Humic acids derived from Leonardite to improve enzymatic activities and bioavailability of nutrients in a calcareous soil

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Abstract: Understanding the role of humic substances in soils is important for developing and utilizing organic fertilizers or soil amendments for sustainable agriculture. The objective of this study was to determine the effects of different fractions of humic acids derived from Leonardite on enzymatic activities and bioavailability of nutrients in a soil. The experiment was carried out by mixing different factions of humic acids with a soil and incubated for 70 d. The treatments included five factions of humic acids (HS1 (low molecular weight), HS2 (medium molecular weight), HS3 (large molecular weight, SED (sediment of fractions), HS (mixture of HS1, HS2, and HS3)), raw Leonardite (IM) and a control (no addition of humic acid). Experimental results showed that application of humic acids significantly improved acid and alkaline phosphatase activities, especially with HS1. Humic substances with high molecular weights significantly inhibit urease activity, and the optimal application rate was 600 kg/hm^2 of humic substances with the high molecular weights. Concentrations of NH_4^+-N were decreased with increasing humus applications. All treatments (HSmix, HS1, HS2, HS3, IM, SED) did not affect the soil contents of Ca, although soil concentrations of K, P, Cu, Zn were increase significantly when small molecular weight humus (HS1) was applied.

Keywords: humic acid, molecular weight, Leonardite, enzyme, nutrient content

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1 Introduction

It has been long reported for the effects of soil organic matters on plant growth and nutrient uptake[1-4]. Humic acids along with fulvic acids are essential components of soil organic matters and play a critical role in improving soil properties[5-7]. Even though humic acids extracted from soils under widely different pedologic (sandy, loamy or clay soils) and geographic environments had similar analytical characteristics and chemical structures, the fractions with large molecular weights showed the greater effects on enzymatic activity in soils than fractions with low molecular weights[8-12]. This suggested that the sizes of molecular weights were of the importance in determining the magnitude of effects on soil enzyme activity.

A large quantity of humic substances are the main components of organic oxidation products and formed in different molecular weights on coal surfaces depending on oxidation conditions under natural condition. When produced for agricultural use, humic acids have been produced by the process of activating carbon in materials such as leonardite[13-17]. Less research has been performed to show how the humic acids extracted from leonardite affect soil enzymes and bioavailability of nutritions[18]. Hence, the objectives of this study were to determine ideal molecular weights and amounts of humic acids to place within artificial horticultural soils, and to examine their effects on soil enzymatic activities and nutrient contents. The results will provide valuable information for using the Leonardite to improve soils.

2 Materials and methods

2.1 Preparation of humic acids

Humic acids were extracted from Leonardite using the method suggested by the International Humic Substances Society (IHSS)[19]. Briefly, an aqueous solution of 0.1 mol/L KOH was mixed with Leonardite, and let it stand for 24 h. Then the mixture was centrifuged at 25 ℃ and the supernatant was separated by pipetting. The pH of the supernatant was adjusted to 7.0 by adding 0.1 mol/L

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of H_2SO_4. The resulting solution was transferred to a Plexiglas cylinder (70 cm tall × 10 cm diam) and settled for 24 h, then the portions of the top, middle and bottom that correspond to low, moderate and high molecular weights were siphoned sequentially out of the tubes[20]. According to Stokes' law, the rate of settlement is proportional to the size of molecules and therefore these solutions were labeled as HS1 (low molecular weight from the upper portion of the column), HS2 (medium molecular weight from the middle portion of the column), and HS3 (large molecular weight from the lower portion of the column), respectively. The mixture (HS) of HS1, HS2, and HS3, and raw Leonardite (IM) were also included for the study. Potassium sulfate (K_2SO_4) was added into air-dried HSmix, HS1, HS2, HS3, SED, and IM to bring the potassium contents to the same level.

2.2 Soil treatments and incubation

The experiment was the completely randomized design with six application rates (0, 200 mg/kg, 400 mg/kg, 600 mg/kg, 800 mg/kg and 1000 mg/kg) of six humic acid fractions (HSmix, HS1, HS2, HS3, SED, IM) and four replicates. Soil (Krome very gravelly loam (Loamy-skeletal, carbonatic, hypothermic Lithic Udorthents)) was collected (0-15 cm depth) from a research field at the Tropical Research and Education Center (TREC), University of Florida, Homestead, Florida. The soil was air dried, then passed through a sieve (<2 mm mesh). Basic soil properties were pH (7.3), organic carbon (18.7 g/kg), Mehlich-3 extractable K (93.12 mg/kg), P (97.69 mg/kg), Ca (20.45 mg/kg) and Mg (160.99 mg/kg). Each humic acid faction was mixed with the soil (300 g each) and packed in 500 mL plastic bottle. Deionized water was added into each bottle to the field holding capacity. Containers were incubated at the room temperature (25 ℃) for 70 d. Additional water was also added weekly based on changes of weights to make up evaporation loss.

2.3 Soil analyses.

Two soil samples were collected from each bottle at 40 d and 70 d. One sample was kept moist and stored at 4 ℃ for enzyme activity measurements and another sample was air-dried at the room temperature for chemical analysis. For soil enzyme activities, three enzymes (phosphomonoesterases (Acid and alkaline phosphatases), phosphodiesterase and urease) were measured. Urease is measured based on the modified method from Gouglas and Bremner[21] and Zantua and Bremner[22]. Phosphomonoesterases and phosphodiesterase were assayed by the photometric methods[23-25].

For chemicals analyses, soil samples were extracted using Mehlich-3 (M-3) extractant (0.2 M CH_3COOH, 0.25 M NH_4NO_3, 0.015 M NH_4F, 0.013 M HNO_3, 0.001 M EDTA). Phosphorous in the extracts was determined using the ascorbic acid method with a spectrophotometer (DU 640, Beckman Instruments Inc., Fullerton, CA), and the concentrations of K, Ca, Cu and Zn were analyzed by atomic absorption spectrophotometer (AA-6300 Shimadzu, Columbia, MD).

2.4 Statistical analyses

Data were subjected to statistical analyses using the SAS statistical software (version 8.0), and Duncan tests for mean separation ($p \leq 0.05$)[26].

3 Results and discussion

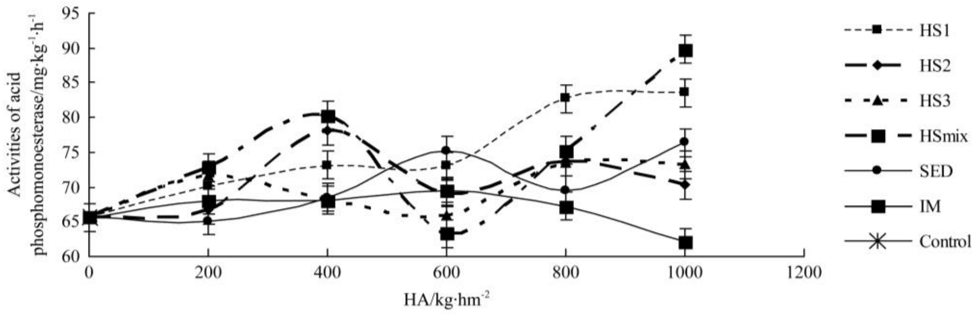
3.1 Soil enzymatic activities at the 40th day following application of humic substances

Humic acids are very important for enzyme functions because they compose a large proportion of soil organic matter and may help to stabilize or inhibit enzymatic activities[27]. After the 40 d, the activity of urease for the control treatment was 69.49 mg/kg ħ with the reduction less than that of HS3 treatment (50.47 mg/kg ħ). Treatments HS1 and HS2 reduced the enzymatic activities compared with that of the control. The phosphodiesterase activity was the highest with HS1 treatment at the addition rate of 600 kg/hm^2. Maximum phosphodiesterase values for the humus treatments (HS1, HS2 and HS3) were 51.0 mg/(kg ħ), 43.0 mg/kg ħ and 48.0 mg/(kg ħ), respectively, which were all higher than the control treatment (35.04 mg/kg ħ). All HS treatments increased acid phosphatase activity at the lower HS application rates (200 kg/hm^2, 400 kg/hm^2) while decreased it to 58.5 mg/(kg ħ) at higher rate of 600 kg/hm^2 (Figure 1). The same trend occurred during the HS1 treatment, when the phosphatase activity peaked (75.6 mg/kg ħ), then declined with increasing application rates. Alkaline phosphatase activity was greater under treatment of HS1 (174.6 mg/kg ħ) than HS2 (158.0 mg/kg ħ) or HS3 (162.0 mg/kg ħ) (Figure 1b). These findings suggest that molecular weights of humic substances strongly affect acid and alkaline phosphatase activities, especially at lower molecular weights, e.g., HS1. However, at the highapplication amount (800 kg/hm^2) of HS treatment, phosphatase activity decreased to 32.10 mg/kg ħ. Overall, humic substances inhibited urease activity, and those with the higher molecular weights such as HS3, had the strongest effect. The treatments SED and IM also stimulated activities of soil phosphodiesterase and of acid and alkaline phosphomonoesterase at levels significantly greater than the control at 40 d.

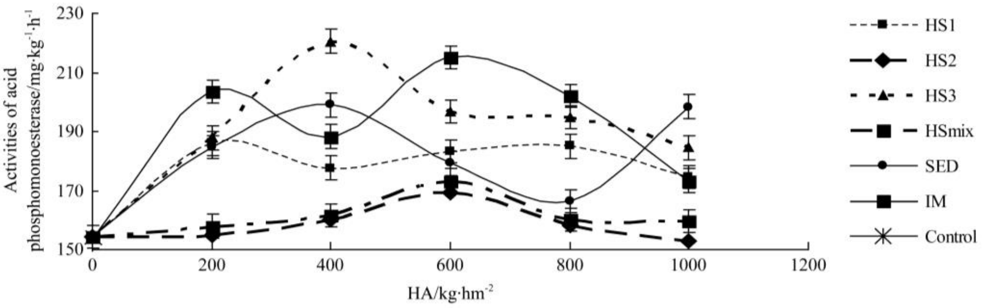
3.2 Soil enzymatic activities at the 70th day following application of humic substances

Responses of soil enzymes to most of humic substances at 70th day following application were significantly higher than these at 40 d. The urease activity for the control was 102.8 mg/(kg ħ) and significantly higher than these for other treatments and the treatment of SED had the lowest urease activity (38.07 mg/(kg ħ)). Compared with the control, phosphodiesterase activity increased significantly with treatments of HS2 and HS3. The highest value of phosphodiesterase activity was 66.2 mg/(kg ħ) for soils treated with HS3.

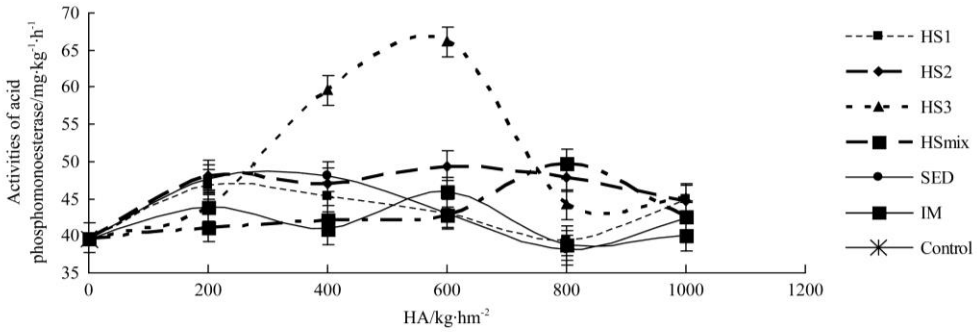
The soil enzymatic activities varied with the increases in the application rates of humic substances (Figures 1 and 2). The activity of urease for HS3 (9.5 mg/(kg ħ)) reached its lowest level at 600 kg/hm^2, and bounced back to 27.42 mg/(kg ħ) (Figure 1b). The SED and IM treatments also inhibited the urease activity. The stimulative effect increased with increasing rates of HS1, but there was no linear relationship between the acid phosphatase and the rates of HS1. Compared with the control, acid phosphatase activity was significantly stimulated by HS2, HSmix, and SED. The HS3 treatmenst yielded the highest activity of alkaline phosphomonoesterase (220.6 mg/(kg ħ)), while HS1 and HS2 led to 186.01 mg/(kg ħ) and 169.21 mg/(kg ħ), respectively. Therefore, the application rate for humic substances at 400 kg/hm^2 was optimal and the lowest molecular weight humus (HS1) was better than HS2 or HS3. Results for the humic treatment with the highest molecular weight (HS3) suggested the higher-mass treatments significantly inhibited urease activity in the soil, and the optimal application rate was 600 kg/hm^2. Similarly, acid and alkaline phosphomonoesterases were significantly increased by HS3 compared with that in the control after 40 d (Table 1).



a. Acid phosphomonoesterase



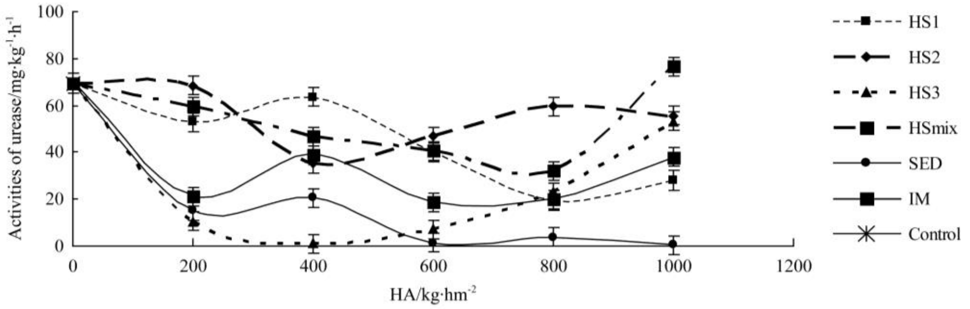
b. Alkaline phosphomonoesterase



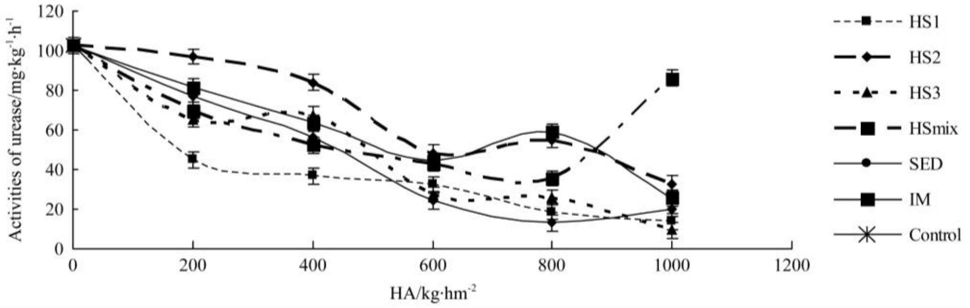
c. Phosphodiesterase

Note: HS1, HS2, HS3, and a mixture of the three (HSmix); SED (insoluble residual), and IM (raw leonardite); and the control. Error bars represent standard errors of the mean (±SEM).

Figure 1 Activities of acid phosphomonoesterase, alkaline phosphomonoesterase and phosphodiesterase in soils collected 70 d after amending with different rates and types of humic acids



a. 40 d



b. 70 d

Note: HS1, HS2, HS3, and a mixture of the three (HSmix); SED (insoluble residual), and IM (raw leonardite); and the control. Error bars represent standard errors of the mean (±SEM).

Figure 2 Activities of urease in soils collected at 40 d and 70 d after amending with different rates and types of humic acids

Table 1 Activities of four enzymes in soils amended with humic acids and a control

Treatment†	Urease /mg (kg h)$^{-1}$††	Acid phospho-monoesterase /mg (kg h)$^{-1}$††	Alkaline phospho-monoesterase /mg (kg h)$^{-1}$††	Phospho-diesterase /mg (kg h)$^{-1}$††
		40 d		
Control	69.49a	58.42c	145.69c	35.04d
HS1	40.79bc	66.17bc	160.13ab	44.16ab
HS2	53.16ab	62.35bc	151.59bc	40.92c
HS3	19.02de	66.99b	155.16b	45.38ab
HSmix	51.16ab	66.62b	155.39b	43.72bc
SED	8.27e	68.76ab	160.07ab	47.39a
IM	27.26cd	76.18a	165.10a	46.44ab
		70 d		
Control	102.76a	65.63b	154.4c	39.71c
HS1	29.42d	76.51a	181.31b	43.89bc
HS2	63.40b	71.57ab	159.10c	47.46ab
HS3	39.15cd	70.49ab	196.89a	51.81a
HSmix	57.40bc	76.29a	162.54c	43.69bc
SED	38.07cd	70.99ab	185.55ab	43.82bc
IM	54.82bc	66.96b	196.42a	41.87bc

Note: † Control, HS1 (low molecular weight), HS2 (medium molecular weight), HS3 (large molecular weight), HSmix (mixture of HS1, HS2, and HS3), SED (insoluble residual), and IM (raw leonardite).

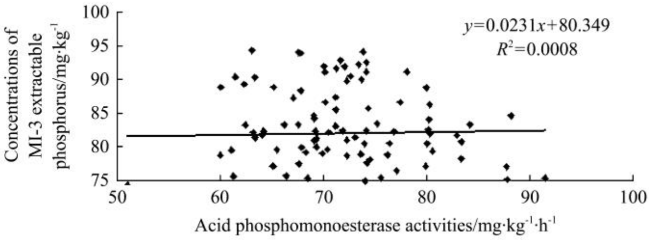
†† means within a column for a given sample date and followed by the same letter were not significantly different ($p \geq 0.05$) based on a one-way ANOVA followed by a *t* test for mean separation.

3.3 Soil nutrients affected by enzymes at the 70th day following application

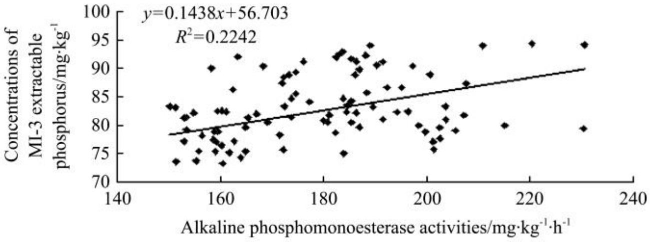
Soil concentrations of phosphorus (P) and copper (Cu) tended to increase, whereas NH$_4^+$-N contents decreased when applied to humic substances, especially those with small molecular weights (HS1) (Figures 3 and 4). HS1a, HS1c and HS1d were 12.08 mg/kg, 10.83 mg/kg and 13.71 mg/kg respectively, all of humic substance fractions treatment had a lower contents of NH4$^+$-N than the control, The results indicated the role of urease which catalyzes the hydrolysis of urea to release ammonia and carbon dioxide. Application of humic substances reduced activities of urease in soils and consequently reduced hydrolysis of urea which applied as a fertilizer. Therefore, these humic substances performed the similar role as a urea inhibitor which is commercially used for improving fertilizer use efficiency[28]. Zinc (Zn) concentration increased more significantly with the application of big molecular weight humus (HS3), HS3b-Zn (2.14 mg/kg) is the highest compared with HS1 and HS2. Application of the treatment with mixed humic masses (HSmix) significantly increased soil concentrations of K, P, Cu, and Zn compared with the applying SED and IM. However, applying HSmix significantly decreased concentrations of NH$_4^+$-N compared with these for SED. The presence of HSmix and SED in the soil may had positive effects on the levels of Cu, HSb- Cu, and SEDc-Cu. Calcium contents of HSmix and the other treatments did not differ significantly from each other. Phosphatases (acid/alkaline phosphatases and phosphodiesterase) perform an important role in soil for mineralizing soil P in organic matter into inorganic P which can be directly uptake by plants. These enzymes are essential for the hydrolysis of pyrophosphate type of fertilizers[29]. The concentrations of extractable P were increased as activities of phosphatases increased after adding humic substances indicated these enzymes enhanced mineralization of organic phosphorus. The process of mineralization of organic matter also released other nutrients and improved soil fertility.

3.4 Coefficients of correlation between soil enzyme activities and extractable nutrients

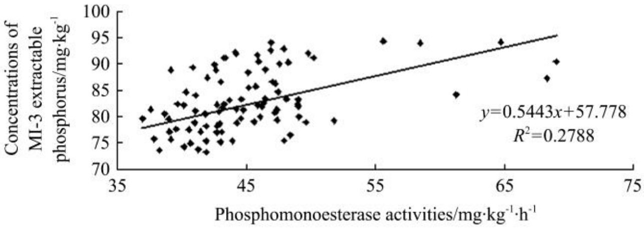
It was observed that urease (U) and acid phosphomonoesterase (AP) had a good influence on K, alkaline phosphomonoesterase (ALP) and phosphodiesterase (PD) had significant effects on P and Zn, ALP had a greater impact on N, PD had a significant effect on Cu (Table 2). However, U, AP, ALP, and PD had no effects on Mg and Ca. By comparing three fitted equations: A: AP, $Y=0.0231X+80.349$, B: ALP, $Y=0.1438X+56.703$, C: PD, $Y=0.5443X+57.778$, we found that the initial concentration of AP, PD and ALP is 80.349, 57.778, and 56.703, respectively, and the activity of PD has a greater effect on the concentration of P with a linear coefficient of 0.5443. By contrast the effect of ALP activity and AP activity on the concentration of P is only 0.1438 and 0.0231 (Figure 3). Meanwhile the initial concentration of PD on Cu, PD on Zn and ALP on Zn is 18.99, 0.4512, and 0.5053, respectively. The activity of PD has a great influence on the concentration of Zn with a linear coefficient of 0.0185. While the effect of ALP activity on the concentration of Zn is 0.0044, and the effect of PD activity on Cu concentration is 0.1352 (Figure 4). Figure 5 shows that the initial concentration of AP to K is 75.863, and the effect of AP activity on Cu concentration is 1.305.



a. Acid phosphomonoesterase



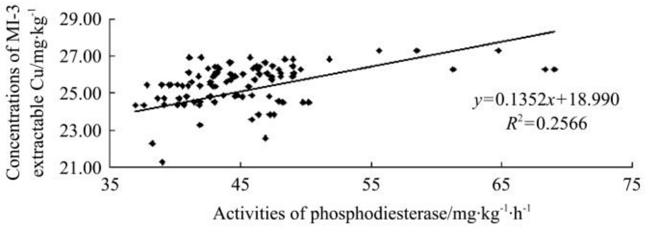
b. Alkaline phosphomonoesterase



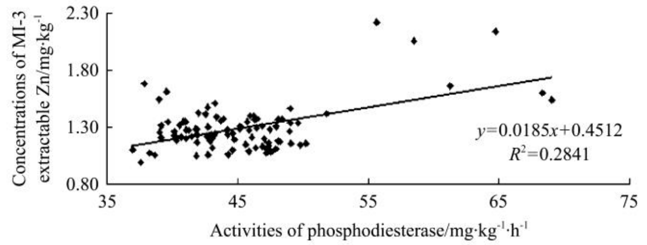
c. Phosphodiesterase

Note: These are from soils collected 70 d after amending with different rates and types of humic acid.

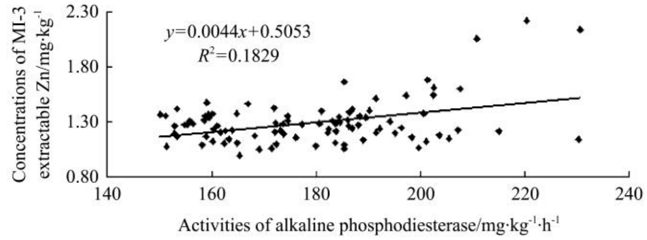
Figure 3 Concentrations of M-3 extractable phosphorus affected by enzymes which react with phosphorus compounds: acid phosphomonoesterase, alkaline phosphomonoesterase, and phosphodiesterase



a. Concentrations of M-3 extractable Cu affected by phosphomonoesterase



b. Concentrations of M-3 extractable Zn affected by phosphomonoesterase



c. Concentrations of M-3 extractable Zn affected by alkaline phosphomonoesterase

Figure 4 Concentrations of M-3 extractable Cu and Zn affected by phosphomonoesterase and of Zn affected by alkaline phosphomonoesterase in soils collected 70 d after amending with different types and rates of humic acids

Table 2 Coefficients of correlation between soil enzyme activities and extractable nutrients

Soil enzyme	K	P	N	Mg	Cu	Zn	Ca
U	–0.41*	–0.21ns	0.32ns	0.07ns	0.04ns	0.13ns	0.29ns
AP	0.56**	–0.16ns	0.28ns	–0.08ns	0.06ns	–0.16ns	–0.15ns
ALP	–0.31ns	0.36*	–0.4*	0.18ns	0.06ns	0.4*	–0.09ns
PD	0.02ns	0.47**	0.03ns	0.29ns	–0.47***	0.55**	0.13ns

Note: *, **, and *** (significant, $p<0.05$, <0.01, and <0.001, respectively); ns (not significant, $p\geq 0.05$).

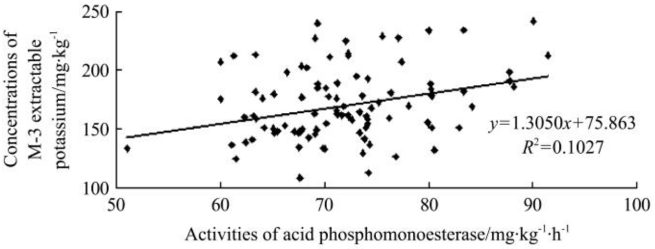


Figure 5 Concentrations of M-3 extractable potassium affected by acid phosphomonoesterase in soils collected 70 d after amending with different rates and types of humic acids

4 Conclusions

Humic acid, especially the higher molecular weight HS3, significantly decreased soil urease activity. While humic acid with moderate molecular weight increased soil acid and alkaline phosphatase activities. Concentrations of NH_4^+-N were decrease with increasing application rates of humus. Not all treatments affected Ca contents, but soil concentrations of K, P, Cu, Zn each increased significantly when applied with the smallest molecular weight humus (HS1). Based on the level of treatments, the optimal application rate for a humic substance was found at 600 kg/hm^2.

Acknowledgements

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