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Running title: ORCHARDS AFFECTED TIBET'S SOIL ECOSYSTEM

How the development of barren land into orchards affects Tibet's soil ecosystem

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ABSTRACT

Fruit production is an important strategy for alleviating poverty on the Tibetan Plateau and leads to the conversion of more natural barren land into orchards. This study aimed to understand how the conversion of barren land to peach (*Prunus persica*) orchards affects soil nutrients, heavy metals and fungal communities in the 0--40 cm profile (at 20--cm intervals). The experiment included three treatments: barren land (BL), peach orchards for 4 years (Y4) and peach orchards for 10 years (Y10). The findings indicated that compared with the results obtained with BL, Y4 reduced the availability of some macronutrients and micronutrients (*i.e.*, N and K, Fe and Mn) due to the exclusive application of chemical fertilizer during the seedling stage. After 6 years of green cultivation management, *e.g.*, the combination of sheep manure and chemical fertilizer with the intercropping of alfalfa (*Medicago sativa Linn*), Y10 effectively improved soil macronutrients but did not enhance the availability of Fe and Mn. Although the investigated heavy metals (*i.e.*, As, Hg, Pb, Cr and Cd) in both the Y4 and Y10 orchard soils were found to pose a low risk to food safety and the soil environment, Hg, Cr and As tended to accumulate in subsoil (20--40 cm). Furthermore, the variations in the fungal community composition and functional groups were mainly driven by the interaction effects of macronutrients, micronutrients and heavy metals, but their independent contribution to specific key functional groups cannot be overlooked. For example, Y4 and Y10 decreased the relative abundance of soil saprotrophic and lichenized fungi mainly due to the loss of micronutrients (*i.e.*, Fe and Mn). However, as a result

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of macronutrient input and dung saprotrophic fungi enrichment, orchard soils promoted the growth of pathogens that play critical roles in fungal cooccurrence networks. These results provide insights linking the variations in soil nutrients and heavy metals to the function of the fungal community during the conversion of barren land to orchards in alpine soil ecosystems. Finally, the findings indicate that supplementation with N or K fertilizer or manure at the seedling stage and fertilizers rich in Fe and Mn throughout the growth period would be beneficial to the balance of soil nutrients. In addition, the risks posed by heavy metal accumulation and fungal pathogen enrichment should be actively prevented.

*Key Word*s: nutrients, heavy metals, fungal community function, cooccurrence network, Tibetan Plateau

INTRODUCTION

As the youngest and highest plateau in the world, the Tibetan Plateau displays weakly developed soils (Qu *et al.*, 2017) and plays a vital role in ecological safety and human health in its surrounding areas (Wu *et al.*, 2016). The risks that intensive agriculture poses to the soil environmental health of fragile plateau ecosystems are being increasingly recognized (Qu *et al.*, 2017; Yang *et al.*, 2020). In recent decades, the expanding human population and their increasing material needs have created constant pressure to convert uncultured land into arable land (Guan *et al.*, 2018). Fruit production has played an important role in providing employment and increasing economic income for township residents (Oldoni *et al.*, 2019), and an increasing number of orchards are being constructed on uncultured lands in Tibet (Wang *et al.*, 2018). Considering the regional food security of Tibet and the ecological functions of grasslands, orchards are usually established on barren land rather than crop land or grassland. However, the impact of converting natural barren land into orchards on the plateau soil environment remains unclear.

There is no doubt that the conversion of barren land to orchards would cause disturbances from tillage and fertilization (Wei *et al.*, 2016), fully change the distribution of solar radiation and precipitation due to the vegetation canopy (Smith and Johnson, 2004), and distinctly alters the root properties (Hazarika *et al.*, 2014; Guan *et al.*, 2018). These man-made and vegetal factors directly or indirectly influence the processes of soil nutrient cycling, heavy metal accumulation and microbial community succession, and changes in these key ecological processes are closely related to the stability and function of the soil ecosystem (Dayamb *et al.*, 2016). Additionally, the issues of soil acidification, fertility decline and heavy metal accumulation caused by the improper use of chemical fertilizers are receiving increasing attention (Zhang *et al.*, 2017). To minimize the environmental risks posed by the fruit industry, the local government vigorously promotes “green cultivation” in mature orchards (Yuan and Chen, 2019), *e.g.*, combining the application of organic fertilizer and chemical fertilizer with the intercropping of green fertilizer crops (Wang *et al.*, 2018; Xue *et al.*, 2018).

However, because orchards at the seeding stage show no profit and generally few manure resources are available for fruit production in Tibet, the application of a chemical fertilizer provided by the local government at the seedling stage is a customary management measure. Therefore, the changes in the abiotic and biological properties of soil from the seedling stage involving the use of a single chemical fertilizer to the mature stage of “green” management remain unclear.

The regulation of soil biodiversity and key processes at the community level are very important strategies for achieving the sustainability of agricultural ecosystems (Lemanceau *et al.*, 2015). Fungi are considered crucial components of belowground biodiversity and play various pivotal functions in the maintenance of ecosystem processes (Hoppe *et al.*, 2016), such as nutrient cycling and belowground carbon transport (Voriskova and Baldrian, 2013) as well as heavy metal transfer and digestion (Lin *et al.*, 2019). These community processes are influenced by facilitative and competitive interactions between functional groups of fungi (Jefwa *et al.*, 2012). Moreover, the interaction patterns among fungal populations with different trophic modes and functions influence the stability of networks and the maintenance of ecological functions (Freilich *et al.*, 2018). The employment of cooccurrence network analysis could provide meaningful information beyond that obtained through sample-level comparisons (Qiu *et al.*, 2019), *e.g.*, the key species in communities can be identified (Barberan *et al.*, 2012). Thus, exploring the response of the soil fungal community cooccurrence network to the conversion of barren land into orchards is conducive to understanding the succession process of microbial community function.

To address the above-described issues, this study assessed the mechanisms of the synergistic changes in soil nutrients, heavy metals and fungal communities due to the conversion of barren land in the middle of the Tibetan Plateau to peach (*Prunus persica*) orchards. We hypothesized that (1) the concentrations of not only nutrients but also heavy metals would be higher in orchard soils than in barren land soil, and these variables would increase with the planting year; (2) the changes in soil nutrients and heavy metals would further lead to variations in the soil fungal community structure and functional groups; and (3) the complexity of the fungal cooccurrence network may be weaker at the seedling stage but stronger at the mature stage.

MATERIALS AND METHODS

Study site and experimental design

This study was conducted at the outdoor peach ecological cultivation demonstration base (E 90°56′79″, N 29°26′05″, and 3608-m elevation) in Lhasa, Tibet, China. The soil is mountain shrubby steppe soil (Wang *et al.*, 2018), which contains 19% clay, 49% silt and 32% sand and is classified as loam according to the USDA system. The Lhasa climate belongs to the plateau temperate semiarid monsoon climate zone. The mean annual temperature and precipitation are 7.4 °C and 426.4 mm, respectively.

Natural barren land (BL) with a history of crop-less cultivation and outdoor peach orchards planted on the BL for four years (Y4) and ten years (Y10) were selected as the study sites in May 2018. The three sample sites (*i.e.*, BL, Y4 and Y10) had the same soil type and altitude and similar slopes and aspects. The total areas of BL, Y4 and Y10 were 0.15, 0.33 and 0.67 ha, respectively. Three plots (15 m × 10 m) which represented replications, were established at each sampling site with > 30 m between the plots. Thus, the experiment included three replications of the following treatments: BL, Y4 and Y10. Peach trees with the Y4 and Y10 treatments were planted in 2014 and 2008, respectively. The Y4 and Y10 planting densities were 1250 plants ha^{-1} (*i.e.*, 2-m plant spacing and 4-m row spacing). During the first four years after tree planting (seedling stage) in the Y4 and Y10 orchards, NPK compound fertilizer (N-P-K, 15-5.6-4.1) was applied once at 900 kg ha^{-1} in early October of each year. In addition, irrigation was performed 6--8 times per year as needed, and weeding was performed 4--5 times per year to prevent weeds from competing with the saplings for nutrients and water. However, in the Y10 orchard, green cultivation management, including the application of sheep manure and intercropping with alfalfa (*Medicago sativa* Linn), was adopted for six consecutive years after the four-year seedling stage. Briefly, sheep manure was applied at 4.8 t ha^{-1} in early October of each year. The same type of NPK compound fertilizer was applied at 450 kg ha^{-1} as the basal fertilizer in early October, and additional fertilizer was applied at 450 kg ha^{-1} in late March of the following year. Both the NPK compound fertilizer and sheep manure were evenly applied in small holes around the trees. Alfalfa was sown around the trees and in alleyways at a sowing rate of 4.0 kg ha^{-1}. From May to September every year, the alfalfa was mowed monthly, and the soil surface was mulched. In addition, according to the soil moisture conditions, irrigation was performed 8-10 times per year.

Soil sampling

Three soil sample sites were sampled using a 4-cm-inner-diameter soil auger in October 2018. In the BL plots, three random soil cores across the 0--40-cm profile (at 20-cm intervals) were sampled. In the Y4 and Y10 orchards, we randomly selected three trees from each plot and sampled six soil cores around trees across the 0--40-cm profile at 20-cm intervals. Soil cores at the same depth from each plot formed one composite sample. The 0-20-cm composite samples of BL, Y4 and Y10 were defined as BLtop, Y4top and Y10top, respectively, and the 20--40-cm composite samples of BL, Y4 and Y10 were defined as BLsub, Y4sub and Y10sub, respectively. After the removal of visible organic debris and sieving to < 2 mm, part of the soil samples was frozen by liquid nitrogen for DNA extraction, another part was stored in a cooled box for the analysis of NO_3^- and NH_4^+, and the remaining part was air-dried for the determination of other physicochemical properties.

Soil physiochemical measurement

The soil pH was measured using a pH meter at a soil:water ratio of 1:2.5 (w/v). The soil organic carbon (SOC) and total N were assayed via dichromate oxidation and the kjeldah method (Bao, 2001), respectively. The total P content was determined using the molybdenum blue method after digestion with $H_2SO_4HClO_4$ at 300 °C for 2 h (Bao, 2001). Alkaline-N (alk-N) was determined by the diffusion-absorption method. NO_3^- and NH_4^+ were extracted with 2 mol L^{-1} KCl solution and analyzed by flow injection analysis (TRAACS 2000, Bran and Luebbe, Norderstedt, Germany). The available P was extracted with 0.5 M $NaHCO_3$ solution (pH 8.5) and quantified using the molybdenum blue method (Bao, 2001). The total K and available K contents were determined by HF-$HClO_4$ digestion and the 1.0 mol L^{-1} CH_3COONH_4 extraction method, respectively (Zheng *et al.*, 2018). The available iron (Fe), manganese (Mn) and zinc (Zn) contents were determined by DTPA extraction and atomic absorption spectrophotometry (Peng *et al.*, 2017). The total chromium (Cr), lead (Pb) and cadmium (Cd) contents were measured by inductively coupled plasma spectrometry. The total arsenic (As) and mercury (Hg) levels were measured by atomic fluorescence spectrometry (Peng *et al.*, 2020).

DNA extraction and sequencing data analysis

Total DNA was extracted from 0.5 g of soil using kits (Mo Bio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The ITS (internal transcribed spacer) regions were amplified using the primers ITS1F (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS2 (5'-TCCTCCGCTTATTGATATGC-3') (Zhang *et al.*, 2015). The PCR conditions for the ITS regions were described by Li et al. (2020), and the PCR products were purified using an AxyPrep DNA Gel Extraction Kit (Axygen Co., Ltd., USA). Sequencing was performed using the Illumina MiSeq PE300 platform at Allwegene Technology Co., Ltd., Beijing, China. The raw reads were uploaded into the NCBI database (PRJNA608235). High-quality sequences were clustered by UCLUST (version 1.2.22) (Edgar, 2010) and assigned by UPARSE (Usearch, version 10.0.240) to operational taxonomic units (OTUs) with a 97% similarity value (Edgar, 2013). Taxonomy assignment was performed using the Silva 128 ribosomal database (http://www.arb-silva.de). The fungal functional groups were assigned using FUNGuild v1.0 (Nguyen *et al.*, 2016).

Construction of fungal functional ecological networks

Based on fungal OTUs with a relative abundance $\geq 0.1\%$, we constructed fungal cooccurrence networks and labeled the guilds and trophic modes for the BL, Y4 and Y10 fungal communities. First, the R package psych (2.0.12 version) was applied to calculate the pairwise correlation coefficient matrix. The matrix of pairwise correlation coefficients between OTUs was constructed using Spearman's correlation method. The pairwise correlations of OTUs with R values higher than 0.7 and P values (adjusted by Benjamini-Hochberg procedure) lower than 0.01 were retained (Benjamini and Hochberg, 1995). Using Gephi 0.9.2, undirected networks were then constructed, and the topological features of the networks were calculated using the Statistics tools in Gephi 0.9.2. The networks were then visualized through the "fruchterman reingold" layout (parameters: area, 10000; gravity, 10; and speed, 1.0).

Statistical analysis

Alpha diversity indicators, including the Chao1, observed species, whole-tree phylogenetic diversity (PD) and Shannon indices, were calculated using the R package Phyloseq 1.12.2. Two-way ANOVA was performed with SPSS (20.0 version) to explore the significant differences in soil physiochemical properties, alpha diversity indicators and fungal functional groups. Multiple comparisons were performed using Duncan's test at a probability level of 0.05 to identify species showing significant differences in abundance among treatments (i.e., biomarkers). To detect differences in the fungal community structure, nonmetric multidimensional scaling (NMDS) and analysis of similarities (ANOSIM) were performed using the vegan package (2.0.3 version). To identify the edaphic factors that are closely related to the fungal community, a redundancy analysis (RDA) was performed using the vegan package (2.0.3 version). The rdacca.hp package (version 0.5-6) was used to calculate the independent contributions of each factor to the explained variation (R-squared) identified by the RDA. In addition, variance partitioning analysis (VPA) was performed to assess the partitions explained by the explanatory variables (classified as macronutrients, micronutrients and heavy metals). Partial least squares path modeling (PLS-PM) was used to illuminate the possible pathways controlling soil abiotic and biological properties. The model was constructed using the "innerplot" function of the "plspm" package. All the analyses in R were performed using R version 3.6.3.

RESULTS

Soil nutrients

The planting year significantly affected the soil pH, macronutrients and micronutrients as well as the heavy metal concentrations (Table I). In addition, most of the investigated parameters were affected by the soil depth and its interaction with the planting year (Table I). The topsoil pH ranged from 7.89 to 8.42 and significantly decreased with the planting year ($P < 0.05$) (Table SI). The SOC and total N of Y10 were significantly higher than those of Y4 and BL ($P < 0.05$), but no significant difference in these variables was found between Y4 and BL ($P > 0.05$; Table SI). The alk-N in Y10top reached 68.10 mg kg^{-1}, and this value was significantly higher than the corresponding levels in BLtop and Y4top by 26.6% and 34.5%, respectively (Table SI). In addition, higher concentrations of total P and available P and lower concentrations of available K were observed in Y4 and Y10 compared with BL. In addition, Fe and Mn were consistently decreased by the Y4 and Y10 treatments (Tables I and SI).

TABLE I

Results of two-way ANOVA (*F* and *P* values) of the soil pH, nutrients and heavy metals

Category	Parameter	Year	Depth	Year × depth

Soil acidity	pH	13.58↓†***	1.13	5.34
Macronutrients	SOC	184.83***	1123.45↓***	11.01**
	N	26.43**	688.66↓***	0.69
	P	140.25↑***	0.03	1.37
	K	66.99↓***	19.26↓***	0.77
	Alkaline-N	55.76***	280.00↓***	52.12***
	NO_3^-	3383.03↓***	1310.49↓***	158.47***
	NH_4^+	162.63↑***	415.21↓***	43.56***
	AP	1093.71↑***	2334.90↓***	348.10***
	AK	3466.92↓***	1886.64↓***	1050.14***
Micronutrients	Fe	1653.83↓***	189.36↓***	260.44***
	Mn	71.50↓***	38.42↓***	16.65***
	Zn	5114.40↑***	2579.21↓***	576.51***
Total heavy metals	As	189.95***	114.45↑***	674.59***
	Hg	370.22***	145.02↑***	22.58***
	Pb	8.79↓**	0.28	3.04**
	Cr	20.14***	196.56↑***	12.69**
	Cd	12.64↓***	1.46	1.73

Note: AP and AK are available phosphorus and available potassium. ** Significant at the 0.01 probability level. *** Significant at the 0.001 probability level. †The arrows after the *F* values show the direction of the significant year or depth effects.

Heavy metal accumulation

The planting year significantly affected the heavy metal concentrations, and the concentrations of As, Hg and Cr increased with increases in the soil depth (Tables I and SI). The concentrations of Pb and Cd in BL and Y4 were significantly higher than those in Y10 ($P < 0.05$; Tables I and SI). However, As, Hg and Cr were clearly accumulated in Y4sub or Y10sub (Table SI). Compared with the results found in BL, the concentration of As (6.08--10.02 mg kg^{-1}) in the orchards showed significantly decreases in topsoil but significant increases in subsoil with the planting year ($P < 0.05$). Y4 markedly increased the concentration of Hg, and this concentration was decreased by Y10 (Table SI). Additionally, the concentrations of Cr in Y4sub and Y10sub were significantly higher than that in BLsub (11.99 mg kg^{-1}) by 28.9% and 31.4%, respectively.

Soil fungal community structure and function

Diversity and composition. Y4top and Y10top exhibit successive increases in the alpha diversity indices of the fungal community, whereas Y4sub had a higher alpha diversity than Y10sub and BLsub (Fig. 1). At the phylum level, the dominant fungi across all soil samples were *Ascomycota* (average relative abundance: 72.6%), *Mortierellomycota* (14.6%) and *Basidiomycota* (3.7%; Fig. S1A). However, obvious differences in the relative abundances of some dominant fungi were detected at different taxonomic levels (Fig. S1). The NMDS plot showed differentiation of the fungal community among BL, Y4 and Y10 as well as between the two soil layers (Fig. 2A), and this finding could also be supported by the ANOSIM results (Table SII). Most of the investigated soil nutrients and heavy metals were correlated with the fungal community composition (Fig. 2B). For example, the concentrations of SOC, NH_4^+ and available P were closely correlated with the fungal community structure of Y4top and Y10top, which was enriched in *Fusarium*, *Gibberella*, *Alternaria*, *Plectosphaerella* and *Aleuria*. However, the fungal community structure of BLtop was correlated with the concentrations of total and available K, available Fe and available Mn. In addition, the concentration of Cr was closely correlated with the fungal communities of Y10sub, which provided a growth advantage to *Cylindrocarpon*, *Dactylonectria* and *Pseudaleuria*.

Fig. 1 Community diversity indices of fungi at 97% sequence similarity. BLtop, Y4top and Y10top represent the topsoil (0-20 cm) of barren land and 4- and 10-year orchards, respectively. BLsub, Y4sub and Y10sub represent the subsoil (20-40 cm) of barren land and 4- and 10-year orchards, respectively.

Fig. 2 Nonmetric multidimensional scaling (NMDS) plot (A) and redundancy analysis (RDA) of fungal community changes with environmental variables (B). The fungi shown in subgraph B are the top 20 genera in terms of average relative abundance. BLtop, Y4top and Y10top represent the topsoil (0-20 cm) of barren land and 4- and 10-year orchards, respectively. BLsub, Y4sub and Y10sub represent the subsoil (20-40 cm) of barren land and 4- and 10-year orchards, respectively.

Trophic modes and functions of the soil fungal community. Overall, the trophic modes and functional groups were affected by the planting year and soil depth (Fig. 3, Tables SIII and SIV). The relative abundance of soil saprotrophic fungi in BLtop was 126 and 16 times higher than those in Y4top and Y10top, respectively. However, Y4sub had a higher abundance of endophytes and mycorrhizae than the other treatments, but preferential accumulation of ectomycorrhiza and fungal parasites was observed in Y10sub (Fig. 3C). In addition, Y10top was enriched in plant saprotrophs, as demonstrated by the finding that the levels of these species in Y10top were 4 times and 5 times higher than those in Y4top and BLtop, respectively. Additionally, Y4 and Y10 were enriched in more pathogens than BL, particularly in topsoil (Fig. 3C). In addition, the RDA plot showed that the fungal functional groups in topsoil and subsoil exhibited obvious differences among BL, Y4 and Y10, and these differences were correlated with some soil nutrients and heavy metals (Fig. S2).

Fig. 3 Trophic modes (A, B) and functional groups (C) of topsoil and subsoil fungi among different treatments. BLtop, Y4top and Y10top represent the topsoil (0-20 cm) of barren land and 4- and 10-year orchards, respectively. BLsub, Y4sub and Y10sub represent the subsoil (20-40 cm) of barren land and 4- and 10-year orchards, respectively.

Contributions of soil nutrients and heavy metals to variations in the fungal composition and function. The independent contributions of soil nutrients and heavy metals to the explained variation in the community composition ranged from 6.96 to 13.97%, and the influence of Fe was prominent (Fig. 4A). In addition, VPA showed that the interaction effects of macronutrients, micronutrients and heavy metals explained 57% of the variation in the fungal community composition (Fig. 4B). Similarly, soil nutrients and heavy metals contributed 4.53--23.28% of the variation in the fungal functional groups, and the highest contributor was Fe (Fig. 4C), whereas the interaction effects of macronutrients, micronutrients and heavy metals explained 59% of the variation in the fungal functional groups (Fig. 4D).

Fig. 4 Independent and integrated effect of the edaphic factor-to-explained variation in the fungal community composition (A, B) and functional groups (C, D). After filtering out some of the variables with strong collinearity, the explanatory variables were divided into three categories: macronutrients (SOC, alk-N, and AP), micronutrients (Fe, Mn, and Zn) and heavy metals (Hg, As, and Cr).

Cascading relationships of fungal functional groups with soil nutrients and heavy metals

The PLS-PM analysis identified the direct and indirect effects of the planting year and soil depth on soil physiochemical properties and fungal functional groups (Fig. 5A). The planting year positively affected the macronutrients (0.520 of the direct effects) and heavy metals (0.296 of the direct effects) but negatively affected the micronutrients (-0.761 of the direct effects). Moreover, micronutrients, macronutrients and heavy metals mediated different groups of saprophytic fungi, lichenized fungi, animal pathogens and fungal parasites. Furthermore, dung saprotrophs positively mediated animal pathogen expression (0.904 of the direct effects). Overall, the planting year negatively affected soil saprotrophs (-0.624 of the total effects) and lichenized fungi (-0.571 of the total effects) but positively affected plant saprotrophs, dung saprotrophs, fungal parasites and animal pathogens (0.401, 0.266, 0.220 and 0.012 of the total effects, respectively; Fig. 5B).

Fig. 5 Cascading relationships of macronutrients, micronutrients, heavy metals and fungal functional groups with the planting year and soil depth. Partial least squares path modeling (PLS-PM) disentangled the major pathways of the influences of the planting year and soil depth on the soil nutrient conditions, heavy metal accumulation and functional groups. The red and blue arrows indicate positive and negative flows of causality, respectively. The numbers on the arrows indicate the significant standardized path coefficients (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). R2 indicates the variance of the dependent variable explained by the model.

Fungal functional cooccurrence networks

The complexity and connectivity of the cooccurrence networks of BL were lower than those of Y4 and Y10 (Fig. 6). This finding could be confirmed by their topological properties: the numbers of nodes, links, and communities and modularity of the fungal networks of Y4 and Y10 were higher than those of BL (Table II). However, the Y4 network had larger numbers of nodes and communities, larger network diameters, increased average clustering coefficients and greater path lengths than the Y10 network (Table II), which indicated that the Y4 network was more complex than the Y10 network. Furthermore, although the network patterns of BL, Y4 and Y10 were dominated by saprotrophs, symbiotrophs and pathotrophs, some differences in the trophic mode and functional group compositions were identified (Fig. 6, Table SV). For example, the proportion of symbiotrophs gradually decreased after planting, whereas the proportion of saprotrophs first increased and then decreased (Table SV). Additionally, lichenized fungi played important roles in the BL network, but animal pathogens and fungal parasites played more important roles in the networks of Y4 and Y10 than in the network of BL (Fig. S3).

Fig. 6 Networks of fungal communities in soils from barren land (BL; A), orchard at the seedling stage (Y4; B) and orchard at the mature stage (Y10; C). The sizes of the nodes represent the relative abundances. Nodes with different trophic modes are labeled with different colors. The node guilds are also labeled as predicted by FUNGuild: EN (endophyte), AM (arbuscular mycorrhizal), EC (ectomycorrhizal), DS (dung saprotroph), US (undefined saprotroph), LS (leaf saprotroph), SS (soil saprotroph), AP (animal pathogen), FP (fungal parasite), and - (undefined). The red lines indicate positive connections between the nodes, and the blue lines indicate negative connections between the nodes.

TABLE II

Global network properties of soil fungi under different treatments[†]

Network indices [‡]	BL	Y4	Y10
Total nodes	111	139	127
Total links	476	546	618
Positive links	274	305	309
Negative links	202	241	309
Network diameter	12	13	11
Average degree	8.595	7.856	9.732
Average clustering coefficient	0.526	0.542	0.537
Modularity	2.526	3.426	9.636
Number of communities	25	31	29
Average path length	3.513	4.358	3.571

Note: [†]BL, Y4 and Y10 represent barren land and orchard soil in which trees have been planted for 4 years and for 10 years, respectively. [‡]The network indices refer to the fungal networks across the 0-40-cm soil profile at each sampling site.

DISCUSSION

Soil macronutrients and micronutrients

Most of the available nutrients in Y4 (*i.e.*, alk-N and available K, Fe and Mn) were significantly lower than those in BL (Table SI), which indicated that the exclusive use of mineral fertilizers decreased soil fertility and may not sustainably supply the nutrients needed by fast-growing trees (Garcia-Orenes *et al.*, 2016). Organic fertilizer application is usually beneficial for improving soil fertility by synchronizing plant needs with nutrient release (Sciubba *et al.*, 2013). In addition, mulch alfalfa can decrease runoff, reduce N leaching, increase water infiltration and improve the organic matter content and quality of soil (Zhong *et al.*, 2018). Hence, the highest contents of SOC, total N and P in both the topsoil and subsoil were recorded with the Y10 treatment, which included 6 years of green cultivation management; in addition, the contents of all investigated available macronutrients were higher in Y10 than in Y4 (Table SI). However, the available Fe and Mn in Y10 were not effectively improved compared with those in Y4, although the application of sheep manure to soil could add a certain amount of Fe and Mn (Abu-Zahra *et al.*, 2010). Furthermore, the PLS-PM analysis showed that the planting year positively affected macronutrients but resulted in a loss of micronutrients (*i.e.*, Fe and Mn; Fig. 5A). In fact, soil micronutrient deficiency is a major constraint for the successful cultivation of fruit trees (Alvarez-Fernandez *et al.*, 2011). For example, Fe deficiency usually induces leaf chlorosis, which leads to reductions in electron transport and photosynthetic efficiency as well as severe fruit yield losses and decreased C fixation (Alvarez-Fernandez *et al.*, 2011). Mn, as a key metallic redox catalyst, governs the behavior of other trace elements in soils (Wang *et al.*, 2016). Hence, the intake of soil micronutrients by fruit trees may cause an imbalance in soil nutrients, which could be alleviated by the application of fertilizers with additional appropriate amounts of Fe and Mn.

It is also worth noting that the conversion of barren land to orchards leads to surface soil acidification. Ten years after the planting of peach trees, the soil pH was decreased by 0.53 units (Table SI). This decrease was attributed to the long-term application of amino-based compound fertilizer combined with alfalfa planting. Ammonium-based fertilizers strongly acidify soils via nitrification (Bolan *et al.*, 2003). In addition, alfalfa is a legume that can fix atmospheric N_2 and form NH_4^+ by nitrogenase in root nodules. This process is accompanied by the absorption of excess cations (particularly K^+) and the net release of H^+ (Goulding, 2016). In addition, the organic acids secreted by roots during nutrient absorption and growth may accelerate localized acidification

Accumulation of heavy metals

Heavy metal accumulation at toxic concentrations in agricultural soils can harm human health and result in long-term ecological damage (Tóth *et al.*, 2016). According to the Chinese national standards of soil contamination of agricultural land (GB 15618-2018), the five investigated heavy metals (*i.e.*, As, Hg, Pb, Cr, and Cd) were all found at levels below the risk screening values (Lu *et al.*, 2019). Additionally, according to the standards in the Finnish legislation for contaminated soil, which have been applied in an international context for agricultural soils (Tóth *et al.*, 2016), the concentrations of these heavy metals were lower than the guideline values (if the concentrations exceed these levels, the area presents ecological or health risks). In general, heavy metals usually enter and accumulate in orchard soils with increasing tilling times and application rates of fertilizers (Zhang *et al.*, 2017; Gil *et al.*, 2018). In our study, the amount and frequency of fertilizer application increased during the mature stage, but the concentrations of the investigated heavy metals did not increase after the seedling stage (Y4; Table I). These results may benefit from green cultivation techniques, that is, the combined application of organic and chemical fertilizers with the intercropping of alfalfa (Abu-Zahra *et al.*, 2010; Zhang *et al.*, 2017). However, we should not neglect the issue that As, Hg and Cr tended to accumulate in the subsoil of orchards, particularly Hg in Y4sub (Table SI), which was mainly attributed to the long-term application of phosphate fertilizer and irrigation (Wang *et al.*, 2016). Hence, the concentrations of heavy metals in orchard soil need to be monitored regularly over the long term.

Variations in the fungal community diversity and structure

The fungal diversity of soil is positively related to ecosystem functioning (Hoppe *et al.*, 2016). Numerous studies have compared the response of the fungal community diversity to the application of organic and chemical fertilizers (Ling *et al.*, 2016; Hu *et al.*, 2017; Lori *et al.*, 2017), but little information about the effects of the conversion of barren land to orchards and shifts in the fertilization strategy on the fungal community diversity is available. In orchard soil ecosystems, the inputs of nutrients, water and plant residues to soil increase with the growth of fruit trees, which could provide a greater diversity of nutrients and habitats for more fungal species (Voriskova and Baldrian, 2013). Additionally, soils receiving manure are generally characterized by microbial guilds related to the degradation of various complex organic compounds (Pereg *et al.*, 2018). Thus, the fungal diversity of orchard soils, particularly topsoil, was higher than that of BL (Fig. 1). This result basically agreed with the findings of previous studies, which reported the positive impact of organic fertilizer on fungal diversity (Hartmann *et al.*, 2015; Hu *et al.*, 2017). However, the observed species and alpha diversity indices of Y10sub were consistently lower than those of Y4sub (Fig. 1). This discrepancy was mainly caused by an insufficient nutrient supply in Y4sub, which promoted fungal growth to obtain more nutrients for the plants through the root system (Carballar-Hernandez *et al.*, 2017). This discrepancy is also likely the cause of the enrichment of widespread root-associated soil microorganisms, such as mycorrhizal fungi in Y4sub (Fig. 3C), which could rely on vigorous extraradical mycelial networks to help the host plant take up more mineral nutrients (Huang *et al.*, 2019).

Obvious differences in the structure of the fungal community were found among BL, Y4 and Y10 as well as between topsoil and subsoil (Fig. 2A, Table SII). This was consistent with previous studies that suggested that the land use type, planting year and physical location are important driving factors for fungal community transformations (Deakin *et al.*, 2018; Lin *et al.*, 2019; Wang *et al.*, 2020). Furthermore, we found that the variations in the fungal community composition were driven mainly by the complex interaction effects of macronutrients, micronutrients and heavy metals (Fig. 4A, B). Soil macronutrient elements such as C, N and P play important roles in the fungal community composition (Fig. 4A, B), as has been widely recognized by related studies (Deakin *et al.*, 2018; Cui *et al.*, 2019; Li *et al.*, 2020). In addition, manure addition can introduce exogenous microorganisms into soil and/or facilitate the growth of specific fungi (Yang *et al.*, 2019), which also contributes to a shift in the soil fungal community. In addition, the fact that micronutrients decline cannot be ignored because these trace elements are necessary for the growth and metabolism of most soil microorganisms (Xue *et al.*, 2018). For instance, in our study, Fe, which can serve as an electron source for iron-oxidizing microorganisms (Weber *et al.*, 2006), was identified as the most prominent contributor to the variation in the fungal community composition (Fig. 4A). Moreover, the accumulation of heavy metals (*i.e.*, Hg, Cr and As) also affected the fungal community composition

(Fig. 4A, B), which may be explained by soil fungi adapting to or resisting heavy metal pollution by driving changes in the community composition and function (Lin *et al.*, 2019).

Variations in fungal community functions

Different types of land use and agricultural practices generally lead to microbial community structure changes, which may not impact community function due to high functional redundancy (Garcia-Orenes *et al.*, 2016). However, the conversion of BL into orchards significantly influenced soil nutrients and heavy metals, and their separate and interaction effects then drive changes in the fungal community function, as supported by the VPA and PLS-PM results (Figs. 4D and 5A). Hence, obvious differentiation among the fungal trophic modes and functional groups were found among BL, Y4 and Y10 (Fig. 3, Tables SIII and SIV). The increase in plant saprotrophic fungi in Y10 due to macronutrient input (Figs. 3C and 5A) may have led to more rapid nutrient cycling than that found in BL and Y4 (Meyling and Eilenberg, 2007). In addition, the loss of micronutrients (*i.e.*, Fe and Mn) in orchard soils led to a lower abundance of lichenized fungi and soil saprotrophic fungi (Figs. 3C and 5A). These findings have been obtained because Fe and Mn are essential cofactors of a wide range of cellular processes and because competition in fungus-plant interactions located in the rhizosphere may result in Fe/Mn shortages, which would likely limit the growth of lichenized and saprotrophic fungi (Paul *et al.*, 2009; Lopez-Berges *et al.*, 2013). The former can thrive in harsh environments and play important roles in covering terrestrial surfaces (Wang *et al.*, 2020), and the latter plays a crucial role in the decomposition of complex organic matter and the conversion of nutrients (Wu *et al.*, 2019). These results hint that orchard soil, due to vegetation cover, plant residues and nutrient inputs, might decrease the microbial transformation of soil native organic matter but may accelerate the decomposition of exogenous labile organic matter (Guan *et al.*, 2018; Luo *et al.*, 2020).

Another notable phenomenon was that Y4top and Y10top provided growth advantages for plant and/or animal pathogens (Fig. 3C). Overall, these pathogens enriched in orchard soil were closely related to the detected increases in SOC and the available nitrogen and phosphorus levels (Fig. 2B), which is consistent with previous studies (Veresoglou *et al.*, 2013; Liu *et al.*, 2021). This finding indicates that improved soil nutrients due to the conversion of BL into orchards likely promoted the growth of pathogenic fungi. Furthermore, the PLS-PM analysis revealed that animal pathogens were mainly and positively affected by dung saprotrophs, which were directly affected by macronutrients but negatively affected by plant saprotrophs and heavy metals (Fig. 5A). Because plant residues and manure may be the host of fungal pathogens, leaving pruned branches and rotten fruit on the soil surface and pouring sheep manure into soil may be the primary cause of the enrichment of pathogenic fungi (Garcia-Orenes *et al.*, 2016). Nevertheless, the combined application of organic and chemical fertilizers could reduce the abundance of pathogens and possibly lessen the probability of plant disease (Ding *et al.*, 2017). In addition, the enriched plant saprotrophs and the downward movement of heavy metals may limit animal pathogen reproduction (Table I, Figs. 3C and 6A). Thus, either plant pathogens or animal pathogens were relatively low in Y10sub (Fig. 3C). Another interesting finding was that the planting year and soil depth positively affected fungal parasites and were directly affected by heavy metals (Figs. 3C and 5). This finding may be attributed to the complex interaction effects between fungi and other microorganisms (*e.g.*, nematodes) in the root zone of orchard soil under the accumulation of heavy metals (Gine *et al.*, 2013), but the relevant hard evidence needs to be further explored.

Fungal functional cooccurrence network patterns and key functional groups

Microbial community network analysis can be used to explore the laws of species co-occurrence and reveal taxa that have a greater impact on community functions (Zheng *et al.*, 2018). The networks of orchard soil exhibit greater complexity than those of BL likely as a result of stronger root-fungal interactions, and more fungal hyphal connections occur in orchard soil (Ling *et al.*, 2016). Regardless of the specific community functions, the presence of more interrelated species likely has positive implications for soil biological fertility (Ling *et al.*, 2016). Therefore, the complex fungal network of orchard soils (both Y4 and Y10) may means higher community stability and stronger resistance to disturbance than that observed in BL (Scheffer *et al.*, 2012). Furthermore, nodes with large hub values and/or large betweenness centrality values are analogous to key microbial species in communities (Montoya *et al.*, 2006), because nodes with large hub values are highly related to numerous species in internal modules, and nodes with large betweenness centrality values are strongly linked to several modules. According to this network theory, the animal and plant pathogens in the networks of Y4 and Y10 (particularly Y4) played more important roles not only within models but also between communities than those in the BL network (Fig. 6, Table SV). Because fungus-based food webs recover slowly after disturbances (Orwin *et al.*, 2006), the potential issues of tree health and the soil environment caused by fungal pathogens need to be considered, and proactive measures should be taken when necessary.

CONCLUSIONS

The present study revealed that the conversion of BL in the alpine system to orchards eventually improved most soil macronutrients (*i.e.*, C, N and P) but consumed many micronutrients (*i.e.*, Fe and Mn). In addition, although the investigated heavy metals (*i.e.*, As, Hg, Pb, Cr and Cd) in orchard soil pose a low risk to food safety and the environment, Hg, Cr and As tended to accumulate in subsoil. The interaction effects of macronutrients, micronutrients and heavy metals were identified as the main drivers of the variation in the fungal community composition and functional groups, but the independent contributions of Fe and Mn, as well as Hg, Cr and As, cannot be overlooked. Furthermore, orchard establishment may enhance fungal community stability due to increases in the fungal diversity and the complexity of cooccurrence networks. However, orchard soil provided unsuitable habitats for soil saprotrophic and lichenized fungi due to the loss of micronutrients but provided suitable habitats for fungal pathogens with important roles in the cooccurrence networks due to the input of macronutrients through the long-term application of fertilizer and/or manure. This study provides important insights linking the variation in soil nutrients and heavy metals to fungal community function and improves our understanding of the mechanisms through which the conversion of BL to orchards causes changes in the ecological functions of alpine soil. For the sustainable use of soil, supplementation with N and K fertilizer or manure at the seedling stage and the application of fertilizers rich in Fe and Mn at both the seedling and mature stages are recommended to maintain the balance of soil nutrients. In addition, the risks caused by the accumulation of heavy metals and the enrichment of fungal pathogens deserve close attention and prevention.

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