A peer-reviewed version of this preprint was published in PeerJ on 2 April 2018.

View the peer-reviewed version (peerj.com/articles/4559), which is the preferred citable publication unless you specifically need to cite this preprint.

Zhang Z, Luo L, Tan X, Kong X, Yang J, Wang D, Zhang D, Jin D, Liu Y. 2018. Pumpkin powdery mildew disease severity influences the fungal diversity of the phyllosphere. PeerJ 6:e4559
https://doi.org/10.7717/peerj.4559

Pumpkin powdery mildew disease severity influences the fungal diversity of the phyllosphere

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Phyllosphere microbiota play a crucial role in plant-environment interactions and are influenced by biotic and abiotic factors. However, there is little research on how pathogens affect the microbial community. In this study, we collected 16 pumpkin (*Cucurbita moschata*) leaf samples showing symptoms of powdery mildew disease with different disease severity levels ranging from L1 (least severe) to L4 (most severe). We examined the fungal community structure and diversity by Illumina MiSeq sequencing of the internal transcribed spacer (ITS) region of ribosomal RNA genes. The fungal communities were dominated by members of the Basidiomycota and Ascomycota. The dominant genus was *Podosphaera* on the diseased leaves, which was the key pathogen responsible for the pumpkin powdery mildew. Ascomycota and *Podosphaera* increased in abundance as disease severity increased from L1 to L4, and were significantly more abundant than other microorganisms at disease severity L4 ($P<0.05$). The richness and diversity of the fungal community increased from L1 to L2, and then declined from L2 to L4, likely due to the biotic pressure at disease severity L4. Maintaining species richness in the phyllosphere will be an important part of managing disease control in this agroecological system and an essential step toward predictable biocontrol of powdery mildew in pumpkin.

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ABSTRACT

Phyllosphere microbiota play a crucial role in plant-environment interactions and are influenced by biotic and abiotic factors. However, there is little research on how pathogens affect the microbial community. In this study, we collected 16 pumpkin (*Cucurbita moschata*) leaf samples showing symptoms of powdery mildew disease with different disease severity levels ranging from L1 (least severe) to L4 (most severe). We examined the fungal community structure and diversity by Illumina MiSeq sequencing of the internal transcribed spacer (ITS) region of ribosomal RNA genes. The fungal communities were dominated by members of the Basidiomycota and Ascomycota. The dominant genus was *Podosphaera* on the diseased leaves, which was the key pathogen responsible for the pumpkin powdery mildew. Ascomycota and *Podosphaera* increased in abundance as disease severity increased from L1 to L4, and were significantly more abundant than other microorganisms at disease severity L4 ($P<0.05$). The richness and diversity of the fungal community increased from L1 to L2, and then declined from L2 to L4, likely due to the biotic pressure at disease severity L4. Maintaining species richness in the phyllosphere will be an important part of managing disease control in this agroecological system and an essential step toward predictable biocontrol of powdery mildew in pumpkin.

Keywords phyllosphere microbiota, powdery mildew, fungal community, community diversity, disease severity, Illumina MiSeq

INTRODUCTION

Powdery mildew is a common fungal disease of cucurbits and the major cause of losses in cucurbit production worldwide. *Golovinomyces cichoracearum* (syn. *Erysiphe cichoracearum*) and *Podosphaera xanthii* (syn. *Sphaerotheca fuliginea*) are main two organisms caused powdery mildew (*Lebeda et al., 2010*). Impacts of powdery mildew on crop production include reduced photosynthesis, impaired growth, premature senescence, and yield loss. The powdery mildew pathogen lives with the obligate biotrophic lifestyle. Powdery mildew symptoms first appear as pale, chlorotic spots on leaves that soon turn powdery-white in appearance (fungal spores) and starts on the crown and lower leaves, mainly on the under-leaf shaded surface. Young plants may turn yellow, stunted, and may die and then severely infected leaves become brown, brittle and die, resulting in foliage loss (*Lebeda et al., 2010*).

The phyllosphere or leaf surface is an important microbial habitat for members of the major bacterial and fungal groups, and Archaea (*Lindow & Leveau, 2002; Lindow and Brandl, 2003*). These microorganisms play a crucial role in helping their host against pathogens (*Lacava et al., 2006; Mejía et al., 2008; Rajendran et al., 2008*). In past years, most of the researchers focused on screening plant growth-promoting microorganisms from plants which can help us manage diseases (*Compant et al., 2005; Everett et al., 2005; Hirano & Upper, 2000; Whipps et al., 2008*). However, not all the microbes in the natural environment are considered culturable. In the past few years, the development of next-generation rRNA sequencing techniques has enabled us to obtain in-depth descriptions of the composition of the microbial communities associated with leaves of *Arabidopsis thaliana* (*Reisberg et al., 2013*), potatoes (*Becker et al., 2008*), rice (*Mwajita et al., 2012*), spinach (*Lopez et al., 2011; Lopez et al., 2013*), grape (*Leveau et al., 2011*), and various tree species including salt cedar (*Redford et al., 2010; Finkel et al., 2011*).

Historically, scholars have begun to study the rhizosphere as a microbial habitat as early as 100 years ago (*Hartmann et al., 2008*) and the importance of microbial communities is well recognized in plant health and growth. Although the root–rhizosphere microbiome is now well known, the phyllosphere microbiome is only partly understood. However, the development of

new high-throughput sequencing technologies is now enabling researchers to focus on the phyllosphere microbiome. It can help us understand the complexity of phyllosphere microbial communities better and study interactions with their host plants and the environment deeply.

As a member of nature, plants are actually affected by various nature's stress factors during their growth period (*Zhang et al., 2014*). The phyllosphere microorganisms are influenced by both biotic and abiotic factors, some of which are fairly stable and constant, such as habitat conditions (*Yang et al., 2016; Fonsecagarcía et al., 2016*), the host genotype (*Sapkota et al., 2015; Bodenhausen et al., 2014; Hunter et al., 2015*), elevation gradient (*Cordier et al., 2012; Zhang et al., 2015*), and seasonal variation (*Copeland et al., 2015; Jackson & Denney, 2011; Davey et al., 2012*). Microbial interactions in the phyllosphere play an important role in the agroecosystem, it not only can affect the health and growth of plants in natural communities, but also the productivity of agricultural crops. There are not only a high proportion of plant-beneficial microorganisms such as antagonists, diazotrophs, and plant growth promoting bacteria (PGPB) in plant-associated habitats, but also plant pathogens and potential human pathogens (*Berg et al., 2005*). Plants can also protect themselves against fungal infection by biological and non-biological inducers by natural means (*Shi et al., 2007*). However, less is known about the colonization and persistence of nonpathogenic microbes on this extensive habitat, as well as their interactions with pathogenic microorganisms, and impact of single strains on the microbial community. The rhizosphere community of specific biocontrol agents have shown minor and only transient effects according to the risk assessment and colonization studies (*Scherwinski et al., 2007; Adesina et al., 2009; Chowdhury et al., 2013; Schmidt et al., 2012*), while impacts of pathogens on the phyllosphere microbiome are largely underexplored. To the best of our knowledge, only one research investigated the relationship between the phyllosphere microbiome and pathogen using Illumina sequencing technology, and the results showed that microbes present on the plant surface play an important role in the resistance to Botrytis cinereal (*Ritpitakphong et al., 2016*).

So far, there have been no studies to analysis of plant microbe–pathogen interactions in the phyllosphere using Illumina MiSeq platform to sequence the internal transcribed spacer (ITS) regions of the rRNA of the fungal communities. In this study, we want to further explore the interaction between the pathogen and other microorganisms and to gain a better understanding of the theoretical basis for disease control in agroecological systems by evaluating whether the diversity and community structure of pumpkin (*Cucurbita moschata* Duchesne ex Poir.) phyllosphere microbiota is influenced by the abundance of the pumpkin powdery mildew pathogen *Podosphaera*. We analyzed the fungal communities of 16 pumpkin leaf samples showing symptoms of powdery mildew disease with different disease severity levels ranging from L1 (least severe) to L4 (most severe) by sequencing the ITS regions of fungal rRNA genes using Illumina MiSeq. The richness and diversity of the fungal community was compared, and statistical analysis based on OUTs or taxonomic classification was also performed. We hope these results could give new perspectives on the function of the leaf microbiome in the control of pumpkin powdery mildew.

MATERIALS AND METHODS

Site and sampling

Leaf samples were randomly collected from pumpkin (*C. moschata*:*nen zao 1*) plants showing symptoms of powdery mildew disease. The samples were collected in June 2015 in the base of Vegetable Research Institute, Changsha, Hunan Province, China. The leaf samples were divided into four groups (L1–L4) based on the proportion of lesion area; L1 (no lesions), 6%<L2<11%, 11%≤L3<20%, L4≥40%, respectively. According to the incidence of powdery mildew of pumpkin (disease grade: 0-4) from 4 different areas, the same size of 10 pumpkin leaves were collected and mixed it into sterile bags, all the leaves are from different pumpkin plants at fruiting stage. Four biological replicates were performed in each treatment group. And each plot was sampled using five-point sampling within an area of 30 m^2**.** Leaf samples were collected in separate bags at refrigerated temperature, and were transferred to the laboratory for processing.

To harvest microbes on the leaf surface, 10 g of leaf were submerged in 100 mL of PBS with 0.01% Tween-80 in a 250 mL sterile conical flask. The flask was shaken at 250 rpm for 30 min at 28°C, and then subjected to ultrasound for 10 min. The microbes were then harvested using air pump filtration using a 0.22 μm filter; the microfiltration membrane was stored at –20°C.

DNA extraction and purification

The MP FastDNA ®SPIN Kit for soil (MP Biochemicals, Solon, OH, USA) was used to extract DNA from the leaf surface samples according to the manufacturer's protocol. DNA was extracted from the microbes harvested from the leaf surface. PCR amplicon libraries were prepared for each sample using the eukaryotic primers ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS2 (5′-GCTGCGTTCTTCA TCGATGC-3′) with the forward primer modified to contain a unique 6 nt barcode at the 5′ end. Fungal ITS1 regions were amplified in a total volume of 50 μL that contained 1 μL (5 μM) of each forward and reverse primer, 1.5 μL of dNTP mix (30 mM each), 0.5 μL of 5 U *Taq* DNA polymerase (TaKaRa), 5 mL of 10 × PCR buffer (with Mg^{2+}) and 1 μL of DNA. Reaction conditions consisted of an initial denaturation step at 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 20 s, primer annealing at 57°C for 25 s, and extension at 68°C for 45 s，and then a final extension at 68°C for 10 min. PCR products with a bright band of between 250 and 450 bp were collected by agarose gel electrophoresis and purified with an E.Z.N.A.® Gel Extraction Kit. The purified PCR amplicons were pooled in equimolar amounts using Qubit (CA，USA) and paired-end sequenced (2×250 bp) on an Illumina MiSeq platform by ANNOROAD Gene Technology Co., Ltd. (Beijing, China) according to standard protocols.

Processing of sequence data

After the MiSeq sequencing machine in fastq format, the raw sequence data reads were collected. Separate files were generated for the forward and reverse directions and the barcodes. Paired end reads were merged using the FLASH program (*Mago et al., 2011*). Sequences containing ambiguous 'N' were removed. Chimera sequences were detected and removed using UCHIME

(*Edgar et al., 2011*). All sequences with 97% similarity were clustered using the USEARCH software to yield operational taxonomic units (OTUs). Low abundance OTUs (≤2 counts) were eliminated from the OTU table. Representative sequences for each OTU were assigned to taxonomic groups using UNITE database (*Abarenkov et al., 2010*). In this study, all the sequences obtained were deposited in the SRA database short-read archive SRR5075731-SRR5075746.

Statistical analysis

The Mothur software was used to calculate rarefaction and diversity indices of all the leaf samples based on resampling of OTUs generated by USEARCH (*Schloss et al., 2009*). Detrended correspondence analysis (DCA) and Venn diagram analysis were performed in subsequent analyses using vegan package in R package v3.1.0. To determine whether the microbial communities present in the phyllosphere of pumpkin leaves with different disease levels were significantly different, the three nonparametric tests (MRPP, Adonis and ANOSIM) were used (*Anderson et al., 2001*). The statistical significance of differences between groups (including the Shannon index, the inverse Simpson index and the relative abundance of the taxonomic subgroups) was assessed by performing a one-way ANOVA followed by Tukey's multiple comparison post hoc test when comparing several groups. The data are presented as the mean ± SE. Besides, a P value of <0.05 was considered to be statistically significant. The software IBM SPSS for Windows, version 22.0 was used to perform statistical analyses.

RESULTS

Composition and structure of the pumpkin phyllosphere fungi

In total, 797,077 quality sequences were obtained for the four disease severity groups. The mean number of sequences per sample was 49,817, with a range of 39,028–62,150 sequences per sample. In total, 399 operational taxonomic units (OTUs) were detected using the UPARSE-OTU algorithm at the 97% identity cut-off. Rarefaction analysis and the Chao1 estimator indicated that the diversity in these leaf samples was within the same range (**Fig. 1**).

The four-way Venn diagrams in **Figure 2** show the distribution of the OTUs in the four disease severity groups. About 2.5% (10), 5.2% (21), 3.5% (14), and 1.2% (5) of all eukaryal OTUs were only found in disease severity group L1, L2, L3 or L4, respectively. And 38.8% (155) were present in the phyllosphere of all the groups. The OTU_2, OTU_3, OTU_5, and OTU_9 were identified as Fungi_sp|SH234328.06FU (https://blast.ncbi.nlm.nih.gov/Blast.cgi, it is matched the sequence NCBI accession KF800560.1, as a uncultured eukaryote clone CMH469 18S ribosomal RNA gene, partial sequence) at the species level, and accounted for 92.42%, 75.41%, 75.85%, and 14.76% of the sequence reads detected in leaves at disease severity levels L1, L2, L3 and L4, respectively. OTU_1 was identified as *Podosphaera_fusca*|SH194415.06FU, and accounted for 1.05%, 1.11%, 10.64%, and 77.9% of the sequence reads detected in leaves at disease severity levels L1, L2, L3 and L4, respectively.

Four fungal phyla, 15 classes and 36 orders were detected in the phyllosphere of the pumpkin samples (**Table 1**). The relative abundance of the main fungal phyllospheric populations at the taxonomic levels of Phyla and Class is shown in **Fig. 3** (a and b, respectively). Overall, the most abundant identifiable phyla were Fungi_unidentified and Ascomycota. The abundance of Fungi_unidentified was decreased while Ascomycota was increased as increased disease severity with leaf. The heatmap of genus level indicated the dominant genus was *Podosphaera* （**Fig. 4**）in the heavy symptoms of mildew infection (L3 and L4), which showed different levels of abundance among four disease severity groups. A lot of common OTUs were observed among these four different kinds of samples.

The multiple-response permutation procedure (MRPP), Adonis and ANOSIM analyses of the microbial communities (**Table 2**) indicate that the structures of the microbial communities detected in the phyllosphere of leaves with different disease levels (L1, L2, L3 and L4) were significantly different ($P < 0.05$). The detrended correspondence analysis (DCA) plot in **Fig. 5** shows that the communities detected in leaves with different disease levels were clearly

207 separated.

208 **Correlation between fungal communities and disease severity**

209 We compared the fungal alpha diversity of the pumpkin leaves using the Shannon and Inverse
210 Simpson diversity indices and OTU numbers (richness). The Shannon index ranged from 0.90 ±
211 0.09 to 1.87 ± 0.19, the Inverse Simpson index ranged from 1.61 ± 0.10 to 3.12 ± 0.53, and the
212 richness ranged from 110.25 ± 6.85 to 217.00 ± 20.84 for the four disease severity groups. The
213 results indicated that the fungal alpha diversity of the pumpkin leaves decreased significantly
214 with increasing disease severity from L2 to L4 (**Table 3**). However, alpha diversity in L2 leaves
215 was higher than in L1 leaves.

216 The fungal communities were dominated by members of the Ascomycota and the most
217 dominant genus was *Podosphaera* (**Fig. 6**). The abundance of *Ascomycota* and *Podosphaera*
218 increased with increasing disease severity. When the disease severity was greatest (L4), there
219 was less fungal diversity but a greater number of OTUs showed a high level of abundance.

220 **DISSCUSION**

221 A number of studies focused on the phyllosphere microorganisms in various plants while the
222 fungal community composition and diversity of pumpkin leaves infected with powdery mildew
223 has not been reported. In our study, amplicon pyrosequencing of the ITS region of rDNA were
224 used to detect the dynamics of fungal communities response to pathogen of pumpkin powdery
225 mildew. The dissimilarity among samples might be owing to the differences in the disease
226 severity, which could select the related fungi colonize pumpkin leaf surface.

227 Microorganisms are the largest population on our planet and participate in the
228 biogeochemical cycling of the Earth as an important component. Microorganisms could also play
229 a crucial role in keeping leaves healthy (*Baker et al., 2010*) and in maintaining the balance of the
230 ecosystem. A variety of beneficial microorganisms colonization on the plant leaves and help to
231 afford plant nutrition and defense against pathogens. Although there are more studies on the
232 plant rhizosphere, it has received considerably more attention in recent years, and interest in the

233 microbiology of leaf surfaces extends beyond pathogens now (*Vorholt, 2012*). Powdery mildew,
234 as a common fungal disease, that affects a wide range of plants, including cucurbits, such as
235 cucumbers, *Luffa* spp., melons and watermelons, leading to huge economic losses annually
236 (*Mcgrath and Shishkoff, 1999*). Among the different species of fungi in the order Erysiphales
237 caused powdery mildew, *Podosphaera xanthii* (a.k.a. *Sphaerotheca fuliginea*) being the most
238 commonly reported cause (*Mcgrath and Shishkoff, 1999*). The development of high-throughout
239 molecular techniques has helped to understand the microbial composition and structure in
240 different environments easily and know how microbial diversity changes as the disease severity
241 changes deeply.

242 Our study has provided new insights into the impact of the plant pathogen *Podosphaera* on
243 the microorganisms inhabiting the pumpkin phyllosphere, a serious pathogen that also causes
244 pumpkin powdery mildew. Previous studies have reported that there are usually more unique
245 OTUs in the rhizosphere of healthy soil than in diseased soil (*Rosenzweig et al., 2012*). In the
246 phyllosphere, there may be same phenomenon as the soil. In our study, the greatest number of
247 unique OTUs was found at disease severity level L2. Fungi_sp|SH234328.06FU was negatively
248 correlated with disease severity. There may be an antagonistic relationship between
249 Fungi_sp|SH234328.06FU and *Podosphaera_fusca*|SH194415.06FU(*Podosphaera_xanthii*). We
250 will investigate this relationship in the future study. The abundance of Ascomycota and
251 *Podosphaera* was positively correlated with disease severity. As the pathogen of pumpkin
252 powdery mildew, *Podosphaera* was the dominant genus in the in the heavy symptoms of mildew
253 infection. DCA, MRPP and adonis revealed significant differences in the composition and
254 structure of the fungal assemblages observed in the four disease severity groups (**Fig. 5, Table**
255 **2**), suggesting that the composition and structure of the fungal assemblages altered as the disease
256 severity increased.

257 The leaf fungal alpha diversity decreased significantly with increasing disease severity from
258 L2 to L4 (**Table 3**). This result agrees with findings reported by Manching et al. (*Manching et al.,*

2014), who analyzed the relationship between southern leaf blight disease severity and maize leaf epiphytic bacterial species richness. And it found that lower species richness (alpha diversity) was correlated with an increase of southern leaf blight disease severity when disease pressure was higher (*Manching et al., 2014*). The decline in overall fungal diversity was enhanced after pathogen stimulation, it also agrees with the results reported by Erlacher et al. (*Erlacher et al., 2014*). Interestingly, leaf fungal alpha diversity increased with increasing disease severity from L1 to L2, which suggests that the pathogen may have caused an increase in the fungal community richness at first and then a decrease when disease pressure was higher. It is well known that powdery mildew fungi are obligate biotrophs and will therefore compete for host nutrient reserves and suppress host defense responses. The growth and reproduction of other fungus could be inhibited when disease pressure was higher in the phyllosphere. This study further increases our understanding of the effect of powdery mildew disease on the microbial communities that inhabit the phyllosphere of pumpkin leaves. In addition, this is merely speculative that maintaining a rich and stable fungal community in the phyllosphere may be an efficient method of managing disease control in agroecological system and an essential step toward predictable biocontrol of powdery mildew.

CONCLUSIONS

In our current study, we demonstrated that the plant pathogen *Podosphaera_fusca* can affect the phyllosphere fungal communities of pumpkin. The pathogen caused an increase in the fungal community richness at first and then a decrease when disease pressure was higher. The decline in overall fungal diversity was enhanced after pathogen stimulation. The abundance of an unidentified genus as Fungi_sp|SH234328.06FU was inversely proportional to pathogen community of *Podosphaera*. In addition, our results showed that maintaining a rich and stable fungal community in the phyllosphere may be an efficient method of managing disease control in agroecological system and an essential step toward predictable biocontrol of powdery mildew.

ACKNOWLEDGMENTS

Illumina sequencing was performed at the ANNOROAD Gene Technology Co., Ltd., Beijing, China.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

This work was supported by the National Science and Technology Pillar Program during the 12th Five-year Plan Period (2014BAD05B04-4), the Agriculture Research System of China (CARS-25-B-05) and the Innovation Platform and Talent Plan (2016RS2019). The authors want to thank ANNOROAD Gene Technology Co., Ltd for data processing.

AUTHOR CONTRIBUTIONS

- ZZ and LL conceived and designed the experiments.
- LL, DJ, XT and XK performed the experiments and analyzed the data.
- LL, YL and DZ drafted the manuscript.
- JY, YL and DW provided the experimental materials.

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Conflicts of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Figure captions

Figure 1 Rarefaction curves for the operational taxonomic units (OTUs).

R01-R04: four replicate samples of the L1 level; R11-R14: four replicate samples of the L2 level; R21-R24: four replicate samples of the L3 level; R31-R34: four replicate samples of the L2 level.

Figure 2 Venn diagram showing unique and shared OTUs detected in the phyllosphere of the four disease severity groups (L1, L2, L3 and L4).

Figure 3 Relative abundance of fungal at the phylum and class level.

Figure 4 Heat map of the top 30 genera detected in all the samples.

R01-R04: four replicate samples of the L1 level; R11-R14: four replicate samples of the L2 level; R21-R24: four replicate samples of the L3 level; R31-R34: four replicate samples of the L2 level. Different colors represent different relative abundances, red represents the high relative abundance, and green represents the low relative abundance.

Figure 5 Detrended correspondence analysis (DCA). L1-L4 indicate the severity level of powdery mildew disease in each pumpkin leaf. N=4.

Figure 6 Relative abundance of Ascomycota and Podosphaera at different severity levels of powdery mildew disease (L1–L4).

Figure 1

Rarefaction curves for the operational taxonomic units (OTUs).

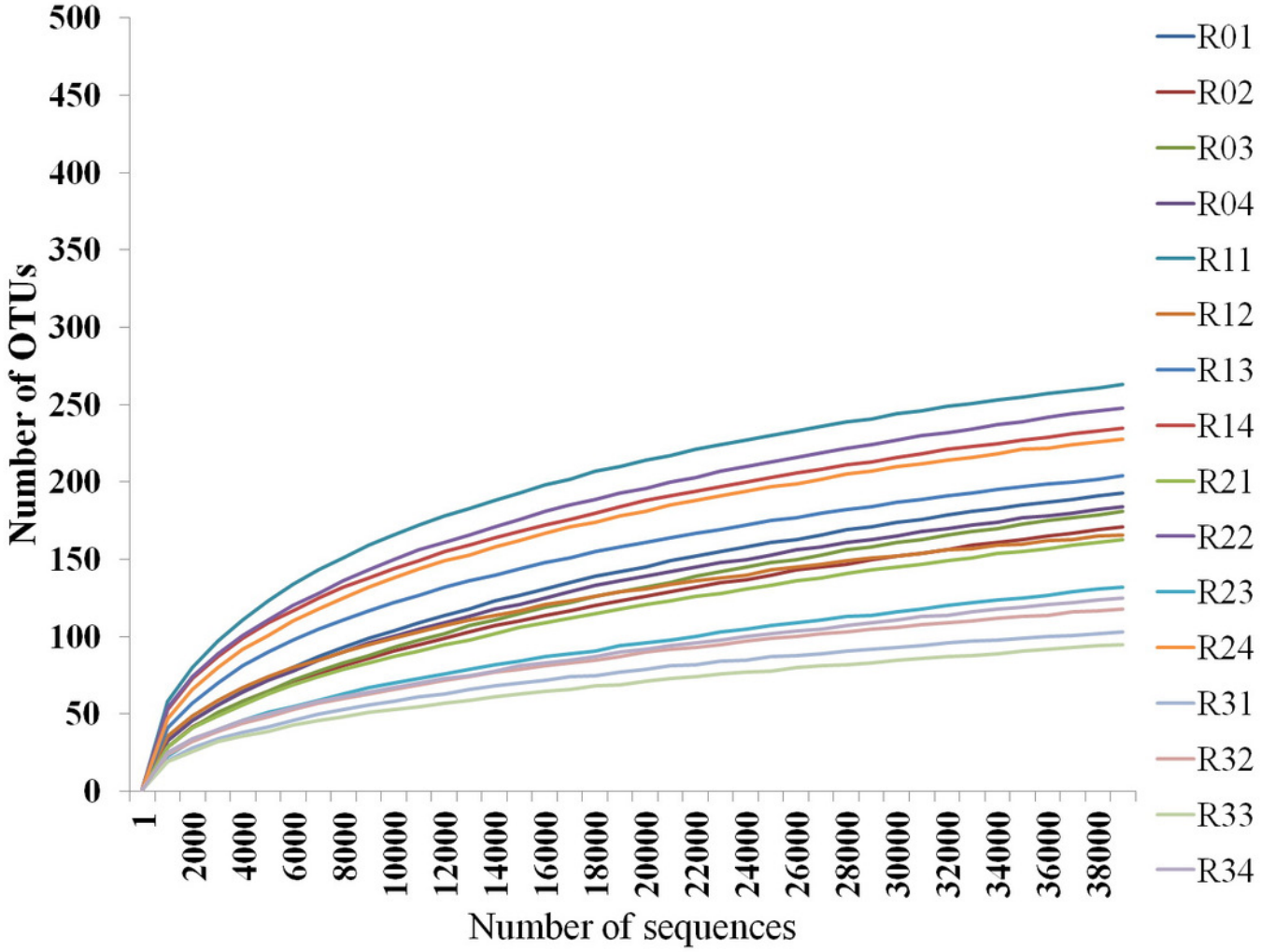

Figure 2

Venn diagram showing unique and shared OTUs detected in the phyllosphere of the four disease severity groups (L1, L2, L3 and L4).

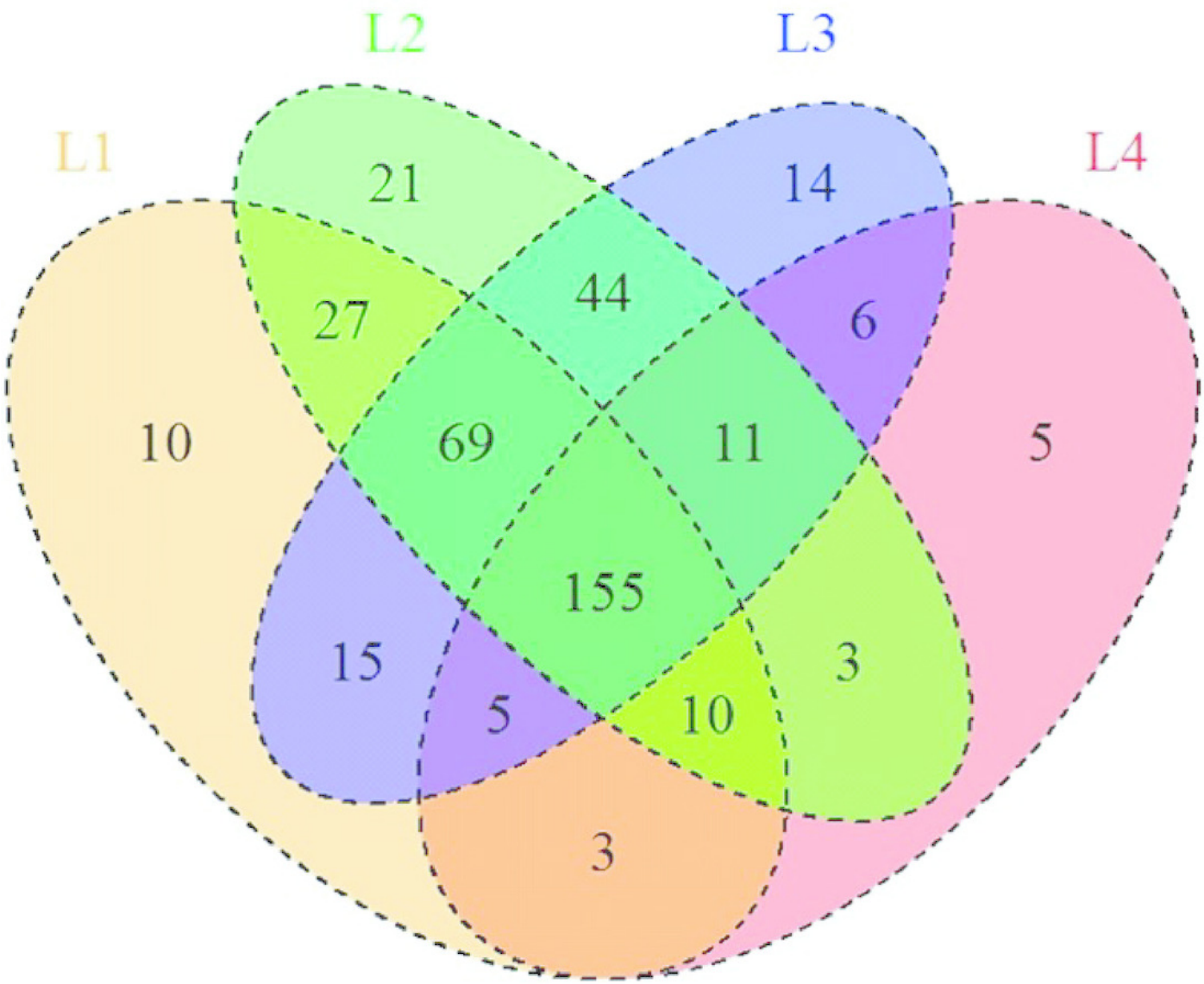

Figure 3

Relative abundance of fungal at the phylum and class level.

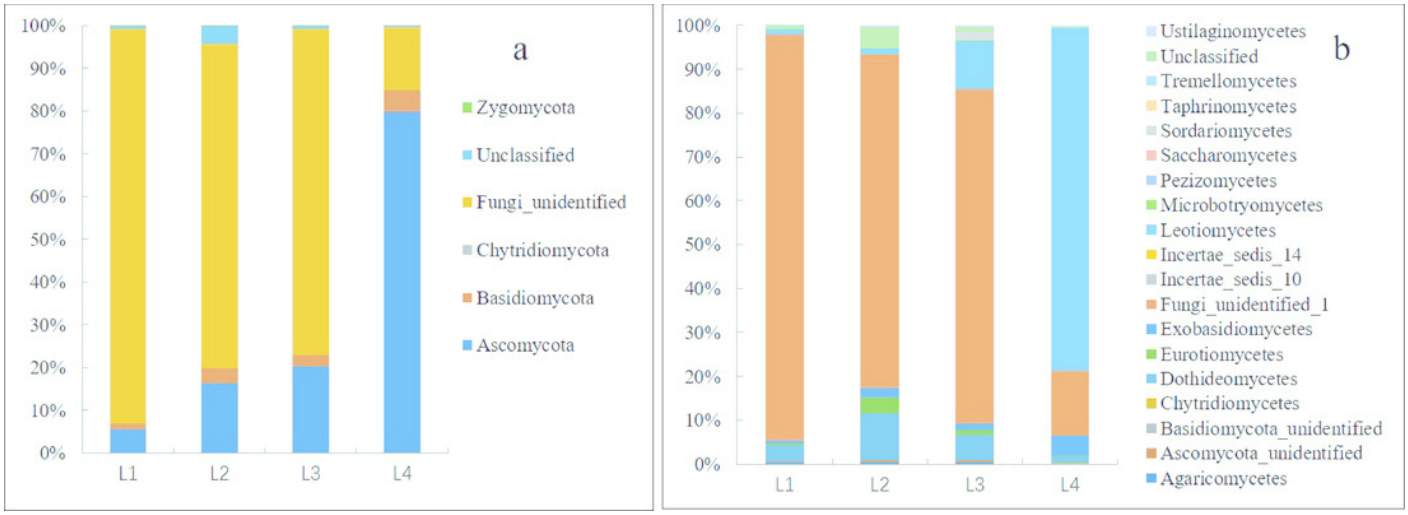

Figure 4

Heat map of the top 30 genera detected in all the samples.

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Figure 5

Detrended correspondence analysis (DCA). L1-L4 indicate the severity level of powdery mildew disease in each pumpkin leaf. N=4.

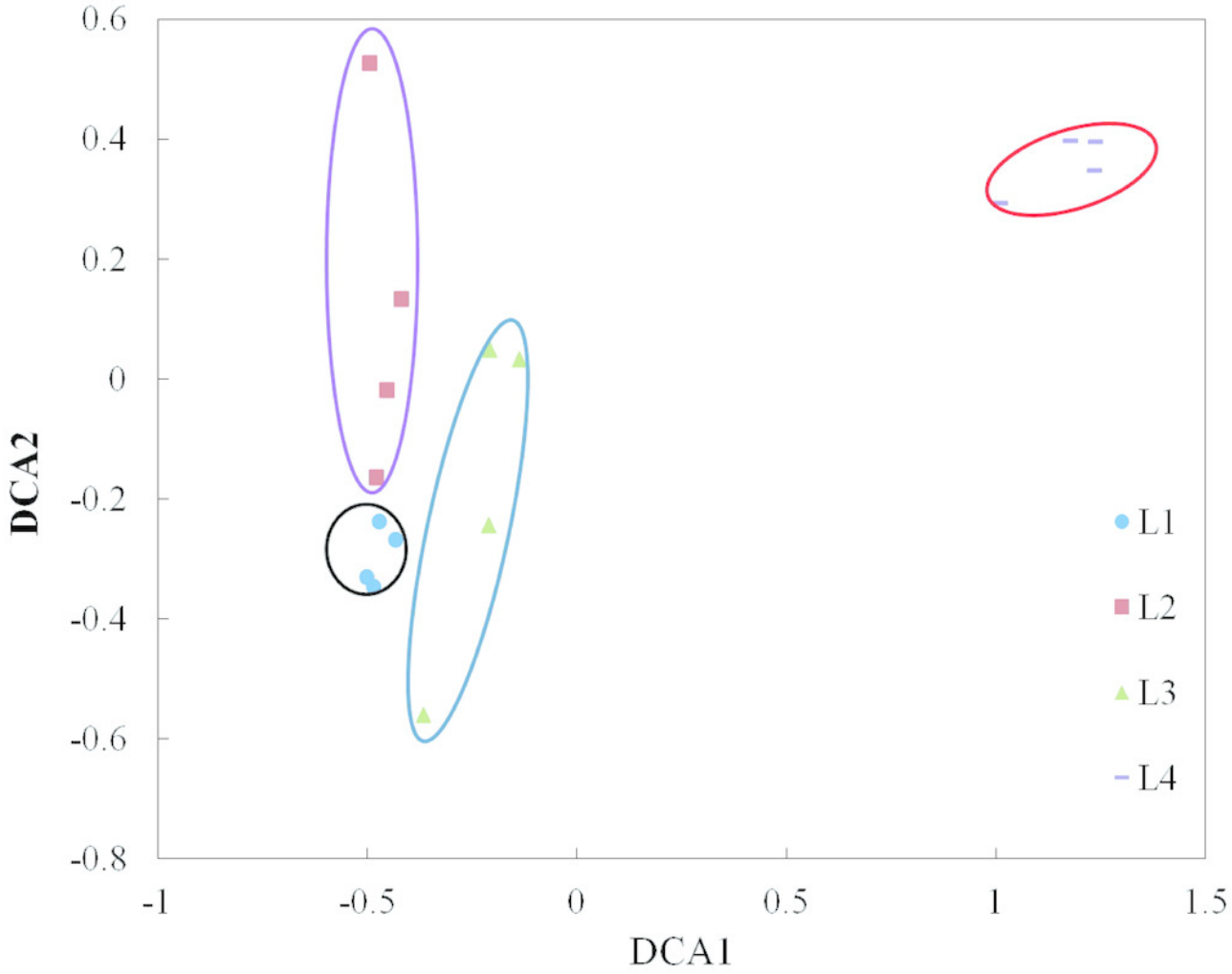

Figure 6

Relative abundance of Ascomycota and Podosphaera at different severity levels of powdery mildew disease (L1–L4).

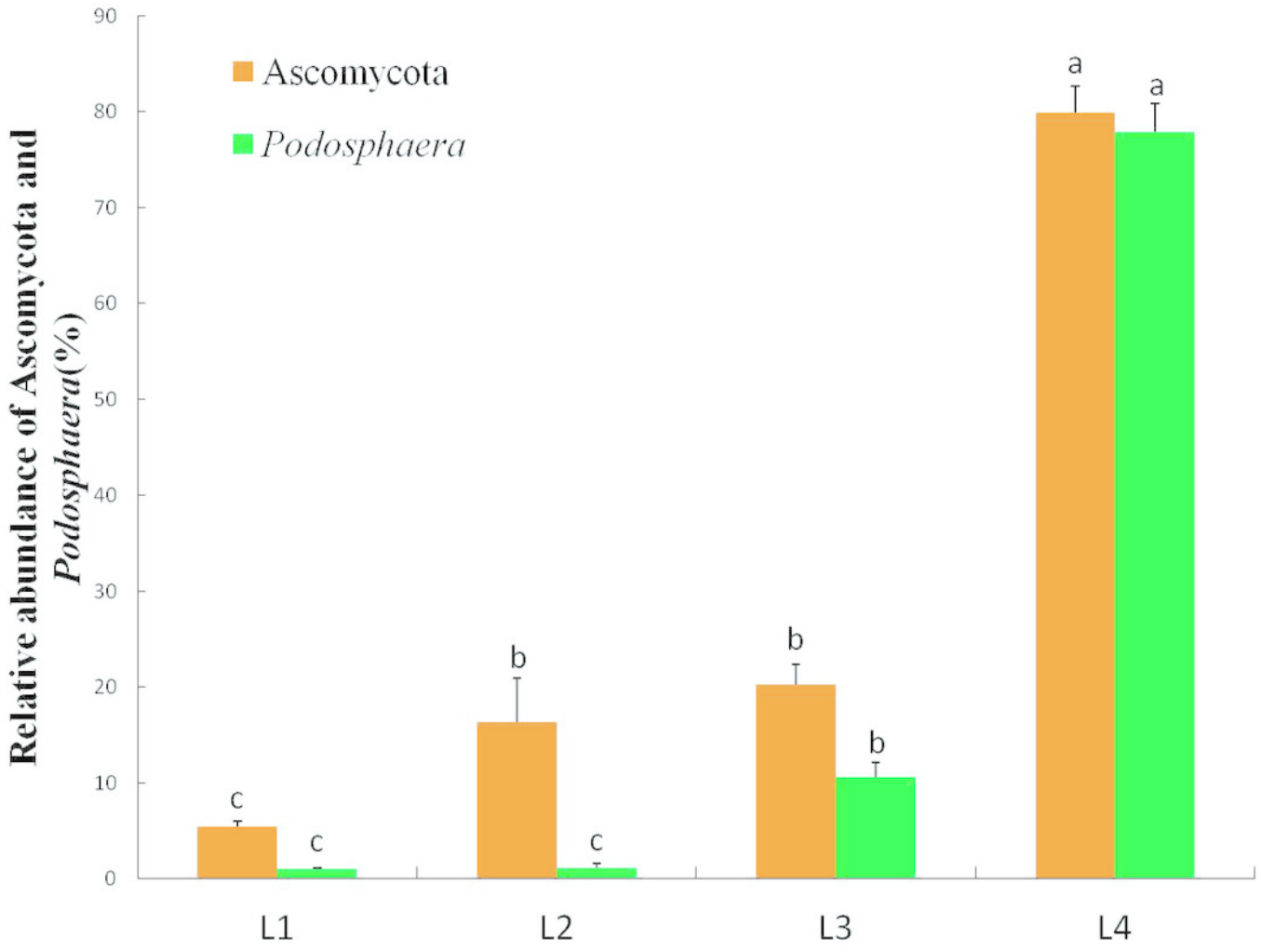

Table 1(on next page)

Number of detected phylotypes classified at different taxonomic levels.

1

Table 1 **Number of detected phylotypes classified at different taxonomic levels.**

Disease severity groups	Phylum	Class	Order	Family	Genus
No. of detected phylotypes	4	15	36	70	101
L1	3	14	35	63	86
L2	3	15	35	66	92
L3	3	14	31	68	87
L4	4	13	30	53	67

2

Table 2(on next page)

The dissimilarity of the fungal community composition in the phyllosphere of pumpkin.

1

2 **Table 2 The dissimilarity of the fungal community composition in the phyllosphere of pumpkin.**

Disease	MRPP		Adonis		Anosim	
severity levels	*P*	Delta	*P*	R^2	*P*	R
L1–L2	0.03	0.145	0.031	0.392	0.026	0.3125
L1–L3	0.037	0.113	0.032	0.539	0.034	0.7188
L1–L4	0.029	0.089	0.026	0.98	0.028	1
L2–L3	0.03	0.178	0.026	0.346	0.024	0.4271
L2–L4	0.038	0.153	0.034	0.936	0.029	1
L3–L4	0.034	0.121	0.035	0.953	0.034	1

3

4

Table 3(on next page)

Diversity indices of the communities on leaf surface showed different disease severity.

Table 3 **Diversity indices of the communities on leaf surface showed different disease severity.**

Group	Richness	Shannon index	Inverse Simpson index	Ace	Chao1
L1	182.25 ± 4.53	1.23 ± 0.03	2.03 ± 0.08	324.47±24.48	279.47±10.47
L2	217.00 ± 20.84	1.87 ± 0.19	3.12 ± 0.53	296.7±24.94	283.08±23.85
L3	192.75 ± 27.19	1.62 ± 0.16	2.64 ± 0.18	341.67±14.09	290.35±22.21
L4	110.25 ± 6.85	0.90 ± 0.09	1.61 ± 0.10	249.61±36.63	181.91±15.71