Influence of ammonium and moisture on survival and nifH transcription in the diazotrophic Pseudomonas mendocina S10

Sushma SHARMA*, Dileep K. SINGH

University of Delhi, Department of Zoology, India; sush.scop28@gmail.com (*corresponding author); dileepksingh@gmail.com

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 nifH transcripts level respectively in presence of ammonium and moisture additives. Strain S10 was capable of growth and expressing nifH transcripts in the presence of 2.5 mM ammonium and 20 percent water availability. Population of isolated strain and it’s nifH mRNA was found at low levels when exposed to 5 mM ammonium for 60 days of incubation period. However, viable bacterial count and nifH 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; nifH; 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).
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.

#### Semi-quantitative 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 *nif*H 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 0th, 30th, 60th, 90th and 120th 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). nifH and 16S rDNA 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 biosystem 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 (Ct) 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^(-ΔΔCt) method was used to study relative changes in nifH gene activity (Livak and Schmittgen, 2001).
Table 1. Details of primer used

| Gene        | Primer | Sequence                  |
|-------------|--------|---------------------------|
| (a) Degenrate primers                               |
| *nifH*      | PolF   | 5'TGCGAYCCSAARGCBGACTC3'   |
|             | PolR   | 5'ATSGCCATCATCTCYCCGGA3'   |
| 16S rDNA    | 357F   | 5'CCTACGGGAGGCAAGCAG3'     |
|             | 518R   | 5'ATTACCGCGGTGCTGG3'       |
| (b) ERIC    | 1      | 5'ATGTAAGCTCCTGGGGATTAC3'  |
|             | 2      | 5'AAAGTAAATGACTTGGGGTGGCG3'|
| (c) Gene specific primers                           |
| *nifH*      | F      | 5'ATGTGTCCTACGACGTGCTG3'   |
|             | R      | 5'CAAATGTTGTTGGCGGTG3'     |
| 16S rDNA    | F      | 5'ATGCAAGTGACGGTAGAG3'     |
|             | R      | 5'GAAGGTTCCCTGCTTCTCC3'    |

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 *nifH* 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 *nifH* gene amplification. From 30 isolates, only 5 strains showed *nifH* amplification which were further subjected to semiquantitative *nifH* gene expression study.

Semi-quantitative RT-PCR to select capable strains

Using relative semi-quantitative PCR, expression level of *nifH* gene was investigated and used to screen an efficient nitrogen fixing bacterium. Band intensities of 360 bp of *nifH* gene were quantified (Figure 1A) and normalized with band intensities of 16S rRNA gene (Figure 1B). *nifH* 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 1.8×10⁸ to 1.9×10⁹ CFU per gram of soil. The experiment was conducted with cell density of 2×10⁸ 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 60th day of incubation, 1.99×10⁸ 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 ($2.1 \times 10^8$ 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.

Quantification of *Psuedomonas mendocina* nitrogenase activity using nifH mRNA

Fixation of atmospheric nitrogen in diazotrophs 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 *Psuedomonas mendocina* S10 under various ammonium and moisture amounts. Initially on 0th 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 *Psuedomonas 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 $nifH$ in *Pseudomonas mendocina* S10 increased sharply when 20 percent of water was present for 30 days of incubation period and stayed $13.45 \pm 0.1$ folds high ($p<0.001$) for at least 60 days. Whereas moisture treatments zero and 10 percent did not affect the $nifH$ gene transcription ($p<0.001$). These findings indicate that ammonium concentration 5 mM represses transcription of $nifH$ 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 $nifH$ mRNA expression levels ($R^2 = 0.739 - 0.571$).

![Figure 5](image1.png)

*Figure 5.* mRNA expression profile of $nifH$ gene in *Pseudomonas 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 $nifH$ 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](image2.png)

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

Each data point represents mRNA abundance of $nifH$ 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 \( \text{nif}H \) gene expression in soil. Semiquantitative PCR revealed that \textit{Pseudomonas mendocina} S10 showed the highest \( \text{nif}H \) 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 \( \text{nif}H \), 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).

\textit{Pseudomonas mendocina} S10 survival and \( \text{nif}H \) mRNA expression were studied in soil. In our study, significant differences in \textit{Pseudomonas mendocina} S10 survival and \( \text{nif}H \) 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 \( \text{nif}H \) expression according to the previous reports. Surprisingly, \textit{Pseudomonas mendocina} S10 cell growth and its \( \text{nif}H \) transcription level was high between incubation period of 30 days and 60 days at 2.5 mM and 5 mM ammonium addition. The \( \text{nif}H \) expression with high transcript level in the presence of ammonium as presented here, for \textit{Pseudomonas mendocina} is also encountered with \textit{Rhodobacter capsulatus} tolerated up to 12.5 mM concentration of ammonium (Hubner et al., 1993) and \textit{A. vinlandii} up to 2M (Burgmann et al., 2003). The reason for high \( \text{nif}H \) expression is may be due to presence of postranscriptional regulation mechanisms in nitrogen-fixing bacteria (Hubner et al., 1993). But in 5 mM ammonium, growth and \( \text{nif}H \) expression showing repression on 90\textsuperscript{th} day which may be due to inhibitory action of ammonium which switch-off the nitrogenase activity.

As earlier reports say, in \textit{Rhodospirillum rubrum}, dinitrogenase reductase activating glycohydrolase (DraG) and dinitrogenase reductase ADP-riboseyltransferase (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 \textit{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- ribosylation- 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 \textit{Pseudomonas mendocina} S10 growth, induced the level of \( \text{nif}H \) transcription. High nitrogenase activity in the presence of greater availability of water was earlier reported by Marshall et al. (1989). The \textit{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 \( \text{nif}H \) transcription of \textit{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). \textit{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 (Shoushti et al., 1985).

According to regression analysis for the expression of \( \text{nif}H \) transcripts with ammonium and moisture treatments, level of \( \text{nif}H \) mRNA showing positive correlation with the number of bacterial counts.
Conclusions

This study demonstrates the survival and level of nifH transcripts of *Pseudomonas mendocina* S10 at variant concentration of ammonium and water in soil. The *Pseudomonas mendocina* S10 population and nifH 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.

Acknowledgements

This work was supported by DBT, India.

Conflict of Interests

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

References

Aaronson S (1970). Experimental microbial ecology. Academic Press, New York pp 236.
Alexander M (1977). An introduction to soil microbiology, John Wiley Sons, New York pp 467.
Burgmann H, Widmer F, Sigler WV, Zeyer J (2003). mRNA extraction and reverse transcription-pcr protocol for detection of *nifH* gene expression by *Azotobacter vinelandii* in soil. Applied and Environmental Microbiology 69(4):1928-1935.
Cattaneo MV, Masson C, Greer CW (1997). The influence of moisture on microbial transport, survival and 2, 4-D biodegradation with a genetically marked *Burkholderia cepacia* in unsaturated soil columns. Biodegradation 8:87-96.
Cleveland CC, Townsend AR, Schimel DS (1999). Global patterns of terrestrial biological nitrogen (N2) fixation in natural ecosystems. Global Biogeochemical Cycles 13(2):623-645.
Cummings SP, Humphry DR, Santos SR, Andrews M, James EK (2006). The potential and pitfalls of exploiting nitrogen fixing bacteria in agricultural soils as a substitute for inorganic fertilizer. Environmental Biotechnology 2(1):1-10.
Fedorov AS, Troshina OY, Laurinavichene TV, Glazer VM, Babykin MM, Zinchenko VV, ... Tsygankov AA (1998). Regulatory effect of ammonium on the nitrogenase activity of *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* is not mediated by ADP-ribosylation of the Fe-protein of nitrogenase. Microbiology 67:610-615.
Fuhrmann J, Davey CB, Wollum AG (1986). Desiccation tolerance in clover rhizobia in sterile soils. Soil Science Society of America Journal 50:639-644.
Guerrero MG, Lara C (1987). Assimilation of inorganic nitrogen. In: The Cyanobacteria. Edited by Fay P and Van Baalen C (Ed), Elsevier Science, Amsterdam pp 163-185.
Hallenbeck PC, Meyer CM, Vignais PM (1982). Nitrogenase from the photosynthetic bacterium *Rhodopseudomonas capsulata*: purification and molecular properties. Journal of Bacteriology 149(2):708-717.
Hallenbeck PC (1992). Mutations affecting nitrogenase switch-off in *Rhodobacter capsulatus*. Biochimica et Biophysica Acta 1118:161-168.
Hsu S, Buckley DH (2009). Evidence for the functional significance of diazotroph community structure in soil. ISME Journal 3:124-136.
Hubner P, Masepohl B, Klipp W, Bickle TA (1993). *nif* gene expression studies in *Rhodobacter capsulatus*: ntrC-independent repression by high ammonium concentrations. Molecular Microbiology 10:123-132.
Indian Agricultural Government (2012). A report on Rajasthan.
Kloepper JW, Beauchamp CJ (1992). A review of issues related to measuring of plant roots by bacteria. Canadian Journal of Microbiology 38:1219-1232.

Lipman LJA, de Nijs A, Lam TJGM, Gastra W (1995). Identification of Escherichia coli strains from cows with clinical mastitis by serotyping and DNA polymorphism patterns with REP and ERIC primers. Veterinary Microbiology 43:13-19.

Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real time quantitative PCR and the 2−ΔΔCT method. Methods 25:402-408.

Ludden PW, Roberts GP (1995). The biochemistry and genetics of nitrogen fixation by photosynthetic bacteria. In: Blankenship RE (Eds). Anoxygenic photosynthetic bacteria. Dordrecht: Kluwer Academic Publishers pp 929-947.

Marshall J, Grobbelaar N, James S (1989). Seasonal changes in the nitrogenase activity and other metabolic parameters of cycad coralloid roots. Botanical Bulletin of Academia Sinica 30(4):285-289.

Ohmori M, Hattori A (1972). Effect of nitrate on nitrogen-fixation by the blue-green alga Anabaena cylindrica. Plant and Cell Physiology 13:589-599.

Okon Y, Labandera-Gonzalez CA (1994). Agronomic applications of Azospirillum: an evaluation of 20 years worldwide field inoculation. Soil Biology and Biochemistry 26:1591-1601.

Orchard VA, Cook FG (1983). Relation between soil respiration and soil moisture. Soil Biology and Biochemistry 15:447-453.

Orr CH, James A, Leifert C, Cooper JM, Cummings SP (2011). Diversity and activity of free-living nitrogen-fixing bacteria and total bacteria in organic and conventionally managed soils. Applied and Environmental Microbiology 77(3):911-919.

Poly F, Montozier IJ, Bally R (2001). Improvement in the RFLP procedure for studying the diversity of nifH genes in communities of nitrogen fixers in soils. Research Microbiology 152:95-103.

Robertst GP, Brill WJ (1980). Gene-product relationships of the nif regulon of Klebsiella pneumoniae. Journal of Bacteriology 144(1):210-216.

Ronen Z, Vasiluk L, Abeliovich A, Nejidat A (2000). Activity and survival of tribromophenol-degrading bacteria in a contaminated desert soil. Soil Biology and Biochemistry 32:1643-1650.

Shoushtari NH, Pepper IL (1985). Mesquite rhizobia isolated from the Sonoran Desert: competitiveness and survival in soil. Soil Biology and Biochemistry 17:803-806.

Versavolic J, Kocurth T, Lupski JR (1991). Distribution of repetitive DNA sequences in eu-bacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Research 19:6823-6831.

Victoria M, Alejandro N, Ricardo G (2017). Use of endophytic and rhizosphere bacteria to improve phytoremediation of arsenic-contaminated industrial soils by autochthonous Betula celtiberica. Applied Environmental Microbiology 15:83(8): e03411-16.

Welbaum G, Sturz AV, Dong Z, Nowak J (2004). Fertilizing soil microorganisms to improve productivity of agroecosystems. Critical Reviews in Plant Science 23:175-193.

Yakunin AF, Hallenbeck PC (1998). Short-term regulation of nitrogenase activity by NH4 in Rhodobacter capsulatus: multiple in vivo nitrogenase responses to NH4 addition. Journal of Bacteriology 180:6392-6395.