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Determinación de organismos endófitos en semillas de *j. Curcas* l y *j. Macrocarpa* griseb

Determination of endophytic organisms in seeds of j. Curcas l and j. Macrocarpa griseb

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ABSTRACT

Endophytes are microorganisms that reside in all or part of their lives in plant tissues in a symbiotic relationship, of parasitism or mutualism, without immediately causing negative effects (Stone et al., 2000). As a result of these long-term associations, endophytic microorganisms and plants have developed great information transfer (Strobel and Daisy, 2003). Certain medicinal plants synthesize chemical substances, providing material for the pharmaceutical and cosmetic industries (Karthikeyan et al., 2008) and endophytics participate in metabolic pathways of these plants and produce bioactive compounds (Zhao et al., 2011).

Keywords: Organisms, Endophytes, Jatropha, Seed.

1 INTRODUCTION

Endophytes are microorganisms that reside in all or part of their lives in plant tissues in a symbiotic relationship, of parasitism or mutualism, without immediately causing negative effects (Stone et al., 2000). As a result of these long-term associations, endophytic microorganisms and plants have developed great information transfer (Strobel and Daisy, 2003). Certain medicinal plants synthesize chemical substances, providing material for the pharmaceutical and cosmetic industries (Karthikeyan et al., 2008) and endophytics participate in metabolic pathways of these plants and produce bioactive compounds (Zhao et al., 2011).

Different studies have revealed various functions of endophytic actinobacteria (Qin et al., 2009). It has been reported in recent years that endophytic actinomycetes produce a large number of secondary metabolites with biological activity, such as antibiotics, antitumor substances and

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plant growth promoters, contributing to mitigate environmental stresses in host plants (Qin et al., 2011). Actinomycetes isolated from the rhizosphere associated with *Trifoliumrepens* L. showed great capacity to solubilize phosphates, fix nitrogen and produce siderophores, all of which are characteristic of plant growth-promoting Rhizobacteria (PGPR), in addition, they increased growth and nutrition benefiting the colonization of roots by mycorrhizal fungi (Franco-Correa et al., 2010). A total of 30 endophytic fungi were isolated from different plant organs, such as leaves, stems, roots and seeds, in the following promising crops for biodiesel: *J. curcas L.,* Pongamia pinnata *(L) Pierre,* Ricinus communis *L.* These fungi accumulated large amounts of intracellular lipids (Susmita et al., 2019). The microorganism KLBMP 1115^{T} was isolated from J. *curcas* roots collected in Southwest China. Sequence analysis of 16 S rRNA genes indicated that it belongs to the genus *Pseudonocardia.* Based on phenotypic characteristics and DNA-DNA hybridization, it was determined that it represents a new species of this genus, for which the name *Pseudonocardiasichuanensissp.nov.,* a Gram-positive and aerobic actinomycete, was proposed (Qin et al., 2011). Another endophyte found in the roots of this species is the KLBMP1111T with the name *Kibdelosporangiumphytohabitanssp*. nov.(Xing et al., 2012). The whole genome of *Enterobacter* sp. has been sequenced. R4-368, endophytic, nitrogen-fixing gammaproteobacterium isolated from *J. curcas* roots. This bacterium shows a strong growth-promoting effect, with an increase in plant biomass and seed production (Madhaiyan et al., 2013).

Endophytes designated as KLBMP 1050T and *Nocardiooidespanzhihuaensissp*. nov. (Qin et al., 2012).

Among the endophytes isolated from *leaves of J. curcas* in New Delhi, India, *Colletotrichum truncatum* EF13 was identified as exhibiting antifungal activity *against Sclerotinia sclerotiorum*, providing protection to the plant against pathogenic fungi, thus being a potential biological control agent. On the other hand, *Colletotrichum truncatum* EF10 produces oils with a fatty acid profile similar to that of its host *J. curcas* (Kumar and Kaushik, 2013).

An endophytic actinomycete isolated from J. *curcas* seeds is the KLBMP1221T for which the name *Amycolatopsisendophyticasp*. nov. (Qian et al., 2011). However, no previous studies were found indicating the presence of endophytes in organs of *J. macrocarpa.*

Microscopic, biochemical and molecular techniques are used for the extraction and identification of microbial communities, among which PCR (polymerase chain reaction), DGGE: gel electrophoresis with denaturation gradient and t-RFLP: analysis of length polymorphisms of terminal restriction fragments are widely used (Stefanis et al., 2013).

OBJECTIVE

To determine the presence of endophytes in seeds of J. *curcas and J.* macrocarpa *and to* identify them.

MATERIALS AND METHODS

Two complementary strategies were used to study endophytic microorganisms. One of them consisted of sowing macerated seeds sterilized superficially, in different culture media. The other strategy was the study of the bacterial ribosomal DNA present in the seed.

CULTIVABLE MICROORGANISMS

For sterilization, grinding and plating, 10 g of J. *curcas* and *J. macrocarpa* seeds were used. They were rinsed with tap water to remove epiphytes and superficially sterilized according to Sun et al. (2008), 70% ethanol for 5 min, sodium hypochlorite 5%, 2 min.Two rinses were performed with sterile distilled water. 3 disinfected seeds of each species were placed in Luria Bertani culture medium (LB), sealed with parafilm and taken to 24/48 h for sterility control. 28 °C

The seeds were crushed in a sterile mortar and pestle, previously cut with a scalpel. The grinding product was transferred to an Erlenmeyer with 90 ml of saline solution (8.5 $^{o}/_{00}$ and suspensions-dilutions up to 10-5).

0.1 ml of each dilution was seeded in plates, in duplicate. Luria Bertani (LB) media were used for 3 days at 30°C and Sodium Propionate Agar (SSI 3) at 28°C for 2 weeks(Shirling and Gottlieb, 1966),liquid and solid. LB contains tryptone 10 g, yeast extract, NaCl, agar, distilled water 1000 ml, pH 7.5 and ISP 3 contains sodium propionate, L-asparagine, KH5 g10 g15 g1 g0.2 g_{2PO4}0.9 g, K2HPO4, MgSO0.6 g_4.7H2O , CaCl0.1 g_2. H2O, agar, distilled water 1000 ml, pH 7.2. 0.02 g18 g

NON-CULTIVABLE MICROORGANISMS

For surface sterilization and grinding, the methodology described for cultivable microorganisms was followed. The grinding product was placed in a Falcon tube and suspended in 2 mL of water + 2 mL of NaH2PO buffer490 mM/g seed PS. In the extraction and quantification of DNA, 1 ml of the aforementioned suspension was used and it was taken to the Eppendorf tube. It was made using the commercial FAST DNA SPIN KIT for SOIL, according to the manufacturer's protocol. 2 µL of solution was used to quantify DNA by spectrophotometry (Nanodrop) at 260 nm. Four independent experiments were conducted.

DNA visualization was performed by gel electrophoresis. An agarose gel with 1% TAE 1X buffer was prepared with the addition of ethidium bromide. The DNA and buffer were mixed in a 5:1 ratio and seeded into the gel. It was run for 1 h at 70 V. It was taken to Gel Doc image digitizer.

Polymerase chain reaction (PCR) was performed with primers specific to the Bacterial Domain. Different amounts of DNA (50, 25, 5, and 2.5 ng) were used in a volume of 50 µL with buffer PCR 1X, $MgCl_2$ 1.5 mM, dNTPs 0.2 µM each, primer 27f 0.08 µM, primer 1495r 0.08 µM, Go Taq DNA polymerase 1.25 U. PCR amplification was performed in Eppendorf Mastercycler Gradient under the following conditions: initial denaturation at 95ºC, 5 min, with DNA denaturation at 95ºC, 30 s; cooling to 55ºC, 30 s and extension to 72ºC, 2 min, followed by 35 cycles and a final extension to 72ºC, 5 min. PCR products encoding 16S ribosomal RNA (Li et al., 2007) were detected by electrophoresis in 1% 70 V agarose gel for 1 h, with 2 repeats for each species. The pure sample and different dilutions were used for J. *curcas*, while only the pure sample was used for *J. macrocarpa.* It was taken to Gel Doc image digitizer.

Restriction Terminal Fragment Length Polymorphism Analysis (T-RFLP)

In PCR, 27f/1495r-specific primers were used for the Bacteria domain that do not amplify mitochondrial or chloroplast DNA from the plant cell (Bianciotto et al., 2004).

Restriction enzyme digestion of the amplification products was performed with RE 10X buffer, Acetyladed BSA 10 µg/µl, 1µg DNA, 10 µg/µl restriction enzyme (Hae III) 4 h.

To check the efficiency of digestion, the analysis of the digestion product was carried out by means of Metaphor 3 gel electrophoresis. The separation of the fragments for fluorescence analysis was carried out in the management service of the INTA Genomics Unit.

The phylogenetic assignment tool (PAT) was used to analyze the digestion products obtained in the t-RFLP procedure (Kent et al., 2003).

Denaturing Gradient Gel Electrophoresis (DGGE)

For this analysis, two PCRs were performed. The first PCR was performed with the fD1/rD1 primers and was detected by agarose gel electrophoresis 1% of the PCR product (1500 bp). The second PCR, with the pair F341/R534, was performed with DNA 27f/1494r PCR, buffer PCR 5X, $MgCl_2$ 7.5 mM, dNTPs 20 µM, first F 341 5ppm/µl, first R 534 5ppm/µl, Go Taq DNA polymerase 5 U/µl. The primers used (27f/1494r) are specific to bacteria (Ogino et al., 2001). PCR amplification was performed in Eppendorf Mastercycler Gradient under the following conditions: initial denaturation at 93ºC, 5 min, with denaturation at 94ºC, 30 s; cooling

to 53º C, 30 s and extension to 72º C, 2 min, followed by 29 cycles and a final extension to 72º C, 10 min. PCR products (200 bp) were analyzed by 0.8% agarose gel electrophoresis. The DGGE gel was assembled using the peristaltic pump system with a denaturing gradient: urea + formamide 40-60%.The gel was assembled and allowed to polymerize until the next day. The run buffer was prepared with 140 ml TAE 1X (Biorad kit), it was brought to 7000 ml with double-distilled water. The buffer was loaded into the tank and heated to 60°C. The gel was placed in the vat.

The product of the second amplification was seeded in a polyacrylamide gel with a urea gradient. Samples were seeded in each lane: 20µl of DNA + 5 µl with 6X loading buffer. It was initially run (pre-run) at 100 V for 20 min and then at 70 V for 16 h (run). The vat was turned off, allowed to cool, and the gel was removed and allowed to cool to room temperature.

The products were detected and visualized by staining with 6 µl of Sybr Green I (1:40 solution in 1X TAE buffer) for 1 h and brought to Gel Doc.

Bands of interest were identified and cut. The sequencing and analysis of the bands allowed the identification of the microorganisms, which was done by comparison with known nucleotide sequences contained in the database (Benson et al., 2013).

RESULTS

CULTIVABLE MICROORGANISMS

Despite testing different media and culture conditions, it was not possible to isolate microorganisms by microbiological methods.

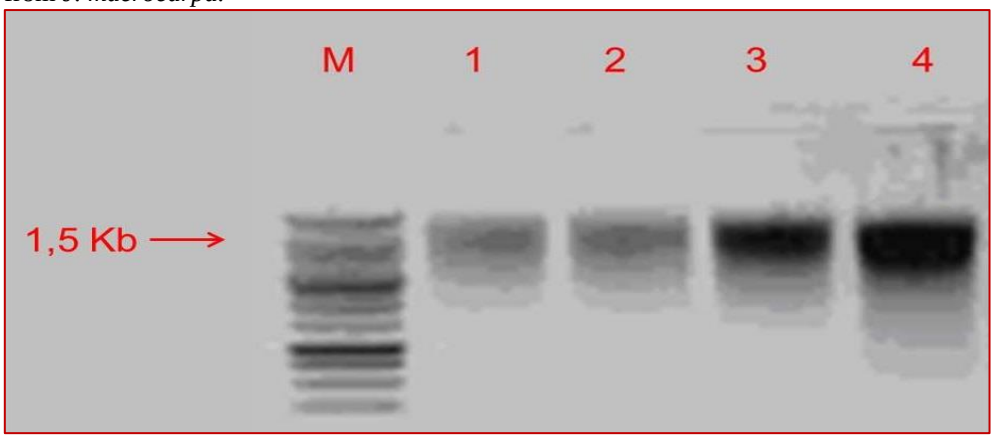
The yields obtained in DNA quantification ranged from 9.4 to 20 µg DNA g $^{seed-1}$. For *J. curcas, 10.0449324 µg DNA g PS seed-1 was obtained, while for* J. macrocarpa the yield was higher, at 17.6030405 µg DNA g PS $_{seed-1}$ (Table 1).

Table 1: DNA yields of J. *curcas* and *J. macrocarpa*

Species	ng/µl DNA	µg DNA/g PS semilla
J. curcas	43,72	10,0449324
J. macrocarpa	76,6	17,6030405

The analysis by agarose gel electrophoresis showed that the size of the extracted DNA is of good quality according to the size of the bands that were present in both *J. curcas* and *J. macrocarpa*, the widest corresponding to 1.5 Kb (Fig. 1).

Fig.1: DNA electrophoresis in J. *curcas* and *J. macrocarpa.* References: M, marker; 1 and 2 DNA from *J. curcas;* 3 and 4 DNA from *J. macrocarpa.*



The electrophoresis carried out in agarose gel of the 16S PCR products of the DNA encoding the 16S ribosomal RNA, useful from the taxonomic and phylogenetic point of view, showed that the quality of the DNA allowed a successful amplification for the primers used in both species. The thickest band was 1.5 Kb. In *J. curcas* the first number represents the sample, the second the repeats (1 and 2) and the third the pure sample or the corresponding dilutions (Fig. 2). In *J. macrocarpa*, only pure samples were used (Fig. 3).

Fig. 2 : Electrophoresis of PCR products in *J. curcas.* References: 1, 1 pure; 2, 1 1:2; 3, 1 1:10; 4, 1 1:20; 5, 2 pure; 6, 2 1:2; 7, 2 1:10; 8, 2 1:20; 9, *E. coli*, control (+); 10, control (-); M, marker

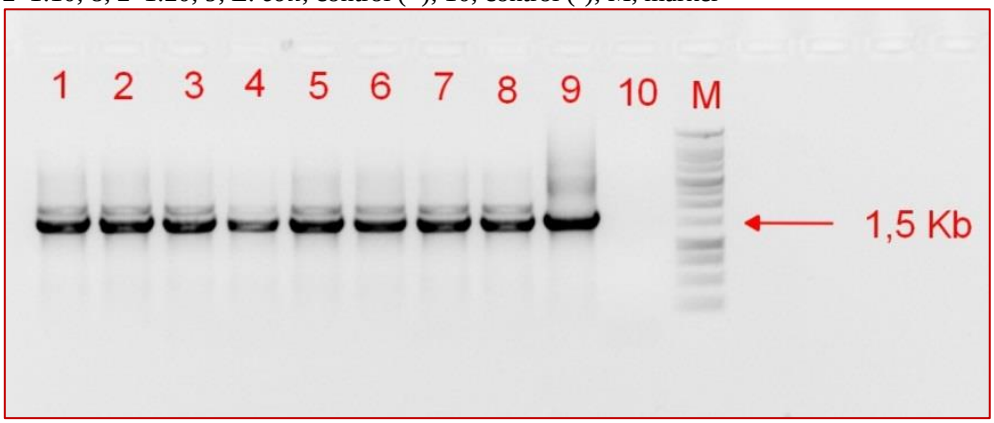
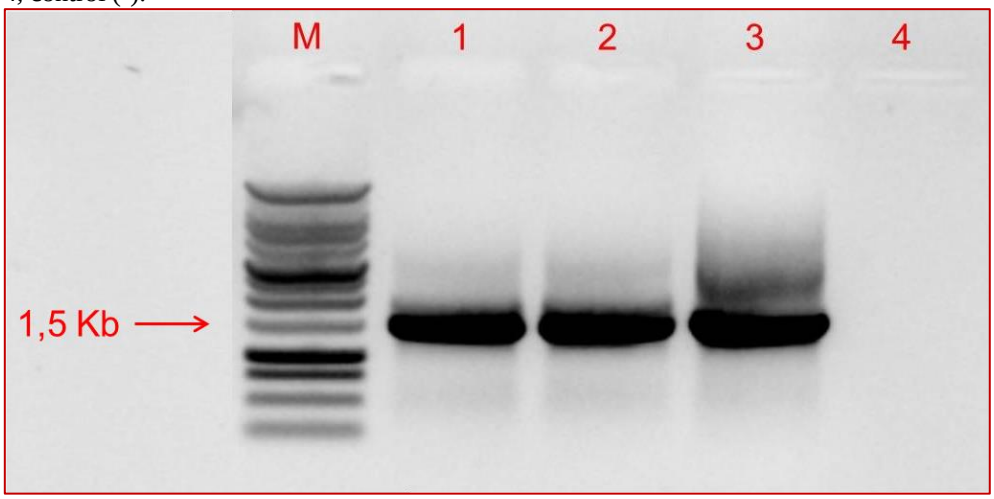


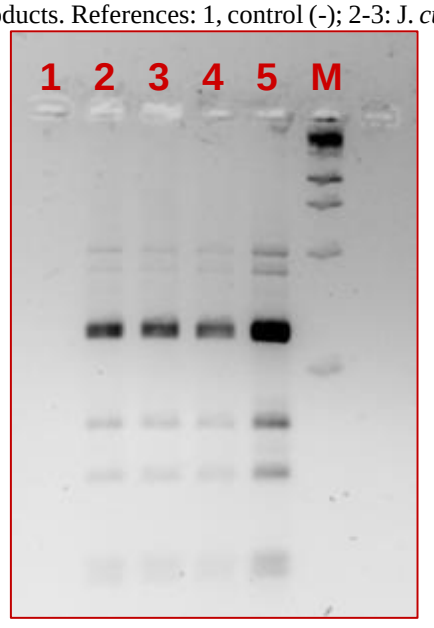
Fig. 3: Electrophoresis of PCR products in *J. macrocarpa.* References: M, marker; 1.1 pure; 2, 2 pure; 3, *E. coli*, control (+); 4, control (-).



Restriction Terminal Fragment Length Polymorphism Analysis (T-RFLP)

After digestion of the amplification products with restriction enzymes, electrophoresis allowed the visualization of the different bands. These indicate the presence of endophyte groups in both J. *curcas* and *J. macrocarpa* (Fig. 4).

Fig. 4: Electrophoresis of digestion products. References: 1, control (-); 2-3: J. *curcas.* 4-5: *J. macrocarpa;* M, marker.



The digestion products obtained in the t-RFLP procedure were analyzed by the phylogenetic mapping tool (PAT), which allowed comparing the TRF fragments with fragments from a database containing known gene sequences of 16S rRNAs. Table 13 shows: the size of the fragment generated in silico from the loaded sequence, the size observed in the experiment (77 ± 1bp), the accession number of the sequence and the list of possible microorganisms for the

fragment obtained, with the homology presented from highest to lowest similarity. The pattern obtained corresponds to non-cultivable bacteria, non-cultivable soil bacteria, *Streptomycesxiamenensis*and other microorganisms of the genus *Streptomyces*, in both species (Table 2).

Table 2. Analysis of TRFs using PAT

Generated Size	Observed Size	Access number	Microorganism
76	77.00	EU881345	Non-cultureable bacteria
76	77.00	EF688396	Non-cultivable soil bacteria
76	77.00	EF492901	Non-cultureable bacteria
76	77.00	EU223962	Non-cultureable bacteria
76	77.00	EU335158	Non-cultureable bacteria
76	77.00	EU669624	Non-cultureable bacteria
76	77.00	EU881177	Non-cultureable bacteria
76	77.00	EU881180	Non-cultureable bacteria
76	77.00	EU786140	Non-cultureable bacteria
76	77.00	GQ488014	Non-cultureable bacteria
76	77.00	FJ444638	Non-cultureable bacteria
76	77.00	FJ444657	Non-cultureable bacteria
76	77.00	FJ444756	Non-cultureable bacteria
76	77.00	FJ444756	Non-cultureable bacteria
76	77.00	EF688354	Non-cultivable soil bacteria
76	77.00	EU881104	Non-cultureable bacteria
76	77.00	EU881271	Non-cultureable bacteria
76	77.00	FJ444736	Non-cultureable bacteria
76	77.00	EF012099	*Streptomyces xiamenensis* (T)
76	77.00	EF012115	*Streptomyces* sp 1A01564
76	77.00	EF012140	*Streptomyces* sp. 13-32
76	77.00	EF056496	*Streptomyces* sp. 1A01646
76	77.00	EF012129	*Streptomyces* sp. z78

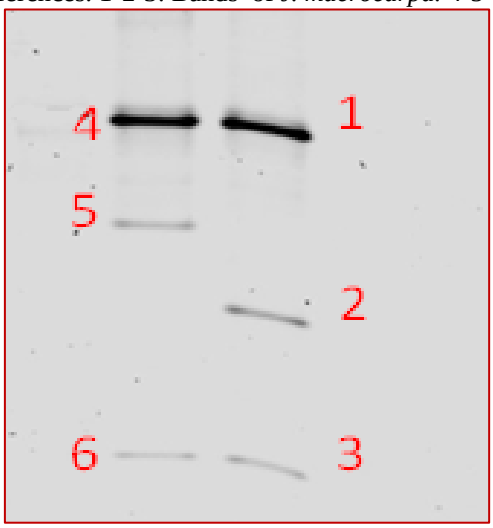
Denaturing Gradient Gel Electrophoresis (DGGE) Analysis

76 77.00 0.012048 EU881345 U881345; uncultured bacteriu

Denaturing Gradient Gel Electrophoresis (DGGE)

The products of the second PCR amplification were seeded in DGGE gel using the peristaltic pump system. In this gel, three bands of interest were identified for each species. Each band represents a different genotype. Bands 1 and 4; 3 and 6 are placed at the same height, indicating the presence of the same microorganisms. Bands 2 and 5 are presented at different levels, showing that there are different microorganisms in one species compared to the other (Fig. 5).

Fig.5: DGGE gel. References: 1-2-3: Bands of J. *macrocarpa.* 4-5-6: Bands of *J. curcas.*



The sequencing and subsequent analysis of the bands obtained by DGGE were analyzed in the Genomics Unit of INTA Castelar, where the sequences of known nucleotides contained in the database are deposited. Table 14 shows the percentage of coverage, extension or mating for each microorganism and the percentage of identity with the sequence analyzed.

Band 1 and 2 had an identity percentage of 84% and 85% and mated 94% and 67% of their length respectively. Band 3 had no matches. Band 4 is 84% identical and paired 100% of its length. Band 5 had no matches. Band 6 had an 86% identity and mated only 35%.

This analysis indicated the presence of the genus*Pseudanabaena* in both species and *Chthonomonas* in *J. curcas.*

Table 3. Sequencing and analysis of DGGE band data

Banda (species)	**Microorganism**	**% Coverage**	**% Identity**
DGGE 1 (*J. macrocarpa)*	*I've never been in a state where I've*	94	84
DGGE 2 (*J. macrocarpa)*	*I've never been in a state where I've*	67	85
DGGE 3 (*J. macrocarpa)*	-	-	-
DGGE 4 (*J. curcas*)	*I've never been in a state where I've*	100	84
DGGE 5 (*J. curcas*)	-	-	-
DGGE 6 *(J. curcas*)	*Chthonomonas calidirosea*	35	86

DISCUSSION

Endophytic microorganisms establish a symbiotic association with plants for a long time, developing a large transfer of information between the two (Ludwig-Műller, 2015). Previous studies indicate the presence of endophytic actinomycetes in vegetative organs of *J. curcas*, such as *Kibdelosporangium and* phitohabitanssp *Nov. (Xing et al., 2012) and Psleudonocardia sichanensis* sp. Nov., isolated from roots (Qin et al., 2011), Nocardiodes panzhihuarensis *sp. Nov., in stems (Qin et al., 2012) and Colletotrichum truncatum* EF13in leaves (Kumar & Kaushik, 2013). In addition, endophytic fungi were found in leaves of this species in 10-day-old leaves (D'Jonsiles et al., 2020). In seeds of *J. curcas*, Qian et al. (2011) isolated *Amycolatopsis endophytica* sp. Nov., a gram-positive, aerobic, immotile, spore-forming actinomycete.In *J. curcas*, the only associated endophytes found so far were actinomycetes and no endophytes were found in the vegetative or reproductive organs of *J. macrocarpa.*

In our research, endophytes were not isolated from seeds of J. *curcas and* J. macrocarpa *by microbiological methods, using* different media and culture conditions. In this sense, Davis et al. (2005) reported that classical microbiological methods limit the knowledge of the diversity of microorganisms, since *only* 1% of them can be isolated and therefore not representative of the microbial community and can provide erroneous information about the non-association with microorganisms of a plant species. A plant completely free of microorganisms represents an exotic exception in nature, rather than a rule of biological relevance (Partida Martínez & Heil, 2011).

Studies with culture-independent molecular techniques: restriction terminal fragment length polymorphism analysis (t-RFLP) and denaturing gradient gel electrophoresis (DGGE) are complementary studies that use different primers to amplify rDNA sequences.

Our results, according to the analysis of t-RFLP in *J. curcas* and J. macrocarpa, *correspond to non-cultivable bacteria associated with plants, non-cultivable bacteria from the soil and several representatives of the genus Streptomyces*, including *Streptomycesxiamenensis*.

The predominance of *Streptomyces* within endophytic actinobacteria coincides with previous research conducted in *J. curcas* (Qin et al., 2015) and other plant species (Zhao et al., 2011; Kim et al., 2012).*Streptomyces* belongs to the group of Actinomycetes, filamentous bacteria, generally Gram-positive, aerobic, spore-forming; many produce natural antibiotics such as streptomycin, such is the case of *Streptomycesgriseus*. Endophytic actinomycetes produce a large number of secondary metabolites with biological activity and antitumor substances (Qin et al., 2011) and generate resistance to biotic and abiotic stress in host plants (Qin et al., 2014). In

previous studies conducted on *J. curcas*, *Streptomyces*, showed plant growth-promoting activity through auxin and siderophores, phosphate solubilization, and nitrogen fixation (Qin et al., 2015). In addition, they inhibit the growth of a wide range of plant pathogens, both fungi and bacteria (Tchinda et al., 2016; Misk and Franco, 2011; Kumar and Kaushik, 2013; Golinska et al., 2015). Endophytic seed-associated bacteria that possess beneficial characteristics, such as stimulation of germination and promotion of seedling establishment, are usually selected by the plant and transferred via seed, thus ensuring these characteristics for future generations (Truyens et al., 2015).

Streptomycesxiamenensis owes its name to the production of a useful drug for the treatment of fibrosis: xyamemimine. This species produces compounds such as terpenoids, siderophores, and ectoins, the latter being protective osmolytes that help the plant survive osmotic stress (Hwang et al., 2014).

In this study, the presence of the genera Pseudanabaena in J. curcas and J. macrocarpa *and Chthonomonas in* J. curcas *was determined* using the denaturing gel electrophoresis *technique.*

Pseudanabaena, belongs to the group of cyanobacteria, formerly called blue-green algae, they are Gram-negative bacteria and the only prokaryotes that perform photosynthesis. Recently, it has been established that cyanobacteria possess nitrogenase enzymes located in heterocysts that fix atmospheric nitrogen and convert it into forms accessible to the plant, with great impact on the cycle of this element (Tsedeke et al., 2016). The type of cyanobacteria-plant association is symbiotic; the prokaryote fixes nitrogen and transfers it in the form of ammonium to plants through unknown molecular mechanisms, and since the photosynthetic activity of cyanobacteria is low, the plant provides carbohydrates (Ekman et al., 2013).

According to recent research, Chthonomonas calidirosea is a Gram-negative, aerobic, thermophilic, motile, non-spore-forming bacterium that was first isolated in New Zealand by 16S rRNA; it is the first representative of the new class Chthonomonadetes and thePhylum Armatimonadetes (Lee et al., 2016). This microorganism is not cultivable by microbiological methods and in terms of its metabolism it does not degrade complex polysaccharides, uses less common carbohydrates and derivatives such as sorbitol and galactans and can establish a mutualistic relationship due to its ability to remove five-carbon sugars that make up hemicellulose from the environment, facilitating the degradation of cellulose that many species cannot perform (Lee et al., 2014).

In this research, the isolation of non-cultivable microorganisms associated with plants and non-cultivable from the soil by means of the molecular technique of length polymorphisms of terminal restriction fragments, would explain the difficulty encountered in isolating microorganisms in crops. Through this technique, it was possible to identify some of the genera potentially present in both types of seeds, some of which are either non-cultivable or may be identified with higher resolution techniques such as deep sequencing andscanning electron microscopy (Ranjard et al., 2000). Laser biospeckle activity (LBSA) to be used for the detection of endophytic colonization of leaves *Jatropha* curcas. Considering that endophytes plays important roles in plant fitness, and traditional techniques to study them are most often destructive, this noninvasive technique could be used as a good indicator of endophytic colonization (D' Jonsiles et al., 2020).

As *J. curcas* and *J. macrocarpa*, they grow in marginal nutrient-poor soils, their nutritional requirements are higher than in other crops. The findings of this research indicate the importance of the presence of native microorganisms present in its seeds, especially the endophytic actinobacteria that promote plant growth and play an important role in the host plant. In addition, we must consider the contribution of symbiotic cyanophytes that contribute to nitrogen fixation. In *J. curcas*, the association with *Chthonomonas calidirosea* adds other pathways in carbohydrate metabolism. Until now, this microorganism had only been found in environments with high temperatures, such as volcanoes. Previous research indicates that the inoculation of endophytes in leaves of *J. curcas* increases desirable agronomic traits in plants (D'Jonsiles et al., 2019).

CONCLUSIONS

The analysis of gel electrophoresis in a denaturing gradient indicates the presence of the genera *Pseudanabaena* in J. curcas and J. macrocarpa *and* Chthonomonas *in J. curcas.* The pattern of restriction terminal fragment length polymorphisms corresponds to non-cultivable microorganisms or microorganisms of the genus *Streptomyces*, in both species.

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