Research Paper

Surface colonization by *Azospirillum brasilense* SM in the indole-3-acetic acid dependent growth improvement of sorghum

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The key to improving plant productivity is successful bacterial-plant interaction in the rhizosphere that can be maintained in the environment. The results presented here confirm *Azospirillum brasilense* strain SM as a competent plant growth promoting bacterium over mid- and long-term associations with sorghum. This study establishes that plant growth can be directly correlated with the associated bacterium's indole-3-acetic acid (IAA) production capability as IAA over-expressing variants, SMp30 and SMΔi3-6 fared better than the wild type strain. The auxin antagonist, p-chlorophenoxy isobutyric acid confirmed the role of bacterial IAA in plant growth promotion and verified the presence of larger amount of IAA available to the seeds on inoculation with IAA over-expressing mutants. Microscopic analysis identified the bacterial association at root tips, root-shoot junction and elongation zone and their surface colonizing nature. Scanning electron microscopy identified larger number of root hairs and extensive exopolysaccharide covering in comparison to untreated ones. In addition, vibroid-shaped Azospirilla attached by means of fibrillar material were dispersed along the elongation zone. The notable difference with IAA over-expressing variants was enhanced number of root hairs. Thus, the variant strains may be more efficient surface colonizers of the sorghum root and used as superior bio-inoculants for improving plant productivity.

Keywords: Indole-3-acetic acid / Plant growth promotion / p-chlorophenoxy isobutyric acid / Scanning electron microscopy

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Introduction

A successful interaction between plants and plant-associated bacteria (PAB) is such that the benefits are mutually accrued. Plant growth-promoting bacteria (PGPB) may not be strictly dependent on their host plant but nevertheless may utilize the products released from the plants either directly or by means of degradation. The reverse is also an exciting phenomenon whereby the secondary metabolites released from the microbes aid the plant to grow, either directly or by evading pathogens [1]. Due to such relationships, bacterial consortia may be found as biofilms, flocs or granules in the heterogenous rhizosphere [2].

There is a continuum of bacterial presence in the soil-rhizosphere-rhizoplane, internal to plant tissues [3]. Soil inhabiting bacteria may be free-living or associative based on whether they can thrive on root exudates for their survival. Rhizospheric bacterial communities have evolved efficient systems for uptake and catabolism of different compounds present in the root exudates [4]. While associative PGPB may attach to the rhizoplane, allowing them to derive maximum benefit from root exudates, some others may penetrate the root tissues (endophytic) gaining direct access to organic compounds present in the apoplast. By occupying this privi-

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leged endophytic location, bacteria do not face competition from their counterparts otherwise encountered in the rhizosphere or bulk soil [5, 6].

There are implicit requirements for the complex process of rhizosphere colonization by PGPB: ability to survive inoculation onto seed, to multiply in the spermosphere in response to seed exudates, attachment to the root surface and colonization of the developing root system [7]. Yet, the field ineffectiveness of PGPB is often attributed to their inability to colonize plant roots [8]. PGPB like *Azospirillum* have been shown to successfully colonize a variety of plants (including pearl millet, maize, wheat, rice, tomato, cotton and soybean) and utilise multiple plant growth promoting (PGP) mechanisms [9–11]. Attachment sites of *Azospirillum* on roots differ with the colonized plant species and initial inoculum concentration. Using constitutive *lacZ* fusions, Arséne and others could not detect differences between the colonization patterns of different *A. brasilense* strains – Sp7, Sp107, Sp245, and Sp246 on wheat roots [12]. PGPB interactions are important as they not only provide a better understanding of plant processes but also help in assessing the likely effect of associated PGPB. At the functional level, PGPB help plants to acquire P and K, along with enhanced N uptake, influencing root morphology and physiology [10, 13].

Many PGPB synthesize various phytohormones (auxins, cytokinins and gibberellins) and other plant growth regulators (PGR) like nitric oxide [14–16]. Of the different PGR produced by PGPB, the most significant is the auxin, indole-3-acetic acid (IAA). Our understanding of the role of phytohormone synthesis as a direct mechanism of plant growth enhancement by PGPB has increased by using molecular approaches to analyze microbial and plant mutants altered in their ability to synthesize or respond to specific phytohormones [14, 17, 18]. The biosynthesis of IAA involves distinct biosynthetic routes in plants and bacteria. Various tryptophan (Trp)-dependent and -independent pathways have been described in bacteria, nevertheless, Trp serves as the major precursor of IAA biosynthesis [17, 19, 20].

Our earlier work established that IAA produced by the bacterial strain *Azospirillum brasilense* SM has a beneficial impact on the development of sorghum roots under controlled conditions [17, 20, 21] as well as under a variety of stresses [21]. In the present study we attempt to identify the association of *Azospirillum brasilense* strain SM with sorghum roots by light and electron microscopy. We also demonstrate that IAA over-expressing mutants of strain SM are beneficial over short to long-term soil exposure on sorghum and hence may be a suitable choice for use as bio-inoculants.

Materials and methods

Bacterial strains and cultivation

The bacterial strain used was *Azospirillum brasilense* SM (MTCC 4037, India). The bacterial cultures were maintained on modified Luria-Bertani (LB*) agar with the appropriate antibiotics (50 μg ml^{-1} ampicillin/kanamycin, 10 μg ml^{-1} tetracycline, as per the requirement of the strain), and purity of the cultures was checked on nitrogen-free basal (Nfb) medium [22]. For IAA estimation, ~2×10^7 colony forming units (cfu ml^{-1}, corresponding to 0.1 OD_{560}) were cultured in the buffered standard succinate medium (SSM) with 5 mM tryptophan (Trp) and IAA was quantified as mentioned earlier [20]. The cells were grown at 30 °C, 200 rpm in all the experiments. The derivatives of strain SM used for analysis during this study, strain SMp30 and SMΔi3-6, were generated by electrotransformation or site-specific mutagenesis [17, 18, 20]. All chemicals used were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA.).

Seed inoculation

Sorghum (var. *Sudex chari*, National Seed Corporation, IARI, New Delhi) seeds were surface inoculated with 2×10^8 cfu ml^{-1} of the bacterial strain [20]. Twelve surface sterilized seeds per set were treated with 20 ml culture from different strains (wild type and IAA overexpressing) for 1 h and monitored subsequently in garden soil/vermiculite mixture (1:1, sterilised by autoclaving) under controlled conditions in the greenhouse at 28 °C for 10 weeks and 16 weeks, respectively. The plants were watered with $1/10^{th}$ strength nitrogen-free solution, NFS, [23]. In order to correlate the plant response with the presence of bacteria, soil suspension at each time point was plated on appropriate antibiotics containing selection media. Soil from the untreated seed sets was used as control.

When the seedlings/plants were harvested at the respective time point, ~1 g sterile soil was collected from the rhizosphere, resuspended in 5ml saline and an aliquot of the suspension was plated on LB containing ampicillin (50 μg/ml), tetracycline (10 μg/ml) or kanamycin (40 μg/ml) to monitor the presence of strain SM, SMp30 and SMΔi3-6, respectively. The total dry weight of all the plants belonging to one treatment set was measured by drying the harvested plants at 75 °C in an oven until a constant weight was obtained.

Seed-germination bioassay for plant growth promoting (PGP) ability

A seed-germination bioassay for confirming the PGP ability of the bacterial strains was designed by treating 50 sorghum seeds with the cultures of the strain SM and its IAA over-expressing mutants, SMp30 and SMΔi3-6 as mentioned above. The treated seeds were placed on sterile Whatman paper moistened with NFS and the subsequent growth was monitored for 4 days in the culture room. A second set of each bacterial treatment was monitored to confirm the involvement of IAA produced by the respective bacteria in growth promotion of sorghum, on sterile Whatman paper discs moistened with the auxin antagonist, p-chlorophenoxy isobutyric acid (PCIB, 1mM). Seeds were then incubated in sterile conditions in the culture room for 4 days and the germination was scored. An untreated control was kept for comparison of results.

Microscopic analysis of plant-bacterial association

To visualize the plant root-bacterial association, the *A. brasilense* SM-derivative strain SMΔpMEi3LF4, expressing β-galactosidase under the native *ipdC* promoter was used to inoculate sorghum seeds and seedlings were harvested at 4 days (2-leaf stage). Roots from harvested seedlings were analyzed by microscopy with an Olympus DX51 Research Microscope fitted with a DP70 Camera. Whole and longitudinal sections of the roots were prepared and stained with either 1% congo-red (for strain SM treated controls) or 5 μg ml^{-1} X-Gal (for SMΔpMEi3LF4 treated seedlings). The roots were stained for 3 h, washed thoroughly with water, fixed with 2% glutaraldehyde and then visualized by microscopy for bacterial-root association.

Scanning electron microscopy

The bacterial association was further analyzed by Scanning Electron Microscopy (SEM). For this purpose, sorghum seeds were inoculated with the wild type and some of the IAA over-expressing variant strains and allowed to grow on NFS-agar for 4 days. After 4 d, the roots were harvested, cut into pieces of 1.5 cm each and fixed in 0.1 M sodium phosphate buffer containing 2.5% glutaraldehyde and 2% paraformaldehyde by vacuum infiltration for 2 h at room temperature and subsequent treatment for 6–12 h at 4 °C. The fixative was removed and the samples were washed twice with the same buffer. Dehydration was carried out in an ascending gradient series of acetone at 4 °C and the samples were dried in a Critical Point Dryer (Jumbo) in a CO_2 atmosphere. The dried samples were fixed to stubs with carbon-cement, sputter-coated with gold (10 nm, Sputter Coating Unit, Balzer Union SCD 020) and examined at different magnification (upto 8000X) by LEO 435 VP (Variable Pressure) SEM fitted with a Zeiss-Leica lens, equipped with digital imaging and 35 mm photography system and operating in high vacuum between 15–30 kV.

Statistical analysis

Each seed inoculation experiment was repeated three times. Data from experiments with bacterial cultures were analyzed for their distribution pattern by the one sample Kolmogorov-Smirnov *Z*-test and all sets were found to be normally distributed. The data were subsequently analyzed for variance by ANOVA followed by Tukey's Post hoc analysis at $P \leq 0.05$ and Student's *t*-test at $P \leq 0.05$. All analysis was performed with Statistical Package for Social Sciences (SPSS ver. 11.0 for Windows). For seed inoculation, the data for shoot lengths of 12 plants per set was analyzed by Student's *t*-test to identify if bacterial inoculation influenced the subsequent growth of sorghum. For germination bioassays, the number of seeds germinated by the 4^{th} d were noted and the percentage was calculated.

Results

We have earlier established the influence of seed inoculation of sorghum with the wild type strain SM at different time points: mid-term (10 weeks) and long-term (16 weeks) duration [21]. Such a study was also conducted for the IAA-over expressing variants of strain SM, SMp30 and SMΔi3-6, to determine the efficacy of this over-expressed bacterial function. These results are presented in Table 1 for 10 and 16 weeks post-treatment along with the control, wild type strain SM. The results presented in Table 1 indicate that both IAA over-expressing strains are capable of boosting the growth of sorghum by not only improving the shoot development but also the weight of the plants. However, a low IAA-expressing variant strain SMIT568s10 showed a decreased PGP effect on sorghum in comparison to the wild type strain SM at 10/16 weeks post-treatment (Table 1), confirming that the plant response is directly correlated with the PGPB's IAA production status and there is much better uptake of nutrients by the IAA over-expressing derivative strains (SMp30 and SMΔi3-6)-treated plants compared to the wild type and untreated ones. That this PGP effect was due to the presence of the bacterial strain used, was confirmed by plating the rhizospheric soil suspension of the treated plants on selection media.

Table 1. Effect of the *Azospirillum brasilense* strain SM and its variants on the growth of sorghum at 10 and 16 weeks post treatment, respectively.

Treatment	Parameter	Untreated	SM	SMp30 (3.2 folds IAA)	SMΔi3-6 (3.1 folds IAA)	SMIT568s10 (0.5 folds IAA)
10 weeks	Average shoot length (cm)	60.80 ± 1.35	68.10 ± 0.87[#]	73.80 ± 1.00[#,*]	72.25 ± 0.90[#,*]	62.36 ± 0.92
	Total dry weight (g)	33.06	40.16	45.56	44.80	36.24
16 weeks	Average shoot length (cm)	86.17 ± 1.08	111.40 ± 2.42[#]	117.50 ± 1.79[#,*]	116.70 ± 2.56[#,*]	90.56 ± 1.10[#,*]
	Total dry weight (g)	72.50	133.00	142.50	145.40	82.60

[#] and [*] represent values that significantly differ from the untreated and strain SM-treated seeds at $P \leq 0.05$, respectively. Each set contained 12 plants per treatment and the values shown are the average ± the standard error from the mean.

Conclusive proof that the PGP effect of strain SM and its derivatives is due to the IAA produced by them was obtained by PCIB treatment. As is clear from Fig. 1, the auxin antagonist, PCIB reduced the PGP effect of strain SM on sorghum in a concentration-dependent manner. The root length response was in the order of strain SM > Untreated > 0.1 mM PCIB > 0.5 mM PCIB > 1 mM PCIB > 5 mM PCIB, with 5 mM PCIB-treated seeds showing no germination, indicating an interference in auxin action. Table 2 shows the effect of 1 mM PCIB on % germination observed after inoculation with strain SM and its IAA over-expressing derivatives. Exposure of strain SM-treated seeds to PCIB dropped germination to 52% in comparison to non PCIB exposed cells. A more drastic drop in % germination was seen with the IAA over-expressing strains, SMp30 (36%) and SMΔi3-6 (32%). On the other hand, only a minor drop of 4% was observed in the germination of the untreated seeds in comparison to the bacteria inoculated seeds. This indicated that PCIB inhibited larger amounts of IAA in case of the IAA over-expressing strains.

To establish that the observed growth beneficial effect on sorghum is due to an association with strain SM, we carried out microscopic analysis of strain SM-treated plants. For this purpose we utilised the strain SM derivative, SMΔpMEi3LF4, that carries a translational fusion of the promoter of the indole-3-pyruvate decarboxylase gene (P_{ipdC}) with the reporter gene-*lacZ* [18]. This reporter strain was employed to study bacterial association with roots by monitoring *lacZ* activity through X-Gal staining of roots. Fig. 2 shows the appearance of blue color at the root tips, root-shoot junction and interspersed along the elongation zone in strain SMΔpMEi3LF4-treated plantlets. This provided evidence for successful association of *A. brasilense* SM with sorghum which was analyzed in detail by light microscopy and SEM.

As is clear from microscopic analysis, the roots of *A. brasilense* SM-treated seeds had root hair tips that were densely stained compared to those observed in case of untreated control plants (Fig. 3e and f in comparison with Fig. 3b and c). Even the unstained root hair tips appeared to contain dense aggregates (Fig. 3d) unlike control roots. In both these cases, however, such densely-stained structures could not be identified within the root cells (Fig. 3d and e). X-Gal staining of SMΔpMEi3LF4-bacterized roots also revealed the same

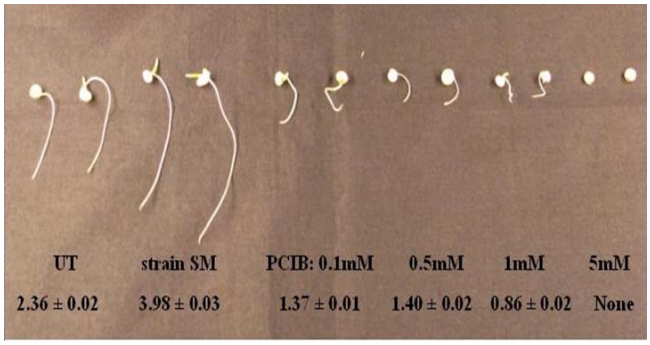

Figure 1. Effect of IAA produced by *Azospirillum brasilense* SM on sorghum root response in the absence and presence of the anti-auxin, PCIB. The seeds were treated with the bacteria and some were further exposed to PCIB as described at the concentrations mentioned. One untreated set (UT) was maintained as a control. The values at the bottom of the picture indicate the average root length of 12 seedlings with their standard error from mean.

Table 2. Effect of the auxin antagonist, PCIB, on the germination of *Azospirillum brasilense* SM and its IAA over-expressing variants treated sorghum seeds.

Treatment	Seeds germinated	Percentage germination
Untreated	50/50	100
Untreated + PCIB	47/50	94
A. brasilense SM	50/50	100
A. brasilense SM + PCIB	26/50	52
SMp30	50/50	100
SMp30 + PCIB	18/50	36
SMΔi3-6	50/50	100
SMΔi3-6 + PCIB	16/50	32

The experiment was repeated at least three times and each set had fifty seeds each. The trend of the results remained same in all the experiments, thus, representative results of only one experiment is shown.

Figure 2. X-Gal staining of *Azospirillum brasilense* SM treated sorghum roots. The plants were treated with 5 µg/ml X-Gal solution for 3 h, washed with water and then visualized by microscopy as described.

findings i.e. root hairs tips were densely stained (Fig. 3g and h) and were curved in many cases (Fig. 3h).

Following the results obtained by light microscopy and X-gal staining, the inoculated roots were further analyzed by SEM so as to confirm the *Azospirillum*-root association. SEM results confirmed the increase in the number of hairs in inoculated roots compared to the untreated control (Fig. 4b, f versus a). Unlike the untreated seedlings which did not show root hairs at 4 days, a large number of the same were observed in case of bacteria-treated roots.

A. brasilense SM cells could be seen dispersed in clusters randomly in the elongation zone of the bacterized roots (Fig. 4c and d), along with the presence of thick mucilaginous masses, mucigel layers or biofilms (BF in Fig. 4e). Although the root hairs appeared to be densely stained as mentioned earlier, the bacterial presence within the root cells could not be confirmed during this study, suggesting that strain SM is not endophytic in nature. *A. brasilense* SM cells exhibited the typical vibroid shape of Azospirilla (VA in Fig. 4c) and had a relatively thick fibrillar material connecting them to the roots as well as each other on the root surface (FM in Fig. 4d). Strain SMp30 and SMΔi3-6 treated-seeds confirmed their association with sorghum roots (Fig. 4f–h). Since the results obtained with strain SMp30 and SMΔi3-6 followed similar pattern, only one set has been shown in Fig. 4. The major difference observed for IAA over-expressing variants was the higher proliferation of root hairs than those observed with the wild type-treated seeds (Fig. 4f versus b). Thus, the IAA over-expressing variant strains seem to be more effi-

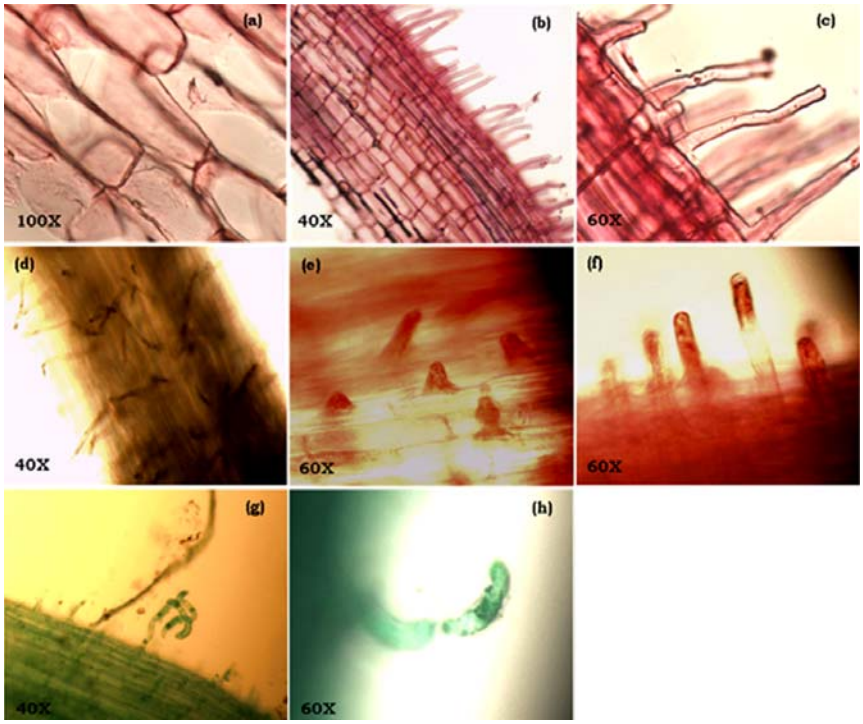

Figure 3. Microscopic view of 4-d old-untreated (a, b, c), strain SM-treated (d, e, f) and SMΔpMEi3LF4-treated (g, h) sorghum roots. Micrographs of roots stained with congo-red are at magnification 10X to 60X. Empty root cells are seen in untreated roots (a, b, c), some of the densely staining tips of root hairs are seen in AbSM treated and stained with congo-red (d, e, f) while SMΔpMEi3LF4 showed some densely staining root hairs (g, h) and some unstained ones (i). The magnification in each case is mentioned with the micrograph.

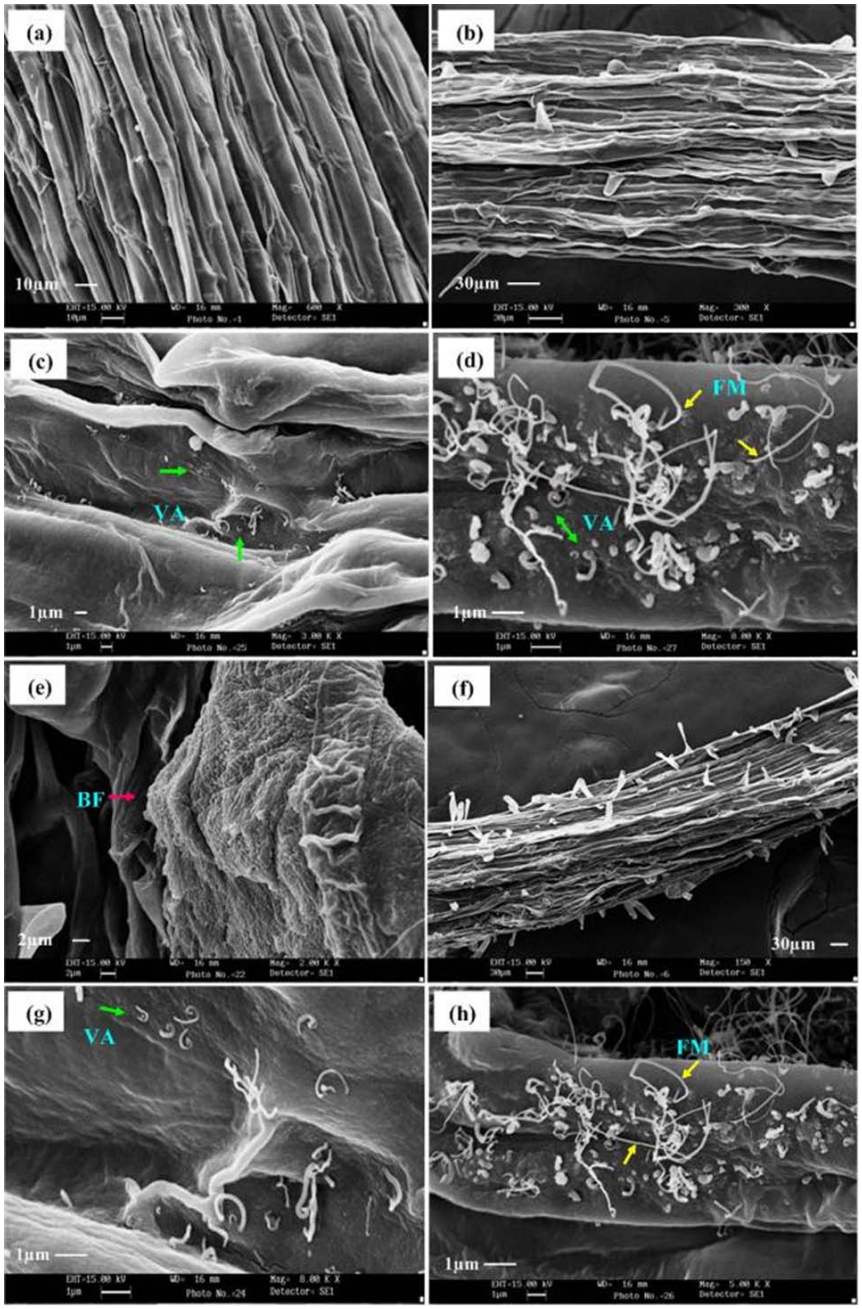

Figure 4. Scanning electron micrographs of sorghum roots: untreated roots (a, 600X), *Azospirillum brasilense* SM treated (b, 300X; c, 3000X; d, 8000X and e, 2000X), SMΔi3-6 treated (f, 300X; g, 5000X and h, 8000X). The treated roots show typical vibroid bacterial cells (VA in c, g and h) embedded in a network connected by fibrillar material (FM in d and g). The scale of magnification is mentioned in each micrograph individually with their scale bars.

cient surface colonizers of the sorghum root and hence better PGPB.

Discussion

A more robust and healthy root system helps the plant to anchor and acquire nutrients more effectively leading to better growth. PGPB, influencing plant growth utilizes various well-known strategies [14, 24]. The beneficial effects of bacterial inoculants can be realized only if they survive competitively in the rhizosphere and produce PGP biomolecules. In this context, *A. brasilense* strain SM is known to tolerate environmental fluctuations and nutrient stresses and IAA biosynthesis is

nutrient-stress inducible [21]. In contrast, it is believed that microbial activity in soils may be restricted by the small amount of available carbon [25]. The strain SM does not possess a functional repressor of IAA biosynthesis encoded by *iaaC*, thus IAA biosynthesis may not be subjected to a strict control as in case of strain Sp245 [18]. Such regulatory differences may also contribute towards selecting the strain to be used as a plant growth-promoting agent.

The improved weight of the plants and PGP response of strain SM derivatives, SMp30 and SMΔi3-6 in comparison with the wild type strain SM observed at 10- and 16-weeks confirmed that increased rhizosphere IAA levels beneficially influence sorghum growth in the long-term. Our earlier studies have shown a short-term benefit (2 weeks post-treatment) to the growth of sorghum in terms of marginally higher root length and significantly higher root branching in case of treatment with IAA over-expressing variant strains of strain SM, SMp30 and lesser root development with the low IAA expressing mutant strain, SMIT568s10 [18, 20]. Our unpublished results also show that strains with IAA accumulation levels similar to that of the wild type strain SM did not show any significant difference from the wild type PGP response (Mandira Kochar, unpublished results). This characteristic of strain SM is important in natural soils and even though limiting conditions may be encountered in nutrient-deficient soils, these bacteria can still grow well and perform their PGP function.

PCIB, the anti-auxin compound impairs the auxin signaling pathway, thereby affecting the auxin regulated physiological response in plants [26]. The concentration-dependent inhibition of root development in the presence of PCIB unambiguously proves that IAA and its over-expression are involved in the improved PGP effect of *A. brasilense* SM and its mutants on sorghum.

Following migration towards the root, the bacterial cells multiply and form small aggregates that provide them a competitive edge with respect to competition for nutrients exuding from the root or even with other bacterial cells. PGPB form biofilms on the root surface where the cells are connected by an exopolysaccharide layer that facilitates a suitable environment for them [27–29]. A similar pattern with the *A. brasilense* cells were also observed in case of strain SM-, SMp30- and SMΔi3-6- treated roots. In addition, curling of root hairs which is known to be a sign of bacterial association with the root, a priori to their penetration was observed and is similar to reports of Rhizobia [30]. Such an active association of root hairs was confirmed by microscopic analysis of sorghum roots with the strain SM-*lacZ* tagged strain SMΔpMEi3LF4.

The endophytic nature of strain SM could not be confirmed by this study and detailed electron microscopy only confirmed its surface-colonizing ability along the elongation zone of the roots and root tips. Thus, it seems to be similar in behaviour to the non-endophytic Sp7 rather than the endophytic strain Sp245. *A. brasilense* cells have been shown to bind to pearl millet, maize, wheat, rice, tomato, pepper, cotton, and soybean roots. They are predominantly known to colonize the root surface and only a few strains e.g., *A. brasilense* Sp245 and FT326 have been reported to penetrate plant cells and xylem [31, 32]. In the case of strain Sp7, the connection between its cells and the plant partner is less pronounced than in the case of the endophytic strain Sp245 [33]. Nevertheless, Azospirilla often attach to cereal roots in higher numbers than other rhizosphere bacteria, and therefore seem to be well equipped for plant root colonization [34].

The mechanism of attachment of Azospirilla to plant roots and sand is thought to involve a fibrillar material [34, 35]. Tomato roots inoculated with *A. brasilense* Cd demonstrate the majority of the surface bacterial population to consist of the typical vibroid shaped single cells surrounded by a relatively thick fibrillar material connecting them to the roots. Unlike tomato roots, colonization of pepper and cotton showed bacteria on the surface of root-hairs. The typical mode of colonization was aggregate formation, containing several cells, randomly dispersed in the elongation and root hair zones and single cells connected by a dense fibrillar network [36, 37]. SEM analysis of *A. brasilense* SM inoculated sorghum roots corroborated these findings of vibroid bacterial cells associated with the elongation zone of the roots and attachment through a fibrillar network. The only difference observed with IAA over-expressing strains SMp30 and SMΔi3-6 was the larger numbers of bacterial cells associated with the root surface and larger number of root hairs. Although similar numbers of bacteria were inoculated and re-isolated from the rhizosphere of treated plantlets (data not shown), larger presence of the IAA over-expressing strains suggested that these were better surface colonizers of sorghum than the wild type.

The root development promoted by PGPB strains like *Azospirillum* and *Pseudomonas* is related to an increase in the mineral and water uptake by inoculated plants [38]. Based on the plant growth parameters monitored, the same could be suggested for strain SM and its IAA over-expressing genetically-modified strains SMp30 and SMΔi3-6. That the IAA production and its positive effect

on sorghum by the IAA over-expressing strains of *A. brasilense* SM is over and above the effect shown by the wild type strain, confirms that metabolic engineering can lead to highly improved bioinoculant strains. Thus, metabolic engineering is a successful approach for strain improvement as improved PGPB strains generated could be used as efficient bio-inoculants to enhance root proliferation and biomass productivity.

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Conflicts of interest

The authors have no conflicts of interest.

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