Urease activity according to the different inhibitors

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ABSTRACT

The objective of this study was to evaluate NBPT, zeolite and humic acid in different concentrations as inhibitors of the activity of the enzyme urease. The activity of the urease enzyme was quantified by the methods proposed by May and Douglas (1976) and Witte and Medina-Escobar (2001). For this reason, two experiments were conducted in a completely randomized design in split plots, in which the incubation periods are the plots and the fertilizers with the inhibitors at the different concentrations are the subplots with three replications. Fifteen fertilizers based on NBPT-coated urea, humic acid and zeolite were used in different concentrations. The two methods of analysis used in the experiment confirmed the efficiency of NBPT in inhibiting the enzymatic action even at low concentration, with 0.02% (m/m) being sufficient to inhibit urease, with no difference between inhibitor concentrations. The addition of humic acid reduced the activity of urease after 36 hours of reaction when the activity of the enzyme was evaluated by the method proposed by May and Douglas (1976). Zeolite did not influence the activity of the urease enzyme when analyzed by the May and Douglas method (1976).

Key words: humic acid, nbpt, zeolite, urea.

INTRODUCTION

Urea is the most commonly used nitrogen fertilizer in agriculture, despite its reduced efficiency particularly due to losses by volatilization of ammonia, which reduce the utilization of the applied nitrogen. For the conversion of N into the ammoniacal form, urea must be hydrolyzed by the enzyme urease, which is found in almost all types of soil, in concentrations that range according to the type of the soil and organic matter content, among other soil and climatic characteristics. In addition, factors that influence the microbiological activity of the soil also influence the urease activity and, consequently, the volatilization rates of ammonia.

Researchers and the fertilizer industry have focused their efforts to reduce the losses of nitrogen applied via urea in order to raise the sustainability of nitrogen fertilization. In this sense, several studies have evaluated the reduction of

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ammonia volatilization by adding urease inhibitor compounds. The addition of micronutrients, especially copper and boron, acidifying products and polymers that interfere in the solubilization and/ or hydrolysis of urea are the objective of several studies, but the treatment of urea with NBPT (N-(n-butyl) thiophosphate triamide) inhibitor stood out in the scientific community due to the achievement of good results.

Moreover, the volatilization of ammonia is reduced as CEC (cation-exchange capacity) of the soil increases, the mixture of urea with high CEC products (Monte and Resende 2005), such as zeolites and humic acids, has been evaluated, with the achievement of few promising results (Reháková et al. 2004, Bernardi et al. 2007, Paiva 2009, Yusuff et al. 2009, Bautista et al. 2011). Because of that, further studies are needed especially on the concentration to be used.

As a result, the objective of this study was to evaluate NBPT, zeolite and humic acid in different concentrations, as inhibitors of urease enzyme activity, by means of the methods of May and Douglas (1976) and Witte and Medina-Escobar (2001).

MATERIALS AND METHODS

The study was conducted at the Soil-Plant Interaction Laboratory (LSP) in the Department of Soils at the UFRRJ Institute of Agronomy, where the activity of the urease enzyme was quantified using the methods proposed by May and Douglas (1976) and Witte and Medina-Escobar (2001).

The experiments were conducted in a completely randomized design in subdivided plots, where the incubation periods are the plots and fertilizers with inhibitors at different concentrations were the subplots with three replications.

In the experiment conducted by using the method proposed by May and Douglas (1976) the treatments consisted of 15 fertilizers, described in Table I.

In the experiment that was carried out using the method proposed by Witte and Medina-Escobar (2001), the treatments with zeolite addition were excluded because the previous tests indicated that the zeolite retains the ammonium and the ammonia of the solution, preventing the reaction of color formation, which impairs the analysis.

Humic acid was obtained from peat which was prepared by drying the material in an oven at 40°C and passing through a 60-mesh sieve. Eighty grams of peat placed in a 1-L centrifuge tube, were weighed. Then, 800 mL of the alkaline potassium hydroxide (KOH) extracting solution was added at a concentration of 0.1 mol L^{-1}, determining a mass/ volume (m/v) ratio of 1:10. The material was left for a two-hour rest and then centrifuged. Extraction was performed at room temperature (25-30°C).

TABLE I
Fertilizers used.

1	commercial pearled urea
2	pearled urea coated with 0.02% of NBPT urease inhibitor
3	pearled urea coated with 0.03% of NBPT urease inhibitor
4	pearled urea coated with 0.04% of NBPT urease inhibitor
5	pearled urea coated with 0.06% of NBPT urease inhibitor
6	pearled urea coated with 0.08% of NBPT urease inhibitor
7	granulated urea with 5% of humic acid
8	granulated urea with 10% of humic acid
9	granulated urea with 20% of humic acid
10	granulated urea with 5% of zeolite
11	granulated urea with 10% of zeolite
12	granulated urea with 20% of zeolite
13	granulated urea with 5% of humic acid and 15% of zeolite
14	granulated urea with 15% of humic acid and 5% of zeolite
15	granulated urea with 10% of humic acid and 10% of zeolite

The fertilizer granulation process was performed using a 40-cm diameter disk granulator.

Initially, the materials used in the formulation of the fertilizers were milled and sieved in a 1-mm mesh sieve, to allow better homogeneity of the final mixture and to facilitate the granulation to obtain regular granules. Mixtures were prepared using urea milled with the different concentrations of the humic acid and or zeolite. Physical mixtures were carried out using sieves, in order to facilitate the homogenization of the solid components. Liquid ingredients were sprayed onto the solid ingredient previously added to the granulator during the granulation process. The fertilizer was removed from the dish and then sifted and classified as to the size of the granules, if it was between 2 and 4 mm it was dried at 45°C in a forced circulation oven. Fertilizers coated with NBPT at different concentrations were supplied by PETROBRAS.

ANALYSIS METHOD PROPOSED BY MAY AND DOUGLAS (1976)

For the execution of analysis by using the method proposed by May and Douglas (1976), samples from the 0-20 cm depth of a Red-Yellow Argisol were collected in a pasture area of Empresa de Pesquisa Agropecuária do Estado do Rio de Janeiro (Agricultural Research Company of the State of Rio de Janeiro) - PESAGRO located on Km 47 of BR-465, Seropédica, Rio de Janeiro. The soil collected was air-dried, and later it was crushed and sifted in a 1-mm mesh sieve. The chemical analysis, performed according to that proposed by EMBRAPA (1997), presented the following results: pH = 5.1; Ca^{2+} = 1.2 cmolc dm^{-3}; Mg^{2+} = 1.6 cmolc dm^{-3}; Al^{3+} = 0.3 cmolc dm^{-3}; Na^+ = 0.0 cmolc dm^{-3}; K^+ = 3.6 cmolc dm^{-3}; H + Al = 3.4 cmolc dm^{-3}; P = 4.5 mg dm^{-3}; SB = 2.9 cmolc dm^{-3}; T = 6.5 cmolc dm^{-3}; V = 45.1%; C = 15.3 g kg^{-1}.

The analyzes were performed by adding 3 g of air dried soil in 50-mL Erlenmeyer's flasks, then 0.5 mL of toluene and 12.0 mL of deionized water

were added. Incubation was carried out at 30°C for 10 minutes in an oven. After that, 3 mL of the urea-containing fertilizer solution at a concentration of 0.1 mol L^{-1} was added, which is equal to 0.2 mol L^{-1} of N for all treatments. The Erlenmeyers were then capped and incubated again at 30°C for 2, 4, 6, 24 and 36 hours. After each incubation time, 15 mL of 2 mol L^{-1} KCl solution was added with 5 mg of phenylmercury acetate, stirred for five minutes, and then filtered. The determination of the exchangeable N-ammoniacal content was carried out by the steam distillation method (Bremner and Keeney 1965).

A blank was performed for each treatment in the manner described above, however, by adding the fertilizer solution after the solution of KCl + phenylmercury acetate. From the filtrate, 10 mL were used for the distillation, then, titrated with standard solution of sulfuric acid in a concentration of approximately 0.0025 mol L^{-1}.

ANALYSIS METHODS PROPOSED BY WITTE AND MEDINA-ESCOBAR (2001)

To perform the analyzes according to the method proposed by Witte and Medina-Escobar (2001), also known as the indophenol method, 7 g of phenol and 34 mg of sodium nitroprusside were dissolved in 80 mL of distilled water. The volume was completed up to 100 mL and the solution was stored at 4°C, protected from light (solution A). Then, 2.96 g of NaOH was dissolved in 140 mL of distilled water, then 29.74 g of $Na_2HPO_4.12H_2O$ were added and the solution was stirred until the reagents were completely dissolved. After that, 40 Ml of NaOCl (4-6%) was added. The pH was adjusted to 12 and the volume completed to 200 mL. The solution was stored at room temperature and protected from light (solution B).

For the preparation of the enzyme solution, 3.6 mg of *Canavalia ensiformis* urease (Urease-U1500) purchased from Sigma Chemical Company dissolved in one liter of distilled water was used.

It was added 520 μL of the enzyme solution and 80 μL of the solution with the fertilizer containing 1 mol L^{-1} of urea, equivalent to 2 mol L^{-1} of N in each test tube, then stirred and left in a water bath at 43°C during the pre-established reaction intervals. After that, 80 μL of the sample was pipetted combined with 3920 μL of distilled water, 40 μL of the reagent A and 400 μL of reagent B. The tubes were capped immediately after addition of reagent B to prevent loss of ammonia. The tubes were then shaken and left in a water bath at 50°C for 20 minutes so the solution would develop color. At the end of the water bath, the tubes remained for an hour and a half at room temperature, so that the solutions stabilized.

For the construction of the calibration curve, 200 μL of ammonium chloride solution at concentrations of 0, 25, 50, 100, 200 and 400 μmol L^{-1} were mixed with 800 μL of water, which were submited to step 2. Ammonium was determined from the spectrophotometer absorbance readings by using the wavelength of 636 nm.

The results were submitted to the F test and the means compared by the Scott-Knott test at 5% of significance.

RESULTS AND DISCUSSION

RESULTS ACHIEVED BY THE MAY AND DOUGLAS METHOD (1976)

The fertilizers added with NBPT resulted in low values of ammonium in solution and after two hours of incubation (Figure 1), the compound had already exerted an inhibitory action upon the enzyme.

The efficiency of NBPT depends on the conversion rate to its oxygen analogue (NBPTO). The details of this reaction are still not known, however, some authors state that the conversion reaction of NBPT to NBPTO is slow or it does not occur in anaerobic environments (Keerthisinghe and Freney 1994, Watson et al. 2008). Pro et al. (2014) suggest that the replacement of sulfur by oxygen in

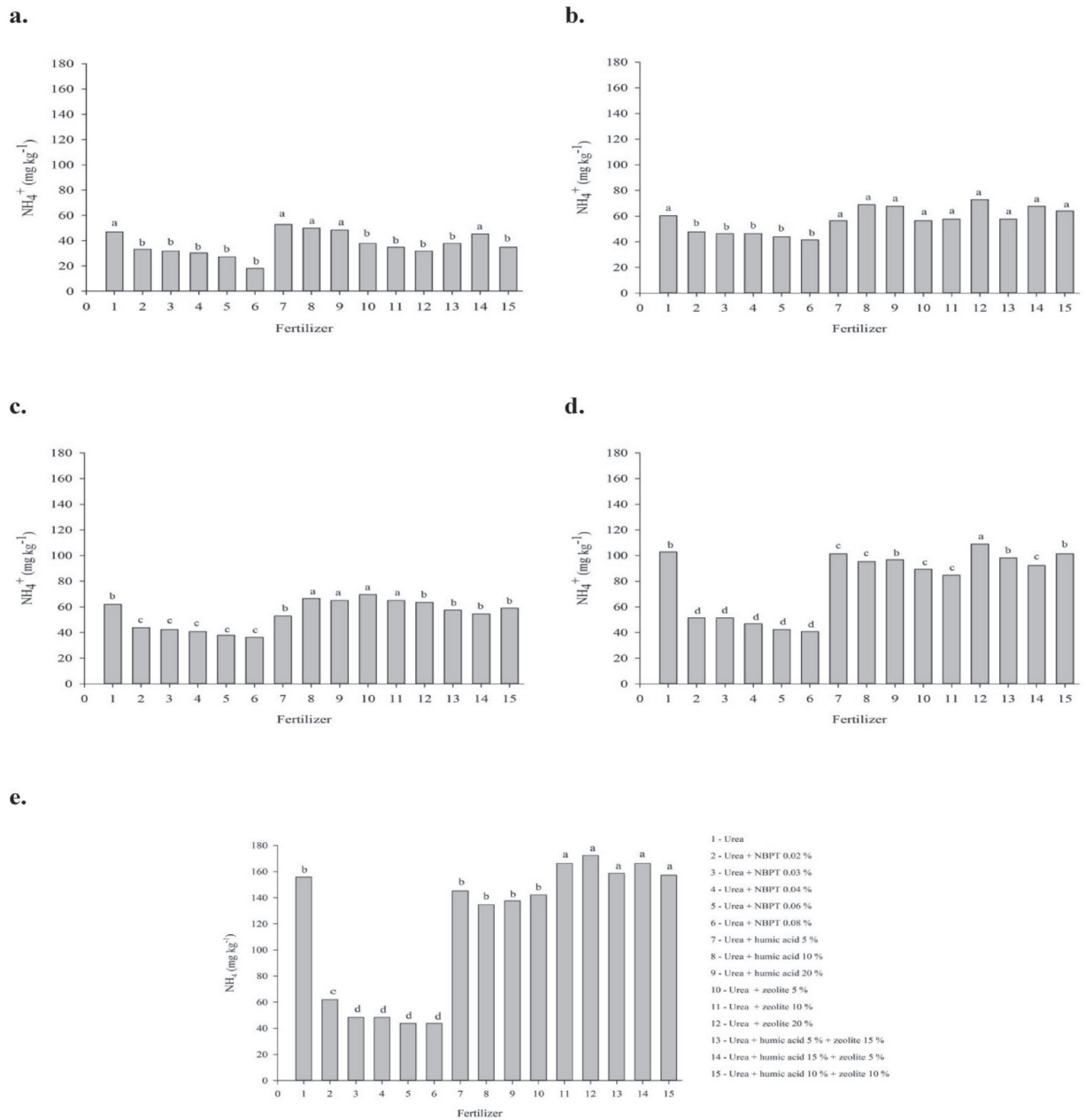the conversion of the compounds only occurs in the soil and probably due to the action of bacteria.

In this study, fertilizers additivited with NBPT were incubated in solution together with the soil. Even in the anaerobic environment and at the lowest dose (0.02%), the compound proved to be efficient in inhibiting the hydrolytic activity of urease. Commercial formulations generally use concentrations ranging from 500 to 2000 mg NBPT kg^{-1} urea (0.05 to 0.2%), although the dose used in Brazil is about 530 mg kg^{-1} (0.053%) (Cantarella and Marcelino 2007).

Keerthisinghe and Blakeley (1995) reported a 48% lower hydrolysis for urea treated with 100 μmol L^{-1} of NBPT, when compared to urea without the compound, incubated for 30 minutes. By reducing the incubation time to 10 min and the concentration of NBPT to 50 μmol L^{-1}, a reduction of 22% was found in urea hydrolysis. The authors considered that the incubation periods were short for the conversion of NBPT to NBPTO and attributed this inhibition to the presence of NBPTO as an impurity present in the NBPT added to urea.

No difference was found in the inhibition rate between compound concentrations at any of the incubation periods (Figure 1). It is recommended to increase the dose of NBPT in situations where urease is present at high concentrations and in conditions that promote it such as at elevated pH and temperature (Bremner and Chai 1986, Schlegel et al. 1986, Carmona et al. 1990, Watson et al. 1994).

It is likely that the amount of soil added for the analyzes provided a low concentration of the enzyme, so the lower NBPT concentration was sufficient to reduce the hydrolysis of the urea. Treatments with urea treated with 0.08% NBPT showed enzymatic activity 61% lower than the common urea treatment. After 2 hours of incubation, this treatment reached urease activity 72% lower than the control, after 36 hours.

The zeolite-added urea treatments and the treatments with zeolite and humic acid mixture (5%

a.

b.

c.

d.

e.

1 - Urea
2 - Urea + NBPT 0.02 %
3 - Urea + NBPT 0.03 %
4 - Urea + NBPT 0.04 %
5 - Urea + NBPT 0.06 %
6 - Urea + NBPT 0.08 %
7 - Urea + humic acid 5 %
8 - Urea + humic acid 10 %
9 - Urea + humic acid 20 %
10 - Urea + zeolite 5 %
11 - Urea + zeolite 10 %
12 - Urea + zeolite 20 %
13 - Urea + humic acid 5 % + zeolite 15 %
14 - Urea + humic acid 15 % + zeolite 5 %
15 - Urea + humic acid 10 % + zeolite 10 %

Figure 1 - Ammonium production, due to the hydrolysis of the conventional and additive urea, submitted to (**a.**) - 2, (**b.**) - 4, (**c.**) - 6, (**d.**) - 24 and (**e.**) - 36 hours of incubation. Different letters between the columns indicate difference by the Scott-Knott test at 5%. Analyzes carried out following proposed methodology May and Douglas (1976).

humic acid, 15% zeolite, 10% humic acid, 10% zeolite) differed from the control (conventional urea) in the two-hour incubation. A difference was found between the treatment of urea with 5% zeolite and the control after 36 hours. These results are probably the response of the low ammonium extraction time

with the KCl solution, where part of the $N-NH_4^+$ may have remained adsorbed to the mineral.

The competitive enzymatic inhibition for a compound is due to the similarity in its chemical structure with the enzymatic substrate, which allows its interaction with the active center of
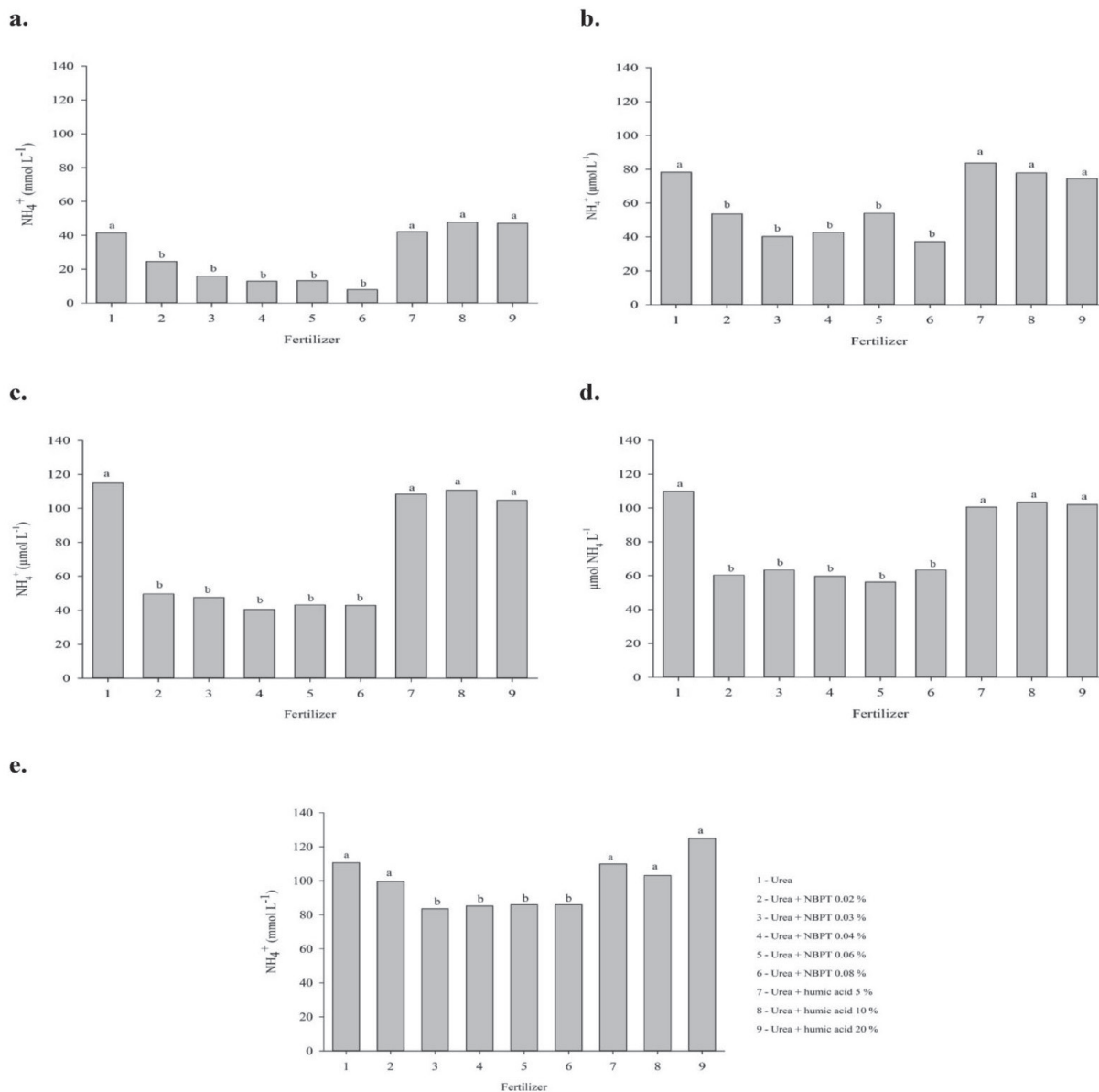
Figure 2 - Ammonium production, due to the hydrolysis of the conventional and additive urea, submitted to (**a.**) - 2, (**b.**) - 4, (**c.**) - 6, (**d.**) - 24 and (**e.**) - 36 hours of incubation. Different letters between the columns indicate difference by the Scott-Knott test at 5%. Analyzes carried out following proposed methodology Witte and Medina-Escobar (2001).

the enzyme (Paiva 2009). Zeolites do not present structural similarity to urea and therefore they are not expected to exert competition for the active site of the enzyme.

The treatments with humic acid differed from the control treatment, with a reduction of up to 14% of the enzymatic activity for the treatment with 10% of humic acid, but the different concentrations

of humic acid did not differ among each other. Probably, the effect of the humic acids on the enzymatic activity is due to the reduction of the pH of the reaction solution, since it was observed that the addition of humic acid to the urea reduced the initial pH of the reaction solutions (unpublished data). Longo and Melo (2005) observed that the enzymatic activity remained 42 to 44% below the

maximum activity of the enzyme at pH 5.2 and the maximum activity was recorded at pH 8.

RESULTS OBTAINED FROM THE WITTE AND MEDINA-ESCOBAR METHOD (2001)

The results obtained by using this method (Figure 2) were similar to the results obtained by the method proposed by May and Douglas (1976), in which the NBPT was effective after the first incubation period, but did not differ between the concentrations of the compound.

It is observed that after 36 hours of incubation, the efficiency of the inhibitor was considerably reduced (Figure 2). In this case, it is possible that the greatest contribution to the reduction of inhibitor efficiency was due to the incubation temperature (43°C). The increase in temperature promotes urease activity (Longo and Melo 2005, Zantua and Bremner 1977), however, it was expected that higher concentrations of NBPT would maintain inhibition of hydrolysis, which was not observed. In this case, the incubation time may have been not sufficient for conversion of the NBPT to the NBPTO, since the reaction is slow in solution.

The maximum inhibition observed occurred in the shortest incubation period (two hours), and the enzymatic activity in treatment with urea treated with 0.08% NBPT was 81% lower than the enzyme activity in the treatment with common urea. After 36 hours of incubation, the urea treated with the lowest concentration of NBPT (0.02%) was already equal to the common urea, showing the reduction of the efficiency of the inhibitor with the increase of the incubation time.

Treatments with urea additivited with humic acid showed no urease activity, differing from the treatment with common urea. In this case, it is possible that the incubation temperature compensated for the reduction of pH promoted by the humic acid.

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