

Original Research Article

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Isolation and Characterization of Plant Growth Promoting Endophytic Diazotrophic Bacteria from Wheat Genotypes and their Influence on Plant Growth Promotion

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ABSTRACT

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Nitrogen (N) is an essential element for the growth and yield of wheat. Some endophytic bacteria can fix N₂ from the air and convert to nitrogen compounds that can be utilized by plants. In this study, endophytic bacteria were isolated from one-month-old seedlings of 13 wheat genotypes growing without nitrogen fertilizer in the ICAR –IARI field. A total of 23 diazotrophic endophytes were isolated from 13 wheat genotypes used. Nitrogenase enzyme activities of endophytes were measured by acetylene reduction assay (ARA). Among 23 isolates, 17 isolates showed the IAA production, three isolates exhibited siderophore production ability and 13 isolates showed ammonia production ability. Thirteen isolates give clear halo zone around the colony on tricalcium phosphate containing Pikovaskaya's agar plates. None of them exhibited HCN production ability. Nitrogenase activity of isolates was ranged from 5 to 30 nmol C₂H₄/mg protein/h. Endophytes inoculation significantly increase the root length, root fresh weight and root dry weight. Likewise, endophytes inoculation also showed significant results on fresh weight and dry weight of shoot. Both the endophytes were identified as *Azospirillum* sp. by 16S rRNA gene sequencing.

Introduction

During the period of green revolution, high yielding varieties of crops have been developed to accomplish food demand for increasing population of India. Synthetic fertilizer and pesticides has applied for increasing production of crops and reduction of production loss because of disease development which create environmental and soil health problems. Cultivation of high-

yielding varieties of crops, intensive cropping system, and imbalance use of chemical fertilizers is the main factors which develops nutrient imbalance in soil, low productivity, decrease soil fertility and low quality of food. Thus, it is becoming a serious problem to develop sustainable approaches for alleviation of detrimental influence of intensive practices used by farmers (Elkoca *et al.*, 2010). Plant growth promoting endophytes mediates

increased growth and yield of crops through direct or indirect mechanism. The direct mechanism of growth promotion of crops includes nitrogen fixation, phosphorus solubilization, production of phyto-hormones (e.g. auxins, gibberellins and cytokinins), siderophore production for iron sequestering and low production of ethylene (Idris *et al.*, 2007; Verma *et al.*, 2013). Application of microorganisms as inoculants enhances the availability of nutrients in soil and decreases the requirement of chemical fertilizers (Cakmakci *et al.*, 2007a). Nitrogen fixing plant growth promoting rhizobacteria/endophytic bacteria plays important role in addition of nitrogen in soil. Available various reports in literature confirmed that diazotrophic endophytes have been isolated from root, stem and leaves of several plants. Vigorous research in this area started after the isolation of the endophytic diazotrophic bacterium *Gluconacetobacter diazotrophicus* from a Brazilian variety of sugarcane (James and Olivares, 1998). Diazotrophic endophytes also trigger the plant growth and soil fertility via production of phytohormones, phosphate (P) solubilization, siderophore production and inhibition of or resistance to, certain pathogens (Hallman *et al.*, 1997; Bottini *et al.*, 2004; Conn and Franco, 2004; Chung *et al.*, 2005; Son *et al.*, 2005). Keeping in view this notion, we attempted the isolation and characterization of diazotrophic bacterial endophytes from wheat genotypes and their influence on plant growth.

Materials and Methods

Wheat genotypes

Seeds of 13 different wheat genotypes, namely CIM 412, WSM 24, 4HPYT 433, 4HPYT404, HD 2967, DPW 621-50, HPW B1, T-297/4HPYT 415, 4HPYT 414, GW-07-112, HD 3086, and T-311/4HPYT 429 were collected from Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi.

Cultivation of different wheat genotype

Different genotypes of wheat were cultivated without nitrogen fertilizers amendment at field of ICAR-IARI, New Delhi for 30 days. Three replicates were maintained for each genotypes.

Isolation of diazotrophic bacterial endophytes and enrichment in nitrogen fixing bacterium medium

After 30 days of sowing, shoots and roots samples were collected from different wheat genotypes for endophytes isolation. Nitrogen free semi solid media was used for isolation of diazotrophic bacterial endophytes. The fresh plant samples (1 g of shoot or root) were surface sterilized sequentially using 0.1% HgCl₂ for 2 minutes and 70% alcohol for 60 seconds; washed with ten changes of sterile distilled water and crushed using mortar pestle under aseptic condition. The suspension was inoculated in nitrogen free semi solid medium for enrichment of nitrogen fixing microorganisms. After 3 subsequent culturing of the microorganisms from pellicle region after serial dilutions, were streaked on nutrient agar medium. The plates were incubated at 30°C for 24-48 hrs and the colony growth was observed.

Plant growth promoting activities of endophytes

IAA production

IAA production was determined by inoculation of endophytic bacteria in tryptophan (5µg/ml) containing nutrient broth and incubated at 28±2°C for 3 days. After incubation period, cultures were centrifuged at 5000 rpm for 10 minute. 10 ml of supernatant was mixed with two drops of orthophosphoric acid and 5 ml Salkowski's reagent (50 ml, 35% perchloric acid; 1 ml 0.5 FeCl₃). Pink to red colour development

indicated of IAA production ability; the optical density (OD) was recorded at 530 nm using a spectrophotometer. The amount of IAA estimated by standard IAA graph and expressed as µg/mL (Patten and Glick, 1996).

Phosphorus solubilization

Phosphate solubilization ability of endophytes was determined by spotting on tricalcium phosphate containing Pikovskaya (1948) agar plates. The plates were incubated at $28 \pm 2^\circ\text{C}$ for 5–7 days. Appearance of a clear zone around endophytes colonies indicated the P-solubilization ability.

HCN production

HCN production ability of endophytes was determined using Castric's method (Castric, 1975). All endophytic bacteria used, were grown in 10% tryptone soy agar supplemented with glycine (4.4 g l^{-1}). A Whatman filter paper No. 1 soaked in 2% sodium carbonate and 0.5% picric acid solution was placed to the underside of the Petri dish lids and the plates were sealed with parafilm and incubated at 30°C for 5-7 days. Change in colour of filter paper from yellow to red-brown indicated HCN production ability of endophytes.

Ammonia production

Detection of ammonia production was done by adding 1 ml of Nessler's reagent to 72 h old cultures grown in peptone broth and positive samples exhibited yellowish brown colour (Dye, 1962).

Siderophore production

Ability to produce siderophore was analysed by spot inoculation of each isolate on nutrient agar medium supplemented with chrome azurol S (CAS) dye solution (Milagres *et al.*, 1999). Plates were incubated at 30°C for 5

days for halo zone formation and the presence of orange halo zone around the colonies was taken as a positive test.

ACC deaminase assay

The ACC deaminase activity of the strains was assayed according to the method of Glick *et al.*, (1995).

Nitrogenase activity

Acetylene reduction assay (ARA) was used for examination of nitrogenase activity of endophytes. Each endophyte culture was inoculated in 20 ml capped tube containing 10 ml of nitrogen free semi solid agar media. The 10% volume of capped tube was replaced with 10% acetylene (v/v) and incubated at 30°C for 24 hours. Ethylene formation was measured by gas chromatography. The nitrogenase activity was calculated in unit $\text{nmol C}_2\text{H}_4/\text{mg protein/h}$ (Lee and Yoshida, 1997).

Hydroponic experiment

For determination of influence of two selected diazotrophic endophytes DS-5 and DS-10 on plant growth a hydroponic experiment was carried out in phytotron at ICAR-Indian agricultural research institute, New Delhi. The wheat seeds were sterilized using 0.1% HgCl_2 and 70% alcohol. The broth cultures of both the endophytes were raised separately such that the inoculum contained 10^9 cfu mL^{-1} .

The sterilized seeds were coated with inoculum by soaking the seeds in broth for 30 minute. Seeds coated only with nutrient broth were used for control treatment. Seeds were germinated on wet cotton and 0.3% agar. After 4 days, the seedlings were transferred into the Hogland solution (1X) containing hydroponic system. Three replications were maintained for each of the three treatments.

Analysis of plant growth and biomass

For observation of root length, shoot length, fresh weight and dry weight of root and shoot plants were harvested from hydroponic system and washed thoroughly with tap water followed by washing with 0.1% HCl and then distilled water and then determined the root length, shoot length and fresh weight of root and shoot.

For determination of dry matter, root and shoot were air dried, after air drying, root and shoot sample were kept in paper bags, dried at 60°C to a constant weight and weighed.

Identification of efficient endophytes

Genomic DNA was extracted by using ZR fungal/bacterial DNA Mini Prep™ genomic DNA isolation kit (ZYMO Research Corporation, USA) and amplified 16S rRNA gene of selected efficient endophytes using PA and PH universal primer. Amplified products of 16S rRNA gene were purified using Qiagen purification Kit.

Sequencing of the purified 16S rRNA gene was done by Sci-Genome Pvt. Ltd., Bangalore. Obtained forward and reverse sequence of 16S rRNA gene was aligned by CAP3 assembly. Aligned sequences were analyzed for maximum homogeneity with available 16S rRNA gene sequence in NCBI database through Blast tools and used the similarity index for their identification.

Statistical analysis

Data was analysed through one way ANOVA and grouping/ranking among various parameters were done by Tukey's method. Significance among parameters was analysed through two way ANOVA at 0.05 levels.

Results and Discussion

Isolation, abundance and distribution of endophytes

A total of 23 diazotrophic endophytes were isolated from 13 wheat genotypes. 4HPYT-404 and K-65 showed maximum abundance of diazotrophic endophytes followed by GW-07-112, HD-3086 and T-311/4HPYT-429. Diazotrophic endophytes distribution was found more in roots in comparison to shoot (Fig. 1).

Characterization of plant growth promoting activities of isolated diazotrophic endophytes

Among 23 isolates, a total seventeen isolates, three isolates and thirteen isolates exhibited IAA production, siderophore production and phosphate solubilization ability respectively. None of them exhibited HCN production ability. Thirteen isolates also showed ammonia production ability. Nitrogenase activity of isolates was ranged from 5 to 30 nmol C₂H₄/mg protein/h (Table 1 and Fig. 2). On the basis of these PGP activities DS-5 and DS-10 were selected for further study.

Influence of endophytes on plant growth

Endophytes inoculation significantly increased the root length, root fresh weight and root dry weight. Likewise, endophytes inoculation also showed significant results on fresh weight and dry weight. Endophytes inoculations were showed no significant result irrespective to shoot length of wheat seedling. There was no significance difference among the treatments. Diazotrophic endophytes inoculation increased the root length, root fresh weight and root dry weight by 61%, 107% and 100% over the control, respectively.

Table.1 Characterization of PGPR activities of diazotrophic endophytes isolated from wheat genotypes

Isolates	IAA (µg/mL)	Siderophore	Phosphate solubilization	HCN	Ammonia	Nitrogenase activity (nmol C ₂ H ₄ /mg protein/h)	ACC deaminase activity
DS-1	5	-	-	-	+	12	-
DS-2	7	-	-	-	+	11	-
DS-3	-	-	-	-	+	21	-
DS-4	5	-	-	-	+	23	-
DS-5	19	+	+++	-	+++	24	-
DS-6	-	-	-	-	+	21	-
DS-7	3	-	-	-	-	12	-
DS-8	11	-	-	-	-	11	-
DS-9	14	-	-	-	+	5	-
DS-10	23	+	++++	-	+++	30	-
DS-11	12.4	-	-	-	-	21	-
DS-12	11.5	-	-	-	-	31	-
DS-13	10	-	-	-	-	15	-
DS-14	-	-	-	-	-	14	-
DS-15	7	-	-	-	-	13	-
DS-16	6	-	-	-	-	12	-
DS-17	12	-	-	-	-	13	-
DS-18	-	-	+	-	-	14	-
DS-19	11.56	+	+	-	+	16	-
DS-22	8.5	-	+	-	+	23	-
DS-21	-	-	-	-	++	27	-
DS-22	6.5	-	-	-	+	28	-
DS-23	-	-	-	-	+	30	-

Table.2 Influence of endophytic diazotrophic bacteria on plant growth

Treatments	Root length (cm)	Root fresh weight (g)	Root dry weight (g)	Shoot length (cm)	Shoot fresh weight (g)	Shoot dry weight (g)
Control	13.33±1.53 ^b	0.46±0.04 ^b	0.17±0.02 ^b	20.33±2.52 ^a	1.50±0.10 ^b	0.54±0.05 ^b
DS-5	20.33±2.52 ^{ab}	0.91±0.09 ^a	0.32±0.04 ^a	25.00±3.00 ^a	3.00±0.26 ^a	1.01±0.14 ^a
DS-10	22.33±2.16 ^a	1.00±0.10 ^a	0.34±0.06 ^a	24.67±2.08 ^a	2.59±0.40 ^a	0.97±0.09 ^a

Fig.1 Abundance and distribution of different endophytic diazotrophic bacterial morphotypes isolated from various wheat genotypes using nitrogen free semi solid media

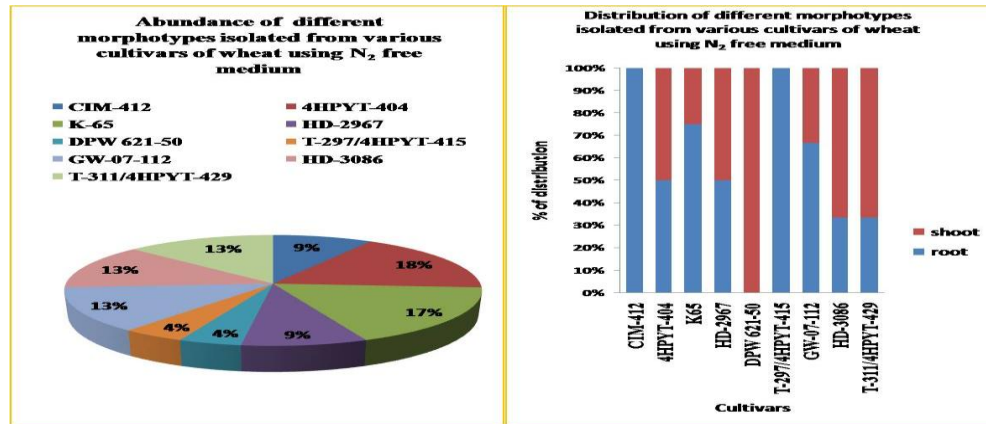
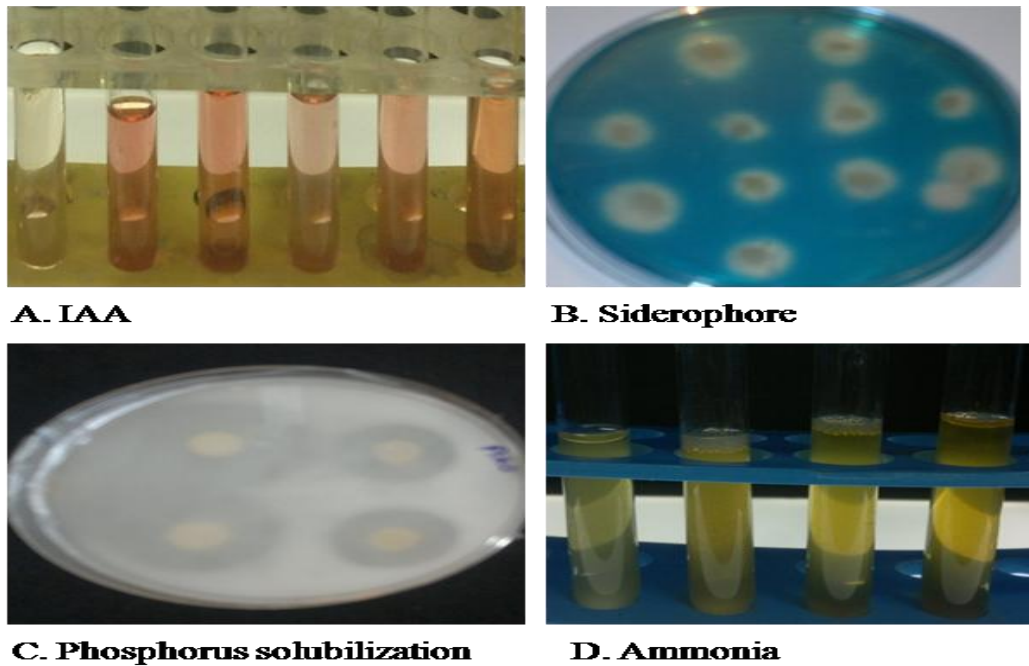


Fig.2 Screening of diazotrophic endophytes for plant growth promoting activities



Likewise, its inoculation increased the shoot fresh weight and shoot dry weight by 86% and 84% over the control respectively (Table 2).

Results of this study clearly demonstrate the presence of diazotrophic endophytic bacteria within the roots and shoots of different wheat genotypes used. Umpteen reports are available in literature on the existence of endophytic bacteria in roots and shoots of

various plants (Hallman *et al.*, 1997; James and Olivares, 1998; Muthukumarasamy *et al.*, 2005). The occurrence of 23 morphotypes from the roots and shoots of 13 wheat genotypes is an interesting observation. However, occurrence of as many as 20 types of endophytic bacteria, comprising both nitrogen-fixing and non-nitrogen-fixing from roots and shoots of rice plants has been reported (Ladha and Reddy, 2000). Existence of higher proportion of diazotrophic

endophytes in roots compared with shoots supports the presumption that roots may be a prime niche for growth and nitrogen fixation by endophytes (James and Olivares, 1998; Ladha and Reddy, 2000). Although there were distinct differences in their pellicle formation, nitrogenase activity, IAA production, P solubilization activity, siderophore production and ammonia production. Sub pellicle formation in nitrogen free semisolid media by diazotrophic endophytes agrees with the reports on several *Azospirillum* species (Dobereiner, 1995). Sub pellicle formation by endophytes indicates that isolates fix nitrogen preferably under semi-anaerobic condition. Acetylene reduction assay confirmed the diazotrophic nature of all 23 isolates. Our results showed that inoculation of diazotrophic endophytes significantly increase the root length, root fresh weight, root dry weight, shoot fresh weight and shoot dry weight. Similar observations have been made in other diazotrophic endophytes, such as *Gluconacetobacter diazotrophicus* in sugarcane (James and Olivares, 1998), *Azoarcus* spp. in rice and kallar grass (Reinhold-Hurek and Hurek, 1998), *Herbaspirillum* spp. in sugarcane and rice (James and Olivares, 1998) and *Serratia marcescens* in rice (Gyaneshwar *et al.*, 2001). Plant growth-promoting effect of DS-5 and DS-10 could be because of nitrogen fixation alone or the combined effects of IAA and P solubilization activity. Further work based on field trials is needed to test the usefulness of these isolates. In conclusion, results of the present study show that wheat genotypes do harbour plant growth-promoting endophytic bacteria.

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