

Indole acetic acid and ACC deaminase-producing *Rhizobium leguminosarum* bv. *trifolii* SN10 promote rice growth, and in the process undergo colonization and chemotaxis

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Abstract This study focuses on the chemotaxis, colonization and rice growth promoting ability of indole acetic acid (IAA) and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase-producing rhizobacteria *Rhizobium leguminosarum* bv. *trifolii* SN10, previously isolated from root nodules of *Trifolium alexandrinum* L. We show here that *R. leguminosarum* bv. *trifolii* SN10 promote the growth of four different rice varieties grown in India in terms of biomass, root branching and N content. In addition, using scanning electron microscopy and viable cell counts, we provide evidence that the bacteria successfully colonize the root surface of the rice variety which showed maximum growth promotion upon inoculation. Not only this, *R. leguminosarum* bv. *trifolii* SN10

exhibit a strong chemotaxis response towards the rice seed and root exudates despite the presence of a bacteriostatic phenolic compound, 7-hydroxycoumarin (umbelliferone). Further, *R. leguminosarum* bv. *trifolii* SN10 secretion of phytohormones such as IAA and ACC deaminase suggest the potential of the plant growth promoting rhizobacteria to be used as biofertilizer to enhance rice crop production in the subcontinent.

Keywords Chemotaxis · Flavonoids · Plant growth promoting rhizobacteria (PGPR) · *Rhizobium* · Rice

Introduction

Nitrogen-fixing bacteria, especially *Rhizobia* and *Bradyrhizobia*, have been known for many years as specific beneficial symbionts for legumes. However, studies mainly in the last decade have revealed that roots of gramineous plant-like rice (Chaintreuil et al. 2000; Yanni et al. 1997), wheat (Hilali et al. 2001), sugarcane and maize (reviewed in Bhattacharjee et al. 2008) can also host as a third niche for the nitrogen-fixing bacteria. Such plant–bacteria association was found to have great potential in improving production of non-leguminous crops, manifested in terms of increased seedling vigor, yield, growth and nutrient uptake, photosynthetic activity, stomatal conductance and N content (reviewed in Bhattacharjee et al. 2008).

The benefits conferred by rhizobial association with non-legumes have been believed to be primarily the result of plant growth promoting rhizobacterial (PGPR) activities (Hilali et al. 2001; Yanni et al. 2001). PGPR exert their growth promoting effect by a wide variety of mechanisms. These include production of growth promoting compounds, such

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as, phytohormones (indole acetic acid (IAA), gibberellins and cytokinins) and siderophores or solubilization of mineral phosphates (Glick 1995; Nelson 2004). In addition, PGPR exert antagonism towards plant pathogens by releasing metabolites like antibiotics, cell wall lytic enzymes or by inducing defense systemic responses (Nelson 2004). PGPR interaction with host plant is complex and not yet fully understood. Plant root colonization by the bacteria is considered as a primary step towards the successful initiation of the plant–microbe interaction (Nelson 2004). Colonization in turn depends on bacterial motility and chemotaxis (specific migration towards chemicals) towards the plant root release or exudates (Bashan et al. 2004). Root exudates are a rich source of nutrients for rhizospheric bacteria that undergo chemotaxis and colonize the plant root surface (Bais et al. 2006). PGPR that make their way to the plant interior are termed endophytes. In either case, the PGPR exert their beneficiary effect on the plant in terms of growth and development.

One such PGPR isolated from rice (*Oryza sativa* L.), cultivated in the field of interior Egypt, was *Rhizobium leguminosarum* bv. *trifolii* (Yanni et al. 1997). This bacterium harbored the potential to improve growth and development of various rice and wheat varieties grown in Egypt. In India, rice forms the staple food crop for more than 60% of the population. With increasing demand, the need to increase production is leading to an excessive application of chemical fertilizers. In the light of growing environmental concerns associated with chemical fertilizer, rhizobial association with cereal is gaining importance as it promises to provide a cleaner path towards maintaining crop production targets. Considering the benefits that would arise from the interaction of rice and PGPR, in this study we have assessed the growth-promoting activities of a phytohormone-producing strain of *R. leguminosarum* bv. *trifolii* (isolated from Berseem clover) on rice. Furthermore, to understand this rice–*Rhizobium* interaction, we have investigated the involvement of rhizobial chemotaxis and plant root colonization.

Materials and methods

Plant seeds and bacterial strains

The strain SN10 of *R. leguminosarum* bv. *trifolii* used in this study was obtained from CCS Haryana Agricultural University, Hisar, India (Gaur et al. 2002). *Escherichia coli* DH5 α strain was provided from Bethesda Research Laboratories (Gaithersburg, MD). The bacterial cultures were maintained on yeast extract mannitol (YEM) medium (Vincent 1970). Rice seeds were obtained from IARI, Delhi India. Seeds of *Trifolium alexandrinum* L. were obtained

from LSTM collection centre, Montpellier, France and IARI, New Delhi, India.

Gnotobiotic rice growth promotion studies

De-hulled seeds of rice were surface-sterilized by soaking in 96% ethanol for 15 min followed by 0.2% HgCl₂ for 5 min. The seeds were then rinsed several times and allowed to imbibe overnight in the dark in sterile water. To allow the germination of the seeds and to check contamination, the surface sterilized seeds were transferred to plates containing Cambel's media (Chaintreuil et al. 2000). The plates were incubated in the dark at 28°C till the plantlets developed 1- to 2-cm long roots. Rice plantlets showing no contamination were soaked for 5 min in an axenic mid-log phase (OD₆₀₀, ~0.6 to 0.8) rhizobial culture in YEM (Vincent 1970). As control, plants were inoculated with suspension of *E. coli* or heat-killed rhizobia in YEM. All the plantlets (inoculated and controls) were then transferred to 25- \times 200-mm tubes (one seedling per tube) containing 20 ml of either N-free Jensen's medium (Vincent 1970) or Jensen's medium containing KNO₃ (10 mM), supplemented with 0.8% agar. The plants were grown for 6 weeks in a growth chamber at 28°C, with a 12-h night and day cycle, adding nutrient solution (N-free Jensen's medium and Jensen's media with 10 mM KNO₃+0.2% agar) every week, under aseptic conditions. Twenty plants were used in each experiment, and three independent experiments were performed. For the dry weight, both shoots and roots were oven-dried at 60°C until the weight became constant (~5 days). The N content of the shoot was estimated by the Kjeldahl method (AOAC 1990).

Bacterial colonization counting and scanning electron microscopy

Rice roots from gnotobiotic seedlings (six plants) after 10 days of growth were carefully removed from each tube, excised, washed with sterile water, blotted dry, divided into two parts. One portion (three roots per plant) was used for viable cell count after taking the fresh weight. The number of bacteria present in the external rooting medium and on the root surface was determined by viable count method as described previously (Yanni et al. 2001). The CFU obtained from three roots in each of the three tubes were then averaged with the SD. The other portion was used for scanning electron microscopy (SEM). Roots for microscopic analysis were carefully rinsed with distilled deionised water and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7) overnight. Tissues were then washed in 0.1 M phosphate buffer, four times for 15 min each. This was followed by distilled H₂O wash 3 \times 5 min. Tissues were dehydrated in graded ethanol series. Samples were then

lyophilized for 3–4 h for critical drying. The moisture-free samples were coated with silver using the Sputter Coater E5100 (Polaron, England) by plasma coating. The thickness of the film was about 100–150 Å. The coated samples were then observed under SEM (Cambridge Setero Scan 360, UK).

Rice seed and root exudates collection

A hundred surface-sterilized seeds of *O. sativa* L. ssp. *japonica* cv. Kdulam, were soaked in 20 ml of sterile water overnight in the dark at 28°C. The solution thus obtained was termed as seed exudates for this study. For root exudates, the germinated seeds were grown on a paper support in 20 tubes containing liquid Jensen media (five seedlings per tube) for 10 days at 28°C with an 18-h light and 6-h dark period in gnotobiotic conditions. The strength of the media was reduced to 1/10 to minimize the interference of nutrient. The nutrient solution was filtered through a 0.2-µm Millipore filter. A batch of rice cultivar Kdulam was inoculated with *R. leguminosarum* bv. *trifolii* SN10. In order to remove the bacteria, root exudates obtained from this batch were centrifuged at 1,200×g for 20 min at 4°C prior to filtration. The exudates were lyophilized and were kept at –20°C for later use.

Chemotaxis assay

The chemotactic responses of the bacterial species towards rice exudates were evaluated using: (1) swarm plate assay (Adler 1973) and (2) Pfeffer's capillary tube assay (Adler et al. 1973). The exudates used for this assay were prepared by dissolving 10 mg of the lyophilized exudates in 0.5 ml of sterile distilled water. In these tests, *T. alexandrium* L. exudates were used as positive control while Phosphate Buffer Saline (PBS, pH 7.0) was used as negative control. Similarly, other chemotaxis assays were conducted by testing chemicals at a final concentration of 10 mM prepared in PBS (pH 7.0)

Detection and quantification of glucose, proline and flavonoid

Detection and quantification of D-glucose was performed using the glucose oxidase peroxidase method. This was done using the GOD–POD Merckotest^R Glucose (Merck) as described in the user's manual. For estimation of L-proline, acid ninhydrin was used as described before (Bates et al. 1973). For the detection of flavonoid compounds, lyophilized seed exudates and the root exudates were extracted with 80% methanol (~160 mg.ml⁻¹) overnight at 4°C, with shaking. The flavonoids were purified by using a Sep-Pak C18 column (Waters) as described in the user's

manual. The high-performance liquid chromatography (HPLC) equipment used for the separation and characterization of the exudates consisted of a Hewlett-Packard 1090 M liquid chromatography system equipped with a diode array detector (200–400 nm). Chromatographic runs were performed on a Waters Symmetry C18 (250×4.6 mm) column. The solvent system and elution profiles used were as described previously (Mathesius et al. 1998). For mass spectrometry (JASCO-1585, Great Dunmow, UK Ltd.) electrospray spectra were obtained using the following: LC/MS system 2790 alliance equipped with a 996 photodiode array detector connected to a Q-TOF I (Micromass UK Ltd., Manchester) electrospray mass spectrometer operated in the positive ionization mode, electrospray; capillary voltage, 3 kV; cone voltage, 40 V; source block temperature, 150°C; desolvation temperature, 250°C. The mass range scanned was 50–1,500 Da, at a rate of 3 s per scan. The column and the conditions used to analyze the samples were the same as used for HPLC/DAD analysis. The synthetic flavonoid used in the identification 7-hydroxycoumarin was obtained from Indofine Chemical Company, Inc., USA. The standards were prepared as indicated by the provider and stored in dark bottles at –20°C.

Detection of 1-aminocyclopropane-1-carboxylic acid deaminase, indole acetic acid

The ACC deaminase activity test was performed as already described (Belimov et al. 2001). Estimation of IAA was done using Salkowski's reagent as described previously (Patten and Glick 2002).

Umbelliferone tolerance test

Bacterial culture dilutions from 10⁻¹ to 10⁻⁹ of either *R. leguminosarum* bv. *trifolii* SN10 or *E. coli* DH5α were prepared from a fresh culture at OD_{600nm}=1, in liquid YEM and plated on YEM containing plates. Sterile filter paper discs similar to those used for antibiograms were treated with 50 µl of umbelliferone concentrations ranging from 1 nM to 10 mM in solution in diluted NaOH (0.01 M), and were placed on the bacteria-plated YEM plates. Diluted NaOH without umbelliferone was used as control. Plates were incubated for 48 h at 30°C. Bacterial tolerance or sensitivity was measured by the radial inhibition of growth reported in millimeters at the lowest concentration tested.

Statistical analysis

Data were analyzed by ANOVA, and the means were compared following Fisher's test of least significant difference to assess the effects of inoculation on plant growth promotion.

Results

IAA and ACC deaminase production by *R. leguminosarum* bv. *trifolii* SN10

In a culture filtrate, *R. leguminosarum* bv. *trifolii* SN10 previously isolated from roots of the leguminous plant *T. alexandrinum* (Berseem clover) synthesize IAA to a final concentration of $2 \pm 0.1 \mu\text{g}\cdot\text{ml}^{-1}$. The IAA production by the bacteria increased in the presence of L-tryptophan in the media. In the presence of this amino acid at $100 \mu\text{g}\cdot\text{ml}^{-1}$ in the media, the IAA production increased from $2 \pm 0.1 \mu\text{g}\cdot\text{ml}^{-1}$ to $25 \pm 1 \mu\text{g}\cdot\text{ml}^{-1}$. *R. leguminosarum* bv. *trifolii* SN10 were able to grow on medium containing ACC as the sole N source, confirming the ability to produce ACC deaminase. The ACC deaminase activity of *R. leguminosarum* bv. *trifolii* SN10 was measured to be $15 \pm 2 (\mu\text{mol NH}_4^+ \cdot \text{mg}^{-1} \text{ of protein} \cdot \text{h}^{-1})$.

Effect of *R. leguminosarum* bv. *trifolii* SN10 inoculation on rice growth

R. leguminosarum bv. *trifolii* SN10 were inoculated to four varieties of rice in the absence of basal N. The performance of the bacteria was scored after 30 days of growth in terms

of plant biomass, root branching and increase in N content. Plants inoculated with heat-killed rhizobia in YEM were used as control. In N-free media, rhizobial inoculation led to an increase in total plant biomass (shoot and root) ranging from 19–61% when compared to heat-killed rhizobia inoculated plants used as the control (Fig. 1a). A similar pattern of increase was observed in the root branching of the four rice varieties upon rhizobial inoculation (Fig. 1b, d). There was minimal change in the N content of the inoculated plants in the absence of basal N in the media (Fig. 1c). Among the four varieties tested, performance of *R. leguminosarum* bv. *trifolii* SN10 was significantly high when inoculated to local variety *Oryza sativa japonica* cv. Kdulam where the increase of plant biomass was almost 60%. To investigate the effect of the presence of a N source on the performance of *R. leguminosarum* bv. *trifolii* SN10, we added 10 mM of KNO_3 to the media. As *O. sativa japonica* cv. Kdulam showed maximum response to *R. leguminosarum* bv. *trifolii* SN10 inoculation, for the rest of the studies, we used this plant microbe combination. Without rhizobial inoculation, plant biomass was increased by 25% in plants grown in the presence of KNO_3 compared to the control plants grown without a N source. However, the biomass increased two times upon the addition of *R. leguminosarum* bv. *trifolii* SN10 to KNO_3 -

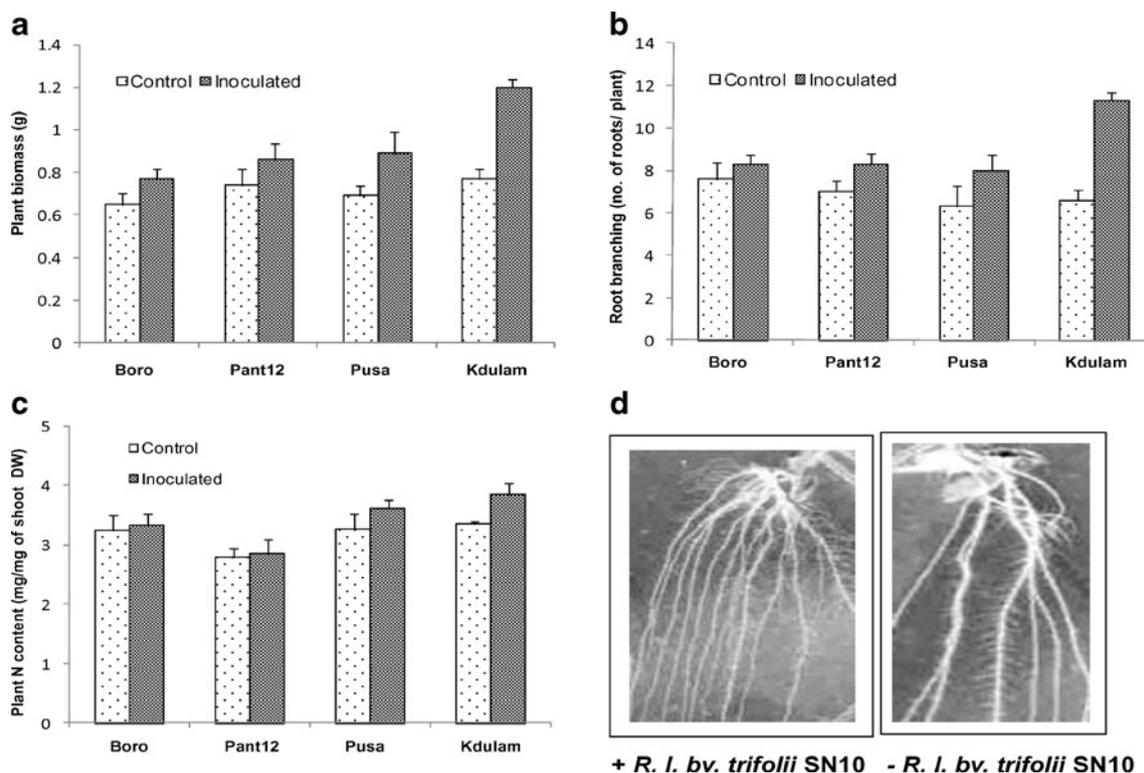


Fig. 1 Growth promotion of different rice varieties after 30 days of inoculation with *R. leguminosarum* bv. *trifolii* SN10 **a** total plant biomass; **b** root branching; **c** plant N content of the four rice varieties

with and without rhizobial inoculation, *DW* dry weight; **d** root branching observed in *O. sativa* L. subsp. *japonica* cv. Kdulam after inoculation with *R. leguminosarum* bv. *trifolii* SN10

containing media when compared to the control (+KNO₃; *R. leguminosarum* bv. *trifolii* SN10) (Table 1). Rhizobial inoculation also led to a change in root morphology in terms of root branching. When compared to the control, root branching increased to 64% in N-free medium, and to 82% in medium supplemented with N (Table 1). Of significant importance is the increase in N content in N-supplemented *R. leguminosarum* bv. *trifolii* SN10-inoculated plants. There was an increase of the shoot N content to 28% when compared to heat-killed rhizobial inoculation (Table 1).

External colonization of rice roots by *R. leguminosarum* bv. *trifolii* SN10

PGPR are known to colonize plant roots while exerting their growth promoting effect (Nelson 2004). We therefore investigated the ability of *R. leguminosarum* bv. *trifolii* SN10 to colonize rice roots by dilution plating and SEM. Roots from inoculated rice were scored for adhering bacteria for 10 days. Uninoculated and *E. coli*-inoculated plants were used as control. Loosely attached bacteria were removed by washing rice roots thoroughly with water. Washing the roots reduced the number of colonies observed by dilution plating in *E. coli*-inoculated plants. In contrast, there was only a 10% reduction in the *R. leguminosarum* bv. *trifolii* SN10-inoculated plants. Overall, the colonization of *R. leguminosarum* bv. *trifolii* SN10 on rice roots were 95–100% more than the *E. coli* and uninoculated plants (Table 2). Colonization was further confirmed by SEM. The roots of 10-day-old rice-inoculated plants were densely populated with *R. leguminosarum* bv. *trifolii* SN10 (Fig. 2).

Chemotaxis towards rice root exudates

Colonization of rice roots by *R. leguminosarum* bv. *trifolii* SN10 prompted us to investigate the in vitro chemotactic motility of the bacteria towards rice root release (exudates) *T. alexandrium* L. is the natural leguminous host plant for

R. leguminosarum bv. *trifolii*, so the root/seed exudates of the plant were used as positive control for this experiment. The average distance moved by *R. leguminosarum* bv. *trifolii* SN10 in rice (Kdulam) seed exudates containing plate was equivalent to the distance traveled in the presence of *T. alexandrium* L. seed exudates and root exudates. When exudates and free compounds were replaced by PBS in the assay, there was no swarm by *R. leguminosarum* bv. *trifolii* (Table 3). *E. coli* did not show any significant movement in the presence of the exudates. Further corroboration of these results was obtained by Pfeffer's capillary tube (Table 3). Pfeffer's capillary tube result showed that seed exudates attracted almost twofold higher bacteria than rice root exudates.

In addition, sugars and amino acids earlier reported to be present in rice seed and root exudates in the first week of germination were individually tested (Bacilio-Jimenez et al. 2003). Among the free sugars and amino acids tested (listed in Table 3), *R. leguminosarum trifolii* SN10 showed a significant chemotactic response towards D-glucose (10 mM) and L-proline (10 mM) in both soft and capillary tube. *R. leguminosarum* bv. *trifolii* SN10 exhibited a positive response even at lower concentrations (2 and 5 mM) of D-glucose and L-proline. The bacterial strain exhibited a significantly lower chemotactic response to other amino acids and sugars in soft agar and capillary tube. However, *E. coli* exhibited a positive chemotaxis towards glucose (data not shown).

Detection of D-glucose and L-proline in rice root and seed exudates

A chemotaxis-promoting compound glucose was found to be an important constituent of *O. sativa* subsp. *japonica* cv. Kdulam seed exudates ($1.16 \pm 0.1 \mu\text{g}\cdot\text{mg}^{-1}$ of seed exudates) and root exudates ($0.25 \pm 0.05 \mu\text{g}\cdot\text{mg}^{-1}$ of root exudates). In addition, proline was also detected but at a lower concentration than glucose in both seed exudates

Table 1 Plant growth and physiological parameters after inoculation of *O. sativa* L. subsp. *japonica* cv. Kdulam with *R. leguminosarum* bv. *trifolii* SN10

Inoculation	Medium	Shoot DW (g)	Root DW (g)	Root Branching (number of roots per plant)	N content (in mg/g of shoot DW)
<i>R. leguminosarum</i> bv. <i>trifolii</i> SN10	N-free	0.84 ± 0.05b	0.40 ± 0.06a	11.3 ± 0.5a	3.85 ± 0.05a
	KNO ₃ (10 mM)	1.00 ± 0.05a	0.42 ± 0.06a	12.0 ± 0.5a	5.25 ± 0.05b
Heat-killed rhizobia in YEM	N-free	0.52 ± 0.04b	0.27 ± 0.05b	6.9 ± 0.1b	3.35 ± 0.05c
	KNO ₃ (10 mM)	0.76 ± 0.05b	0.25 ± 0.05b	6.6 ± 0.1b	4.10 ± 0.05

Plants inoculated with heat-killed inoculum of *R. leguminosarum* bv. *trifolii* SN10 in YEM medium were the control. Results are means ± standard deviation of three independent experiments with each treatment measured on 20 plants. All data with the same letters are not significantly different ($P \leq 0.05$)

DW dry weight

Table 2 Bacterial colonization of *O. sativa* sp. *japonica* cv. Kdulam after inoculation with either *R. leguminosarum* bv. *trifolii* SN10, *E. coli* DH5 α , killed inocula or sterile YEM (controls)

	Rhizosphere (growth media) in CFU/ml	Rice root in CFU/gfw of tissue
<i>R. leguminosarum</i> bv. <i>trifolii</i> SN10	6.3×10^7 ($\pm 0.4 \times 10^7$) a	6.7×10^5 ($\pm 0.7 \times 10^5$) b
<i>E. coli</i> DH5 α	1.2×10^3 ($\pm 0.5 \times 10^3$) c	ND
Controls	ND	ND

Results are means \pm standard deviation of three independent experiments with each treatment measured on three repetitions. All data with the same letters are not significantly different ($P \leq 0.05$)

CFU colony forming unit, gfw gram fresh weight, ND not detected

($0.66 \pm 0.05 \mu\text{g}\cdot\text{mg}^{-1}$) and root exudates ($0.25 \pm 0.05 \mu\text{g}\cdot\text{mg}^{-1}$). These concentrations corresponded from 13.2 to 23.2 mM of D-glucose and 5 mM of L-proline in root and seed exudates respectively, which are in the range of concentrations of free D-glucose and L-proline tested for positive chemotaxis of *R. leguminosarum* bv. *trifolii* SN10.

Rice root releases a phenolic compound that inhibit *E. coli* growth

Phenolic compounds released by plants can act as biocontrol for microbial growth (Siqueira et al. 1991). We therefore investigated the rice root exudates for the presence of phenolic compounds that inhibited *E. coli* chemotaxis in seed and root exudates despite of the presence of a high concentration of glucose in the latter. HPLC-MS analysis indicated that a phenolic compound 7-hydroxycoumarin (retention time of 15.40 min and absorption maxima of 322.85 nm) is present at a detectable concentration in uninfected exudates (Fig. 3a). The mass spectrometry analysis

of both molecular ions (M+H)⁺ and (M+AcN)⁺ of this compound gives m/z 163.06 and 204.08 which is in agreement with the structure of umbelliferone (data not shown). However, its concentration steeply increases from $15 \pm 1.5 \mu\text{g}\cdot\text{g}^{-1}$ (without rhizobia) to $58 \pm 2 \mu\text{g}\cdot\text{g}^{-1}$ in *E. coli* and *R. leguminosarum* bv. *trifolii* SN10-infected exudates (Fig. 3b). We therefore checked the sensitivity of *R. leguminosarum* bv. *trifolii* SN10 towards different concentrations of 7-hydroxycoumarin in vitro. At a high concentration of 10 mM, 7-hydroxycoumarin does not have any adverse effect on rhizobial survival. However, in the case of *E. coli*, hollow zones indicating bacterial inhibitions were observed at all concentrations tested.

Discussion

Over the years, the growing demand of rice has led to the indiscriminate use of chemical fertilizers around the world. With arising concerns about the effects of fertilizer use on the

Fig. 2 Colonization of roots of *O. sativa* L. subsp. *japonica* cv. Kdulam, *R. leguminosarum* bv. *trifolii* SN10, 10 days after inoculation. SEM photograph showing general colonization of the surface of secondary roots **a** uninoculated rice plant, **b–d** rice plant inoculated with *R. leguminosarum* bv. *trifolii* SN10

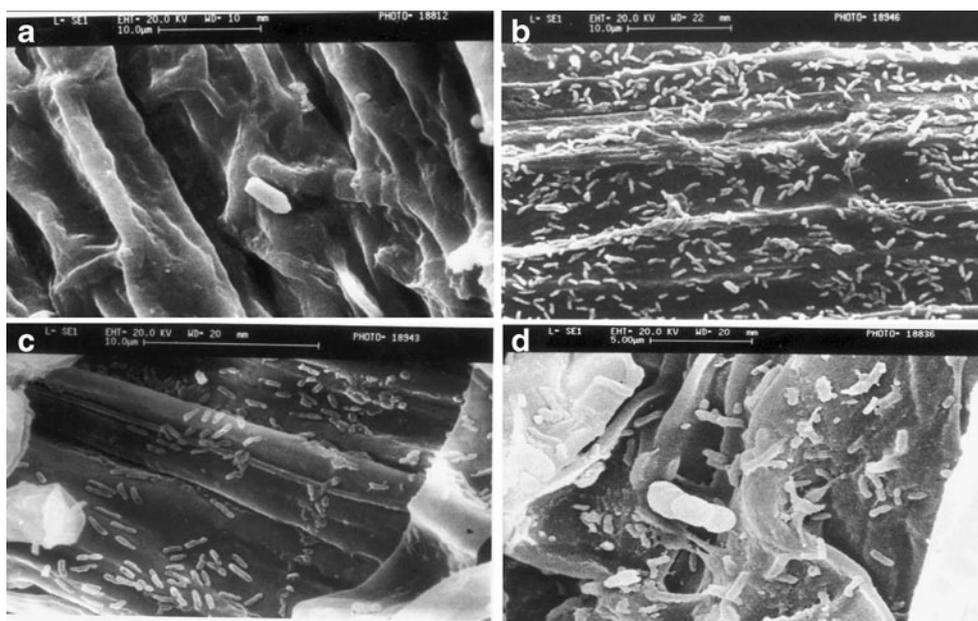


Table 3 Induction of chemotaxis of *Rhizobium leguminosarum* bv. *trifolii* SN10

Chemo attractant	Swarm diameter ^a	Number of bacteria ($\times 10^3$) in Pfeffer capillary tube (Adler 1973)
Plant exudates		
<i>Oryza sativa</i> L. ssp. <i>japonica</i> cv. Kdulam		
Seed exudates	++	25 \pm 1
Root exudates	++	17 \pm 1.5
<i>Trifolium alexandrinum</i>		
Seed exudates (20 mg/ml)	++	30 \pm 2.75
Root exudates (20 mg/ml)	++	27 \pm 2.5
Chemicals		
Amino acids (10 mM)		
L-Proline	+	12 \pm 2
L-Histidine	–	0.05 \pm 0.1
L-Valine	–	0.25 \pm 0.05
L-Alanine	–	0.20 \pm 0.07
L-Glycine	–	0.07 \pm 0.02
Carbohydrates (10 mM)		
D-Glucose	++	21 \pm 1.5
D-Mannose	–	1.5 \pm 0.5
D-Galactose, D-arabinose, D-xylose	–	2.7 \pm 0.25
D-Glucuronic acid	–	0.04 \pm 0.01
Control		
Phosphate buffer (pH 7, 10 mM)	–	0.02 \pm 0.05

++ swarm diameter between 6 and 8 mm, + swarm diameter between 3 and 6 mm, – swarm diameter between 1 and 2 mm

^aRefers to the movement of the swarm diameter on the 2nd day after inoculation

environment, the quest for non-consequential alternatives is being promoted. India is one of the largest rice producers in the world. In this context, we found that a clover symbiont synthesizing ACC deaminase and IAA, *R. leguminosarum* bv. *trifolii* SN10, could enhance growth of various varieties of rice grown in the subcontinent. In this association, *R. leguminosarum* bv. *trifolii* SN10 acts as a PGPR, undergoes chemotaxis and successfully colonizes the plant resisting the inhibitory effect of an antimicrobial compound released by the plant.

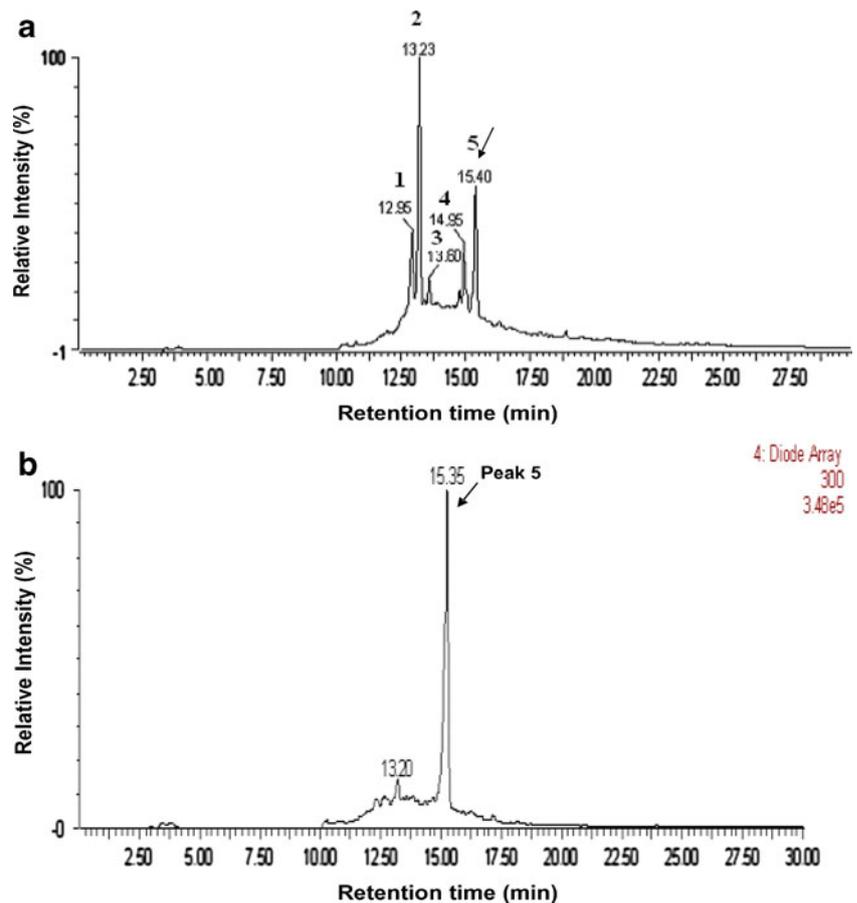
As a general feature, we observed that *R. leguminosarum* bv. *trifolii* SN10 isolated from the legume *T. alexandrinum*, has stimulatory effects on different rice varieties evaluated in terms of shoot and root biomass and root branching. However, the effect of the bacteria is dependent on the variety of rice. Emphasis on the selection of the combination of bacteria and plant for obtaining optimum response has been made previously by other workers (Biswas et al. 2000a; Pedraza et al. 2010; Yanni et al. 2001). *R. leguminosarum* bv. *trifolii* are known to fix N in vitro as well as in association with legume (O'gara and Shanmugam 1978; Kishinevsky et al. 1992). However, it has been observed that *R. leguminosarum* bv. *trifolii* does not fix N in association with rice (Biswas et al. 2000; Yanni et al. 1997, 2001). Thus, the performance of the bacteria improved further when used in supplementation to N in the media. We can therefore

speculate that IAA and ACC deaminase production by *R. leguminosarum* bv. *trifolii* SN10 may account for the observed increase in plant biomass in biotised plants (Glick 1995; Khalid et al. 2005; Shaharoon et al. 2006; Zahir et al. 2011). Root branching would provide an increased surface area for adsorption of nutrients (Yanni et al. 1997) in soil explaining the additional gain in rice plant biomass and N content upon *R. leguminosarum* bv. *trifolii* SN10 inoculation.

In addition to phytohormone production by the bacteria, plant growth promotion also depends on the establishment of the inoculated strain in the rhizosphere. Thus colonization of the host plant by PGPR is a prerequisite for exerting a growth-promoting effect on the host plant (Larcher et al. 2003). A huge colonization on the surface of *O. sativa japonica* roots by *R. leguminosarum* bv. *trifolii* SN10 as against the *E. coli*-inoculated batch, indicates the specific rice root colonizing capacity of the bacteria. *R. leguminosarum* bv. *trifolii* SN10 adherence to the root was significant as loosely attached bacteria could be removed by washing. Our observation is thus in accordance with other studies which have shown that bacteria that promote plant growth also colonize the host plant (Larcher et al. 2003; Pedraza et al. 2010; Vande Broek et al. 1998).

It has been suggested that a prerequisite for the effective colonization of roots is positive chemotaxis towards root exudates (Vande Broek et al. 1998). A positive chemotaxis

Fig. 3 Flavonoid analysis of rice root exudates. HPLC profile of rice root exudates **a** without rhizobial inoculation, **b** with rhizobial inoculation



of *R. leguminosarum* bv. *trifolii* SN10 towards *O. sativa japonica* cv. Kdulam exudates therefore suggests that rice seed and root exudates release compounds that attract bacteria towards the plant leading to colonization. We also observed that *R. leguminosarum* bv. *trifolii* SN10 showed a significant chemotaxis towards glucose and proline which constituted considerable percentages of the seed and root exudates. The higher chemotaxis of *R. leguminosarum* bv. *trifolii* SN10 towards seed exudates than root exudates similar to the observation of Kato and Yasuhiro (2006) may be due to the higher concentration of glucose and proline in seed exudates. Most microorganisms use glucose, and in the case of proline as a source of C, thus *E. coli* showed positive chemotaxis towards glucose in the plate assay (Adler et al. 1973; Vilchez et al. 2000). Chemotaxis was also observed by some endophytic bacteria towards rice root exudates (Bacilio-Jimenez et al. 2003), *Azospirillum* towards strawberry (Pedraza et al. 2010) and *Nostoc* towards non-host *Trifolium repens* root extract (Nilsson et al. 2006). Our results suggest that in the case of this rice–*Rhizobium* interaction, biomolecules like glucose and proline function as major chemoattractants for the chemotaxis of *R. leguminosarum* bv. *trifolii* SN10.

The accumulation of high concentrations of 7-hydroxycoumarin upon inoculation with *R. leguminosarum* bv. *trifolii* SN10 in rice (*O. sativa japonica* cv. Kdulam) root exudates is a stress response towards microbial invasion. The coumarins are known to inhibit the growth and sporulation of fungal plant pathogens and to provide as well a defense tool against hostile microorganisms (Matern et al. 1999; Weinmann 1997). This high concentration of 7-hydroxycoumarin in these rice exudates produced upon microbial invasion might be the inhibitory compound that restricts *E. coli* chemotaxis (Duncan et al. 1998) as *E. coli* is sensitive to umbelliferone at any concentration.

The present results clearly indicate that *R. leguminosarum* bv. *trifolii* SN10, a symbiont of legumes can colonize rice root surfaces and confer beneficiary effects by enhancing root branching, increasing the biomass and N content of the plant, under laboratory conditions. Their colonization is subject to chemotaxis towards biomolecules released by rice root exudates. *R. leguminosarum* bv. *trifolii* SN10 confers the growth promoting activity to *O. sativa japonica* cv. Kdulam as a PGPR and resists the antimicrobial activity of the root release. Thus, *R. leguminosarum* bv. *trifolii* SN10 stands as a potential candidate for further field

studies which would be helpful in extending their use as a PGPR for rice in India.

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