Plant Growth-Promoting Nitrogen-Fixing Enterobacteria Are in Association with Sugarcane Plants Growing in Guangxi, China

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The current nitrogen fertilization for sugarcane production in Guangxi, the major sugarcane-producing area in China, is very high. We aim to reduce nitrogen fertilization and improve sugarcane production in Guangxi with the help of indigenous sugarcane-associated nitrogen-fixing bacteria. We initially obtained 196 fast-growing bacterial isolates associated with the main sugarcane cultivar ROC22 plants in fields using a nitrogen-deficient minimal medium and screened out 43 nitrogen-fixing isolates. Analysis of 16S rRNA gene sequences revealed that 42 of the 43 nitrogen-fixing isolates were affiliated with the genera Enterobacter and Klebsiella. Most of the nitrogen-fixing enterobacteria possessed two other plant growth-promoting activities of IAA production, siderophore production and phosphate solubilization. Two Enterobacter spp. strains of NN145S and NN143E isolated from rhizosphere soil and surface-sterilized roots, respectively, of the same ROC22 plant were used to inoculate micropropagated sugarcane plantlets. Both strains increased the biomass and nitrogen content of the sugarcane seedlings grown with nitrogen fertilization equivalent to 180 kg urea ha⁻¹, the recommended nitrogen fertilization for ROC22 cane crops at the seedling stage.¹¹N isotope dilution assays demonstrated that biological nitrogen fixation contributed to plant growth promotion. These results suggested that indigenous nitrogen-fixing enterobacteria have the potential to fix N₂ associated with sugarcane plants grown in fields in Guangxi and to improve sugarcane production.

Key words: biological nitrogen fixation, enterobacteria, nifH, plant growth-promoting bacteria, sugarcane

Guangxi is the major sugarcane- and sugar-producing area in China and produces about 60% of China’s sugarcane and sugar. The present sugarcane mean yields are between 70 and 80 Mg ha⁻¹. The cost of sugarcane production in Guangxi is much higher than in Brazil. One of the major factors of the high cost is high N-fertilization. Over 60% of the sugarcane fields are applied with urea at over 600 kg ha⁻¹, but N-fertilizer mean applications are between 70 and 80 Mg ha⁻¹, which is very high. We aim to reduce nitrogen fertilization and improve sugarcane production in Guangxi with the help of indigenous sugarcane-associated nitrogen-fixing bacteria. We initially obtained 196 fast-growing bacterial isolates associated with the main sugarcane cultivar ROC22 plants in fields using a nitrogen-deficient minimal medium and screened out 43 nitrogen-fixing isolates. Analysis of 16S rRNA gene sequences revealed that 42 of the 43 nitrogen-fixing isolates were affiliated with the genera Enterobacter and Klebsiella. Most of the nitrogen-fixing enterobacteria possessed two other plant growth-promoting activities of IAA production, siderophore production and phosphate solubilization. Two Enterobacter spp. strains of NN145S and NN143E isolated from rhizosphere soil and surface-sterilized roots, respectively, of the same ROC22 plant were used to inoculate micropropagated sugarcane plantlets. Both strains increased the biomass and nitrogen content of the sugarcane seedlings grown with nitrogen fertilization equivalent to 180 kg urea ha⁻¹, the recommended nitrogen fertilization for ROC22 cane crops at the seedling stage.¹¹N isotope dilution assays demonstrated that biological nitrogen fixation contributed to plant growth promotion. These results suggested that indigenous nitrogen-fixing enterobacteria have the potential to fix N₂ associated with sugarcane plants grown in fields in Guangxi and to improve sugarcane production.

BNF may help farmers to maintain sugarcane yields under reduced N-fertilization and develop environmentally benign sugarcane production in Guangxi. At present, little is known about the diversity and predominant population of nitrogen-fixing bacteria associated with the sugarcane plants growing in Guangxi. Recently, some nitrogen-fixing bacteria have been isolated from sugarcane plants grown in Guangxi (26, 28, 44, 52, 53) using NFb, JNFb and LGI-P media that were respectively used to isolate Azospirillum (10), Herbaspirillum (6) and Gluconacetobacter diazotrophicus (8); however, nitrogen-fixing bacteria belonging to the genera Azospirillum, Herbaspirillum, and Gluconacetobacter, which are predominantly associated with sugarcane plants in Brazil, have not been isolated. The diversity of nitrogen-fixing bacteria associated with sugarcane plants grown with high N-fertilization in Guangxi may be different from in Brazil.

The ROC22 cultivar is the main sugarcane cultivar, growing in over 60% of sugarcane-planting areas in Guangxi. It is sensitive to low nitrogen stress and requires at least 150 kg ha⁻¹ urea fertilization at the seedling stage for tillering and elongation of the plant cane crops (23). The recommended dose of urea fertilization for plant cane crops at the seedling stage is 180 kg ha⁻¹, 30% of urea fertilization for a season (46). Recent studies have shown that nitrogen-fixing bacterial strains isolated from other sugarcane cultivars are able to provide nitrogen to micropropagated ROC22 sugarcane seedlings via BNF and promote sugarcane growth (26, 29); however, neither indigenous nitrogen-fixing bacteria associ-
ated with ROC22 sugarcane plants nor their associative BNF under recommended N-fertilization have been investigated.

Here, we attempted to isolate a large number of nitrogen-fixing bacteria associated with ROC22 sugarcane plants, investigate their diversity and predominant affiliation, and evaluate their potential for plant-growth promotion and associative BNF under the recommended N-fertilization. We initially obtained 196 fast-growing isolates from rhizosphere soil and roots of ROC22 sugarcane plants grown in 14 production areas. Nitrogen-fixing isolates were screened using the acetylene reduction assay (ARA) and PCR amplification of the \textit{nifH} gene encoding the iron protein of nitrogenase (55). We found that enterobacteria were predominant among the obtained nitrogen-fixing bacteria by analyzing their 16S rRNA gene (\textit{rrs}) sequences. We further screened their plant growth-promoting activities, including the production of indole acetic acids (IAA) and siderophores, phosphate solubilization and ACC (1-aminocyclopropane-1-carboxylic acid) deamination. Finally, we chose two \textit{Enterobacter} spp. strains isolated from the same ROC22 plant to inoculate micropropagated ROC22 sugarcane seedlings and investigate their plant growth-promoting and associative BNF activities under the recommended N-fertilization for ROC22 crops using the $^{15}$N isotope dilution technique.

### Materials and Methods

#### Bacterial isolation

Root samples were taken from ROC22 sugarcane plants grown for five to eight months in the fields in 14 production areas (Table 1). Root systems of six sugarcane plants in each production area were dug out; bulk soil loosely adhering to the roots was shaken

| Isolates   | Isolation site | Isolation source | Genus affiliation | IAA production | Siderophore production | Phosphate solubilization | ACC deaminase |
|------------|----------------|------------------|-------------------|----------------|------------------------|--------------------------|--------------|
| CZ150S     | Chongzuo       | soil             | \textit{Klebsiella} | +             | +                      | +                        | –            |
| CZ152S     | Chongzuo       | soil             | \textit{Burkholderia} | –             | +                      | +                        | –            |
| CZ186S     | Chongzuo       | soil             | \textit{Klebsiella} | +             | +                      | +                        | –            |
| GG164S     | Guigang        | soil             | \textit{Klebsiella} | +             | +                      | +                        | –            |
| HG148S     | Hengshian      | soil             | \textit{Enterobacter} | –             | +                      | +                        | –            |
| HG149S     | Hengshian      | soil             | \textit{Klebsiella} | +             | +                      | +                        | –            |
| LA16S      | Longan         | soil             | \textit{Klebsiella} | +             | +                      | +                        | –            |
| LC55S      | Liuxi          | soil             | \textit{Klebsiella} | +             | +                      | +                        | –            |
| NN145S     | Nanning        | soil             | \textit{Enterobacter} | –             | +                      | +                        | –            |
| GG132S     | Pingguo        | soil             | \textit{Enterobacter} | +             | +                      | +                        | –            |
| QQ25S      | Qinzhou        | soil             | \textit{Enterobacter} | –             | +                      | +                        | –            |
| QQ33S      | Qingzhou       | soil             | \textit{Klebsiella} | –             | +                      | +                        | –            |
| TD153S     | Tiandong       | soil             | \textit{Klebsiella} | +             | +                      | +                        | –            |
| YL34S      | Yulin          | soil             | \textit{Enterobacter} | –             | +                      | +                        | –            |
| YK115S     | Yangxi         | soil             | \textit{Klebsiella} | +             | +                      | +                        | –            |
| YK116S     | Yangxi         | soil             | \textit{Klebsiella} | +             | +                      | +                        | –            |
| YK117S     | Yangxi         | soil             | \textit{Klebsiella} | +             | +                      | +                        | –            |
| YX118S     | Yangxi         | soil             | \textit{Klebsiella} | +             | +                      | +                        | –            |
| DX120E     | Daxin          | root             | \textit{Klebsiella} | +             | +                      | +                        | –            |
| DX194E     | Daxin          | root             | \textit{Klebsiella} | +             | –                      | +                        | –            |
| GG151E     | Guigang        | root             | \textit{Klebsiella} | –             | +                      | +                        | –            |
| GG152E     | Guigang        | root             | \textit{Klebsiella} | –             | +                      | +                        | –            |
| GG153E     | Guigang        | root             | \textit{Enterobacter} | +             | +                      | +                        | –            |
| GG154E     | Guigang        | root             | \textit{Klebsiella} | –             | +                      | +                        | –            |
| GG155E     | Guigang        | root             | \textit{Klebsiella} | –             | +                      | +                        | –            |
| GG156E     | Guigang        | root             | \textit{Klebsiella} | –             | +                      | +                        | –            |
| GG157E     | Guigang        | root             | \textit{Klebsiella} | –             | +                      | +                        | –            |
| HH120E     | Huxian         | root             | \textit{Klebsiella} | –             | +                      | +                        | –            |
| LA11E      | Longan         | root             | \textit{Klebsiella} | +             | +                      | +                        | –            |
| LA14E      | Longan         | root             | \textit{Enterobacter} | –             | +                      | +                        | –            |
| NN143E     | Nanning        | root             | \textit{Enterobacter} | –             | +                      | +                        | –            |
| NN144E     | Nanning        | root             | \textit{Enterobacter} | –             | +                      | +                        | –            |
| NN208E     | Nanning        | root             | \textit{Enterobacter} | –             | +                      | +                        | –            |
| PNG12E     | Pingguo        | root             | \textit{Klebsiella} | +             | +                      | +                        | –            |
| QQ20E      | Qinzhou        | root             | \textit{Enterobacter} | +             | –                      | +                        | –            |
| SS107E     | Shangsi        | root             | \textit{Klebsiella} | +             | +                      | +                        | –            |
| SS82E      | Shangsi        | root             | \textit{Klebsiella} | –             | +                      | +                        | –            |

“+” presents positive, “−” presents negative
off; rhizosphere soil tightly adhering to the roots was suspended in autoclaved distilled water; roots were washed in sequence once by autoclaved distilled water, 70% (v/v) ethanol for 30 s, 0.1% (v/v) HgCl₂, for 1 min and 70% (v/v) ethanol for 30 s, five times by autoclaved distilled water, and ground with autoclaved quartz sand and phosphate-buffered saline (40) with a mortar and pestle. Soil suspensions and root homogenates were 10-fold serially diluted using autoclaved distilled water. One hundred microliters of each suspension were spread on modified nitrogen-deficient Ashby’s agar medium (per liter contains 10 g sucrose, 0.2 g NaCl, 0.2 g K₂HPO₄, 0.2 g MgSO₄·7H₂O, 0.1 g CaSO₄·2H₂O, 5 g CaCO₃, 15 g agar, pH 7.0) (3). After incubation at 30°C for 3–5 d, colonies with distinguished morphology were purified three times by streaking on Ashby’s agar. Purified isolates were maintained on Luria-Bertani (LB) agar (40). Their liquid LB cultures were stored with 15% (v/v) glycerol at −70°C.

**ARA**

One milliliter of each isolate grown overnight in liquid LB medium was harvested, washed twice, and suspended in 10 mL of liquid Ashby’s medium in a 60-mL Erlenmeyer flask. After static incubation at 28°C for 24 h, the flask was sealed with a rubber stopper and then 5 mL (10%) gas volume in the flask was replaced with acetylene. After incubation for another 24 h, ethylene was detected with a Shimadzu GC-9A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a column filled with GDX-502 (Boru Jianhie Chromatography Technology, Tianjin, China) (53). To ARA-negative in initial ARA screening but nifH-positive isolates of LA165S, DX194E and LC89E, each isolate was suspended in modified liquid Ashby’s medium supplemented with 0.05% (w/v) yeast extract in flasks at 28°C; ARA was performed every 2 h on bacterial cultures during a 24 h growth period.

**Colony PCR**

A colony approximately 1 mm in diameter grown on LB agar was picked up with an autoclaved 10-μL pipette tip and transferred to 10 μL sterilized Millipore water in a PCR tube. The bacterial suspension was heated in a P7021TP-6 microwave oven (Galanz, Foshan, China) at full power for 3 min. After centrifugation, 1 μL of the bacterial lysate was used as the template for PCR. The G. diazotrophicus strain PAL5 isolated from sugarcane plants (13) and the Escherichia coli strain DH5α were respectively used as positive and negative controls for nifH amplification. The full-length partial nifH sequences were performed with the degenerate Z-primers (55). Amplification of near full-length nifH sequences was performed with universal 27F/1492R primers (22). The primers were synthesized by Sangon (Sangon Biotech, Shanghai, China) and Takara (Takara Biotechnology, Dalian, China). The primers synthesized by Sangon were purified by ULTRAPAGE and determined by mass spectrometry. The primers synthesized by Takara were purified by HPLC. The Z-primers were used at 2 μM. The 27F/1492R primers were used at 0.25 μM. Ready-to-use 2× concentrated PCR masters (0.1 μL−1 Tag DNA polymerase, 0.2 mM dNTP, 3 mM MgCl₂, 2×PCR buffer) produced by Sangon and Tiangen (Tiangen Biotech, Beijing, China) were used for reactions. PCR amplification was performed in a PTC-200 DNA Engine thermal cycler and an S1000 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). A touchdown PCR strategy was used for nifH amplification to improve amplification specificity. In the 20 touchdown cycles, the annealing temperature was decreased by 0.5°C every cycle from 67 to 57°C. Fifteen additional cycles were performed at the annealing temperature of 57°C. PCR products were electrophoresed on agarose gels, stained with ethidium bromide, visualized and recorded with a JS-680B Gel Documentation and Analysis System (Shanghai Peiqing Science and Technology, Shanghai, China), and compared with molecular weight markers (Takara).

**Cloning and sequencing of nifH and rrs fragments**

The expected amplicons were excised from agarose gels, purified by a TIANgel Midi Purification Kit (Tiangen), cloned into the pMD18-T vector (Takara) and screened as described by the manufacturers. Three to six clones containing inserts of the correct size for each amplicon were chosen for sequencing (Invitrogen, Carlsbad, CA, USA).

**Sequence analysis**

The sequence segments corresponding to Z-primers were removed from the amplified nifH sequences. The partial nifH sequences were translated using the MEQA 4.0 program (45). The deduced amino acid sequences were aligned using the MUSCLE program (11) implemented in the MEGA 4.0 program. The rrs sequences were screened with the Mallard program (4) and BLASTed (1). The rrn 5S, 16S, 23S and 28S rRNAs sequences were BLASTed (1). The rrn 5S, 16S, 23S and 28S rRNAs sequences were screened with the Mallard program (4) and BLASTed (1). The rrs sequences of enterobacteria were aligned with those from the type strains of the species belonged to the genera Enterobacter and Klebsiella in the List of Prokaryotic names with Standing in Nomenclature (http://www.bacterio.cict.fr/mi/ microbacterium.html) using the MUSCLE program. Phylogenetic analysis was performed using the PhyML 3.0 program based on the maximum-likelihood principle (16). The best-fit TIM1+G model of nucleotide substitution was estimated using the jModelTest program based on the Akaike information criterion (38). The branch support measurement was assessed by a nonparametric, Shimodaira-Hasegawa-like test implemented in the PhyML 3.0 program.

**Determination of IAA production, siderophore production, phosphate solubilization, and ACC deaminase activity**

IAA production from a 3-d culture in DF medium (36) supplemented with 0.02% (w/v) L-tryptophan was determined by a microplate colorimetric assay (41). Siderophore production was determined by the Chrome Azurol S plate assay (42). Mineral phosphate solubilization activity was determined using Pikovskaya’s agar medium containing 0.5% (w/v) Ca₃(PO₄)₂ (37). Bacterial utilization of ACC was screened using a colorimetric assay of ACC based on the ninhydrin reaction (24); bacterial ACC deaminase activity was measured as described by Penrose and Glick (2003) (36).

**Inoculation and acclimatization of micropropagated sugarcane plantlets**

Sugarcane micropropagated plantlets were developed from stem apical meristems of cultivar ROC22 (51). Two nitrogen-fixing Enterobacter spp. strains of NN145S and NN143E isolated from rhizosphere soil and surface-sterilized roots, respectively, of the same ROC22 plant were used to inoculate micropropagated plantlets. Both strains were also able to produce siderophores and solubilize Ca₃(PO₄)₂. Inoculation and acclimatization of micropropagated plantlets were performed as described by Lin et al. (26). Rooted plantlets were co-cultured with bacteria in liquid one-tenth MS medium (sucrose and salt mixture) (39). Initial density of the bacteria was approximately 2·10¹⁰ cells per milliliter medium. Plantlets without inoculation were prepared as the control. Seven days after inoculation, plantlets were transferred to autoclaved sands and acclimatized for 14 d (26).

**Counting of NN145S and NN143E cells colonizing sugarcane plantlets**

Seven days after inoculation, plantlets were briefly washed with autoclaved distilled water, dried with autoclaved tissue paper, weighed, and ground with autoclaved quartz sand and phosphate-buffered saline in a mortar and pestle. The homogenates were serially diluted and spread on Ashby’s agar. Bacterial colonies were counted after incubation at 30°C for 3 d.

**15N isotope dilution assay**

Sand and perlite were mixed 1:1 (v:v), autoclaved twice at 121°C for 2 h, and cooled for 1 d at room temperature after each autoclave. The soil mixtures were added to 10 mg (NH₄)₂SO₄ (10.11 atom %
of ground root and shoot materials were respectively analyzed for water to remove the attached soil mixtures. Roots and shoots were Fifty-five days after transplant, plant roots were washed by distilled at 26±2°C, and watered as needed with autoclaved distilled water. Results

The partial nifH, anfH (encoding the iron protein of Fe-only alternative nitrogenase; 20) and rrs sequences were deposited in GenBank under accession numbers HQ204222 to HQ204264, HQ204265 to HQ204276, and HQ204277 to HQ204319, respectively (Table S1).

PCR amplification and sequencing confirmation of partial nifH genes

PCR amplification of nifH (nifF PCR) based on Z-primers amplified approximately 360 bp fragments from all 40 ARA-positive isolates (Fig. S1A). Cloning and sequencing of the amplicons showed that all 40 ARA-positive isolates contained sequences that were the closest match to nifH (Table S1). In addition, sequences matching anfH encoding the iron protein of Fe-only alternative nitrogenase (20) were obtained from 12 isolates (Table S1). The conserved residues of C86, C98, R101, D126, D130, and C133 (Azotobacter vinelandii OP NiiH numbering; GenBank accession number AAA64709) for nitrogenase iron protein were present in all the obtained partial NifH and AnfH sequences (data not shown).

Application of Z-primer-based colony nifH PCR

In the initial screening of the 196 bacterial isolates, ARA did not detect nitrogenase activity from 156 isolates; however, ARA may miss true nitrogen-fixing bacteria whose nitroge-

nase activities would be induced under more favorable growth conditions or at more active growth stages. To verify this assumption, we used Z-primer-based colony nifH PCR for the 156 ARA-negative isolates. Positive amplification was obtained from three isolates of LA16S, DX194E, and LC89E (Fig. S1B). Subsequent cloning and sequencing confirmed that the three isolates contained nifH (Table S1). We further performed ARA on the three isolates grown in liquid Ashby’s medium supplemented with 0.05% (w/v) yeast extract. Bacterial growth was promoted by yeast extract and reached the exponential phase after 4 h. Weak acetylene reduction activity was detected from the cultures of three isolates grown for 8–16 h (data not shown); therefore, Z-primer-based colony nifH PCR is able to rapidly screen nifH-positive isolates from a large number of bacterial isolates and largely reduce the number of isolates for further ARA determination.

Analysis of rrs sequences

Approximately 1,500 bp of rrs sequences for all 43 nitrogen-fixing isolates were obtained by PCR amplification and confirmed by subsequent cloning and sequencing. Sequence BLASTing suggested that 28 isolates belonged to the genus Klebsiella, 14 isolates belonged to the genus Enterobacter, and isolate CZ152S belonged to the genus Burkholderia (Table 1 and S1). The rrs sequence of CZ152S showed the highest sequence similarity to those of B. cepacia strain LMG 12614 (99.8%) and B. cenocepacia strain J2315 (99.8%). The phylogenetic tree of the rrs sequences of the 28 Klebsiella spp. isolates, 14 Enterobacter isolates, and the type strains of the Klebsiella and Enterobacter species are shown in Fig. 1.

Inoculation effects on micropropagated sugarcane seedlings

Seven days after inoculation, approximately 6.5×10⁷ NN145S cells and 8.0×10⁷ NN143E cells per gram of the micropropagated sugarcane plantlets were counted, whereas no bacterial colonies were formed from uninoculated controls. After 14 d acclimatization without N-fertilization and 55 d growth with 8.82 mg N kg⁻¹ soil including ¹⁵N isotope, inoculated sugarcane seedlings showed higher dry weights and nitrogen contents than those of uninoculated controls (Fig. 2). Inoculation of strain NN145S increased the dry weights of roots, shoots, and whole seedlings at 22.7%, 30.6%, and 26.7%, respectively (Fig. 2A), and increased nitrogen contents of roots, shoots, and whole seedlings at 13.7%, 20.0%, and 17.9%, respectively (Fig. 2B); all the increases were statistically significant at the 95% confidence level (Fig. 2A and B). Inoculation of strain NN143E increased
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...and increased nitrogen contents of roots, shoots, and whole seedlings at 17.2%, 27.4%, and 24.0%, respectively (Fig. 2E); the increases were statistically significant at the 95% confidence level, except that the increase of the root nitrogen content was significant at the 90% confidence level (Fig. 2D and E). Moreover, the $^{15}$N isotope concentrations of the inoculated plants were significantly lower than those of the uninoculated controls (Fig. 2C and F) as a result of dilution by N<sub>2</sub> fixation.

Seedlings inoculated with NN145S received 7.3%, 5.0%, and 6.1% nitrogen in roots, shoots, and whole plants from N<sub>2</sub>, respectively; seedlings inoculated with NN143E received 8.3%, 5.3%, and 6.7% nitrogen in roots, shoot, and whole plants from N<sub>2</sub>, respectively. The dry weight (0.77 g plant<sup>−1</sup>), nitrogen content (10.79 mg plant<sup>−1</sup>), and %Ndfa (6.7) of sugarcane seedlings inoculated with NN143E were respectively higher than those (0.74 g plant<sup>−1</sup>, 10.26 mg plant<sup>−1</sup>, and 6.1) of seedlings inoculated with NN145S but were not statistically significant.

Discussion

This initial large-scale isolation of nitrogen-fixing bacteria from root samples of the main sugarcane cultivar ROC22 grown in Guangxi obtained a limited number of nitrogen-fixing isolates that showed limited diversity and predominantly belonged to the genera Klebsiella and Enterobacter.
Previous studies showed that high levels of N-fertilization reduced the bacterial cell number (12, 34) and genetic diversity (7) of *G. diazotrophicus* in sugarcane plants. The high levels of N-fertilization in the sugarcane fields in Guangxi may act as a selective factor to reduce the population and diversity of nitrogen-fixing bacteria.

Enterobacteria are seemingly predominant in the fast-growing cultivable nitrogen-fixing bacteria associated with ROC22 sugarcane plants growing in Guangxi. On the one hand, the selective nitrogen-deficient Ashby’s medium used in the isolation procedure may lead to the predominant isolation of enterobacteria. On the other hand, nitrogen-fixing enterobacteria have been isolated from sugarcane plants cultivated in Guangxi using NFb and JNFb media (28, 44, 53) and also isolated from sugarcane plants cultivated in other countries (15, 27, 31, 33, 48). Mehnaz *et al.* (31) found that enterobacteria were predominant among the nitrogen-fixing bacteria isolated from sugarcane plants in Pakistan. Taulé *et al.* (48) found that enterobacteria formed a major group among the endophytic nitrogen-fixing bacteria isolated from sugarcane plants in Uruguay. Magnani *et al.* (30) showed that enterobacteria, including three nitrogen-fixing isolates, comprised a major group of endophytic bacteria isolated from Brazilian sugarcane using potato agar medium. Moreover, the detection of *nifH* sequences in sugarcane plants grown in Japan without culture showed that *nifH* sequences homologous to those of enterobacteria were predominant (2). The frequent association between nitrogen-fixing enterobacteria and sugarcane crops may develop from the long use of organic manures in agriculture (21).

Recent studies have shown that nitrogen-fixing enterobacteria can fix N2 associated with sugarcane plants and promote sugarcane growth. Luo *et al.* (29) showed that a *Klebsiella* sp. strain fixed N2 and increased nitrogen content of the ROC22 seedlings under gnotobiotic conditions. Wu *et al.* (52) showed that a nitrogen-fixing *Pantoea* sp. strain promoted the growth of sugarcane plants grown in non-sterile sand. Govindarajan *et al.* (15) showed that a nitrogen-fixing *Klebsiella* sp. strain increased the biomass and nitrogen content of sugarcane plants grown in non-sterile soil. Mirza *et al.* (33) showed that a *Klebsiella* sp. isolate SC20 (identified on the basis of its *rrs* sequence [GenBank accession number AJ278447]) fixed N2 and increased the nitrogen content of sugarcane plants under gnotobiotic conditions. Here, we showed that two *Enterobacter* spp. strains provided nitrogen to sugarcane plants via BNF and promoted sugarcane growth under gnotobiotic conditions and relatively high N-fertilization (equivalent to 180 kg urea ha⁻¹) recommended for sugarcane plant crops at the seedling stage in Guangxi. To our knowledge, this is the first report that indigenous nitrogen-fixing *Enterobacter* spp. strains can fix N2 associated with the hosts of sugarcane plants and promote their growth.

Using the method of introducing endophytic bacteria into the rooted micropropagated sugarcane plantlets established by Reis *et al.* (39), both the rhizosphere soil isolate NN145S and the root isolate NN143E obtained from the same ROC22 sugarcane plant were able to heavily colonize the ROC22 seedlings at respective densities of 6.5×10⁷ and 8.0×10⁷ cells per gram fresh weight. It would be interesting to study their colonization patterns, interactions and coordinated BNF contributions after coinoculation into the host ROC22 plant.

ARA is a sensitive method to detect nitrogenase activity of microbial cultures and has been widely used to identify nitrogen-fixing isolates (17). It is also known that bacterial nitrogenase activities vary with the media, culture conditions, and growth stages; therefore, using ARA to screen diazotrophs from a large number of isolates may miss true diazotrophs in the silent state of N₂ fixation and is rather time-consuming. Here, we have shown that colony *nifH* PCR can circumvent the disadvantages of ARA and rapidly screen nitrogen-fixing bacteria from a large number of isolates. Firstly, *nifH* PCR amplification based on Z-primers is sensitive and specific for the identification of ARA-positive isolates belonging to broad taxonomic classes (9, 18, 47). Demba Diallo *et al.* (9) have revealed that Z-primers have the highest rate of match to *nifH* sequences in the databases.
among the widely used universal nifH primers. Moreover, Z-primer-based PCR can amplify the corresponding fragments of nifH, vnfH, and anfH (9, this study). Secondly, colony nifH PCR skipping DNA extraction is easy to perform, rapid, reproducible, and not related to bacterial growth stages. Commercially available reagents, such as the ready-to-use 2× concentrated PCR masters, can facilitate PCR manipulation. In a modern microbiology laboratory, a thermal cycler for PCR is usually more available than a gas chromatograph for ARA. Using colony nifH PCR for the initial screening can largely reduce the number of isolates for further ARA determination; however, great care should be taken regarding the contamination of primers and other PCR reagents by trace levels of nifH (14, 54).

In conclusion, colony nifH PCR followed by ARA determination of nifH-positive isolates enables highly efficient workflow to screen and identify diazotrophs. The initial large-scale isolation of nitrogen-fixing bacteria from ROC22 sugarcane root samples obtained predominantly nitrogen-fixing enterobacteria that possessed multiple plant growth-promoting activities of IAA production, siderophore production, or phosphate solubilization. The initial inoculation of two Enterobacter spp. strains showed the potential for nitrogen-fixing enterobacteria to provide nitrogen via BNF to sugarcane cultivars requiring a high level of N-fertilization and increase sugarcane production in fields with relative high N-fertilization in Guangxi.

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