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# 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.

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

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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; Fonseca *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 cinerea* (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),  $6\% < L2 < 11\%$ ,  $11\% \leq L3 < 20\%$ ,  $L4 \geq 40\%$ , 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<sup>2</sup>. 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 µL of 10 × PCR buffer (with Mg<sup>2+</sup>) 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 ( $\leq 2$  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  $\pm$  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

separated.

### Correlation between fungal communities and disease severity

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

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

## DISCUSSION

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

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

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

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

The leaf fungal alpha diversity decreased significantly with increasing disease severity from 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.

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### 293 **AUTHOR CONTRIBUTIONS**

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

## 298 **REFERENCES**

- 299 **Abarenkov K, Nilsson RH, Larsson KH, Alexander LJ, Eberhardt U, Erland S, Høiland K,**  
300 **Kjøller R, Larsson E. 2010.** The UNITE database for molecular identification of fungi-recent  
301 updates and future perspectives. *New Phytologist* **186(2)**:281–285 DOI: [10.1111/j.1469-](https://doi.org/10.1111/j.1469-8137.2009.03160.x)  
302 [8137.2009.03160.x](https://doi.org/10.1111/j.1469-8137.2009.03160.x).
- 303 **Adesina MF, Grosch R, Lembke A, Vatchev TD, Smalla K. 2009.** In vitro antagonists of  
304 *Rhizoctonia solani* tested on lettuce: rhizosphere competence, biocontrol efficiency and  
305 rhizosphere microbial community response. *FEMS Microbiology Ecology* **69(1)**: 62–74  
306 DOI:[10.1111/j.1574-6941.2009.00685.x](https://doi.org/10.1111/j.1574-6941.2009.00685.x).
- 307 **Anderson MJ. 2001.** A new method for non-parametric multivariate analysis of variance.  
308 *Austral Ecology* **26(1)**:32–46 DOI: [10.1111/j.1442-9993.2001.01070.pp.x](https://doi.org/10.1111/j.1442-9993.2001.01070.pp.x).
- 309 **Baker CM, Chitrakar R, Obulareddy N, Panchal S, Williams P, Melotto M. 2010.**  
310 Molecular battles between plant and pathogenic bacteria in the phyllosphere. *Brazilian*  
311 *Journal of Medical & Biological Research* **43(8)**:698–704.

- 312 **Becker R, Behrendt U, Hommel B, Kropf S, Ulrich A. 2008.** Effects of transgenic fructan-  
313 producing potatoes on the community structure of rhizosphere and phyllosphere bacteria.  
314 *FEMS Microbiology Ecology* **66(2)**: 411–425 DOI: [10.1111/j.1574-6941.2008.00562.x](https://doi.org/10.1111/j.1574-6941.2008.00562.x).
- 315 **Berg G, Eberl L, Hartmann A. 2005.** The rhizosphere as a reservoir for opportunistic human  
316 pathogenic bacteria. *Environmental Microbiology* **7(11)**:1673–1685 DOI: [10.1111/j.1462-2920.2005.00891.x](https://doi.org/10.1111/j.1462-2920.2005.00891.x).
- 317
- 318 **Bodenhause N, Bortfeldmiller M, Ackermann M, Vorholt J. A. 2014.** A synthetic  
319 community approach reveals plant genotypes affecting the phyllosphere microbiota. *Plos*  
320 *Genetics* **10(4)**: 72–73 DOI: [10.1371/journal.pgen.1004283](https://doi.org/10.1371/journal.pgen.1004283).
- 321 **Chowdhury SP, Dietel K, Rändler M, Schmid M, Junge H, Borriss R, Hartmann A, Grosch**  
322 **R. 2013.** Effects of *Bacillus amyloliquefaciens* FZB42 on lettuce growth and health under  
323 pathogen pressure and its impact on the rhizosphere bacterial community. *Plos One* **8(7)**:  
324 e68818 DOI:[10.1371/journal.pone.0068818](https://doi.org/10.1371/journal.pone.0068818).
- 325 **Compant S, Duffy B, Nowak J, Clement C, Barka EA. 2005.** Use of plant growth-promoting  
326 bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future  
327 prospects. *Applied and Environmental Microbiology* **71(9)**: 4951–4959 DOI:  
328 [10.1128/AEM.71.9.4951-4959.2005](https://doi.org/10.1128/AEM.71.9.4951-4959.2005).
- 329 **Copeland JK, Yuan L, Layeghifard M, Wang PW, Guttman DS. 2015.** Seasonal community  
330 succession of the phyllosphere microbiome. *Molecular Plant-Microbe Interactions* **28(3)**:  
331 274–285 DOI: [10.1094/MPMI-10-14-0331-FI](https://doi.org/10.1094/MPMI-10-14-0331-FI).
- 332 **Cordier T, Robin C, Capdevielle X, Fabreguettes O, Desprez-Loustau ML, Vacher C. 2012.**  
333 The composition of phyllosphere fungal assemblages of European beech (*Fagus sylvatica*)  
334 varies significantly along an elevation gradient. *New Phytologist* **196(2)**: 510–519 DOI:  
335 [10.1111/j.1469-8137.2012.04284.x](https://doi.org/10.1111/j.1469-8137.2012.04284.x).
- 336 **Davey ML, Heegaard E, Halvorsen R, Ohlson M, Kauserud H. 2012.** Seasonal trends in the  
337 biomass and structure of bryophyte-associated fungal communities explored by 454  
338 pyrosequencing. *New Phytologist* **195(4)**: 844–856 DOI: [10.1111/j.1469-8137.2012.04215.x](https://doi.org/10.1111/j.1469-8137.2012.04215.x).
- 339 **Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. 2011.** UCHIME improves sensitivity  
340 and speed of chimera detection. *Bioinformatics* **27(16)**: 2194–2200 DOI:  
341 [10.1093/bioinformatics/btr381](https://doi.org/10.1093/bioinformatics/btr381).



- 342 **Erlacher A, Cardinale M, Grosch R, Grube M, Berg G. 2014.** The impact of the pathogen  
343 *Rhizoctonia solani* and its beneficial counterpart *Bacillus amyloliquefaciens* on the indigenous  
344 lettuce microbiome. *Front Microbiology* **5**: 175 DOI: [10.3389/fmicb.2014.00175](https://doi.org/10.3389/fmicb.2014.00175).
- 345 **Everett KR, Vanneste JL, Hallett IC, Walter M. 2005.** Ecological alternatives for disease  
346 management of fruit rot pathogens. *New Zealand Plant Protection* **58(2005)**, 55–61
- 347 **Finkel OM, Burch AY, Lindow SE, Post AF, Belkin S. 2011.** Geographical location  
348 determines the population structure in phyllosphere microbial communities of a salt-excreting  
349 desert tree. *Applied and Environmental Microbiology* **77(21)**: 7647–7655 DOI:  
350 [10.1128/AEM.05565-11](https://doi.org/10.1128/AEM.05565-11).
- 351 **Fonsecagarcía C, Colemanderr D, Garrido E, Visel A, Tringe SG, Partidamartínez LP.**  
352 **2016.** The cacti microbiome: interplay between habitat-filtering and host-specificity. *Front*  
353 *Microbiology* **7(287)**: 150 DOI: [10.3389/fmicb.2016.00150](https://doi.org/10.3389/fmicb.2016.00150).
- 354 **Hartmann A, Rothballer M, Schmid M. 2008.** Lorenz Hiltner, a pioneer in rhizosphere  
355 microbial ecology and soil bacteriology research. *Plant Soil* **312(1-2)**: 7–14  
356 DOI:[10.1007/s11104-007-9514-z](https://doi.org/10.1007/s11104-007-9514-z).
- 357 **Hirano SS, Upper CD. 2000.** Bacteria in the leaf ecosystem with emphasis on *Pseudomonas*  
358 *syringae*-a pathogen, ice nucleus, and epiphyte. *Microbiology and Molecular Biology Reviews*  
359 **64(3)**: 624–653 DOI: [10.1128/MMBR.64.3.624-653.2000](https://doi.org/10.1128/MMBR.64.3.624-653.2000).
- 360 **Hunter PJ, Pink DAC, Bending GD. 2015.** Cultivar-level genotype differences influence  
361 diversity and composition of lettuce (*Lactuca* sp.) phyllosphere fungal communities. *Fungal*  
362 *Ecology* **17(137)**: 41–56 DOI: [org/10.1016/j.funeco.2015.05.007](https://doi.org/10.1016/j.funeco.2015.05.007).
- 363 **Jackson CR, Denney WC. 2011.** Annual and seasonal variation in the phyllosphere bacterial  
364 community associated with leaves of the southern magnolia (*Magnolia grandiflora*).  
365 *Microbial Ecology* **61(1)**: 113–122 DOI:[10.1007/s00248-010-9742-2](https://doi.org/10.1007/s00248-010-9742-2).
- 366 **Lacava PT, Araújo WL, Azevedo JL, Hartung JS. 2006.** Rapid, specific and quantitative  
367 assays for the detection of the endophytic bacterium *Methylobacterium mesophilicum* in  
368 plants. *Journal of Microbiological Methods* **65(3)**: 535–541 DOI:  
369 [10.1016/j.mimet.2005.09.015](https://doi.org/10.1016/j.mimet.2005.09.015).
- 370 **Lebeda A, Mgrgrath MT, Sedlakova B. 2010.** Fungicide Resistance in Cucurbit Powdery  
371 Mildew Fungi. *Fungicides* **11**, 221-246 DOI: [10.5772/14080](https://doi.org/10.5772/14080).



- 372 **Leveau JHJ, Tech JJ. 2011.** Grapevine microbiomics: bacterial diversity on grape leaves and  
373 berries revealed by high-throughput sequence analysis of 16S rRNA amplicons. *Acta*  
374 *Horticulturae* **905(905)**: 31–42 DOI: [10.17660/ActaHortic.2011.905.2](https://doi.org/10.17660/ActaHortic.2011.905.2).
- 375 **Lindow SE, Brandl MT. 2003.** Microbiology of the phyllosphere. *Applied and Environmental*  
376 *Microbiology* **69(4)**: 1875–1883.
- 377 **Lindow SE, Leveau JH. 2002.** Phyllosphere microbiology. *Current Opinion Biotechnology*  
378 **13(3)** :238–243 DOI: [10.1016/S0958-1669\(02\)00313-0](https://doi.org/10.1016/S0958-1669(02)00313-0).
- 379 **Lopez-Velasco G, Carder PA, Welbaum GE, Ponder MA. 2013.** Diversity of the spinach  
380 (*Spinacia oleracea*) spermosphere and phyllosphere bacterial communities. *FEMS*  
381 *Microbiology Letters* **346(2)**: 146–154 DOI: [10.1111/1574-6968.12216](https://doi.org/10.1111/1574-6968.12216).
- 382 **Lopez-Velasco G, Welbaum GE, Boyer RR, Mane SP, Ponder MA. 2011.** Changes in  
383 spinach phylloepiphytic bacteria communities following minimal processing and refrigerated  
384 storage described using pyrosequencing of 16S rRNA amplicons. *Journal of Applied*  
385 *Microbiology* **110(5)**: 1203–1214 DOI: [10.1111/j.1365-2672.2011.04969.x](https://doi.org/10.1111/j.1365-2672.2011.04969.x).
- 386 **Mago T, Salzberg SL. 2011.** FLASH: fast length adjustment of short reads to improve genome  
387 assemblies. *Bioinformatics* **27(21)**: 2957–2963 DOI: [10.1093/bioinformatics/btr507](https://doi.org/10.1093/bioinformatics/btr507).
- 388 **Manching HC, Balintkurti PJ, Stapleton AE. 2014.** Southern leaf blight disease severity is  
389 correlated with decreased maize leaf epiphytic bacterial species richness and the phyllosphere  
390 bacterial diversity decline is enhanced by nitrogen fertilization. *Frontiers in Plant Science*  
391 **5**: 403 DOI: [10.3389/fpls.2014.00403](https://doi.org/10.3389/fpls.2014.00403).
- 392 **Mcgrath MT, Shishkoff N. 1999.** Evaluation of biocompatible products for managing cucurbit  
393 powdery mildew. *Crop Protection* **18(7)**: 471–478 DOI: [10.1016/S0261-2194\(99\)00048-4](https://doi.org/10.1016/S0261-2194(99)00048-4).
- 394 **Mejía LC, Rojas EI, Maynard Z, Bael V, Arnold AE, Hebbar P, et al. 2008.** Endophytic  
395 fungi as biocontrol agents of *Theobroma cacao* pathogens. *Biological Control* **46(1)**: 4–14  
396 DOI: [10.1016/j.biocontrol.2008.01.012](https://doi.org/10.1016/j.biocontrol.2008.01.012).
- 397 **Mwajita MR, Murage H, Tani A, Kahangi EM. 2012.** Evaluation of rhizosphere, rhizoplane  
398 and phyllosphere bacteria and fungi isolated from rice in Kenya for plant growth promoters.  
399 *Springerplus* **2(1)**: 1–9 DOI: [10.1186/2193-1801-2-606](https://doi.org/10.1186/2193-1801-2-606).
- 400 **Rajendran L, Ramanathan A, Durairaj C, Samiyappan R. 2011.** Endophytic *Bacillus subtilis*  
401 enriched with chitin offer induced systemic resistance in cotton against aphid infestation.

- Archives of Phytopathology and Plant Protection. **44(14)**: 1375–1389 DOI: <https://doi.org/10.1080/03235408.2010.499719>.
- Redford AJ, Bowers RM, Knight R, Linhart Y, Fierer N. 2010.** The ecology of the phyllosphere: geographic and phylogenetic variability in the distribution of bacteria on tree leaves. *Environmental Microbiology* **12(11)**: 2885–2893 DOI: [10.1111/j.1462-2920.2010.02258.x](https://doi.org/10.1111/j.1462-2920.2010.02258.x).
- Reisberg EE, Hildebrandt U, Riederer M, Hentschel U. 2013.** Distinct phyllosphere bacterial communities on Arabidopsis wax mutant leaves. *Plos One* **8(11)**: e78613 DOI: [10.1371/journal.pone.0078613](https://doi.org/10.1371/journal.pone.0078613).
- Rosenzweig N, Tiedje JM, Quensen III JF, Meng Q, Hao JJ. 2012.** Microbial communities associated with potato common scab-suppressive soil determined by pyrosequencing analyses. *Plant Disease* **96**, 718–725 DOI: <https://doi.org/10.1094/PDIS-07-11-0571>.
- Sapkota R, Knorr K, Jørgensen L N, O'Hanlon KA, Nicolaisen M. 2015.** Host genotype is an important determinant of the cereal phyllosphere mycobiome. *New Phytologist* **207(4)**: 1134–1144 DOI: [10.1111/nph.13418](https://doi.org/10.1111/nph.13418).
- Scherwinski K., Wolf A, Berg G. 2007.** Assessing the risk of biological control agents on the indigenous microbial communities: *Serratia plymuthica* HRO-C48 and *Streptomyces* sp. HRO-71 as model bacteria. *BioControl* **52(1)**: 87–112 DOI: [10.1007/s10526-006-9006-8](https://doi.org/10.1007/s10526-006-9006-8).
- Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF. 2009.** Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology* **75(23)**: 7537–7541 DOI: [10.1128/AEM.01541-09](https://doi.org/10.1128/AEM.01541-09).
- Schmidt CS, Alavi M, Cardinale M, Müller H, Berg, G. 2012.** *Stenotrophomonas rhizophila* DSM14405<sup>T</sup> promotes plant growth probably by altering fungal communities in the rhizosphere. *Biology and Fertility of Soils* **48(8)**: 947–960 DOI: [10.1007/s00374-012-0688-z](https://doi.org/10.1007/s00374-012-0688-z).
- Shi Z, Wang F, Zhou W, Zhang P, Fan Y. 2007.** Application of osthonol induces a resistance response against powdery mildew in pumpkin leaves. *International Journal of Molecular Sciences* **8(9)**: 1001–1012.
- Ritpitakphong U, Falquet L, Vimoltust A, Berger A, Métraux JP, L'Haridon F. 2016.** The microbiome of the leaf surface of arabidopsis protects against a fungal pathogen. *New Phytologist* **210(3)**: 1033–1043 DOI: [10.1111/nph.13808](https://doi.org/10.1111/nph.13808).

**Vorholt JA. 2012.** Microbial life in the phyllosphere. *Nature Reviews Microbiology* **10(12):** 828–840 DOI:10.1038/nrmicro2910.

**Whipps JM, Hand P, Pink D, Bending GD. 2008.** Phyllosphere microbiology with special reference to diversity and plant genotype. *Journal of Applied Microbiology* **105(6):** 1744–1755 DOI: 10.1111/j.1365-2672.2008.03906.x.

**Yang T, Sun HB, Shen CC, Chu HY. 2016.** Fungal assemblages in different habitats in an Erman's birch forest. *Frontiers in Microbiology* **7:** 244 DOI: 10.3389/fmicb.2016.01368.

**Zhang Y, Cong J, Lu H, Li GL, Xue YD, Deng Y, Li H, Zhou JZ, Li DQ. 2015.** Soil bacterial diversity patterns and drivers along an elevational gradient on Shennongjia Mountain, China. *Microbial Biotechnology* **8(4):** 739–746 DOI: 10.1111/1751-7915.12288.

**Zhang Z, Wei J, Han X, Liang L, Yang Y, Meng H, Xu YH, Gao ZH. 2014.** The sesquiterpene biosynthesis and vessel-occlusion formation in stems of *Aquilaria sinensis* (Lour.) Gilg trees induced by wounding treatments without variation of microbial communities. *International Journal of Molecular Sciences* **15(12):** 23589–23603 DOI:10.3390/ijms151223589.

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466 **Figure captions**

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

468 R01-R04: four replicate samples of the L1 level; R11-R14: four replicate samples of the L2 level;

469 R21-R24: four replicate samples of the L3 level; R31-R34: four replicate samples of the L2 level.

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

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

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

474 R01-R04: four replicate samples of the L1 level; R11-R14: four replicate samples of the L2 level;

475 R21-R24: four replicate samples of the L3 level; R31-R34: four replicate samples of the L2 level.

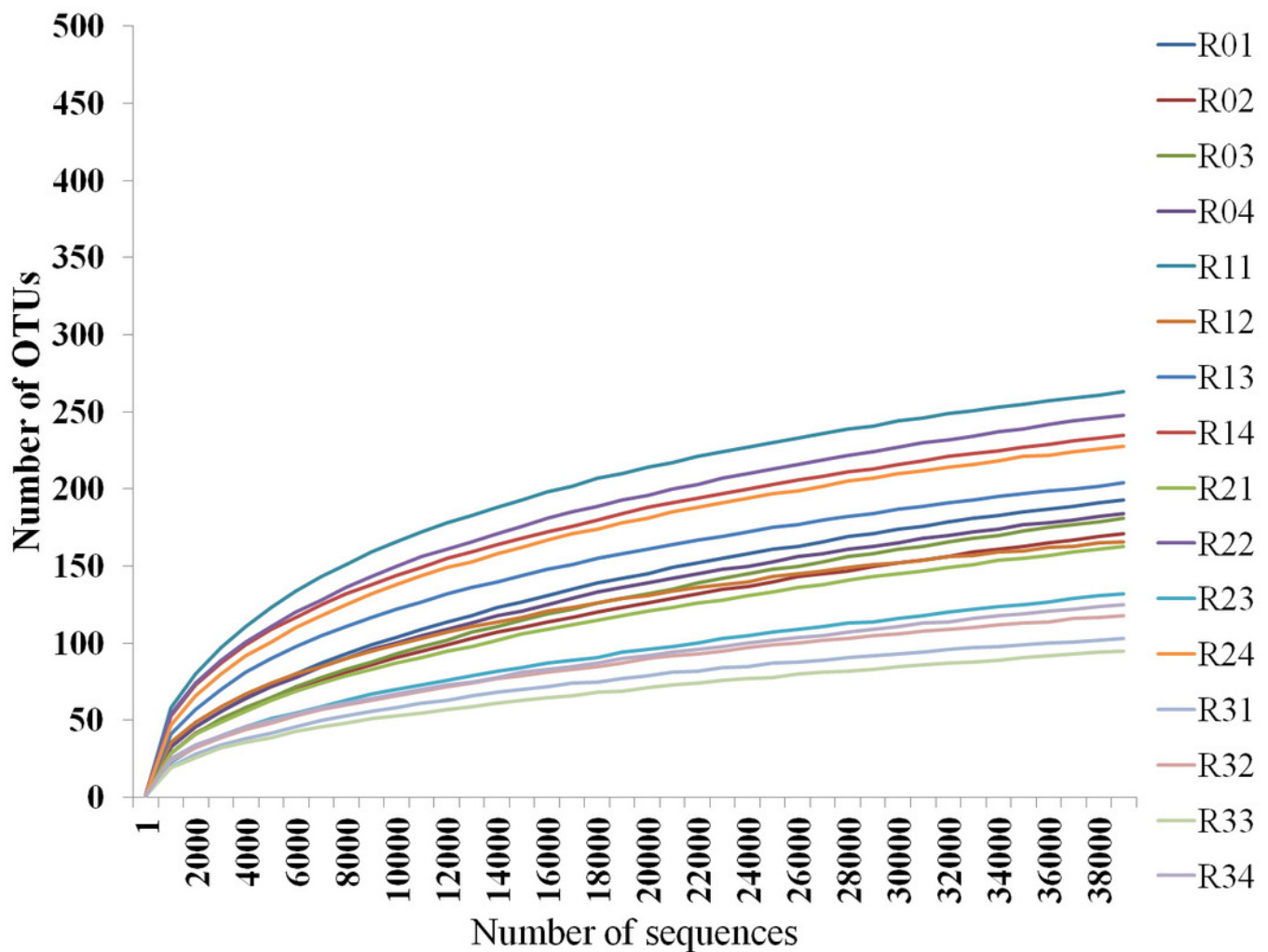
476 Different colors represent different relative abundances, red represents the high relative  
477 abundance, and green represents the low relative abundance.

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

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

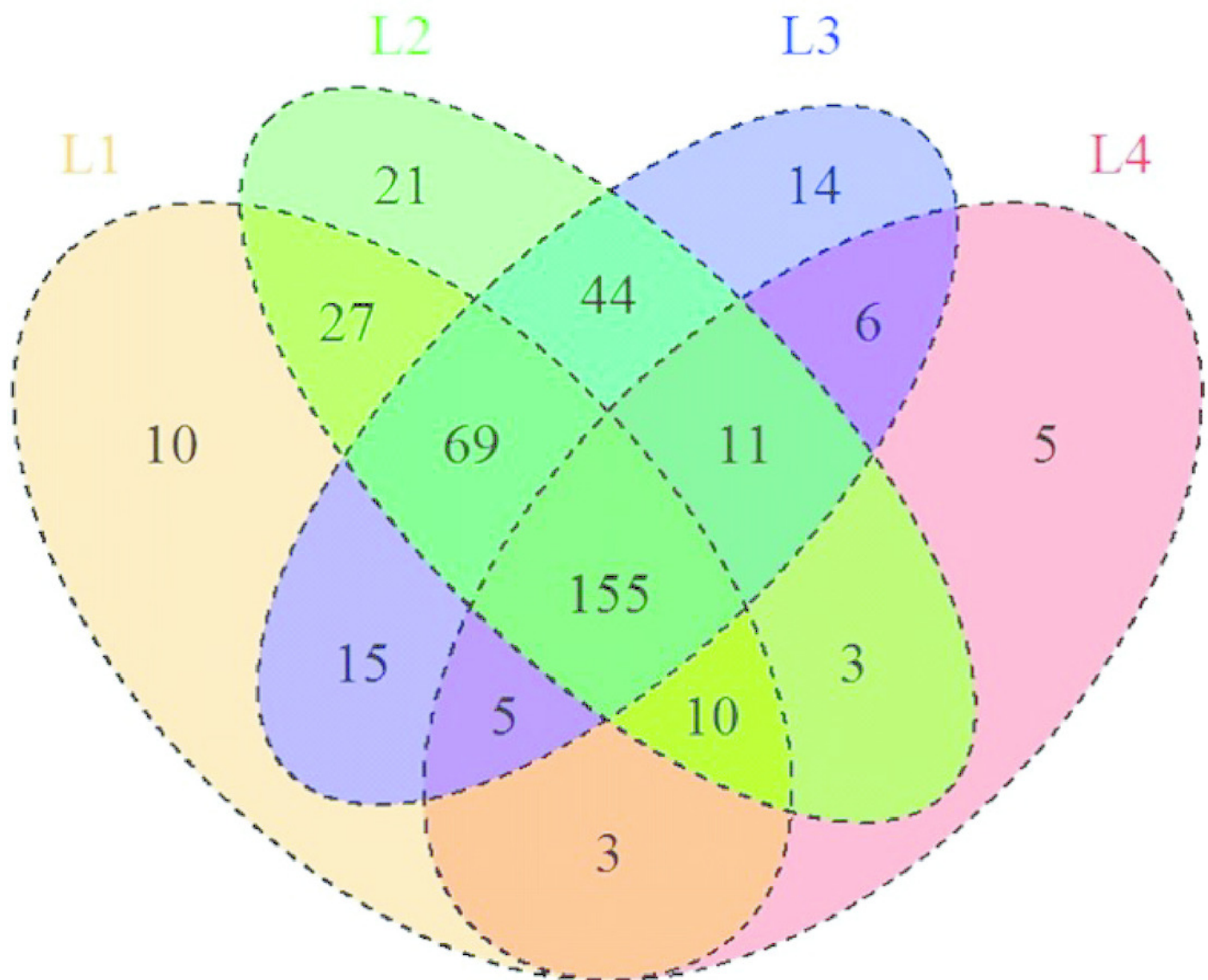
# Figure 1

Rarefaction curves for the operational taxonomic units (OTUs).



# Figure 2

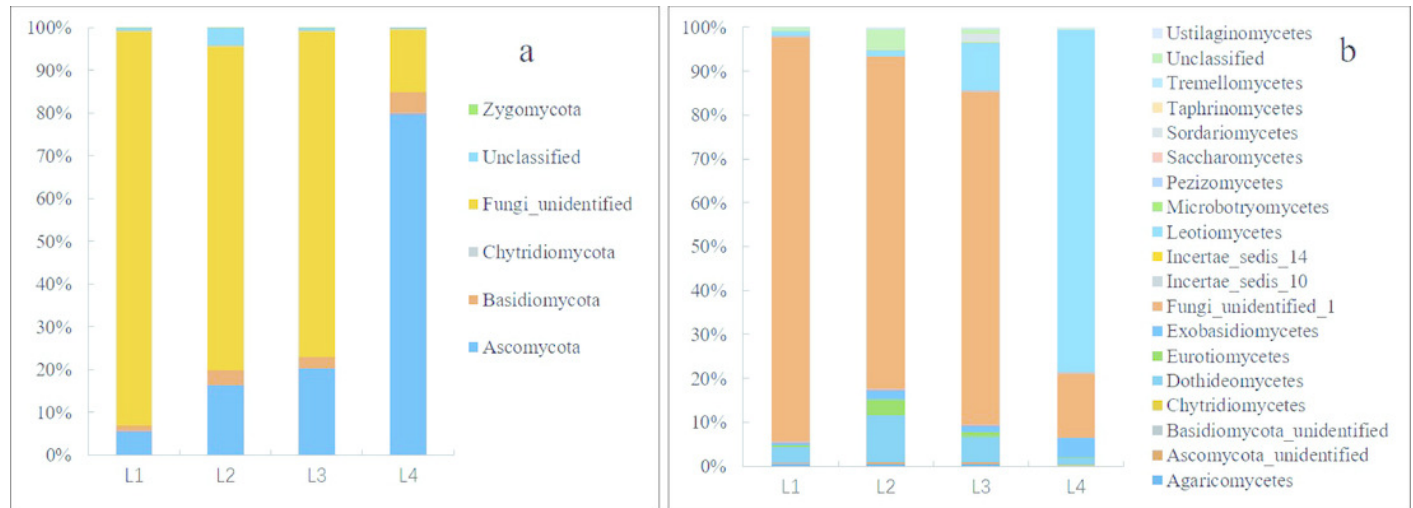
Venn diagram showing unique and shared OTUs detected in the phyllosphere of the four disease severity groups (L1, L2, L3 and L4).





# Figure 3

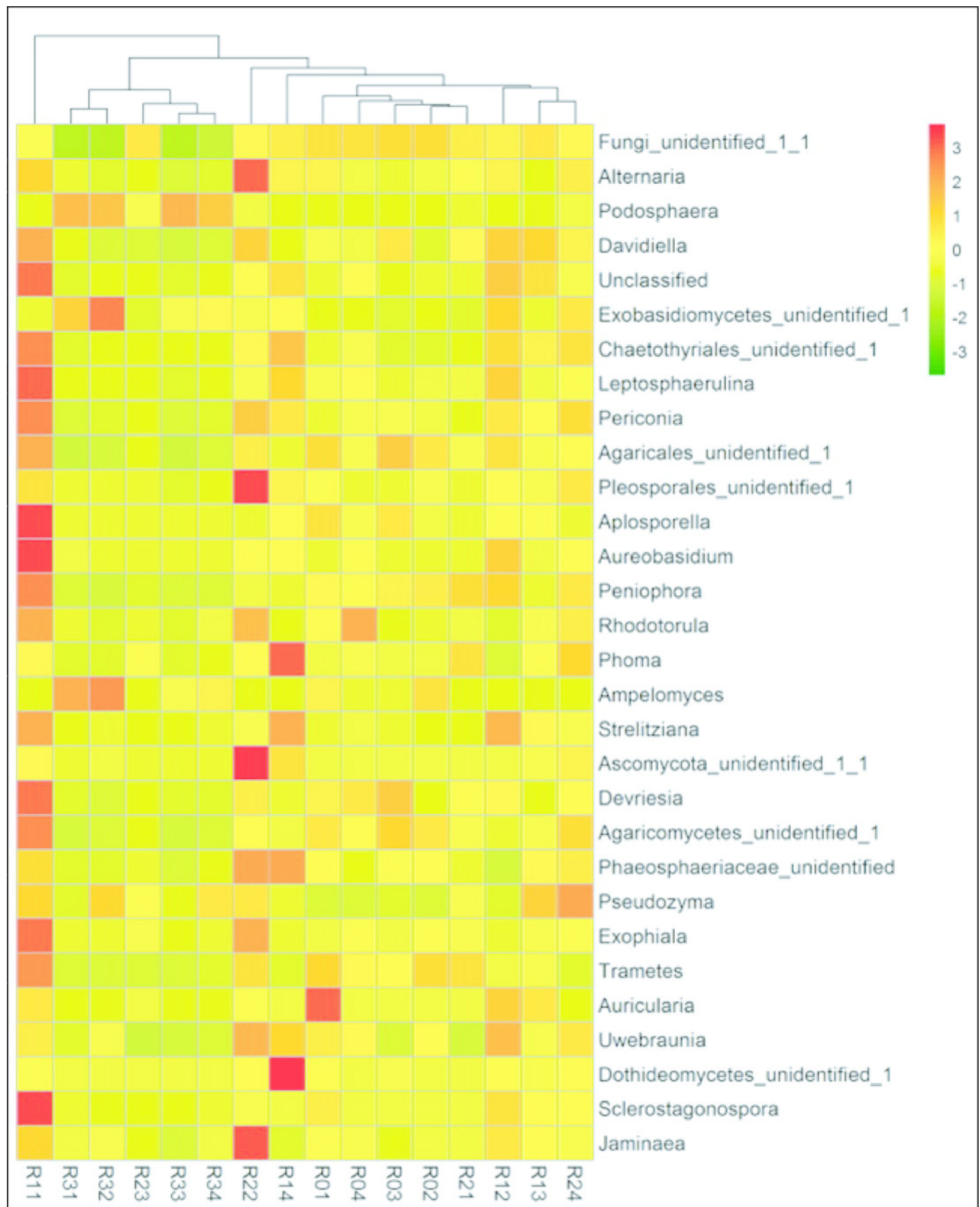
Relative abundance of fungal at the phylum and class level.



# Figure 4

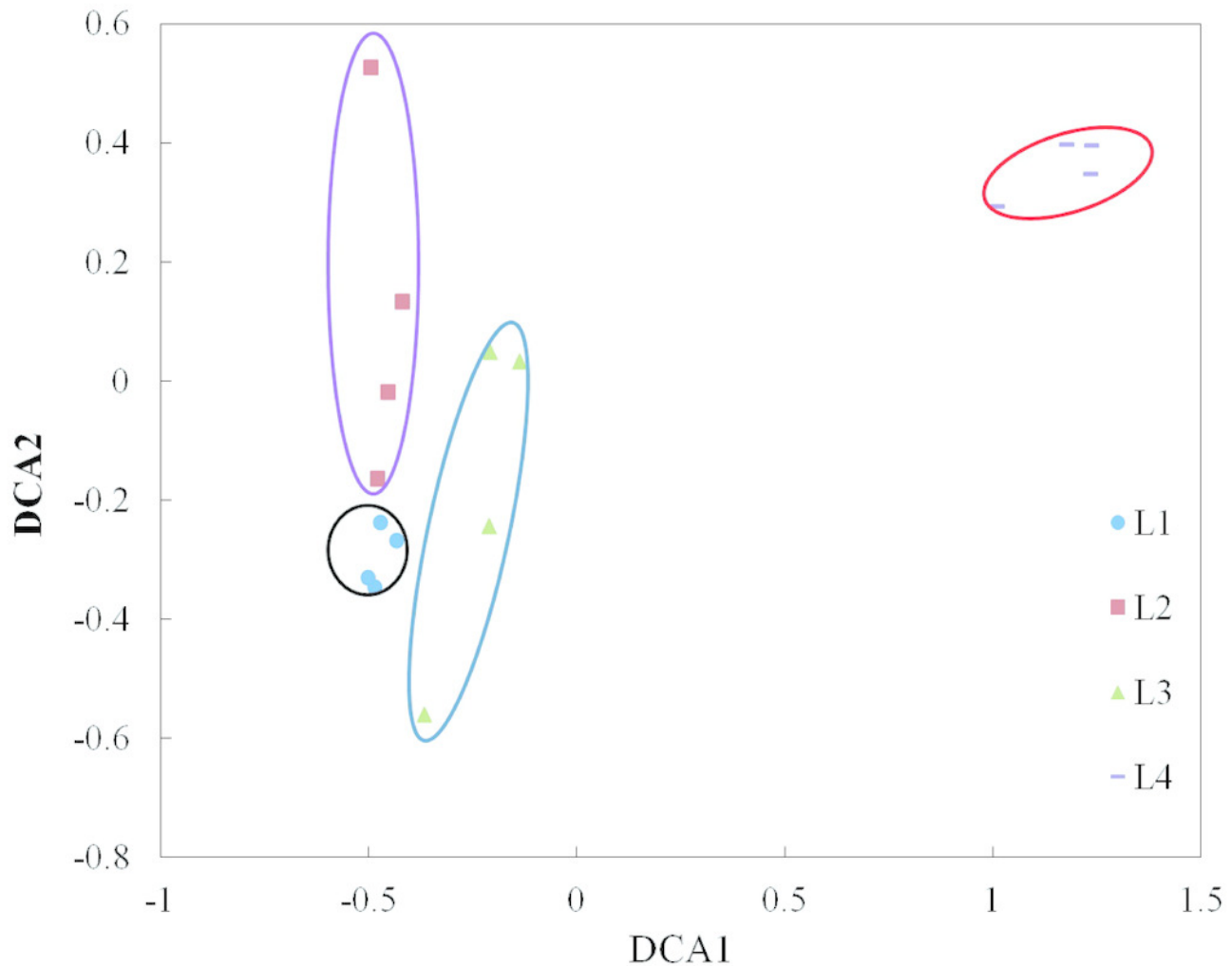
Heat map of the top 30 genera detected in all the samples.





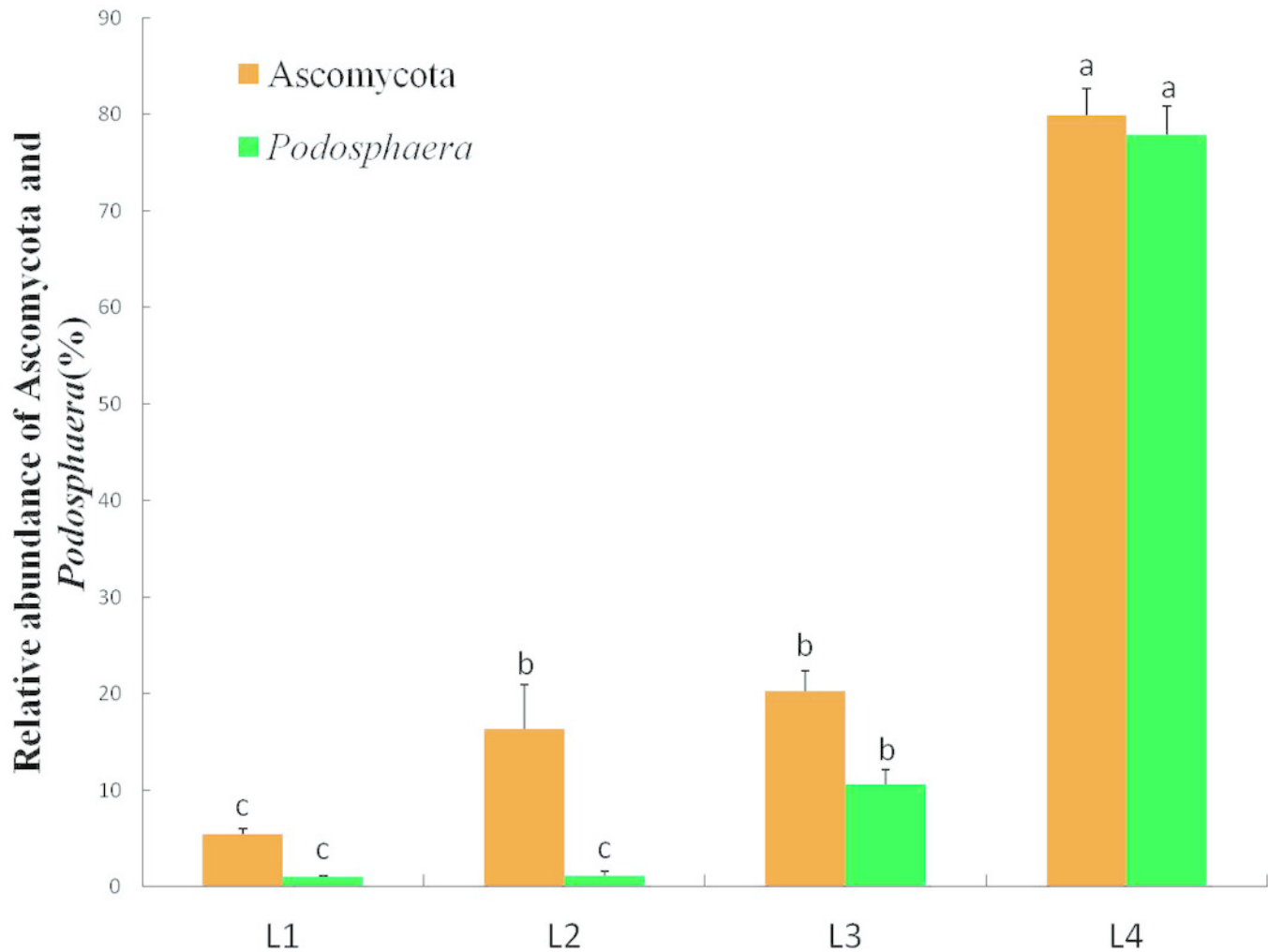
# Figure 5

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.

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**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.

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

| Disease<br>severity levels | MRPP     |       | Adonis   |                       | Anosim   |          |
|----------------------------|----------|-------|----------|-----------------------|----------|----------|
|                            | <i>P</i> | Delta | <i>P</i> | <i>R</i> <sup>2</sup> | <i>P</i> | <i>R</i> |
| 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        |

### **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<br>index | Inverse<br>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 |