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A New Bacterial Disease of Cassava in Venezuela Caused by *Enterobacter cloacae*

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ABSTRACT

In this article we report a new cassava (*Manihot esculenta* Crantz) bacterial disease in Venezuela. The disease has been observed in different Venezuelan regions since 2001. Leaves of the collected samples showed angular water-soaked lesions on the leaf lamina, which became later necrotic with a chlorotic halo. Advanced disease stages induce an early senescence of leaves leaving the stem bare. Bacteria isolated formed small beige, glistening, smooth colonies with regular margins within 24 h incubation at 30°C on LB agar or semi-selective threhalose agar. All isolates were Gram negative, facultative anaerobic with several peritrichious flagella observed under the Electron Microscope. The bacterium was identified as *Enterobacter cloacae* by biochemical assays, but 16S rRNA gene sequence analysis did not allow discrimination between *E. cloacae* and *Pantoea agglomerans*. Other PCR based identification methods such as ERIC and GIRRN-LIRRN ribotyping, and *hsp60* sequence identified the strains as *E. cloacae*. Pathogenicity test on cassava plants reproduced the disease symptoms and allowed the re-isolation of *E. cloacae* from the infected tissues. This is the first report of a disease caused by *E. cloacae* on cassava and it may have an impact on cassava seed production.

Key Words: Cassava; *Enterobacter*; Pathogenicity; Venezuela

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a tropical starchy root crop, native to South America, especially important as staple food in sub-Saharan Africa, Asia and America, representing the main sources of calories and income for more than 500 million people worldwide. Cassava is a perennial plant, propagated by 30 cm long stem sections, with a storage root producing cycle ranging between 8-18 months depending upon the varieties. In South America cassava is mostly grown on small farms, without fertilization and usually intercropped with other edible plants such as yams, sweet potato, maize and beans. However, cassava grows better than any other major food plant in poor soils and under drought conditions (Ospina & Ceballos, 2002).

Despite the adaptability of the crop to poor conditions, and the estimated potential of productivity of 80 tonnes per hectare, the world average yield for cassava has remained in 12-13 tonnes per hectare (FAOSTAT, 2007). However, yields can be improved by using healthy and well adapted genetic material as vegetative seed, sandy well drained soils, fertilization and adequate agriculture practices such as disease and pest control (Ospina & Ceballos, 2002;).

Several bacterial diseases have been reported in cassava including Cassava Bacterial Blight (CBB) caused by *Xanthomonas axonopodis* pv. *manihotis* (*Xam*), bacterial angular leaf spot caused by *X. campestris* pv. *cassavae*, bacterial stem gall caused by *Agrobacterium tumefaciens* Biovar 1, bacterial stem rot caused by *Pectobacterium carotovorum* sbsp. *carotovorum* (*Erwinia carotovora* pv. *carotovora*) and bacterial wilt caused by *E. herbicola*. *E. herbicola* was renamed *E. agglomerans* by Beji *et al.* (1988) and later replaced in genus Pantoea as *Pantoea agglomerans* (Gavini *et al.*, 1989) (http://www.apsnet.org/online/common/names/cassava.asp).

The major cassava bacterial disease limiting productivity in different parts of South America (Colombia & Venezuela) and Africa is cassava bacterial blight (CBB). Yield losses due to CBB can reach up to 80-90% during

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high epiphytotic periods such as those reported in Zaire and Colombia (Lozano, 1986; Sánchez & Verdier, 1998; Ospina & Ceballos, 2002). CBB in cassava has been extensively studied and a high genetic diversity in the different population of *Xam* in Colombia and Venezuela has been found (Verdier *et al.*, 1998b; Restrepo *et al.*, 2000). With the idea to understand the dynamic of the genetic variability of *Xam* population, we attempted to study isolation and characterization of the genetic diversity of different Venezuelan populations of *Xam*, initiated previously by Verdier *et al*. (1998a), and found a new bacteriosis present on cassava fields. In this study we report the identification and characterization of the causal agent of this bacterial disease in Venezuela.

MATERIALS AND METHODS

Bacterial isolation and culture: Cassava leaves showing angular water-soaked lesions or necrotic lesions on the leaf lamina were collected in 2001, 2005 and 2008, from different Venezuelan regions (Anzoátegui, Monagas & Portuguesa). Pieces of water-soaked lesions were transferred to a sterile saline solution (0.85%) and the bacteria were allowed to diffuse into the solution from the edge of the dissected tissue. Bacterial strains were isolated by streaking on LB agar or on semiselective trehalose agar media plates used for the isolation of *X. axonopodis* pv *manihotis* (Schaad *et al.*, 2001). Plates were incubated 24 h at 30°C. Single colonies were restreaked at least two times to ensure purity. All isolates were stored at -80°C in LB supplemented with 20% glycerol.

Physiological and biochemical characterization: Bacteria were grown in LB at 30°C to an optical density of 1 OD. Freshly grown bacterial cultures were used for all the assays. Gram stain and the 3% KOH test were performed. Flagella were observed under an EM (Philips Cryo CM120) from an o/n bacterial culture carefully re-suspended in water ($1:10^4$ dilution factor) and negative stained (Servicio de Microscopía Electrónica del Departamento de Biología Estructural, Instituto Venezolano de Investigaciones Científicas). Biochemical identification was performed using API20E strips (bioMerieux, Inc.) and the MicroScan Autoscan4 system. All tests were conducted more than once to confirm the strain identification. Fatty Acid Analysis was performed using the MIDI Inc microbial identification system by the service of the University of Florida, Plant Pathology Department, Gainesville, USA, (Dr Ellen Dickstein).

Pathogenicity test on cassava plants: Pathogenicity studies were performed using cultures of the different isolated strains, grown in LB at 30°C for 24 h. Cultures were adjusted to an estimated density of 10^8 CFU/mL by optical density previous to plant inoculation. Healthy cassava *in vitro* plants from the IDEA Germoplasm Bank, greenhouse plantlets or stem cutting plants were used for the pathogenicity test. All cassava plant material was initially grown from *in vitro* plants using cassava micropropagation media containing Murashige and Skoog (1962) salts supplemented with thiamine 10 mg/L, myo-inositol 100 mg/L, 2% sucrose, 0.027 µM α-naphthaleneacetic acid, 0.023 µM gibberellic acid and 0.18% Phytagel™. Leaf inoculations were done with a sterile tooth stick previously dipped in the bacteria culture. Plants were also inoculated with sterile LB medium as a negative control. Initially, 28 cassava cultivars were assayed to study their susceptibility to the pathogen. All the experiments were conducted in triplicate. Plants were evaluated for the presence of disease symptoms during 2-4 weeks. Individual plants were evaluated using the disease severity rating scale of 1 to 6 where 1, no symptoms; 2, less than 50% leaf area with lesions; 3, 50% or more leaf area with lesions; 4, 100% of leaf area with lesions; 5, several dead leaves and 6, symptoms on the stem or dead plant. Plant tissue with disease symptoms were sampled and strains isolated and identified again. Experiments with *in vitro* plants, greenhouse plantlets or stem cutting plants were performed in triplicates. At least three sets of independent experiments were conducted with each plant material.

Genomic DNA and Plasmid Isolation: DNA was isolated from o/n cultures either by the procedure described by Chen and Kuo (1993) or using Wizard® Genomic DNA purification kit from Promega. Total DNA was electrophoresed in 0.8% agarose gel containing Tris-borate-EDTA (TBE) buffer as described by Sambrook *et al.* (1989). DNA was stained with 0.5 µg/mL of ethidium bromide and visualized and photographed under UV light with the Molecular Imager Chemidoc System from BioRad.

PCR amplification: Partial 16S ribosomal RNA gene amplification was performed as described by Lu *et al*. (2000), using U1 and U2 universal primers. Reactions were performed in a final volume of 25 µL containing 0.2 µM of each primer, 200 µM dNTPs, 10 mM Tris-HCl [pH 8.4], 3 mM $MgCl_2$, 1 U GoTaq® Flexi DNA polymerase from Promega and 100 ng of template DNA. Amplification reactions were carried out in a PT-100 MJ Research thermocycler. After 10 min of denaturation at 94°C, 35 cycles of 60 sec of denaturation at 94°C, 60 sec of annealing at 55°C and 2 min of extension at 72°C were performed, with a final extension at 72°C for 10 min.

ERIC-PCR analysis and PCR ribotyping were performed as described by Versalovic *et al.* (1991) and Appuhamy *et al.* (1998). The primers were ERIC IR 5'ATGTAAGCTCCTGGGGATTCAC 3' and ERIC2 5'AAGTAAGTGACTGGGGTGAGCG 3'for ERIC PCR analysis; and GIRRN 5'GAAGTCGTAACAAGG3' and LIRRN 5'CAAGGCATCCACCGT 3' for ribotyping. Reactions were performed in a final volume of 25 µL containing 0.2 µM of each primer, 200 µM dNTPs, 10 mM Tris-HCl [pH 8.4], 3 mM $MgCl_2$, 1 U GoTaq® Flexi DNA polymerase from Promega and 100 ng of template DNA. Amplification reactions were carried out in a PT-100 MJ

thermocycler. After 5 min of denaturation at 95°C, 35 cycles of 30 sec of denaturation at 94°C, 30 sec of annealing at 50°C and 6 min of extension at 72°C were performed, with a final extension at 72°C for 6 min. *Enterobacter cloacae* strain ATCC 13047 and *P. agglomerans* USBCU1 (Biotechnology Lab. collection) were used as reference for PCR experiments.

PCR reactions of *hsp60* were performed as reported by Hoffmann and Roggenkamp (2003), using primers Hsp60-F 5´GGTAGAAGAAGGCGTGGTTGC 3´and Hsp60-R 5´ ATGCATTCGGTGGTGATCATCAG 3´. *X. axonopodis* pv. *manihotis* containing the gene encoding for pathogenicity protein PathB was detected by PCR using XV and XK specific primers (Verdier *et al.*, 1998a; Schaad *et al.*, 2001).

DNA products were electrophoresed in 1-2% agarose gel containing either Tris-acetate-EDTA or Tris-borate-EDTA buffer as described by Sambrook *et al.* (1989). DNA fragments were stained with 0.5 µg/mL of ethidium bromide and visualized and photographed under UV light with the Molecular Imager Chemidoc System from BioRad. Amplified DNA fragments were purified using the AxyPrep™ PCR Cleanup kit from Axygen and then sequenced by the sequencing service of the Biotechnology Center, Instituto de Estudios Avanzados (IDEA), Caracas, Venezuela. Following sequencing, DNA sequences were analyzed using DNAMAN and a NCBI BLAST was conducted to compare the sequenced fragment (~341 bp for *hsp60* & ~850 bp for *pathB*) with sequences published in the Genebank. (http://www.ncbi.nlm.nih.gov/BLAST/).

Table I: Physiological and biochemical characteristic of the *E cloacae* isolated strains. *E. cloacae* ATCC 13047 was use as a reference strain

Characteristic	Anz03	Gua56	Mon11	Mon536	*E. cloacae*
Gram stain	-	-	-	-	-
Motility	+	+	+	+	+
Yellow Pigment	-	-	-	-	-
β-Galactosidase	+	+	+	+	+
Arginina dihydrolase	+	+	+	+	+
Lysine decarboxylase	-	-	-	-	-
Ornithine decarboxylase	+	+	+	+	+
Citrate utilization	+	+	+	+	+
Hydrogen sulfide	-	-	-	-	-
Ureasa	-	-	-	-	-
Tryptophan deaminase	-	-	-	-	-
Indole	-	-	-	-	-
Voges-Proskauer	+	+	+	+	+
Gelatin liquefaction	-	-	-	-	-
Acid from:					
Glucose	+	+	+	+	+
Mannitol	+	+	+	+	+
Inositol	-	-	-	-	-
Sorbitol	+	+	+	+	+
Rhamnose	+	+	+	-	+
Sucrose	+	+	+	+	+
Melibiose	+	+	+	+	+
Amygdalin	+	+	+	+	+
Arabinose	+	+	+	+	+
Trehalose	+	+	+	+	+
Oxidase	-	-	-	-	-
Nitrate reduction	+	+	+	+	+
Catalase	+	+	+	+	+
Anaerobic growth	+	+	+	+	+
Growth at 42ºC	+	+	+	+	NE
Growth at 5% NaCl	+	+	+	+	NE

NE means no essayed

RESULTS

Symptoms on diseased plants: Fig. 1 shows plants from the field with leaves showing necrotic lesions with a chlorotic halo. Advanced stages of the disease induced an early senescence of leaves leaving the stem bare.

Isolation and physiological and biochemical identification: Samples from Anzoátegui, Portuguesa and Monagas states yielded small beige, glistening, smooth colonies with regular margins on LB plates after 24 h incubation at 30ºC. Longer incubations (48-72 h) allow the growth of *Xam,* when present, recognized as a mucoid, cream shining colonies, with no antagonist between them. Fig. 2 is an electron microscopy photograph of strain Gua56 showing rod-shaped bacterial cells with rounded ends with several peritrichous flagella.

Table I shows the results of physiological and biochemical characterization of the bacterial isolates as well as the results for *E. cloacae*. Biochemical tests indicated that the strains isolated belong to the species *E. cloacae* (99% identity with the results expected for *E. cloacae*). Identical results were obtained with API20E tests and MicroScan Autoscan4 system. However, compared with Anz03, Gua56 and Mon11, Mon536 was negative for the production of acid from rhamnose. Resistance to ampicillin and cefazolin was detected in Anz03. Fatty acids profile of Gua56 was not conclusive; allowing the inclusion of the strain GUA56 in the family group of Enterobacteraceas, genus *Enterobacter sp*. Gua 56 strain had a similarity index of 0.917, 0.696 and 0.651 with *E. cancerogenus*, *E. asburiae* and *E. hormaechei*, respectively.

Molecular identification: In order to confirm the biochemical results, molecular techniques were applied. The first approach for the molecular identification of the isolates was sequencing of 16S ribosomal RNA gene. Partial gene sequences of the four strains were compared with the sequences present in GenBank. Table II shows the percentage of identity of our sequences with the sequences of the 16S ribosomal RNA gene of different species. Since 16S rRNA gene sequence analysis was not conclusive and did not allow the discrimination between *E. cloacae* and *P. agglomerans,* enterobacterial repetitive intergenic consensus primers (ERIC) and PCR ribotyping identification methods were performed. The results of these experiments are presented in Fig. 3. From the results of ERIC-PCR analysis and PCR ribotyping with GIRRN-LIRRN primers it can be conclude that the isolated strains have identical fragment mass than one of the *E. cloacae* strain used as control.

In addition *hsp60* DNA sequence of GUA56 showed

Table II: Percentage of maximum identity found for the 16S rRNA gene sequences of the isolated strains when compared with the sequences on the GeneBank

	Pantoea agglomerans				*Enterobacter cloacae*			
Strain	**Total Score**	**Q. Coverage**	**Max. Ident.**	**Gaps**	**Total Score**	**Q. Coverge**	**Max.Ident.**	**Gaps**
Anz03	1299	718/730 (94%)	98%	0	1297	718/730 (94%)	98%	0
Gua56	1478	833/852 (99%)	97%	4	1487	835/852 (99%)	98%	4
Mon11	935	563/589 (82%)	95%	15	933	563/589 (82%)	95%	15
Mon46	1613	881/885 (100%)	99%	0	1613	881/885 (100%)	99%	0
MonA4	1607	878/884 (99%)	99%	0	1618	880/884 (99%)	99%	0

Table III: Susceptibility of cassava varieties to Gua56 strain

	Week 1	**Week 2**	**Week 3**	**Week 4**
Control	**1**	**1**	**1**	**1**
22	1	2	2	2
24	1	2	2	2
35	3	5	5	5
46	1	2	3	3
47	1	2	2	3
48	1	2	2	3
49	1	1	3	5
50	2	5	5	5
51	1	2	3	5
52	2	5	5	6
53	1	1	2	3
54	1	2	2	3
55	2	2	3	5
56	2	5	5	5
57	1	2	2	2
58	1	1	2	3
59	2	2	3	5
60	2	2	2	3
61	1	2	2	3
62	2	2	3	3
63	2	3	3	3
64	2	2	2	3
65	1	2	2	3
85	1	2	3	5
86	3	5	5	5
89	2	3	5	6
90	2	2	3	5

Plants were evaluated for the presence of disease symptoms during 4 weeks. Individual plants were evaluated using the disease severity rating scale of 1 to 6 where 1= no symptoms, 2= less than 50% of leaf lesion, 3= 50% or more of leaf damage, 4= 100% of leaf damage, 5= several dead leaves and 6= symptoms on the stem or dead plant. The values represent the absolute values of the average between replicas (n=3).

99,4% identity in a fragment of 342 bp with chaperonin GroL (Hsp60) partial sequence of *E. cloacae* ssp. *cloacae* NCTC9394 published in GenBank under the accession number FP929040.1, and 100% identity with a fragment of 333 bp of *E. cloacae* partial sequence of *hsp60* gene for strains numbers 8, 40, 32, 157 and 131, accession numbers AJ417137.1, AJ417133.1, AJ417130.1, AJ417121.1 and AJ417120.1, respectively.

Representative isolates Gua56 and Anz03 have been deposited into the Venezuelan Collection of Microorganism, Universidad Central de Venezuela, under accession number CVCM 1992 and CVCM 1993. 16S rRNA gene partial sequences of these strains were introduced in the GenBank under the accession numbers GU593270 and GU593272.

Pathogenicity tests: The pathogenicity tests were performed with the different *E. cloacae* strains isolated, and cassava plants grown either from stem cutting in the greenhouse or *in vitro* plants (Fig. 4). *E. cloacae* strains reproduced the symptoms described for the disease on susceptible cassava varieties. Using *in vitro* cassava plants, a test of susceptibility to *E. cloacae* of 28 cassava varieties of IDEA germoplasm bank was performed with the Gua56 isolate (Table III). Cassava varieties 52 and 89 were the most susceptible to the infection with *E. cloacae*. Several varieties such as 22, 24 and 57 however, were resistant to the infection.

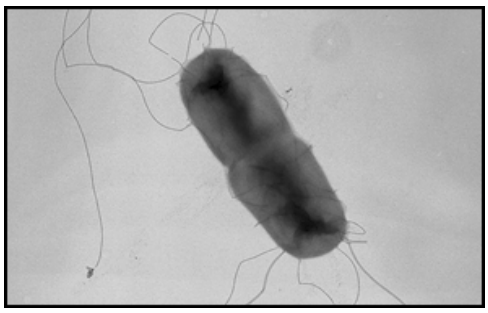
Plasmid profile: The pathogenicity of *Xanthomonas axonopodis* pv. *manihotis* is associated with a plasmid of 44 kbp (p44) carrying the effector gene *path*B (Verdier *et al.*, 1998a; González *et al.*, 2002). In order to study the origin of the pathogenicity of the isolated *E. cloacae* strains, total DNA was isolated and separated on agarose gels. Plasmids were shown to be present (Fig. 5). A *Xam* DNA sample characterized in our lab was also included in order to compare the plasmid profile between the two cassava pathogens. *E. cloacae* strains contained 3 plasmids. Two plasmids were larger than the ones present in *Xam,* which are very close in size (Fig. 5). Also a third plasmid, hard to separate from the genomic DNA, was observed in *E. cloacae* strains (not shown). In order to check on the possibility that none of the plasmids present in *E. cloacae* strains have the gene enconding PathB reported by Verdier *et al.* (1998a), a PCR with specific primers were performed (Fig. 6), and only DNA from *Xam* amplified a fragment of the expected size (898 bp) indicating that a different mechanism of pathogenicity than the one present in *Xam* is present in *E. cloacae*.

DISCUSSION

In the results presented here, isolation and characterization of the causal agent of a new cassava bacterial disease is described. Physiological and biochemical characterization indicated that the causal agent of the disease was a species of *E.* cloacae complex. Several members of the family *Enterobacteriaceae* such as *P. agglomerans*, *E. cloacae*, *E. cancerogenus*, *P. carotovorum* and *S. marcescens* are pathogens of plants, sharing many characteristic as being rod shaped, Gram negative, non-spore forming and facultatively anaerobic

Fig. 1: Diseased cassava plant in the field showing necrotic leaf lesions. Typically the watersoaked lesions become necrotic and form a chlorotic margin around it

Fig. 2: Electron microscopy of GUA56 strain isolated from cassava showing rod-shaped cells and peritrichous flagella (15.000X)



among others. However, only *P. carotovorum* and *P. agglomerans* were previously described as causal agent of diseases in cassava. In the present study molecular methodologies were used to confirm the identification of the isolates. 16S rRNA gene sequencing was not useful in the identification of the strains because it has a poor discrimination power between *Pantoea* and *Enterobacter* species. Similar results have been obtained by other authors (Spröer *et al.*, 1999; Janda & Abbott, 2007, Rodriguez *et al*., 2008). On the other hand, ERIC and PCR ribotyping with GIRRN-LIRRN primers showed an identical profile to that of the *E. cloacae* reference strain used in this study. A different band pattern was obtained in GUA56 when compared to the *P. agglomerans* reference strain used. Sequencing of *tuf* and *atpD* conserved genes has been recommend by Paradis *et al.* (2005) since they provide a better discrimination between pairs of species belonging to the family *Enterobacteriaceae*. On the other hand, Hoffmann and Roggenkamp (2003), working on the population genetics of the nomenspecies *E. cloacae*, used a combination of sequence and PCR-restriction fragment length polymorphism analysis of three housekeeping genes *hsp60*, *rpoB* and *hemB*, as well as *ampC* gene, in order to analyze the genetic structure and the phylogenetic

Fig. 3: Fingerprints obtained by ERIC PCR (A) and PCR Ribotyping (B). Lane 1: 1kb plus DNA ladder from Invitrogen™, Lane 2: Gua56, Lane 3: *Enterobacter cloacae* ATTC 13047, Lane 4: *Pantoea agglomerans* USBCU1

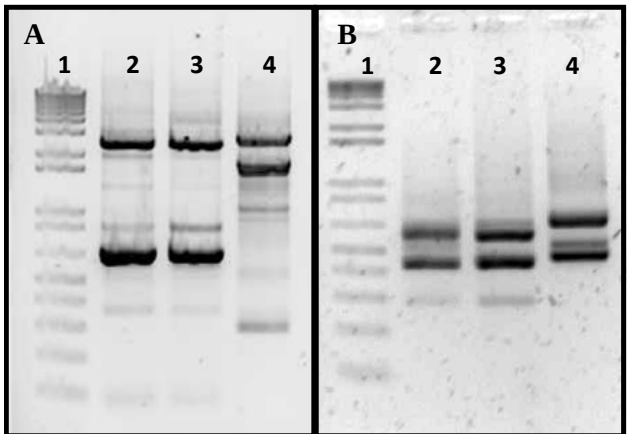
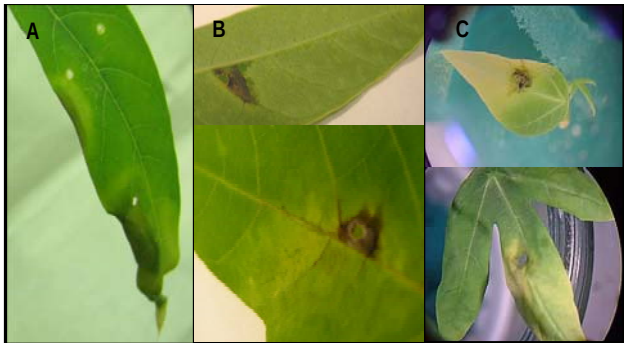


Fig. 4: Pathogenicity tests. A: Pathogenicity tests on cassava vegetative stem cuttings, B: cassava plants grown in growth chambers and C: on *in vitro* cassava plants inoculated with isolated bacterial strains

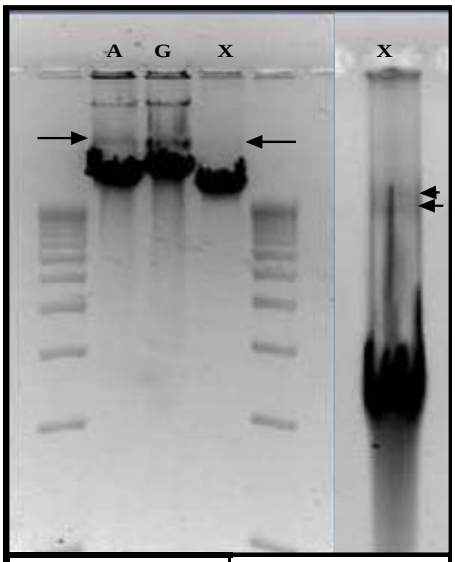


relationship between the clusters of the *E. cloacae* complex. These authors based on the neighbor-joining tree of the *hsp60* sequences, defined 13 genetic clusters in the *E. cloacae* complex. GUA56 *hsp60* partial gene sequence confirms the identification of the isolated strains as *E. cloacae* ssp. *cloacae*.

E. cloacae, an opportunistic human pathogenic bacterium, has been found as endophyte of several plant species with no deleterious effect on the host plant (Hinton & Bacon, 1995) and as seed associated bacterium suppressing seed infections (Kageyama & Nelson, 2003). *E. cloacae* has been also reported as pathogenic bacteria of economic important crops such as onion, papaya, ginger, apple, macadamia, dragon fruit, mulberry and mung bean sprouts (Nishijima *et al.*, 1987; Bishop & Davis, 1990; Nishijima *et al.*, 2004; Nishijima *et al.*, 2007; Masyahit *et al.*, 2009; Wang *et al*., 2010) and different species of orchids (Takahashi *et al.*, 1997).

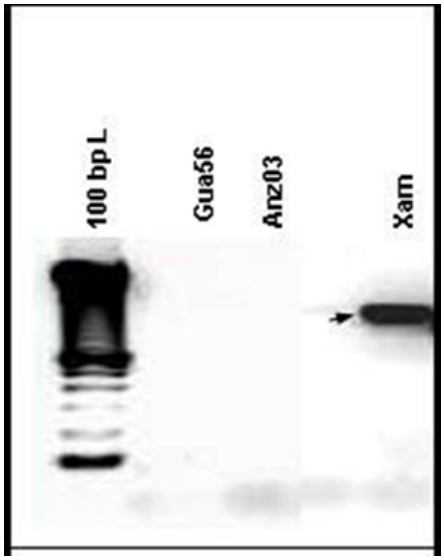
The bacterial pathogenicity of an endophyte may be

Fig. 5: Genomic and plasmid DNA isolated from *E. cloacae* strains (A: Anz03 & G: Gua56) and *Xam* (X), and loaded on 0.8% agarose gels (0.5 x TBE)



Bacteria	# Plasmids
E. cloacae	3
Xam	2

Fig. 6: Detection of *path*B encoding gene by PCR reactions. Amplified DNA was loaded on 1% agarose gels (0.5 x TBE). Arrow indicates the 898 bp expected DNA fragment amplified in *Xanthomonas axonopodis* pv *manihotis*. 100 bpL: DNA marker 100bp ladder; Gua56 and Anz03: *E. cloacae* strains; *Xam*: *Xanthomonas axonopodis* pv. *Manihotis*



related to the interaction and horizontal transfer of DNA encoding pathogenicity factors, with other microorganisms. In Venezuela, *Xam* is endemic in areas where cassava is traditionally grown. Very often, when the rainy season starts, watersoaked leaf lesions caused by *Xam* can be observed in susceptible cultivars. So, it could be of special interest to study the interaction of *E. cloacae* infection and *Xam* infection in the Anzoategui and Monagas states, where it is known that there is a high infectivity of *Xam.* The results obtained however, indicate that at least there is no transfer of *Xam path*B gene to *E. cloacae*. Other pathogenicity mechanisms such as the presence of quorum sensing, biofilm formation and the production of proteolytic enzymes need to be addressed in the strains isolated, as well as the conditions that induce the expression of factors that mediates the pathogenicity of *E. cloacae* in plants.

CONCLUSION

This is the first report of a disease caused by *Enterobacter cloacae* on cassava in Venezuela. Biochemical assays and PCR based identification methods such as ribotyping, ERIC and *hsp60* gene sequence identified the different isolated strains as *E. cloacae.*

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