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Kinetics of Soil Urease in Four Agricultural Soils Affected by Urease Inhibitor PPD at Contrasting Moisture Regimes

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Kinetics of Soil Urease in Four Agricultural Soils Affected by Urease Inhibitor PPD at Contrasting Moisture Regimes

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An incubation test was conducted on four agricultural soils to investigate effects of phenyl phosphorodiamidate (PPD) on soil urease kinetics at contrasting moisture regimes with contrasting fertility levels. The PPD made the Michaelis constant (Km) increase and the maximum enzyme reaction velocity (Vmax) decrease, behaving as a mixed inhibitor. With incubation time, Km first increased and then decreased under saturation condition, whereas it decreased under field-moist condition, the opposite of the changes of Vmax. Compared with black soil and albic soils, brown and cinnamon soils had larger Km and lower Vmax and Vmax/Km ratios, indicating that the effectiveness of PPD was greater in soils with low fertility. Compared with brown soil and cinnamon soil, black soil and albic soil showed more increases in Km and decreases in Vmax. To apply PPD under waterlogged, saturated, and low-fertility conditions could be a reasonable way to increase fertilizer nitrogen (N)–use efficiency.

Keywords Kinetic parameters, mixed inhibition, moisture regime, phenyl phosphorodiamidate

Introduction

Urea is the predominant source of inorganic nitrogen (N) fertilizer used in agriculture throughout the world, accounting for 46% of the total world fertilizer N consumption (Watson 2000). However, the rapid hydrolysis of urea in soils by action with soil urease can result in significant N losses through ammonia (NH₃) volatilization, nitrate (NO₃⁻) leaching, and nitrogenous gaseous emission, having both economic and environmental implications (Bolan et al. 2004) and lowering use efficiency of urea N (Harrison and Webb 2001; Sanz-Cobena et al. 2008). It is very important, therefore, to minimize such losses using some strategies, such as coating or treating N fertilizers with urease inhibitors, nitrification inhibitors, polymers, and elemental sulfur (S) to optimize fertilizer N efficiency (Sommer, Schjoerring, and Denmead 2004; Webb et al. 2005).

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Among these strategies, application of urease inhibitors has been proposed as one of the most promising strategies by inhibiting soil urease activity in soils (Wang et al. 1995; Grant et al. 1996; Grant and Bailey 1999). Slowing urea hydrolysis would allow more time for urea to diffuse into soil from the application point and would hence reduce ammonium (NH_4^+) concentration present in soil solution and the potential for NH_3 volatilization and nitrification (Grant et al. 1996). Among various types of urease inhibitors that have been identified and tested, phenyl phosphorodiamidate (PPD) has been found significantly effective at relatively low concentrations under laboratory conditions (Keerthisinghe and Freney 1994; Byrnes and Freney 1995; Krajewska and Zaborska 2007). This inhibitor forms stable complexes with urease and is among the most efficient inhibitors of the enzyme after the conversion to its hydrolyzed product, diamidophosphate (DAP) (Krajewska and Zaborska 2007). As urease is an enzyme present in a wide variety of fungi and bacterial species of soil, the efficiency of PPD to decrease urease activity may depend on its diffusion from the application point and its concentration maintained in microsites. Under laboratory and field conditions, PPD has been tested for its ability to retard urea hydrolysis and to reduce the loss of urea N (Wang, Van Cleemput, and Demeyer 1991a; Freney et al. 1993b; Lou et al. 1994). However, information about its effect on the kinetics of soil urease is very limited, especially at differing water regimes.

Most studies on the kinetics of the test enzyme focus on urease extracted from soil (Nannipieri et al. 1982) and of immobilized urease (Nannipieri et al. 1978; Makboul and Ottow 1979; Boyd and Mortland 1985; Burns 1986; Vaughan and Ord 1991; Gianfreda, Rao, and Violante 1992; Lai and Tabatabai 1992). However, little information is known on the kinetics of immobilized urease influenced by multiple factors. A study of the kinetic behaviors of urease affected by urease inhibitors should reveal the different inhibition mechanisms of urease inhibitors on urease, and it can also be used to evaluate the effectiveness of urease inhibitors on urease.

The objective of this study was therefore to evaluate the effect of PPD on the kinetics of soil urease at contrasting water regimes (water-logged saturation, field moisture), by an incubation test with four different fertility agricultural soils in northeastern China, to provide information about the mechanism of enzymatic reaction and the selection of promising urease inhibitors for certain soils.

Materials and Methods

Soil and Inhibitors

Four test surface soils (0–20 cm) (black soil, albic soil, brown soil, and cinnamon soil; Haplic Phaeozems, Mollic Albi–Boric Luvisols, Hapli-Udic Luvisols, and Hapli-Ustic Luvisols, respectively; WRB 1988) were sampled from the Hailun Experimental Station of Ecology, Chinese Academy of Sciences, in Heilongjiang Province (47° 25′ N, 126° 46′ E); 853 Farm, in Heilongjiang Province (46° 27′ N, 132° 56′ E); the station of the long-term fertilizer test, Shenyang Agricultural University in Liaoning Province (41° 82′ N, 123° 57′ E); and the station of Soil and Fertilizer of Chaoyang City in Liaoning Province (41° 41′ N, 120° 33′ E). After removing plant roots and debris, the composite samples were air dried at ambient temperature, sieved to 2 mm, and stored at 4 °C. All physicochemical characteristics of test soils are shown in Table 1.

Urease inhibitor PPD (97%) was purchased from ACROS (Janssen-Pharmaceuticaan, Geel, Belgium). Except for phosphoric acid and sulfuric acid as guaranteed grade reagents, other reagents were of analytical grade.
Table 1  
Basic properties of four test soils

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Black soil</th>
<th>Albic soil</th>
<th>Brown soil</th>
<th>Cinnamon soil</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH H_2O (1:2.5)</td>
<td>6.45</td>
<td>5.74</td>
<td>5.84</td>
<td>7.25</td>
</tr>
<tr>
<td>Organic matter (g kg(^{-1}))</td>
<td>50.32</td>
<td>35.42</td>
<td>14.84</td>
<td>16.25</td>
</tr>
<tr>
<td>Total N (g kg(^{-1}))</td>
<td>2.43</td>
<td>1.84</td>
<td>0.89</td>
<td>1.02</td>
</tr>
<tr>
<td>Available N (mg kg(^{-1}))</td>
<td>145.16</td>
<td>48.42</td>
<td>30.14</td>
<td>38.79</td>
</tr>
<tr>
<td>Sand (%)</td>
<td>13.9</td>
<td>16.6</td>
<td>29.0</td>
<td>45.4</td>
</tr>
<tr>
<td>Clay (%)</td>
<td>34.6</td>
<td>18.6</td>
<td>20.2</td>
<td>18.9</td>
</tr>
<tr>
<td>Silt (%)</td>
<td>51.5</td>
<td>64.8</td>
<td>50.8</td>
<td>35.7</td>
</tr>
</tbody>
</table>

**Incubation Test**

The air-dried soil samples with 15% moisture content were pre-incubated at 25 °C for 21 days to resume microbial activities (Bandick and Dick 1999; Zornoza et al. 2006). Then, the samples were amended with PPD at a rate of 50 mg kg\(^{-1}\) dry soil (about 1% on a urea weight basis), and incubated at 25 °C under field-moist (20% moisture content) and water-logged and saturated (with a 3- to 5-cm water layer) conditions. The samples incubated under field-moist conditions were in plastic bags, while those incubated under waterlogged saturation were in 150-ml stoppered Erlenmeyer flasks (5 g soil for each flask). During incubation, water loss (assessed by weight) was compensated daily by adding distilled water. Controls were run without amendment of test urease inhibitor and specific substrate. Three replicates were used for each moisture regime.

**Urease (EC 3.5.1.5) Activity Assay**

At 1, 10, and 30 days after incubation, 5 g soil was thoroughly mixed with 5 ml urea solution with a series of concentrations (5, 10, 15, 25, 35, and 45 mmol L\(^{-1}\)), and then incubated at 37 ± 1 °C for 5 h. After incubation, the residual urea was extracted by 50 ml of 2 mol L\(^{-1}\) M potassium chloride (KCl)–acetic phenyl mercury solution for 1 h on a constant-temperature shaker, followed by filtration with quantitative filter paper (φ15 cm), and analyzed by a continuum flow autoanalyzer (AA3, Bran + Luebbe, SEAL Analytical, Inc., Mequon, Wisc.), using the reaction of urea with diacetylmonoxime (DAM) in the presence of thiosemicarbazide (TSC), phosphoric acid (H\(_3\)PO\(_4\)), and sulfuric acid (H\(_2\)SO\(_4\)) under heating. The intensity of the red color formed as a result of this reaction was measured at 527 nm wavelength. Soil urease activity was expressed as mg hydrolyzed urea-N kg\(^{-1}\) dry soil 5 h\(^{-1}\).

**Michaelis Kinetic Parameters Measurement**

The kinetic parameters Km and Vmax were calculated by the Lineweaver–Burk equation, the linear transformation of Michaelis–Menten equation (Segel 1975):

\[
\frac{1}{V} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}
\]  
(1)
where \( V \) is the enzyme reaction velocity (mg hydrolyzed urea-N·kg\(^{-1}\) dry soil·5 h\(^{-1}\)), \([S]\) is the concentration of substrate (mmol L\(^{-1}\)), \( K_m \) is the Michaelis constant (mmol L\(^{-1}\)), and \( V_{max} \) is the maximum enzyme reaction velocity (mg hydrolyzed urea-N·kg\(^{-1}\) dry soil·5 h\(^{-1}\)). \( K_m \) indicates the affinity of urease to its specific substrate urea, and gives the substrate concentration at which the reaction rate reaches half of its maximum value (\( V_{max}/2 \)).

Statistical Analysis

All data were calculated on the basis of oven-dried soil and represented as means ± standard deviation of 3 × 3 data. The effects of incubation time, soil type, water regime, and their interactions on the kinetic parameters of soil urease were analyzed by a two-way analysis of variance (ANOVA) with the General Linear Models (GLM) procedure of SPSS 11.5 for Windows, and the differences among treatment means were performed by Duncan’s multiple-range test at \( P < 0.05 \) (SPSS Inc, Chicago, Ill., USA).

Results and Discussion

The four agricultural soils had different physicochemical properties. Organic matter, total N, and available N contents all followed in the order of black soil > albic soil > cinnamon soil > brown soil, and on view of texture, black soil, albic soil, and brown soil were considered as silt soil, whereas cinnamon soil was considered as sand soil (Table 1), indicating black soil and albic soil had greater soil fertility than cinnamon soil and brown soil.

The two-way ANOVA of kinetic parameters showed that incubation time, soil type, water regime and their combinations mostly had significant effects on \( K_m \), \( V_{max} \), and \( V_{max}/K_m \) of soil urease (Table 2).

Under contrasting water regimes, \( K_m \) values of test enzyme in four agricultural soils varied from 21.48 to 53.95 mmol L\(^{-1}\) (Figure 1), which was in the range of that observed in other studies (Ladd 1985; Gianfreda, Rao, and Violante 1992). Compared with the control, PPD significantly increased \( K_m \), irrespective of soil type and water regime. This may be due to the formation of the inhibitor–urease complex, decreasing the affinity of urease to its

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>Km</th>
<th>Vmax</th>
<th>Vmax/Km</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( F )</td>
<td>( P )</td>
<td>( F )</td>
</tr>
<tr>
<td>A(^a)</td>
<td>129.475</td>
<td>0.000***</td>
<td>415.483</td>
</tr>
<tr>
<td>B</td>
<td>79.719</td>
<td>0.000***</td>
<td>4.891</td>
</tr>
<tr>
<td>C</td>
<td>63.358</td>
<td>0.000***</td>
<td>2.732</td>
</tr>
<tr>
<td>AB</td>
<td>3.420</td>
<td>0.007**</td>
<td>22.501</td>
</tr>
<tr>
<td>AC</td>
<td>6.732</td>
<td>0.003**</td>
<td>14.921</td>
</tr>
<tr>
<td>BC</td>
<td>29.740</td>
<td>0.000***</td>
<td>26.044</td>
</tr>
<tr>
<td>ABC</td>
<td>4.577</td>
<td>0.001***</td>
<td>20.454</td>
</tr>
</tbody>
</table>

\(^a\)A, B, and C represent incubation time, soil type, and water regime, respectively.

**Significant at \( P < 0.01 \).

***Significant at \( P < 0.001 \).
specific substrate urea, or the conformational changes in the tertiary structure of enzymatic protein making active sites less accessible to its substrate (Vieth and Venkatasubramanian 1973).

The effect of PPD on $K_m$ differed with incubation time, soil type, and water regime. Under waterlogged and saturated conditions, $K_m$ first increased up to the 10 d of incubation and then decreased with incubation time in three of the four soils, while under field-moist conditions, $K_m$ decreased with time, indicating the greater effectiveness of PPD under waterlogged conditions. The decrease in effectiveness of PPD with incubation time could be due to the changes in its structural and functional properties, concentration maintained in microsites, and diffusion of its effective forms from the application point in soils. At the prophase of incubation, the formation of more inhibitor–urease complexes may significantly block the formation of urease–substrate complexes. With incubation time, PPD was gradually decomposed, and especially under saturated conditions released a more effectively hydrolyzed product, diamidophosphate (DAP) (Krajewska and Zaborska 2007). In water-logged treatment, the effectiveness of PPD on urease also depended on the rate and the time of DAP formation, and the synergetic effects of the two compounds, PPD and DAP. It was found that the time taken for $K_m$ to recover to the control level was almost the same in both water regimes, demonstrating that PPD was a promising urease inhibitor, especially under saturation.

Soil type also influenced the changes of $K_m$. Under both saturated and field-moist conditions, the $K_m$ values of brown soil and cinnamon soil were larger than those of black soil and albic soil, indicating that soils with high fertility have lower $K_m$ values. This may be due to (1) high organic matter and clay protecting the enzyme against destruction by environmental factors (Burns 1977) and (2) high organic matter in soils supplying nutrition for microorganisms, to accelerate their growth and proliferation, resulting in more free enzyme excreted (He et al. 2002). However, black soil and albic soil increased $K_m$ more than brown soil and cinnamon soil under both water regimes, probably due to the presence of more free enzymes in soils with greater fertility. The variations of $K_m$ with soil type

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**Figure 1.** $K_m$ values of soil urease affected by phenyl phosphorodiamidate (PPD) at contrasting water regimes. Values sharing the same lowercase letter are not significantly different at $P < 0.05$ (Duncan’s test). WL and NM represent saturated and normal moisture content, respectively.
indicated that PPD could effectively inhibit urease activity and reduce the urea-N losses in soils with lower fertility.

**Vmax**

Under experiment conditions, Vmax values of test enzyme varied from 147.49 to 238.35 mg hydrolyzed urea-N·kg$^{-1}$ dry soil·5 h$^{-1}$ (Figure 2), being similar to the previous report (Ladd 1985). Compared with the control, PPD decreased Vmax significantly in all soils with both moisture regimes. This is likely due to the formation of the inhibitor–enzyme complex decreasing the formation and dissociation of the enzyme–substrate complex (Lai and Tabatabai 1992).

The changes of Vmax affected by PPD differed with incubation time, soil type, and water regimes (Figure 2; Table 2). Under saturation, the Vmax value firstly decreased up to 10 d and then increased with incubation time, while under field-moist condition, Vmax continued to increase, which could be due to their structural and functional characteristics, as mentioned previously. In addition, the time taken for Vmax to recover to the control level was observed within 30 d under both water regimes, which confirmed the results of previous studies (Lou et al. 1994) that PPD could more effectively retard urea hydrolysis and reduce the urea-N losses, especially under saturation.

In general, under both saturated and field-moist conditions, the Vmax value of cinnamon soil was lower than that of black soil, albic soil, and brown soil, suggesting that soils with high fertility accelerated the dissociation of the enzyme–substrate complex, due to greater organic C content (Table 1). Simultaneously, black soil and albic soil decreased of Vmax more than that of brown soil and cinnamon soil under both water regimes, demonstrating that Km was likely affected by soil type.

**Vmax/Km**

Vmax/Km has been considered as an index of the catalytic capacity of enzymes in enzymatic reactions. In general, compared with the control, the test inhibitor significantly

![Figure 2](image_url)

**Figure 2.** Vmax values of soil urease affected by phenyl phosphorodiamidate (PPD) at contrasting water regimes. Values sharing the same lowercase letter are not significantly different at $P < 0.05$ (Duncan’s test). WL and NM represent saturated and normal moisture content, respectively.
Figure 3. $V_{\text{max}}/K_m$ values of soil urease affected by phenyl phosphorodiamidate (PPD) at contrasting water regimes. Values sharing the same lowercase letter are not significantly different at $P < 0.05$ (Duncan’s test). WL and NM represent saturated and normal moisture content, respectively.

decreased $V_{\text{max}}/K_m$ (Figure 3), indicating the decrease of catalytic ability of urease in the process of enzymatic reactions, due to the synergetic effects between the decrease of the urease’s affinity to the substrate and the dissociation rate of the enzyme–substrate complex.

With incubation time, $V_{\text{max}}/K_m$ significantly increased under both saturated and field-moist conditions, indicating the increase of catalytic capacity of test enzyme and the decrease in effectiveness of PPD. Compared with brown soil and cinnamon soil, black soil and albic soil had larger $V_{\text{max}}/K_m$ ratios under both water regimes, implying that the catalytic capacity of the test enzyme is larger in soils with greater fertility. Compared with field-moist condition, saturation decreased $V_{\text{max}}/K_m$, again indicating the increased effectiveness of PPD on urease under saturation.

It has been shown from the kinetic behaviors of test enzyme in four agricultural soils of northeastern China that the mechanism of PPD on urease was of mixed inhibition, in accordance with the observation of Krajewska and Zaborska (2007).

Conclusion

This study has demonstrated that PPD was of mixed inhibitor on soil urease. Water regime and fertility level significantly influenced the changes of kinetic parameters of the test enzyme. Under saturation, $K_m$ first increased and then decreased with incubation time, whereas it decreased under field-moist conditions, the opposite to the changes of $V_{\text{max}}$. The $K_m$ values of brown soil and cinnamon soil were larger than those of black soil and albic soil, and the changes of $V_{\text{max}}$ and $V_{\text{max}}/K_m$ ratios were the reverse. Applying urease inhibitor PPD could be a feasible way to increase fertilizer N-use efficiency, especially under waterlogged, saturated, or low-fertility conditions.

Results also suggested that further research is needed to study the mechanisms of urease inhibitors on urease to control the release of fertilizer N according to the characteristics of agricultural crop. Despite the fact that only a urease inhibitor was studied in
Soil Urease Kinetics as Affected by PPD

this report, the results obtained are of significance to make reliable conclusions of general interest that are presumably extendable to other inhibitors and management practices. Simultaneously, a new method for choosing a promising urease inhibitor may be studying its effects on urease kinetics.

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