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Original article

Short-term effects of exogenous protease application on soil fertility with rice straw incorporation

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A B S T R A C T

Rice straw incorporation increases soil fertility in the long-term. However, it may cause N deficiency in the short-term. The aim of this study was to use exogenous protease to reduce N deficiency in short-term. A two factor incubation experiment was conducted to test the effects of protease addition (at six levels ranging from 0 to 3%) to the soil with or without incorporated rice straw at 5%. After a 120-day incubation period, soil protease activity, available N, available P and electrical conductivity of treatments with at least 0.5% added protease were significantly ($P < 0.05$) greater than the no-protease control, with increases ranging from 82.4 to 168.8, 35.3 to 52.3, 10.9 to 21.9, and 107.1 to 173.9%, respectively. Soil organic matter and pH of treatments with added protease were lower than the no-protease control. Without straw incorporation, protease amendments only affected the soil protease activity itself, not other soil properties. In an orthogonal experiment designed to test the effects of temperature (15–35 °C), pH (5.5–7.5), water content (40% field capacity to submerged), and protease concentration (1–3%), fuzzy comprehensive evaluation was used for overall consideration of soil fertility, the optimum conditions are as follows: temperature 35 °C, pH 7.5, protease concentration 1%, and 100% field capacity. When protease was added to soil under conditions similar to rice field conditions, soil fertility was only slightly lower than that under optimal conditions. It is concluded that protease can reduce N deficiency, the effects of protease are influenced by environmental conditions, and exogenous protease may be used in field operations.

1. Introduction

Straw management impacts soil fertility and plays an important role in sustainable agriculture. In rice cropping systems, the traditional way of straw disposal is open-field burning [19], which, however, causes air pollution and losses of straw N, P, and K from the straw material [24]. Therefore, it is important to seek alternative disposal options, such as incorporation of straw into soil. Straw incorporation is a common practice in the world. It has been reported that straw incorporation can enhance soil fertility in the long-term [32,45]. However, immediately after straw addition to soil, the high C/N ratio may cause N deficiency due to microbial immobilization [8,29].

Extracellular enzymes in soil catalyze nutrient mineralization and the initial, rate-limiting step of straw decomposition [2]. Protease in soil can promote degradation of protein from straw [46]. According to a series of studies on the reaction mechanism of protease-catalyzed protein hydrolysis [15,46], they showed that oxygen has no effect on protease activity, so protease is expected to function normally in flooded rice-growing areas. Currently, there are numerous available microorganisms for agricultural uses [11,26,37], and protease is widely used in many fields for different purposes such as the textile industry, food industry, and pharmaceutical industry [1,18]. However, little attention has been paid to the short-term effects of exogenous protease application with straw incorporation in soil.

The hypothesis of this study was that the protease application with straw incorporation would increase soil available N by accelerating straw protease degradation [15]. The increase in soil available N may lead to an increase in microbial activity [13] and then organic matter (OM) and organic P are likely to be mineralized faster by more rapid microbial attack [6]. This suggested potential benefits of using protease as bio-fertilizer to enhance soil fertility. Because of these reasons, the objectives of this study were: (i) to investigate the effects of exogenous protease application on soil fertility by analysis of available N, P, K, OM, pH, electrical conductivity (EC), and soil protease activity; and (ii) to determine the optimum pH, temperature, water content and protease concentration for soil fertility with protease application.

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2. Materials and methods

2.1. Materials

The soil used in this study was collected from the top 15 cm of a rice cultivated area in Minhang District, Shanghai, China. The soil properties were determined by the methods described by Cao et al. [5]. Available N (akali-hydrolyzable N) was determined by the micro-diffusion technique; the released NH_3 from alkaline hydrolysis in soil by NaOH was absorbed by boric acid, and then titrated by H_2SO_4. Available P was determined by the Olsen method [23]. Available K was extracted with 1 M ammonium acetate (pH 7) and analyzed by a flame photometer. Soil OM was determined by the oil bath $K_2Cr_2O_7$ titration method. Soil pH and EC were analyzed with a 1:5 soil-to-water ratio. The available N, P, and K of the soil used in this study were 38.5, 6.9, and 127.2 mg kg^{-1}, respectively; the OM was 13.0 g kg^{-1}; the pH was 7.32 and the EC was 0.20 dS m^{-1}.

The rice straw was taken from the same cultivated area after harvesting. It consisted of whole–plant above ground material without seeds. The rice straw was ground to pass through a 0.1-cm screen to thoroughly mix with soil as described by Chidthaisong et al. [10]. General characteristics of the rice straw were determined by methods described by Cao et al. [5]. The total C was 39.3%; the total N, P, and K was 10.5, 3.4, and 9.9 g kg^{-1}, respectively; the C/N ratio was 37.4.

The neutral protease used in this study was a commercial *Bacillus subtilis* neutral protease powder from Donghua Biotechnology Ltd. (Beijing, China). Protease activity of the sample was 58,820 U g^{-1} measured as the tyrosine glycine released during hydrolysis of 0.5% casein at pH 7.5 and 30 °C for 10 min with the Folin–phenol method [34].

2.2. Experimental design

2.2.1. Two factor experiment

The effects of protease at different concentrations on soil fertility were investigated by a two factor experiment. The first factor is the presence of straw incorporation with two levels (yes or no), and the second factor is the protease concentration with six levels (0, 0.25, 0.5, 1, 2, and 3%). Soil (50 g) and rice straw (2.5 g) with the addition of protease at concentrations of 0, 0.25, 0.5, 1, 2, and 3% of straw dry weight were mixed thoroughly and filled into 100-ml polyvinyl containers, respectively. The use of such a large amount of straw would make it easier to measure nutrient changes after protease addition. Other works using a similar high ratio of straw were carried out by Wu et al. and Potthoff et al. [25,38]. Different amounts of protease were added to soil without straw to investigate soil fertility in the presence of protease but the absence of straw. The soil water content was at 100% field capacity for favor of straw decomposition [31] and for ease of handling, which was regulated by weight loss every 2 days through a small sponge-filled hole at the bottom of the containers. The containers were loosely capped and incubated for up to 120 days at 25 °C in dark. Each treatment was replicated four times, which were arranged in a completely randomized design.

2.2.2. Orthogonal experiment

Temperature, pH, protease concentration, and water content are the main factors expected to affect soil fertility with protease application, because protease activity is affected by temperature and pH [46], while the release and immobilization of N, P, and K are affected by temperature, pH and water content [9,12,30]. Using a full factorial design, 81 experiments would be required to effectively determine the optimum conditions. However, using an $L_9(3^4)$ orthogonal design, only a limited number of experiments (9) were required. The factors and their levels were designed as shown in Table 1, treatment 1–9 (T1–T9) were conducted. At the beginning of the experiment, soil pH was regulated to the desired value with 0.01 M HCl or NaOH. Soil (50 g) and rice straw (2.5 g) with the addition of protease at given concentrations were incubated under the conditions described in Table 1 for 40 days in dark (from the two factor experiment, most soil properties of treatments with protease application reached a plateau after 40 days of incubation). Each treatment was replicated four times, which were arranged in a completely randomized design.

2.3. Sampling and assay

In the two factor experiment, soil samples were collected at day 5, 10, 20, 40, 80, and 120 of the incubation by destructive sampling. In the orthogonal experiment, soil samples were collected at the end of the incubation. All the soil samples were air-dried, passed through a 2-mm sieve before analysis. Selected soil properties were determined according to Cao et al. [5] as described above. Soil protease activity was assayed by determination of glycine released from 4 g air-dried soil sample in 24 h at 37 °C with the ninhydrin colorimetry, the protease activity was expressed as μg glycine caused by 1 g dry soil in 24 h [14].

Table 1
Orthogonal design, results of treatment 1–9 (T1–T9) in orthogonal experiment, and statistical analysis of results.

No.	Temperature (°C)	pH	Protease concentration (%)	Water content	Available N (mg kg^{-1})	Available P (mg kg^{-1})	Available K (mg kg^{-1})	OM (g kg^{-1})	Evaluated value
T1	15	5.5	1%	40% FC	55.1e	16.7b	212.4c	44.2b	0.087
T2	15	6.5	2%	100% FC	63.3cd	18.8a	211.2c	42.1c	0.597
T3	15	7.5	3%	Submerged	65.3abc	18.9a	210.4c	47.0a	0.578
T4	25	5.5	2%	Submerged	61.8d	18.0ab	216.2b	42.6bc	0.551
T5	25	6.5	3%	40% FC	61.7d	18.4ab	212.8c	41.0cd	0.549
T6	25	7.5	1%	100% FC	63.9bcd	18.7a	212.2c	38.2e	0.689
T7	35	5.5	3%	100% FC	66.8a	19.1a	220.3a	37.9e	0.998
T8	35	6.5	1%	Submerged	65.7ab	18.0ab	220.4a	39.9de	0.816
T9	35	7.5	2%	40% FC	65.2abc	18.2ab	216.8b	38.5e	0.767
k_1	0.421	0.545	0.531	0.468					
k_2	0.596	0.654	0.638	0.761					
k_3	0.860	0.678	0.708	0.648					
R	0.440	0.133	0.178	0.294					

Means in the same column followed by different letters are significantly different at $P < 0.05$ ($n = 4$). OM: organic matter; FC: soil holding water equivalent to field capacity; Evaluated value: value evaluated by the fuzzy comprehensive evaluation of soil fertility of each treatment. k_1, k_2, and k_3: k_i represents the average of evaluated nutrient value at level i of the parameter in the corresponding column; R: maximum difference among k_1, k_2, and k_3. A higher k value would indicate a preferred level for the chosen parameter, while a higher R value would indicate a greater influence of the parameter.

2.4. Statistical analyses

2.4.1. Variance analysis and regression analysis

Data of the two factor experiment were statistically evaluated using a two-way repeated measures analysis of variance (ANOVA) and Duncan's multiple range test. Quadratic polynomial regression was used to determine the correlations between soil parameters and protease concentration. All statistical analyses were considered significant at the $P < 0.05$ level.

2.4.2. Fuzzy comprehensive evaluation

In orthogonal experiment, the soil parameters can be evaluated by fuzzy comprehensive evaluation. Fuzzy comprehensive evaluation refers to the overall evaluation of a phenomenon affected by multiple parameters, which was used for overall consideration of the soil fertility in this study [7].

Assessment indices for fuzzy comprehensive evaluation were selected. The pH and EC were obviously changed by acid or alkali because of regulating the pH of soil, while available N, P, K, and OM were selected to evaluate soil fertility. Therefore, an assessment indices set was as

$U = (U_1, U_2, \ldots, U_m) =$ (available N, available P, available K, OM)

There were n treatments that were evaluated. X_{mn} is the value of each treatment of U_m

$U_m = (X_{m1}, X_{m2}, \ldots, X_{mn})$

According to the report on the relationship between the indices and soil fertility [33], membership grade function values of each index can be calculated by Eqs. (1) or (2). In this study, the soil available N, P, and K were not high enough to meet the demand of plant growth, the higher soil available nutrient concentration was more favorable for rice growth [20], so the available N, P, and K were calculated by Eq. (1). However, in this study, the soil OM was much higher than the optimal category for soil fertility with rice straw incorporation [20]. Excessive OM content had many negative effects on rice growth [24,35], so the OM was calculated by Eq. (2).

$$\mu(X_{mn}) = r_{mn} = (X - X_{\min})/(X_{\max} - X_{\min}) \quad (1)$$

$$\mu(X_{mn}) = r_{mn} = (X_{\max} - X)/(X_{\max} - X_{\min}) \quad (2)$$

Where X is the value of each index of each treatment; $X_{\min}$ and $X_{\max}$ are minimal and maximal values of each index, respectively.

Then the fuzzy transformation matrix R was obtained, where r_{mn} is the grade function value of each index.

$$R = \left\{ \begin{matrix} r_{11} & r_{12} & \cdots & r_{1n} \\ r_{21} & r_{22} & \cdots & r_{2n} \\ r_{m1} & r_{m2} & \cdots & r_{mn} \end{matrix} \right\}$$

Weighting coefficient for each assessment index was determined by the method recommended by Chen [7] of taking advice from experts in soil science. The set A of weighting coefficients was formed as

$A = (a_1, a_2, \ldots, a_m) = \{0.4_{(\text{available N})}, 0.25_{(\text{available P})}, 0.2_{(\text{available K})}, 0.15_{(\text{OM})}\}$

The results of fuzzy comprehensive evaluation were obtained as

$$B = A \cdot R \quad (3)$$

3. Results

3.1. Soil biochemical properties of the two factor experiment

3.1.1. Soil biochemical properties after protease application with straw incorporation

Protease application had a positive effect on soil available N (Fig. 1A). Soil available N concentrations of treatments with protease application were significantly higher than those of the control throughout the incubation. After 120 days incubation period, available N concentrations of treatments with protease application were 22.0–52.3% higher than that of the control. Available N increased with the increase of protease concentration, the available N of treatments with the highest protease concentration was the highest among all treatments throughout the incubation. But the differences of available N among different treatments narrowed when protease concentration ranged from 1 to 3% (treatments C3–C5). After 120 days incubation period, a high correlation was obtained between available N and protease concentration ($r^2 = 0.908^*$, * represent significance at $P < 0.05$). In the control treatment, the available N declined during the first 20 days and was lower than the original level (38.5 mg kg^{-1}), then it increased and was a little higher than the original level after 120 days incubation. However, in treatments with protease application, the available N increased during the first 40 days and then declined, the available N concentrations were all higher than the original level throughout the incubation.

Protease application also had a positive effect on soil available P (Fig. 1B). Available P concentrations of treatments with protease application were significantly higher than those of the control throughout the incubation. After 120 days incubation period, the available P concentrations of treatments with protease application were 3.1–21.9% higher than that of the control. Available P increased with the increase of protease concentration, but there were no significant differences for available P when protease concentration ranged from 1 to 3%. Available P increased until day 10 and then slightly decreased.

Protease application had no significant effect on soil available K (Fig. 1C). The available K concentrations of all treatments were much higher than the original level (127.2 mg kg^{-1}) after 5 days incubation.

Protease application accelerated OM decomposition (Fig. 1D). Although the relationship between OM and protease concentration was ambiguous, OM degraded more rapidly with protease application. After 120 days incubation period, the OM of treatments with protease application were 5.6–12.4% lower than that of the control treatment.

Protease application decreased soil pH (Fig. 1E). Soil pH values of treatments with protease application were higher than those of the control in the initial 20 days and then significantly lower than those of the control. In treatments with or without protease application, soil pH increased in the first 20 days, and then decreased sharply until day 40.

Protease application increased soil EC (Fig. 1F). The soil EC values of treatments with protease application were significantly higher than those of the control throughout the incubation. After 120 days incubation period, the EC of treatments with protease application were 64.9–173.9% higher than that of the control. Soil EC increased with the increase of protease concentration.

Exogenous protease application increased soil protease activity markedly (Fig. 2). The soil protease activities of treatments with protease application were all significantly higher than those of the control throughout the incubation, but their differences narrowed with time. Soil protease activity increased with the increase of protease concentration. After 120 days incubation period, a high correlation ($r^2 = 0.972^*$, * represent significance at $P < 0.05$) was obtained between soil protease activity and protease concentration. Clearly, soil protease activity depended upon the exogenous protease concentration.

3.1.2. Soil biochemical properties after protease application without straw incorporation

As shown in Table 2, the addition of protease to soil without straw had no significant effect on soil biochemical properties, only soil protease activity increased with the increase of protease concentration. From two-way ANOVA, a significant interaction effect was

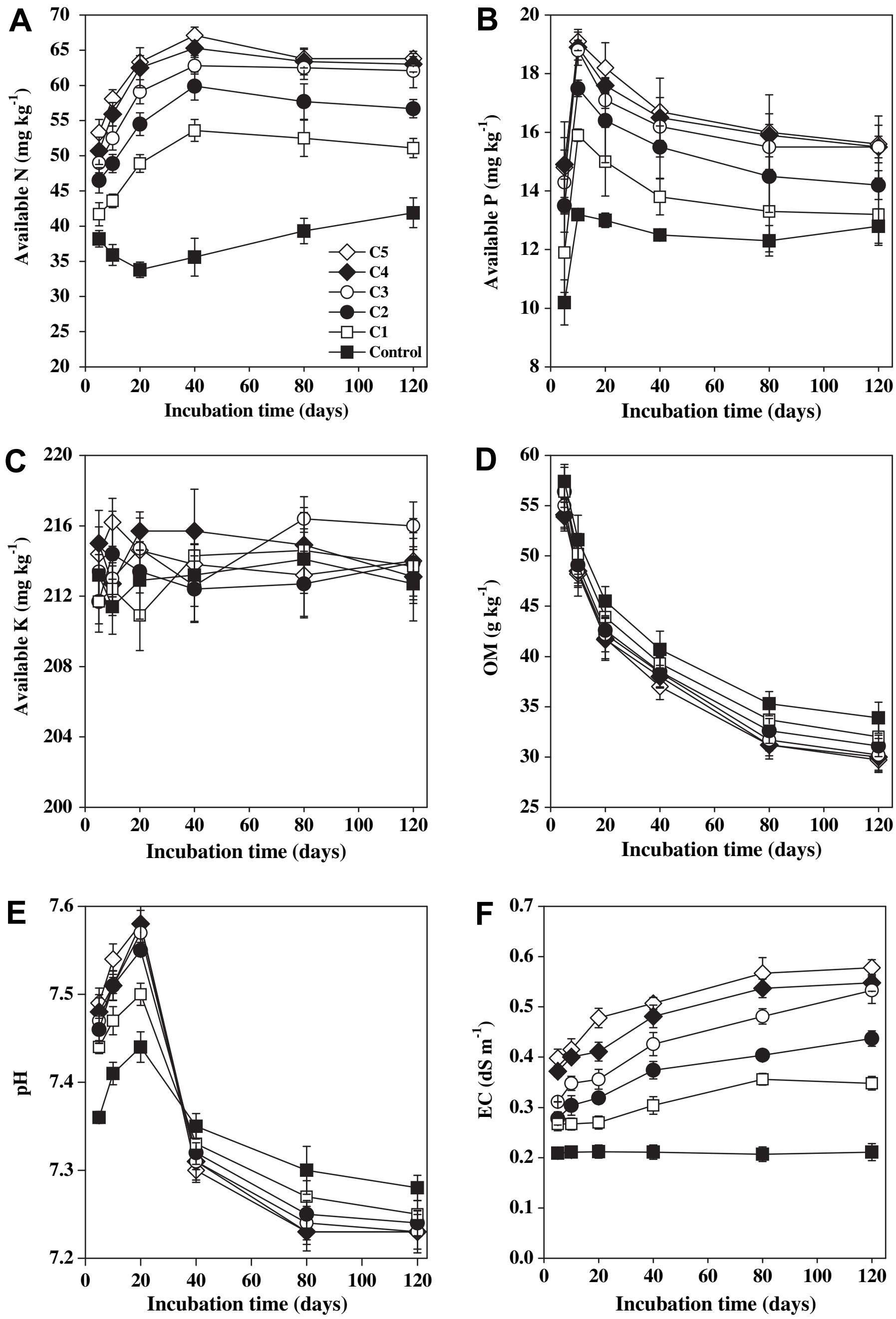

Fig. 1. The changes of available N (A), available P (B), available K (C), OM (D), pH (E) and EC (F) in soils applied by different protease concentrations. OM: organic matter; EC: electrical conductivity. Treatments applied rice straw with protease concentrations of 0, 0.25, 0.5, 1, 2, and 3% were referred as control, C1, C2, C3, C4, and C5, respectively. Error bars represent the standard deviations.

found between straw incorporation and protease application for soil induces except soil protease activity.

3.2. The determination of the optimum conditions of orthogonal experiment

Table 1 shows the results of the orthogonal experiment. The values of each index (Table 1) were calculated by Eqs. (1) or (2), respectively, and then the fuzzy transformation matrix R (Table 3) was obtained. From Eq. (3), we can draw the fertility values of the fuzzy comprehensive evaluation: $B = (0.087, 0.597, 0.578, 0.551, 0.549, 0.689, 0.998, 0.816, 0.767)$.

Fertility values of the fuzzy comprehensive evaluation were evaluated by analysis of variance (Table 1). The temperature was the most important factor influencing soil fertility. The influence of factors on soil fertility from large to small was: temperature, water content, protease concentration, and pH. The optimum environmental condition would be at a temperature of 35 °C, pH 7.5, and 100% field capacity.

Based on the two factor experiment, protease application increased soil fertility significantly, but the difference of most soil properties narrowed when protease concentration ranged from 1 to 3%. In order to save the cost of protease, 1% protease concentration was chosen. A test was conducted with 1% protease concentration,

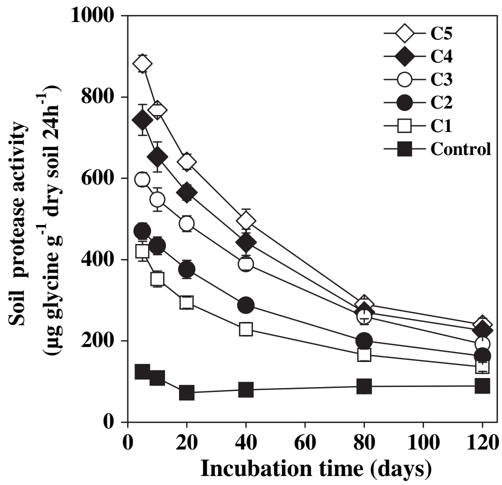

Fig. 2. Soil protease activity with rice straw incorporation after an application of protease with different concentrations. Treatments applied rice straw with protease concentrations of 0, 0.25, 0.5, 1, 2, and 3% were referred as control, C1, C2, C3, C4, and C5, respectively. Error bars represent the standard deviations.

Table 3
Fuzzy transformation matrix *R* of assessment indexes of treatment 1–9 (T1–T9) in orthogonal experiment.

No.	T1	T2	T3	T4	T5	T6	T7	T8	T9
U_1	0.00	0.70	0.87	0.57	0.56	0.75	1.00	0.91	0.86
U_2	0.00	0.88	0.92	0.54	0.71	0.83	1.00	0.54	0.62
U_3	0.20	0.08	0.00	0.58	0.24	0.18	0.99	1.00	0.64
U_4	0.31	0.54	0.00	0.48	0.66	0.97	1.00	0.78	0.93

U_1, U_2, U_3, and U_4: assessment indexes of available N, P, K, and OM, respectively.

temperature 35 °C, pH 7.5, and water content of 100% field capacity. Through a confirmatory test of this optimal combination of factors, which was not included in the orthogonal design, the amounts of available N, P, K were 65.9, 18.1, 218.2 mg kg^{-1}, and the OM was 38.0 g kg^{-1}. By fuzzy comprehensive evaluation, the evaluated value of soil fertility for the optimum treatment combination was higher than those of T1, T2, T3, T4, T5, T6, T8, T9, and almost the same to that of T7.

In order to certify the potential of protease application in the rice field, a test was conducted under conditions as follows: temperature 35 °C, pH 7.5, protease concentration 1%, and submerged. The amounts of available N, P, K were 65.7, 18.5, 218.0 mg kg^{-1}, and the OM was 39.8 g kg^{-1}, respectably. By fuzzy comprehensive evaluation, the evaluated value of soil fertility was only very slightly lower than that under the optimal conditions.

4. Discussion

4.1. Effects of protease application at different concentrations on soil fertility

4.1.1. Effects with straw incorporation

Protease applications potentially increase soil available N because they promote the release of amino acids from straw protein by hydrolysis [43]. Amino acids are part of the available N and can be utilized by plants and microorganisms [5,17,36]. Even if the amount of microorganisms can increase with protease application due to growth with this additional substrate used as a carbon, nitrogen and energy source, according to the increase in available N in our study, the amount of N immobilized by the microorganisms may be less than the amount of N released from straw. This may be due to the limitation of carbon source. The difference in the amount of available N among different treatments narrowed when the protease concentration ranged from 1 to 3%, which may be due to the limitation of substrate concentration, as reported previously [47]. In the control treatment, the initial decrease in available N resulted from the rapid immobilization of nitrogen by microorganisms and the relatively slow straw N mineralization [8,29]. In treatments with protease application, the initial increase in available N could result from the faster straw organic N mineralization, and the later decline was possibly attributed to denitrification [3,22].

Protease application increased available P concentration because microbial activity can increase as a result of the increase in available N [13]. Therefore, more organic P of straw may change into available P by microbial activity. The initial increase in available P may result from the mineralization of organic P from straw, and the following decrease may be attributed to immobilization of P by microorganisms and soil [28]. Protease application did not significantly affect the available K (Fig. 1C) because all of straw K is in inorganic form, and plant residues can release large amounts of soluble K without microbial transformation after incorporation [41]. The available K was much higher than the original level after 5 days of incubation, which was due to the rapid release of straw K. This is consistent with the report of Johnson et al. [16].

Soil OM decreased faster with protease application. This was caused by the increase in available N that may promote the growth of microorganisms, leading to the faster decomposition of OM used as an energy substrate. The initial increase in soil pH was due to ammonification, yielding NH_3 from soil proteins or peptides. It is known that with a p*K* value of less than 9.2 at pH 7–8 for ammonium dissociation, any NH_3 released will protonate and produce an alkaline effect in soil [42]. The later decrease in pH is due to the nitrification of mineralized N, which causes a decrease in pH owing to H^+ production [39,40]. The pH values of treatments with protease application were higher than those of the control in the first 20 days but later became lower than those of the control treatment. The probable reason was that straw N was mineralized faster in treatments with protease application, and the nitrification of mineralized

Table 2
Soil biochemical properties of treatments with protease application without rice straw incorporation.

Treatments	Available N (mg kg^{-1})	Available P (mg kg^{-1})	Available K (mg kg^{-1})	OM (g kg^{-1})	pH	Electrical conductivity (dS m^{-1})	Protease activity (μg glycine g^{-1} dry soil 24 h^{-1})
N0	37.3a	6.7a	127.0a	13.2a	7.3a	0.20a	97.5a
N1	38.0a	6.8a	127.3a	13.0a	7.3a	0.20a	269.0b
N2	37.6a	6.9a	127.0a	13.1a	7.3a	0.20a	317.9c
N3	37.7a	6.9a	127.3a	13.0a	7.3a	0.20a	387.3d
N4	38.1a	6.9a	126.8a	13.1a	7.3a	0.20a	480.4e
N5	38.1a	6.9a	127.2a	13.2a	7.3a	0.21a	564.8f

Results are the average values of six samples (four replicates) collected at indicated times (5, 10, 20, 40, 80, and 120 days of the incubation). Treatments of 50 g soil added with 0, 0.25, 0.5, 1, 2, and 3% of 2.5 g straw (equivalent to the protease addition amounts of treatments with straw incorporation) but without straw incorporation were referred as N0, N1, N2, N3, N4, and N5, respectively. Numbers followed by the same letters are not significantly different ($P < 0.05$).

N advanced and produced more H^+ earlier. The EC increased with the increase in protease concentration because more ions were released from the faster mineralization of straw [27]. However, the result showed that the EC values were still below the threshold EC for salinity suggested by the Food and Agriculture Organization of the United Nations even at 3% protease concentration. According to previous reports [21,44], EC has a positive correlation with soil fertility when it is lower than the threshold of salinization. The soil protease activity of treatments with protease application was always higher than of the control throughout the incubation period (Fig. 2), but their differences narrowed with time, which was probably due to the hydrolysis, sorption, and inactivation of enzymes in soil [46].

4.1.2. Effects without straw incorporation

Protease autolysis releases N [4]; therefore, in this laboratory study, the increase in soil available nutrients may involve two main processes: the mineralization of substrate (rice straw) and the hydrolyzation of protease. Protease application without straw had no significant effect on soil properties and only increased, as expected, soil protease activity (Table 2). One reason is that the additional amount of protease was negligible; the other is that although soil protease activity increased significantly after protease application without straw, the substrate in soil was little without a significant effect on soil fertility. The results indicate that protease has no significant effect on soil fertility without straw incorporation, and the effects of protease on soil fertility mainly depend on the protease action on straw.

4.2. Effects of protease application with straw on the soil fertility of orthogonal experiment

A temperature of 35 °C and a pH 7.5 were the optimum environmental conditions for soil fertility with protease application. This can be explained by the properties of the neutral protease used in this study; the protease activity was highest at 35 °C and pH 7.5. The 100% field capacity was optimum for soil fertility with protease application; this is reasonable since 100% field capacity is the optimum water content for straw nutrient mineralization [31]. Based on the results of a two factor experiment, protease concentration was found to be a non-significant factor of soil fertility when the enzyme concentration ranged from 1 to 3%. High fertility was observed in the test conducted under conditions similar to those of natural rice-growing areas. This suggests that the field conditions may be suitable for protease application in most rice-growing areas.

We therefore conclude from the results of this study in regard to straw degradation rates and no unintended effect on soil parameters that protease applications have a great potential to enhance soil fertility in rice-growing areas.

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