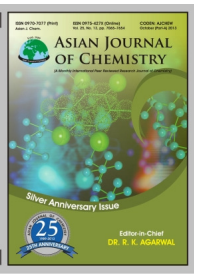
Asian Journal of Chemistry; Vol. 25, No. 18 (2013), 10335-10338

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2013.15384



Effects of Nitrogen Deposition on Typical Hydrolytic Enzyme Activities by Fluorimetric Assay

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(*Received*: 26 February 2013; *Accepted*: 14 November 2013) AJC-14397

Soil properties and five hydrolytic enzyme activities under different simulated nitrogen deposition treatments were determined under semi-arid grassland in Northern China. Significant acidification was observed under N addition treatments. Highest values of soil total carbon, phosphorus and olsen-P concentration was observed in medium level of N application. Soil acid phosphomonoesterase, phosphodiesterase and N-acetyl-β-D-glucosaminidase activities increased with N amendment. The β-D-glucosidase and arylsulfatase activities were stimulated under medium level of N addition. The principal components analysis showed that N deposition might influence soil enzyme activities indirectly through soil pH, total carbon, total phosphorus and olsen-P concentration. Furthermore, the principal component analysis also showed similarity of $N_{2.8}$ and $N_{4.0}$ treatments, which indicated N saturation at 2.8 mol N m^{-2} yr^{-1} in the study area.

Key Words: Hydrolytic enzyme activities, Nitrogen deposition, Fluorimetric assay, Nutrient cycling.

INTRODUCTION

Widespread nitrogen deposition and fertilization caused by anthropogenic activities are changing ecosystem processes, structure and functioning[1,3]. Atmospheric nitrogen deposition had been reported to enhance phosphorus nutrition limitation[4,5], change plant and microbial communities[6,7]. In addition, high inorganic N addition generally increase rates of mass loss for litter and decrease the content of lignin, tannin and other secondary plant compounds[8-10]. Thus, potential influence of nitrogen deposition on soil enzyme activities might exit in semi-arid grassland ecosystems.

Enzymes catalyze biochemical reactions in soil, including organic degradation, mineralization and nutrient cycling[11,12]. Hydrolytic enzymes are the main drivers for degrading substrate to be available for microbial and plant uptake[12]. The mostly studied β-D-glucosidase, N-acetyl-β-D-glucosaminidase, phosphomonoesterase, phosphodiesterase and arylsulfatase play important role in C, N, P and S nutrient cycling, respectively. In detail, β-D-glucosidase, which hydrolyses glucose from cellobiose is main functioned in cellulose degradation; N-Acetyl-β-D-glucosaminidase, which hydrolyses glucosamine from chitobiose is main functioned in chitin and peptidoglycan degradation; phosphomonoesterase and phosphodiesterase, which catalyze the hydrolysis of phosphate from esters and anhydrides; arylsulfatase, which catalyzes the hydrolysis of arylsulfate anion on O-S bond in S mineralization[11,13]. The potential activities of these enzymes are often used as indicators of microbial metabolism and biogeochemical processes[14,15].

Measurements of soil enzyme activities include colorimetric, fluorimetric and radiolabelled methods and with different substrate, assay conditions and incubation time[12]. The fluorimetric method could reduce the incubation time and cost, allow rapid and sensitive determination of soil enzyme activities[12]. We used fluorimetric method to study the effect of 4-year multi-level simulating nitrogen deposition on five typical hydrolytic soil enzyme activities in Inner Mongolia grassland, Northern China. Main soil physicochemical factors were also measured, which had been documented to be significantly affected by nitrogen addition to investigate the indirect effects on soil enzyme assays in response to nitrogen addition.

EXPERIMENTAL

The experiment field locates at the Inner Mongolia Grassland Ecosystem Research Station (IMGERS), Xilin river Basin, Inner Mongolia Autonomous Region of China (43º262N, 116º042E, 1200 m above sea level). The mean annual temperature in the study area is 0.3 ºC, ranging from -21.6 ºC in January to 19.0 ºC in July. The mean annual

precipitation is 346 mm with 60-80 % falling between May and August. The soil was classified as dark chestnut (Calcic Chernozem according to ISSS Working Group RB, 1998).

Experimental design: Experimental field was established in 2006. There were 6 replicates for each of the 7 treatments, which included a Control (No nutrient addition) and 6 treatments with different rate of N addition. Total 42 of 6 m × 6 m plots were laid out in a randomized block design separated by 1 m walkways. Nitrogen was added at the early growing season (May 1-3) with 0, 0.4, 0.8, 1.6, 2.8 and 4.0 mol N m^{-2} yr^{-1} as commercial urea. In the study treatments were referred as control, N_0, $N_{0.4}$, $N_{0.8}$, $N_{1.6}$, $N_{2.8}$ and $N_{4.0}$. To assure that N was the only limiting nutrient, each plot except control also received 0.05 mol P m^{-2} yr^{-1} as KH_2PO_4.

Surface (0-15 cm) soil samples were collected from three soil cores on September 19, 2009 after 4 years of experiment. Moisture samples were sieved (< 2 mm) and stored at 4 ºC. Phosphatase activities were analyzed within one week. Subsamples were air-dried at ambient temperature for one week and stored prior to analysis.

Soil moisture was determined after drying the soils at 105 ºC for 24 h. Soil pH was measured with a glass electrode (soil:water ratio = 1:2.5). Soil total carbon (TC) and total nitrogen (TN) were determined using an automatic elemental analyzer (Vario EL III, Elementar, Germany). Available P was extracted with 0.5 M $NaHCO_3$ at pH 8.5[16]. Total phosphorus (TP) was digested from 2.0 g air-dried soil (< 0.15 mm) in 30 mL of 70 % perchloric acid[17]. Concentration of P_i in all extracts was determined colorimetrically using acid-molybdate colorimetry[18]. Soil texture was determined according to the method of Ryan *et al.*[19].

Soil enzyme assays: Five hydrolytic enzyme activities involved in the cycles of carbon, nitrogen, phosphorus and sulfur were determined using fluorogenic substrates based on a method described by Marx *et al.*[12]. The enzymes and substrates were: (i) β-D-glucosidase (β-D-Gase, EC 3.2.1.21) assayed with 4-methylumbelliferyl β-D -glucopyranoside; (ii) N-acetyl-β-D-glucosaminidase (NAGase, EC 3.2.1.52) assayed with 4-methylumbelliferyl N-acetyl-β-D-glucosaminide; (iii) acid phosphomonoesterase (PMase, EC 3.1.3.2) assayed with 4-methylumbelliferyl phosphate; (iv) phosphodiesterase (PDase, EC 3.1.4.1) assayed with *bis*-(4-methylumbelliferyl) phosphate; and (v) arylsulfatase (ARSase, EC 3.1.6.1) assayed with 4-methylumbelliferyl sulfate potassium salt. Substrates were dissolved in 0.4 % methylcellosolve (2-methoxyethanol; 0.1 % final concentration in the assay).

For each sample, soil suspensions were prepared with 1:100 soil to deionized water (including 1 mM NaN_3 in deionized water to prevent microbial activity). Stir for 15 min on a magnetic stir-plate. Soil suspension of 50 µL was then pipetted into microplate of 96 wells (8 wells per substrate) containing 100 µL substrate and 50 µL acetate buffer (pH 5.0). Plates were incubated for 2 h at 30 ºC fit for the soil samples. The reaction was terminated by 50 µL of 0.5 M NaOH. The fluorescence products were determined immediately on a FLUO star Optima multi-detection plate reader (BMG Labtech, Offenburg, Germany), with excitation at 360 nm and emission at 460 nm. Control wells were prepared containing substrate, buffer and 1 mM NaN_3 without soil suspension for each substrate. Blank wells were prepared containing soil suspension and buffer without substrate.

Statistical analyses: Results are presented as means ± SD (standard deviation, n = 6). The differences among the treatments were tested using the one-way ANOVA of SPSS 11.5 for Windows, with the mean separation performed using the Duncan test and were considered significant at a 5 % level ($p < 0.05$). Principal components analysis (PCA) was applied to identify the effects of N deposition on soil properties and soil enzyme activities using Canoco Software 4.5 (Microcomputer Power, USA). Figure was prepared with sigmaplot 10.0 (Systat, Point Richmond, CA).

RESULTS AND DISCUSSION

The values of soil properties influenced by 4-year-N deposition were given in Table-1, which included soil pH, total carbon, total nitrogen, total phosphorus, Olsen-P, C/N and soil texture information. Soil enzyme activities were shown in Fig. 1. The relationship of hydrolytic enzyme assays with soil properties responding to N deposition were summed up in Fig. 2.

Soil properties: The soil pH ranged from 5.81 to 7.48 and decreased significantly under the N deposition treatments after 4 years of N application, which has been reported extensively[20,21]. Lower total carbon concentrations were found in control, $N_{0.4}$ and $N_{4.0}$ treatments. Similar as our study the total carbon concentration increased with soil fertilization was reported by many researchers. For example, soil total carbon was reported to increase with N fertilization[22,23].

TABLE-1
SOIL pH, TOTAL CARBON (TC), TOTAL NITROGEN (TN), TOTAL PHOSPHORUS (TP), OLSEN-P, C/N AND SOIL TEXTURE TREATED FOR 4 YEARS WITH (1) CONTROL, WITHOUT FERTILIZER; (2) N_0, AN ANNUAL APPLICATION OF 0.05 mol P m^{-2} AS KH_2PO_4; (3) $N_{0.4}$, AN ANNUAL APPLICATION OF 0.4 mol N m^{-2} AS UREA, 0.05 mol P m^{-2} AS KH_2PO_4; (4) $N_{0.8}$, 0.8 mol N m^{-2} AS UREA, 0.05 mol P m^{-2} as KH_2PO_4; (5) $N_{1.6}$, 1.6 mol N m^{-2} AS UREA, 0.05 mol P m^{-2} AS KH_2PO_4; (6) $N_{2.8}$, 2.8 mol N m^{-2} AS UREA, 0.05 mol P m^{-2} AS KH_2PO_4; AND (6) $N_{4.0}$, 4.0 mol N m^{-2} AS UREA, 0.05 mol P m^{-2} AS KH_2PO_4

Treatment	pH (1:2.5)	TC (g kg^{-1})	TN (g kg^{-1})	TP (mg kg^{-1})	Olsen-P (mg kg^{-1})	C/N	Sand (%)	Silt (%)	Clay (%)
Control	7.48 ± 0.03 a	22 ± 1.0 bc	1.8 ± 0.09 a	379 ± 18 d	19 ± 2.4 bc	12.2	46	39	15
N_0	7.39 ± 0.11 a	32 ± 5.4 a	1.8 ± 0.12 a	356 ± 19 d	18 ± 3.2 c	17.7	44	39	17
$N_{0.4}$	6.87 ± 0.03 b	24 ± 2.3 b	1.8 ± 0.08 a	403 ± 24 cd	19 ± 1.5 bc	13.2	48	28	24
$N_{0.8}$	6.58 ± 0.05 c	31 ± 5.8 ab	1.9 ± 0.06 a	511 ± 44 a	22 ± 2.6 abc	16.4	50	33	17
$N_{1.6}$	6.08 ± 0.02 d	32 ± 2.7 a	2.0 ± 0.04 a	460 ± 16 b	23 ± 4.0 ab	16.5	43	34	23
$N_{2.8}$	5.81 ± 0.05 e	29 ± 2.6 ab	2.0 ± 0.06 a	448 ± 38 bc	23 ± 2.8 a	14.9	51	30	19
$N_{4.0}$	5.94 ± 0.14 de	20 ± 0.9 c	1.9 ± 0.04 a	448 ± 29 bc	23 ± 2.3 ab	10.4	47	32	21

Values are shown as mean ± standard deviation of six replicates. Values within one column with the same letter are not significant at 5 % level.

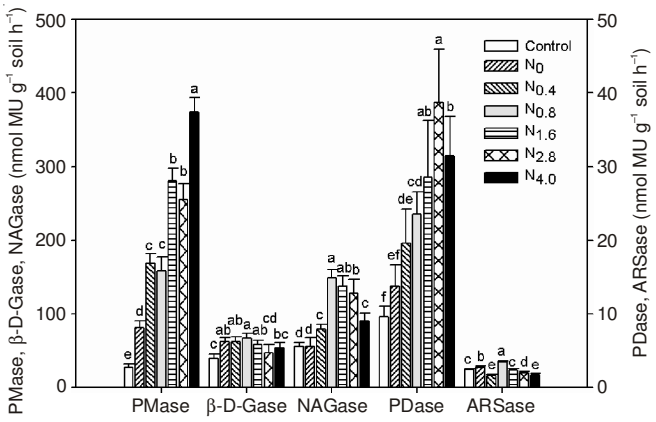

Fig. 1. Five hydrolytic enzymes activities affected by various treatments for 4 years under semi-arid grassland in Northern China. These treatments were (1) control, without fertilizer; (2) N_0, an annual application of 0.05 mol P m^{-2} as KH_2PO_4; (3) $N_{0.4}$, an annual application of 0.4 mol N m^{-2} as urea, 0.05 mol P m^{-2} as KH_2PO_4; (4) $N_{0.8}$, 0.8 mol N m^{-2} as urea, 0.05 mol P m^{-2} as KH_2PO_4; (5) $N_{1.6}$, 1.6 mol N m^{-2} as urea, 0.05 mol P m^{-2} as KH_2PO_4; (6) $N_{2.8}$, 2.8 mol N m^{-2} as urea, 0.05 mol P m^{-2} as KH_2PO_4 and (6) $N_{4.0}$, 4.0 mol N m^{-2} as urea, 0.05 mol P m^{-2} as KH_2PO_4. β-D-Gase = β-D-glucosidase, NAGase = N-acetyl-β-D-glucosaminidase, PMase-acid phosphomonoesterase, PDase = phosphodiesterase and ARSase = arylsulfatase. Values are presented as mean ± standard deviation of six replicates, values with the same letter are not significant at 5 % level

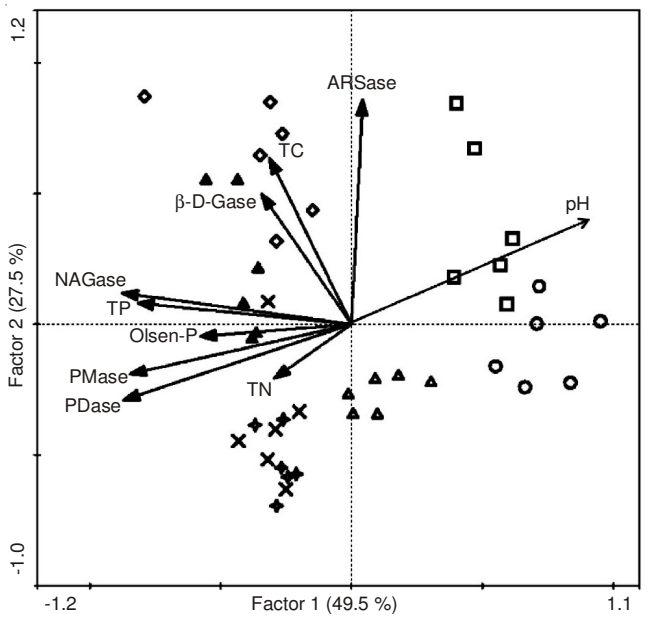

Fig. 2. Principal components analysis (PCA) of soil properties and five hydrolytic enzymes activities determined by fluorimetric assay. β-D-Gase = β-D-glucosidase, NAGase = N-acetyl-β-D-glucosaminidase, PMase-acid phosphomonoesterase, PDase-phosphodiesterase and ARSase-arylsulfatase; ○: Control; □: N_0; △: $N_{0.4}$; ◇: $N_{0.8}$; ▲: $N_{1.6}$; ×, $N_{2.8}$; ❖, $N_{4.0}$

Treatments of $N_{0.4}$ and $N_{4.0}$ did not change soil total carbon might caused by the decomposition rate of total carbon was nearly the same as the accumulation rate of total carbon, which had been reported by Poirier *et al.*[24]. The total nitrogen concentrations were not affected by simulating N deposition. The soil total phosphorus ranged between 356 and 511 mg kg^{-1} soil and treatments received N more than 0.8 mol m^{-2} yr^{-1} significantly increased soil total phosphorus. The concentrations of Olsen-P ranged between 18 and 23 mg kg^{-1} soil, with the higher values found in high N addition ($N_{1.6}$, $N_{2.8}$ and $N_{4.0}$). Higher total phosphorus and Olsen-P concentration with more N application was attributed to the input of P from dead microbes and dry plant tissue[24,25] as grass biomass and soil microbial biomass could increase with N addition[26,27]. As expected, lower value of C/N was found in $N_{4.0}$ treatments due to high N addition. The studied soils had 43-51 % of sand (0.053-2.0 mm), 28-39 % of silt (0.002-0.053 mm) and 15-24 % of clay (< 0.002 mm) (Table-1).

Soil enzyme assays: The PMase, β-D-Gase and NAGase activities were higher than PDase and ARSase activities. The PMase, NAGase and PDase activities increased with N addition and showed significantly higher activities than control and N_0 treatments. Medium N deposition treatment ($N_{0.8}$) increased soil β-D-Gase and ARSase (Figs. 1 and 2). Similar as our results β-D-Gase, NAGase, PMase and ARSase activities were reported to increase with N amendment[28-30]. Reasons of β-D-Gase, NAGase increased with N amendment was attributed to inorganic N addition stimulate soil labile organic matter decomposition and fungal mass[29,31]. Medium N application treatment increase β-D-Gase and ARSase could be the indirect effect of N addition on soil carbon (Fig. 2). Similar relationship of β-D-Gase and total carbon had been reported by Tabatabai[11]. Reasons of PMase and PDase stimulated by N amendment were due to the coping mechanism to biological P limitation[4] or the effect on soil total phosphorus and Olsen-P (Fig. 2).

Principal component analysis of soil properties, phosphatase activities: The principal component analysis application based on soil properties and five hydrolytic enzymes activities determined by fluorimetric assay, explained 77 % of the data variation (Fig. 2). Factor 1 explained 49.5 % of the total variability and was related to total phosphorus, total nitrogen, Olsen-P and soil pH. This factor distinguished low level N addition treatments (control, N_0 and $N_{0.4}$) from medium and high level ones ($N_{0.8}$, $N_{1.6}$, $N_{2.8}$ and $N_{4.0}$). Factor 2 explained 27.5 % of the total variability and was related to soil total carbon content. This factor distinguished the medium level N application treatments ($N_{0.8}$ and $N_{1.6}$) from low (control, N_0 and $N_{0.4}$) and high level ones ($N_{2.8}$ and $N_{4.0}$) (Fig. 2). The principal component analysis showed that treatments without N addition (control and N_0) had more similarity with higher soil pH. Medium level N application treatments ($N_{0.8}$ and $N_{1.6}$) had more similarity that were observed higher values of soil total carbon, β-D-glucosidase and N-acetyl-β-D-glucosaminidase activities, which indicated that appropriate N addition could increase the carbon and nitrogen cycling. High level N application ($N_{2.8}$ and $N_{4.0}$) had more similarity that were observed with higher total phosphorus, Olsen-P, PMase and PDase indicated higher N addition could increase P cycling.

The principal component analysis also showed negative relationship of soil pH with NAGase, PMase and PDase, which suggested the effect of N addition on those enzyme activities could be indirect effect on soil pH. The pH effect on soil enzyme activities has been studied intensively, which showed soil pH could influence enzyme stability[11]. Furthermore, there were positive and intimate relationships of soil total carbon with β-D-Gase and ARSase, soil total phosphorus and Olsen-P with phosphatase activities as well as NAGase with soil total nitrogen.

Conclusion

Indirect effect of nitrogen deposition on soil total phosphorus, Olsen-P, total nitrogen and soil pH influence the acid phosphomonoesterase, phosphodiesterase and N-acetyl-β-D-gluco-saminidase activities. The β-D-glucosidase and arylsulfatase activities were stimulated under medium level of nitrogen addition by the change of soil carbon. The PCA showed similarity of $N_{0.8}$ and $N_{1.6}$, $N_{2.8}$ and $N_{4.0}$ treatments. Thus, the grassland in this area could fertilize with the relative low level of nitrogen application to minimize production cost and environmental pollution.

ACKNOWLEDGEMENTS

The authors thank Prof. Xingguo Han in Institute of Applied Ecology, Chinese Academy of Sciences for field and sampling assistance. The study was supported by the National Basic Research Program of China (2011CB100506, 2011CB100504), the National Natural Science Foundation of China (41171241) and the National Key Technology R&D Program of China (2012BAD14B04).

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