophan as a precursor of IAA (Gordon and Weber 1951; Leveau and Lindow 2005). The isolates were incubated at 28 °C for 4 days. The supernatant was obtained after 15 min of centrifugation at 18,516×*g*. The reaction was performed by mixing 0.5 mL of the supernatant with 1 mL of Salkowski's reagent. The mixture was incubated in darkness for 30 min. The absorbance was estimated at 530 nm, and the IAA concentration was determined with an IAA standard curve (Szkop et al. 2012).

ACC deaminase activity

Isolates were tested to growth on a medium with 1-aminocyclopropane-1-carboxylic acid (ACC) as the sole source of nitrogen. Liquid Glucose Ivo medium (LGI) broth was supplemented with 1 µL of 2 mol L^{-1} ACC solution prepared in deionized water and sterilized by filtration (Johnston-Monje and Raizada 2011). Non-supplemented LGI broth was used as a control of nitrogen fixation activity. A total of 10 µL of the bacterial suspension (OD600 of 0.8) was added to 190 µL of the LGI broth in a sterile 96-well plate. The plate was incubated at 28 °C for 10 days, and the optical density (OD600) was measured every 24 h. Those isolates growing exclusively in supplemented LGI media were considered to display a deaminase activity for the substrate ACC.

In vitro nitrogen-fixation activity

The nitrogen-fixation activity of the isolates was measured by the acetylene reduction assay (ARA) using Haahtela's medium. Isolates were grown in closed vessels (10 mL) containing 5 mL of semi-solid media with 50 µL of bacterial suspension (OD600 of 0.8). The tubes were sealed, and after 72 h of incubation at 28 °C, 1 mL (*v*/*v*) of the head atmosphere of the test tube was exchanged with an equal volume of acetylene gas, followed by another 24 h of incubation at 28 °C. Closed vessels without bacteria and vessels inoculated with *Klebsiella variicola* 6A3 were used as negative and positive controls, respectively.

The ethylene concentration was measured on a Varian 3300 (Walnut Creek, CA, USA) gas chromatograph fitted with a hydrogen flame ionization detector and a

3.2 mm × 2 m stainless steel column packed with Poropak N (80–100 mesh). The column was operated isothermally at 95 °C with nitrogen as the carrier gas at a flow rate of 30 mL min^{-1}. The detector temperature was 105 °C. Ethylene production was estimated by integration of the area under the curve and transformed into nmol of ethylene h^{-1} units.

PCR amplification of the *nif*D gene

Based on the identity of bacteria from this work, a pair of primers nifD-JalaF (5′-CGBGGCTGCGCCTAYGC-3′) and nifD-JalaR (5′-CGGGCRAAGATGGCGAAGC-3′) was designed to amplify *nif*D genes present among the isolates from the collection, using the following conditions: 1 min at 94 °C; 1 min at 55 °C; and 1 min at 72 °C for 35 cycles, using 100 ng of template DNA, 10 pmol L^{-1} of each primer and 3.0 mmol L^{-1} of $MgCl_2$. The expected size of the amplicon was approximately 1.2 kb. PCR products were purified, sequenced, and analysed.

Plant growth promotion effect on maize

A total of 12 relevant PGPB strains belonging to five species were tested on "Conejo" maize plants. Bacterial suspensions (OD600 of 1.0) were applied as a seed drench to surface sterilized seeds at a ratio of 10 mL per 20 seeds. Sterile distilled water was used as the untreated negative control.

Seedlings were grown in a 1-kg plastic bag with vermiculite, under a 12.5 h light/11.5 dark cycle in greenhouse conditions at an average temperature of 20 °C. The seedlings were watered every 2 days with 150 mL per bag of Hoagland solution (Hoagland and Arnon 1950). After 50 days, growth parameters such as height and dry weight of stems and roots were measured. Each experiment included three plants per bacterial strain treatment with three replications.

Statistical analysis

Indoleacetic quantification and phosphate solubilisation were expressed as average calculation and its standard deviation calculations. Differences between treatment data from plant height and weight were determined using a two-way ANOVA test followed by a Dunnett's multiple comparison test using GraphPad Prism software version 6.00 for Windows (GraphPad Software, La Jolla California USA; www.graphpad.com).

Detection of antagonistic isolates

Antagonistic effects against the maize fungal pathogen *Fusarium* sp. 50 was tested through fungi–bacteria dual culture. Forty-eight-hour bacterial cultures were harvested from Tryptic Soy Agar (TSA) and adjusted to an OD600 of 0.8 with sterile distilled water. Bacterial suspension spots of 5 µL were placed peripherally on a Yeast Peptone Dextrose (YPD) plate. A 5 mm cylinder taken from the mycelial fungal growth plate was placed at the centre of a YPD plate. Plates were incubated at 28 °C for 96 h. Fungal growth inhibition zones indicated an antagonistic effect of bacteria against fungi.

Maize protection against fungal infection

The potential antagonist capabilities of PGPB against *Fusarium* sp. 50 were evaluated in a maize cob model. Fresh maize cobs (*Zea mays* var. Conejo) were surface disinfected as described above. A total of 10 µL of bacterial suspensions adjusted to an OD600 of 1.0 (approximately 8×10^6 CFU mL^{-1}) were injected in four equidistant points to each cob and incubated in plastic boxes for 24 h at room temperature. After incubation, conidial suspensions (10^5 per 10 µL) of *Fusarium* sp. 50 were inoculated at the centre of a cob and marked with a toothpick. Cobs were incubated at room temperature and monitored daily until the appearance of signs of fungal infection. Each experiment included three cobs per treatment.

Results

Soil characterization

Soil of Jala is a neutral substrate (pH of 6.6–7.3) with a high saturation point (52%), which is a desirable feature according to the maize crop demands. This soil is fertile and rich in organic matter, nitrogen, phosphorus, and other minerals. It presents a low level of salinity (pH = 7.05, CE = 0.74 dS m^{-1}, RAS = 1.07, and PSI = 1.31). These characteristics explain why this soil is suitable to sustain the cultivation of maize.

Isolation of endophytic and rhizospheric PGPB from maize Jala

A total of 374 strains were isolated from the soil, grain, and roots of 10 Jala maize landrace plants (Table 1). Among the isolates, 2 rhizospheric: *Klebsiella variicola* R3J3HD7 (MK855126), *Burkholderia* sp. R3J3HD10 (MK855131); 5 grain endophytic: *Herbaspirillum seropedicae* E2WL3 (MK855130), *Pantoea* sp. E2K4 (MK855135), *Pseudomonas protegens* E1BL2 (MK855121), *Pseudomonas protegens* E2HL9 (MK855122), *Rhizobium pusense* E2K3 (MK855124); and 9 root endophytic strains: *Achromobacter xylosoxidans* Z2K8 (MK855127), *Achyromobacter xylosoxidans* Z3AD5a (MK855134), *Burkholderia* sp. Z2K9 (MK855132), *Chryseobacterium gleum* Z1HL3

Table 1 Bacterial strains isolated from the soil and tissue of Jala maize cultivars using four different nitrogen-free media

Sample	Number of isolates (*n*)		ID code of the relevant isolates*
	Direct	Enrichment	
Rhizospheric soil	93	162	R3J3HD10, R3J3HD7
Grain tissue	12	33	E2K4, E2HL9, E1BL2, E2WL3, E2K3,
Root tissue	28	46	Z2K3, Z2WD1, Z2WD2, Z2K9, Z1HL3, Z2K8, Z3AD5a, Z2WL1, Z1K6

(MK855133), *Kosakonia* sp. Z2WD1 (MK855128), *Pantoea* sp. Z2WD2 (MK855136), *Phytobacter diazotrophicus* Z2WL1 (MK855129), *Pseudomonas alcaligenes* Z1K6 (MK855125), *Pseudomonas japonica* Z2K3 (MK855123) were selected by their PGP capabilities and identified by 16S rRNA gene analysis. The identity percentages of the isolates are shown in Table 2.

Isolate characterization as potential PGPB

Phosphate solubilization

All 374 isolates were cultured in NBRIP, and the isolates that displayed a solubilization halo were cultured in broth media to confirm and quantify the solubilization rate. Consistent semi-qualitative and quantitative results of phosphate

Table 2 Identification by 16S rRNA and sequence analysis of strains isolated from Jala maize cultivars

Identified microorganism (GeneBank accession number)	Closest NCBI match of type strains (GeneBank accession number)	Identity percentage (%)
Achromobacter xylosoxidans Z2K8 (MK855127)	*Achromobacter xylosoxidans* NBRC15126 NR_113733	99.79
Achyromobacter xylosoxidans Z3AD5a (MK855134)	*Achromobacter xylosoxidans* NBRC15126 NR_113733	99.47
Burkholderia sp. Z2K9 (MK855132)	*Burkholderia cepacia* strain NBRC14074 NR_113645	98.25
Burkholderia sp. R3J3HD10 (MK855131)	*Burkholderia metallica* R-16017T AM747632	98.54
Chryseobacterium gleum Z1HL3 (MK855133)	*Chryseobacterium gleum* ATCC 35910 NR_042506	98.83
Herbaspirillum seropedicae E2WL3 (MK855130)	*Herbaspirillum seropedicae* Z67 NR114720	99.51
Klebsiella variicola R3J3HD7 (MK855126)	*Klebsiella variicola* F2R9 NR_025635	99.86
Kosakonia sp. Z2WD1 (MK855128)	*Kosakonia pseudosacchari* JM-387 NR_135211	99.38
Pantoea sp. E2K4 (MK855135)	*Pantoea anthophila* LMG2558 NR_116749	99.02
Pantoea sp. Z2WD2 (MK855136)	*Pantoea anthophila* LMG2558 NR_116749	98.27
Phytobacter diazotrophicus Z2WL1 (MK855129)	*Phytobacter diazotrophicus* LS 8 NR_115869	99.00
Pseudomonas protegens E1BL2 (MK855121)	*Pseudomonas protegens* CHA0 NR_114749	100
Pseudomonas protegens E2HL9 (MK855122)	*Pseudomonas protegens* CHA0 NR_114749	99.87
Pseudomonas alcaligenes Z1K6 (MK855125)	*Pseudomonas alcaligenes* ATCC14909 NR_114472	98.89
Pseudomonas japonica Z2K3 (MK855123)	*Pseudomonas japonica* NBRC103040 NR_114192	99.43
Rhizobium pusense E2K3 (MK855124)	*Rhizobium pusense* NRCPB10 NR_116874	99.48

solubilization were found for the strains *Pantoea* sp. Z2WD2, *P. protegens* E2HL9, *Pantoea* sp. E2K4, *P. japonica* Z2K3, *H. seropedicae* E2WL3, *P. protegens* E1BL2, *Kosakonia* sp. Z2WD1, and *P. diazotrophicus* Z2WL1 (Table 3). The rhizospheric strains *Kosakonia* sp. Z2WD1 and *Pantoea* sp. Z2WD2 were the most efficient phosphate solubilizers (Fig. 1).

Siderophore production

Almost all 374 strains were able to secrete some type of siderophore. However, *A. xylosoxidans* Z2K8 and Z3AD5a, *P. japonica* Z2K3, *Chryseobacterium gleum* Z1HL3, and *P. protegens* E2HL9 and E1BL2 strains exhibited higher ion-chelating capabilities (Table 3) (Fig. 2).

Auxin production (IAA)

Auxin production was quantified at the stationary growth phase. More than 50 isolates demonstrated IAA production. *Pantoea* sp. Z2WD2, E2K4, and *P. diazotrophicus* Z2WL1 were the best producers of auxins, accumulating more than 20 µg mL^{-1} in the culture media. All the strains could produce IAA with and without the addition of tryptophan; among them, *P. diazotrophicus* Z2WL1 doubled its production with the addition of tryptophan, while *H. seropedicae*

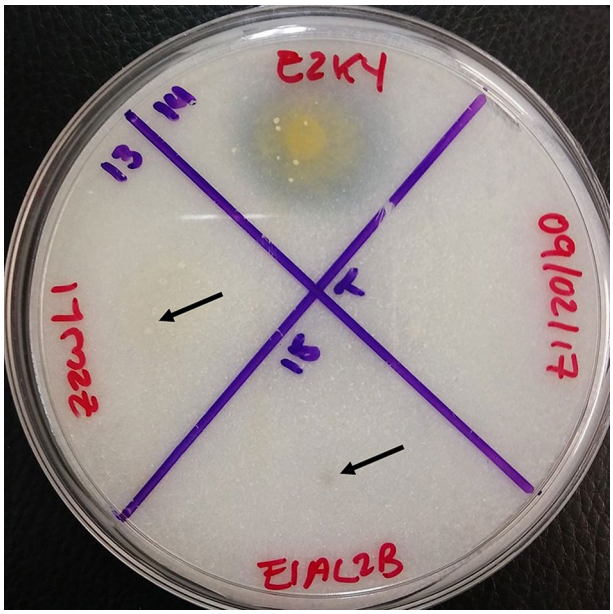


Fig. 1 Phosphate-solubilizing activity of bacteria isolated from Jala maize landrace. Production of clear halo zones of solubilization on NBRIP agar plates by *Pantoea* sp. E2K4. Weak halos formed by *Pseudomonas protegens* E1BL2 and *Phytobacter diazotrophicus* Z2WL1 strains, and no halo was observed with *Rhizobium pusense* E2K3, which was used as the negative control

Table 3 Plant-growth-promoting features of bacteria isolated from Jala maize landrace

Strain	Siderophore production (captured ion)	Growth with ACC	Phosphate solubilization		IAA production (mg mL^{-1})	
			Index in the agar assay	Orthophosphate detection	+trp	–trp
Achromobacter xylosoxidans Z2K8	Co^{2+}, Fe^{3+}, Mo^{2+}, V^{5+}, Zn^{2+}	+	1.0	ND	1.7 ± 0.55	0.003 ± 0.006
Achromobacter xylosoxidans Z3AD5a	Co^{2+}, Fe^{3+}, Mo^{2+}, V^{5+}, Zn^{2+}	+	1.0	ND	0.0	0.0
Burkholderia sp. Z2K9	Fe^{3+}, Mo^{2+}, V^{5+}, Zn^{2+}	+	1.5	64.9	9.6 ± 0.8	7.4 ± 1.6
Burkholderia sp. R3J3HD10	Fe^{3+}, Mo^{2+}, V^{5+}, Zn^{2+}	–	2.0	81.8	9.7 ± 0.7	30.1 ± 3.1
Chryseobacterium gleum Z1HL3	Co^{2+}, Fe^{3+}, Mo^{2+}, V^{5+}, Zn^{2+}	–	1.0	ND	5.38 ± 0.9	3.31 ± 1.4
Herbaspirillum seropedicae E2WL3	Mo^{2+}, V^{5+}, Zn^{2+}	–	1.5	105.03 ± 3.7	12.2 ± 0.5	2.2 ± 1.2
Klebsiella variicola R3J3HD7	Co^{2+}, Mo^{2+}, V^{5+}, Zn^{2+}	–	1.3	ND	9.03 ± 0.61	8.77 ± 2.62
Kosakonia sp. Z2WD1	Fe^{3+}, V^{5+}, Zn^{2+}	–	2.1	142.31 ± 4.2	1.02 ± 0.3	3.73 ± 0.4
Pantoea sp. E2K4	Mo^{2+}, Zn^{2+}	+	2.2	105.84 ± 4.3	25.0 ± 2.03	31.7 ± 2.3
Pantoea sp. Z2WD2	Co^{2+}, Fe^{3+}, Mo^{2+}, V^{5+}, Zn^{2+}	+	3.2	109.25 ± 1.3	55.8 ± 2.4	56.5 ± 2.6
Phytobacter diazotrophicus Z2WL1	Fe^{3+}, Mo^{2+}, V^{5+}, Zn^{2+}	–	1.2	50.21	33.05 ± 5.5	69.8 ± 1.8
Pseudomonas protegens E1BL2	Co^{2+}, Fe^{3+}, Mo^{2+}, V^{5+}, Zn^{2+}	+	1.1	41.82	1.2 ± 0.6	4.3 ± 0.2
Pseudomonas protegens E2HL9	Co^{2+}, Fe^{3+}, Mo^{2+}, V^{5+}, Zn^{2+}	–	1.6	106.54 ± 0.3	2.38 ± 0.8	1.8 ± 0.6
Pseudomonas alcaligenes Z1K6	Co^{2+}, Fe^{3+}, Mo^{2+}, V^{5+}	–	1.0	ND	5.38 ± 0.90	5.64 ± 0.83
Pseudomonas japonica Z2K3	Co^{2+}, Fe^{3+}, Mo^{2+}, V^{5+}, Zn^{2+}	–	2.3	99.71 ± 1.8	1.1 ± 0.3	5.7 ± 1.3
Rhizobium pusense E2K3	Mo^{2+}, V^{5+}, Zn^{2+}	+	1.0	ND	53.40 ± 3 2.07	30.49 ± 23.24

ND stands for not detected for orthophosphate detection was performed only on liquid media on strains that depicted a halo in the NBRIP test

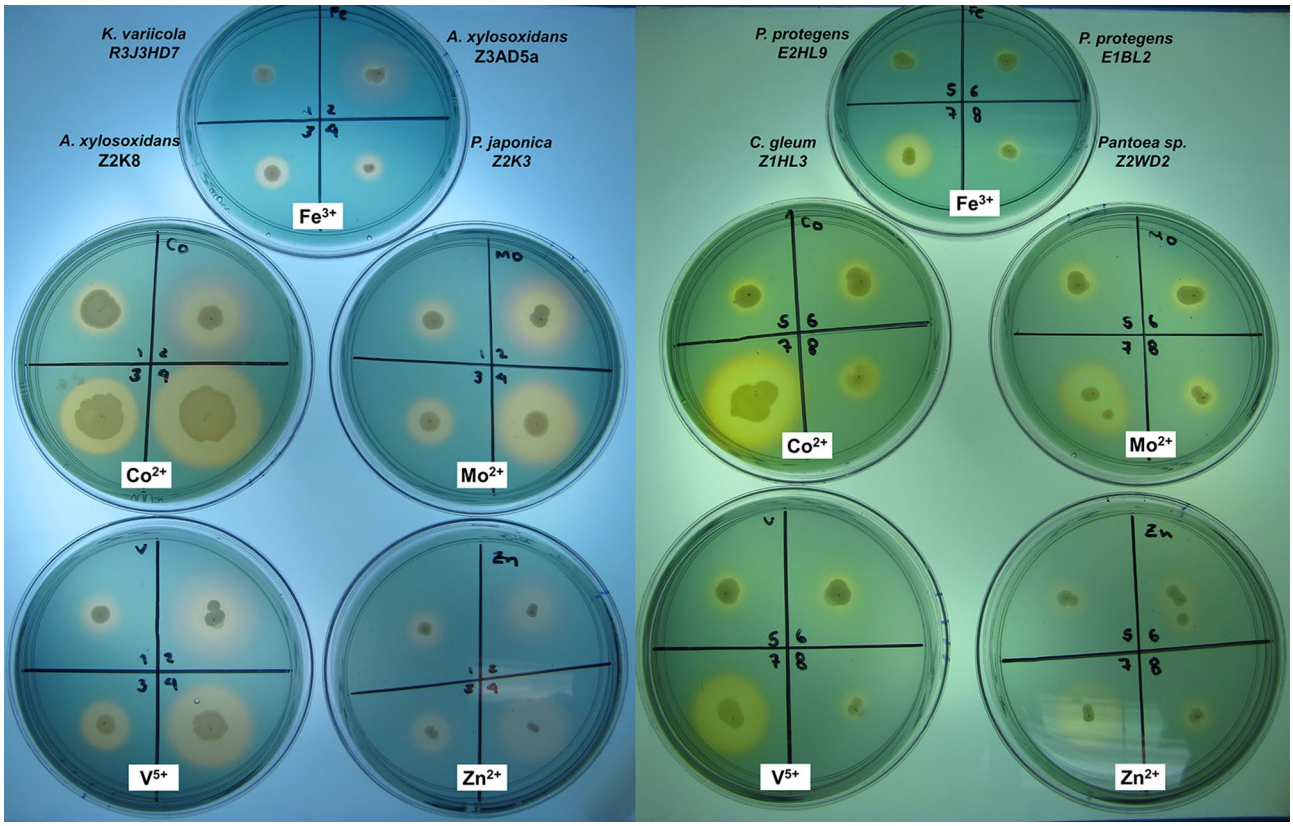


Fig. 2 Detection of siderophore production by colour change on CAS-blue agar supplemented with five different ions (Co^{2+}, Fe^{3+}, Mo^{2+}, V^{5+}, Zn^{2+}) after 48 h of incubation by the strains (clockwise sense) (1) *Klebsiella variicola* R3J3HD7, (2) *Achromobacter xylosoxidans* Z3AD5a, (3) *Pseudomonas japonica* Z2K3, (4) *Achromobacter xylosoxidans* Z2K8, (5) *Pseudomonas protegens* E2HL9, (6) *Pseudomonas protegens* E1BL2, (7) *Pantoea* sp. Z2WD2 and (8) *Chryseobacterium gleum* Z1HL3

E2WL3 produced more auxin-like molecules in medium without tryptophan (Table 3).

A total of 16 strains with the best potential PGP phenotypes (IAA and siderophore production and phosphate solubilization) were selected for evaluation under a second stage of tests that included the ACC deaminase activity test, ARA assay, and amplification and sequencing of *nif*D genes.

Growth in ACC as a sole nitrogen source

Pantoea sp. Z2WD2, *P. protegens* E1BL2, *R. pusense* E2K3, *Pantoea* sp. E2K4, *Burkholderia* sp. Z2K9, and *A. xylosoxidans* Z3AD5a and Z2K8 grew on ACC as a sole nitrogen source. *K. varicola* R3J3HD7 is a diazotrophic bacteria that grew in the control medium without any nitrogen source. For this reason, its deamination activity cannot be recognized (Fig. 3).

In vitro nitrogen-fixation activity

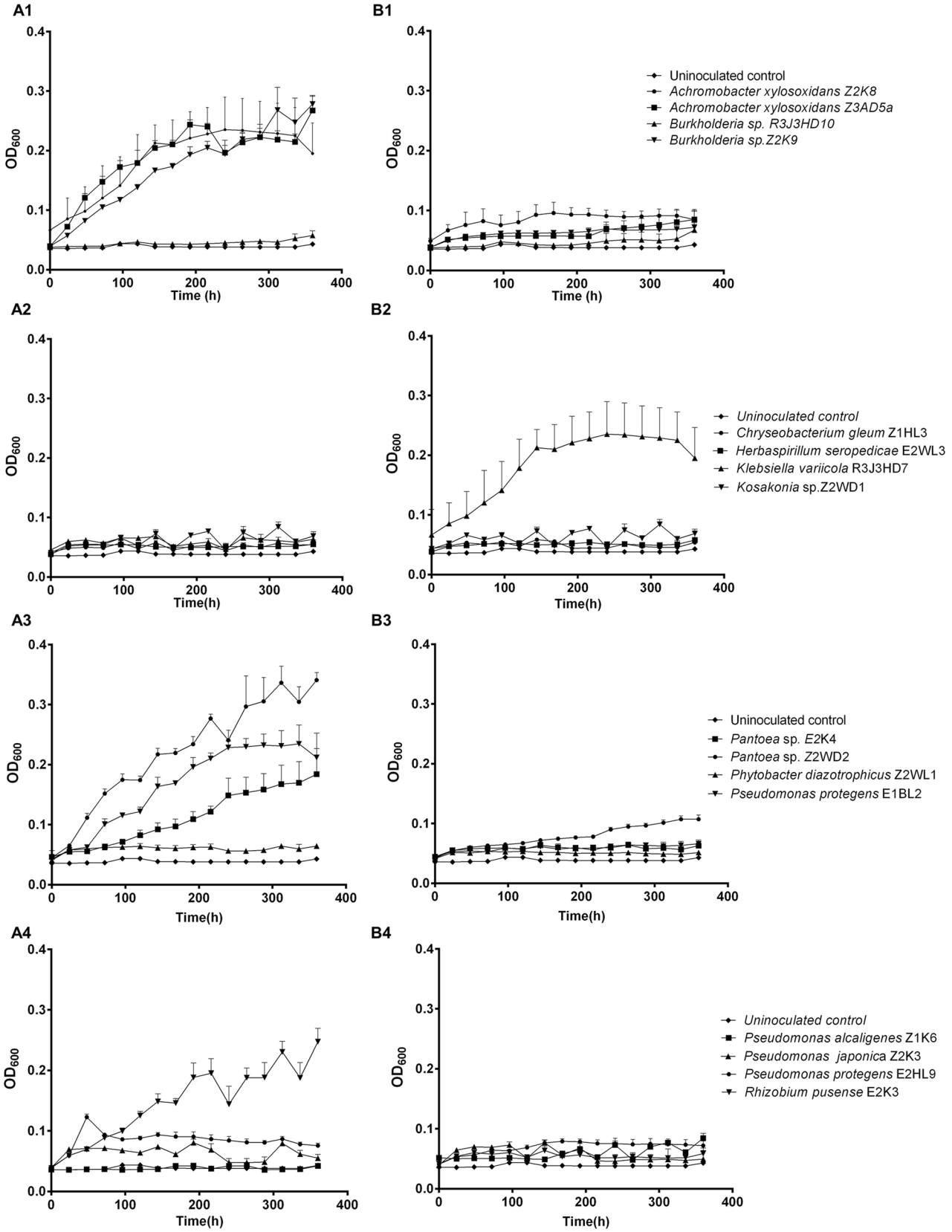
A total of four strains were able to fix nitrogen in vitro; however, only *H. seropedicae* and *K. variicola* R3J3HD7 exhibited acetylene reduction rates higher than 19 nmol of ethylene h^{-1} (Table 4).

Amplification, sequencing, and phylogeny of the *nif*D gene

The gene *nif*D was amplified on *H. seropedicae* E2WL3, *P. diazotrophicus* Z2WL1, and *P. protegens* E2HL9 strains from root or grain endophytes and *K. variicola* R3J3HD7 from the rhizosphere. All the ARA-positive isolates harboured *nif*D genes (Table 4) except for the *P. protegens* isolate E2HL9, which was unable to reduce acetylene under the assayed conditions.

Plant growth promotion of bacteria on Conejo landrace maize

Differences within the inoculated plants were measured and registered. The plant-growth-promoting (PGP) effect of the strains was evident after 50 days in a greenhouse. The *Burkholderia* sp. isolate Z2K9, *P. protegens* E2HL9 and E1BL2, *K. variicola* R3J3HD7, and *H. seropedicae* E2WL3 significantly increased the length of the roots, leaves, and


A1
B1
A2
B2
A3
B3
A4
B4
OD600
Time (h)
Time(h)
0.0
0.1
0.2
0.3
0.4
0
100
200
300
400
Uninoculated control
Achromobacter xylosoxidans Z2K8
Achromobacter xylosoxidans Z3AD5a
Burkholderia sp. R3J3HD10
Burkholderia sp.Z2K9
Uninoculated control
Chryseobacterium gleum Z1HL3
Herbaspirillum seropedicae E2WL3
Klebsiella variicola R3J3HD7
Kosakonia sp.Z2WD1
Uninoculated control
Pantoea sp. E2K4
Pantoea sp. Z2WD2
Phytobacter diazotrophicus Z2WL1
Pseudomonas protegens E1BL2
Uninoculated control
Pseudomonas alcaligenes Z1K6
Pseudomonas japonica Z2K3
Pseudomonas protegens E2HL9
Rhizobium pusense E2K3

◂**Fig. 3** Growth kinetics of the utilization of ACC as a sole nitrogen source by bacteria isolated from Jala maize landrace. Panels **A1** to **4** LGI medium supplemented with ACC as the sole source of nitrogen. Panels **B1** to **4** show bacteria growing on LGI medium without any source of nitrogen (nitrogen fixation control)

dry weight of entire maize plants when compared to the uninoculated control (Fig. 4).

Antagonistic interaction tests

P. protegens E1BL2 and E2HL9, *K. variicola* R3J3HD7, and *Burkholderia* sp. R3J3HD10 demonstrated the broadest and highest growth control of *Fusarium* sp. 50 in vitro. In addition, *P. protegens* E1BL2 and *Burkholderia* sp. R3J3HD10 protected Conejo maize cobs from infection and rot by *Fusarium* sp. 50 (Fig. 5).

Discussion

In this study, the PGP potential of endophytic and rhizospheric bacteria associated with Jala landrace native Mexican maize was explored as an effort to highlight the importance and potential of the maize-bacteria symbiosis. The soil where the Jala maize grows has been described as poor with low N, K, and organic matter contents and low cation-exchange capacity (Aguilar-Castillo et al. 2006). Though, in this work, the samples of Jala maize soils showed silt, fertile, and well-structured soil with low salinity and high water-holding ability. These differences are possibly derived from the differences between the sites, the time of year, and the sampling strategies.

The isolation of bacteria performed in this work involved the use of three solid media without a nitrogen source to promote the biased isolation of nitrogen-fixing bacteria. However, many other non-nitrogen-fixing bacteria were isolated in these "nitrogen-free media," probably, because agar contains between 1 and 178 mmol L^{-1} (0.1%) of total nitrogen (Scholten and Pierik 1998). The nitrogen content in aboveground and belowground tissues of grasses range be between 0.2 and 1.1% (Zhang et al. 2014), but this nitrogen source is not easily available for endophytic bacteria, which face a restrictive environment of assimilable inorganic or organic nitrogen sources. This could explain the high frequency of isolation of diverse endophytic/diazotrophic and non-diazotrophic bacteria from maize plant tissues (Baldani et al. 2014; Ji et al. 2014; Pérez-Montaño et al. 2014).

The nitrogen-fixing capability of the isolated bacteria was confirmed by PCR detection and sequencing of *nif*D genes and by the acetylene reduction test, which is an indirect test to demonstrate nitrogen fixation. Diazotrophic bacteria from both rhizosphere soil and plant tissues were isolated and identified to the genus or species level by phylogenetic and similitude analyses. Though there is no consensus on the threshold for differentiating bacterial species, in this work a nucleotide similitude in the 16S rRNA sequence above ≥ 98.65% was used to define the species (Kim et al. 2014). *Klebsiella variicola* R3J3HD7 and *Herbaspirillum seropedicae* E2WL3 had high acetylene reduction rates in vitro, and *Pantoea* sp. E2K4 and *Phytobacter diazotrophicus* Z2WL1 had more modest rates under the assayed conditions. These quantitative differences are not important and have no relevant biological meaning, because these in vitro estimations depend on the nitrogen-free media used and the environmental conditions assayed to find the best expression of diazotrophic phenotype (Rao 1978; Raven 1988; Morales-Jiménez et al. 2013).

Among the strains associated with Jala maize, rhizospheric *K. variicola* R3J3HD7 showed important in vitro PGP features, the growth promotion of "Conejo" maize landrace, and the highest rate of ethylene production. This diazotrophic enterobacteria of the *Klebsiella pneumoniae* complex has also been frequently found in the rhizosphere and as an endophyte of roots, stems, and leaves of sugarcane, banana, rice, and maize, among other plants (Wei et al. 2014; Lin et al. 2015; Reyna-Flores et al. 2018).

The endophytic isolates *Pantoea* sp. E2K4 and Z2WD2 are located in the *Pantoea ananatis*/*Pantoea allii* clade. Both PGPB species exhibited prominent features, including siderophore and IAA production, ACC utilization as a sole nitrogen source, phosphate solubilization, and growth promotion of "Conejo" maize landrace, additionally *Pantoea* sp. E2K4 was also able to reduce acetylene and harboured the *nif*D gene. *P. ananatis* has been reported mainly as a phytopathogen of a wide variety of hosts, such as maize, rice, onion, and eucalyptus, but as PGPB of maize, potato, and pepper (Coutinho and Venter 2009; Brady et al. 2011; Ikeda et al. 2013). The strains isolated in our study were not phytopathogenic for maize in the greenhouse assay. Instead, they exhibited a PGP phenotype. The *P. ananatis* pan-genome harbour a sizeable accessory genome of 1690 protein coding sequences, which reveals a wide intraspecific diversity and multiple niche occupation (Weller-Stuart et al. 2017).

The endophytic strain *H. seropedicae* E2WL3 was isolated from grains and displayed several in vitro and greenhouse PGP properties including nitrogen fixation, IAA and siderophore production, phosphate solubilization, and growth promotion of "Conejo" maize landrace. All these phenotypic traits have been commonly reported in *H. seropedicae* isolated from tissues or the rhizosphere of maize (Balsanelli et al. 2016; da Fonseca Breda et al. 2019; Dall'Asta et al. 2019).

Among the strains in this study, the highest yield of phosphate solubilization in the hydroxyapatite assay was detected in the root endophyte *Kosakonia* sp. Z2WD1

Table 4 Amplification of *nif*D gen and acetylene reduction rates of bacteria isolated from Jala maize landrace

Strain	*nif*D	Acetylene reduction rate (nmol of ethylene h^{-1})
Positive control *Klebsiella variicola* ATCC BAA-830T	+	21.7 ± 1.75
Achromobacter xylosoxidans Z2K8	–	ND
Achyromobacter xylosoxidans Z3AD5a	–	ND
Burkholderia sp. Z2K9	–	ND
Burkholderia sp. R3J3HD10	–	ND
Chryseobacterium gleum Z1HL3	–	ND
Herbaspirillum seropedicae E2WL3	+	19.77 ± 3.53
Klebsiella variicola R3J3HD7	+	27.7 ± 14.19
Kosakonia sp. Z2WD1	–	ND
Pantoea sp. E2K4	+	7.75 ± 1.97
Pantoea sp. Z2WD2	–	ND
Phytobacter diazotrophicus Z2WL1	+	1.81 ± 0.58
Pseudomonas protegens E1BL2	–	ND
Pseudomonas protegens E2HL9	–	0.0
Pseudomonas alcaligenes Z1K6	–	ND
Pseudomonas japonica Z2K3	–	ND
Rhizobium pusense E2K3	–	ND

ND stands for not determined for acetylene reduction assay was performed only on strains that presented the *nif*D gene

(142 mg mL^{-1}). After nitrogen, phosphorus is the most important plant nutrient and the phosphate solubilization capacity is a common phenotype feature among rhizospheric isolates. The low similitude of the 16S RNA gene of the strain with other formally described species suggests that this strain is a new species within the genus. Other soil, endophytic, phyllospheric, and rhizospheric *Kosakonia* species stand out for their ability to promote plant growth through several mechanisms, including nitrogen fixation, inorganic phosphate solubilization, mineralization of organic phosphate, siderophore production, and auxin synthesis and efflux. Strains of *Kosakonia radicincitans, Kosakonia arachidis, Kosakonia oryzae, Kosakonia sacchari*, and *Kosakonia pseudosacchari* have been isolated from wheat, maize, groundnut, rice, sugar cane, banana, and yerba mate (Peng et al. 2009; Madhaiyan et al. 2010; Witzel et al. 2012; Zhu et al. 2013; Bergottini et al. 2015; Kämpfer et al. 2016; Cruz Barrera et al. 2019). To our knowledge, only one *K. radicincitans* strain was associated with bacterial wilt in banana plants (Suhaimi et al. 2014). Although most species of *Kosakonia* harbour functional *nif* genes and fix nitrogen (Madhaiyan et al. 2010; Witzel et al. 2012; Zhu et al. 2013), any *nif* gene nor acetylene reduction activity was detected for the strain of *Kosakonia* sp. Z2WD1.

Several strains of three species of *Pseudomonas* were isolated from grain and root maize tissues, but only *P. protegens* E2HL9 and E1BL2 expressed PGP features. Both *P. protegens* E2HL9 and E1BL2 strains isolated from grain tissue exhibited notable PGP characteristics and fungal antagonism against *Fusarium* sp. 50, but they were unable to fix nitrogen. *P. protegens* and many other *Pseudomonas* species have never been recognized as nitrogen-fixing bacteria, only *Pseudomonas azotifigens* and *P. stutzeri* A15 harbour an entire set of functional nitrogen-fixation genes that may have been horizontally acquired from other diazotrophs (Desnoues et al. 2003; Yan et al. 2008). The ability of both strains to produce siderophores has also been recognized in other *Pseudomonas* species (Sharma and Johri 2003). The main function of the siderophores pyoverdine and enantiopyochelin of *P. protegens* is iron scavenging, but they also increase the survival of the strains in soil, confer resistance to fungal fusaric acid, and promote competence for iron uptake with other bacteria (Ruiz et al. 2015; Drehe et al. 2018). The strain *P. protegens* E1BL2 exhibited antifungal properties against *Fusarium* sp. 50, both in vitro and in a cob infection model, and produced HCN (data not shown), a volatile antifungal agent. Actual evidence suggests that *P. protegens* could be worth exploitation as an efficient biocontrol agent to reduce citrus canker caused by *Xanthomonas citri* subsp. citri. (Michavila et al. 2017).

Although root-associated *Phytobacter diazotrophicus* Z2WL1 showed some phenotypic traits usually associated with PGPB, such as being able to reduce acetylene and harbour the *nif*D gene, it did not promote the growth of maize plants in any of the greenhouse tests. There is not much information about this species but a report in wild rice and the sequenced genome of an isolate from the oil palm without further details (Salha et al. 2020; Zhang et al. 2008). For these reasons, more studies are needed to evaluate the importance of this species isolated from plants.

Rhizobium pusense E2K3 isolated from tissue grain exhibited modest PGP in vitro features, but the *nif*D gene and acetylene reduction were not detected. This species (syn. *Agrobacterium pusense*) has been isolated from bean nodules and chickpea rhizosphere (Panday et al. 2011; Aguilar et al. 2016) and was reported as the main human pathogen within the genus *Agrobacterium*/*Rhizobium* (Aujoulat, et al. 2015). For this reason, it was not considered for the PGP experiments in maize. On the other hand, neither *Chryseobacterium gleum* Z1HL3 nor *Achromobacter xylosoxidans* Z2K8 and Z3AD5a strains were included in the maize growth promotion experiments due to the frequent association of these species with human infections, and their PGP potential is scarce (Swenson and Sadikot 2015; Arouna et al. 2017).

Burkholderia sp. Z2K9 and *Burkholderia* sp. R3J3HD10 are two closely related strains. Both strains

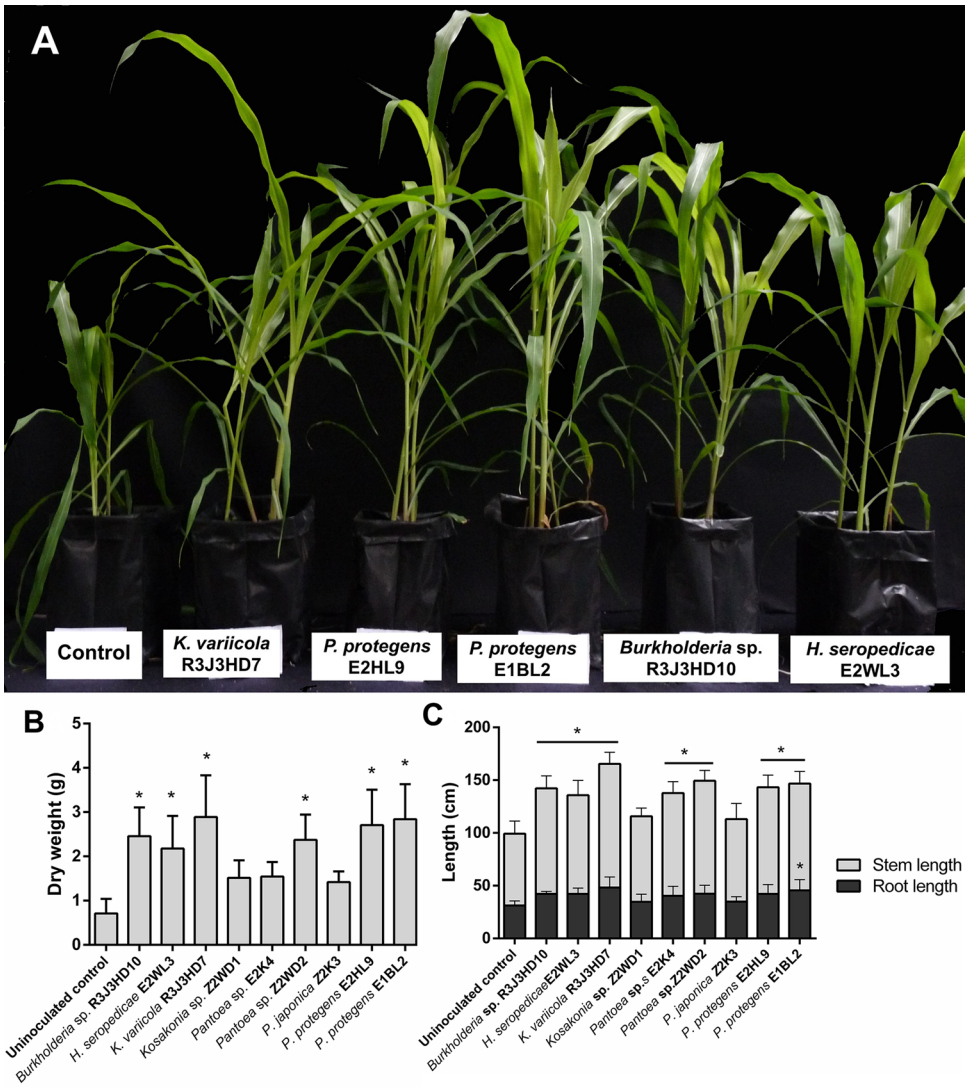


Fig. 4 Growth promotion assay on maize plants from the landrace "Conejo" inoculated individually with either endophytic or rhizospheric PGPB isolated from Jala maize. **A** Maize plants of 50 days inoculated with PGPB under the following scheme (from left to right): non-inoculated plant, *K. variicola* R3J3HD7, *P. protegens* E2HL9, *P. protegens* E1BL2, *Burkholderia* sp. R3J3HD10, and *H. seropedicae* E2WL3. **B** Dry weight of the maize plants on 50th day. **C** Stem and root length of treated plants after 50 days. Data analysis was performed using GraphPad PRISM statistics program. Differences between treatments and control means were determined using two-way ANOVA with multiple comparison ($*p < 0.05$)

expressed some PGP features in vitro, but only *Burkholderia* sp. R3J3HD10 was able to promote maize growth in the greenhouse assay and to antagonize *Fusarium* sp. 50. There are many papers that report the fungal antagonism and/or PGP capacities of *Burkholderia* spp. in many plants, including maize and teosinte of non-identified landraces (Ikeda et al. 2013; Zhao et al. 2014; Pande et al. 2017; Esmaeel et al. 2018; Tagele et al. 2018, 2019; You et al. 2020). *Burkholderia* sp. R3J3HD10 was closely related to *Burkholderia contaminans*, a PGPB strain isolated from the rhizospheric soil of maize reported to control banded leaf and sheath blight of maize caused by *Rhizoctonia solani* in greenhouse assays (Tagele et al. 2018). The potential of *Burkholderia* spp. in agriculture is a debated subject due to the disadvantages reported by its pathogenic potential for humans despite its clear PGP and antifungal suppression phenotype (Eberl and Vandamme 2016; Rosenblueth et al. 2018).

This concern is not exclusive for *Burkholderia* spp. for practically all the species isolated in this work, have been associated to clinical cases from immunocompromised and elderly patients (Aujoulat et al. 2015; Bhatti et al. 2017; Pillonetto et al. 2018; Rodríguez-Medina et al. 2019; Mertschnigg et al. 2020). The potential pathogenic features, the molecular epidemiology registers, and the high resemblance among strains isolated from plants and human infections, support the idea of a cross-kingdom bacteria that might follow a route of transmission from plants to humans (Barrios-Camacho et al. 2019). Although clinical cases of these species vary from very rare to infrequent, it has been proposed that their use in agriculture should not be encouraged (Rosenblueth et al. 2018). In our opinion, since there are few cases of human infections related to PGPB species from agricultural origin, the potential use as a biofertilizer, bioinoculant, or antagonistic of each species must be evaluated on a case-by-case basis through a comparative whole-genome analysis to identify putative virulence factors

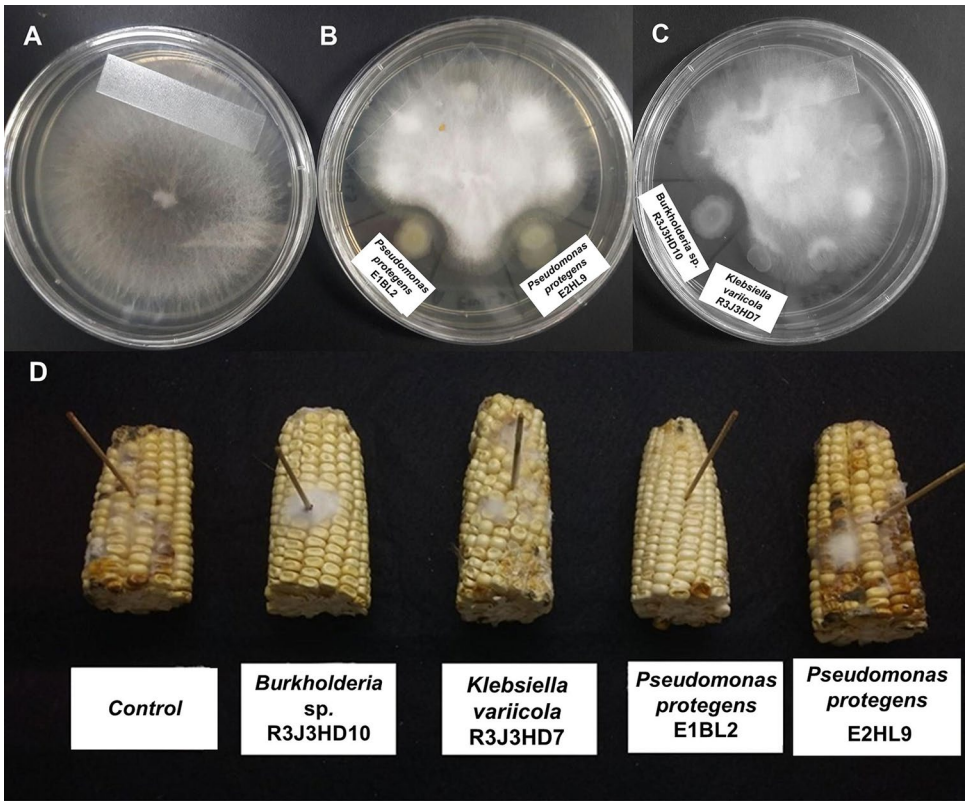


Fig. 5 Antagonistic interaction of *Fusarium* sp. 50 with the bacterial isolates *Burkholderia* sp. R3J3HD10, *K. variicola* R3J3HD7, and two strains of *P. protegens* E1BL2 and E2HL9. **A** Growth of *Fusarium* sp. 50 in potato dextrose agar (PDA). **B** Inhibition of fungal growth by *P. protegens* E1BL2 and E2HL9. **C** Inhibition of fungal growth by *Burkholderia* sp. R3J3HD10 and *K. variicola* R3J3HD7. **D** Maize cob protection assay on "Conejo" maize cobs inoculated with PGPB, after 7 days of infection with *Fusarium* sp. 50. Toothpicks mark the inoculation point of *Fusarium* sp. 50

that discern the plant beneficial strains from potential human pathogenic strains (Mertschnigg et al. 2020). In general, many potential virulence genes associated to genomic islands have been detected only in clinical strains (Faoro et al. 2019). In the future, pan-genomic and pathogenesis tests in animal models should be mandatory, to determine the strains innocuousness before incorporating them into a bioinoculants formulation. In this study, maize plants responded both positively and neutrally to individual bacterial inoculation, discarding the possibility of handling pathogenic bacteria. The main PGPB in the greenhouse assays were *Burkholderia* sp. R3J3HD10, *H. seropedicae* E2WL3, *Pantoea* sp. Z2WD2, *P. protegens* E2HL9, *P. protegens* E1BL2, and *K. variicola* R3J3HD7.

If the plant is considered a holobiont, it could be expected that its microbiome is also diverse and represents an important potential as a PGP resource, as demonstrated in this study. Face to the obstacles derived from the social opposition to the use of genetically modified organisms, culturable bacteria represents an important reserve of germplasm that can contribute to a more sustainable agriculture.

Conclusions

Jala landrace is colonized by bacteria that display PGP features, such as nitrogen fixation, phosphate solubilization, utilization of ACC as a sole nitrogen source, and siderophore and auxin production. These features were detected for both rhizospheric and endophytic bacteria except for the production of ACC deaminase, found exclusively in the endophytic bacteria from grain and root tissue. Most of the outstanding strains were isolated from root and grain tissues and gather more than one PGP ability. Maize plants responded both positively and neutrally to the individual bacterial inoculation, discarding the possibility of having isolated pathogenic bacteria. Under greenhouse conditions, maize plants growth is improved by *Burkholderia* sp. R3J3HD10, *H. seropedicae* E2WL3, *Pantoea* sp. Z2WD2, *P. protegens* E2HL9, *P. protegens* E1BL2, and *K. variicola* R3J3HD7.

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Author contributions BRG, CVG and JEGC performed the isolation and bacterial characterization; BRG, NMJ and EVC performed greenhouse experiments; RIAG, LVT and CHHR provided the samples, designed, and coordinated the study; BRG wrote the first draft

manuscript; EVC RIAG, NMJ and CHHR contributed to manuscript editing. All authors read and approved the final manuscript.

Availability of data and material Not applicable for this section.

Accession Numbers: Uploaded to the National Center for Biotechnology Information database (NCBI): K855127—*Achromobacter xylosoxidans* Z2K8; MK855134—*Achyromobacter xylosoxidans* Z3AD5a; MK855132—*Burkholderia* sp. Z2K9; MK855131—*Burkholderia* sp. R3J3HD10; MK855133—*Chryseobacterium gleum* Z1HL3; MK855130—*Herbaspirillum seropedicae* E2WL3; MK855126—*Klebsiella variicola* R3J3HD7; MK855128—*Kosakonia* sp. Z2WD1; MK855135—*Pantoea* sp. E2K4; MK855136—*Pantoea* sp. Z2WD2; MK855129—*Phytobacter diazotrophicus* Z2WL1; MK855121—*Pseudomonas protegens* E1BL2; MK855122—*Pseudomonas protegens* E2HL9; MK855125—*Pseudomonas alcaligenes* Z1K6; MK855123—*Pseudomonas japonica* Z2K3; MK855124—*Rhizobium pusense* E2K3.

Code availability Not applicable for this section.

Declarations

Conflict of interest The authors declare that they have no conflict of interest in the publication.

Ethics approval Not applicable for this section.

Consent to participate This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication Not applicable for this section.

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