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In vitro characterization of endophytic bacteria associated with physic nut (*Jatropha curcas* L.) and their potential for plant-growth promotion and biocontrol

Caracterização in vitro de bactérias endofíticas associadas ao pinhão-manso (*Jatropha curcas* L.) e seu potencial de promoção de crescimento vegetal e biocontrole

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Paula Cristiane Machado
Doutora em Biotecnologia
Institution: Programa de Pós-Graduação em Biotecnologia, Universidade Federal de São Carlos-UFSCar, CEP.13565-905. São Carlos, São Paulo, Brasil
E-mail: machadopc16@gmail.com

Paulo Henrique Marques Andrade
Doutor em Genética e Evolução
Institution: Programa de Pós-Graduação em Genética Evolutiva e Biologia Molecular, Universidade Federal de São Carlos-UFSCar, CEP.13565-905. São Carlos, São Paulo, Brasil
E-mail: pandrad012@gmail.com

Cristina Paiva de Sousa
Doutor em Ciências Biológicas – Microbiologia
Institution: Programa de Pós-Graduação em Biotecnologia, Universidade Federal de São Carlos-UFSCar, CEP.13565-905. São Carlos, São Paulo, Brasil
E-mail: prokarya@gmail.com

Clovis Wesley Oliveira de Souza
Doutor em Ciências Biológicas – Microbiologia
Institution: Programa de Pós-Graduação em Biotecnologia, Universidade Federal de São Carlos-UFSCar, CEP.13565-905. São Carlos, São Paulo, Brasil
E-mail: clovis@ufscar.br

Paulo Teixeira Lacava
Doutor em Ciências – Energia Nuclear na Agricultura
Institution: Programa de Pós-Graduação em Biotecnologia, Universidade Federal de São Carlos-UFSCar, CEP.13565-905. São Carlos, São Paulo, Brasil
E-mail: ptlacava@ufscar.br

ABSTRACT
The physic nut (*Jatropha curcas* L.) is a shrubby plant of perennial cycle, belonging to the family Euphorbiaceae, from Central America and currently vegetates spontaneously in diverse regions of the planet. The commercial interest in Brazil occurred due to the desirable characteristics of that crop as an agricultural option for renewal of the Brazilian energy base, being a promising raw material for biodiesel production. Oil plants that have a high biotechnological potential may have a genetically

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diverse microbial population with characteristics of promoting the growth of multifunctional plants. P*lant growth-promoting endophytes* (PGPE) are of biotechnological interest since they can improve the growth of several important agronomical crops. The present study aimed the biochemistry characterization of thirty-seven endophytic bacteria strains associated with *J. curcas* plants, with the potential of plant growth promotion. Of this total of evaluated strains, 75% showed positive results for fixation of nitrogen, 62% produced IAA in the presence of the tryptophan precursor, 32% solubilized inorganic phosphate and 35% exhibited antagonistic activities against phytopathogenic fungi (*Lasiodiplodia. subglobosa*, *L. euphorbicola*, and *L. pseudotheobromae*) in physic nut. To our knowledge, this is the first report of this potential of biocontrol against *Lasiodiplodia* species. Among the thirty-seven bacterial isolates identified by partial sequencing of the 16S gene, the presence of the genera *Arthrobacter*, *Bacillus*, *Citrobacter*, *Curtobacterium*, *Enterococcus*, *Klebsiella*, *Leucobacter*, *Lysinibacillus*, *Microbacterium*, *Rhodococcus*, and *Serratia* was observed. Our results indicated that the cultivable endophytic bacteria isolated from *J. curcas* have the potential to demonstrate multiple characteristics of PGPE *in vitro* and have the potential for other large-scale assays such as biofertilizer and biopesticides.

Keywords: Biofertilizers, Plant growth promotion, Sustainable agriculture.

RESUMO

O pinhão-manso (*Jatropha curcas* L.) é uma planta arbustiva de ciclo perene, pertencente à família Euphorbiaceae, da América Central e atualmente vegeta espontaneamente em diversas regiões do planeta. O interesse comercial no Brasil ocorreu devido às características desejáveis dessa cultura como opção agrícola para renovação da base energética brasileira, sendo uma promissora matéria-prima para a produção de biodiesel. As plantas oleaginosas com alto potencial biotecnológico podem apresentar uma população microbiana geneticamente diversa com características de promoção do crescimento de plantas multifuncionais. Endófitos promotores de crescimento de plantas (PGPE) são de interesse biotecnológico, uma vez que podem melhorar o crescimento de várias culturas agronômicas importantes. O presente trabalho teve como objetivo, a caracterização bioquímica de trinta e sete bactérias endofiticas associadas à cultura do pinhão-manso, com potencial para promoção de crescimento vegetal. Desse total de isolados avaliados, 75% das bactérias avaliadas fixaram nitrogênio, 62% dos isolados foram capazes de sintetizar o fitohormônio IAA na presença do precursor do triptofano, 32% dos isolados avaliados tiveram capacidade de solubilização de fosfato inorgânico e 35% exibiram atividades antagônicas contra fungos fitopatogênicos em pinhão-manso (*L. subglobosa*, *L. euphorbicola* e *L. pseudotheobromae*). Até onde sabemos, este é o primeiro relato desse potencial de biocontrole contra espécies de *Lasiodiplodia*.Dentre os trinta e sete isolados identificadas pelo sequenciamento parcial do gene 16S, foi observado a presença dos gêneros *Arthrobacter*, *Bacillus*, *Citrobacter*, *Curtobacterium*, *Enterococcus*, *Klebsiella*, *Leucobacter*, *Lysinibacillus*, *Microbacterium*, *Rhodococcus* e *Serratia*. Nossos resultados indicaram que as bactérias endofíticas cultiváveis isoladas de *J. curcas* possuem potencial para demonstrar múltiplas características de PGP *in vitro* e tem potencial para outros ensaios em larga escala como biofertilizante e biopesticidas.

Palavras-chave: Biofertilizantes, Promoção de crescimento vegetal, Agricultura Sustentável.

1 INTRODUCTION

The physic nut (*Jatropha curcas* L.), is a plant belongs to the Euphorbiaceae family, that is genetically close to the castor plant (*Ricinus communis* L.); it originates from Central America and is currently distributed in all tropical regions of the globe (Kumar and Tewari, 2015; Kumar et al., 2016). The physic nut is a plant that produces seeds containing inedible oil with biotechnological potential.

This culture has received special attention since it could be used as renewable feedstocks has to supply energy and alternative fuels. The detoxified cake by-product from oil extraction can be used for fish and animal feed, biogas, or as organic fertilizer. (Nahar and Ozores-Hampton, 2011; Laviola et al. 2015).

Jatropha curcas is a tropical plant and can be grown in low to high rainfall, diverse soil types, with limited nutrients, rapid growth, easy propagation, and adaptation to a wide range of environmental stress conditions, being able to take advantage of anthropic/unproductive areas (Abhilash et al. 2011). Due to these characteristics, there was a great incentive for the commercial cultivation of physic nut in Brazil as a renewable raw material to produce biodiesel. However, the great expansion of cultivated areas has been accompanied by the occurrence of new pathogens and the appearance of diseases called descending dryness and stem base rot, caused by species of the *Lasiodiplodia* fungus, causing losses in productivity and causing mortality of 80% of physic nut plants (Machado, Pine, Pereira; 2014).

In this scenario, farmers have been *abandoned* of the cultivation of physic nut in different regions of Brazil. Chemical control alone does not provide protection or curative control when the damage comes from species of *Lasidioplodia*. The adoption of a series of additional measures is being recommended, such as the management of the culture and biological control (Tavares, 1995; Silva et al., 2015).

In Brazil, several studies on climatic adaptation and productivity have been developed on the genetic variability of physic nut, however, as it is a perennial cycle culture, it takes a long period to achieve the expected results (Edrisi et al. 2015; Laviola et al., 2015). The study of the biotechnological and agricultural potential of the endophytic microbiota associated with physic nut presents itself as an alternative that can contribute to research aimed at increasing productivity and establishing this crop. Mohanty et al. (2017) hypothesize that its ability to adapt to environmental stresses could be due to its endophytes (Madhaiyan et al. 2013; Qin et al. 2012;).

Endophytic microorganisms are potential sources of natural, bioactive, and chemically new products for exploitation in medicine, agriculture, and industry (Gao, Li, Lou, 2018; Rajamanikyam et al., 2017; Soares et al., 2017). Generally, plants have an endophytic microbiota that is important for their health and maintenance (Azevedo and Araújo 2007; Rodriguez et al., 2019). Endophytic bacteria bring many benefits to plants, such as biological control (Lacava and Azevedo, 2014; Eljounaidi, Lee, Bae, 2016), including competition of nutrients or with the production of toxins harmful to pathogens (Hazarika et al., 2019), decreasing disease susceptibility (Busby et al. 2016; Christian et al. 2019; Compant et al. 2005), increasing resistance to abiotic stressors (Márquez et al. 2007; Rodriguez et al. 2008), shaping phytochemical profiles (Kusari et al. 2012; Panaccione et al. 2014), and mediating plant functional trait expression (Harrison and Griffin 2020). In addition, these microorganisms can

influence the growth of plants, by increasing the availability of nutrients, such as biological fixation nitrogen, phosphate solubilization, and production of siderophores; or in the production of compounds that function as plant regulators, such as phytohormone indole-3-acetic acid (IAA) (Santoyo et al., 2016; Yadav and Yadav, 2019).

Only a few studies in Brazil have focused on the analysis of the culturable endophytic microbial communities associated with *J. curcas* (Moniruzzaman et al. 2016). Therefore, this study aimed to identify partial 16S rRNA gene and characterize cultivable endophytic bacteria associated with *J. curcas* L. with potential applications in agriculture as biofertilizer and biopesticides.

2 MATERIAL AND METHODS

2.1 BACTERIAL ISOLATES

Thirty-seven bacterial strains endophytically isolated (Araújo et al., 2014) from leaves of *J. curcas* trees were tested in this study. These strains are part of the Bacterial Collection of the Laboratory of Microbiology and Biomolecules, Federal University of São Carlos, São Carlos, SP, Brazil.

2.2 BACTERIAL MOLECULAR IDENTIFICATION

Endophytic strains were grown in 100% trypticase soy broth (TSA-Merck, Sigma-Aldrich, USA) for 48 h at 28 °C. Genomic DNA was extracted using a modified protocol of salt extraction from Aljanabi and Martinez (1997). Partial 16S rDNA was amplified using the primers 1500R 5'-GGTTACCTTGTTACGACTT- 27F 5'-AGAGTTTGATCCTGGCTCAG-3' (Heuer et al., 1997). PCR was performed using 0.2 mM each dNTP, 1 pmol each primer, 1,25 U Taq DNA polymerase, and 1× buffer in a final volume of 15 µl. Amplifications were carried out in a thermocycler (GeneAmp PCR System 9700, Applied Biosystems) with an initial denaturation step at 95 °C for 3 min; 31 cycles at 95°C for 30 s, 56°C for 30 s, and 72 °C for 1 min; and one final elongation cycle at 72 °C for 10 min.. As a negative control, we replaced the DNA template with sterile DNase-free water. The 16S rDNA gene PCR products were purified using the polyethylene glycol method described by Lis (1975) and sequenced at the DNA Consult Genética e Biotecnologia Ltda (São Carlos, SP, Brazil - https://www.dnaconsult.com.br/). Sequencing was made using the primer 27F. Bacterial identification was performed by comparing the obtained sequences against those deposited in the GenBank from the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) using the BLASTn tool (Altschul et al., 1997).

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2.3 PLANT GROWTH PROMOTING TRAITS

2.3.1. Growth capacity of the isolates in nitrogen-free culture medium

The evidence of the strains to fix atmospheric nitrogen was assessed as described by Baldani et al. (2014) by growing the strains on a semisolid nitrogen-free medium seven consecutive times. After 96 h of incubation at 28 °C in the dark, the formation of a white growth film near the surface of the tubes indicated a positive result *Burkholderia ambifaria* strain RZ2MS16, was used as a positive control (Batista et al., 2018). This procedure was performed in triplicate.

2.3.2. Auxin production

The IAA production was evaluated using the colorimetric method developed by Bric et al. (1991) with modifications and adapted for the quantitative method described by Husen (2013). All thirty-seven isolates were inoculated in tubes containing 5 mL of liquid 10% TSB (w/v) containing 50 μg mL^{-1} L-tryptophan, in triplicate. Tubes were incubated for 72 h at 28 ± 2 °C. They were then centrifuged at 12,000 rpm for 5 min. Subsequently, IAA concentration in the culture's supernatant was estimated using Salkowski reagent. The OD values obtained were interpolated from a standard curve to determine the IAA concentration per ml basis (Sarwar and Kremer, 1995). Additionally, as a positive control, we measured the auxin produced by *B. ambifaria* strain RZ2MS16, which was previously reported as an IAA producer (Batista et al., 2018).

2.3.3 phosphate solubilization

The ability of bacterial strains to solubilize inorganic calcium phosphate was indicated by a halo obtained after cultivation of the bacteria on culture medium supplemented with $Ca_3(PO_4)_2$ at 28 °C for 72 h according to methods described by Verma et al. (2001). *B. ambifaria* strain RZ2MS16, was used as a positive control for phosphate solubilization (Batista et al., 2018). The ratio between the halo diameter (cm) and the colony diameter (cm) was calculated, generating an Index of Phosphate Solubilization (IPS) that was useful for ranking the bacteria into three categories (low, medium and high IPS) as proposed by Silva Filho and Vidor (2000).

2.4 ANTAGONISTIC ACTIVITY AGAINST PHYTOPATHOGENIC FUNGI

The bacterial strains were evaluated *in vitro* using a parity method against the new species of phytopathogenic fungi, *Lasiodiplodia subglobosa*, *L. euphorbicola*, and *L. pseudotheobromae*, of *J. curcas*. These phytopathogenic isolates were kindly provided by Prof. Dr. Olinto Liparini Pereira, Department of Phytopathology, Federal University of Viçosa – UFV, Minas Gerais, Brazil (www.dfp.ufv.br/departamento/laboratorios).

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A preliminary assay was performed to select bacteria with antagonistic activity. The bacteria were inoculated two-by-two in the extremities of Petri dishes (9 cm diameter) containing Potato Dextrose Agar medium (PDA) (KASVI) The phytopathogenic fungus was inoculated with a 0.5 cm agar disc in the center of the dishes. The plates were incubated for 7 days at 28 °C. Only bacteria that inhibited fungal growth were evaluated for antagonistic activity. Thirteen selected bacterial strain was inoculated onto Tryptic Soy Agar (TSA) (KASVI) medium and after 48 h transferred to 5 mL of sterilized water and the absorbance of thirteen bacterial suspensions was measured and adjusted to OD_{600} = 0.1. Approximately 10 µL of the bacterial suspension was inoculated on one extremity of the plates, and on the same day, the fungus (0.5 cm diameter disc) was inoculated in the center of the plate. The experiments were performed in triplicate. After incubation for 7 days at 28 °C the percentage inhibition (IA%) was calculated using the formula:

$$\%IA = 100 - \{\frac{X1}{\frac{[X2+X2\prime+X2"]\ x\ 100}{3}}\}$$

2.5 STATISTICAL ANALYSIS

Data were subjected to analysis of variance, and means were compared by the *Scott Knott* test for phosphorus solubilization index (<0.05) and the *Tukey* Test for plant experiments (<0.05). Statistical analyses were performed with Sisvar software (version 5.6).

3 RESULTS

3.2 MOLECULAR IDENTIFICATION

All thirty-seven selected strains were sequenced partial 16S rDNA gene, after comparison of sequences obtained with the registered sequences in the international bank of genes (GenBank). Firmicutes were the most abundant phylum represented mostly by *Bacillus* strains, followed by phylum Actinobacteria and minor contributions from the phyla Proteobacteria. The strains were identified as belonging to the genera *Arthrobacter*, *Bacillus*; *Citrobacter*; *Curtobacterium*; *Enterococcus*; *Klebsiella*; *Leucobacter*; *Lysinibacillus*; *Microbacterium*; *Rhodococcus* and *Serratia*, and nucleotide sequences were deposited at the GenBank with accession numbers MH037575 – MH037610 and MH208710 (Table 1).

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3.3 PLANT GROWTH-PROMOTING TRAITS

All thirty-seven endophytic bacteria strains were biochemically tested for three PGP traits (Table 2). Evidence of nitrogen-fixing ability was observed in 21 strains, and most of them also belonged to the *Bacillus* genus (71%) (Table 2). It was observed that 23 strains (62%) were able to produce IAA in the presence of the precursor tryptophan. The IAA production ranged from 2,4 to 84,11 $\mu g.mL^{-1}$, highlight a lineage of the genus *Citrobacter* (EPM-63B) with a value of 84.11 $\mu g.mL^{-1}$, two strains of the genus *Klebsiella* (EPM-4 and EPM-63), three strains of the genus *Bacillus* (EPM-10, EPM-92 and EPM-54) with values of 58,25 $\mu g.mL^{-1}$, 45$\mu g.mL^{-1}$ and 47.07$\mu g.mL^{-1}$ respectively and two strains of the genus *Microbacterium* (EPM-96 and EPM-50C) with respective values of 56.34 µg.mL-1 and 45.25µg.mL-1 of IAA. About the capacity to solubilize phosphate, 12 strains demonstrated able to solubilize phosphate *in vitro*. Five strains bacterial, showed the highest IPS (> 3.0), 2 of the genus *Serratia* (EPM-2; EPM-75), 2 of the genus *Klebsiella* (EPM-4 and EPM-63), and 1 of the genus *Citrobacter* (EPM-63B) (Table 2). Four strains showed the three PGPE traits evaluated. Four strains demonstrated two of the PGPE traits evaluated.

Table 1. Identification by partial sequencing of 16S rDNA of the endophytic bacteria strains isolated from the *Jatropha curcas*.

Strains	GenBank accession Nº.	Identification	ID (%)	Reference accession Nº.
PM-78	MH037604	*Arthrobacter* sp.	100%	KR906431.1
EPM-5	MH037577	*Bacillus* sp.	100%	MG062868.1
EPM-10	MH037580	*Bacillus* sp.	99%	MG027677.1
EPM-36A	MH037585	*Bacillus* sp.	100%	KY621950.1
EPM-37	MH037586	*Bacillus* sp.	100%	MG062841.1
EPM-41A	MH037587	*Bacillus* sp.	99%	MF187637.1
EPM-41B	MH037588	*Bacillus* sp.	100%	MG988282.1
EPM-53	MH037593	*Bacillus* sp.	100%	NR_112637.1
EPM-54	MH037594	*Bacillus* sp.	100%	LC372639.1
EPM-55A	MH037595	*Bacillus* sp.	100%	KX090189.1
EPM-58	MH037597	*Bacillus* sp.	100%	NR_113265.1
EPM-63D	MH037599	*Bacillus* sp.	100%	MG526971.1
EPM-70	MH037602	*Bacillus* sp.	100%	KX618337.1
EPM-88	MH037607	*Bacillus* sp.	100%	MG585942.1
EPM-89	MH037608	*Bacillus* sp.	100%	MG799433.1
EPM-92	MH037609	*Bacillus* sp.	100%	MG016492.1
EPM-63B	MH208710	*Citrobacter* sp.	99%	JF935085.1
EPM-33	MH037583	*Curtobacterium* sp.	100%	MF526513.1
EPM-34	MH037584	*Curtobacterium* sp.	100%	KY908492.1
EPM-6	MH037578	*Enterococcus* sp.	100%	MF424691.1
EPM-87A	MH037605	*Enterococcus* sp.	100%	KR364767.1
EPM-87B	MH037606	*Enterococcus* sp.	100%	MF424509.1

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EPM-4	MH037576	*Klebsiella* sp.	100%	MF804987.1
EPM-63	MH037598	*Klebsiella* sp.	100%	MF461048.1
EPM-46B	MH037590	*Leucobacter* sp.	100%	LC065352.1
EPM-55B	MH037596	*Lysinibacillus* sp	100%	KY848326.1
EPM-7	MH037579	*Microbacterium* sp.	99%	MF524142.1
EPM-42	MH037589	*Microbacterium* sp.	100%	KP027812.1
EPM-47	MH037591	*Microbacterium* sp.	100%	NR_117294.1
EPM-50C	MH037592	*Microbacterium* sp.	100%	MF526709.1
EPM-96	MH037610	*Microbacterium* sp.	100%	JX471120.1
EPM-25	MH037581	*Rhodococcus* sp.	99%	KX062526.1
EPM-28	MH037582	*Rhodococcus* sp.	99%	KT936133.1
EPM-2	MH037575	*Serratia* sp.	99%	KX821734.1
EPM-66	MH037600	*Serratia* sp.	99%	MF927589.1
EPM-66B	MH037601	*Serratia* sp.	100%	NR_036886.1
EPM-75	MH037603	*Serratia* sp.	99%	NR_114043.1

Table 2. Evaluation of *in vitro* tests for plant growth promoter bacteria isolated from leaves of *Jatropha curcas*.

Identification[a]	**Strains**	**IAA[b](µg/ml)**	**IPF[c]**	**BNF[d]**
Arthrobacter sp.	PM78	2.40	-	-
	EPM5	-	-	+
	EPM10	58.25	-	+
	EPM36A	6.21	1.51	+
	EPM37	-	-	+
	EPM41A	15.97	1.58	+
	EPM41B	15.99	1.63	+
	EPM53	-	-	+
Bacillus sp.	EPM54	47.07	1.64	+
	EPM55A	-	-	+
	EPM58	-	-	+
	EPM63D	-	-	+
	EPM70	-	-	+
	EPM88	-	-	+
	EPM89	-	-	+
	EPM92	48.45	-	+
Citrobacter sp.	EPM63B	84.11	4.53	-
Curtobacterium sp.	EPM33	7.71	-	+
	EPM34	12.83	-	+
	EPM6	-	2.39	-
Enterococcus sp.	EPM87A	-	-	-
	EPM87B	-	-	-
Klebsiella sp.	EPM4	79.87	3.05	-
	EPM63	65.37	4.44	-
Leucobacter sp.	EPM46B	8.77	-	-
Lysinibacillus sp.	EPM55B	9.49	-	+
	EPM7	27.5	-	-
Microbacterium sp.	EPM42	19.72	-	-
	EPM47	20.14	-	-

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	EPM50C	45.25	-	-
	EPM96	56.34	-	+
Rhodococcus sp.	EPM25	-	-	+
	EPM28	-	-	+
	EPM2	38.93	3.39	-
Serratia sp.	EPM66	6.98	2.92	-
	EPM66B	6.82	2.86	-
	EPM75	5.40	3.01	-

[a]The identification was done according to BLASTn analysis of complete 16S rDNA sequences and phylogenetic analysis; [b]Indole-3-acetic acid production (µg ml); Phosphate Solubilization Index; [d]Biological Nitrogen Fixation (with '+' = positive and with '-' = negative).

3.4 ANTAGONISTIC ACTIVITY AGAINST PHYTOPATHOGENIC FUNGI

Among the thirty-seven bacterial strains evaluated against phytopathogenic fungi *L. subglobosa*, *L. euphorbicola*, and *L. pseudotheobromae* (Figure 1), 35% exhibited antagonism the three species of phytopathogenic fungi evaluated and submitted to semi-quantitative tests and analyzed statistically by Scott-knott (Table 3).

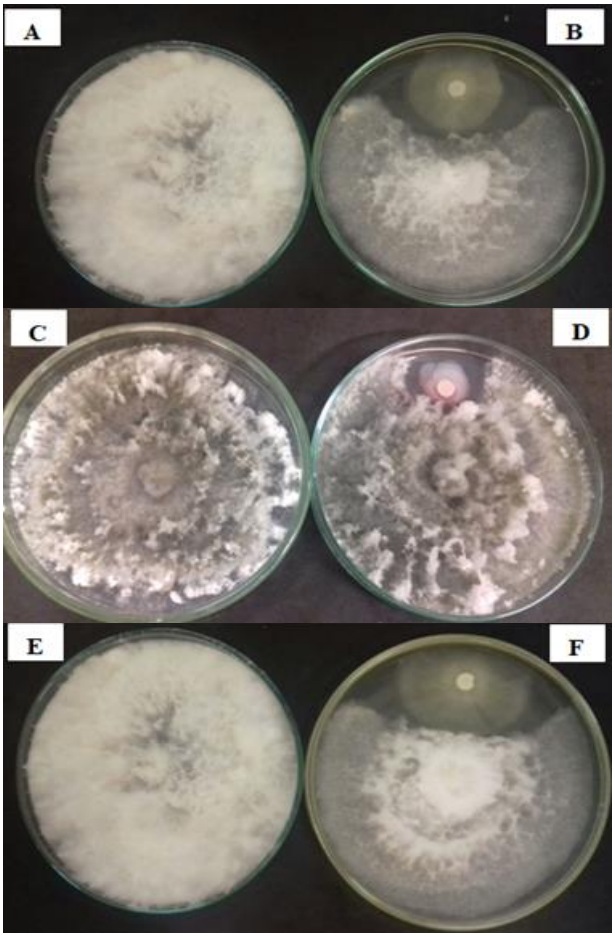

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Figure 1. Antagonism examples of the initial screening of endophytic bacteria against p*hytopathogenic fungi reported to cause diseases root or collar rot in J. curcas* (*Lasiodiplodia subglobosa, L. euphorbicola,* and *L. pseudotheobromae*). (A) Growth of the phytopathogen *L. subglobosa* in culture medium (control); (B) Antifungal activity of the endophytic bacterium *Bacillus* sp. EPM-55A against *L. subglobosa*; (C) Growth of the phytopathogen *L. euphorbicola* in culture medium (control), (D) Antifungal activity of the EPM-55A against *L. subglobosa*; (E) Growth of the phytopathogen *L. pseudotheobromae* in culture medium (control); and (F) Antifungal activity of the EPM-55A against *L. pseudotheobromae*.

Table 3. Statistical grouping of the index of growth reduction of phytopathogenic fungi by endophytic bacteria isolated from the *Jatropha curcas*.

Strains	Identification	% growth reduction of phytopathogenic fungi*		
		Lasiodiplodia subglobosa	***Lasiodiplodia euphorbicola***	***Lasiodiplodia pseudotheobromae***
EPM2	*Serratia* sp.	38.33% A	35.00% A	39.16%A
EPM5	*Bacillus* sp.	69.16% D	48.33% C	63.33%C
EPM37	*Bacillus* sp.	65.00% C	48.33% C	65.00% C
EPM53	*Bacillus* sp.	37.50% A	36.66% A	40.00% A
EPM55A	*Bacillus* sp.	74.00% E	51.66% C	76.66% D
EPM58	*Bacillus* sp.	67.50% C	49.16% C	70.00% C
EPM63D	*Bacillus* sp.	64.16% C	49.16% C	65.83% C
EPM66	*Serratia* sp.	44.16% B	40.83% B	45.83% B
EPM66B	*Serratia* sp.	46.66% B	40.83% B	46.66% B
EPM70	*Bacillus* sp.	66.66% C	56.66% D	66.66% C
EPM75	*Serratia* sp.	44.16% B	40.83% B	45.83% B
EPM88	*Bacillus* sp.	34.16% A	34.16% A	38.33% A
EPM89	*Bacillus* sp.	37.50% A	35.00% A	38.33% A

*Values with the same letter do not differ at a 5% significance level (*Scott Knott* test).

4 DISCUSSION

The Ministry of Agriculture, Livestock, and Supply (MAPA) recently launched the National Bioinsumption Program and aims to sustainably exploit the potential of Brazilian biodiversity to reduce the dependence on producers of imported inputs and expand the supply of raw materials for the sector (Brasil, 2020). In this context, physic nut easily grows under harsh environment and limited nutrient availability can be considered a promising source of microorganisms with biotechnological potential for the use of biological resources in Brazilian agriculture (Mohanty et al., 2017).

Although endophytes are prevalent, the literature about this group of microorganisms from renewable energy plants *J. curcas* is scarce. Some studies of the endophytic and rhizospheric bacteria diversity associated with *J. curcas* found the phyla Actinobacteria, Acidobacteria, Chlroflexi, Firmicutes, and Verrucomicrobia (Jha; Annapurna; Saraf, 2012; Dubey et al. 2016; Madhaiyan et al., 2012; Qin et al., 2015); which demonstrates the ability of the phyla found in the present study in endophytic colonization of physic nut plants. According to recent references, the main described genera associated with physic nut crop are *Acinetobacter, Bacillus, Burkholderia, Curtobacterium, Enterobacter, Enterococcus, Microbacterium, Pleomorphomonas, Pseudomonas,*

Procmicromonosporaceae, *Salmonella*, *Sanguibacter*, *Staphylococcus* e *Serratia* (Jha; Annapurna; Saraf, 2012; Madhaiyan et al., 2012; Madhaiyan et al., 2013). These genera have already been described as capable of promoting plant growth in various other crops (Chauhan, Bagyaraj, Sharma, 2013; Castro et al., 2017; Batista et al., 2018).

Some studies highlighted that the endophytic bacteria associated with physic nut possess plant growth-promoting attributes that can be explored as microbial inoculants for crops (Madhaiyan et al., 2012; Mohanty et al. 2017). In this way, Madhaiyan et al. (2012) reported that the endophytic strain of *Enterobacter arachidis* (R4-368) was shown to be able to colonize root tissues and promote the growth of physic nut seedlings.

Biological nitrogen fixation (BNF) is a process carried out by groups of microorganisms that have the functional enzyme nitrogenase, which can be used as a source of nitrogen (N) for plant nutrition. In agricultural areas, there are reports that 10 to 60% of the N accumulated in the plant may come from atmospheric N_2, in corn, rice, sugarcane, some forage grasses, and others, not legumes, being due values, allowing for substitution partial nitrogen fertilizers used in agriculture, by BNF processes (Munees and Kibret, 2014; Reis Junior; Mendes; Hungria, 2018). This gives us a future perspective on the BNF assays in which 56.75% of the bacteria evaluated in the present study could fix nitrogen *in vitro*, which mostly belong to the genus *Bacillus* and also by the genera *Curtobacterium*, *Lysinibacillus*, and *Rhodococcus*. Several genera and species of free-living bacteria capable of fixing nitrogen have been reported in association with a large number of non-leguminous plants in the most diverse biomes, including *Azotobacter*, *Azospirillum*, *Azoarcus*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Derxia*, *Enterobacter*, *Gluconacetobacter*, *Herbaspirillum*, *Klebsiella*, *Paenibacillus*, and *Pseudomonas* (Ahemad and Kibret, 2014; Rodrigues et al., 2016; Batista et al., 2018; Trinh et al., 2018).

In the present study, 62% of the endophytic strains were able to synthesize the phytohormone IAA in the presence of the tryptophan precursor. A strain of the genus *Citrobacter* (EPM-63B) with a value of 84.11 $\mu g.mL^{-1}$, two strains of the genus *Klebsiella* (EPM-4 and EPM-63) with respective values of 79.87 $\mu g.mL^{-1}$ and 65.37 $\mu g.mL^{-1}$, three strains of the genus *Bacillus* (EPM-10, EPM-92 and EPM-54) with values of 58,25 $\mu g.mL^{-1}$, 48,45$\mu g.mL^{-1}$ and 47.07$\mu g.mL^{-1}$ respectively and two strains of the genus *Microbacterium* (EPM-96 and EPM-50C) with respective values of 56.34 $\mu g.mL^{-1}$ and 45.25$\mu g.mL^{-1}$. The values obtained in the present study are higher than reported by Jha; Annapurna; Saraf (2012) in studies evaluating the potential of promoting growth of *Enterobacter cloacae* (MSA1) and *Enterobacter cancerogenus* (MSA2) associated with physic nut.

In the present study, was isolated one strain *Citrobacter* sp. (EPM-63B), producing five times more of that IAA concentration reported by Assumpção et al. (2009), that in a similar study ,isolated and characterized endophytic bacteria from soybean seeds an report a strain of *Citrobacter* sp.

producing16.7 μg. mL^{-1} of IAA and demonstrating the tendency to increase all parameters evaluated in the development of soybean plants, although it did not differ statistically from the uninoculated control. The inoculation with *Klebsiella* sp. (strain Sal 1- sweet potato bacterial endophyte) IAA-producing 65 μg. mL^{-1}, increased fresh root weight of tomato and radish plants (Dhungana and Itoh, 2018). Kim et al (2017), reported were of isolate AY-13 (*Klebsiella variicola*) producing IAA (84.27±3.55 μg/mL) was inoculated in soybean seedlings to examine its potential for promoting growth and reprograming after flooding stress. AY-13 application not only mitigated the flooding stress, but also significantly improved the plants' growth, enhanced chlorophyll contents, and improved the quantum efficiency of chlorophyll fluorescence during and after flooding stress. These values corroborate with our results obtained for this genus in present study.

The response of plants to the IAA released bacteria may range from beneficial to deleterious effects, depending on its concentration. When in low concentrations the IAA can stimulate growth and when in high concentrations can inhibit the development of the root (Lambrecht et al., 2000). Therefore, all isolates producing IAA are eligible for *in vivo* studies since they have shown other mechanisms for promoting plant-growth that can be combined with IAA production, generating promising results (Devi et al., 2016; Olayemi and Odedara, 2017).

Other mechanism evaluated in this study was the solubilization of insoluble inorganic phosphate *in vitro*, where 32% of the evaluated endophytic strains had this capacity. Twelve showed phosphate solubilization index >3 highlighting strains belonging to the genera *Citrobacter* (EPM-63B); *Klebsiella (*EPM-4 and EPM-63); and *Serratia* (EPM-2; EPM-75) that solubilized high levels of phosphate. In a similar study, Mohanty et al. (2017) characterized the endophytic bacteria of *J. curcas* and evaluated their plant growth-promoting effect on maize (*Zea mays* L.), and the inoculation of these endophytic strains on maize seeds significantly increased the shoot and root length of seedlings compared with those of noninoculated. These authors presume that phosphatase is exuded from the root tissue and mediates P mineralization in the rhizosphere zone for P assimilation. Promising results of microorganisms associated with physic nut have been observed in promoting plant growth for isolated *Terribacillus saccharophilus* strain 002-048 (Mohanty et al., 2017).

Assumpção et al. (2009) reported a high phosphate solubilization index (ISF 4.7) of a *Citrobacter* sp. strain, endophytically isolated from soybean seeds, with an index similar to that obtained in the present study. Ji et al. (2014) isolated endophytic *Klebsiella* sp. (KW7-S06 strain) from leaves, stem, and roots of rice plants with phosphate solubilization activity ranging from 1.3 to 3.3 mg/mL promoted plant growth when inoculated in rice seeds.

Devi et al., (2016) reported that *Serratia marcescens* (AL2-16 strain) isolated from the *Achyranthes aspera* L., showing phosphate solubilization index was found to be in the range of 2.5-

4.5, when inoculated in *A. aspera* L., increased all growth parameters of plant and higher P content for the plant.

Two strains, representing *Bacillus* sp. EGI 63071 and EGI 63106 endophytically isolated from *Lycium ruthenicum* by Liu et al (2019), were effective in terms of stimulated wheat growth under salt stress. The authors speculate that these results were probably due to the ability of tested strains to convert unusable nitrogen and phosphorus to accessible forms. The understanding of the ability and efficiency of microorganisms in solubilizing phosphates can select lines with a high potential of use for the inoculation in plants as biofertilizers. This can replace or reduce the use of soluble phosphate fertilizers, using a better use of the existing natural phosphates or added to the soil (Abreu, et al., 2017).

A total of thirteen endophytic bacterial strains, tested *in vitro* against the fungi *Lasiodiplodia subglobosa*, *L. euphorbicola*, and *L. pseudotheobromae*. The *Bacillus* genus showed higher antagonistic activity compared with other strains, demonstrating an IA% between 34.16 and 76.66%. The strains EPM-55A and EPM-70, both *Bacillus* sp. genus, showed an IA% greater than 50% mycelial growth reduction of species of *Lasidiplodia* tested.

According to Machado et al. (2014), species of *Lasiodiplodia* were isolated from *J. curcas* with symptoms of wilt, leaf fall, and yellowing due to root or collar rot. From morphological and phylogenetic studies and inoculated in 6-months-old *J. curcas*, and all the *Lasiodiplodia* species produced symptoms reproduced symptoms similar to those observed in the field. From the lesions, it was possible to isolate and retrieve the inoculated fungus, completing the *Koch's postulates* (Machado et al., 2014). This disease is associated with root and collar rot, in which the initial symptoms are wilting and yellowing of the leaves, ending with leaf fall and death of the plant. Currently, this disease is associated with *Lasiodiplodia* species in Brazil (Pereira et al.2009; Latha and Prakasam, 2012).

Studies by Che et al. (2015) have demonstrated the antagonistic ability of a *Bacillus brevis* isolated from the soil of Yongtai County in Fujian, China against the fungus *L. theobromae*, disease-causing apples rot. Sajitha et al., (2014), demonstrated the efficacy of the antagonistic property of *B. subtilis* strains present in aerobic compounds from several sources against *L. theobromae*. There is no record of studies aiming at the evaluation of the antagonistic activity of endophytic bacteria strains, isolated from *J. curcas*, to the phytopathogenic fungi evaluated in the present study. To our knowledge, this is the first report of this potential of biocontrol against *Lasiodiplodia* species. Our results were accordance to the findings of literature. The bacterial group's *Bacillus* and *Serratia* isolated endophytically from several plants demonstrate the potential to be used in the biocontrol of other diverse phytopathogenic fungi (Lacava and Azevedo, 2014; Li et al., 2015; Ahmad, et al., 2017; Shahzad et al., 2017).

Endophytic bacteria have the potential to act adversely directly against plant pathogens due to their unique symbiotic relationships within their hosts. Also, endophytes may act indirectly against diseases, benefiting their hosts, increasing overall plant growth or plant protection responses, as in the case of induced systemic resistance (Lacava, Melo, Pereira, 2018; Silva et al., 2019). Besides, antagonism tests can indicate the potential for biological control of diseases and pathogens, using endophytic bacteria that can aid in combating diseases and pests without harming the environment with toxic substances, such as some agrochemicals (Sobral et al., 2014).

5 CONCLUSION

A total of thirty-seven endophytic bacterial strains of physic nut were screened for plant growth-promoting *in vitro* and genetically identified by partial sequencing of 16S rDNA, demonstrating a genetic diversity of bacterial strains belonging to the genera *Arthrobacter*, *Bacillus*, *Citrobacter*, *Curtobacterium*, *Enterococcus*, *Klebsiella*, *Leucobacter*, *Lysinibacillus*, *Microbacterium*, *Rhodococcus* and *Serratia*. Besides, 56.75% of the bacteria evaluated could fix nitrogen, 62% of the isolates were able to synthesize the phytohormone IAA in the presence of the tryptophan precursor, 32% of the evaluated isolates had capacity solubilization of insoluble inorganic phosphate, and 35% exhibited antagonistic activities against phytopathogenic fungi *L. subglobosa*, *L. euphorbicola*, and *L. pseudotheobromae*. The next steps of this study will move towards the inoculation of endophytic isolates, with the best *in vitro* results for promoting growth, in physic nut seedlings to verify performance *in vivo*.

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