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Original Article

Insights into cross-host colonization and nitrogen fixation in wheat by *Populus euphratica*-associated endophytic diazotrophs

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Abstract

Endophytic diazotrophic bacteria from non-host crops represent a promising source for developing novel bioinoculants. This study isolated and characterized nitrogen-fixing bacteria from the leaves and twigs of *Populus euphratica* and assessed their ability to colonize and promote growth in Wheat. From fifty-two isolates obtained on nitrogen-free media, five potent strains—identified by 16S rRNA sequencing as Beijerinckia fluminensis FA-7, Stenotrophomonas maltophilia FA-9, Pseudomonas aeruginosa FA-16, Klebsiella pneumoniae FA-4, and Enterobacter cloacae FA-8 were selected for their nitrogen fixation and plant growth-promoting (PGP) traits. These strains exhibited multiple PGP activities, including phosphate solubilization, indole-3-acetic acid (IAA) production, and the production of siderophores and exopolysaccharides. In growth chamber and wirehouse experiments under varying nitrogen regimes [full (N+), limiting (N-), and zero (N0)], all strains enhanced at least one wheat growth parameter. Notably, Beijerinckia fluminensis FA-7 significantly improved early growth, nutrient uptake, and root architecture—increasing root length, surface area, and fine root development—as confirmed by WinRhizo analysis. Our findings demonstrate that endophytic diazotrophs from Populus can successfully colonize wheat and enhance its growth, highlighting their potential as effective microbial inoculants for agriculture.

Keywords: Endophytes, PGPR, Driazotroph, Nitrogen fixation, Wheat

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Introduction

Nitrogen fixing endophytes are utilized as bio-inoculants for improving crop yield, as well as part of integrated plant nutrition management systems (Ranjan and Yadav, 2019).All cereal crops, especially wheat require an adequate supply of nutrients in the form of fertilizer and thus increasing the cost of cereal crop production. The excessive and inappropriate use of nitrogen fertilizer results in low N use efficiency (NUE), where 50–70% of applied nitrogen fertilizer is lost to the environment, resulting in major environmental issues. As a result, diazotrophs are receiving a lot of attention these days as an environmentally acceptable instrument for enhancing productivity in agricultural and environmental research (Rashid et al., 2019). These bacteria are a natural way of delivering fixed nitrogen to plants, into a form that can be used by plants. In terms of aiding their host through nitrogen fixation, endophytic bacteria outperform their rhizospheric and rhizoplanic counterparts because they may supply fixed nitrogen directly to their host (Preyanga et al., 2021). Endophytic bacteria, in addition to fixing nitrogen, may promote plant development by one or more pathways, by improving the availability of key plant nutrients for cellular activity and metabolic activity such as N, P, Fe and Zn; by modulating growth hormones such as auxin, ethylene (ACC deaminase), cytokinins, and gibberellins; by producing siderophores such as thiamine, biotin, riboflavin and niacin, vitamins and solubilizing phosphorus(Ranawat et al., 2021). Trees are not provided with any fertilizer and irrigation like other field crops (Dey et al., 2004). Some trees, thousands of years old are growing in nutrient limited soil even in climate that are suboptimal for plant growth, besides the fact that tree nitrogen requirement is high. A number of trees have evolved multiple strategies in association with diazotrophs to deal with nitrogen deficiency. Diazotrophic bacteria colonizing trees have a tremendous scope to increase crop yield by supplying nitrogen to support sustainable agriculture. Populus (*Populus euphratica*) growing in harsh and nutrient-limited environments across Pakistan can be the potential candidates for hosting diazotrophic endophytes with role in nitrogen fixation and abiotic stress tolerance(Vandana et al., 2021). Their ability to grow in low nitrogen conditions is generally attributed to the presence of diazotrophic endophytic bacteria. These findings strengthen the hypothesis that tree diazotrophs may endophytically colonize roots, stems and leaves of the cereals and may provide a part of fixed nitrogen to plant. Adding biological fractions of nitrogen to cereal crop can enhance its production in sustainable way as chemical fertilizers have already been overused.

Material and Methods

Isolation of endophytes on nitrogen free media and its biochemical characterization

Healthy field-grown tree twigs and leaves of populus that were healthy and free of disease were collected from various places at the University of Agriculture, Faisalabad. After being moved right away to the lab, the samples were cleaned under running water, cut into 10cm pieces, and then dried on paper towels. Every macerated tree sample was serially diluted to 10^{-4} or 10^{-7} in order to isolate and count endophytic bacteria from the tree's aerial portions. A leaf suspension was produced. Each test tube containing nitrogen-free semisolid media, such as Lipman growth Ivo medium (LGI), nitrogen-free bromothymol blue medium (NFB), and combined carbon medium (CCM), which are made by adding agar 1.5 to 1.8 g L^{-1}.100 µL of each dilution, was inoculated in order to isolate nitrogen fixing endophytes.

Potassium (K), qualitative & quantitative phosphate (P) solubilization activity and Indole 3-acetic acid (IAA)

The amount of IAA (indole 3-acetic acid) generated by endophytic diazotrophs with and without L-TRP was determined by using a spectrophotometer. The ability of the colonies to solubilize potassium and phosphorus was demonstrated by the presence of a halo zone surrounding them called potassium solubilization index (KSI) and phosphorus solubilization index (PSI). This was determined by measuring the zone diameter at different intervals while the isolates were incubated on their particular media. Alexandrov agar medium was used to evaluate K solubilization, while Pikovskaya agar plates were used to test P. Another indicator of P solubilization efficiency is the P solubilization efficiency (PSE). Using a tricalcium phosphate (TCP) bioleaching assay to prepare a fresh culture of isolates, the P solubilization activity of the chosen endophytes was also quantitatively assessed. Soluble P in culture supernatant was estimated using the Molybdenum blue method, compared to

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uninoculated control and expressed as equivalent P as mg L^{-1}.

Identification of strains through 16S_r RNA

Each bacterial isolate was identified using the molecular approach of 16S rRNA gene sequencing. The DNA Data Bank of Japan (DDJB) supplied the accession numbers, and Macrogen Korea (http://dna.macrogen.com/eng) received the pure isolated strains in for gene sequencing. Each sample was sent in a single 1.5mL centrifuge tube in agar stab culture and placed in the Ziplock bag. The 16S rDNA gene sequences of all isolates were obtained and compared to the organisms' 16S rDNA gene sequences found in the GenBank databases. It was discovered that the five isolates contained a range of rhizosphere bacteria with various physiological and biochemical traits.

Growth room experiments under axenic conditions

The best five strains selected as inoculation for wheat plant; their ability to fix nitrogen and promote development led to their selection for the growth chamber investigation. At the Nuclear Institute for Agriculture and Biology (NIAB), a controlled experiment lasting 15 days was carried out in a growth chamber. The chosen bacteria were tested for colonization efficiency and growth promotion activities at lower nitrogen levels in an axenic environment in a growth chamber investigation. Five isolates were selected for the wheat crop in the growth chamber experiment: FA-7, FA-9, FA-16, FA-4, and FA-8. One millilitre of a suspension containing 106–107 CFU/mL was used to inoculate the plants. 200 g of sterilized sand were put into 500 mL plastic cups. Cups were kept in a growth room with a regulated climate. Three centimetres of sterilized sand was used to plant six seeds each pot, which were then culled to produce one seedling per container. All inoculated plants were fertilized with three levels of nitrogen using ammonium nitrate (NH_4NO_3). The treatments included Inoculation (nitrogen fixing bacteria) + (0 mg N kg^{-1}) 0% of the recommended N fertilizer, bacterial Inoculation (nitrogen fixing bacteria) + (20 mg N kg^{-1}) 50% of the recommended N fertilizer and Inoculation (nitrogen fixing bacteria) + (40 mg N kg^{-1}) +100% of the recommended N fertilizer. 50 mL of N-free Hoagland nutrition solution was added to each cup in a 1:2 ratio after sterile distil water was used for irrigation. Seedling biomass (fresh, dry mass of root and shoots) and seedling length (length of root and shoots) were measured during harvest.

Evaluation of rhizosphere and endophytic colonization

Seedlings from each treatment were harvested ten days after inoculation. Roots and shoots were separated to evaluate rhizosphere colonization. Following serial dilutions, a 100 mL aliquot was plated on agar plates. After the plates were incubated for seven days at room temperature, colonies were counted. To evaluate endophytic colonization, seedlings were surface sterilized. The tissues of the stem, roots, and leaves were serially diluted and macerated on CCM plates. Following seven days of culture at room temperature, colonies were counted.

Estimating the abundance of ^{15}N in wheat plants

The nitrogen fixation of bacterial strains and the effect of inoculation on plant growth output and nitrogen levels were evaluated using the 15N abundance method. With a 5% a.e. (atomic access), ^{15}N was utilized as urea. For 15N analysis, wheat seedlings were cultivated in plastic pots for this investigation. The Kjeldahl method was used to calculate the total nitrogen in plants. The plants were first mixed with a digesting mixture containing K_2SO_4, $CuSO_4$, and Se before being digested. A gas isotope ratio mass spectrometer (IRMS) was used to test for 15N after all samples were evaporated to a volume of 5 mL and delivered to the Pakistan Institute of Nuclear Science and Technology (PINSTECH) in Islamabad. The sodium hypobromite was used to liberate the 15N. Nitrogen fixation was determined using % ^{15}N a.e (atomic excess) formula. The equations utilized are listed below.

Natural abundance:

$$\%Ndfa = (\delta^{15}N_{NFS} - {}^{15}N_{FS} / \delta^{15}N_{NFS}) \times 100$$

^{15}N isotope dilution method:

$$\%Ndfa = (1 - \%\ {}^{15}N_{FS} / \%\ 15N_{NFS}) \times 100$$

where FS stands for fixing system, NFS for non-fixing system, $\delta^{15}N$ for natural abundance of ^{15}N, and %Ndfa for nitrogen fixation.

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Root analysis using WinRhizo image analysis system

WinRHIZO is an image analysis system created primarily for root morphorlogy analysis in various formats. Morphology, including root length, area, volume, number of tips, topology, architecture, and color, can be analyzed by it. It is made up of components for collecting images and a computer application that can be modified to meet different needs. Clean roots can be automatically or interactively analyzed using WinRHIZO. Excel data files provide a thorough summary of the sample's measurement data, which is also displayed on the screen. The WinRhizo image analysis system was used to analysed the roots in a pot experiment under gnobiotic environment. For the purpose of root examination, wheat seedlings were cultivated in tiny glass jars for this investigation. Wet the root and gently wash it to release it from the soil. To stop root loss during washing, a steel filter is positioned underneath the sample. The organic matter and soil particles should be thoroughly cleansed before photographing the roots. After dipping the roots for three minutes in 0.1% methyl violet made with 8 mM $CaSO_4$, they were washed with distilled water. On the scanner was a clear tray that was partially filled. Water is necessary to spread the roots and organize the cleaned roots in the transparent tray.

The analysis is completed and saved in the text and image files.

Wire house experiment

A pot trial was conducted to assess the effectiveness of endophytic diazotrophs in promoting wheat growth in soil at the University of Agriculture, Faisalabad. The best five strains selected on the basis of growth promotion activity in axenic study were used to inoculate the wheat. Each plastic pot had twelve kg of soil for the wheat plant. After a bacterial suspension containing 108 $CFUmL^{-1}$ was inoculated, four seeds were planted in each pot. Fresh isolate inoculum (1 mL per seed) was applied to wheat seeds. Only two plants remained viable after ten days. In the wire house, a randomized full block design was used for the trial. The treatments included Inoculation (nitrogen fixing bacteria) + 0% of the recommended N fertilizer (0 g N kg^{-1}), Inoculation (nitrogen fixing bacteria) + 50% of the recommended N fertilizer (0.6 g N kg^{-1}) and Inoculation (nitrogen fixing bacteria) + 100% of the recommended N fertilizer (1.2 g N kg^{-1}). Harvesting was carried out at 30 days after planting to determine seedling biomass (dry mass of root and shoots) and Seedling length (length of root and shoots).

Statistical analysis

Analysis of variance (ANOVA) was used to evaluate the obtained data in a completely randomized design (CRD), and Statistix 8.1 software was used to compare the mean values using least significant design (LSD) at a 0.5% probability level. The graphs were created using the Prism program (GraphPad, 8.0.1).

Results

Screening for *in vitro* plant growth-promoting characteristics such as potassium solubilization index (KSI), phosphorus solubilization index (PSI), Quantitative Phosphorus solubilization and indole-3-acetic acid (IAA) synthesis

Numerous traits that are known to promote plant growth, stress tolerance, or biocontrol were investigated. The results of the characterisation are summarised in Table 1. Isolates of nitrogen-fixing endophytes were examined for K solubilisation. With the exception of isolate FA-4, the majority of isolates tested positive for K solubilisation, according to the results. The isolate FA-9 generated the largest PSI of 1.65, while the isolate FA-7 displayed the maximum K halo zone diameter (1.42 cm). The findings showed that all isolates had the ability to solubilise tri-calcium phosphate, or inorganic phosphate, with a range of 2.04 to 2.82 mg L^{-1}. The minimum quantity of insoluble phosphate was solubilized by FA-4 (2.04 mg L^{-1}) and the maximum was solubilized by FA-7 (2.82 mg L^{-1}), followed by FA-16 (2.70 mg L^{-1}), FA 9-2.27 (2.2.07 mg L^{-1}), and FA-8 (2.26 g L^{-1}). Isolated bacterial strains were screened for their ability to synthesize IAA quantitatively. Without tryptophan, the maximum production of IAA was carried out by FA-7 (2.70 mg L^{-1}), followed by FA-8 and FA-4 which synthesized IAA in the range of 2.46 mg L^{-1} and 2.63 mg L^{-1}, respectively. Significant differences in IAA production were recorded with the addition of tryptophan, with the maximum IAA production (2.56 mg L^{-1}) was observed by FA-7, followed by FA-8 (2.44 mg L^{-1} and FA-4 (2.22 mg L^{-1}), indicating the ability of tryptophan to act as a precursor in IAA production (Table 1).

Table-1. Growth-promoting characteristics of wheat endophytes.

	Strains				
Characteristics	**FA-4 Klebsiella pneumoniae**	**FA-7 Beijerinckia fluminensis**	**FA-8 Enterobacter cloacae**	**FA-16 Pseudomonas aeruginosa**	**FA-9 Stenotrophomonas maltophilia**
Potassium solubilization index (KSI)	0.21±0.05	1.42±0.00	0.32±0.00	0.26±0.00	0.20±0.04
Phosphorus solubilization index (PSI)	0.69±0.03	1.73±0.07	1.50±0.00	1.45±0.08	1.65±0.03
Quantitative Phosphorus solubilization	2.04±0.37	2.82±0.18	2.26±0.22	2.70±0.21	2.27±0.19
IAA (μg ml^{-1}) with tryptophan	2.22±0.09	2.56±0.35	2.44±0.28	2.15±0.20	1.88±0.10
IAA (μg ml^{-1}) without tryptophan	2.46±0.22	2.70±0.61	2.63±0.47	2.37±0.07	2.01±0.32

Identification of strains through 16Sr RNA

All of the bacterial isolates were identified using molecular techniques for 16S rDNA gene sequencing. The DNA Data Bank of Japan (DDJB) supplied the accession numbers as stated, and each strain was sent to Macrogen Korea (http://dna.macrogen.com/eng) for gene sequencing. The 16S rDNA gene sequences of all isolates were obtained and compared to the species' 16S rDNA gene sequences found in the GenBank databases. It was discovered that the five isolates contained a range of endophytic bacteria with various physiological and biochemical traits. Five strains were identified: FA-7 (Beijerinckia fluminensis), FA-9 (Stenotrophomonas maltophilia), FA-16 (Pseudomonas aeruginosa), FA-4 (Klebsiella pneumoniae), and FA-8 (Enterobacter cloacae) (Table 2).

Table-2. Identification of Strains Through 16Sr RNA.

Strain ID	Genus	Specie
FA-7	Beijerinckia	Beijerinckia fluminensis
FA-9	Stenotrophomonas	Stenotrophomonas maltophilia
FA-16	Pseudomonas	Pseudomonas aeruginosa
FA-4	Klebsiella	Klebsiella pneumoniae
FA-8	Enterobacter	Enterobacter cloacae

Endophytic and rhizospheric colonization

Beijerinckia fluminensis colonized the wheat rhizosphere in all growth trials, FA-7, resulting in 4.24×10^5 cfu g^{-1} in the rhizosphere. 4.69×10^5 cfu g_{-1} in roots, 3.82×10^3 cfu g^{-1}. FA-7 was also detected in shoots on the TSA and CCM root, stem, and leaf imprint plates. In both growth trials, there was no indication of FA-4 strain endophytic colonization in the stem and leaf tissues, whereas FA-4 Klebsiella pneumoniae endophytically colonized wheat roots with 2.27×10^5 cfu/g fresh weight (Table 3).

Table-3. Colonization of isolated nitrogen fixing bacteria in Rhizosphere, roots and shoots.

Strain ID	Specie	Rhizosphere Endophytes (cfu g^{-1})	Root Endophytes (cfu g^{-1})	Shoot Endophytes (cfu g^{-1})
FA-7	Beijerinckia fluminensis	4.24×10^5	4.69×10^5	3.82×10^4
FA-9	Stenotrophomonas maltophilia	4.00×10^5	3.46×10^5	3.49×10^4
FA-16	Pseudomonas aeruginosa	4.16×10^5	3.38×10^5	3.44×10^4
FA-4	Klebsiella pneumoniae	0.68×10^5	2.27×10^5	0.06×10^4
FA-8	Enterobacter cloacae	2.71×10^5	2.83410^5	2.06×10^4

Image analysis system to assess the effect of nitrogen fixing endophytes on wheat root morphology

The chemistry of the rhizosphere and the responses of the wheat crop's roots to root growth are significantly influenced by nitrogen-fixing bacteria and varying nitrogen dosages. The results clearly demonstrate that both nitrogen fertilizers and inoculation significantly enhanced the wheat's root volume (cm^3), length per volume (cm/m^3), surf area, average diameter (mm), root tips, and root length (cm). In comparison to the non-inoculated control, the morphology of wheat roots was significantly altered by five chosen strains applied to wheat seeds. Applying FA-7 to wheat seeds resulted in a substantial increase of 44%, 23%, and 25% in root length, surface area, and fine roots, respectively ($p < 0.05$). Wheat seeds treated with Stenotrophomonas maltophilia FA-16 showed a substantial 21% increase in fine root length ($p < 0.05$) (Table 4). Doses of nitrogen fertilizer and bacterial treatment were found to interact on morphology of wheat root (Figure 1).

Table-4. Effect of nitrogen fixing endophytic bacterial isolates on morphology of wheat roots.

	Strains				
Growth parameters	**FA4 Klebsiella pneumoniae**	**FA-7 Beijerinckia fluminensis**	**FA-8 Enterobacter cloacae**	**FA-16 Pseudomonas aeruginosa**	**FA-9 Stenotrophomonas maltophilia**
Root length (cm)					
No nitrogen	78.0±0.012	212.3±0.049	135.2±0.015	138.9±0.136	165.2±0.036
Half nitrogen	132.4±0.006	273.4±0.023	229.3±0.003	158.9±0.003	192.2±0.009
Full nitrogen	183.2±0.012	332.5±0.024	379.4±0.022	256.6±0.022	191.9±0.001
Surf area					
No nitrogen	12.45±0.73	24.70±0.44	14.83±0.60	16.86±0.88	18.72±0.29
Half nitrogen	15.62±0.87	31.96±0.60	26.51±0.58	20.33±0.33	22.79±1.01
Full nitrogen	21.65±1.15	39.05±1.15	38.57±0.00	31.63±0.73	23.06±0.58
Average diameter (mm)					
No nitrogen	0.32±0.00	0.38±0.73	0.36±0.58	0.37±0.58	0.39±0.58
Half nitrogen	0.37±0.29	0.38±0.60	0.38±0.58	0.37±0.44	0.41±0.33
Full nitrogen	0.35±0.88	0.51±0.29	0.38±0.29	0.37±0.60	0.39±0.76
Root tips					
No nitrogen	480±0.33	606±0.43	911±0.30	1104±0.62	542±0.80
Half nitrogen	1377±0.69	639±0.57	801±0.98	1058±0.17	735±2.38
Full nitrogen	2338±0.01	840±0.47	937±0.71	836±0.73	1215±0.95
Length per volume (cm/m^3)					
No nitrogen	183.17±0.05	332.51±0.02	191.87±0.03	256.64±0.02	379.45±0.00
Half nitrogen	132.38±0.02	273.43±0.05	192.24±0.06	158.86±0.06	229.34±0.03
Full nitrogen	78.00±0.02	212.27±0.09	165.17±0.01	138.88±0.06	135.20±0.03
Root volume (cm^3).					
No nitrogen	0.204±0.07	0.365±0.20	0.221±0.01	0.31±0.23	0.312±0.00
Half nitrogen	0.147±0.11	0.297±0.01	0.215±0.05	0.207±0.02	0.244±0.03
Full nitrogen	0.158±0.03	0.229±0.16	0.169±0.10	0.163±0.06	0.129±0.01

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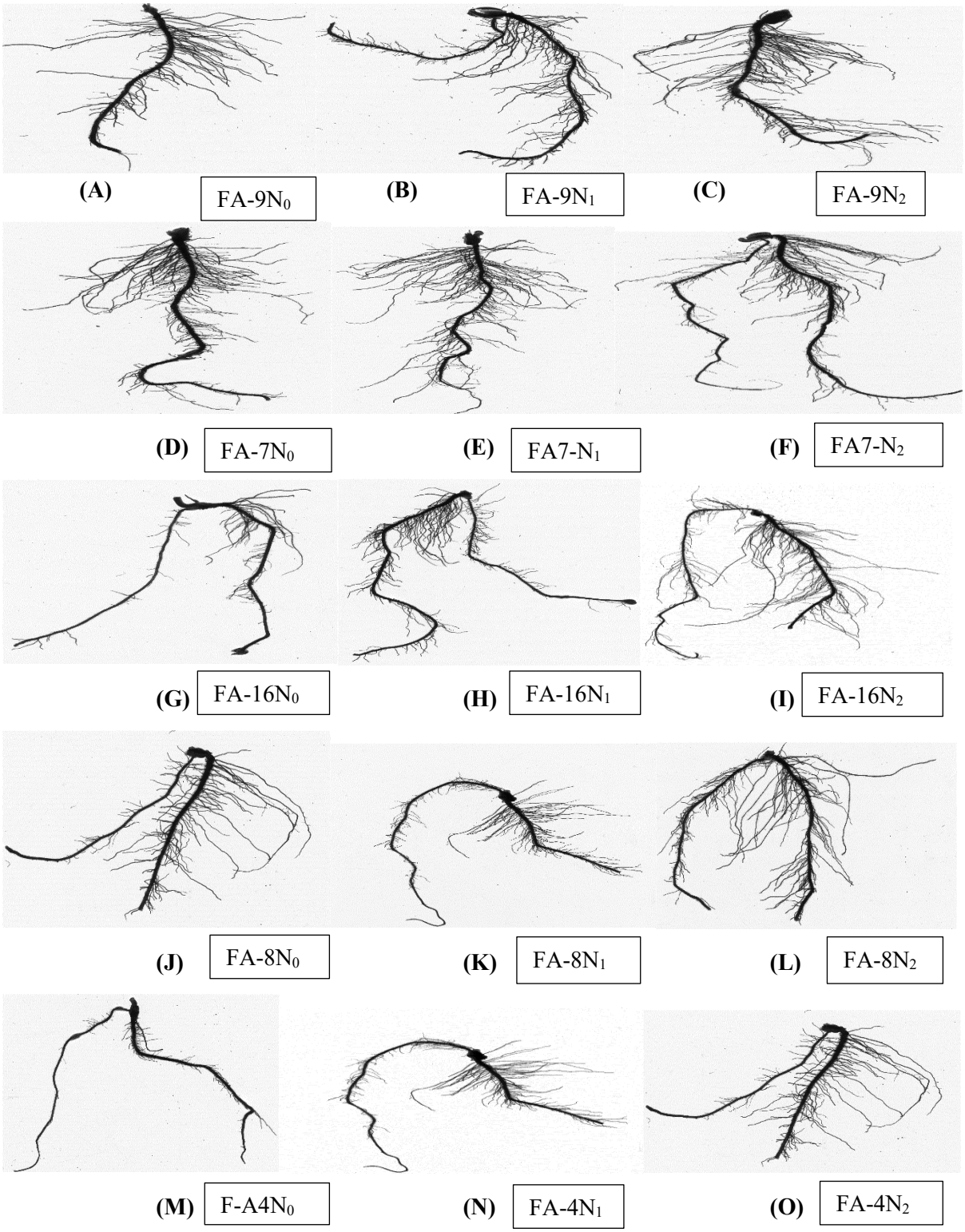

Figure-1. WinRHIZO pro-software root scan study of wheat root treated with bacterial strains (A)FA-9 at N_o (unfertilized rate) (B) FA-9 at N_1 (half-recommended rate) (C) FA-9 at N_2 (full-recommended rate) (D) FA-7 at N_o (unfertilized rate) (E) FA-7 at N_1 (half-recommended rate) (F) FA-7 at N_2 (full-recommended rate) (G) FA-16 at N_o (unfertilized rate) (H) FA-16 at N_1 (half-recommended rate) (I) FA-16 at N_2 (full-recommended rate (J) FA-8 at N_o (unfertilized rate) (K) FA-8 at N_1 (half-recommended rate) (L) FA-8 at N_2 (full-recommended rate) (M) FA-4 at N_o (unfertilized rate) (N) FA-4 at N_1 (half-recommended rate) (O) FA-4 at N_2 (full-recommended rate) compared with control.

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^{15}N analysis of in vivo N_2 fixation

All the inoculated plants had lower ^{15}N% abundance than their corresponding uninoculated counterparts, suggesting that biologically fixed nitrogen intake (%Ndfa) is the cause of the ^{15}N dilution. In all three trials, FA7 displayed the lowest ^{15}N dilution. Each bacterial strain has a different %Ndfa number, which can range from 2% to 9%. With the exception of FA 4, which showed no discernible impact on the percentage of Ndfa following the addition of N fertilizer, external N fertilization decreased Ndfa. The maximum proportion of Ndfa was found in FA7 + No nitrogen (11.89%), while the lowest percentage was found in FA4 + N_0 (2.94%). Data on the percentage of foliar 15N atom surplus for inoculated and control seedlings in the axenic growth trial showed that seedlings inoculated with FA-7 drew 8.35% of the foliar N from atmosphere (Ndfa) in the full nitrogen treatment, 9.25% in the second treatment of half nitrogen harvest, and 11.89% in the third treatment of no chemical nitrogen (Table 5). Seedlings inoculated with FA-9 obtained 6.88% of the foliar N from the atmosphere in the full nitrogen, 8.48% in the half nitrogen, and 9.38% in the no nitrogen treatment.

Table-5. The effect of nitrogen-fixing strain inoculation on wheat plant growth, its N contents, and ^{15}N abundance in sterilized soil, as well as estimations of biologically fixed nitrogen**.**

	%15N abundance			% N dfa		
Isolates	**No nitrogen**	**Half nitrogen**	**Full nitrogen**	**No Nitrogen**	**Half nitrogen**	**Full nitrogen**
FA-4 Klebsiella pneumoniae	0.56±0.20	1.12±0.03	1.83±0.04	4.14±0.20	4.23±0.07	2.94±0.06
FA-9 Stenotrophomonas maltophilia	0.54±0.09	0.97±0.03	1.61±0.03	9.38±0.05	8.48±0.08	6.88±0.21
FA-8 Enterobacter cloacae	0.53±0.02	0.94±0.02	0.91±0.04	6.72±0.02	5.63±0.02	3.88±0.02
FA-16 Pseudomonas aeruginosa	0.52±0.02	0.88±0.02	0.97±0.03	8.59±0.02	6.72±0.03	4.25±0.46
FA-7 Beijerinckia fluminensis	0.51±0.04	0.77±0.05	0.94±0.02	11.89±0.02	9.25±0.02	8.35±0.06

Growth promoting activity of selected endophytic bacteria under axenic conditions

Inoculation of nitrogen-fixing bacterial strains resulted in a considerable surge shoot length, root length, shoot fresh weight and root fresh weight of seedlings as shown by results. The responses of the bacterial strains varied greatly in terms of shoot length. When compared to the uninoculated reference, all five isolates under study showed a significant increase in shoot length, both with and without nitrogen treatments, with the exception of two (FA-7 and FA-16). FA-7 (23.19 cm) produced the most increase in shoot length when infected with a full dose of nitrogen, with up to 93% more shoot length than the uninoculated control. Compared to the uninoculated control, FA-16 (20.08 cm) produced up to 66% more shoot length. With two isolates (FA-4, FA-8) at zero nitrogen dose, all of the selected nitrogen-fixing bacterial strains significantly increased root length after inoculation. The other three isolates (FA-9, FA-7, and FA-16) all performed better than the uninoculated control at N_0, N_1, and N_2 half and full dosages of nitrogen in terms of growing root length, with an 80% increase in root length. The complete nitrogen dose of FA-7 and FA-9 inoculation at N0

resulted in a statistically significant increase in root length of 16 cm and 17 cm, respectively, in comparison to the control. Isolate FA-7 inoculation produced the greatest increase in shoot fresh weight (94 percent over the uninoculated control). Following FA-16 and FA-8 at N2, the fresh weight of the shoots increased by 88% and 72%, respectively, above the uninoculated control. When given a full dose of nitrogen, isolate (FA-7, 3.20 cm) outperformed the uninoculated control, increasing root fresh weight by 99 percent. FA-7 and FA-16 were the next most successful isolates, increasing root fresh weight by 93% and 86%, respectively, over the control. According to data on the shoot dry weight of wheat saplings under axenic settings, five isolates (FA-4, FA-8, FA-9, FA-16, and FA-7) significantly increased the dry weight of wheat shoots at zero dose of nitrogen than the control treatment. After receiving a half dose of nitrogen, isolates FA-7 and FA-9 considerably boosted the dry weight of the shoot by up to 81% as compared to the control. The findings demonstrated that different levels of root dry weight were raised by inoculation with nitrogen-fixing bacterial strains. The isolates that increased root dry weight the most were FA-7, FA-9, FA-8, FA-4, and FA-16. These isolates were infected with the full amount of nitrogen. FA-7 and FA-9 were the most effective, causing a statistically equal and up to 83% increase in dry root weight when compared to the uninoculated control (Table 6).

Table-6. Effect of selected nitrogen fixing endophytic bacterial isolates on Shoot length, root length, shoot fresh weight, root fresh weight, shoot dry weight, root dry weight of wheat cultivars. Means are given with standard error of three replicates and LSD is statistically significant differences at probability of $p \leq 0.05$.

	Strains					
	Without strain	**FA-4 Klebsiella pneumoniae**	**FA-9 Stenotrophomonas maltophilia**	**FA-8 Enterobacter cloacae**	**FA-7 Beijerinckia fluminensis**	**FA-16 Pseudomonas aeruginosa**
No nitrogen	7.66±0.28	16.92±0.36	14.28±0.67	14.54±0.05	16.66±0.15	15.78±0.60
Half nitrogen	10.87±0.40	17.31±0.96	15.92±0.58	15.44±1.06	20.74±0.69	17.33±0.96
Full nitrogen	13.91±1.01	20.04±0.31	16.96±1.40	18.26±0.53	23.19±1.10	20.08±0.24
Root length(cm)						
No nitrogen	3.91±0.01	13.45±1.24	14.81±1.20	12.50±0.52	17.53±0.61	16.94±1.10
Half nitrogen	7.20±0.95	15.96±1.37	15.88±0.33	14.51±0.79	18.51±0.88	16.53±0.62
Full nitrogen	8.55±1.22	15.58±1.49	16.11±0.63	16.43±1.08	20.22±1.09	19.79±0.26
Shoot fresh weight(g)						
No nitrogen	0.86±0.09	1.08±0.03	1.14±0.01	1.37±0.16	0.75±0.03	1.54±0.09
Half nitrogen	0.95±0.03	1.74±0.02	1.67±0.04	1.88±0.02	0.96±0.21	2.02±0.23
Full nitrogen	1.64±0.03	2.04±0.11	2.83±0.04	3.09±0.01	1.20±0.31	3.20±0.06
Root fresh weight (g)						
No nitrogen	0.53±0.02	1.08±0.01	1.05±0.02	1.13±0.29	1.2±0.19	0.68±0.11
Half nitrogen	0.67±0.03	1.13±0.06	1.13±0.04	1.24±0.20	1.29±0.38	1.26±0.07
Full nitrogen	0.80±0.08	1.54±0.22	1.57±0.03	1.73±0.04	2.43±0.27	0.87±0.14

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Shoot dry weight(g)						
No nitrogen	0.26±0.02	0.35±0.02	0.33±0.01	0.49±0.03	0.54±0.03	0.30±0.02
Half nitrogen	0.32±0.03	0.45±0.01	0.44±0.02	0.56±0.01	0.58±0.03	0.46±0.21
Full nitrogen	0.45±0.02	0.54±0.02	0.61±0.02	0.83±0.03	0.92±0.01	0.53±0.16
Root dry weight (g)						
No nitrogen	0.21±0.003	0.32±0.058	0.30±0.034	0.31±0.012	0.40±0.023	0.28±0.024
Half nitrogen	0.42±0.003	0.62±0.004	0.61±0.002	0.73±0.003	0.77±0.035	0.65±0.079
Full nitrogen	0.53±0.033	0.71±0.004	0.74±0.024	0.83±0.008	0.95±0.004	0.88±0.006

Screening beneficial nitrogen fixing endophytic bacteria to promote wheat plant growth in a wire house study

The data showed significant increases in wheat plant height and shoot fresh weight following inoculation with all of the identified nitrogen-fixing bacterial strains. Inoculation with three isolates (FA-7, FA-9, and FA-16) at a nitrogen level of 0 kg ha^{-1} resulted in a significant increase in shoot length, with plant height increases of up to 25%, 21%, and 17%, respectively, compared to the control. At a nitrogen level of 60 kg ha^{-1}, the isolate FA-7 showed a maximum increase in shoot length of 34% over the corresponding control. At the same nitrogen level and after inoculation at a nitrogen level of 120 kg ha^{-1}, it was statistically comparable to FA-9. According to data on shoot fresh weight, all isolates were able to increase fresh shoot weight both with and without nitrogen, with notable outcomes at a nitrogen level of 0 kg ha^{-1}. At zero nitrogen, FA-7 reached a maximum fresh shoot weight of 123% more than the control, followed by FA-9, which caused a 115% increase in shoot fresh biomass. FA-4 produced the smallest increase in fresh shoot weight, which was 44% higher than the control and statistically equivalent to both the control and FA-8. The effect of the inoculation with certain bacterial strains was significant at nitrogen levels of 0 kg ha^{-1} when compared to each other and the applicable control. In the absence of nitrogen, three isolates (FA-7, FA-9, and FA-8) yielded notable outcomes in terms of 100-grain weight. When compared to the control, all of the other isolates produced a 48% increase in 100-grain weight and were statistically identical to FA-4. The data in the image contrasts the number of spikes of wheat grown in pots with the impact of adding nitrogen fertilizer either by itself or in combination with the diazotroph inoculants FA-7 and FA-9. According to the results, applying nitrogen fertilizers by themselves resulted in a significantly higher number of tillers (27.3%) than using unfertilized treatments (control). However, applying nitrogen fertilizer in conjunction with the inoculation FA-7 improved this difference from 27.3% to 112.1%. When used in conjunction with the concurrent inoculation of FA-7 at zero dose of nitrogen fertilizer, the number of tillers was statistically equivalent to the control treatment (Figure 2).

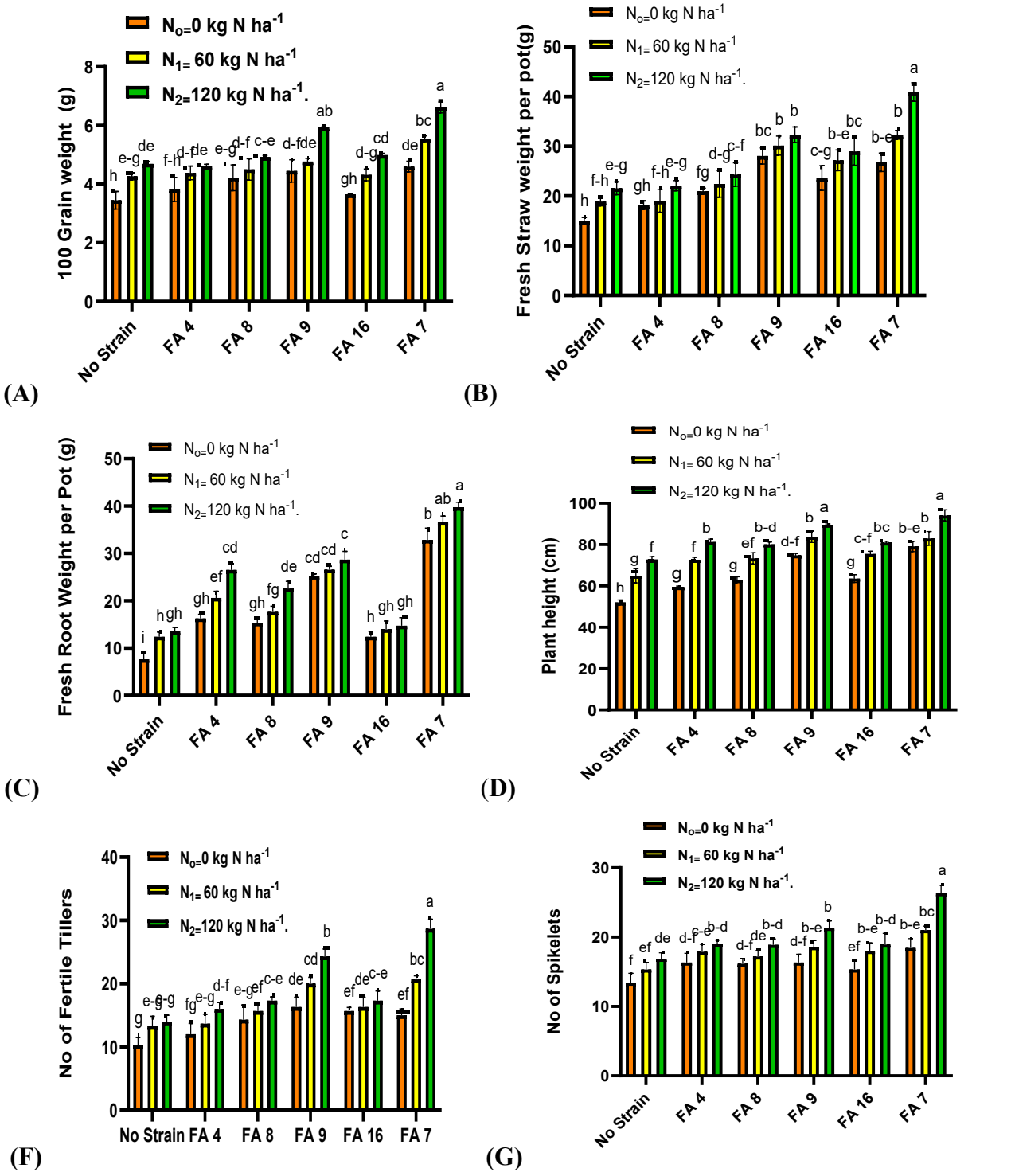

Figure-2. Plant growth-promoting (PGP) endophytic bacteria with nitrogen-fixing activity in boosting wheat growth in a wire house trial (A) plant height (cm), (B) fresh shoot weight (g), (C)dry shoot weight (g), (D) fresh root weight (g), (E) root length (cm), (F) 100 Grain weight (g). Columns are showing the mean values (n = 3) whereas, bars are indicating the standard errors (S.D). Different letters are representing the significant difference among all means value according to least significant design (LSD) at $p < 0.05$.

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Discussion

In the current study, under zero, half and full doses of nitrogen fertilization (N_o, N_1, N_2), the application of Beijerinckia fluminensis FA-7, Stenotrophomonas maltophilia FA-9, Pseudomonas aeruginosa FA-16, Klebsiella pneumoniae FA-4 and Enterobacter cloacae FA-8 significantly improved wheat shoot growth, nitrogen fixation and yield. A growth room and wire house study was conducted to assess the response of nitrogen-fixing bacteria isolated from trees with the hypothesis that inoculation with nitrogen-fixing endophytes would aid in wheat development. Nitrogen is the most essential nutrient required for plant growth, development, and progress in addition to being component of chlorophyll, proteins, energy transfer, and genetic material. To meet plant nitrogen requirements and get maximum output, farmers apply chemical fertilizers (Abdul Sattar et al., 2025; Ollio et al., 2025). Nitrogen fixing bacterial inoculants has emerged as a potential technology with a wide range of benefits for enhancing plant development, because it is a relatively easy and inexpensive alternative technique (Dhali et al., 2021; Ali et al., 2025). A total of fifty-two (52) isolates were isolated from populus on nitrogen free media, out of which five potential strains were selected including Beijerinckia fluminensis FA-7, Stenotrophomonas maltophilia FA-9, Pseudomonas aeruginosa FA-16, Klebsiella pneumoniae FA-4 and Enterobacter cloacae FA-8. These five isolates with N fixing ability, P and K solubilization and IAA synthesis activities were chosen for growth studies. These plant growth-promoting bacterial traits, such as nutrient solubilization, may have contributed to growth promotion by increasing nutrients levels in the rhizosphere, thereby increasing root elongation (Tariq et al., 2025) by auxin production, resulting in increased absorptive surface area for uptake (Khan et al., 2019). By altering the physiology of the roots, Beijerinckia fluminensis FA-7 considerably improved the root length, surface area, and fine roots of wheat, according to the WinRhizo image analysis system. According to the study, diazotrophic bacteria of the populus have characteristics that promote plant growth and may be used as microbial inoculants for crops. All infected plants had lower 15N% abundance than their corresponding uninoculated counterparts, suggesting that biologically fixed nitrogen intake (%Ndfa) is the cause of the ^{15}N dilution. In each of the three trials, FA7 displayed the highest ^{15}N dilution. Because microbial inoculants have the capacity to enhance plant growth, increase nutrient availability and uptake, and support plant health both directly and indirectly, they are a natural tool for the integrated management of agro-environmental issues(Rodrigues et al., 2018; Liu et al., 2025). Dey et al. (2004) studies stated that vigour index of possible strains was significantly higher than that of other cereals and wheat crops (Burragoni and Jeon, 2021; Jiang et al., 2021). Kammar et al. (2021) found that applying a strain isolated from the wheat rhizosphere increased the shoot length of wheat by 70.5%. The mineralization of nutrients that are readily available to plants and the nitrogen fixation by endophytes may be the causes of this increase in shoot length (Geries and Elsadany, 2021; Nguyen et al., 2021). According to Guo et al. (2021) applying endophytic bacteria increased the dry weight of wheat plants. According to Nascimento et al. (2019) the application of diazotrophs increased the number of tillers in wheat by 25%. The diazotrophs also had a favourable influence on the number of tillers. Although it has been suggested that the diazotroph bacteria's production of IAA is the reason for the plant's increased number of tillers, this cannot be the sole explanation. Another explanation would be that endophytes mentioned by Photolo et al. (2021) improved the plants' overall growth. Based on their nitrogen fixation activity, each strain was chosen, and tests were conducted on them separately with varying concentrations of chemical nitrogen fertilizers. The findings of this study lend credence to the idea that combining tree endophytes can increase crop growth and production. Additionally, all crop development parameters and crop yield were equivalent to those with the full recommended dose of nitrogen fertilizer when inoculants were treated at a half rate of the required dose(Haroon et al., 2019; Heijo et al., 2020; Li et al., 2021;). The results also demonstrated that the crop growth parameter and yield were lower when endophytes inoculants were applied in conjunction with the full recommended dose of nitrogen fertilizer than when the recommended fertilizer and endophyte inoculants were applied at a half dose rate. Our results were comparable to those of studies (Nascimento et al., 2019; Dhali et al., 2021; Fouda et al., 2021). According to their findings, the dry weight of tomatoes grown in greenhouse settings with 75% fertilizer and two endophyte inoculants was noticeably higher than that of tomatoes grown with the full recommended dosage of fertilizers and no inoculants. According to Dhali et al. (2021) the application of bacteria results in

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a notable increase in root length; they also claim that the generation of phyto-hormones by endophytes may be a primary factor in the growth of plant roots. Diazotrophs have been shown to increase wheat plant grain yield (Singh et al., 2021; Taulé et al., 2021). Grain yield may be impacted since the application of diazotrophs increased the number of tillers per plant (Aasfar et al., 2021; Faria et al., 2021).

Conclusion

The effectiveness of various strategies for utilizing nitrogen-fixing endophytic bacteria as a potential source of nitrogen to improve the growth and yield of cereal crops was investigated through a series of wire house and laboratory experiments. According to the study, diazotrophic bacteria of the populus have characteristics that promote plant growth and may be used as microbial inoculants for crops. The strains that were inoculated outperformed the uninoculated control. The isolate FA-7 proved to be the most successful in both growth room and pot testing, leading to a significant increase in both growth and yield. Following FA-7, strain FA-9 also markedly enhanced growth and yield in both axenic and potted environments.

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Contribution of Authors

Bhatti FA: Conceived idea, designed research methodology, collected and analyzed data and wrote the first draft of manuscript.

Naveed M: Conceptualized and supervised study, interpreted data and edited the manuscript

Asghar HN, Ishaque W & Basra SMA: Reviewed literature, assisted in laboratory experiments and edited the manuscript

All authors read and approved the final draft of the manuscript.

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