

DENITRIFICATION BY *AZOSPIRILLUM BRASILENSE* AND *SINORHIZOBIUM SP* IN THE PRESENCE OF THE NITRIFICATION INHIBITOR

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ABSTRACT

Investigation the effects of nitrification inhibitor viz, 3,4dimethylpyrazolephosphate (DMPP on denitrification and N_2O/N_2 productions by N-fixers bacteria (*Azospirillum brasilense* Az204 and *Sinorhizobium sp* TNAU14 a model experiment was carried out in laboratory conditions. As a model experiments consisted of 3 treatments, control, 1% v/v C_2H_2 and 1 μg DMPP mL^{-1} in combination with the inoculation of *Azospirillum brasilense* or *Sinorhizobium sp*. The concentration of N_2O and CO_2 , the population density, and the level of nitrate, nitrite, and ammonium in the medium solution were analyzed. The results showed that the kinetic of N_2O production by *Azospirillum brasilense* in the presence and absence of 1 % acetylene ranged about 2–18 μg N_2O $mL^{-1} day^{-1}$, and 2–17 μg N_2O $mL^{-1} day^{-1}$. The rate of N_2O production by *Sinorhizobium sp* was about 4–15 μg N_2O $mL^{-1} day^{-1}$ and 4–12 μg N_2O $mL^{-1} day^{-1}$ in the presence and absence of 1% v/v acetylene. The N_2O portion of total-denitrification loss by *Azospirillum brasilense* or *Sinorhizobium sp* was about 70–98% and 80–95% and decreased until about 50–51% due to the decreasing of nitrate supply. The application of 1 μg DMPP mL^{-1} decreased both N-fixers population. The CO_2 -production by *Azospirillum brasilense* decreased significantly. DMPP may influence the population of non-target-microorganism in soils.

Key words : *Azospirillum brasilense*, *Sinorhizobium sp*, nitrification inhibitor

PENGARUH INHIBITOR NITRIFIKASI TERHADAP AKTIVITAS DENITRIFIKASI OLEH *AZOSPIRILLUM BRASILENSE* DAN *SINORHIZOBIUM SP*

ABSTRAK

Kajian pengaruh inhibitor nitrifikasi 3,4 -dimethylpyrazolephosphat (DMPP) terhadap denitrifikasi dan produksi N_2O/N_2 oleh bakteri pemfiksasi nitrogen (*Azospirillum brasilense* Az₂₀₄ and *Sinorhizobium sp* TNAU14) telah dilakukan dalam bentuk percobaan model pada kondisi laboratorium. Percobaan terdiri dari 3 perlakuan yaitu kontrol: pemberian 1% v/v C_2H_2 dan pemberian 1 μg DMPP mL^{-1} yang dikombinasikan dengan inokulasi *Azospirillum brasilense* atau *Sinorhizobium sp*. Pengukuran dilakukan terhadap konsentrasi gas yang dihasilkan yaitu N_2O dan CO_2 , kerapatan populasi, kandungan nitrat, nitrit, dan ammonium dalam larutan media. Hasil penelitian menunjukkan bahwa produksi N_2O oleh *Azospirillum brasilense* dengan perlakuan dan tanpa perlakuan 1% asetilen masing-masing sekitar 2–18 μg N_2O $mL^{-1} hari^{-1}$, dan 2–17 μg N_2O $mL^{-1} hari^{-1}$. Produksi N_2O oleh *Sinorhizobium sp* berkisar 4–15 μg N_2O $mL^{-1} hari^{-1}$ dan 4–12 μg N_2O $mL^{-1} hari^{-1}$ yaitu dengan pemberian dan tanpa pemberian 1% asetilen. Kehilangan nitrat dalam bentuk emisi N_2O melalui denitrifikasi oleh *Azospirillum brasilense* atau *Sinorhizobium sp* berkisar 70–98% dan 80–95% dan menurun hingga sekitar 50–51% akibat penurunan suplai nitrat. Perlakuan 1 μg DMPP mL^{-1} pada media pertumbuhan dapat menurunkan jumlah populasi kedua bakteri pemfiksasi nitrogen. Produksi CO_2 oleh *Azospirillum brasilense* menurun secara signifikan. DMPP dapat mempengaruhi populasi mikroorganisme non target di dalam tanah.

Kata kunci: *Azospirillum brasilense*, *Sinorhizobium sp*, inhibitor nitrifikasi

INTRODUCTION

Nitrous oxide is a biogenic greenhouse gas emitted to the atmosphere from soils and it stimulated by agricultural management practices. Optimization of agricultural resources for improved and sustainable agriculture involves the use of nitrification inhibitors. Nitrous oxide (N_2O) is produced by biological processes in the soil such as denitrification and nitrification (Stark and Richards, 2008). The use of nitrification inhibitors has been shown to be a useful technique to reduce N_2O emissions and NO_3^- leaching from soil, when mineral or high organic-N fertilizers are applied. Further, the application of these compounds that retard nitrification is used to improve N recovery and N use efficiency in agricultural soils, while the same time limiting the environmental impacts of N loss and thus improving sustainability (Fillery, 2007). It have beneficial effect on nitrous oxide emission to the atmosphere or denitrification (Weiske *et al.* 2001; Di *et al.* 2006; 2007), and reducing nitrate leaching into ground water (Di *et al.* 2004; 2005) and affect N retention in the root zone and microbial biomass and activity in the rhizosphere and as a result increase plant growth (Douma *et.al.* 2005; Malla *et. al.* 2005, Moir, *et al.* 2007). The use of nitrification inhibitors (NIs) show a reduction of about 60% in NO_3^- -leaching, 70% in N_2O emissions and an increase of more than 20% in crop and pasture yield can be achieved (Pasda, *at. al.* 2001: Sahrawat, 2004; Douma *et al.* 2005; Di. *et al.* 2007; Moir *et al.* 2007; Singh & Verma, 2007). Nitrification results in the formation of highly mobile nitrate, which is susceptible to loss from root zone by leaching and/or gaseous emissions of di-nitrogen or nitrous oxide through denitrification. As the loss of soil N in solution or gaseous form can cause pollution as well as N deficiencies in crops and pastures, the prospect of actively regulating these soil processes has major implication for improving efficiency of fertilizer nitrogen in agriculture and for plant productivity (Chen *et al.* 2008; Li *et al.* 2008: Stark & Richards, 2008). The

reduced nitrification can have significant impacts on the soil carbon cycle and, for example, decrease organic decomposition. N species (i.e. ammonium vs. nitrate) may a more important driver of carbon cycling and ecosystem functioning than the quantity of N present in the system (Austin *et al.* 2006, Stark & Richards, 2008).

Nitrification inhibitors use in agriculture should be recommended in low concentration and capable to control nitrate supply to crop so that avoid the excess of nitrate supply in soils. The inhibitor has the specific influence that is only inhibit the nitrification and not for nitrification so that accumulation can be avoid. The Inhibitor should be *bacteriostatic* and not a *bactericide* which killing certain microorganism in soils like *Nitrosobacter* sp, *Nitrosococcus* sp. Ammonium-recommended fertilizers are the most widely used source of N for crop production and keeping the applied fertilizer N in NH_4^+ form by using nitrification inhibitors (NIs) is a well documented strategy for reducing N loss and to minimize negative environmental impacts of the fertilizer-use. Three compounds have been commercialized as NIs for agricultural use including (i) nitrapyrin (2-chloro-6-trichloromethyl-pyridin, trade name N-Serve), (ii) dicyandiamide or DCD (trade name Didin, Alzon or and Ensan), and (iii) more recently DMPP (a pyrazole derivative, 3,4-dimethylpyrazole phosphate; trade name ENTEC) (Zerulla *et al.* 2001; Weiske *et al.* 2001; Barth *et. al* 2006; 2008; Ali, *et al.* 2008). The application DMPP under field conditions reduced the emission of N_2O and CO_2 significantly (Weiske *et al.* 2001). However, the site effect on non target microorganism activity in soils is still unclear, particularly on diazotrophs N-fixer bacteria.

Recently, increasing attention is being paid to diazotrophs bacteria, this due to their ability in fixing nitrogen and produce the plant growth promoting factor (phytohormones) and as well as on their ability to denitrify simultaneously (Biro, *et al.* 2000; Cassan, *et al.* 2009: Naher, *et al.* 2009). It was also reported that arbuscular mycorrhizal fungi (AMF) colonization and AM fungi activity

is enhanced by diazotrophs (*Azospirillum* and *Rhizobium*) (Barassi, *et al.* 2007; Mia & Shamsuddin, 2010; Molina-Favero, *et al.* 2008:) The highly energy consumption during the nitrogen fixation may be provided from nitrate respiration. The energy gain by using nitrate as an alternative electron acceptor under anaerobic condition is nearly as high during oxygen respiration (Stephan, *et al.* 1984; Ottow & Benckiser 1994). Consequently, the combination of highly energy consuming of nitrogen fixation with the ATP providing nitrate respiration seems to be widespread under N-binding bacteria. Recently work showed that the nitrogen fixing denitrifiers are detected under N-diazotrophic families of *Aquaspirillum*, *Azospirillum*, *Azoarcus*, *Bacillus*, *Bradyrhizobium*, *Sinorhizobium*, *Pseudomonas*, *Rhodobacter* and *Rhodopseudomonas* (Yassin & Patwardhan, 2007; Cassan, *et al.* 2009; Mia & Shamsuddin, 2010). The quantification of potential N_2O released by N-fixing-denitrifying bacteria is important in estimating the contribution of agricultural practice to the global N_2O -emission. In addition, the percentage of a climate relevant trace gas (N_2O) or green house effect gas on the total denitrification losses is still unclear.

Objective of the present study was to evaluate under laboratory conditions the effect of the nitrification inhibitor viz., 3,4-dimethylpyrazole-phosphate (DMPP) on the activity of N-fixer-denitrifiers bacteria *Azospirillum brasilense* Az204 and *Sinorhizobium sp* TNAU14 (population number, kinetic of N_2O - and CO_2 -emission and as well as on the N_2O/N_2 -ratio).

MATERIALS AND METHODS

Used NI concentrations

The used nitrification inhibitor DMPP (purity 99.9 %), was obtained from the BASF, Ludwigshafen, Germany. In experiments utilizing this NI, stock solution of the inhibitor was prepared in distilled water by mixing the inhibitor in solution, whereas for experiment with NI as pure active ingredient as well as the control. Portions of this stock solution were used to achieve the

desired level in soil. Recommended rates for the application of DMPP is $0.36 \mu\text{g g}^{-1}$ dry soils, respectively. In agriculture the recommended NI concentrations correspond to 90 kg N ha^{-1} N-fertilizer application.

Culture enrichments

The *Azospirillum brasilense* Az 204 and *Sinorhizobium sp* TNAU used were obtained from culture collection of Department of Agricultural Microbiology Tamil Nadu University of Agriculture (TNAU), Coimbatore, India and was isolated from sugarcane rhizosphere, while the *Sinorhizobium sp* TNAU 14 was isolated from peanut nodule (*Arachys hypogaea*). Both *Azospirillum* and *Sinorhizobium* stock culture were maintained on solid yeast-manitol growth medium for *Sinorhizobium* and solid Lactat-biotin-nicotin acid-pantothenic growth medium for *Azospirillum* at 4°C (Dreyfus *et al.* 1988). The enrichment was successfully by inoculated those culture in to 250 ml Flask volume containing 100 ml nutrient mediums (Table 1). The amount of about $10^8 \text{ cell mL}^{-1}$ was obtained after 3 days of incubation at 25°C and was used as inoculants in model experiments.

Model experiments and analyses

The experiments were consisted of three treatments, as follows; (1) control, (2) 1% v/v C_2H_2 and (3) $1 \mu\text{g mL}^{-1}$ DMPP in combination with the inoculation of *Azospirillum brasinlense* Az204 or *Sinorhizobium sp* TNAU 14 and provided with three replication. One milliliter of inoculants (about $10^8 \text{ cell mL}^{-1}$) of *Azospirillum* or *Sinorhizobium* was transferred inoculated in to 2,5 L flask (Fisherband, Fisher Scientific, Germany) equipped with firmly capped and rubber septum (Verneret, French) containing 100 ml medium which was treated formerly with $1 \mu\text{g mL}^{-1}$ DMPP or about 3 times of field concentration used (3-4 dimethylpyrazolephosphate) under sterile condition. Subsequently the bottle was flushed with N_2 -gases (about 10-min) to obtained N_2 -atmospheric conditions (anaerobic conditions): Finally, the 1% v/v C_2H_2 was added in to the suitable flask

using 70 ml volume of air tight syringes (Becton & Dickinson, Irland) and those treatments were incubated for 12 days at 25 °C. The concentration of N_2O and CO_2 in the headspace were measured regularly using a gas chromatography equipped with electron capture detector (Perkin Elmer 8500, Überlingen, Germany; Porapak Q Alltech, column 3 + 1m precolumn, 1/8", 80-100 mesh, N_2 carrier gas, detector 350 °C, injector 40 °C, oven 50 °C, flow 30 ml min^{-1}). Before each measurement, the flask was flushed about 5 min with N_2 gas and the measurement was done after 30 min incubation. About 40 ml of gas sample was taken using air tight syringes (Plastipak-Syringes, Becton and Dickinson, Irland) for N_2O and CO_2 analyses. The released N_2O and CO_2 were calculated after integration of peak area using the computer program peak simple gaschromatography data system for GC and LC (SRI Instruments, USA) using and the kinetic of N_2O - and CO_2 -production expressed in $\mu g\ mL^{-1}\ d^{-1}$ were calculated according to Gay-Lussack for ideal gas, $n = (P \times V)/(R \times T)$. The N_2O or CO_2 retained in medium solution were estimated according to Regina *et al.* (1998). The population density was calculated according to the MPN method (Lorch *et al.* 1995) and as well as the chemical properties (Nitrate, nitrite, and ammonium content) in the medium solution were analyzed regularly during the incubation times (DEV, 1981:1983, Navone, 1964).

Quantification of actual N_2O -emission and total denitrification losses ($N_2O + N_2$)

Denitrification as measured via the acetylene-inhibition-method. This method can be used to determine either actual denitrification under field conditions or potential denitrification under optimized laboratory conditions (anaerobiosis, addition of substrates, optimum temperatures). The N_2O released in absent of acetylene was pointed as actual denitrification, where as its concentration in present of 1% v/v acetylene was pointed as total denitrification (Pell, *et al.* 1998)

RESULTS AND DISCUSSION

Results

Potential of N_2O -emission and Nitrate Reduction

In Fig. 1 the N_2O -emission potential (control) and total denitrification ($N_2O + N_2$, in the present of 1% C_2H_2) of N-fixing *Azospirillum brasilense* Az204 (A) and *Sinorhizobium sp* TNAU14 is presented, where as the nitrate reduction is presented in Fig. 2.

This results revealed that either *Azospirillum brasilense* Az204 or *Sinorhizobium* were able to use the nitrate as electron acceptor. The N_2O -emission of the treatments inoculated with *Azospirillum brasilense* Az204 was increased rapidly and reached the highest emission (about 16-18 on 2-3th days), while the highest N_2O -

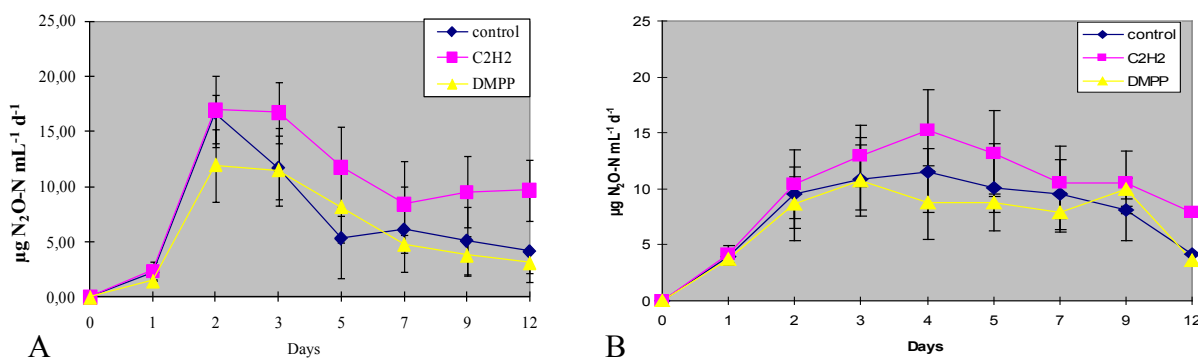


Figure. 1. The N_2O -release of *Azospirillum brasilense* Az204 (A) and *Sinorhizobium sp* TNAU14 (B) in the present of C_2H_2 and DMPP in model experiments (25 °C, N_2 -atmosphere)

Table. 1 Actual N_2O -release (without acetylene) and total denitrification (in the present of 1% acetylene) during 12 days of incubation time (N_2 -Atmosphere, 25°C) by *Azospirillum brasilense* Az204- und *Sinorhizobium sp* TNAU14.

Waktu (hari)	N_2O ($\mu\text{g N}_2\text{O-N mL}^{-1}$)		Total denitrifikasi ($\text{N}_2\text{O} + \text{N}_2$) ($\mu\text{g N}_2\text{O-N mL}^{-1}$)		Persentase N_2O (%)	
	<i>Azospirillum brasilense</i>	<i>Sinorhizobium sp</i>	<i>Azospirillum brasilense</i>	<i>Sinorhizobium sp</i>	<i>Azospirillum brasilense</i>	<i>Sinorhizobium sp</i>
1	2.19	4.00	2.37	4.17	92.53	95.8
2	16.67	8.49	16.98	9.42	98.17	90.1
3	11.65	10.87	16.70	12.92	69.79	84.1
5	5.31	11.54	11.77	15.25	45.11	75.7
7	6.09	10.11	8.42	13.18	72.36	76.7
9	5.10	8.16	9.52	10.56	53.55	77.3
12	4.13	4.12	9.67	7.94	42.70	51.9

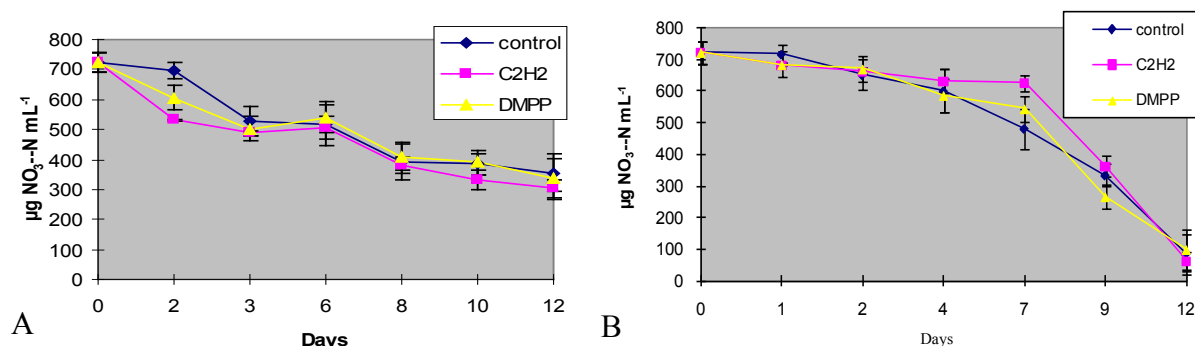


Figure 2. The reduction nitrate by *Azospirillum brasilense* Az204 (A) and *Sinorhizobium sp* TNAU14 (B) in the present of C_2H_2 and DMPP in model experiments (25°C , N_2 -atmosphere)

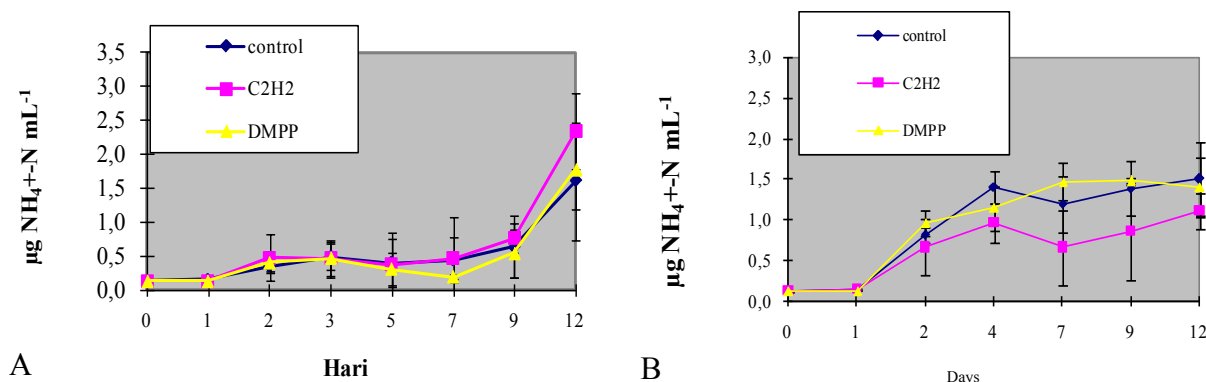


Figure 3. The ammonium formation by *Azospirillum brasilense* Az204 (A) and *Sinorhizobium sp* TNAU14 (B) in the present of C_2H_2 and DMPP in model experiments (25°C , N_2 -atmosphere)

emission ($15\text{-}16 \mu\text{g N}_2\text{O-N mL}^{-1}$ medium) of the treatments inoculated with *Sinorhizobium sp* TNAU14 was reached on 3-4th days after incubation. The N_2O percentage of total denitrification were relatively high (80-90%) at the beginning of the experiments and it's decreased slowly until 40-50% (Tab.

1). This behavior was depended on nitrate availability (Fig. 2). The relatively high of nitrate supply (about $700 \mu\text{g NO}_3\text{-N mL}^{-1}$ at the beginning of the experiments) lead to the higher production of N_2O) and the total denitrification losses (the quality of denitrification gas) was dominated by N_2O .

In addition, the decreasing of nitrate content was followed by the reduction the N_2O -emission and the dynamic nitrate reduction and the N_2O -production of both N-fixers bacteria showed relative similar pattern.

Nitrite and Ammonium Formation

The pattern of the ammonium (Figure 3) and nitrite (Figure 4) formation either inoculated with *Azospirillum brasilense* Az204 or *Sinorhizobium* were relative different. The nitrite concentration of treatments inoculated with *Azospirillum brasilense* Az204 were relative low. In contrast, the nitrite contents of treatments inoculated with *Sinorhizobium sp* TNAU14 were clearly increased. This may lead to a differently denitrification potential. An increasing of the ammonium contents in medium of both treatments may be derived from nitrogen fixation. Consequently, both N-fixers have the ability to denitrify and to bind the nitrogen simultaneously under a

relative high of nitrate content.

CO_2 Emission and Population Density

In Figure 5. the CO_2 -production of *Azospirillum brasilense* Az204 (A) and *Sinorhizobium sp* TNAU14 (B) in the present of acetylene and DMPP is presented. Fig. 6 showed that even pattern of CO_2 -emission were relative similar, but the inoculation with *Sinorhizobium sp* lead to much higher production of CO_2 . The relative higher production was supported by the high population density of *Sinorhizobium sp* (Fig. 6). The population of *Sinorhizobium sp* TNAU was much higher than *Azospirillum brasilense* Az204. This may be expected, because the *Sinorhizobium* is belong to fast growing rhizobacteria.

The effect of DMPP on the nitrogen fixer activity

The activity of *Azospirillum brasilense* Az204 and *Sinorhizobium sp* TNAU 14

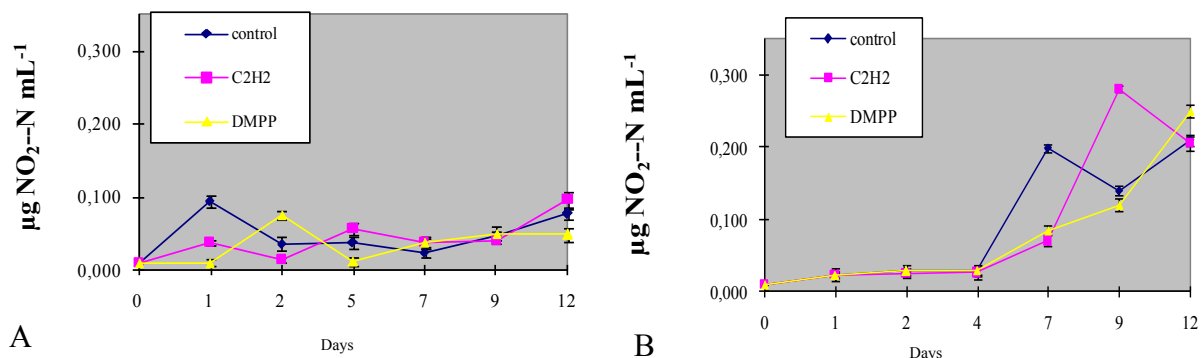


Figure 4. The nitrite formation by *Azospirillum brasilense* Az204 (A) and *Sinorhizobium sp* TNAU14 (B) in the present of C₂H₂ and DMPP in model experiments (25 oC, N₂-atmosphere)

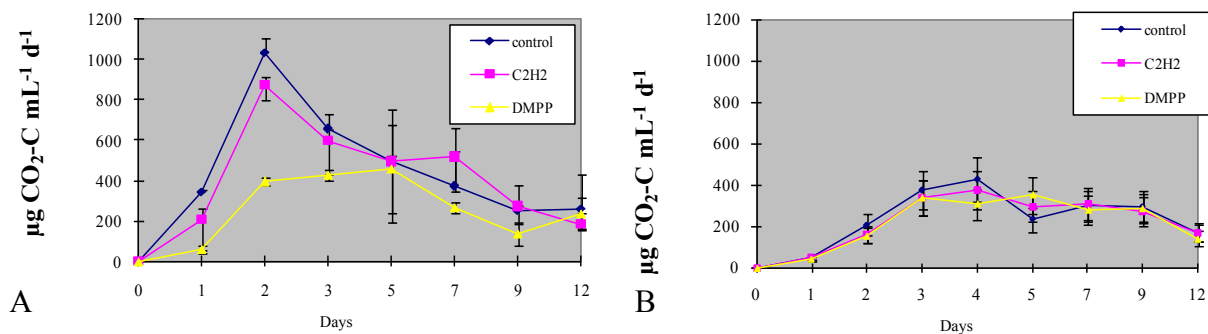


Figure 5. The CO_2 -emission of of N-fixing *Azospirillum brasilense* Az204 (A) and *Sinorhizobium sp* TNAU 14 (B) in the present of acetylene or DMPP model experiments (25 oC, N₂-atmosphere)

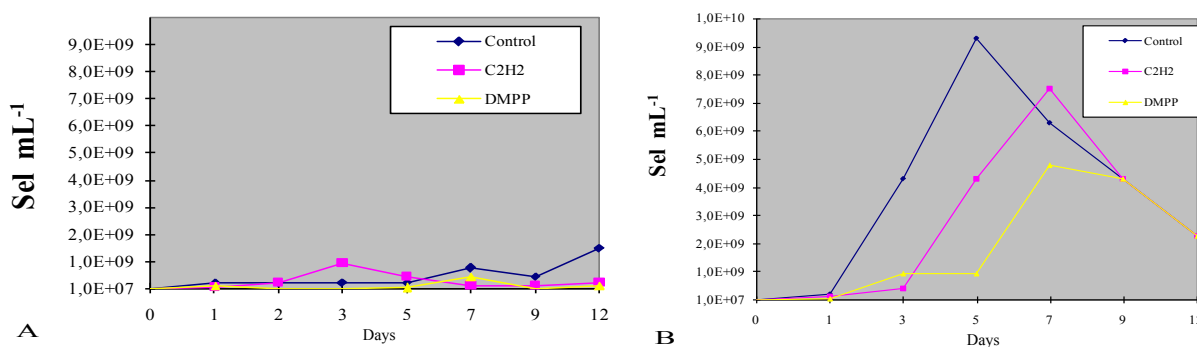


Figure 6. The population density of *Azospirillum brasilense* Az204 (A) and *Sinorhizobium* sp TNAU14 (B) in the present of DMPP and acetylene in model experiments (25 oC, N₂-atmosphere)

in the present of 1 $\mu\text{g mL}^{-1}$ (about 3 times recommended field application) DMPP (dimethyl pyrazolephosphate) are presented in Figure. 1, 5 and Figure. 6, respectively. The N₂O emission in present of DMPP was relative lower than control either inoculated with *Azospirillum brasilense* or *Sinorhizobium* sp. These results were supported by the CO₂-formation and population density. The population density and as well as the CO₂ formation of both N₂-fixers in the present of DMPP were relative lower than control. Consequently, the application of DMPP may give a negative side effect on other microbes (*non target microorganisms*). However, Its effect under field conditions will be influenced on the soil physical and chemical properties and as well as the biological interactions.

Discussion

Thus far, the potential side effects of nitrification inhibitors on the soil ecosystem have not been established in detail. The existing literature suggests that their use does not negatively affect the soil microbial community, and there is evidence that nitrification inhibitors have no effects on microbial biomass, respiration and enzymatic activities (Di *et al.* 2007; Mahmood, *et al.* 2005; Muller *et al.* 2002). Molecular analysis of the soil bacterial community indicated that application of nitrification inhibitor (dicyandiamide :DCD) to soil did not affect the composition of the predominant bacterial phyla present in soil (Callaghan, *et al.* 2010). In agreement

with these findings, Egamberdiyeva *et al.* (2001) reported increased numbers of oligonitrophilic bacteria and cellulose degradation activity and a decrease in the number of nitrifying and denitrifying bacteria after application of potassium oxalate as nitrification inhibitor, while at the same time availability of fertilizer N to plants was increased. They concluded that the combination of potassium oxalate and mineral fertilization showed promising potential concerning nitrification inhibition. While their study did not assess the specific effects of synthetic nitrification inhibitors on soil microbial populations. Austin *et al.* (2006) showed that reduced nitrification can have significant impacts on the soil carbon cycle and, for example, decrease organic matter decomposition, when applying nitrapyrin to an undisturbed semi-arid steppe. Their results indicated that N species (i.e. ammonium vs. nitrate) may be a more important driver of carbon cycling and ecosystem functioning than the quantity of N present in the system. This underlines the significance of different forms of N in terms of carbon turnover in soils and highlights the need for further studies into the effects of chemical nitrification inhibitors on all nutrient turnover processes and their interactions in soil ecosystems.

More recent studies demonstrated that the efficacy of DMPP was closely related to soil organic constituent and that the adsorption of DMPP to the soil fraction played a major role in controlling inhibition effect (Azam & Farooq, 2003; Austin, *et.*

al, 2006: Barth, *et al.* 2006:2008). Once the nitrification inhibitor is in the soil, it may be gradually broken down by soil microbes and its efficacy slowly disappears. A timely nitrification inhibition is wished and an incubation of DMPP in the sandy loam showed that after 12 days of incubation the DMPP-concentration significantly decreased and at day 35 only small amounts of DMPP were still detectable. A major role hereby may play the temperature and availability of organic carbon (Irigoyen, *et al.* 2003: Sahrawat, 2004). However, performance of NIs can be highly variable in different agro ecosystems. Granulated DMPP-fertilizer application is apparently superior to liquid DMPP-application and under wet conditions, favorable for nitrate leaching, most effective in the sandy loam (Barth, *et al.* 2008: 2008: Irigoyen, *et al.* 2003: Li, *et al.* 2008).

In practice, however, DMPP as aggregates (with a grain diameter of about 4mm) formulated on ammonium-N applied, therefore it would not in a homogeneous distribution (Azam, *et al.* 2001; Barth, *et al.* 2001: Zerulla, *et al.* 2001: Di *et al.* 2006). It can be assumed that the granules of ENTEC (N-fertilizer and DMPP) after rain hydrolyzed gradually with the result that ammonium and possibly DMPP (also CIMPP) to diffuse rapidly among the granules into the soil. It took consequently in the field, temporally and spatially different concentration gradients of DMPP (or CIMPP) and ammonium, which for DMPP between 0 and about 100 $\mu\text{g g}^{-1}$ dry soil at a granulate distance (in the 0-5 mm zone of the granule center) are expected (Azam *et al.* 2001). A comparison of the inhibitory concentrations for further inhibition of nitrification in the laboratory showed that a complete inhibition of ammonium oxidation was temporally and spatially under the pellets and around them is around given the least, especially since about 80% of DMPPs remains in a loamy soil over a 10-days period in around during 0-5 mm of the granules (AZAM *et al.* 2001). However, it remains very difficult to know the real, locally and time and the process of granules to inhibit nitrification in the field, because the concentrations of DMPP and ammonium

because of the different diffusion rates should change continuously. It is likely that DMPP in soils with usual pH values from 5 to 6.5 predominantly active cation (diffuse through Protonation) and due to their molecular sizes significantly slower than the ammonium. In Laboratory experiments, which were carried out under standard conditions and at different temperatures (4, 15, 25 °C) and soil moisture (18 or 20% of the mWHC), let the example of a silty clay showed that only 5-15% of DMPPs after 10 days were in the 25-40 mm zone around the granules, which confirmed the very low mobility DMPPs (Azam *et al.* 2001, Di, *et al.* 2007). In soils, the ratio of ammonium was changed to DMPP in the course of the time probably constantly. The inhibitions of Nitrification in the field were depending on such conditions, which in model experiment are hardly possible to create them. From the above considerations' can be concluded that the nitrification inhibition must take place substantially in close contact with the DMPP-granules. To clear up this point, some more field experiments in different soil types should be conducted.

CONCLUSION

Based on the obtained results above, the following conclusion can be drawn; The kinetic of N_2O production by *A. brasilense* in the present of 1% v/v acetylene or in absent of acetylene ranged about 2-18 $\mu\text{g N}_2\text{O mL}^{-1} \text{ day}^{-1}$, and 2-17 $\mu\text{g N}_2\text{O mL}^{-1} \text{ day}^{-1}$, respectively, while the rate of N_2O production by *Sinorhizobium sp* was about 4-15 $\mu\text{g N}_2\text{O mL}^{-1} \text{ day}^{-1}$ and 4-12 $\mu\text{g N}_2\text{O mL}^{-1} \text{ day}^{-1}$. The N_2O portion of total denitrification losses at the beginning of experiment (700 $\mu\text{g N-NO}_3^- \text{ mL}^{-1}$) by *Azospirillum brasilense* AZ204 or *Sinorhizobium sp* was about 70-98% and 80-95%, respectively and its decreased gradually until about 50- 51% due to the decreasing of nitrate supply. The application of 1 $\mu\text{g mL}^{-1}$ DMPP (3 times of field concentration) has lead to the decreasing of both nitrogen fixers population, particularly; the CO_2 -production (carbon mineralisation) by *Azospirillum brasilense* was decreased significantly.

DMPP may influence the population of non target microorganism in soils.

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REFERENCES

- Ali, R., Iqbal, J., Tahir G. R., & Mahmood T. 2008. Effect of o 3-5 dimethylpyrazole and nitrapyrin on nitrification under high soil temperature. *Pak. J. Bot.*, 40:1053-1062.
- Austin A. T., Sala, O. E., & Jackson R. B. 2006. Inhibition of nitrification alters carbon turnover in the Patagonian steppe. *Ecosystems.*, 9:1257-1265.
- Azam, F., Benckiser, G., Muller, C & J. C. G. Ottow. 2001. Release, movement and recovery of 3-4 dimethylpyrazole phosphate (DMPP), ammonium and nitrate from stabilized fertilizer granules in a silty clay soil under laboratory conditions. *Biol. Fertil. Soils.*, 34:118-125.
- Barth, G., Tucher, S von & Schmidhalter, U. 2001. Influence of soil parameter on the effects of 3-4 dimethylpyrazole phosphate (DMPP) as nitrification inhibitor. *Biol. Fertil. Soil.* 34:98-102.
- Barth, G. 2006. Influence of soil properties on the effect of 3,4 - dimethylpyrazole - phosphate as nitrification inhibitor. *Technischen Universität München.*
- Barth, G. 2008. Effectiveness of 3,4-Dimethylpyrazole Phosphate as Nitrification Inhibitor in Soil as Influenced by Inhibitor Concentration, Application Form, and Soil Matrix Potential. *Pedosphere.*, 18:378-385
- Barassi, C. A., Sueldo. R. J., Creus, C. M., Carrozi, L. E., Casanovas, E. M & M. A. Preyra. 2007.: *Azospirillum spp.*, a Dynamic Soil Bacterium Favourable to Vegetavble Crop Production. *Dynamic Soil, Dynamic Plant.*, 1:68-82.
- Callaghan, M. O., Nelson, T, Lardner, R, Carter, P, Gerard.E, & Brownbridge, M. 2010. Non-Target impact of The Nitrification Inhibitor Dicyandiamide on Soil Biota. *World Congress of Soil Science. Brisbane, Australia*, 1-6 August 2010.
- Cassan, G., Perrig, D., Sgroy, V., Masciarelli., Penna, C., & Luna V., 2009. *Azospirillum brasilense* Az39 and *Bradyrhizobium japonicum* E109, inoculated singly or in combination, promote seed germination and early seedling growth in corn (*Zea mays* L.) and soybean (*Glycine max* L.). *European Journal of Soil Biology.*, 45: 28-35.
- Chen D., Suter H. C., Islam A, Edis R, & Freney J.R. 2008. Prospects of improving of fertilizer nitrogen in Australian agriculture: a review of enhanced efficiency fertilizers. *Australia Journal of Soil Research.*, 46: 289-301.
- DIN. 1997 *Die Ammonium-N und Nitritbestimmung.*
- Di, H. J & Cameron K. C. 2004. Effects of temperature and application rate of a nitrification inhibitor, dicyandiamide (DCD) on nitrification rate and microbial biomass in a grazed pasture soil. *Aust. J Soil Res*, 42: 927-932.

- Di, H. J., & Cameron K. C. 2005. Reducing environmental impacts of agriculture by using a fine particle suspension nitrification inhibitor to decrease nitrate leaching from grazed pastures. *Agric Ecosyst. Environ.*, 109: 202-212.
- Di, H. J., & Cameron K. C. 2006. Nitrous oxide emissions from two dairy pastures soils as affected by different rate of fine particle suspension nitrification inhibitor- a lysimeter study. *Nutr. Cycling Agroecosys.*, 79: 281-290.
- Di, H. J. Cameron, K. C., & Sherlock, R. R. 2007. Comparison of the effectiveness of a nitrification inhibitor, dicyandiamide (DCD), in reducing nitrous oxide emissions in four different soils under different climatic and management conditions. *Soil Use Manag.*, 23: 1-9.
- Douma, A. C., Polychronaki, E. A., Giourga, C., & Loumu, A. 2005. Effect of fertilizers with the nitrification inhibitor DMPP (3,4 dimethylpyrazolophosphate) on yield and soil quality. *Proc. Of the 9th International Conference on Environmental Science and Technology, Rhodes, Greece.*, 1-3 September 2005.
- Dreyfus, B., Garcia, J. L., & Gillis, M. 1988. Characterisation of *Azorhizobium caulinodans* gen. nov., sp. nov., a stem-nodulating, nitrogen Fixing bacterium isolated from *Sesbania rostrata*. *Int. J. Syst. Bacteriol.*, 38:89-98.
- Fillery I. 2007. Plant-recommended manipulation of nitrification in soil: a new approach to managing N loss? *Plant and Soil.*, 294: 1-4.
- Egamberdiyeva D., Mamiev M., & Poberejskaya S. 2001. The influence of mineral fertilizer combined with a nitrification inhibitor on microbial populations and activities in calcareous Uzbekistanian soil under cotton cultivation. *The Sci. World Journ.*, 1, 108-113.
- Irigoyen I., Muro J., Azpilikueta M., Aparicio-Tejo A., & Lamsfus C. 2003. Ammonium oxidation kinetics in the presence of nitrification inhibitors DCD and DMPP at various temperatures. *Aust. J. Soil Res.*, 41, 1177-1183.
- Li, H., Liang, Xi, Chen, Y., Tian, G & Ni, W. 2008. Effect of nitrification inhibitor DMPP on nitrogen leaching, nitrifying organisms and enzyme activities in a rice-oil seed rape cropping system. *Journ. Environ. Sci.*, 20: 149-155.
- Lorch, J. H., Benckiser G., & Ottow J. C. G. 1995. Basic methods for counting microorganisms in soil and water. In: Alef K and Nanniperi (eds). *Methods in applied soil microbiology and biochemistry.* pp. 146-161. Academic London-Toronto: Press, Sandiego New York Boston Sydney, Tokyo.
- Mahmood, T., Kaiser W., Ali R., Ashraf M., Gulnaz A., & Iqbal Z. 2005. Ammonium versus nitrate nutrition of plants stimulates microbial activity in the rhizosphere. *Plant and Soil.*, 277:233-243.
- Malla, G., Bathia, A., Pathak, H., Prasad, S., Jain, N., & Singh, J. 2005. Mitigating nitrous oxide and methane emissions from soil in rice-wheat system of the Indo-Gangetic plain with nitrification and urease inhibitors. *Chemosphere.*, 58:141-147.
- Mia, B. M. A., & Z. H. Shamsuddin, 2010. *Rhizobium* as crop enhancer and biofertilizer for increased cereal production. *Afr. J. Biotechnol.*, 37: 6001-6009.

- Moir, J., Cameron, K. C., & Di, H. 2007. Effects of the nitrification inhibitor dicyandiamide on soil mineral N, pasture yield, nutrient uptake and pasture quality in a grazed pasture system. *Soil Use Manag.*, 23: 111-120.
- Molina-Favero, C., Creus, C. M., Simontacchi, M., Puntarulo, S., & Lamattina, L. 2008. Aerobic Nitric Oxide Production by *Azospirillum brasilense* SP245 and Its Influence on Root Architecture in Tomato. *MPMI.*, 21:1001-1009.
- Muller, C., Stevens R. J., Laughlin, R. J., Azam F., & Ottow J.C.G. 2002. The nitrification inhibitor DMPP had no effect on denitrifying enzyme activity. *Soil Biology and Biochemistry.*, 34:1825-1827.
- Naher, U. A., Radziah. O., Shamsuddin, Z. H., Halim, M. S., & M. I. Razi. 2009. Isolation of Diazotrophs from Different Soils of Tanjong Karang Rice Growing Area in Malaysia. *Int. J.Agric.Biol.*, 11: 547-552.
- Ottow J. C. G., & Benckiser G. 1994. Effect of ecological conditions on total denitrification and N_2O -release from soil. *Nova Acta Leopoldina NF 17 Nr.*, 288, 251-262.
- Pasda, G. K., Hanhdel, G., & Zerulla, W. 2001. Effect of fertilizers with new nitrification inhibitor DMPP 3-4 dimethylpyrazole phosphate on yield and quality of agricultural and horticultural crops. *Biol. Fertil. Soils.*, 34:85-97.
- Pell, M., Stenberg, B., & Torstensson, L. 1998. Potential denitrification and nitrification tests for evaluation of pesticide effects in soil. *Ambio.*, 37:24-28.
- Regina, K., Silvova, J., Martikainen, P. J. 1998. Mechanism of N_2O & NO production in the soil profile of drained and forested peat land, as studied with acetylene, nitrapyrin and dimethyl ether. *Biol. Fertil. Soils.*, 27: 205-210.
- Rome, S., Fernandez, M. P., Brunei, B., Normand P & J. C. Cleyet-Marel. 1996. *Sinorhizobium medicae* sp. nov., isolated from annual *Medicago* spp. *Int.J.Syst.Bacteriol.*, 46:972-980.
- Sahrawat, K. L. 2004. Nitrification inhibitors for controlling methane emission from submerged rice soils. *Current Science.*, 87:1084-1087.
- Singh. S. N., & A. Verma. 2007. The Potential of Nitrification Inhibitors to Manage the Pollution Effect of Nitrogen Fertilizers in Agricultural and Other Soils: A Review *Environmental Practice.*, 9:266-279.
- Stark. C. H & Richards, K. G. 2008. The continuing challenge of agricultural nitrogen loss to the environment in the context of global change and advancing research. *Dynamic Soil, Dynamic Plant.*, 2: 1-12.
- Stephan, M. P., Zimmer, W., & H. Bo the H. 1984. Denitrification by *Azospirillum brasilense* Sp7. II. Growth with nitrous oxide respiratory electron acceptor. *Arch., Microbiol.* 138: 212-216.
- Suter, H., Chen, D., Li, H., Edis, R., & C. Walker. 2010. Reducing N_2 emission from nitrogen fertilisers with the nitrification inhibitor DMPP. *World Congress of Soil Science. Soil Solution for a Changing World. Brisbane.*, Australia 1-6 August 2010.
- Weiske, A., Benckiser, G., Herbert. T., & Ottow, J. C. G. 2001. Influence of nitrification inhibitor 3-4 dimethylpyrazole phosphate (DMPP) in comparison to dicyandiamide (DCD) on nitrous oxide emission

- and methane oxidation during 3 years repeated application in field experiments. *Biol. Fertil. Soils*: 34: 109-117.
- Yasari, E., & Patwardhan, A. M. 2007: Effects of (*Azotobacter* and *Azospirillum*) Inoculants and Chemical Fertilizers on Growth and Productivity of Canola (*Brassica napus L.*) *Asian J. Plant Sci.*, 6: 77-82.
- Zerulla, W., Barth, T., Dressel, J, Erhardt, K., Horschler von Loqueghien, K., Pasda, G., Raedle, M & Weissmeier, A. H, 2001. 3-4 dimethylpyrazole phosphate (DMPP) a new nitrification inhibitor for agriculture and horticulture. An Introduction. *Biol. Fertil. Soils*: 34:79-84