Effects of Wine-cap *Stropharia* Cultivation on Soil Nutrients and Bacterial Communities in Forestlands of Northern China

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Abstract

Background. Cultivating the wine-cap mushroom (*Stropharia rugosoannulata*) on forestland has become popular in China. However, the effects of wine-cap *Stropharia* cultivation on soil nutrients and bacterial communities are poorly understood.

Methods. We employed chemical analyses and high-throughput sequencing to determine the impact of cultivating the wine-cap *Stropharia* on soil nutrients and bacterial communities of forestland.

Results. Cultivation regimes (Y010, Y011, Y001 and Y101) of *Stropharia* on forestland resulted in consistent increases of soil organic matter (OM) and available phosphorus (AP) content. Among the cultivation regimes, the greatest soil nutrient contents was found in regime Y101 and the lowest total N and alkaline hydrolysable N contents was observed in regime Y001. No significant differences were observed in alpha diversity among all cultivation regimes. Specific soil bacterial groups, such as Acidobacteria, increased in abundance after cultivation of *Stropharia rugosoannulata*.

Discussion. Given the numerous positive effects exerted by OM on soil physical and chemical properties, and the consistent increase in OM content for all cultivation regimes, we suggest that mushroom cultivation is beneficial to forest soil nutrient conditions through increasing OM content. Based on the fact that the regime Y101 had the highest soil nutrient content as compared with other cultivation regimes, we recommend this regime for application in farming practice. The spent mushroom compost appeared to be more influential than the hyphae of *S. rugosoannulata* on the soil nutrients and bacterial communities; however, this requires further study. This research provides insight into understanding the

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effects of mushroom cultivation on the forest soil ecosystem and suggests a relevant cultivation strategy that reduces its negative impacts.

Introduction

The wine-cap *Stropharia* mushroom (*Stropharia rugosoannulata* Farlow ex. Murrill) is one of the top ten mushrooms traded internationally and is recommended by the UN Food and Agriculture Organization for export to developing countries (Murrill 1922; Hawksworth *et al.* 1996). This mushroom is sciophilous and can be cultivated with different kinds of raw materials, such as straw, sawdust, rice husk and corncobs (CUCEDH 2013; Domondon and Poppe 2000; Gong *et al.* 2016). It is easy to cultivate and can reach a high yield with extensive management. These features make *S. rugosoannulata* suitable for under-forest cultivation. In practice, this mushroom has been cultivated in large gardens with trees and shrubs (Domondon and Poppe 2000) and under hardwood shade (Bruhn *et al.* 2010). Many experiments have been carried out to increase mushroom production (Bonenfant-Magne´ *et al.* 2000; Domondon *et al.* 2004; Bruhn *et al.* 2010; Zeng 2013), which have enabled the large-scale cultivation of *S. rugosoannulata*.

Cultivating mushrooms in forestlands, including under the shade of nursery stocks, has become popular in China. This kind of mushroom cultivation can efficiently use the large expanses of space under nursery stocks. Meanwhile, the straw by-products, which are usually incinerated or discarded in the field (Lu *et al.* 2018), can be consumed by the mushrooms, thereby reducing waste and air pollution. Due to this, the Chinese government has encouraged the cultivation of economically valuable mushrooms in forestland. Thus, the wine-cap

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Stropharia is cultivated under the forest in several Chinese provinces, including Shandong, Fujian (Zeng 2013) and Yunnan (Yang *et al.* 2015).

In China, nursery soil has suffered from improper management, including flood irrigation and excessive inputs of synthetic nitrogen fertilizer. Additionally, topsoil is removed with seedings and nursery stock transactions each year. All these can cause soil erosion, degradation (Wang *et al.* 2004), pollution (Dissanayake and Rajapaksha 2013) and acidification (Conyers *et al.* 2011; Geng *et al.* 2016). Fortunately, the importance of these problems has now become apparent, and several attempts have been made to improve soil conditions (Chadwick *et al.* 2015; Zheng *et al.* 2016; Sihi *et al.* 2017). In several studies, the residual compost waste generated by the mushroom production, i.e., spent mushroom compost, is used in soil bioremediation to improve soil aeration, maintain soil structure (Kadiri and Mustapha 2010), balance soil nutrient (Uzun 2004; Jonathan *et al.* 2011), and increase soil biological activity (Li *et al.* 2012). Growing mushrooms under nursey stocks can be a good alternative, as a considerable amount of spent mushroom compost will be left in the soil after mushroom harvesting. However, there is currently very limited understanding of the effects on soil nutrients that are caused by mushroom cultivation. Additionally, how mushroom cultivation will influence microbial community composition is also worthy of attention, given that the hyphae of these mushrooms can select certain bacterial taxa in the soil (Nazir *et al.* 2010). Finally, there is concern that the cultivation of *S. rugosoannulata* on forestland might lead to soil nutrient loss (Socolow 1999). In this study, we investigated how different cultivation regimes affect the sustainable development of *S. rugosoannulata* stocks

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under nursery stock shade. Specifically, we cultivated *S. rugosoannulata* under nursery stocks in Liying (Jining, Shandong, China), one of the largest centres for seedling production in China. We used four cultivation regimes, based on common methods: (i)…. (Y010), (ii)…. (Y011)…etc, to test the effects of growing *S. rugosoannulata* on influencing soil nutrients and soil microbial community composition.

Materials and Methods

Experimental site

The experimental forestland was an area of 20 × 150 m, located in Liying Town, Jining City, Shandong Province (116°37′E, 35°30′N, 43 m above sea level). The nursery stock is made up by 7- to 10-year-old trees of horse chestnut (*Aesculus chinensis* Bunge), which were planted with 2 m spaces between plants in rows and 5 m between rows to achieve a canopy density of 0.7. This location is considered a warm temperate, semi-humid monsoon climate characterized by hot, rainy summers and cold, dry winters, with an annual average temperature of 13.2–14.1 °C. The highest temperature in July exceeded 27°C, and the annual average temperatures above 10 °C accumulated to 4060.7 °C (growing degree days). The annual precipitation is 650–700 mm, with rainfall from May to August accounting for more than 65% of the total rainfall for the whole year. The soil type was non-calcareous cinnamon tide with a clay loam texture. All these data were obtained from the Jining Soil and Fertilizer Workstations, China (1990).

Sample plots and S. rugosoannulata *cultivation*

The experimental forestland was divided into five, 20 × 30 m grids, which were marked as

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Y000, Y010, Y011, Y001, and Y101, respectively. Among them, Y010, Y011, Y001, and Y101 were used for mushroom cultivation with different regimes, and each of them was divided into three, 10 × 20 m plots for independent replicates; Y000 was used as a no-cultivation control. The cultivation year of each grid is shown in Table 1.

The cultivation of *S. rugosoannulata* began in 2013 and was performed every November as described by Gong *et al.* (2016). The basic materials included 48.9% rice husk, 30% corncobs crushed into particles with a diameter of 0.5 cm to 1 cm, 20% sawdust, which was a mixture that contained a variety of hardwood chips, 1% soil acquired from each plot before cultivation and 0.1% lime. These materials were mixed, stacking fermentation was performed, and then distributed onto the sample plots between the plant rows with a thickness of approximately 25 cm. The *S. rugosoannulata* spawn was divided into blocks approximately 3 cm in length and inoculated into the fermented material using superimposed square planting. Then, 3 cm of the forest surface soil was sprinkled onto the surface of the fungal bed. The fungal bed was vented and kept moist by a 2-3 cm cover of straw under black plastic film. A micro-spray system was installed in each plot, and the ditch between the cultivation beds drained into a stagnant water well. By April of the next year, fruiting had begun, and by late June, the harvest was complete. The soil was subjected to rotary tillage in November, i.e., the material rotting stage (MRS).

Sample collection and measurements of soil properties

A five-point sampling method was used to collect soil samples in October 2016. The surface organic materials of Y011, Y001, and Y101, and 1 cm of the surface soil of Y000 and Y010

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were removed to distinguish the effect of the raw organic materials added from the mushroom cultivation. Five soil cores (5 cm diameter) were collected from each plot with a depth of 30 cm, fully pooled and then sifted using a 2-mm sieve. Subsequently, each soil sample was divided evenly into two portions: one was air dried and used for soil nutrient measurements, and the other was stored at -20 °C before soil DNA extraction.

The soil properties were measured in the Shandong Provincial Key Laboratory of Soil Erosion and Ecological Restoration (Tai'an, Shandong, China). The soil organic matter (OM) content was determined with the potassium dichromate external heating method (Ciavatta *et al.* 1991). The total nitrogen (TN) content was determined by the dichromate oxidization method (Bremner 1965). The total phosphorus (TP) content was determined by molybdenum-blue colorimetry after digestion by HF-HClO4 (Jackson 1958). The alkaline hydrolysable nitrogen (AN) content was determined using the alkaline-hydrolysable diffusion method (Xiong *et al.* 2008). The available phosphorus (AP) was extracted with sodium bicarbonate and determined using the molybdenum-blue method (Olsen 1954). The available potassium (AK) was extracted by ammonium acetate and then determined by flame photometry (Carson 1980). The soil pH was determined according to the international standard with a soil/water ratio of 1:5 (ISO 10390: 2005). The soil field capacity (FC) was measured using the laboratory Wilcox method (Duan *et al.* 2010).

Soil DNA extraction and polymerase chain reaction (PCR) amplification

The hexadecyl trimethyl ammonium bromide (CTAB) method was used for the soil DNA extraction (Zhou *et al.* 1996), and the purity and concentration of genomic DNA was

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monitored by 1% agarose gel electrophoresis. DNA was diluted to 1 ng/μL using sterile water for the PCR. The forward specific primer 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) (Turner *et al.* 1999) and reverse specific primer 907R (5′-CCGTCAATTCMTTTRAGTTT-3′) (Lane *et al.* 1991) were employed to amplify the V4-V5 region of 16S RNA. PCR-based amplifications were performed using Phusion® High-Fidelity PCR Master Mix with GC Buffer and high-fidelity DNA polymerase (New England Biolabs, USA) following an amplification programme of 1 cycle at 98 °C for 1 min, 30 cycles composed of three steps for each cycle (98 °C for 10 s, 50 °C for 30 s, and 72 °C for 30 s), and a final elongation step of 72 °C for 5 min.

Equal volumes of 1X loading buffer (containing SYBR green) and PCR products were mixed and electrophoresed on a 2% agarose gel. Samples with bright main bands between 400 and 450 bp were selected for further experimentation. The PCR products were mixed in equidensity ratios and then purified with a Qiagen Gel Extraction Kit (Qiagen, Germany). The library was constructed using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA), and the library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 systems. The library was sequenced on an Illumina HiSeq 2500 platform at Novogene Bioinformatics Technology Co., Ltd, Beijing, China., and 250 bp paired-end reads were generated. All paired-end reads were deposited in Sequence Read Archive (SRA), BioProject: PRJNA453134.

Bioinformatic analysis

Paired-end reads were assigned to samples based on their unique barcodes and truncated by

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The OTU abundance data were rarefied using a standard sequence number corresponding to the sample with the fewest sequences. Subsequent analyses of the alpha diversity and beta diversity were performed based on the rarefied output data. The alpha diversity indices, including Good's coverage estimator and the Shannon and Simpson diversity indices, were calculated using QIIME (Version 1.7.0). The differences in taxonomic composition were

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evaluated using a beta diversity analysis. The methods of principal component analysis (PCA), principal co-ordinates analysis (PCoA) and non-metric multi-dimensional scaling (NMDS) were used to illustrate the clustering of different samples. PCA was calculated in the R packages FactoMineR (Lê *et al.* 2008) and ggplot2 packages (Wickham et al. 2010), and the Hellinger transformation method (Rao 1995) was used for PCA. PCoA of the weighted and unweighted UniFrac distances was calculated in the R package “ape” (Lozupone and Knight, 2005). An NMDS of the weighted and unweighted UniFrac distances was calculated according to Peck (2010). A canonical correspondence analysis (CCA) calculated using the R package “vegan” (Oksanen et al. 2007) was used to visualize the relationship between edaphic factors and the bacterial community structure in each sample plot. Prior to performing the CCA, we filtered out intercorrelated the environmental factors that affected sample distribution by using a variance inflation factor (VIF) analysis (Gross et al. 2003).

Statistical analysis

The soil chemical concentration, dominant taxa and alpha diversity indices were measured, and a one-way analysis of variance (ANOVA) was performed to determine whether differences existed among treatment means at a significance level of α = 0.05. Multiple comparisons were conducted for significant effects using the Tukey's test at α = 0.05, and FDR of Benjamini-Hochberg (Benjamini and Hochberg 1995) was used for Tukey’s test. These statistical analyses were implemented using the Statistical Program for Social Sciences SPSS (Version 22; IBM, USA).

The linear discriminant analysis (LDA) effect size (LEfSe) (Segata *et al.* 2011) was used

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to identify significantly different taxa among groups using the LEfSe software with a default LDA score value of 4. An analysis of molecular variance (AMOVA, Excoffier *et al.* 1992), analysis of similarities (ANOSIM, Clarke 1993) and permutational multivariate analysis of variance (PERMANOVA or ADONIS, Anderson 2001) was used to determine differences in the microbial community structure between the groups using the amova function in Mothur software (https://www.mothur.org/). Correlations among edaphic factors with estimated diversity levels were tested for significance via Spearman's correlations (Algina *et al.* 1999) performed in R (Version 2.15.3).

Results

Soil properties

As shown in Table 2, the cultivation of *S. rugosoannulata* in forestland changed the soil FC, pH, OM, TN, TP, AN, AP and AK contents. The ANOVA showed that the soil OM and AP increased significantly in all cultivating regimes (Y010, Y011, Y001 and Y101) of *S. rugosoannulata* compared with the no-cultivation control. The soil nutrient concentrations in Y101 were the highest among all grids. Additionally, the soil TP and AN in Y010, the soil TP, AN, and AK in Y011 and the soil TN and AN in Y001 decreased significantly compared with those of the control. In addition, the soil FC and pH in all cultivating regimes were not significantly changed (P<0.05).

Bacterial community composition

After removing potential chimaeras, a total of 1,127,888 high-quality V4-V5 16S rDNA sequences were analysed across the 5 grids. These sequences were assigned to 8,751 OTUs.

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The number of OTUs in the grids ranged from 4,756 to 5,011 (Table S1).

The phylogenetic relationship of different OTUs of each sample was illustrated in Figure S3-17. The top ten most abundant phyla represented 94% of the sequences (Fig. 1a), of which, Proteobacteria and Acidobacteria were the most dominant phyla in all groups, representing 55–61% of the total sequences. Among them, only Acidobacteria, Planctomycetes and Gemmatimonadetes showed significant changes in relative abundance between forestlands with one of the cultivation regimes (Y010, Y011, Y001 and Y101) and the no-cultivation control (Y000) (Fig. 1a, Table S2). Acidobacteria in Y001 was the most abundant, and was significantly more abundant than in the control. The abundance of Planctomycetes in forestland with cultivation was greater than that of the no-cultivation control and was greatest in Y101. Significantly fewer Gemmatimonadetes were observed in Y101 than in the other sample groups.

The LEfSe analysis was used to identify the specific bacterial groups in the soil from forestland with the cultivation regimes (Y010, Y011, Y001 and Y101) and in the no-cultivation control (Y000). Major differences were observed in the bacterial groups among the samples. Notably, Acidobacteria was the most common group in the soil with cultivation (Fig. 1b-e). The most frequently observed differences were between Y101 and Y000 (Table S3).

Bacterial α-diversity

Before performing the α-diversity analysis, the OTU abundance data were normalized with a cutoff value of 59,458. In all samples, the Good's coverage values reached 0.98 (Table S1),

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indicating that the normalised sequencing data was sufficient to capture the bacterial diversity. The Shannon and Simpson indexes were calculated to evaluate the bacterial diversity (Table S1), and no significant difference was observed among the five grids (p=0.05), even though slightly lower Shannon and Simpson values were observed in the forestlands with cultivation than in the no-cultivation control.

OTU-level bacterial β-diversity analysis

The PCA of the bacterial community construction in different samples is shown in Fig. 2a. The five treatments were clearly distinguished in the PCA. The first two principal components, PC1 and PC2, best reflected the differences between these treatments and represented variations of 12.35% and 10.04% in the bacterial community, respectively. Within the PC1 axis, Y101 was distinct from the other samples. Within the PC2 axis, Y000 was distinct. Similarly, the weighted Unifrac-based analysis of PCoA and NMDS (Figure S1) and unweighted Unifrac-based analysis of PCoA and NMDS (Figure S2) all showed that Y101 was distinct from the other samples. These data indicated that the bacterial community composition in Y101 was relatively distinct from other grids (Y000, Y010, Y011, and Y001). However, significant differences in the bacterial community composition were not found between Y000 and Y101 via the AMOVA (Fs = 4.05, P = 0.074), the ANOSIM (R = 1, P = 0.1) or the ADONIS (R2 = 0.52, P = 0.1) (Table S4).

The VIF analysis suggested that the FC, pH, OM, TP, and AN were the uncorrelated edaphic factors in the CCA that could represent the relationship between soil physicochemical properties and bacterial community composition. Based on this model, a total of 60.39% of

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the variance was explained by CCA1 (24.46%) and CCA2 (19.49%), which were the first two constrained axes of the CCA (Fig. 2b). The CCA suggested that the OM, TP, AN were the determinants among the edaphic factors. The correlation analysis showed that only OM was significantly associated with the soil bacterial composition (r = -0.579, *P* = 0.024) and the Shannon index (r = -0.571, *P* = 0.026).

Discussion

Effects of cultivating S. rugosoannulata *under nursery stock shade on soil properties*

Recently, growing *S. rugosoannulata* under nursery stock shade has been considered a win-win agricultural practice that can improve the quality of nursery stock soil in China. In this study, higher organic matter and AP content were observed in forestlands with cultivation of *S. rugosoannulata* compared with the no-cultivation control. However, other soil nutrients did not increase consistently in the forestlands with cultivation; some significantly decreased (Table 2). These results were unexpected because a positive correlation has been observed between the OM content and soil fertility (Sadikhani *et al.* 2014). However, the acute angles between the arrow line representing organic matter content and the arrow lines representing other nutrients in the CCA (TN, TP, AN, AP and AK; Fig. 2b) indicate that organic matter content was positively correlated with the other nutrients in this study also. These results are consistent with those of other studies (Sihi *et al.* 2017; Zhou *et al.* 2017).

Cultivation in temperate climates usually results in a significant loss of mineralized organic N in soil (Tiessen *et al.* 1994). Although farming *S. rugosoannulata* in forestland is a type of agricultural practice, it is distinct from traditional crop cultivation. The decrease in AN

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content in forestland under cultivation (except for Y101) indicated that the following cultivation regimes resulted in nitrogen loss: fallow for 1 year after prior cultivation (Y010), 2 years of continuous cultivation (Y011) and current-year cultivation (Y001). The overyear cultivation (Y101) regime effectively suppressed the nitrogen loss and significantly increased the AN content. In addition, the Y101 regime performed well in maintaining soil fertility and had the highest soil nutrient content (Table 2). In contrast, the Y011 regime resulted in a loss of soil nutrients with a significant decrease in TP, AN and AK content (Table 2). The Y001 regime resulted in a significant decrease in TN and AN content.

Soil use and management, such as less intensive management, can cause the loss of phosphorus (Leinweber et al. 1999). Herein, total phosphorus loss was found in Y010, Y011, and Y001. This loss may have resulted from the use of a large amount of water during the fruiting stage of *S. rugosoannulata*. In contrast, a significant increase in total phosphorus was observed in Y101, indicating that the amount of phosphorus increase was greater than the amount of phosphorus lost in Y101. We hypothesize that spent mushroom compost left after the harvesting of fruiting bodies would add a certain amount of macronutrients like nitrogen, phosphorous and potassium (NPK) (Kim et al. 2011); however, this hypothesis needs to be further tested in the future.

It has been suggested a higher OM content may lower the soil pH (Hodes 1996) and increase the water content at field capacity (Hudson 1994; Tale and Ingole 2015). Inconsistent with this, Y011 and Y001 showed an increase of OM content, together with a decrease in the FC and an increase in the pH. The decreased FC may be related to disturbances from farming

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Effects of cultivating S. rugosoannulata *under nursery stock shade on the soil bacterial community composition*

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Using high-throughput sequencing analyses, we observed a consistently higher abundance of Acidobacteria and a consistently lower abundance of Actinobacteria and Firmicutes in the forestlands with cultivation (Y010, Y011, Y001, and Y101; Table S1). However, reports (Ramirez *et al.* 2012; Zhou *et al.* 2017) showed that the abundance of Acidobacteria was reduced with increased nutrient inputs because of the oligotrophic properties of these organisms, and the abundance of Actinobacteria and Firmicutes was increased with increased nutrient inputs because of the copiotrophic properties of these organisms. These inconsistencies may be ascribed to the increased organic matter in forestlands with cultivation that creates an oligotrophic soil environment due to its ability to slowly release nutrients (Tiessen *et al.* 1994). In addition, we observed a consistently increased abundance of Planctomycetes and Bacteroidetes and a consistently reduced abundance of Chloroflexi and Nitrospirae in our study (Table S2). The further study is needed to reveal the underlying mechanisms for this phenomenon.

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Interactions between soil fungi and bacteria are common in nature. For example, fungus-released compounds may impact bacterial selection (Warmink *et al.* 2009; Nazir *et al.* 2010). During the cultivation cycle of *S. rugosoannulata*, high-density hyphae are observed in the culture substrate for long periods and are even found in the spent mushroom compost. Additionally, the soil contains a considerable amount of hyphae. Therefore, the changes in bacterial communities in the soil after the incorporation of spent mushroom compost would be consistent with changes in bacterial communities in soil environments that surround the dense fungal hyphae, such as soil microhabitats, i.e. hyphospheres or mycospheres (Johansson *et al.* 2004; Nazir *et al.* 2010), that more or less are densely permeated by the fungal hyphae. In our study, a decrease in bacterial diversity was found in forestlands with cultivation (Table S1), which was consistent with reports showing that the bacterial community diversity is lower than that of bulk soil (Warmink *et al.* 2009; Halsey *et al.* 2016). Additionally, the selection of bacteria by the hyphae of *S. rugosoannulata* may represent a factor that contributes to the emergence of specific bacterial groups, such as Acidobacteria and Subgroup_6 which also belongs to Acidobacteria in forestlands (Fig. 1b-e). However, it is possible that spent mushroom compost could be more influential on the soil nutrients and bacterial communities because the hyphae of the wine-cap *Stropharia* disappear along with the deposition of spent mushroom compost (data not published). Further study is needed to understand the impacts of spent mushroom compost and fungal hyphae on soil texture and microbial communities.

Conclusion

Overall, the increased soil content of organic matter and available phosphorus and the changes in soil bacterial community composition and diversity in the forestland soil with cultivation suggest that *S. rugosoannulata* cultivation changed the nursery stock soil

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properties. Given the positive effects on soil physical and chemical properties of organic matter, the highest contents of soil organic matter in the Y101 cultivation regime suggested that this regime is most appropriate for forestland soils. In addition, this research suggests that (1) organic matter content is the dominant factor affecting soil bacterial community composition, and (2) the spent mushroom compost after harvesting the fruiting bodies of *S. rugosoannulata* is important for improving both soil nutrient content and soil bacterial community composition and diversity, due to the more abundant organic matter and hyphae of *S. rugosoannulata.*

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