# Plant growth-promoting bacteria isolated from sugarcane improve the survival of micropropagated plants during acclimatisation

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## Highlights

- *A total of 162 isolates obtained from the rhizosphere, rhizoplane, roots, and stems of sugarcane were characterised for plant growth-promoting features and identified by partial 16S rDNA sequencing.*
- *Two PGPBs strains isolated from sugarcane (182-Bacillus and 336-Pseudomonas) significantly improved survival rates of micropropagated seedlings during the acclimatisation stage.*
- *Under different stress conditions, the 336-Pseudomonas strain improved the survival of micropropagated plants during the acclimatisation stage.*

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*See online Appendix for additional materials.*

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## Abstract

The plant microbiome plays an important role in nutrient acquisition and buffering plant hosts against abiotic and biotic stress. During *in vitro* propagation of sugarcane, pathogenic microorganisms are eliminated and most of the beneficial endophytic microorganisms. The objective of this study was to isolate and characterise potential plant growth-promoting bacteria (PGPB) from sugarcane and to analyse their ability to improve the survival of micropropagated sugarcane plantlets during the acclimatisation stage. First, bacterial isolates from sugarcane were identified by partial *16S rDNA* sequencing and tested for plant growth-promoting (PGP) features, such as inorganic and organic phosphate solubilisation nitrogen fixation, siderophore synthesis, indole-3-acetic acid production, tolerance to abiotic stress and antibiotics production. Then three bacterial strains with multiple PGP traits were independently applied to micropropagated seedlings of the sugarcane variety TUC 03-12 when the plants were transferred to a nursery for *ex vitro* acclimatisation. The effect of selected PGPB on survival rates of micropropagated plantlets was evaluated in three independent assays, using different batches of seedlings. Thirty days after inoculation, 182-*Bacillus* and 336-*Pseudomonas* isolates significantly improved the transferred plants survival rate. High variability in plant survival among independent experiments was observed, but treatments with the 336-*Pseudomonas* strain showed a low mortality rate (20%) in all assays. This procedure constitutes a biological tool to improve the survival of micropropagated plants during greenhouse acclimatisation. Furthermore, it provides an initial tool for selecting bacteria with possible PGP effects in the field.

## Introduction

Sugarcane (*Saccharum* spp. hybrids) is an important crop in

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tropical and subtropical areas responsible for more than 80% of world sugar production (OECD/FAO 2018). Furthermore, due to the high biomass production and its efficiency in converting solar energy into chemical energy (Lam *et al*., 2010), sugarcane is a valuable source for the production of renewable fuels (Neves *et al*., 2015).

Sugarcane is commercially multiplied through cane setts, called 'seed cane.' This agamic propagation favours the spread of systemic diseases, which cause important production losses (Noguera *et al*., 2013). Traditionally, cane growers use their own cane as seed for planting without taking enough precautions about seed cane health, vigour, and genetic identity. High-quality seed cane, *i.e*., with genetic purity and guaranteed health, can be obtained through meristem culture techniques and micropropagation. *In vitro* obtained seedlings are acclimatised to *ex vitro* conditions in a greenhouse and later multiplied in field nurseries to make enough seed cane available for commercial planting. Throughout the *in vitro* propagation process, plants are grown mainly under heterotrophic conditions (Kozai *et al*., 2005; Rocha *et al*., 2013) but suffer severe stress when they are transferred to *ex vitro* autotrophic growth conditions during the acclimatisation stage. Consequently, significant seedling loss can occur even though all proper precautions are taken (Murashige, 1974). One of the major reported causes of high shoot mortality is desiccation due to uncontrolled transpiration and serious nutritional deficiencies during this transition period (Oliveira *et al*., 2002; Yadav *et al*., 2019). Additionally, considering that the meristem micropropagation technique eliminates the pathogenic microorganisms and most of the beneficial endophytic microorganisms (Oliveira *et al*., 2002), the acclimatisation stage could be a useful scenario to test the beneficial effects of potential plant growth-promoting bacteria (PGPB). Using PGPB could constitute a sustainable strategy to improve plant survival during the acclimatisation process of micropropagated seedlings. PGBP can act on the growth and development of plants by promoting and improving the availability of nutrients through i) nitrogen fixation; ii) solubilisation of inorganic phosphate and micronutrients; iii) mineralising organic phosphate; iv) production of phytohormones and vitamins. In addition, PGPB can improve stress resilience, providing increased tolerance to abiotic stress and protection against pests and diseases (Numan *et al*., 2018; Kumar *et al*., 2020).

Several studies have been carried out in sugarcane with PGPB to evaluate their potential capacity as biofertilisers (Fuentes-Ramirez *et al*., 1993; Boddey *et al*., 1995; Mirza *et al*., 2001; Oliveira *et al*., 2002; Inui-Kishi *et al*., 2012; Pedula *et al*., 2016). However, the ability of PGPB to provide nutrients and stimulate plant growth depends on their successful establishment as part of the plant microbiome and their ability to adapt to different environmental factors such as climate, soil composition, and soil management (Rilling *et al*., 2019). In most cases, an initial selection of PGPB is carried out by *in vitro* tests since these are fast, simple, reproducible, and relatively inexpensive, but they are conducted in growing conditions very different from those found in the field (Figueiredo *et al*., 2017). Therefore, the expression of certain characters expressed under *in vitro* conditions could be lost or reduced when tested under *in vivo* conditions since the combination of environmental and physiological conditions determines, in most cases, a final phenotype. Therefore, in conjunction with *in vitro* testing, a rapid and reliable *in vivo* method is desirable to better assess the beneficial effects of bacterial isolates on plants.

In the present study, it was hypothesised that bacteria isolated from sugarcane niches could increase the survival of plantlets at the acclimatisation stage, being a valuable tool for the success of the process. Furthermore, these assays could be useful for *in vivo* testing of PGPB by allowing the selection of promising bacteria for further studies related to their potential growth-promoting effects. Therefore, the objective of this study was to isolate and characterise PGPB from different sugarcane habitats and analyse their potential use as a biological tool to improve micropropagated sugarcane plant survival during *ex vitro* acclimatisation. Those strains that improve plant survival in the acclimatisation stage will be selected for future comprehensive growth promotion studies.

## Materials and methods

### Sampling

The starting material for the bacterial isolates was collected in 2014 and 2016 in an experimental sugarcane field of the EEAOC, Tucumán, Argentina. Each year, five sugarcane plants of commercial cultivar LCP 85-384 were randomly collected and grouped to obtain a representative composite sample of bacterial isolates. Samples from the rhizosphere, rhizoplane, roots, and stems were processed separately in the laboratory for bacterial isolation, as described below.

### Bacterial isolation

*Isolation of rhizosphere bacteria*: samples were processed by homogenising 5 g of rhizosphere with 45 mL of a sterile physiological solution (NaCl 0.9%) by shaking for 30 min. Shaken homogenised samples were left to settle for 45 min, and diluted supernatants (1/100 and 1/1000) were plated on trypticase soy agar (TSA) plates and incubated at 28°C for 72 h.

*Isolation of rhizoplane bacteria:* Samples were processed by washing roots twice with a sterile physiological solution (NaCl 0.9%) supplemented with Tween 0.1%. After the second wash, dilutions of 1/100 and 1/1000 were plated on TSA plates and incubated at 28°C for 72 h.

*Root endophyte isolation:* After washing twice with 0.9% NaCl, sugarcane roots were weighed and disinfected superficially with ethanol 70%, 30 s; sodium hypochlorite 5%, 1 min; and three washes with sterile distilled water. Roots (1 g) were ground with a pestle in a mortar containing 2 mL of a sterile physiological solution (2:1 v/w). After homogenisation, 1 mL of the grounded material was used to make dilutions of 1/100 and 1/1000 that were plated on TSA plates and incubated at 28°C for 72 h.

*Stem endophyte isolation:* Pieces of 10 cm in length of sugarcane stalks from the middle section of sugarcane plants were superficially disinfected (ethanol 70%, 1 min; sodium hypochlorite 5%, 10 min; and three washes with sterile distilled water). Sterilized plant material was after that cut into disks of approximately 10 mm thickness, and disks were ground using a pestle and mortar in a sterile physiological solution (2 mL). Homogenized plant tissue (1 mL) was used to make dilutions of 1/100 and 1/1000, which were plated on TSA plates and incubated at 28°C for 72 h.

### Sequencing of the *16S rDNA* gene and phylogenetic analysis of isolates

To identify the bacterial isolates to genus level, colony PCR was performed using the commonly used universal primer 27F and 1492R (Lane, 1991), amplifying nearly the entire length of the gene 16S ribosomal DNA (Frank *et al*., 2008). The amplification conditions used were described previously (Gallo *et al*., 2012;



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Milanesi *et al*., 2015). The amplicons were purified using PCR clean up kits (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's instructions, while BMR Genomics S.r.l performed the sequencing (Italy, http://www.bmr-genomics.it/). The quality of the obtained chromatograms was checked through Geospiza's FinchTV software (Perkin Elmer Inc., Waltham, USA; www.geospiza.com/Products/finchtv.html), and isolates were identified by partial *16S rDNA* sequencing and alignment by BLAST against the Genbank of the National Centre for Biotechnology Information (NCBI) database. A phylogenetic analysis was performed by aligning the *16S rDNA* gene partial sequences to the nearest BLAST matches and to other reference sequences of each identified genus, using the Muscle alignment implemented in the MegaX software (Kumar *et al*., 2018).

Phylogenetic trees were built using the maximum likelihood method, employing RaxML (maximum randomised axillary probability) implemented in the open-source CIPRES Science Gateway (https://www.phylo.org/). One-thousand bootstraps resampling per tree were generated, and values above 50% are shown at the branches. Trees were visualised and edited using the open-source iTOL (Interactive tree of life, https://itol.embl.de/).

## *In vitro* screening for potential plant growth-promoting activities

Twenty-four isolates were evaluated for the following plant growth-promoting (PGP) traits: inorganic and organic phosphate solubilisation, potential nitrogen fixation (measured as growth on nitrogen-free medium), siderophore, and indol acetic acid (IAA) production. Each test was performed in triplicate.

### *Inorganic and organic phosphate solubilisation*

Isolates were cultivated on broth media tryptic soy broth (TSB) for 24 h at 28°C. Ten µL of the bacterial cultures (containing approximately $10^6$ colony forming units (CFUs) $mL^{-1}$) were spotted on National Botanical Research Institute's phosphate growth agar medium (NBRIP), as described by Nautiyal (1999), with one source of insoluble inorganic (calcium, aluminium or iron) or organic (phytate) phosphate for each assay. After 4 days of incubation at 28°C, plates were checked for bacterial growth and for developing a solubilisation halo around the colonies. As many bacterial isolates could solubilise different phosphate sources without forming a clear halo around the colony, bromophenol blue (0.075 g $L^{-1}$) was added to the culture medium to enhance its visibility. *Pseudomonas* spp. and *Kokuria rhizophila* were used as positive and negative controls, respectively.

### *Growth on nitrogen-free medium*

Bacterial isolates were plated in Jensen (1942) medium (without a nitrogen source) and incubated at 28°C for 7 days. Previously, the bacterial suspensions were washed with saline solution to remove nitrogen from the culture medium. Bacterial growth in the medium was considered putative evidence for the capacity for atmospheric nitrogen fixation. *Rhizobium spp.* and *K. rhizophila* were used as positive and negative controls, respectively.

### *Siderophore synthesis*

Siderophore synthesis was determined using the universal chrome azurol sulfonate (CAS) assay described by Tortora *et al*. (2011). In short, 5 µL of a bacterial suspension was spotted on the surface of the CAS-blue agar medium and incubated at 28°C for 48 h. As a result, a change of colour from blue to orange was observed around siderophore-producing colonies. *Pseudomonas* spp. was used as a positive control.

### *Indole-3-acetic acid production*

Indole-3-acetic production was quantified by the colorimetric method described by Glickmann and Dessaux (1995). Bacterial cultures grown in TSB L-tryptophan supplement (Sezonov *et al*., 2007) were centrifuged at 3000 *g* for 10 min, and 100 µL of the supernatant was mixed with 100 µL of Salkowski reagent (12.5 g of $FeCl_3$ in $H_2SO_4$ 7.5 M) and incubated in the dark for 30 min at room temperature. Auxin production was verified by a supernatant colour change (yellow to pink) due to the reaction of the Salkowsky reagent with the IAA. A known auxin strain producer from the genus *Pseudomonas* was used as a positive control. A culture of *K. rhizophila* which does not produce auxin, was used as a negative control.

## Bacterial growth under abiotic stress conditions

Tolerance of isolates to high salinity and dehydration was evaluated by measuring bacterial growth on TBS agar medium plates supplemented with NaCl (5 or 7.5%) and 5% polyethylene glycol (PEG 6000) respectively (Alibrandi *et al*., 2018). Isolates were plated and incubated at 28°C. To determine the tolerance of individual isolates, growth under abiotic stress conditions was compared with growth on TSB medium agar without any supplementation. Bacterial growth was monitored after three, five, and seven days of incubation and expressed according to the following criteria: (+) tolerant, same or better growth than in non-stress conditions; (+/–) moderately tolerant, no single colonies or single smaller than on control plates; (–) susceptible, no visible growth.

## Antimicrobial activity

*In vitro* inhibitory activity against *Escherichia coli* (Gram-negative) and *K. rhizophila* (Gram-positive) of the selected isolates was evaluated. For each isolate, 5 µL of a cell suspension ($10^6$ CFUs $mL^{-1}$) were spotted on the surface of LB medium agar plates and incubated for 24 h at 28°C. After incubation, an overlay of LB soft agar containing *E. coli* DH5α™ (Invitrogen) or *K. rhizophila* ATTC 10,240 was applied. After 16 h of incubation at 37°C, antibiotic production was evidenced as growth inhibition halos in the surrounding zone of the bacterial isolate patches.

## Inoculation of micropropagated sugarcane seedlings

Micropropagated sugarcane seedlings of the TUC 03-12 variety were obtained by *in vitro* cultivation of meristems, according to the EEAOC's high-quality seed cane production programme (Noguera *et al*., 2015). After the *in vitro* multiplication and rooting process, seedlings were transferred to *ex vitro* conditions in a nursery for acclimatisation. The acclimatisation procedure included the following steps: First, individualised seedlings were placed in trays with a fungicide solution (Captan 2%) and kept at room temperature for 16 h. Then, seedlings were classified into four categories according to size (<3 cm; 3-5 cm; 5-7 cm, and >7 cm) and planted in trays with a disinfected commercial substrate based on peat and perlite. After planting, trays were kept in a specially conditioned greenhouse with high humidity (RH=80-100%) and low light to avoid dehydration of the plants. After two weeks of acclimatisation, the irradiance was increased, and the humidity gradually decreased (Díaz Romero *et al*., 2005).

For the inoculation of seedlings, three bacterial strains, 182-*Bacillus* (GenBank accession number MT581442), 221-*Paenibacillus* (GenBank accession number MT581441), and 336-*Pseudomonas* (GenBank accession number MT581443), were selected based on their biochemical marker profile, as long as they belong to harmless genera for humans. To carry out inoculation,

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individual bacterial colonies were placed in LB liquid medium and cultured at 28°C with shaking for 24 h; subsequently, cells were harvested by centrifugation at 2100 g for 10 min at 4°C. Finally, the supernatant was discarded, and the cell pellet was re-suspended in sterile distilled water and diluted to an $OD_{600}$ of 0.1 (corresponding to approximately $10^8$ CFU $mL^{-1}$). This bacterial suspension was used to immerse the roots of micropropagated seedlings of the sugarcane variety TUC 03-12, selected for its relatively high mortality rate during *ex vitro* acclimation. Plant roots were immersed in the suspension for 30 min prior to transfer to the substrate, and seedlings immersed in sterile distilled water were included as a control.

### Measurement of the survival rate in acclimatisation of micropropagated sugarcane seedlings

The trial was carried out with a completely randomised experimental design using three trays with 25 seedlings for each treatment. All experiments (bacterial treatment) were repeated three times, using different batches of micropropagated plants and bacterial growth suspensions. After treatment with bacteria or water, seedlings were transferred to a nursery for acclimatisation. Plant survival (rate of surviving plants) was evaluated every three days for 30 days.

**Table 1. Identification of potential plant growth-promoting bacteria isolated from sugarcane, based on *16S rDNA* partial sequences analysis.**

| Sample name (accession number) | *16S rDNA* sequence length (nts) | Best BLAST hit(s) (accession number) | Sequence similarity (%) | E-value |
|---|---|---|---|---|
| 182 (MT581442) | 813 | *Bacillus megaterium* strain IAM 13418 (NR_043401.1) | 100.00 | 0.0 |
| | | *Bacillus aryabhattai* B8W22 (NR_115953.1) | 100.00 | 0.0 |
| | | *Bacillus flexus* strain NBRC 15715 (NR_113800.1) | 100.00 | 0.0 |
| 188 (MN394157) | 862 | *Escherichia fergusonii* ATCC 35469 (NR_074902.1) | 99.54 | 0.0 |
| 189 (MN394158) | 801 | *Klebsiella variicola* strain F2R9 (NR_025635.1) | 99.88 | 0.0 |
| 210 (MN394159) | 865 | *Bacillus zhangzhouensis* strain MCCC 1A08372(NR_148786.1) | 99.88 | 0.0 |
| | | *Bacillus safensis* strain NBRC 100820 (NR_113945.1) | 99.88 | 0.0 |
| | | *Bacillus pumilus* strain NBRC 12092 (NR_112637.1) | 99.88 | 0.0 |
| 212 (MN394161) | 768 | *Paenibacillus pabuli* strain NBRC 13638 (NR_113627.1) | 99.48 | 0.0 |
| 221 (MT581441) | | *Paenibacillus taichungensis* strain BCRC 17757 (NR_044428.1) | 99.48 | 0.0 |
| | | *Paenibacillus xylanilyticus* strain XIL14 (NR_029109.1) | 99.48 | 0.0 |
| 215 (MN394160) | 816 | *Rhizobium tropici* CIAT 899 (NR_102511.1) | 100.00 | 0.0 |
| | | *Rhizobium freirei* PRF 81 (NR_116336.1) | 100.00 | 0.0 |
| | | *Rhizobium hainanense* strain I66 (NR_029195.1) | 100.00 | 0.0 |
| | | *Rhizobium multihospitium* strain CCBAU 83401 (NR_044053.1) | 100.00 | 0.0 |
| | | *Rhizobium miluonense* strain CCBAU 41251 (NR_044063.1) | 100.00 | 0.0 |
| 226 (ON081964) | 803 | *Bacillus gibsonii* strain DSM 8722 (NR_026143.1) | 99.75 | 0.0 |
| 243 (ON081965) | 940 | *Achromobacter mucicolens* strain R-46658 (NR_117613.1) | 99.68 | 0.0 |
| 258 (ON081966) | 857 | *Bacillus halotolerans* strain DSM 8802 (NR_115063.1) | 99.53 | 0.0 |
| 260 (ON081967) | | *Bacillus mojavensis* strain m15718 (NR_118290.1) | 99.53 | 0.0 |
| 263 (ON081968) | | *Bacillus nakamurai* strain NRRL B-41091 (NR_151897.1) | 99.53 | 0.0 |
| | | *Bacillus subtilis* subsp. *subtilis* strain (NR_102783.2) | 99.53 | 0.0 |
| | | *Bacillus tequilensis* strain 10b (NR_104919.1) | 99.53 | 0.0 |
| | | *Bacillus vallismortis* strain NBRC 101236 (NR_113994.1) | 99.53 | 0.0 |
| 274 (ON081969) | 862 | *Bacillus marisflavi* strain TF-11 (NR_118437.1) | 99.42 | 0.0 |
| 287 (ON081970) | 661 | *Bacillus albus* strain MCCC 1A02146 (NR_157729.1) | 100.00 | 0.0 |
| 301 (ON081971) | 921 | *Bacillus subtilis* subsp. subtilis strain 168 (NR_102783.2) | 99.67 | 0.0 |
| | | *Bacillus nakamurai* strain NRRL B-41091 (NR_151897.1) | 99.67 | 0.0 |
| | | *Bacillus vallismortis* strain NBRC 101236 (NR_113994.1) | 99.67 | 0.0 |
| | | *Bacillus tequilensis* strain 10b (NR_104919.1) | 99.67 | 0.0 |
| 311 (MN394162) | 897 | *Bacillus pseudomycoides* strain NBRC 101232 (NR_113991.1) | 100.00 | 0.0 |
| 315 (ON081972) | 913 | *Brevibacterium frigoritolerans* strain DSM 880 (NR_117474.1) | 99.78 | 0.0 |
| *317 (MN394163)* | *848* | *Acinetobacter soli* strain B1 (NR_044454.1) | 99.88 | 0.0 |
| 323 (MN394164) | 820 | *Pseudomonas batumici* strain UCM B-321 (NR_118125.1) | 99.63 | 0.0 |
| 325 (ON081973) | 776 | *Acinetobacter soli* strain B1 (NR_044454.1) | 100.00 | 0.0 |
| 329 (MN394165) | 833 | *Kosakonia radicincitans* DSM 16656 (NR_117704.1) | 98.46 | 0.0 |
| 333 (ON081974) | 872 | *Acinetobacter soli* strain B1 (NR_044454.1) | 99.77 | 0.0 |
| 334 (ON081975) | 907 | *Bacillus luciferensis* strain LMG 18422 (NR_025511.1) | 99.78 | 0.0 |
| 336 (MT581443) | 778 | *Pseudomonas koreensis* strain Ps 9-14 (NR_025228.1) | 99.74 | 0.0 |

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### Statistical analysis

Each seedling tray containing 25 plants was considered as one experimental unit. Plant mortality rate by tray was evaluated every three days throughout a month. Three trays were evaluated for each treatment factor (Control, 182-*Bacillus*, 221-*Paenibacillus,* and 336-*Pseudomonas*). This trial was repeated three times independently, using different batches of seedlings. Data are presented as mean±standard error. To analyse mortality rate progress, a generalised linear model (GLM) under binomial distribution and link logit was adjusted for the treatment factor and days (fixed factors) and their interaction.

Focusing on day 30, a GLM under binomial distribution and link logit was conducted with treatment factor as the fixed effect and the assay as the random effect. Additional analyses were conducted to evaluate differences in mortality rate among treatment factors within each assay. Multiple comparisons were performed with *a posteriori* method DGC (Di Rienzo *et al*., 2002); different letters indicate statistically significant differences (P<0.05). All analyses were conducted using Infostat v. 2020 (Di Rienzo *et al*., 2020).

## Results

### Bacterial isolation and identification

After 3 days of incubation at 28°C, 162 isolates were obtained from the rhizosphere, rhizoplane, roots, and stems of sugarcane. Based on colony morphology differences and growth patterns, 24 isolates were selected for further experiments and identified from a partial *16S rDNA* gene sequence. It was found that all sequences showed high homology (>98.5%) with bacterial sequences deposited in the NCBI database. According to BLAST alignments, the 24 isolates belonged to the genera: *Achromobacter, Acinetobacter, Bacillus, Brevibacterium, Escherichia, Klebsiella, Kosakonia, Paenibacillus, Pseudomonas,* and *Rhizobium* (Table 1). In most bacterial isolates, phylogenetic analysis confirmed the taxonomic affiliation at the genus level indicated by a high homology to a single species by the BLAST alignment, while some isolates could not be grouped unequivocally into a single species. A phylogenetic tree for all sequenced isolates is shown in Supplementary Figure 1. *Bacillus* was the only genus isolated from all the studied ecological niches. *Paenibacillus, Acinetobacter,* and *Pseudomonas* were isolated from rhizoplane and roots, while *Escherichia* and *Klebsiella* were found only in rhizosphere samples.

### Characterisation of bacteria as PGPB

*In vitro* tests were carried out to detect potential growth-promoting characteristics. In addition, the ability to increase nutrient availability by solubilising inorganic and organic phosphate, potential nitrogen fixation, and the production of siderophores and IAA were evaluated. The majority of the 24 selected bacterial isolates possessed more than one characteristic associated with PGP activity (Table 2).

Regarding the solubilisation of phosphates, 14 isolates exhibited the ability to solubilise at least one of the four phosphate tested sources, out of which three isolates (188-*Escherichia,* 329-*Kosakonia,* and 336-*Pseudomonas*) could solubilise phosphate from all the evaluated sources.

Every studied isolate could grow on Jensen medium without a nitrogen source, evidencing their putative capacity for atmospheric nitrogen fixation (Table 2). However, only four siderophore-producing strains were observed, while 11 isolates showed evidence of the production of IAA (Table 2).

### Salt and water stress tolerance assays and antimicrobial activity

Bacterial isolates showed variable tolerance to salt and water stress (Table 2). Concerning salinity tolerance, ten isolates were classified as susceptible at the lower salt concentration (NaCl 5%), six were found to be moderately tolerant, and eight were classified as tolerant. However, at the highest concentration, NaCl (7.5%), 20 isolates were classified as susceptible, four moderately tolerant, whereas no isolate was considered tolerant.

Evaluation of tolerance to dehydration by growing bacteria in media supplemented with PEG (5% final concentration) revealed ten susceptible isolates, six moderately tolerant, and eight tolerant.

The genera *Paenibacillus* isolated from rhizoplane (212) and roots (221) showed antibacterial activity against *E. coli* and *K. rhi-*

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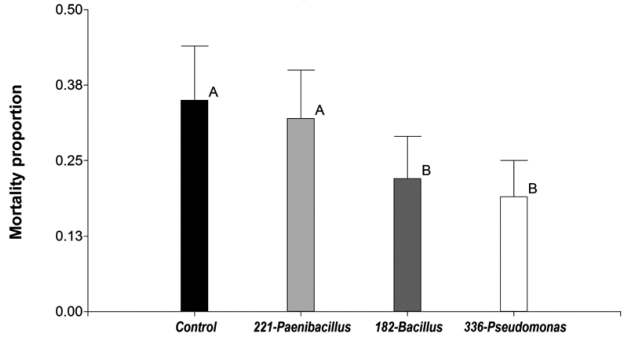


**Figure 1. Mortality of micropropagated sugarcane seedlings 30 days after treatments. The data show mean values ±SE of the mortality of seedlings 30 days after inoculation with 182-*Bacillus*, 221-*Paenibacillus*, 336-*Pseudomonas* or water (control). Different letters in the graph indicate statistically significant differences among treatments (P<0.05).**

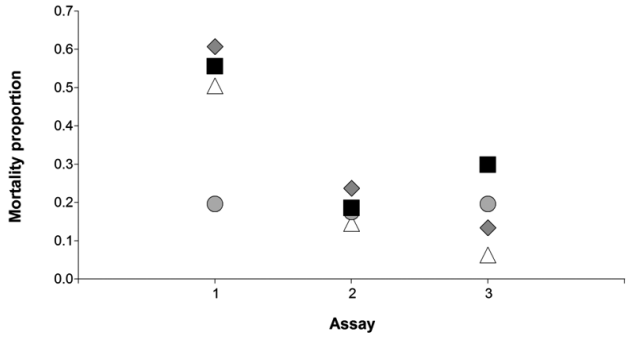


**Figure 2. Plant mortality 30 days after treatments in three independent experiments. Mortality proportion for sugarcane seedlings treated with water (control) (■), 182-*Bacillus* (▲), 221-*Paenibacillus* (◆), and 336-*Pseudomonas* (●) are shown in the graph. Data are presented as a proportion of dead seedlings 30 days after treatment in each experiment.**

*zophyla,* while strain 287-*Bacillus* isolated from rhizosphere inhibited growth of *K. rhizophyla* (Table 2).

## Effects of bacterial isolates on micropropagated sugarcane seedlings

From 24 isolates tested, three isolates were selected to study their potential ability to improve the survival of micropropagated sugarcane seedlings of the TUC 03-12 variety during the *ex vitro* acclimatisation stage. To aid future technological development, bacteria were chosen from different niches based on their biochemical properties, but isolates that showed high similarity to possible human pathogens were discarded (*i.e.*, *Acinetobacter*, *Achromobacter*, *Escherichia*, *Kosakonia*). By these parameters, the 336-*Pseudomonas* isolate from roots was chosen since it presented all the tested PGP characteristics, although it was susceptible to salt stress, tolerant to water stress, and did not present any antimicrobial activity. In addition, 221-*Paenibacilus* from the rhizoplane and 182-*Bacillus* from the rhizosphere were selected for further tests. When analysing the effect of each bacterium using a GLM, considering three independent trials and their triplicate jointly, a significant interaction (P=0.0053) between treatment and time was observed. Mortality rate progress through time was found to be significantly different in each bacterial treatment compared with the mortality rate of the seedlings treated with water (control) (Figure S2).

At the end of the experiments (day 30), significant differences were observed between treatments (P=0.0011). Two of the evaluated strains (182-*Bacillus* and 336-*Pseudomonas*) significantly improved the survival rate of seedlings in acclimatisation compared to control plants (Figure 1 and Table S1). When each assay was analysed independently on day 30 (Figure 2), seedling survival was found to vary significantly between the three experiments. This was confirmed by the statistical analysis showing a significant interaction between treatment and assay (P=0.0017). In the assay using the first batch of seedlings, the highest mortality rate was obtained for the control plants treated with water (0.56±0.06), indicating that they had a high-stress levels. Under these conditions, only the 336-*Pseudomonas* strain was able to improve significantly (P=0.0001) the survival of seedlings compared to the control plants, showing the lowest mortality proportion (0.21±0.05). Treatments with strains 182-*Bacillus* and 221-*Paenibacillus* showed a mortality of 0.51±0.06 and 0.61±0.06, respectively, with no significant differences compared to the control. In the assay using the second batch of seedlings, it was observed a low mortality rate (between 0.16 and 0.25) for all treatments (including control), responding adequately to the acclimatisation conditions showing no significant differences between them (P>0.05). Finally, using the third batch of seedlings, the three bacterial treatments showed statistically significant differences

**Table 2. Plant growth-promoting activities, stress-tolerance, and antimicrobial activity of bacteria isolated from sugarcane microhabitats.**

| Habitat | Isolate | Taxon group | PGP tests | | | | | | | Stress test§ | | | Antimicrobial activity^ | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Phosphate solubilisation* | | | | Putative diazotrophs° | Siderofore production* | IAA production# | NaCl (%) | | PEG (%) | | |
| | | | Ca-P | Fe-P | Al-P | Fy-P | | | | 5 | 7.5 | 5 | *Kokuria rhizophila* | *Escherichia coli* |
| **Rhizosphere** | **182** | ***Bacillus*** | - | - | - | - | + | - | + | - | - | + | - | - |
| Rhizosphere | 188 | *Escherichia* | + | + | + | + | + | + | + | - | - | +/- | - | - |
| Rhizosphere | 189 | *Klebsiella* | - | + | - | - | + | + | - | - | - | - | - | - |
| Rhizosphere | 287 | *Bacillus* | - | - | - | - | + | - | - | +/- | - | +/- | + | - |
| Rhizosphere | 301 | *Bacillus* | - | + | - | - | + | - | ND | - | - | +/- | - | - |
| Rhizosphere | 311 | *Bacillus* | - | - | - | - | + | - | - | + | - | - | - | - |
| Rhizosphere | 315 | *Brevibacterium* | - | - | - | - | + | - | - | + | - | - | - | - |
| Rhizoplane | 210 | *Bacillus* | - | - | - | - | + | - | - | +/- | +/- | + | - | - |
| Rhizoplane | 215 | *Rhizobium* | - | + | - | - | + | - | ND | +/- | - | - | - | - |
| **Rhizoplane** | **221** | ***Paenibacillus*** | - | + | - | - | + | - | + | +/- | +/- | + | + | + |
| Rhizoplane | 317 | *Acinetobacter* | + | + | + | - | + | - | + | + | - | - | - | - |
| Rhizoplane | 323 | *Pseudomonas* | - | - | - | - | + | + | - | +/- | - | - | - | - |
| Root | 212 | *Paenibacillus* | - | + | - | - | + | - | + | +/- | +/- | + | + | + |
| Root | 226 | *Bacillus* | - | - | - | - | ND | - | - | - | - | +/- | - | - |
| Root | 243 | *Achromobacter* | - | - | - | - | + | - | - | + | - | + | - | - |
| Root | 274 | *Bacillus* | - | - | - | - | + | - | - | + | - | + | - | - |
| Root | 325 | *Acinetobacter* | + | + | - | + | + | - | + | + | - | + | - | - |
| Root | 329 | *Kosakonia* | + | + | + | + | + | - | - | + | +/- | +/- | - | - |
| Root | 333 | *Acinetobacter* | + | + | - | - | + | - | + | + | - | + | - | - |
| Root | 334 | *Bacillus* | - | - | - | - | + | - | ND | - | - | - | - | - |
| **Root** | **336** | ***Pseudomonas*** | + | + | + | + | + | + | + | - | - | +/- | - | - |
| Stem | 258 | *Bacillus* | - | + | - | - | + | - | + | - | - | - | - | - |
| Stem | 260 | *Bacillus* | - | + | - | - | + | - | + | - | - | - | - | - |
| Stem | 263 | *Bacillus* | - | + | - | - | + | - | + | - | - | - | - | - |

PGP, plant-growth promoting; ND, not determined. *(+) growth and halo zone; (-) no growth, no halo zone; °(+) growth; (-) no growth; #IAA liquid assay: (+) auxin producers (colour change of the supernatant from yellow to pink); (-) no auxin producers; § + Tolerant (same or better growth than on control plates without NaCl/PEG); – Susceptible, (no growth); +/– moderately tolerant (reduced growth, no single colonies or single colonies smaller than on control plates without NaCl/PEG); ^+ Presence of inhibition halo; – Absence of inhibition halo.

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($P<0.001$) compared to control plants, improving the survival of transferred plants. In this last trial, the mortality rate of the control plants (0.31±0.05) was intermediate compared to trials 1 and 2, while the bacterial treatments showed a rate of 0.21±0.04 for 336-*Pseudomonas*, 0.15±0.04 for 221-*Paenibacillus* and only 0.08±0.03 for 182-*Bacillus*.

It must be highlighted that the treatment of the plants with the 336-*Pseudomonas* strain showed a low mortality rate in all three assays carried out (0.21, 0.19, and 0.21, respectively).

## Discussion

Micropropagated sugarcane plantlets suffering stress from nutritional deficiencies could be aided by PGPB capable of facilitating nutrient capture, thus increasing plant survival.

In the present work, 162 bacteria were isolated from the rhizosphere, rhizoplane, roots, and stems of sugarcane plants, and 24 isolates were identified by partial *16S rDNA* gene sequencing. In agreement with Lamizadeh *et al*. (2016), it was found that bacterial isolates belonging to the genus *Bacillus* were predominant (46%), considering all isolates obtained from the four ecological niches of sugarcane.

Growth-promoting biochemical properties allowed for an initial and rapid selection of strains with growth promotion characteristics in plants. Several isolates were capable of solubilising different phosphates sources, belonging to genera previously reported as phosphate solubilisers isolated from sugarcane (Lamizadeh *et al*., 2016; Bhardwaj *et al*., 2017; Kaur and Putatunda, 2018; Pirhadi *et al*., 2018; Patel *et al*., 2019). In the present study, the majority of the isolates strains were able to fix N, reflecting the importance of this macronutrient for the crop. On the other hand, only four isolates belonging to the genera *Escherichia, Klebsiella,* and *Pseudomonas* produced siderophores, suggesting lower importance of the absorption of iron (Fe) in sugarcane, although complementary studies are needed to draw further conclusions. Many bacteria isolated from sugarcane have been reported to produce IAA (Beneduzi *et al*., 2012), consistent with our study since out of the 24, 11 were identified as IAA-producing strains belonging to the genus *Acinetobacter*, *Bacillus*, *Escherichia*, *Paenibacillus* and *Pseudomonas*.

Even though bacterial growth under high salinity conditions and/or dehydration does not indicate the ability to provide improved tolerance to the plant, it is the first requirement for such an effect. As desiccation is a possible cause of seedling mortality rate at the *ex vitro* acclimatisation stage, the three selected bacterial strains (182-*Bacillus*, 221-*Paenibacillus,* and 336-*Pseudomonas*) were all tolerant or moderately tolerant to salinity/dehydration. However, further studies of the application of stress-tolerant bacteria to plants exposed to water scarcity/high salinity are necessary before a protective effect of a specific isolate can be concluded.

The acclimatisation stage in producing healthy sugarcane seedlings is critical since it determines the commercial viability of the whole process (Noguera *et al*., 2013). Therefore, the use of PGPB in micropropagation systems has been previously studied in several plants, and bacterial inoculation at the beginning of the acclimatisation has been suggested since it could be essential to favour the establishment of beneficial microbiota of the rhizosphere (Garcia *et al*., 2004; Russo *et al*., 2008).

Studies carried out in sugarcane regarding the use of PGPB in micropropagated seedlings have focused on the contribution of N-fixation caused by bacterial inoculation at the multiplication or root formation stage (Mirza *et al*., 2001; Oliveira *et al*., 2002). Oliveira *et al*. (2002) evaluated the effect of the inoculation of endophytic N-fixing bacteria in the micropropagation stage and observed an increase of around 30% of total nitrogen accumulated in seedlings inoculated with a mixture of strains. However, a strains mixture negative effect on the survival of seedlings was observed after 45 days from the inoculation during *ex vitro* acclimatisation.

To reduce negative impacts during acclimatisation, isolated bacteria with multiple PGP characteristics were inoculated at the beginning of this phase to study possible beneficial effects on seedling survival rates. Treatment with strains 182-*Bacillus* and 336-*Pseudomonas* improved the survival rate of seedlings 30 days after initiating the acclimatisation process. These results agree with studies by Jibu *et al*. (2010), who demonstrated that the inoculation of tea plants with *Pseudomonas sp.* during acclimatisation improved the survival of plants by 40% compared to control plants. Likewise, Zayed *et al*. (2017) demonstrated that inoculation of Stevia plants with endomycorrhizal spores and a strain of *Pseudomonas fluorescens* increased the survival rate by 60%, in addition to improving other growth parameters such as total leaf number and root and shoot length. Similarly, previous studies have shown that strains of the genus *Bacillus* can improve the health and survival of micropropagated banana plants (Jaizme-Vega *et al*., 2004).

The high variability of mortality rate observed among the three experiments for water-treated control plants reflects what usually happens during the acclimatisation process of TUC 03-12 seedlings, even though all possible precautions are considered in the laboratory and greenhouse. In this study, the first experiment showed the most stressful condition, as reflected by the lowest survival rate of control plants (44%). In contrast, the second assay produced a strong seedling establishment in soil for all treatments, as mortality rates were much lower (~20%), and no significant differences were observed between bacterial treatments and negative control. Finally, the third experiment showed moderate stress with an intermediate mortality rate of 0.31 for the water-treated control plant. Considering all three scenarios, it was concluded that at moderate stress, all bacterial treatments demonstrated a higher survival rate than water treatment.

Furthermore, plants inoculated with strain 336-*Pseudomonas* showed a good seedling establishment and survival in all three assays, providing a robust response and survival of plants during *ex vitro* acclimatisation. Since it was beyond the initial objective of this work, the presence of the inoculated bacteria on the plant has not yet been revealed. However, future research will be carried out to elucidate the colonisation and the action mode underlying the beneficial effect found in this study. In our laboratory, micropropagated sugarcane seedlings are produced to obtain healthy seedlings with high-quality plant material (pathogen-free and with genetic purity) under ISO 9001/2015 - 9000-7036 - IRAM certification (Perera *et al*., 2015; Díaz *et al*., 2020). However, seedlings of the TUC 03-12 variety have presented a low survival percentage in the *ex vitro* acclimatisation stage compared to other varieties. This causes a decrease in the availability of plant material for planting in seedbeds in the field, making rapid distribution difficult for farmers. In 2019, 19,070 *in vitro*-micropropagated seedlings of the TUC 03-12 variety (Díaz *et al*., 2020) were produced at the EEAOC, but only 9575 plants survived the initial *ex vitro* acclimatisation process (49.8% mortality). In the present work, a similar mortality rate was observed in the control plants (56%) for the first trial, while the inoculation treatment with the 336-*Pseudomonas* strain improved the survival rate of seedlings showing a total mortality rate of only 21%.

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Considering that the treatment with 336-*Pseudomonas* significantly improved survival rates in all three assays (20% mortality), it could greatly impact the number of successfully acclimatised seedlings and, therefore, in the production of seed cane of variety TUC 03-12 offered to growers. The improved survival rate achieved by inoculating micropropagated seedlings of this sugarcane variety with PGPB shows a strong potential economic impact, as it could produce an increase of up to 25 tons of seed cane in the first stage of propagation in the field (Basic seedbeds). Moreover, bacterial strains could also have a substantial beneficial impact during the adaptation and growth of seedlings in the propagation of seedbeds under field conditions. If they have such an impact, it would contribute to an increased seed cane yield and provide a more significant number of commercial hectares with healthy seedlings each year.

## Conclusions

Research on the use of PGPB is becoming increasingly important to achieve more sustainable agricultural production. In this work, inoculation of micropropagated sugarcane seedlings with 336-*Pseudomonas* strain at the beginning of the acclimatisation stage improved survival by 20%, allowing its adaptation to different stress conditions occurring during the most critical stage of the whole process. These results potentially significantly impact the production of sanitised and true-to-type seed cane of variety TUC 03-12 offered to growers. Future studies will be conducted on the effects of bacterial mix, detection of inoculated strains in plantlets, measurement of parameters of plant growth promotion, and adaptation of inoculated plants to field condition.

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