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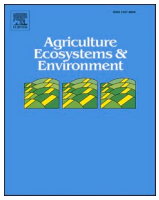

Intercropping with *Trifolium repens* contributes disease suppression of banana Fusarium wilt by reshaping soil protistan communities

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ABSTRACT

Fusarium wilt disease of bananas, caused by the soil-borne pathogen *Fusarium oxysporum*, threatens banana production. Intercropping, cultivation of more than one crop simultaneously on the same field, has emerged as efficient and sustainable land management for suppressing Fusarium wilt disease. Although previous studies have proven the changes in soil microbial communities including bacteria and fungi under intercropping contributed to disease suppression, little is known about the role of protistan communities in driving this effect. In a field experiment, we assessed microbiome shifts with a focus on protists under intercropping of banana with the legume *Trifolium repens*. Our results showed that the reduced Fusarium wilt disease incidence and the decreased *Fusarium* pathogen density under intercropping could be attributed to the changes in protistan community compositions. Specially, we observed a significant negative correlation between the relative abundance of phagotrophic protists and *Fusarium oxysporum*. We further conducted a pot experiment to examine the impacts of the legume crop on phagotrophic protists across different spatial distances, which showing that intercropping enriched the relative abundance of phagotrophic protists, especially *Cercomonas*, with members of this group showing the capacity to directly inhibit the growth of *Fusarium* pathogen *in vitro*. We highlight that predatory protists are important agents underlying disease suppression in intercropping system, which can offer new venues to promote plant health in sustainable agriculture.

1. Introduction

Bananas (*Musa* spp.) are globally important fruit crops (Dita et al., 2010), but banana production faces significant challenges due to Fusarium wilt, a destructive disease caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *cubense* (Butler, 2013; Ploetz, 2015). Fusarium wilt occurs in almost all banana-producing areas across China, posing a significant constraint on banana yields (Bubici et al., 2019; Jie et al., 2009). Banana Fusarium wilt is managed by cultivating disease-resistant varieties, applying chemical fungicides (Ismaila et al., 2023; Sun et al., 2018), using of biological control agents, or intercropping (Hong et al., 2020; Nel et al., 2007; Shen et al., 2019). Some resistant varieties show efficient resistance to banana Fusarium wilt (Liu et al., 2023; Smith et al., 2006), but these varieties often exhibit poor fruit quality. The overused chemical fungicides cause environmental problems (Chen et al., 2022; Li et al., 2021; Swarupa et al., 2014). As a result, the restoration of healthy banana production requires a more environmentally friendly and effective approach to suppress Fusarium wilt disease.

The implementation of intercropping measures has been reported to provide economically viable and effective means to reduce the occurrence of soil-borne diseases of diverse crops (Zhang et al., 2023; Zhou et al., 2023). For instance, intercropping with rice (Ren et al., 2008) and wheat (Lv et al., 2018) can significantly reduce Fusarium wilt in watermelon. Intercropping with a legume crop has been reported to not

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only help suppress soil borne pathogens (Tian et al., 2019; Were et al., 2022; Zhou et al., 2019), but also to improve crop nutrient status (Gao et al., 2014). These effects have been linked to intercropping-induced changes in soil microbial composition and function. In addition, intercropping with legume facilitates microbial community dispersal across different compartments between the different crops, thereby promoting interactions among adjacent compartments (Fernández-Aparicio et al., 2010; Liang et al., 2022). Tiemann et al. (2015) demonstrated that intercropping with a legume crop significantly modulated microbial community composition to enhance host plant functional resilience and tolerance to stress. Several studies have shown that intercropping can lead to the recruitment of beneficial indigenous soil microbial taxa via root exudates leading to increased host protection against pathogens (van Elsas et al., 2012; Zhalnina et al., 2018; Zhu and Morel, 2019). A previous study also found that intercropping with the legume *Trifolium repens* effectively reduced the incidence of banana wilt disease by regulating soil microorganisms and enriching beneficial microorganisms of bacteria and fungi (Yang et al., 2022). However, it still remains to be determined whether protistan communities, the important soil microbial components, contribute to the disease suppression under intercropping management schemes.

Protists, especially microbial-feeding predators (Adl and Gupta, 2006; Geisen et al., 2018), are important consumers of bacteria and fungi in the soil food web, thereby impacting soil microbial composition and function. They can feed selectively on microbial prey (Glücksman et al., 2010; Schulz-Bohm et al., 2017), leading to differential impacts on soil microbial communities (Amacker et al., 2022). Through this selective predation and activity induction, protists can promote some resistant bacteria to increase pathogen-suppressive secondary metabolites (Gao et al., 2019; Jousset et al., 2006). For instance, predatory protists can potentially reduce banana Fusarium wilt by promoting the expression of disease-suppressing secondary metabolite synthesis genes e.g., non-ribosomal peptide synthetase gene in pathogen-suppressive bacteria (Guo et al., 2022). Also, it has been shown that some of these phagotrophic protists can directly prey on plant pathogens (Geisen et al., 2016), including *Fusarium* strains. Nonetheless, the effect of predatory protists on the pathogenic fungi under banana-legume intercropping systems remains unclear.

To examine the impact of protists on banana Fusarium wilt disease suppression under legume intercropping management, we took a holistic soil microbial community approach including protistan community under legume intercropping versus banana monoculture management. Via this comparison, we sought to identify key protist taxa linked to disease suppression under legume intercropping. We subsequently conducted a pot experiment to further examine the potential impacts of the legume on predatory protists across different spatial distances. We hypothesized that the disease suppression associated with intercropping of *Trifolium repens* could be at least partly explained by changes in protistan community composition and specific protistan taxa. We further hypothesized that the specific protistan taxa enriched during legume intercropping can have direct inhibitory effects on soil-borne *Fusarium* pathogen.

2. Materials and methods

2.1. Intercropping field experiment

The experimental site was located in Qiaotou town (18°38′N, 108°45′E), Hainan Province, China. This region has sandy loam soil and a tropical monsoon climate with an average annual temperature of 24 °C and precipitation of 1786 mm, respectively. The intercropping field experiment was performed according to previous study (Yang et al., 2022). The intercropping treatment consisted of 60 banana plants in one column (the spacing between plants and rows was 2 m) with 1 kg of seeds per column of the legume *Trifolium repens*. Bananas planted from October 2019 to August 2020, and treatments were established in a randomized complete block design with three replicates for each treatment. *Trifolium repens* seeds were sown one week after banana planting, approximately 30 cm from the banana column (Fig. 1a). The same configuration was used for the banana monoculture treatment, but without seeding of the legume. Soil samples were collected from both treatments at the flowering (Inter_FS: intercropping with *Trifolium repens* at flowering stage; Mono_FS: monoculture at flowering stage) and at maturity stages of crop development (Inter_MS: intercropping with *Trifolium repens* at maturity stage; Mono_MS: monoculture at maturity stage). The banana flowering stage occurred approximately 6 months after the banana planting and the maturity stage occurred approximately 9 months.

We traced the incidence of banana wilt disease during the banana growth period based on the appearance of typical wilt symptoms, including brown discoloration of vascular tissues, pseudo stem splitting, leaf yellowing and plant death (Deltour, 2017). The level of disease incidence was calculated as the percentage of infected plants in relation to total number of banana plants until the incidence stabilized (Wang et al., 2015).

2.2. Pot experiments to test the effects of the legume on pathogens and protists over different spatial distances

We used a planting box (90×30×30 cm, length×width×height), which was separated by a 30 μm nylon mesh into 3 section (as shown in Fig. S1), to test the effects of the intercropping plant, *Trifolium repens,* on soil-borne pathogens and protists. We filled the boxes evenly with 30 kg of soil, planted two-month-old banana seedling in the left section and sowed *Trifolium repens* seeds (15 g per pot) in the right section. This resulted in the two crops being separated by a 30 cm. The nylon mesh separating the compartments, constituted a barrier for root growth across the central compartment, but did allow for the movement of root exudates and microorganisms (Fig. S1) (Li et al., 2007; Zhou et al., 2023). The soil for pot experiments was collected from Chengmai County, Hainan Province, with a 20-yr history of banana monoculture that suffers from serious Fusarium wilt disease. Pot experiments were conducted in a greenhouse at Hainan University from May 2020 through September 2020, Banana seedlings (*Musa* AAA *Cavendish* cv *Brazil*) were provided by the Chinese Academy of Tropical Agricultural Sciences at the Danzhou Campus of Hainan University.

2.3. Soil sampling, DNA extraction and Illumina MiSeq tag sequencing

For intercropping treatment, we randomly selected 5 banana plants and collected 1 soil core (~15 cm in depth and 5 cm in diameter) between banana and the legume, and further mixed the 5 soil cores into one composite sample as one replicate during the flowering stage and maturity stage. Each treatment was replicated three times. During sampling, we also collected soil samples in banana columns in banana monoculture treatment, using same methods as the intercropping treatment. One portion of each sample was air-dried for chemical analyses, and the other portion was stored at −80 °C for subsequent DNA extraction.

Soil physicochemical properties were measured according to a previous study (Yang et al., 2022). The pH value was measured using a pH meter, with a soil-to-water suspension ratio of 1: 2.5 (m/v). The soil organic matter (OM) content was determined following Ciavatta's method (Ciavatta et al., 1991). Ammonium nitrogen (NH_4^+-N) was extracted with potassium chloride (2 mol/L) and determined by indophenol blue colorimetry. Nitrate nitrogen (NO_3^--N) was extracted using potassium chloride and analyzed with an ultraviolet spectrophotometer (Wang et al., 2015). The instrument model used for this analysis is "AA3 Auto Analyzer" by Shenzhen E-Zheng tech Co., Ltd, with wavelengths used are 660 nm and 550 nm, respectively. The available phosphorus (AP) in the soil was extracted with ammonium fluoride-hydrochloric acid and assayed using the molybdenum-antimony anti-colorimetric

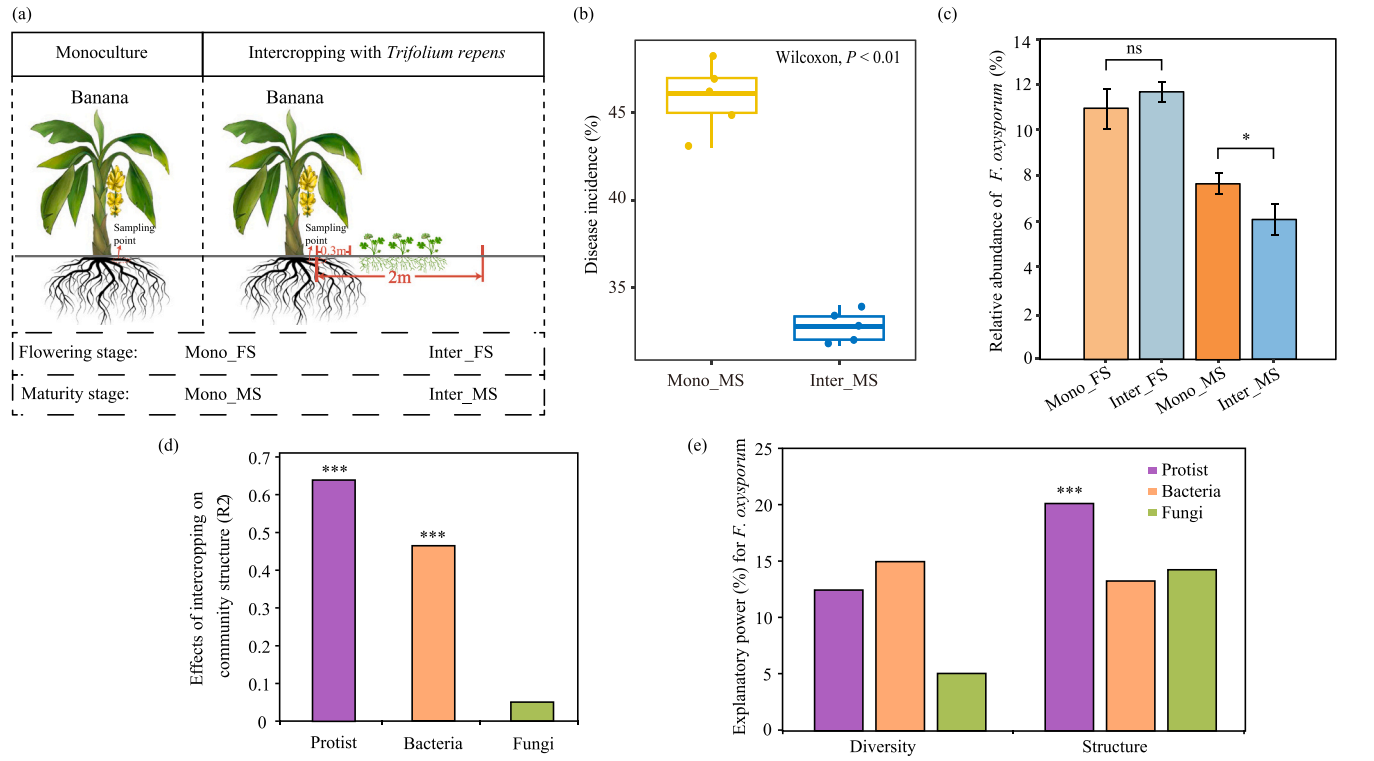

Fig. 1. Schematic diagram of banana intercropping field experiment design and sampling (a). The disease incidence of banana Fusarium wilt under banana intercropping and monoculture systems (b). The relative abundance of *Fusarium oxysporum* under banana intercropping and monoculture systems (c). The effects of intercropping on bacterial, fungal and protistan community structure (d). The relative importance of bacterial, fungal and protistan community for *Fusarium oxysporum* under banana intercropping and monoculture systems (e). Mono_FS: monoculture at flowering stage; Inter_FS: intercropping with *Trifolium repens* at flowering stage; Mono_MS: monoculture at maturity stage; Inter_MS: intercropping with *Trifolium repens* at maturity stage. One asterisk means $P < 0.05$, three asterisks mean $P < 0.001$.

method at a wavelength 700 nm (Shen et al., 2019). The instrument model used for this analysis was "FP 640", manufactured by Shanghai Jingmi Co., Ltd. Available potassium (AK) was extracted with ammonium acetate and measured using a flame photometer. The instrument model is "722 N", also manufactured by Shanghai Jingmi Co., Ltd.

In pot experiment, soil samples were collected from the middle of the box between the two nylon meshes after 120 days of cultivation. As shown in Fig. S1, five points (labeled A-E) were identified with a 6 cm interval. We collected five soil samples equidistant from the vertical line of each point, resulting in a total of 25 soil samples per pot, and this was replicated three times. Three soil samples at corresponding locations were mixed into one composite sample.

Soil DNA was extracted from 0.5 g soil using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories Inc., USA), according to the manufacturer's instructions. Bacterial-, fungal-, and eukaryote-universal primers sets were used for high-throughput Illumina MiSeq sequencing: 520 F (5′-AYTGGGYDTAAAGNG-3′) and 802 R (5′-TACNVGGGTATCTAATCC-3′) were selected to amplify the V4 region of bacterial 16 S rRNA genes (Schoch et al., 2012); ITS5F (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS1R (5′-GCTGCGTTCTTCATCGATGC-3′) were used to target the ITS1 region for fungi (Hong et al., 2023), and 528 F (5′-GCGGTAATTCC AGCTCCAA-3′) and 706 R (5′-AATCCRAGAATTTCACCTCT-3′) to amplify eukaryotic 18 S rRNA gene V4 regions (Heidelberg et al., 2013; Ohore et al., 2022). PCR amplification was performed in a 25 μl volume: 5 μl of 5 × reaction buffer, 5 μl of 5 × GC buffer, 2 μl dNTPs (2.5 mM), 1 μl of each primer (10 μM), 0.25 μl of high-fidelity DNA polymerase, 2 μl of DNA template and 8.75 μl of ddH_2O. The thermal cycling conditions were as follows: initial denaturation (98 °C for 2 min), followed by 30 cycles of denaturation (98 °C for 15 s), annealing (55 °C for 30 s), extension (72 °C for 30 s), and a final extension (72 °C for 5 min). Each sample was amplified in triplicate, pooled in equimolar concentrations of 20 ng·μl^{-1}, and then purified with a PCR Purification Kit (Axygen Bio, USA). The raw sequence data has been deposited in the NCBI Sequence Read Archive (SRA) database under accession number PRJNA992755.

2.4. Bioinformatic analyses

The bacterial, fungal, and protistan raw sequences were processed according to previously established protocols (Xiong et al., 2020; Yang et al., 2022). Briefly, low-quality sequences and singletons were removed. After that, the remaining sequences were assigned at a 97% similarity threshold, and chimeras were filtered using UCHIME (Edgar et al., 2011). Finally, representative sequences for bacterial and fungal OTUs were classified using the RDP classifier against the RDP Bacterial 16 S rRNA gene and the UNITE Fungal ITS databases, respectively (Yang et al., 2022). *Fusarium oxysporum* was used as a surrogate for pathogenic *Fusarium oxysporum* f. sp. *cubense* (Foc), as previous study showed that the abundance of *Fusarium oxysporum* is positively correlated with Fusarium wilt disease (Yuan et al., 2021; Yang et al., 2022). The 18 S rRNA gene sequences were matched against the Protist Ribosomal Reference database (PR2) (Guillou et al., 2012). We removed OTUs assigned as Rhodophyta, Streptophyta, Metazoa, Fungi, and unclassified Opisthokonta sequences for protistan community analyses. Taxonomic protistan OTUs was assigned into different functional groups according to their predicted feeding mode (i.e., phagotrophs, parasites, phototrophs, plant pathogens and saprotrophs) (Adl et al., 2019; Dumack et al., 2020; Xiong et al., 2019). Relative abundances of each protistan taxonomic and functional group in relation to total protistan reads were used for later analyses.

2.5. Testing the inhibition of Fusarium oxysporum by Cercomonas isolates

To examine potential direct inhibition of the *Fusarium oxysporum*

pathogen by members of the *Cercomonas* genus, we conducted a direct predation assay using *Cercomonas* sp. strain S24D2 isolated and identified previously (Amacker et al., 2020; Gao, 2020). The co-culture experiment consisted of the inoculation of the *Cercomonas* strain and the pathogen *Fusarium oxysporum* f. sp. *cubense*, with inoculation of only *F. oxysporum* as a control. *F. oxysporum* spore solution was prepared by filtering through 100 μm filter paper. The fungal density was then estimated using a hemocytometer and was adjusted to 400 cells μl^{-1}. Prior to the experiment, protists were cultivated with *E. coli* (*Escherichia coli*) as food source at 15 °C These cultures were then washed two times with PAS (Page's Amoeba Saline) by centrifugation (800 g, 5 min) to remove *E. coli*. The numbers of protists were evaluated under an inverted microscope and adjusted to 40 individuals μl^{-1}. A mixed inoculum of 50 μl of the *Cercomonas* sp. solution and 50 μl of the *F. oxysporum* spore solution was added to wells in a 96-well plate, and 1 μl aliquots were transferred from the suspension to PDA medium. As a control, another mixed inoculum of 50 μl of a *F. oxysporum* spore solution and 50 μl PAS was added to another 96-well plate, and 1 μl aliquots from the suspension were transferred to PDA medium. We recorded the length/diameter of *F. oxysporum* hyphae at different time intervals (24 h, 48 h, 72 h and 96 h).

2.6. Statistical analyses

Wilcoxon test was used to compare the disease incidence between treatments under intercropping and monoculture. Student's *t* test was used to compare the relative abundance of *Fusarium oxysporum* between treatments under intercropping and monoculture at two different periods. The α-diversity of bacterial, fungal and protistan communities was estimated using non-parametric Shannon indexes. A principal coordinate analysis (PCoA) based on Bray–Curtis distance metrics was used to explore the differences in bacterial, fungal and protistan community compositions. The α-diversity and PCoA of bacterial, fungal and protistan communities were calculated using MOTHUR (Schloss et al., 2009). One-way ANOVA with Tukey's HSD test was performed to assess the differences of the relative abundance of *F. oxysporum* in different treatments. The permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001) was performed to assess the effects of intercropping on soil microbiome community structures through the "Adonis" function with 999 permutations in the "vegan" package in R (version 3.4.3) (Guo et al., 2021). We used the "relaimpo" package (Grömping, 2006) in R (version 3.4.4) to calculate the relative importance of main microbial parameters to evaluate the explanatory power for *F. oxysporum*. Abundant protistan OTUs (with average relative abundance across all samples > 0.1%) were used to identify potential indicator taxa, which were assessed in LEfSe (Segata et al., 2011) through the "lefse command" in Mothur. A pairwise Spearman correlation matrix was calculated with the "corr.test" function in the package "psych" in R (version 3.4.4). Network properties were characterized by the "igraph" package in R (version 3.4.4). Networks of indicator protists linked with the *F. oxysporum* were visualized in Cytoscape (v3.5.1). In addition, since the enrichment of phagotrophs was found in the presence of the legume, only phagotrophic protists were selected to analyze the relative change. The relative change of protistan OTUs enriched by legume was calculated using the following formula: (point E – point C) / point C (Table S3).

3. Results

Legume intercropping (Inter_MS) has a cumulative disease incidence value of 32.78%, which was significantly lower (Wilcoxon test, $P < 0.01$) than the value of 46.11% observed for the Mono_MS (banana monoculture) (Fig. 1b). We also found that the relative abundance of *Fusarium oxysporum* under Inter_MS was lower than Mono_MS at the maturity stage (student's *t* test, $P < 0.05$), but not at the flowering stage (Fig. 1c).

The community structure of protistan and bacterial communities differed across the two cropping systems, with the strongest impacts on protists (PERMANOVA: $P < 0.001$; Fig. 1d). We also found that the community structure of protists better predicted the density of the *Fusarium oxysporum* pathogen compared to bacterial and fungal community structure (Fig. 1e). We focused subsequent analyses on protists, since protists were more sensitive to legume intercropping (Fig. 1d) and showed the best prediction on the development of *Fusarium oxysporum* pathogens (Fig. 1e). Phagotrophic protists were the most abundant functional protistan group, and the cercozoan protists were the most dominant protistan taxa across all soil samples (Fig. S2). Indicator species analysis revealed 56 protistan indicator OTUs in the intercropping system, with almost half of these identified as phagotrophs and 37.5% as cercozoan protists (Table S2). However, the overall predatory protists did not differ between treatments under intercropping and monoculture (Fig. 2a and Fig. S2). We found that legume intercropping enriched the relative abundance of Cercozoa compared to monoculture at the flowering stage (student's *t* test, $P < 0.05$), but no significant difference was observed at the maturity stage (Fig. 2a and Fig. S2). Moreover, regression analysis revealed that both phagotrophic and cercozoan protists were negatively correlated with disease incidence (phagotroph: $R^2 = 0.809$, $P < 0.001$; Cercozoa: $R^2 = 0.753$, $P < 0.001$; Fig. 2b, c). Together, these findings suggest that phagotrophic protists of cercozoan taxa were contributed to banana Fusarium wilt disease suppression under intercropping with legume during early stages of crop growth.

The co-occurrence network comprising *Fusarium oxysporum* and indicator protistan OTUs showed that the intercropping system exhibited a more complex network structure with more microbial links (Fig. S3). A simplified co-occurrence network focusing on the indicator protistan OTUs with negative correlation with *Fusarium oxysporum* is presented in Fig. 2d. The intercropping network consisted of 33 protistan OTUs, including 12 phagotrophic protists, whereas the monoculture network exhibited a lower number of protistan OTUs with 14 protistan OTUs, including 6 phagotrophic protists (Fig. 2d).

Our pot experiment confirmed that legume planting reduced the relative abundance of *Fusarium oxysporum* (Fig. S4). We further examined the changes of the relative abundance of protistan community composition at different distances from the *Trifolium repens* plant. We found that 13 indicator protistan OTUs were enriched by the legume, including 8 phagotrophic protists and 4 phototrophic protists (Fig. 3a and Table S3). P_OTU58 (the most abundant phagotrophic protist OTU accounting for 2.12%, belonging to *Cercomonas*) increased of 102% by legume selection (Fig. 3a). We found that the relative abundance of phagotrophic protists significantly increased with decreasing distance to the legume plant ($R^2 = 0.061$, $P < 0.001$; Fig. S5), while the relative abundance of phototrophic protists decreased ($R^2 = 0.063$, $P < 0.001$; Fig. S5). In addition, the relative abundance of *Cercomonas* (P_OTU58) significantly increased with decreasing distance to the *Trifolium repens* plant ($R^2 = 0.293$, $P < 0.001$; Fig. 3b). We further confirmed that the relative abundance of *Cercomonas* was significantly selected by the legume (*Cercomonas*: $R^2 = 0.232$, $P < 0.001$, Fig. 3c). In addition, we tested the predation effects of *Cercomonas* strain (*Cercomonas* sp. S24D2) on *Fusarium oxysporum* f. sp. *cubense* growth on the PDA culture medium. We observed *Cercomonas* strain significantly inhibited *Fusarium oxysporum* growth on the PDA culture medium compared with the control treatment (Fig. 3d).

4. Discussion

Previous studies have reported that intercropping with a legume can effectively reduce the incidence of banana wilt disease (McIntyre et al., 2001; Yang et al., 2022). Although it has been shown that disease reduction is related to changes in soil microbial communities of bacteria and fungi (Wu et al., 2020), little is known about how such intercropping affects other soil microbiome components, protists. It has been shown that protist communities can be highly indicative for disease

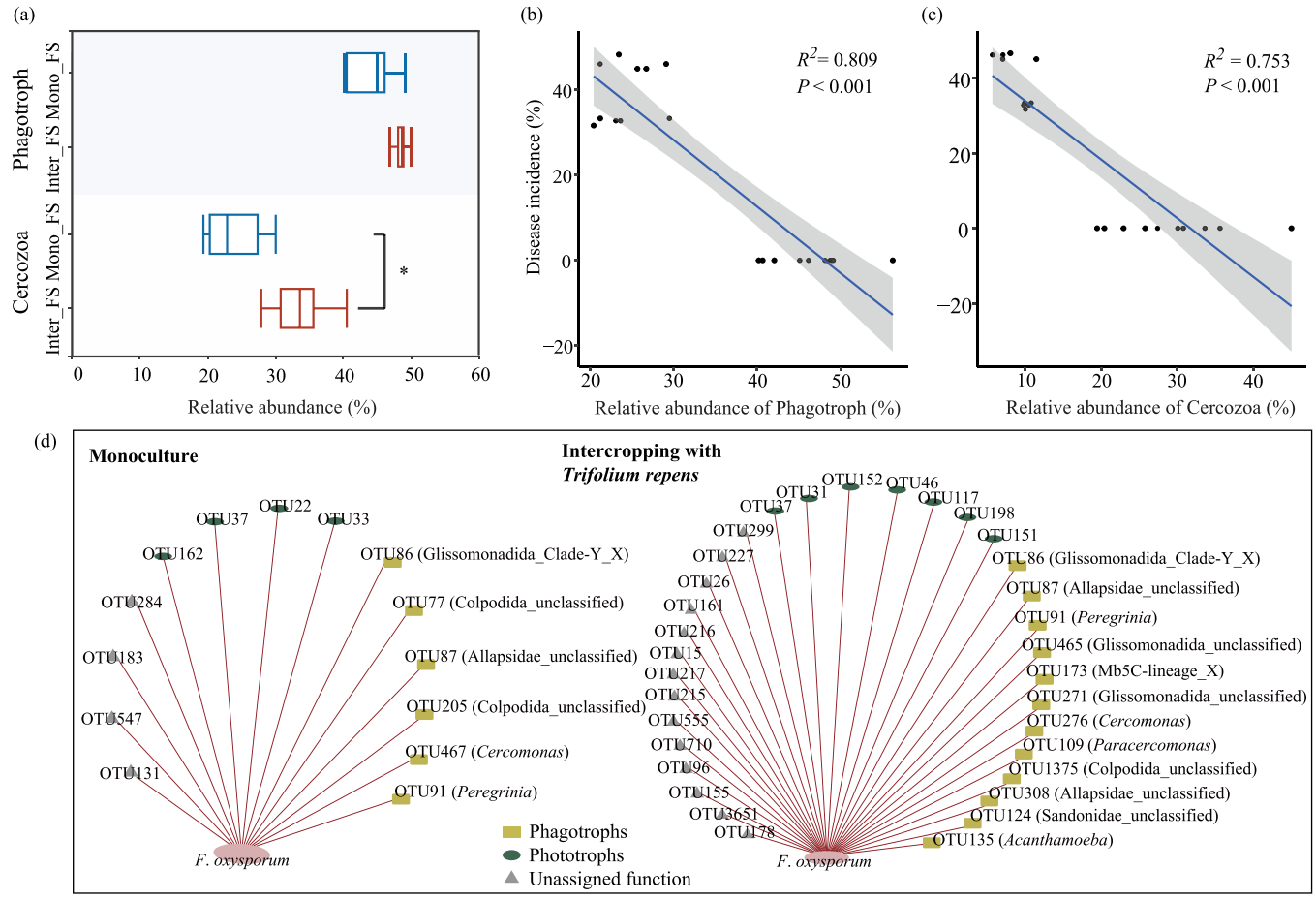

Fig. 2. The relative abundance of key protists under banana intercropping and monoculture systems (a). Correlations between the relative abundance of phagotrophic protists (b), cercozoan protists (c) and disease incidence across plant growth under banana intercropping and monoculture systems. Co-occurrence networks between indicator protistan OTUs and *Fusarium oxysporum* under intercropping *Trifolium repens* and monoculture (d). In panel a, almost half of protistan indicator OTUs identified as phagotrophs and 37.5% as cercozoan protists under intercropping via indicator species analysis. Asterisk means $P < 0.05$. In panel d, only indicator protistan OTUs negatively related with *Fusarium oxysporum* were selected. Red lines indicate negative correlations; detailed annotation of protistan OTUs are provided in Table S2.

suppression abilities (Ren et al., 2022). In this study, we demonstrate that the suppression of banana Fusarium wilt disease associated with legume intercropping (*Trifolium repens*) management is linked with changes in protistan community composition and that specific indicator protistan taxa of *Cercomonas* can directly inhibit the Fusarium pathogen. Our findings indicate that the protistan community, especially predatory protists, plays a previously neglected role in banana Fusarium wilt disease suppression, in addition to the application of plant-beneficial bacteria and fungi, we propose that predatory protists might offer new venues to support plant health in sustainable agriculture.

The intercropping system was found to decrease the pathogen density in the soil and incidence of the Fusarium wilt disease compared to the monoculture. This finding is in accordance with our previous study that showed that the use of a banana–*Trifolium repens* intercropping effectively reduces the incidence of this soil-borne fungal pathogen (Yang et al., 2022). Increasing plant diversity via legume intercropping has been shown to have positive effects on disease suppression for neighboring plants (Gao et al., 2014; Ren et al., 2008) potentially through increasing the diversity of root exudates. In addition, intercropping can reduce plant disease through shaping soil microbial communities (Zhou et al., 2019), for example enriching antagonistic microbes to support plant health (Mwakilili et al., 2021). The focal crops can perceive chemical signals released by neighboring plants to assemble a disease-suppressive microbiome enriched in plant-beneficial bacteria, such as those producing the antifungal compounds (*i.e.*, 2, 4-diacetylphloroglucinol and pyrrolnitrin) (Latz et al., 2012; Zhou et al., 2023). Legume intercropping has been shown to regulate fungal community composition, resulting in increased abundance of certain fungal genera, such as *Trichoderma* (Lian et al., 2018), which also contribute to pathogen inhibition (Wu et al., 2018).

Unlike previous research investigating the role of soil microbial communities in intercropping-induced disease suppression, which exclusively focused on bacteria and fungi (Ablimit et al., 2022; Yang et al., 2022), we emphasized the importance of protistan communities in controlling Fusarium wilt disease under intercropping system. Our results support the notion that protists serve as an important indicator within microbial communities in predicting plant health (Xiong et al., 2020). Intercropping with the legume alters the composition of the protistan community, enriching certain protistan taxa of the Cercozoa that were linked to disease suppression, especially during the early stage of crop development. This finding supports previous research that the initial microbial community of predatory protists (Xiong et al., 2020), as well as microbial functions (Wei et al., 2019), at the early stages of plant development can determine future plant health.

Our results also indicate that an increase in microbial community complexity upon intercropping, that might contribute to soil ecosystem services of disease control (Zhao et al., 2022). The changes in protist communities under legume intercropping may exert important top-down control of other soil-borne microbial populations (Asiloglu et al., 2021). Legume intercropping could enhance more potential

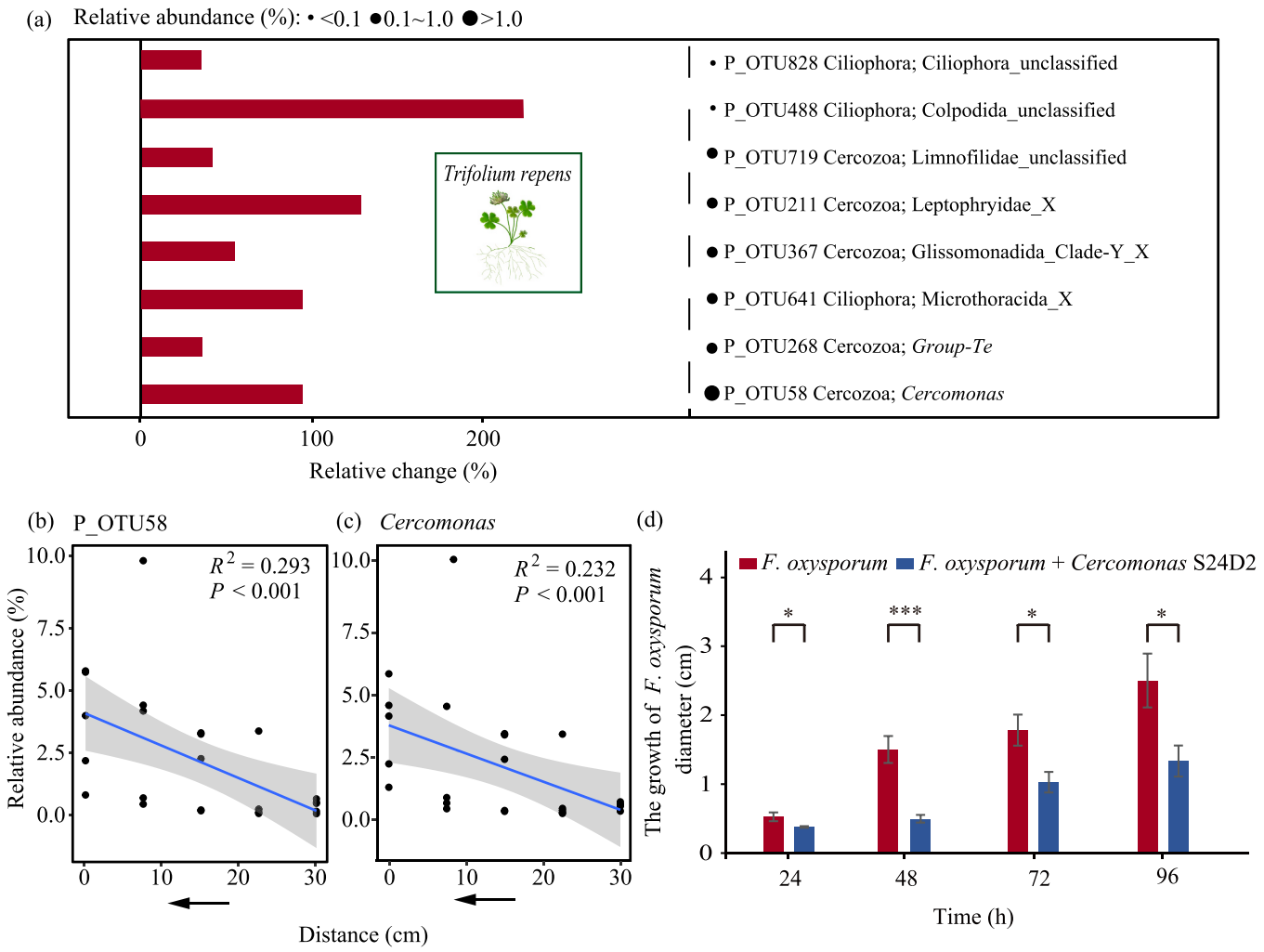

Fig. 3. The effects of different distance from *Trifolium repens* on the relative abundance of phagotrophic protists (a). Correlations between distances from *Trifolium repens* and the relative abundance of P_OTU58 (b), *Cercomonas* (c). Bar chart displaying the hypha diameter (cm) of *Fusarium oxysporum* in two treatments at different time intervals (d).
In panel a, Relative change (enriched by *Trifolium repens*): (point E - point C) / point C. In panels b,c, the relative abundance of P_OTU58 accounts for 92.24% of *Cercomonas*. Distance indicates the distance from *Trifolium repens* and "P_" represents protistan OTUs in the pot experiment, distinguished from protistan OTUs in the field experiment. Asterisk (*) indicates $P < 0.05$, tree asterisks (***) indicate $P < 0.001$.

pathogen-suppressive protists that showing significantly negative correlations with *Fusarium oxysporum* pathogen, supporting the role that phagotrophic protists in disease suppression (Ren et al., 2023; Wu et al., 2022). Phagotrophic protists can suppress disease via a range of mechanisms, including directly preying on fungal pathogens (Geisen et al., 2016), selectively enriching beneficial microbes with antagonistic functions (Gao et al., 2019; Guo et al., 2022), or activating bacterial functions responsible for suppressing plant pathogens (Bonkowski, 2004; Jousset et al., 2006).

The phagotrophic protists (Cercozoa taxa) enriched under intercropping with legume might be explained by species-specific predator-prey relationships (Pedersen et al., 2011; Schulz-Bohm et al., 2017) through the enriched bacterial and fungal populations by legume intercropping (Yang et al., 2022), as the majority of the protistan predators do not directly utilize root nutrients. These findings indicate the potential prey under intercropping system would indirectly increase predatory protists which might further promote disease-suppression. In addition, the predatory effect of *Cercomonas* on *Fusarium* pathogen was validated *in vitro* experiments, supporting previous findings that *Cercomonas* sp. can grow by utilization on plant pathogenic fungus, *Fusarium culmorum* (Geisen et al., 2016). Since the disease suppressive effects of legume intercropping are partly explained by the enrichment of the specific protistan taxa, such as *Cercomonas* that contribute to pathogen suppression via direct predation, we propose that these organisms would be optional agents for the development of new biocontrol strategies.

We demonstrated that in addition to bacteria and fungi, phagotrophic protists play key roles in banana Fusarium wilt disease suppression under intercropping system. Phagotrophic protists, especially *Cercomonas,* enriched by legume intercropping play an important role in *Fusarium* pathogen suppression and this impact depends on the spatial distribution of the legume plants. Future studies should focus on the isolation of keystone protistan taxa, thereby to unlock the trophic interactions and potential microbial functions involved in soil borne disease suppression. We propose predatory protists could serve as cornerstone agents in targeted microbiome engineering to promote plant health thereby reducing dependency on exogenous pesticide applications.

5. Conclusions

We highlight the role of protists, especially predatory protists, in contributing to reducing disease incidence of banana Fusarium wilt under legume intercropping management. Our results demonstrate that protistan communities are sensitive to intercropping legume, and

phagotrophic protists, especially cercozoan protists, recruited by the legume serve as the important microbial determinants of banana Fusarium wilt disease suppression. We propose that predatory protists may provide the leverage controller for targeted soil microbiome, that contributing to soil-borne disease suppression and ultimately promoting crop performance.

CRediT authorship contribution statement

Xiangyu Ren: Conceptualization, Methodology, Software, Visualization, Writing – review & editing. **Zeyuan Zhou:** Resources, Methodology, Writing – review & editing. **Manyi Liu:** Resources, Writing – review & editing. **Zongzhuan Shen:** Resources, Supervision. **Beibei Wang:** Investigation, Methodology, Writing – review & editing, Supervision, Funding acquisition. **Alexandre Jousset:** Writing – review & editing. **Stefan Geisen:** Writing – review & editing. **Mohammadhossein Ravanbakhsh:** Writing – review & editing. **George A. Kowalchuk:** Writing – review & editing. **Rong Li:** Resources, Supervision, Funding acquisition. **Qirong Shen:** Resources, Supervision. **Wu Xiong:** Investigation, Conceptualization, Methodology, Visualization, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agee.2023.108797.

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