Effects of Freezing-Thawing on Microbial Community Structure in the Soil of Old Apple Orchards

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Abstract. The study here aimed to investigate the effects of pre-winter ditching and freezing-thawing on soil microbial structure in different soil layers of old apple orchards. A total of 30 samples were obtained from 3 Nov. 2016 to 9 Mar. 2017. The relative abundance, alpha diversity, community structure of fungi, and the relationship between environmental factors and microbial community structure were analyzed, and the greenhouse experiments were used for further verification. Results showed that the number of actinomycete and total bacterial colonies decreased, whereas the number of fungi sustained decreased, resulting in a higher bacteria/fungi ratio. The percentage of *Fusarium* initially decreased, then later increased by 11.38%, 3.469%, 2.35%, 2.29%, and 3.09%. However, *Fusarium* levels were still 9% lower on 9 Mar. 2017 than on 3 Nov. 2016. Both the abundance and diversity of the community were higher in the upper soil than in the lower. The main environmental factor contributing to the percentage of *Fusarium* change was average temperature (AT), although highest temperature (HT) and water content (WC) also had an impact. The *Malus hupehensis* Rehd. seedlings growing in lower soil were more vigorous than that in upper soil. In sum, pre-winter ditching and freezing-thawing in old apple orchards can reduce the abundance percentage of harmful *Fusarium* and promote the growth of *M. hupehensis* Rehd. seedlings.

Apple replant disease (ARD), also known as apple continuous cropping disorder, occurs when apple trees are re-cultivated (Mazzola and Manici, 2012; Yim et al., 2013). It is generally believed that the imbalance of microbial community structure, such as *Rhizoctonia*, *Phytophthora*, *Pythium*, and *Fusarium* are the main causes of ARD (Tewoldemedhin et al., 2011; Zhang et al., 2012). Moreover, *Fusarium* is considered one of the main harmful fungi causing ARD in the Bohai Gulf of China (Wang et al., 2018). The changes of soil microbial community structure are closely related to environmental factors (Liu et al., 2014), so

exploring the relationship between environmental factors and soil microorganisms to seek measures to improve soil microbial community structure has gradually become a hot topic of research.

Pre-winter ditching, freezing-thawing, and replacing soil layers are environmental protection measures. Pre-winter ditching can cause the soil temperature to fluctuate up and down through 0°C due to seasonal or day-to-day heat changes, resulting in frequent freezing-thawing processes in the topsoil and below a certain depth (Doney and Schimel, 2007), which affects the microbial community structure. Seasonal freezing-thawing is common in northern China (Li and Meng, 2013). Currently, there are many measures to prevent ARD, although most of them are based on deep pre-winter ditching (Ali et al., 2004). Freezing-thawing has been previously shown to affect the migration and transformation of soil phosphorus by changing the physical and chemical properties and microliving activities of the soil (Li and Meng, 2013). Importantly, the physiological activity and community structure of soil microbes were changed after freezing-thawing, with their cell structures being destroyed (Li et al., 2018), which was similar to the effect of the dry-wet cycle and chloroform fumigation (Warren, 2014). Freeze-thaw cycles can affect the soil microbial population and community composition (Fan et al., 2012; Meisner et al., 2021) by reducing the fluidity of the lipid membrane of soil microbes, causing

cell rupture (Fan et al., 2012), and by forming a relatively anaerobic environment (Warren, 2014; Zhang et al., 2018).

However, the effects of freezing-thawing on the changes in soil microbial community structure of old apple orchards under pre-winter ditching, especially the dynamic changes of microbial community structure in different soil layers, has been less studied. To address the lack of previous study, our research focused on this particular topic. We incorporated high-throughput sequencing technology to explore, in depth, the dynamic changes in soil microbial community structure of old apple orchards, before and after ditching, with its own front-to-back control, and their relationship with environmental factors.

Materials and Methods

Experimental materials

The experiment was carried out from Sept. 2016 to June 2017. The test soil samples were obtained from the 35-year-old apple orchard in Manzhuang, Daiyue District, Tai'an City, Shandong Province. The soil texture was brown loam. The contents of available potassium, the available phosphorus, the nitrate, ammonium nitrogen, and the organic matter were 106.74 mg·kg⁻¹, 9.13 mg·kg⁻¹, 12.11 mg·kg⁻¹, 4.39 mg·kg⁻¹, and 5.16 g·kg⁻¹, respectively.

On 3 Nov. 2016, the apple trees in the old orchard were removed, and their roots were removed. The original tree hole was taken as the center and the planting ditch, which was 200 cm long, 50 cm wide, and 80 cm deep. The 0 to 40 cm of cultivated soil layer was evenly placed on the left side of the planting ditch (upper layer soil), and the noncultivated soil layer of 40 to 80 cm was uniformly placed on the right side of the planting ditch (lower layer soil), as shown in Fig. 1.

Experimental designs

On 3 Nov. 2016, 1 Dec. 2016, 14 Dec. 2016, 16 Jan. 2017, and 9 Mar. 2017 at ≈10 AM, three samples were taken from the upper and the lower layer soil. The weather was recorded 1 week before sampling (special weather conditions were also noted). When sampling, the topsoil (0–3 cm) was first removed, then points in the upper layer soil (0–40 cm) and the lower layer soil (40–80 cm) were then randomly selected, and the samples were separately collected by using a ring knife. Soil samples were filtered through a 12-mesh sieve, mixed well, and then packed into three sterile sealed pouches. The first one was quickly put into liquid nitrogen, placed in a –80°C refrigerator for DNA extraction, and saved for high-throughput sequencing; the second one was stored in a 4°C refrigerator and used to determine the number of microorganisms; and the last one was naturally dried and stored at room temperature for basic soil physical and chemical property determination only on 3 Nov. 2016.

After sampling in Mar. 2017, the experimental soils were separately transported to the experimental station of Shandong Agricultural University for a greenhouse test with

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Fig. 1. Schematic diagram of the ditching before winter in the old orchard in Manzhuang, Shandong.

M. hupehensis Rehd seedlings. The upper and the lower soil samples were mixed thoroughly separately then transported to pots (14 cm × 9 cm × 15 cm, capable of holding 3 kg of soil). *M. hupehensis* Rehd seedlings with five to six leaves were planted in pots containing the upper soil and in pots containing the lower soil, with 25 replicates for each type of soil. The seedlings were managed under conditions of 16 h of light, 8 h of darkness, and 60% moisture content. The biomass of five seedlings was taken in mid-May and mid-June, respectively.

Experimental methods

Determination of the number of soil culturable microorganisms. The number of culturable microorganisms was determined by agar plate dilution method (Hua et al., 2012). Beef peptone medium was used for the culture of bacteria before counting after 24 h of incubation at 37 °C, and PDA medium was used for the culture of fungi before counting after 48 h of incubation at 28 °C. Moreover, Gao 1 agar medium was used for the culture of actinomycetes before counting after 96 h of incubation at 28 °C.

Soil genomic DNA extraction and polymerase chain reaction amplification. The soil microbial genomic DNA was extracted using Fast DNA SPIN for soil kit (MP Biomedicals, Solon, OH). The fungal internal transcribed spacer (ITS) region was double-ended sequenced on the Illumina MiSeq platform. The sequences of the primers were ITS1F (5'-CTTGGTCATTAGAGGAAGTAA-3'), and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') (Adams et al., 2013). Trans Start Fast Pfu DNA Polymerase was used for polymerase chain reaction (PCR) amplification in a 20-μL reaction system containing 4 μL of 5×FastPfu Buffer, 2 μL of 2.5 mM dNTPs, forward primer (5 μM), reverse primer (5 μM) of 0.8 μL each, 0.4 μL FastPfu Polymerase, 0.2 μL bovine serum albumin, and 10 ng template DNA, supplemented with double distilled H₂O to a final volume of 20 μL. The PCR reaction conditions were set as follows: pre-denaturation at 95 °C for 3 min; denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 45 s for 35 cycles, and finally extension at 72 °C for 10 min (ABI GeneAmp 9700 PCR machine). Using high-throughput sequencing technology, a total of 360,654 optimized sequences, 834 operational taxonomic units (OTUs), and 249 genera were obtained in this experiment.

Determination the biomass of *M. hupehensis* Rehd. seedlings. The plant height and the stem diameter of *M. hupehensis* Rehd. seedlings were measured with a meter ruler and a vernier caliper, respectively. The dry weight and the fresh weight were measured by an electronic balance.

Data analysis

Analysis of variance, *t* test, and differential significance were performed with IBM (Armonk, NY) SPSS19.0 software, and the effects of sampling times and soil levels on the number of culturable microorganisms were determined by a two-way analysis of variance and spherical test. Based on the OTU abundance table, R language tools were used to obtain the community Heatmap. R language and software Circos-0.67-7 tools were used to analyze the relative abundance percentage of different soil strains at different sampling times. Based on the results of OTU, the diversity of Shannon index, Chao1 index, Ace index, Simpson index, and coverage (Coverage) were calculated by Mothur software. To analyze the influence of environmental factors on community distribution, redundancy analysis (RDA), principal coordinates analysis (PCoA), and similarity analyses (ANOSIM) were performed by the R language vegan package. The interpretation and the significant differences of community differences at different sampling times were also determined.

Results

Analysis of environmental factors at different samplings. As seen in Table 1, the WC of the upper layer of soil is lower than that in the lower layer of soil from the first to fourth samplings, as the water contents varied in different samplings ($P < 0.01$). The AT showed a declining trend with a drop of 96.8% from the first to the fourth samplings (8.53 to 0.27 °C), and the AT was increased slightly during the fifth (early spring) sampling. The lowest WC appeared in the third (deep winter) sampling (4.40%). Due to the snowfall before the fourth sampling, the WC showed a short period rise, the AT of the fourth

sampling was the lowest, fifth sampling showed gradual warming.

Analysis of quantitative changes of culturable microorganisms at different samplings. The cultured bacterial amount, actinomycetes amount, and fungi amount showed significant differences between the upper and lower layers in bacterial and actinomycetes ($P < 0.05$), and extremely significant differences in fungi amount ($P < 0.01$) (Table 2). As the sampling time progressed, the bacteria, actinomycetes, and the bacteria/fungi showed an initial decrease then increase in trend. During the third and fourth samplings, they were significantly reduced, followed by a slight increase, but the number of fungi continued to decrease from 107.33×10^3 colony-forming units (CFU)/g at the first (early winter) sampling to 30.67×10^3 CFU/g at the fifth (early spring) sampling, the downward trend reached 71%. The number of fungi in lower soil was decreased by 62.5%, so the trend was decreased more in the upper soil. The sampling time affected the microbial biomass and the bacteria/fungi, and there was also a significant interaction between different soil layers and sampling times (Supplemental Table 1).

Analysis on species compositions of different samplings. PCoA analysis between samples indicated differences between fungi communities in both the upper and lower layers (Fig. 2). The first two principal component scores accounted for 66.28%, and 74.87% of the total variation in the upper layer (Fig. 2A), and the lower layer (Fig. 2B), respectively, suggesting that freezing-thawing may be one of the important factors accounting for the change in microbial community structure, especially in the upper layer (Fig. 2A).

The heatmap at the genus level showed that the soil fungi community after freezing-thawing varied, which was consistent with the PCoA (Fig. 3). The heatmap also indicated that there were differences in the abundance of the soil fungi community. The top 20 dominant genera in all samples were used to construct a heatmap; at the genus level, the percentages of *Mortierella*, *Guehomyces*, *Fusarium*, *Penicillium*, and *Microascus* in the upper soil were

Table 1. Environmental factors in different sampling times.

Sample name	Water content (%)	Avg temp (°C)	Highest temp (°C)	Lowest temp (°C)
U1	12.85 ± 0.00 b	8.53 ± 0.21 a	17	4
D1	15.34 ± 0.01 a	8.50 ± 0.10 a		
U2	8.34 ± 0.00 e	4.63 ± 0.21 b	11	-2
D2	8.92 ± 0.01 cd	4.63 ± 0.12 b		
U3	4.40 ± 0.00 f	4.20 ± 0.20 c	12	-2
D3	4.77 ± 0.00 f	4.17 ± 0.06 c		
U4	8.57 ± 0.00 de	0.27 ± 0.06 e	10	-5
D4	9.33 ± 0.01 cd	0.23 ± 0.06 e		
U5	3.75 ± 0.00 g	3.63 ± 0.15 d	13	-1
D5	3.53 ± 0.00 g	3.57 ± 0.15 d		

U1 = The upper layer soil sampled on 3 Nov. 2016. D1 = The lower layer soil sampled on 3 Nov. 2016. U2 = The upper layer soil sampled on 1 Dec. 2016. D2 = The lower layer soil sampled on 1 Dec. 2016. U3 = The upper layer soil sampled on 14 Dec. 2016. D3 = The lower layer soil sampled on 14 Dec. 2016. U4 = The upper layer soil sampled on 16 Jan. 2017. D4 = The lower layer soil sampled on 16 Jan. 2017. U5 = The upper layer soil sampled on 9 Mar. 2017. D5 = The lower layer soil sampled on 9 Mar. 2017.

Data shown are means ± sd. Different lowercase letters in the same column indicate a significant difference at the $P < 0.05$ level by Duncan's new multiple range test.

Table 2. The influence of different sampling times on microorganisms in different soil layers.

		Bacterial amount ($\times 10^5$ CFU/g)			Actinomycetes amount ($\times 10^4$ CFU/g)			Fungi amount ($\times 10^3$ CFU/g)			Bacteria/fungi		
		Avg	Standard deviation	F value	Avg	Standard deviation	F value	Avg	Standard deviation	F value	Avg	Standard deviation	F
Sampling time $\times$ Soil level	U1	54.00	2.70	62.2*	95.70	7.60	45.8*	107.00	1.00	1164**	50.30	2.90	0.80
	U2	29.00	2.70		66.30	2.50		55.30	4.20		52.70	7.20	
	U3	23.30	3.10		56.00	3.50		36.30	3.20		64.40	8.60	
	U4	20.70	1.50		37.30	3.20		32.70	2.10		63.20	2.20	
	U5	22.70	2.50		40.30	3.10		30.70	1.50		74.20	11.00	
	D1	4.67	0.58		24.30	4.20		23.30	3.10		20.40	4.80	
	D2	3.33	0.58		12.30	2.10		12.70	2.50		26.60	3.50	
	D3	3.00	1.00		10.30	3.10		9.67	1.53		32.70	16.10	
	D4	2.67	0.58		11.00	1.00		12.30	1.50		21.80	5.30	
	D5	3.67	0.58		15.30	1.50		8.67	0.58		42.10	4.00	

U1 = The upper layer soil sampled on 3 Nov. 2016. D1 = The lower layer soil sampled on 3 Nov. 2016. U2 = The upper layer soil sampled on 1 Dec. 2016. D2 = The lower layer soil sampled on 1 Dec. 2016. U3 = The upper layer soil sampled on 14 Dec. 2016. D3 = The lower layer soil sampled on 14 Dec. 2016. U4 = The upper layer soil sampled on 16 Jan. 2017. D4 = The lower layer soil sampled on 16 Jan. 2017. U5 = The upper layer soil sampled on 9 Mar. 2017. D5 = The lower layer soil sampled on 9 Mar. 2017.

The fungi, actinomycetes, and the bacteria/fungi all conform to the spherical test ($P > 0.05$). The analysis of variance table was obtained by using the "Spherical degree adopted" in the one-way analysis of variance and referring to the "Pillai Tracking" data after "Greenhouse-Geisser" correction.

*Indicates a significant difference of the same area between the soil level and sampling time at the 0.05 level.

**Indicates extremely significant difference of the same area between the soil level and sampling time at the 0.01 level.

listed in the top 5 (Fig. 3A), and the percentages of *Alternaria*, *Fusarium*, *Mortierella*, *Trichoderma*, and *Penicillium* were listed in the top 5 in the lower layer (Fig. 3B).

Analysis on soil microbial community structure with a special focus on *Fusarium*. At the genus level, the microbial community structure of the upper soil and the lower soil belong to different types (Fig. 4C), among which the proportion of *Fusarium* in upper soils was 7.65%, 5.16%, 1.42%, 3.81%, and 4.61%, respectively (Fig. 4A), and the proportion of *Fusarium* in lower soils was 17.26%, 2.87%, 2.87%, 0.46%, and 0.08%, respectively (Fig. 4B). The percentage of abundance was decreased first and then increased in upper soils, although the percentage of abundance of

Fusarium in the fifth sampling (4.61%) was still lower than that of the first sampling (7.65%), whereas the percentage of *Fusarium* in the lower soils was decreased all the time.

Analysis of fungal diversity at different sampling times. Based on Table 3, the coverage values of all samples were all greater than 99%. The results showed that the soil fungal community richness index and diversity index were basically larger in the upper soil than in the lower soil ($U > D$). Among them, the Chao1 index of 1U was the highest, reaching 583.20, and the Shannon index of 5U was the highest, reaching 4.00. There were no significant differences between other sampling times in each of the same soil layers, there was a significant difference between the upper and lower

soil layers, which indicates that the community composition of fungi in the upper layer of soil is more complex and diverse than that in the lower layers.

Typical correspondence analysis of different samples. The results showed that the distribution patterns of the upper and lower layers of the first three sampling times were closer on the 1-axis, and their difference in community composition was small (Fig. 5). While the fourth and the fifth samplings were taken, the community compositions in the upper layers and lower layers were distributed in two quadrants of the 1-axis and 2-axis, showing large separation and large differences in community composition (Fig. 5A). The RDA analysis also showed that the

Fig. 2. Principal coordinates analysis (PCoA) in community structure in different soil layers. (A) PCoA of the unweighted UniFrac distance matrix representing differences in community structure in the upper layer soil. (B) PCoA of the unweighted UniFrac distance matrix representing differences in community structure in the lower layer soil.

Fig. 3. Community heatmap analysis on the genus level in different soil layers. (A) Community heatmap analysis on genus level in the upper layer soil. (B) Community heatmap analysis on genus level in the lower layer soil.

AT had the greatest effect on the changes of the percentage of *Fusarium*, followed by the HT, and that both were negatively correlated (Fig. 5A). The WC in the soil also had a certain impact on the change in community structure. The preceding results indicate that the AT is an important factor affecting the

community distribution in different sampling periods. The HT and WC also play a role. At the genus level, *Fusarium* has a significant correlation with AT, WC, and HT (Fig. 5B). The difference in community compositions between the upper and lower soil layers of the first sampling (early winter) and the fifth

sampling (early spring) was significantly greater than the intragroup difference.

Biomass analysis of *M. hupehensis* Rehd. seedlings in different soil layers. As seen in Table 4, the biomass of *M. hupehensis* Rehd. seedlings growing in the lower soil is significantly higher than the upper soil. The dry

Fig. 4. Typing analysis and relative abundance of *Fusarium* in different soil layers. (A) Analysis of the differences in the relative abundance of *Fusarium* in the upper layer soil. (B) Analysis of the differences in the relative abundance of *Fusarium* in the lower layer soil. (C) Typing analysis on genus level in different soil layers.

weight of *M. hupehensis* Rehd. seedlings in the lower soil in May and June increased by 30.59% and 20.31%, respectively, compared with seedlings in the upper soil.

Discussion

The results of this experiment showed that soil microorganisms are mainly concentrated in the upper soil, which is consistent with the results of Zhang's analysis of soil microorganisms in the apple orchard root area of Weibei dry plateau, where microorganisms were mainly concentrated in the 0 to 40-cm soil layer and decreased with increasing depth (Zhang et al., 2020). It is believed that more humus may accumulate in the surface soil, thus the hydrothermal and aeration conditions in the upper layer soil were conducive to the growth and reproduction of microorganisms, whereas the lower soil may have fewer nutrients and poorer ventilation conditions (Liu et al., 2019).

Compared with the sampling on 3 Nov. 2016, the number of microorganisms, especially the fungi in soils, were decreased on 9 Mar. 2017, the bacteria/fungi were increased. The number of fungi decreased with decline in AT and the WC. This was consistent with the previous results that showed that freezing-thawing cycles affected the soil microbial population and community composition (Fan et al., 2012; Meisner et al., 2021). The analysis suggested that the seasonal freezing-thawing cycles can reduce the fluidity of the lipid membrane of soil microbes, causing cell rupture (Fan et al., 2012). Moreover, soil freezing formed a relatively anaerobic environment, which can cause unfavorable conditions for some microbes; air drying can reduce soil WC and reduce microbial activity by reducing microbial respiration, thereby reducing the number of microorganisms (Zhang et al., 2018).

Species diversity usually reflects the difference in community structure at different

taxonomic levels (Caro-Quintero and Ochman, 2015). In addition to the unidentified fungi, the relative abundance of Ascomycota (contains the sexual reproduction of *Fusarium*) reached 54%. At the genus level, *Fusarium* was dominant, which may be related to the ecological function of *Fusarium* in continuous soil. It was indicated that *Fusarium oxysporum* had become a dominant flora with the potential of cucumber continuous cropping (Leoni et al., 2013). The difference in competitiveness between different strains and the ability to adapt to survival and so on will have an impact on the microbial community structure (Philipot et al., 2013). Our results showed that the percentage of *Fusarium* is higher in the upper soil and the overall percentage of *Fusarium* abundance was decreased after ditching and freezing. This could be explained by the fact that *Fusarium* is an aerobic microorganism in the soil. Borrego-Benjumea et al. (2014) also found that the number of *Fusarium* species

Table 3. Alpha diversity index of fungi at different sampling times.

Sample name	Shannon index	Simpson index	Ace index	Chao1 index	Coverage (%)
U1	3.70 ± 0.21 ab	0.06 ± 0.02 bc	581.50 ± 38.48 a	583.20 ± 34.49 a	99.63 ± 0.00 d
D1	3.16 ± 0.20 bcd	0.09 ± 0.02 bc	308.77 ± 8.43 c	310.01 ± 5.06 c	99.86 ± 0.00 ab
U2	3.51 ± 0.11 abc	0.08 ± 0.01 bc	468.37 ± 31.70 b	467.79 ± 34.88 b	99.77 ± 0.00 bcd
D2	3.24 ± 0.16 bcd	0.10 ± 0.02 bc	246.12 ± 25.49 c	255.99 ± 17.69 c	99.92 ± 0.00 a
U3	3.11 ± 0.11 cde	0.11 ± 0.01 bc	508.50 ± 65.05 ab	490.88 ± 37.19 a	99.68 ± 0.00 d
D3	3.14 ± 0.28 cde	0.13 ± 0.03 b	257.41 ± 27.14 c	269.31 ± 51.60 c	99.91 ± 0.00 a
U4	3.61 ± 0.09 abc	0.06 ± 0.00 bc	489.14 ± 12.80 ab	497.79 ± 21.33 ab	99.73 ± 0.00 bcd
D4	2.74 ± 0.21 de	0.21 ± 0.04 a	333.64 ± 5.26 c	334.69 ± 11.26 c	99.82 ± 0.00 abc
U5	4.00 ± 0.04 a	0.04 ± 0.00 c	549.98 ± 7.40 ab	547.08 ± 16.69 ab	99.71 ± 0.00 cd
D5	2.58 ± 0.15 e	0.23 ± 0.04 a	286.36 ± 18.16 c	291.68 ± 15.46 c	99.84 ± 0.00 abc

U1 = The upper layer soil sampled on 3 Nov. 2016. D1 = The lower layer soil sampled on 3 Nov. 2016. U2 = The upper layer soil sampled on 1 Dec. 2016. D2 = The lower layer soil sampled on 1 Dec. 2016. U3 = The upper layer soil sampled on 14 Dec. 2016. D3 = The lower layer soil sampled on 14 Dec. 2016. U4 = The upper layer soil sampled on 16 Jan. 2017. D4 = The lower layer soil sampled on 16 Jan. 2017. U5 = The upper layer soil sampled on 9 Mar. 2017. D5 = The lower layer soil sampled on 9 Mar. 2017.

Data shown are means ± SD. Different lowercase letters in the same column indicate a significant difference at $P < 0.05$ level by Duncan's new multiple range test.

Fig. 5. Redundancy analysis (RDA) on the genus level in different soil layers. (A) RDA on genus level in different soil layers. (B) Spearman correlation heatmap on genus level. The average temperature has an F value of 3.75, an interpretation of 0.118, and a *P* value of 0.01; the maximum temperature has an F value of 3.51, an interpretation of 0.112, and a *P* value of 0.016; the water content has an F value of 2.23, an interpretation of 0.0738, and a *P* value of 0.063.

gradually decreased with the increase of soil depth, which was consistent with the temperature trend, indicating that low temperature imposed an inhibitory effect to a certain extent on the growth of *Fusarium* (Liu et al., 2016).

In addition, the results of this experiment also showed that richness index and diversity index of the soil fungal community were larger in the upper soil than in the lower soil (U>D). According to the analysis, when the

temperature and WC were in the middle state, it is more suitable for the growth and reproduction of most microorganisms, so there was no obvious dominant microorganism. In the upper soil, the conditions were appropriate due to ventilation conditions and nutrients. Microbes had a relatively stable relationship with soil, and the diversity and richness index were higher in the upper soil (Qin et al., 2010; Wang et al., 2017). The Chao1 index and Shannon index initially decreased and then increased with sampling time. The trend, contrary to the change in the Simpson index, showed that in addition to multiple freezing-thawing cycles, the number of microbes can be reduced, and that alternating wet and dry changes can impact soil microbial diversity (Pajares et al., 2018).

Some physicochemical factors, such as pH, temperature, moisture, and inorganic salts in the environment, may also affect the microbial community structure (Kan et al., 2016). RDA analysis showed that the AT, HT, and WC were correlated with the sample flora, and the AT had the greatest influence on the community composition. Extreme temperatures were not suitable for the growth and development of microorganisms (Onwuka, 2018). At the genus level, *Fusarium* levels were significantly correlated with the AT, WC, and HT, so proper reduction of temperature and control of moisture content can effectively inhibit the growth and reproduction of microorganisms.

The different soil layers cause differences in the exchange of microbial community structure (especially in *Fusarium*) within the soil, and thereby are closely linked to soil environmental conditions (Zeng, 2014), which in turn, play an important role in the healthy growth of plants (Yim et al., 2013). It is tempting to speculate that the biomass of *M. hupehensis* Rehd. seedlings grown in the lower soil is increased due to the more suitable soil microecological environment for growth. Therefore, we have reason to believe that opening the ditch before winter and replacing the soil layer after freezing and thawing can alleviate the obstacles to continuous apple cropping to a certain extent.

Conclusion

During pre-winter ditching and freezing-thawing in old apple orchards, the dynamic changes of environmental factors, such as temperature and moisture content, could significantly reduce the number of harmful *Fusarium*, and the lower layer of soil after pre-winter ditching and freezing-thawing could better promote the growth of *M. hupehensis* Rehd. seedlings.

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Table 4. The biomass of *Malus hupehensis* Rehd. seedlings in different soil layers.

Sample name	Ht (cm)	Stem diam (mm)	Fresh wt (g)	Dry wt (g)
May Upper layer soil	8.03 ± 0.12	1.70 ± 0.07	7.22 ± 0.07	3.04 ± 0.04
Lower layer soil	10.14 ± 0.13**	1.85 ± 0.06*	8.62 ± 0.27**	3.97 ± 0.20*
June Upper layer soil	12.22 ± 0.74	1.97 ± 0.05	8.85 ± 0.11	3.84 ± 0.16
Lower layer soil	13.79 ± 0.45*	2.13 ± 0.03**	10.69 ± 0.33**	4.62 ± 0.13**

The *t* test was used to determine the significance of the difference between the two assays.

*Indicates a significant difference of the same area at the 0.05 level.

**Indicates extremely significant difference of the same area at the 0.01 level.

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Supplemental Table 1. Analysis of variance table.

		Sum of square	Degrees of freedom	Mean square error
Sampling time	Bacteria	1,261	1.60	790
	Actinomycetes	4,787	4.00	1,197
	Fungi	8,607	4.00	2,152
	Bacteria/fungi	1,881	4.00	470
Soil level	Bacteria	5,253	1.00	5,254
	Actinomycetes	14,830	1.00	14,830
	Fungi	4,242	4.00	1,060
	Bacteria/fungi	7,799	1.00	7,799

The fungi, actinomycetes and the bacteria/fungi all conform to the spherical test ($P > 0.05$). The analysis of variance table was obtained by using the “Spherical degree adopted” in the one-way analysis of variance and referring to the “Pillai Tracking” data after “Greenhouse-Geisser” correction.