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HIGHLIGHTS

- We studied the effects of CP and AZO on soil microeukaryotes and P solubilizers.
- CP shifted soil microeukaryotic communities from fungi to protist dominance.
- CP reduced the phoC/phoD gene copy number and related phosphatase activities.
- *Sinorhizobium* and *Streptomyces* are the key species to improve soil P availability.
- CP and AZO had no synergistic effects on soil microeukaryotes and P solubilizers.

GRAPHICAL ABSTRACT
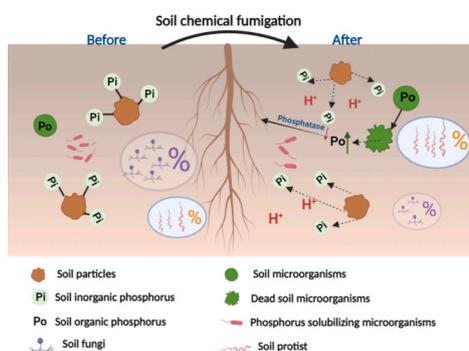
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ABSTRACT

Fumigants and fungicides are effective at controlling soil-borne pathogens but might also adversely affect soil beneficial microbes, such as soil phosphorus (P) solubilizing microbes, further altering nutrient cycling processes. Therefore, this study investigated the effects of the fumigant chloropicrin (CP) and the fungicide azoxystrobin (AZO) on soil microeukaryotes and P-cycling related soil bacteria through a greenhouse experiment. Soil microeukaryotic communities and bacterial communities containing two phosphomonoesterase encoding genes (*phoC* and *phoD*) were analysed using high-throughput sequencing methods. Results showed that, when applied at the field recommended application dosage, the fungicide AZO had no significant influence on the community structure of soil microeukaryotes and *phoD*-containing bacteria. However, in CP-fumigated soils, the soil microeukaryotic community composition changed from fungi-dominated to protist-dominated. CP fumigation significantly decreased the total *phoC/phoD* gene copy number but increased the relative abundance of some

phoC/phoD-containing bacteria (such as *Sinorhizobium* and *Streptomyces*), which are significantly positively correlated to available P compositions in soil. The structural equation model (SEM) confirmed that CP fumigation could affect soil available P content directly by altering *phoC*-/*phoD*-containing bacteria, or indirectly by affecting *phoC/phoD* gene abundance and acid/alkaline phosphatases activity in soil. The inconsistent changes in *phoC/phoD*-containing bacteria, *phoC/phoD* gene number, and the phosphomonoesterase activities indicated that enzyme secretion may not be the only way for P solubilizing soil microorganisms to regulate P availability after soil fumigation. The outcome of this study can provide theoretical support for the design of soil beneficial microorganism recovery strategies and the regulation of phosphate fertilizer after soil fumigation.

1. Introduction

Soil-borne diseases caused by soil-borne pathogens have become one of the most serious obstacles in the cultivation of high-value crops such as strawberries, ginger, and herbs (Xiong et al., 2023). Soil fumigation can effectively prevent the occurrence of soil-borne diseases by injecting chemical fumigants such as chloropicrin (CP) into the soil to kill soil-borne pathogens (Rokunuzzaman et al., 2016). CP fumigation has been proven to effectively reduce the number of soil pathogens and significantly increase the marketable yield of crops (Zhang et al., 2019; Zhu et al., 2021). However, due to the broad killing spectrum of fumigants, soil fumigation could have deleterious effects on non-target soil microorganisms, such as Proteobacteria, Chloroflexi and Acidobacteria. Some of these non-target organisms are beneficial microorganisms such as *Bacillus* (Zhang et al., 2019; Fang et al., 2020).

Apart from soil bacteria, non-target soil microeukaryotes, including fungi, protists and metazoans, can also be affected by soil fumigation. A study found that short-term chloroform fumigation decreased the soil microbial biomass size, promoted the turnover of soil microeukaryotic community composition, and ultimately helped to maintain soil organic carbon mineralization processes by releasing hydrolytic enzymes (Chen et al., 2015). Regarding soil microbial community composition, soil fumigation could have different effects on different microeukaryotes. For example, Li et al. (2021) found that CP fumigation reduced the relative abundance of Ascomycota, while another study conducted by Zhang et al. (2019) found that CP fumigation at 30 g m^{-2} for 10 days in a strawberry greenhouse improved the relative abundance of Basidiomycota. Among soil microeukaryotes, soil protists can prey on soil bacteria and fungi or decompose soil organic carbon (Geisen et al., 2018). Therefore, necromass released from cell lyses could provide nutrient sources for those surviving soil protists and promote their recovery in fumigated soils. However, the recovered soil protists might further prey on the remaining soil bacteria or fungi and slow down their growth. When designing microbial restoration scheme for fumigated fields, previous studies have usually focused on the composition assembly of soil beneficial bacteria, ignoring the changes occurring in soil microeukaryotes.

Changes in soil functional microorganisms after soil fumigation could further change related biochemical soil processes, such as soil nutrient cycling (Li et al., 2017). Among soil nutrients, phosphorus (P) is one of the most essential elements for plant growth, and its availability is largely controlled by soil phosphorus solubilizing microorganisms (PSMs), which can be affected by soil fumigation (Huang et al., 2020a, 2020b). PSMs can solubilize inorganic P via acidification by releasing protons and organic acids, via chelation and exchange reactions of bounded P (Tian et al., 2021), or mineralize organic P to inorganic P by various extracellular phosphatases, such as acid and alkaline phosphatases (AiP and AlP) (Nannipieri et al., 2011). AiP and AlP are non-specific enzymes that catalyse the hydrolysis of ester-phosphate bonds to release orthophosphate (Fraser et al., 2017) and increase P availability for plant uptake. Bacterial *phoC* and *phoD* genes have been identified as being responsible for the synthesis of AiP and AlP, respectively (Luo et al., 2019). Since fumigants can disturb *phoC/phoD*-containing bacteria, soil fumigation may also change the number and expression of *phoC/phoD* genes, thereby changing the soil P cycling process (Castellano-Hinojosa et al., 2022).

In addition to pre-treatment with soil fumigation, other fungicides with specific targets have also been used to control pathogen recovery during farming (Huang et al., 2019; Wang et al., 2022a). These fungicides were also found to inhibit the populations of non-target bacteria and actinomycetes, as well as the activities of soil enzymes such as urease, protease and dehydrogenase (Wang et al., 2018). For example, Han et al. (2020) found that the application of the fungicide azoxystrobin (AZO) at 5.0 mg kg^{-1} significantly decreased the relative abundance of *Streptomyces Actinomadura*, *Bacillus*, *Sphingomonas*, *Haliangium*, *Streptococcus*, *Nitrospira*, *Lysobacter*, and *Altererythrobacter* by 1.1 to 95.1 %. Therefore, application of AZO fungicide might disrupt soil microbiome recovery pathways in fumigated soils. Conversely, the effect of AZO on the soil microbiome might be altered by pre-treating soils with fumigation, as previous studies found that CP fumigation inhibited the adsorption and degradation of AZO in the soil system (Huang et al., 2019). Therefore, the combined application of CP and AZO may have different effects on the soil microbiome as opposed to using them individually. Our previous study showed that the using a CP fumigant and an AZO fungicide together led to more profound effects on the P uptake in ginger and soil P fractions, which could have been due to the variations in P-cycling related soil microbes (Wang et al., 2022a; Wang et al., 2023). In order to explore the underlying microbial mechanisms in soil P cycling processes with the application of CP fumigant and AZO fungicide, this study further investigated two phosphomonoesterase genes (*phoC* and *phoD*) containing bacteria, using the same greenhouse experiments as those performed in our previous study. The current study focused on the knowledge gaps that surround the unclear combined effects of CP fumigant and AZO fungicide on P-cycling related soil microorganisms to provide information relevant to phosphate fertilizer adjustments after soil fumigation. The soil microeukaryotic community was also analysed to provide theoretical support for the development of soil biological amendments to improve crop growth after fumigation. Based on our previous findings, we can hypothesis that at the field recommended application rates, CP fumigation has much stronger effects on the soil microbiome than AZO fungicides, which results in no significant comprehensive effects when CP and AZO are applied together.

2. Materials and methods

2.1. Experiment materials and design

The soil used in this experiment was surface soil (sandy clay loam) collected from fields in An'qiu, Shandong Province, one of the most important vegetable production areas in China. Ginger (zingiber officinale) was used as model plant due to the intensive use of soil fumigation and fungicides in ginger fields. Pesticide usage surveys among local farmers found that fumigant chloropicrin and fungicide azoxystrobin are among the most commonly used fumigants and fungicides in the region. Detailed methods of soil pretreatment and experimental design were described in our previous study (Wang et al., 2022a). This study aimed to simulate the effects of chloropicrin fumigation processes and azoxystrobin fungicide application on soil functional microorganisms under real farmland condition. Therefore, before the experiment, a field (1.2

m × 2.2 m) that had never been planted with ginger was pretreated with in-situ chloropicrin fumigation for one week. For the soil fumigation, chloropicrin (CP; Dalian Lvfeng Chemical Co. Ltd.) was injected into the surface soil layers (0-15 cm) at the field recommended dosage of 37.1 g m^{-2}. The field was immediately covered with plastic film for one week and then ventilated for another week by removing the plastic film. Another field adjacent to the fumigated field was selected as the source of unfumigated soil. After fumigation, surface soils (0–20 cm) from both unfumigated and fumigated fields were collected and taken to the greenhouse for use as unfumigated and CP-fumigated soils.

In the greenhouse, three levels of azoxystrobin (AZO, Hebei Zhongbaolv Crop Technology company) were added into unfumigated and CP-fumigated soils, respectively, making 6 treatments in total including: CK (untreated soil), AZO1 (once application of AZO (azoxystrobin)), AZO2 (twice applications of AZO), CP (CP fumigated soil without AZO), CP + AZO1 (CP fumigated soil combined with AZO1) and CP + AZO2 (CP fumigated soil combined with AZO2) with 5 pot replications for each treatment and every sampling occasion. At the beginning of the experiment, 6 kg of soil and 100 g of healthy germinated ginger rhizome were put into each pot (diameter 30 cm, height 25 cm). The soil was spread evenly, and the ginger rhizome was buried 10 cm below the soil surface. AZO was applied using a spray method with a field recommended dosage of 47.1 mg m^{-2} 8 weeks after planting (WAP) for AZO1 and CP + AZO1 treatments, while the same amount of AZO was applied again at 16 WAP for AZO2 and CP + AZO2 treatments. During the experiment, soil samples were collected twice using a disruptive sampling method. The first batch of soil samples were collected after soil fumigation but before ginger planting (BP: 12/04/2019) as the initial soils to study the effects of CP fumigation process on the soil microbiome. The second soil sampling was conducted at 17 WAP (flourishing growth stage), one week after the application of AZO2, which includes all of the six treatments (CK, AZO1, AZO2, CP, COP+AZO1 and CP + AZO2). In addition, our previous study analysed the half-life of AZO in our experiment and found that it was about 7 days, which means that AZO fungicide would have the strongest effects on soil microorganisms at this time (Wang et al., 2022a). Therefore, we selected 17 WAP samples to study the effects of AZO fungicide, CP fumigate and their combination on soil microorganisms. When sampling, all roots and other debris in soil were separated and sorted out. Then, the soil sample was collected and stored at −80 °C for DNA extraction.

The soil acid and alkaline phosphatase (AiP and AlP) activity were analysed according to the production of ρ-nitrophenol by hydrolysis of ρ-nitrophenyl, while soil P fractions were analysed using a modified Hedley's sequential extraction method. All analysis methods and results were published in our previous study (Wang et al., 2023).

2.2. DNA extraction and quantification of phoC and phoD genes

Total soil DNA was extracted from 0.50 g of fresh soil sample using the Fast DNA® SPIN Kit for Soil (MP Biomedicals, California, US), according to the manufacturer's protocol, and then stored at −20 °C for sequencing.

The amount of *phoC* and *phoD* was determined by quantitative polymerase chain reaction (qPCR) using the Bio-Rad CFFX96 Real-Time System (Bio-Rad Laboratory, CA, USA) with SYBP Select Master Mix (2×) (Applied Biosystems, USA). The primers, qPCR program and reaction composition are listed in Table S1. The amount of *phoC* and *phoD* was expressed as gene copy numbers per gram dry soil (copies g^{-1} dry soil) and were calculated using standard curves established by serial dilutions of 1E3, 1E4, 1E5, 1E6, 1E7 and 1E8 of plasmid DNA containing *phoC* and *phoD* fragments. The amplification efficiencies of our methods were 85 %–105 % with R^2 values > 0.995.

2.3. High-through sequencing of 18S rRNA and phoC/phoD genes

The structure of microeukaryotic communities and *phoC-/phoD-*

containing microbial communities were assessed using high-throughput sequencing. The 18S rRNA gene of the V4 region was amplified using the universal primer (528F(GCGGTAATTCCAGCTCCAA)/706R (AATC-CRAGAATTTCACCTCT)). The primers for *phoC* and *phoD* genes are shown in Table S1. PCR reactions were conducted using BioRad S1000 (Bio-Rad Laboratory, CA, USA). PCR products were checked for quality using agarose gel electrophoresis and purified with the E.Z.N.A. Gel Extraction Kit (Omega, USA). Sequencing libraries were generated using the NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® (New England Biolabs, MA, USA), and were assessed on the Qubit@ 2.0 Fluorometer (Thermo Fisher Scientific, MA, USA). After the quality check, the libraries were sequenced on an Illumina Nova6000 platform with a PE250 mode for the 18S rRNA and p*hoD* genes, while the PE150 mode was used for the *phoC* gene (Guangdong Magigene Biotechnology Co., Ltd. Guangzhou, China).

2.4. Sequencing data processing

The Illumina MiSeq sequencing reads for the 18S rRNA, *phoC* and *phoD* were processed using the DADA2 pipeline v1.8 (Callahan et al., 2016). Forward reads were trimmed at 240 bp, and reverse reads were trimmed at 220 bp. Reads with an error rate higher than 2 were discarded from the dataset (maxEE = 2). Reads that mapped against the phiX genome were discarded as well. The remaining reads were processed through the main DADA2 pipeline at the default settings. After merging forward and reverse reads, an amplicon sequence variant table (ASV) was produced. Chimeric ASVs were removed prior to taxonomic assignment. Micro-eukaryote samples were identified using the DECI-PHER package (Wright, 2016) against the SILVA SSU database (SSU_r138) at the default settings (Quast et al., 2012). Taxonomy of *phoC* and *phoD* ASVs were assigned using Assign-Taxonomy-with-BLAST (https://github.com/Joseph7e/Assign-Taxonomy-with-BLAST) based on blast searches against the NCBI nucleotide (NT) database. For the BLAST-based taxonomy assignment, identity thresholds were set at 97 % for species, 90 % for family, and 80 % for phylum. All of the OTU data (for microeukaryotes, *phoC-/phoD*-containing bacteria) and qPCR data for *phoC* and *phoD* genes are available from the Dryad Digital Repository: doi:https://doi.org/10.5061/dryad.t4b8gtj71.

2.5. Statistical analyses

To estimate differences in soil microbial community structure, the Shannon and Chao1 were calculated using the "*vegan*" package in R (version 4.3.1) to quantify the alpha diversity of each sample. Beta diversity was calculated based on Bray-Curtis distance using the "*ape*" package in R (version 4.3.1), and PERMANOVA was conducted using the "*vega*n" package in R (version 4.3.1) to quantify the difference between different groups. The Beta diversity was then visualized by principal coordinate analysis (PCoA) in Origin 2021.

In order to reduce redundancy, OTU sequences that could be annotated at the phylum level were selected as valuable OTU sequences for further analysis. The top 10 phyla based on relative abundance were visualized in Origin 20. For the microeukaryotic communities, valuable OTU sequences that present in >20 samples (30 samples in total) were then analysed by Linear discriminant analysis Effect Size (LEfSe) with a linear discriminant analysis (LDA) score > 5.0 to determine the biomarkers from phylum to genus levels (*p* > 0.05) (http://huttenhower. sph.harvard.edu/galaxy) (Zhao et al., 2021). For *phoC-/phoD*-containing bacteria, correlation coefficients between the top 10 *phoC-/phoD*-containing bacteria and soil P fractions (Table S2) (Wang et al., 2023) were calculated in IBM SPSS Statistic 20 and displayed using a heatmap in Origin 20. Structural equation modelling (SEM) was established using IBM SPSS AMOS 25 to quantify the direct and indirect effects of *phoC-/phoD*-containing bacteria on the *phoC/phoD* gene copy number, acid and alkaline phosphatase (AiP and AlP) activity (Table S3) and different soil P fractions (Wang et al., 2023).

The normality and homogeneity of all variances in the soil microbial community structure (such as the relative abundance, α-diversity, *phoC*/*phoD* gene copy number) were tested using the Kolmogorov-Smirnov and Levene tests ($p > 0.05$), respectively, using IBM SPSS Statistic 20. For non-normally distributed data, non-parametric Kruskal–Wallis analysis was used for the variance and the Wilcoxon test was applied to compare the differences between each pair of treatments. For normally distributed data, one-way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) at $p < 0.05$ were used for the difference comparison and significance detection, respectively.

3. Results

3.1. The microeukaryotic community structure

For samples collected at BP, no significant difference in the diversity of the microeukaryotic community was detected among different treatments. At 17 WAP, only CP-fumigated treatments significantly decreased Shannon and Chao1 indices (0.6–0.8 and 504.5–705.2, respectively), as compared with CK treatment (Table 1). Similarly, based on principal coordinate analysis (PCoA) and PERMANOVA analysis, the microeukaryotic community was significantly separated in treatments with CP fumigation (CP, CP + AZO1 and CP + AZO2) and treatments without CP fumigation (CK, AZO1 and AZO2). AZO application did not significantly affect the microeukaryotic community compositions (Fig. 1 (A)).

Regarding taxa, for samples collected at BP, the microeukaryotic community was dominated by fungi Ascomycota, accounting for >50 % of the relative abundance, followed by the protists Ciliophora and Cercozoa (Fig. 1(B)). CP fumigation significantly decreased the relative abundances of the fungi Mucoromycota, Basidiomycota and Chytridiomycota by 6.7 %, 6.2 % and 3.5 %, respectively. At 17 WAP, the microeukaryotic community was still dominated by fungi Ascomycota in non CP-fumigated treatments (26.1 %–30 %), while the protist Ciliophora dominated the microeukaryotic community in CP-fumigated treatments (49.1 %–55.1 %). CP fumigation significantly decreased the relative abundances of Mucoromycota, Basidiomycota and Nematozoa by 12.2 %–13.0 %, 10.6 %–10.8 % and 3.2 %–5.7 %, respectively, while significantly increasing the relative abundance of the protist Hyphochytriomycetes by 2.6 %–5.3 % (Fig. 1(B)).

The LEfSe results based on LDA score > 5.0 are used to identify the differently abundant taxa among different treatments (Fig. S2). For samples collected at BP, at genus level, there were 9 significant biomarkers in CK treatment, while there were 4 significant biomarkers in CP treatment (Fig. 2). At 17 WAP, only CK, AZO2 and CP treatments showed biomarkers that were significantly different from other treatments at the genus levels, such as *Holosticha* and *Tausonia* in CK, *Phascolodon* in CP, and *Bryometopus*, *Geastrum*, *Mortierella* and *Rhogostoma* in AZO2 (Fig. 2).

3.2. phoC-/phoD-containing bacterial community structure

3.2.1. phoC/phoD gene copy number

No significant difference was found in gene copy numbers of *phoC* and *phoD* between CK, AZO1 and AZO2 treatments, nor between CP, CP + AZO1 and CP + AZO2 treatments (Fig. 3). CP fumigation significantly reduced the gene copy numbers of *phoC* and *phoD* throughout the whole experiment by 0.8×10^7–2.8×10^7 copies g^{-1} dry soil and 27.8×10^7–33.0×10^7 copies g^{-1} dry soil, respectively.

3.2.2. α- and β diversity

After quality filtering, a total of 12,370,611 *phoC*-containing and 4,001,341 *phoD*-containing bacterial sequences were obtained from all samples, which were clustered into 4016 *phoC*-containing bacterial OTUs and 3475 *phoD*-containing bacterial OTUs. For *phoC*-containing bacteria, for the samples collected at BP, CP fumigation had no significant effect on the Chao1 index, but significantly reduced the Shannon index by 0.7 as compared to CK treatment. In contrast, for samples collected at 17 WAP, CP fumigation significantly increased the Shannon and Chao1 index by 1.1 and 128.0, respectively. AZO2 treatment also led to a significantly higher Shannon index as compared to CK treatment. Similarly, for *phoD*-containing bacteria, for samples collected at BP, there was no significant difference in the Shannon and Chao1 indices between CK and CP treatments. At 17 WAP, CP and CP + AZO1 treatments significantly increased the Shannon index by 0.4 and 0.5. respectively, while AZO2 treatment significantly increased the Chao1 index by 272 as compared to CK treatment (Table 2).

PCoA and PERMANOVA analysis showed that the *phoC*-containing bacterial communities were not significantly separated between different treatments regardless of the sampling time (Fig. 4 (A)), while the *phoD*-containing bacterial communities in non CP-fumigated treatments (CK, AZO1 and AZO2) were significantly separated from CP-fumigated treatments (CP, CP + AZO1 and CP + AZO2).

3.2.3. phoC/phoD-containing bacterial community composition

For *phoC*-containing bacteria, Acidobacteria was the most dominant phylum, followed by Actinobacteria, Proteobacteria and Abditibacteriota (Fig. 5 (A)). The AZO1 treatment had no significant effect on the *phoC*-containing microbial community structure throughout the entirety of the experiment. For samples collected at BP, CP fumigation significantly increased the relative abundances of Proteobacteria, Firmicutes and Planctomycetes by 5.3 %, 1.2 % and 0.7 %, respectively. However, at 17 WAP, all or some of the treatments with AZO2 and CP fumigation significantly decreased the relative abundance of Proteobacteria, Chloroflexi and Firmicutes, but significantly increased the relative abundance of Acidobacteria and Abditibacteriota.

For *phoD*-containing bacteria, Proteobacteria, Actinobacteria and Planctomycetes were the top three dominant phyla, accounting for >90 % of the relative abundance (Fig. S3). Since *phoD*-containing bacteria could not be separated at phylum level, we focalized to genus level. The top 15 dominant genera of *phoD*-containing bacteria are shown in Fig. 5 (B). The AZO1 treatment also had no significant influence on the *phoD*-containing microbial community structure throughout the whole experiment. For samples collected at BP, there was no significant difference in the relative abundance of *phoD*-containing bacteria between CK and CP treatments, only the relative abundance of *Sinorhizobium* was significantly increased by 1.4 % in CP treatment. At 17 WAP, in all or part of the AZO2 treatment and CP-fumigated treatments, the relative abundances of *Sinorhizobium*, *Ralstonia* and *Streptomyces* significantly increased by 1.1 %–3.1 %, 1.2 %–4.2 % and 4.9 %–8.3 %, respectively. A significant decrease of the relative abundance of *Pseudonocardia* (17.3 %–21.0 %) was observed under CP fumigation as compared to CK

Table 1
Shannon and Chao1 indices of microeukaryotic communities. Treatments include: CK (untreated soil), AZO1 (a single application of AZO 8 weeks after planting (WAP)), AZO2 (double applications of AZO at 8 and 16 WAP), CP (CP fumigated soil without AZO), CP + AZO1 (CP combined with AZO1) and CP + AZO2 (CP combined with AZO2). Sampling times were BP (Before planting) and 17 WAP (17 weeks after planting). Data represents the means of replicates with standard deviations (SD). Lowercase letters (a and b) indicate the significant difference between CK and other treatments at each sampling time (Kruskal–Wallis analysis with the Wilcoxon test, $p < 0.05$).

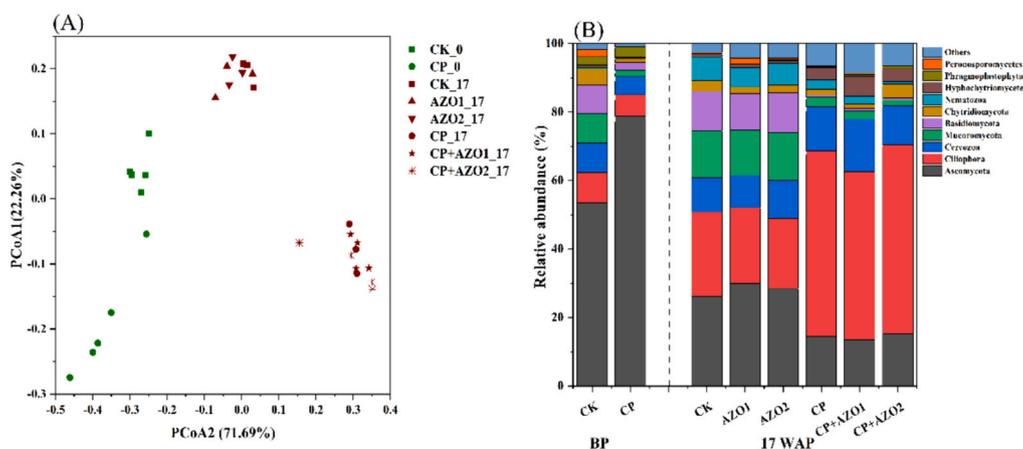| Sampling time	Treatments	Shannon	Chao1
BP	CK	5.3 ± 0.2a	1498.6 ± 107.3a
	CP	3.9 ± 1.1a	1207.7 ± 332.1a
17WAP	CK	5.9 ± 0.0a	1691.2 ± 162.9a
	AZO1	5.8 ± 0.0ab	1594.3 ± 37.5ab
	AZO2	6.0 ± 0.0a	1747.6 ± 194.3a
	CP	5.1 ± 0.4b	1061.5 ± 76.4bc
	CP + AZO1	5.3 ± 0.1b	986.0 ± 292.2bc
	CP + AZO2	5.2 ± 0.2b	1186.7 ± 132.4b

Fig. 1. Principal coordinate analysis (PCoA) of micro-eukaryote composition (A), and the relative abundance of the top 10 micro-eukaryotes at the phylum level (B). Green symbols represent samples collected before planting (BP), while red symbols are samples collected 17 weeks after planting (WAP). Treatments include CK (untreated soil, square), AZO1 (a single application of AZO 8 weeks after planting (WAP), up triangle), AZO2 (double applications of AZO at 8 and 16 WAP, down triangle), CP (CP fumigated soil without AZO, circle), CP + AZO1 (CP combined with AZO1, star) and CP + AZO2 (CP combined with AZO2, asterisk).
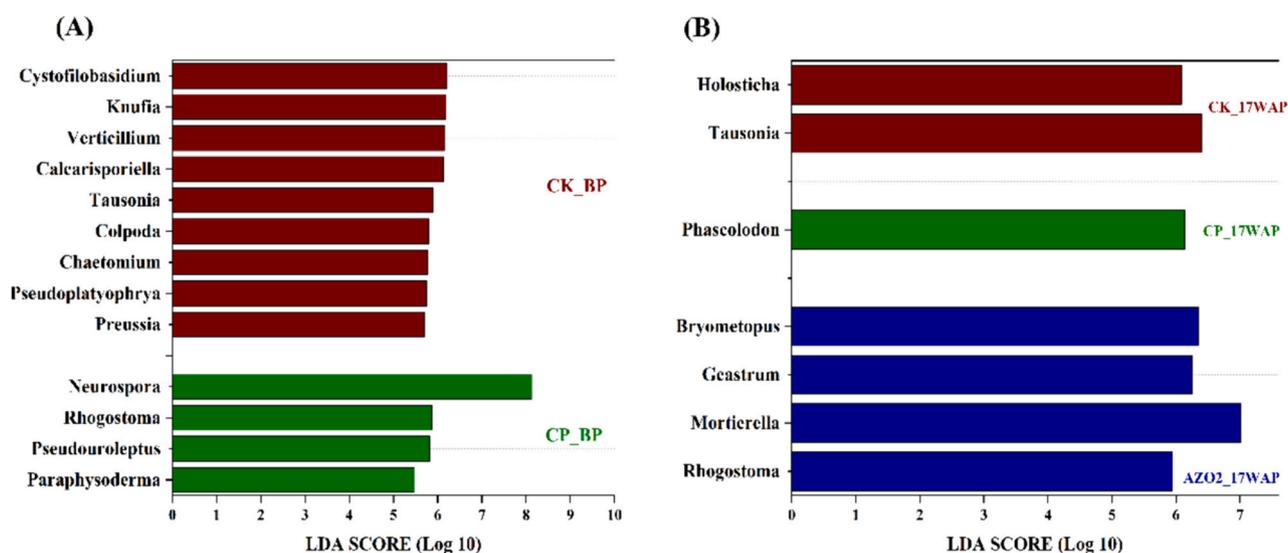

Fig. 2. Microbial biomarkers at genus level with linear discriminant analysis (LDA) score > 5.0 from linear discriminant analysis effect size (LEfSe), for samples collected before fumigation (A) and 17 weeks after planting (B). Treatments include CK (untreated soil), AZO1 (a single application of AZO 8 weeks after planting (WAP)), AZO2 (double applications of AZO at 8 and 16 WAP), CP (CP fumigated soil without AZO), CP + AZO1 (CP combined with AZO1) and CP + AZO2 (CP combined with AZO2).

treatment.

3.3. Relationships between phoC-/phoD-containing bacteria, phoC/phoD gene copy number, acid/alkaline phosphatase activity and different soil P fractions

Our previous study found that CP fumigation significantly increased the proportions of soil available P (Resin-P + NaHCO$_3$-P) to total P by 9.0 % to 15.5 %, and the combined application of CP and AZO released more available P compared with the individual application of these compounds. However, CP and AZO application significantly decreased soil phosphomonoesterase activities (Wang et al., 2023). In this study, a significant positive correlation was found between AiP activity and *phoC* gene copy number ($p < 0.01$; R^2: 0.71), and between AlP activity and *phoD* gene copy number ($p < 0.01$; R^2: 0.64) (Fig. 6). The correlation heatmap showed that the abundance of *phoD*-containing bacteria, such as *Sinorhizobium* and *Streptomyces*, were significantly positively related to the proportions of different soil available P fractions, while the

abundance of *Pseudonocardia* showed significant negative correlation with the proportions of moderately labile P fractions, such as NaHCO$_3$-Pi ($p < 0.05$), NaHCO$_3$-Po ($p < 0.01$) and NaOH-Pi ($p < 0.05$). The NaHCO$_3$-Po showed most correlations with different *phoC*-/*phoD*-containing bacteria, such as *phoC*-containing bacteria Actinobacteria ($p < 0.01$) and *phoD*-containing bacteria *Limnoglobus* ($p < 0.05$) (Fig. 7).

Structural equation modelling (SEM) indicated that the diversity of *phoC*-containing bacteria had a significant positive effect on the AiP activity ($p < 0.001$) through positively affecting the *phoC* gene copy number ($p < 0.05$). The activity of AiP then showed a significant positive effect on the AP content ($p < 0.001$). In contrast, the diversity of *phoD*-containing bacteria led to a significant negative effect on the *phoD* gene copy number ($p < 0.001$) which negatively affected the AlP activity ($p < 0.01$). The activity of AlP showed a slightly negative effect on the AP content ($p > 0.05$). Both the diversity of *phoC*-/*phoD*-containing bacteria showed a significant direct positive effects on the content of soil potential available organic P fractions ($p < 0.01$), and the latter had a significant positive effect on the AP content ($p < 0.05$) (Fig. 8).
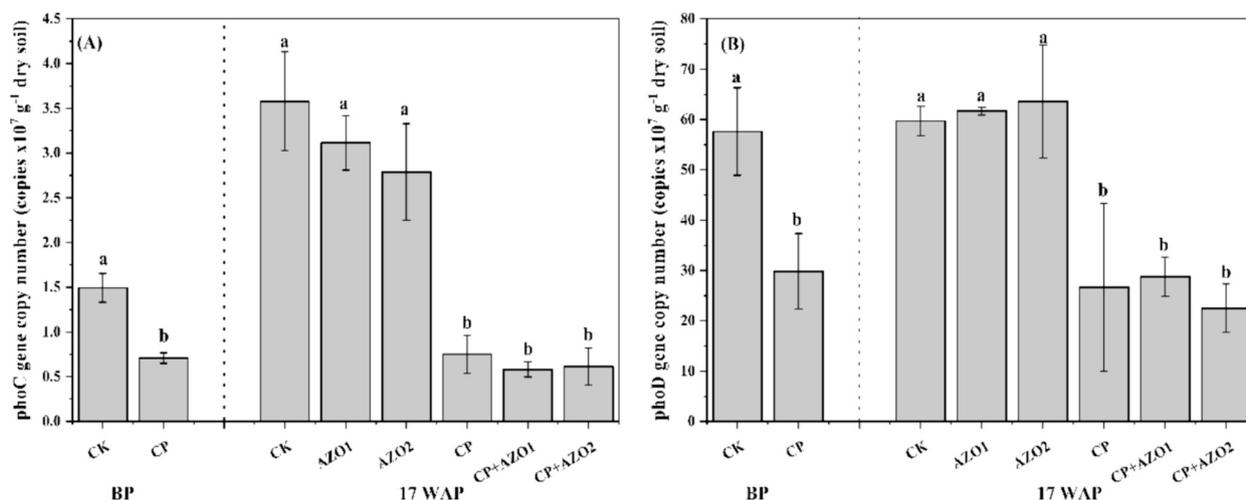
Fig. 3. *phoC* (A) and *phoD* (B) gene copy number (copies g^{-1} soil). Treatments include CK (untreated soil), AZO1 (a single application of AZO 8 weeks after planting (WAP)), AZO2 (double applications of AZO at 8 and 16 WAP), CP (CP fumigated soil without AZO), CP + AZO1 (CP combined with AZO1) and CP + AZO2 (CP combined with AZO2). Sampling times were BP (Before planting) and 17 WAP (17 weeks after planting). Data represents the means of replicates with standard deviation (SD). Lowercase letters (a and b) indicate the significant difference between CK and the other treatments at each sampling time (ANOVA with LSD test, *p < 0.05*).

Table 2

Shannon and Chao1 indices of *phoC*- and *phoD*-containing microorganisms. Treatments include CK (untreated soil), AZO1 (a single application of AZO 8 weeks after planting (WAP)), AZO2 (double applications of AZO at 8 and 16 WAP), CP (CP fumigated soil without AZO), CP + AZO1 (CP combined with AZO1) and CP + AZO2 (CP combined with AZO2). Sampling times were BP (Before planting) and 17 WAP (17 weeks after planting). The values represent the average values of replicates with standard deviation (SD). Lowercase letters (a and b) indicate the significant difference between CK and other treatments for every sampling time (ANOVA with LSD test, *p < 0.05*).

Sampling time	Treatments	Shannon		Chao1	
		phoC-microbe	*phoD*-microbe	*phoC*-microbe	*phoD*-microbe
BP	CK	2.5 ± 0.5a	5.5 ± 0.0a	327.0 ± 106.4a	1273.8 ± 74.9a
	CP	1.8 ± 0.7b	5.5 ± 0.1a	248.2 ± 53.0a	1361.6 ± 70.4a
17 WAP	CK	1.5 ± 0.1c	5.0 ± 0.2b	215.0 ± 63.7bc	818.3 ± 229.8bc
	AZO1	1.8 ± 0.5bc	5.1 ± 0.1ab	254.7 ± 23.5ab	1017.0 ± 89.7ab
	AZO2	2.4 ± 0.2ab	5.2 ± 0.0ab	306.0 ± 66.6ab	1090.3 ± 36.4a
	CP	2.6 ± 0.4a	5.4 ± 0.2a	343.0 ± 38.4a	967.0 ± 122.5abc
	CP + AZO1	2.2 ± 0.2abc	5.3 ± 0.2a	226.5 ± 44.0bc	1033.5 ± 106.5ab
	CP + AZO2	2.0 ± 0.2bc	5.2 ± 0.3ab	149.8 ± 19.4c	784.8 ± 254.5c

4. Discussion

4.1. Effects of CP fumigation and AZO application on the soil microeukaryotic community

In our study, over 85 % of the microeukaryotic communities consisted of soil fungi and protists, mainly including the fungi Ascomycota, Mucoromycota and Basidiomycota, and the protists Ciliophora and Cercozoa. Regardless of application times (once or twice) or CP fumigation (with or without), AZO application had no effect on soil microeukaryotic community structure, which is consistent with the study conducted by Álvarez-Martín et al. (2016) who found that the application of AZO at 2 and 26 mg kg^{-1} had little influence on the soil microeukaryotic community structure. This lack of effect might be attributed to the low application rates of AZO in combination with the rapid degradation rates (0.55 mg kg^{-1}) (Wang et al., 2022a).

However, CP fumigation significantly changed the structure of soil microeukaryotic communities and enhanced the species turnover across our study. For instance, the relative abundance of Ascomycota increased significantly in CP-fumigated soils at BP, but at 17 WAP, the values became significantly lower in CP-fumigated soils compared to the untreated soils. This suggests that Ascomycota was more resistant to CP fumigation, but with the growth of ginger, other microbes recovered faster than Ascomycota in CP-fumigated soils. Other studies also suggest that many Ascomycota species have special morphological characteristics that help them survive under those extensive stress conditions (Chen et al., 2015). CP fumigation also significantly decreased the relative abundance of Mucoromycota, especially the genus Mortierella. In fumigated soils, the relative abundance of Mortierella decreased by 20.4 % for samples collected at BP and by 11.9 % to 15.7 % for samples collected at 17 WAP (Fig. S1). Mortierella consists of non-pathogenic plant microbes which are able to inhibit plant diseases (Zhang et al., 2019), degrade many pesticides (Zhang et al., 2021) and promote solubilizing soil phosphorus (Li et al., 2021). Therefore, the reduction of Mortierella may negatively affect plant health and soil P solubilization. Zhang et al. (2019) also found that Mortierella decreased after CP fumigation at 30 g m^{-2} for 10 days, while Zhu et al. (2021) found that CP fumigation at 40 mg kg^{-1} for 7 days significantly increased the relative abundance of Mortierella. These inconsistent results may be due to the different initial soil microbial composition, experimental incubation conditions or plant interruptions in the different experiments.

In contrast to the soil fungal community, the relative abundance of protists Ciliophora and Cercozoa decreased by 28.8 % to 38.3 % at BP, while the values were 27.0 % to 34.6 % and 0.4 % to 5.9 % higher in CP-fumigated soils than non-fumigated soils at 17 WAP. Soil protists have proven to be more sensitive to organic pollutants than fungi (Wu et al., 2022b). Ciliophora and Cercozoa, two important phagocytic protists (consumers), are able to control soil-borne diseases by directly preying on pathogenic bacteria or fungi, or by secreting bacteriostatic metabolites (Wu et al., 2022a). The increases in Ascomycetes in CP-treated soil at BP might yield more prey for protist consumers (Ciliophora and Cercozoa), which would then increase the relative abundance of protists. In addition, as nutrient sources, the necromass released from cell lyses during CP fumigation might also enhance the recovery of the
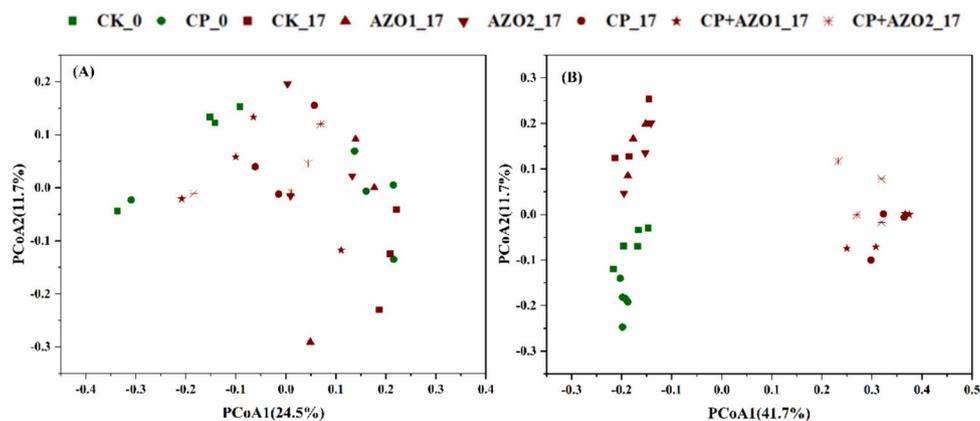
Fig. 4. Principal coordinate analysis (PCoA) of the composition of *phoC*-containing bacteria (A) and *phoD*-containing bacteria (B). Green symbols represent samples collected before planting (BP), while the red symbols represent samples collected at 17 weeks after planting (WAP). Treatments include CK (untreated soil, square), AZO1 (a single application of AZO at 8 WAP, up triangle), AZO2 (double applications of AZO at 8 and 16 WAP, down triangle), CP (CP fumigated soil without AZO, circle), CP + AZO1 (CP combined with AZO1, star) and CP + AZO2 (CP combined with AZO2, asterisk).
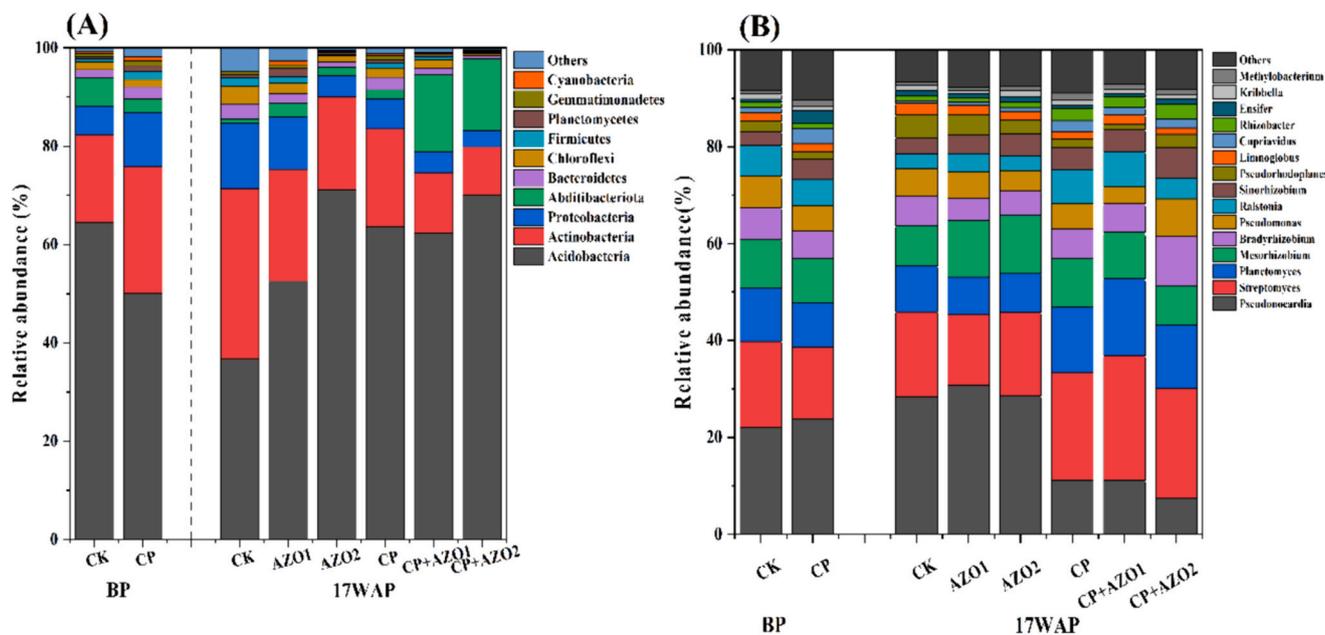

Fig. 5. Relative abundance of the top 10 phyla of *phoC*-containing bacteria (A) and top 15 genera of *phoD*-containing bacteria (B). Treatments include CK (untreated soil), AZO1 (a single application of AZO 8 weeks after planting (WAP)), AZO2 (double applications of AZO at 8 and 16 WAP), CP (CP fumigated soil without AZO), CP + AZO1 (CP combined with AZO1) and CP + AZO2 (CP combined with AZO2). Sample times were BP (before planting) and 17 WAP (17 weeks after planting).

surviving Ciliophora and Cercozoa (Xiong et al., 2023). Finally, CP changed the soil microeukaryotic community from fungi-dominated to protist-dominated, which might improve soil health by reducing plant pathogen populations and increasing pathogen consumer populations, thereby increasing ginger yield (Wang et al., 2022a). However, further studies are needed to clarify the functional changes in the soil microeukaryotic communities caused by soil fumigation.

4.2. Effects of CP fumigation and AZO application on the soil phosphorus solubilizing bacterial community

Since both plant roots and soil microorganisms can secrete acid phosphatase, most previous studies that have explored the effects of pesticides on soil P solubilizing microbes have focused only on alkaline phosphatase secreted only by soil microorganisms (Huang et al., 2020a, 2020b). However, acid phosphatase is also an important enzyme that catalyses the mineralization of organic P, especially in acidic soils

(Acosta-Martínez et al., 2011). Therefore, in this study, two representative phosphomonoesterase code genes (*phoC* and *phoD*) responsible for the production of acid and alkaline phosphatase (Fraser et al., 2017), respectively, were used to explore soil P solubilizing bacteria.

The results show that CP fumigation significantly decreased the gene copy number of *phoC* and *phoD*, whereas no significant effect was observed after AZO application, regardless of whether the soil was CP fumigated or not. A previous study conducted by Huang et al. (2020a) also found that CP fumigation at 53 mg kg^{-1} and 106 mg kg^{-1} significantly reduced the gene copy number of *phoD* even after 77 days of incubation. In our study, the significant positive correlation between the *phoC/phoD* gene copy number and the AiP/AlP activity suggested that the decreases in the amount of *phoC/phoD* may be the direct cause of the decreased AiP/AlP activity in CP-fumigated soil (Fig. 6).

In order to identify the microbes containing *phoC/phoD* genes, we performed high-throughput sequencing with *phoC/phoD* as the target gene region. These results showed that both CP fumigation and AZO
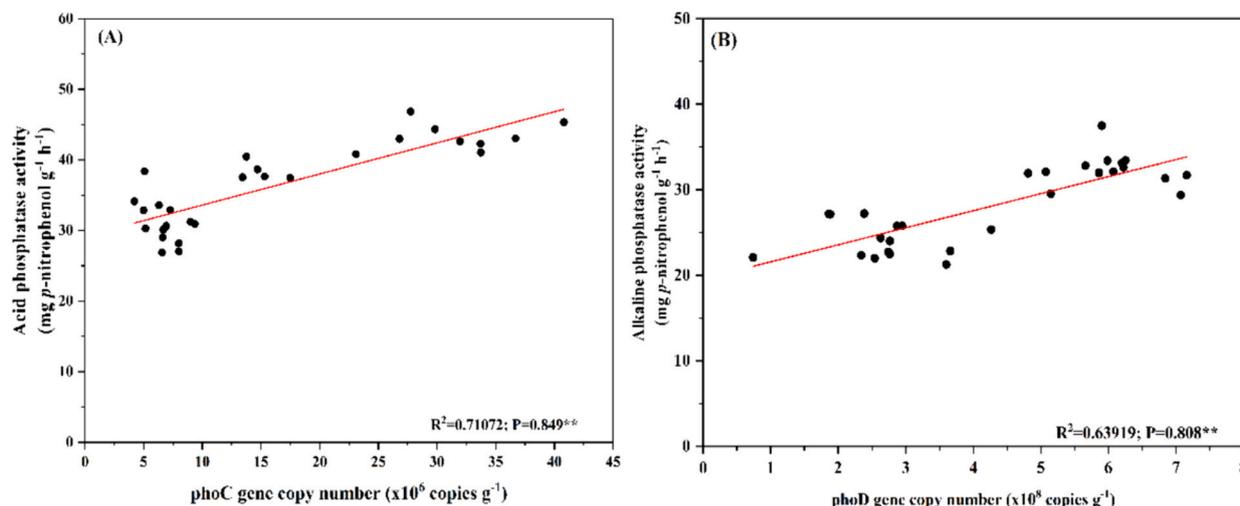
Fig. 6. Relationship between acid phosphatase activity and *phoC* gene copy number (A), and between alkaline phosphatase activity and *phoD* gene copy number (B).
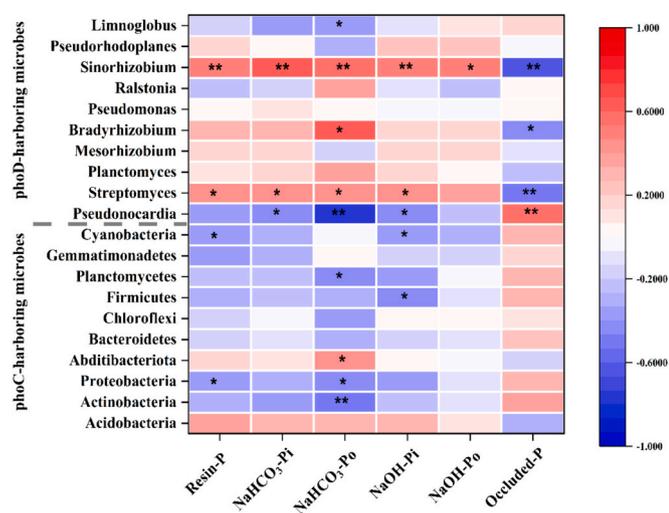

Fig. 7. Heatmap of the correlation between *phoC*-/*phoD*-containing bacteria and the proportions of different soil P fractions to TP including Resin-P, NaHCO$_3$-Pi, NaHCO$_3$-Po, NaOH-Pi, NaOH-Po and Occluded-P. ** means significant correlation at $p < 0.01$; * means significant correlation at $p < 0.05$.

application significantly altered the *phoC*-containing bacterial community composition, while only CP fumigation significantly changed the *phoD*-containing bacterial community composition. The different responses of different soil microorganisms to CP fumigation and AZO application may be due to their difference in tolerance to CP and AZO toxicity and their abilities to remediate CP and AZO contamination (Huang et al., 2020a). For example, the relative abundance of Firmicutes increased significantly after CP fumigation, possibly due to the ability of the microorganisms to form spores to resist environmental stress (Castellano-Hinojosa et al., 2022). In our study, AZO and CP application significantly decreased soil pH, resulting in a significant increase in the relative abundance of some acidophiles such as Acidobacteria (Rousk et al., 2010). The relative abundance of Abditibacterium, which is highly resistant to antibiotics and toxic compounds (Tahon et al., 2018), was significantly higher in CP + AZO1 and CP + AZO2 than in CK. A single application of AZO did not have any effect on *phoC*-containing bacteria, while two applications of AZO significantly reduced some *phoC*-containing bacteria such as Actinobacteria, Chloroflexi and Firmicutes. This suggests that the application of AZO needs to be frequent to pose a toxic effect to these microbes. For *phoD*-containing bacteria, the relative

abundances of *Streptomyces*, *Ralstonia* and *Sinorhizobium* were significantly higher in CP-fumigated soil, likely due to their ability to degrade CP and use it as a nutrient source (Apel et al., 2007). Huang et al. (2020a) also found that *Sinorhizobium* was significantly enriched in CP-fumigated soil.

Changes in soil functional microorganisms, especially soil beneficial microorganisms, may further change soil biochemical processes, ultimately affecting plant growth. For example, Actinobacteria includes many beneficial species responsible for soil organic matter decomposition and soil-borne pathogen suppression (Li et al., 2021; Ma et al., 2021). In addition, Firmicutes are also important decomposers of soil organic matter and promote soil nutrient cycling processes (Ma et al., 2021). Therefore, by utilizing the microbial necromass released by CP and AZO application, the surviving soil beneficial microorganisms may promote the turnover of soil organic matter and other soil nutrient cycling processes. In addition, *Sinorhizobium* has been shown to produce organic acids, especially indole-3-acetic acid (IAA), to dissolve phosphate rock into available phosphorus (Bianco and Defez, 2010), while *Streptomyces* are biocontrol agents used against soil-borne pathogens (Zhang et al., 2021; Wang et al., 2022a). In our study, heatmap analysis showed that the relative abundances of *Sinorhizobium* and *Streptomyces* were significantly positively correlated with soil available P contents. Therefore, the increase of *Sinorhizobium* and *Streptomyces* in CP-fumigated soil might be an important reason for the increased levels of soil available P and ginger yields. The heatmap also showed that NaHCO$_3$-Po was the P fraction that was the most susceptible to the effects of different types of soil P solubilizing microorganisms, which may be because NaHCO$_3$-Po are P fractions that slightly complex with organic matter surfaces and are easily mineralized by phosphatases (Wang et al., 2022b).

From the SEM, the diversity of *phoD*-containing bacteria has a significant negative influence on the *phoD* gene copy number. The *phoD* gene copy number has a significant positive influence on the activity of AlP. AlP activity showed a negative influence on the potential available organic P content. This means although the diversity of *phoD*-containing bacteria increased with the ginger growth and soil microbiome recovery, the *phoD* gene abundance might decrease among those remaining or recovered *phoD*-containing bacteria, resulting in a total decrease in the *phoD* gene copy number.

The decrease in the *phoD* gene copy number induces a decrease in AlP activity, which increases the potential available organic P fractions (NaHCO$_3$-Po and NaOH-Po). The results also show that the diversity of *phoD*-containing bacteria has a significant positive influence on AP contents. CP fumigation decreases the diversity of *phoC*-containing bacteria, which then also decreases the *phoC* gene copy number and AiP
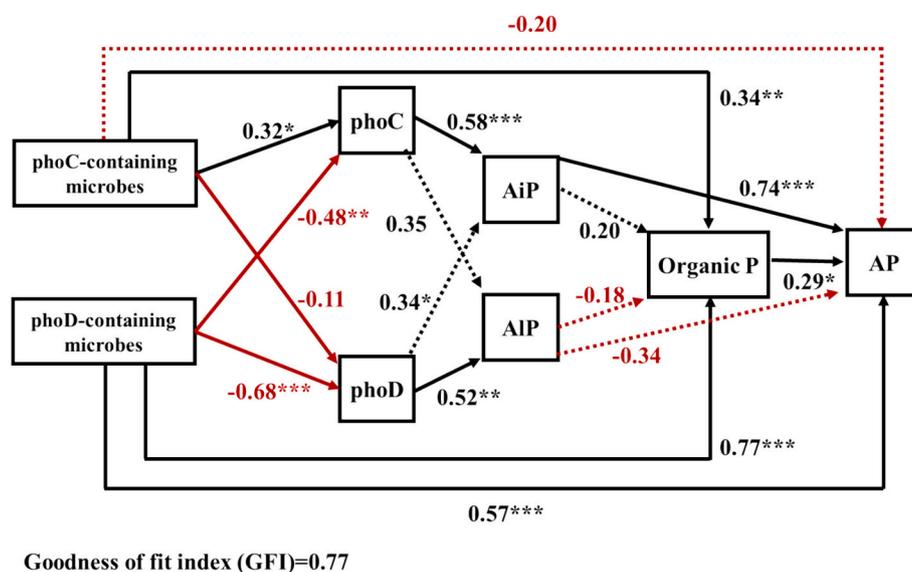
Fig. 8. Structural equation modelling (SEM) of the direct and indirect effects of the diversity of *phoC-*/*phoD*-containing microorganisms on different soil P fractions. To simplify the SEM model, the first axis of PCoA scores for each sample were used to represent the diversity of *phoC-*/*phoD*-containing microorganisms. Organic P refers to the sum of NaHCO₃-Po and NaOH-Po, while AP refers to the sum of Resin-P and NaHCO₃-Pi. Red arrows refer to negative effects while black arrows refer to positive effects. Dotted arrows represent non-significant paths (p > 0.05). Significant path was: *: $0.01 < p < 0.05$; **: $0.001 < p < 0.01$; ***: $p < 0.001$. Numbers adjacent to the arrows are standardized path coefficients.

activity. The diversity of *phoC*-containing bacteria had a negative influence on AP, which also means that a higher AP content in the soil indicates a lower diversity of *phoC*-containing bacteria. Assuming that a higher AP content in the soil has some negative causative effect on the diversity of *phoC*-containing bacteria, the application of P fertilizers to increase the AP content in the fields should be done thoughtfully, to avoid potentially harmful effects on the *phoC*-containing microbes. One key question to be answered is: how can farmers optimally adjust the amount of P fertilizer to achieve P balance in fields with soil fumigation?

5. Conclusion

This study investigated the effects of CP fumigation and AZO application on soil micro-eukaryotes and *phoC-*/*phoD*-containing bacteria. Regardless of the frequency of AZO application and CP fumigation, AZO application had no significant effect on soil micro-eukaryotic and *phoD*-containing bacterial community structure. CP fumigation shifted micro-eukaryotic communities from fungal dominance to protist dominance, suggesting that CP fumigation may reduce soil-borne diseases by killing pathogens and promoting the growth of pathogen consumers. CP fumigation significantly reduced the *phoC/phoD* gene copy number and influenced soil available P contents by altering the *phoC-*/*phoD*-containing bacterial community structures. It must be noted that this study was performed in a greenhouse with pure AZO and CP applications. The results may differ in farms where a cocktail effect arises, from the simultaneous addition of multiple chemical compounds. However, this study can provide practical information for soil beneficial microorganisms recovery strategies in fumigated soils and for adjusting phosphate fertilizer application during crop growth to promote plant growth and improve nutrient use efficiency. Furthermore, *Sinorhizobium*, *Streptomyces* and *Pseudonocardia* are considered to be the three genera that control soil P fractions in CP-fumigated soils; methods to exploit these key microbial species to optimize soil P management in fumigated soils needs to be further explored.

CRediT authorship contribution statement

Yan Wang: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Xiaomei Yang:** Writing – review & editing, Supervision, Funding acquisition. **Paula Harkes:** Writing – review & editing, Methodology. **Joris J.M. van Steenbrugge:** Methodology, Investigation, Formal analysis. **Minggang Xu:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Violette Geissen:** Writing – review & editing, Supervision, Project administration, Conceptualization.

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.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.172871.

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