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Effect of different long-term fertilization regimes on the viral community in an agricultural soil of Southern China

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ABSTRACT

Fertilization plays a pivotal role on soil biological process and affects the soil bacterial community, which act as hosts for viruses. The effect of fertilization on soil viral community has not been well explored. In this study, a Haplic Acrisol soil, which is the soil type for 13 provinces in Southern China, was analyzed after 22 years different fertilization regimes for their viral composition. The soil responded to organic fertilizations with an increased amount of soil organic matter (SOM) and pH (increased from 5.7 to 6.6), while with the decreased SOM and pH for chemical fertilization, especially for single nitrogen fertilization. The combined effects of SOM and pH caused by long-term different fertilization regimes on soil viral communities were investigated by direct calculation of virus-like particles (VLPs) through epifluorescence microscopy. The highest VLP abundance (13.1×10^7 per gram dry soil) was detected in soil applied with chemical and organic fertilizers. The viral and bacterial abundances of organic soil were 4 and 5 times higher than those of inorganic soil respectively. Transmission electron microscopy observation revealed a higher frequency of *Myoviridae* viruses in soil with organic amendments than without organic amendments, and vice versa for *Podoviridae* viruses. These results demonstrate that organic fertilizer could increase viral abundance and morphological diversity through suppressing soil acidification and improving soil organic matter.

1. Introduction

Viruses are widely distributed in all ecosystems, and it is likely that their presence has important implications for maintaining the ecosystem functions. In soil, viruses, and among them especially bacteriophages influence the soil microbial community through various mechanisms, e.g. by directly influencing bacterial mortality through lysis, infection and energy acquisition from host cells [1–3], and the effect of the host genetic composition by phage-mediated processes [4], such as lysogenic conversion and transduction after attaching and gene transfer (transduction) [5–8]. Soil viral adsorption and desorption can be influenced by many factors, including virus characteristics, land use, plant diversity, or soil physic-chemical features [9].

Several studies have focused on the viral abundance in different environments. Srinivasiah et al. [10] found that the viral abundance changed more than 2000 folds across marine system ranging from the deep sea to freshwater marshes. Another research revealed that viral abundance changed only 16 folds across different soils [11]. Williamson and co-workers [9] reported that a forest soil which contained more organic matter than an agricultural soil, also harbored more virus-like particles (VLPs). But comparison of viral abundances in the same agricultural soil with different management practices has, to your knowledge, not yet been well studied.

In China, red soil, which belongs to the Haplic Acrisol (according to FAO-UNESICO WORLD MAP Revised Legend [12]) is one of the most important soil types which in fact covers 13 provinces in Southern China. The color of this soil is brick red or brown red, the fertility and productivity are usually low. The soil pH typically ranges from 5 to 6, and unbalanced fertilization especially excess nitrogen input may decrease the soil pH even further (acidification). In contrast, organic fertilization can stabilize the soil pH and increase crop yields through soil quality improvement [13]. Therefore, organic fertilization and chemical/organic combined fertilization are encouraged in this agricultural soil area.

Soil pH is a key factor to drive the composition and activity of the soil bacterial community [14], which are the main hosts of soil

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viruses. Hence soil pH should also have strong effects on the soil viral community. For this study, a long-term (since 1990) fertilization research site located in Hunan Province of Southern China was selected. Different fertilization regimes for 22 years affected the soil in different ways: organic fertilizations not only increased the soil organic matter (SOM), but also stabilized or even increased the soil pH, while chemical fertilization, especially single nitrogen fertilization decreased SOM and soil pH. The combined effects of soil pH and SOM caused by long-term different fertilization regimes on soil viral communities were investigated by direct calculation of virus-like particles (VLPs) through epifluorescense microscopy (EFM). The morphological diversity of viruses was determined by direct observation using transmission electron microscopy (TEM). The results of this study demonstrate correlations between viral abundance and soil properties, especially SOM and pH.

2. Materials and methods

2.1. Field description and experimental design

The field experiment is located in Qiyang, Hunan Province, in Southern China (111° 52′ 32″E, 26° 45′ 12″N), where the climate is subtropical monsoon climate with an average annual rainfall of 1250 mm and a mean annual temperature of 18 °C. The potential evapotranspiration rates reach 1470 mm/year. This fertilization experiment was started in 1990 and included annual rotations of winter wheat (*Triticum aestivum* L.) and summer maize (*Zea mays* L.). Different fertilization treatments were implemented with two replicates in a random block design. Five fertilization regimes were chosen for this study: Control without fertilizers (CK), chemical fertilizers (nitrogen, phosphate, and potassium fertilizer, NPK), single nitrogen fertilizer (N), organic fertilizer (M), and chemical and organic fertilizer (NPKM). N, NPK, NPKM and M treatments received the same amount of nitrogen (456 kg/ha). Each plot was 20 m × 40 m and the fertilization information is listed in Table 1. Soil properties were measured by standard methods [15].

2.2. Sample collection and soil analysis

Soil samples (depth of 0–20 cm) were collected in May, 2012. The temperature was between 23 and 28 °C when sampling. Twelve randomly selected soil cores (approximately 5 cm in diameter) were taken from each plot and mixed to form a composite sample. During the period of sending soil samples from Qiyang to the laboratory, fresh soil samples were stored with sterile bags, airtight and regular ventilation. The soil samples were stored at 4 °C in the laboratory. Fresh, field-moist soils were stored at 4 °C in the laboratory and processed within one week. All soil samples were homogenized and passed through a 4-mm sieve prior to use.

Table 1
Detailed fertilization of different treatments (kg ha^{-1}).

	Element	CK[a]	N	NPK	NPKM	M
Maize	N (urea)[b]	0	213(456)	213(456)	64(137)	0
	N (pig manure)	0	0	0	149(29200)	213(42000)
	P (calcium superphosphate)	0	0	185(699)	185(699)	–
	K (potassium chloride)	0	0	73(140)	73(140)	–
Wheat	N (urea)	0	91(195)	91(195)	27.3(58.5)	0
	N (pig manure)	0	0	0	63.7(12500)	91(18000)
	P (calcium superphosphate)	0	0	78.9(298)	78.9(298)	–
	K (potassium chloride)	0	0	31.4(60)	31.4(60)	–

[a] Control without fertilizers (CK), single nitrogen fertilizer (N), chemical fertilizers (nitrogen, phosphate, and potassium fertilizer, NPK), chemical and organic fertilizer (NPKM), organic fertilizer (M).

[b] Inside the brackets represent fertilizer applicated, and outsides indicate the N, P or K element content.Twenty-two-year annual fertilization of these soils were performed before soil sampling. Wheat (*Triticum aestivum* L.) was planted in winter while maize (*Zea mays* L.) in summer as rotation for every year on every soil field. 30% was fertilized in wheat season while 70% in maize season. In addition to control, the other 4 kinds of fertilizing soil receive the same rate of nitrogen.

2.3. Extraction conditions

Soil viruses were extracted from 10 subsamples of each soil following the methods reported by Wommack et al. [9,16]. In detail, samples (5.0 g) were weighed into 50-ml Teflon-coated polyethylene centrifuge tubes, and 15 ml of 1% potassium citrate solution (containing per liter, 10 g of potassium citrate, 1.44 g of $Na_2HPO_4{\cdot}7H_2O$, and 0.24 g of KH_2PO_4, pH 7, as described by Paul et al. [17]) was added into each tube. All tubes were vortexed for 3–5 min (with each 10 s interrupted by 15 s of settling on ice) and allowed to stand for 15 min. After centrifugation at 5000 rpm (equal to 2991 × *g*) for 15 min, the supernatants were passed through paper filter (Wo Hua) to remove small soil impurities. Subsequently, all tubes were centrifuged at 11,000 rpm (equal to 14,475 × *g*) for 5 min to settle down some bacteria and soil particles. To eliminate bacteria and small soil impurities, supernatants were passed through 0.20-μm pore size mixed cellulose ester microporous membrane filter (Pall Corporation). To improve the extraction efficiency, soil particles, settled down by centrifugation at 5000 rpm, were resuspended in fresh eluant solution and the extraction process was repeated once. Finally, the combined filter liquor from double extractions was ultracentrifuged at 29,000 rpm (equal to 144000 × *g*) for 60 min with a Beckman SW32Ti rotor, and the sedimentary bacteriophage was resuspended in SM buffer (100 mM NaCl, 10 mM $MgSO_4{\cdot}6H_2O$, 50 mM Tris-Cl [pH 7.5] [16]) for further study.

2.4. Epifluorescence microscopy (EFM)

VLPs were enumerated as described by Patel et al. [18]. After 100-fold dilution, the phage aliquots (10 μl) were suspended in 5 ml of 0.02 μm filter-sterilized MilliQ water. A 0.8-μm pore size, 25 mm diameter mixed cellulose ester membrane filter was wetted with filtered MilliQ water and placed onto the center of the filter holder. Next, a 0.02-μm pore size, 25 mm diameter Anodisc filter (Whatman International, Ltd., Dassel, Germany) was placed on top of the 0.8-μm pore size filter. The phage dilution was vacuum filtered through both filters. After drying by rubbing with a clean Kimwipe, the anodisc filter containing captured virus particles was stained by adding 100 μl of 1:400 SYBR Green I solution (1 μl SYBR Green I mixed with 400 μl 0.02 mm filter-sterilized Milli Q water). Filters were incubated for 18 min in the dark in a closed drawer. Then the filters were carefully picked up and dried with a clean Kimwipe in the dark, 30 μl of Fluorescence protectant (0.1% (vol/vol) p-phenylenediamine, 50% glycerin, 50% PBS (0.13 mol/L NaCl, 7.0 mmol/L Na_2HPO_4, 3.0 mmol/L NaH_2PO_4)) anti-fade mounting medium was used to fix the filters on the glass slide. The slides were analyzed by EFM with an upright biological microscope (Leica DM 5000 B). Twenty fields per sample were photographed at a magnification of 1000.

2.5. Transmission electron microscopy (TEM)

VLPs morphological categorization was conducted as described by Williamson et al. [9,16,19]. Aliquots (5 μl) of re-suspended viruses in the SM buffer were dried on Formvar-coated 400 copper mesh Cu grids (3.05-mm diameter) using an incandescent lamp.