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Applicability of the microbial inoculation method in the study on litter decomposition

Wenxing ZHOU[1], Siran ZHANG[1], Lijie WANG[1], Hongyan HUI[1], Xiaoxi ZHANG[1,2,*]

1. College of Life Sciences, Yan'an University, Yan'an 716000, China.
2. Shaanxi Engineering and Technological Research Center for Conservation and Utilization of Regional Biological Resources, Yan'an University, Yan'an 716000, China.
[*]Corresponding author's E-mail: zhangxiaoxi712100@gmail.com; Tel: +86 15209290640

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ABSTRACT: The decomposition of litter is one of the core links of the material cycling of ecosystem, studying this process is helpful for understanding the indirect effects of environmental alterations on the stability of ecosystems. In the traditional method litter is placed into litterbags which are placed outdoor on or in soil. Since under these conditions it is very difficult to analyze the parameters involved in litter decomposition separately, these experiments are also done indoor under controlled conditions. Inoculating the litter with soil or phyllosphere microorganisms and preventing any other interaction of the litter samples with the surrounding soil is an emerging method in the indoor investigation of plant litter decomposition, in order to experimentally minimize the impacts of invading soil constituents on litter decomposition parameters. However, few parallel-experiments were conducted to compare this inoculation method with the traditional litterbag method. Hence, it is unclear whether the inoculation method, when compared to the litterbag method, would cause artifacts with respect to the decomposition rate and microbial communities under controlled indoor conditions. In the present study, litters of *Pinus tabuliformis*, *Salix babylonica* and mixed litter from a *Robinia pseudoacacia* forest, representing litters with different decomposabilities, were chosen for a comparative experiment. Each type of litter was incubated under controlled indoor conditions for six months using the litterbag method and soil microorganism inoculation method. The decomposition rates and the litter fungal communities of the same litters were compared. Using microbial inoculation method, the decomposition rates exhibited no significant differences relative to those observed in the litterbag method. More importantly, the disturbance by biotic and abiotic factors from external soil on the fungal community in the samples could be excluded.

KEY WORDS: Fungal communities, litter decomposition, litterbag method, microbial inoculation method.

INTRODUCTION

Plant litter is a crucial interface for the material cycling and energy transfer within the continuum of plants, decomposers and soil. The key nutrients released from litter, such as N and P, meet over 70% of the demands for the plant growth in some cases; additionally, plant litters also play important roles in the formation of soil, the release of greenhouse gases, the carbon storage and the ecological hydrologic process (Bani *et al*., 2018; Levi *et al*., 2020; Liu *et al*., 2019; Otaki and Tsuyuzaki, 2019). The human-made effects on the natural ecosystem are rapidly increasing. When evaluating the environmental changes caused by human activities, such as vegetation degradation and restoration, global warming, changes in precipitation, increasing UV radiation, contamination or nitrogen deposition (Chen *et al*., 2019; Jing *et al*., 2019; Lv *et al*., 2020; Pieristè *et al*., 2019; Zhang *et al*., 2015; Zhong *et al*., 2018), their effects on plant litter decomposition can be seen as key indicators. Therefore, in the fields of soil science, ecology, biogeochemistry and etc., litter decomposition is one of the research hotspots.

To investigate the decomposition process of litter, the in-situ decomposition is undoubtedly the method that is the closest to the actual status of litter decomposition. However, all the uncontrollable disturbances in the filed environment, such as other types of litter, root invasion, and the random changes in microenvironmental factors (i.e., temperature, humidity or light), can significantly affect the decomposition of the research object (Huang *et al*., 2020; Tian *et al*., 2018; Zhang *et al*., 2019). Therefore, it is difficult to accurately and focally study the influence of specific factors on litter decomposition by simply adopting in-situ experiments. In these cases, indoor-simulated decomposition experiments can be conducted to reduce some variables and to focus on certain other variables for comparison (Li *et al*., 2017; Zhang *et al*., 2019). In addition, as the field environment usually does not provide the most favorable conditions for the decomposers, the litter decomposition in the in-situ experiments might take quite a long time. In the boreal forests, the complete decomposition of litter might take several decades (Moore *et al*., 2017). Considering the generally short decomposition period in the published investigations, the decomposition rates are quite limited, thus it is difficult to estimate the whole decomposition process. Additionally, the extrapolation of the experimental results might cause unacceptable errors in estimating the decomposition rate, especially that in the

late stages of decomposition (Moore *et al*., 2017). The environmental conditions-controlled indoor experiments are helpful in shortening the time of decomposition (Chae *et al*., 2019), in order to study the whole litter decomposition process in limited period to the greatest extent.

Currently, the litterbag method (LB) is the most widely used in the investigations of litter decomposition in both outdoor and indoor experiments (Panteleit *et al*., 2018; Smith *et al*., 2014; Krishna and Mohan, 2017; Moore *et al*., 2017; Otaki and Tsuyuzaki, 2019). During the indoor experiment, in addition to maintaining suitable temperature and humidity, burying the litterbags in the soil medium can also further accelerate the decomposition (Levi *et al*., 2020; Zhang *et al*., 2015), and thus allows to complete the research in brief time. However, it is impossible to prevent external soil particles and organisms from entering the litterbag during the treatment, regardless whether the litterbags are buried in soil or fixed on the soil surface. The invading soil particles and the nutrients and microorganisms carried by them will certainly affect the determination of litter decomposition indicators, such as remaining mass, nutrient release rate, litter enzymatic activity and microbial community structure, etc. However, washing of the litter residues might lead to further errors in determinations, especially for the well decomposed litters. To solve these problems, researchers developed a new approach to study the decomposition of litter, that is the microbial inoculation method (IN). In this method, the litter or soil is washed with water and the resulting suspension used to inoculate the litter with soil-derived microorganisms; the inoculated litter is then incubated under soil-free conditions (Chae *et al*., 2019; Chen *et al*., 2019; Jurkšienė *et al*., 2017). By doing this, the impacts of invading external abiotic and biotic soil constituents from the surrounding soil on the study on the one hand will be minimized, on the other hand, however, the decomposers in the litter might also be more similar to those in the field conditions (containing all sources from endophytic, phyllosphere and soil -derived species), and the excessive disturbance occurring in the field can be avoided as well.

The IN is increasingly used in litter decomposition studies (Chae *et al*., 2019; Chen *et al*., 2019; Jurkšienė *et al*., 2017). However, few experiments are conducted to compare the data obtained by IN and traditional LB, including the basic indicators, e.g., observed decomposition and nutrient release rates, and the microbial indicators, such as the litter fungal community characteristics. The observed decomposition rates are the basis for almost any study on litter decomposition to get conclusions (Bärlocher *et al*., 2020). Fungi do not only play important roles in the decomposition of the main components of litter (e.g., cellulose and lignin), but in helping other decomposers (bacteria mainly) to enter and colonize the litter, and thus they usually are seen as the most important microbes involved in litter decomposition (Purahong *et al*. 2016; Wang *et al*. 2020). Hence, these data are of great importance in analyzing how the treatments in studies affect the decomposition of litter, both in the view of phenomena and mechanism. Therefore, comparatives-experiments are necessary to find (1) whether IN would cause similar changes in litter decomposition rates as in the traditional buried LB, and (2) whether IN is more advantageous in studying the litter-decomposing fungal communities than LB.

In the present study, we compared the differences in decomposition rates of three litter types incubated in different indoor conditions: litterbags in soil medium and litter with microbial inoculums. We also compare the composition and structure of fungal communities of the different types of litter incubated with both methods (Lu *et al*. 2017; Wang *et al*. 2020). We hypothesized that IN would not obviously change the litter decomposition rate relative to LB; however, the litter fungal community may be more different with that in soil after the decomposition experiment.

MATERIAL AND METHODS

The sampling region

Litters of *Robinia pseudoacacia* and the understory species were collected from an artificial forest located at Ansai District (E 108°5′44″–109°26′18″, N 36°30′45″–37°19′3″). Soil samples for the LB and IN were collected nearby from a tree-free grassland abandoned for ten years. Litter samples of *Pinus tabuliformis* and *Salix babylonica* were collected from the urban area located at Baota Distinct (E 109°14′10″–110°50′43″, N 36°10′33″–37°2′5″) of Yan'an City, China. The studied area is classified as the hilly region of the Loess Plateau, with an altitude of 860–1525 m, loess soil, and temperate continental semi-arid monsoon climate, with an annual average precipitation of 506.6–550 mm, an annual average temperature of 7.0–9.1 °C, an annual sunshine duration of 2448.6 h and a frost-free season of 150–160 d.

Sampling and processing of the litter and soil

In the late autumn 2019, 20 g of the persistent intact dead needles were collected from ten mature individuals of *P. tabuliformis*. All needles were cut into 5 cm length and mixed to form a composite sample. The litter of *S. babylonica* was collected from ten matures individuals (100 g for each one) using litter traps by shaking the trees. All litter collected from the same tree species was mixed in a composite sample. For the *R. pseudoacacia* forest, five plots of 1 × 1 m were established and litter of *R. pseudoacacia* and the dominant understory plants, *Bidens parviflora* and *Setaria viridis*, was collected by litter traps and cutting, and mixed to form a composite sample according to the observed mass proportion of the different

Table 1. The substrate quality of the tested litters.

Litter types	Nutrient concentration (mg g^{-1})			Ecological stoichiometry	
	C	N	P	C/N	C/P
S. babylonica	288.41±4.98^{c}	21.33±0.15^{a}	1.10±0.19^{a}	13.52±3.32^{c}	262.19±2.21^{c}
P. tabuliformis	464.15±6.91^{a}	4.97±0.36^{c}	0.60±0.05^{b}	94.17±5.44^{a}	779.65±57.1^{a}
Mixture in *R. pseudoacacia* forest	331.02±0.51^{b}	10.42±0.04^{b}	0.98±0.05^{a}	31.75±0.37^{b}	337.78±0.26^{b}

The different letters in the same column indicated significant differences among litter types.

plants in the natural conditions (8:1:1). According to their substrate quality properties, species composition and original habitat, the mentioned 3 litter types could be seen as the representative of at least the following litter types: labile/recalcitrant monospecific litters, mixed litter, and litters from forest and urban habitats, aiming to draw general conclusions. In the laboratory, all types of litters were air-dried in sterile conditions to preserve the endogenous and phyllosphere microorganisms that participate in the decomposition of litter (Chapman *et al*., 2013).

For collecting the soil samples from the grassland nearby the *R. pseudoacacia* forest in Ansai Distinct, ten 1 m × 1 m quadrats were established in the grassland and all the soil of the 0–10 cm layer was collected, mixed, and larger debris, such as roots and stones, was removed. The water content and saturation moisture capacity of the soil were measured for the following treatments.

The dried litter subsamples of all types of litter were smashed to pass through a 1 mm sieve, and then their C content was measured using the sulfuric acid-potassium dichromate oxidation method (Bao, 2000, Table 1). The N and P contents were measured using phenol-blue colorimetry and vanadium-molybdenum yellow colorimetry after the litter was digested by sulfuric acid and hydrogen peroxide (Bao, 2000, Table 1). Subsequently, the litters of *P. tabuliformis*, *S. babylonica* and the mixed litter from the *R. pseudoacacia* forest were used in the following experiments. Briefly, 36 subsamples with a mass of 5 g of each type of the litters were prepared, 18 of them were sealed into 15 × 10 cm litterbags with 1 mm mesh size and buried into 22 cm × 16.3 cm × 9 cm sized incubation pots containing 500 g of soil (one litterbag in each pot), and the pots were then covered with plastic film with 3 air-vents. These litters were marked as litterbag group. The remaining 18 subsamples were placed into 480 mL plastic breathable PP plastic jars, the diameter of the jars was 9 cm, and marked as inoculation group.

Litter decomposition experiments

According to a previous study (Zhang *et al*., 2015), after the litterbags were buried into soil, sterilized distilled water was sprayed in all pots for several times, until the soil moisture reached 50% of the saturation moisture capacity. The mass of water added was calculated according to the difference between the measured actual moisture and the saturation moisture capacity of the soil samples and controlled by the weight of pots. Each 6 pots containing the same litter were connected using rubber band, and used to simulate an independent decomposition process. A total of three decomposition processes was simulated (three replicates).

For the inoculation group, the same soil as for the LB experiment kept in 4 °C was mixed with sterilized normal saline with the portion of 1 g/1.5 mL and stirred for 0.5 h, the supernatant obtained was then diluted by five times and sprayed into the jars (Chen *et al*., 2019). A total of 5.00 g of the diluted supernatant was separately sprayed to fully wet the litter. Each 6 jars containing the same litter were connected using a rubber band as well, and used to simulate an independent decomposition process. A total of three decomposition processes was simulated (three replicates).

In the LB as well as IN treatments, the indoor temperature was controlled by an air conditioner at 25 °C, and the litters were incubated for 180 days. During the incubation, the pots or jars were weighed twice a week and sterilized distilled water was added to maintain the humidity constant. In the LB group, three litterbags were harvested from the three bundles of connected pots, respectively, 30, 60, 90, 120 and 180 d after incubation, and in each sampling so that each type of litter had three replicates. The litter residues were brushed and sieved (0.25 mm mesh) to remove the adhering soil. Then the litter samples were oven-dried at 65 °C, sieved again, and weighed. In the IN group, the litter residues harvest was conducted with the same sampling method used in the LB group. Alternatively, the visible mycelium was removed with sterilized tools, air-dried, and weighed. For both groups, three extra litter samples (three replicates) of each type were harvested at the fifth sampling. For the litterbag group, the harvested litter residues were only carefully sieved using sterilized tools to remove the adhering soil particles as much as possible, and additionally, the corresponding decomposition medium (soil) of each litter samples and the original soil sample were also sampled. All the extra fresh litter and soil samples were placed in dry ice boxes and sent to Novogene Co., Ltd., Beijing, China for the following analyses of litter fungal community.

Sequencing

Briefly, the DNA of litter fungi was extracted using the CTAB/SDS method using a fast DNA SPIN kit (MP Biomedicals, USA). Then, the ITS1 genes of fungi were amplified after integrity checking. The PCR program contained one minute of initial denaturation at 98 °C, 30

cycles of ten seconds of denaturation at 98 °C, 30 seconds of annealing at 50 °C, 30 seconds of elongation at 72 °C, and a final elongation step at 72 °C for 5 min. The quality of products was detected by 2% agarose gel electrophoresis, and then the products were purified using a gel extraction kit (Qiagen, Germany). According to the instructions from the manufacturer, sequencing libraries were generated and the index codes were added. The quality of the library was checked using a Qubit@ 2.0 fluorimeter (Thermo Scientific, Wilmington, NC, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, United States), and subsequently, the sequencing was performed on a NovaSeq platform (Illumina; United States). The original sequences were filtrated to remove chimeras, and then the OTU clustering and species annotation were performed using UPARSE based on the SILVA data base with a similarity of sequence of ≥ 97%). Based on these data, the relative abundance of the OTUs, and the Chao1, ACE, Shannon and Simpson diversity indexes were obtained by QIIME. Additionally, a NMDS analysis was performed based on the Bray-Curtis distance, and the PERMANOVA test was employed to detect the significance of community differences.

Data processing

The alteration of litter mass remaining over the decomposition duration were fitted with the following model (Eq. 1, Moore *et al*., 2017), and the annual decomposition rate of litter (k) and the limit of litter decomposition (R_0) were calculated using SigmaPlot 12.5:

$$M_t/M_0=R_0+e^{-kt} \quad (1),$$

where M_t is the mass remaining of litter at time t, M_0 is 5.00 g, and e is the base of natural logarithm.

As there were three independent decomposition processes simulated of each type of litter in the experiments using different methods, three k values could be obtained as replicates for the following analyses.

The k values, and the mass remaining rate of the same litter in difference experiments were compared by a t test at each sampling time, and a one-way analysis of variance followed with LSD *post hoc* test was conducted to detect the differences in the decomposition rate of the three types of litter in the same decomposition experiment using IBM SPSS 23.0. All significance levels for the mentioned analyses were α=0.05. Sigmaplot 12.5 was employed for the drafting.

RESULTS

The decomposition rates of litter

The observation of the decomposition processes indicated that IN caused a significant (P<0.05, the same hereafter) higher mass remaining rate of *S. babylonica* litter than LB at the 6th month in decomposition (Fig. 1), while it caused significant increases in the remaining rate of the mixed litter at the first two months. According to the fitting results (Table 2), in the experiment using litterbag method, both *S. babylonica* and mixed litters decomposed significantly faster than *P. tabuliformis* litter, however, in the IN experiment, mixed litter and *P. tabuliformis* litter exhibited a statistically equal decomposition rate, which was markedly slower than that of the *S. babylonica* litter (P<0.05). However, the same type of litter exhibited no significant difference in the decomposition rate in the experiments using different methods (P>0.05).

Table 2 The annual decomposition rate (k) of the tested litters in experiments using different method.

Litter types	Litterbag method	Microbial inoculation method
S. babylonica	23.84±0.93^{a}	19.74±2.20^{a}NS
P. tabuliformis	7.87±2.84^{b}	6.72±1.70^{b}NS
Mixture in *R. pseudoacacia* forest	14.96±2.96^{a}	9.25±3.39^{b}NS

Note: the different letters in the same column indicated significant differences among litter types based on one-way ANOVA (P<0.05), while NS indicated no significant differences between the k values of the same litter type in experiments using the two methods (P>0.05).

Properties of litter fungi

Species composition

In order to conveniently describe the results, the abbreviation LBS was used for representing the corresponding soil medium, while OS was used for representing the original status of the soil medium. Additionally, 1, 2 and 3 were used for representing *S. babylonica* litter, *P. tabuliformis* litter and the mixed litter from the *R. pseudoacacia* forest, respectively.

At the phylum level (Fig. 2A), Ascomycota was the most dominant fungi in the litters harvested from litterbag, it occupied 79.14%, 92.96% and 44.06% of the fungal species in LB1/2/3 litters, and there were significant or very significant differences in their relative abundance in different types of litter (P<0.001–0.019 based on LSD *pos-hoc* analysis). For the inoculated litters, Ascomycota were also the most dominant fungi (89.37%, 90.86% and 61.40% in IN1/2/3 litters), while their relative abundance in IN1/2 litter was significantly higher than that in IN3 litter (P<0.01). As for the differences in the experimental methods, the relative abundance of Ascomycota in IN1/3 litters was significantly higher than that harvested from litterbags (P=0.021–0.023). The fungal composition of the litters harvested from litterbags and their corresponding soil medium also exhibited significant differences. The relative abundances of Ascomycota in LBS1 and LBS2 soils (47.70% and 55.25%) were significantly lower than those in LB1 and LB2 litters (P <0.020–0.043), while in LBS3 soil, there was a significantly higher relative abundance of Ascomycota than in LB3 litter (P <0.001). In addition, the relative

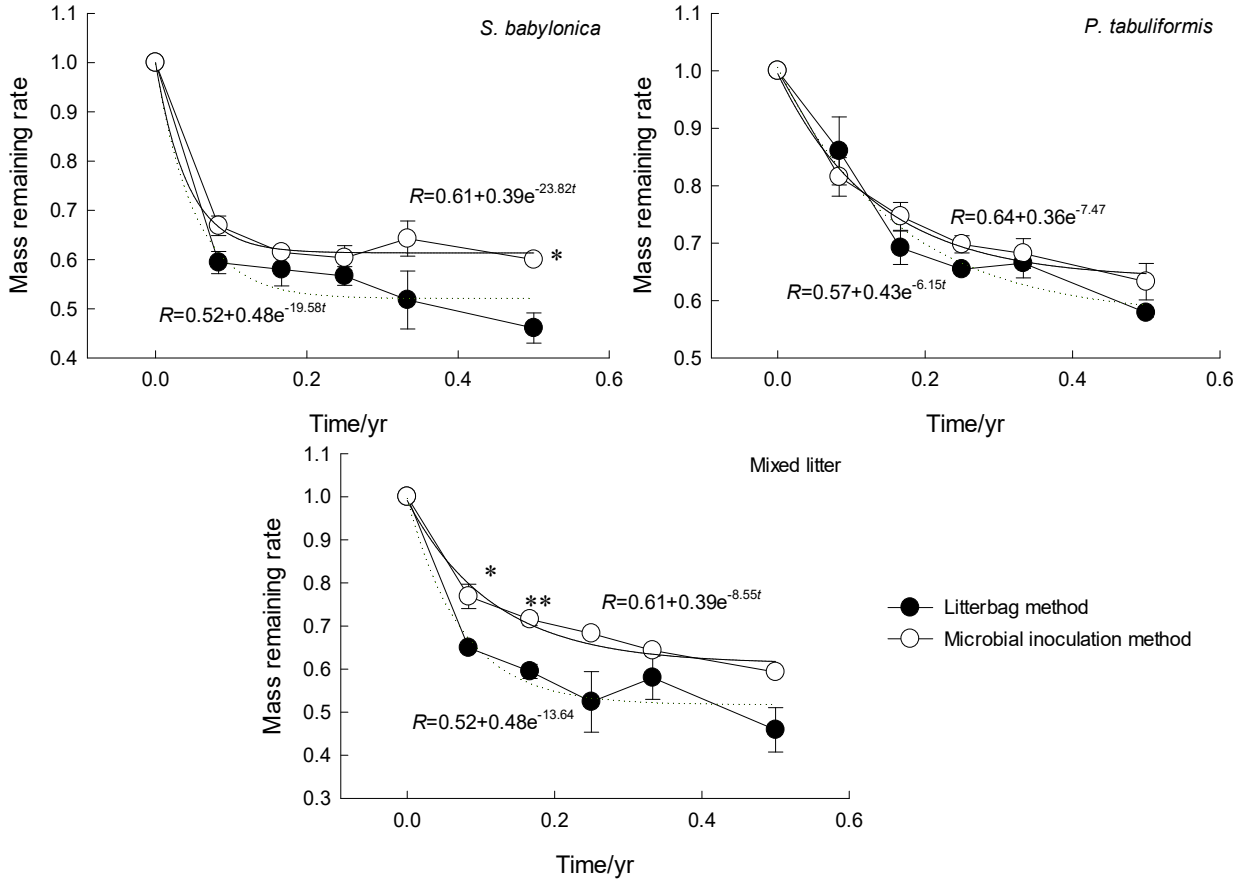

Fig. 1. The alteration of the mass remaining rates and the fit of the decomposition processes for three different types of litters tested with the litterbag method and the microbial inoculation method (both under controlled indoor conditions). **Note**: * or ** in the same column indicated significant or very significant differences between the two methods (P<0.05 or 0.01). The decomposition of a given litter type was fitted using the alteration of the average mass remaining rate over the decomposition duration, all the p values of the fitted equations were less than 0.01, and their R^2 fell between in 0.93 – 0.98.

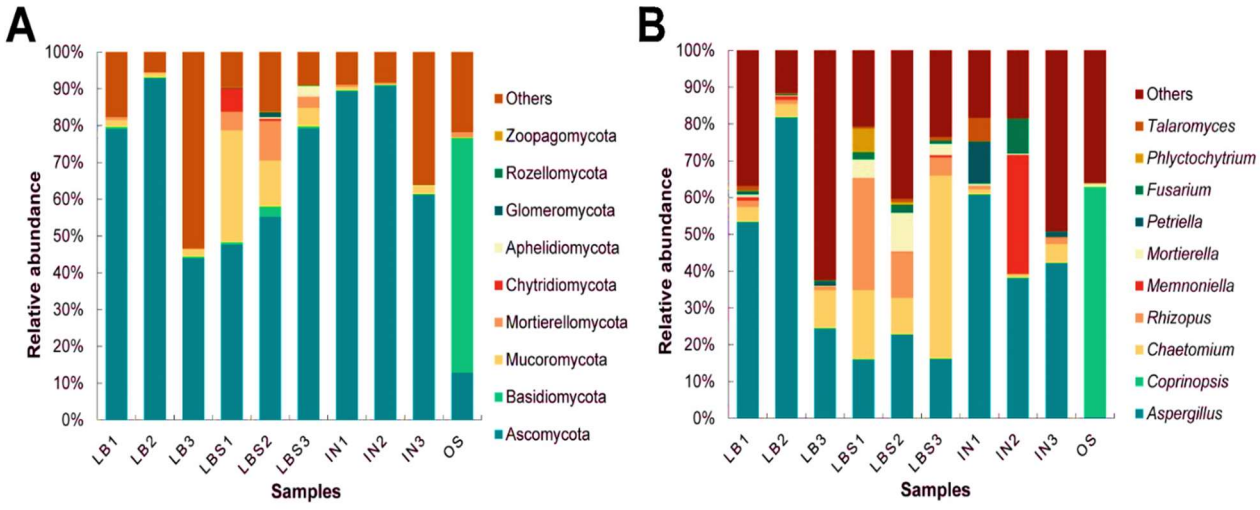

Fig. 2. The relative abundances of the dominant fungal phylum (**A**) or genera (**B**) in litter and soil samples after 180 days. **Note**: LB: litter residues obtained in the experiment using litterbag method; LBS: the corresponding soil medium; IN: litter residues obtained in the experiment using inoculation method; OS: the original status of the soil medium; 1: *S. babylonica* litter; 2: *P. tabuliformis* litter; and 3: mixed litter from the *R. pseudoacacia* forest.

Table 3. The α diversity of the fungi in different samples.

Samples	ACE index	Chao1 index	Shannon-Weiner index	Simpson index
LB1	499.35±29.05^{b}	488.96±34.79^{b}	4.41±0.06^{b}	0.83±0.01^{bc}
LB2	321.84±10.40^{cd}	294.78±18.10^{cd}	3.43±0.05^{de}	0.78±0.01^{bc}
LB3	361.82±6.57^{c}	345.14±9.36^{cd}	3.86±0.06^{bcd}	0.81±0.01^{bc}
LBS1	506.92±17.71^{b}	476.69±17.94^{b}	4.22±0.12^{bc}	0.85±0.03^{b}
LBS2	653.49±27.57^{a}	623.96±26.23^{a}	5.32±0.11^{a}	0.94±0.00^{a}
LBS3	530.58±36.22^{b}	499.85±34.74^{b}	3.84±0.10^{bcd}	0.75±0.03^{cd}
IN1	391.29±23.63^{c}	377.40±26.14^{c}	2.88±0.08^{e}	0.67±0.02^{de}
IN2	343.48±33.71^{c}	321.22±27.42^{c}	3.61±0.14^{cd}	0.82±0.02^{bc}
IN3	239.48±7.47^{d}	226.43±3.27^{d}	3.31±0.04^{de}	0.80±0.01^{bc}
OS	510.79±62.43^{b}	497.71±59.09^{b}	3.32±0.62^{de}	0.60±0.07^{e}

Note: The different letters in the same column indicated significant differences among the samples, $P<0.05$.

abundances of Mucoromycota (LBS1/2), Mortierellomycota (LBS1/3) and Basidiomycota (LBS3) in the soil media were also significantly higher than those in the corresponding litters (P=0.018-0.049), and were remarkably different from the fungal composition of the original soil sample (dominated by 12.81% of Ascomycota and 63.92% of Basidiomycota).

At the genus level (Fig. 2B), *Aspergillus* was the most dominant genus in the litters harvested from the litterbags, it occupied 53.45%, 81.83% and 24.54% of the fungal species in LB1/2/3 litters, and there were very significant differences in its relative abundance among the different types of litter ($P<0.005$). Besides, *Chaetomium* (the relative abundance in LB3 litter was significantly higher than that in LB1/2 litters) and *Rhizopus* (no significant differences among LB1/2/3 litters) were also sub-dominant species. For all inoculated litters, *Aspergillus* was also the most dominant fungal species (60.92%, 38.24% and 42.30% in IN1/2/3 litters), while in IN1 and IN2, *Petriella* and *Talaromyces*, and *Memnoniella* and *Fusarium* were also sub-dominant species, which exhibited significant differences to other litter types. Generally, the differences among litter fungal composition at the genus level was more obvious than at the phylum level. Concerning the differences in the experimental methods, the relative abundance of *Aspergillus* in IN2 litter was significantly lower than that in LB2 litter ($P<0.001$), while its relative abundance in IN3 litter was significantly higher than that in LB3 litter ($P=0.036$). The relative abundances of *Chaetomium* (lower in IN1/2/3 litters than in LB1/2/3 litters, $P=0.002$–0.045), *Petriella* and *Talaromyces* (higher in IN1 litter than in LB1 litter), and *Memnoniella* and *Fusarium* (higher in IN2 litter than in LB2 litter) in the same type of litters obtained using different methods also exhibited significant differences ($P<0.05$). Similar to those at the phylum level, the fungal genus composition of the litter harvested from the litterbags and their corresponding soil medium also exhibited significant differences, mainly observed as the significantly lower relative abundance of *Aspergillus* in the LBS1/2/3 soils than that in LB1/2/3 litters ($P=0.001$–0.014), and the much higher relative abundances of *Chaetomium* (higher in LBS1/3 than that in LB1/3, $P=0.007$–0.034), and *Rhizopus* and *Mortierella* (higher in LBS1/2 than that in LB1/2, $P=0.038$–0.049). Additionally, the fungal genus composition of the decomposition medium was remarkably different from that of the original soil sample, which was dominated by 62.53% of *Coprinopsis*.

α and β diversity

The analysis of α diversity of the fungi (Table 3) indicated that there were significant differences in the fungal species richness among the litters harvested from litterbags, their corresponding soil medium, or among the inoculated litters based on the ACE and Chao1 data. For the litters from litterbags, LB1 litter had a significantly higher species richness than LB2/3 litters ($P<0.05$), for the inoculated litters, IN1/2 litters had significantly higher species richness than IN3 litter, while for the soil media, the species richness of LBS2 was the highest ($P<0.05$). As for the differences in experimental methods, *S. babylonica* and the mixed litters from litterbag had significantly higher species richness than the inoculated litters. In addition, the species richness of all the soil media were generally higher than that in litters or the original soil sample.

For the fungal diversity, which was comprehensively considered based on Shannon-Weiner and Simpson indexes, the three litter types harvested from litterbag exhibited no significant difference ($P>0.05$), while in the inoculated litters, only IN1 exhibited slightly lower fungal diversity than other litters ($P<0.05$). For the soil media, the fungal diversity of LBS2 was the highest ($P<0.05$). As for the differences in experimental methods, only *S. babylonica* litter harvested from litterbag had significantly higher fungal diversity than the inoculated litter ($P<0.05$). Being different from those observed in the species richness, the differences in the fungal diversity among the litters and the corresponding soil medium were usually negligible ($P>0.05$).

The results of PCoA (Fig. 3) and PERMANOVA indicated that there were significant differences in the fungal community structure among all the litter types harvested from litterbag ($P<0.05$), while for the

inoculated litters, only the fungal community structure between IN1 and IN3 exhibited significant difference (P<0.01), and the fungal community structure of the same litter type exhibited significant differences as they were treated using LB or IN (P<0.05). In addition, the fungal community structure in the litters harvested from litterbag and their corresponding soil medium were also quite different (P<0.05), however, the fungal communities in the *S. babylonica* and mixed litter harvested from litterbags were obviously more similar with those in their corresponding soil medium as shown in Fig. 3.

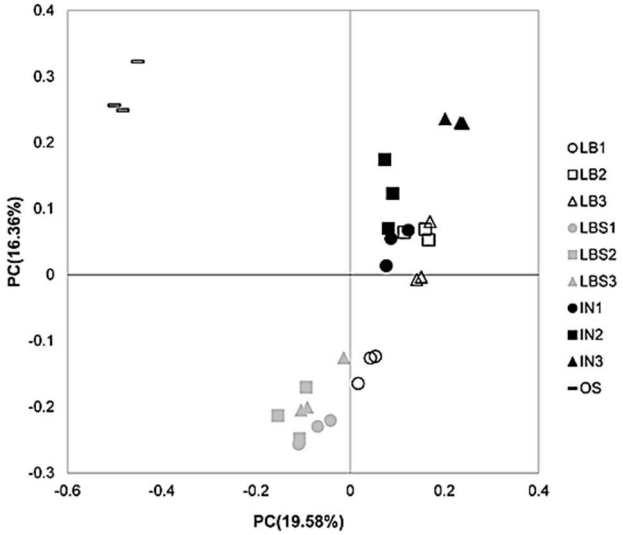

Fig. 3. Results of the PCoA of the fungal community in different samples.

DISCUSSION

The decomposition rates of litter

The results indicated that for all litter types in the present study, including the labile litter (*S. babylonica* litter, also used for representing for the litter produced in the urban conditions), recalcitrant litter (*P. tabuliformis* litter) and mixed litter (which is the most common form of litter in forest conditions), the calculated litter decomposition rate exhibited no significant difference in both experiments conducted using LB or IN. This means that investigations based on IN will not change the decomposition rate, which ensures the comparability of the results obtained from experiments using different methods. In another view, it can at least maintain the decomposition rate of litter observed in the LB experiments. The similar decomposition rate in both experiments might be attributed to the same conditions in both the experiments (including litter substrate quality, the microenvironmental properties and the source of decomposers; Berg and McClaugherty, 2014; Chae *et al*., 2019; Tu *et al*., 2011; Yan *et al*., 2010), which was in line with previous similar comparative-experiments (Gu *et al*., 2020). In the present study, the components of the same type of litter were sufficiently homogenized before decomposition, including those with different substrate quality such as leaf, total petiole and stalk (Osono *et al*., 2011), and then was divided into subsamples for the experiment. Hence, the difference in the substrate quality of litter used in different experiments could be excluded to great extent. For the microenvironmental conditions, the temperature was the same in both experiments. In the LB experiment, the soil water content was adjusted to a relative high level to avoid the moisture limits, while in the IN experiment, the litter was also sufficiently wetted. These methods were also extensively used in previous studies (Zhang *et al*., 2019; Chen *et al*., 2019). Hence, the difference of moisture was not the main factor causing the differences in the decomposition rate as well. For the decomposers, the fungal decomposers mainly came from the soil besides the original phyllosphere fungi in the LB experiment, while in the IN experiment, the microorganisms also came from the suspension of the same soil medium. Although the microbial communities in litters harvested from litterbag might be continuously affected by the soil communities, the fungal communities in the same type of litter might still exhibit similar carbon resources utilization trends or abilities (Bani *et al*., 2018; Fioretto *et al*., 2007; García-Palacios *et al*., 2016) as the "screening effects" of the chemical properties of litter on the microbes (Sauvadet *et al*. 2019; Wang *et al*. 2020). For all the mentioned reasons, the same litter in different experiments exhibited no significant differences in their overall decomposition rate.

In the same experiment, the 3 types of litter exhibited significantly different decomposition rates, which might be mainly attributed to the differences in their substrate quality. Generally, the litter with higher contents of N and P, lower ratios of C/N, C/P and lignin/N and lower contents of lignin decomposes faster (Berg and McClaugherty, 2014; Jia, 2019). Being similar to the previous studies (Berg and Mcclaugherty, 2014; Jia, 2019), the recalcitrant *P. tabuliformis* litter exhibited the slowest decomposition in both experiments. Furthermore, *P. tabuliformis* litter has higher density, lower specific surface area, and thicker cuticle than the other 2 types of litter, which also contribute to its slow decomposition (Chae *et al*., 2019). Notably, the statistical ordering of the decomposition rate for the 3 types of litter was not the same in different experiments. The observed mass loss during litter decomposition also indicated that the mixed litter in the litterbags decomposed significantly faster than those were inoculated during in the first 2 months of decomposition. Because the soil medium was collected nearby the *R. pseudoacacia* forest, which might cause the home-field advantage effects (Prescott and Grayston, 2013), and consequently, accelerate the decomposition of the mixed litter from *R. pseudoacacia* forest. However, in the IN experiment, the mixed litter was not continuously affected by the soil medium, and thus exhibited a

decomposition rate that was statistically equal to that of the *P. tabuliformis* litter. These differences indicated that in the LB experiment, the inappropriate soil medium would significantly affect the decomposition rate, while misestimates could be excluded to great extent with the inoculation method. Additionally, as only the decomposition of mixed litter was affected by different experimental methods, it was speculated that in the unburied conditions, the increase in the spatial distance and the changes in the decomposition medium might hinder the nutrient transfer among litters, which might alter the mixed decomposition effects, and consequently, the overall decomposition rate (Chen *et al*., 2017).

Properties of litter fungi

For the litter fungi, most of the results based on the high-throughput sequencing technology or the cultivation-taxonomy approaches indicated that Ascomycota is the most dominant fungal phylum in decomposing litter, especially the moderately decomposed litter (Jatav *et al*., 2020; Purahong *et al*., 2016), while *Penicillium*, *Aspergillus*, *Trichoderma*, *Mucor*, *Chaetomium* and *Cladosporium* are the dominant genera observed in previous investigations (Song *et al*., 2004). In the present study, Ascomycota was also the most dominant fungal group in all types of litter and in the experiments using two methods, which was in line with the previous studies (Jatav *et al*., 2020; Purahong *et al*., 2016). The dominance of Ascomycota species might be attributed to their ability of decomposing lignocellulose, which helps them to take place of sugar fungi to be the dominant fungal species, until the nutrient conditions become worse in the late stage of decomposition (Purahong *et al*., 2016). On the genus level, *Aspergillus* exhibited obvious dominance in all types of litter and in the experiments using two methods, which was in line with the findings of some of the previous studies from tropical areas (Jatav *et al*., 2020), but was different from others from temperate regions (Gołębiewski *et al*., 2019). That might be firstly attributed to their ability to utilize main recalcitrant substrates such as lignin, tannins and cellulose (Song *et al*., 2010; Cesco *et al*., 2012). In addition, it was speculated that the litter substrate quality properties when sampling the litters were more suitable for the growth of this genus (Jatav *et al*., 2020; Zhao *et al*., 2019). Certainly, the environmetal factors also contribute to the high relative abundance of *Aspergillus* species, because they are more common under warmer conditions (Klich, 2002) as they were applied in our experimental setting. However, high throughput sequencing is biased by the primer selection, particularly since we chose the ITS1 barcode and not the complete ITS region. Even the complete ITS region has limited significance due to the lack of DNA data for the majority of fungal species, low resolution on the species level in many genera and a high proportion of wrongly labeled sequences in databases (Hofstetter *et al*., 2019). In following studies, more advanced technology should be used for the determination of litter fungal community, and traditional isolation and identification technology might also be helpful in this issue.

As hypothesized, the same type of litter obtained by different experimental methods exhibited significant differences in the fungal community, especially on the genus level. For instances, except for the significant differences in the relative abundance of *Aspergillus*, *Petriella* and *Talaromyces*, which exhibited high relative abundance in *S. babylonica* litter, and *Memnoniella* and *Fusarium*, which exhibited high relative abundance in *P. tabuliformis* litter in the experiment using IN were almost not found in the same litters harvested from litterbags (Fig. 2). In addition, all litters harvested from litterbags exhibited higher *Chaetomium* abundance than those obtained using inoculation method, which was a dominant genus detected in the soil media (Fig. 2). The results of PCoA also showed that the fungal community of the litters harvested from litterbag was more similar with their corresponding soil medium (Fig. 3). This indicated that LB might lead to significant changes in the fungal community, and the importance of the specific species, which were "selected" by given litter (Asplund *et al*., 2018; Song *et al*., 2004) and might play crucial roles in litter decomposition, could not be observed. This would certainly cause misunderstandings. The alterations in the fungal community in the litters might be firstly attributed to the soil particles adhered on the litters. These soil particles would harbor extra fungi and thus affect the sequencing results, making the fungal communities more similar with those in soil. Second, in the experiment using LB, soil fungi might transfer nutrients, e.g., soil nitrogen, to litter and alter the substrate quality of litter (Zheng and Han, 2016). Therefore, soil medium will change the composition of litter fungi, because the succession of litter fungi is affected by litter changes to great extent (Chapman *et al*., 2013). Third, burying litterbags in soil would significantly affect the air environment of litter, while the colonization of some fungal species depends on spore dispersal by air, thus their abundances might decrease obviously in buried conditions, regardless of the decomposition stage of litters (Osono *et al*., 2006). Finally, the soil fungi might invade the litter by hyphae (Zuo *et al*., 2020; Osono *et al*., 2006), which might also alter the fungal community and make them more similar with those in soil. Certainly, not all the species in soil could successfully colonize litter, e.g., those of *Rhizopus* and *Memnoniella.* That might be attributed to their lower competitive capacity than other species, or higher sensibility to specific secondary metabolites, or they might prefer to colonize the more decomposed litters. (Chomel *et al*., 2016; Purahong *et al*., 2016; Osono *et al*., 2006).

CONCLISION AND OUTLOOK

In the present study, we conducted a comparative experiment to assess the applicability of IN in the study of litter decomposition, since few such experiments have been published. It was found that when using IN to investigate the decomposition of three types of representative litter, the decomposition rates of the litter exhibited no significant differences relative to those observed in the experiment using LB. More importantly, the disturbance of invading particles or organisms from the surrounding soil on litter fungal community could be excluded. With respect to assessing the decomposition rate, IN was more appropriate for the study of litter decomposition than LB. In addition, IN could be used to study the litter decomposition characteristics in different layers (such as the interface between litter and soil), or to separately analyze the roles played by endophytic, phyllosphere and soil fungi in the decomposition of litters, and their relationships and contributions during litter decomposition. Further modifications of the experimental setting could include using different temperature and water regimes for comparing the effects of abiotic conditions, or taking the soil from below the litter for making the wash suspension and IN in order to avoid introducing fungi which do not occur in that site. Other biotic factors such as bacteria and soil fauna also playing roles in litter decomposition, and getting increasing attention, could be included for testing whether IN would affect the measuring results of these decomposers in further studies.

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LITERATURE CITED

Asplund, J., H. Kauserud, S. Bokhorst, M.H. Lie, M. Ohlson and L. Nybakken. 2018. Fungal communities influence decomposition rates of plant litter from two dominant tree species. Fungal. Ecol. **32:** 1–8.

Bani, A., S. Pioli, M. Ventura, P. Panzacchi, L. Borruso, R. Tognetti, G. Tonon and L. Brusetti. 2018. The role of microbial community in the decomposition of leaf litter and deadwood. Appl. Soil Ecol. **126:** 75–86.

Bao, S. 2000. Soil agro-chemistrical analysis. China Agriculture Press, Beijing.

Bärlocher, F., M.O. Gessner and M.O.S. Graça. 2020. Methods to study litter decomposition: A Practical Guide. Berlin: Springer International Publishing.

Berg, B. and C. MCclaugherty. 2014. Plant litter. Decomposition, humus formation, carbon sequestration. Third ed. Springer-Verlag Berlin Heidelberg.

Cesco, S., T. Mimmo, G. Tonon, N. Tomasi, R. Pinton, R. Terzano, G. Neumann, L. Weisskopf, G. Renella, L. Landiand and P. Nannipieri. 2012. Plant-borne flavonoids released into the rhizosphere: impact on soil bio-activities related to plant nutrition. A review. Biol. Fertil. Soils. **48(2)**: 123–149.

Chae, H.M., S.H. Choi, S.H. Lee, S. Cha, K.C. Yang and J.K. Shim. 2019. Effect of litter quality on needle decomposition for four pine species in Korea. Forests **10(5):** e371.

Chapman, S.K., G.S. Newman, S.C. Hart, J.A. Schweitzer and G.W. Koch. 2013. Leaf litter mixtures alter microbial community development: Mechanisms for non-additive effects in litter decomposition. PLoS One **8(4):** e62671.

Chen, F., G.G. Wang, X. Fang, S. Wan, Y. Zhang and C. Liang. 2019. Nitrogen deposition effect on forest litter decomposition is interactively regulated by endogenous litter quality and exogenous resource supply. Plant Soil **437(1-2):** 413–426.

Chen, Y., S. Ma, J. Sun, X. Wang, G. Cheng and X. Lu. 2017. Chemical diversity and incubation time affect non-additive responses of soil carbon and nitrogen cycling to litter mixtures from an alpine steppe soil. Soil Biol. Biochem. **109:** 124–134.

Chomel, M., M. Guittonny-Larchevêque, C. Fernandez, C. Gallet, A. DesRochers, D. Paré, B. G. Jackson and V. Baldy. 2016. Plant secondary metabolites: A key driver of litter decomposition and soil nutrient cycling. J. Ecol. **104(6):** 1527–1541.

Fioretto, A., S. Papa, A. Pellegrino and A. Fuggi. 2007. Decomposition dynamics of *Myrtus communis* and *Quercus ilex* leaf litter: Mass loss, microbial activity and quality change. Appl. Soil Ecol. **36(1):** 32–40.

García-Palacios, P., E.A. Shaw, D.H. Wall and S. Hättenschwiler. 2016. Temporal dynamics of biotic and abiotic drivers of litter decomposition. Ecol. letter **19(5):** 554–563.

Golębiewski, M., A. Tarasek, M. Sikora, E. Deja-Sikora, A. Tretyn and M. Niklińska. 2019. Rapid microbial community changes during initial stages of pine litter decomposition. Microbial Ecol. **77(1)**: 56–75

Gu, Y., T. Li, Q. Tao and L. Wang. 2020. Comparison on early decomposition process of forest litter from litter bag and natural environment. J. Zhejiang For. Sci. Technol. **40:** 1-8.

Hofstetter, V., B. Buyck, G. Eyssartier, S. Schnee and K. Gindro. 2019. The unbearable lightness of sequenced-based identification. Fungal Divers. **96(1):** 243–284.

Huang, Y., X. Yang, D. Zhang and J. Zhang. 2020. The effects of gap size and litter species on colonization of soil fauna during litter decomposition in *Pinus massoniana* plantations. Appl. Soil Ecol. **155:** 103611.

Jatav, B., T. Sharma and S. Dassani. 2020. Succession of microfungi on leaf litter of *Acacia catechu* in Datia, Madhya Pradesh, India. J. Pure Appl. Microbiol. **14(1):** 581–590.

Jia, B. 2019. Litter decomposition and its underlying mechanisms. Chinese J. Plant Ecol. **43(8):** 648–657.

Jing, H., P. Zhang, J. Li, X. Yao, G. Liu and G. Wang. 2019. Effect of nitrogen addition on the decomposition and release of compounds from fine roots with different diameters: The

importance of initial substrate chemistry. Plant Soil **438(1-2):** 281–296.

Jurkšienė, G., D. Janušauskaitė, K. Armolaitis and V. Baliuckas. 2017. Leaf litterfall decomposition of pedunculate (*Quercus robur* l.) and sessile (*Q. Petraea* [matt.] liebl.) oaks and their hybrids and its impact on soil microbiota. Dendrobiology **78:** 51–62.

Klich, M.A. 2002. Biogeography of *Aspergillus* species in soil and litter. Mycologia **94(1):** 21–27.

Krishna, M. and M. Mohan. 2017. Litter decomposition in forest ecosystems: A review. Energy Ecol. Environ. **2(4):** 236–249.

Levi, E., S. Archer, H. Throop and C. Rasmussen. 2020. Soil-litter mixing promotes decomposition and soil aggregate formation on contrasting geomorphic surfaces in a shrub-invaded sonoran desert grassland. Plant Soil **450(1-2):** 397–415.

Li, S., Y. Tong and Z. Wang. 2017. Species and genetic diversity affect leaf litter decomposition in subtropical broadleaved forest in southern China. J. Plant Ecol. **10(1):** 232–241.

Liu, Y., Z. Cui, Z. Huang, H.-T. Miao and G.-L. Wu. 2019. The influence of litter crusts on soil properties and hydrological processes in a sandy ecosystem. Hydrol. Earth Sys. Sci. **23(5):** 2481–2490.

Lu, W., N. Liu, Y. Zhang, J. Zhou, Y. Guo and X. Yang. 2017. Impact of vegetation community on litter decomposition: Evidence from a reciprocal transplant study with 13C labeled plant litter. Soil Biol. Biochem. **112:** 248–257.

Lv, W., L. Zhang, H. Niu, B. Li, Q. Wang, Y. Zhou, Y. Wang, L. Jiang, P. Liu, H. Hong, S. Jia, C. Luo, D. Tsechoe, H. Zhou and H. Hong. 2020. Non-linear temperature sensitivity of litter component decomposition under warming gradient with precipitation addition on the Tibetan Plateau. Plant Soil **448(1-2):** 335–351.

Moore, T.R., J. Trofymow, C. Prescott, B. Titus and C. W. Group. 2017. Can short-term litter-bag measurements predict long-term decomposition in northern forests? Plant Soil **416(1-2):** 419–426.

Osono, T., C. To-Anun, Y. Hagiwara and D. Hirose. 2011. Decomposition of wood, petiole and leaf litter by Xylaria species from northern Thailand. Fungal. Ecol. **4(3)**: 210–218.

Osono, T., D. Hirose and R. Fujimaki. 2006. Fungal colonization as affected by litter depth and decomposition stage of needle litter. Soil Biol. Biochem. **38(9):** 2743–2752.

Otaki, M. and S. Tsuyuzaki. 2019. Succession of litter-decomposing microbial organisms in deciduous birch and oak forests, northern Japan. Acta Oecol. **101:** 103485.

Panteleit, J., F.G. Horgan, M. Türke, A. Schmidt, M. Schädler, M. Bacht, R. Brandl and S. Hotes. 2018. Effects of detritivorous invertebrates on the decomposition of rice straw: evidence from a microcosm experiment. Paddy Water Environ. **16(2):** 279–286.

Pieristè, M., M. Chauvat, T.K. Kotilainen, A.G. Jones, M. Aubert, T.M. Robson and E. Forey. 2019. Solar UV-A radiation and blue light enhance tree leaf litter decomposition in a temperate forest. Oecologia **191(1):** 191–203.

Prescott, C.E. and S.J. Grayston. 2013. Tree species influence on microbial communities in litter and soil: Current knowledge and research needs. Forest Ecol. Manag. **309:** 19–27.

Purahong, W., T. Wubet, G. Lentendu, M. Schloter, M.J. Pecyna, D. Kapturska, M. Hofrichter, D. Kruger and F. Buscot. 2016. Life in leaf litter: Novel insights into community dynamics of bacteria and fungi during litter decomposition. Molecular Ecol. **25(16):** 4059–4074.

Sauvadet, M., N. Fanin, M. Chauvat and I. Bertrand. 2019. Can the comparison of above-and below-ground litter decomposition improve our understanding of bacterial and fungal successions? Soil Biol. Biochem. **132:** 24–27.

Smith, S.W., S.J. Woodin, R.J. Pakeman, D. Johnson and R. Van Der Wal. 2014. Root traits predict decomposition across a landscape-scale grazing experiment. New Phytol. **203(3):** 851–862.

Song, F., X. Tian, X. Fan and X. He. 2010. Decomposing ability of filamentous fungi on litter is involved in a subtropical mixed forest. Mycologia. **102(1)**: 20–26.

Song, F., X. Tian, Z. Li, C. Yang, B. Chen, J. Hao and J. Zhu. 2004. Diversity of filamentous fungi in organic layers of two forests in Zijin Mountain. J. For. Res. **15(4):** 273–279.

Tian, K., X. Kong, J. Gao, Y. Jia, H. Lin, Z. He, Y. Ji, Z. Bei and X. Tian. 2018. Local root status: A neglected bio-factor that regulates the home-field advantage of leaf litter decomposition. Plant Soil **431(1-2):** 175–189.

Tu, L., H. Hu, T. Hu, J. Zhang, L. Liu, R. Li, H. Dai and S. Luo. 2011. Decomposition of different litter fractions in a subtropical bamboo ecosystem as affected by experimental nitrogen deposition. Pedosphere **21(6):** 685–695.

Wang, W., Q. Zhang, X. Sun, D. Chen, H. Insam, R.T. Koide and S. Zhang. 2020. Effects of mixed-species litter on bacterial and fungal lignocellulose degradation functions during litter decomposition. Soil Biol. Biochem. **141:** e107690.

Yan, H., X. Gu and H. Shen. 2010. Microbial decomposition of forest litter: A review. Chinese J. Ecol. **29:** 1827–1835.

Zhang, X., B. Wang and Z. Liu. 2019. Impacts of plant secondary metabolites from conifer litter on the decomposition of *Populus purdomii* litter. J. For. Res. **30(6):** 2237–2245.

Zhang, X., Z. Liu, Q. Yu, N.T. Luc, Y. Bing, B. Zhu and W. Wang. 2015. Effect of petroleum on decomposition of shrub-grass litters in soil in northern Shaanxi of China. J. Environ. Sci. **33:** 245–253.

Zhao, C., J. Long, H. Liao, C. Zheng, J. Li, L. Liu and M. Zhang. 2019. Dynamics of soil microbial communities following vegetation succession in a karst mountain ecosystem, southwest China. Sci. Rep. **9(1):** e2160.

Zheng, J. and S. Han. 2016. Nitrogen transfer in the litter-soil interface ontinuum of the temperate forest. J. Beijing Forestry Univ. **38**: 116–122.

Zhong, Y., W. Yan, R. Wang, W. Wang and Z. Shangguan. 2018. Decreased occurrence of carbon cycle functions in microbial communities along with long-term secondary succession. Soil Biol. Biochem. **123:** 207–217.

Zuo, L., R. Huang, Q. Zhang, P. Chen and L. Li. 2020. The diversity of endophytic and epiphytic fungi of the litter in differently aged *Casuarina equisetifolia* forests in Hainan island. Acta Ecol. Sin. **40(17):** 6086–6095.