Drivers of soil microbial metabolic limitation changes along a vegetation restoration gradient on the Loess Plateau, China

Lei Deng, Changhui Peng, Chunbo Huang, Kaibo Wang, Qiuyu Liu, Yulin Liu, Xuying Hai, Zhourping Shangguan

Abstract

Soil extracellular enzymatic activity (EEA) stoichiometry could reflect the biogeochemical equilibrium between the metabolic requirements of microbial communities and environmental nutrients availability. However, the drivers of soil microbial metabolic limitation (SMML) changes remain poorly understood following vegetation restoration. We compared sites along a vegetation restoration chronosequence over a 30-year period on the Loess Plateau, China, and measured the potential activities of two C-acquiring enzymes (β-1,4-glucosidase (BG) and β-1,4-N-acetylglucosaminidase (NAG)), two N-acquiring enzymes (β-1,4-N-acetylglucosaminidase (NAG) and β-leucine aminopeptidase (LAP)), and one organic-P-acquiring enzyme (alkaline phosphatase (AP)), to quantify and compare the variations in metabolic limitations for soil microorganisms using EEA stoichiometry. The results showed constant microbial P limitation, but not N limitation, and an open downward “unimodal” trend in microbial C limitation; however, the microbial P limitation displayed exactly the opposite trend during vegetation restoration. Restoration age and properties of plant, soil, and microorganisms contributed to 82.9% of microbial C limitation and 84.6% of microbial P limitation, with soil presenting the highest relative effects of 76.1% and 59.6% on microbial C and P limitations, respectively. Plant productivity and species diversity decreased microbial C limitation owing to increasing plant C inputs, but increased microbial P limitation owing to plant nutrients competition with soil microorganisms. When the fungi:bacteria ratio in the soil increased, the SMML decreased. Vegetation restoration increased the soil nutrients content and reduced SMML, and a decrease in the soil water content increased microbial P limitation. Thus, the effects of long-term vegetation restoration on SMML were the result of combined influences of plants, soil, and microorganisms.

1. Introduction

Soil microorganisms (e.g., fungi and bacteria) occupy a central position in global biogeochemical cycles by mineralizing dead organic matter (i.e., cellulose, hemicellulose, and lignin) to its constitutive elements (Sinsabaugh et al., 2008; Moorhead et al., 2013). Most of the free-living microbial communities are limited by energy and nutrients (e.g., C, N, and P) (Kuzyakov and Xu, 2013; Moorhead et al., 2016). Soil extracellular enzymes produced by microorganisms are the proximate agents of organic matter decomposition (Sinsabaugh et al., 2008; Peng and Wang, 2016), and can deconstruct plant and microbial cell walls, depolymerize macromolecules, and ultimately produce soluble substrates for microbial assimilation (Burns and Dick, 2002). Changes in the relative abundances of extracellular enzymatic activity (EEA) during C, N, and P cycling are the result of cellular metabolism (Peng and Wang, 2016), and reflect the biogeochemical equilibrium between the metabolic and nutrient requirements of the microbial community and environmental nutrients availability (Moorhead et al., 2013; Fanin et al., 2016). Thus, soil EEA stoichiometry can be a useful tool for identifying the microbial resource allocation during acquisition of energy and nutrients (e.g., C, N, and P) (Fanin et al., 2016).

Soil EEA stoichiometry method employs EEA ratios and stoichiometric invariance to predict nutrient availability in the environment.
and metabolic activity of microorganisms (Sinsabaugh et al., 2009; Moorhead et al., 2013, 2016). According to Sinsabaugh et al. (2009), EEA stoichiometry represents an intersection of Ecological Stoichiometry Theory (EST) with Metabolic Theory of Ecology (MTE), and can improve our understanding of the effects of energy and nutrients on microbial metabolism (Sinsabaugh et al., 2012). Ecological studies have quantified the activities of extracellular enzymes that produce assimilated products from the principal C, N, and P sources, such as β-1,4-glucosidase (BG), β-α-cellulobiose (CBH), β-1,4-N-acetylglucosaminidase (NAG), -l-leucine aminopeptidase (LAP), and alkaline phosphatase (AP) (Sinsabaugh et al., 2009; Fanin et al., 2016). These enzymes activities can serve as proxy indicators of microbial resource allocation for C, N, and P acquisition (Schimmel and Weintraub, 2003; Fanin et al., 2016), because the activities of other related enzymes are typically lower than and correlated with these enzymes (Sinsabaugh, 1994). Within this context, Sinsabaugh et al. (2008) suggested that the relative activities between BG/(NAG+LAP) and BG/AP reflect nutrients (N and P) limitation. Moorhead et al. (2016) proposed calculating the “vector length” and “vector angle” created by connecting a line between the plot origin and a point represented by the C:P vs. C:N ratios to acquire the enzyme activities of a microbial community. This would quantify the relative investments in C vs. nutrient acquisition (vector length) or P vs. N acquisition (vector angle). For example, Cui et al. (2019a) used the method to determine that microorganisms in the grasslands shifted from P to N limitation with the increasing precipitation gradient. Therefore, application of EEA stoichiometry method can facilitate a better understanding of microbial metabolic limitation in soils.

Soil microorganisms regulate the response of EEA to soil resource availability to fulfill their nutrients needs (Sinsabaugh et al., 2009; Burns et al., 2013; Fanin et al., 2016); i.e., when soil nutrients availability changes, the EEA modifies enzymatic allocations to acquire energy (C) and nutrients (N, P) from the soil. Soil nutrients are co-determined by plant, soil, and microbial composition in most of the ecosystems (Zhang et al., 2011; Büemann et al., 2012; Deng et al., 2013, 2018). For example, changes in litter inputs and quality may modify the soil C content and P and N stoichiometry (Cross and Schlesinger, 2001; Córdova et al., 2018), which may alter microbial composition and activities as C and nutrients requirements change during decomposition (Kaiser et al., 2014; Li et al., 2019). Bacteria and fungi in soil significantly differ in their growth forms, life strategies, competitiveness, and resources utilizing abilities (Li et al., 2019). Previous studies have reported that fungi have lower nutrients requirements than bacteria and exhibit a high C-use efficiency (CUE) in poor quality substrates (Keiblinger et al., 2016; McGuire et al., 2010), whereas bacteria require higher N and P concentrations for maintaining their high turnover rates (Elser et al., 2003). Hence, the variations in soil nutrients can influence soil microbial composition (Schneider et al., 2012; Kaiser et al., 2014). Soil microbial communities further adjust their physiological metabolism, as reflected by different degrees of exoenzyme expression (Sinsabaugh et al., 2009). In general, EEA is related to the quality of available organic matter and nutrients demands of the microbial biomass (Sinsabaugh et al., 2015). In addition, soil physicochemical properties, such as pH, water, and clay content, have direct and indirect effects on soil microbial metabolism by altering the concentrations of available substrate and soil C, N, and P stoichiometry (Peng and Wang, 2016; Romanowicz et al., 2016). However, the impact of plant, soil, and microbial composition on soil microbial metabolic limitation (SMML) has received little attention, and their relative contribution and mechanisms through which they affect SMML have been rarely investigated.

Vegetation restoration in degraded landscapes is an effective measure to improve soil quality in many locations worldwide (Fanin et al., 2016; Deng et al., 2016; Deng and Shangguan, 2017; Zhu et al., 2018, 2019). However, the soil quality changes slowly and stabilizes within a short time, whereas the soil biological characteristics, such as soil EEA, are sensitive to changes in soil internal and external environments (Burns et al., 2013; Ren et al., 2016a). Thus, soil EEA is often used as an indicator in the evaluation of soil recovery conditions in different ecosystems (Lino et al., 2016; Ren et al., 2016a; Raiesi and Salek-Gilani, 2018). Previous studies have implied that vegetation restoration creates a suitable growth environment for soil microorganisms and further leads to an increase in microbial biomass and enzymatic activity (Ren et al., 2016a; Raiesi and Salek-Gilani, 2018; Zhang et al., 2018). Although vegetation restoration is known to improve soil quality (Zhu et al., 2018; Deng et al., 2019) and increase microbial activity (Ren et al., 2016a; Raiesi and Salek-Gilani, 2018; Zhang et al., 2018), its effect on SMML remains poorly understood. Some indirect observations of soil microbial C, N, and P stoichiometry have shown that soil microbial activity may be limited by P deficiency as a consequence of increased microbial biomass and diversity following afforestation (Ren et al., 2016b). However, the dynamics and mechanisms through which SMML operates during vegetation restoration are still unclear, because the SMML is not determined by the absolute amount of EEA, but can be indicated by the relative ratios of enzymes (Moorhead et al., 2013, 2016).

Loess Plateau, located in Northwest China, comprises some of the most vulnerable ecological systems in the world owing to the arid or semi-arid condition of majority of this area (Jiang et al., 2018; Deng et al., 2019). To prevent soil erosion and land degradation, “Grain for Green” Program has been implemented in this region (Deng et al., 2013, 2019), which provides an ideal platform to study SMML following vegetation restoration. However, most of the previous studies had only focused on soil microbial composition (Ren et al., 2016b; Xiao et al., 2017), soil enzymes activities (Wang et al., 2012; Ren et al., 2016a), or soil nutrients (Zhang et al., 2018; Zhu et al., 2018), with little information available on microbial metabolic limitations and the combined effects of plants, soil, and microorganisms following vegetation restoration. Therefore, the objectives of the present study were to: 1) explore the dynamics of SMML following vegetation restoration; 2) determine the relative contributions of plants, soil, and microorganisms on SMML during vegetation restoration; 3) identify the potential mechanisms by which plants, soil, and microorganisms affect SMML during vegetation restoration. The results obtained could be beneficial for soil nutrients identification and ecosystem stabilization in critical ecological zones.

2. Methods and materials

2.1. Study area

This study was conducted in the Zhifanggou catchment, located in Ansai County in the middle of the Loess Plateau, China (36°46′28″–36°46′42″N, 109°13′46″–109°16′03″E; 1010–1400 m a.s.l., 8.27 km²). The study area is characterized by a semi-arid climate and a deeply incised hilly-gully Loess landscape. Slopes vary between 0° and 65°. The Zhifanggou watershed is a popular case study area for comprehensive soil and water conservation studies in the Loess Plateau. The mean annual temperature is 9.1 °C (from 1970 to 2010), average frost-free period is 157 days, duration of sunshine per year is 2415 h, and mean annual precipitation is 503 mm (from 1970 to 2010, with 70% rainfall between July and September). The soil is mainly highly erodible Huangmei soil (Calcaric Cambisols, FAO), and the Loess-derived soils are fertile but extremely susceptible to erosion. The Zhifanggou catchment has been an experimental site of the Institute of Soil and Water Conservation, Chinese Academy of Science (CAS), since 1973. Widespread vegetation restoration has been undertaken over the past decade to prevent soil degradation, and complete land use history and vegetation restoration information is available. The main herbaceous plants are Stipa bungeana, Bothriochloa ischaemum, Artemisia sacrorum, Potentilla acuclus, Stipa grandis, Androsace erecta, Heteropappus altaicus, Lespedeza bicolor, Artemisia capitillaries, and
Artemisia frigida, of which S. hungeana is the most widely distributed through natural restoration on abandoned farmlands. In addition, shrubs such as Rosa xanthina, Spiraea pubescens, Amorpha fruticosa, and Hippophae rhamnoides can be found in gullies. The main replanted trees in the study area are Robinia pseudoacacia, Caragana microphylla, Pinus tabuliformis, and Platycladus orientalis.

2.2. Experimental design

The “space” for “time” method was used to study vegetation restoration. This chronosequence scheme monitors plants and soils under similar climatic conditions following the sequence of vegetation development (Bhojvaid and Timmer, 1998), and has been widely adopted in vegetation restoration research on the Loess Plateau (Xiao et al., 2017; Deng et al., 2018; Zhu et al., 2018). An abandoned farmland chronosequence in the catchment was selected for study after the history of the sites was determined by consulting the Soil and Water Conservation Experiment Station, CAS. Six restoration ages (RAs) of 3, 8, 13, 18, 23, and 30 years were selected. In August 2015, six sites were established for each RA to conduct field survey. The sites were separated by a distance of 0.3–1 km. At each site, a plot of 20 × 20 m was established. In each plot, three quadrats (1 m × 1 m) were randomly selected. In total, six plots with 18 quadrats for each RA were selected. In each quadrant, the plant community coverage (PCC, %), plant community height (PCH, cm), and plant species richness (PSR, number of species per m²) were observed as described in our previous study (Deng et al., 2013). The morphological traits of the herbage in each RA are listed in Table 1. The plots were all located near the top of Loess mounds, and there were slight differences among the sites in terms of altitude (1185–1340 m), aspect, gradient (18°–30°), and previous land use history. For comparison, six sites on maize farmland (CK) were selected. Before the farmland was abandoned, maize (Zea mays) had been widely seeded. The average amount of fertilizer applied was 225–300 kg ha⁻¹, sheep manure with 1.7–2.4 kg N ha⁻¹ base fertilizer in April and 300–450 kg ha⁻¹ urea with 140.1–210.2 kg N ha⁻¹ base fertilizer in June as topdressing. A total of 42 plots were sampled in this field survey.

2.3. Plant and soil samples collection

In each quadrant, all the aboveground biomass (AB) of the green plants and litter biomass (LB) above the ground were harvested (Deng et al., 2013). To measure belowground biomass (BB), the soil was sampled three times in each quadrat using a 9-cm-diameter root auger. Most of the roots were collected within the soil samples and then isolated using a 2-mm sieve. The remaining fine roots collected from the soil samples were isolated by spreading the samples in shallow trays, overfilling the trays with water, and allowing the outflow from the trays to pass through a 0.5-mm mesh sieve. No attempt was made to distinguish between living and dead roots, and all the roots were oven-dried at 65 °C and weighed to within 0.01 g. Soil samples were collected at the center points in the quadrats of each plot. The ground litter layer was first removed before soil sampling. Soil sampling in the 0–20-cm layer was achieved using a soil drilling sampler (9-cm inner diameter). The collected soils from each plot were mixed together to make one sample. All the samples were sieved through a 2-mm screen, and roots and other debris were removed. Then the samples were divided into two groups: one group was air-dried and stored at room temperature for the determination of soil physical and chemical properties, while the other group was stored at 4 °C in a portable refrigerator during field sampling. Once in the laboratory, the samples were stored at 4 °C until analyses. Soils were analyzed for enzymes activities immediately after the samples reached the laboratory (Xu et al., 2015). The soil bulk density (BD) (g cm⁻³) was measured (in triplicates) using a soil bulk sampler with a stainless steel cutting ring (5-cm diameter and 5-cm height) at points adjacent to the soil sampling quadrats. The original volume of each soil core and its dry mass after oven-drying at 105 °C were measured.

2.4. Measurement of soil physical and chemical properties

The soil organic C (SOC) content was determined using the Walkley-Black method (Nelson and Sommers, 1996). The total N (TN) content was determined by the Kjeldahl method (Bremner, 1996). The available nitrogen (AN) was determined by continuous alkalii-hydrolyzed reduction diffusing method (Cornfield, 1966). The total P (TP) and available P (AP) were determined by Olsen method (Olsen and Sommers, 1982). For the determination of dissolved organic C (DOC) content, the field-fresh soil samples (equivalent to 15 g of oven-dried soil) were extracted with 60 ml of 0.5 M K₂SO₄ (soil:solution ratio, 1:4) for 1 h. After centrifuging at 4000 rpm for 25 min, the supernatant was filtered through a 0.45-μm membrane filter and measured in a TOC analyzer (TOC-VPCH, Shimadzu, Japan) (Li et al., 2015). Soil pH was determined at a soil-water ratio of 1:2.5 (PHSJ-4A pH meter, Zhangqiu Meihua International Trading Co., China). Soil BD (g cm⁻³) was determined from the volume of the core sampler and oven-dried weight of the bulk soil samples. Soil water content (SW, %) was determined gravimetrically by drying the samples at 105 °C. Microbial biomass C, N, and P content (MBC, MBN, and MBP, respectively) were analyzed by chloroform fumigation-extraction method (Brookes et al., 1982, 1985; Vance et al., 1987). Soil basal respiration (BR) was estimated via CO₂ evolution at 25.8 °C in samples incubated for 14 days, and adjusted to 50% of the field water holding capacity (Jenkinson and Powlson, 1976). The soil microbial community structure (fungi (F) and bacteria (B)) was investigated by determining the relative abundances of the phospholipid fatty acids (PLFAs) of the different microbial groups in the soils (Bardgett et al., 1996). Soil microbial CUE was ascertained as the ratio of MBC to DOC (Insam et al., 1989).

2.5. Enzymatic activity assays

The potential activities of two C-acquiring enzymes (BG and CBH), two N-acquiring enzymes (NAG and LAP), and one organic-P-acquiring enzyme (AP) were measured using modified versions of standard fluorometric techniques (Siaya-Cork et al., 2002; Sinsabaugh et al., 2008, 2009; Steinweg et al., 2012, Table 2). The enzymatic activity was expressed as nanomoles of substrate released per hour per gram of soil organic matter (SOM) (nmol g OM⁻¹ h⁻¹) (Sinsabaugh et al., 2008). These five enzymes were selected owing to two reasons (Peng and Wang, 2016). First, the potential activities of these five enzymes are frequently linked to rates of microbial metabolism and biogeochemical processes, and are generally used as indicators of microbial nutrient demand (Schimel and Weintraub, 2003; Moorhead and Sinsabaugh, 2006; Sinsabaugh et al., 2009). Second, these five enzymes have also been used as indicators of C-, N-, and P-acquiring enzymes in previous studies on EEA stoichiometry at the global scale (Sinsabaugh et al., 2008) and regional scale (Waring et al., 2013; Peng and Wang, 2016; Cui et al., 2019a).

2.6. Quantification of microbial metabolic limitation

Sinsabaugh et al. (2008) suggested that the relative activities of C- vs. P-acquiring enzymes and C- vs. N-acquiring enzymes could reflect soil microbial nutrient limitation (N or P). Moorhead et al. (2016) proposed calculating the length and angle of the vector created by connecting a line between the plot origin and a point represented by the coordinates of C- vs. P-acquiring enzymes and C- vs. N-acquiring enzymes. The length of the vector quantifies the relative C limitation and the angle quantifies the relative P vs. N limitation (Appendix Fig. S1). In this study, microbial metabolic limitation was quantified by calculating the vector lengths and angles of enzymatic activity for all data based on untransformed proportional activities. The vector length, representing
<table>
<thead>
<tr>
<th>Restoration age (years since farmland abandonment)</th>
<th>Altitude (m)</th>
<th>Slope (°)</th>
<th>Aspect</th>
<th>Soil type</th>
<th>PCH (cm)</th>
<th>PCC (%)</th>
<th>PSR (species m(^{-2}))</th>
<th>AGB (g m(^{-2}))</th>
<th>BGB (g m(^{-2}))</th>
<th>LB (g m(^{-2}))</th>
<th>pH</th>
<th>BD (g cm(^{-3}))</th>
<th>SW (%)</th>
<th>Main species</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL (0 yr)</td>
<td>1222–1274</td>
<td>21–22</td>
<td>Sunny</td>
<td>LS</td>
<td>66.0 ± 5.7 a</td>
<td>8.8 ± 0.5 cd</td>
<td>4.5 ± 0.6 d</td>
<td>119.3 ± 22.6 bc</td>
<td>58.3 ± 4.2 de</td>
<td>29.0 ± 3.4 d</td>
<td>8.62 ± 0.08 a</td>
<td>1.21 ± 0.08 ab</td>
<td>6.17 ± 0.40 b</td>
<td>Maize, Artemisia capillaris, Heteropappus altaicus, Salvia collina</td>
</tr>
<tr>
<td>3 yr</td>
<td>1268–1300</td>
<td>20–23</td>
<td>Sunny</td>
<td>LS</td>
<td>41.6 ± 6.4 c</td>
<td>18.7 ± 2.4 bc</td>
<td>7.0 ± 0.5 c</td>
<td>73.7 ± 6.6 c</td>
<td>98.0 ± 5.6 de</td>
<td>118.5 ± 12.2 c</td>
<td>8.61 ± 0.08 a</td>
<td>1.23 ± 0.03 ab</td>
<td>6.45 ± 0.04 ab</td>
<td>Artemisia capillaris, Artemisia sacrorum, Potentilla bifida, Bothriochloa ischaemum, Lespedeza davurica</td>
</tr>
<tr>
<td>8 yr</td>
<td>1260–1312</td>
<td>18–25</td>
<td>Sunny</td>
<td>LS</td>
<td>68.2 ± 4.9 a</td>
<td>23.5 ± 4.7 b</td>
<td>8.5 ± 0.6 bc</td>
<td>91.0 ± 10.0 c</td>
<td>158.5 ± 27.5 d</td>
<td>167.2 ± 26.1 c</td>
<td>8.57 ± 0.04 a</td>
<td>1.18 ± 0.02 abc</td>
<td>6.08 ± 0.11 b</td>
<td>Bothriochloa ischaemum, Artemisia sacrorum, Lespedeza davurica</td>
</tr>
<tr>
<td>13 yr</td>
<td>1185–1261</td>
<td>20–28</td>
<td>Sunny</td>
<td>LS</td>
<td>60.1 ± 2.0 ab</td>
<td>40.7 ± 8.8 a</td>
<td>9.0 ± 0.7 b</td>
<td>100.3 ± 15.5 c</td>
<td>322.3 ± 62.3 c</td>
<td>130.8 ± 27.1 bc</td>
<td>8.55 ± 0.08 a</td>
<td>1.17 ± 0.03 abc</td>
<td>5.51 ± 0.14 c</td>
<td>Bothriochloa ischaemum, Artemisia sacrorum, Lespedeza davurica</td>
</tr>
<tr>
<td>18 yr</td>
<td>1246–1294</td>
<td>23–30</td>
<td>Sunny</td>
<td>LS</td>
<td>52.0 ± 1.7 bc</td>
<td>49.2 ± 5.1 a</td>
<td>10.0 ± 0.4 ab</td>
<td>177.0 ± 26.3 ab</td>
<td>486.3 ± 99.4 b</td>
<td>209.7 ± 29.3 b</td>
<td>8.51 ± 0.09 a</td>
<td>1.15 ± 0.05 bc</td>
<td>5.05 ± 0.31 c</td>
<td>Artemisia sacrorum, Stipa bungeana, Lespedeza davurica</td>
</tr>
<tr>
<td>23 yr</td>
<td>1256–1287</td>
<td>22–25</td>
<td>Sunny</td>
<td>LS</td>
<td>69.0 ± 4.7 a</td>
<td>54.2 ± 5.1 a</td>
<td>11.2 ± 0.6 a</td>
<td>159.0 ± 9.8 a</td>
<td>722.7 ± 48.8 a</td>
<td>281.8 ± 21.1 a</td>
<td>8.58 ± 0.03 a</td>
<td>1.18 ± 0.07 c</td>
<td>4.37 ± 0.26 d</td>
<td>Stipa bungeana, Artemisia sacrorum, Lespedeza davurica</td>
</tr>
<tr>
<td>30 yr</td>
<td>1228–1340</td>
<td>22–28</td>
<td>Sunny</td>
<td>LS</td>
<td>8.62 ± 0.08 a</td>
<td>1.21 ± 0.08 ab</td>
<td>6.17 ± 0.40 b</td>
<td>Maize</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: FL, farmland; GL, grassland; LS, loess soil; PCH, plant community height; PCC, plant community coverage; PSR, plant species richness; AGB, aboveground biomass; BGB, belowground biomass; LB, litter biomass; pH, soil pH; BD, bulk density; SW, soil water. Different lowercase letters below the data indicate statistically significant differences among different restoration ages (p < 0.05). Data indicates Means ± Standard error (SE). n = 6. “–” indicates no data.
Table 2
Ecoenzymes included in this study.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Abbreviation</th>
<th>EC</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-1, 4-Glucosidase</td>
<td>BG</td>
<td>3.2.1.21</td>
<td>Cellulose degradation: hydrolyses glucose from cellulobiose</td>
</tr>
<tr>
<td>β-1, 4-Cellobioseidase</td>
<td>CBH</td>
<td>3.2.1.91</td>
<td>Cellulose degradation: hydrolyses cellulobiose dimers from non-reducing ends of cellulose molecules</td>
</tr>
<tr>
<td>β-1, 4-N-Acetylglucosaminidase</td>
<td>NAG</td>
<td>3.2.1.14</td>
<td>Chitin and peptido glycogen degradation: hydrolyses glucosamine from chitinobiose</td>
</tr>
<tr>
<td>s-Leucine aminopeptidase</td>
<td>LAP</td>
<td>3.4.11.1</td>
<td>Proteolysis: hydrolyses leucine and other hydrophobic amino acids from the N terminus of polypeptides</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>AP</td>
<td>3.1.3.1</td>
<td>Hydrolyses phosphate from phosphonooxadiazides and phospholipids</td>
</tr>
</tbody>
</table>

Note: * Enzyme commission classification (Sinsabaugh et al., 2008, 2009).

C limitation, was calculated as the square root of the sum of $x^2$ and $y^2$ (Eq. (1)):

$$\text{Length} = \text{SQR}T(x^2 + y^2)$$

where $x$ represents the relative activity of C- vs. P-acquiring enzymes (i.e., $(BG + CBH)/(BG + CBH + AP)$) and $y$ denotes the relative activity of C- vs. N-acquiring enzymes (i.e., $(BG + CBH)/(BG + CBH + NAG + LAP)$) (Moorhead et al., 2016).

Vector angle (Eq. (2)), representing N/P limitation, was calculated as the arctangent of the line extending from the plot origin to point $(x, y)$:

$$\text{Angle}^{(*)} = \text{DEGREES}(\text{ATAN}2(x,y))$$

Based on enzymatic stoichiometry theory (EST), soil microbial C limitation is considered to increase as the vector lengths increase and soil microbial N/P limitation is represented by vector angles as follows: angles $< 45^\circ$ denote N limitation and angles $> 45^\circ$ represent P limitation (Moorhead et al., 2013). The rationale for interpreting the relative limitations of C, N, and P to soil microbial activity using relative activities of C-, N-, and P-acquiring enzymes is based on stoichiometric and metabolic theories of ecological systems (Sterner and Elser, 2002, Appendix Fig. S1).

2.7. Statistical analysis

The statistical distributions of enzymes activities and vector characteristics (length and angle) were examined with normal probability plots, and both skewness and kurtosis were calculated for each metric. Analysis of variance (ANOVA) was used to determine the differences in enzymes activities among various RAs when homogeneity of variance was confirmed and significance was observed at $p < 0.05$. Tukey’s test was used for multiple comparisons using the multcomp package in the R v.3.3.2 software (R Development Core Team, 2014). Pearson’s test was employed to determine the correlations among all variables assessed in the study. Linear regressions were used to examine the relationships among vector characteristics and RAs. Multivariable linear regression analysis (MLRA) was conducted using SPSS ver. 17.0 (SPSS Inc., Chicago, IL, USA) to quantify the contributions of relevant factors to the variations in vector characteristics. A backward stepwise regression model was applied to detect the key factors of RAs as well as plant, soil, and microbial properties that affected soil microbial C and P limitations (Appendix Table S1). Partial least squares path modeling (PLS-PM) was then applied to further identify the possible pathways by which various attributes control C and P limitations. The model was constructed using “plspm” package in R software. The boxplot and linear regression figures were generated using “ggplot2” package in R software.

3. Results

3.1. Variations in soil enzymatic activity and enzymatic stoichiometry

Vegetation restoration had a significant impact on soil enzymatic activity since farmland abandonment (Fig. 1). Along with vegetation restoration, C-acquiring enzymes activities (BG + CBH) significantly increased in the first 23 years after farmland abandonment, and then decreased overall (Fig. 1a). The N-acquiring enzymes (NAG + LAP) and P-acquiring enzymes (AP) displayed the same trend, with an initial decrease, followed by an increase and a decrease over the whole period of vegetation restoration (Fig. 1b and c). The lowest soil enzymatic activity occurred after 8 years and the peak soil enzymatic activity was noted after 23 years (Fig. 1b and c). The ratios of C:N (BG + CBH)/(NAG + LAP), C:P ((BG + CBH)/AP), and N:P ((NAG + LAP)/AP) increased in the initial stages (< 13 years) and then decreased (Fig. 1d–f).

3.2. Variations in SMML

The vector length indicated the microbial C limitation. It was found that microbial C limitation increased in the first 13 years of vegetation restoration and then decreased, displaying an open downward “unimodal” trend over the whole period of vegetation restoration (Figs. 2a and 3a). In contrast, the vector angle indicated that microbial N/P limitation decreased in the first 13 years of vegetation restoration and then increased, displaying an open downward “unimodal” trend over the whole period of vegetation restoration (Figs. 2b and 3b). Fig. 4 shows the relationship between soil microbial C and N/P limitations, with most of the study sites listed in the upper left area (Fig. 4) and vector angles of $> 45^\circ$ (Figs. 2 and 4), suggesting that soil microbial activity was P-limited rather than N-limited in the study area.

3.3. Factors affecting SMML

The contributions of RA as well as plant, soil, and microbial properties could explain 82.9% of the microbial C limitation and 84.6% of the microbial P limitation (Fig. 5), with soil properties presenting the maximum effect. The relative effects of RA as well as plant, soil, and microbial properties on soil microbial C limitation were 1.0%, 6.7%, 76.1%, and 16.2%, respectively, while those on microbial P limitation were 2.7%, 16.1%, 59.5%, and 21.7%, respectively (Fig. 5). Backward stepwise regression models indicated that RA, LB, soil nutrients (SOC, TN, TP, C/P, DOC, AN, and AP), and soil microbial properties (F, B, and F/B) had strong effects on microbial C limitation; while RA, plant biomass (AB and LB), PCH, PCC, and PSR, soil nutrients (SOC, C/P, AN, and AP), soil water, and soil microbial properties (F, F/B, and MBP) had strong effects on microbial P limitation (Appendix Table S1).

4. Discussion

4.1. Effects of vegetation restoration on soil enzymatic activity

Soil enzymatic activity plays an important role in C cycling, nutrient dynamics, and soil structure and function, and is closely linked to the primary productivity of an ecosystem (Raiesi and Salek-Gilani, 2018). As sensitive indicators of the influence of land use changes or vegetation restoration on soil (Wang et al, 2012; Raiesi and Salek-Gilani, 2018), variations in plant cover, SOC, soil structure, and environmental conditions (e.g., pH and water) after farmland abandonment could change the soil microbial composition and enzymatic activity since farmland abandonment.
activity (Raiesi, 2012). In the present study, the C-acquiring enzymes (BG + CBH) activities after the re-establishment of natural species were higher than those in the farmland (Fig. 1), similar to the findings of Garcia et al. (1997) in a semi-arid Mediterranean region; however, the enzymatic activities decreased in the later stage during vegetation restoration (Fig. 1). Furthermore, N-acquiring enzymes (NAG + LAP) and P-acquiring enzymes (AP) showed the same trend of an initial decrease (< 8 years), followed by an increase (8–23 years), and then a decrease (> 23 years), which was similar to the "bimodal" trend over the whole period of vegetation restoration (Fig. 1b and c). In the early stage of vegetation restoration (ESVR), although the biomass of plants increased (Table 1), it did not change significantly, when compared with that in the farmland stage, which might be owing to the continuous application of organic fertilizer to farmland for agricultural production (Deng et al., 2013). As the RA increased, the plant biomass significantly improved and competed with the microorganisms for soil resources (Bardgett et al., 2005). Besides, fungal activity also significantly increased, requiring more nutrients to meet its own growth requirements (Fanin et al., 2016). As a result, microorganisms began to secrete more N- and P-acquiring enzymes into the soil. Owing to the continuous inputs from plants, the N and P nutrients in the soil significantly increased until the late stage of vegetation restoration (LSVR) (> 23 years) (Table 1), resulting in sufficient availability of these nutrients for the growth and metabolism of microorganisms. Therefore, the microorganisms did not secrete more enzymes to obtain additional nutrients, because the efficiency of enzymes is negatively correlated with nutrients availability.

Fig. 1. Variations of soil enzymatic activity and enzymatic stoichiometry at different vegetation restoration ages (RAs) since farmland abandonment. Note: (a), C-acquiring enzymes: BG + CBH; (b), N-acquiring enzymes: NAG + LAP, (c), P-acquiring enzymes AP; (d), (BG + CBH)/(NAG + LAP); (e), (BG + CBH)/AP; (f), (NAG + LAP)/AP. In the box plot, the black boxes show 25% and 75% quantiles, the whiskers are min-max values, the yellow small square is the mean value, the horizontal line is the median, and the yellow error bar is standard deviation (SD). Different lowercase letters below the data indicate statistically significant differences among the different RAs (p < 0.05). n = 6. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
4.2. Effects of vegetation restoration on SMML

The results of the present study revealed that microbial C limitation (vector length) increased in the first 13 years since vegetation restoration and then decreased (Figs. 2a and 3a), indicating an increase in the relative energy requirement (Fanin et al., 2016) with respect to nutrients in the LSVR. Although plant C inputs (e.g., litter and roots) increased following vegetation restoration (Table 1, Ruiz-Jaén and Aide, 2005; Deng et al., 2013), the SOC did not change in the early stages (Table 3). The increase in fungal activity was accompanied by an

(Allison et al., 2007).

Fig. 2. Variations of soil microbial C limitation (a) and microbial N/P limitation (b) at different vegetation restoration ages (RAs) since farmland abandonment. Note: In the box plot, the black boxes show the 25th and 75th quantiles, the whiskers are min-max values, the yellow small square is the mean value, the horizontal line is the median, and the yellow error bar is the standard deviation (SD). Soil microbial C limitation is represented by vector lengths; microbial C limitation increases as the values increase. Soil microbial N/P limitation is represented by vector angles: angles < 45° represent N limitation and angles > 45° represent P limitation. Different lowercase letters below the data indicate statistically significant differences among the different RAs (p < 0.05), n = 6. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. Linear regression relationships of soil microbial C limitation (a) and microbial P limitation with vegetation restoration, and the linear regression relationship between soil microbial C limitation and microbial P limitation (c). Note: red lines indicate linear model fits, and gray lines are the 95% confidence intervals of these linear models. The significance level is p < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
increased demand for energy, but the microbial CUE decreased to a certain extent (Table 3), leading to a gradual increase in microbial C limitation. In the LSVR (> 13 years), SOC and DOC significantly increased (Table 3) and the microbial CUE also significantly improved (Table 3), resulting in a decrease in microbial C limitation. These results indicated that the microbial C limitation had the same trend as that of the soil C-acquiring enzymes activity (Figs. 1 and 2), further confirming that soil microbial communities could adjust their physiological metabolism, reflecting different degrees of exo-enzyme expression, to adapt to changes in the external environment (Sinsabaugh et al., 2009).

In addition, the present study also showed that soil microbial activity was always limited by P rather than N throughout the entire process of vegetation restoration on the Loess Plateau (Figs. 2 and 4). The main reason for this may be the lack of available P in the local soil regions, which is an important factor leading to microbial P limitation (Wang et al., 2009; Liu et al., 2013), and can cause strong competition between soil microbial and vegetation communities during vegetation development (Cui et al., 2019b). The soil P content significantly increased in the LSVR (Table 3), possibly owing to the presence of legumes (*Lespedeza davurica*), resulting in relatively higher soil N sequestration than soil P sequestration; therefore, the microbial P limitation was higher in the LSVR. In the present study, microbial P limitation decreased in the first 13 years and then increased over the remaining period of vegetation restoration (Figs. 2b and 3b). Although soil bacterial activity slightly decreased along with a decrease in BR (Table 3), the soil C content remained unchanged since farmland abandonment (Table 3). This finding indicated that the soil P content was sufficient for soil microbial growth, resulting in a decrease in soil microbial P limitation in the ESR (< 13 years). In the LSVR (> 13 years), although the soil P content significantly increased (Table 3), the soil microbial biomass significantly improved (Table 3) along with obvious increase in the accumulation of plant biomass, when compared with those in the ESR (Table 1), which increased the competition for P. The soil microbial activity resulted in increases in nutrients requirements to sustain high growth rates (Sterner and Elser, 2002), thus leading to an increase in microbial P limitation. Previous studies have also reported P limitation in soils in the LSVR on the Loess Plateau (Jiao et al., 2013; Ren et al., 2016a, b), especially in areas where soil erosion had accelerated the loss of soil P (Feng et al., 2013).

4.3. Factors affecting SMML during vegetation restoration

Although vegetation restoration affected SMML (Cui et al., 2019a, b), the RA only played a relatively minor role in plant, soil, and microbial systems (PSMS). It was found that RA, plant, soil, and microbial properties co-contributed to 82.9% and 84.6% of the microbial C and P limitations, respectively, whereas the RA alone made a relative contribution of 1% and 2.7% to the microbial C and P limitations, respectively (Fig. 5). This finding indicated that RA was not a key factor affecting soil microbial activity, similar to that reported by Harris (2003), who also suggested that RA only had an indirect effect on soil microbial activity. In general, changes to plant and soil properties affect soil microbial structure and function (Giai and Boerner, 2007). Besides, RA, as an individual factor, is largely irrelevant to microbial C and P limitations, because it is not a driving variable, but just a category in the data analysis.

The present study found that soil properties had the strongest effect on SMML among the PSMS (Fig. 5). Soil provides nutrients and water to maintain soil microbial growth (Ruiz-Jaén and Aide, 2005; Liu et al., 2013). As a result of vegetation restoration, the soil C pool is improved owing to increased plant C inputs, such as litter and root turnover (Zhang et al., 2011; Deng and Shangguan, 2017; Deng et al., 2018). Previous studies have reported that plant composition and litter quality have strong effects on EEA and soil microbial activity (Weand et al., 2010), as well as an indirect impact on microbial nutrients limitation by affecting microbial composition and soil nutrients supply (Cui et al., 2019b). Besides, the SOC content in different vegetation restoration stages also strongly affects the supply of C available to microorganisms (Xiao et al., 2017; Zhang et al., 2018). In the present study, SOC and C/P were found to have the strongest effect on soil microbial C and P limitations (Fig. 5). It has been reported that vegetation restoration results in more C sources to feed soil microorganisms (Zhu et al., 2010), subsequently reducing C restrictions for microbial communities (Cui et al., 2019b). In the current study, the soil C/P ratio exhibited a strong negative correlation with microbial C limitation (Appendix Table S2), confirming that soil P had negative effects on microbial C limitation (Appendix Table S2). Previous studies have indicated that ecological stoichiometric ratios can predict ecosystem stability and limitation during biogeochemical cycling (Ollinger et al., 2002). Cui et al. (2019b) found that microbial C and P limitations in grasslands were strongly affected by soil C:N, C:P, and N:P ratios, which can be attributed to soil C:N:P stoichiometry that can effectively affect the structure and activity of microbial communities (Sinsabaugh et al., 2009; Taylor and Townsend, 2010; Ren et al., 2016b). Soil microbial activity requires maintenance of elemental stoichiometric balance, with homeostasis in the nutrients supply environment under various types of vegetation (Cui et al., 2018). In the present study, soil water was noted to be an important factor affecting microbial P limitation (Fig. 5). Bell et al. (2008) found that soil water is a crucial factor affecting litter decomposition and microbial activity; therefore, the low moisture content in the LSVR could have affected microbial metabolism and nutrients acquisition, leading to a higher microbial P limitation in the LSVR (Fig. 2).

Soil microbial structure and composition were observed to be the second most important factors affecting microbial metabolic limitation (Fig. 5). According to the growth rate hypothesis (Sterner and Elser, 2002), the growth of soil microbial activity is associated with a large demand for P for the synthesis of ribosomal RNA, resulting in MBP having a strong direct effect on microbial P limitation (Fig. 5). Previous studies have also reported that the relative abundance of soil microorganisms is very sensitive to changes in the C:N:P ratio in soil (Turner and Haygarth, 2005; van der Heijden et al., 2008). The fundamental differences in the growth forms and life strategies of bacteria and fungi (Strickland and Rousk, 2010) can influence C:N:P demands depending on the relative proportion of each microbial group in the community.
Effects of restoration age (RA), and plant, soil, and microbial properties on (a) soil microbial C limitation and (b) soil microbial P limitation, and the relative effect of RA, and plant, soil, and microbial properties on soil microbial C and P limitations (small figure in the upper right corner). Note: AB, aboveground biomass; BB, belowground biomass; LB, litter biomass; PCH, plant community height; PCC, plant community coverage; PSR, plant species richness; SOC, soil organic carbon; TN, total nitrogen; TP, total phosphorus; DOC, dissolved organic carbon; AN, available nitrogen; AP, available phosphorus; pH, soil pH; BD, bulk density; SW, soil water; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen; MBP, microbial biomass phosphorus; F, fungi; B, bacteria; F/B, the ratio of fungi to bacteria; BR, basic respiration; CUE, microbial carbon use efficiency. Plants: AB, BB, LB, PCH and PCR; Soil properties: SOC, TN, TP, SOC:TN, SOC:TP, TN:TP, DOC, AN, AP, pH, BD, SW; Microbial properties: MBC, MBN, MBP, F, B, F/B, BR and CUE. Soil microbial C limitation is represented by vector lengths and microbial C limitation increases as the values increase. Soil microbial P limitation is represented by vector angles (angles > 45° in Fig. 3).

Table 3
Variations of soil C, N, and P, and C, N, and P stoichiometry, soil microbial biomass C, N, and P and microbial composition properties at different vegetation restoration ages (RAs) since farmland abandonment. Note: (a), SOC, soil organic carbon content; (b), TN, total nitrogen content; (c), TP, total phosphorus content; (d), C/N, the ratio of SOC to TN; (e), C/P, the ratio of SOC to TP; (f), N/P, the ratio of TN to TP; (g) DOC, dissolved organic carbon content; (h) AN, available nitrogen; (i) AP, available phosphorus. MBC, microbial biomass carbon; (b), MBN, microbial biomass nitrogen; (c); MBP, microbial biomass phosphorus; (d), F, fungi; (e), B, bacteria; (f), F/B; (g), BR, basic respiration; (h), CUE, microbial carbon use efficiency. Different lowercase letters below the box in the same line indicate statistically significant differences among the different RAs (p < 0.05). The values are the mean ± standard deviation (SD). n = 6.

<table>
<thead>
<tr>
<th>Restoration age (years since farmland abandonment)</th>
<th>FL (0 yr)</th>
<th>3 yr</th>
<th>8 yr</th>
<th>13 yr</th>
<th>18 yr</th>
<th>23 yr</th>
<th>30 yr</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOC (g kg(^{-1}))</td>
<td>3.41 ± 0.40c</td>
<td>3.05 ± 0.19c</td>
<td>2.98 ± 0.71c</td>
<td>3.28 ± 0.38c</td>
<td>3.55 ± 0.26c</td>
<td>5.07 ± 0.88b</td>
<td>5.89 ± 0.15a</td>
</tr>
<tr>
<td>TN (g kg(^{-1}))</td>
<td>0.40 ± 0.04cd</td>
<td>0.33 ± 0.13d</td>
<td>0.34 ± 0.06d</td>
<td>0.36 ± 0.02cd</td>
<td>0.44 ± 0.04bc</td>
<td>0.51 ± 0.01b</td>
<td>0.65 ± 0.03a</td>
</tr>
<tr>
<td>TP (g kg(^{-1}))</td>
<td>0.50 ± 0.04bc</td>
<td>0.48 ± 0.02c</td>
<td>0.49 ± 0.02bc</td>
<td>0.50 ± 0.01bc</td>
<td>0.51 ± 0.03ab</td>
<td>0.51 ± 0.03ab</td>
<td>0.53 ± 0.01a</td>
</tr>
<tr>
<td>C/N</td>
<td>8.55 ± 0.84ab</td>
<td>8.46 ± 1.01ab</td>
<td>8.85 ± 0.98ab</td>
<td>9.05 ± 3.10ab</td>
<td>8.16 ± 0.38b</td>
<td>10.02 ± 0.27a</td>
<td>9.02 ± 0.52ab</td>
</tr>
<tr>
<td>C/P</td>
<td>6.78 ± 0.67b</td>
<td>6.32 ± 0.26b</td>
<td>6.10 ± 1.40b</td>
<td>6.52 ± 0.79b</td>
<td>6.95 ± 0.67b</td>
<td>9.92 ± 0.36a</td>
<td>11.03 ± 0.13a</td>
</tr>
<tr>
<td>N/P</td>
<td>0.79 ± 0.06b</td>
<td>0.69 ± 0.06b</td>
<td>0.70 ± 0.17b</td>
<td>0.74 ± 0.17b</td>
<td>0.85 ± 0.09b</td>
<td>1.24 ± 0.02a</td>
<td>1.22 ± 0.06a</td>
</tr>
<tr>
<td>DOC (g kg(^{-1}))</td>
<td>0.28 ± 0.03c</td>
<td>0.28 ± 0.02c</td>
<td>0.29 ± 0.02c</td>
<td>0.37 ± 0.09b</td>
<td>0.45 ± 0.05a</td>
<td>0.49 ± 0.08a</td>
<td>0.49 ± 0.04a</td>
</tr>
<tr>
<td>AN (mg kg(^{-1}))</td>
<td>33.34 ± 3.89b</td>
<td>30.27 ± 1.83b</td>
<td>32.23 ± 4.28b</td>
<td>32.44 ± 2.56b</td>
<td>35.99 ± 1.82b</td>
<td>45.57 ± 2.87a</td>
<td>512.26 ± 6.75a</td>
</tr>
<tr>
<td>AP (mg kg(^{-1}))</td>
<td>32.09 ± 6.13bc</td>
<td>25.27 ± 1.79bc</td>
<td>25.27 ± 3.63bc</td>
<td>25.53 ± 3.68bc</td>
<td>33.24 ± 2.76b</td>
<td>49.86 ± 6.67a</td>
<td>57.92 ± 5.93a</td>
</tr>
<tr>
<td>MBC (mg kg(^{-1}))</td>
<td>54.38 ± 13.60c</td>
<td>69.70 ± 18.73c</td>
<td>56.23 ± 6.18c</td>
<td>61.03 ± 19.79c</td>
<td>59.29 ± 10.40c</td>
<td>111.13 ± 35.52c</td>
<td>145.52 ± 19.60a</td>
</tr>
<tr>
<td>MBN (mg kg(^{-1}))</td>
<td>16.20 ± 2.29c</td>
<td>12.39 ± 4.85cd</td>
<td>10.32 ± 0.78d</td>
<td>13.90 ± 2.85cd</td>
<td>16.06 ± 1.71c</td>
<td>22.07 ± 4.70b</td>
<td>30.86 ± 2.85a</td>
</tr>
<tr>
<td>MBB (mg kg(^{-1}))</td>
<td>2.16 ± 0.21ab</td>
<td>1.70 ± 0.22c</td>
<td>1.63 ± 0.23c</td>
<td>1.96 ± 0.33bc</td>
<td>1.89 ± 0.36bc</td>
<td>2.25 ± 0.41ab</td>
<td>2.46 ± 0.28a</td>
</tr>
<tr>
<td>F (nmol g(^{-1}) soil)</td>
<td>0.31 ± 0.07d</td>
<td>0.26 ± 0.02b</td>
<td>0.32 ± 0.03c</td>
<td>0.34 ± 0.04cd</td>
<td>0.42 ± 0.11bc</td>
<td>0.47 ± 0.13ab</td>
<td>0.54 ± 0.08a</td>
</tr>
<tr>
<td>B (nmol g(^{-1}) soil)</td>
<td>8.32 ± 1.80a</td>
<td>7.49 ± 1.86a</td>
<td>6.71 ± 1.72a</td>
<td>7.61 ± 1.22a</td>
<td>7.20 ± 1.48a</td>
<td>7.45 ± 2.03a</td>
<td>7.30 ± 0.97a</td>
</tr>
<tr>
<td>F/B</td>
<td>0.04 ± 0.01cd</td>
<td>0.04 ± 0.01cd</td>
<td>0.05 ± 0.01cd</td>
<td>0.04 ± 0.01cd</td>
<td>0.06 ± 0.01bc</td>
<td>0.07 ± 0.03ab</td>
<td>0.08 ± 0.01a</td>
</tr>
<tr>
<td>BR (mg g(^{-1}) d(^{-1}))</td>
<td>53.45 ± 6.16a</td>
<td>41.19 ± 5.42ab</td>
<td>32.50 ± 10.14b</td>
<td>41.66 ± 14.22ab</td>
<td>51.18 ± 11.56a</td>
<td>42.49 ± 10.44b</td>
<td>42.64 ± 7.51ab</td>
</tr>
<tr>
<td>CUE (mg g(^{-1}))</td>
<td>0.20 ± 0.06bc</td>
<td>0.25 ± 0.08ab</td>
<td>0.19 ± 0.03bc</td>
<td>0.17 ± 0.07bc</td>
<td>0.13 ± 0.03c</td>
<td>0.23 ± 0.06ab</td>
<td>0.30 ± 0.05a</td>
</tr>
</tbody>
</table>

n = 42.
(Güsewell and Gessner, 2009), The physiological metabolism of these microorganisms can be further altered by the secretion of extracellular enzymes to acquire nutrients that satisfy their own growth (Sinsabaugh et al., 2009; Schneider et al., 2012). For example, the EEA may be higher in soils with large fungal populations owing to the longer hyphal length (Burke et al., 2011; Kivlin and Treseder, 2014). However, whether the production of some enzymes is differentially controlled by specific groups of soil microorganisms is still unclear, and is central to understanding the role of community structure in ecosystem functioning (Fanin et al., 2016), requiring further research.

4.4. Potential mechanism of the effect of vegetation restoration on SMML

The present study showed that plant, soil, and microbial processes had a combined effect on microbial metabolism, and further affected microbial metabolic limitation (Fig. 6). In the vegetation restoration process, three major mechanisms affect soil microbial C limitation. First, vegetation restoration improves the soil pool (Deng et al., 2018) and soil nutrients content (Zhang et al., 2011; Deng et al., 2013; Jiao et al., 2013) owing to long-term plant C inputs. This provides sufficient C sources to fulfill microbial growth and reproduction, and decreases microbial C limitation in the long term. The decomposition of SOC is an important microbial-mediated process (Demisie et al., 2014). Soil microorganisms produce extracellular enzymes that catalyze the

Fig. 6. The conceptual model of the effect of restoration age (RA), plant, soil, and microbial properties on microbial C and P limitations following vegetation restoration were determined by partial least squares path modeling. Note: Plants: AB, aboveground biomass; LB, litter biomass; PCH, plant community height; PCC, plant community coverage; PSR, plant species richness. Soil: SOC, soil organic carbon; TN, total nitrogen; TP, total phosphorus; DOC, dissolved organic carbon; AN, available nitrogen; AP, available phosphorus; SW, soil water; Soil microbes: MBP, microbial biomass phosphorus; F, fungi; B, bacteria; F/B, the ratio of fungi to bacteria. The red single arrow lines indicate the direct effects of independent variables on soil microbial C and P limitation in the backward stepwise model (Appendix Table S1), and the path coefficient on the lines. The dashed blue line indicates the indirect effects on soil microbial C limitation. The black double arrow line indicates negative correlation. The thicker the single arrow line the greater the effect on soil microbial C and P limitation. “+” indicates positive effect, and “−” indicates negative effect. “r” is the Pearson correlation coefficient. RE is the residual error of the path modeling. N = 42. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
mineralization of SOC and convert nutrients from organic into inorganic forms (Demisse et al., 2014). When the available C sources and nutrients are limited, soil microorganisms increase the secretion of extracellular enzymes; thus, the SOC and its fractions have an important effect on soil microbial composition and enzymatic activity (Xu et al., 2015; Yu et al., 2017). The significant effects of soil SOC, TN, and TP on microbial metabolic limitation identified in the present study confirmed the importance of soil properties and nutrients in maintaining EEA (Fig. 6). Second, vegetation restoration supplies plant litter to soil, directly providing nutrition (C sources) for soil microbial growth and further decreasing microbial C limitation. Plant litter can stimulate the microbial production of enzymes involved in C and nutrients cycling (Tian and Shi, 2014). Non-substrates and simple compounds in litter, such as glucose, may also enhance the microbial production of BG (Hernández and Hobbie, 2010). As a readily available molecule, glucose can be directly assimilated into microorganisms, thus stimulating microbial growth (Kwabia et al., 2003). Third, vegetation restoration increases microbial biomass and changes soil microbial structure, leading to a greater demand for labile C and hence an increased microbial C limitation (Fig. 6). In the present study, the soil fungal activity significantly increased with long-term vegetation restoration (Table 3), which confirmed that soils with high C levels tend to become increasingly dominated by fungi (Waring et al., 2013; Ng et al., 2014). Fungi generally require more C per unit biomass than bacteria, and physiological constraints may favor fungi over bacteria in soils with a high C:N ratio (Keiblinger et al., 2010); otherwise, a high fungi:bacteria ratio would increase the microbial C limitation.

Unlike microbial C limitation, microbial P limitation presented some different drivers. While plants, soil nutrients, and soil microbial structure affected microbial P limitation, soil water content had a strong negative effect on microbial P limitation (Fig. 6). The potential mechanisms of microbial P limitation included three processes that led to an increase in microbial P limitation and one process that led to a decrease in microbial P limitation during vegetation restoration. First, plant productivity and species diversity improved following vegetation restoration (Table 1, Deng et al., 2013), thus increasing the absorption of nutrients (e.g., N and P). Vegetation restoration increased microbial P limitation owing to the relatively small increase in soil TP, when compared with soil C and N (Table 3), and the rapid growth of plants leading to nutrients competition with soil microorganisms. Second, vegetation restoration not only increased plant production, but also enhanced soil microbial biomass, especially soil fungi. The growth of soil microorganisms is associated with a higher demand for P for the synthesis of ribosomal RNA (Sterner and Elser, 2002), and thus increased microbial P limitation (Fig. 6). Third, vegetation restoration had a negative effect on soil water on the Loess Plateau (Table 1), consistent with those reported in arid and semi-arid regions (Yang et al., 2015; Ye et al., 2019). A lower soil moisture content was not conducive to the infiltration of AP into microorganisms (Bell et al., 2008), making it difficult for microorganisms to absorb soil P, which increased microbial P limitation. However, soil nutrient levels, including available C, AN, and AP, were increased by vegetation restoration (Table 3), which provided more nutrients for microbial production and metabolism (Ren et al., 2016b) and reduced microbial P limitation to a certain extent. Therefore, the increased microbial P limitation associated with long-term vegetation restoration was the result of a combination of effects induced by plants, soil, and microorganisms.

5. Conclusion

As a consequence of vegetation restoration following farmland abandonment on the Loess Plateau, soil microbial communities were co-limited by C and P from the perspective of microbial metabolism and plant-microbe nutrients competition. In particular, soil microorganisms in the ESVR (< 13 years) were mainly restricted by C, while those in the LSVR (> 13 years) were mainly restricted by P. The effects of vegetation restoration on microbial C and P limitation were the result of a combination of effects induced by plants, soil, and microorganisms. Plant properties, such as plant productivity and species diversity, decreased microbial C limitation owing to increasing plant C inputs, but increased microbial P limitation owing to plant nutrient competition with soil microorganisms. With regard to soil microbial composition, the increasing fungi:bacteria ratio enhanced microbial C and P limitation. However, continuous increase in soil nutrients slowed down microbial metabolic limitation, whereas vegetation restoration (that decreased the soil water content in arid and semi-arid regions) increased microbial P limitation. Owing to the different mechanisms driving microbial C and P limitations, a negative correlation was noted between them over the 30-year period of vegetation restoration since farmland abandonment. Thus, the findings of this study provide an indirect theoretical basis for sustainable management of ecological restoration, and reveal the plant nutrients limitations that occur during vegetation restoration in arid and semi-arid regions. Acknowledgements This study was sponsored by the National Natural Science Foundation of China (41730638, 41877538, ), the Strategic Priority Research Program of the Chinese Academy of Sciences (XDA23070201), the Funding of Special Support Plan of Young Talents Project of Shaanxi Province in China, and the Funding of Promoting Plan to Creative Talents of “Youth Science and Technology Star” in Shaanxi Province of China (2018KJXX-088). We are grateful to the Soil and Water Conservation Experiment Station, Chinese Academy of Sciences, for their help during the field investigation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.geoderma.2019.06.037.

References


