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Endophytic bacteria mitigate mercury toxicity to host plants

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Abstract

Plant communities growing in metal-contaminated areas can develop resistance mechanisms by establishing symbiotic associations with endophytic microorganisms. The functionality and diversity of endophytic communities depend on the amount and type of metal present in the soil. To characterise the response of endophytic bacterial communities to mercury-induced abiotic stress, we analysed the colonization frequency and number of bacterial isolates in the roots of *Aeschynomene fluminensis* (Joint Vetch) and *Polygonum acuminatum* (Smartweed), which represent the families Fabaceae and Polygonaceae, respectively. These two plant species are found in many mercury-contaminated areas. The isolates were characterised by morpho- and genotyping and identified by 16S rDNA gene sequencing. The bacteria belonged to the phyla Actinobacteria, Bacteriodetes, Firmicutes, and Proteobacteria. The Hill series and Venn diagram provided evidence that mercury affects the composition, diversity, and richness of the endophytic bacterial communities. Inoculation with *Bacillus*_sp_BacI34, *Burkholderia*_sp_BacI45, *Enterobacter*_sp_BacI14, *Enterobacter*_sp_BacI26, *Enterobacter*_sp_BacI18, *Klebsiella_pneumoniae*_BacI20, *Lysobacter_soli*_BacI39, *Pantoea*_sp_BacI16, and *Pantoea*_sp_BacI23 promoted the growth of corn (*Zea mays*) plants in mercury-supplemented substrata. It is noteworthy that *Pantoea sp*_BacI23 increased the host plant length (root and shoot) by 117.09 ± 0.28%. Endophytic bacterial strains may well provide important inoculants for plant growth promotion on metal-contaminated sites and in metal bioremediation programs.

Keywords Endophytes · Trace metal · Wetland · Bioremediation · Plant growth promotion

1 Introduction

Mercury is a class B metal (Nieboer and Richardson 1980) naturally found in the earth crust that occurs in soil, water, and air in several chemical forms, including metallic (Hg^0), ionic (Hg^+, Hg^{2+}), organometallic ($(CH_3)_2Hg$, CH_3Hg^+) forms (Carrasco-Gil et al. 2013). Transformation of this metal via methylation, demethylation, and reduction depends on its distribution and the environment conditions (Asaduzzaman et al. 2019). Mercury is among the 20 substances that the United States Environmental Protection Agency and the Agency for Toxic Substances and Disease Registry classify as highly toxic to humans and aquatic organisms (Ullah et al. 2015; Darko et al. 2016). It therefore threatens not only human and animal health, but also ecosystems (Román-Ponce et al. 2016). The high toxicity of mercury has prompted the search for strategies that minimise its detrimental effects or contamination levels to the environment (Farias et al. 2012; Seccatore et al. 2014; Oliveira et al. 2015).

The Pantanal is the largest tropical wetland in the world and comprises one of the largest and most biodiverse biomes in Brazil (Junk et al. 2014). Anthropic influences, such as deforestation, erosion, and gold mining have led to severe mercury contamination in many parts of the Pantanal (Ceccatto et al. 2016), where the mercury concentration in suspended sediments has ranged from 0.02 to 0.61 $mg.kg^{-1}$ (Lacerda et al. 1991). The Brazilian legislation (BRASIL 2009) and World Health Organization (WHO, 2003) recommend that mercury concentrations should be lower than 0.5 $mg.kg^{-1}$ for urban areas. The main problem is illegal mining activity that has increased the mercury contamination of soil, water, and biota (Ceccatto et al. 2016; Cebalho et al. 2017). Mercury levels in

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the soil samples near a gold-mining site of Poconé, Mato Grosso State, Brazil, are 6.48 times greater than the limit established by the Brazilian legislation (Pietro-Souza et al. 2017). Mercury contamination is of public interest because it threatens humans via various exposure routes (Seccatore et al. 2014), and this metal has the capacity to bioaccumulate in the food chain (Vishnivetskaya et al. 2011; Zhang et al. 2013; Mani and Kumar 2014; Alanoca et al. 2016).

Mercury contamination of natural areas exerts a strong selective pressure for the development of mercury-resistant plants (Fidalgo et al. 2016). The association with endophytes enables the host plants to adapt and face adverse conditions including attack by phytopathogens (Soares et al. 2016a), high levels of metal contamination (Shen et al. 2013; Manohari and Yogalakshmi 2016), and other physical and chemical stresses (Rodriguez et al. 2008; Soares et al. 2016c). Endophytic microorganisms inhabit the internal organs of the host plant without causing any disease or infection (Schulz and Boyle 2005; White et al. 2014). These microorganisms have functional traits that promote host plant growth, such as phosphate solubilization and the production and release of ammonia, cyanuric acid, hydrocyanic acid, indoleacetic acid (IAA), nitrogen, siderophores, and enzymes (amylase, cellulase, esterase, and protease) (Cuzzi et al. 2011; Glick 2015; Mathew et al. 2015; Manohari and Yogalakshmi 2016; Soares et al. 2016b).

Plant-associated endophytes can remove, transform, and even assimilate the contaminants present in sediments, soil, water, and air as a strategy to mitigate the toxic effects of metals (Zhang et al. 2013, 2016). Bacteria have mercury resistance mechanisms mediated by enzymes encoded in the operon *mer* that are capable of reducing this metal (Harichová et al. 2012; Yu et al. 2014). Other mechanisms of bacterial resistance to toxic metals involve alteration of plasma membrane permeability, cell morphology, and efflux systems; biosorption, complexation, demethylation, oxidation, precipitation, reduction, and volatilization of metals; and production of exopolysaccharides (Yu et al. 2014; Ullah et al. 2015; Xie et al. 2015; Naik and Dubey 2016).

Plants can host mercury-resistant endophytic bacteria (Pérez et al. 2016; Durand et al. 2018) and fungi (Pietro-Souza et al. 2017). Soil mercury contamination influences the composition and structure of root endophytic fungal communities of *Aeschynomene fluminensis* Vell and *Polygonum acuminatum* Kunth. that colonize wetland environments (Pietro-Souza et al. 2017). We hypothesise that plants growing in mercury-contaminated environments host endophytic bacterial communities. The objectives of the present study were a) to characterise the endophytic bacterial community isolated from roots of *Aeschynomene fluminensis* Vell. and *Polygonum acuminatum* Kunth. collected at environments contaminated or not with mercury; b) to identify the mercury-resistant community in the collected samples; c) to characterise functional traits important for bioremediation and host plant growth; and d) to examine to what extent bacteria inoculation improves plant growth.

2 Materials and methods

2.1 Sampling and processing

The biological material and soil samples were collected in September 2014, at three sites of Poconé, a typical Pantanal region from the State of Mato Grosso, Brazil: Site 1: S 16°15′ 42.7" W 056°38′43.6"; Site 2: S 16°21′19.7" W 056°20′13.9"; and Site 3: S 16°15′51.3 "W056°38′54.3". This area is characterised by a rainy period from October to April, a drought period from June to December, a long flooding period from December to May (Junk et al. 2016), annual average rainfall of 1239 mm, and temperature of 26 °C (Alvares et al. 2013). The climate is classified as Aw (Köppen 1930). Data from previous chemical analyses that determined the total soil mercury concentration were used to select the collection sites (Pietro-Souza et al. 2017).

Endophytic bacteria were isolated from roots of five adult plants of *A. fluminensis* (named Asc) and *P. acuminatum* (named Pol) collected at areas contaminated or not with mercury (named HgY and HgN, with Hg^{2+} levels of 3.24 and < 0.0017 mg/kg, respectively). These plant species were chosen due to their capacity to colonize HgY environments abundantly (Pietro-Souza et al. 2017). The soil and vegetal material were packaged in plastic bags, identified according to the collection site, and stored at 4 °C until processing. The samples were superficially cleaned with neutral detergent, washed with tap water, and further superficially disinfected with ethanol 70% (1 min) and sodium hypochlorite 2.5% (5 min). They were then rinsed five times with sterile distilled water (Pietro-Souza et al. 2017).

Three bacterial isolation procedures (de Souza et al. 2013; Franchi et al. 2017) were then used: fragmentation, maceration, and enrichment. 1) Fragmentation: 120 root fragments of each sample were transferred to Petri dishes ($N = 10$) containing Luria Bertani (LB) medium supplemented with 30 $\mu g.mL^{-1}$ of $HgCl_2$ (LB + Hg). 2) Maceration: the disinfected roots were macerated and diluted (10^{-1} to 10^{-3}) in 0.87% NaCl, and further plated in triplicate in solid LB medium not supplemented with $HgCl_2$. 3) Enrichment: 5 mL of the macerate were inoculated in 45 mL of LB + Hg broth and shaken (100 rpm; 72 h); 5 mL of this culture were inoculated in 45 mL of LB + Hg broth and incubated under the same conditions; finally, the culture was diluted (10^{-1} to 10^{-8}) in 0.87% NaCl and plated in triplicate in solid LB + Hg medium (Cabral et al. 2013). In the three, the Petri dishes were incubated at 28 °C and analysed daily. The colonies were characterised macroscopically and grouped morphologically after purification. The strains were stored in 20% glycerol, at −20 °C.

2.2 Identification of root endophytic bacteria

DNA was extracted from the isolated strains using the Wizard Genomic DNA Purification Kit (Promega) following the manufacturer's protocol. The morphological groups were confirmed by ERIC-PCR fingerprinting of the products using the initiator oligos ERIC-1R (5′-ATGTAAGCTCCTGG GGATTCAC-3′) and ERIC-2 (5′-AAGTAAGTGACTGG GGTGAGCG-3′) (Vandamme et al. 1993).

One lineage from each ERIC-PCR group was identified through 16S rDNA gene sequencing, using the primers 27F and 1492R to amplify the 16S gene region (Lane 1991). The amplicons were enzymatically purified using ExoSap-it PCR Product Cleanup Reagent (GE Healthcare) and sequenced by the Sanger method using the BigDye™ Terminator Cycle Sequencing kit. The sequences were edited using BioEdit software (version 7.2.5) and compared to sequences deposited at GenBank using the nBLAST tool (http://www.ncbi.nlm.nih.gov/). The nucleotide sequences were deposited at GenBank under the accession numbers KX641492 to KX641588.

2.3 Plant growth-promoting properties and mercury resistance of the isolates

The isolated bacterial strains were characterised with respect to their capacity to solubilize inorganic phosphates (Podile and Kishore 2007), fix nitrogen (Cavalcante and Dobereiner 1988), synthesize ammonia (Pandey et al. 2015) and IAA (Cuzzi et al. 2011), and secrete hydrocyanic acid (HCN) (Lorck 1948), siderophores (Milagres et al. 1999), and the hydrolytic enzymes amylase, cellulase, esterase, and protease (Carrim et al. 2006). The presence of halo, color change and/or colony growth was analysed for each methodology. The minimal inhibitory concentration (MIC) of mercury was determined in LB broth containing serial concentrations of Hg^{2+} (0–500 μg/mL) (El-deeb et al. 2012).

2.4 Mitigation of mercury toxicity to host plants by endophytic bacteria

Asc and Pol seed germination and growth in the greenhouse are very irregular, making it very difficult to use them in bioremediation assays. Corn (*Zea mays* hybrid maize AG 1051) plants were chosen due to their agronomic importance to this region of Brazil (Duarte and Pasa 2016) and their effectiveness for bioremediation of contaminants and metals (Mani and Kumar 2014; Dixit et al. 2015; Ullah et al. 2015; Shinwari et al. 2015), including mercury remediation (Pietro-Souza et al. 2017).

Endophytic bacterial strains isolated using the fragmentation and enrichment methods in mercury-supplemented medium were selected for the assays of corn plant growth promotion. First, corn seeds were disinfected by immersion in 70% ethanol (1 min) and 2.5% sodium hypochlorite (5 min), rinsed in sterile distilled water, and submerged for 1 h in the test bacterial suspension previously activated in LB broth (OD: 10^8 CFU.mL^{-1}). Next, the seeds were transferred to 1.0 dm^3 vessels containing vermiculite:sand 1:1 (m:m) supplemented with 40 mg.kg^{-1} of $HgCl_2$. Seven days after sowing, bacteria were reinoculated by adding 1 mL of bacterial inoculum (OD: 10^8 CFU.mL^{-1}) to the soil near the plants. The field capacity of the substratum was maintained at 70%, and it was irrigated weekly with 70% ionic strength Hoagland solution (Hoagland and Arnon 1950). The control groups consisted of plants not inoculated with endophytic bacteria (P), and plants inoculated with endophytic bacteria grown in vessels with (CHgY) or without (CHgN) addition of mercury. After 20 days of cultivation, the length of aerial shoots and roots was measured. The growth promotion efficiency was calculated to determine how effectively endophytic bacteria promoted plant growth (Almoneafy et al. 2014).

2.5 Data analysis

The colonization frequency in root fragments inoculated with bacteria was calculated and data were analysed using the *F* and *Student's t* tests ($p < 0.05$) (Harris and Sommers 1968). The diversity of bacterial communities was analysed using the Hill Series (Hill 1973). The species composition of the communities (AscHgN, AscHgY, PolHgN, PolHgY) was visualized in the Venn diagram constructed with the aid of the online software DrawVenn (http://bioinformatics.psb.ugent.be/webtools/Venn/).

The co-occurrence patterns of microbial taxa within the host and contaminated soil was explored by Network analysis. A Spearman's correlation between two genera was considered statistically robust if $p < 0.05$ (Vegan package on R). Bacterial modules or sub-communities of the community were calculated using the Louvain algorithm (Blondel et al. 2008) and network properties were calculated using the statistics tools implemented in Gephi 0.9.1 (Bastian et al. 2009).

Results from the qualitative functional characterisation were expressed as positive (+) or negative (−) when the production of functional traits were detected or not, respectively. Differences between treatments in the corn growth parameters were analysed by the Dunnett's test, using the softwares R (version 3.2.5) and Assistant 7.7.

3 Results

3.1 Structure of the endophytic bacterial community of *A. fluminense* and *P. acuminatum*

This section comprises data from four endophytic bacterial communities isolated from roots of *A. fluminense* (Asc) and *P. acuminatum* (Pol) collected at areas contaminated (HgY) or not (HgN) with mercury, which were named as AscHgN, AscHgY, PolHgN, and PolHgY.

Fragmentation of root tissues allowed estimation of the extent of plant tissue colonization by endophytes. The percentage of root fragments colonized by endophytic bacteria in the HgY area (41.88 ± 34.56%) was higher than the percentage found in the HgN area (14.12% ± 13.49) ($p < 0.05$), regardless the plant species. Colonization rate in Pol roots (48.10% ± 29.59) was greater than colonization rate in Asc roots (7.92% ± 7.79) collected at both sites ($p < 0.05$).

A total of 207 bacterial strains were isolated from root fragments of AscHgN, AscHgY, PolHgN, and PolHgY inoculated in mercury-supplemented medium: 34, 44, 59, and 70 strains, respectively. The isolated strains belonged to phyla Proteobacteria (PolHgY = AsHgY = 100%, PolHgN = 98.31%, and AscHgN = 76.47%) and Firmicutes (PolHgN = 1.69% and AscHgN = 23.53%) (Table 1); the last phylum was exclusively found in HgN areas. The classes identified were: Alphaproteobacteria, Bacilli, Betaproteobacteria, and Gammaproteobacteria; the two last ones were isolated from all the communities evaluated, and they were abundant in Pol and Asc roots, respectively. The class Bacilli was exclusively found in HgN areas, especially in Asc (23.53%). Nine genera distributed in 23 species were identified, among which *Enterobacter* was the most frequent in AscHgY (47.73%) and PolHgY (51.43%), and *Burkholderia* was the most frequent in AscHgN (66.7%) and PolHgN (44.12%). The dominant species were *Burkholderia*_sp_BacI41 (32.35%) in AscHgN, *Burkholderia_cepacia*_BacI47 and *Pantoea*_sp_BacI16 (64.40%) in AscHgY, *Enterobacter*_sp1_X (29.55%) in PolHgN, and *Enterobacter_cloacae*_X (51.43%) in PolHgY (Table 1).

The Hill series demonstrated that richness and diversity indices depended on the plant species and the presence or absence of mercury in the soil (Fig. 1). Endophytic bacterial communities from the HgN site had richness (when $a = 0$, AscHgN = 7 and PolHgN = 8) and diversity α greater than communities from the HgY site (AscHgY = 4 and PolHgY = 7). The Shannon diversity indice confirmed these parameters ($e^{H'}$ when $a = 1$; AscHgN = 1.54, AscHgY = 1.38, PolHgN = 1.63, and PolHgY = 1.28). The Simpson diversity indice (1/D when $a = 2$) evidenced that Pol was represented by dominant species and low diversity as compared with Asc, regardless mercury contamination (AscHgN and AscHgY = 0.75, PolHgN = 0.76, PolHgY = 0.64) (Fig. 1a).

Pol roots had lower number of cultivable endophytic bacteria per gram of macerated root tissue (PolHgN = $1.04 \times 10^5 \pm 5.02 \times 10^3$ UFC/g and PolHgY = $3.27 \times 10^5 \pm 1.36 \times 10^4$ UFC/g) than Asc roots (AscHgN = $1.11 \times 10^5 \pm 6.82 \times 10^3$ UFC/g and AscHgY = $5.72 \times 10^5 \pm 3.23 \times 10^4$ UFC/g) ($p < 0.05$). Fifty-one morphotypes were differentiated on the basis of the growth characteristics in culture medium. They belonged to phyla Bacteriodetes (0.39%), Actinobacteria (8.05%), Proteobacteria (21.58%), and Firmicutes (69.98%), and were distributed in 18 genera and 39 species (Table 1), as determined by ERIC-PCR fingerprinting and 16S rDNA gene sequencing. Bacteriodetes was exclusively found in the PolHgN community while Actinobacteria was exclusively found in Pol, independently of the environment. The most abundant species in AscHgN, AscHgY, PolHgN, and PolHgY communities were *Burkholderia_kururiensis*_BacI100 (20.65%), *Ralstonia*_sp_X (22.49%), *Bacillus_subtilis*_BacI75 (17.96%), and *Enterobacter*_sp3_X (23.08%), respectively (Table 1). The Hill Series of diversity indices provided evidence that endophytic bacterial communities from plants grown in HgY areas had richness, diversity, and dominance indices (AscHgY: richness = 8, Shannon = 1.99, Simpson = 0.85; PolHgY: richness = 18, Shannon = 2.67, Simpson = 0.91) greater than communities from plants grown in HgN areas (AscHgN: richness = 6, Shannon = 1.78, Simpson = 0.83; PolHgN: richness = 13, Shannon = 2.14, Simpson = 0.86). Pol had richness, diversity, and dominance indices greater than Asc, regardless the collection site (Fig. 1b).

The four endophytic bacterial communities differed markedly with respect to their composition (Fig. 2). Data from both isolation methods revealed that more species were specific to one host than were shared (Fig. 2). *Enterobacter*_sp1_X specifically colonized Asc roots while *Enterobacter_cloacae*_X and *Klebsiella_pneumoniae*_X specifically colonized Pol roots, as evidenced by fragmentation of root tissues (Table 1). *Enterobacter_ludwigii*_X was isolated from Asc and Pol roots collected at contaminated sites (AscHgY and PolHgY), using the maceration technique (Table 1).

The endophytic bacterial communities of plants collected at HgY areas were enriched after three cycles of passage in culture media supplemented with mercury. Inoculation with root samples from hosts collected at the HgN site did not result in microbial growth, but inoculation with root samples collected at the HgY site resulted in bacterial isolates from the phyla Proteobacteria (84.61%) and Firmicutes (7.69%), that included 8 genera and 12 species (Table S1). This method also resulted in the isolation of the yeast *Rhodotorula mucilaginosa*_X from Asc roots.

The Asc roots had higher species richness than Pol roots: 8 and 5 species, respectively. The relative abundance of these species in AscHgY was *Rhodotorula mucilaginosa*_X (71.60%), *Pseudomonas*_sp_BacI38 (10.76%), *Klebsiella*_sp_BacI31 (8.52%), *Enterobacter*_sp_BacI32

Table 1 Relative frequency of bacteria identified in endophytic isolates from fragments (F) or macerates (M) of *Aeschynomene fluminensis* (Asc) and *Polygonum acuminatum* (Pol) roots collected at areas contaminated (HgY) or not (HgN) with mercury

Phylum	Species	NCBI ID	%	PolHgY	PolHgN	AscHgY	AscHgN
Actinobacteria	*Methylobacterium_ fujisawaense*_BacI84	KX641567	99	0.00/0.00**	0.00/12.98	0.00/0.00	0.00/0.00
	*Microbacterium*_sp_BacI80	KX641563	98	0.00/0.00	0.00/1.65	0.00/0.00	0.00/0.00
	*Streptomyces_novaecaesareae*_BacI81	KX641564	99	0.00/0.00	0.00/13.98	0.00/0.00	0.00/0.00
	*Streptomyces_recifensis*_BacI66	KX641550	99	0.00/2.17	0.00/0.00	0.00/0.00	0.00/0.00
Bacteroidetes	*Chitinophaga_pinensis*_BacI77	KX641561	99	0.00/0.00	0.00/1.50	0.00/0.00	0.00/0.00
Firmicutes	*Bacillus_amyloliquefaciens*_BacI76	KX641560	99	0.00/0.00	0.00/1.6	0.00/0.00	0.00/0.00
	*Bacillus_cereus*_X	KX641526	98	0.00/5.93	0.00/0.00	0.00/19.38	23.53/15.96
		KX641545					
		KX641569					
		KX641570					
		KX641578					
		KX641579					
	*Bacillus_megaterium*_BacI64	KX641548	99	0.00/4.24	0.00/0.00	0.00/0.00	0.00/0.00
	*Bacillus_ velezensis*_BacI82	KX641565	99	0.00/0.00	0.00/13.65	0.00/0.00	0.00/0.00
	*Fictibacillus nanhaiensis*_BacI69	KX641553	99	0.00/1.60	0.00/0.00	0.00/0.00	0.00/0.00
	*Bacillus_pumilus*_BacI67	KX641551	99	0.00/2.18	0.00/0.00	0.00/0.00	0.00/0.00
	*Bacillus*_sp_BacI19	KX641508	99	0.00/0.00	1.69/0.00	0.00/0.00	0.00/0.00
	*Bacillus_subtilis*_BacI75	KX641559	99	0.00/0.00	0.00/17.96	0.00/0.00	0.00/0.00
Proteobacteria	*Acinetobacter_baumannii*_BacI43	KX641527	99	0.00/0.00	0.00/0.00	0.00/0.00	29.41/0.00
	*Acinetobacter_calcoaceticus*_BacI97	KX641580	99	0.00/0.00	0.00/0.00	0.00/0.00	0.00/14.87
	*Acinetobacter*_sp1_BacI21	KX641510	99	0.00/0.00	8.47/0.00	0.00/0.00	0.00/0.00
	*Acinetobacter*_sp2_BacI53	KX641537	99	0.00/4.83	0.00/0.00	0.00/0.00	0.00/0.00
	*Acinetobacter_tandoii*_BacI52	KX641536	99	0.00/4.69	0.00/0.00	0.00/0.00	0.00/0.00
	*Burkholderia_ambifaria*_BacI90	KX641573	99	0.00/0.00	0.00/0.00	0.00/9.90	0.00/0.00
	*Burkholderia_cenocepacia*_BacI71	KX641555	99	0.00/0.00	0.00/2.57	0.00/0.00	0.00/0.00
	*Burkholderia_cepacia*_X	KX641531	99	0.00/6.21	32.20/2.18	0.00/0.00	0.00/18.72
		KX641547					
		KX641557					
		KX641582					
	*Burkholderia_kururiensis*_BacI100	KX641583	99	0.00/0.00	0.00/0.00	0.00/0.00	5.88/20.65
	*Burkholderia_nodosa*_BacI5	KX641496	99	8.57/0.00	0.00/0.00	0.00/0.00	0.00/0.00
	*Burkholderia_seminalis*_BacI48	KX641532	99	0.00/0.00	0.00/0.00	25.00/0.00	0.00/0.00
	*Burkholderia*_sp1_BacI28	KX641516	99	0.00/0.00	0.00/0.00	22.72/0.00	0.00/0.00
	*Burkholderia*_sp2_BacI92	KX641575	99	0.00/0.00	0.00/0.00	0.00/11.25	0.00/0.00
	*Burkholderia*_sp3_BacI41	KX641525	99	0.00/0.00	0.00/0.00	0.00/0.00	32.35/0.00
	*Burkholderia*_sp4_BacI98	KX641581	99	0.00/0.00	0.00/0.00	0.00/0.00	0.00/16.80
	*Burkholderia*_sp5_X	KX641528	99	0.00/0.00	0.00/0.00	0.00/0.00	2.94/0.00
		KX641529					
	*Cronobacter_sakazakii*_BacI54	KX641538	99	4.97/0.00	0.00/0.00	0.00/0.00	0.00/0.00
	*Dyella_jiangningensis*_BacI74	KX641558	99	0.00/0.00	1.99/0.00	0.00/0.00	0.00/0.00
	*Dyella_marensis*_BacI94	KX641577	99	0.00/0.00	0.00/0.00	0.00/12.58	0.00/0.00
	*Enterobacter_cloacae*_X	KX641492	99	51.43/0.00	13.56/0.00	0.00/0.00	0.00/0.00
		KX641494					
		KX641507					
		KX641513					
	*Enterobacter_ludwigii*_X	KX641539	99	0.00/5.10	0.00/0.00	0.00/8.57	0.00/0.00
		KX641571					

Table 1 (continued)

Phylum	Species	NCBI ID	%	PolHgY	PolHgN	AscHgY	AscHgN
	*Enterobacter*_sp_BacI22	KX641511	99	0.00/0.00	1.69/0.00	0.00/0.00	
	*Enterobacte*r_sp1_BacI85	KX641568	99	0.00/0.00	0.00/0.00	0.00/6.57	0.00/0.00
	*Enterobacter*_sp2_X	KX641514	99	0.00/0.00	0.00/0.00	29.54/0.00	2.94/0.00
		KX641515					
		KX641530					
	*Enterobacter*_sp3_X	KX641534	99	0.00/23.08	0.00/0.00	0.00/0.00	0.00/0.00
		KX641541					
		KX641552					
		KX641554					
	*Dickeya_chrysanthemi*_BacI59	KX641543	99	0.00/5.66	0.00/0.00	0.00/0.00	0.00/0.00
	*Escherichia*_sp_X	KX641495	99	7.14/0.00	0.00/0.00	0.00/0.00	0.00/0.00
		KX641499					
	*Klebsiella_pneumoniae*_X	KX641505	99	28.57/0.00	8.47/0.00	0.00/0.00	0.00/0.00
		KX641509					
	*Kosakonia_cowanii*_BacI60	KX641544	99	0.00/5.80	0.00/0.00	0.00/0.00	0.00/0.00
	*Mycobacterium*_sp_BacI83	KX641566	98	0.00/0.00	0.00/15.30	0.00/0.00	0.00/0.00
	*Pantoea*_sp1_BacI16	KX641506	99	0.00/0.00	32.20/0.00	0.00/0.00	0.00/0.00
	*Pantoea*_sp_BacI51	KX641535	99	0.00/4.55	0.00/0.00	0.00/0.00	0.00/0.00
		KX641572					
	*Pantoea*_sp1_I23	KX641512	99	0.00/0.00	1.69/0.00	0.00/0.00	0.00/0.00
	*Pantoea*_sp2_BacI65	KX641549	99	0.00/2.15	0.00/0.00	0.00/0.00	0.00/0.00
	*Pantoea_dispersa*_BacI89		99	0.00/2.15	0.00/0.00	0.00/9.24	0.00/0.00
	*Pseudomonas_knackmussii*_X	KX641500	99	1.43/0.00	0.00/1.47	0.00/0.00	0.00/0.00
		KX641562					
	*Pseudomonas_monteilii*_BacI6	KX641497	99	1.43/0.00	0.00/0.00	0.00/0.00	0.00/0.00
	*Pseudomonas_putida*_BacI58	KX641542	99	0.00/5.52	0.00/0.00	0.00/0.00	0.00/0.00
	*Pseudomonas*_sp_BacI7	KX641498	99	1.43/0.00	0.00/0.00	0.00/0.00	0.00/0.00
	*Ralstonia_mannitolilytica*_BacI72	KX641556	99	0.00/0.00	0.00/2.38	0.00/0.00	0.00/0.00
	*Ralstonia_pickettii*_BacI62	KX641546	99	0.00/6.07	0.00/0.00	0.00/0.00	0.00/0.00
	*Ralstonia*_sp_X	KX641574	99	0.00/0.00	0.00/0.00	0.00/22.49	0.00/0.00
		KX641576					
	*Rhizobium*_sp_BacI49	KX641533	99	0.00/0.00	0.00/0.00	22.73/0.00	0.00/0.00
	*Rhizobium_tropici*_BacI102	KX641584	99	0.00/0.00	0.00/0.00	0.00/0.00	0.00/12.99
	*Serratia_marcescens*_BacI56	KX641540	99	0.00/5.24	0.00/0.00	0.00/0.00	0.00/0.00

*% identity, ** data from fragments (F)/macerates (M)

(3.14%), *Pseudomonas_stutzeri*_BacI36 (1.94%), *Bacillus*_sp_X (1.79%), *Sphingomonas*_sp_X (1.79%), and *Lysobacter_soli*_BacI39 (0.45%), while the relative abundance of species in PolHgY was *Enterobacter*_sp_BacI14 (97.16%), *Klebsiella_pneumoniae*_BacI15 (2.65%), *Enterobacter*_sp_BacI12 (0.06%), *Novosphingobium*_sp_BacI10 (0.06%), and *Pantoea_agglomerans*_BacI11 (0.06%).

A correlation matrix was constructed using qualitative data from the presence or absence of the species identified through the three isolation methods – fragmentation, maceration, and enrichment. The data were also used to construct interaction networks to compare the hosts and collection sites (Table 2). A total of 66 species were present in the communities AscHgN, AscHgY, PolHgN, and PolHgY: 11, 20, 20, and 28, respectively. The parameters of the interaction network of endophytic bacteria (Table 2) evidenced that (i) Pol had a more structured network; (ii) the presence or absence of mercury had a determining force on the interaction and connectivity among endophytic species; and (iii) endophytic bacterial communities from plants collected at HgY environments were centralized with less modularity (Table 2).

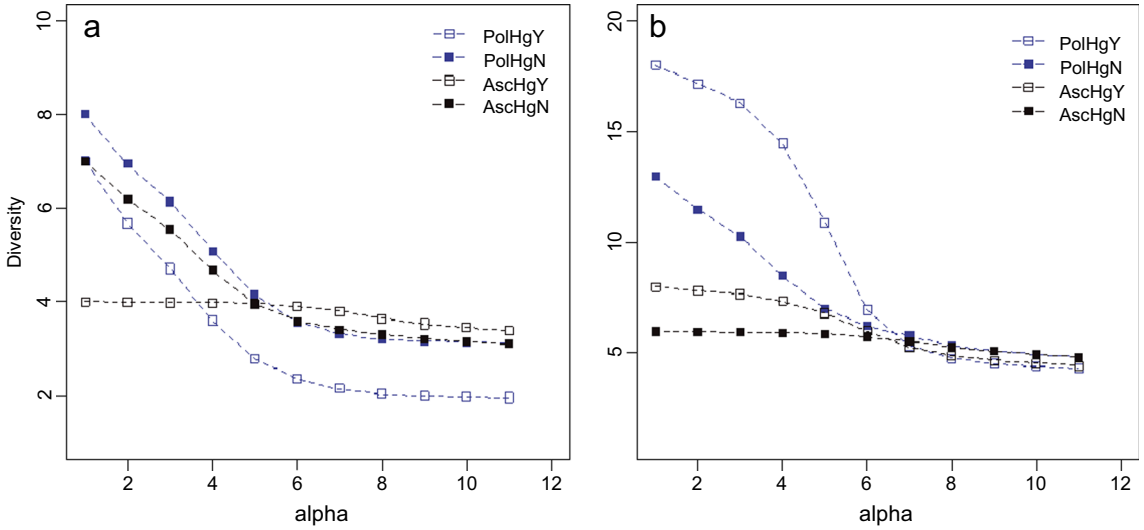

Fig. 1 Diversity of endophytic bacterial communities from *Aeschynomene fluminensis* (Asc) and *Polygonum acuminatum* (Pol) collected at areas contaminated (HgY) or not (HgN) with mercury, as examined through the Hill series, using root **a**) fragments or **b**) macerates

3.2 Functional characterisation of endophytic bacterial communities

Thirteen endophytic bacterial strains had score = 7 for the promising plant growth-promoting functional traits (Table S1), while six strains had low score (2) for ammonia and IAA. Strains isolated from plants collected at HgY environments exhibited greater proportion of functional traits than strains isolated from plants collected at HgN areas (Table 3). PolHgY roots hosted three amylase-secreting strains (*Bacillus_nanhaiensis*_BacI69, *Enterobacter*_sp_BacI14, and *Klebsiella*_sp_BacI2), and three siderophore-producing strains (*Bacillus_megaterium*_BacI64, *Enterobacter*_sp_BacI14, and *Kosakonia_cowanii*_BacI60). AscHgY and PolHgN roots hosted the cyanide acid-producing bacterial strains *Burkholderia_seminalis*_BacI48 and *Enterobacter*_sp_BacI22, respectively. Ammonia was produced by 27.63%, 26.80%, 22.68%, and 35.50% of the bacterial strains isolated from roots of AscHgN, AscHgY, PolHgN, and PolHgY, respectively (Table 3). IAA-secreting and nitrogen-fixing bacterial strains predominated in plants collected at HgY areas (Table 3).

Maceration of root tissues provided isolation of endophytic bacterial strains that were more sensitive to mercury, with MIC values ranging from 0 to 62 μg/mL of Hg^{2+}; MIC values of most of the strains ranged from 0 to 7.5 μg/mL of Hg^{2+} (Fig. 3 and Table S1). Endophytic bacterial strains isolated using the fragmentation and enrichment techniques exhibited broader ranges of MIC values: 0–250 μg/mL and 15–500 μg/mL of Hg^{2+}, respectively. The last technique provided isolation of mercury-resistant strains with high MIC values.

3.3 Host growth promotion in the presence of mercury

Addition of 40 $mg.kg^{-1}$ of $HgCl_2$ to the substrate reduced the corn plant length (CHgY = 25.16 ± 2.65 cm) by approximately

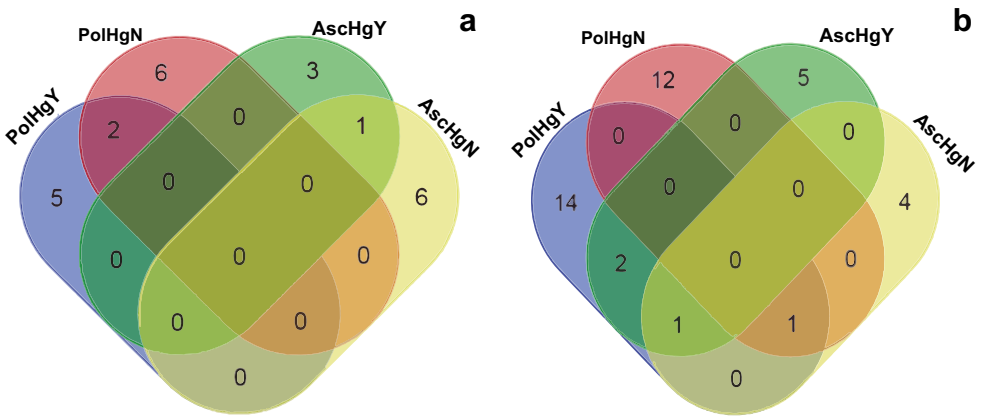

Fig. 2 Venn diagram of endophytic bacterial communities isolated from *Aeschynomene fluminensis* (Asc) and *Polygonum acuminatum* (Pol) roots collected at areas contaminated (HgY) or not (HgN) with mercury, and submitted to **a**) fragmentation or **b**) maceration

Table 2 Statistical parameters of undirected interpretation of the interaction networks from endophytic bacteria isolated from *Aeschynomene fluminensis* (Asc) and *Polygonum acuminatum* (Pol) collected at sites contaminated (HgY) or not (HgN) with mercury

Parameter	Pol	Asc	HgN	HgY
Average Degree	20.977	14.786	15.600	21.689
Diameter	2	3	2	2
Density	0.499	0.548	0.538	0.493
Edges	451	207	234	488
Modularity	0.448	0.279	0.311	0.476
Nodes	43	28	30	45
Sum change	0.01075	0.00223	0.00253	0.0136

40% relative to the plants grown in the absence of this metal (CHgN = 43.03 ± 4.80 cm) (Dunnett's test, $p < 0.05$) (Fig. 4). Growth reduction was more pronounced in the shoot (43.5% reduction) than in the root (38.4% reduction) (Fig. 4). Corn plant inoculation with 27 endophytic bacterial strains promoted growth of plants seeded in the $HgCl_2$-supplemented substrate (Fig. 4); 36.36% and 63.64% of such strains were isolated using the enrichment and fragmentation techniques, respectively. Inoculation with *B. cereus*_BacI42 and *Pantoea*_sp_BacI23 increased the plant length by 57.48 ± 5.45 and 117.09 ± 0.28%, respectively. Inoculation with *Bacillus*_sp_BacI34, *Burkholderia*_sp_BacI45, *Enterobacter*_sp_BacI14, *Enterobacter*_sp_BacI26, *Enterobacter*_sp_BacI18, *K. pneumoniae*_BacI20, *L. soli*_BacI39, *Pantoea*_sp_BacI23, and *Pantoea*_sp_BacI16 increased the plant length by more than 70% in $HgCl_2$-supplemented substrate when compared with non-inoculated plants grown in the same substrate.

Table 3 Number of endophytic bacterial strains with plant growth-promoting functional traits, isolated from *Aeschynomene fluminensis* (Asc) and *Polygonum acuminatum* (Pol) roots collected at areas contaminated (HgY) or not (HgN) with mercury

Functional trats	AscHgN	AscHgY	PolHgN	PolHgY
Ammonia	12(12.37)*	27(27.84)	21(21.65)	33(34.02)
Cyanidric Acid	0 (0.00)	1 (1.03)	1 (1.03)	0 (0.00)
IAA	12(12.37)	25(25.77)	21(21.65)	33(34.02)
Nitrogen	11(11.34)	26(26.80)	18(18.56)	32(32.99)
Siderophores	0 (0.00)	0 (0.00)	0 (0.00)	3 (3.09)
Enzymes				
Amylase	0 (0.00)	0 (0.00)	0 (0.00)	3 (3.09)
Cellulase	0 (0.00)	7 (7.22)	8 (825)	8 (8.25)
Esterase	4 (4.12)	2 (2.06)	8 (8.25)	5 (5.15)
Phosphatase	8 (8.25)	8 (8.25)	7 (7.22)	17(17.53)
Protease	5 (5.15)	9 (9.28)	10(10.31)	10(10.31)

(*) percentage of isolates analysed

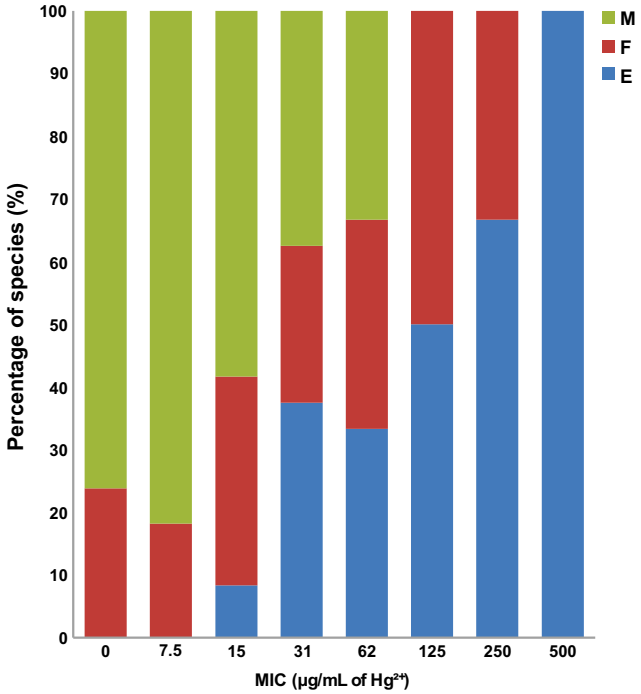

Fig. 3 Minimal inhibitory concentration (MIC) for endophytic bacteria isolated from *Aeschynomene fluminensis* and *Polygonum acuminatum* roots collected at areas contaminated or not with mercury. The species were isolated using the enrichment (E), fragmentation (F), and maceration (M) techniques

4 Discussion

We examined how mercury contamination influenced the diversity of endophytic bacteria in *A. fluminensis* (Asc) and *P. acuminatum* (Pol) roots. The predominance of these two plant species in the community grown in the selected HgY area suggests that they have developed mechanisms to limit soil mercury toxicity, and root endophytic fungi communities appear to play important roles (Pietro-Souza et al. 2017). It is also clear that, in the case of Asc and Pol, the roots host endophytic bacteria, regardless the site of plant collection. Endophytic bacteria colonize root tissues, can migrate to other plant organs (Jha et al. 2013), and play roles in plant adaptation and growth in contaminated soils (Afzal et al. 2017). Analysis of endophyte colonization of host plants growing in environments contaminated with elements such as arsenic, copper, chrome, nickel, and zinc has shown the presence of metal-resistant strains with potential in bioremediation (Sun et al. 2010; Fidalgo et al. 2016; Román-Ponce et al. 2016; Sánchez-López et al. 2018).

The endophytic bacteria population density in plants collected at HgY sites ranged from 5.72×10^5 to 3.27×10^5 CFU.g^{-1} of tissue, which is smaller than the range reported in the literature: 2.7×10^7 to 1.2×10^8 CFU.g^{-1} (Pérez et al. 2016). Plant roots collected at HgY sites had greater colonization frequency and richness than plant roots collected at HgN sites. Such variations

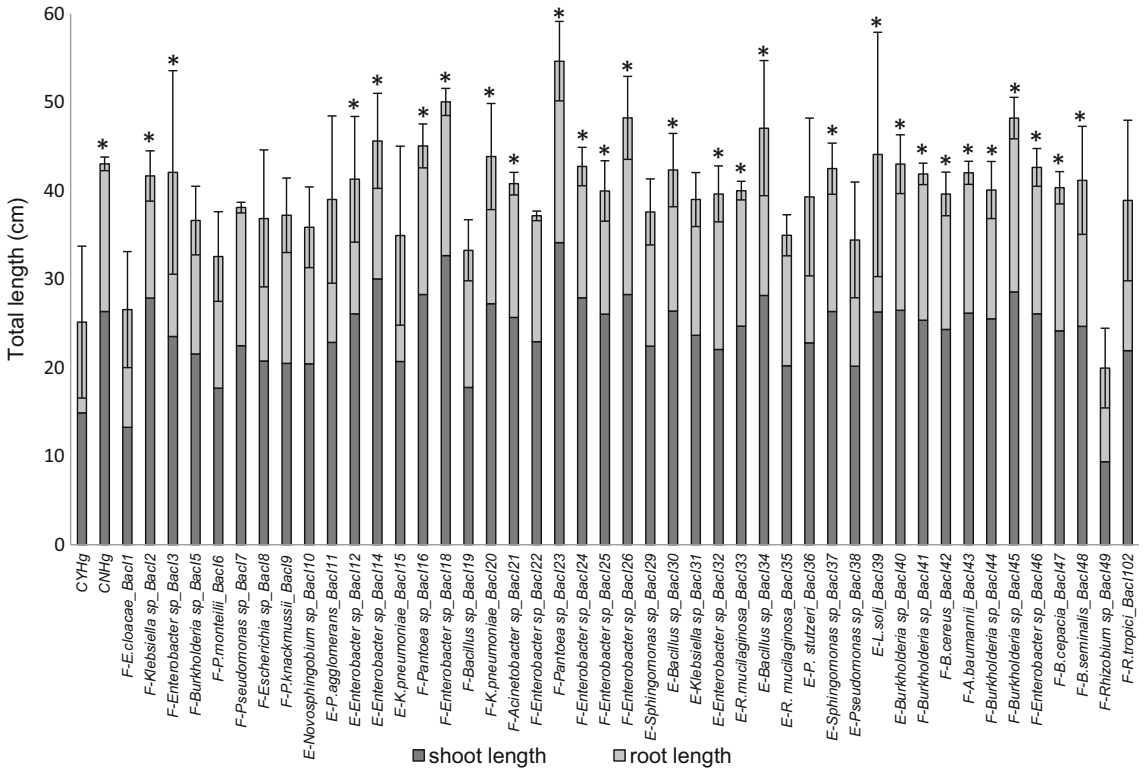

Fig. 4 Growth rate of corn plants (*Zea mays*) inoculated with endophytic microorganisms and seeded in mercury-supplemented substrate. Data are presented as the mean ± standard deviation of four replicates of plants. CNHg = non-inoculated corn plants grown in substrate non-supplemented with mercury; CYHg = non-inoculated corn plants grown in mercury-supplemented substrate. $*p < 0.05$ (analysis of variance followed by the Dunnett's test - control CYHg)

indicate that environment composition determines the associated community more strongly than the plant species (Teixeira et al. 2010). The host roots usually have greater richness and diversity of endophytes than the leaves, bark, flowers, and fruits (Gaiero et al. 2013), which are determined by edaphic factors (Hardoim et al. 2008). Soil mercury contamination increased the richness of the root endophytic bacterial community as has been shown for fungal communities (Pietro-Souza et al. 2017).

The high soil mercury concentration influenced the diversity and structure of endophytic bacterial communities (Figs. 1and 2), corroborating another report on the composition and diversity of endophytes (Sun et al. 2010). The interaction among endophytes, host plants, and the environment promotes diversity variation, increases richness, and provides competitive advantages to the host plant over native species (Mallon et al. 2015).

Analysis of the interaction network identified alterations in the co-occurrence patterns from microorganisms undergoing different treatments (Long et al. 2018). The most compact and complex networks – from Pol and HgY areas – indicate that the species keep a microbial community that is more stable, with strong correlation and that respond to mercury presence in the environment (Stegen et al. 2012; Jiao et al. 2016). The networks from Asc and HgN areas maintained weaker interspecific cooperation, which can be associated with the lower number of positive correlations found among the analysed species.

Isolation of cultivable bacteria represents only a small fraction of the real diversity that exists in the plant (Tanaka et al. 2014; Fidalgo et al. 2016). Actinobacteria, Bacteriodetes, Firmicutes, and Proteobacteria were the predominant phyla in Asc and Pol roots (Table 1). These phyla are often associated with the two host plants studied (Pereira and PML 2014; Maida et al. 2015; Maropola and Ramond 2015; Fidalgo et al. 2016; Román-Ponce et al. 2016; Sánchez-López et al. 2018), including those growing in metal-contaminated environments (Luo et al. 2011; Mesa et al. 2017; Durand et al. 2018; Gu et al. 2018). The species *Bacillus_cereus*_X, *Burkholderia_cepacia*_BacI47, and *Enterobacter_cloacae*_X were detected with high abundance; they are usually found in endophytic bacterial communities. The exclusive presence of the genus *Enterobacter* in communities from HgY areas (Table 1) was probably associated with mercury resistance mechanisms (Mosa et al. 2016) (see Table S1) that could include increasing the solubility, reducing or oxidizing the metal to less toxic forms (Mani and Kumar 2014).

Endophytic bacteria isolated from host plants collected at HgY sites produce more plant growth-promoting functional traits than those collected at HgN sites (Table 3; Table S1).

Bacillus, *Burkkolderia*, *Enterobacter*, *Klebsiella*, *Pantoea*, and *Pseudomonas* are bacterial genera that bear a variety of plant growth-promoting functional traits (da Costa et al. 2014; Ullah et al. 2015; Meng et al. 2015).

Endophytic bacteria that carry plant growth-promoting traits and are resistant to metals can be used to enhance plant growth (Sun et al. 2010). It is noteworthy that 60.47% of the isolated bacterial strains mitigated mercury toxicity in corn plants (Fig. 4). Our data and those of others show that host plants and endophytes probably established a mutualistic symbiotic relationship that increases plant growth in the presence of mercury (Rodriguez et al. 2008). In conclusion, we demonstrated that mercury-resistant endophytic bacterial strains – especially *Pantoea sp*_BacI23 – promote host plant growth. However, further research on mercury remediation under field conditions, as well as the elucidation of resistance mechanisms are still required.

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