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# Ureic Nitrogen Transformation in Multi-Layer Soil Columns Treated with Urease and Nitrification Inhibitors

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The use of *N*-(*n*-butyl)thiophosphoric triamide (NBPT), as a urease inhibitor, is one of the most successful strategies utilized to increase the efficiency of urea-based fertilization. To date, NBPT has been added to the soil incorporated in fertilizers containing either urea or the inhibitor at a fixed percentage on the urea weight. The possibility of using NBPT physically separated from urea-based fertilizers could make its use more flexible. In particular, a granulated product containing NBPT could be utilized in soils treated with different urea-based fertilizers including livestock urine, the amount depending on soil characteristics and/or the urea source (e.g., mineral fertilizer, organo-mineral fertilizer, or animal slurry). In this study, a multilayer soil column device was used to investigate the influence of an experimental granular product (RV) containing NBPT and a garlic extract, combining the ability to protect NBPT by oxidation and nitrification inhibition activity, on (a) spatial variability of soil urease and nitrification activities and (b) timing of urea hydrolysis and mineral-N form accumulation ($NO_2^-$, $NO_3^-$, $NH_4^+$) in soil treated with urea. The results clearly demonstrated that RV can, effectively, inhibit the soil urease activity along the soil column profile up to 8−10 cm soil layer depth and that the inhibition power of RV was dependent on time and soil depth. However, nitrification activity is not significantly influenced by RV addition. In addition, the soil N transformations were clearly affected by RV; in fact, RV retarded urea hydrolysis and reduced the accumulation of $NH_4^+$-N and $NO_2^-$-N ions along the soil profile. The RV product was demonstrated to be an innovative additive able to modify some key ureic N trasformation processes correlated with the efficiency of the urea-based fertilization, in a soil column higher than 10 cm.

**KEYWORDS: Urease inhibitor; nitrification inhibitor; *N*-(*n*-butyl)thiophosphoric triamide (NBPT); urea fertilizer; mineral N**

## INTRODUCTION

Nitrogen (N) is the nutrient that most influences crop production, and it is generally applied to soil with fertilization, representing the largest amount. However, the efficiency of N utilization by plants is generally low, depending on many factors: soil characteristics, climatic factors, crop type, and agronomical practices and fertilization management (*1*, *2*). The N use efficiency (NUE) for cereal production, for example, is approximately evaluated, on a worldwide scale, as being close to 33% (*3*), causing serious warnings about the economical and environmental aspects of N fertilization.

Urea constitutes the predominant source of industrial N fertilizer used in agriculture and represents 46% of the total world consumption of nitrogenous fertilizers (*4*). In addition, the urea transferred to the soil with animal slurries must also be considered. Urea is in fact the greatest nitrogenous component in urine, up to 97% of urinary N (*5*), but to date, it has been very difficult to estimate the contribution of livestock urine to soil urea addition.

Urea can be an inefficient N source due to its rapid hydrolysis caused by soil urease (*4*). This reaction leads to an increase in soil pH and to an accumulation of $NH_4^+$-N and $NO_2^-$-N. Ammonia accumulation can cause $NH_3$ losses by volatilization that, especially in sand alkaline soils and with urea surface application, can exceed 50% of the N applied (*6*). Nitrite accumulation, however, could be toxic for germinating seeds, seedlings, young plants, and soil microorganisms: in addition, it could favor gaseous N losses by chemical denitrification (*7*). The rate of hydrolysis of urea in soil is related to urease activity, availability of water, pH, temperature, organic C content, air humidity, and the form in which urea is applied (*8*).

The use of urease inhibitors, compounds delaying the hydrolysis of urea when applied to soil together with the fertilizer, may be a strategy for reducing the problems associated with the use of urea-based fertilizers. Urease inhibitors, slowing down urea hydrolysis, allow time for the surface broadcasted urea to move and dilute into the soil solution by diffusion or convection (*1*).

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**Table 1.** Physical and Chemical Properties of the Soil

| | texture | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| pH[a] | sand % | silt % | clay % | total $CaCO_3$ (g $kg^{-1}$) | $HCO_3^-CO_3^{2-}$ (g $kg^{-1}$) | CEC ($cmol_c$ $(kg^{-1})$ | organic carbon (g $kg^{-1}$) | humic carbon (g $kg^{-1}$) | total N (g $kg^{-1}$) | C/N ratio | available P (mg $kg^{-1}$) | K (mg $kg^{-1}$) |
| 8.1 | 75 | 14 | 11 | 620 | 160 | 18.0 | 12.7 | 4.6 | 0.82 | 15.5 | 49.8 | 218 |

[a] Soil to water ratio is 1:2.5.

This avoids an excess in $NH_4^+$-N accumulation at the microsite level of urea transformation with consequent undesired pH increasing $NH_3$ volatilization and $NO_2^-$-N accumulation.

Among the tested inhibitors, *N*-(*n*-butyl)thiophosphoric triamide (NBPT) was found to be one of the most effective (*9*−*11*). It is efficient at low concentrations and reduces the rate of urea hydrolysis and volatilization losses in a wide variety of soils. It also reduces the rise in pH favoring the nitrification process.

In a laboratory soil incubation experiment, Vittori Antisari and co-workers (*9*) added urea fertilizer containing different amounts of NBPT and tested these compounds in soils with different chemical−physical characteristics. They demonstrated that, in general, NBPT had reduced $NH_3$ losses due to volatilization after urea fertilization for 15 days, but the effectiveness of the inhibitor was strongly influenced by soil characteristics. Sandy and organic C content rich soils needed higher rates of NBPT (calculated as % w/w of added urea) to obtain significant urea hydrolysis and a reduction of $NH_3$ volatilization (*9*). Furthermore, the use of NBPT caused a considerable reduction of the formation of $NO_2^-$-N and favored an accumulation of $NO_3^-$-N proportional to the NBPT concentration employed.

NBPT was very efficient in reducing $NH_3$ losses also when incorporated into organic mineral fertilizers by a blend of urea and peat or leather meal as organic matrix. (*10*).

Sanz-Cobena and co-workers (*11*) carried out a field experiment on sunflower crop to verify the effect of a mixture of urea and NBPT (0.14%, w/w) on the mitigation of volatilized $NH_3$ and evolution of mineral N, with respect to urea alone. $NH_3$ emissions, in plots fertilized with urea + NBPT, were lower than those treated with urea alone because of a reduction in urease activity during the first 9 days after inhibitor application. This reduction in enzymatic activity promoted a decrease in the exchangeable $NH_4^+$-N and $NO_2^-$-N pools, while it caused an increase in the $NO_3^-$-N pool.

Combining the use of urease and nitrification inhibitors has sometimes been suggested in order to reduce the risk of $NO_3^-$ losses by leaching (*12*, *13*). However, the literature contains very few examples of the combined use of urease and nitrification inhibitors in soil urea fertilization.

Gioacchini and co-workers (*14*), for example, tested the use of NBPT and DCD (dicyandiamide) together to reduce N losses through volatilization ($NH_3$ gas) and leaching ($NO_3^-$) in two soils fertilized with urea. Their results showed that 8 months after the fertilization treatments the $NO_3^-$-N leaching process was significantly higher in the soil treated with urea + NBPT + DCD versus the soil treated with urea + NBPT and urea alone. These findings suggest that more research could be done to optimize the utilization of the urease and nitrification inhibitors together to improve ureic-N plant use efficiency and reduce the environmental N losses.

Until now in agriculture, NBPT has been added to the soil incorporated in fertilizers containing both urea and the inhibitor, and the inhibitor concentration was calculated as a percentage of urea weight, usually between 0.5 and 0.01% (*9*, *10*, *14*).

However, this approach meant that few types of fertilizers (urea + NBPT) were available on the market with no possibility to adapt the % of the inhibitor to the soil characteristics. The possibility of using a granular product containing NBPT physically separated from urea-based fertilizers could make its use more flexible. In particular, this new type of product could be utilized in soils treated with different urea-based fertilizers, including livestock urine, the amount depending on soil characteristics (e.g., organic matter content, sand content, and pH) and/or the urea source (e.g., mineral fertilizer, organic mineral fertilizer, or animal slurry).

Taking into account the above considerations, a multilayer soil column system was used to study the effect of a new experimental granular product, containing NBPT and a natural nitrification inhibitor (garlic extract), on (a) spatial variability of soil urease and nitrification activities and (b) urea hydrolysis and mineral-N forms ($NO_2^-$, $NO_3^-$, and $NH_4^+$) accumulation in soil treated with urea.

## MATERIALS AND METHODS

**Experimental Soil and Product.** An Aquic Xeropsamment soil (*15*), representative of an important agricultural area located southeast of the Po valley (Rimini, Italy) was chosen for the experiment. The soil, sampled from the ploughed profile (0−25 cm depth), was wet sieved to 2 mm and then air-dried. Soil chemical−physical characterization (**Table 1**) was carried out using the official methods of the Italian Ministry of Agriculture and Forestry (*16*).

The experimental product, Rhizovit (RV), consists of a molecular complex (LCN) able to control urea hydrolysis and to delay ammonium nitrification. This complex contains a urease inhibitor (NBPT) and an essential oil extracted from garlic with antioxidant activity and able to delay ammonium nitrification (French Patent No. 2922220).

The preparation of the product was carried out by spraying a liquid formulation containing the NBPT and the garlic oil. This liquid product (LCN) is applied on the surface of granules of a calcareous amendment containing 30% CaO, 10% MgO, and 15% $SO_3$. This amendment is produced by mixing and granulating calcium carbonate, magnesium oxide, and calcium sulfate. The size of the granules is between 1.5 and 3 mm. The synthesis of the product consists of two successive steps: in the first, the mineral component was granulated and dried, and LCN was then sprayed on the granule surface. The amounts of NBPT and of the essential oil were 1.25 and 0.5 g per kg of RV, respectively. The RV product was supplied by Timac-Agro International (Fench Patent No. 0765455).

**Multilayer Soil Columns.** Plexiglas columns (4 cm external diameter, 22 cm height, and 0.5 cm thickness) were prepared by gently and uniformly packing 160 g of soil to achieve a 15 cm soil column. At the bottom, the soil was separated, using a glass wool septum, by a 1 cm layer of sand (white quartz, 50−70 mesh, Aldrich, USA) to ensure water drainage. On the top, each column was sealed with holed parafilm to avoid the formation of an anaerobic environment and excessive humidity losses. Each column was placed in a beaker, and all of the columns were then stored for the duration of the experiment in a growth chamber in the dark at a constant temperature of 25 °C and at a relative humidity of 80%. The soil in the columns was kept at a constant moisture of 30% corresponding to hold field capacity. The humidity was checked three times a week by gravimetry, and deionized water, if necessary, was added from the top of each soil column to maintain the settled moisture. Before starting the experiment, the columns were left for 14 days to let the dried soil equilibrate with the settled moisture.

**Evaluation of Urease and Nitrification Activities.** Two sets of 24 columns each were subjected to one of the following treatments:

(1) no application of product (C); (2) application of granular RV (RV). RV was applied by adding a granule in the first centimeter of the soil at the top of the column at the rate of 1 ton $ha^{-1}$, corresponding to an addition of 1.25 kg of NBPT and 0.5 kg of nitrification inhibitors $ha^{-1}$.

The experiment lasted 29 days, and after 0, 1, 2, 4, 7, 10, 16, and 29 days, the soil was taken out from three columns for each treatment and cut from the top, using a suitable piston, to obtain 6 soil layers, each 2 cm high (0−2, 2−4, 4−6, 6−8, 8−10, and 10−12 cm). The soil layers were separately analyzed for urease and nitrification activities, and humidity.

The urease activity in the soil samples was measured according to ref *17*. This method consists of urease activity determination by calculating the $NH_4^+$-N quantity produced during 2 h of incubation at 37 °C of the soil samples in the presence of a buffered urea solution. The $NH_4^+$-N quantity produced was extracted with a KCl (1 N) + HCl (0.01 N) solution and was then colorimetrically determined (Jasco 7800 UV/vis spectrophotometer).

The urease activity was expressed as $\mu$g of $NH_4^+$-N produced by 1 g of dried soil in 2 h of incubation. The potential nitrification activity was measured following the method (*18*) based on the determination of the amount of $NO_2^-$-N produced during the incubation of soil samples with $NaClO_3$ in the presence of $(NH_4)_2SO_3$ for 5 h at 25 °C.

The amount of $NO_2^-$-N produced was extracted with a KCl (2 N) solution and was then colorimetrically determined (Jasco 7800 UV/vis spectrophotometer). The nitrification potential activity was expressed as $\mu$g of $NO_2^-$-N produced by 1 g of dried soil in 5 h of incubation.

The results for both the enzymatic activities were expressed as the average and standard deviation of three replicates and plotted as % of residual enzyme activity in the samples treated with RV versus the control (C) using this expression: % of residual enzyme activity = (enzyme activity RV)/(enzyme activity C) × 100

**Urea Hydrolysis and Distribution of Mineral Nitrogen Forms.** Three sets of 24 columns each were subjected to one of the following treatments: (1) no application of product (C); (2) application of urea (210 kg $ha^{-1}$ N) at the soil surface of the top of the columns (U); (3) application of urea (210 kg $ha^{-1}$ N) + RV (1 t $ha^{-1}$) (corresponding to an addition of NBPT and garlic extract, respectively, of 0.27% and 0.11% w/w on the applied urea) (U + RV).

The experiment lasted 29 days, and after 0, 1, 2, 4, 7, 10, 16, and 29 days, three columns for each treatment were analyzed for residual urea, $NH_4^+$-N, $NO_3^-$-N, $NO_2^-$-N, and humidity at the different soil layers. Soil layers were obtained as previously described.

The determination of the residual urea was carried out according to ref *19*. Residual urea was extracted from soil samples with a KCl (2 N) solution containing phenylmercuric acetate to stop the urease activity and was colorimetrically measured (Jasco 7800 UV/vis spectrophotometer).

For the determination of the nitrogen mineral forms, soil samples were extracted with a $CaCl_2$ (0.01 M) solution and filtered, and the $NH_4^+$-N, $NO_3^-$-N, and $NO_2^-$-N were colorimetrically determined in the extracts by means of an Autoanalyzer II (Technicon).

The results for each nitrogen form analyzed are expressed as the average and standard deviation of three replicates.

**Statistical Analysis.** Treatments were compared on the basis of data collected, and the GLM procedure of SAS statistical package was used for the ANOVA of the split plot design (*20*).

## RESULTS AND DISCUSSION

**Evaluation of Urease and Nitrification Activities.** The % of residual urease activity measured at different soil layers and periods of time in the RV columns with respect to the urease activity content in the C columns is shown in **Figure 1**.

The results clearly demonstrated that, in general, the RV product had significantly influenced the soil urease activity along the soil column profile up to 8−10 cm soil layer depth and that the inhibition power of the RV product was dependent on time and soil layer depth ($p < 0.0001$).

The greatest inhibition power of RV was observed in the surface layer (0−2 cm). In fact, in this layer, 24 h after RV addition, the urease activity was reduced by 50% with respect to the control, and after a week, urease activity sank to 0. At the end of the experiment, after 29 days, the residual urease activity in the same layer increased to 50% of the control value.

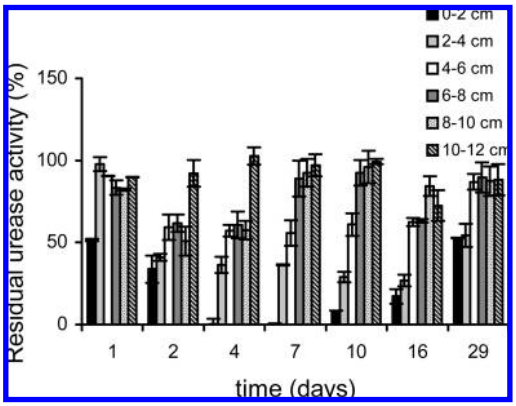


**Figure 1.** Residual urease activity measured in the columns treated with RV with respect to the urease activity measured in the control columns.

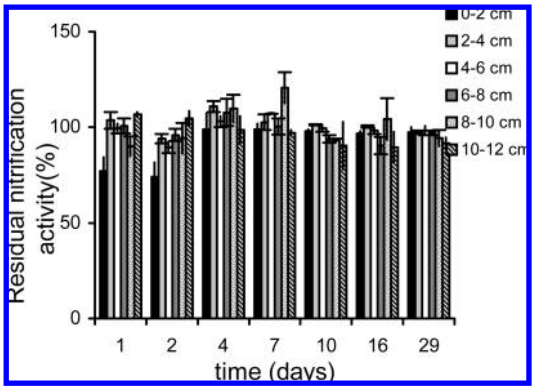


**Figure 2.** Residual potential nitrification activity measured in the columns treated with RV with respect to the nitrification activity measured in the control columns.

In the 2−4 cm soil layer, the RV urease inhibition power was still high, but lower than that in the top layer. The RV inhibition effect became significant only 2 days after RV addition. The maximum inhibition was reached between days 2 and 16 in this soil layer, when the residual urease activity measured between 30 and 40% of the control activity. At the end of the experiment, the residual urease activity increased to 55% of the control value.

In the 4−6 cm soil layer, the urease activity in the RV columns was reduced to 60% of the enzyme activity measured in the C columns from days 2 to 16. In the soil layers included between 6 and 8 cm and 8 and 10 cm of the RV columns, the urease activity inhibition trend was similar; in fact, from days 2 to 4 after the beginning of the experiment, the enzyme activity measured around 60% of control C; however, the differences in urease activity values between RV and C treatments then became nonsignificant. In the deeper soil layer (10−12 cm), we did not observe any significant urease inhibition effect.

The % of nitrification potential activity measured in the RV treatment with respect to the nitrification potential activity content in the C columns is shown in **Figure 2**.

A significant inhibitory effect of the nitrification process can be observed only from days 1 to 2 in the top layer of RV columns ($p < 0.05$). In fact, in the 0−2 cm layer, during the first 2 days of incubation, in the presence of RV product, the nitrification potential activity slowed down to 77% of the C value. In all other analyzed soil samples, no significant differences were found for nitrification activity between RV and C treatments.

**Urea Hydrolysis and Mineral Nitrogen Form Distribution.** The effects of the RV product on urea hydrolysis and soil

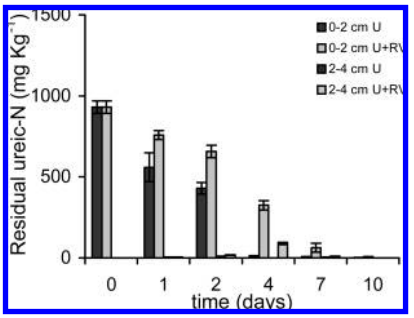


**Figure 3.** Residual ureic-N (mg $kg^{-1}$) measured in the columns treated with urea + RV and in the columns treated with urea only.

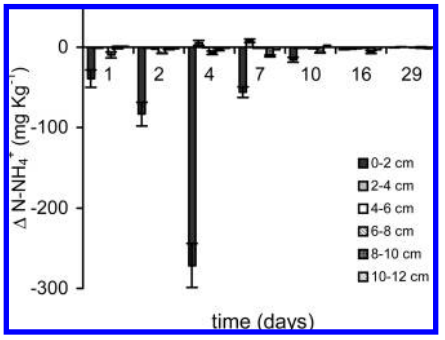


**Figure 4.** Extra accumulation of $NH_4^+$-N in the columns expressed as $\Delta NH_4^+$-N = $NH_4^+$-N (U + RV columns) − $NH_4^+$-N (U columns).

accumulation of $NH_4^+$-N, $NO_2^-$-N, and $NO_3^-$-N are shown in **Figures 3**, **4**, **5**, and **6**, respectively.

In the absence of the RV granule, (treatment U), urea hydrolysis occurred rapidly, and after 4 days, urea was almost completely hydrolyzed. The presence of the RV granule (U + RV treatment), instead, was successful in delaying urea hydrolysis (**Figure 3**). A significant accumulation of ureic-N in the top layer of the U + RV columns, with respect to the U columns, was in fact observed until day 7. The differences in ureic-N accumulation between U + RV columns and U columns were significant ($p < 0.0001$) right from the first day of incubation. In fact, an extra accumulation of ureic-N of 165, 228, 320, and 63 mg/kg was measured in U + RV columns 1, 2, 4, and 7 days after the beginning of the experiment, respectively. After this period of time, the accumulation of ureic-N was almost undetectable in any column.

The results highlighted that, despite being an uncharged molecule that could easily move along the soil profile, urea was actually detected only in the upper soil layer of the experimental columns regardless of the treatments. Only in the U + RV columns was a small portion of ureic-N detected in the 2−4 cm layer 4 days after the beginning of the experiment (**Figure 3**). The presence of NBPT, in the U + RV column, could have played a role in this finding. In fact, NBPT, inhibiting urease activity up to 10 cm of depth (**Figure 1**), could have favored a slight diffusion of the unhydrolyzed urea through the soil profile up to 2−4 cm depth. Anyway, in the absence of significant water additions, simulating a rain or an irrigation event (*1*, *11*), the diffusion and hydrolysis processes of urea remained confined to the upper soil layer where, however, NBPT displayed the highest inhibition power (**Figure 1**).

**Figures 4**, **5**, and **6** show the differences (Δ) measured among the concentrations of $NH_4^+$-N, $NO_2^-$-N, and $NO_3^-$-N in U + RV and U columns. As a consequence, positive or negative Δ values are representative of extra accumulation of $NH_4^+$-N, $NO_2^-$-N, and $NO_3^-$-N in U + RV or U columns, respectively.

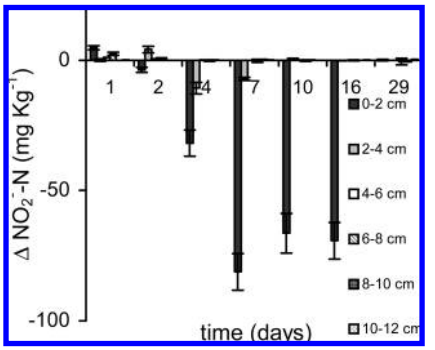


**Figure 5.** Extra accumulation of $NO_2^-$-N in the columns expressed as $\Delta NO_2^-$-N = $NO_2^-$-N (U + RV columns) − $NO_2^-$-N (U columns).

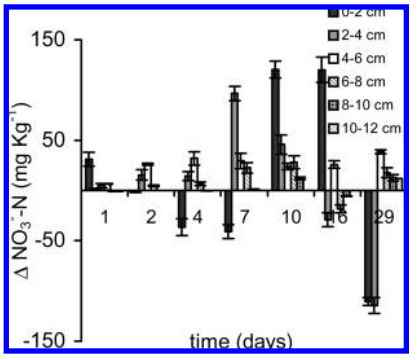


**Figure 6.** Extra accumulation of $NO_3^-$-N in the columns expressed as $\Delta NO_3^-$-N = $NO_3^-$-N (U + RV columns) − $NO_3^-$-N (U columns).

**Figure 4** shows the differences between $NH_4^+$-N accumulated in U + RV and U columns. The results suggest that, in U columns, from 0 to 2 cm, a high extra accumulation of $NH_4^+$-N is favored probably due to a more rapid urea hydrolysis with respect to U + RV columns. In fact, the highest $NH_4^+$-N extra accumulation was observed, in U columns, 4 days after starting urea incubation, when urea was practically all hydrolyzed (**Figures 3** and **4**). In this soil layer, the Δ values of $NH_4^+$-N are significantly different over time ($p < 0.0001$). In general, $NH_4^+$-N moved poorly toward the bottom of the columns, and also for this reason, the $NH_4^+$-N level in the other soil layers, from 4 to 12 cm depth, was never significantly influenced by the different treatments, Δ nearly 0.

Like $NH_4^+$-N, $NO_2^-$-N also tended to be extra accumulated in U columns. This behavior was more evident between days 4 and 16 in the 0−2 cm soil layer depth (**Figure 5**). In U columns, a little $NO_2^-$-N extra accumulation was also detected in the second soil layer (2−4 cm) between days 4 and 7. In the two first layers from the top of the column, in fact, the Δ values of $NO_2^-$-N are significantly different over time ($p < 0.0001$), while in all of the other soil layers, no significant $NO_2^-$-N extra accumulation was observed for either treatment. In U + RV columns, according to Bremner and co-workers (*21*), the addition of a urea inhibitor (NBPT) slowed down urea hydrolysis (**Figure 3**), reducing $NH_4^+$-N and $NO_2^-$-N soil accumulation (**Figures 4** and **5**) with respect to U columns. In fact, a high accumulation of $NH_4^+$-N, as happened in the U columns, and the correlated pH increase could inhibit the oxidation to $NO_3^-$-N of the higly toxic $NO_2^-$-N form, favoring its dangerous accumulation(*22*).

**Figure 6** shows the $NO_3^-$-N extra accumulations. Unlike the other mineral nitrogen forms, the Δ values of $NO_3^-$-N are significantly different with time and soil depth ($p < 0.0001$); in fact, an extra accumulation of $NO_3^-$-N was observed in many soil layers in both treatments. It is probable that, in the case of this very mobile N form, the humidity of the soil columns was

sufficient to provoke a vertical mobilization (figure **6**). We did not observe a clear trend of $NO_3^-$-N extra accumulation. However, in general, we had the highest extra accumulation of $NO_3^-$-N in U + RV columns during the first 16 days and in U columns at day 29 (particularly in 0−4 cm depth). The poor effect of the nitrification inhibitor contained in the RV pellets together with the positive effect on the progress of the nitrification process exerted by NBPT were probably the reasons for the $NO_3^-$-N extra accumulation trend found (*23*). In fact, we only observed an extra accumulation of $NO_3^-$- N in U columns 16 days after the beginning of the experiment, probably due to a delay of the $NO_2^-$-N nitrification process. These findings are very similar to that obtained by Vittori Antisari and co-workers (*9*) and by Sanz-Cobena and co-workers (*11*) that determined both an increase in nitrate concentration in urea + NBPT soil treatment compared to urea alone.

In conclusion, the RV product showed a significant capacity to influence urease activity in time and space, and this is a great achievement for an inhibitor thought to be spread out in the soil separately from the urea pellet. This result, in addition, was obtained by adding an amount of NBPT to the soil, precisely 0.27% w/w of the added urea, similar to that usually added to NBPT + urea formulations, between 0.01 to 0.5% w/w of the added urea (*9*, *10*, *14*).

The RV product, furthermore, positively influenced the ureic N transformations along the soil profile. In fact, by retarding urea hydrolysis and reducing the $NH_4^+$-N accumulation, the RV product was able to contribute to preventing volatilization loss of $NH_3$−N and soil accumulation of $NO_2^-$-N toxic ions. However, the RV product was not able to exert a significant inhibition of the nitrification activity. This could be due to different reasons: first, a lower concentration of the garlic extract, used as a nitrification inhibitor, than was needed; second, a lower soil stability and a rapid microbial degradation of the garlic extract. However, we must consider that the garlic extract was inserted in the product principally to protect the NBPT from microbial oxidation than for its nitrification inhibitory properties.

## ACKNOWLEDGMENT

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