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# Control of Apple Replant Disease Using Mixed Cropping with *Brassica juncea* or *Allium fistulosum*

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**Abstract:** Evidence indicates that *Allium* and *Brassica* species which release bioactive compounds are widely used in bio-fumigation to suppress soil-borne diseases. However, the active molecules of such plant residues are easily volatilized. In this study, we conducted mixed cropping of the apple tree with *Allium fistulosum* or *Brassica juncea*; the results demonstrated that such mixed cropping significantly improved the growth of the grafted apple seedlings and alleviated apple replant disease (ARD) for two years. The terminal-restriction fragment length polymorphism profile results showed that the soil fungal community demonstrated distinct variation and diversity in terms of composition. *A. fistulosum* and *B. juncea* significantly improved the Margalef, Pielou, and Shannon indices. In addition, the analyses of clone libraries showed that *A. fistulosum* and *B. juncea* promoted the proliferation of antagonistic fungi such as *Mortierella*, *Trichoderma*, and *Penicillium*, and inhibited the proliferation of pathogens such as *Fusarium*. *Fusarium. Proliferatum (F. proliferatum)* was abundant in replanted soil and proved to be an aggressive pathogen of apple seedlings. Our findings thus indicate that apple tree mixed cropping with *A. fistulosum* and *B. juncea* was an effective long-term method for modifying the resident fungal community and alleviating ARD.

**Keywords:** apple replanted disease; *F. proliferatum*; T-RFLP; qPCR; fungal community



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## 1. Introduction

Apple replant disease (ARD) occurs in all major apple-growing regions worldwide [1]. Necrosis of fine feeder roots stunted tree growth both above and below ground, and drought and nutrient stress leading to yield reduction, are considered to be the main symptoms of ARD [2]. This disease affects apple yield and quality for the entirety of the orchard's lifetime. Indeed, due to limited land resources, ARD is expected to dramatically restrict the development of the modern apple industry in China.

ARD can be caused by a variety of abiotic factors such as pH, phytotoxins, soil problems, toxicity of heavy metals in soil, and the stress brought on by drought or cold. Nevertheless, biotic factors appear to be the main cause of ARD, as the disease is usually controlled by pre-plant soil fumigation [3]. Microorganisms are considered to be an important part of soil function, as they are involved in the release of soil mineral nutrients, plant disease resistance, and so on [4]. The change of the microbial community structure is the main reason contributing to the occurrence of ARD [5]. Thus, the microbial communities in soil need to be better understood for ARD management [6]. With the development of molecular biology, the techniques of terminal-restriction fragment length polymorphism (T-RFLP) profiling and quantification by real-time quantitative PCR (qPCR) are usually applied to analyze the fungal communities in the soil affected by ARD [2–7].

Fungi of the genera *Cylindrocarpon*, *Rhizoctonia*, *Phytophthora,* and *Pythium* are found frequently in ARD-affected soils and have proved to be crucial in the aetiology of ARD [8,9]. Although *Fusarium* spp. are also frequently isolated from diseased roots of replanted apple trees [9] and negative correlations abound regarding this genus and ARD [10], the role of *Fusarium* species as a pathogen is controversial [11]. Manici proved that *F. acuminatum* was non-pathogenic in ARD [12]. However, *F. oxysporum* and *F. solani* were shown to be aggressive pathogens of replanted trees [13,14].

Methyl bromide (MB) is banned in many regions and countries as it can deplete atmospheric ozone. Crop rotation is an organic method used to combat disease in many farming systems [15], but it typically takes several years for crop rotation to modify the soil. The application of organic amendments can alter soil microbial communities by producing a disease-suppressive soil or growth medium [16], which reduces potential hazards and controls chemical pollution. At present, soil amendments based on organic residues, such as compost, have been promoted for crop production [17,18]. Bioactive plant products, including those of *Brassicaceae* family [7], *Azadirachta indica* [19], and *Tagetes erecta* [20], introduced either as cover crops or for soil amendment, have also been used as effective measures for soil-borne disease control. *Brassica* plant releases volatile compounds and specific isothiocyanate products, which have been shown to inhibit many soil-borne pathogens [21–23]. The application of mustard seed powder not only inhibited the growth of *Pythium spp*. pathogens in replanted apple orchard soil, but also promoted *Trichoderma* and changed the fungal community structure, reduced the infection of *Pythium* on apple roots and alleviated ARD for a long time [7]. The large sulfur compounds released by *Allium* were effective against many genera of bacteria, fungi, and insects [24–26]. The by-products of onion contain a large amount of dimethyl disulphide and dipropyl disulphide. The application of onion compost can not only inhibit the growth and reproduction of Pythium, but also significantly improve the yield of aloe vera and strawberry [27]. The dimethyl trisulfide present in leek volatiles was found to have a strong inhibitory effect on *Fusarium oxysporum* and can be used to control banana fusarium wilt [4].

Organic residue-based soil amendments are a useful method in crop production; however, the active molecules of plant residue are easily volatilized or consumed [4,16]. In apple orchards, plant pathogenic microorganisms accumulate in the rhizosphere and roots within 1–2 years after the establishment of the orchard [28]. Therefore, it is essential to develop an environmentally friendly, durable, and effective approach to relieve ARD.

This study aimed to explore the effects of a new cultivation model on ARD: mixed cropping with *A. fistulosum* and *B. juncea*. The purpose of this study is to determine: (1) the influence of mixed cropping on the growth of grafted seedlings; (2) the effect of mixed cropping on the fungal community; (3) the pathogenicity of *Fusarium. Proliferatum;* and (4) the long-term suppressive effect of mixed cropping on ARD.

## 2. Materials and Methods

### *2.1. Experimental Materials and Treatment*

The study was conducted at an experimental station in Shandong Agricultural University from 2015 to 2016. Soil samples were collected from a 30-year-old apple orchard located in Tai'an (Shandong, China). All soil samples were from the rhizosphere soil of apple trees with a depth ranging from 5 to 50 cm. The soil samples were sandy and had the following chemical content: $NO_3N$ ($mg{\cdot}kg^{-1}$), 7.8; $NH_4N$ ($mg{\cdot}kg^{-1}$), 5.1; available P ($mg{\cdot}kg^{-1}$), 26.7; available K ($mg{\cdot}kg^{-1}$), 28.39; organic matter ($mg{\cdot}kg^{-1}$), 23.1; pH 5.9. The soil samples were mixed thoroughly before use. In early March 2015, 2-year-old apple (Fuji) trees on *Malus hupehensis* Rehd. rootstocks were used to replant apple in plastic containers (height: 42 cm, diameter: 45 cm) filled with 80 kg of soil. Plastic containers were placed in the natural environment outdoors. The experiment contained four different treatments: T1 (replanted soil fumigated with methyl bromide), T2 (replanted soil cropping with about 20 plants of *A. fistulosum*), T3 (replanted soil with about 20 plants of *B. juncea*) and CK (replanted soil). Nine apple seedlings were planted in each replicate and a total

of 36 seedlings were planted. *A. fistulosum* and *B. juncea* were planted in mid-May and harvested in mid-December, and approximately 20 plants were planted in each plastic container. Normal water and fertilizer management was carried out during the experiment; the following year, the planting of *A. fistulosum* and *B. juncea* was continued. The plant biomass was measured in October 2015 and October 2016. Rulers and vernier calipers were used to measure the plant height, total length of the branch, and the trunk diameter of young apple trees. Electronic scales were used to measure the dry weight. Soil samples were collected by the five-point sampling method in October 2016. After removing the soil around the upper layer, a ring knife was used to preserve the soil samples. Approximately 160 g of soil was obtained from each ring knife. All soil samples were brought back to the laboratory in an ice box, and the soil was mixed and passed through a 2 mm sieve. The sieved soil samples were divided into three parts: one part was frozen at −80 °C for soil microbial analysis, the sample for DNA extraction was frozen at −20 °C, and the remaining part was stored in a refrigerator at 4 °C.

### *2.2. Fungal Pathogen Isolation, Identification and Pathogenicity Testing*

Infected roots were collected from replanted apple trees. The roots were washed with deionized water, disinfected with 1% sodium hypochlorite for 1 min, and rinsed twice with sterile water. The infected roots were cut into root segments (0.2–0.3 cm) and placed on fresh potato-dextrose agar (PDA) in Petri dishes (9 cm diameter) for 2 days at 28 °C under natural light. The infected roots were cut into root segments (0.2–0.3 cm) and placed in fresh potato-dextrose agar (PDA) (9 cm diameter), then the Petri dishes were placed in natural light at 28 °C for 2 days. Subsequently, they were transferred to new PDA for purification. After 5 days of incubation, the morphological characteristics of colonies, conidium terriers, and conidia were preliminarily identified using a microscope.

The spores of *F. proliferatum* were isolated from cultures in liquid PDA medium (without agar) at 28 °C in the dark for 4 to 7 days and the conidia and mycelia were separated by filtering the conidial suspensions through three layers of gauze. The spores were regulated to the desired concentration ($10^4$ spores·mL$^{-1}$) by counting them in a hemocytometer and then tested for pathogenicity. The planting medium was inoculated with 100 ml conidial suspensions ($10^4$ spores·mL$^{-1}$) and sterile water was used as the control. Four-leaf-old *M. hupenhensis* Rehd. seedlings were planted in the planting medium for pathogenicity testing, and 30 replicates were used for each treatment. The seedlings were grown in a glasshouse at 20–25 °C for 4 weeks, and their survival was recorded.

### *2.3. DNA Extraction and PCR Amplification*

The PowerSoil® DNA Isolation Kit (MO BIO, Carlsbad, CA, USA) was used to obtain the fungal DNA following the manufacturer's instructions. Fungal internal transcribed spacer (ITS) regions were amplified with primers ITS1F (5′-CTTGGTCATTTAGACGAAGTAA-3′) and ITS4 (5′-TCCTCCGC TTATTGATATGC-3′) [29]. The PCR amplification reaction contained 12.5 µL of 2 × Easy Tap PCR Super Mix (Novozan Biotechnology Co., Ltd., Nanjing, China),1.5 µL of DNA,1 µL of ITS1F (10 M), 1 µL of ITS4 (10 M) and 9 µL of dd$H_2O$. PCR amplifications were carried out in a 2720 thermocycler (Applied Biosystems, USA) for an initial 3 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C, followed by a final extension at 72 °C for 10 min. The DNA Fragment Purification Kit (Takara Biotech Co., Ltd., Dalian, China) was used to confirm and purify the PCR products on 2.0% agarose gel.

### *2.4. Terminal-Restriction Fragment Length Polymorphism (T-RFLP) Analysis*

We used T-RFLP analysis to detect changes in the fungal diversity in response to the soil treatment. The PCR amplification reaction is: 12.5 µL of 2 × Easy Tap PCR Super Mix (Novozan Biotechnology Co., Ltd., Nanjing, China),1.5 µL of DNA, 1 µL of ITS1F (10 M) with 5′HEX labeled by 6-carboxyfluorescein (FAM), 1 µL of ITS4 (10 M)) and 9 µL of dd$H_2O$. Purified PCR products of each sample were digested in a total volume of 20 µL,

containing 10 μL of purified PCR product, 1 μL of Hha I (Novozan Biotechnology Co., Ltd., Nanjing, China), 2 μL of 10 × M Buffer and 7 μL of $ddH_2O$, at 37 °C for 1 h. The 3730 ABI electrophoretic capillary sequencer (Applied Biosystems) was used to determine the lengths of the fluorescently labeled terminal restriction fragments (TRFs) and the Peak Scan Program was used for analysis. The TRFs from the ITS genes with sizes between 50 and 500 bp were used for further analysis. Each TRF was treated as an operational taxonomic unit (OTU). The peak area was standardized with respect to the sum of all peak areas in each sample. Peaks comprising <1% of the total chromatogram area were excluded from the analysis [29]. BIO-DAP (http://nhsbig.inhs.uiuc.edu/wes/populations.html accessed on 15 September 2017) was used in the Alpha diversity index analysis. The Alpha diversity index mainly comprises the Margalef, Shannon, Pielou, and Simpson indices. The SPSS v 19.0 statistical software (IBM, New York, USA) was used to evaluate the microbial community associated with soil in each treatment.

### *2.5. Sequence Analysis of Clone Libraries*

Fungal clone libraries were constructed from the samples of each treatment. Purified PCR products of each sample were ligated into the pMD18-T vector (Novozan Biotechnology Co., Ltd., Nanjing, China). Plasmids were transformed into competent *Escherichia coli* cells (DH5a, Novozan Biotechnology Co., Ltd., Nanjing, China), and LB agar plates containing 100 $g \cdot mL^{-1}$ ampicillin, 40 $g \cdot mL^{-1}$ X-Gal and 24 $g \cdot mL^{-1}$ IPTG were used to conduct blue-white screening of cloned plasmids. The selected transformants were grown overnight in 0.8 mL LB broth containing 100 $g \cdot mL^{-1}$ ampicillin, and primers M13F (5′-GGTAACGCCAGGGTTTCC-3′) and M13R (5′-CGCCAGGGTTTCCCAGTCAGGA-3′) were used for PCR amplification to check for the presence of the ITS rRNA gene in randomly selected clones. Selected clones containing the PCR product inserts were sequenced by Beijing Qingke Biology Co., Ltd. Sequences (Beijing, China) were identified by comparing with those in the GenBank database.

### *2.6. Quantification of F. proliferatum in Replanted Soil by Real-Time Quantitative PCR (qPCR)*

The specific primers CR and CF were designed based on the rDNA ITS sequence of *F. proliferatum*. All the primers were synthesized by Sangon Biotech Co. (Shanghai, China) at HPLC purification grade. The Premier Primer 5.0 software was used to design specific primer sequences, CF: 5′-GATCGGCGAGCCCTTGCGGCAAG-3′, and CR: 5′-CGCCGCGTACCAGTTGCGAGGGT-3′. The amplified target fragment was 101 bp in length. The qPCR was performed on CF × 96 TM Real-Time PCR Detection System (Bio-Rad, California, USA) containing the following components: 12.5 μL of SYBR green PCR Master Mix (Novozan Biotechnology Co., Ltd., Nanjing, China), 1 μL of CF (10 μM), 1 μL of CR (10 μM), 1.5 μL of DNA, and 9 μL of $ddH_2O$. The cycling parameters consisted of an initial 30 s at 95 °C, followed by 40 cycles of 5 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C with a detection step at the end of each cycle. To ensure that only products at the desired melting temperature were generated from the SYBR Green qPCR, a melting curve was obtained after each assay. The standard curves for quantifying gene copy numbers were determined by cloning the PCR products in a plasmid, using the procedures in the manufacturer's instructions for the pMD18-T vector (Novozan Biotechnology Co., Ltd., Nanjing, China). The resulting recombinant plasmid was subjected to 10-fold serial dilution ($1 \times 10^0$ to $1 \times 10^7$ copies). For all runs, the R2 value of the standard curve was 0.99 or better.

### *2.7. Statistical Analysis*

The SPSS v 19.0 statistical software was used for statistical analysis. The statistical significance of the difference between treatments was determined by one-way analysis of variance (ANOVA) and Student's *t*-test followed by Duncan's multiple range test, with $p < 0.05$ assuming statistical significance.

## 3. Results

### *3.1. Effect of Mixed Cropping with A. fistulosum and B. juncea on the Growth of Replanted Seedlings*

The plant height, trunk diameter, branch length, and dry weight of grafted seedlings were measured in 2015 and 2016. The analyses of variance showed that the soil fumigated by methyl bromide resulted in the greatest growth, followed by that with mixed cropping treatments (Table 1). After the first growing season, the dry weight of grafted seedlings mixed with *A. fistulosum* and *B. juncea* was greatly improved (23.89% and 26.29%, respectively), compared to those planted in untreated replanted soil ($p < 0.05$). In addition, the plant height, trunk diameter and length of branch were also increased (12.72%, 15.42%, 14.67%; 12.06%, 52.88%, 71.79%, respectively). Results were similar for the second growing season: the growth of grafting seedlings, mixed cropping with *A. fistulosum* and *B. juncea* were improved significantly. The plant height, trunk diameter, the total length of the branch, and the dry weight of grafted seedlings upon mixed cropping with *A. fistulosum* improved by 6.50%, 9.19%, 36.04%, and 24.28%, respectively. For the *B. juncea*-treated seedlings, the trunk diameter, the total length of the branch, and the dry weight were increased by 7.04%, 7.84%, 34.38%, and 31.63%, respectively.

**Table 1.** Effect of mixed cropping with *A. fistulosum* and *B. juncea* on biomass of apple grafted seedlings. T1, grafted seedlings replanted on the soil fumigated with methyl bromide; T2, grafted seedlings replanted on the soil mixed cropping with *A. fistulosum*; T3, grafted seedlings replanted on the soil mixed cropping with *B. juncea*.; CK: grafted seedlings replanted. Data of biomass were calculated in October 2015 and in October 2016. Data are the means of three replicates (±SE), different letters indicate significant differences at $p < 0.05$.

| Date | Treatment | Plant Height (cm) | Trunk Diameter (mm) | Total Length of Branch (cm) | Dry Weight (g) |
|---|---|---|---|---|---|
| 15 October 2015 | CK | 152.00 ± 2.31c | 19.91 ± 0.35 c | 312.67 ± 15.17 c | 540.24 ± 25.55 c |
| | T1 | 204.67 ± 6.33 a | 26.08 ± 1.40 a | 589.67 ± 32.11 a | 890.07 ± 69.07 a |
| | T2 | 171.33 ± 3.53 b | 22.98 ± 0.47 b | 477.00 ± 47.35 b | 669.14 ± 38.41 b |
| | T3 | 170.33 ± 3.76 b | 22.83 ± 0.30 b | 536.00 ± 19.70 ab | 682.28 ± 34.27 b |
| 15 October 2016 | CK | 184.67 ± 3.48 c | 123.33 ± 1.20 c | 455.67 ± 34.36 c | 834.67 ± 11.85 c |
| | T1 | 218.33 ± 0.88 a | 143.33 ± 3.18 a | 678.00 ± 18.00 a | 1167.00 ± 34.49 a |
| | T2 | 196.67 ± 2.91 b | 134.67 ± 0.67 b | 574.33 ± 34.84 b | 1037.33 ± 32.44 b |
| | T3 | 197.67 ± 1.67 b | 133.00 ± 3.06 b | 612.33 ± 20.22 ab | 1098.67 ± 20.18 b |

### *3.2. Effects of Mixed Cropping with A. fistulosum and B. juncea on the Fungal Community*

Through T-RFLP analysis, we found significant differences in the diversity of the fungal community between various treatments (Figure 1). The replanted soil, upon mixed cropping with *A. fistulosum* and *B. juncea,* was higher in the Shannon, Pielou, and Margalef indices and lower in the Simpson index, than the untreated replanted soil. The fumigated soil had the lowest Shannon index, Pielou index, and Margalef index, and the highest Simpson index, due to the lack of measurable diversity. Compared to the replanted soil, the fungus Margalef index in the replanted soil mixed with *A. fistulosum* and *B. juncea* improved by 36.87% and 53.26%, respectively. Results were similar for the Shannon index: mixed cropping with *A. fistulosum* and *B. juncea* improved the Shannon index by 51.09% and 54.59%, respectively. The fungus Pielou index in the replanted soil mixed with *A. fistulosum* and *B. juncea* was improved by 38.81% and 37.31%, respectively. However, the fungus Simpson index of this group was reduced by 88.75%, compared to that of the untreated soil.

The effect of mixed cropping with *A. fistulosum* and *B. juncea* on the fungal community structure in replanted soils was reflected in the cluster analysis of the ITS rDNA terminal-restriction fragments (Figure 2). The cluster analysis showed that the fungal community structure was significantly different among the treatments. Three repetitions of each treatment were clustered together. The fumigated soil was an independent group and was far from the other three treatments. In addition, the fungal community of *A. fistulosum*-

treated soil was more closely related to the community of the *B. juncea*-treated soil rather than that of the replanted soil (Figure 2).

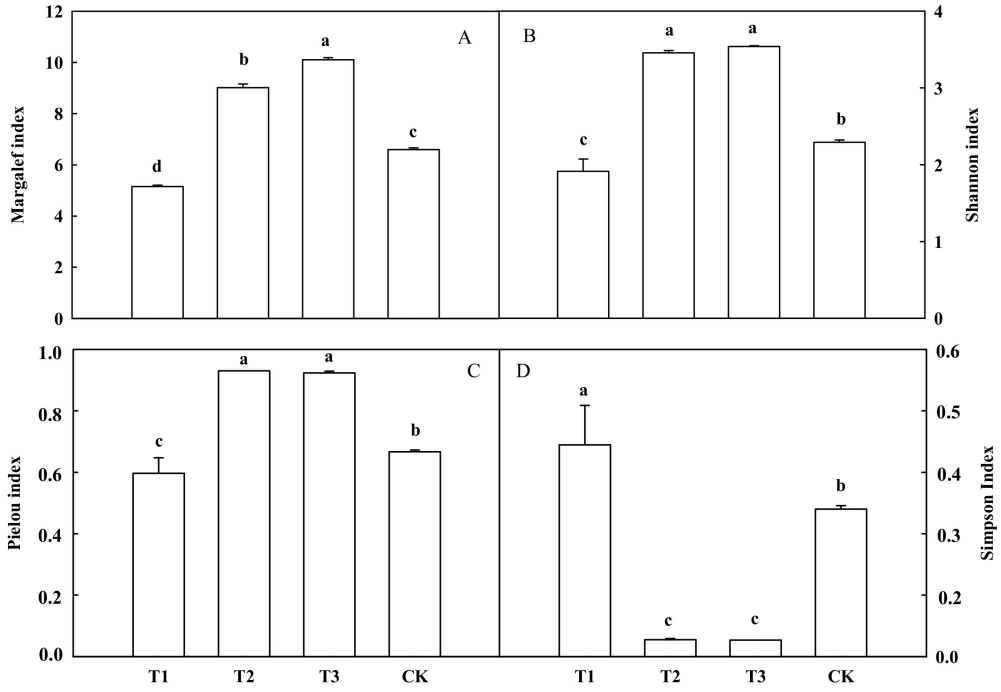


**Figure 1.** Species diversity of soil fungi. T1, the replanted soil fumigated with methyl bromide; T2, the replanted soil mixed cropping with *A. fistulosum*; T3, the replanted soil mixed cropping with *B. juncea*; CK: the replanted soil; (**A**), Margalef index of different soil samples; (**B**), Shannon index of different soil samples; (**C**), Pielou index of different soil samples; (**D**), Simpson index of different soil samples. Data are the means of three replicates (±SE), different letters indicate significant differences at $p < 0.05$.

The dominant fungal genera in the four treatment groups were identified by DNA sequencing of the clone libraries (Figure 3). In the replanted soil, *Fusarium* (20.91%) *Guehomyces* (13.64%), *Mortierella* (8.18%), and *Cryptococcus* (6.36%) were dominant. After mixed cropping for 17 months, the fungal community composition changed significantly. *A. fistulosum* increased the relative abundance of *Mortierella* (19.51%), *Trichoderma* (20.33%), *Penicillium* (15.45%), and *Sordariomycetes* (19.20%). In addition, the relative abundance of *Fusarium* (5.69%), and *Guehomyces* (4.06%) were decreased in the replanted soil mixed with *A. fistulosum*. For the *B. juncea*, *Mortierella*, *Trichoderma,* and *Penicillium* were also increased (16.10%, 12.71%, and 19.49%, respectively). In the *B. juncea* treated soil, *Fusarium* was decreased (6.80%). After fumigation by methyl bromide, fumigated soil recovered more *Mortierella* (49.13%) and *Trichoderma* (8.62%).

### *3.3. Pathogenicity Testing of F. proliferatum and Real-Time Quantitative PCR (qPCR) Assay*

*F. proliferatum* was isolated and used for pathogenicity testing as it was abundant in replanted soil, based on the analysis of clone libraries. The results showed that *F. proliferatum* significantly suppressed the growth of *M. hupenhensis* Rehd. seedlings (Table 2). After the conidial suspension was irrigated with the planting medium, the seedlings stopped growing gradually. Compared to the uninoculated seedlings, the plant height of the inoculated seedlings was decreased by 74.85%. Likewise, the dry weight of seedlings also decreased by 62.86%. Furthermore, upon survival analysis, it was found that 56.67% of the seedlings (17/30) had died.

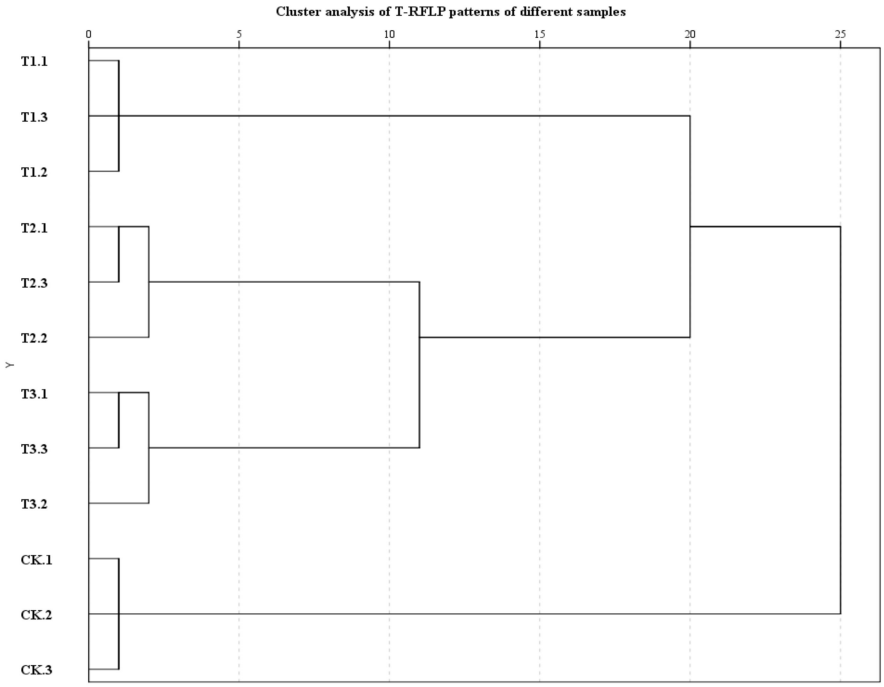


**Figure 2.** Cluster analysis of T-RFLP derived data for the fungal community. T1, the replanted soil fumigated with methyl bromide; T2, the replanted soil mixed cropping with *A. fistulosum*; T3, the replanted soil mixed cropping with *B. juncea*.; CK: the replanted soil. Three replicates were calculated in every treatment.

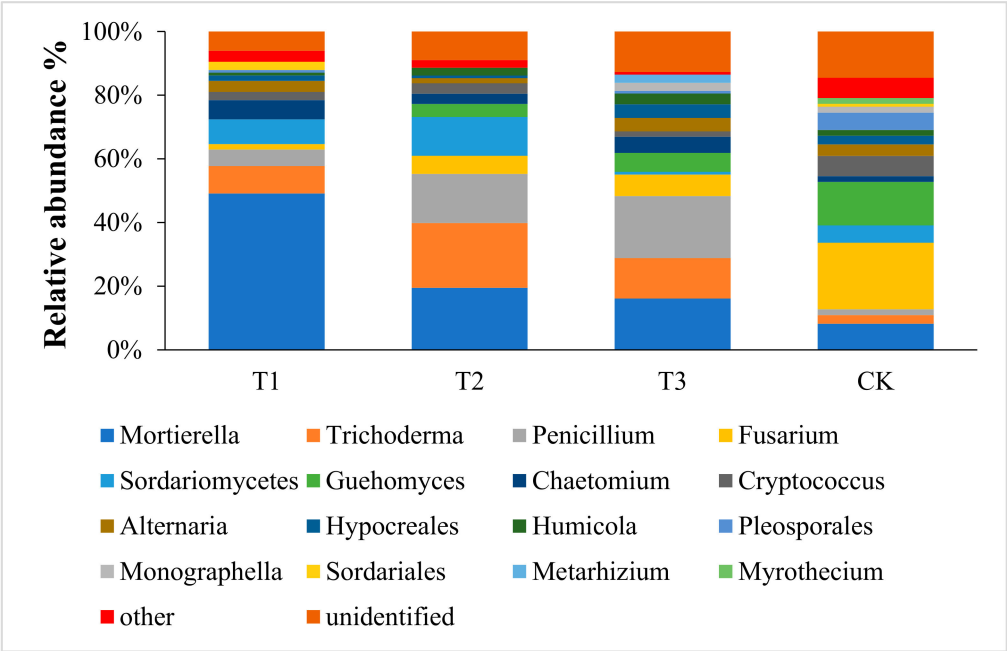


**Figure 3.** Relative abundances of the fungi in different soil samples at the fungal genus level. T1, the replanted soil fumigated with methyl bromide; T2, the replanted soil mixed cropping with *A. fistulosum*; T3, the replanted soil mixed cropping with *B. juncea*.; CK: the replanted soil. In the fumigated soil, *A. fistulosum* treated soil, *B. juncea* treated soil, and replanted soil, Total 116,123,118 and 110 clones were identified, respectively. Relative abundances of fungi >1% were used for analysis. Relative abundances of fungi <1% were classified into others.

**Table 2.** Effect of *F. proliferatum* on *M. hupenhensis* Rehd. seedlings. Inoculated: 100 mL conidia suspensions ($10^4$ spores/mL) inoculated for 4 weeks; Uninoculated: 100 mL sterile water as control. Data are the means of thirty replicates (±SE), different letters indicate significant differences at $p < 0.05$.

| | Plant Height (cm) | Dry Weight (cm) | Death Rate (%) |
|---|---|---|---|
| Inoculated | 8.43 ± 0.18 b | 2.80 ± 0.57 b | 56.67% |
| Uninoculated | 14.74 ± 0.15 a | 4.56 ± 0.07 a | NF |

The real-time fluorescence units were plotted against the initial concentration of plasmid DNA ranging from 26.3 ng/μL to 2.63 × $10^{-5}$ ng/μL. The standard curve y = −3.675 x + 9.128 ($R^2$ = 0.999) was used for our qPCR analysis. The standard curve produced by the real-time PCR assay revealed good linearity within the detection limit and a high correlation between the Ct values ($R^2$ > 0.99). According to the standard curve, the absolute copy number of DNA could be calculated. The results of qPCR showed that the copy number of *F. proliferatum* in replanted soil was the highest at up to 1.53 × $10^4$ copies. In contrast, the methyl bromide-fumigation treatment had the lowest copy number of *F. proliferatum*. Compared to the control, the copy number of *F. proliferatum* in the replanted soil mixed cropping with *A. fistulosum* and *B. juncea* was reduced by 29.73% and 27.85%, respectively.

## 4. Discussion

Many studies have shown that the aetiology of ARD was mostly biological, as the growth of replanted trees significantly increased in fumigated and pasteurized soil [5,6]. Consistent with this, fumigation led to the highest observed growth of replanted seedlings within two years in our study (Table 1). Previous studies suggested that *Brassica* and *Allium* were kinds of bioactive plants which could suppress soil-borne diseases and promote crop growth [27,30]. Thus, these bioactive plants are generally regarded as ideal materials for crop rotation, intercropping, and bio-fumigation [27,31]. Our results showed that mixed cropping with *A. fistulosum* and *B. juncea* could alleviate ARD in replanted apple seedlings and lead to more growth (Table 1). On the one hand, the root exudates of *A. fistulosum* and *B. juncea* could produce substances that kill soil-borne harmful fungi and promote the healthy growth of apple roots [16,28,30]; on the other hand, the root residues of *A. fistulosum* and *B. juncea* are decomposed to produce organic matter and other nutrients that promote apple tree growth [32]. This study provides a novel method of mixed cropping of apple seedlings with *Allium* and *Brassica* to suppress ARD.

Fungi and fungal communities play important roles in the soil ecosystem, and the diversity of fungal communities can naturally be antagonistic to various plant pathogens [33]. Wang et al. reported that the addition of seaweed fertilizer significantly improved the composition of the fungal community to control ARD [34]. Dmn et al. found that Brassicaceae seed meals modified the resident fungal community by augmenting *Trichoderma* spp. [7]. The T-RFLP results of our study indicated that mixed cropping with *A. fistulosum* and *B. juncea* significantly altered the fungal community. The Margalef, Pielou, and Shannon indices were improved, indicating that the diversity of the fungal community was enriched (Figure 3). The results of clone libraries further proved that the dominant fungal species in replanted soil were significantly influenced by *A. fistulosum* and *B. juncea*. Although *Fusarium* was the dominant genus in replanted soil, *A. fistulosum* and B. *juncea* appeared to preferentially enhance certain fungi such as *Mortierella*, *Trichoderma* and *Penicillium*. *Trichoderma* is a well-known antagonist against soil-borne plant pathogens and has been reported to protect against many plant diseases incited by *F. oxysporum*, *F. proliferatum,* and *F. solani* [35,36]. Based on our previous study, *Penicillium* was an antagonistic fungus that inhibited growth of *F. oxysporum*, *F. moniliforme*, *F proliferatum,* and *F. solani* [37]. It was noteworthy that the relative abundance of *Mortierella* changed significantly across different treatments in our study. *Mortierella* was found to be the most abundant in fumigant-treated soil, followed by *A. fistulosum*- and *B. juncea*- treated soils. Li et al. reported that the

relative abundance of *Mortierella* was negatively correlated with the incidence of *Fusarium* wilt disease [38]. In addition, Melo et al. reported that *Mortierella* was an antagonistic fungus that suppressed soil disease by competing with pathogens for resources, producing antibiotics [39].

Earlier studies found that long-term replanting not only changed the rhizosphere microbial community structure and function, but also increased the quantity of harmful fungi [40]. Pathogens accumulated and invaded the roots of young apple trees. *Fusarium* was found to be the dominant genus in replanted soil and *F. proliferatum* was abundant in infected roots. Chang et al. reported that *F. proliferatum* was an aggressive pathogen for soybean, causing severe root rot and reduction of seedling emergence [41]. In addition, *F. proliferatum* also was suspected as the causative pathogen of ARD in China [42]. The result of pathogenicity testing indeed demonstrated that *F. proliferatum* was an aggressive pathogen causing ARD, leading to a reduction in growth and, ultimately, in death (Table 2).

Previous studies suggested that *Brassica* and *Allium* were kinds of bioactive plants that could release bioactive compounds to suppress soil-borne pests and diseases [30,32]. In this study, the qPCR results showed that *A. fistulosum* and *B. juncea* reduced the proliferation of *F. proliferatum* in replanted soil (Figure 2), relying on their active compounds. Members of the plant family *Brassicaceae* produce glucosinolates, upon which hydrolysis yields biologically active compounds including isothiocyanates. Isothiocyanates have a broad spectrum of antimicrobial activity and suppress pathogenic fungi, such as *Pythium* spp., *Phytothphora* spp., *Sclerotinia* spp., *Cylindrocarpon* spp., and *Rhizoctonia* spp. [43,44]. The pronounced biocidal properties of *Allium* species are tightly linked to the complex biochemistry of the sulphur compounds they contain [45]. Dimethyl disulphide emission from *Allium* was also proven to be effective in controlling fungal soil phytopathogens such as *Pythium ultimum*, *Verticilium dahlia*, *Tylenchulus semipenetrans*, and *F. oxysporum* [46] as well as nematodes [47]. Other studies have also suggested that mechanisms other than isothiocyanate production may be important in reducing soil-borne diseases by *Brassica crops* [48]. Research demonstrated that suppression of *R. solani* by *Brassica napus* seed meal was associated with specific changes in soil microbial communities and was unrelated to levels of glucosinolate [49]. Other researchers have also noted the role of the stimulation of soil microbial activity or the alteration of soil microbial communities in the suppression of specific diseases [50,51].

However, the active molecules of plant residue are easily volatilised or consumed; *B. juncea* seed meal releases allyl isothiocyanate within the first 24–48 h [16], and *Allium* by-products release dimethyl disulphide for up to one month [27]. Control of soil-borne fungi is extremely difficult owing to their extensive mycelia and numerous spores. After bio-fumigation, soil-borne fungi can recover and lead to root infections [43]. Nevertheless, our study proves that replanted seedlings, upon mixed cropping with *A. fistulosum* and *B. juncea*, can suppress *F. proliferatum* for a long time, relying on live plants to continuously release bioactive compounds. Thus, mixed cropping of replanted apple with *B. juncea* and *A. fistulosum* presents a new cultivation pattern that may provide long-term suppression of pathogenic *F. proliferatum*.

## 5. Conclusions

This study evaluated the effects of mixed cropping of apple trees with *B. juncea* and *A. fistulosum* on ARD and on the fungal community structure. The diversity of the soil fungal community of apple trees was improved distinctly by mixed cropping with *B. juncea* and *A. fistulosum*, as shown in the Margalef, Pielou, and Shannon indices. In addition, the results of clone libraries showed that mixed cropping with *A. fistulosum* promoted the proliferation of antagonistic fungi such as *Mortierella*, *Trichoderma*, and *Penicillium*, and inhibited the proliferation of Fusarium. *F. proliferatum* was abundant in replanted soil and proved to be an aggressive pathogen of apple seedlings. This study proves that mixed cropping with *A. fistulosum* and *B. juncea* was an effective method for alleviation of ARD. Due to the biological nature of ARD aetiology, the mixed cropping pattern with

*A. fistulosum* and *B. juncea* could be a sustainable method for ARD management. Over the course of two years, we observed that *A. fistulosum* and *B. juncea*, as bioactive plants, could efficiently alleviate ARD and inhibit the growth of *F. proliferatum* over the long term. Future studies will focus on the effects of apple trees mixed with *A. fistulosum* and *B. juncea* on soil nutrients and bacterial community, analyze and determine the effective ingredients of *A. fistulosum* and *B. juncea* to inhibit *F. proliferatum*, which will further explain the effects of this planting pattern. In the meantime, this is just one case-by-case study, and more cultivation patterns can be tried to determine which may have the best effect on the alleviation of ARD. Even so, this study still provides valuable information for modifying the resident fungal community and is expected to provide theoretical guidance and data support for the application of mixed cropping patterns in the alleviation of ARD.

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