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Research Article

Successful colonization of roots and Plant growth promotion of sorghum (*Sorghum bicolor* L.) by seed treatment with *Pseudomonas putida* and *Azotobacter chroococcum*

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Pseudomonas putida **(P29) and** ***Azotobacter chroococcum*** **(Azb19) are the efficient promising strains selected from** ***in vitro*** **plant growth promoting studies. These two strains were tested for their ability to promote growth of sorghum and colonize sorghum roots. Seed bacterization with P29 and Azb19 resulted in increased plant height, shoot height, root volume, leaf area and total plant dry mass. Further, bacterial inoculation also significantly increased macro-and micro-nutrient uptake by sorghum plants. Using electroporation method, pure cultures of P29 and Azb19 were transformed with pHC 60 plasmid containing gfp gene. Transformants detected by colony PCR were used to study the colonization pattern on roots of sorghum. Confocal fluorescence scanning microscope (CLSM) was used to locate the inoculants on or inside roots. Root colonization in sorghum by P29 was internal whereas Azb19 was detected on root surface. GFP-tagged** ***Pseudomonas*** **was predominantly detected at the root differentiation zone. In case of Azb19 small aggregates of micro-colonies were observed on the surface of the roots. The efficient sorghum root colonization by these inoculants clearly demonstrated that the introduced strains could successfully inhabit the rhizosphere and thus resulting in increased nutrient uptake. Inoculation with P29 resulted in increased uptake of P (288.5%), K (179.1%), Fe (242.7%), and Zn (168.1%) as compared to Azb19 where the uptake of P, K, Fe, Mn, and Zn increased by 142.6%, 161.6%, 199.5%, and 121.9%, respectively. On the other hand, inoculation with Azb19 could enhance better uptake of N (163.6%) as compared to P29 (133.3%). The strains also differed in their mode of root colonization.**

Key words: Root colonization, *Pseudomonas*, *Azotobacter*, GFP, PGPR, Sorghum, Confocal, laser scanning microscope

INTRODUCTION

Sorghum (*Sorghum bicolor* L.) is an important rainfed crop, grown globally on 41.9 million ha. In India, sorghum is grown in a wide range of environments. It occupies around 7.7 million hectares especially grown in semi-arid regions of the country producing 7.0 million metric tons of grain (FAO). Plant growth promoting rhizobacteria (PGPR) have originally been defined as root-colonizing bacteria that cause either plant growth promotion or biological control of plant diseases.

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Generally, PGPR promote plant growth directly by either facilitating resource acquisition (nitrogen, phosphorus and essential minerals) or modulating plant hormone levels and indirectly by decreasing the inhibitory effects of various pathogens on plant growth and development in the form of biocontrol agents. Indian agro ecosystems are not only thirsty but also hungry. Resource-poor small and marginal farmers fail to meet the nutrient requirements of crops through external supplementation Entire quantity of nutrients applied to the soil are not immediately taken up by the plants and thus are fixed in the soil and thereby becoming unavailable to the plants. Microbe-mediated plant growth promotion and nutrient supplementation is a well-established phenomenon (Kloepper et al., 2004; Desai et al., 2007; Praveen Kumar et al., 2012).

Phosphorus, potassium, zinc, sulphur and iron supplementing microbes are already available in commercial forms as bioinoculants (Mazid 2014). Candidate PGPRs are usually obtained by screening large collections of bacterial isolates seeking biochemical or genetic traits known to be involved in plant beneficial activity. However, to be effective in plants, the candidate strains need to be able to establish and maintain a sufficient population in the host plant. Competitive rhizosphere colonization is crucial for plant-PGPR interactions. The efficiency of nutrient uptake and catabolism by bacteria is an important factor in competitiveness (Chin-A-Woeng et al., 2003). A key determinant for the ability of a root system to acquire nutrients from the soil is the extent to which it is colonized by appropriate plant growth promoting bacteria. A variety of cell surface molecules contribute to the colonization process. Application of such microorganisms in agriculture is expected to reduce the use of chemical fertilizers and pesticides. Plant growth conditions, including soil type and occurrence of stress factors, also plays a part in determining the composition of plant microflora. This complex network of interdependence makes it problematic to study and predict plant-microbe interactions, especially in case of soil-grown plants. Hence, the colonization potential of each PGPR candidate has to be experimentally determined. Therefore to track the presence of such bacterial strains in the rhizosphere, requires selective detection methods like using a marker system, which is stably maintained and expressed in the environment. Hence this study was undertaken to understand the ability of stable transformants of *Pseudomonas putida* (P29) and *Azotobacter chroococcum* (Azb19) containing *gfp* gene to colonize the rhizosphere of sorghum. From the previous studies on characterization of bacterial isolates for plant growth promoting traits and nutrient mobilizing ability, *Pseudomonas putida* (P29) and *Azotobacter chroococcum* (Azb19) were selected for pot culture studies as well as root colonization studies. To visually demonstrate colonization such as the location of *Pseudomonas putida* and *Azotobacter chroococum* on sorghum roots, confocal laser scanning microscopy was used.

MATERIALS AND METHODS

Vector

pHC 60 vector (Fig 1) was kindly provided by Plant Molecular Biology laboratory, University of Hyderabad, Hyderabad, India. pHC 60 vector contains *gfp* gene and a tetracycline marker. This vector was maintained in *Escherichia coli* DH5α cells. pHC 60 vector was isolated by standard method using Nucleospin plasmid kit (Macherey-Nagel GmbH,Germany). Amplification of *gfp* gene was done for confirmation of the *gfp* gene in the plasmid by PCR using *gfp* specific primers.

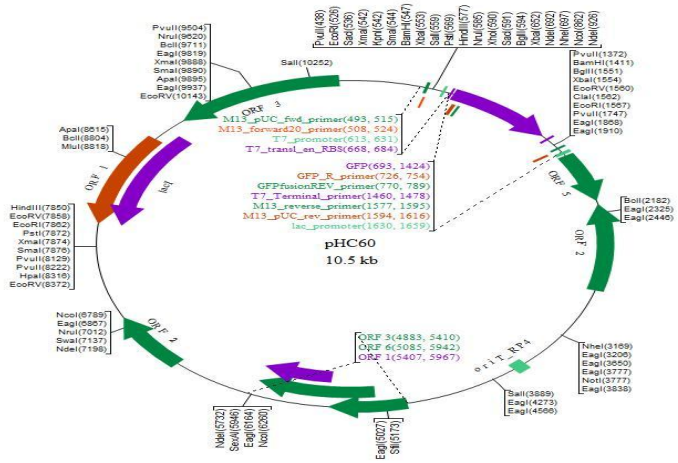

Figure 1. pHC 60 Plasmid vector

Transformation of test bacterial strains

*Pseudomonas*29 and *Azotobacter*19 were made electro-competent using the protocol of Xue et al. (1999) with some modifications. Five mL of LB broth was inoculated with a single colony of bacteria for electroporation and incubated overnight at 37°C on an orbital shaker at 250 rpm. On the following day, cells were harvested by centrifugation at 5152 g for five minutes and the pellet was suspended in 600 µL of 10% glycerol. This step was repeated twice. A volume of 80 µL of the electro-competent cells was collected and suspended in 400 µL of 10% glycerol with 60 µL of plasmid (1µg). Plasmid DNA and cells were transferred to a chilled two mm gap electroporation cuvette (Bio-Rad, Hercules) and incubated on ice for five min. The cells were then electroporated in a Gene Pulser Xcell electroporator (Bio-Rad) at a voltage of three kV, capacitance of 25 µF, resistance of 200Ω and a time constant of nine milli seconds. The cells were then transferred immediately to one mL of LB broth and incubated at 37°C for two h on an orbital shaker at 250 rpm. Transformants were

selected by plating the serially-diluted suspension on LB plates amended with 10µgmL^{-1} tetracycline and incubated overnight at 37°C. Positive clones were then further confirmed by colony PCR. Individual colonies from the antibiotic amended plates were picked and amplified using *gfp* primer. PCR product was resolved on 1% agarose gel to detect the presence of amplified 730 bp *gfp* gene. For amplification, forward primer 5´-ATAGAATTCATGGCTAGCAAGGAGAA3` and reverse primer 5´AATCTCGAGGCAGCCGGTTCCTTTGTA-3` were used.

Colony PCR conditions

Colony PCR steps included i) denaturation for three min at 95°C ii) 30 cycles of denaturation for one min each at 94°C iii) annealing for 30 sec at 57°C iv) elongation for one min at 72°C and v) a final extension step of 2 min at 72°C. The PCR products were then held at 10°C.

Sorghum seedlings treatment with bacteria and plant growth conditions

Sorghum seeds were surface sterilized with 0.1% $HgCl_2$ for 3 min and then washed three times with sterile double-distilled water. After surface sterilization, seeds were placed on water agar and were incubated. After the emergence of primary roots, seedlings were uprooted carefully from the water agar. Excess agar was removed by carefully rinsing the roots in sterile double-distilled water. Bacterial cells from an overnight culture on LB agar plates, grown at 37°C were harvested. The final bacterial inoculum used for root dip inoculation of sorghum seedlings was 4×10^8 cfuml^{-1} determined both spectrophotometrically (600nm) and by counting viable tetracycline resistant (TcR) colonies.
Sorghum (crop variety SPV 462) seedlings from water agar were dipped in the bacterial suspension and were transferred to 22cm diameter pots filled with sterile soil. Six replications for each treatment were maintained. Seedlings without bacteria were potted to serve as control samples. Plants were grown for three weeks with growth conditions as follows: temperature 26±1°C, humidity 70±2%, 200µmol m^{-2} s^{-1} light intensity and a photoperiod of 16 h light/8h dark. After three weeks, plants were uprooted carefully and microbial count on root surfaces was taken using dilution plating method.

Sample Preparation for Microscopy

Plants were uprooted carefully from the pots and excess soil was removed by shaking. Root samples (sections of root tip) were cut, soaked in sterilized ddH_2O and placed in sterile plastic petri dishes.

Evaluation of plant growth promoting ability

Agronomic parameters such as shoot length, shoot dry mass, root dry mass and root volume were recorded following standard procedures. Chlorophyll content was estimated in terms of SPAD reading using a SPAD meter.

Nutrient Analysis

Nutrient analysis of the plant samples was done as described by (Tandon, 2001) which is briefly described here. Oven-dried plants were finely ground in a mortar and pestle to amorphous powder and 100 mg was taken in 150 mL conical flask containing 10 mL nitric acid (HNO_3) and perchloric acid ($HClO_4$) in 9:4 ratio. The flasks were placed on a hot plate and digested at 300°C until the entire plant material was digested and turned colorless. The extract was taken in 100 mL volumetric flask and the volume was made up to 100 mL with distilled water. These samples were used for estimation of sodium, potassium, and calcium by flame photometer. Phosphorus was quantified by sulphomolybdic acid method. Total nitrogen content of the plants was estimated by micro Kjeldahl method. Similarly, micronutrients such as iron, copper, manganese, zinc and magnesium were estimated by atomic absorption spectrophotometer.

Statistical analysis

The data were analyzed by using the appropriate statistical methods wherever needed. The pot culture studies data were analyzed by analysis of variance (ANOVA). The means were compared using LSD.

RESULTS

pHC 60 vector with *gfp* gene and tetracycline resistant marker was extracted from *E. coli* DH5α cells. After successful extraction, *Pseudomonas*29 and *Azotobacter*19 were transformed with pHC 60 by electroporation technique. pHC 60 vector was isolated from the positive clones and the *gfp* gene was amplified (Fig 2).

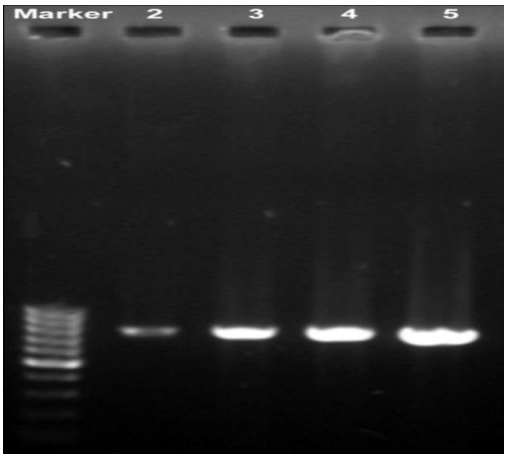

Fig2. PCR amplification of *gfp* gene from pHC 60 of transformed clones of Azb19 (lanes 2&3) and P29 (lanes 4&5)

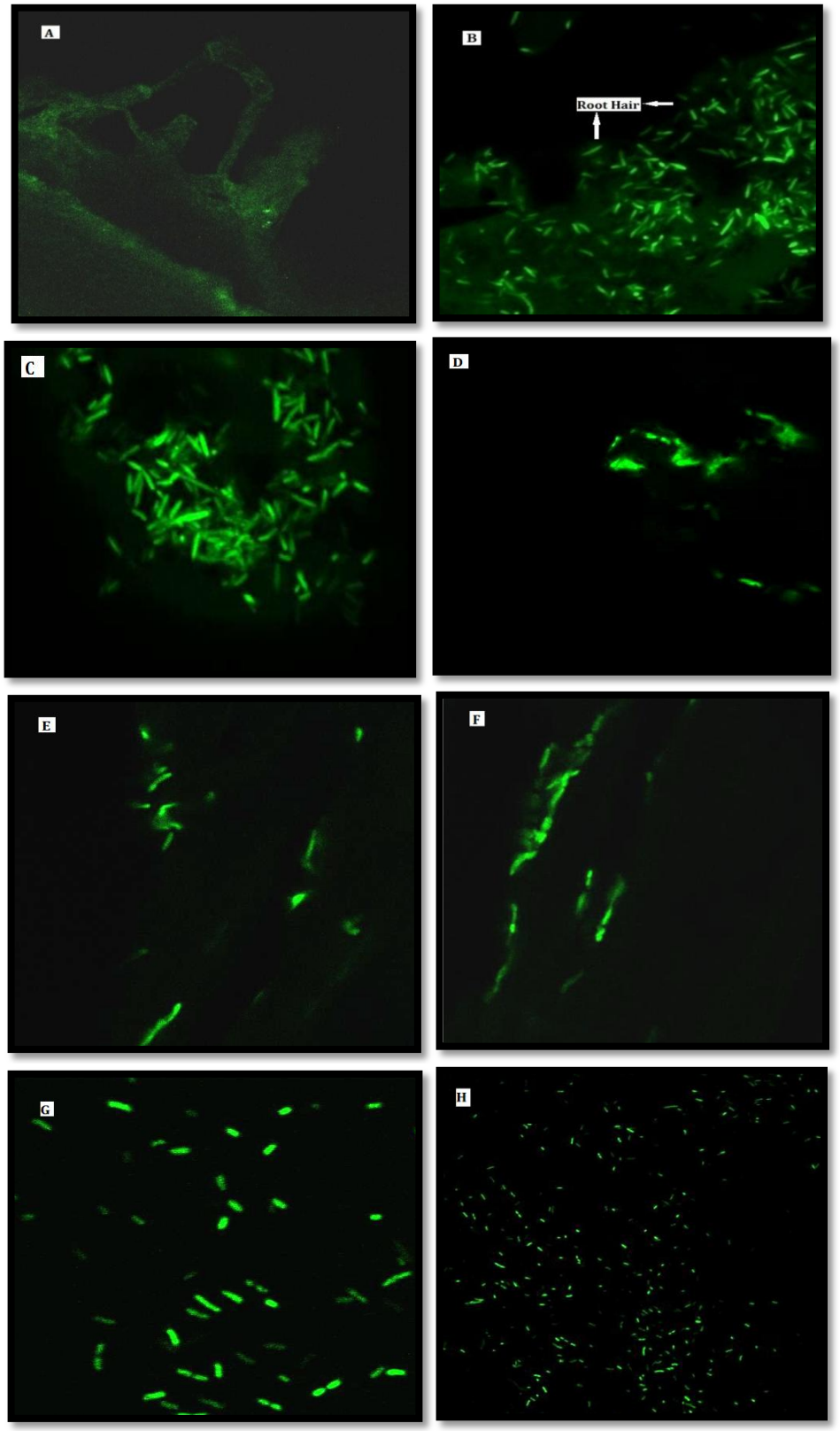

Figure 3. CLSM images of root colonization of Sorghum. Confocal analysis was performed on 1–2cm long root longitudinal sections to show internal colonization (A, B, C) by *Pseudomonas* and surface colonization (D, E, F) by *Azotobacter*. A) Confocal optical sections of root hairs, B) Detection of *gfp*-tagged *Pseudomonas* inside the root hairs, C) Cells of *Pseudomonas* near the boundary of a root hair, D) *gfp*-tagged *Azotobacter* attached to root surface of Sorghum, E) Cells of *Azotobacter* observed as single cells, F) as micro colonies (arrowed). Image G & H represents the pure culture of GFP tagged *Azotobacter* and *Pseudomonas.*

Table1. Effect of seed bacterization of sorghum with Azb19 and P29 on plant growth parameters

Treatments	RV (cc)	SL (cm)	RDM (gm)	SDM (gm)	SPAD reading
Azb19	23.39^{a}(±6.1)	24.35^{ab}(±6.2)	1.2^{a}(±0.0)	5.78^{a}(±1.96)	23.23^{a} (±0.25)
P29	19.8^{b}(±3.2)	26.3^{a}(±5.1)	0.8^{ab}(±0.0)	6.40^{a}(±2.03)	24.40^{a}(±1.5)
Control	16.01^{c}(±3.7)	20.46^{b}(±3.2)	0.1^{b}(±0.0)	4.09^{b}(±0.09)	23.10^{a}(±0.21)
CV%	20.39	18.27	24.15	16.89	15.57
LSD	1.83	4.90	0.36	0.8	4.13

Values in the brackets are standard errors
Treatments superscribed with same letter do not differ significantly (P<0.05)

Table 2. Nutrient analysis of sorghum plant samples inoculated with Azb19 and P29

Treatments	N%	P%	K%	Fe (ppm)	Cu (ppm)	Mn (ppm)	Zn (ppm)
Azb19	4.81^{a}(±1.5)	0.87^{ab}(±0.4)	1.39^{a}(±0.4)	441.6^{b}(±15.5)	11.6^{a}(±0.0)	115^{c}(±5.4)	256^{b}(±14.5)
P29	3.92^{b}(±0.9)	1.76 ^{a}(±0.6)	1.54^{a}(±0.3)	537.2^{a}(±23.7)	8.0^{b}(±0.0)	141^{a}(±8.0)	353^{a}(±19.5)
Control	2.94^{b}(±0.8)	0.61^{b}(±0.2)	0.86^{a}(±0.1)	221.3^{c}(±0.3)	13.0^{a}(±0.0)	125^{b}(±6.0)	210^{bc}(±12.5)
CV%	23.38	10.84	22.99	15.5	17.05	4.14	1.32
LSD	0.8	1	0.87	90	4.42	9.15	3.28

Values in the brackets are standard errors
Treatments superscribed with same letter do not differ significantly (P<0.05).

Colonization of sorghum rhizosphere by P29

A time-course colonization process of *gfp*-tagged plant growth promoting P29 including bacterial adhesion to root hair surface and the colonization of the intercellular spaces within the cortex of the differentiation zone was recorded using confocal laser scanning microscopy (Fig 3). It was observed that bacteria were attached along the entire root hair surface, mostly at random making it imperative that root hairs were required for the efficient root colonization of sorghum plants. Interestingly, often bacterial cells seemed to adapt themselves to the shape of root hairs orienting the bacterial cells in such a way that an intimate contact between bacterial cells and root hair surfaces was established. Further, often boundary regions of root surfaces were covered with significant portion of bacteria. After rapid colonization of sorghum rhizoplane, *gfp*-tagged P29 was predominantly detected at the root differentiation zone. Observation of primary roots revealed that the segments within one cm distance from plant basal sites were highly colonized by the inoculant. Few bacterial cells were observed within the range of two cm distance from the root tip. In general, the bacterial adhesion was very prominent on the lower root portions as compared to upper root portions.

Colonization of sorghum rhizosphere by Azb19

Root colonization by Azb19 was restricted to surface of rhizoplane (Fig 3). The first site of colonization was the zone of cell elongation at the root tip and tip area of emerging lateral roots where bacteria started to accumulate. *gfp*-tagged cells of Azb19 were not evenly distributed along the root; instead they were found as micro-colonies located either at the surface of the roots or on root hairs. On the highly colonized segments, a number of micro-colonies have been easily observed around root surfaces.

Plant growth promoting activity of bacterial strains

Seed bacterization of sorghum with P29 and Azb19 enhanced the plant growth significantly. Both the isolates showed plant growth promotion as compared to respective un-inoculated controls. Increase in root volume, shoot length and plant biomass were significantly higher in treated plants as compared to un-inoculated control. Inoculation of sorghum with Azb19 was superior than P29 in terms of increase in root volume (23.39 cm^3) and root dry mass (1.2g) (Table 1).

Nutrient uptake by the Plants

There was a significant increase in the uptake of both major- and minor-nutrients when sorghum seeds were bacterized with P29 and Azb19 as compared to controls (Table 2). However, both strains had differentially influenced uptake of different nutrient elements which was reflected selective enhancement of uptake. Inoculation with P29 resulted in increased uptake of P (288.5%), K (179.1%), Fe (242.7%), and Zn (168.1%) as compared to Azb19 where the uptake of P, K, Fe, Mn, and Zn increased by 142.6%, 161.6%, 199.5%, and 121.9%, respectively. On the other hand, inoculation with Azb19 could enhance better uptake of N (163.6%) as compared to P29 (133.3%). Interestingly, uptake of both Mn and Cu appeared to be hindered in both treatments as compared to control (Table 2).

DISCUSSION

Microbe-mediated plant growth promotion has been proved successfully in many crop plants (Stephen 2009). Efficient root colonization and rhizosphere competence are two important parameters in determining the fate of introduced microorganism in the rhizosphere and thereby show its effects on plants through nutrient solubilization and plant growth promotion. For monitoring introduced microorganisms, it is a routine practice to tag these micro orgainsms with suitable marker gene so that they can be easily detected in the rhizosphere. Transformation of both P29 and Azb19 by electroporation using the plasmid pHC 60 resulted in stable transformants and showed satisfactory levels of *gfp* expression.

It is established that efficient colonization on plant roots is a critical step for PGPR for plant-microbe interactions (Chin-A-Woeng et al., 2000; Lugtenberg et al. 2001; Kamilova et al. 2005; Timmusk et al. 2005; Ongena et al. 2008). Our results have demonstrated surface and internal root hair colonization by Azb19 and P29 in sorghum. Similar observations were made by Pilar et al (2011) based on the investigations on olive roots inoculated with *Pseudomonas* spp. There is a strong aggregation pattern only at specific sites on the roots like root hairs, probably due to variations in root exudation at different places within the root system (Lugtenberg 2001). Number of investigations demonstrated a non-uniform distribution of *Pseudomonas* on plant root: heavily colonized areas are usually found at junctions between epidermal root cells, concave parts of the epidermal surface or sites where side roots appear (Bloemberg et al. 1997; Chin-A-Woeng et al. 1997), all assumed sites of exudation. There are increasing evidences that root exudates play a key role in plant-microbe interactions (Somers et al. 2004). Hiltner 1904 observed the abundance of microorganisms in the rhizosphere, which was later found to be related with root exudation (Hartmann et al. 2008). In agreement with these published results, mode of colonization of *Azotobacter* is small-aggregate, micro colonies and single-cell colonization. Plant growth conditions, including soil type and occurrence of stress factors, also play a part in determining the colonization of plant growth promoting microflora. Soil factors such as soil acidity, soil humidity and soil nutrients like aluminum and potassium levels, etc., influence root hair density, length, physiology and development (Hansen 2005 and R. Lanna Filho). Moreover, under soil drought conditions, some PGPR may stimulate cellular division in roots and increase root hairs (Mattos 2005). This further leads to enhanced water and nutrient uptake by plant roots, especially from deeper soil layers (Compant 2010). Accordingly, it is necessary to be aware of soil factors influencing root hairs that may affect bacteria-root hair interactions and subsequently, plant growth promotion and nutrient management abilities deployed by bacteria. Root colonization is successful if the introduced bacteria are able to spread and propagate along the root in the presence of competitive indigenous rhizosphere microorganisms. Compared with *Pseudomonas*, however, so far only little was known about the colonization pattern of *Azotobacter*. In the current study, seed bacterization with plant growth promoting bacteria resulted in increased plant height (root volume and shoot height), leaf area and drymass. Similar increases in plant parameters were observed in different crops inoculated with *gfp*-tagged *Azospirillum* in wheat seedlings under salt stress conditions (Macario Bacilio 2004). This improved growth by PGPR is due to efficient root colonization and nutrient solubilization. The present study indicated that bacterial inoculation of sorghum with P29 and Azb19 significantly increased the N and P content in leaves of sorghum. Higher uptake of essential nutrients like phosphorous and zinc compared to un-inoculated control plants could be justified from the fact that the unavailable forms of these nutrients were solubilized and made available in the root region by applying PGPR. Plants which are inoculated with plant growth promoting rhizobacteria usually have higher N-P-K content than that of un-inoculated plants. Results showed that bacterial treated plants showed significant differences in Fe, Cu, Mn and Zn content in Sorghum leaves. The enhancement of macro and micronutrient uptake, except Cu and Mn by inoculation with PGPR may be due to efficient root colonization which has impact on initiation and development of lateral roots and increased root weight (Mattos 2008). The availability of Mn to plants is governed by oxidation and reduction processes (Marschner 1988). Decrease in Mn uptake in sorghum on inoculation with *Azotobacter* in this study could be attributed to their possible Mn oxidizing and reducing activities (Marschner 2003). Similarly lower uptake of Mn by mycorrhizal plants has been found by Pacovsky (1986) and Kothari etal (1991). The relative importance of root exudates and microorganisms in oxidation and reduction processes is still not clear.

CONCLUSION

In summary, genetically tagged strains of *Pseudomonas* and *Azotobacter* were capable of colonizing the roots of sorghum. Fluorescently tagged bacteria, CLSM and sorghum plants provided a powerful approach to study sorghum–bacteria interaction. The importance of efficient Sorghum root colonization by plant growth promoting *Azotobacter*19 and *Pseudomonas*29 has clearly demonstrated that colonization is the key factor for nutrient uptake and plant growth promotion.

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