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The effect of nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) on nitrifying organism populations under *in vitro* conditions

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ABSTRACT

The application of nitrification inhibitors is a technique to reduce the nitrate concentration on leachates that delay ammonium oxidation by reducing the activity of ammonium oxidizing bacteria in soils. Two experiments were carried out in order to estimate the influence of DMPP on the population of ammonium oxidization bacteria under *in vitro* conditions. In both experiments, three treatments were established. The treatments were the following: a) ammonium oxidization bacteria established in a growing media without fertilizers, b) ammonium oxidization bacteria established in a growing media with Urea, and c) ammonium oxidization bacteria established in a growing media with DMPP. Results obtained showed that the population of the ammonia oxidizing bacteria diminished in the DMPP treatment as compared with the urea and control treatments. In conclusion, DMMP influences on ammonium oxidization bacteria activity being a useful tool in fertilizers strategies to reduce the contamination by nitrates in groundwater.

Keywords: Ammonium Oxidizing Bacteria; Fertilizers; Nitrogen; Nitrification Inhibitors; Winogradsky

1. INTRODUCTION

Nitrogen (N) plays an important role on the growth and yield, since it is required in highest amounts by plants and, hence; constitutes the basis of fertilization strategies for agronomic and horticultural crops [1]. In actual agricultural practices, nitrogen is usually used in greater quantities than those needed in order to guarantee a high yield [2]. As a consequence, nitrogen over fertilization may cause environmental degradation due to nitrogen losses [3]. Nitrogen losses are caused by Nitrate (NO_3^-) and amonnium (NH_4^+) leaching, erosion, volatilization, denitrification and fixation in soil organic matter [4]. NO_3^- leaching from agricultural soils is one of the important global environmental concerns [5]. These losses contribute to NO_3^--N contamination of groundwater [6]. A high NO_3^--N content in groundwater and drinking water does harm people and livestock [5].

A technique to diminish NO_3^--N leaching into groundwater and to conserve NH_4^+ fertilizers applied to soils is the retardation of biological oxidation of NH_4^+-N to NO_3^--N [5,6]. Actually, there are compounds that effectively inhibit nitrification when applied to soils in conjunction with NH_4^+ fertilizers or NH_4^+-producing compounds, such as urea or ammonium sulphate [6,7]. These compounds are called nitrification inhibitors (NI_s). NI_S delay ammonium oxidation by reducing the activity of *Nitrosomonas* bacteria (ammonium oxidizing bacteria) in the soil. Ammonium oxidization bacteria transform NH_4^+ into NO_2^-, which in turn is oxidized to NO_3^- by *Nitrobacter* bacteria [8]. Recently, DMPP have been introduced in Colombia to be used in nitrogen nutrition of different crops [9]. Likewise, contamination of surface water and/or groundwater by nitrates leaching has obtained importance in managing of crops, mainly; in rose crops in Colombia [10]. In particular, little is known about the effect of fertilizers, especially, NI_s on microorganisms present in tropical soils. For that reason, the aim of this study was to estimate the influence of DMPP on the population of ammonium oxidization bacteria collected from tropical soil under *in vitro* conditions.

2. MATERIAL AND METHODS

2.1. Isolation Bacteria

In our study, two experiments were carried out in May 2010. Ammonium oxidization bacteria were obtained by the preparation of Winogradsky's columns [11]. Soil for columns was collected on 10 December 2009 from upper 10 cm of a rose crop established in Mosquera, Colombia (4˚42′28″ N and 74˚13′58″ W). For bacteria extraction, 10 ml from middle of Winogradsky's column was diluted in 250-ml Erlenmeyer flask containing 90 ml of a NH_4^+ salt solution for ammonium oxidization bacteria which had the following composition: $NaHPO_4$ (13.5 g), KH_2PO_4 (0.7 g), $MgSO_4 \cdot 7H_2O$ (0.1 g), $NaHCO_3$ (0.5 g), $FeCl_3 \cdot 6H_2O$ (0.014 g), $CaCl_2 \cdot H_2O$ (0.18 g) and $(NH_4)_2SO_4$ (0.5 g) per liter of water [12]. Three growing media were established in a shaker incubator (Labline 3527, Lab-Line instrument, Inc. USA) during 15 days at 28˚C and 150 rpm, to achieve fully aerobic conditions. After the period of incubation, 10 ml of solution were taken from the growth media to determine the existence of ammonium oxidization bacteria by the presence of NO_2^--N and NO_3^--N using the Griess's reagent, respectively [5]. Then, NH_3 oxidizers were obtained by the technique described by Skinner and Walker [13]. Consequently, isolated colonies of ammonium oxidization bacteria from agar were taken by a handle. Next, those colonies were diluted in a salt solution of NaCl at 20% w/v. To estimate the initial concentration of inoculum, twofold dilutions series from 10^2 to 10^6 were done realized, and after that, inoculum was set in agar during 48 h. Subsequently, inoculum concentration was calculated by counting colonies as described by Madigan et al. [14]. The initial concentrations were 6.2×10^5 cfu/ml and 3.7×10^4 cfu/ml for each experiment, respectively.

2.2. Treatments

After that, 10 ml of inoculum solution at 20% of NaCl were diluted in 250-ml Erlenmeyer flask containing 90 ml of a salt solution for ammonium oxidization bacteria (concentration is mentioned above) for each experiment, respectively. In both experiments, three treatments were established. The treatments were the following: i) a 250-ml Erlenmeyer flask containing 90 ml of an ammonium salt solution and 10 ml of NaCl at 20% with Urea plus DMMP at 1%. 37 mg of fertilizer were added by growing media. This amount is equivalent to 170 ppm that it is the commercial dose used at fertirrigation programs in rose crops. ii) a 250-ml Erlenmeyer flask containing 90 ml of a salt solution and 10 ml of NaCl at 20% with Urea (37 mg of fertilizer), and iii) a 250-ml Erlenmeyer flask containing 90 ml of a salt solution and 10 ml of NaCl at 20% without fertilizer (control). Each treatment was placed in a shaker incubator (Labline 3527, Lab-Line instrument, Inc, USA) during 14 days at 28˚C and 150 rpm. Additionally, fertilizer was added to treatments with urea or Urea + DMPP at a dose mentioned above every 2 days during the incubation. To determine the concentration of inoculum at each sample point, 2 ml of each Erlenmeyer were taken to perform twofold dilution series up to 10^7. Afterwards, inoculum was placed in plates with agar. Consequently, inoculum concentration was estimated by counting the colonies as described by Madigan et al. [14]. Samples were done every 2 days during 14 days. The same methodology was used in both experiments.

2.3. Statistical Analysis

Analyses of variance were carried out on the data to evaluate the effect of different treatments. Both experiments were analyzed together as a series of experiments. Values were transformed using the Log_{10} transformation before analysis. Data were evaluated using Statistix Version 8.0 (Analytical Software, Tallahassee, FL, USA). Four replicates for each treatment were used.

3. RESULTS AND DISCUSSION

An increasing ammonium oxidization bacteria population was observed during first 4 days after the beginning of treatments. Significant differences were found on ammonium oxidization bacteria population in both experiments at 6 days after the treatments started. Ammonium oxidization bacteria cultivated in a growing media with DMPP had a less population than bacteria established in urea or control treatments. After this period, ammonium oxidization bacteria population started diminishing in all treatments. At 14 days after the beginning of experiments, bacteria established in a media with urea had a higher population than DMPP and control treatments in both experiment 1and experiment 2 (**Figures 1(a)** and **(b)**).

Differences were found on ammonium oxidization bacteria population in the double interaction fertilizer treatments and the different experiments (**Figure 2**). Treatments with DMPP showed a lower amount of ammonium oxidization bacteria population than control and urea treatments at 6 days after beginning both experiments. DMPP inhibited the mean ammonium oxidization bacteria population by 5% and 12% compared to urea treatments in experiments 1 and 2, respectively. A similar trend was observed at 14 days after the treatments started, but the DMPP had a greater percentage of inhibition than at 6 days after the beginning of treatments. DMPP reduced the mean ammonium oxidization bacteria populations by 31% and 33% regarding urea in experiments 1 and 2, respectively. Also, DMPP diminished

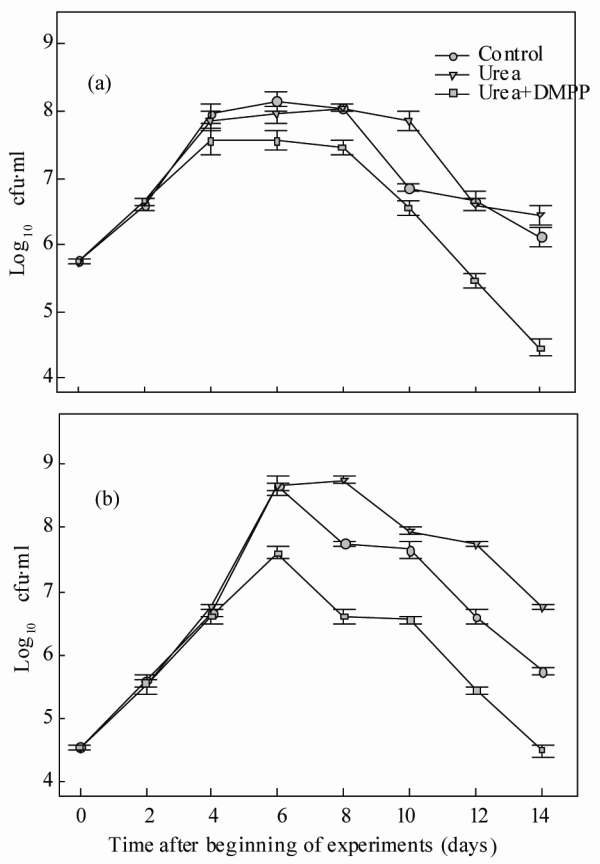

Figure 1. Evolution of ammonium oxidizing bacteria population from control treatment (◯), from bacteria that received urea (▽), and bacteria that received DMPP (□) during 14 days. Each point represents the mean four values. Vertical bars represent ± S.E.

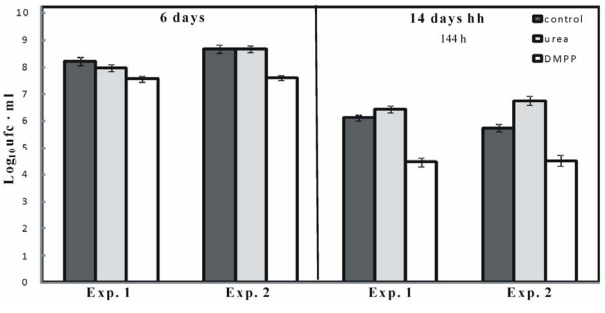

Figure 2. Evolution of ammonium oxidizing bacteria population from control bacteria (■), from bacteria that received urea (▩), and bacteria that received DMPP (▭) at 6 and 14 days. Each bar chart represents the mean four values. Vertical bars represent ± S.E.

ammonium oxidization bacteria populations by 27% and 22% compared to control treatments in experiments 1 and 2, respectively. Similar observations were found by Li *et al.* [5], who reported that ammonium oxidization bacteria populations were significantly reduced in soils fertilized with DMMP compared to soils fertilized with urea in rice crops. Likewise, our results showed that DMPP depressed the activities of ammonium oxidization bacteria as was also stated by Zerulla *et al.* [15] and Irigoyen *et al.* [16]. Li *et al.* [5] and Fernandez-Escobar *et al.* [17] also concluded that the NI_s inhibited ammonium oxidization bacteria activity, causing NO_3^--N reduction in leachates. Finally, the lack of growth in bacteria cultivated with DMPP during the experiment is mainly due to the effect bacteriostatic (not bactericide) of this molecule, since DMPP diminishes the growth of ammonium oxidizing bacteria, causing a reduction in the concentration of nitrate in the growing media [15].

In conclusion, the activity of the ammonium oxidization bacteria came from a tropical soil was inhibited by DMPP treatment as compared to the urea and control treatments. DMPP fertilizers could be considered an useful tool in fertilization programs of rose plants in order to reduce the contamination in surfacewater and/or groundwater by nitrates leaching, since studies conducted by Henao and Florez [14] estimated that that NO_3^--N concentrations in leachates came from rose plants cultivated were above the drinking water quality standards (maximum contamination limit of 10 ppm NO_3^--N) [18].

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