XXII International Workshop on Smuts and Bunts

Microbiome signature of endophytes in wheat seed response to wheat dwarf bunt caused by Tilletia controversa Kühn

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http://www.doc88.com/p-5837177986268.html
Significance of smut diseases in Colorado

Fig 1. Healthy and bunted (*T. controversa*).
（B.J. Goates）

Fig 2. Close-up of wheat ear smutted by *T. controversa*, showing smutted grains removed and broken open (Priekule, 2007)

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1. Introduction

- The seed microbiota can influence seed germination and seedling phenotype or drive root microbiota assembly, with the potential to promote plant growth and sustainably protect crops from pathogens (Matsumoto et al., 2021). Assessing the impacts of seed treatments on microbial diversity will be one of the key factors in controlling pathogen invasion.
- The traditional control of wheat dwarf bunt depends on stirring a fungicide together with the seeds, which are not environmentally friendly. Here, we analyzed the seed microbiome signatures of endophytes between resistant and susceptible cultivars after infection with *T. controversa* based on a combination of amplicon sequencing and isolation approaches.
- This study will explore the microbiome signature of endophytes in the seed to increase the understanding of endophytic community structure and supply information that will provide a promising selection and contribute to the control of wheat seedborne disease in an eco-friendly manner.

2. Materials and Methods

- **Seed treatment, plant culture, and pathogen inoculation**
- **Endophytic microorganism detection based on isolation**
- **Endophytic microorganism detection based on amplicon sequencing**
- **Suppression of the germination rates of the teliospores of *T. controversa* by potential antagonist isolates**

2.1 Seed treatment, plant culture, and pathogen inoculation

Disinfected seeds of 24 wheat cultivars were germinated in sterile moist muslin cloth for 1 month, planted in pots (mixed with organic matter and soil at a ratio of 1:2), and placed into growth chambers (LT-36VL; Percival Scientific, USA) under 24 h of light and 60 % relative humidity.

Spores of T. controversa were inoculated into the root zone of wheat plants, and this process was repeated 5 times at 1-day intervals. Plants that received ddH2O inoculation were used as controls. Finally, seven spikes each were taken from the inoculated and uninoculated wheat of each cultivar.

Table 1. List of the varieties used in this study

Variety name	Treatment	Groups	Sample ID
Resistant cultivar 1	Infected	RI	S1
Resistant cultivar 2	Noninfected	RH	S2
Resistant cultivar 3	Infected	RI	S3
Resistant cultivar 4	Noninfected	RH	S4
Resistant cultivar 5	Infected	RI	S5
Resistant cultivar 6	Noninfected	RH	S6
Resistant cultivar 7	Infected	RI	S7
Resistant cultivar 8	Noninfected	RH	S8
Resistant cultivar 9	Infected	RI	S9
Resistant cultivar 10	Noninfected	RH	S10
Resistant cultivar 11	Infected	RI	S11
Resistant cultivar 12	Noninfected	RH	S12
Susceptible cultivar1	Infected	SI	S13
Susceptible cultivar2	Noninfected	SH	S14
Susceptible cultivar3	Infected	SI	S15
Susceptible cultivar4	Noninfected	SH	S16
Susceptible cultivar5	Infected	SI	S17
Susceptible cultivar6	Noninfected	SH	S18
Susceptible cultivar7	Infected	SI	S19
Susceptible cultivar8	Noninfected	SH	S20
Susceptible cultivar9	Infected	SI	S21
Susceptible cultivar10	Noninfected	SH	S22
Susceptible cultivar11	Infected	SI	S23
Susceptible cultivar12	Noninfected	SH	S24

2.2 Endophytic microorganism detection based on isolation

After crushing the seed samples, 9 mL of 0.01% Tween 80 was added, and the sample was vortexed at 1,500 rpm for 1 min and sonicated in an ultrasonic cleaner for 3 min to obtain the first sample diluent.

One milliliter from the first sample diluent was added to 9 mL of 0.01% Tween 80 and vortexed to obtain the second sample diluent, and this process was repeated for the third sample diluent.

0.1 (mL) of the first, second, and third sample diluents was cultured on the bacterial isolation medium separately, and 0.1 mL of the first and second sample diluents was cultured on the fungal isolation medium separately.

Table 2. Medium formation used in this study.

Medium	Formation
LB	Tryptone 10 g/L; Yeast extract 5 g/L; NaCl 10 g/L; Agar 15 g/L.
Per 25 NA	Peptone 1.25 g/L; Beef extract 0.75 g/L; NaCl 1.25 g/L; Agar 15 g/L.
PDA	Potato extract 200 g/L; glucose 20 g/L; Agar 15 g/L.
Per 25 PDA	Potato extract 200 g/L; glucose 20 g/L; Agar 15 g/L.
TSA	Tryptone 15 g/L; Soy papain hydrolyzate 5 g/L; NaCl5 g/L; Agar 15 g/L.
R2A	Yeast extract powder 0.5 g/L; Peptone 0.5 g/L; Casein Hydrolyzate 0.5 g/L; Glucose 0.5 g/L; Soluble Starch 0.5 g/L; $KH_2PO_4$0.3 g/L; $MgSO_4$0.024 g/L; $C_3H_3NaO_3$ 0.3 g/L; Agar 15 g/L.
Rice	Rice 300 g/L, Agar 20g/L.
TWYE	Yeast extract0. 25 g/L;$KH_2PO_4$0. 5 g/L;Agar 15 g/L.
CMA	Maize flour 5 g/L; Peptone 0.1 g/L; Glucose 1 g/L
DG 18	Casein peptone 5g/L; Anhydrous dextrose 10 g/L; $KH_2PO_4$1 g/L; $MgSO_4$0.5 g/L; Chlornitramine 0.002 g/L; Chloramphenicol 0.1 g/L; Agar 15 g/L.
MEA	Malt extract 30 g/L; Soybean peptone 3 g/L, Agar 15 g/L.
RBM	Peptone 5 g/L; Glucose 10 g/L; $KH_2PO_4$1 g/L; MgSO4 0.5 g/L; Bengal Red 0.03 g/L; Chloramphenicol 0.1 g/L; Agar 15 g/L.
V8	V-8 Juice 200 g/L $CaCO_3$g/L; Agar 15 g/L.

The CFU (Colony-forming units, CFU) with different phenotypes was extracted from each culture medium for PCR amplification. Primers and PCR conditions were as follows:

Primers used for bacterial identification	Primers used for fungal identification
27F: AGAGTTTGATCMTGGCTCAG	ITS4: TCCTCCGCTTATTGATATGC
1492R: TACGGYTACCTTGTTACGACTT	ITS5: GGAAGTAAAAGTCGTAACAAGG

PCR conditions		
Pre-denaturation	94 ℃ for 4 min	
Denaturation	94 ℃ for 40 s	35 cycles
Annealing	55 ℃ for 40 s	
Extension	72 ℃ for 60 s	
Finally extension	72 ℃ for 10 min	

2.3 Endophytic microorganism detection based on amplicon sequencing.

The FastDNA spin kit for soil was used to extract DNA from seeds samples by following the manufacturer's instructions. DNA was then purified and sent to Shanghai Personal Biotechnology Co., Ltd., China, for MiSeq sequencing. Primers were as follows:

Primers used for bacterial identification	Primers used for fungal identification
799F: AACMGGATTAGATACCCKG	ITS4: CTTGGTCATTTAGAGGAAGTAA
1193R: ACGTCATCCCCACCTTCC	ITS2: GCTGCGTTCTTCATCGATGC

Data analysis:

- Raw paired-end sequence reads were filtered with FASTQ (v.0.19.7)
- High-quality reads were analyzed with Quantitative Insights into Microbial Ecology (QIIME2)
- Generate amplicon sequence variants (ASVs) with DADA2 qiime dada2 denoise-single.
- UNITE v8_99 ITS and the SILVA v132_99 16S rRNA gene database were used to train a naive Bayesian classifier to classify ASVs taxonomically
- q2-diversity plugin was used to calculate the biodiversity of the samples
- The abundance of genus showed with heatmap were generated with pheatmap (v1.0.12)
- Significantly different genera were visualized with a volcano plot using ggplot2 package.
- Cytoscape was used to construct the network with Spearman correlation > 0.3 and *P* value < 0.01

2.4 Suppression of the germination rates by potential antagonist isolates

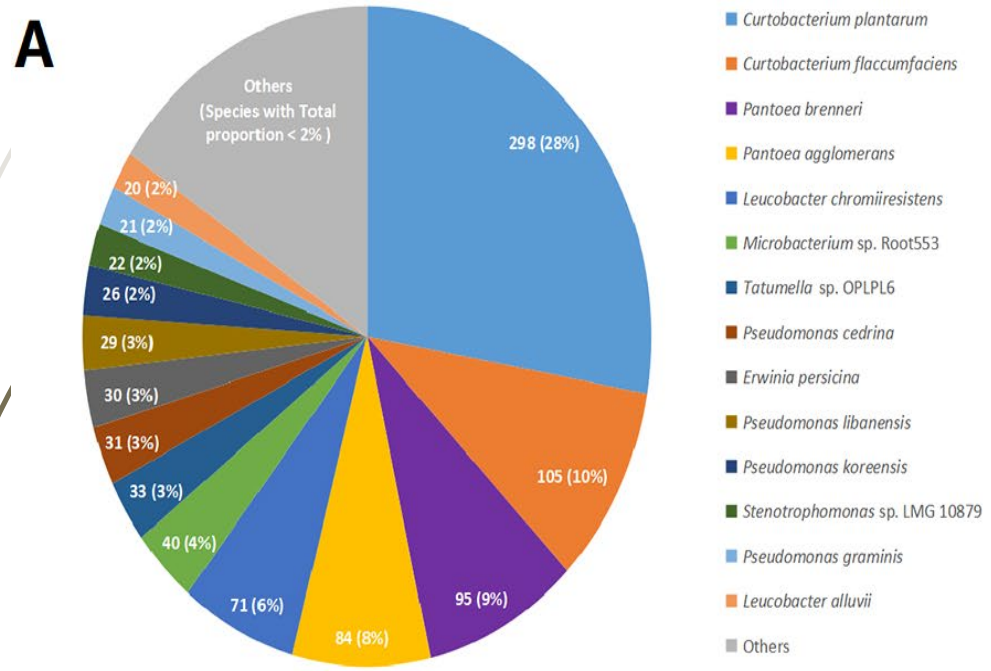
- Teliospores were sterilized with 0.25% NaClO for 1 min and then washed 3 times with sterile ddH_2O. The concentration was adjusted to 10^5 teliospores/mL by a hemocytometer and then 200 mL of suspension was added to the soil-agar medium.
- Media were maintained with a 24-h light cycle at 4 °C in an incubator for 20 days.
- Then, each plate was divided into two equal areas: half of the plate was treated with antagonist isolate (10^3 CFU/mL), and the same volume of sterile ddH_2O was added to the other half for the control and then incubated at 4 °C for 10 days. The procedure was repeated for each potential antagonist isolate for 7 biological replicates. The germination rates of *T. controversa* were then calculated with each antagonist cocultivation under a microscope, and analysis of variance (ANOVA) was conducted on the germination rates using SPSS 17.0.

3. Results

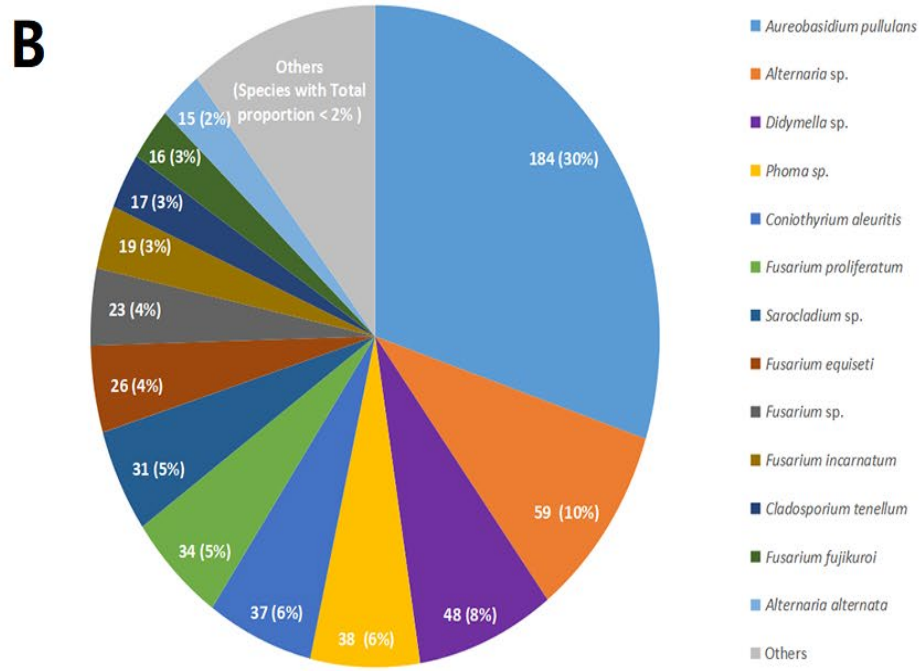
- **Endophytic microorganisms obtained based on isolation**
- **Relative abundances of endophytic microorganisms**
- **Amplicon sequencing and bioinformatics pipeline**
- **Diversity analysis of endophytic community composition**
- **Cross-kingdom connectivity of endophytic microbiota**
- **Overlapping endophytic microorganisms from isolation and amplification sequencing**
- **Antagonists against the germination of teliospores of *T. controversa***

3.1 Endophytic microorganisms obtained based on isolation

- Endophytic bacteria (A): 1,392 isolates
- Endophytic fungi (B): 636 isolates



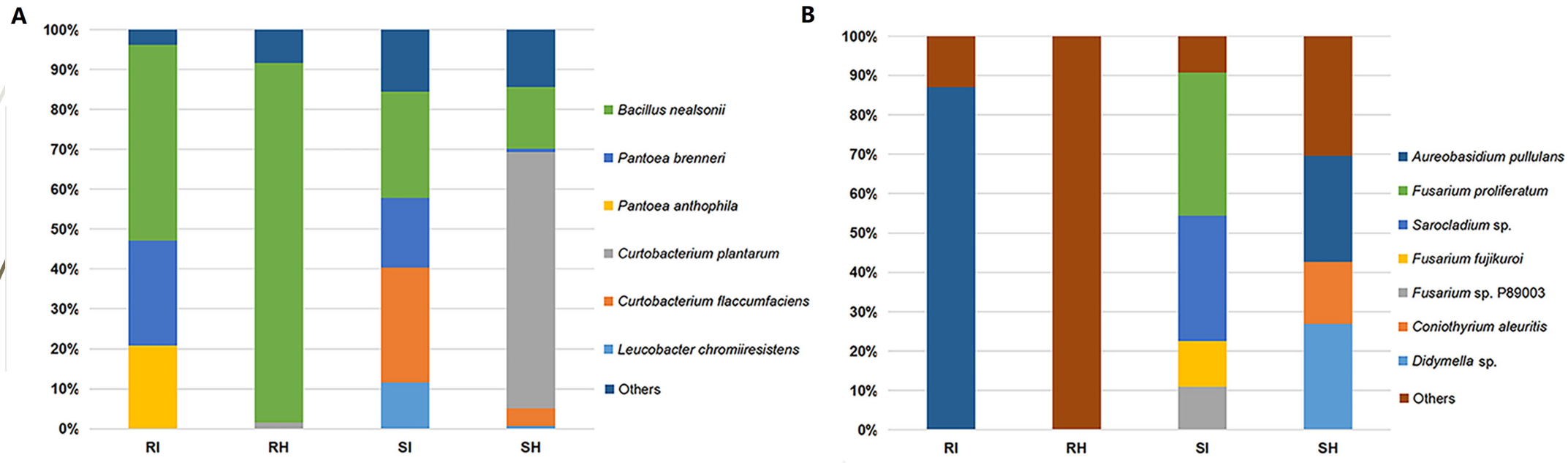
Bacterial isolates



Fungal isolates

3.2 Relative abundances of endophytic microorganisms

The total relative abundance of the main endophytic microorganisms were visualized, revealing that the relative abundances of endophytic microorganisms varied among cultivars and changed after *T. controversa* infection.

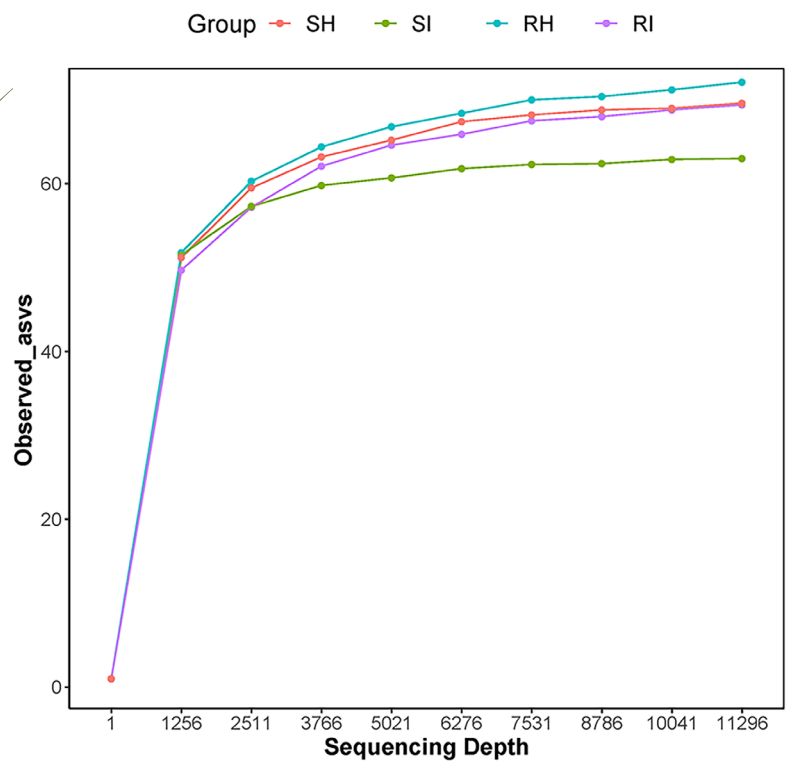


Bacterial communities

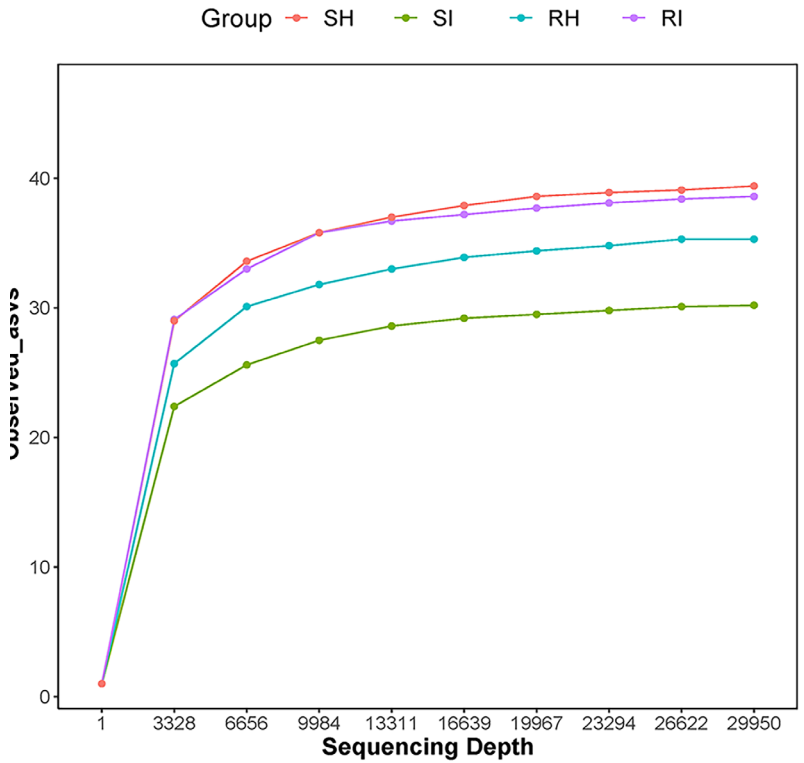
Fungal communities

3.3 Amplicon sequencing and bioinformatics pipeline

As the sequence depth increased, the number of ASVs increased rapidly and leveled off, which indicated that the sequencing depth meets the requirements.



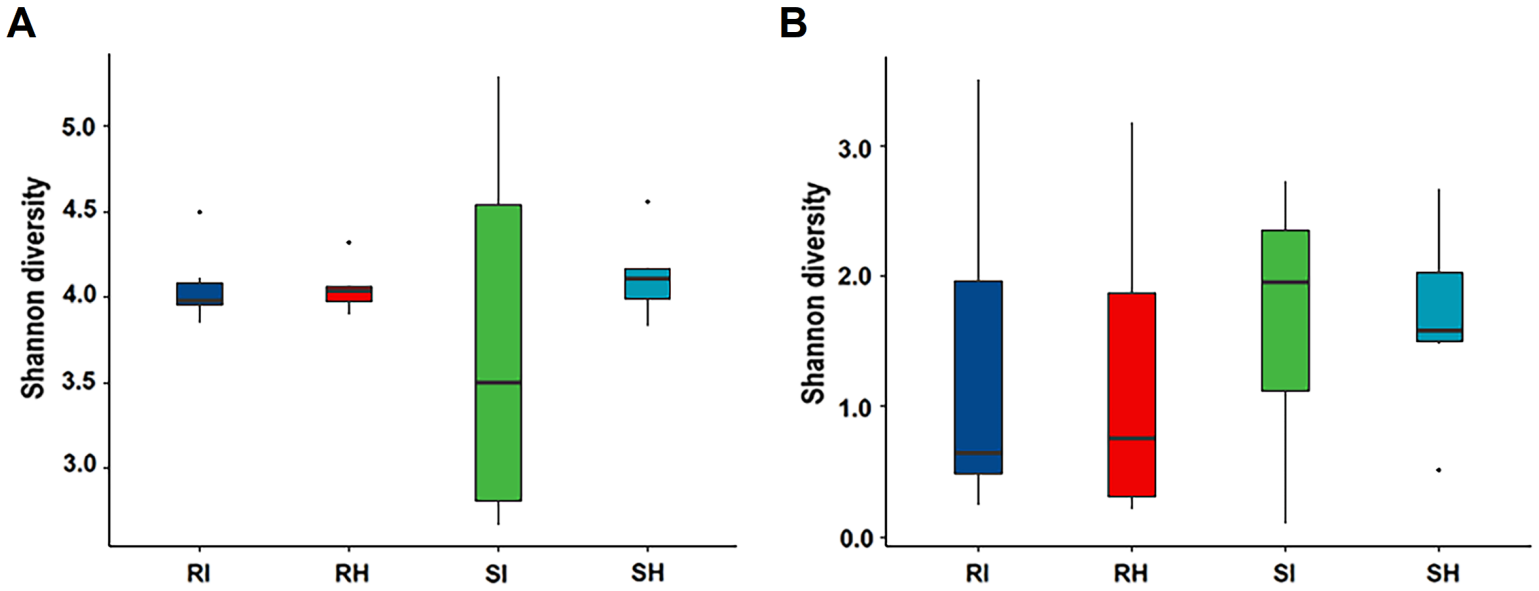
Rarefaction curves of bacterial communities



Rarefaction curves of fungi communities

3.4 Diversity analysis of endophytic community composition

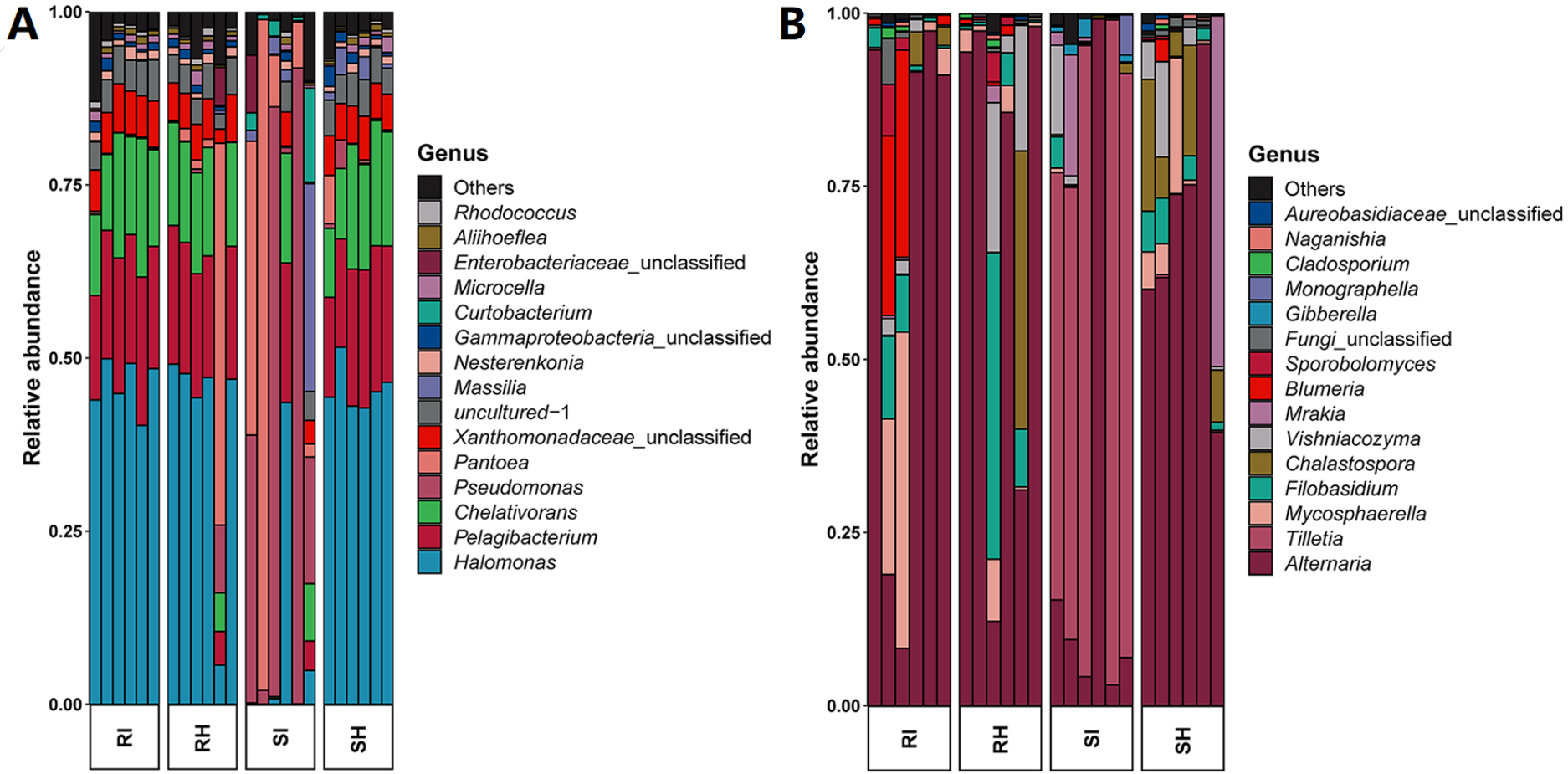
Shannon diversity analysis suggested that infection with *T. controversa* decreased the diversity of infection resistance (RI) and susceptibility (SI) for bacteria, while it caused higher fungi diversity in susceptible infected cultivars (SI) than in resistant infected cultivars (RI).



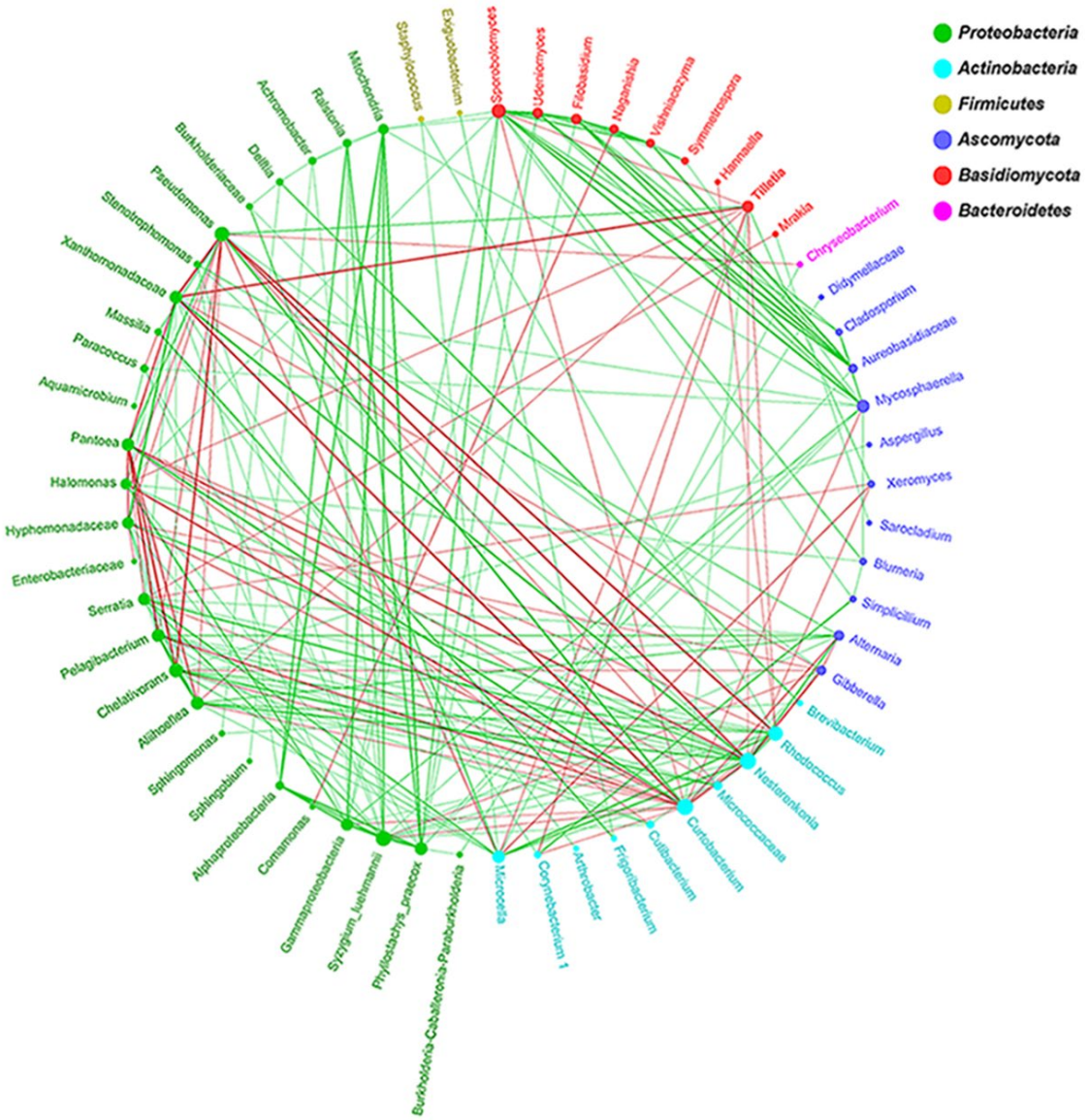
Bacterial communities

Fungal communities

3.5 Cultivars and infection-associated seed endophytes.



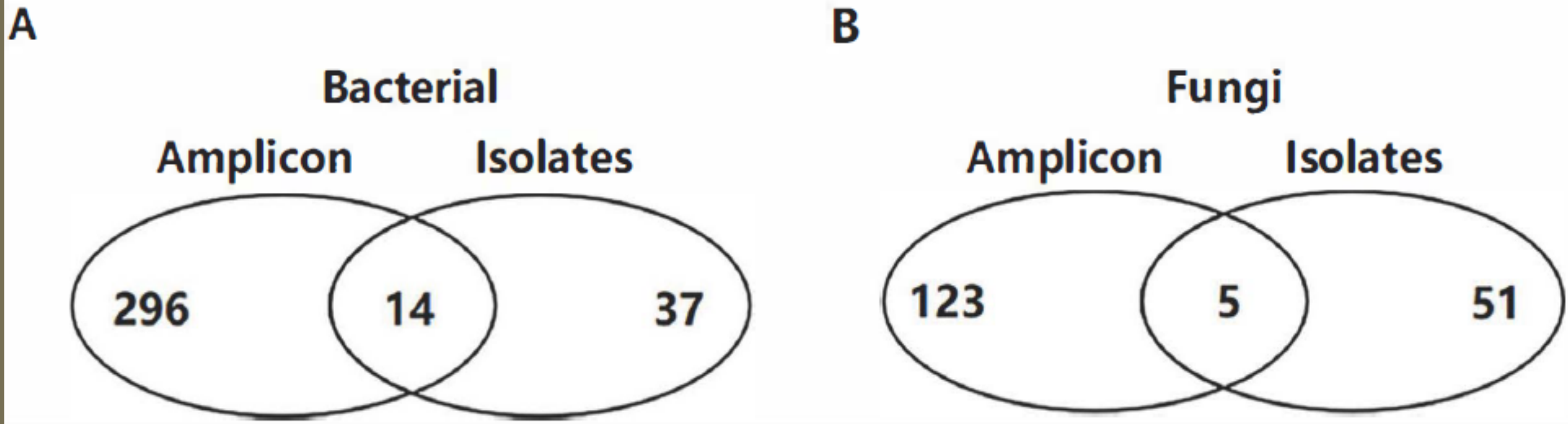
3.6 Cross-kingdom connectivity of endophytic microbiota



- Separate co-occurrence networks were constructed for all groups. The green lines indicated positive correlated and red lines indicated negative correlated.
- Results showed that *Xanthomonadaceae*, *Halomonas*, *Aliihoeflea*, *Microcella*, *Corynebacterium*, *Nesterenkonia* and *Rhodococcus* were negatively correlated with *Tilletia*.

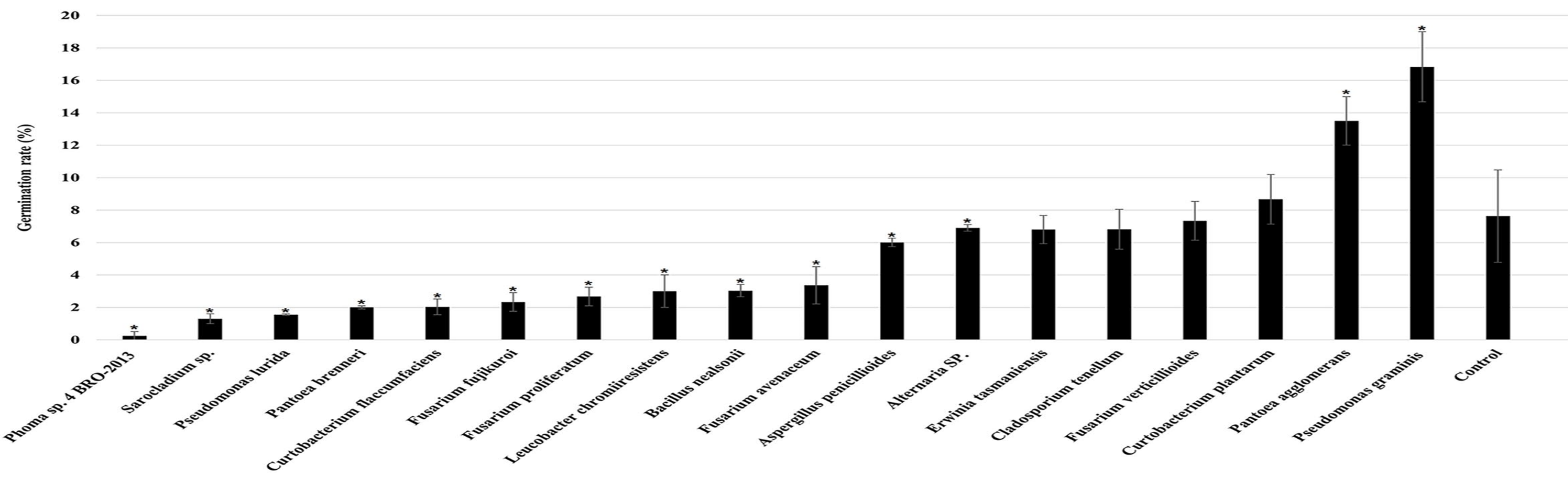
3.7 Overlapping endophytic microorganisms from isolation and amplification sequencing

- For bacterial species: 51 bacterial were isolated, 310 species were obtained by amplicon sequencing, 14 species were overlapping.
- For fungi species: 56 bacterial were isolated, 128 species were obtained by amplicon sequencing, 5 species were overlapping.



3.8 Antagonists against the germination of teliospores of *T. controversa*

The germination rates indicated that *Phoma* sp. strain 4 BRO-2013, *Sarocladium* sp., *P. lurida*, *P. brenneri*, *C. flaccumfaciens*, *F. fujikuroi*, *F. proliferatum*, *L. chromiiresistens*, *B. nealsonii*, *F. avenaceum*, *A. penicillioides*, and *Alternaria* sp. inhibited the germination of teliospores significantly.



4. Discussion

- In our study, amplicon sequencing (310 bacterial species in 155 genera and 128 fungal species in 81 genera) and microbial isolation (66 bacterial species in 29 genera and 52 fungal species in 23 genera) approaches were carried out to identify endophytes in wheat seeds; 14 bacterial species and 5 fungal species overlapped in both methods, suggesting a complementary relationship between the two approaches.

- Shannon diversity analysis showed that the bacterial diversity was lower in the susceptible cultivar than in the resistant cultivar (RI) after *T. controversa* infection, which indicates that bacterial diversity contributes to resistance to *T. controversa*.

4. Discussion

- Network analysis suggested that *Xanthomonadaceae*, *Halomonas*, *Aliihoeflea*, *Microcella*, *Corynebacterium* and *Rhodococcus* were negatively correlated with *Tilletia*.

- Antagonistic experiment showed that *Phoma* sp. strain 4 BRO-2013, *Sarocladium* sp., *P. lurida*, *P. brenneri*, *C. flaccumfaciens*, *F. fujikuroi*, *F. proliferatum*, *L. chromiiresistens*, *B. nealsonii*, *F. avenaceum*, *A. penicillioides*, and *Alternaria* sp. inhibited the germination of teliospores significantly, which were expected to be used in the biocontrol of *T. controversa* in the future.

- In conclusion, these results suggest that *T. controversa* prevalence was lower in resistant cultivars in which higher microbial diversity was featured, and specific promising antagonists potentially contributed to their pathogen suppression ability.

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RESEARCH ARTICLE

Microbiome Signature of Endophytes in Wheat Seed Response to Wheat Dwarf Bunt Caused by *Tilletia controversa* Kühn

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