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Research Article

# Influence of ammonium and moisture on survival and *nif*H transcription in the diazotrophic *Pseudomonas mendocina* S10

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**Abstract**

Nitrogen is important for crop productivity and usually added in form of urea into the soil which negatively affects the environment. It is important to utilize nitrogen fixing bacteria for improving the nitrogen content of soil in India. Here, we have isolated nitrogen fixing-bacteria *Pseudomonas mendocina* S10 from rhizospheric soil and studied its nitrogenase activity along with its survival under sterile soil conditions. Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR and Real-time quantitative PCR were employed to investigate the population and *nif*H transcripts level respectively in presence of ammonium and moisture additives. Strain S10 was capable of growth and expressing *nif*H transcripts in the presence of 2.5 mM ammonium and 20 percent water availability. Population of isolated strain and it's *nif*H mRNA was found at low levels when exposed to 5 mM ammonium for 60 days of incubation period. However, viable bacterial count and *nif*H transcript levels remained low in the presence of 1.25 mM ammonium and zero percent water content. These findings indicate that isolated strain could tolerate ammonium up to 5 mM for 60 days and can maintained their cell viability in low moisture conditions. Results revealed the advantage of using gene expression to evaluate the physiological state of microorganism's population in soil.

***Keywords:*** ERIC-PCR; nitrogenase; *nif*H; *Pseudomonas mendocina* S10; real time PCR

## Introduction

Nitrogen is considered to be the crucial nutrient after water and carbon that restricts the ecosystem's productivity (Cummings *et al*., 2006). To increase production, nitrogen is applied in the form of chemical fertilizers which have a negative impact on the environment. This affects the quality of soil and water, contributes to the emission of greenhouse gases, and employs consumption of non-renewable fossil fuels (Orr *et al*., 2010). Therefore, Biological Nitrogen Fixation (BNF) remains a viable alternative for inorganic nitrogen fertilizers. BNF is the reduction of atmospheric nitrogen to fill nitrogen pool in the soil for consumption of plants and animals, useable form which refills the nitrogen pool in the soil (Cleveland *et al*., 1999). Majority of BNF is accomplished by bacteria called diazotrophs. The total N content of different ecosystems is primarily done by symbiotic and free-living diazotrophs (Hsu *et al*., 2009).

From past few years, the microbial inoculants of N-fixing bacteria have been used as expression substitute for chemically synthesized N fertilizers (Welbaum *et al*., 2004). In the last decade, microbial

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inoculants have been successfully used to improve crop production. *Azospirillum, Azotobacter* and *Klebsiella* are few genera that have been investigated to raise the soil N content (Kleopper *et al*., 1992; Okon *et al*., 1994).

Moisture, temperature, pH, texture, oxygen and nutrient availability are the soil environmental factors that regulate the growth of introduced bacterial strains (Alexander *et al*., 1977). The potential of non-native strains to survive in soil is generally limited which results in the reduced plant growth. Thus, it is essential to isolate and characterize native strains which have been acclimatized to the prevailing factors, for the enhancement of soil productivity (Victoria *et al*., 2012).

According to the report of Indian Agriculture Government in 2012, soil of Rajasthan is deficient in nitrogen and its requirement is fulfilled by chemical fertilizers. As a consequence, the selection and use of bacterial strains should be executed considering the adaptive potentiality. Therefore, isolation, identification, screening, and selection of expeditious strains are major steps for making them economical. Before the field examination, it is crucial to conduct a study on the simulated natural environment. The aim of this study is to isolate nitrogen-fixing bacteria and to investigate the possible changes in its endurance and *nif*H gene expression under stress conditions like inorganic N and moisture.

## Materials and Methods

*Isolation of nitrogen-fixing bacteria*

Soil sampling was done from the experimental field of Agriculture Research Institute, Jaipur, Rajasthan, India. Top soil was obtained from 10 cm depth, air dried, sieved and stored at 4 °C until used for the experiment. Nitrogen-fixing bacteria were isolated by enrichment of soil in nitrogen free medium (broth) (Aaronson, 1970). Bacterial strains were picked based on features like shape, size, color, margin, surface etc. Further purification of colonies was done by streaking on nitrogen free medium agar plates. Selected bacterial colonies were screened by amplification of *nif*H gene which could be indirect evidence for nitrogenase activity. *nif*H gene positive strains were identified by 16S rDNA sequencing.

*Semiquantitative expression of nif*H *gene of isolated strains*

Bacterial RNA was isolated from mid-log phase culture using Tri reagent (Ambion AM9738; USA), as per manufacturer's protocol. cDNA synthesis carried out by cDNA synthesis kit (Thermo scientific, K1622; Lithuania, Europe).

For amplification of *nif*H and 16S rDNA, 1 µl of RT PCR product was used in subsequent PCR. Reaction conditions were 95 °C for 5 min, followed by 25 cycles at 95 °C for 1 min (denaturation), 57 °C for 1 min (annealing), and 72 °C for 2 min (extension), followed by 7 min at 72 °C (final extension). Primers were used in the PCR, for *nif*H gene PolF/PolR (Poly *et al*., 2001) and 357F/518R for 16S rDNA (Table 1a). PCR was carried out in C1000 Thermal Cycler (BIO-RAD). Reaction products were seen on agarose gel electrophoresis. RT-PCR products were quantified with help of Gel Documentation system and Image lab software (BIORAD).

*Survival and nifH gene expression of isolated strain S10*

*Preparation of Pseudomonas mendocina S10 inoculum*

Culture of *Pseudomonas mendocina* S10 was grown in nitrogen free medium till mid-log phase and resuspended in sterile saline (0.9% NaCl). This suspension was used to inoculate the soil.

*Soil microcosm*

Soil for microcosm study was autoclaved at 121 °C at 15 psi for 15 min and was subjected to drying oven for 24 hours at 105 °C to remove any residual moisture. Soil moisture was adjusted to field conditions by adding 5 ml of sterilised deionized water. $2*10^8$ cells of S10 strain were inoculated per gram of soil. Isolated strain in soil was exposed to three concentrations of nitrogen and moisture. All microcosms were incubated at 25 °C in dark. Soil samples were collected on $0^{th}$, $30^{th}$, $60^{th}$, $90^{th}$ and $120^{th}$ day of the experiment.

*ERIC-PCR*

This was done to examine the survival of inoculated strain in the soil. Inoculated strain was isolated from soil by serial dilutions on LB agar plates containing antibiotics, Vancomycin (30 µg/ml), Cefadroxil (30 µg/ml) and Penicillin (10 µg/ml) and obtained colonies were subjected to ERIC-PCR for confirming the identity of strain. Primers were used (Versalovic *et al*., 1991) (Table 1b). PCR conditions were 95 °C for 5 min (initial denaturation) followed by 35 cycles of denaturation (45 sec at 92 °C), annealing (1 min at 52 °C), and extension (10 min at 70 °C), with a final extension (20 min at 70 °C). PCR product was analyzed on agarose gel (0.8%). Banding pattern was evaluated using gel doc.

*RNA extraction*

Total RNA was extracted using MOBIO soil RNA kit (USA) as per manufacturer's instruction. RNA samples were stored at -80 °C and quantified by nanodrop ND1000 (Thermo scientific). RNA quality was checked on 2% agarose gel with supermix DNA ladder.

*Primer designing*

Further degenerate primers of conserved regions were employed (Table 1a). *nif*H and 16Sr DNA genes were amplified from cDNA of *Pseudomonas mendocina* S10. This involved the PCR cycles as follows: 1 cycle at 94 °C (5 min), 30 cycles at 94 °C (1 min), 57 °C (1 min), 72 °C (2 min), 1 cycle at 72 °C (15 min), and finally hold at 4 °C. PCR products were checked on 1% agarose gel (Banglore Genei, USA) and gel images were taken. PCR product of interest was purified using a gel extraction kit (Promega, USA), ligated into a pGEMT vector (Promega, USA) and transformed into *Escherichia coli* DH5α. Universal M13 primers were used to check positive inserts and then sequenced (Scigenome, Kerala, India). NCBI (National Center for Biotechnology Information) nucleotide BLAST program was utilized to identity of gene.

The gene specific primers (Table 1c) from the cDNA sequences were designed using online program (Eurofins MWG Operon).

*Quantitative (real time) PCR*

One µg of RNA used for cDNA formation as per manufacturer's instruction (Thermo scientific). SYBER green (Applied biosystem) used for qPCR Reactions were set up with Applied bisystem Viia 7 system. Reaction mixtures were kept at 95 °C (heating) for 15 min (activation of SYBER green) prior to complete 40 cycles of denaturation (95 °C, 15 sec), annealing (60 °C, 15 sec) and extension (72 °C, 15 sec). *Pseudomonas mendocina* S10 gene specific primers were used and a standard curve was set up using 5-fold dilutions of all samples cDNA pool. Standard deviation was determined (software) on the replicate threshold cycles ($C_T$) scores. The standard curve produced was linear (r2 = 0.98) and the PCR efficiency was 94%. To check unique PCR product, a melt-curve was run at the end of 40 cycles. Reactions with no cDNA were done to check absence of nonspecific-primer dimers.

16S rRNA gene was an internal standard (for normalization) and primers were used (Table 1c). Applied Biosystems Viia software was used to calculate threshold values of cycles. $2^{(-\Delta\Delta Ct)}$ method was used to study relative changes in *nif*H gene activity (Livak and Schmittgen, 2001).

**Table 1.** Details of primer used

| | Gene | Primer | Sequence |
|---|---|---|---|
| (a) | Degenrate primers | | |
| | *nif*H | PolF | 5'TGCGAYCCSAARGCBGACTC3' |
| | | PolR | 5'ATSGCCATCATCTCYCCGGA3' |
| | 16S rDNA | 357F | 5'CCTACGGGAGGCAGCAG3' |
| | | 518R | 5'ATTACCGCGGCTGCTGG3' |
| (b) | ERIC | 1 | 5'ATGTAAGCTCCTGGGGATTCAC3' |
| | | 2 | 5'AAGTAAGTGACTGGGGTGGCG3' |
| (c) | Gene specific primers | | |
| | *nif*H | F | 5'ATGTGTCCTACGACGTGCTG 3' |
| | | R | 5'CAAATGTTGTTGGCCGCGTA 3' |
| | 16S rDNA | F | 5'ATGCAAGTCGAGCGGTAGAG3' |
| | | R | 5'GAAGGTCCCTGCTTTCTCC3' |

Forward primer; R: reverse primer. Modified bases: I=Inosine, Y=CT, S=CG, R=AG, B=GCT.

*Statistical analysis*

Statistical analysis of viable colony count and *nif*H transcript level was performed by using analysis of variance (ANOVA) with $p<0.05$ using the Sigmaplot version 12.5. For comparison of bacterial colony count and gene transcript level target, linear regression analysis was performed.

## Results

*Isolation of bacteria*

We have isolated 30 bacterial strains from the enriched soil cultures. Isolated strains were screened by *nif*H gene amplification. From 30 isolates, only 5 strains showed *nif*H amplification which were further subjected to semiquantitative *nif*H gene expression study.

*Semi-quantitative RT-PCR to select capable strains*

Using relative semi-quantitative PCR, expression level of *nif*H gene was investigated and used to screen an efficient nitrogen fixing bacterium. Band intensities of 360 bp of *nif*H gene were quantified (Figure 1A) and normalized with band intensities of 16S rRNA gene (Figure 1B). *nif*H gene mRNA level was significantly high in bacterial strain S10 ($p<0.001$) than other bacterial strains (Figure 2). 16S rDNA sequence revealed that strain S10 as *Pseudomonas mendocina* with accession no. KM015513.

*Survival of Pseudomonas mendocina S10 in an in-vitro study*

DNA finger printing technique, ERIC PCR was utilized to confirm the band patterning of the viable bacterial colonies. The initial population of *Pseudomonas mendocina* S10 was to be $1.8*10^8$ to $1.9*10^8$ CFU per gram of soil. The experiment was conducted with cell density of $2*10^8$ CFU per gram of soil.

Following incubation at 25 °C, survival was monitored regularly for 120 days. A significant decrease in cell density was reported during initial 30 days of incubation period. *Pseudomonas mendocina* S10 grew at a faster rate in samples amended with 2.5 mM with its peak growth on $60^{th}$ day of incubation, $1.99*10^8$ CFU per gram of soil ($p<0.001$) (Figure 3). However, its growth was retarded in samples with 1.25 mM ammonium whereas, at 5 mM ammonium addition, moderate growth was observed.

On analyzing samples supplemented with different moisture levels, high viable bacterial count was determined in sample with 20 percent moisture availability ($2.1*10^8$ CFU per gram) ($p<0.001$). In other moisture conditions, viable bacterial count was retarded (Figure 4).

After 120 days of incubation period significantly low population of the strain S10 ($p<0.001$) was observed in all variants.

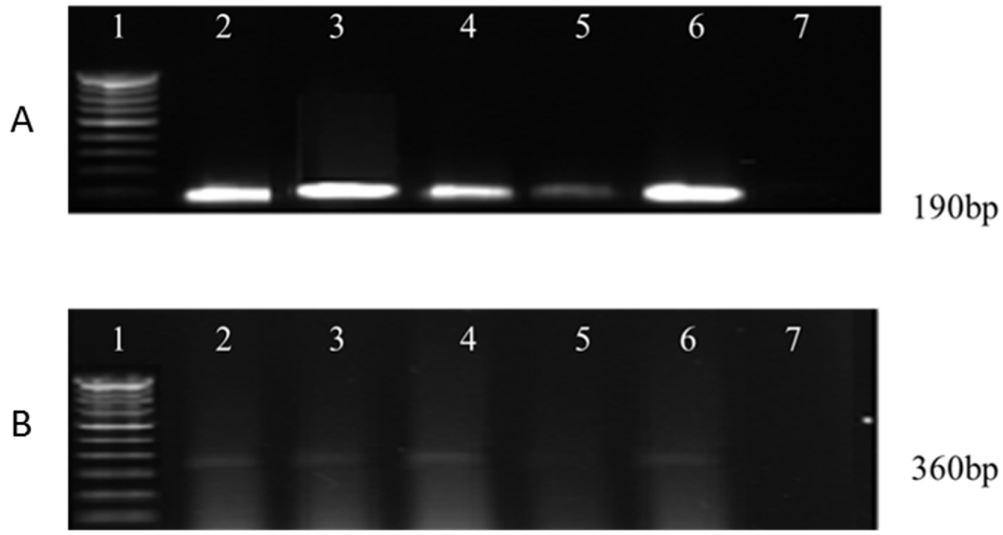


**Figure 1.** Gel image illustrating the semi-quantitative RT-PCR of *nif*H gene transcripts. Lane 1: 100 bp DNA marker, Lane 2 to 6: 16S rDNA (A) and *nif*H gene (B) of isolated strains N2, S10, N2, N8 and N11 respectively, Lane 7: negative control

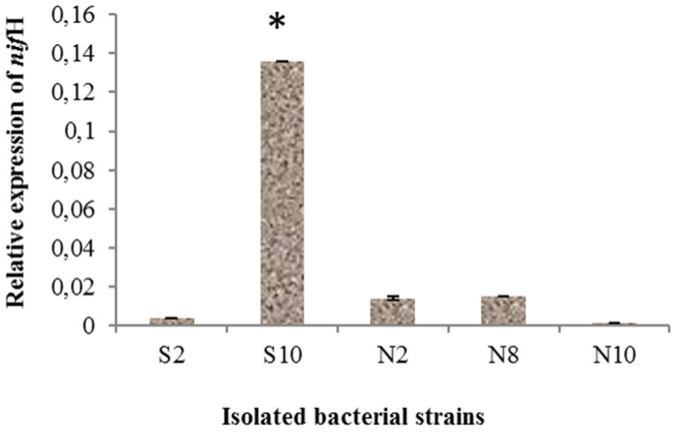


**Figure 2.** Semi-quantitative RT-PCR confirmation of *nif*H gene in the isolated bacterial strains. Each data point represents *nif*H transcripts levels. One-way ANOVA determined the significance of difference. Each data point represents mean and the vertical line on it indicates the standard error

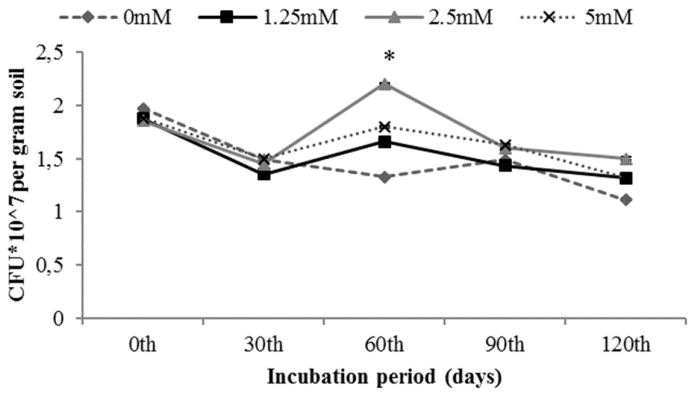


**Figure 3.** Growth response of *Psuedomonas mendocina* S10 in soil treated with variant ammonium concentrations 0 mM, 1.25 mM, 2.5 mM, and 5 mM

Each data point represents no. of bacterial colonies per gram of soil. One-way ANOVA determined the significance of difference. Each data point represents mean and the vertical line on it indicates the standard error. The asterisk shows significance level: $p<0.05$ with 0-day samples.

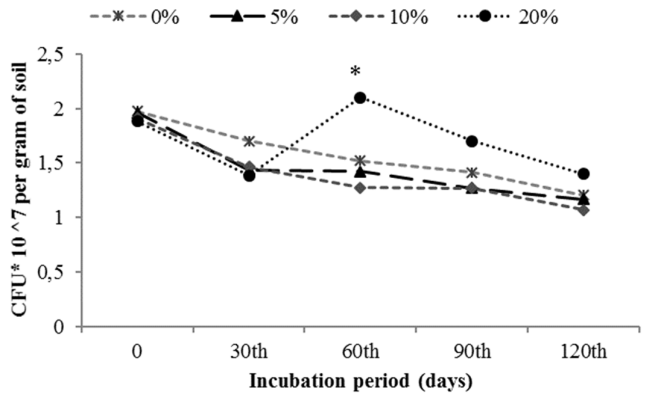


**Figure 4.** Growth response of *Psuedomonas mendocina* S10 in soil treated with moisture percentages 0%, 2.5%, 5%, and 10%

Each data point represents no. of bacterial colonies per gram of soil. One-way ANOVA determined the significance of difference. Each data point represents mean and the vertical line on it indicates the standard error. The asterisk shows significance level: $p<0.05$ with 0-day samples

*Quantification of Pseudomonas mendocina nitrogenase activity using nifH mRNA*

Fixation of atmospheric nitrogen in diazotrophes requires the expression of nitrogenase enzyme encoding gene *nif*H, which is component of *nif*HDK operon (Robert *et al*., 1980). Comparative ΔΔcT method of real time PCR was employed to assess *nif*H gene activity in *Pseudomonas mendocina* S10 under various ammonium and moisture amounts. Initially on $0^{th}$ day, expression level was similar in both ammonium and moisture treated samples (Figures 5 and 6).

Addition of 2.5 mM ammonium enhanced the *nif*H transcripts level ($p<0.001$) in *Pseudomonas mendocina* S10, showing 8.2 folds upregulation after 60 days of incubation (Figure 5). Similarly, ammonium addition of 5 mM caused an approximate 89% increase in *nif*H transcripts level, 7.3 folds upregulation. The effect of ammonium additive on *nif*H transcription was not transient and transcripts levels stayed high for at least 45 days (Figure 5). However, no *nif*H transcripts were detected after 90 days of incubation with 5 mM ammonium stress. For 1.25 mM ammonia concentration, low *nif*H mRNA levels were detected ($p<0.001$).

In moisture treated soil samples, transcripts level of *nif*H in *Pseudomonas mendocina* S10 increased sharply when 20 percent of water was present for 30 days of incubation period and stayed 13.45±0.1 folds high ($p<0.001$) for at least 60 days. Whereas moisture treatments zero and 10 percent did not affect the *nif*H gene transcription ($p<0.001$). These findings indicate that ammonium concentration 5 mM represses transcription of *nif*H to certain extent in comparison with 2.5 mM and water is further enhancing its expression.

*Comparison of bacterial counts and relative mRNA expression level*

Regression from *Pseudomonas mendocina* S10 colony count from treated samples indicates a positive correlation with relative *nif*H mRNA expression levels ($R^2 = 0.739 - 0.571$).

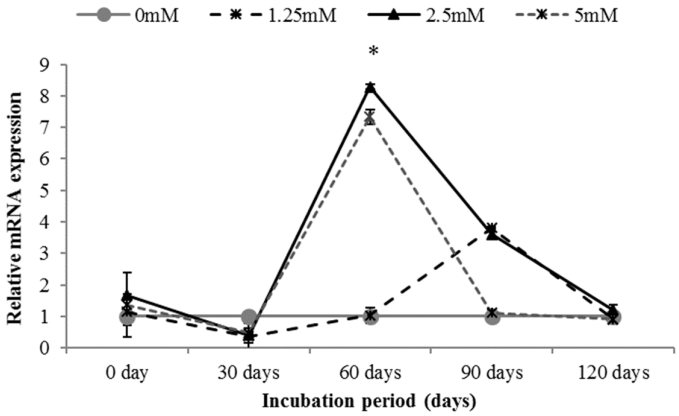


**Figure 5.** mRNA expression profile of *nif*H gene in *Psuedomonas mendocina* S10 inoculated in soil treated with ammonium concentrations 0 mM, 1.25 mM 2.5 mM, and 5 mM

Each data point represents mRNA abundance of *nif*H gene normalized to 16S rRNA, which was used as a housekeeping gene. One-way ANOVA determined the significance of difference. Each data point represents mean and the vertical line on it indicates the standard error. The asterisk shows significance level: $p<0.05$ with 0-day samples

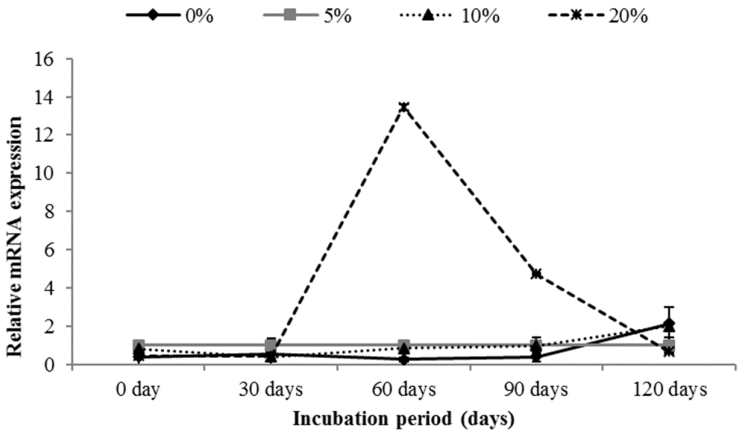


**Figure 6.** mRNA expression profile of *nif*H gene in *Psuedomonas mendocina* S10 inoculated in soil treated with moisture percentage 0%, 5%, 10%, and 20%

Each data point represents mRNA abundance of *nif*H gene normalized to 16S rRNA, which was used as a housekeeping gene. One-way ANOVA determined the significance of difference. Each data point represents mean and the vertical line on it indicates the standard error. The asterisk shows significance level: $p<0.05$ with 0-day samples

**Discussion**

The increasing importance of nitrogen-fixing bacteria in agriculture has resulted in many efforts to isolate and identify bacteria from soil. Here we isolate the nitrogen-fixing bacteria and evaluated its survival and *nif*H gene expression in soil. Semiquantitative PCR revealed that *Pseudomonas mendocina* S10 showed the highest *nif*H mRNA level which was used for further work.

Ammonium is the crucial factor which regulates nitrogen fixation. It generally represses the growth and expression of *nif*H, which is strain-dependent (Guerrero and Lara, 1987; Ohmori and Hattori, 1972). Among physical factors, the percentage of water saturation is a vital factor controlling bacterial activity and its survival in soil (Cattaneo *et al*., 1997).

*Pseudomonas mendocina* S10 survival and *nif*H mRNA expression were studied in soil. In our study, significant differences in *Pseudomonas mendocina* S10 survival and *nif*H transcripts at different concentrations of ammonium and moisture were observed by using finger printing ERIC-PCR and qPCR respectively.

Transcriptional and post-translational are two regulatory levels of nitrogen fixation. Ammonium represses the nitrogen fixation by inhibiting *nif*H expression according to the previous reports. Surprisingly, *Pseudomonas mendocina* S10 cell growth and its *nif*H transcription level was high between incubation period of 30 days and 60 days at 2.5 mM and 5 mM ammonium addition. The *nif*H expression with high transcript level in the presence of ammonium as presented here, for *Pseudomonas mendocina* is also encountered with *Rhodobacter capsulatus* tolerated up to 12.5 mM concentration of ammonium (Hubner *et al*., 1993) and *A. vinlandii* up to 2M (Burgamann *et al*., 2003). The reason for high *nif*H expression is may be due to presence of posttranscriptional regulation mechanisms in nitrogen-fixing bacteria (Hubner *et al*., 1993). But in 5 mM ammonium, growth and *nif*H expression showing repression on 90$^{th}$ day which may be due to inhibitory action of ammonium which switch-off the nitrogenase activity.

As earlier reports say, in *Rhodospirillum rubrum,* dinitrogenase reductase activating glycohydrolase (DraG) and dinitrogenase reductase ADP-ribosyltransferase (DraT) unambiguously interceded the nitrogenase inhibition by ammonium, due to covalent modification/demodification of Fe-protein via ADP-ribosylation (Ludden *et al*., 1995). In the *Rhodobacter capsulatus* a photosynthetic bacterium, extrinsic ammonium causes three different responses of nitrogenase: an ADP-ribosylation of Fe-protein (Hallenbeck *et al*., 1992, 1982), an ADP- bosylation- independent switch-off effect (Fedorov *et al*., 1988; Yakunin *et al*., 1998), and an ADP-ribosylation-independent magnitude response, where the concentration of added ammonium affects the inhibition intensity (Yakunin *et al*., 1998).

Water plays a crucial role in survival and activity of bacteria in soil (Ronen *et al*., 2000). In our study, high moisture availability in soil provided the favorable condition for *Pseudomonas mendocina* S10 growth, induced the level of *nif*H transcription. High nitrogenase activity in the presence of greater availability of water was earlier reported by Marshall *et al*. (1989). The *Bradyrhizobium* bacterium isolated from sandy-loam soil require a high percentage of soil moisture for its survival and activity (Orchard *et al*., 1983).

Low moisture conditions were undesirable for the growth and *nif*H transcription of *Pseudomonas mendocina* S10. Some free-living rhizobia (saprophytic) can tolerate drought conditions or low water potential and symbiotic rhizobia bacterium can survive in soils with limiting moisture level (Fuhrmann *et al*., 1986). *Prosopis* (mesquite), a desert soil rhizobium which was active in desert soil for period of one month, but modified strain was not capable to survive under such conditions (Shoushtari *et al*., 1985).

According to regression analysis for the expression of *nif*H transcripts with ammonium and moisture treatments, level of *nif*H mRNA showing positive correlation with the number of bacterial counts.

## Conclusions

This study demonstrates the survival and level of *nif*H transcripts of *Pseudomonas mendocina* S10 at variant concentration of ammonium and water in soil. The *Pseudomonas mendocina* S10 population and *nif*H transcripts level indicate that this strain could tolerate 5 mM concentration of ammonium and showing peak expression at 2.5 mM ammonium, whereas high moisture condition is favourable for its growth. This study proposes the efficient nitrogen-fixing bacteria which could be employed to enhance soil productivity. *Pseudomonas mendocina* 10 the potential to improve the soil nitrogen content in field is yet to be determined.

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## Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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