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## SHORT COMMUNICATION

# Influence of External Nitrogen on Nitrogenase Enzyme Activity and Auxin Production in *Herbaspirillum seropedicae* (Z78)

Tan Tzy Yin, Ui Li Pin and Amir Hamzah Ahmad Ghazali*

School of Biological Sciences, Universiti Sains Malaysia, 11800 USM, Pulau Pinang, Malaysia

**Abstrak:** Keupayaan diazotrof hidup bebas untuk menghasilkan enzim nitrogenase dan auksin boleh mempengaruhi pertumbuhan tumbuhan perumah. Dalam kajian ini, diazotrof dibiakkan di dalam medium pertumbuhan yang mengandungi pelbagai kepekatan nitrogen (N) untuk menentukan kepekatan optimum N bagi menggalakkan pertumbuhan mikrob, peningkatan pengikatan gas N ($N_2$) dan penghasilan fitohormon. Oleh itu, kajian melihat sama ada tahap N yang berbeza yang dibekalkan kepada *Herbaspirillum seropedicae* (Z78) akan mempunyai apa-apa kesan yang signifikan kepada aktiviti nitrogenase dan pengeluaran auksin. Aktiviti nitrogenase tertinggi dan pengeluaran auksin terendah *H. seropedicae* (Z78) dicatatkan pada 0 $gL^{-1}$ $NH_4Cl$. Apabila tahap N luaran ditingkatkan, ia menyebabkan penurunan yang ketara dalam aktiviti nitrogenase, dengan penghasilan auksin yang lebih tinggi. Dalam ujian berikutnya, dua saiz inokulum berbeza Z78 ($10^6$ dan $10^{12}$ cfu/ml) telah dipilih untuk mengkaji kesan peratusan berbeza asetilena terhadap aktiviti nitrogenase daripada inokulum melalui asai penurunan asetilena (ARA). Hasil kajian menunjukkan bahawa, pada $10^6$ cfu/ml inokulum, jumlah yang paling optimum asetilena diperlukan untuk asai enzim nitrogenase adalah 5%, sedangkan pada saiz inoculum yang lebih tinggi ($10^{12}$ cfu/ml) sekurang-kurangnya 10% daripada asetilena diperlukan untuk aktiviti nitrogenase optimum. Penemuan ini menjelaskan kesan tahap N berbeza terhadap aktiviti nitrogenase diazotrof dan penghasilan auksin serta faktor-faktor penting yang mempengaruhi pertumbuhan tumbuhan perumah.

**Kata kunci:** *Herbaspirillum seropedicae* (Z78), Nitrogen Luaran, Asetilena, Saiz Inokulum, Aktiviti Nitrogenase, Penghasilan Auksin

**Abstract:** The production of nitrogenase enzyme and auxins by free living diazotrophs has the potential to influence the growth of host plants. In this study, diazotrophs were grown in the presence of various concentrations of nitogen (N) to determine the optimal concentration of N for microbial growth stimulation, promotion of gaseous N ($N_2$) fixation, and phytohormone production. Therefore, we investigate whether different levels of N supplied to *Herbaspirillum seropedicae* (Z78) have significant effects on nitrogenase activity and auxin production. The highest nitrogenase activity and the lowest auxin production of *H. seropedicae* (Z78) were both recorded at 0 $gL^{-1}$ of $NH_4Cl$. Higher levels of external N caused a significant decrease in the nitrogenase activity and an increased production of auxins. In a subsequent test, two different inoculum sizes of Z78 ($10^6$ and $10^{12}$ cfu/ml) were used to study the effect of different percentages of acetylene on nitrogenase activity of the inoculum *via* the acetylene reduction assay (ARA). The results showed that the optimal amount of acetylene required for nitrogenase enzyme activity was 5% for the $10^6$ cfu/ml inoculum, whereas the higher inoculum size ($10^{12}$ cfu/ml) required at least 10% of acetylene for optimal nitrogenase activity. These findings provide a clearer

*Corresponding author: amirhg@usm.my

understanding of the effects of N levels on diazotrophic nitrogenase activity and auxin production, which are important factors influencing plant growth.

**Keywords:** *Herbaspirillum seropedicae* (Z78), External Nitrogen, Acetylene, Inoculum Sizes, Nitrogenase Activity, Auxins Production

Nitrogen (N) is an essential nutrient for all life, and the vast majority of organisms have adapted to process N through various environmental sources. In plants, N is taken up only in the reduced form of ammonia ($NH_3$). The reduction takes place *via* biological N fixation (BNF), a high-energy process requiring hydrolysis of many adenosine triphosphate (ATP) molecules within the cell (Hartmann *et al.* 1986). BNF involves symbiotic or associative relationships between the diazotroph and the host plant, as the diazotrophic nitrogenase enzyme (encoded by the diazotroph *nifHDK* genes) catalyses the reduction of gaseous nitrogen ($N_2$) to $NH_3$ (de Campos *et al*. 2006). However, the process is suppressed in many bacterial species when an ample or excess supply of fixed N is available. In the presence of excess fixed N, the diazotrophic nitrogenase enzyme is no longer active, either due to down-regulation of protein synthesis and/or inactivation of the protein (Rudnick *et al.* 1997). Early findings by Eady *et al*. (1978) and Postgate (1982) reported that nitrogenase enzyme is not synthesised by *Azospirillum brasilense* when supplied with high concentrations of ammonium chloride ($NH_4Cl$). It has also been shown that when diazotrophs are exposed to even low amounts of fixed N (including $NH_3$, ammonium [$NH_4^+$] and nitrate ion [$NO_3^-$]), the BNF process can be suppressed by a strict regulatory control mechanism (Eady *et al*. 1978; Postgate 1982; Merrick & Edwards 1995). The fixation process and its regulation are known to be controlled *via* transcriptional repression of the *nif* A gene, which encodes the N fixation activator protein (Yan *et al*. 2010). The presence of fixed forms of N in the cell prevents $N_2$ fixation through the action of N regulatory protein C (NtrC), which prevents nitrogenase enzyme synthesis by repression of the *nif* A gene. The *nif* A gene encodes a positive regulatory protein that activates transcription of other *nif* genes leading to nitrogenase enzyme synthesis. Under limited N conditions, NtrC is active and allows transcription of *nif* A, leading to the synthesis of genes required for N fixation. A similar inactivation mechanism is employed by *Rhodospirillum rubrum*, in which the presence of $NH_4^+$ leads to the suppression of nitrogenous enzyme transcription through covalent modification of an Fe protein (Pope *et al.* 1985). In this case, an excess of $NH_4^+$ causes the addition of an adenosine diphosphate (ADP) molecule to dinitrogenase reductase (Fe protein), which results in loss of enzyme activity. In addition, the amount of fixed N influences the colonisation and growth of diazotrophs on the root surfaces. Excess of $NH_4^+$ causes a reduction in the number of diazotrophs and a decrease in BNF activity (Rivera *et al.* 1991). Decreased nitrogenase enzyme activity of *A. diazotrophicus* due to high concentrations of $NH_4Cl$ and ammonium nitrate ($NH_4NO_3$) has long been recorded (Muthukumarasamy *et al.* 2002). These various findings clarify the reasons why optimal N concentrations are crucial for maximising $N_2$ fixation activities of diazotrophs. In addition to fixation of $N_2$, diazotrophs promote rooting and growth of host plants by synthesising different growth-promoting hormones

such as gibberellins and auxins (indole-3-acetic acid, IAA) (Asghar *et al.* 2002; Ozturk *et al.* 2003). Previous reports show that 80% of bacteria isolated from the rhizosphere are capable of producing phytohormones essential for plant growth (Patten & Glick 1996). For example, IAA production is actively stimulated in plants and increases the cell elongation of roots. Moreover, IAA promotes cell division and differentiation of the vascular tissues of the plant (Tien *et al.* 1979). The secretion of auxins is influenced by microbial culture conditions, diazotroph growth stage and availability of substrates (Frankenberger & Arshad 1995). Several experiments have involved inoculating free-living diazotrophs such as *Azospirillum brasilense* onto paddy plants, sugar cane and in vitro oil palm shoots, showing the promotion of host-plant root growth by auxin secretion (Keyeo *et al.* 2011; Noor Ai'shah *et al.* 2013). However, it is unclear whether phytohormone production of any diazotroph is also influenced by external N concentrations and, if so, what level of external N constitutes the optimal amount. Thus, the objectives of the present experiment are as follows: 1) to observe the effects of external N sources on $N_2$ fixation activities and auxin production of *Herbaspirillum seropedicae* (Z78) and 2) to determine the optimal external N concentration for maximising $N_2$ fixation and phytohormone production by Z78.

The bacteria *H. seropedicae* Z78 (ATCC 35893) was cultured in 250 ml Erlenmeyer flasks containing minimal N medium (Okon *et al.* 1977). The culture was shaken continuously at 160 rpm (28°C) for 48–72 hours on a rotary shaker until the medium became turbid. The optical density for the inoculum was measured at 530 nm, using a spectrophotometer (Lambda Bio + spectrophotometer, Perkin Elmer, USA). A total of 20 µl of broth culture was transferred to semisolid culture media with varied N concentrations and prepared for nitrogenase enzyme analysis. In addition, a total of 1 ml of bacterial culture was inoculated to fresh minimal N medium containing L-tryptophan for auxin production analysis.

The nitrogenase enzyme activity of *H. seropedicae* (Z78) was determined using the acetylene reduction assay (ARA) (Hardy *et al*. 1968; Ohyama & Pham 2006). The assay was performed in airtight 30 ml universal bottles containing 10 ml N-free semisolid media (NFb) with different N concentrations (0, 0.25, 0.75 and 1.0 $gL^{-1}$) of $NH_4Cl$. A total volume of 5% air was removed from the headspace of each universal bottle and replaced with acetylene gas ($C_2H_2$) (99.8% purity), followed by 24 hours incubation at 30°C (Elbeltagy *et al.* 2001). At the end of incubation, a total of 1 ml gas mixture was withdrawn and transferred into a vacuum tube before it was injected into a GC-2014 gas chromatograph (Shimadzu, USA) to assay for the presence of ethylene ($C_2H_4$) gas. The gas chromatograph was fitted with a Supelco Carboxen 1004 stainless steel micropacked column, 2 m × 0.76 mm ID, and equipped with a flame ionization detector (FID). N was used as the carrier gas at a flow rate of 30 ml $min^{-1}$, while the column, injection and FID temperatures were maintained at 80°C, 180°C and 180°C, respectively. The actual concentration of ethylene produced was determined based on the prepared standard curve of $C_2H_4$ gas and the peak area (Somasegaran & Hoben 1985; Elbeltagy *et al.* 2001). The N fixation activity ($\mu$mol $C_2H_4$ cfu $^{-1}$ $h^{-1}$) was defined based on the $C_2H_4$ concentration ($\mu$mol $C_2H_4$) and the viable cell number (cfu) of Z78.

The auxin production of Z78 was assayed based on Salkowski's colourimetric technique (Asghar *et al.* 2002; Patten & Glick 2002). The inocula were cultured in a 250 ml Erlenmeyer flask containing 100 ml of minimal N medium (Okon *et al.* 1977) and treated with different concentrations of $NH_4Cl$ (0, 0.25, 0.75 and 1.0 $gL^{-1}$ of $NH_4Cl$). All of the media preparations were supplemented with 0.5 $gL^{-1}$ L-tryptophan as a precursor for auxin biosynthesis (Glickmann & Dessaux 1995; Theunis *et al*. 2004; Zahir *et al*. 2010; Noor Ai'shah *et al.* 2013). The presence of auxins in the broth culture was detected through the appearance of pinkish red colour (Barazani & Friedman 1999). The colour intensity was measured by a spectrophotometer at 530 nm (Dobbelaere *et al.* 1999).

The results were analysed statistically by analysis of variance (ANOVA) using SPSS 15.0 for data analysis. The ANOVA was performed to test the significance of the treatment effects. One-way analysis of variance for a split-plot factorial design with Duncan's multiple-range test was used to compare the means.

The ability of Z78 to fix N and to produce auxins when supplied with different levels of $NH_4Cl$ was studied. The highest nitrogenase activity ($2.54 \times 10^{-7}$ µmol $C_2H_4$/cfu/h) was recorded at 0 $gL^{-1}$ of $NH_4Cl$ supply relative to the cultures treated with 0.25, 0.75 and 1.0 $gL^{-1}$ of $NH_4Cl$ (Fig. 1). This result indicates that higher levels of N sources have an impact on the acetylene reduction activity, while the nitrogenase enzyme activity of the diazotroph decreases with increasing N supply in the growth medium. The results were different for auxin production, where the highest activity was recorded for inoculum supplied with higher amounts of N (0.25–1.0 $gL^{-1}$ of $NH_4Cl$) (Fig. 2). A trend of increased production was noted when N was supplied to the growth medium. A concentration of 0 $gL^{-1}$ of $NH_4Cl$ resulted in the lowest production of auxins (2.43 µg/ml), while 1.0 $gL^{-1}$ of $NH_4Cl$ resulted in the highest level of auxin production at 11.93 µg/ml.

Previous studies have shown that the BNF activity of diazotrophic microorganisms is influenced by fixed N input because $NH_4^+$ transport consumes an inducible and energy-dependent system that is repressed by $NH_3$ (Hartmann & Kleiner 1982; Pedrosa & Yates 1983). For example, Rasmussen *et al.* (2012) describe the influence of cattle slurry-N fertiliser on suppressed $N_2$-fixation activity of white and red clover inoculated with selected diazotrophs. Another finding by Naudin *et al.* (2011) highlighted that extensive N fertilisation reduced the biomass and $N_2$ fixed by peas in an intercropping system of wheat and pea. In the present study, the process might increase the biomass of wheat but not of pea, and it is likely that the diazotroph population had decreased. Rivera *et al.* (1991) found that higher mineral N concentration decreased the population size of diazotrophic microorganisms in sugar cane and led to a reduction in nitrogenase enzyme activity. The use of $NH_4Cl$ and $NH_4NO_3$ is said to reduce both the colonisation and the acetylene-reduction activity of *Acetobacter diazotrophicus.* Even in small amounts, exogenous $NH_4^+$ is known to quickly and reversibly inhibit the nitrogenase activity in the whole cells of *H. seropedicae* (Fu & Burris 1989). All of these studies contribute to the interpretation of our results, wherein the highest nitrogenase activity was recorded for Z78 grown in medium

treated with 0 $gL^{-1}$ of $NH_4Cl$ and wherein higher levels of N sources (0.25, 0.75 and 1.0 $gL^{-1}$ of $NH_4Cl$) influenced the nitrogenase enzyme activity. A similar response to N input on nitrogenase activity was reported by Klassen *et al.* (2001), in which the addition of 0.2 mmol $L^{-1}$ $NH_4Cl$ caused almost immediate inhibition of the nitrogenase activity of *A. brasilense* FP2. However, the activity was fully recovered following exhaustion of ammonium ions from the medium. The N sources (e.g., $NH_4Cl$ and $NH_4NO_3$) also have an impact on root colonisation and the acetylene-reduction activity of *A. diazotrophicus* (Muthukumarasamy *et al.* 2002). These various findings also explain why the nitrogenase activity is the highest when the N supply is 0 $gL^{-1}$ of $NH_4Cl$ and decreases once the N supply increases. Our experimental results are best explained by the regulatory control mechanism of the nitrogenase enzyme at the transcriptional and post-translational levels (Chubatsu *et al.* 2012). In addition, culture conditions, which include nutrient availability, pH and bacterial strain, will also influence the "$NH_4^+$ switch off" mechanisms (Hartmann *et al.* 1986). Vose *et al.* (1981); Rudnick *et al.* (1997), and Steenhoudt and Vanderleyden (2000) also highlighted the inhibition of nitrogenase activity for *A. brasilense* and *H. seropedicae* due to inactivation of NifA under excess N conditions. Upon exhaustion of N sources from the medium, the NifA was activated through a process that involved the signal transduction protein PII. Recent findings by Yan *et al.* (2010) show that the transcriptional regulation of the *nif* gene depends on both *nif*-specific and *ntr* gene regulatory systems. Suppression of the nitrogenase activity may be due to the inhibition of *nif* A gene expression (Souza *et al.* 1999). The *nif* A gene is important for inducing other *nif* gene expression including the iron-molybdenum cofactor (FeMo-co) nitrogenase structural genes.

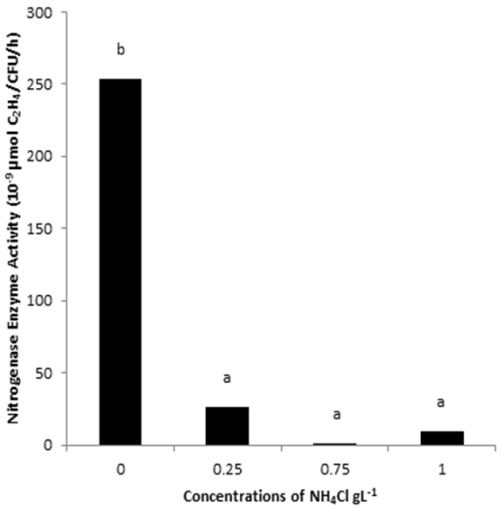


**Figure 1:** Nitrogenase enzyme activity with different $NH_4Cl$ concentrations ($gL^{-1}$) for Z78 (*H. seropedicae).*

*Notes:* Data shown are the means of triplicate tests. Means accompanied by different letters are significantly different (Duncan's test, $p<0.05$).

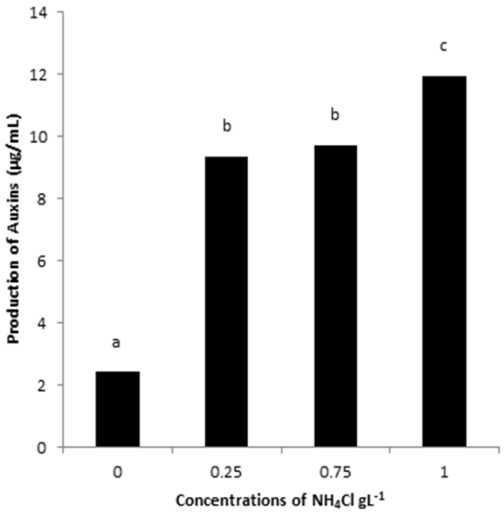


**Figure 2:** Production of auxins for Z78 (*H. seropedicae*) treated with different $NH_4Cl$ concentrations ($gL^{-1}$).
*Notes:* Data shown are the means of triplicate tests. Means accompanied by different letters are significantly different (Duncan's test, $p<0.05$).

It has been demonstrated that diazotrophs can be beneficial to plants not only for their ability to fix $N_2$ but also for their ability to produce beneficial phytohormones such as IAA (Rodrigues *et al.* 2008). Auxin production is important and reportedly plays a vital role in stem and root elongation in higher plants and growth stimulation of microorganisms (Goodwin 1978; Tsavkelova *et al.* 2006). Auxin production is also reported to affect photosynthesis in inoculated host plants. In addition, the resistance of plants to stress factors is also influenced by auxin biosynthesis (Tsavkelova *et al.* 2006). IAA is synthesised by microbes including the epiphytic and tissue-colonising bacteria in soil (Patten & Glick 1996). Auxins are also produced by isolates from the rhizosphere as secondary metabolites due to a rich supply of substrates available (Strzelczyk & Pokojska-Burdziej 1984). One of the most prominent diazotrophs and auxin producers is *Azospirillum* spp., which is reported to form an association with both cereal and non-cereal plants (Bashan *et al.* 2004). Thus, auxin-synthesising rhizobacteria are better studied than other rhizobacteria (Tsavkelova *et al.* 2006; Spaepen *et al.* 2007). Our results have shown that higher levels of auxins were produced by Z78 when the media was supplied with a higher amount of N. An increasing trend is observed, with 0 $gL^{-1}$ of $NH_4Cl$ resulting in the lowest production of auxins and 1.0 $gL^{-1}$ of $NH_4Cl$ resulting in the highest. Similar findings by Tharwat *et al.* (2004) showed that application of various N sources such as $NH_4Cl$, ammonium sulphate [$(NH_4)_2SO_4$], ammonium phosphate monobasic ($NH_4H_2PO_4$) and $NH_4NO_3$ in low concentrations stimulated indole production in *Azospirillum* strains. However, application of potassium nitrate ($KNO_3$), sodium nitrate ($NaNO_3$) and potassium ($KNO_2$) inhibited the production of indole compounds for all bacteria tested. Noor Ai'shah *et al.* (2013) reported

that the viable cell numbers of diazotrophs also influenced the production of optimum IAA and can be related to $N_2$ fixation capacity of diazotrophs. It was reported that higher viable cell numbers of diazotrophs (up to $10^{10}$ cfu $ml^{-1}$) influenced the production of optimum IAA. The overall IAA productivity of Z78 was recorded at 0.165 µg $ml^{-1}$ $hr^{-1}$, while the viable cell count increased gradually (Noor Ai'shah *et al.* 2013). Any declines in IAA production reflected the decline in viable cell numbers of inoculum tested. Although the diazotroph could produce more IAA, under in vitro conditions, IAA production may show detrimental effects to the host plants. Tharwat *et al.* (2004) noted that high amounts of indoles produced by *Azospirillum* strains reduced the length of roots and stems of the host plants. Similarly, Keyeo *et al.* (2011) found that plants inoculated with *A. brasilense* (Sp7) and *H. seropedicae* (Z78) both showed inferior growth due to excess auxin production. Higher concentrations of IAA in the range of 0.001–1.0 µg/ml can also inhibit nitrogenase activity of *Azospirillum lipoferum* (Silveira & Drozdowicz 1983). These findings clearly support the conclusion that the optimal concentration of N required by Z78 to stimulate $N_2$ fixation activity was recorded at 0 $gL^{-1}$ of $NH_4Cl$. A higher supply of exogenous N would suppress nitrogenase enzyme activity. The optimal concentration of N needed for auxin production is also 0.25 $gL^{-1}$ of $NH_4Cl$.

In conclusion, *H. seropedicae* (Z78) shows the highest nitrogenase activity when supplied with 0 $gL^{-1}$ of $NH_4Cl$ based on the ARA test. When higher levels of N were supplied, a significant decrease in the nitrogenase activity of these bacteria resulted. The production of auxins requires at least 0.25 $gL^{-1}$ of $NH_4Cl$. High auxin production therefore does not correspond to high nitrogenase activity.

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