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Growth response of maize plantlets inoculated with *Enterobacter* spp., as a model for alternative agriculture

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ABSTRACT

A maize rhizosphere isolate was phenotypically and genotypically characterized and identified as *Enterobacter* spp. bacterium. Germinated seeds were inoculated, the plantlets were sown in vermiculite and in soil and grown under laboratory and field conditions, respectively. The adherence, colonization and plant growth promotion capability of *Enterobacter* sp. UAPS03001 was evaluated in "Rojo-Criollo" maize under laboratory conditions. Twenty days after inoculation, the treated plantlets showed larger biomass than non-inoculated ones. In field grown plants, the kernel biomass was also greater in inoculated than in non-inoculated plants. The inoculation of maize sprouts with plant growth- promoting bacteria before their sowing in the field would be an alternative practice for achieving successful yield in temporal agriculture.

Key words: inoculated plantlets, PGPR, maize, *Enterobacter* spp.

RESUMEN

Respuesta de plántulas de maíz inoculadas con *Enterobacter* spp. como un modelo de agricultura alternativa. En este trabajo se aisló una bacteria de la rizósfera de maíz, que fue caracterizada mediante métodos fenotípicos y genotípicos e identificada como *Enterobacter* sp. UAPS03001. La bacteria fue inoculada en semillas de maíz "Rojo-Criollo" germinadas en forma axénica. Las semillas germinadas e inoculadas se plantaron en vermiculita y posteriormente las plántulas fueron cultivadas en vermiculita o en suelo, para evaluar el efecto promotor del crecimiento vegetal de dicha bacteria, bajo condiciones de laboratorio y de campo. Bajo condiciones de laboratorio, también se evaluó la capacidad de esta cepa para adherirse a las plantas de maíz y colonizarlas. Veinte días después de la inoculación, las plántulas inoculadas mostraron una biomasa mayor con referencia a las no inoculadas. En campo, la biomasa de la mazorca fue también mayor en las plantas inoculadas respecto de las plantas no inoculadas. La inoculación de germinados de maíz con una bacteria promotora del crecimiento vegetal y su posterior transferencia a campo podría ser una práctica alternativa para llevar a cabo una producción exitosa en agricultura de temporal.

Palabras clave: plántulas inoculadas, PGPR, maíz, *Enterobacter* spp.

INTRODUCTION

Zea mays (Poaceae) apparently evolved from Teocintle (*Zea perennis*), (6, 48) in a geographic region that comprises the Central and Southeast regions of Mexico. Its origin as a crop dates back to 5400 (31) to 7000 years BC (32, 33) and was eventually disseminated to other regions in the Americas and the entire world (19, 36). This plant forms the basis of the Mexican diet. For instance, the grains are used for preparing diverse traditional foods and beverages like *tortillas, molotes, tamales, gorditas, tostadas, tesgüino*, and *tejuino* (13, 27). Nowadays, maize uses include modern exploitation for producing fructose syrup (46), bio-oil, and others (18). The local "Rojo-Criollo" maize cultivar is used for preparing traditional candies (Secretaría de Turismo de Tlaxcala, México 2010: Tlaxcala pre-hispanicgastronomy, http://www.tlaxcala.gob.mx/turismo/anexo/gastronomico/b_mestiza.html) and a kind of red *tortillas*. Bacterial inoculation of maize, mainly with *Azospirillum,* has been a successful common practice (5, 23, 30). Additionally, growth enhancement has also been observed by experimental inoculation with many other bacteria, for instance *Pseudomonas* (17, 23, 39), *Klebsiella pneumoniae*, *Pantoea agglomerans*, *Gluconacetobacter diazotrophicus* and *Herbaspirillum seropedicae* (40). Temporal agriculture is the most common farming for maize cultivation in Central Mexico, which is also characterized by poor income. This and other facts have provoked a

continuous reduction of traditionally cultivated maize fields (22). One of the greatest problems is related to the erratic start of the rainy season, especially in current times, which is probably due to global climatic change (16, 34, 45). The deficiency of water on time and the presence of seed-feeding fauna have forced farmers to invest more in seed (38). In this work, a bacterial strain was isolated from the maize rhizosphere, and was further characterized by phenotypic and genotypic assays. The aim of this work was to inoculate axenic maize seedlings with the isolated bacteria, and to assess their capacity to promote the growth of plants both at growth chamber level and in the field. The growth of corn plants was stimulated under the conditions explored in this work, so it is proposed that the inoculation of germinated axenic maize sprouts with beneficial bacteria, their subsequent growth, and the introduction of inoculated plantlets to the fields, could be an alternative production system in temporal crops.

MATERIAL AND METHODS

Bacterial isolation and characterization

Strain UAPS03001 was isolated from the rhizosphere of "Rojo Criollo" maize .This variety has been cultivated by some people in San Diego Buena Vista Tlaxcala (Figure 1) for a long time, but, similarly to many other native varieties from Mexico, it has not been genetically characterized yet. Five plants grown for one month were extracted from soil (19° 10' 30.59" N, 98° 09' 50.05" W, Elevation: 2408 MASL) and transported to the laboratory in sterile conditions where soil adhered to their roots (considered rhizosphere) was resuspended in water (1:10 w/v). This mixture was vortexed at 3000 rpm for 3 min and one hundred microliters of serial dilutions (1:10 factor) were plated on Congo Red agar media (37). Bacteria from high dilution were isolated. The strain UAPS03001 was selected for testing its plant growth promoting activity. This strain was characterized by phenotypic characteristics and by 16S rDNA similarity. Biochemical testing was performed with MARK 20 panels from DADE Behring, analyzing the bacterial growth/activity for 24 biochemical tests (including the use of carbon sources) and resistance to 23 antibiotics. Bacterial response was detected with the automated system MicroScan 4 (Baxter Inc. Mexico). The phenotypic tests performed are listed in Table 1. The sequence of the 16S rDNA gene was compared with public sequences. Briefly, genomic DNA was extracted and purified from bacterial cells grown to stationary phase with a Wizard Genomic DNA Isolation kit (Promega). A fragment of ca. 1365 bp of the 16S rDNA gene was amplified with the universal primers: UN27F 5'-TAGAGTTTGATCCTGGCTCAG-3' and UN1392R 5'-CAGGGGCGGTGTGTACA-3' (Biodiversa Inc., Mexico). The PCR product was sequenced with the same primers at the Instituto de Biotecnología (UNAM). The sequence was initially analyzed with BLAST, and subsequently with DNA Star. The GenBank accession number of the 16S rDNA sequence of UAPS03001 is HM355806.

Antibiotic resistance

Antibiotic resistance was tested for designing media for the reisolation of the inoculated strain. UAPS03001 was plated on LB and Congo Red media (37). Antibiotic multidiscs (Bio Rad, Mexico) were placed in duplicate on inoculated agar plates.

Germination of maize seeds

Seeds of the "Rojo-Criollo" maize variety were germinated as follows: the seeds were washed with distilled water and rinsed with 70% ethanol for 10 min and immersed under agitation in 6.5% sodium hypochlorite for 20 min. The seeds were washed eight times with sterile distilled water under sterile conditions, and incubated for three days at 30°C under high environmental humidity on MS solid medium (29) supplemented with 20 mM glucose and 30 mM sucrose (medium MSJ3). Seeds showing microbial growth after incubation were discarded.

Growth promotion experiment in environmental chamber

Strain UAPS03001 was grown overnight in LB broth. Bacterial growth was inoculated in the same medium and grown until stationary phase. The cells were washed and resuspended in the same volume in sterile distilled water. Cell quantity was determined in triplicate with the drop plate method (12). Forty germinated maize seeds were immersed in the bacterial suspension for 1 h and sown in tubes containing 5 g of sterile vermiculite.

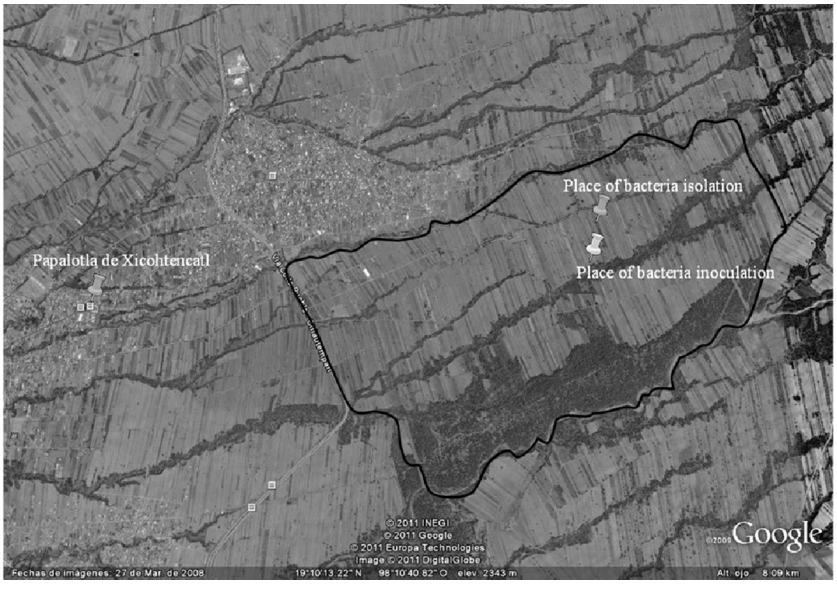

Figure 1. Aerial view of San Diego Buenavista (closed in bold line) belongs to "Papalotla de Xicohtencatl" community. It is a place located to the south of Tlaxcala in the Central Mexican Plateau (Altiplano de México). Photo obtained from Google Earth.

As a control, other forty germinated maize seeds were immersed in sterile distilled water under the conditions mentioned above. The sprouts were grown under the following conditions: 25 to 28°C, light/darkness cycle 16/8 h, 70% humidity. The plantlets were watered once with 25 ml of MS solution without carbon source (medium MSJ1).

a) Quantification of bacterial adherence. The number of cells adhering to the sprouts was quantified after 12 h. Six replicates and six controls were vigorously vortexed in a volume of sterile distilled water. Cell numbers were determined by the drop plate method on agar selective media.

b) Quantification of bacterial colonization. Ten and 20 days after inoculation, the roots of ten plants for each treatment were immersed in 20 ml of sterile distilled water and vigorously vortexed for 40 s. Rhizospheric bacteria were determined with serial dilutions of the bacterial suspension by dropping 20 µl on selective media plates and 24-hour incubation.

c) Aerial plant biomass. The aerial region of 20 plants was chopped into small pieces, dehydrated at 70°C for four days, and weighed. Data were compared with the Student's t-test.

Field experiment

Plantlet production. Two hundred "Rojo-Criollo" maize variety seeds were germinated in MSJ3 medium as explained above. One-half of the sprouts was inoculated with bacterial cells as mentioned whereas the other half was treated as control. The sprouts were seeded in sterile vermiculite and watered with MSJ1 as described above. The plantlets were incubated for five days under the conditions of the previous experiment and subsequently transferred to the field.

Plant growth . Sowing was at the start of the rainy season in May 2007. The field, located in Papalotla, Tlaxcala, Mexico (19° 10' 19.93" N, 98° 09' 52.74" W, Elevation: 2394 MASL), was ploughed and the plantlets were sown with 0.8 m of distance between them, placing 10 plants by furrow, with a total of 10 rows for each treatment. The space between rows was 70 cm and each experimental block was in a space of 10 m in length (distance from the furrow) by 8 m wide. The border between inoculated and non-inoculated plantlets was 3 m in length. Holes, ca. 15 cm in depth and 20 cm in diameter were made with the aid of a spade. The plantlets were introduced in the holes (one plantlet per hole), covered with soil, and watered with 500 ml distilled water. Two days later the plantlets were watered as before. One month after seeded, each plant was fertilized with 7.5 g of NPK 17:17:17 (containing 1.7 g of nitrogen, 1.7 g of P_2O_5, and1.7 g of K_2O per 10 g) (15), and a final plough was made. Eight month-old plants were harvested and the maize kernels were weighed.

Data Analysis

Statistical analysis for rhizospheric colonization and aerial dry biomass was performed using Sigma Plot (Handel Scientific Software). Differences were considered according to the Student´s t test results.

RESULTS

Isolation and characteristics of rhizospheric bacteria

The strain designated UAPS03001 was isolated from the rhizosphere of "Rojo-Criollo"maize. The morphology of this strain in Congo Red agar corresponded to small red circular translucent colonies, showing regular margin, convex elevation and smooth texture. Phenotypic tests of strain UAPS03001 showed 95% similarity to *Enterobacter cloacae* (Table 1) and the sequence comparison of the 16S rDNA gene showed 96.3% identity with the same species (Table 2). This strain was resistant to some antibiotics (Table 3), and ampicillin (Ap) and erythromycin (E) were chosen for the selective media added with Ap (50 µg/ml) and E (50 µg/ml). Bacterial growth was comparable in selective and non-selective media (results not shown).

Table 1. Phenotypical traits and antibiotic pattern of strain UAPS03001, obtained with the panel MARK 20 from Dade Behring (ID = *Enterobacter cloacae* with 99.99% of probability)

Biochemical test	Result	*E. cloacae*
Glucose	+	+[3]
Sucrose	+	+[1]
Sorbitol	+	+[1]
Raffinose	+	+[1]
Arabinose	+	+[3]
Inositol	-	+[1]
Adonitol	-	-[1]
Melobiose	+	+[1]
Urea use	-	-[1]
H2S	-	-[3]
Indole	-	-[3]
Lysine decarboxylase	-	-[1]
Arginine dihydrolase	+	+[1]
Ornithine decarboxylase	+	+[1]
Tryptophan deaminase	-	-[3]
Esculin hydrolysis	+	V[1]
Voges-Proskauer	+	+[2]
Citrate	+	+[3]
Malonate	+	+[1]
O-nitrophenyl-β-D-galactopyranoside (β-galactosidase activity)	+	+[1]
Oxidase activity	-	-[3]

[1]means reference 11, [2]means reference 42, [3]means reference 44. V means variable.

Adherence and colonization assays

Adherence and colonization assays were developed in triplicate with similar results for each independent experiment. We are showing results for one of them. The sprouts were inoculated with a suspension containing 9×10^8 CFU/ml, showing an adherence of 1.05×10^7 CFU per plant (SD 7×10^6). The bacterial population in the rhizosphere 10 days after inoculation (dai) was 7.12 $\times 10^8$ CFU/g vermiculite and at 20 days had increased to 1.86×10^9 UFC/g vermiculite (Fig. 2). Several microbial morphologies, different from those of strain UAPS03001 were detected from non-inoculated plantlets. Those microorganisms were recovered in low quantities (1×10^3 CFU/g vermiculite) and were not further characterized.

Growth promotion experiments both in environmental chamber and in the field

Adherence and colonization assays were developed in triplicate with similar results for each independent experiment. We are showing results for one of them. The

Table 2. Sequence similarity with 16S rRNA of *Enterobacteriaceae* species [% identity]

	UAPS01001 (GQ267502)
Averyella dalhousiensis (DQ481464)	93.4
Brenneria alni (AJ223468)	89.8
Budvicia aquatica (AJ233407)	91.0
Buttiauxella agrestis (DQ223871)	93.4
Edwarsiella hoshinae (AB050825)	91.8
Enterobacter aerogenes (AB004750)	94.7
Enterobacter amnigenus (AB004749)	94.7
Enterobacter asburiae (AB004744)	94.9
Enterobacter cancerogenus (Z96078)	95.0
Enterobacter cloacae (AJ251469)	96.3
Enterobacter gergoviae (AB004748)	93.9
Enterobacter helveticus (DQ273688)	94.1
Enterobacter hormaechei (AJ853889)	94.8
Enterobacter kobei (AJ508301)	95.5
Enterobacter ludwigii (AJ853891)	95.9
Enterobacter nimipresuralis (Z96077)	94.7
Enterobacter radicincitans (AY563134)	94.6
Enterobacter turicensis (DQ273681)	94.4
Enterobacter cowanii (AJ508303)	94.6
Erwinia billingiae (AM055711)	93.2
Ewingella americana (DQ383802)	91.1
Hafnia alvei (DQ412565)	91.8
Pantoea ananatis (U80196)	92.5
Tatumella ptyseos (AJ233437)	92.9

The accession number of each bacterial species is shown in parentheses and *Enterobacter* species identities in bold letters.

inoculated plants showed statistically significant greater biomass than the controls (Figure 3) 0.168 g (±0.034) for inoculated plants and 0.080 g (±0.049) for non-inoculated plants. All the plants survived and showed strong vigor throughout the experiment (Results not shown). In inoculated plants, the flowering started ca. 200 days after transplantation. The total kernel biomass of eight month-plants, obtained from 100 plants of each treatment, was higher in inoculated than in non-inoculated plants, 19.6 and 12.86 kg, respectively.

DISCUSSION

The strain UAPS03001 isolated from the rhizosphere of "Rojo-Criollo" maize was designated as *Enterobacter* spp. because phenotypic tests of the strain showed 95% similarity to *Enterobacter cloacae* (Table 1) and the sequence comparison of the 16S rDNA gene showed high identity value (96.3%) with the same species (Table 2). However, according to polyphasic taxonomy, more studies are needed to designate the species name (47). *Enterobacter* spp. has also been isolated from the rhizosphere of other plants, e.g. from *Lolium perenne* rhizosphere (41), rice rhizosphere (26) and from soil near the roots of leguminous plants (49), showing its natural association to this environment. It is desirable to find native strains for inoculation attempts. The first advantage would be the adaptation to local biological and non-biological conditions, whereas the second advantage would be the use of indigenous organisms that would prevent environmental disturbance.

Table 3. Resistance of *Enterobacter* spp. to antibiotics from Multidiscs Biorad

Antibiotic	Biorad abbreviation	µg in the disc	Resistance (R) or Susceptibility (S) with diameter of effect (cm)
Ampicillin	AMP	10	R
Erythromycin	E	15	R
Cephalotin	CF	30	R
Penicillin	PE	10	R
Dicloxacillin	DC	1	R
Ceftriaxone	CRO	30	S (2.7)
Cefotaxime	CTX	30	S (2.4)
Netilmicin	NET	30	S (1.8)
Nitrofurantoin	NF	300	S (1.2)
Chloramphenicol	CL	30	S (1.8)
Amikacin	AK	30	S (1.3)
Carbencillin	CB	100	S (2.0)
Trimethoprim/ Sulfamethoxazole	SXT	25	S (2.5)
Cefuroxime	CXM	30	S (1.8)
Pefloxacin	PEF	5	S (3.0)
Gentamicin	GE	10	S (2.3)
Tetracicline	TE	30	S (2.2)
Ceftazidime	CAZ	30	S (2.1)

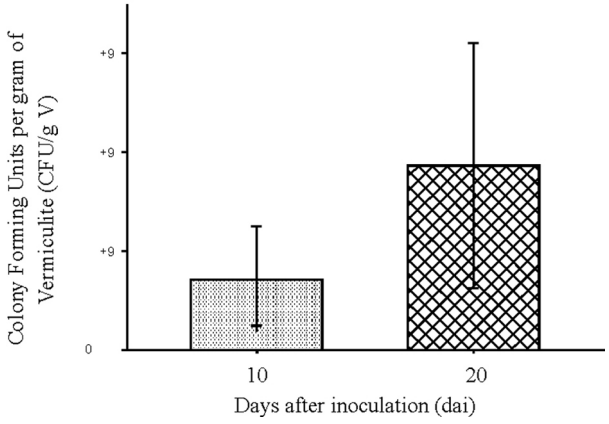

Figure 2. *Enterobacter* spp. UAPS03001 cell numbers colonizing maize rhizosphere. Quantifications were done at 10 and 20 days after inoculation (dai) in selective medium. Mean of ten replicates and standard deviations are presented. Differences were statistically significant with the Student's t-test ($p \leq 0.05$).

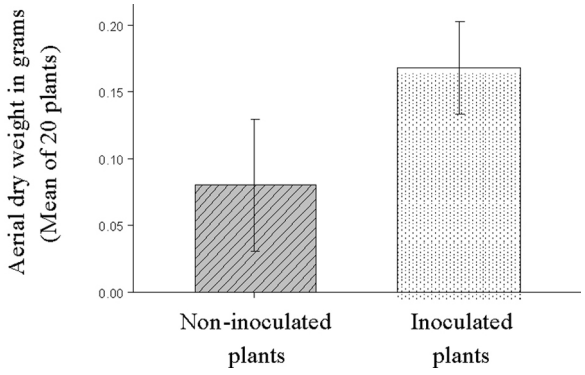

Figure 3. Aerial dry biomass of maize plants at twenty days after inoculation. Dark grey bar, non-inoculated controls; grey bar, inoculated plants. Mean of twenty plants and standard deviations are presented. Differences were statistically significant with the Student's t test ($p \leq 0.05$).

The strain herein isolated showed good adherence to seed sprouts and root colonization capability, similar to other rhizospheric bacteria (3, 4, 8, 28). Inoculation experiments were performed under environmental chamber conditions, where non-inoculated plants showed low quantities of other microorganisms different from strain UAPS03001 after 20 days. This means that a natural bacteria population is present inside the maize seed, which could not be eliminated by superficial sterilization, as suggested by Bresan and Borges (2). The inoculated plants showed statistically significant greater biomass than the controls under environmental chamber conditions and the total kernel biomass of eight month plants was higher in inoculated than in non-inoculated plants. Other experiments have demonstrated growth promotion by some *Enterobacter* strains on different plants, i. e. stimulation of the growth of tomato, pepper and mung bean plants by the plant growth-promoting bacterium *Enterobacter cloacae* CAL3 (25) or the stimulation of the growth of *Brassica oleracea* by the nitrogen-fixing endophytic bacteria *Enterobacter* spp. strain 35 (50). In the present work, the mechanisms for promoting plant growth by *Enterobacter* spp. are not definite but as in other growth promoting bacteria, N-fixation, production of IAA and cytokinins, phosphate solubilization, and phytopathogen antagonism could participate in this activity (24), as has been suggested for *Enterobacter ludwigii* (41) and *Enterobacter radicincitans* (14).

Temporal agriculture is dependent on climate, so it is also highly susceptible to even minor environmental alterations (16). Particularly, plant germination is extremely vulnerable to lack of water. Most of the inoculation experiments have been done with seeds, so the bacterial survival capability in the soil had been determinative for plant colonization (1, 43). In this work, the inoculation was done in sprouts instead, in an attempt to increase bacterial and plant survival. Under our conditions, *Enterobacter* spp. UAPS03001 was proficient at promoting the growth of inoculated maize sprouts. Even though some *Enterobacter* strains have been designed as beneficial bacteria given their capability to suppress plant disease (20) and their biocontrol properties (21), some species of *Enterobacter* have been reported as opportunistic human pathogens (10). Hence, the results presented in this work have to be taken only as a model of study of inoculated plantlets, because they could be a latent danger when applied extensively. More studies are needed to determine potential hazards or benefits of *Enterobacter* spp. UAPS03001. At the present time, other beneficial bacteria such as *Azospirillum brasilense* could result more suitable to that end (5, 8, 24, 30). Sprout inoculation with beneficial bacteria as an alternative to seed inoculation could be advantegeous to maize sown in temporal agriculture and could possibly ameliorate foraging by seed-feeding fauna (9, 38). In this work, the maize seeds were germinated in MSJ3 medium, but less expensive substrates, such as vermiculite, could also be used. The scaling-up of this procedure for application in productive farms is a problem for maize but has been currently developed in other crops such as strawberry (7). Nevertheless, the process can be used for green house plantlet production without scaling-up. This procedure of inoculation in other crops is under test in our lab. A future variant of particular interest is the remediation of polluted temporal soils with plants inoculated via sprouts, for instance, with *P. putida* KT2440, which is able to use a variety of carbon sources (35), including xenobiotic compounds.

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