Expl Agric. (2013), volume 49 (1), pp. 43–52 © *Cambridge University Press 2012*
doi:10.1017/S0014479712001019

PLANT GROWTH-PROMOTING BACTERIAL ENDOPHYTES FROM SUGARCANE AND THEIR POTENTIAL IN PROMOTING GROWTH OF THE HOST UNDER FIELD CONDITIONS

By HEMLATA CHAUHAN†‡, D. J. BAGYARAJ§ *and* ANITA SHARMA†

†*Department of Microbiology, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, Uttarakhand 263145, India and* §*Centre for Natural Biological Resources and Community Development, No. 41, RBI Colony, Anand Nagar, Bangalore 560024, India*

(*Accepted 13 October 2012; First published online 6 November 2012*)

SUMMARY

Ten endophytic bacteria were isolated from different sugarcane varieties growing in the Crop Research Centre, Pantnagar on nitrogen-free medium. Plant growth-promoting potential of the isolates was reported in terms of indole acetic acid (IAA) production, phosphorus solubilization, siderophore production and antagonistic action against the pathogen *Colletotrichum falcatum*, which causes red rot disease in sugarcane *in vitro*. All the isolates were able to produce IAA (4.8–9 μg ml^{-1}); three isolates (H3, H5 and H14) solubilized insoluble phosphorus on Pikovaskaya's agar; two isolates (H10 and H14) showed siderophore production on Chrome-azurol S (CAS) agar and antagonism against *C. falcatum* was exhibited by two isolates (H14 and H15) in a dual plate assay. 16 S rRNA sequencing identified isolates H3 and H12 as *Pseudomonas* spp., and H8, H14 and H15 as *Bacillus* spp. A field experiment on sugarcane was conducted with five plant growth-promoting bacterial endophytes *Pseudomonas* spp. (H3 and H12) and *Bacillus* spp. (H8, H14 and H15) along with standard strains of *Gluconacetobacter* and *Azospirillum* spp. Plant height, chlorophyll content, total nitrogen and cane length were significantly higher in almost all inoculated plants compared with the uninoculated control. An increase of 40% in cane yield over the control was obtained after inoculation with isolate H15 (*Bacillus* spp.). This was statistically on par with the standard endophyte *Gluconacetobacter diazotrophicus*, which resulted in 42% increased cane yield. Identification of new diazotrophs and their promising results towards improving plant growth in the field suggest their use as inoculants in future.

INTRODUCTION

Sugarcane is one of the world's major sugar crops, providing about 75% of the sugar for human consumption. India is the second largest producer of sugar in the world. The sugar industry greatly contributes (1% GDP) to the growth and prosperity of the rural agricultural economy in India. Sugarcane is a high nutrient-demanding crop. An estimated requirement of nitrogen (N) for sugarcane is about 150 Kg N ha^{-1} for sugar cane production. It is vital to replenish the soil with plant nutrients to increase crop productivity along with the maintenance of soil health. This can be done by use of bacterial supplements either partially or in an integrated way of nutrient management.

The potential of plant-associated bacteria in stimulation of plant growth with soil/plant health management has been described by various workers (Nihorimbere *et al.*, 2011). Different microorganisms have been utilized as biofertilizers. They exert

‡Corresponding author. Email: chauhan.hemlata@gmail.com

beneficial effects on plants directly or indirectly through transfer of biologically fixed nitrogen to the plant, production of phytohormones or other compounds and by enhancement of mineral uptake. Several nitrogen fixing species such as *Enterobacter cloacae*, *Bacillus polymyxa*, *Klebsiella pneumoniae*, *Azotobacter vinelandii*, *Azospirillum* spp., *Herbaspirillum seropedicae* and *Acetobacter diazotrophicus* have been isolated from internal or external parts of sugarcane plants (Baldani *et al.*, 1987; Cavalcante and Döbereiner, 1988; Olivares *et al.*, 1996). It was estimated that up to 70% of the plant nitrogen originated from biological nitrogen fixation for certain Brazilian sugarcane cultivars.

Identifying the diazotrophic bacteria responsible for nitrogen gain is important for agricultural application as well as for understanding ecosystem processes. The prospect of manipulating crop rhizosphere microbial populations by inoculation of beneficial bacteria to increase plant growth has shown considerable promise in laboratory and greenhouse studies; however, responses have been variable in the field (Bowen and Rovira, 1999). It is necessary to discover new plant growth-promoting rhizobacteria (PGPR) and explore their potential and interaction with other organisms and the host under field conditions.

Uttarakhand is a newly formed state of India, and sugarcane is one of the important economic crops of this developing state. Role of microbial inoculants in improving crop productivity is well documented (Souza *et al.*, 1994). In the present study, bacterial endophytes from sugarcane varieties were isolated, characterized and tested for plant growth-promotion activities. The potential of selected endophytes on the growth and yield of sugarcane under field conditions was investigated.

MATERIAL AND METHODS

Isolation

Endophytic bacteria were isolated from different sugarcane varieties growing at the Crop Research Centre, Pantnagar, North India. Root and stem samples of sugarcane were collected. For bacterial isolation, the outer layer of stem was removed, washed thoroughly with tap water, cut into thin sections of 2–3 cm, washed with sterile distilled water, surface sterilized with 75% alcohol for 5 min, washed three times with sterile distilled water, followed by 3% Chloramine T treatment for 3 min, washed again with sterile distilled water and then macerated in a sterilized pestle and mortar with sterile water containing 10% sucrose. Plant suspension, 100 μL, from each sample was poured into vials containing semisolid nitrogen-free LGIP medium (Cavalcante and Döbereiner, 1988) in triplicate and incubated at 30 $\pm$ 2 °C for 7–10 days. A similar protocol was also applied to root samples. The vials were observed for yellow orange coloured growth. Vials showing growth were restreaked on solid LGIP agar plates. Cultures were maintained on LGIP slants for routine use and in glycerol stocks for long-term preservation at –80 °C.

In vitro *plant growth-promoting properties*

Isolates were qualitatively tested for P-solubilizing activity on Pikovaskaya agar (Pikovaskaya, 1948). Quantitative estimation of indole acetic acid (IAA) production

was done colorimetrically by growing the cultures with or without tryptophan (100 μg ml^{-1}) for 48 h at 30 °C in triplicates. The IAA content was measured by the standard procedure (Gordon and Webber, 1951). Siderophore production by the isolates was qualitatively estimated by the Chrome-azurol S assay (Schwyn and Neilands, 1987) in Petriplates and the diameter of the clearing zone was measured. The red-rot fungus (*Colletotrichum falcatum)*, isolated from infected tissues of sugarcane plants, was identified using cultural and morphological characters. The efficiency of sugarcane endophytes to inhibit the growth of red-rot pathogen was checked using plate assay, growing bacterial culture and *C. falcatum* on potato dextrose agar (PDA) supplemented with yeast extract peptone mannitol agar (YPM; 1:1) at 30 °C. All *in vitro* assays had three replications.

Bacterial identification

Based on the laboratory studies of plant growth-promotion traits, five bacterial isolates (H3, H14, H8, H12 and H15) were selected for field experiment and sequenced (partial 16 S ribosomal RNA gene). This was performed at the National Centre for Cell Sciences (NCCS), Pune. The identities of the isolates were determined through a BLAST search. The Gene Bank accession numbers of the isolates are FJ357337, FJ357338, FJ357339, FJ357340 and FJ357342.

Field experiment

A field experiment employing endophytic isolates along with two standard organisms (*Gluconoacetobacter diazotrophicus* (MTCC1224) and *Azospirillum lipoferum* (MTCC2306)) was conducted on sugarcane variety Co-Pant 90223 during year 2007–2008 at the Agronomy Block, Crop Research Centre, Pantnagar in a randomized block design with three replications and eight treatments. Pantnagar is situated at 29°N latitude and 79.9°E longitude and at an altitude of 243.84 m above the mean sea level in foothills of Shivalik range of Himalayas in the Tarai region of Uttarakhand. The experimental area falls in humid subtropical zone and receives about 1500–1700-mm annual rainfall out of which 80–90% is received during the months of June to September. The minimum and maximum temperature usually ranges from 6.2–36.5 °C. The mean humidity varies from 42–90%. Cropping history of field includes 2003–2004, sugarcane; 2004–2005, Sesbania; 2005–2006, sugarcane and 2006–2007, sugarcane. Soil samples from 0–15-cm depth from the experimental plot were collected randomly before sowing and mixed together to form a composite sample. The soil was air-dried, processed by passing through 2-mm sieve and chemical analysis of soil was done using standard procedures (Jackson, 1973; Olsen *et al.*, 1954). Soil was clay loam in texture with pH 7.2, organic carbon 1.41%, total nitrogen 0.10%, available P_2O_5 17.1 kg ha^{-1} and available K_2O 280.6 kg ha^{-1}. The plot size was 18 m^2 for each treatment. Uniform doses of potassium 40 kg ha^{-1} and 60 kg ha^{-1} phosphorus were applied to all the plots at the time of planting in furrows. Only 50% (60 kg ha^{-1}) of the basal-recommended nitrogen (120 kg ha^{-1}) was applied at the time of planting. No further nitrogen was added during the whole experiment. Bacterial inocula were

prepared by growing the isolates in YPM broth to a cell density of 10^8 CFU ml^{-1}. Sugarcane setts (stem cuttings with three buds) were soaked in bacterial suspensions for 1 h. The treated setts (with three buds) were planted manually in five rows per plot with 24 setts in each row. Standard agronomical operations were followed till harvest. The crop was maintained in the field for about a year. Percentage germination was recorded at 30 and 45 days after planting (DAP). Shoot counts were calculated on hectare basis at 90, 120,150 and 180 days after planting. The height was measured at an interval of 30 days, starting from 90 days till 210 days after planting. Physiological parameters, such as chlorophyll *a*, chlorophyll *b*, total chlorophyll content and total nitrogen content in leaves and stems, were also recorded using standard procedures (Arnon 1949; Tondon, 1998). Cane length, cane girth, number of millable canes and cane yield were recorded at the time of harvest. Juice analysis was also performed after harvest. Data were analyzed statistically using analysis of variance (ANOVA) and treatment differences were tested by 'F' test of significance on the basis of null hypothesis (Cochran and Cox, 1959).

RESULTS

Endophytic bacteria were isolated from stems and roots of sugarcane after thorough surface sterilization. Ten endophytic bacterial isolates from different sugarcane varieties were recovered, purified and coded as H3, H5, H8, H9, H10, H12, H13, H14, H15 and H16. The variety and part of the sugarcane from which the isolates were obtained are as follows: Co-S-8436 root and stem (isolate H3 and H5 respectively), Co-1148 root (isolate H8), Co-Pant 842111 root (isolate H9) and stem (isolates H10 and H12), Co-Pant 97222 root (isolate H13), Co-Pant 84212 stem (isolate H14), Co-Pant 3220 root (isolate H15) and Co-Pant 1216 stem (isolate H16). Standard strains of the nitrogen-fixing bacteria *Gluconacetobacter diazotrophicus* MTCC 1224, *Azospirillum lipoferum* MTCC 2306 and *Azospirillum brasilense* MTCC 125 were obtained from the Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh, India.

All the isolates isolated from sugarcane and used in this study were found to produce IAA. The IAA production ranged from 4.8–9 μg ml^{-1}. On Pikovaskaya medium, formation of clear zones (2–5 mm) around bacterial colonies was observed in isolates H5, H13, H14 and H15. Production of siderophore was observed in H10 and H14. Isolates H14 and H15 inhibited the *in vitro* growth of pathogen *Colletotrichum falcatum* (Table 1).

The 16 S rRNA gene sequences of the isolates and BLAST search revealed that isolates H3 and H12 had 99% similarity to *Pseudomonas aeruginosa*, while isolates H8, H14 and H15 showed similarity to *Bacillus* spp. The field experiment conducted with five selected isolates brought out that percentage germination was significantly higher in all the inoculated treatments than the uninoculated control. Shoot counts per plot were higher for plants inoculated with sugarcane endophytes than the two standard organisms used. The highest number of shoots was observed in the *Bacillus* (H15) treatment. Interestingly, all the isolates enhanced plant growth significantly in terms of height compared with the uninoculated control (Table 2). Maximum plant height (382 cm) was observed in *G. diazotrophicus* followed by *Pseudomonas* H12 (358 cm) at harvest.

Table 1. *In vitro* plant growth-promoting properties of bacterial endophytes.

Isolate	IAA production (μg ml^{-1})	P solubilization zone (mm)	Siderophore production	Antifungal property against *Colletotrichum falcatum*
H3	4.8 ± 0.02	–	–	–
H5	9.18 ± 0.04	4	–	–
H8	7.07 ± 0.04	–	–	–
H9	9.64 ± 0.006	–	–	–
H10	8.61 ± 0.01	–	+	–
H12	6.9 ± 0.006	–	–	–
H13	7.9 ± 0.002	2	–	–
H14	6.17 ± 0.02	5	+	+
H15	5.3 ± 0.02	3	–	+
H16	7.1 ± 0.007	–	–	–

Note: In column 2 ± indicates standard error; in columns 3, 4 and 5 + indicates positive and – indicates negative.

Table 2. Effect of inoculation with bacterial endophytes on growth parameters of sugarcane in the field (2007–2008).

Treatments	% Germination (45 DAP)	Shoot count ha^{-1} (120 DAP)*	Plant height (cm) (150 DAP)	chlorophyll *a*, chlorophyll *b*,* total chlorophyll (mg g^{-1} fresh weight) (90 DAP)		
Uninoculated control	27^{c}	524	295.5^{d}	0.668^{d}	0.533	1.425^{d}
P. aeruginosa H3	35^{b,c}	565	350.2^{b}	1.584^{b}	0.821	2.533^{b}
Bacillus H14	37^{b}	528	347.1^{c}	1.32^{c}	0.725	2.305^{c}
Bacillus H8	35^{b,c}	546	338.3^{c}	1.28^{c}	0.724	2.694^{b}
Pseudomonas H12	35^{b,c}	563	358.8^{b}	1.26^{c}	0.784	2.172^{c}
Bacillus H15	42^{a,b}	633	346.0^{c}	1.56^{b}	0.753	2.104^{c}
G. d.	46^{a}	626	382.1^{a}	1.60^{b}	0.787	2.685^{b}
A. l.	40^{a,b}	550	356.6^{b}	2.13^{a}	0.947	3.091^{a}

Notes: *G. d.* = *Gluconacetobacter diazotrophicus*, *A. l.* = *Azospirillum lipoferum*.
DAP = Days after planting; *nonsignificant.
Values followed by the same superscript in each column do not differ significantly at $p < 0.05$.

Bacterial inoculation affected photosynthetic pigments in that chlorophyll *a* content was significantly higher in all the inoculated treatments compared with the control. The highest chlorophyll *a* level was found in plants inoculated with *A. lipoferum* (74 mg g^{-1} fresh wt) followed by the plants inoculated with *G. diazotrophicus*.

Chlorophyll *b* content was also significantly higher in inoculated plants compared with the uninoculated control. Maximum chlorophyll *b* was observed in the plants inoculated with *A. lipoferum* and *Bacillus* H14 (0.93 and 0.89 mg g^{-1} fresh wt respectively).

Total chlorophyll was also higher in all the inoculated treatments compared with the control. Total chlorophyll values obtained with plants inoculated with *A. lipoferum* and *G. diazotrophicus* were 3.1 and 2.7 mg g^{-1} fresh wt respectively. Nitrogen content in leaves was significantly higher in all the treatments with bacterial endophytes than the control and was highest in the *A. lipoferum* treatment. Nitrogen content of the stem was

https://doi.org/10.1017/S0014479712001019 Published online by Cambridge University Press

Table 3. Effect of inoculation with bacterial endophytes on sugarcane yield and other agronomic parameters at harvest (270 DAP) in a field experiment.

Treatments	Cane length (cm)*	Cane girth (cm)	Single cane weight (kg)	Available sugar (%)	NMC (ha^{-1})	Cane yield (t ha^{-1})	Leaf nitrogen (%)	Stem nitrogen (%)
Uninoculated control	174	8.55^{b}	0.80^{d}	12.22^{c}	36.6^{b}	27.59^{c}	0.477^{b}	0.136^{b}
P. aeruginosa H3	191	8.59^{b}	0.96^{c}	12.59^{b}	46.6^{a}	34.9^{a}	0.544^{b}	0.212^{b}
Bacillus H14	214	9.28^{a}	1.21^{a}	12.78^{a}	42.4^{b}	30.37^{bc}	0.576^{b}	0.197^{b}
Bacillus H8	219	9.50^{a}	0.95^{c}	12.61^{a}	39.2^{b}	31.10^{bc}	0.546^{b}	0.174^{b}
Pseudomonas H12	195	8.82^{b}	0.89^{c}	12.72^{a}	41.4^{b}	33.61^{b}	0.575^{b}	0.169^{b}
Bacillus H15	205	9.05^{a}	1.21^{a}	13.04^{a}	46.8^{a}	38.51^{a}	0.614^{b}	0.275^{a}
G. d.	207	9.48^{a}	1.27^{a}	12.59^{b}	49.4^{a}	39.20^{a}	0.574^{b}	0.275^{a}
A. l.	219	9.27^{a}	1.08^{b}	12.80^{a}	46.2^{a}	36.20^{a}	0.928^{a}	0.339^{a}

Notes: *G. d.* = *Gluconacetobacter diazotrophicu*, *A. l.* = *Azospirillum lipoferum*.
NMC = number of millable canes; *nonsignificant.
Values followed by the same superscript in each column do not differ significantly at $p < 0.05$.

also higher in all the inoculated treatments compared with the uninoculated control. In general, the nitrogen content of the leaf was higher than that of the stem.

Five representative samples from each plot were collected at the time of harvest (270 DAP) and cane length, girth, weight, green top (green leafy top part of sugarcane after harvesting cane, and used as fodder), sucrose content and yield were calculated on area basis. The cane length was significantly higher in all the inoculated treatments compared with the control and was highest in the *A. lipoferum* treatment (218 cm) followed by *Bacillus* H8-treated plants (214 cm) (Table 3). The cane girth was also higher in all the inoculated treatments than in the control. However, the difference was not significant in plants inoculated with *P. aeruginosa* isolates H3 and H12. Single cane weight was significantly higher in all the inoculated treatments compared with the control, except for plants inoculated with *P. aeruginosa* H12. Maximum cane weight was 1.27 kg in *G. diazotrophicus*-treated setts and 1.21 kg in *Bacillus* H15-treated plants. Percentage available sugar was higher in all the inoculated treatments compared with the control. However, a significant difference was observed only in plants inoculated with *Bacillus* H14 and H15, *P. aeruginosa* H12 and *A. lipoferum* having 12.8, 12.7, 13.0 and 12.9% available sugar respectively. Number of millable canes (NMC) was counted from each net plot at the time of harvest. NMC were higher in all the inoculated treatments compared with the control but a significant difference was observed only in *P. aeruginosa* H3, *Bacillus* H15, *G. diazotrophicus* and *A. lipoferum* treatments. Cane yield was higher in all the inoculated treatments compared with the control and was highest (39 t ha^{-1}) in the *G. diazotrophicus* treatment but did not differ significantly from the *Bacillus* H15 (38.5 t ha^{-1}) and *A. lipoferum* (36.2 t ha^{-1}) treatments (Table 3).

DISCUSSION

Presently great interest is being shown in the introduction and manipulation of indigenous soil and rhizosphere microflora in order to provide a consistent and

https://doi.org/10.1017/S0014479712001019 Published online by Cambridge University Press

effective increase in crop productivity. Bacteria on roots and in the rhizosphere are benefitted from root exudates, but some bacteria and fungi are capable of entering the plants as endophytes. Endophytes are the organisms (bacteria and fungi) inhabiting the interior of the plants and live most of their lifecycle inside the plant tissue without eliciting any pathogenic symptoms. Several endophytic bacteria were reported to promote growth of plants by different mechanisms (Baldani *et al.*, 1987; Döbereiner, 1992. In this study 10 bacterial endophytes were recovered from stem and roots of different sugarcane varieties commonly grown in North India after surface sterilization. Increase in crop yield does not depend only on fixed nitrogen, but other mechanisms could also play an important role in making the microorganism more potent to be used as PGPR. Endophytic bacteria can promote plant growth by secreting plant growth regulators (Lee *et al.*, 2004), phosphate solubilization (Wakelin *et al.*, 2004), enhancing hyphal growth and mycorrhizal colonization (Will and Sylvia, 1990) and by producing siderophores (iron chelating molecules which increase its availability to plants) (Costa and Loper, 1994). In addition, endophytic bacteria also supply essential vitamins/growth factors and resistance against plant pathogens (Bandara *et al.*, 2006). In the present study the IAA production *in vitro* was observed in all the isolates. P solubilization was observed in H5, H13, H14, H15 and standard *G. diazotrophicus* on Pikovaskaya agar. Mahesh Kumar *et al.* (1999) working with endophytes from sugarcane also reported P solubilization by *G. diazotrophicus.* Biocontrol is an indirect mechanism of plant growth promotion shown by rhizobacteria especially studied in *Pseudomonas* and *Bacillus*. In a dual plate assay, isolates H14 and H15 from sugarcane and standard *G. diazotrophicus* inhibited the pathogen *C. falcatum*.

16 S rRNA sequencing showed similarity of many of the isolates with either *Pseudomonas* or *Bacillus*. Isolates H3 and H12 showed 99% similarity to *Pseudomonas aeruginosa*, and isolates H8, H14 and H15 to *Bacillus* spp. Endophytic *Bacillus* spp. from maize, wheat and rye grass with nitrogen-fixing ability and plant growth-promoting activities have been reported by several authors (Melnick *et al.*, 2008; Sorokin *et al.*, 2008). Nitrogen-fixing *Pseudomonas* has also been isolated from the roots of sorghum (Krotkzy and Werner, 1987) and as rice endophytes in China (You and Zhou 1989; You *et al.*, 1991).

In field experiment inoculated sugarcane plants were significantly superior in terms of plant height and shoot counts. *Bacillus* spp. and *Pseudomonas* spp. have been reported to promote plant growth in grape wine, tomato, maize, rice and sugar beet through various mechanisms (Mehnaz, 2011; Mirza *et al.*, 2006; Wang *et al.*, 2009). In the present study, the number of shoots was higher in inoculated treatments than in the control. Positive and significant effects of inoculation of endophytes other than *Pseudomonas* spp. and *Bacillus* spp. on sugarcane have been reported by Sevilla *et al.* (1998).

The efficiency of nitrogen fertilization is related to the performance of photosynthetic apparatus of the plant. Nitrogen is one of the most important constituents of chlorophyll and thus chlorophyll content can be used as an indicator of nitrogen level in leaves. Low chlorophyll values indicate low nitrogen in leaf and *vice versa* (Rutge, 1991). Chlorophyll *a*, chlorophyll *b* and total chlorophyll contents were significantly higher in all the inoculated treatments compared with the control

at all the time intervals studied. Higher chlorophyll content in sugarcane treated with bacteria was also reported by Muthukumarasamy *et al.* (1999), Peng *et al.* (2002) and Sevilla *et al.* (1998).

The bacterial inoculations also increase the total plant nitrogen content (Muthukumarasamy *et al.*, 1999; Oliveira *et al.*, 2002). The nitrogen content was high in plants treated with bacteria compared with the uninoculated control at the time of harvest. Döbereiner (1992) reported that bacterial inoculation of micro-propagated sugarcane seedlings could increase plant growth and nitrogen fixation. Muthukumarasamy *et al.* (1999) reported that plants co-cultivated with endophytes accumulated more nitrogen in comparison to plants fertilized with commercial nitrogen fertilizers under field conditions. In our study the nitrogen content was highest with the *A. lipoferum*-treated sugarcane, followed by the *Bacillus* H15 and *G. diazotrophicus* treatment.

Increase in sugarcane yield due to inoculation with endodiazotrophs has been reported by several authors (Govindarajan *et al.*, 2007; Mehnaz, 2011; Oliveira *et al.*, 2003; Sevilla *et al.*, 1998, 2001). In our study, cane length was significantly higher in all the inoculated treatments. Percentage increase over the control in cane length showed that maximum increase of 25.5% was in the treatment with H8. Sucrose concentration increased up to 4% in the bacterial treatment *Bacillus* H15 and *A. lipoferum* compared with the control. Increase in cane yield was 42% in *G. diazotrophicus*-treated plants and 40% in H15-treated plants. Increase in cane weight was up to 51% and 59% following inoculation with H15 and *G. diazotrophicus* respectively. An increase in growth parameters and yield attributes of sugarcane by inoculation of nitrogen-fixing endophytes indicates the potential of these bacteria to provide a sustainable alternative to inorganic nitrogen fertilizer in sugarcane.

The search for microorganisms that improve soil fertility and enhance plant nutrition has continued to attract attention due to increased cost of fertilizers, pesticides and some of the negative environmental impacts of these chemicals. Sustaining and enhancing the growth and yield of sugarcane have become a major focus of research keeping in mind the use of ethanol as an alternative fuel. Studies on endophytic bacteria in sugarcane so far have been focused on *G. diazotrophicus*. The present study identifies some plant growth-promoting *Pseudomonas* and *Bacillus* spp. from sugarcane and shows their significant contribution towards growth and yield under field conditions. Percentage increase in yield over the uninoculated control for isolate *Bacillus* H15 was 40% not differing significantly from the standard *G. diazotrophicus* (42%) suggesting that *Bacillus* H15 can be used as a bioinoculant for sugarcane, thereby reducing the need for nitrogen fertilizer application.

Acknowledgements. We are grateful to Dean Student Welfare (DSW), Pantnagar for financial support during the study, and Mr. Yadav, field staff of the Department of Agronomy, for technical assistance during the field experiment. We are also grateful to Dr. Yogesh Souche, National Centre for Cell Sciences (NCCS), Pune, for sequencing. We are also thankful to Drs. Sanjeev Agarwal and Shefali Dhobal (Dept. of Biochemistry) for assistance.

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