Plant and Soil **237:** 47–54, 2001.

Isolation, partial characterization, and the effect of plant growth-promoting bacteria (PGPB) on micro-propagated sugarcane *in vitro*

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Received 4 December 2000. Accepted in revised form 29 July 2001

Key words: Enterobacter, nitrogen fixation, phytohormones, 16S rRNA

Abstract

We report the isolation of nitrogen fixing, phytohormone producing bacteria from sugarcane and their beneficial effects on the growth of micropropagated sugarcane plantlets. Detection of the nitrogen fixing bacteria by ARA-based MPN (acetylene reduction assay-based most probable number) method indicated the presence of up to 10^6 bacteria per gram dry weight of stem and 10^7 bacteria per gram dry weight of root of field-grown sugarcane. Two nitrogen fixing bacterial isolates were obtained from stem (SC11, SC20) and two from the roots (SR12, SR13) of field-grown plants. These isolates were identified as *Enterobacter* sp. strains on the basis of their morphological characteristics and biochemical tests. The isolate SC20 was further characterized by 16S rRNA sequence analysis, which showed high sequence similarity to the sequence of *Enterobacter cloacae* and *Klebsiella oxytoca*. All the isolates produced the phytohormone indoleacetic acid (IAA) in pure culture and this IAA production was enhanced in growth medium containing tryptophan. The bacterial isolates were used to inoculate micro-propagated sugarcane *in vitro* where maximum increase in the root and shoot weight over control was observed in the plantlets inoculated with strain SC20. By using the ^{15}N isotope dilution technique, maximum nitrogen fixation contribution (28% of total plant nitrogen) was detected in plantlets inoculated with isolate SC20.

Introduction

Roots of the important crops like rice, wheat and sugarcane are frequently colonized by nitrogen fixing, plant growth-promoting bacteria (PGPB; Bashan and Holguin, 1998). Numerous nitrogen fixing bacteria of the genera *Acetobacter, Arthrobacter, Azoarcus, Azospirillum, Azotobacter, Bacillus, Enterobacter, Herbaspirillum, Klebsiella* and *Zoogloea* have been isolated from these crops and other graminaceous plants (Barraquio et al., 2000; Bilal and Malik, 1987; Boddey et al., 1995a; James and Olivares, 1997; James et al., 2000; Malik et al., 1997; Reinhold-Hurek et al., 1993). These bacteria are present in the rhizosphere and also in the interior regions of the plant roots and stem and usually cause no disease symptoms (James and Olivares, 1997). Diazotrophic bacteria have been linked with the high nitrogen fixation reported particularly in sugarcane where these bacteria were found in high numbers (Boddey et al., 1991, 1995a,b; Döbereiner et al., 1995a,b; James and Olivares, 1997). The beneficial effects of PGPB on plants have also been attributed to the production of phytohormones that promote root development and proliferation resulting in efficient uptake of water and nutrients (Haahtela et al., 1990; Tien et al., 1979).

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Among the phytohormones, production of auxins by a wide, diverse group of soil microbial isolates has been demonstrated (Arshad and Frankenberger, 1998). Microbial isolates from the rhizosphere of various crops appear to have a greater potential to synthesize and release auxins as secondary metabolites because of rich supply of substrates available in root exudates (Strezelczyk and Pokojska-Burdzeij, 1984). Production of auxins by microbial isolates varies greatly among different species and strains of the same species and is also influenced by culture condition, growth stage and availability of the substrate(s). IAA production by a number of *Acetobacter diazotrophicus* isolates from sugarcane was confirmed by Fuentes-Ramirez et al. (1993) who also suggested that, in addition to a role in nitrogen fixation, this bacterium could promote rooting and improve sugarcane growth by direct effects on metabolic processes.

In Brazil, continuous sugarcane cultivation, with low nitrogen inputs and almost total removal of plant biomass at each harvest, does not deplete soil nitrogen resources (Boddey, 1995b; Urguiaga et al., 1992). These observations suggest that sugarcane crop may obtain considerable nitrogen from biological nitrogen fixation. Association of nitrogen fixing bacteria with the roots of sugarcane was first demonstrated by Döbereiner and her co-workers (Döbereiner 1959, 1961; Döbereiner et al., 1972) and later confirmed by several workers by using acetylene reduction assay (Hegazi et al., 1979; Ruschel, 1981; Zafar et al., 1986). Diazotrophic bacteria such as *Azospirillum, Acetobacter, Beijerinckia* and *Herbaspirillum* have also been obtained in pure culture from sugarcane (Döbereiner et al., 1995a; Fuentes-Ramirez et al., 1993; Gillis et al., 1989, 1991; Rennie et al., 1983). A nitrogen balance study performed by Lima et al. (1987) provided direct evidence of a significant contribution of plant-associated nitrogen fixation to sugarcane. The results of other studies (Boddey et al., 1991; Urquiaga et al., 1992) also showed that some sugarcane varieties can obtain large contributions of plant-associated nitrogen fixation, ranging from 60 to 80% of total plant N, equivalent to over 200 KgN/ha/year. Additional beneficial effects to sugarcane due to phytohormone production by diazotrophic bacteria (*A. diazotrophicus*), have also been suggested (Sevilla et al., 1998)

This study aims at isolation and identification of diazotrophs from roots and aerial parts of sugarcane and partial characterization of the isolates by measuring nitrogen fixing ability and phytohormone production in pure culture as well as quantification of nitrogen fixation in inoculated sugarcane plantlets *in vitro*.

Materials and methods

Detection of nitrogen fixing activity in sugarcane

Nitrogen fixing activity in field-grown sugarcane (*Saccharum officinarum* L. Var. CP - 43/33) of Ayub Agricultural Research Institute (AARI), Faisalabad was detected by acetylene reduction assay (ARA). The roots and stem (internodes, 20 cm above soil) were washed thoroughly with sterilized water and stem samples were further divided into (i) epidermal and hypodermal layers of rind (ii) outer part of pith and (iii) central part of pith. Root and stem samples (4.0 g) were taken in triplicate and were collected from three different plants. ARA was measured by the method described by Zafar et al. (1986).

Enumeration of diazotrophic bacteria associated with sugarcane

The population of diazotrophic bacteria associated with stem and roots of field grown sugarcane was determined by ARA-based MPN method (Alexander, 1965). The roots and stem samples (1.0 g) were collected in triplicate from three different plants and washed with sterile water to remove adhering soil particles. The samples were finely ground with the pestle and mortar in 10 ml of sterile water. Serial dilutions (10×) of this suspension were prepared and from each dilution of the series, 100 μL suspension was inoculated in five replicate vials containing N-free semi solid Combined Carbon medium (CCM; Rennie, 1981). The vials were incubated at 30 °C for 48 h and ARA was measured by a gas chromatograph. The population of diazotrophs was determined using the probability table of Cochran (1950).

Isolation of diazotrophic bacteria from sugarcane

Serial dilutions of the bacterial growth in the semi solid medium in ARA positive vials (nitrogen-free CCM vials used for enumeration of diazotrophic bacteria) were spread on LB agar plates and incubated at 30 °C for 24–48 h. Different types of colonies appearing on plates were picked and streaked on fresh LB agar plates. All the different types of colonies were again inoculated in N-free semisolid media and

were assayed for confirmation of acetylene reduction activity. Finally, four isolates showing high ARA were selected for detailed studies.

Physiological and biochemical tests

Physiological and biochemical tests were performed using the QTS-20 miniaturized identification system (DESTO Laboratories, Karachi, Pakistan). These kits have been devised for identification of the members of family Enterbacteriaceae. Two additional tests, Oxidation-fermentation test (Hugh and Leifson, 1953) and catalase test (MacFaddin 1980) that are not included in QTS-20 were also performed for the identification of the isolates.

PCR-amplification and 16S rRNA sequence analysis

Cells of the isolate SC20 were grown in LB broth for 24 h at 30 °C. The cell pellets from 1.5 mL cultures were obtained by centrifugation at 9000 *g* for 5 min and washed with TE buffer (10 m*M* Tris.Cl; 1 m*M* EDTA, pH 8). The cell pellets were then suspended in 200 μL of TE buffer. Cells were lysed by placing in boiling water for 5 min in the presence of sodium dodecyl sulfate (1% SDS). The lysate was extracted twice with phenol/chloroform followed by two extractions with chloroform/isoamyl alcohol (24:1). After adding 0.1 volume of sodium acetate (3 m, pH 5.2) and 0.5 volume of isopropanol, the supernatant was kept at −20 °C for 30 min. The nucleic acids were then precipitated by centrifugation at 9000 *g* for 20 min and the resulting pellet was washed with 70% ethanol before drying under vacuum. The nucleic acid pellets were then dissolved in 100 μL TE buffer and used as template for PCR amplification of 16S rRNA gene. Each reaction mixture (50 μL) contained 0.5 μL Taq DNA polymerase (5U /μL; Gibco/BRL), 5 μL Taq buffer, 5 μL dNTPs (200 μM), 5 μL (100 ng /μL) of each primer (Primer FGPS4-281 bis: AGA GTT TGA TCC TGG CTC AG; Primer FGPS1509'-153: AAG GAG GTG ATC CAG CCG CA; Normand, 1995), 24.5 μL sterile water and 1 μL of template. After denaturation of the template at 94 °C for 4 min, 30 rounds of temperature cycling (94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min) were followed by incubation at 72 °C for 7 min. The PCR products were gel purified (NuSieve, 1.2%) by using QIAquick spin (QIAGEN) kits. Both strands of the DNA were sequenced on an automated sequencer (Perkin-Elmer ABI PRISM Model 373) by using amplification primers as well as internal primers (Normand, 1995). The sequence has been deposited in the EMBL databank (Accession no. AJ278447).

Identification of plant growth hormones by high-pressure liquid chromatography

The growth medium (agar-free CCM supplemented with 1.0 g/L NH_4NO_3) was inoculated with bacterial cultures and incubated at 30 °C on an orbital shaker (Lab-Line Instruments Inc. USA) at 80 rpm. The bacterial cells were harvested after 7 or 14 days of growth by centrifugation at 6000 *g* for 10 min and the cell-free supernatant was used for the detection of IAA. For extraction of IAA from the cell-free supernatant, the method described by Tien et al. (1979) was used. For identification and quantification of the IAA (Rasul et al., 1998), the samples were analysed on HPLC (Perkin-Elmer, USA; Series 200 LC pump; Turbochrom Navigator software).

Micro-propagation of sugarcane and quantification of nitrogen fixation by ^{15}N isotopic dilution technique

Sugarcane variety CP 43/33 (AARI, Faisalabad) was used for *in-vitro* culture studies. The explants were obtained from the apical portion of stem of the field grown sugarcane plants collected from Sugarcane Research Institute, AARI, Faisalabad. The outer layers of leaf sheath were peeled off until the inner most leaves with the thickness of 5 mm were exposed. The explant of 3–4 mm size was dissected by making cuts with sterilized tools under aseptic conditions. The sliced tissues were placed in magenta jars (Sigma) containing 25 ml modified MS medium (Murashige and Skoog, 1962). The cultures were incubated in the dark at 25 ± 2 °C and the induced calli were maintained on the same medium for callus induction by serial transfers. Then 5–6-week-old embryogenic calli were placed on the MS regeneration medium for differentiation and regeneration (Murashige and Skoog, 1962) at a photoperiod of 16 h. The regenerated plantlets were then transferred to the MS rooting media for root induction (Murashige and Skoog, 1962) for 3–4 weeks. The roots of the plantlets were washed with sterilized water under aseptic conditions to remove the rooting medium and then transferred to glass tubes (25 cm × 3.5 cm) containing sterilized vermiculite and Hoagland solution (half strength; Hoagland and Arnon, 1950) as nutrient source.

The micro-propagated plantlets of sugarcane at three-leaf growth stage were transferred to glass tubes

(25 cm × 3.5 cm) containing sterilized vermiculite and nitrogen-free Hoagland solution (half strength) as nutrient source. ^{15}N labelled ammonium sulfate (0.75 mg N /tube) of 10 atom% excess was added as a tracer to quantify nitrogen fixation. Three plantlets were transferred to each tube and inoculated after 2 days. The tubes were closed with cotton plugs to avoid contaminations, allowing the aerial parts to grow outside the tubes. The bacterial cultures for inoculation were grown in LB broth for 16 h at 30 °C. The cell pellets were obtained by centrifugation at 6000 *g* for 5 min, washed and resuspeded in sterile water. One mL of bacterial suspension ($10^8 - 10^9$ cells /mL) was added near the roots of each plantlet. Heat-killed cells were used to inoculate the plantlets used as non-fixing control. Another set of plantlets was kept as non-inoculated control. For each treatment nine plantlets (three tubes) were used. Plantlets were grown at 30 ± 2 °C day, 25 ± 2 °C night temperature and harvested after 8 weeks. For measuring root surface area of the plantlets, the root system was excised and washed to remove adhering vermiculite particles. Excessive water was removed with the blotting paper and root system was spread on a transparent plastic sheet and placed on a scanner attached with the computer. The Root Image Analysis Program, developed by Washington State University Research Foundation, Washington State University, USA) was used for the measurement of the root surface area. The plant material was dried to a constant weight in an oven at 70 °C for 72 h. The dried plant samples were ground to a fine powder and total N in these samples was determined by using a semi micro-Kjeldahl method based on wet combustion in a Rapid Kjeldahl System (Labconco, Kansas City, Missouri). The analysis for ^{15}N excess was carried out on a double inlet Mass Spectrometer (Varian MAT GD 150). Quantification of nitrogen fixation based on isotope dilution was calculated by the formula of Fried and Middleboe (1977), which is:

$$\% \text{ N fixed} = 1 - \frac{(^{15}\text{N atom\% excess})\ \text{fs}}{(^{15}\text{N atom\% excess})\ \text{nfs}} \times 100,$$

where fs is fixing system and nfs is non fixing system.

Statistical analyses

Results of the measurements were subjected to analysis of variance (ANOVA) and significance at the 5% level was tested by Duncan's Multiple Range Test (DMRT) by using a computer software programme. Mean values and the standard error were calculated.

Table 1. Nitrogen fixing activity (ARA) and population of diazotrophic bacteria in field grown sugarcane plants

Plant parts	Acetylene reduction activity (nmol C_2H_4 /g dry wt./ h)	Population of diazotrophs [a] (MPN /g dry wt.)
Root	122 (± 30)	$5.20 (\pm 0.87) \times 10^7$
Rind	40 (±10)	$3.44 (\pm 0.40) \times 10^3$
Outer pith	45 (±12)	$4.08 (\pm 0.51) \times 10^6$
Inner pith	18 (±6)	$2.37 (\pm 0.45) \times 10^3$

Values are the average of 3 replicates. The values in the parenthesis indicate standard error.
[a] ARA-based MPN counts.

The experimental design was Randomised Complete Block Design (RCBD).

Results and discussion

Acetylene reduction activity (ARA) was detected in both the roots as well as in the tillers of sugarcane (Table 1), indicating the presence of nitrogen-fixing bacteria in both the roots and the aerial parts of sugarcane. Among the aerial parts, acetylene reduction activity was highest in the outermost region of the pith just below the epidermal layer of rind. Relatively lower ARA values of washed roots of sugarcane were obtained as compared to those reported by Zafar et al. (1986) for another sugarcane variety. In the same study made by Zafar et al. (1986), reduction or complete inhibition of acetylene reduction by the washed roots as compared to unwashed roots was observed and it was concluded that nitrogen-fixing bacteria were loosely associated with the roots and thus easily removed during the washing steps. In the present study, in which washed plant materials were used, a much closer association of diazotrophs with plant tissues was indicated by the detection of 10^7 diazotrophic bacteria/ g dry weight of root and 10^6 diazotrophic bacteria/ g dry weight of stem (outer pith) tissues (Table 1). The presence of diazotrophic bacteria in the range of 10^3–10^6 in surface layers (rind) as well as in internal parts (pith) of stem was also observed. Colonization of the aerial parts of sugarcane by nitrogen fixing bacteria has been demonstrated by isolation of diazotrophs from the sugar solution in intercellular spaces of stem (Dong et al., 1995) and also by using light and electron microscopy coupled with immunogold labelling (James and Olivares, 1997).

Isolation of the N_2-fixing bacteria was made from roots and the outermost part of pith showing high ARA and population of diazotrophs. Four colonies

showing high ARA in semi-solid N-free media were selected for detailed studies and were labelled as SC11, SC20, SR12 and SR13. The isolates SR12 and SR13 were obtained from the roots and SC11 and SC20 were obtained from the stem. Isolation of diazotrophic bacteria from both the roots and the stem of sugarcane has been reported previously (Cavalcante and Döbereiner, 1988; Caballero–Mellado and Martinez-Romero, 1994; Dong et al., 1995).

All the isolates formed off-white round colonies on LB agar plates and the cells were Gram negative, short motile rods. The isolates were positive for the following tests: Acid and gas production from D-glucose, catalase, nitrate reduction, H_2S production, utilization of sodium malonate and sodium citrate, urea hydrolysis, Voges Proskauer test and acid production from sugars (glucose, sucrose, mannitol, maltose, rhamnose, sorbitol). All the isolates were negative for the following tests: oxidase, indole, lysine decarboxylase, arginine dihydrolase and acid production from inositol. The isolates SC11, SC20 and SR11 were negative for ONPG (o-nitrophenyl-ß-D-galactopyranoside) and ornithine decarboxylase tests and the isolate SR12 was positive for both these tests. Partial 16S rRNA gene sequence of one of the isolates (SC20) was also determined by PCR amplification and direct sequencing of the PCR product (Figure 1). The nucleotide sequence showed high similarity to those of *Klebsiella oxytoca* (Accession no. Y17661; 98.3% similarity) and *Enterobacter cloacae* (Accession no. AJ251469; 97.8% similarity). However, the isolate SC20 shared more morphological and physiological characters with *Enterobacter cloacae* (Grimont and Grimont, 1992; Holt et al., 1994) as compared to *Klebsiella oxytoca* (Grimont et al., 1992). Slightly lower sequence similarity observed between the sequence of SC20 and *Enterobacter cloacae* as compared to *Klebsiella oxytoca* may be due to the fact that a partial 16S rRNA sequence rather than a complete sequence was compared.

The production of plant growth hormones by bacteria and their beneficial effects on plant growth have been reported (Bashan and Holguin, 1997; Patten and Glick, 1996). Among the bacterial strains tested for indoleacetic acid production, only *Enterobacter* sp. SR13 produced significant amounts of IAA when tryptophan was not added to the growth medium as precursor of IAA biosynthesis (Table 2). In this tryptophan-free medium, *Enterobacter* strains SC11, SC20 and SR12 produced only traces of IAA. However, all strains produced significant amount of IAA

Table 2. Indoleacetic acid production by the bacterial isolates from sugarcane

Bacterial strain	IAA in tryptophan-free medium (µg/L)		IAA production in the presence of tryptophan (µg/L)	
	7 days	14 days	7 days	14 days
Enterobacter SC11	0.15 A	0.28 B	94.7 D	1177.5 B
Enterobacter SC20	0.10 A	11.3 B	688.0 A	750.0 C
Enterobacter SR12	0.15 A	0.50 B	201.5 C	2211.7 A
Enterobacter SR13	0.13 A	253.2 A	507.7 B	1183.2 B

Means followed by the same letter are not statistically different at 5% level. Values are average of 3 replicates.

Table 3. Effect of inoculations with plant growth-promoting bacteria on micro-propagated sugarcane *in vitro*

Bacterial strain	Shoot dry weight (g/plant)	Root dry weight (g/plant)	Root surface area (cm^2/plant)	Nitrogen fixed [a](%Ndfa)
Enterobacter SC11	0.79 A	0.25 AB	164.3 C	22.8 B
Enterobacter SC20	0.83 A	0.29 A	269.4 B	28.2 A
Enterobacter SR12	0.55 BC	0.19 B	499.8 A	17.9 B
Enterobacter SR13	0.75 AB	0.25 AB	547.4 A	20.6 B
[b]Control	0.50 C	0.19 B	490.6 A	–

Means followed by the same letter are not statistically different at 5% level. Values are average of 9 plantlets.
[a]Percent nitrogen derived from air.
[b]Plantlets inoculated with heat-killed cells.

in the presence of tryptophan in the medium, especially during later growth stages (i.e. after 14 days of growth). Among the isolates, SR12 produced the highest amount (2211 µg/L) of IAA in the presence of tryptophan after 2 weeks of growth. Production of similar amounts of IAA by *Azospirillum* strain ER2 has been reported by Rasul et al. (1998). Higher amounts of IAA were detected in the cell-free culture medium of all the strains tested after 7 days of growth as compared to 14 days of growth. Similar results i.e. increase in IAA production in the presence of tryptophan and with the age of the bacterial cultures have been reported (Gonzalez–Lopez et al., 1986; Rasul et al., 1998).

Application of bacterial inoculants as biofertilizer has been reported to result in improved growth and increased yield of cereal crops (Bashan and Holguin, 1997; Okon and Labandera–Gonzalez, 1994). In the present study, in which micro-propagated sugarcane plantlets were used, an increase in the biomass of all inoculated plantlets as compared to controls was observed (Table 3). The difference between the two types of control plantlets (non-inoculated control and the plantlets inoculated with heat-killed cells) in the growth parameters studied in the present study was

Fragment A: First nucleotide of the sequence corresponds to position 25 on *E.Coli* 16S rRNA.

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                                                                    70
1
CAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAACGGTAGCACAGAGAGCTTGCTCTCGGGT
                                                                   140
71
GACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGT
                                                                   210
141
AGCTAATACCGCATAACGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCATGCCATCAGATGTGCCCA
                                                                   280
211
GATGGGATTAGCTAGTAGGTGGGGTAAAGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGAC
                                                                   350
281
CAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGG
                                                                   420
351
GCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGG
                                                                   490
421
AGGAAGGCGTTAAGGTTAATAACCTTGGXGATTGACGTTACCCGCAGAAGAAGCACCGGCTAAACTCCGT
                                                                   560
491
GCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGC
                                                                   630
561
GGTCTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTTGAAACTGGCAGGCTTGAG
                                                                   700
631
TCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGC
                                                                   770
701
GAAGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCC
                                                                   826
771
TGGTAGTCCACGCTGTAAACGATGTCGACTTGGAGGXTGTTACCCTTGAGAAGTGG
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Fragment B: First nucleotide of the sequence corresponds to position 1012 on *E.Coli* 16S rRNA.

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1
                                                                    70
CAGAGATGCTTTGGTGCCTTCGGGAGCTCTGAGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGT
71
                                                                   140
GAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAACT
141
                                                                   210
CAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGT
211
                                                                   280
AGGGCTACACACGTGCTACAATGGCATATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAA
281
                                                                   350
AGTATGTCGTAGTCCGGATTGGGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATC
351
                                                                   420
AGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAA
421
                                                                   490
AAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACCACTTTGTGATTCATGACTGGGGTGAAGTCGTA
491        504
ACAAGGTAACCGTA
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Figure 1. 16S rRNA sequence of the nitrogen fixing bacterial isolate (SC20) from sugarcane.

not significant. Maximum increase in the biomass of the plantlets occurred with isolate SC20. The increase in the plant biomass was 55% in roots and 70% in shoots over the control (inoculated with heat-killed cells). Among the bacterial strains compared, maximum increase in root surface area was noted in the plantlets inoculated with strain SR13. Increase in the root length of the plantlets due to inoculation with the phytohormone producing bacteria has been reported (Bashan and Holguin, 1997; Haahtela et al., 1990; Tien et al., 1979). In the plantlets inoculated with the isolates SC20 and SC11, which gave the greatest increase in plant biomass (root and shoot weight), less root surface area was recorded due to the fact that the roots were healthier (thicker) and shorter than other treatments. This may be due to over production of phytohormones by the inoculated bacteria under the experimental conditions as higher concentrations of growth hormones are known to inhibit root elongation in some crops (Harari et al., 1988; Rasul et al., 1998; Tien et al., 1979; Zelena et al., 1988). Nitrogen fixation as measured by ^{15}N isotopic dilution

method showed up to 28% Ndfa (nitrogen derived from air) in the inoculated plantlets (Table 3). In Brazil, certain sugarcane varieties were found to obtain large contributions of nitrogen (up to 80% of plant N) from plant-associated N_2-fixation (Boddey et al., 1991, 1995a,b; Urquiaga et al., 1992).

Nitrogen fixing, phytohormone producing *Enterobacter* strains isolated in the present study showed beneficial effects on micro-propagated sugarcane plantlets and thus have potential for use in the commercial production of a bio-fertilizer for sugarcane.

Acknowledgements

Thanks are due to Dr. M. I. Sajjad, PINSTECH, Islamabad for his help in ^{15}N analysis. The technical help and guidence provided by Dr. Yusuf Zafar and Saif-ur-Rasheed, Plant Biotechnology Division, NIBGE to raise the sugarcane seedlings by micropropagation is thankfully acknowledged. The research work reported here was partially supported by ICGEB, Trieste, Italy under the project CRP/PAK 96-01.

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Section editor: T.C. Paulitz