Response of soil phosphorus to fumigation in ginger production

Yan Wang

Propositions

1. Fumigation effectively prevents soil-borne diseases in ginger during the first three years of application.
   (this thesis)

2. Soil fumigation is an unsustainable way to improve soil phosphorus availability.
   (this thesis)

3. If the direction of scientific research is determined by popularity, then science becomes performative.

4. The distinction between scientific theory and practice is tautological.

5. Communication is essential for productivity in numbers, just as commutativity is essential for the product of numbers.

6. Stereotypes hinder communication and exacerbate discrimination.

Propositions belonging to the thesis, entitled:

Response of soil phosphorus to fumigation in ginger production

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Response of soil phosphorus to fumigation in ginger production

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Response of soil phosphorus to fumigation in ginger production

Yan Wang

Thesis

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Chapter 1

General introduction

1.1 Soil Phosphorus Cycling

Phosphorus (P) is one of the most important nutrient elements for plant growth, accounting for nearly 0.2% of plant dry weight (Achary et al., 2017). It is involved in energy storage and transfer in metabolic pathways (e.g., photosynthesis, respiration, cell division and reproduction) in the form of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADP) (Abolfazli et al., 2012). P is needed in the construction of important cellular constituents (e.g., DNA, RNA, phosphoproteins, phospholipids, sugar phosphates and enzymes) (Huang et al., 2017). It also plays an important role in cell signalling through phosphorylation and dephosphorylation to protect cells (Abolfazli et al., 2012). In addition, P is essential for seed germination, flowering and fruiting, and promotes root development and improves the quality of crops (Krishnaraj et al., 2014).

Soil P is the sole source of P for plant growth (Achary et al., 2017). However, only dissolved orthophosphate ($H_2PO_4^-$, HPO_4^{2-}, PO_4^{3-}) can be directly uptake by plants, and more than 99% of total P cannot be absorbed before being solubilized or mineralized (Sharma et al., 2013), causing 43% of soil to be P deficient for plant growth (Zhao et al., 2019). To maximize crop yields and meet the needs of a rapidly growing population, large amounts of phosphate fertilizers have been produced and used. China used 10,330,187 tonnes of P_2O_5 for agricultural production in 2019 (FAOSTAT, 2020). Unfortunately, phosphate fertilizers are made from phosphate rock, a non-renewable resource, and the reserves are declining rapidly (Weihrauch et al., 2018). It is estimated that the current supply will only last 70-200 years if current consumption is maintained (Achary et al., 2017). Therefore, there is an urgent need to improve soil P efficiency and avoid a potential P crisis that may lead to global famine and starvation-related mortality (Lemming et al., 2019; Weihrauch et al., 2018).

In soil, the balance between dissolved P and bound P on the surface of soil particles, which is involved in adsorption/desorption and precipitation/dissolution processes, determines the bioavailability of soil inorganic P (Weihrauch et al., 2018). Inorganic P (Pi) exists in soil solution as orthophosphate ($H_2PO_4^-$, HPO_4^{2-}, PO_4^{3-}), or bound to soil particles through physisorption and chemisorption processes (Jiang et al., 2015). The transformation of soil inorganic P is highly dependent on soil pH (Hinsinger, 2001; Zhou et al., 2018). In acidic soils, dissolved P is mainly adsorbed to Fe/Al/Mn-oxyhydroxides, clay minerals and metal cations in humic substances through surface electrical charges (Shen et al., 2011). When the concentration of P anions in the soil solution reaches saturation, Fe/Al-P-minerals could form precipitates in highly acidic soils. As soil pH increases, soil particles become gradually negatively charged, and bound P is exchanged with OH^- through ligand exchange or ligand-promoted dissolution and released freely into the soil solution (Weihrauch et al., 2018). In alkaline soils, the binding of orthophosphate to Ca^{2+} dominates due to the increased replacement of H^+ by Ca^{2+} on the oxyhydroxides' surface, leading to Ca-P-mineral precipitation when P anion concentration becomes high enough in the soil solution (Weihrauch et al., 2018). Precipitated Ca-P-minerals can also dissolve and become bioavailable by reacting with carbonic acid when the pH values decrease (Shen et al., 2011; Weihrauch et al., 2018). Hence, Pi is most soluble in soil solution at pH 6.0~6.5 (Abolfazli et al., 2012). Organic P (Po) contains carbon-hydrogen bonds which are grouped according to the phosphorus bonds (Huang et al., 2017): 1) Phosphate esters, including C-O-P functional group, are classified into phosphate monoesters and diesters according to the number of moieties per phosphorus. Phosphate esters

(mainly in the form of inositol phosphates) account for more than 50% of soil organic P but are sparingly soluble and slowly mineralized. Phosphate diesters, referring to nucleic acids (such as DNA and RNA), phospholipids and teichoic acids, are more easily broken down and mineralized. 2) Phosphonates, characterized by functional group C-P, are bound and present mainly in the form of 2-aminoethylphosphonic acid. They are less abundant and fairly stable in soil systems. 3) Phosphoric acid anhydrides, containing anhydride bonds and phosphate monoesters, play crucial roles in biochemical energy transfer in the form of ATP or DANP.

Different fractions of soil P can be extracted and analysed by Hedley's sequential extraction method (Hedley et al., 1982). Based on its solubility in different extractants, soil P can be divided into 1) Resin-P (easily available P), a soluble inorganic orthophosphate ($H_2PO_4^{2-}$ or $H_2PO_4^-$) in the soil solution and the only form that can be taken up by plants directly (Hedley et al., 1982) ; 2) $NaHCO_3$-P ($NaHCO_3$-Pi and $NaHCO_3$-Po, labile P), inorganic P fractions slightly complexed with Fe /Al minerals and easily mineralized Po fractions mainly consist of orthophosphate diesters (Negassa et al., 2009); 3) NaOH-P (NaOH-Pi and NaOH-Po, moderately labile P), P fractions strongly complexed with Fe /Al minerals and calcic compounds, as well as organic P fractions more strongly associated with soil organic matter, mainly represent the orthophosphate monoesters (Negassa et al., 2009); and 4) Occluded P (unavailable P), which is occluded by soil aggregate and cannot be used by plants (Fan et al., 2019; Motavalli et al., 2002). Among these soil P fractions, inorganic P fractions ($NaHCO_3$-Pi and NaOH-Pi) are available for plants after solubilization, while organic P fractions ($NaHCO_3$-Po and NaOH-Po) need to be mineralized for plant uptake (Spohn et al., 2013). Inorganic P solubilization and organic P mineralization are largely controlled by soil phosphorus solubilizing microorganisms (PSMs) that secrete organic acid and synthesize various phosphatase enzymes (Richardson et al., 2009).

For PSMs, phosphorus solubilizing bacteria (PSB; e.g., *Bacillus, Burkholderia, Microbacterium, Pseudomonas* and *Rhizobium*) account for 1% to 50% of the total soil microbial population, while phosphorus solubilizing fungi (PSF; e.g., *Penicillium*, *Aspergillus* and *Rhizopus*) only contribute 0.1 - 0.5% to the P solubilisation potential (Khan, 2009). PSB can convert the insoluble inorganic phosphate to soluble $H_2PO_4^-$ and HPO_4^{2-} through lowering the soil pH by releasing protons and organic acids (e.g., gluconic acid) (Bhattacharyya et al., 2012). Organic acids secreted by PSB are also able to solubilize Ca- or Fe-/Al- bounded P by chelating the iron and aluminium cations which P is bound to (Liu et al., 2014; Spohn et al., 2015). PSB can mineralize organic P to soluble inorganic P by producing extracellular phosphatases that can catalyse the hydrolysis of esters and anhydrides of phosphoric acid (Nannipieri et al., 2011). There are three major phosphatases existing in the soil system (Rodríguez et al., 2006): 1) nonspecific phosphatases involved in dephosphorylation of phospho-eater or phosphoanhydride from organic matter, 2) phytases that release P from phytic acid, and 3) phosphonatases that break C-P from organophosphonates. Among these phosphatases, acid phosphatase (AiP, EC 3.1.3.2) and alkaline phosphatase (AlP, EC 3.1.3.1) are two nonspecific phosphohydrolases able to hydrolyse simple phosphate esters to orthophosphate (Fraser et al., 2017). The AiP can be excreted by plant roots, nodules and soil microbes, while the production of AlP is carried out by soil bacteria and some fungi (Fraser et al., 2017). In addition to PSB, arbuscular mycorrhizal fungi (AMF)are responsible for the mycorrhizal uptake pathway that transfers soil P to plants and promotes plant P uptake (Ipsilantis et al., 2012; Smith et al., 2011). AMF can extend the nutrient uptake range for root systems by forming a

symbiotic interface between roots and arbuscular or hyphal coils, or by altering plant hormones to promote root growth (Smith et al., 2011).

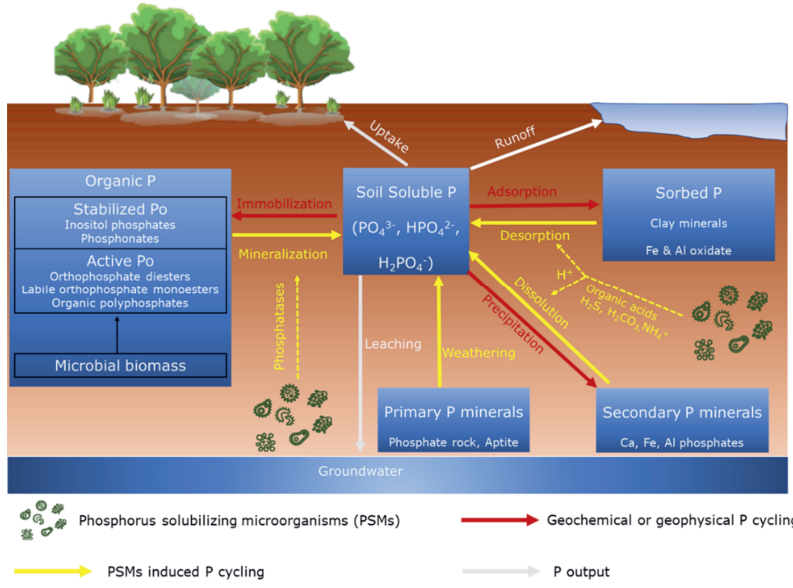

Fig 1.1 Phosphorus cycling processes in the soil system. Modified based on (Shen et al., 2011; Tian et al., 2021)

Limited phosphate sources and lower bioavailability of P in the soil systems are reinforcing the urgency to find economic and environmentally friendly ways to optimize soil P management. The availability of soil P is largely limited by the rapid reactions of P anions with soil particles, as well as by abundant soil microbial activity that converts orthophosphate into diverse organic forms (Achary et al., 2017). Soil properties, especially pH, determine adsorption/desorption, precipitation/dissolution processes, as well as soil microbiome and play a key role in the immobilization and mineralization processes of soil P (**Fig 1.1**). Therefore, any agricultural management practices that can alter the soil microbiome, especially the soil P solubilizing microbial community structures, might consequently change the P cycling processes in the soil system. Among those agricultural management practices, soil fumigation is one of the most important methods to reduce soil-borne diseases by disturbing the soil microbiome.

1.2 Soil fumigation

Fumigants are broad-spectrum chemicals that exist in liquid, solid and gas forms that once injected, vaporize and easily diffuse through the soil pores, eliminating a large proportion of the soil organisms (Rokunuzzaman et al., 2016; Zuckerman, 2017). Based on the chemical structure, physical properties and injury mechanisms, a large number of fumigants, such as methyl bromide (MeBr), 1,3-dichloropropene (1,3-D), chloropicrin, metam-sodium, dazomet, have been developed and used in agricultural systems (Zuckerman, 2017). MeBr dominated the fumigant family until 2015, when it was phased out due to its damage to the ozone layer (Sun et al., 2018b). Alternative candidates, such as 1,3-D, chloropicrin, metam-sodium, and dazomet, appeared and gradually took over the chemical fumigant markets in the 21st century (Ruzo, 2006).

These fumigants effectively control soil-borne diseases through a variety of modes of action as described in Yang et al. (2011): 1) destroying microbial membrane systems by interfering with the synthesis of lipids, sterol and other components; 2) interfering with the biosynthesis of amino acids and protein; 3) breaking signal transduction taking place in the cell membranes and the functions of certain proteins; 4) affecting microbial respiration by inhibiting the activities of some enzymes such as NADH oxidoreductase, succinate-dehydrogenase and cytochrome bc1; and 5) affecting microbial nucleic acid synthesis, mitosis and cell division.

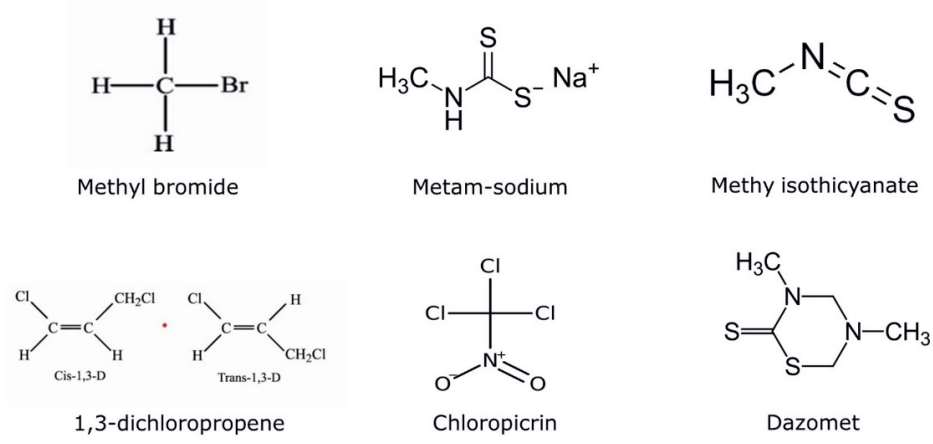

Fig. 1.2 Chemical structures of different fumigants.

With various modes of action, fumigants can be antimicrobial sterilizers, fungicides, herbicides, insecticides, and nematicides, efficiently controlling nematodes, soil-borne pathogens and weeds and ultimately increasing crop yield. Previous study found that, although chloropicrin could be degraded into nitrogenous compounds after several days in the soil (Neilson et al., 2020), chloropicrin fumigation could significantly inhibit the growth of pathogens and thus, promote ginger production (Mao et al., 2014). Research showed that the colony forming units of *R. solanacearum* were reduced by 76% to 85% with chloropicrin fumigation at a rate of 50 g m^{-2}, and that the mortality of ginger was reduced from 60% in untreated soil to 0.4% in chloropicrin-fumigated soil, thereby increasing yields by 4.77 kg m^{-1}

(Mao et al., 2014). Chen et al.(2022) found that after dazomet fumigation at 600 kg hm^{-2} for 4 months, apple seedling height increased by 24.77 cm, stem diameter increased by 4.52 mm and fresh weight increased by 25.63 g. In this study, the gene copy number of *Fusarium solani* was significantly reduced by 91.19% immediately after fumigation, 67.31% 4 months after fumigation, and 54.19% 16 months after fumigation, indicating that the amounts of *Fuarium solani* recovered with time.

Fumigant products are available in the form of gases (e.g., methyl bromide), liquids (e.g., chloropicrin) and solids (e.g., zinc phosphide) (Zuckerman, 2017), which determine their application methods. Liquid fumigants can be applied together with water irrigation via drip pipes (Ślusarski et al., 2016; Yu et al., 2020), or injected directly into the soil layers (usually 15 – 30 cm) using a shank or machine injection (Wang et al., 2022b). In order to reduce the potential exposure risk to farmers, some volatile fumigants (e.g., chloropicrin) can also be made into gelatine capsules and inserted into the soil (Yan et al., 2012). Researchers found that chloropicrin gelatine capsules and chloropicrin injection exhibited similar inhibitory effects on soil-borne pathogens (Yan et al., 2012). The chloropicrin gelatine capsule was also found to be more efficient at controlling soil-borne diseases when applied at a depth of 15 cm than when it was applied at a depth of 5 cm. The deeper the fumigants are applied, the more efficient they are at controlling soil-borne diseases (Yan et al., 2017a). Typically, regardless of the application method, fumigated soils need to be immediately covered with plastic films to create an enclosed soil space to prevent emission of fumigants into the air (Wang et al., 2022a). A more impermeable film can reduce the emission of fumigants, thereby improving the inhibitory effects of the fumigant on soil-borne pathogens. For example, Fang et al.(2017) found that the diffusion rate of the fumigant methyl isothiocyanate through totally impermeable film was 0.0034 cm h^{-1} compared to 7.97 cm h^{-1} through a low-density polyethylene film. Mao et al. (2014) concluded that the reduction of *R.solanacearum* after chloropicrin fumigation at 50 g m^{-2} was higher in fields covered with totally impermeable films (85.3%) than that in fields covered with polyethylene film (75.9%).

Since soil microorganisms are the target of soil fumigation, in addition to the properties of fumigants, action modes and application methods, other factors that affect soil microorganisms, will also affect the efficiency of soil fumigation. For example, different fumigants are usually applied simultaneously to increase the spectrum to kill soil-borne pathogens and maximize crop yield (Mao et al., 2016), or in combination with other fungicides or nematicides (Huang et al., 2019a). Although the combined application of different fumigants may accelerate or retard the degradation of one fumigant in the mixture (Zheng et al., 2004), in general, the combined application of different fumigants showed higher efficiency in controlling soil-borne pathogens than a single application. For example, Mao et al. (2016) found that the reduction of *Fusarium oxysporum* and *Phytophythora* spp. were significantly higher in treatments with combined applications of 1,3-D+dimethyl disulfide (fungal reduction: 95.5% ~ 99.2%) than treatments with a single application of 1,3-D or dimethyl disulfide (fungal reduction: 65.7% ~ 85.2%). Li et al. (2021) also found that a first-year application of dazomet and a second-year application of chloropicrin significantly increased strawberry yield and the relative abundance of beneficial microorganisms such as *Bacillus* and *Firmicutes* as compared to two consecutive years of chloropicrin application.

In addition to a mixed application of different fumigants, other fungicides, such as strobilurin fungicide azoxystrobin (AZO), with specific targets are also applied during the crop growth to reduce soil-borne diseases that can occur even after initial soil fumigation (Huang 2019b). Fungicides can kill fungal pathogens and imperfect fungi through various actions modes such as disrupting ATP production in fungal mitochondria (Howell et al., 2014; Sun et al., 2018b), significantly delaying plant senescence caused by saprophytic fungi (Bertelsen et al., 2001) and increasing the crop yield (Zhang et al., 2010). Huang et al. (2019a) found that chloropicrin or dazomet combined with a fosthiazate nematicide increased cucumber yield as compared to the application of the fumigant or nematicide alone. Therefore, the addition of fungicides to fumigated fields during crop growth may result in different effects on soil-borne disease control and crop growth than when applied alone. However, the combined effects of fumigants and fungicides on soil health remain largely unclear.

With the increases in the times of application, soil fumigation may cause irreversible effects on the soil microbiome, leading to the gradual disapperance of poorly resistant microorganisms and the predominance of microorganisms with a stronger resistance (Dangi et al. 2017; Li et al. 2017; Zhang et al. 2020b; Zhou et al. 2020). Zhang et al. (2017b) found that after 3 years of continuous chloropicrin fumigation, seven genera from *Actinobacteria* and one genus from *Bacteroidetes* disappeared, while *Saccharibacteria* increased significantly. Dangi et al. (2017) concluded that there were significant differences in soil microbial communities between untreated sites and sites with 15 and 30 years of annual fumigation with methyl bromide. Arbuscular mycorrhizal fungi (AMF) were especially lower in all fumigated sites compared to non-fumigation sites. Yao et al. (2006) found that apple tree growth and yield were not affected by pre-plant fumigation with a mixture of 78% dichloropropene+17% chloropicrin as compared to untreated controls after two years. They suggested that this result may be related to the reduced inhibitory effects of chloropicrin on soil-borne diseases. Zhang et al. (2020b) found that the strawberry yield increment varied with different consecutive years of chloropicrin fumigation, with a maximum of 2.0 kg m^{-2} after 2 years, which then decreased to 0.9 kg m^{-2} after 5 years.

Apart from soil harmful microorganisms, there are also microorganisms that have beneficial effects on plant health and growth (Mendes et al., 2013) by promoting nutrient uptake as biofertilizers (Vejan et al., 2016), inducing systemic resistance through plant hormones (Pieterse et al., 2014) and controlling plant disease (Compant et al., 2005). The bacteria which can promote plant growth are called plant growth-promoting bacteria (PGPB), such as nitrogen-fixing bacteria (Vejan et al., 2016), phosphorus solubilizing microorganisms (PSMs) (Khan, 2009), and other plants growth-promoting rhizobacteria (Ahemad et al., 2014).

In addition to targeting pathogenic fungi, oomycetes and nematodes, fumigants may also influence non-target microbes due to their broad biocidal activities (Castellano-Hinojosa et al., 2021; Dangi et al., 2015). Rokunuzzaman et al. (2016) found that in chloropicrin fumigated soil, the gram-positive bacteria *Firmicutes,* which has thick cell walls, became the dominant population, accounting for 75% of the bacteria. After two months, although the proportion of *Firmicutes* decreased to the basal level and the *Bacteroidetes* and *Proteobacteria* returned to their dominance, the microbial community structure was still different from that in soils without chloropicrin. Zhang et al. (2019b) found that soil beneficial bacteria, such as the organic material decomposer *Actinobacteria*, and plant disease-controlling fungal genera such as *Humicola*, *Chaetomium* and *Mortierella*, were reduced by chloropicrin fumigation,

negatively affecting crop yield. Species associated with biodegradation such as *Sphingomonas* spp., *Rhodococcu* spp. (Li et al., 2017) and *Chloroflexi*, which can use fumigants as nutrient sources and degrade the fumigants, were increased by soil fumigation (Zhang et al., 2019b). Li et al. (2017) found that chloropicrin fumigation significantly decreased the number of some other plant growth-promoting microorganisms, such as *Streptomyces*, involved in the processes of soil nitrogen denitrification (Fang et al., 2022) and phosphorus solubilization (Sharma et al., 2013).

Soil phosphorus-cycling related genes and microorganisms may be affected by soil fumigation. A large number of researchers have found that pesticides, especially fungicides, adversely affect AMF colonization and hyphal uptake of P (Burrows et al., 2007; Kjoller et al., 2000; Schweiger et al., 2001). For example, Dangi et al. (2017) found that soil fumigation significantly decreased the relative abundance of AMF which may cause detrimental effects on the crop P uptake. However, studies conducted by Huang et al. (2020a; 2020b) have found that soil fumigation using chloropicrin and dazomet changed the abundance and diversity of *phoD*-containing microorganisms and increased the amounts of soil available phosphorus. They suggested that P fertilizers could be reduced at the early stage (< 30 days). Pu et al. (2022) also found that chloropicrin fumigation at 60 g m^{-2} significantly increased soil available phosphorus content by 7.11% as compared to that in untreated soil. Therefore, the increase in soil available P content and the decrease in the proportions of AMF may cause uncertain effects on the P uptake by plants. However, there is still a knowledge gap regarding the changes in plant P uptake after soil fumigation.

1.3 Ginger cultivation in China: fumigation application

Ginger (*Zingiber offinale Rosc.*) is an important commercial spice crop with high agricultural and medical value in tropical and subtropical countries including China (Zhang et al., 2017a). China has become the world's second-largest producer of ginger, accounting for 26% ginger production of the world (Srinivasan et al., 2018). There are 6.0×10^4 ha of agricultural land planting ginger, yielding up to 6.4×10^5 tons of ginger in 2020 (FAOSTAT, 2020).

However, due to intensification and monoculture, ginger is suffering from soil-borne diseases, namely soft rot, bacterial wilt, leaf spot and yellow disease caused by soil-borne pathogens, such as *Pythium*, *Pseudomonas solaracearum* and *Ralstonia solanacearum*, *Phyllisticta* and *Fusarium* (Jiang et al., 2018). These soil-borne pathogens can invade ginger roots and stems and what's worse, if surrounding allows, pathogens could also break through the protective microbial shield and destroy resistance to soil-borne diseases (Mansfield et al., 2012; Mendes et al. 2013). After being attacked by these soil-borne diseases, ginger roots start rotting and leaves wilt, turning yellow and dry until the whole plant dead (Meenu et al., 2017).

Soil-borne diseases seriously affect soil health, resulting in yield reduction or ginger survival, leading to tremendous economic losses for ginger farmers. Therefore, numerous agricultural management practices have been developed to control soil-borne pathogens and reduce soil-borne diseases. These include (Gupta et al. 2017; Meenu and Kaushal 2017): 1) cultivation practices such as crop rotation, tillage and organic amendment; 2) biological control using biofertilizers containing beneficial microorganisms such as *Trichoderma spp*. and *Bacillus cepacian*; 3) genetic tools to cultivate crops that

are insensitive or resistant to soil-borne diseases; and 4) chemical control by organic and inorganic fungicides. With all of these practices, pre-treating soil with chemical fumigants has become one of the most efficient methods to control plant diseases (Mao et al., 2017).

1.4 Knowledge gap and scientific question

Soil fumigation is an efficient method to control soil-borne diseases and improve crop yields. The side effects of soil fumigation on soil ecosystems have been extensively studied. However, due to the limitations of detection technology, lack of interdisciplinary and other factors, there are still many knowledge gaps that have not been solved in terms of the side effects of soil fumigation on soil health.

1). Previous studies focused on the level of the soil microbial community structure, such as changes in soil microbial community composition and relative abundance, and did not go deep into soil microbial functions and ecosystem services. Changes in the soil microbiome may further alter the soil microbial functions, such as soil nutrient cycling. However, the side effects of soil fumigation on soil P cycling-related microbes, P transformation processes, and plant P uptake remain largely unknown due to the non-specificity of the microorganisms and genes responsible for P transformation and the diverse forms of soil P. Changes in soil P cycling in fumigated soils may ultimately alter the crop P uptake. However, the effect of soil fumigation on crop P use efficiency has not been well-studied.

2). Soil microbial function is the result of the interaction of different soil microorganisms with the surrounding environment, and determines soil health and crop production. Most studies were conducted in bare soils under controlled conditions, while in farmlands soil fumigation is used before planting. Plants can shape the microbial community structure and disrupt microbial activities by secreting root exudates (Doornbos et al., 2011). Therefore, the growth of crops in fumigated soils might alter changes in the soil microbiome, which is not well-stated.

Meanwhile, it was shown that the soil microbiome could recover quickly after a single soil fumigation, even if it sometimes fails to return to the initial level (Dangi et al., 2015; Dangi et al., 2014; Fang et al., 2018; Liu et al., 2015; Rokunuzzaman et al., 2016; Zhang et al., 2019a). However, repeated soil fumigation may cause irreversible damage to the soil microbiome by eliminating sensitive species and accumulating tolerant species (Li et al., 2017), which might ultimately alter the soil functions such as soil P cycling. How and to what extent long-term repeated soil fumigation affects the crop growth and soil P availability remain to be answered.

In actual farmland, fungicides with specific targets are also applied during crop growth to prevent soil-borne diseases that may arise after initial soil fumigation, resulting in multiple pesticide contamination of agricultural system (Huang et al., 2019b). However, to date, little is known about the side effects of the combined applications of different fumigants and fungicides on the crop growth and soil P availability.

Thus, the side effects of soil fumigation on soil P cycling processes under different agroecosystems, which are critical for improving soil health and food production, remain largely unknown. Therefore, based on the above knowledge gaps, our scientific question was how soil fumigation affects the soil

phosphorus availability under ginger cultivation. To answer this question, we designed the following four research objectives.

1.5 Research objectives and outlines

This PhD thesis aims at improve our understanding of the side effects of soil fumigation on soil P availability under different agroecosystems including real farmlands with different fumigation histories, and soils with combined application of fumigants and fungicides. Ginger was used as the model plant due to the wide application of soil fumigation in ginger cultivation.

The following research objectives will be addressed:

1). To study the response of soil phosphorus availability and ginger growth to the long-term continuous chloropicrin fumigation.

2). To determine the effects of the combined application of chloropicrin fumigant and azoxystrobin fungicide on ginger growth and phosphorus uptake.

3). To examine the variations of soil phosphorus fractions and related phosphorus enzyme activity under the combined application of chloropicrin fumigation and azoxystrobin fungicide.

4). To study research the changes in the soil microeukaryotic community and phosphorus solubilizing microorganisms caused by the combined application of chloropicrin fumigation and azoxystrobin fungicide.

For objective 1, field observations were conducted in ginger fields with continuous chloropicrin (CP) fumigation for 0, 3 and 7 years to study the variation of ginger growth and soil P availability in real farmlands with different CP fumigation history. Ginger yields, P uptake, soil P fractions and two phosphatase (acid and alkaline phosphatase) activity were test.

After field observation, a greenhouse experiment was conducted under the control conditions for objectives 2 to 4 to study the effects of chloropicrin (CP) fumigate and (AZO) fungicide on ginger growth, soil P availability and its microbial mechanisms. In the greenhouse experiment, CP and AZO were applied individually and in combination. We also used single and double applications of AZO to reveal the effects of the recurrent use of AZO on ginger growth and soil P availability.

In this greenhouse experiment, we first wanted to understand ginger growth conditions and P uptake (Objective 2). We then went deep into the soil system to analyse soil phosphorus fractions and two different phosphatase activities (Objective 3). After clarifying the soil P availability in the soil system from objective 3, we further analysed the soil microbial community structure, especially phosphorus solubilizing microorganisms in objective 4. We then used the soil chemical properties (such as pH and SOM) and soil microbiome (objective 4) to explain the variation of soil P availability (objective 3), and the changes in ginger growth and phosphorus uptake (objective 2).

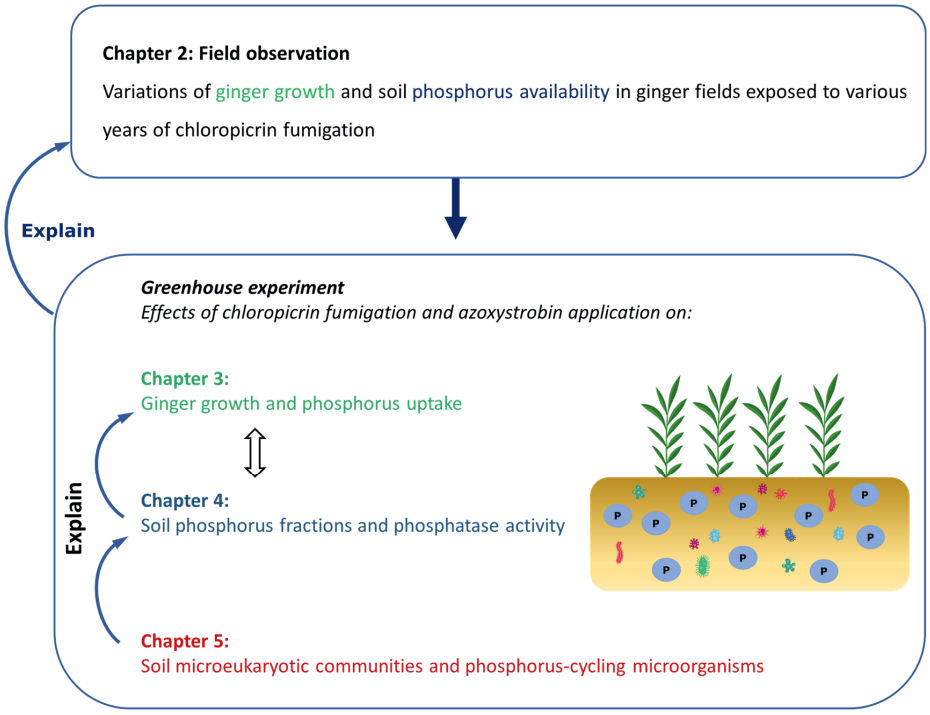

Fig. 1.3 Outline of this PhD thesis


Spring

Chapter 2

Variations of soil phosphatase activity and phosphorus fractions in ginger fields exposed to different years of chloropicrin fumigation

Abstract**:** *Although soil fumigation efficiently controls soil-borne diseases, the effects of repeated soil fumigation on soil phosphorus (P) cycling are still largely unknown. In this study we conducted a field experiment to explore soil phosphorus availability in ginger fields with 0, 3 and 7 years (F0, F3 and F7) of annual chloropicrin (CP) fumigation history in Shandong Province of China. Soil samples (0-20 cm) were collected at four different times in 2019. Ginger yield, soil phosphatase (acid and alkaline) activities and soil P fractions were measured. Results showed that ginger rhizome yield was similar in F0 and F3 (70.0 t* ha^{-1}*), but significantly lower in F7 (37.5 t* ha^{-1}*). The acid phosphatase (AiP) activity was significantly higher in F0, while alkaline phosphatase (AlP) activity was the highest in F3. There was no significant difference in the labile P (Resin-P+*$NaHCO_3$*-P+NaOH-P) between F0 and F7, with 33.6% to 57.5% of total P (TP), while the labile P was significantly lower in F3, being less than 30% of TP. Redundancy analysis (RDA) showed that the highest pH values in F3 contributed to the lowest soil P availability there. AiP activities showed highly positive effects on the soil labile P contents. Results suggested that more P fertilizers are needed after 3 years of CP fumigation to avoid soil P deficiency for ginger growth. The death of ginger became the main limitation for ginger production after 7 years of CP fumigation, at which time, comprehensive agricultural practices should be considered to control ginger soil-borne diseases.*

Based on:

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2.1 Introduction

Ginger (*Zingiber offinale Rosc.*) is an important commercial crop in China with high agricultural and medical value (Zhang et al., 2017a). During cultivation, ginger is prone to soft rot, bacterial wilt, leaf spot and yellowing caused by soil-borne pathogens such as *Pythium*, *Pseudomonas solaracearum* and *Ralstonia solanacearum*, *Phyllisticta* and *Fusarium* (Jiang et al., 2018). Under appropriate environmental conditions, soil-borne pathogens can invade ginger roots and stem bases, causing ginger diseases and reducing production (Mansfield et al., 2012).

To prevent soil-borne diseases in ginger crops, soil fumigation has become a key step prior to ginger cultivation in China (Rokunuzzaman et al., 2016). Among the various fumigants, chloropicrin (Trichloronitromethane, CP) is one of the most widely used compounds owing to its advantageously high inhibitory efficiency on soil-borne pathogens and low environmental residues (Sun et al., 2018b). Within several days after application, CP is broken down into nitrogenous compounds via both chemical and microbial processes (Neilson et al., 2020). The degradation rates of CP can be influenced by the application rate, soil moisture, organic matter content and other soil properties (Ashworth et al., 2019; Ashworth et al., 2018). Soil microorganisms that can metabolize and use CP as a source of energy and nutrients are main players in CP biodegradation (Qin et al., 2016). CP has received increasing attention over the last few years due to its broad antimicrobial activities, environmental behaviors and ecological effects on the soil microbiome, soil enzymes and ultimately on nutrient cycling and soil health (Huang et al., 2020a; Li et al., 2017; Pecina et al., 2016).

Previous studies have shown that soil microbial communities recovered relatively rapidly after soil fumigation (Dangi et al., 2015; Dangi et al., 2014; Fang et al., 2018; Liu et al., 2015; Rokunuzzaman et al., 2016; Zhang et al., 2019a). Zhang et al. (2017b) found that after repeated application of soil fumigants, susceptible soil microbes could disappear while the proportion of those tolerant microbes could increase gradually and even eventually dominate the soil microbiome. They concluded that after 3 years of continuous chloropicrin fumigation, seven genera of *Actinobacteria* and one genus in *Bacteroidetes* disappeared while *Saccharibacteria* increased significantly. Dangi et al. (2017) also found that there were significant differences in soil microbial communities between untreated sites and sites exposed to 15 and 30 years of annual fumigation with methyl bromide. All of these long-term studies revealed that the changes in the soil microbiome after long-term repeated soil fumigation were irreversible. Scientists do not yet know how long it would take for the soil microbial community to recover from repeated fumigation if it can recover at all.

With the potential for the reduced activity of soil organisms after soil fumigation, one major question is how nutrient cycles in soil could be affected. Among the diverse nutrient cycling processes in soil, many researchers have explored the detrimental effects of CP on nitrogen (N) cycling and concluded that soil nitrification processes could be inhibited by soil fumigation in various soil types (Fang et al., 2018; Li et al., 2017; Sun et al., 2018b; Yan et al., 2017b).

Soil phosphorus (P) is the second most important plant growth-limiting nutrient after nitrogen Although the soil system is rich in P, 95% - 99% of the soil total P is present in an insoluble form (Wan et al., 2020) due to binding to the inorganic minerals or organic matter (Schaller et al., 2019), causing about 43% of

the soils to be deficient in phosphorus available for plant growth (Zhao et al., 2019). On the other hand, phosphate rock as a raw material for phosphate fertilizer is a limited resource, which also emphasizes the urgent need to improve the availability of soil phosphorus for plant growth (Lemming et al., 2019). The soil P availability for plant uptake depends on the composition of different forms of P existing in the soil. Hedley's sequential extraction method for P (Hedley et al., 1982), based on the P availability for plants, classifies soil P fractions as 1) Resin-P (easily available P), a soluble inorganic orthophosphate ($H_2PO_4^{2-}$ or $H_2PO_4^-$) in the soil solution and the only form that can be taken up by plants directly (Wang et al., 2011); 2) $NaHCO_3$-P ($NaHCO_3$-Pi and $NaHCO_3$-Po, labile P), inorganic P fractions slightly complexed with Fe /Al minerals and labile organic P fractions complex with organic matter surfaces; 3) NaOH-P (NaOH-Pi and NaOH-Po, moderately labile P), P fractions strongly complexed with Fe /Al minerals and calcic compounds, as well as organic P fractions more strongly associated with soil organic matter; and 4) Occluded P (unavailable P), which is occluded by soil aggregate and cannot be used by plants (Fan et al., 2019; Koch et al., 2018; Motavalli et al., 2002). Among these soil P fractions, inorganic P fractions ($NaHCO_3$-Pi and NaOH-Pi) are available for plants after solubilization, while organic P fractions ($NaHCO_3$-Po and NaOH-Po) need to be mineralized for plant uptake (Spohn et al., 2013). Inorganic P solubilization and organic P mineralization are mainly controlled by phosphorus solubilizing microorganisms (PSMs) that secrete organic acid and synthesize various phosphatase enzymes (Richardson et al., 2009). Acid phosphatase (AiP, EC 3.1.3.2) and alkaline phosphatase (AlP, EC 3.1.3.1) are two non-specific phosphohydrolases able to hydrolyze simple phosphate monoesters to orthophosphate. The AiP can be excreted by plant roots, nodules and soil microbes, while the production of AlP is carried out by soil bacteria and some fungi (Fraser et al., 2017).

Many studies have found that PSM species showed various responses to fumigant composition (Huang et al., 2020a; Pecina et al., 2016; Zhang et al., 2019b) and application rates (Huang et al., 2020b). For example, Pecina et al. (2016) proved that the population of *Pseudomonas ssp.* and *Bacilus ssp.* increased immediately after CP fumigation in intensive tomato production fields with alkaline soil. Dangi et al. (2017) concluded that the proportion of arbuscular mycorrhizal fungi (AMF) was lower in sites with 15, 33 and 39 years of annual methyl bromide fumigation as compared to non-fumigated sites. The variation of PSMs leads to a change in P solubilizing genes and phosphatase enzyme activities, which essentially determine soil P availability. Recently, researchers like Huang et al. (2020a; 2020b) found that soil fumigation using chloropicrin and dazomet significantly increased the plant available P content in soil and the amount of leached soil P during the first two weeks of incubation. No significant difference was observed for the amounts of available P and leached P after 30 days, regardless of the concentrations of chloropicrin and dazomet applied.

However, the response patterns of soil P cycling to long-term repeated soil fumigation in agricultural systems are still far from clear. Understanding the soil P transformation in fields exposed to long-term soil fumigation is crucial in providing deeper insight into the interaction between soil fumigation and soil phosphorus availability. Therefore, in this study, we aimed to understand the effects of repeated chloropicrin fumigation on ginger yield, P uptake, soil phosphatase activity and soil P fractions in a real-world field. We hypothesized that the repeated chloropicrin fumigation could increase ginger yield, but decrease the soil available P contents by increasing ginger P uptake".

2.2 Material and methods

2.2.1 Experiment design

The sampling sites were located in Anqiu, in the Shandong Province of China (N36°21 '42'', E119°10 '38'', **Fig. 2.1**), a region with a temperate continental monsoon climate. The mean temperature is 12.2℃ and the mean annual precipitation is 646.3 mm.

Ginger production fields belonging to the local smallholder farmers in a plain region were selected (**Fig. 2.1**). The historical cropping systems in all farms were wheat-corn rotations. These systems were changed at different years to monoculture ginger production in 2012, 2016 and 2019. The application of the soil fumigant chloropicrin to prevent soil-borne diseases has started with the cultivation of ginger production. We selected fields with 0, 3 and 7 years of annual chloropicrin fumigation (F0, F3 and F7; 3 replicates each). The size of the replicate field ranges from 530 to 1300 m^2. Five soil samples were taken from each replicate field to take soil heterogeneity into consideration and were analyzed separately.

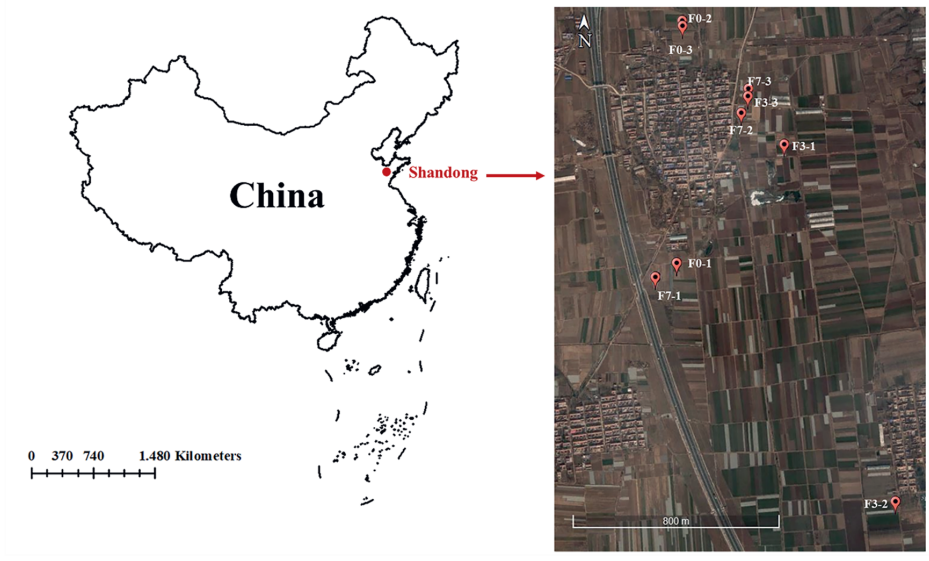


Fig. 2.1 The locations of sampling sites. The red dot names with Shandong indicates the location of the sampling region in China. The picture on the right is a screenshot of the sampling fields from Google Earth. F0, F3 and F7 represent fields with 0, 3 and 7 years of chloropicrin fumigation history before 2019, with three replicated fields for each of them (F0-1, F0-2, F0-3; F3-1, F3-2, F3-3; F7-1, F7-2, F7-3).

Agricultural management practices, such as tillage, then fumigation, rides and furrows, fertilization with biofertilizers (microbial biocontrol agent) before plantation; fertilization with compound mineral (N-P_2O_5-K_2O:18-15-22) and organic fertilizers (soybean meal), open ditch irrigation during a vegetation period of 8 months, were similar in all fields. During the entire ginger growth period, the total P input

was about 48 kg ha^{-1}. The soils are classified as sandy loam (Sand: 52.4% ~ 69.8%; Silt: 16.1% ~ 37.4%; Clay: 10.3% ~ 20.7%. International soil classification system). The initial soil properties are presented in **Table 2.1**.

Table 2.1 Soil physiochemical properties in the selected fields at the begin of the experiment in 2019. F0, F3 and F7 represent fields with 0, 3- and 7-years chloropicrin fumigation history before 2019, with 3 replicated fields for each of them. Data represents the means of five replicates in each field with standard deviation.

Field	pH (H_2O) [a]	SOM (g kg^{-1}) [b]	TP (g kg^{-1}) [c]	TN (g kg^{-1}) [d]
F0-1	5.1 ± 0.1	18.3 ± 2.4	1.5 ± 0.3	0.7 ± 0.0
F0-2	4.9 ± 0.2	25.3 ± 3.4	1.6 ± 0.2	0.6 ± 0.1
F0-3	6.3 ± 0.3	20.8 ± 1.1	1.2 ± 0.1	0.3 ± 0.2
F3-1	6.6 ± 0.2	21.4 ± 5.8	2.2 ± 0.3	0.7 ± 0.1
F3-2	7.7 ± 0.2	24.9 ± 3.0	2.4 ± 0.3	0.8 ± 0.1
F3-3	7.1 ± 0.1	23.2 ± 3.0	2.3 ± 0.2	0.6 ± 0.1
F7-1	5.7 ± 0.3	17.2 ± 1.5	1.4 ± 0.2	0.8 ± 0.1
F7-2	6.1 ± 0.2	24.4 ± 2.4	2.4 ± 0.1	1.2 ± 0.1
F7-3	5.5 ± 0.3	27.0 ± 2.5	1.7 ± 1.5	1.0 ± 0.1

[a] pH (H_2O): soil: ultra-pure water with a ratio of 1:2.5; [b] SOM: Soil organic matter (g kg^{-1}), colorimetric method after H_2SO_4-K_2CrO_7 oxidation; [c] TP: Total phosphorus (g kg^{-1}), analyzed by inductively coupled plasma-optical emission spectroscopy (ICP-OES) after HNO_3-HF-H_2O_2 digestion at 180 °C using a MARS 5 Xpress microwave system (CEM, USA); [d] TN: Total nitrogen (g kg^{-1}), measured using an elemental analyzer.

Chloropicrin (Dalian Lvfeng Chemical Co. Ltd. (Dalian, China)) was applied in all selected fields before ginger planting in March 2019. Chloropicrin was injected 15 to 30 cm into the soil using a specialized machine and the average application rate was 375 kg ha^{-1}. The fields were then covered by plastic films for 2 weeks. The plastic films were removed to release built-up gas one week before planting. A previous study showed that the half-life of CP was about 6 days under the following conditions: an application rate of 392 kg ha^{-1} under 10℃, 8% soil moisture content and 1% soil organic matter content (Ashworth et al., 2019), which was similar to conditions in our sampling region.

Topsoil samples from 0-20 cm depth were collected at four stages: Before fumigation (05/03/2019), After fumigation (28/03/2019), Middle growth period (24/08/2019) and Harvest (23/10/2019). Each sample was divided into two subsamples: one was air-dried for soil chemical properties and the other was stored at 4℃ for analysis of soil phosphatase enzyme activities.

2.2.2 The ginger yield and P uptake

Ginger rhizome yields were estimated at the harvesting stage. Three ginger plants were selected randomly in every field except for the plot F7-3 in which all ginger plants died due to a soil-borne disease before sampling could be carried out. For ginger plant samples, the biomass weight of fresh shoots and rhizomes were measured by gravimetric analysis. The yield of ginger rhizome was estimated by local

farmers, while the yield of ginger shoot was calculated based on the ratio of shoot biomass and rhizome biomass measured from ginger plant samples. The shoot and rhizome samples were separated, and the total P content was measured using inductively coupled plasma-optical emission spectroscopy (ICP-OES) after HNO_3-H_2O_2 digestion (Rosa et al., 2020). The total P uptake was calculated by multiplying the P content per unit biomass by the total biomass in each field (Zhao et al., 2019).

2.2.3 Soil phosphatase activity

Soil AiP was measured according to the protocol described by Tabatabai (1969). Briefly, 1.0 g of fresh soil was added (< 2 mm) to a 50 mL flask, along with 0.2 mL methylbenzene, 1.0 mL 0.05 M *p*-nitrophenyl phosphate and 4.0 mL modified universal buffer of pH 6.5, mixed and incubated at 37 °C for 1 h. Then, 1.0 mL 0.5 M $CaCl_2$ and 4.0 mL 0.5 M NaOH were added to terminate the reaction and then the mixture was filtrated. Absorbance of the filtrate was measured using a spectrophotometer at 410 nm. To measure AlP activity, the universal buffer of pH 6.5 was replaced by a universal buffer of pH 11(Zhao et al., 2019).

2.2.4 P fractions in soil

Soil Olsen-P proposed by Olsen (1954.) was analyzed colorimetrically using the molybdate blue method after extraction with 0.5 mol L^{-1} $NaHCO_3$ at pH 8.5 for 30 min (Hu et al., 2012). Soil P fractions were sequentially extracted using the methods slightly modified according to Tiessen et al. (2006). Briefly, 0.5 g of air-dried soil (< 0.25 mm) was put into a 50 mL centrifuge tube and sequentially extracted with 1) ultrapure water with two anion exchange resin membrane strips (1 cm × 2 cm) converted to the bicarbonate form; 2) 0.5 M $NaHCO_3$; 3) 0.1 M NaOH and 1 M HCl at 25℃ for 16 h (180 rpm). The soil suspension was centrifuged at 10000 g for 10 min at 0℃ and decanted. After adjusting pH, inorganic P (Pi) fractions in each extract were measured using the molybdate ion colorimetry method at 700 nm (Costa et al., 2016; Zhou et al., 2018). Total P in the extracts was determined using ICP-OES while the organic P fractions (Po) were calculated as the difference between TP and Pi. The P fractions were interpreted as Resin-P, $NaHCO_3$-P ($NaHCO_3$-Pi and $NaHCO_3$-Po), NaOH-P (NaOH-Pi and NaOH-Po) and Occluded P (HCl-Pi and residual P) (Hedley et al., 1982). The concentration of Occluded P was estimated by subtracting the sum of the total other P fractions from the TP concentrations of samples (Koch et al., 2018).

2.2.5 Statistical analysis

The statistical analysis was performed by IBM SPSS Statistic 20. Normality of the measured data and homogeneity of variance were tested using the Kolmogorov-Smirnov and Levene test ($p > 0.05$). Due to the non-normal distribution of Olsen-P, P fraction composition as well as AiP and AlP activity values, the non-parametric Kruskal -Wallis analysis with Wilcoxon test ($p < 0.05$) was used to facilitate the comparisons between the different treatments. One-way analysis of variance (ANOVA) with Fisher's least significant difference (LSD) at $p < 0.05$ was applied to normally distributed data (ginger P uptake). Redundancy analysis (RDA) was performed to establish the relationship among P fractions (response variables) and soil properties (explanatory variables) using Origin 2020. All of the figures were created using Origin 2020.

2.3 Results

2.3.1 The ginger yield and P uptake

The average ginger rhizome yields in F0 and F3 were almost the same at 70.0 t ha^{-1}, while the rhizome yield of F7 was the lowest at 37.5 t ha^{-1} (**Fig. 2.2 (a)**) The total ginger P uptake showed similar trends as ginger biomass (**Fig. 2.2 (b)**). There was no significant difference in shoot P uptakes between F0 and F3 with 11.0 and 10.1 kg ha^{-1}, respectively, which were significantly higher than shoot P uptake in F7 (5.9 kg ha^{-1}). No significant difference was observed for root P uptake between F0 and F3 (F0: 19.3 kg ha^{-1}; F3: 23.1 kg ha^{-1}), while the root P uptake was significantly lower in F7 (10.9 kg ha^{-1}).

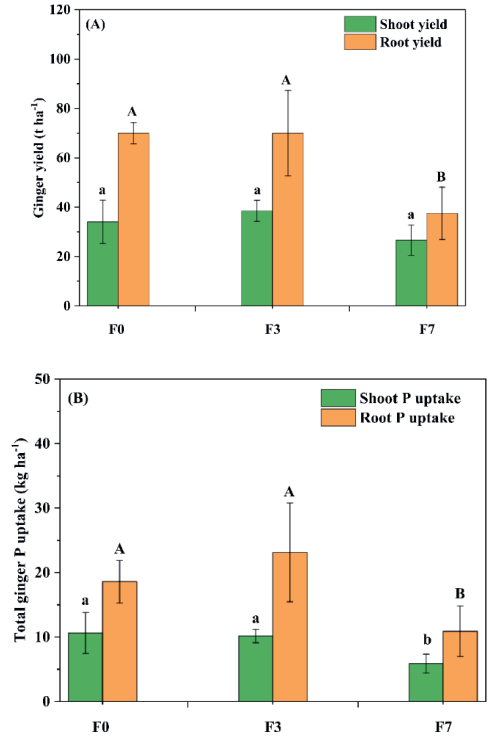


Fig. 2.2 The average of ginger yield (a) and total ginger P uptake (b) after harvest. F0, F3 and F7 represent fields with 0, 3- and 7-years of chloropicrin fumigation history before 2019, with 3 replicated fields for each of them. Legends indicate the average ginger shoot (light green) and root (light orange) yield per hectare in Fig.2.2 (a) and the total P content in ginger shoots (light green) and roots (light orange) per hectare in Fig.2.2 (b). Column represents the average of replicates with standard deviation. In Fig. 2.2 (a), the uppercase letters (A and B) and lowercase letters (a and b) indicate the significant difference in ginger root and shoot yield among three treatments, respectively. In Fig.2.2 (b), the uppercase letters (A and B) and lowercase letters (a and b) indicate the significant difference in ginger root and shoot P uptake among three treatments, respectively. Treatments with the same letter had no significant difference (ANOVA with LSD test, $p < 0.05$)

2.3.2 Phosphatase activity

The average AiP activity was significantly higher in F0 than F3 and F7 during the entire ginger growth period (**Fig. 2.3 (a)**). In F0, the average AiP activity changed from 24.0 mg *p*-nitrophenol g^{-1} h^{-1} before fumigation to 14.6 mg *p*-nitrophenol g^{-1} h^{-1} after fumigation, and recovered to 19.6 mg *p*-nitrophenol g^{-1} h^{-1} after harvest. There were no significant differences between F3 and F7 (12.1 to 13.8 mg *p*-nitrophenol g^{-1} h^{-1}) except for the samples taken after fumigation. For samples collected after fumigation, the average AiP activity was significantly higher in F3 (13.9 mg *p*-nitrophenol g^{-1} h^{-1}) than in F7 (9.4 mg *p*-nitrophenol g^{-1} h^{-1}).

For samples collected before fumigation, the average AlP activity was significantly higher in F3 (14.1 mg *p*-nitrophenol g^{-1} h^{-1}) than in F0 (6.1 mg *p*-nitrophenol g^{-1} h^{-1}) and F7 (6.5 mg *p*-nitrophenol g^{-1} h^{-1}), while there was no significant difference between F0 and F7 (**Fig. 2.3 (b)**). For samples collected after fumigation, the average AlP activity in F3 (20.6 mg *p*-nitrophenol g^{-1} h^{-1}) was still significantly higher than in F0 (7.7 mg *p*-nitrophenol g^{-1} h^{-1}). For samples collected during the middle growth period and harvest, no significant difference in the average AlP activity (14.9 to 21.6 mg *p*-nitrophenol g^{-1} h^{-1}) was observed among three treatments.

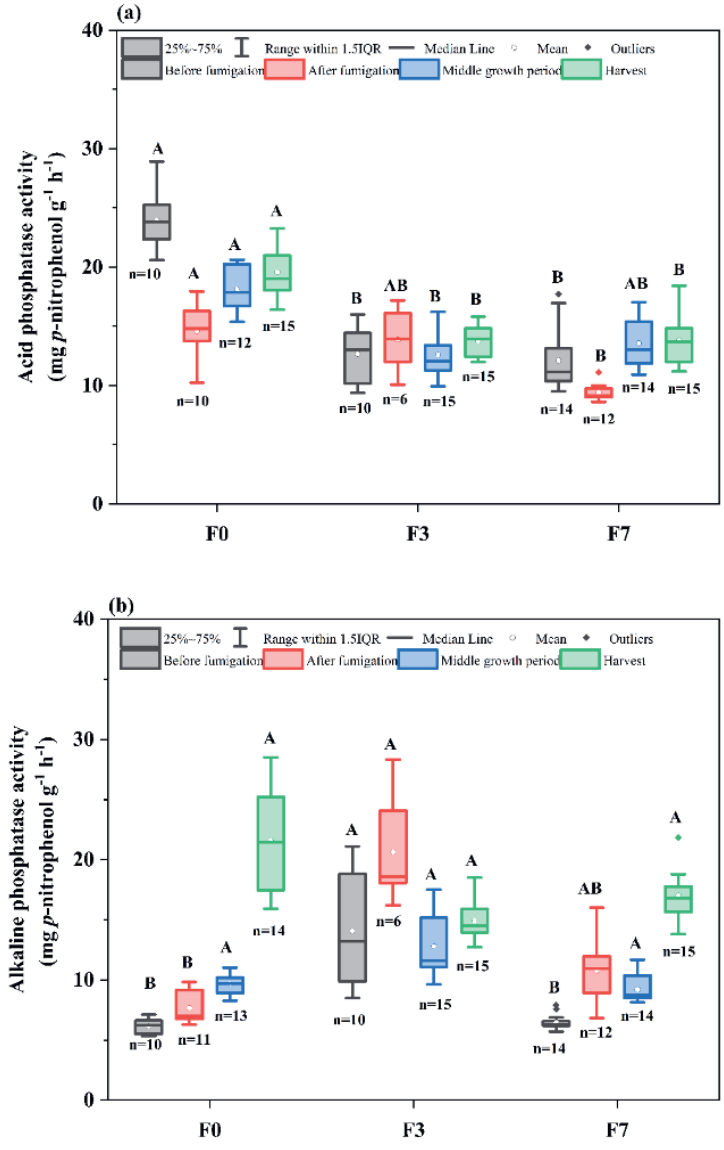


Fig. 2.3 Soil acid (a) and alkaline (b) phosphatase activity in the three treatments and sampling times. F0, F3 and F7 represent fields with 0, 3- and 7-years of chloropicrin fumigation history before 2019, with 3 replicated fields for each of them. Legend indicates the sampling time, including Before fumigation (gray), After fumigation (red), Middle growth period (blue) and Harvest (green). n is the number of actual samples in the corresponding box. The uppercase letters (A and B) indicate the significant difference among the three treatments during each sampling time (Wilcoxon test, $p < 0.05$)

2.3.3 Variation of soil P in the different treatments

For samples collected before fumigation, the content of soil Olsen-P were 129.8, 106.2 and 148.3 mg kg^{-1} in F0, F3 and F7, respectively, and the differences among the three fumigated fields were not statistically significant (**Fig. 2.4**). During the whole ginger growth period, the application of chemical fertilizers by local farmers and the P uptake by ginger caused the variation of soil Olsen-P content to be 111.1 ~ 162.1, 87.1 ~ 127.2 and 137.8 ~ 203.6 mg kg^{-1} in F0, F3 and F7, respectively. The average TP contents were significantly lower in F0 (1179 to 1523 mg kg^{-1}) than that in F3 (1994 to 2487 mg kg^{-1}) and F7 (1967 to 2257 mg kg^{-1}) during the whole growing period. After measuring the total phosphorus (TP) contents, different soil P fractions were extracted using modified Hedley's sequential extraction method (**Fig. S2.1**).

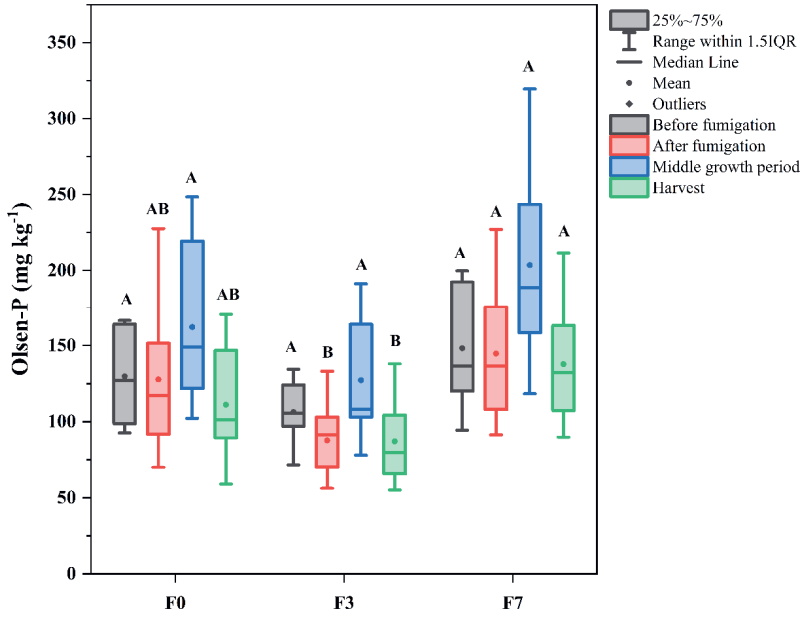


Fig. 2.4 Soil Olsen-P in different fields and sampling times. F0, F3 and F7 represent fields with 0, 3- and 7-years of chloropicrin fumigation history before 2019, with 3 replicated fields for each of them. Legend indicates the sampling time, including Before fumigation (gray), After fumigation (red), Middle growth period (blue) and Harvest (green). The number of samples for every box were 15 (n = 15) except for samples of F0 and F3 before fumigation (n = 10). The uppercase letters (A and B) indicate the significant difference among the three treatments during each sampling time (Wilcoxon test, $p < 0.05$)

For each of the P fractions, unavailable Occluded-P showed similar variation as TP with significantly lower contents in F0 (461 to 738 mg kg^{-1}) than in F3 (1485 to 2019 mg kg^{-1}) and F7 (1237 to 1522 mg kg^{-1}). Significantly lower contents of NaOH-Po and NaOH-Pi were found in F3 (NaOH-Po: 20 to 153 mg kg^{-1}; NaOH-Pi: 65 to 89 mg kg^{-1}) as compared to F0 (NaOH-Po: 32 to 273 mg kg^{-1}; NaOH-Pi: 159 to 204 mg kg^{-1}) and F7 (NaOH-Po: 28 to 275mg kg^{-1}; NaOH-Pi: 133 to 159 mg kg^{-1}). There was no significant difference in the contents of $NaHCO_3$-Po among the three treatments, while the contents of $NaHCO_3$-Pi and Resin-P were significantly higher in F7 than in F0 and F3 (**Fig. S2.1**).

The proportions of soil P fraction to total P were also calculated to eliminate the basic differences among fields by dividing the soil total P contents by every P fraction content (**Fig. 2.5**). For samples collected before fumigation, the proportion of Resin-P was significantly lower in F3 (7.6%) than in F0 (12.1%) and F7 (12.4%). The $NaHCO_3$-Pi proportion appeared to have the same variation as Resin-P with 6.7%, 4.5% and 6.6% in F0, F3 and F7, respectively. The proportion of Resin-P and $NaHCO_3$-Pi remained significantly lower in F3 than F0 and F7 during the whole growth period. The proportion of

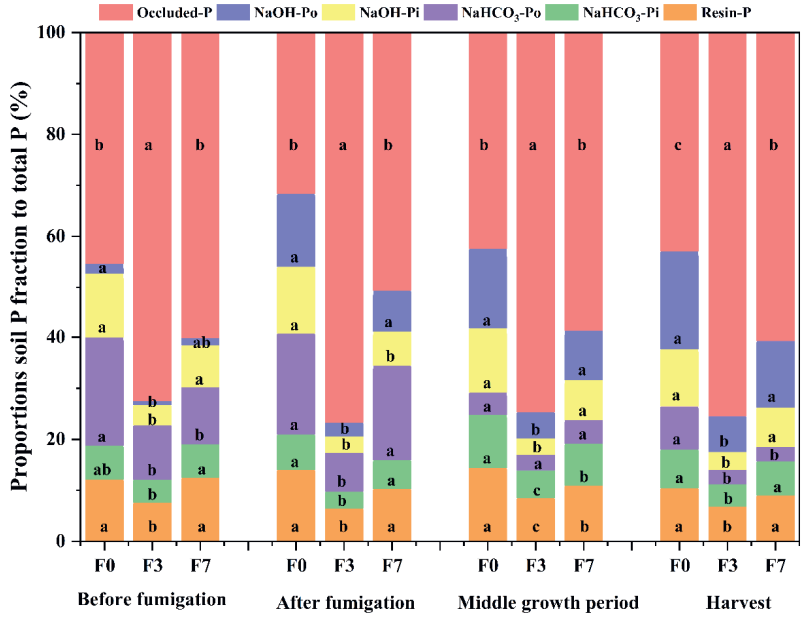


Fig. 2.5 Proportions of various soil P fraction to total P in the three treatments and four sampling times. F0, F3 and F7 represent fields with 0, 3- and 7-years chloropicrin fumigation history before 2019, with 3 replicated fields for each of them. Sampling times were 1) Before fumigation; 2) After fumigation; 3) Middle growth period and 4) Harvest. Legend indicates the soil P fractions, including Resin-P (orange), $NaHCO_3$-Pi (green), $NaHCO_3$-Po (purple), NaOH-Pi (yellow), NaOH-Po (blue) and Occluded-P (red). The lowercase letters (a, b and c) indicate the significant difference of each P fraction among the three treatments for every sampling time (Wilcoxon test, $p < 0.05$)

$NaHCO_3$-Po was significantly higher in F0 with 21.2% as compared to the proportions measured in F3 (10.6%) and F7 (11.2%) before fumigation. After fumigation, the proportions of $NaHCO_3$-Po became 19.7%, 7.7% and 18.1% in F0, F3 and F7, respectively. During the middle growth period, there was no significant difference in the proportions of $NaHCO_3$-Po among three treatments. However, the proportions of $NaHCO_3$-Po were 8.4% in F0 but still remained at 2.8% and 2.9% in F3 and F7 after harvest. The proportion of NaOH-Pi was significantly lower in F3 with 3.9% and there were no significant differences between F0 (12.7%) and F7 (8.2%) before fumigation. The proportion of NaOH-Pi remained almost constant through the whole sampling time in all three treatments. However, NaOH-Po showed similar changes as $NaHCO_3$-Po among different CP fumigation fields and sampling times. The proportions of NaOH-Po were 2.0%, 0.9% and 1.4% before fumigation which increased to 7.5%, 2.5% and 5.0% in F0, F3 and F7 fields, respectively after fumigation. The NaOH-Po proportion increased during the whole ginger growth period and reached up to 19.4%, 6.9% and 13.0% in F0, F3 and F7 fields, respectively after harvest. The proportion of Occluded-P showed opposite trends to labile P fractions with significantly higher values in F3 with 72.5% and there were no significant differences between F0 (45.3%) and F7 (60.1%) for samples before fumigation. The sampling time did not have a significant influence on the proportion of Occluded-P.

2.3.4 Correlation analysis among soil properties, P fractions and phosphatase enzymes

The effects of selected soil properties on the composition of soil P fractions were further analyzed using redundancy analysis (**Fig. 2.6**). The first two axes explained over 99% of the total variation. The results showed that Occluded-P fractions were the main forms in F3, while other labile P fractions took the main place in F0 and F7 and no significant differences were observed among these fractions.

According to the indicating arrows, pH, AlP activity, SOM and TN were highly positively correlated with the proportion of Occluded-P to TP. Labile P fractions (especially $NaHCO_3$-Po) were highly associated with AiP activity.

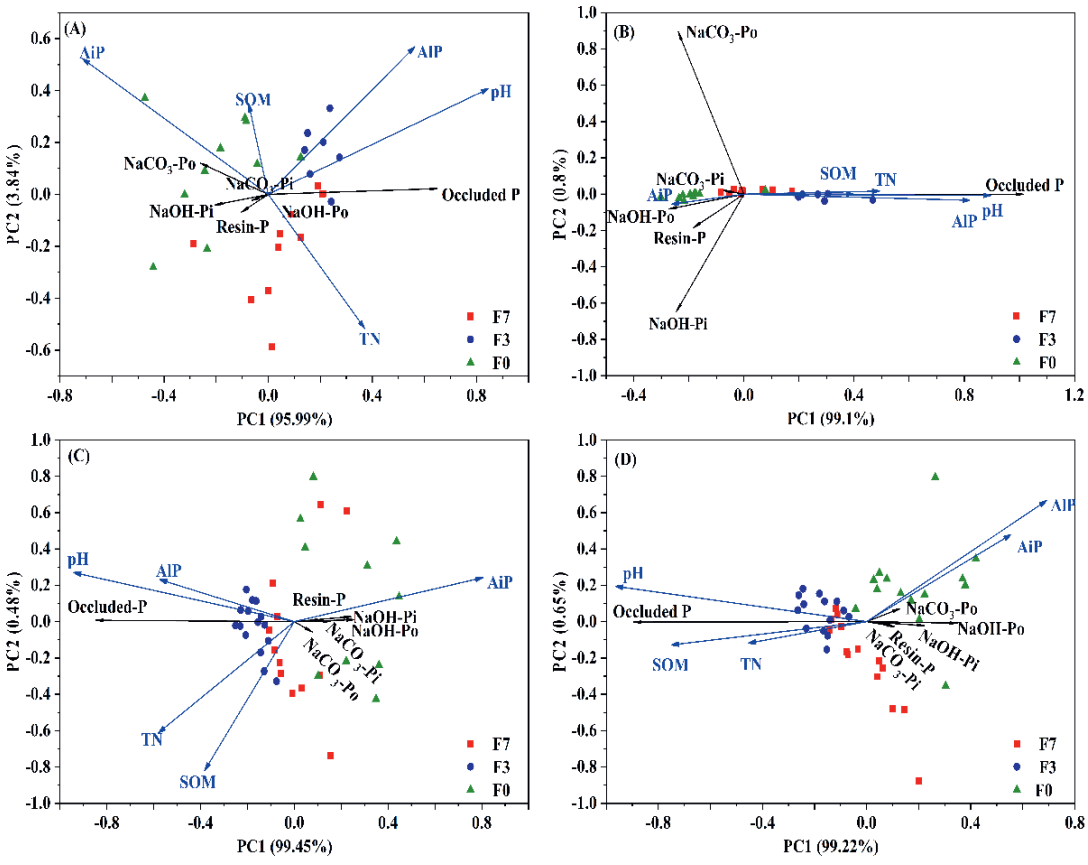


Fig. 2.6 Redundancy analysis (RDA) of P fractions and soil properties. Every figure refers to the RDA analysis for different sampling times including (A): Before fumigation, (B): After fumigation, (C): Middle growth period and (D): Harvest. Legend indicates the samples from F0 (green triangle), F3(blue dot) and F7(red square) treatments. The blue arrows are soil properties including pH, SOM, AiP, AlP and TN. Black arrows are soil P fractions including Resin-P, $NaHCO_3$-Pi, $NaHCO_3$-Po, NaOH-Pi, NaOH-Po and Occluded-P. The position and length of arrows indicate the direction and strengths of the effects of soil properties on P fractions

2.4. Discussion

2.4.1 The effects of different years of CP fumigation on ginger yield and P uptake

In this study, we found no significant differences in ginger yields among F0 and F3, while the yields decreased significantly in F7 due to the high level of soil-borne diseases. Similar results were also found in a study by Yao et al. (2006), in which they concluded that apple tree growth and yield were not affected by pre-plant fumigation with a mixture of 78% dichloropropene+17% chloropicrin as compared to untreated controls after two years. They suggested the results might have been related to the decreased inhibitory effects of CP on soil-borne diseases. Our study is also in line with a study conducted by Zhang et al. (2020b), who found that the strawberry yield increment varied with different consecutive years of CP fumigation with a maximum of 2.0 kg m^{-2} after 2 years, then decreased to 0.9 kg m^{-2} after 5 years. In the current study, all of the ginger plants in F7-3 field died gradually during the later ginger growth period because of the severe soil-borne diseases occurring there. It was also confirmed by the local ginger growers that the inhibitory effects of CP fumigation on soil-borne diseases decreased greatly after several times of CP applications. This finding underlines the need for other comprehensive agricultural management practices, rather than sole CP fumigation, to control soil-borne diseases in ginger crops.

The calculated ginger P uptake showed the same variation as ginger yields with no significant difference among F0 and F3, while F7 was significantly decreased in this study, which also perhaps contributed to the decrease of arbuscular mycorrhizal fungi (AMF) in CP fumigated soil (Smith et al., 2011). There is another long-term study based on chronosequence sites which showed that proportions of AMF were significantly lower for sites exposed to 15, 33 and 39 years of fumigation as compared to their non-fumigated counterparts. AMF has been proved to contribute to the mycorrhizal uptake pathway in delivering soil phosphorus to plants (Smith et al., 2011), so the decrease of AMF could have detrimental effects on plant P uptake and soil P availability.

2.4.2 The effects of different CP fumigation years on phosphatase activities

Our study showed that soil AiP activities were significantly lower in F3 and F7 than in F0 except for samples taken after fumigation. The first time of CP fumigation in F0 put the most evident inhibitory effects on AiP. Even so, AiP activities recovered more quickly in F0 during the ginger growth. AiP activities in F3 and F7 remained almost constant among the different sampling times, meaning that soil CP fumigation did not have an obvious influence on AiP activities in F3 and F7.

On the other hand, the highest values for soil AlP activities were observed in F3 except for the samples gathered after harvest. Similar AlP activity values were observed for F0 and F7. It has been proved that AlP increases with the increase of soil pH (Dick, 2000). In this study, AlP activity was also significantly positively correlated with soil pH (**Fig. S2.2**). Therefore, we assume that the highest pH values in F3 are one of the most important reasons for the highest AlP activity there. On the other hand, AlP mainly is originated from soil microorganisms (Acosta-Martínez et al., 2015). A study by Huang, et al. (2020b) found the application of dazomet fumigant at 50 mg kg^{-1} showed a transient inhibitory effect on AlP activity before 14 days, which may be due to the decrease of soil *phoD* gene abundance which is

response for the production of AlP. However, in our research, the microbial mechanism of AlP activity changes in different fields needs further study.

2.4.3 The effects of different years of CP fumigation on P fractions

Soil P fractions extracted using sequential methods with different extracting agents are indicators of their solubility in soil solution and their availability for plants (Milić et al., 2019). In our experimental fields, the historical cropping system was maize-wheat rotation before the ginger planting. As a cash crop, ginger planting requires more fertilizers input than cereal crops (maize and wheat), causing significantly higher total P levels in F3 and F7 than in F0 (**Table. 2.1**). Fertilization increased the concentration of P in the soil solution, thereby enhancing the adsorption of phosphorus by soil particles (Weihrauch et al., 2018), making soil P unavailable for ginger plants in F3 and F7 (**Fig. S2.1**). Therefore, we did not refer to the total content in this study but we focused on the proportion of each P fraction to total P to compare the composition of soil phosphorus (**Fig. 2.5)**.

The proportion of plant labile phosphorus (Resin-P+$NaHCO_3$-P+NaOH-P) was the lowest in F3, which may be due to the higher pH value in F3 (pH: 6.6 to 7.7) than F0 (pH: 4.9 to 6.3) and F7 (pH: 5.5 to 6.1). The optimum pH for the highest soil P availability is 5.5 to 6.0, because when the soil pH is lower than 5.0, soil P can form insoluble Fe/Al-P-minerals (Zhou et al., 2018), while in alkaline soil (pH>7), soil P combined with Ca^{2+} and precipitates in the form of Ca-P-minerals (Zhou et al., 2018). The RDA analysis in our study also showed that the proportions of soil Occluded-P are highly positively associated with soil pH. Compared with F7, the significantly higher ginger yield and ginger P uptake in F3 may also attribute to the lower proportions of soil labile P. The lowest proportion of soil labile P in F3 indicates that, although the yields of ginger rhizomes were similar between F0 and F3, the significantly lower proportion of soil labile P in F3 may be a key limiting factor for higher ginger yields.

On the other hand, the significant decrease in ginger yield and P uptake, and the lack of reduction in the application of phosphate fertilizer in F7, may further cause more labile P fractions to be delayed on the soil surface, because plant uptake is one of the most important outputs of P in the soil system. Therefore, the low P use efficiency and high proportions of easily labile P (Resin-P), labile P ($NaHCO_3$-P) and moderately labile P (NaOH-P) in F7 may cause severe environmental problems such as water eutrophication washed by surface runoff or P leaching into the groundwater system (Zhang et al., 2021b).

Among different soil P fractions, the proportions of organic P (especially NaOH-Po) increased significantly after CP fumigation again in 2019, which may be caused by the release of microbial organic P from the lysis of soil microorganisms (Fang et al., 2018). The released microbial organic P fractions are then mineralized into available inorganic P by various soil phosphatase (Acosta-Martínez et al., 2011). However, CP fumigation also reduced soil phosphatase activity (Huang et al., 2020a), especially AiP in our study, which may then retard the mineralization of organic P and eventually lead to the accumulation of organic P in the soil (**Fig.2.7**). However, the detail microbial mechanisms also need to be studied further.

A previous lab incubation study conducted by Huang et al. (2020a) found that CP fumigation significantly

increased the proportion of $NaHCO_3$-Po for up to 14 days in acidic soil, and significantly decreased the proportion of $NaHCO_3$-Po up to 49 days in alkaline soil. All of the soil P fractions recovered to a similar level after 49 days among all CP treatments in two kinds of soil. They suggested that farmers could reduce the P fertilizers application in the early stage (< 30 days) due to the increase of soil available P. However, our study found that the release of available P by the lysis of dead microbes may not satisfy the increasing demand for soil phosphorus by ginger, at which time, P deficiency may become a major limitation for getting a higher yield in F3. With the increasing of CP fumigation years, soil-borne pathogens may become resistant to CP (Qiao et al., 2010), leading to a serious ginger death in F7. At this time, we should first consider finding another effective method to prevent soil-borne diseases.

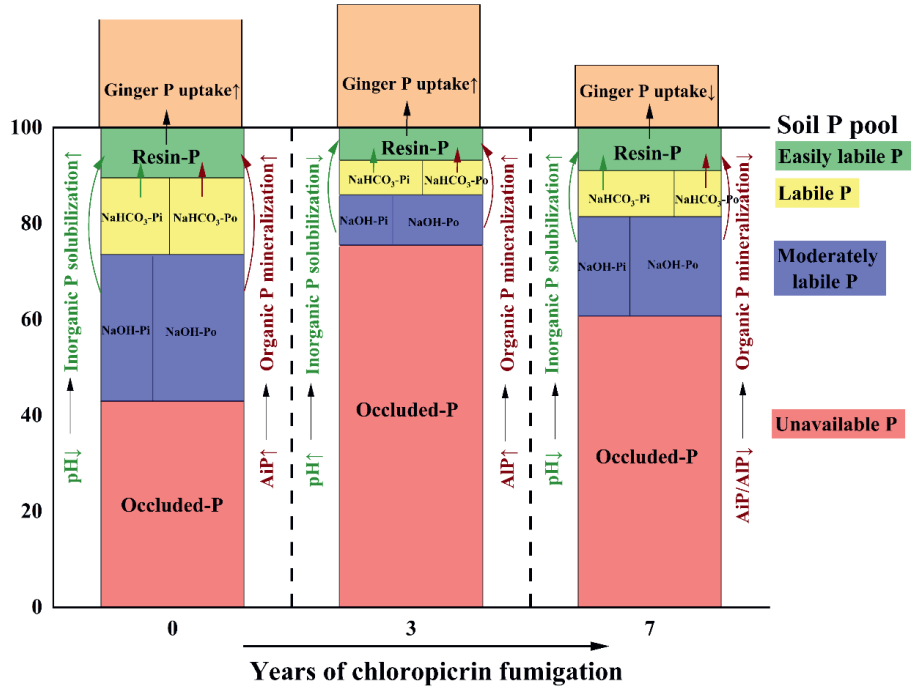


Fig. 2.7. Schematic diagram of the composition of soil P pool, the role of soil pH and phosphatase activity in soil P transformation, and the ginger P uptake. Data used in this figure is the data measured for the samples collected at Harvest. 0, 3 and 7 represent fields with 0, 3 and 7 years of chloropicrin fumigation before 2019. The numbers at Y axis means the proportions of each P fraction to total P. P fractions are Resin-P, $NaHCO_3$-Pi, $NaHCO_3$-Po, NaOH-Pi, NaOH-Po and Occluded-P. The distribution of NaOH-Pi and NaOH-Po, as well as the distribution of $NaHCO_3$-Pi and $NaHCO_3$-Po in the figure were decided according to their own proportions to TP. The soil P pool was divided into Easily labile P, labile P, moderately labile P and unavailable P according to their solubility and availability for plant

2.5. Conclusion

In this field study, we tested the effects of different years of chloropicrin fumigation on ginger yield, phosphatase activities and soil P availability. The results showed, compared with the ginger yield in fields with the first CP fumigation, no significant difference was observed on the ginger yields in the fields with 3 years of CP fumigation. However, the contents of soil labile P decreased significantly after 3 years of CP fumigation, which may become an important limiting factor for the ginger yields. The ginger yield decreased significantly after 7 years of repeated CP fumigation, at which time, ginger death became the main limiting factor for ginger production, and controlling soil-borne diseases using a better method rather than sole CP fumigation should be the first consideration. The lower phosphatase activities after repeated CP fumigation were also important influencing factors for the reduced soil P availability, which highlighted the fact that microbial mechanisms, especially P solubilizing microbes, need to be studied further to understand the variations in ginger yield and soil P availability after long-term repeated CP fumigation.

2.6. Acknowledgement

We want to thank local farmers and workers who kindly supported to finish the field observation. Robin Palmer is also highly appreciated for language editing. This research was supported by National Natural Science Foundation of China (41877072) and National R and D key program of China (grant no:2016YFE011270).

Supplementary materials

Table S2.1. Soil basic properties in the three treatments and four sampling time. F0, F3 and F7 represent fields with 0, 3- and 7-years chloropicrin fumigation history before 2019, with 3 replicated fields for each of them. Sampling time were 1) Before fumigation; 2) After fumigation; 3) Middle growth period and 4) Harvest. Data represents the means of replicates with standard deviation.

Sampling time	Treatment	pH	SOM (mg kg^{-1})	TP (mg kg^{-1})	TN (g kg^{-1})
Before fumigation	F0	5.1 ± 0.3	22.4 ± 4.7	1523 ± 253	0.7 ± 0.1
	F3	7.0 ± 0.4	21.4 ± 4.3	2277 ± 228	0.6 ± 0.1
	F7	5.8 ± 0.4	22.9 ± 4.7	2128 ± 595	1.0 ± 0.2
After fumigation	F0	5.3 ± 0.7	22.2 ± 2.6	1288 ± 199	0.4 ± 0.2
	F3	7.1 ± 0.6	24.4 ± 3.3	2487 ± 430	0.9 ± 0.2
	F7	5.6 ± 0.4	25.5 ± 4.0	2257 ± 629	0.9 ± 0.2
Middle growth period	F0	5.6 ± 0.6	17.4 ± 2.6	1179 ± 157	0.5 ± 0.1
	F3	7.2 ± 0.5	20.8 ± 2.2	1994 ± 261	0.8 ± 0.2
	F7	5.9 ± 0.3	19.7 ± 3.7	1967 ± 561	0.8 ± 0.2
Harvest	F0	5.4 ± 0.7	11.2 ± 1.2	1385 ± 150	0.6 ± 0.1
	F3	7.2 ± 0.4	16.5 ± 2.2	2183 ± 265	0.8 ± 0.2
	F7	5.8 ± 0.4	15.2 ± 3.1	2257 ± 620	0.8 ± 0.2

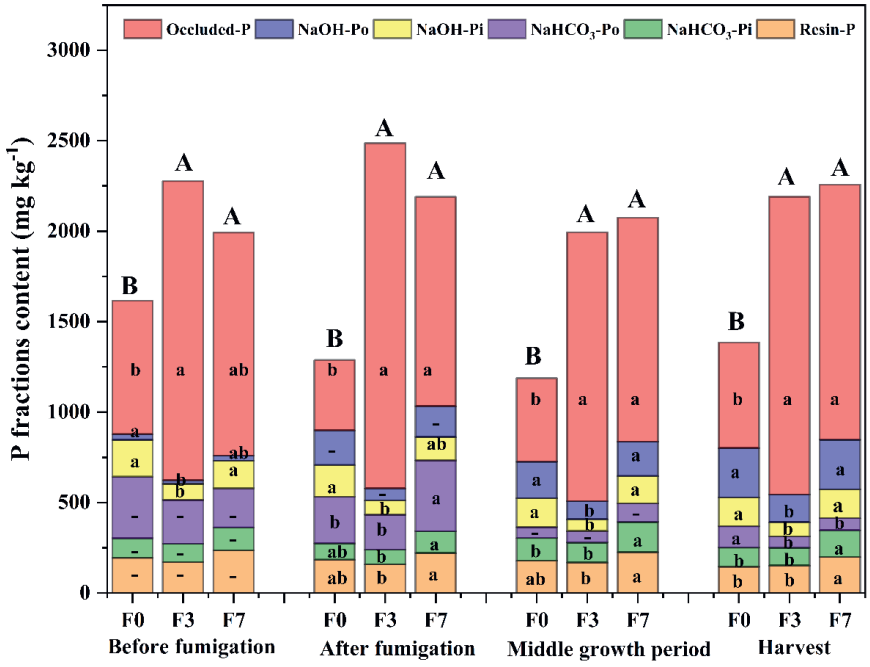


Fig. S2.1. Soil P fractions content in the three treatments and four sampling time. F0, F3 and F7 represent fields with 0, 3- and 7-years chloropicrin fumigation history before 2019, with 3 replicated fields for each of them. Sampling time were 1) before fumigation; 2) after fumigation; 3) middle growth period and 4) after harvest. Legend indicates the soil P fractions, including Resin-P (orange), $NaHCO_3$-Pi (green), $NaHCO_3$-Po (purple), NaOH-Pi (yellow), NaOH-Po (blue) and Occluded-P (red). The lowercase letters (a and b) indicated the significant difference of each P fraction among the three treatments for every sampling time. The uppercase letters (A and B) indicated the significant difference of total P among three treatments for every sampling time. "–" means no significant differences (Kruskal - Wallis analysis, p <0.05)

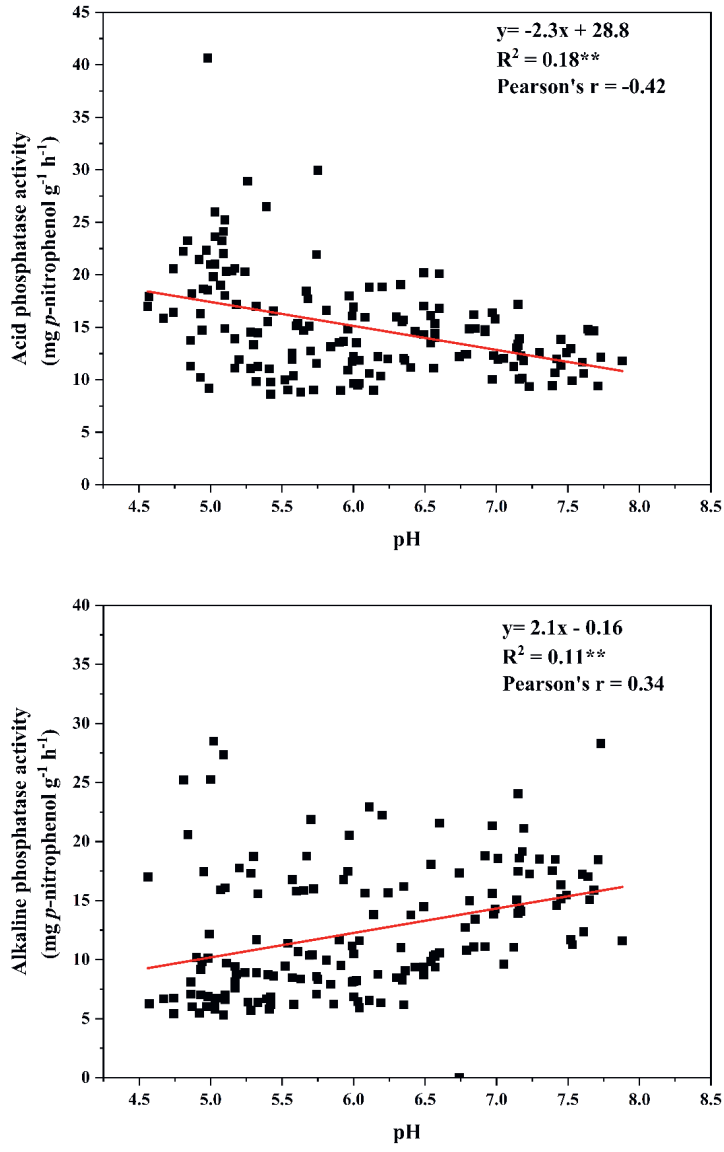


Fig. S2.2. The relationship between acid/alkaline phosphatase activity and soil pH values. ** means the acid/alkaline phosphatase activity and pH values were significantly correlated at 0.01 level.

Fig S2.3 Ginger fields before soil fumigation

Fig S2.4 Ginger fields during soil fumigation

Fig S2.5 Ginger fields after soil fumigation

Fig S2.6 Ginger field furrows after soil fumigation

Fig S2.7 Ginger planting


Summer

Chapter 3

Effects of chloropicrin fumigation and azoxystrobin application on ginger growth and phosphorus uptake

Abstract: *Soil chloropicrin (CP) fumigation helps to increase crop yields by eliminating soil-borne diseases which inhibit plant growth. However, little is known about the effect of the CP fumigation combined with fungicide application on plant growth and nutrient uptake. In this study, we conducted a mesocosm experiment with six treatments: CK (untreated soil), AZO1 (a single application of azoxystrobin (AZO)), AZO2 (double applications of AZO), CP (CP fumigation with no AZO), CP+AZO1 (CP combined with AZO1) and CP+AZO2 (CP combined with AZO2) to investigate the effects of CP fumigation and AZO application on ginger growth and phosphorus (P) uptake. Results showed that a single application of AZO had no significant effect on ginger height, biomass and P uptake whether treated with or without CP fumigation, whereas double applications of AZO combined with CP fumigation significantly improved ginger height and the total amount of P in root ($P<0.05$). Meanwhile, AZO residues were similar in all treatments with the same number of applications, with less than 50% remaining in the soil after 7 days applied, indicating that CP fumigation treatment did not influence AZO degradation in ginger cultivation. In addition, although the differences in P use efficiency observed across the different treatments were not significant, they nevertheless suggest that the P budget and soil microbial activity may contribute to those differences. Therefore, further studies should be done to link P cycling with microbial communities, and how these related to fumigation and fungicide application.*

Based on:

Wang Y, Yang X, Xu M, Geissen V (2022) Effects of chloropicrin fumigation and azoxystrobin application on ginger growth and phosphorus uptake. Ecotoxicol Environ Saf 232: 113246. doi: 10.1016/j.ecoenv.2022.113246

3.1 Introduction

Ginger (*Zingi*ber offinale Rosc.) is an important commercial spice crop in tropical and subtropical countries including China (Zhang et al., 2017a). China is the world's second-largest producer of ginger, accounting for 26% of the world's ginger production (Srinivasan et al., 2018). 5.6×10^4 ha of agricultural land in China is devoted to ginger production, yielding 6.0×10^5 tons of ginger in 2019 (FAOSTAT, 2019). The health and growth of the ginger plants are strongly limited by many soil-borne diseases such as soft rot, bacterial wilt and yellows diseases induced by soil-borne pathogenic bacteria and fungi (Jiang et al., 2018), leading to severe production and economic losses for farmers (Mansfield et al., 2012).

In order to eliminate soil-borne pathogens to reduce the occurrence of ginger diseases and to increase profits, ginger farmers treat the soil prior to planting by injecting fumigants into the soil pores at a depth of 30 - 40 cm using specialized equipment. Soil fumigants are broad-spectrum chemicals that may be antimicrobial sterilizers, fungicides, herbicides, insecticides, and nematicides, that once injected, vaporize and easily diffuses through the soil pores, thereby eliminating a large proportion of the organisms within (Rokunuzzaman et al., 2016). Chloropicrin (trichloronitromethane, CP) is one of the most widely used fumigants owing to its high efficiency in killing soil-borne pathogens and low environmental residues (Xie et al., 2015). Although CP could be degraded into nitrogenous compounds after several days in the soil (Neilson et al., 2020), CP fumigation could significantly inhibit the growth of pathogens and thus promote ginger production (Mao et al., 2014). It was showed that the colony forming units of *R. solanacearum* were reduced by 76% to 85% with CP fumigation at a rate of 50 g m^{-2}, and that the mortality of ginger was reduced from 60% in untreated soil to 0.4% in CP fumigated soil, thereby increasing yields by 4.77 kg m^{-1}. Therefore, there practice of CP fumigation is intensively used and extended in ginger cultivation.

Along with soil fumigation prior to ginger planting, ginger growers also add other fungicides such as azoxystrobin (AZO) to the soil during the growing period to enhance the inhibition of soil-borne pathogens (Sun et al., 2018b). In 1996, AZO became the first strobilurin fungicide, inhibiting fungal pathogens such as *Ascomycetes*, *Basidiomycetes*, *oomycete* and imperfect fungi by disrupting ATP production in fungal mitochondria (Howell et al., 2014; Sun et al., 2018b). Previous studies showed that AZO application could significantly delay plant senescence caused by saprophytic fungi (Bertelsen et al., 2001) and increase the yield of winter wheat (Zhang et al., 2010). Kunova et al. (2013) also found that the growth of *Magnaporthe oryzae* mycelium on the surface of rice could be reduced by 50% by using AZO at 0.044 mg L^{-1}. However, the effects of the combined application of CP and AZO on ginger growth are still largely unknown. Ginger farmers usually add AZO by irrigating or spraying it on the ginger rhizomes after the appearance of symptoms of soil-borne diseases such as ginger wilt and rot during the ginger growth period. (Liu et al., 2021) found that if sugar beet seedlings have been infected by *R.* solani, the application of AZO at 167 g a. i. ha^{-1} did not prevent seedling death. In addition, the necessity of applying additional fungicide after initial soil fumigation which kills almost all soil microbes, has not been well examined. It is vital to further examine this issue in order to avoid the overuse of pesticides and reduce unnecessary costs to farmers, while also preventing crop loss.

Due to their broad-spectrum destruction, CP and AZO also have detrimental impacts on soil beneficial microbes which are responsible for soil nutrient transformation (Guo et al., 2015; Wang et al., 2018).

Among soil nutrients, phosphorus (P) is one of the most important elements for the plant growth, taking part in energy transfer for cellular metablolism and the construction of cell membranes and nucleic acids (Abolfazli et al., 2012). Only dissolved P in the soil solution can be used by plants directly, and over 99% of the total P is unavailable for plant uptake before being solubilized or mineralized (Sharma et al., 2013). Studies conducted by Huang et al. (2020a; 2020b) found that soil fumigation using chloropicrin and dazomet increased the contents of soil available P fractions significantly. They suggested that P fertilizers could be reduced at the early stage (< 30 days). On the other hand, Dangi et al. (2017) found that soil fumigation significantly decreased the relative abundance of arbuscular mycorrhizal fungi (AMF) which are responsible for the mycorrhizal uptake pathway to transfer soil P to plants (Smith et al., 2011). Therefore, after the application of CP and AZO, the increase of soil available P fractions and the decrease of the proportions of AMF may cause uncertain effects on the P uptake by plants. However, there is still a knowledge gap regarding to the changes in plant P uptake after soil fumigation or fungicide application separately, in addition to the changes that take place in soil exposed to the combined application of soil fumigation and fungicides.

Therefore, the objective of this study was to explore the effects of CP fumigation and AZO application on ginger growth and P uptake using a mesocosm experiment. We investigated a single application and double applications of AZO to observe whether the number of AZO applications made a significant difference. By comparing the ginger growth indicators (height and biomass) and P uptake among various treatments, our study will yield practical information on CP fumigation and AZO application in ginger cultivation.

3.2 Materials and methods

3.2.1 Experiment materials and design

3.2.1.1 Preparation

Before setting up the mesocosm experiment, 2.64 m^2 (1.2 m × 2.2 m) of soil from a previously untreated field (sandy clay loam according to the international classification system) in An'qiu, in the Shandong Province of China was selected. The soil in the field was treated with a fumigant before being collected for the experiment. Chloropicrin (CP) [CCl_3NO_2] (Dalian Lvfeng Chemical Co. Ltd.) was used in this study at the field recommended application rate of 37.1 g m^{-2}. 100 g of chloropicrin was injected into the soil in the field at a depth of roughly 15 cm using an injector. After injection, the treated soil was immediately covered with plastic tarp for one week. The plastic tarp was then removed to allow the gas to dissipate for another week. A field adjacent to the fumigated field was selected as the source for unfumigated soil. After the gas was allowed to escape, surface soil (0 - 20 cm) from fumigated and unfumigated field was collected and taken to the greenhouse, where soil was then passed through 4 mm sieve and mixed separately in preparation for the treatments. The soil properties are listed in **Table S3.1**.

3.2.1.2 Experimental design

Ginger (*Zingiber officinale*) was used as the model plant due to the wide use of CP and AZO on ginger.

Before planting, the ginger rhizome was put on a breeding bed to germinate. The healthy germinated ginger rhizome was then put into the mesocosm. Briefly, 6 kg of treated soil (calculated based on the measured water content of fresh soil) and 100 g of 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.

There were six treatments: 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 with no AZO), CP+AZO1 (CP combined with AZO1) and CP+AZO2 (CP combined with AZO2). For AZO1 and CP+AZO1 treatments, AZO was applied using a suspension spray 8 weeks after planting. For AZO2 and CP+AZO2 treatments, the plants were treated the same as in the previous treatments with an additional spray of AZO applied 16 weeks after planting. Each dose of azoxystrobin (AZO) (Hebei Zhongbaolv Crop Technology company) was applied at the field recommended application rate of 0.55 mg kg^{-1} soil (47.1 mg AZO m^{-2}).

Sampling was conducted four times: 1) before planting for CK and CP treatments (before planting, BP;12/04/2019); 2) one week after the first AZO application (9 WAP: 17/06/2019) for CK, AZO1, CP and CP+AZO1 treatments; 3) one week after the second AZO application (17 WAP: 10/08/2019) for CK, AZO1, AZO2, CP, CP+AZO1 and CP+AZO2 treatments; 4) after harvest (28 WAP:15/10/2019) for all of the treatments. There were five replicates of each treatment made for every sampling occasion, tallying to a total of 90 pots. Destructive sampling was carried out on each pot. After the 17 WAP sampling, 5.0 g (about 70.8 g m^{-2}) of compound chemical fertilizer (N-P-K: 16-12-2; N ≥ 16%, P_2O_5 ≥ 12%, K_2O ≥ 20%) was applied to each pot to supply nutrients for the growth of ginger. When sampling, all roots and other debris in soil were separated and sorted out. Then, the soil sample was collected and divided into two subsamples: one subsample was air-dried and sieved for soil property analysis; while the other subsample was stored at -80 °C for AZO residue analysis.

3.2.2 Soil sample analysis

3.2.2.1 Soil properties

pH was determined in soil: water suspension with a ratio of 1:2.5. SOM was measured using colorimetric method after H_2SO_4-K_2CrO_7 oxidation (Sun et al., 2018a). Total P (TP) was measured using inductively coupled plasma-optical emission spectroscopy (ICP-OES) after HNO_3-HF-H_2O_2 digestion at 180 °C using a MARS 5 Xpress microwave system (CEM, USA). Soil Olsen P was decided colorimetrically using the molybdate blue method after extraction with 0.5 mol L^{-1} $NaHCO_3$ at pH 8.5 for 30 mins (Zhou et al., 2018). Total nitrogen (TN) was measured using an elemental analyzer.

3.2.2.2 Azoxystrobin residues analysis

Extraction of azoxystrobin in the soil samples

A modified QuEChERS method was used for samples extraction and clean-up (Huang et al., 2019b): 10 g of a soil sample was added to a 50 mL centrifuge tube. 5 mL of pure water and 10 mL of ACN were added to the tube and the mixture was shaken vigorously for 3 min using a shaker and sonicated for 15

mins in an ultrasound bath. 4 g of anhydrous $MgSO_4$ and 1 g of NaCl were added to the mixture. The tube was shaken for 3 mins and centrifuged for 5 mins at an RCF of 3000 g. The supernatant was cleaned using a 6 mL cleanert Florisil-SPE column and then filtered through a 0.22 µm filter membrane for high performance liquid chromatography (HPLC) analysis.

High Performance Liquid Chromatography (HPLC) Analysis of AZO

Quantitative determination of azoxystrobin in the extracted solution was analyzed using an HPLC (Agilent 1100 series, Agilent Technologies, Santa Clara, California, USA) equipped with an autosampler, quaternary pump, degasser, and variable wavelength UV detector (VWD). Agilent ZORBA × SB-C18 column (250 mm × 4.6 mm, 5 µm, Agilent Technologies, Santa Clara, California, USA) was used as the analytical column.

The chromatographic separation was carried out using an acetonitrile/water mobile phase at a flow rate of 70:30 (v/v), flow rate: 1 mL min^{-1}, injection volume: 10 µL, assay time: 11 min., column temperature: 25 °C. The signals were recorded at a wavelength of 210 nm. Chromatographic data were collected and integrated using ChemStation rev. B.03.02 software (Marczewska et al., 2020).

Quality control

Stock solutions of the AZO standard at 1000 µg mL^{-1} were prepared in acetonitrile by weighting 0.0500 g of AZO into a 50 mL volumetric flask and diluting it with acetonitrile to a volume of 50 mL. Then 1 mL of AZO stock solution was taken and transferred to a 100 mL flask and diluted with acetonitrile, such that the AZO working solution attained a concentration of 10 µg mL^{-1}. The stock and working solution were kept in dark brown vials at -20 °C (Abdelraheem et al., 2015). 0, 0.5, 1.0, 2.5, 5.0, 10.0, 25.0 and 50.0 mL of AZO working solution were taken and transferred into 50 mL volumetric flasks and diluted with acetonitrile to 50 mL, obtaining concentrations of 0, 0.1, 0.2, 0.5, 1.0, 2.0. 5.0, 10.0 µg mL^{-1} of AZO. A typical HPLC-VWD chromatogram of AZO standard solution at the 5.0 µg mL^{-1} level can be seen in **Fig. S3.1**, showing that the retention time was about 2.87 minutes. Linearity was tested using a scatter diagram plotted using the standard solution concentrations as the x axis, while the measured peak area was the y axis (Marczewska et al., 2020). The scatters were linearly fitted and strong linearity was observed, with coefficient of determination $R^2 > 0.999$ for AZO in the calibration range between 0-10.0 µg mL^{-1} (**Table S3.2**).

Recovery was calculated by using 10.0 g of blank soil samples in five replicates that were spiked with 1 mL of AZO standard solution (1.0 and 5.0 µg mL^{-1}) at 100 and 500 µg kg^{-1} and then extracted using the chosen method. The recovery values were calculated as the percentage of the spiked analyte recovered (Abdelraheem et al., 2015). The mean recoveries of AZO in soil at 0.1 and 0.5 mg kg^{-1} levels were 69.2% and 83.6% (**Table S3.3**), respectively, and were almost in the acceptable range of 70-120% (Saha et al., 2020).

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated using the residual standard deviation of the standard curves and the slope of the regression line (Abdelraheem et al., 2015). The result showed that the calculated LOD and LOQ in soil were 0.01 and 0.04 mg kg^{-1},

respectively (**Table S3.3**). In addition, the matrix effect was evaluated at the spiking level of 500 μg kg^{-1} for AZO (Saha et al., 2020): we found that the soil only caused a 16.4% (< 20%) negative matrix effect at the 0.5 mg kg^{-1} spike level.

3.2.3 Ginger growth and P uptake

At 28 WAP, the ginger plants were removed from the pots and cleaned. Ginger height was measured with a ruler. Fresh shoot and rhizome biomass weight was measured gravimetrically (Liu et al., 2018).

The P concentrations in shoots and rhizomes were measured separately using ICP-OES after HNO_3-H_2O_2 digestion. The P amounts of ginger shoots and rhizomes were calculated by multiplying the P contents by the corresponding ginger shoot and rhizome biomass (Tang et al., 2008). Total ginger P amount was calculated as the sum of shoot and rhizome P amount

Partial factor productivity of P (PFP_p) (kg kg^{-1}) was calculated by dividing the ginger rhizome biomass by the applied phosphorus amount in each pot using the following equation (1) (Si et al., 2018)

$$PFP_p = Y_t / P_f \quad (1)$$

where, Y_t is rhizome yield at harvest (g); P_f is the total amount of P fertilizer that was applied to each pot. Higher values of PFP_p means that ginger was able to produce higher yields with the same amount of inorganic P fertilizer.

The physiological P use efficiency (PPUE, g^2 DM g^{-1} P) was calculated as the ratio between ginger rhizome yield and ginger rhizome P content (DM = dry matter) (Gu et al., 2018; Hammond et al., 2009):

$$PPUE = Y_t / P_c \quad (2)$$

where, Y_t is rhizome yield at harvest (g); P_c is the ginger rhizome P content (g g^{-1}DM). The larger the PPUE value, the less physiological P requirements and tissue content needed for the same yield (Akhtar et al., 2014).

The P budget was estimated by subtracting the P that was removed from each pot by the whole ginger plant from the P applied as mineral fertilizer (kg ha^{-1}) (Gu et al., 2018; Hammond et al., 2009). The P budget represents the difference between P output (ginger P uptake) and P input (P fertilizer). Lower values of the P budget indicate that more P fertilizer was remained in the soil instead of being taken up by the ginger (Hu et al., 2012).

3.2.4 Statistical analysis

IBM SPSS Statistics 20 was used for statistical analysis. Normality of the measured data and homogeneity of variance were first tested using the Kolmogorov-Smirnov and Levene test ($p > 0.05$), respectively. After testing, some of the measured data were normal distributed (such as soil properties, ginger growth parameters and P use efficiency), while others were normal distributed after *log*

translation (such as soil AZO residues). Therefore, one-way analysis of variance (ANOVA) was conducted to compare the differences between each pair of treatments. Fisher's least significant difference (LSD) at $p < 0.05$ was used for significance comparison. All the figures were made using Origin 2020.

3.3 Results

3.3.1 Soil properties

The average soil pH ranged from 6.5 to 6.9 with no significant difference between CK, AZO1 and AZO2 treatments at all sampling times. However, after 9 WAP, the average values of pH for CP, CP+AZO1 and CP+AZO2 treatments were significantly lower (0.15 ~ 0.30) than in the CK treatment. SOM ranged from 16.0 ~ 17.7 mg kg^{-1} up to 9 WAP, after which it increased to 24.6 ~ 29.3 mg kg^{-1}. However, there was no significant difference between any of the treatments. The values of TP varied from 1.1 to 2.0 g kg^{-1} during the whole growth period, while no significant difference between different treatments. Up until 17 WAP, soil Olsen-P content was 79.6 to 88.8 mg kg^{-1}, except for the CP+AZO2 which dropped to 22.6 mg kg^{-1}. At 28 WAP, the value of Olsen-P increased in all treatments to 96.5~111.6 mg kg^{-1}, while TP increased to 2.0 g kg^{-1} due to the application of fertilizer at 17 WAP. The value of Olsen-P was significantly higher in CP+AZO1 (111.6 mg kg^{-1}) than in any of the other treatments (**Table S3.1**).

3.3.2 AZO residues in the soil

Soil samples from CK and CP treatments were considered to be blank samples because no AZO was added to these treatments during the entire experiment. AZO residues in soil from the other treatments were calculated by subtracting the background values of blank soil samples from the measured values of AZO from treated soil samples (**Table 3.1**). Results showed that, at 9 WAP, there was no significant difference in the average AZO residues between AZO1 (0.243 ± 0.079 mg kg^{-1}) and CP+AZO1 (0.235 ± 0.007 mg kg^{-1}). At 17 WAP, the AZO residues decreased significantly to 0.045 ± 0.007 and 0.030 ± 0.0152 mg kg^{-1} in AZO1 and CP+AZO1, respectively. The AZO residues were significantly higher in AZO2 (0.167 ± 0.088 mg kg^{-1}soil) and CP+AZO2 (0.195 ± 0.044 mg kg^{-1}soil) than in AZO1 and CP+AZO1. At 28 WAP, the AZO residues decreased significantly to LOD levels in AZO2 (0.027 ± 0.009 mg kg^{-1} soil) and CP+AZO2 (0.050 ± 0.008 mg kg^{-1} soil). However, the level of AZO residue was significantly higher in CP+AZO2 than in AZO1 (0.015 ± 0.005 mg kg^{-1} soil).

Table 3.1 Residue levels of azoxystrobin (AZO) in soil samples from different treatments (CK (untreated soil), AZO1 (A single application of AZO at 8 weeks after planting (WAP)), AZO2 (double applications of AZO at 8 and 16 WAP), CP (chloropicrin (CP) fumigated with no AZO), CP+AZO1 (CP combined with AZO1) and CP+AZO2 (CP combined with AZO2)). Data represents the means of replicates with standard errors (SE). n is the number of soil samples corresponding to the treatment. The lowercase letters (a, b and c) indicate the significant difference between treatments for each sampling period, while uppercase letters (A, B and C) indicate the significant difference between the same treatment among different sampling times (ANOVA with LSD test, p <0.05).

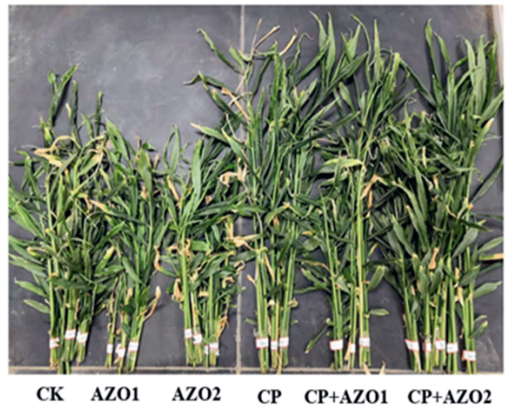
Sampling time	Treatments	AZO residue (mg kg^{-1}soil)
9 WAP	AZO1 (n=3)	0.243 ± 0.079 a (A)
	CP+AZO1 (n=3)	0.235 ± 0.007 a (A)
17 WAP	AZO1 (n=3)	0.045 ± 0.007 bc (B)
	AZO2 (n=3)	0.167 ± 0.088 ab (A)
	CP+AZO1 (n=3)	0.030 ± 0.0152 c (B)
	CP+AZO2 (n=4)	0.195 ± 0.044a (A)
28 WAP	AZO1 (n=3)	0.015 ± 0.005 b (C)
	AZO2 (n=4)	0.027 ± 0.009 ab (B)
	CP+AZO1 (n=3)	0.027 ± 0.007 ab (B)
	CP+AZO2 (n=5)	0.050 ± 0.008 a (B)

3.3.3. Ginger growth

At 28 WAP, no significant difference in the average height of ginger plants was observed among CK, AZO1, AZO2 and CP+AZO1 treatments with all shoots measuring from 47.9 ± 9.0 to 67.3 ± 9.0 cm. Ginger height was significantly higher in CP (86.0 ± 1.3 cm) and CP+AZO2 (72.1 ± 4.6 cm) than in CK (**Fig. 3.1**).

The average fresh shoot biomass was 35.4 ± 11.5 g for CK and there was no significant difference observed between CK and any of the other treatments. However, the fresh shoot biomass was

significantly lower under AZO1 treatment (21.0 ± 14.3 g) than under CP (65.1 ± 7.0 g) and CP+AZO2 (65.7 ± 13.2 g) treatments. The fresh ginger rhizome biomass showed similar variations to the shoot biomass. No significant difference was observed between CK treatment (19.6 ± 6.1 g) and any of the other treatments, while the fresh rhizome biomass was significantly lower in AZO1 (13.5 ± 6.4 g) than in CP (34.7 ± 1.6 g) and CP+AZO2 (33.0 ± 6.6 g) (**Fig. 3. 2**).



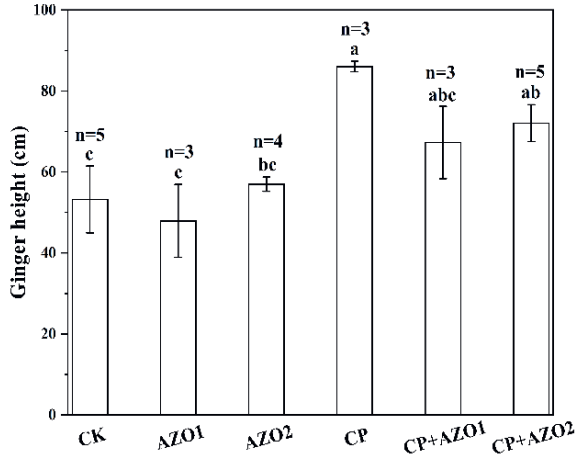


Fig. 3.1. The ginger height at 28 weeks after planting (WAP) among six treatments, including: CK (untreated soil), AZO1 (A single application of azoxystrobin (AZO) at 8 WAP), AZO2 (double applications of AZO at 8 and 16 WAP), CP (chloropicrin (CP) fumigated with no AZO), CP+AZO1 (CP combined with AZO1) and CP+AZO2 (CP combined with AZO2). Data represents the means of replicates with standard error (SE). n is the number of plant samples corresponding to the box. Lowercase letters (a, b and c) indicate significant differences between CK and other treatments (ANOVA with LSD test, $p < 0.05$).

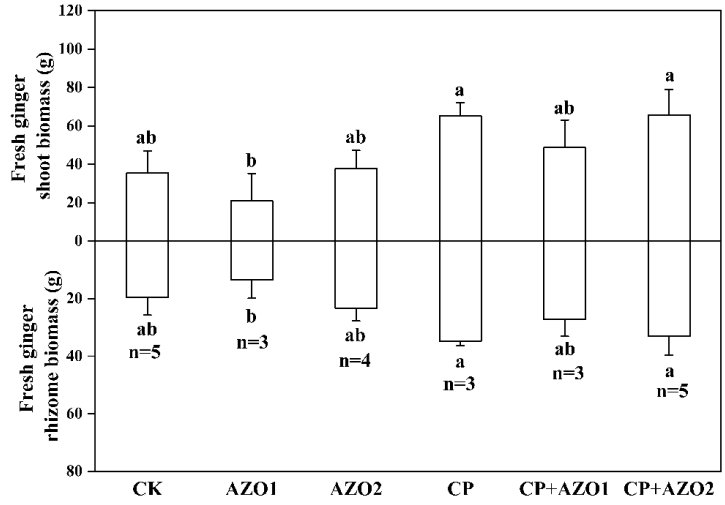


Fig. 3.2. The fresh ginger shoot and rhizome biomass 28 weeks after planting (WAP) among six treatments, including: CK (untreated soil), AZO1 (A single application of azoxystrobin (AZO) at 8 WAP), AZO2 (double applications of AZO at 8 and 16 WAP), CP (chloropicrin (CP) fumigated with no AZO), CP+AZO1 (CP combined with AZO1) and CP+AZO2 (CP combined with AZO2). Data represents the means of replicates with standard error (SE). n is the number of plant samples in the corresponding box. Lowercase letters (a and b) indicate significant differences between CK and other treatments (ANOVA with LSD test, $p < 0.05$).

3.3.4. P uptake in ginger

The amount of P in ginger shoots and roots was calculated by multiplying the P content by the total ginger shoot and root biomass, respectively. The total ginger P amount was the sum of the P amount of the ginger shoots and roots (**Fig. 3.3**). No significant difference was observed in the average shoot P amount between CK (13.5 ± 4.5 mg) and all other treatments (9.0 ± 6.4 to 26.4 ± 3.3 mg). However, the average P amount of ginger rhizome was significantly lower in CK treatment (6.1 ± 1.6 mg) than in CP+AZO2 treatment (18.3 ± 6.3 mg) (**Fig. 3.3 (A)**). The total P amount of ginger was significantly lower in CK treatment (20.0 ± 5.9 mg) than in CP (43.6 ± 5.2 mg) and CP+AZO2 (42.0 ± 9.6 mg) treatments (**Fig. 3.3 (B)**).

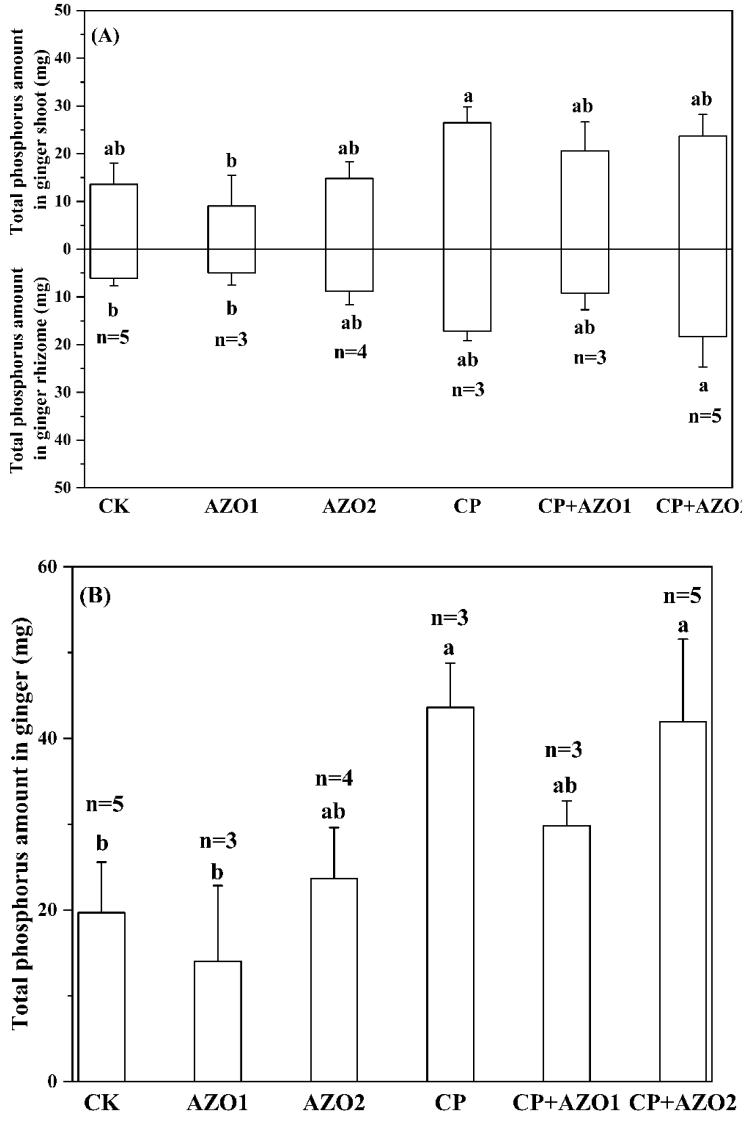


Fig. 3.3 Total phosphorus (P) amount in ginger shoots and rhizomes (A), and total P amount in ginger plant (B) after 28 weeks after planting (WAP) among the six treatments, including: CK (untreated soil), AZO1 (A single application of azoxystrobin (AZO) at 8 WAP), AZO2 (double applications of AZO at 8 and 16 WAP), CP (chloropicrin (CP) fumigated with no AZO), CP+AZO1 (CP combined with AZO1) and CP+AZO2 (CP combined with AZO2). Data represents the means of replicates with standard error (SE). n is the number of plant samples in the corresponding box. Lowercase letters (a and b) indicate significant difference between CK and other treatments (ANOVA with LSD test, p <0.05).

The P use efficiency was quantified using the three indicators shown in **Table 3.2**. The average value of partial factor productivity (PFPp) of CK was 77.6 ± 24.4 kg kg^{-1}, and there was no significant difference between different treatments. The average value of the physiological P use efficiency (PPUE) showed a decreasing trend from CK (1799.8 ± 751.6 $g^2DMg^{-1}P$) to CP+AZO2 (589.9 ± 84.8 $g^2DMg^{-1}P$), although these differences were not statistically significant. The P budget was -32.8 ± 0.8 kg ha^{-1} under CK, which was significantly lower than the values under CP (-29.4 ± 0.7 kg ha^{-1}) and CP+ZAO2 (-29.7 ± 1.4 kg ha^{-1}) treatments.

Treatments without CP fumigation (CK, AZO1 and AZO2) and with CP fumigation (CP, CP+AZO1 and CP+AZO2) were separated by the principal component analysis (PCA) (**Fig. 3.4**). The components 1 (64.5%) and 2 (14.1%) explained 78.6% of total variation in the data sets. In the PCA, CP, CP+AZO1 and CP+AZO2 treatments were positively correlated with ginger biomass, ginger P uptake and PFPp. The PPUE was negatively correlated to Olsen-P and strongly positively correlated with CK. None of the treatment was significantly correlated with Olsen-P contents.

Table 3.2. Different ginger phosphorus (P) use efficiency in six treatments including: CK (untreated soil), AZO1 (A single application of azoxystrobin (AZO) at 8 weeks after planting (WAP)), AZO2 (double applications of AZO at 8 and 16 WAP), CP (chloropicrin (CP) fumigated with no AZO), CP+AZO1 (CP combined with AZO1) and CP+AZO2 (CP combined with AZO2). PFPp (kg kg^{-1}) refers to partial factor productivity; PPUE (g^2 DM $g^{-1}P$) means the physiological P use efficiency (DM=dry matter). n is the number of plant samples corresponding to the treatment. Data represents the means of replicates with standard errors (SE). The lowercase letters (a and b) indicate the significant difference between treatments during each sampling period (ANOVA with LSD test, $p < 0.05$).

Treatments	PFP_p (kg kg^{-1})	PPUE (g^2 DM $g^{-1}P$)	P budget (kg ha^{-1})
CK (n=5)	77.6 ± 24.4 ab	1799.8 ± 751.6a	-32.8 ± 0.8b
AZO1 (n=3)	53.6 ± 25.3 b	894.5 ± 173.2a	-33.6 ± 1.3b
AZO2 (n=4)	92.5 ± 17.6 ab	714.7 ± 105.3a	-32.3 ± 0.8ab
CP (n=3)	137.8 ± 6.5 a	723.0 ± 60.9a	-29.4 ± 0.7a
CP+AZO1 (n=3)	108.2 ± 23.1ab	598.2 ± 202.3a	-31.4 ± 0.4ab
CP+AZO2 (n=5)	131.0 ± 26.1a	589.9 ± 84.8a	-29.7 ± 1.4a

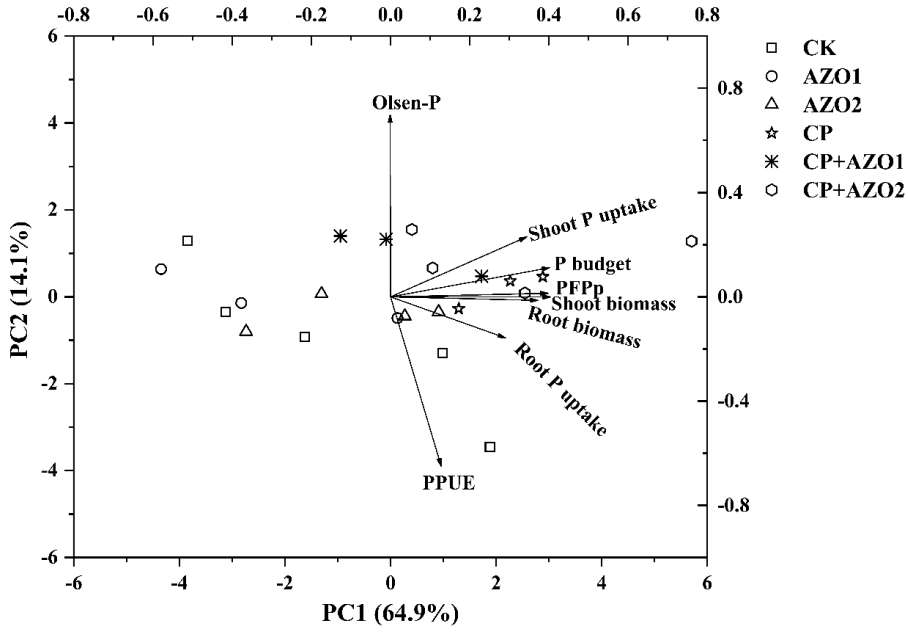


Fig. 3.4. Principal component analysis of soil Olsen-P, ginger growth indicators and ginger P use efficiency parameters for samples collected at 28 weeks after planting (WAP). Treatments: CK (untreated soil, square), AZO1 (A single application of azoxystrobin (AZO) at 8 WAP, circle), AZO2 (double applications of AZO at 8 and 16 WAP, up triangle), CP (chloropicrin (CP) fumigated with no AZO, star), CP+AZO1 (CP combined with AZO1, star (*)) and CP+AZO2 (CP combined with AZO2, Hexagon). Arrows are Olsen-P, ginger shoot and fresh root biomass, ginger shoot and root P amount, PFPp (partial factor productivity (kg kg^{-1})), PPUE (physiological P use efficiency (DM=dry matter) (g^2 DM g^{-1}P)) and P budget (difference values between total P removed by ginger plants and input P amounts).

3.4 Discussion

3.4.1 Effects of fumigation on azoxystrobin residues in the soil

This study explored the effects of the CP fumigation on soil AZO residues. The results showed that, at 9 WAP (7 days after the first application of AZO), the average AZO residues were 0.24 and 0.23 mg kg^{-1} in AZO1 and CP+AZO1, respectively, which were about 50% of the applied AZO amount (0.55 mg kg^{-1}). At 17 WAP (7 days after the second application), the AZO residues were 0.24 and 0.19 mg kg^{-1} in AZO2 and CP+AZO2, respectively, which were about 25% of the total amount of AZO applied (1.10 mg kg^{-1}). These results indicated that the degradation half-life of AZO in the soil in this experiment was about 7 days. These findings were in agreement with Saha et al. (2020) who found that the half-life of AZO was 8.4 ~ 10.0 days in farm soil under an application dose of 5.75 ~ 11.5 g m^{-2}.

However, no significant difference was found in the AZO residues between AZO1 and CP+AZO1 treatments, or AZO2 and CP+AZO2 treatments, suggesting that CP fumigation had no significant influence on AZO degradation in our study. Our results are different from the findings of Huang et al., (2019a), who concluded that CP fumigation (53 mg kg^{-1}) extended the half-life values of AZO from 52.9 to 64.2 days. The enhanced dissipation of AZO in our experiment may have been caused by the much higher temperature in our greenhouse (Purnama et al., 2015), more solarization during the summer season (Fenoll et al., 2010) and the phytoremediation by ginger plants in our experiment (Romeh, 2017) compared to the 25℃, dark incubator conditions in Huang's study. The AZO residues in CP+AZO2 were significantly higher than in AZO1 at 28 WAP, suggesting that the combined application of CP fumigation and double applications of AZO caused AZO accumulation compared to one AZO application.

3.4.2 Effects of CP fumigation and AZO application on ginger growth

In our study, regardless of whether the soil was fumigated, a single application of AZO did not cause significant changes in the height and biomass of ginger, which may be caused by the low application rate of AZO and the rapid degradation of AZO in the soil. The ginger height was significantly higher in CP and CP+AZO2 treatments than in the CK treatment. However, no significant difference was observed for the ginger shoot and rhizome biomass between CK and other treatments. Neilson et al., (2020) found similar results, that is, fumigation using CP at 64 l per hectare was effective at reducing wilt ratings, but no marketable yield was observed in potato crops. Different results were observed in a field study conducted by Mao et al. (2014), who found that fumigation with CP using 50 g m^{-2} significantly reduced the mortality of ginger by 59% and increased the ginger yields by 4.77 kg m^{-2}. Zhang, et al.(2019b) also found that soil CP fumigation using 30 g m^{-2} increased strawberry yield by 1.5 ~ 2.0 kg m^{-2}.

One of the main reasons for the difference may be the different qualities of the initial soils used in the different studies. In soils seriously infected by soil-borne pathogens, the application of CP could inhibit soil-borne pathogens effectively and improve the survival rates of crops significantly, causing significantly higher crop yields (Mao et al., 2014; Zhang et al., 2019b). However, the soil in our experiment was selected from fields that never been planted with ginger before and had no soil-borne diseases specific to ginger. The difference in soil quality between untreated and CP fumigated soil may not have been so large that it would have led to a significant difference in ginger yield. Therefore,

although the ginger plant grew taller under CP fumigation treatments, the ginger rhizome biomass was not significantly different from those collected from unfumigated treatments. The results indicate that soil fumigation and fungicide application in soils that were initially already health may be not necessary for ginger growth.

3.4.3 Effects of CP fumigation and AZO application on ginger P uptake

Ginger P amount was calculated by multiplying the P content with the ginger biomass. The results showed that the variations of ginger P amount and the partial factor productivity (PFPp) were similar to the ginger biomass, indicating that ginger biomass was an important determinant of the P uptake of the ginger plant.

The average ginger physiological P use efficiency (PPUE) values represent the ability to generate ginger rhizome yield under same rhizome tissue P levels. Higher PPUE means that more P taken up by ginger was transported to the harvestable parts of the plants (ginger rhizome), and thus, there are lower physiological P requirements and tissue contents necessary to produce the same yield (Akhtar et al., 2014). In our study, PPUE was higher under CK (1799.8 ± 751.6 g DM g^{-1}P) than CP+AZO2 (589.0 ± 84.8 g DM g^{-1}P). Although the difference was not statistically significant, the results indicate that the application of CP and AZO could decrease the ginger's physiological P use efficiency (PPUE). Although CP fumigation could increase the soil available P content at early stages (in the equivalent of seedling stage) (Huang et al., 2020a), tissue grow was primarily concentrated in the shoots (Nair, 2019), thus the lower PPUE indicates that more of the P taken up was used for shoot growth instead of rhizome growth. The decrease in the PPUE of ginger in CP-treated soil may also be attributed to the possible decrease of AMF which can increase the accessibility of soil P to ginger rhizomes (Dangi et al., 2017; Smith et al., 2011). However, the details of this mechanism require further research to be addressed.

3.5 Conclusion

In this study, we tested the effects of the application of CP and AZO on ginger growth and P uptake. We found that, whether applied individually or in combination with CP fumigation, a single application of AZO led to negligible effects on ginger growth, whereas double applications of AZO combined with CP fumigation could promote ginger growth and the P amount of ginger rhizomes. In addition, the negative P budget for all treatments indicates that the added P fertilizer was left in the soil, which may cause serious environmental problems such as eutrophication, and financial losses for farmers. The insignificant differences in P use efficiency among the treatments may be related to soil phosphorus transformation and soil microbial activity, but further research is necessary to ascertain this.

3.6 Acknowledgement

We want to thank local farmers in Anqiu, Shandong Province of China, especially Yujia Li, who kindly helped with the preparation of soil. Colleagues in Chinese Academy of Agricultural Sciences, such as Ran Li and Guoxi Wang, are highly appreciated for mesocosm setup. We also want to thank Robin Palmer and Darrell Tang (Wageningen University) for language editing. This research was supported by the National Natural Science Foundation of China (41877072, 41620104006).

Supplementary material

Table S3.1. Soil basic properties in different treatments CK (untreated soil), AZO1 (a single application of azoxystrobin (AZO) at 8 weeks after planting (WAP)), AZO2 (double applications of AZO at 8 and 16 WAP), CP (chloropicrin (CP) fumigated with no AZO), CP+AZO1 (CP combined with AZO1) and CP+AZO2 (CP combined with AZO2)); Sampling time were BP: Before planting; 9, 17 and 28 weeks after planting (WAP). n is the number of soil samples corresponding to the treatment. Data represents the means of replicates with standard deviation (SD). The lowercases indicate the significant difference between treatments during each period (ANOVA LSD test, p<0.05).

Sampling time	Treatments	pH	SOM ($g\ kg^{-1}$)	Olsen-P ($mg\ kg^{-1}$)	TP ($g\ kg^{-1}$)	TN ($g\ kg^{-1}$)
BP	CK (n=5)	6.7±0.0a	16.9±0.8a	82.5±8.1a	1.7±0.1a	1.7±0.1a
	CP (n=5)	6.7±0.1a	17.7±1.3a	83.1±5.2a	1.7±0.1a	1.6±0.1a
9WAP	CK (n=3)	6.9±0.1a	17.7±2.1a	79.9±2.0a	1.8±0.0a	1.5±0.1a
	AZO1 (n=3)	6.8±0.1a	17.2±0.9a	81.6±7.1a	1.2±0.1a	1.5±0.2a
	CP (n=5)	6.6±0.1b	16.5±0.5a	83.5±3.6a	1.3±0.0a	1.5±0.1a
	CP+AZO1 (n=5)	6.5±0.1b	16.0±0.4a	79.7±3.9a	1.2±0.0a	1.5±0.1a
17WAP	CK (n=3)	6.8±0.0a	26.7±0. 8a	81.1±5.4a	1.3±0.1a	1.6±0.1a
	AZO1 (n=3)	6.9±0.1a	25.8±4.4a	87.1±3.0a	1.2±0.0a	1.6±0.2a
	AZO2 (n=3)	6.9±0.1a	28.5±1.1a	82.0±2.7a	1.2±0.1a	1.5±0.1a
	CP (n=3)	6.6±0.1b	26.7±1.2a	84.0±2.5a	1.1±0.2a	1.4±0.1a
	CP+AZO1 (n=4)	6.7±0.1b	27.1±1.4a	88.8±3.4a	1.2±0.1a	1.4±0.1a
	CP+AZO2 (n=4)	6.7±0.1b	26.3±1.4a	22.6±1.4b	1.2±0.0a	1.5±0.1a
28WAP	CK (n=5)	6.7±0.1a	25.7±0.5a	100.2±8.2b	2.0±0.1a	1.5±0.1a
	AZO1 (n=4)	6.6±0.1a	29.3±6.7a	101.3±2.9b	2.0±0.1a	1.7±0.1a
	AZO2 (n=4)	6.6±0.1a	28.8±5.0a	96.5±4.5b	1.9±0.0a	1.6±0.1a
	CP (n=3)	6.5±0.0b	24.6±1.5a	102.0±1.8b	2.0±0.1a	1.6±0.1a
	CP+AZO1 (n=4)	6.5±0.2b	24.8±1.4a	111.6±2.8a	1.9±0.0a	1.5±0.0a
	CP+AZO2 (n=5)	6.5±0.1b	27.3±4.4a	102.9±9.4b	1.9±0.1a	1.5±0.1a

[a] pH (H_2O): soil: ultra-pure water with a ratio of 1:2.5; [b] SOM: soil organic matter ($g\ kg^{-1}$), colorimetric method after H_2SO_4-K_2CrO_7 oxidation); [c] Soil Olsen-P was decided colorimetrically using the molybdate blue method after extraction with 0.5 mol L^{-1} $NaHCO_3$ at pH 8.5 for 30 mins; [d] TP: Total phosphorus ($g\ kg^{-1}$) , analyzed by inductively coupled plasma-optical emission spectroscopy (ICP-OES) after HNO_3-HF-H_2O_2 digestion at 180℃ using a MARS 5 Xpress microwave system (CEM, USA); [e] TN: Total nitrogen ($g\ kg^{-1}$), measured using an elemental analyzer.

Table S3.2. Linearity parameters obtained by High Performance Liquid Chromatography analysis with variable wavelength UV detector (HPLC-VWD) analysis of azoxystrobin standard solution

Substance	Calibration levels	Slope	Intercept	Correlation coefficient	Residual st.dev.Sy/x	Concentration range (µg mL^{-1})
Azoxystrobin	8	70.038	3.8055	0.9997	0.15	0-10

Table S3.3. The average recovery (%) of the spike and the matrix effect. RSD: Relative standard deviation of the recovery; LOD: Limit of detection; LOQ: Limit of quantification.

Substance	Fortification level (mg kg^{-1})	Recovery (%)	RSD (%)	Matrix effects	LOD (mg kg^{-1})	LOQ (mg kg^{-1})
Azoxystrobin	0.1	69.2	6.0	-16.4%	0.01	0.04
	0.5	83.6	7.5			

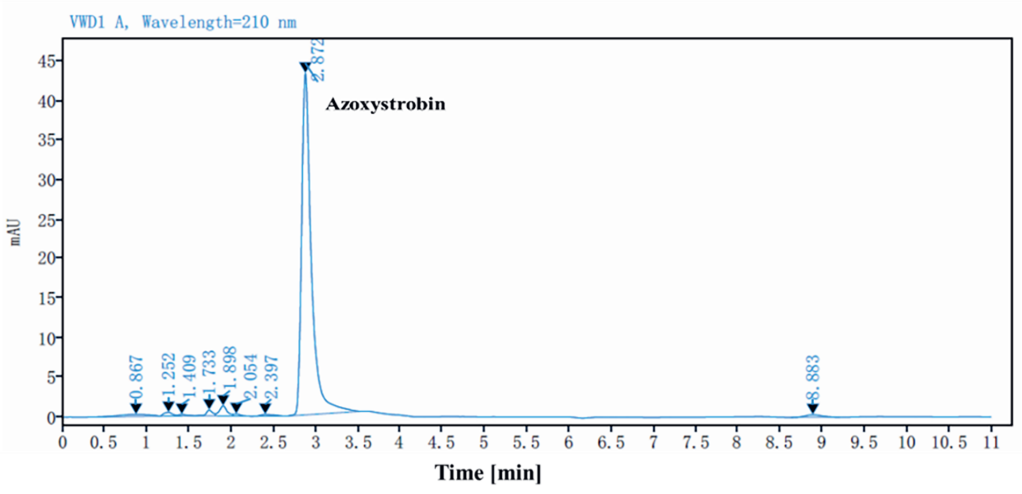


Fig. S3.1. High Performance Liquid Chromatography analysis with variable wavelength UV detector (HPLC-VWD) chromatogram of azoxystrobin standard solution at a concentration level of 5.0 µg mL^{-1}.

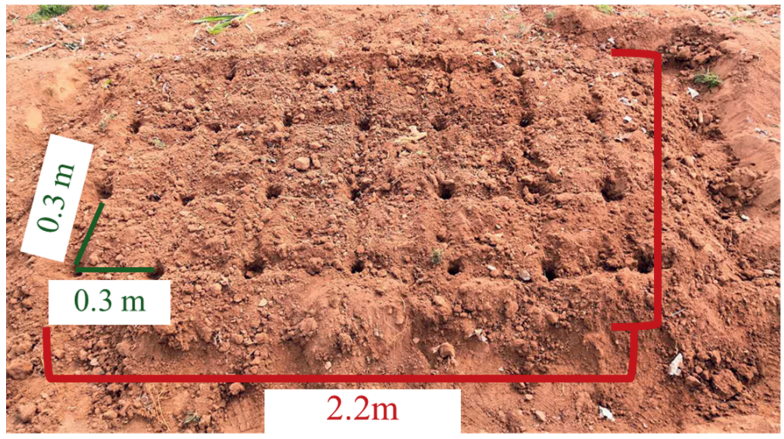


Fig. S3.2. On-site soil fumigation setup

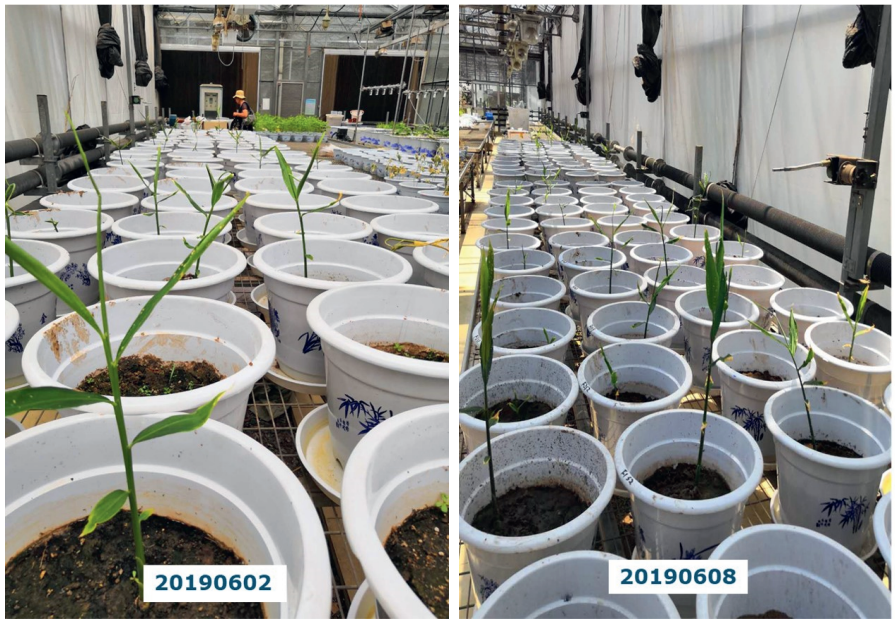


Fig. S3.3. Ginger growth condition at the greenhouse experiment

Autumn

Chapter 4

Response of phosphatase activity and soil phosphorus fractions to the application of chloropicrin and azoxystrobin in ginger cultivation

Abstract: *Although fumigants and fungicides are widely used to control soil-borne pathogens, they can also change the cycling processes of soil nutrients (such as nitrogen and phosphorus) by affecting soil beneficial microorganisms, which is a key issue for soil fertility. However, the effects of combined application of fumigants and fungicides on soil phosphorus (P) availability remains largely unclear. We investigated the effects of chloropicrin (CP) fumigation and azoxystrobin (AZO) application on soil P phosphatase activity and soil P fractions in ginger production during a 28-week pot experiment using six treatments: CK (control), AZO1 (a single application of AZO), AZO2 (double applications of AZO), CP (CP fumigated soil without AZO), CP+AZO1 (CP combined with AZO1) and CP+AZO2 (CP combined with AZO2).Results showed that a single application of AZO did not affect soil phosphatase activity and the composition of soil P fractions regardless of with or without CP fumigation. CP fumigation significantly reduced soil phosphatase activity, but increased the proportions of soil available P (Resin-P, $NaHCO_3$-Pi and $NaHCO_3$-Po) to total P (TP) by 9.0% ~ 15.5%. The combined application of CP and AZO did not produce any synergistic effects on the soil P availability. The lower soil phosphatase activity in CP-fumigated soil indicated that soil CP fumigation is not a sustainable way to improve soil P availability in the long run. The soil microbial activities, especially microorganisms related to P cycling, may be responsible for the variations of soil P availability, but further research is needed.*

Based on:

Wang Y, Yang X, Xu M, Geissen V (2022) Response of phosphatase activity and soil phosphorus fractions to the application of chloropicrin and azoxystrobin in ginger cultivation, plant and soil, under review.

4.1 Introduction

Soil fumigation has increased steadily over the last decades, driven by the demand for control crop diseases caused by soil-borne pathogens (Jiang et al., 2018) and achieve a high crop yield. For example, Ślusarski et al. (2016) found that chloropicrin (CP), applied using drip irrigation at 40 g m^{-2}, reduced the inoculum density of *Verticillium dahlia* in the soil by 86% and improved the average pepper marketable yields by 10.9% to 90.0% in different trials. In addition to the soil fumigation before planting, other fungicides such as azoxystrobin (AZO) are also applied during crop growth to enhance the inhibitory effects on soil-borne diseases such as ginger wilt and rot diseases by inhibiting respiration in fungal mitochondria (Feng et al., 2020; Huang et al., 2019b; Zhang et al., 2020a).

However, due to its broad antimicrobial activity, CP is indiscriminately poisonous to all organisms not only targeted harmful pests, pathogens, and nematodes, but also beneficial soil microorganisms (Li et al., 2017), which may negatively affect the associated nutrient cycling processes. Previous studies have found that CP fumigation could significantly inhibit the nitrification processes in various soils (Li et al., 2017; Sun et al., 2018b; Yan et al., 2017b), but significantly increased the amount of soil available phosphorus (P) and leached P by altering the structure of the microbial community encoding the alkaline phosphatase *phoD* gene (Huang et al., 2020). AZO fungicide was also found to have detrimental effects on non-target functional microorganisms and their governed enzyme activities such as soil urease, invertase and phosphatase (Adak et al., 2019; Wang et al., 2018). However, up to now, the combined effects of CP fumigation and AZO application on soil phosphatase activity and the governed soil P cycling process still remains largely unclear due to the great diversity and non-specific existence forms of soil P.

Soil P exists in different chemical forms including inorganic P (Pi) and organic P (Po), which determines the soil P availability for plant uptake (Weihrauch et al., 2018). According to the solubility in the soil solution and the availability for the plant uptake, soil P is interpreted by Hedley's sequential extraction methods as Resin-P (Easily available P), $NaHCO_3$-P ($NaHCO_3$-Pi and $NaHCO_3$-Po: Labile P), NaOH-P (NaOH-Pi and NaOH-Po: Moderately labile P), and Occluded P (HCl-Pi and residual P: Unavailable P) (Hedley et al., 1982). Only dissolved inorganic P fractions in the soil solution such as Resin-P can be taken up by plants directly. Other insoluble inorganic P fractions such as $NaHCO_3$-Pi and NaOH-Pi need to be solubilized by organic acids, while insoluble organic P factions such $NaHCO_3$-Po and NaOH-Po need to be mineralized by phosphatase, before being taken up by plants (Fan et al., 2019; Koch et al., 2018). Acid phos phatase (AiP, EC3.1.3.2) and alkaline phosphatase (AlP, EC3.1.3.1) are two important phosphomonoesterases that can hydrolyze simple phosphate monoesters ($NaHCO_3$-Po and NaOH-Po) into orthophosphate (PO_4^{3-}) which can be uptaken by the plants (Fraser et al., 2017). Accordingly, CP fumigation and AZO application may potentially alter the soil P fractions either by killing soil microbes and releasing the phosphorus inside microorganisms (Achat et al., 2012), or by changing the composition of soil P solubilizing microorganisms (PSMs) such as *Pseudomonas, Bacillus, Rhizobium*, and *Enterobacter* and fungi like *Penicillium* and *Aspergillus* (Huang et al., 2020a; 2020b; Pecina et al., 2016; Zhang et al., 2019b) which can secret organic acids to dissolve Pi and produce phosphatase to mineralize Po (Wang et al., 2011). Therefore, understanding the composition of different soil P fractions and phosphatase activity is a key part of understanding microbial functions in soil contaminated by fumigants and fungicides.

Nonetheless, most previous studies focused only on the individual fumigant or fungicide application in the soil, ignoring the fact that fumigants and other fungicides coexist in real agricultural systems (Han et al., 2020). Some studies have found that CP fumigation could decrease the adsorption of AZO fungicide on soil particles and extend the degradation time of AZO (Huang et al., 2019b). Soil microbiome recovery after initial soil fumigation may be affected by AZO application during crop growth, resulting in different effects on soil P cycling. In addition, our previous study found that only CP fumigation combined with double application of AZO increased the ginger height and root P uptake, while a single application of AZO did not affect the ginger growth regardless of with or with CP fumigation (Wang et al., 2022). Therefore, in this study, we want to go further in exploring the variations of soil phosphatase activity and soil P fractions under the combined application of CP fumigant and AZO fungicide and try to answer whether CP and AZO application could change the soil P availability. We hypothesize that CP and AZO can increase the soil available P contents, and the dual application of AZO and the combined application of CP and AZO can enhance this effect.

4.2 Material and methods

4.2.1 Experiment materials and design

From March to October of 2019, a greenhouse experiment was conducted at the Chinese Academy of Agricultural Sciences, Beijing, China. The details of the experiment were described in our previous study (Y. Wang et al., 2022) and summarized in **Table 4.1**. Briefly, before the experiment, CP (Dalian Lvfeng Chemical Co. Ltd.) was injected at 37.1 g m^{-2} in a field (1.2 m × 2.2 m) that had never been CP fumigated before. After CP fumigation, the top soils (0-20 cm) of the CP-fumigated field and an adjacent field without CP fumigation were taken into the greenhouse for the next step. In the greenhouse, ginger (*Zingiber officinale*) was used as the model plant. 6 kg of soil and 100 g of germinated ginger rhizome were put into each pot (diameter 30 cm, height 25 cm), making 90 pots in total including six treatments with five replicates of each treatment made for every sampling time (**Table 4.1**). Treatments include: CK (control), AZO1 (a single application of AZO), AZO2 (double applications of AZO), CP (CP fumigated soil without AZO), CP+AZO1 (CP combined with AZO1) and CP+AZO2 (CP combined with AZO2). For AZO1 and CP+AZO1, AZO (Hebei Zhongbaolv Crop Technology Company) was applied at 8 weeks after planting (WAP) using a suspension spray method, while for AZO2 and CP+AZO2, AZO was applied again at 16 WAP. Each application amount of AZO was 47.1 mg m^{-2}.

The soil was destructively sampled four times during the different growth periods of ginger, including BP: before planting (12th of April); 9 WAP (seedling stage, 17th of June); 17 WAP (flourishing growing stage, 10th of August) and 28 WAP (harvest stage, 15th of October). After the 17 WAP sampling, 5.0 g (about 70.8 g m^{-2}) of compound chemical fertilizer (N-P-K: 16–12–2; N ≥ 16%, P_2O_5 ≥ 12%, K_2O ≥ 20%) was applied to each pot.

For the soil samples, one part was air-dried and sieved to 2 mm for the analysis of basic soil properties using the methods described in (Y. Wang et al., 2022), and to 0.25 mm for the analysis of soil P fractions. Another part was directly sieved to 2 mm and stored at 4℃ for phosphatase activity analysis.

Table 4.1. Chloropicrin, azoxystrobin and fertilizer application. Treatments include: CK (control), AZO1 (a single application of AZO), AZO2 (double applications of AZO), CP (CP fumigated soil without AZO), CP+AZO1 (CP combined with AZO1) and CP+AZO2 (CP combined with AZO2). WAP: weeks after planting.

Treatments	Chloropicrin ($g\ m^{-2}$)	Azoxystrobin ($mg\ m^{-2}$)		Compound chemical fertilizer (N ≥ 16%, P_2O_5 ≥ 12%, K_2O ≥ 20%, g)
	(28^{th}, Mar.)	8 WAP (08^{th}, Jun.)	16 WAP (29^{th}, Jul.)	17 WAP (11^{th}, Aug.)
CK	-	-	-	5.0
AZO1	-	47.1	-	5.0
AZO2	-	47.1	47.1	5.0
CP	37.1	-	-	5.0
CP+AZO1	37.1	47.1	-	5.0
CP+AZO2	37.1	47.1	47.1	5.0

4.2.2 Soil phosphatase activity

Soil acid (AiP) and alkaline (AlP) phosphatase activity was measured using the method modified by Tabatabai (Tabatabai, 1969). 1.0 g of a fresh soil sample (< 2 mm) was incubated with 0.2 mL of toluene, 1.0 mL of 0.05 M *p*-nitrophenyl phosphate and 4.0 mL of modified universal buffer (pH 6.5 for acid phosphatase and pH 11 for alkaline phosphatase) in a 50 mL centrifuge tube at 37℃ for 1 h. After 1 h, the reaction was ended by adding 1.0 mL of 0.5 M $CaCl_2$ and 4.0 mL of 0.5 M NaOH solution. The mixture was then filtrated and the absorbance of the filtrate at 410 nm was measured using a spectrophotometer. The concentration of *p*-nitrophenol in soil extract was calculated according to the *p*-nitrophenol standard curve and the phosphatase activity was quantified by the amount of *p*-nitrophenol produced per soil per hour (mg *p*-nitrophenol $g^{-1}\ h^{-1}$).

4.2.3 Soil phosphorus fractions

A modified method described by Tiessen & Moir, (2006) and Hedley et al., (1982) was used to fractionate the soil P. Briefly, 0.5 g of air-dried soil (< 0.25 mm) was added into a 50 mL centrifuge tube and extracted by the following sequential order: 1) 30 mL of ultra-pure water with two anion exchange resin membrane strips (1 cm × 2 cm) converted to the bicarbonate form (Resin-P, easily available P); 2) 30 mL of 0.5 M $NaHCO_3$ solution ($NaHCO_3$-Pi+$NaHCO_3$-Po, labile P); and 3) 30 mL of 0.1 M NaOH (NaOH-Pi and NaOH-Po, moderately labile P). For each extraction step, the mixture of soil and extractant was shaken at 25℃ for 16 h (180 rpm). The soil suspension was then centrifuged at 10000 g at 0℃ for 10 minutes and decanted. Inorganic P fractions (Pi) in each extract were measured at 700 nm using the molybdate ion colorimetry method (Costa et al., 2016; Zhou et al., 2018). Total P (TP) in the extracts was determined using inductively coupled plasma-optical emission spectroscopy (ICP-OES) while the organic P fractions (Po) were calculated as the difference between TP and Pi. The concentration of Occluded P was estimated by subtracting the sum of the total of other P fractions from the total P concentrations of samples (Koch et al., 2018).

4.2.4 Statistical analysis

The ratio of the sum of Resin-P, $NaHCO_3$-Pi and NaOH-Pi to the sum of $NaHCO_3$-Po and NaOH-Po (Pi/Po = (Resin-P +$NaHCO_3$-Pi+NaOH-Pi)/($NaHCO_3$-Po+NaOH-Po)) were calculated to evaluate the transformation between these plant inorganic and organic P fractions.

The statistical analysis was performed using IBM SPSS Statistic 20. Normality and homogeneity of variance of the measured data were tested using the Kolmogorov-Smirnov and Levene test ($p > 0.05$). The non-parametric Kruskal-Wallis analysis was used for the variance of Pi/Po and the Wilcoxon test was applied to compare the differences between each pair of treatments. For the normally distributed values (soil P fraction contents and proportions, phosphatase activity), one-way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) at $p < 0.05$ were used for significance comparison among treatments.

Random forest (RF) analyses were conducted to predict the importance of different soil properties (explanatory variables, mainly as TP (total P), AP (available P), SOM (soil organic matter), pH, AiP and AlP) on each of the soil P fractions (response variables) using the "*randomForest*" package in RStudio. Redundancy analysis (RDA) was also performed to establish the relationship among P fractions (response variables) and soil properties (explanatory variables) using Origin 2020. All of the figures were made using Origin 2020.

4.3 Results

4.3.1 Soil phosphatase activity

The variation of soil AiP and AlP activity among different treatments and sampling times are shown in **Fig. 4.1**. The AiP activity was significantly lower in CP, CP+AZO1 and CP+AZO2 (27.1 ~ 35.5 mg *p*-nitrophenol g^{-1} h^{-1}) as compared to the activity in the CK (38.3 ~ 44.2 mg *p*-nitrophenol g^{-1} h^{-1}) during the whole experiment. There was no significant difference in the average AiP activity among CK, AZO1 and AZO2 until 17 WAP. At 28 WAP, the average AiP activity was significantly lower in AZO1 and AZO2 (35.3 ~ 36.7 mg *p*-nitrophenol g^{-1} h^{-1}) as compared to that in CK (39.8 mg *p*-nitrophenol g^{-1} h^{-1}). In CP-fumigated soil, no significant difference in the average AiP activity was observed between CP and CP+AZO1, while average AiP activity in CP+AZO2 (32.3 ~ 34.3 mg *p*-nitrophenol g^{-1} h^{-1}) was significantly higher than that in the CP (28.4 ~ 29.4 mg *p*-nitrophenol g^{-1} h^{-1}) from 17 WAP onwards (**Fig. 4.1 (A)**).

The average AlP activity showed similar variations as the average AiP activity (**Fig. 4.1 (B)**) with significantly lower values in CP, CP+AZO1 and CP+AZO2 (17.1 ~ 26.8 mg *p*-nitrophenol g^{-1} h^{-1}) than that in CK (22.7 ~ 33.2 mg *p*-nitrophenol g^{-1} h^{-1}) during the whole experiment. No significant difference was observed in the average AlP activity between CK, AZO1 and AZO2 until 28 WAP when the average AlP activity in the AZO2 (21.4 mg *p*-nitrophenol g^{-1} h^{-1}) was significantly lower than that in CK (22.7 mg *p*-nitrophenol g^{-1} h^{-1}). However, in CP-fumigated soil, the average AlP activity in CP+AZO2 (18.7 ~ 26.8 mg *p*-nitrophenol g^{-1} h^{-1}) was significantly higher than that in CP (17.1 ~ 22.1 mg *p*-nitrophenol g^{-1} h^{-1}) from 17 WAP onwards. There was still no significant difference between CP and CP+AZO1 treatments.

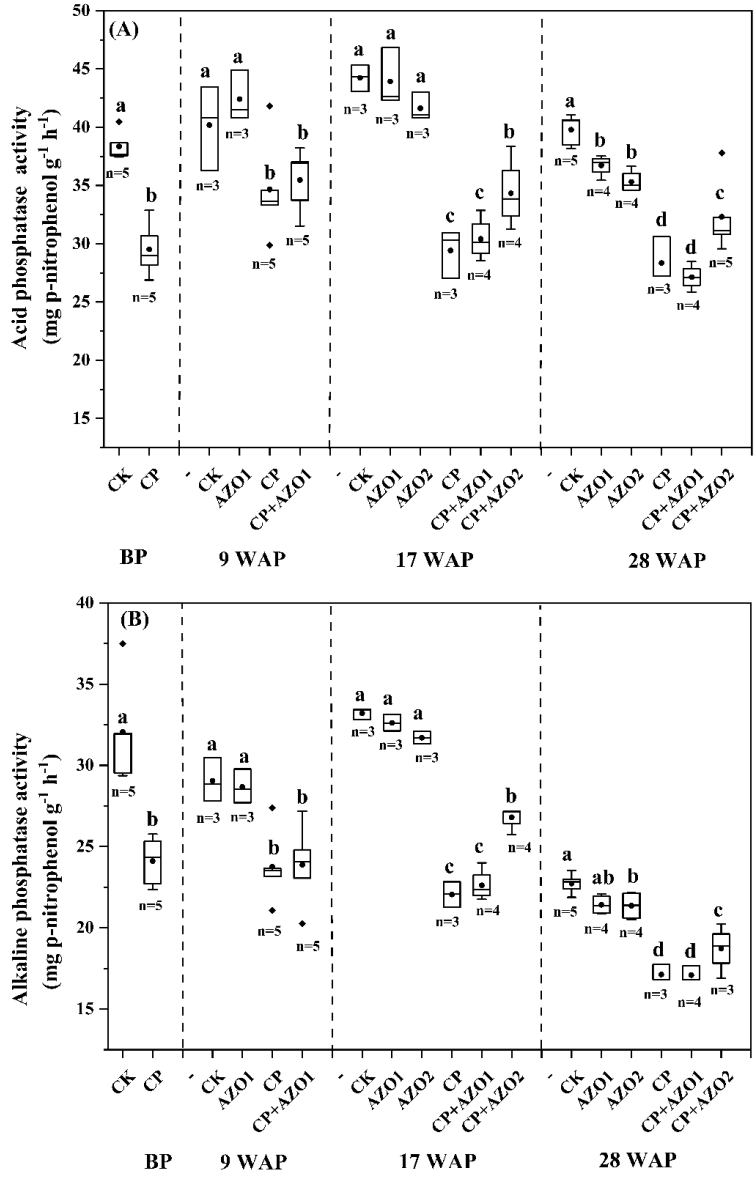


Fig. 4.1 Soil acid (A) and alkaline (B) phosphatase activity in different treatments and sampling times. Treatments include CK (control), AZO1 (a single application of AZO), AZO2 (double applications of AZO), CP (CP fumigated soil without AZO), CP+AZO1 (CP combined with AZO1) and CP+AZO2 (CP combined with AZO2). Sample times were: Before planting; 9, 17 and 28 weeks after planting (BP, 9 WAP, 17 WAP and 28 WAP). Columns represent the mean values of replicates with standard deviation (SD). n is the actual number of samples from the corresponding column. The lowercase letters indicate the significant difference between treatments during each sampling time (ANOVA with LSD test, $p < 0.05$).

4.3.2 Soil P fractions

The values of TP were from 1.1 to 2.0 g kg^{-1} during the whole experiment and no significant difference was observed between different treatments (**Table S3.1**). Except for the content of $NaHCO_3$-Po at 17 WAP, no significant difference was detected between different treatments with respect to the different P fraction contents. For samples collected at 17 WAP, the content of $NaHCO_3$-Po was significantly higher in CP+AZO2 (161.0 mg kg^{-1}) than that in CK (27.0 mg kg^{-1}) (**Fig. S4.1**).

The proportions of each P fraction to TP are shown in **Fig. 4.2**, and there was no significant difference between CK and CP before fumigation (BP). For the samples collected at 9 WAP, the proportions of Resin-P, $NaHCO_3$-Pi and NaOH-Pi to TP were significantly higher in AZO1, CP and CP+AZO1 (Resin-P: 11.0% ~ 12.0% of TP; $NaHCO_3$-Pi: 8.3% ~ 9.2% of TP; NaOH-Pi: 9.3% ~ 9.7% of TP) as compared to that in CK (Resin-P: 8.0% of TP; $NaHCO_3$-Pi: 6.0% of TP; NaOH-Pi: 6.5% of TP). The proportions of $NaHCO_3$-Po to TP were significantly higher in CP (14.7% of TP) and CP+AZO1 (15.2% of TP) than that in CK (6.5% of TP). No significant difference in the proportions of NaOH-Po to TP was observed between different treatments (19.2% ~ 23.2% of TP).

At 17 WAP, no significant difference in the proportions of all P fractions to TP was measured between CK, AZO1 and AZO2. The proportions of Resin-P, $NaHCO_3$-Pi and $NaHCO_3$-Po to TP were significantly higher in CP, CP+AZO1 and CP+AZO2 (Resin-P: 12.0% ~ 12.7% of TP; $NaHCO_3$-Pi: 9.8% ~ 10.2% of TP; $NaHCO_3$-Po: 8.7% ~ 13.8% of TP) than that in CK (Resin-P: 10.7% of TP; $NaHCO_3$-Pi: 8.3% of TP; $NaHCO_3$-Po: 2.0% of TP). No significant difference in the proportions of NaOH-Pi to TP was observed between CK (9.0% of TP) and all of the other treatments except for that in the CP (11.0% of TP). There was no significant difference in the proportions of NaOH-Po to TP between all of the treatments (24.0% ~ 26.3% of TP). The soil P fraction composition changed dramatically from 17 WAP to 28 WAP due to the application of chemical fertilizer. However, only the proportions of $NaHCO_3$-Po to TP were significantly higher in CP+AZO1 (4.1% of TP) and CP+AZO2 (4.6% of TP) than that in the CK (0.3% of TP). Until 17 WAP, the Pi/Po ratio was about 1.0 in all treatments. At 28 WAP, the Pi/Po values increased to 4.5 in the CK which was significantly higher than in AZO2 (3.0), CP (2.7), CP+AZO1(2.5) and CP+AZO2 (2.3) (**Fig. 4.3**).

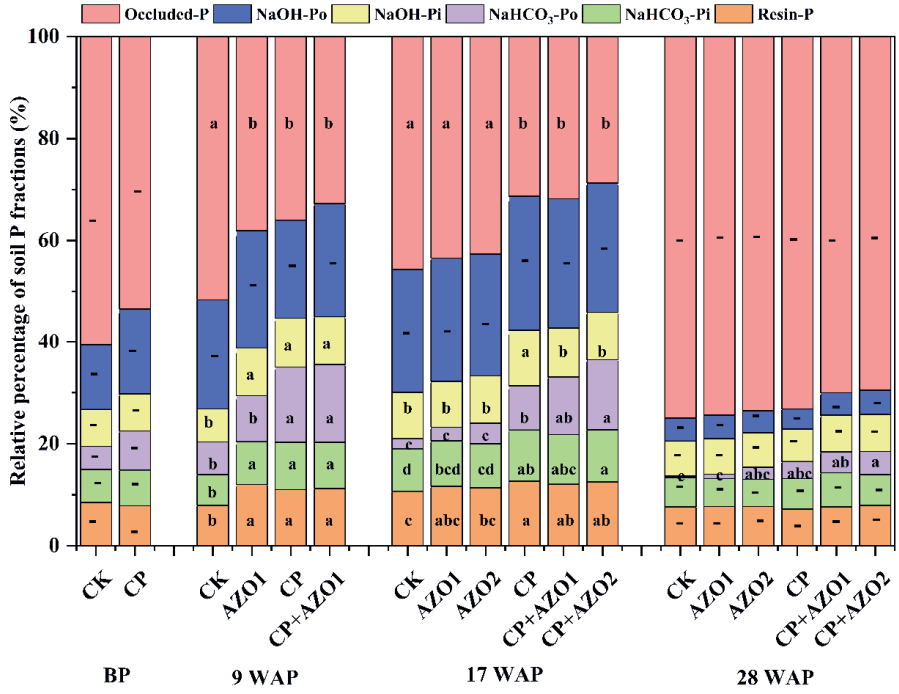


Fig. 4.2 Relative percentages of soil P fractions in different treatments and sampling times. Treatments include CK (control), AZO1 (a single application of AZO), AZO2 (double applications of AZO), CP (CP fumigated soil without AZO), CP+AZO1 (CP combined with AZO1) and CP+AZO2 (CP combined with AZO2). Sample times were Before planting; 9, 17 and 28 weeks after planting (BP, 9 WAP, 17 WAP and 28 WAP). Resin-P (orange, inorganic P fractions extracted by water), $NaHCO_3$-Pi (green, inorganic P fractions extracted by 0.5 M $NaHCO_3$ solution), $NaHCO_3$-Po (purple, organic P fractions extracted by 0.5 M $NaHCO_3$ solution), NaOH-Pi (yellow, inorganic P fractions extracted by 0.5 M NaOH solution), NaOH-Po (blue, organic P fractions extracted by 0.5 M NaOH solution) and Occluded-P (red, unavailable P). Columns represent the mean values of replicates. The lowercase letters (a. b. c and d) indicate the significant difference in each P fraction between treatments during each sampling time. '-' means no significant differences (ANOVA with LSD test, $p < 0.05$)

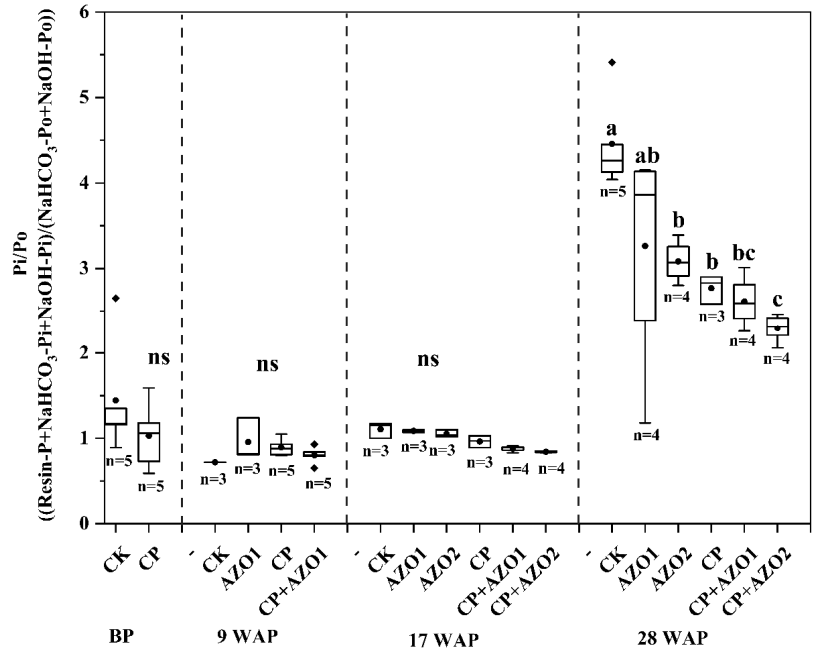


Fig. 4.3 The ratio of labile Pi (Resin-P+$NaHCO_3$-Pi+NaOH-Pi) to Po ($NaHCO_3$-Po+NaOH-Po) among different treatments for each sampling time. Pi: inorganic P; Po: organic P; Resin-P, $NaHCO_3$-P and NaOH-P are P fractions extracted sequentially using water, 0.5 M $NaHCO_3$ solution and 0.5 M NaOH solution. Treatments include CK (control), AZO1 (a single application of AZO), AZO2 (double applications of AZO), CP (CP fumigated soil without AZO), CP+AZO1 (CP combined with AZO1) and CP+AZO2 (CP combined with AZO2). Sample times were Before planting; 9, 17 and 28 weeks after planting (BP, 9 WAP, 17 WAP and 28 WAP). n is the actual number of samples from the corresponding boxes. Lowercase letters (a, b and c) indicate significant differences between CK and other treatments for each sampling, while 'ns' means no significant differences (Wilcoxon test, $p < 0.05$)

4.3.3 Soil P fractions in relation to phosphatase activity and soil properties

Based on the data collected during the whole experiment, random forest analysis was used to calculate the importance of selected soil chemical properties (TP, AP, pH, SOM, **Table S3.1**), and soil AiP/AlP activity on the proportions of different soil P fraction (**Fig. 4.4**). The larger values of **Increase of mean square error (MSE)** indicate the greater importance. TP was ranked the most important for soil P fractions, followed by AP, except for $NaHCO_3$-Po. In addition to TP and AP, other soil properties were found to be of different importance for different soil P fractions. For the organic P fractions, SOM (16.0 ~ 29.3 mg kg^{-1}) was the most important influencing factor for $NaHCO_3$-Po (19.8% of increase of MSE), followed by AiP, AlP and pH (6.5 ~ 6.9) which were of similar importance (11.9% ~ 13.8% of increased of MSE). AlP was of the highest importance for NaOH-Po (14.3% of increase of MSE), while AiP was the least important (0.8% of increase of MSE).

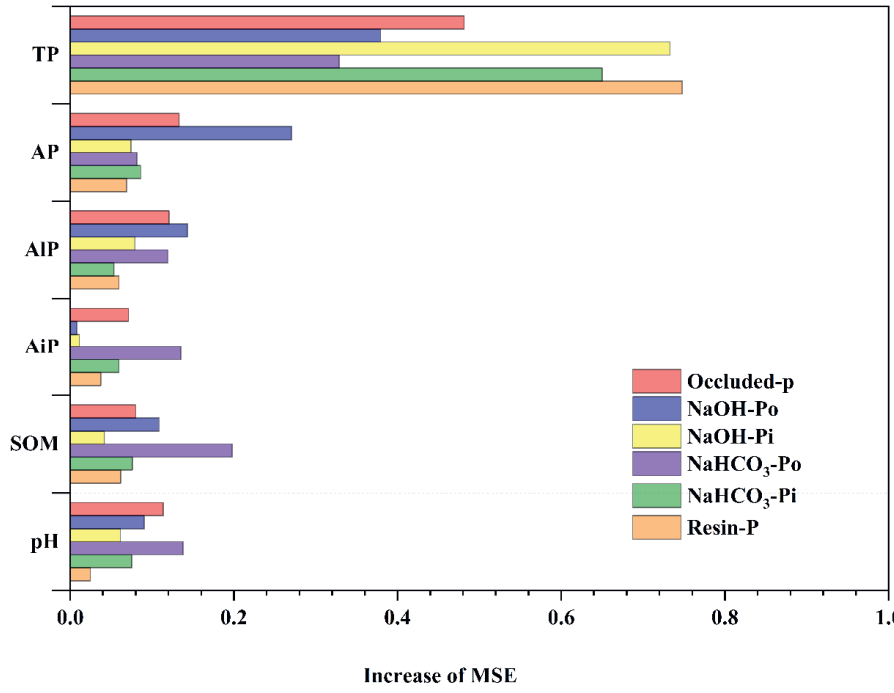


Fig. 4.4 Random Forest mean predictor importance (percentage of increase of mean square error, MSE) of major soil properties as drivers of the soil P fractions. The variables are shown in importance descending order. An increase of MSE indicates the contribution to RF prediction accuracy for the variable. Soil properties include TP (total phosphorus), AP (available phosphorus), AlP (alkaline phosphatase), AiP (acid phosphatase), SOM (soil organic matters), and pH. Legend indicates the soil P fractions, including Resin-P (orange, inorganic P fractions extracted by water), $NaHCO_3$-Pi (green, inorganic P fractions extracted by 0.5 M $NaHCO_3$ solution), $NaHCO_3$-Po (purple, organic P fractions extracted by 0.5 M $NaHCO_3$ solution), NaOH-Pi (yellow, inorganic P fractions extracted by 0.5 M NaOH solution), NaOH-Po (blue, organic P fractions extracted by 0.5M NaOH solution) and Occluded-P (red, unavailable P).

Nonetheless, selected soil properties showed less influence on soil inorganic P fractions than organic P fractions. pH and AlP were considered to be the most important factors for NaOH-Pi (6.1% and 7.9% of increase of MSE) and Occluded-P (11.4% and 12.1% of increase of MSE), while SOM and pH were the most important for $NaHCO_3$-Pi (7.5% of increase of MSE). All selected soil properties had much less important impacts on Resin-P (< 7% of increase of MSE).

The results of redundancy analysis (RDA) showed that the first two principal components explained more than 89% of the variations of soil P fraction, with 86.86% in the first axis and 2.32% in the second axis (**Fig. 4.5**). For samples collected at the same time, distinct separation was observed between no CP-fumigated soils (CK, AZO1 and AZO2) and CP-fumigated soils (CP, CP+AZO1 and CP+AZO2). Along RDA1, soil TP, AP and SOM showed highly positive relationships with Occluded-P, and negative relationships

with NaOH-Po. Along RDA2, higher values of $NaHCO_3$-Po appeared in CP-fumigated soil (CP, CP+AZO1 and CP+AZO2), which were negatively related to soil pH, AiP and AlP activities. The inorganic P fractions (Resin-P, $NaHCO_3$-Pi and NaOH-Pi) did not show significant differences among treatments and sampling time.

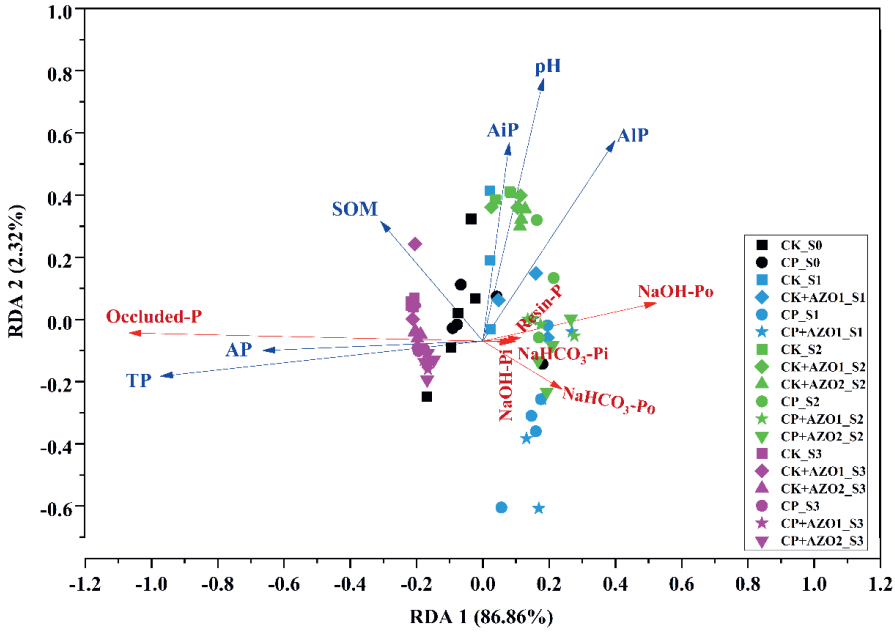


Fig. 4.5 Redundancy analysis (RDA) of P fractions and soil basic properties. Symbols with the same shape were samples of the same treatment, including CK (control), AZO1 (a single application of AZO), AZO2 (double applications of AZO), CP (CP fumigated soil without AZO), CP+AZO1 (CP combined with AZO1) and CP+AZO2 (CP combined with AZO2). Symbols with the same color represent samples of the same sampling time, including S0: Before planting; S1, S2 and S3 were 9,17 and 28 weeks after planting. The red arrows were different soil P fractions including Resin-P (inorganic P fractions extracted by water), $NaHCO_3$-Pi (inorganic P fractions extracted by 0.5 M $NaHCO_3$ solution), $NaHCO_3$-Po (organic P fractions extracted by 0.5 M $NaHCO_3$ solution), NaOH-Pi (inorganic P fractions extracted by 0.5 M NaOH solution), NaOH-Po (organic P fractions extracted by 0.5 M NaOH solution) and Occluded-P (unavailable P). Blue arrows were soil properties, including pH, SOM (soil organic matter), AiP (acid phosphatase), AlP (alkaline phosphatase), AP (available phosphorus), TP (total phosphorus). The position and length of arrows indicated the direction and strengths of the effects of soil properties on P fractions.

4.4 Discussion

4.4.1 Soil phosphatase activity

In this study, we found that AZO application did not affect soil phosphatase activity before 17 WAP regardless of whether the soil was CP fumigated or not, which may be caused by the low application rate of AZO (47.1 mg m^{-2}). This result was also in line with a previous study conducted by Wang et al. (2019) who found that the application of AZO at a rate of 2.0 mg kg^{-1} had no significant effect on the phosphatase activity on the 7th day of incubation in Spodosols. Similarly, in our study, soil samples were collected one week after the AZO application. The short period between application and sampling could be another reason why no significant difference was seen. In addition, for samples collected at 28 WAP, compared with CK, the AiP activity of AZO1 and AZO2 treatment was significantly reduced, which confirmed that AZO may take a longer time to show its effect on phosphatase activity.

CP fumigation significantly inhibited the AiP and AlP activities, which was also consistent with other studies. For example, a study conducted by Huang et al. (2020a) also found that, compared with CK, AlP activity in CP-fumigated soil (application amount was 53 mg kg^{-1}) was significantly lower by 32.2%. We also found that, compared with AZO1 and AZO2, the phosphatase activity was significantly lower in CP+AZO1 and CP+AZO2, which may be mainly due to the strong inhibitory effect of CP. No significant difference in the soil phosphatase activity between CP and CP+AZO1 treatment was observed. However, compared with the values in CP, the soil phosphatase activity was significantly higher in CP+AZO2, which may be due to the changes in soil microbes. Previous studies have shown that CP fumigation increased the abundance of *Bacillus sp.* that can degrade AZO and use AZO as carbon and nitrogen sources (Feng et al., 2020; Li et al., 2017), so the two applications of AZO may promote the growth of *Bacillus sp.* in CP fumigated soil. *Bacillus sp.* are also important soil P solubilizing microbes that secret phosphatase into the soil (Hayat et al., 2010), causing higher phosphatase activities in CP+AZO2 treatment. However, further studies are needed for the detail microbial mechanisms.

4.4.2 Soil P fractions

In this study, there was no significant difference in the absolute content of Resin-Pi and $NaHCO_3$-Pi among different treatments during the whole experiment (**Fig. S4.1**), which was different from the results of incubation experiments conducted by Huang et al.(2020a; 2020b), that is, soil fumigation by chloropicrin and dazomet significantly increased the soil available P contents. The different findings may be due to the P uptake by ginger in our experiment (**Fig. 3.3**). From 17 WAP to 28 WAP, the ginger plants were in the flouring growth and mature stages. At this time, ginger plants absorbed a large amount of soil available P because the P in the ginger seeds cannot meet the needs of ginger growth. However, although CP fumigation could increase the content of soil available P by killing soil microbes (Huang et al., 2020a), the increased P uptake by ginger in CP-fumigated soil resulted in insignificant differences between different treatments.

Nonetheless, this study found that soil organic P fractions (mainly as $NaHCO_3$-Po) were accumulated in the CP-fumigated soils during the whole ginger growth period. The ratio of inorganic P (Resin-P + $NaHCO_3$-Pi + NaOH-Pi) to organic P ($NaHCO_3$-Po + NaOH-Po) content also confirmed that the proportions

of soil organic P increased in CP-fumigated soil after 17 WAP. The RF and RDA results showed that the proportions of $NaHCO_3$-Po to TP were highly negatively correlated with the activity of AiP and AlP, suggesting that the decreased activity of AiP and AlP may be one of the most important reasons for the accumulation of $NaHCO_3$-Po in CP-fumigated soil.

Among soil microbes, 50 - 75% of microbial P exists in the form of nucleic acid, 20% exists in P monoesters and 5% exist in phospholipids (Weihrauch et al., 2018). Previous studies suggested that CP fumigation could release the organic P inside microbial cells by killing and breaking down the microbial cells, and the released organic P could be subsequently extracted using 0.5 M $NaHCO_3$ ($NaHCO_3$-Po) and 0.1 M NaOH (NaOH-Po) (Huang et al., 2017). AiP and AlP then hydrolyze these phosphomonoesters into orthophosphate (Acosta-Martínez et al., 2011). However, in CP-fumigated soils, the decreased activity of AiP and AlP might slow down the mineralization process of soil organic P and lead to the accumulation of organic P fractions (mainly as $NaHCO_3$-Po) (**Fig. 4.6**).

In addition, in our previous study, the average values of physiological P use efficiency (PPUE) of ginger showed a decreasing trend in CP, CP+AZO1 and CP+AZO2 treatments (589.9 to 723.0 $g^2DM\ g^{-1}P$) as compared to the values in CK (1799.8 g^2 DM $g^{-1}P$) (**Table 3.2**), which may be due to the reduction of arbuscular mycorrhizal fungi (AMF) after CP fumigation (Smith et al., 2011). Studies have found that soil fumigation significantly decreased the amount of AMF which plays vital role in the mycorrhizal uptake pathway to deliver soil P to ginger plants (Dangi et al., 2017). Therefore, although CP fumigation could increase the proportion of soil available P, the ginger may not uptake more P from the soil due to the lack of soil phosphorus solubilizing microorganisms (especially AMF).

Besides, AZO application had no significant effect on the soil P fractions regardless of with or without CP fumigation, suggesting that, when applied at the recommended amounts in the field, the combined application of CP and AZO did not produce synergistic effects on the soil P availability. Overall, the results of this study showed that soil CP fumigation could increase the amount of soil labile P fractions. However, since the reduction of soil phosphatase activity may hinder the mineralization of soil organic P, this is not a sustainable way to improve soil fertility in the long run.

4.5 Conclusion

A single application of AZO at the recommended application rates does not affect soil phosphatase activity and soil P fractions, regardless of whether the soil was CP fumigated or not. CP fumigation increases the relative percentage of soil labile P, but decreases the soil phosphatase activity, which might hinder the mineralization of soil organic P. This result indicates that soil CP fumigation is not a sustainable way to improve soil fertility in the long run. The decreased soil phosphatase activity is also indirect evidence for the absence of phosphorus solubilizing microorganisms. Therefore, the current research also emphasizes the need for further research on microbial mechanisms, especially P solubilizing microbes, to explain the variation of soil P availability and ginger P uptake.

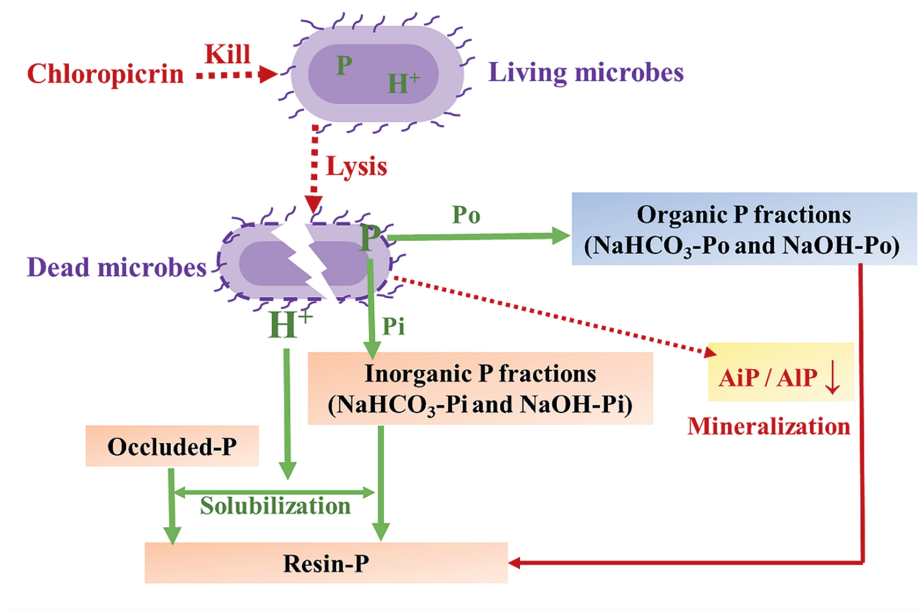


Fig. 4.6 Schematic diagram of soil phosphorus (P) transformation process in CP fumigated soil. Pi: inorganic P; Po: organic P; Resin-P: inorganic P fractions extracted by water; $NaHCO_3$-Pi: inorganic P fractions extracted by 0.5 M $NaHCO_3$ solution; $NaHCO_3$-Po: organic P fractions extracted by 0.5 M $NaHCO_3$ solution; NaOH-Pi: inorganic P fractions extracted by 0.5 M NaOH solution; NaOH-Po: organic P fractions extracted by 0.5 M NaOH solution and Occluded-P (unavailable P). AiP: acid phosphatase; AlP: alkaline phosphatase

4.6 Acknowledgement

We want to thank local farmers in Anqiu, Shandong Province of China, especially Yujia Li, who kindly helped with the preparation of soil. We also want to thank colleagues from Chinese Academy of Agricultural Sciences for mesocosm setup. Robin Palmer is highly appreciated for language editing. This research was supported by the National Natural Science Foundation of China (41877072,41620104006).

Supplementary material

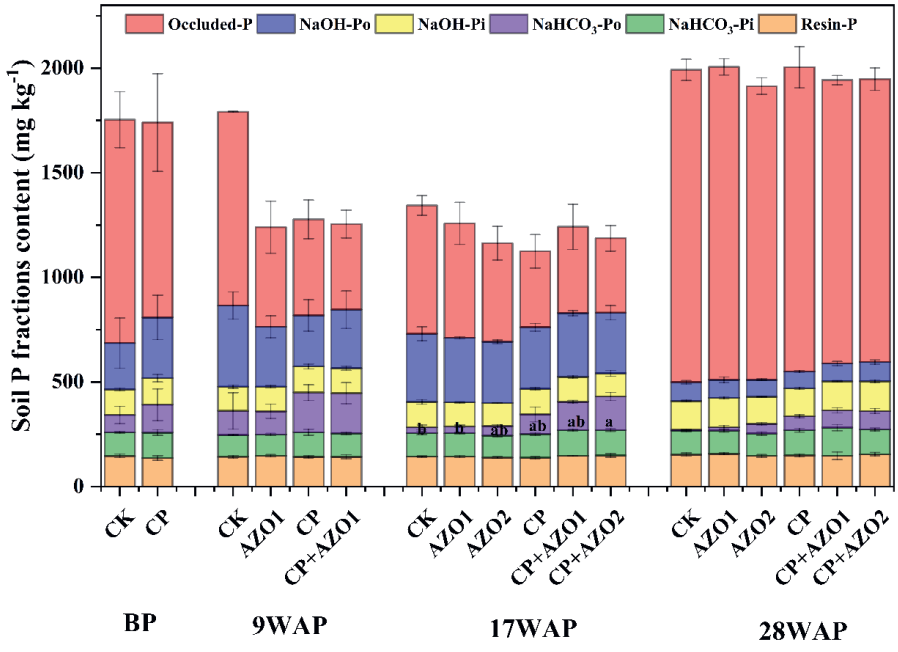


Fig. S4.1. The contents of soil phosphorus (P) fraction for different treatments and sampling times. Treatments include CK (control soil), AZO1 (a single application of AZO at 8 weeks after planting (WAP)), AZO2 (double applications of AZO at 8 and 16 WAP), CP (CP fumigated soil without AZO), CP+AZO1 (CP combined with AZO1) and CP+AZO2 (CP combined with AZO2). Sample times were Before planting; 9, 17 and 28 weeks after planting (BP, 9WAP, 17WAP and 28WAP). Legend indicates the soil P fractions, including Resin-P (orange), $NaHCO_3$-Pi (green), $NaHCO_3$-Po (purple), NaOH-Pi (yellow), NaOH-Po (blue) and Occluded-P (red). Columns represent the mean values of replicates with stand deviation. The lowercase letters (a and b) indicate the significant difference of $NaHCO_3$-Po between treatments at 17WAP. Apart from that, there were no significant differences in all of the soil fractions among different treatments at each sampling time (ANOVA with LSD test, $p < 0.05$)

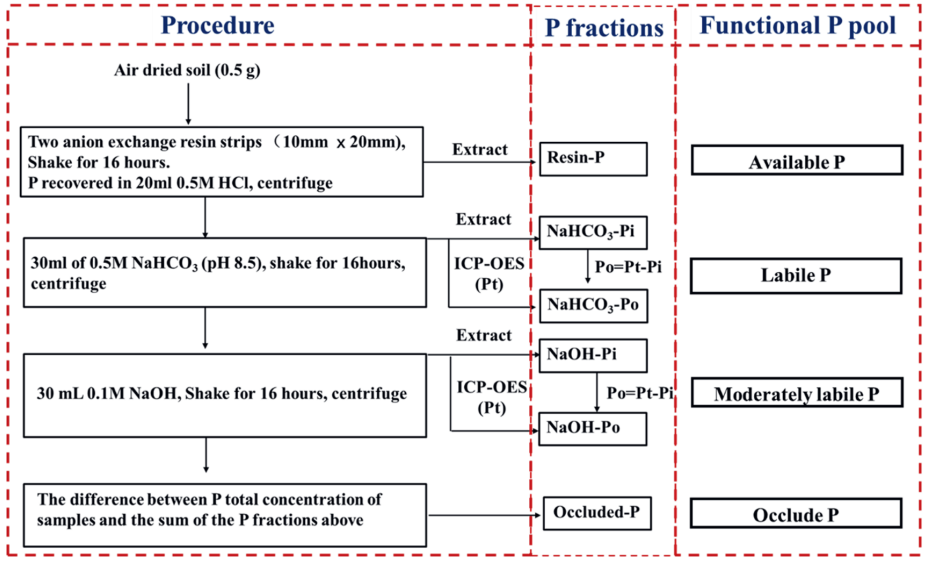


Fig. S4.2. Flowchart of Hedley sequential method for extraction and analysis of soil phosphorus


Winter

Chapter 5

Effects of chloropicrin fumigation and azoxystrobin application on soil microeukaryotic communities and phosphorus-cycling microorganisms

Abstract: *Soil fumigants and fungicides are effective at controlling soil-borne pathogens but might also adversely affect beneficial non-target soil microbes, such as phosphorus solubilizing microbes. Therefore, this study investigated the effects of chloropicrin (CP) fumigant and azoxystrobin (AZO) fungicide on soil microeukaryotic communities and bacteria containing two phosphatase encoding genes (phoC and phoD) in a greenhouse experiment using six treatments: CK (untreated soil); AZO1 (a single application of AZO); AZO2 (double applications of AZO); CP (CP fumigated soil without AZO application), CP+AZO1 (CP combined with AZO1) and CP+AZO2 (CP combined with AZO2). Results showed that one or two applications of AZO did not affect soil microeukaryotic and phoD-containing bacterial community structure with or without CP fumigation. In CP-fumigated soils, the relative abundance of fungi was significantly decreased, while the relative abundance of protists was significantly increased, suggesting that CP fumigation can reduce soil-borne diseases by killing pathogens or promoting the growth of pathogen consumers. CP fumigation significantly decreased the phoC/phoD gene copy number, but significantly increased the relative abundance of Sinorhizobium and Streptomyces which have significant positive effects on the soil labile P compositions. The structural equational model (SEM) confirmed that CP fumigation could affect soil labile P content directly by altering phoC-/phoD-containing bacteria, or indirectly by affecting soil phoC/phoD gene abundance and phosphatase activity. However, balancing the use of pesticides and fertilizers still requires further research.*

Based on:

Wang Y, Yang X, Xu M, Geissen V (2022) Effects of chloropicrin fumigation and azoxystrobin application on soil microeukaryotic communities and phosphorus-cycling microorganisms, *Soil Biology and Biochemistry*, to be submitted

5.1 Introduction

Chloropicrin (CP) is one of the most commonly used fumigants for controlling soil-borne diseases during the cultivation of high-value crops such as strawberries, ginger, and Chinese herbs (Rokunuzzaman et al., 2016). Many studies have found that CP fumigation can inhibit the growth of soil-borne pathogens and increase crop production (Zhang et al., 2019b, Zhu et al., 2021). For example, Zhu et al. (2021) found that the gene copy number of *Fusarium oxysporum* significantly decreased by 1.5×10^{10} after the application of CP at 40 mg kg^{-1}. Zhang et al. (2019b) found that CP fumigation at 30 g m^{-2} significantly increased the marketable yield of strawberry crops by 1.6 ~ 2.0 kg m^{-2}. Besides the target pathogens, CP fumigation could also induce deleterious effects on non-target soil microorganisms due to the broad-spectrum action of CP (Zhang et al., 2019b).

Previous studies have explored the side effects of CP fumigation on soil bacterial community structures and genes encoding key enzymes involved in soil nitrogen cycling. For example, Fang et al. (2020) found that CP fumigation at 500 kg ha^{-1} significantly decreased the proportions of *Proteobacteria* by 33%, *Chloroflexi* by 75% and *Acidobacteria* by 76%, but increased the proportions of *Firmicutes* by 137%, *Gemmatimonadetes* by 89%, and *Actinobacteria* by 366% 59 days after fumigation. Li et al. (2017) found that CP can decrease the abundance of ammonia-oxidizing archaea (AOA) and increase the abundance of ammonia-oxidizing bacteria (AOB) and denitrification significantly. However, little is known about the effects of CP fumigation on soil microeukaryotes such as fungi, protists, and metazoans, or on microbes involved in phosphorus (P) cycling processes.

Soil microeukaryotes play important roles in primary production, element cycling (Geisen et al., 2018) and the maintenance of soil fertility (Chen et al., 2015). P solubilizing microorganisms (PSMs), such as P solubilizing bacteria (PSB) like *Bacillus*, *Burkholderia*, and *Pseudomonas* (Bhattacharyya et al., 2012) as well as some fungi like *Penicillium*, *Aspergillus* and *Rhizopus* and actinomycete like *Streptomyces* (Alori et al., 2017), are the main conductors of soil P solubilization and mineralization. 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 (Nannipieri et al., 2011). During the mineralization process of soil organic P, acid and alkaline phosphomonoesterases (AiP and AlP) are non-specific enzymes that catalyse the hydrolysis of ester-phosphate bonds to release orthophosphate (Fraser et al., 2017) and increase the soil P availability for plant uptake. In bacteria, *phoC* and *phoD* genes have been identified as being responsible for the synthesis of AiP and AlP, respectively (Luo et al., 2019). Previous studies have found that soil fumigation using chloropicrin and dazomet changed the abundance and diversity of *phoD*-containing bacteria and increased the amount of soil available P (Huang et al., 2020a; 2020b). However, due to the great diversity and unspecific property of PSMs, different studies have found inconsistent effects of soil fumigation on these PSMs. Zhang et al. (2019a) found that the application of 1,3-D at 50, 40 and 30 g m^{-2} increased the abundance of *Pseudomonas* but decreased the abundance of *Bacillus*. However, Pecina et al. (2016) found that both *Pseudomonas* ssp. and *Bacillus* ssp. total population counts increased immediately after CP fumigation. Therefore, the changes in *phoC*/*phoD* genes and related *phoC*/*phoD*-containing bacteria under soil fumigation are still far from clear (Castellano-Hinojosa et al., 2021).

In addition, other fungicides with specific targets have also been used for pathogen control after pre-treatment with soil fumigation during farming (Huang et al., 2019b, Wang et al., 2022). 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, 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% (Han et al., 2020). The effect of AZO on the soil microbiome could be altered by the coexistence of other fungicides (Saha et al., 2020, Liu et al., 2021, Silva et al., 2021). Researchers found that CP fumigation inhibited the dissipation and degradation of AZO in the soil system (Huang et al., 2019b), which means that the combined application of CP and AZO may have different effects on the soil microbiome as opposed to using them alone. Our previous study showed that the coexistence of CP and AZO can influence ginger P uptake and soil P fractions which could be due to the variations of soil P-cycling related soil microbes (Wang et al., 2022). However, there is little research focusing the combined effects of CP and AZO on soil P-cycling related microbes. Therefore, we hypothesized that CP fumigation and AZO application could alter the microbial community structure, especially P-cycling related microbes. In this study, we aim to investigate the effects of CP fumigation and AZO application on soil microeukaryotic communities and two phosphatase genes (*phoC* and *phoD*) containing microorganism.

5.2 Materials and methods

5.2.1 Experiment design

The details of the greenhouse experiment were described in our previous study (Wang et al., 2022). Briefly, a field (1.2 m × 2.2 m) that had never been planted with ginger in An'qiu, Shandong Province of China was fumigated using CP (chloropicrin; Dalian Lvfeng Chemical Co. Ltd.) at 37.1 g m^{-2} for one week. A field adjacent to the fumigated field was selected as the source for unfumigated soil. After fumigation, surface soils (0-20 cm) from the fumigated and unfumigated fields were collected and taken to the greenhouse.

6 kg of treated 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. There were six treatments: 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) and 5 replicates of each treatment made for every sampling occasion. Each dose of AZO (azoxystrobin; Hebei Zhongbaolv Crop Technology company) was applied at 47.1 mg AZO m^{-2}. Soil samples were taken using a disruptive sampling method before planting (BP: 12/04/2019) and 17 weeks after planting (17 WAP: 10/08/2019). When sampling, all roots and other debris in soil were separated and sorted out. Then, the soil sample was collected and stored at -80℃ for DNA extraction.

5.2.2 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℃ 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 (2X) (Applied Biosystems, USA). The primers, qPCR program and reaction composition are listed in **Table S 5.1**. The amount of *phoC* and *phoD* was expressed as gene copy numbers per gram soil (copies g^{-1}) 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.

5.2.3 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 (AATCCRAGAATTTCACCTCT). The primers for *phoC* and *phoD* genes are shown in **Table S5.1**. 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 PE250 mode for the 18S rRNA and *phoD* genes, while the PE150 mode was used for the *phoC* gene (Guangdong Magigene Biotechnology Co.,Ltd. Guangzhou, China).

5.2.4 Sequencing data processing

The Illumina MiSeq sequencing reads for the 18S rRNA, *phoC* and *phoD* were processed using the DADA2 pipeline v1.8 (Callahan, McMurdie et al. 2016). Forward reads were trimmed at 240bp, and reverse reads were trimmed at 220bp. 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. Microeukaryote samples were identified using the DECIPHER package (Wright 2016) against the SILVA SSU database (SSU_r138) at the default settings (Quast, Pruesse et al. 2013). 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.

5.2.5 Statistical analyses

To eliminate differences between samples caused by sequencing depth, OTU reads were rarefied for further analysis. The Shannon and Chao1 were calculated using the "*vegan*" package in RStudio to

quantify the alpha diversity of each sample. Beta diversity was calculated based on Bray-Curtis distance using the "*ape*" package in RStudio, and visualized by principal coordinate analysis (PCoA) in Origin 2021.

In order to reduce redundancy in the OTU table, sequences of annotations not available at the phylum level were discarded. The relative abundance of the top 10 phyla was calculated and visualized in Origin 20. For the microeukaryotic communities, OTUs present in more than 20 samples (30 samples in total) were analysed by Linear discriminant analysis Effect Size (LEfSe) with a linear discriminant analysis (LDA) score > 2 to determine the biomarkers from phylum to genus ($p > 0.05$) (http://huttenhower.sph.harvard.edu/galaxy) (Zhao, et al., 2021). For *phoC*-/*phoD*-containing microorganisms, correlation coefficients between the top 10 *phoC*-/*phoD*-containing microorganisms and soil P fractions (**Fig. 4.2**) 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 microorganisms on the *phoC*/*phoD* gene copy number, acid and alkaline phosphatase (AiP and AlP) activity (**Fig. 4.1**) and different soil P fractions.

All variance, normality and homogeneity of variance were tested using the Kolmogorov-Smirnov and Levene tests ($p > 0.05$), respectively, in 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 comparation and significance detection, respectively.

5.3 Results

5.3.1 The microeukaryotic community structure

For samples collected before planting (BP), there was no significant difference in Shannon (5.3 ~ 3.9) and Chao1(1498.6 ~ 1207.7) indices between CK and CP. At 17 WAP, the values of Shannon and Chao 1 were significantly lower in CP-fumigated soils (Shannon: 5.1~ 5.3; Chao 1: 986.0 ~ 1186.7) than in CK (Shannon: 5.9; Chao 1: 1691.2) (**Table 5.1**).

Principal coordinate analysis (PCoA) showed that the first two axes, PC1 and PC2, explained 71.69% and 22.26% of the variation in soil microeukaryotic communities, respectively (**Fig. 5.1(A)**). For the samples collected at the same time, treatments with CP fumigation (CP, CP+AZO1 and CP+AZO2) were significantly separated from treatments without CP fumigation (CK, AZO1 and AZO2). However, the CK, AZO1 and AZO2 treatments were not seperated from each other. CP, CP+AZO1 and CP+AZO2 treatments as well were not seperated from each other either.

Table 5.1 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

For samples collected before planting (BP), fungi *Ascomycota* dominated the microeukaryotic communities, accounting for 53.4% in CK and 78.7% in CP followed by *Ciliophora* (CK: 8.8%; CP: 6.3%) and *Cercozoa* (CK: 8.6%; CP: 5.3%). The relative abundances of *Mucoromycota*, *Basidiomycota* and *Chytridiomycota* were significantly lower in CP than in CK. At 17 WAP, the relative abundances of *Ascomycota* decreased to 26.1% ~ 30.0% in non CP-fumigated treatments (CK, AZO1 and AZO2) and to 13.4% ~ 15.3% in CP-fumigated treatments (CP, CP+AZO1 and CP+AZO2), while the relative abundances of *Ciliophora* increased to 20.4% ~ 24.6% in non CP-fumigated treatments and to 49.1% ~ 55.1% in CP-fumigated treatments. *Cercozoa* accounted for 9.4% ~ 15.3%. The relative abundances of *Mucoromycota* and *Basidiomycota, Nematozoa* were significantly lower in CP-fumigated treatments (*Mucoromycota*:1.5% ~ 2.7%; *Basidiomycota*: 0.7% ~ 0.8%; *Nematozo*a: 0.8% ~ 2.7%) than in non CP-fumigated treatments (*Mucoromycota*:13.2% ~ 13.9%; *Basidiomycota*: 10.7% ~ 11.6%; *Nematozo*a: 5.6% ~ 6.9%), while the relative abundance of *Hyphochytriomycets* was significantly higher in CP-fumigated treatments (3.7% ~ 5.7%) than in non CP-fumigated treatments (0.8% ~ 1.0%) (**Fig. 5.1(B)**).

The LEfSe results showed that, for samples collected before planting, there were 37 significant biomarkers in CK (3 phylum categories, 7 class categories, 9 order categories, 9 family categories and 9 genus categories), while there were 44 significant biomarkers in CP (9 phyla, 11 classes, 11 orders, 9 families and 4 genera). *Cystofilobasidum, knufia, Verticillum, Calcarisporiella, Tausonia, Colpoda, Chaetomium, Pseudoplatyophrya* and *Preussia* were the most prominent genus in CK. *Neurospora, Rhogostoma, Pseudouroleptus* and *Paraphysoderma* were significantly enriched in CP. At 17 WAP, there were 24 significant biomarkers in CK (8 phyla, 8 classes, 4 orders, 2 families and 2 genera), 33 significant biomarkers in AZO2 (7 phyla, 8 classes, 8 orders, 6 families and 4 genera), 32 significant biomarkers in CP (10 phyla, 10 classes, 9 orders, 2 families and 1 genus) and 6 significant biomarkers in CP+AZO2 (3

phyla, 2 classes, 1 order). *Holosticha* and *Tausonia* were significantly enriched in CK, and *Phascolodon* was significantly enriched in CP, while *Bryometopus, Geastrum, Mortierella* and *Rhogostoma* were significantly enriched in AZO2 (**Fig. 5.2**).

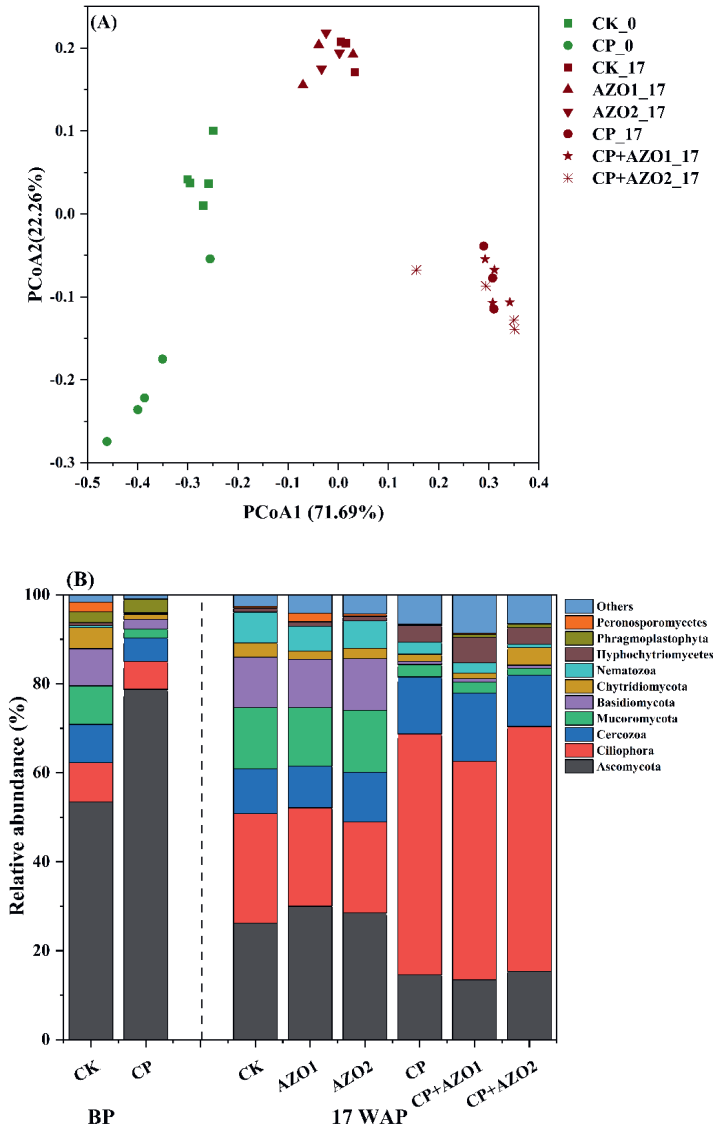


Fig. 5.1 Principal coordinate analysis (PCoA) of microeukaryote composition (A), and the relative abundance of the top 10 microeukaryotes at the phylum level (B). Green symbols represent samples collected before planting (BP), while red symbols are samples collected 17 weeks after planting (WAP). Treatments 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, star (*)).

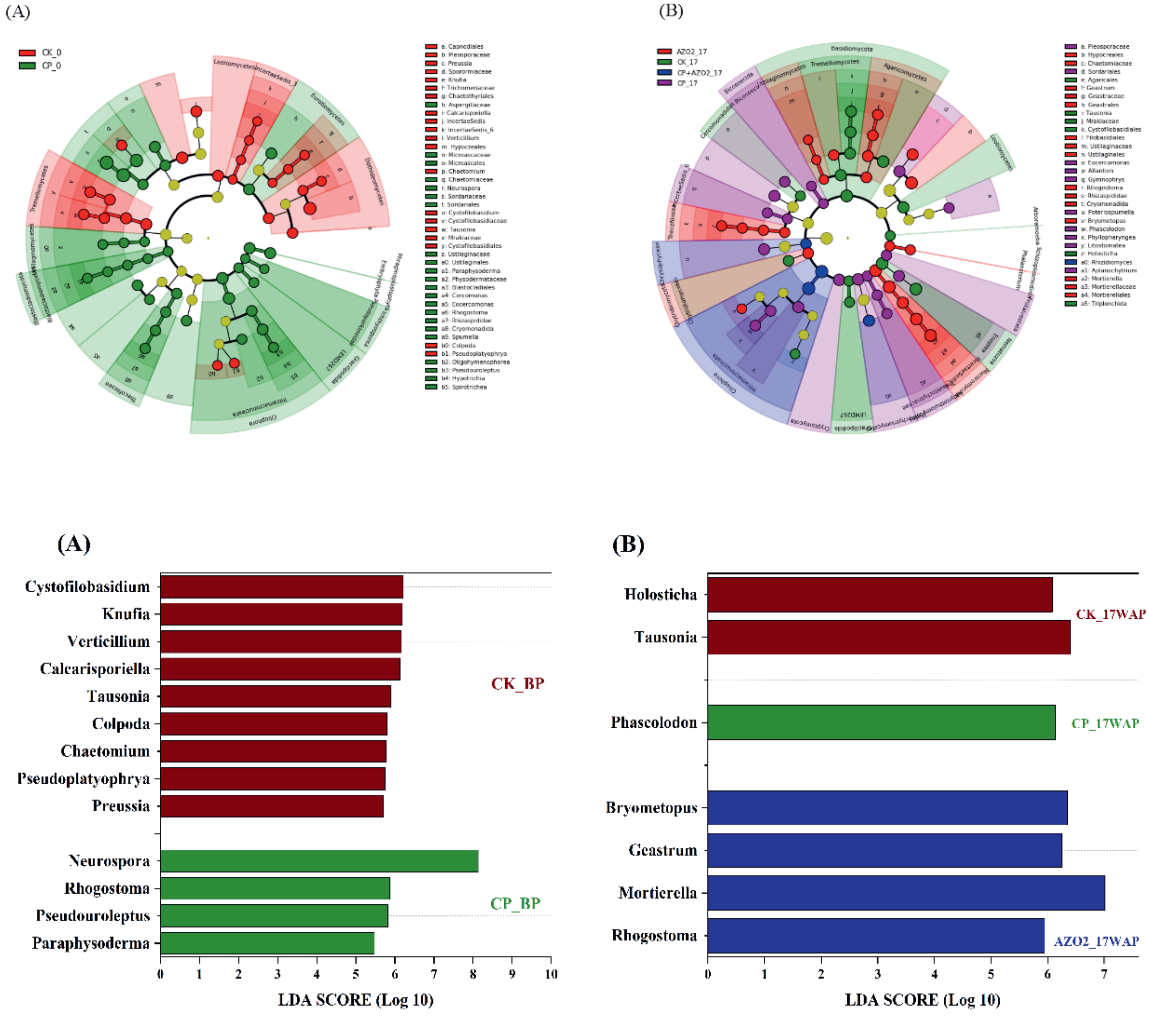


Fig. 5.2 Linear discriminant analysis Effect Size (LEfSe) with a linear discriminant analysis (LDA) score for samples collected before fumigation (A) and 17 weeks after planting (B). Treatments include CK (untreated soil), AZO1 (a single application of AZO 8 weeks after planting (WAP)), AZO2 (double 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). For the LDA score (at the genus level), the red bars are the CK treatment; green bars are the CP treatment; Blue bars are the AZO2 treatment.

5.3.2 *phoC*/*phoD* gene copy number

The gene copy numbers of *phoC* and *phoD* are presented in **Fig. 5.3**. For samples collected before planting, the gene copy numbers of *phoC* and *phoD* were significantly lower in CP (*phoC*: 0.7 x 10^7 copies g^{-1}; *phoD*: 29.8 x10^7 copies g^{-1}) than in CK (*phoC*: 1.5 x 10^7 copies g^{-1}; *phoD*:57.6 x10^7 copies g^{-1}). At 17 WAP, the gene copy numbers of *phoC* and *phoD* were significantly lower for CP-fumigated treatments (*phoC*: 0.6 x 10^7 ~ 0.8 x 10^7 copies g^{-1}; *phoD*: 22.5 x 10^7 ~ 28.8 x 10^7 copies g^{-1}) than in non CP-fumigated treatments (*phoC*: 2.8 x 10^7 ~ 3.6 x 10^7 copies g^{-1}; *phoD*: 59.7 x 10^7 ~ 63.6 x 10^7 copies g^{-1}). There was no significant difference between CK, AZO1 and AZO2, nor between CP, CP+AZO1 and CP+AZO2.

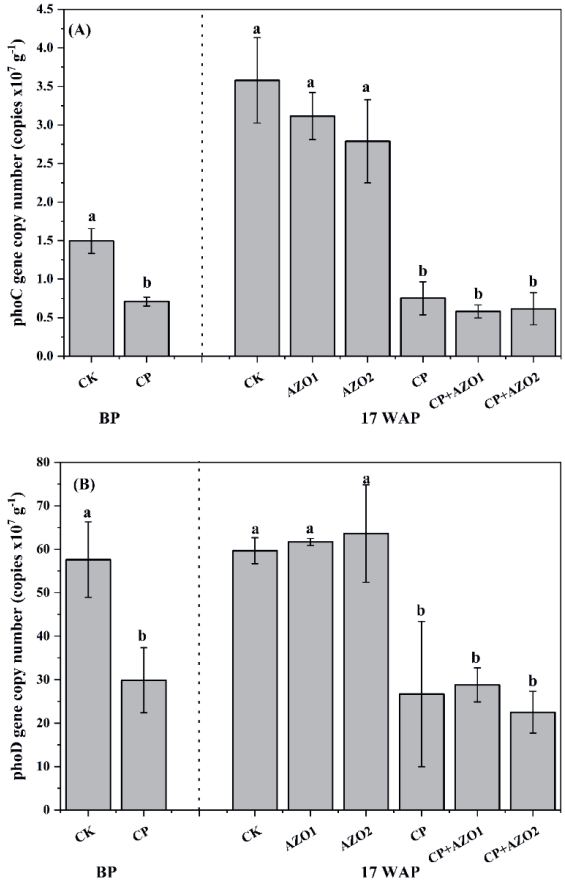


Fig. 5.3 *phoC* (A) and *phoD* (B) gene copy number (copies g^{-1} soil). Treatments include CK (untreated soil), AZO1 (a single application of AZO 8 weeks after planting (WAP)), AZO2 (double applications of AZO at 8 and 16 WAP), CP (CP fumigated soil without AZO), CP+AZO1 (CP combined with AZO1) and 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, $p<0.05$).

5.3.3 *phoC*-/*phoD*-containing bacterial community structure

5.3.3.1 *α*-diversity

After quality filtering, a total of 12370611 *phoC*-containing and 4001341 *phoD*-containing bacterial effective 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 before planting, the Shannon index was significantly lower in CP (2.5) than in CK (1.8), while no significant difference was observed in the Chao1 index. At 17 WAP, the Shannon index was significantly lower in CK (1.5) than in AZO2 (2.4) and CP (2.6), while the Chao1 index was significantly lower in CK (215.0) than in CP (343.0). For *phoD*-containing bacteria, there was no significant difference in the Shannon and Chao1 indices between CK and CP for samples collected before planting. At 17 WAP, the Shannon index was significantly lower in CK (5.0) than in CP (5.4) and CP+AZO1(5.3), while the Chao1 index was significantly lower in CK (818.3) than in AZO2 (1090.3) (**Table 5.2**).

5.3.3.2 *β*-diversity

PCoA results showed that, for *phoC*-containing bacteria, PC1 and PC2 accounted for 24.5% and 11.7% of the variations, respectively (**Fig. 5.4**). The *phoC*-containing bacterial communities in samples collected before planting could be significantly separated between CK and CP treatments, whereas PC1 and PC2 could not separate the microbial communities between different treatments at 17 WAP. For *phoD*-containing bacteria, PC1 and PC2 accounted for 41.7% and 11.7% of the variations, respectively. For samples collected before planting, the *phoD*-containing bacterial communities could not be separated by PC1 and PC2, 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) at 17 WAP.

Table 5.2 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

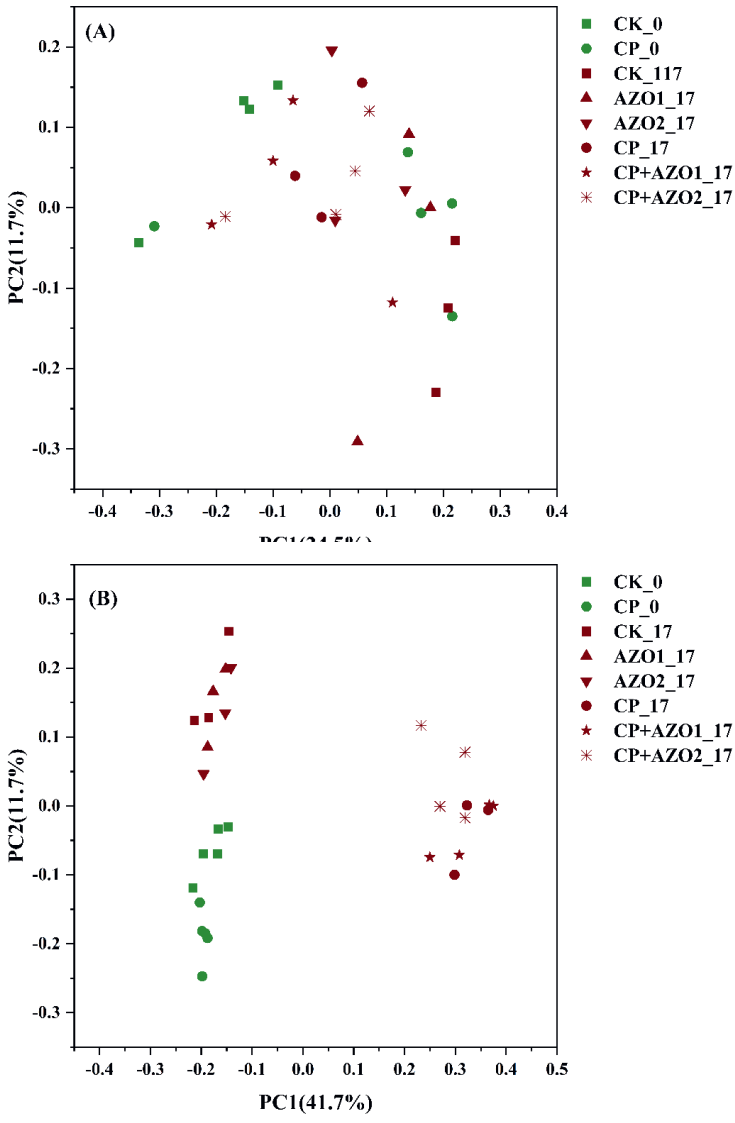


Fig. 5.4 Principal coordinate analysis (PCoA) of the composition of *phoC*-containing bacteria (A) and *phoD*-containing bacteria (B). Green symbols represent samples collected before planting (BP), while the red symbols represent samples collected at 17 weeks after planting (WAP). Treatments include 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, star (*)).

5.3.3.3 *phoC*-/*phoD*-containing bacterial community structure

For *phoC*-containing bacteria, *Acidobacteria* (36.7% ~ 71.2%) was the most dominant phylum, followed by *Actinobacteria* (12.2% ~ 34.7%), *Proteobacteria* (3.4% ~ 13.3%) and *Abditibacteriota* (0.8% ~ 15.7%) (**Fig. 5.5 (A)**). For samples collected before planting (BP), there was no significant difference in the relative abundances of *Acidobacteria* and *Actinobacteria*. CP fumigation significantly increased the relative abundances of *Proteobacteria*, *Firmicutes* and *Planctomycetes* by 5.3%, 1.2% and 0.7%, respectively. At 17 WAP, the relative abundance of *Acidobacteria* was significantly higher and *Proteobacteria* was significantly lower, in AZO2 and CP-fumigated treatments (*Acidobacteria*: 64.4% ~ 71.2%; *Proteobacteria*: 3.4% ~ 6.1%) than that in CK (*Acidobacteria*: 36.7%; *Proteobacteria*: 13.3%). The relative abundance of *Abditibacteriota* was significantly higher in CP+AZO1 and CP+AZO2 (14.5% ~ 15.7%) than in CK (0.8%). The relative abundances of *Chloroflexi* and *Firmicutes* were significantly lower in AZO2, CP+AZO1 and CP+AZO2 (*Chloroflexi*: 0.4% ~ 1.7%; *Firmicutes*: 0.4% ~ 0.6%) than in CK (*Chloroflexi*: 3.7%; *Firmicutes*: 1.6%). Compared to CP, CP+AZO2 significantly decreased the relative abundances of *Actinobacteria*, *Bacteroidetes*, *Chloroflexi* and *Firmicutes* by 10.0%, 1.9%, 1.6% and 0.6%, respectively. The relative abundance of *Abditibacteriota* was significantly higher in CP+AZO1 and CP+AZO2 (14.5% ~ 15.7%) than in CP (1.9%).

For *phoD*-containing bacteria, *Proteobacteria* (57.9% ~ 65.3%), *Actinobacteria* (30.8% ~ 38.5%) and *Planctomycetes* (3.3% ~ 4.5%) were the top three dominant phyla **(Fig. S5.2**). Since *phoD*-containing bacteria could not be separated at the phylum level, we downscaled to the genus level. The top 10 dominant genera of *phoD*-containing bacteria are shown in **Fig. 5.5 (B).** There was no significant difference in the relative abundance of *phoD*-containing bacteria between CK and CP before planting, but the relative abundance of *Sinorhizobium* was significantly higher in CP (4.2%) than in CK (2.8%). At 17 WAP, the relative abundance of *Pesudonocardia* was significantly lower, while the relative abundance of *Streptomyces* was significantly higher, in CP-fumigated treatments (*Pesudonocardia*:7.3% ~ 11.1%; *Streptomyces*: 22.3% ~ 25.8%) than in CK (*Pesudonocardia*: 28.4%; *Streptomyces*: 17.5%). The relative abundance of *Ralstonia* was significantly higher in CP (7.0%) and CP+AZO1(7.1%) treatments than in CK (3.0%). The relative abundance of *Sinorhizobium* was higher in AZO2 (4.7%) and CP-fumigated treatments (4.5% ~ 6.5%) than in CK (3.4%).

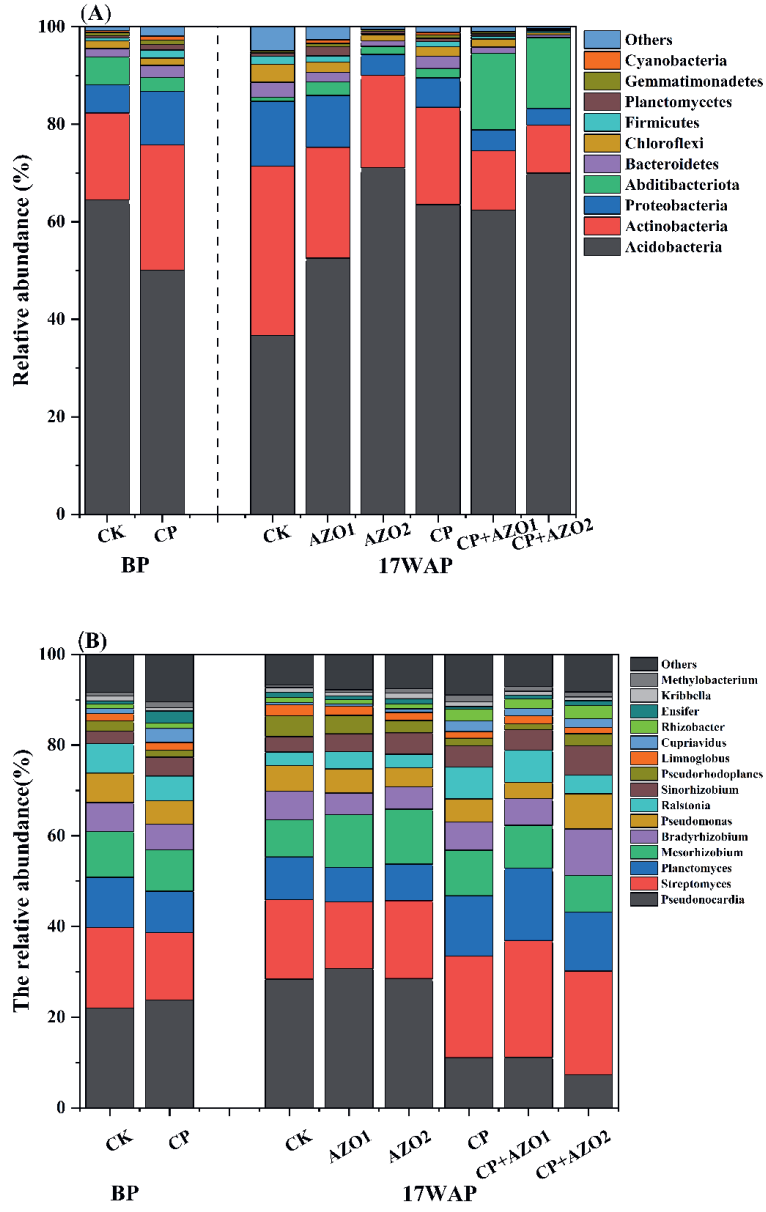


Fig. 5.5 Relative abundance of the top 10 phyla of *phoC*-containing bacteria (A) and top 15 genera of *phoD*-containing bacteria (B). Treatments include CK (untreated soil), AZO1 (a single application of AZO 8 weeks after planting (WAP)), AZO2 (double applications of AZO at 8 and 16 WAP), CP (CP fumigated 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).

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

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. 5.6**). The correlation heatmap showed that *phoD*-containing bacteria such as *Sinorhizobium*, *Streptomyces* and *Pseudonocardia* were the bacteria that had the greatest impact on different soil P fractions. *Sinorhizobium* and *Streptomyces* had significant positive effects on soil P fractions except for Occluded-P, while *Pseudonocardia* had significant negative effects on the proportions of $NaHCO_3$-Pi ($p < 0.05$), $NaHCO_3$-Po ($p < 0.01$) and NaOH-Pi ($p < 0.05$). The proportion of $NaHCO_3$-Po to total P (TP) was negatively affected by *phoC*-containing bacteria such as *Actinobacteria* ($p < 0.01$), *Proteobacteria* ($p < 0.05$), *Planctomycetes* ($p < 0.05$) and the *phoD*-containing bacteria *Limnoglobus* ($p < 0.05$). *Bradyrhizonium* had significant positive influence on the proportion of $NaHCO_3$-Po to TP ($p < 0.05$) (**Fig. 5.7**).

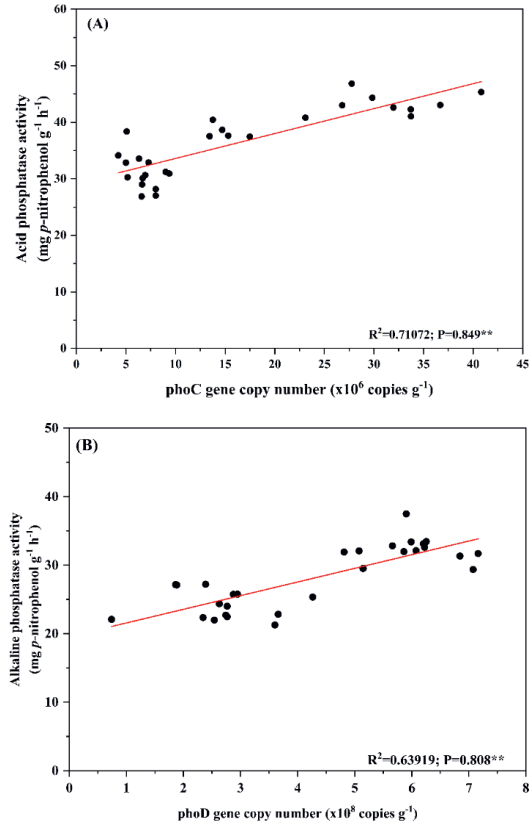


Fig. 5.6 Relationship between acid phosphatase activity and *phoC* gene copy number (A), and between alkaline phosphatase activity and *phoD* gene copy number (B).

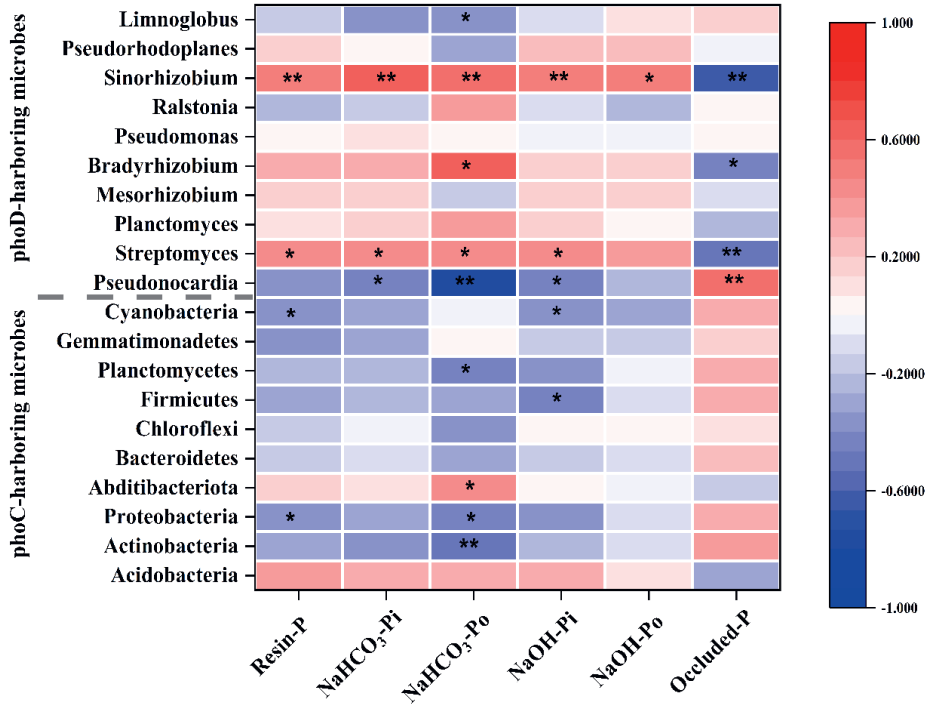


Fig. 5.7 Heatmap of the correlation between *phoC*-/*phoD*-containing bacteria and the proportions of different soil P fractions to TP including Resin-P, $NaHCO_3$-Pi, $NaHCO_3$-Po, NaOH-Pi, NaOH-Po and Occluded-P. ** means significant correlation at $p < 0.01$; * means significant correlation at $p < 0.05$.

Structural equational modelling (SEM) indicated that the diversity of *phoC*-containing bacteria had a significant positive effect on the *phoC* gene copy number ($p < 0.05$) which had a positive effect on AiP activity ($p < 0.001$). The activity of AiP showed a significant positive effect on the AP content ($p < 0.001$). The diversity of *phoC*-containing bacteria showed a significant positive effect on the content of soil potential organic P fractions ($p < 0.01$), and the latter had a significant positive effect on the AP content ($p < 0.05$). The diversity of *phoD*-containing bacteria had a significant negative effect on the *phoD* gene copy number ($p < 0.001$), and the *phoD* gene copy number had a significant positive effect on the AlP activity ($p < 0.01$). The diversity of *phoD*-containing bacteria had a significant positive effect on the contents of the soil potential organic P fraction ($p < 0.001$) and AP content ($p < 0.001$) (**Fig. 5.8**).

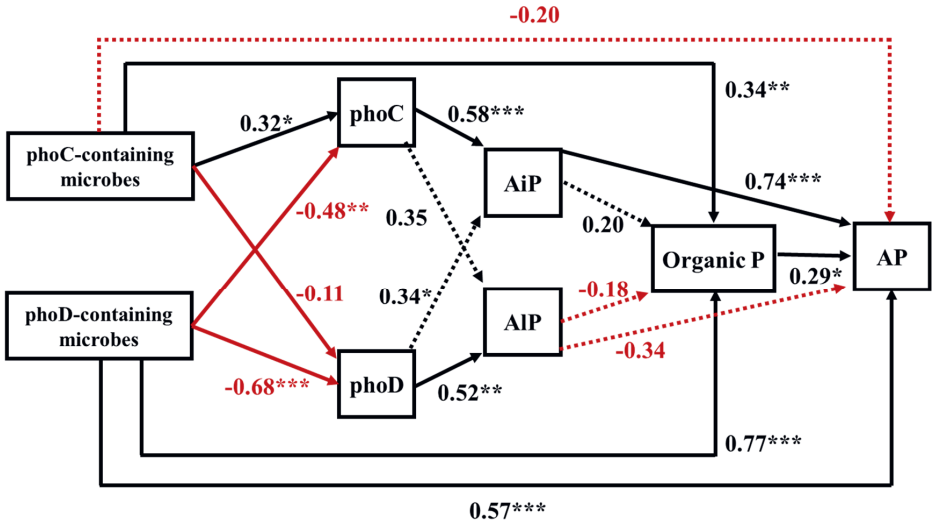


Fig. 5.8 Structural equation modelling (SEM) of the direct and indirect effects of the diversity of *phoC*-/*phoD*-containing microorganisms on different soil P fractions. To simplify the SEM model, the first axis of PCoA scores for each sample were used to represent the diversity of *phoC*-/*phoD*-containing microorganisms. Organic P refers to the sum of $NaHCO_3$-Po and NaOH-Po, while AP refers to the sum of Resin-P and $NaHCO_3$-Pi. Red arrows refer to negative effects while black arrows refer to positive effects. Dotted arrows represent nonsignificant 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.

5.4 Discussion

5.4.1 Effects of CP fumigation and AZO application on the soil micro-eukaryotic community

In our study, over 85% of the microeukaryotic communities consisted of soil fungi and protists. Results showed that AZO application had no effect on soil microeukaryotic community diversity or structure regardless of whether it was applied once or twice, with or without CP fumigation, which may be attributed to the rapid degradation rates of AZO (Wang et al., 2022). Our results were in agreement with 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.

CP fumigation significantly changed the structure of soil microeukaryotic communities before planting and 17 weeks after planting. For the fungal community, CP fumigation significantly increased the relative abundance of *Ascomycota*, while it decreased the relative abundance of *Mucoromycota*, *Basidiomycota* and *Chytridiomycota* before planting. However, for samples collected at 17 WAP, CP fumigation significantly decreased the relative abundance of *Ascomycota* and *Basidiomycota*. *Ascomycota* contains many species with pathogenic, saprophytic and endophytic properties, and cause serious plant rot diseases (Pu et al., 2022), while *Basidiomycota* also includes plant pathogens that cause rusts and smuts, which can reduce plant yield and even cause plant death (Volk 2013), suggesting that CP fumigation could effectively inhibit the recovery of soil pathogenic fungi in our study. Previous studies also found that CP fumigation decreased the relative abundance of *Ascomycota* (Li et al., 2021), while another study conducted by Zhang et al. (2019b) found that CP fumigation at 30 g m^{-2} for 10 days in a strawberry greenhouse increased the relative abundance of *Basidiomycota*.

In this study, CP fumigation also significantly decreased the relative abundance of *Mucoromycota*, especially the genus *Mortierella*. Among fungal communities, the relative abundance of *Mortierella* decreased by 20.4% before planting and 11.9% ~ 15.7% for samples collected at 17 WAP **(Fig. S5.1).** *Mortierella* is reported to inhibit plant diseases (Zhang et al., 2019), degrade many pesticides (Zhang et al., 2021a) and promote solubilizing soil phosphorus (Li et al., 2021). Therefore, the reduction of *Mortierella* may negatively affect plant health and soil P solubilization. Some studies also found that *Mortierella* decreased after CP fumigation at 30 g m^{-2} for 10 days (Zhang et al., 2019b), while others found that CP fumigation at 40 mg kg^{-1} for 7 days significantly increased the relative abundance of *Mortierella* (Zhu et al., 2021). These inconsistent results may be due to the different incubation conditions and plant interruptions in the different experiments.

Soil protists have been proven to be more sensitive than fungi to organic pollutants (Wu et al., 2022b). Unlike the soil fungal community, the soil protist community showed increasing trends in CP-fumigated soil, especially for samples collected at 17 WAP. The relative abundance of *Ciliophora* and *Cercozoa* in CP-fumigated soils were 27.0% ~ 34.6% and 0.4% ~ 5.9% higher than those in non CP-fumigated soils, respectively. Therefore, from the results, we can see that the soil microeukaryotic community changed from fungal dominance to protist dominance after CP fumigation.

Soil protists are an important part of the ecologically functional soil microbiomes. For example, phagotrophic protists are consumers of soil bacteria, fungi, and other microeukaryotes that could

release nutrients from their prey through predation. The primary production by these photosynthetic soil protists is an important carbon input into the soil system, and the parasitic protists are also the main conductors of soil organic carbon decomposition (Geisen et al., 2018). *Ciliophora* and *Cercozoa*, the two important phagocytic protists (consumers) and 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 before planting provided more prey to protist consumers (*Ciliophora* and *Cercozoa*), which increased the relative abundance of protists and decreased the relative abundance of fungi at 17 WAP. Therefore, this result suggests that CP fumigation can improve soil health by reducing plant pathogens or increasing pathogen consumers, thereby increasing ginger yield (Wang et al., 2022).

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

Since both plant root and soil microorganisms can secrete acid phosphatase, most previous studies that explored the effects of pesticides on soil P solubilizing microbes have only focused on alkaline phosphatase secreted only by soil microorganisms (Huang et al., 2020a; 2020b). However, acid phosphatase is also an important enzyme that catalyzes the mineralization of organic P, especially in acidic soils (Acosta-Martínez et al., 2011). Therefore, in this study, two representative phosphatase regulators (*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 showed 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. 5.6**).

In order to find the microbes containing *phoC*/*phoD* genes, we performed high-throughput sequencing with *phoC*/*phoD* as the target gene region. The sequencing results showed that both CP fumigation and AZO application significantly altered the *phoC*-containing bacterial community structure, while only CP fumigation significantly changed the *phoD*-containing bacterial community structure. The different responses of different soil microorganisms to CP fumigation and AZO application may be due to their different tolerances to pesticide toxicity and their abilities to remediate pesticide contamination (Huang et al., 2020a). For example, the relative abundance of *Firmicutes* increased significantly after CP fumigation, possibly due to the ability of the organisms to form spores to resist environmental stress (Castellano-Hinojosa et al., 2021). The relative abundance of *Abditibacterium*, which is highly resistant to antibiotics and toxic compounds (Tahon, 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 *Acidobacteria*, *Chloroflexi* and *Firmicutes*. This suggested that the application of AZO needs to be high to show the toxicity to these microbes.

For *phoD*-containing bacteria, the relative abundance of *Streptomyces*, *Ralstonia* and *Sinorhizobium* were significantly higher in CP-fumigated soil, likely due to their ability to degrade CP and use CP as a nutrient source (Apel et al., 2007). For example, Huang et al. (2020a) also found that *Sinorhizobium* was significantly enriched in CP-fumigated soil and suggested that these fumigation enriched species could degrade or bioremediate contaminants.

In our study, heatmap analysis showed that *Sinorhizobium* and *Streptomyces* had significant positive effects on soil labile P contents, indicating that the increase of *Sinorhizobium* and *Streptomyces* in CP-fumigated soil was an important reason for the increased levels of soil labile P. *Sinorhizobium* has been shown to produce organic acids, especially indole-3-acetic acid (IAA), to dissolve phosphate rock into available phosphorus (Bianco et al., 2010). In addition, *Streptomyces* are biocontrol agents used against soil-borne pathogens, increasing crop yields (Zhang et al., 2021a, Wang et al., 2022). The heatmap also showed that $NaHCO_3$-Po was the most susceptible to the effects of different types of soil PSMs, which may be because $NaHCO_3$-Po are P fractions that are slightly complexed with the surface of organic matter and are easily mineralized by phosphatases (Wang et al., 2022). s

From the SEM, the diversity of *phoD*-containing bacteria had a significant negative influence on the amount of *phoD* gene copy number, while the *phoD* gene copy number had a significant positive influence on the activity of AlP, and the AlP activity showed negative influence on the potential available organic P contents. This means that CP fumigation increased the diversity of *phoD*-containing bacteria, leading to the decrease of *phoD* gene copy number. The decrease of *phoD* gene copy number induced the decrease of AlP activity, which caused the increase of potential available P fractions. Results also showed that the diversity of *phoD*-containing bacteria had a significant positive influence on the AP contents. CP fumigation decreased the diversity of *phoC*-containing bacteria, then decrease the *phoC* gene copy number and AiP activity and the decrease of organic P amounts (not significant). The diversity of *phoC*-containing microbes had a negative influence on AP, which also means that the amount of AP had negative feedback on *phoC*-containing bacteria. The more P fertilizers applied, the higher AP content and the lower diversity of *phoC*-containing bacteria. One key question to be answered is how to adjust the amount of P fertilizers to get a P balance in fields with soil fumigation.

5.5 Conclusion

This study investigated the effects of CP fumigation and AZO application on soil micro-eukaryotes and *phoC*-/*phoD*-containing bacteria. Regardless of AZO application frequency and CP fumigation, AZO application had no effect on soil micro-eukaryotic and *phoD*-containing bacterial community structure. CP fumigation shifted microeukaryotic communities from fungal dominance to protist dominance, suggesting that CP fumigation could reduce soil-borne diseases by killing pathogens or promoting the growth of pathogen consumers. CP fumigation significantly reduced the *phoC*/*phoD* gene copy number, and influenced soil labile P contents by altering the *phoC*-/*phoD*-containing bacterial community structures. *Sinorhizobium*, *Streptomyces* and *Pseudonocardia* are the three genera that control the soil P fractions in CP-fumigated soils, but how to use these key microbial species to reduce the use of pesticides and fertilizers needs to be further studied.

Supplementary materials

Table S5.1 Primers, program and reaction composition used for qPCR for *phoC* and *phoD* genes

Target gene	Primer	Sequence (5' – 3')	Size	Reference	Program	Reaction composition
phoC	*phoC*F *phoC*R	CGGCTCCTATCCGTCCGG CAACATCGCTTTGCCAGTG	155 bp	(Fraser et al., 2017)	95 ℃ 10min; 95 ℃ 15S; 60/65 ℃ 30S; 72 ℃ 30S; 95 ℃ 15S; 60 ℃ 60S; 95 ℃ 15S. 40 cycles	10µl 2 x qPCRmix; 0.5µl forward primer (5pmol/µl); 0.5µl reverse primer (5pmol/µl); 2µl DNA template; 7µl sterile distill water
phoD	*alpsF*	*CAGTGGGACGACCACGAGGT*	370 bp	(Fraser et al., 2017; Zhang et al., 2020c)		

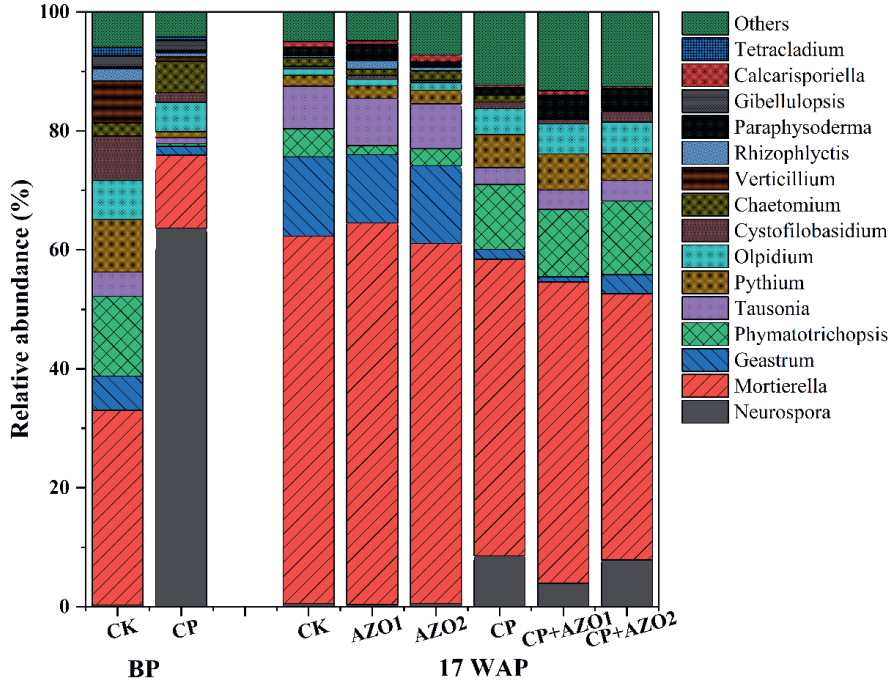


Fig. S5.1 Relative abundance of the top 15 genera of fungi. Treatments include CK (untreated soil), AZO1 (a single application of AZO 8 weeks after planting (WAP)), AZO2 (double applications of AZO at 8 and 16 WAP), CP (CP fumigated soil without AZO), CP+AZO1 (CP combined with AZO1) and CP+AZO2 (CP combined with AZO2). Sample times were BP (before planting) and 17 WAP (17 weeks after planting).

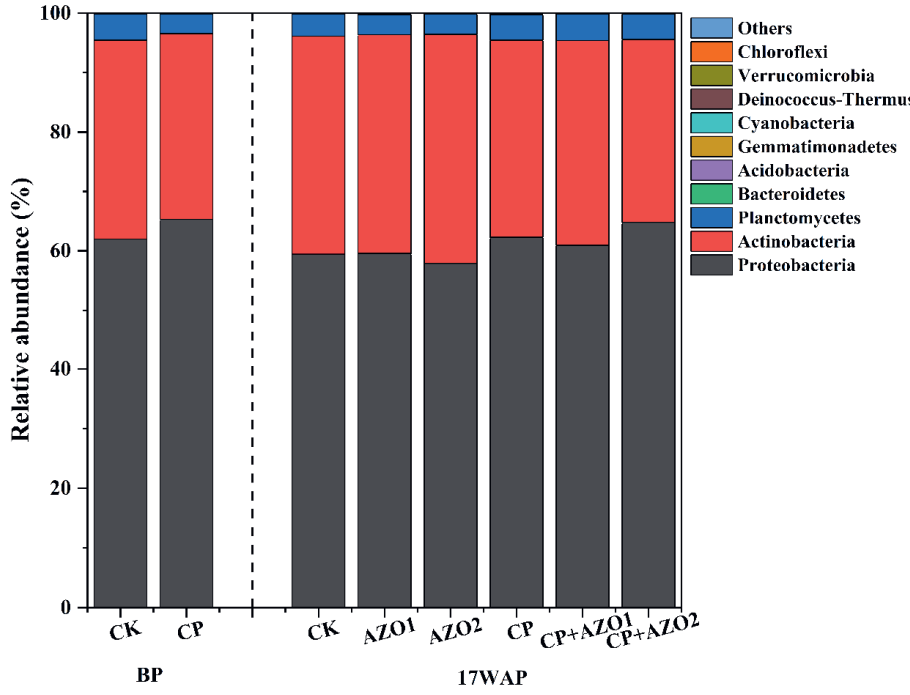


Fig. S5.2 Relative abundance of the top 10 phyla of *phoD*-containing bacteria. Treatments include CK (untreated soil), AZO1 (a single application of AZO 8 weeks after planting (WAP)), AZO2 (double applications of AZO at 8 and 16 WAP), CP (CP fumigated soil without AZO), CP+AZO1 (CP combined with AZO1) and CP+AZO2 (CP combined with AZO2). Sample times were BP (before planting) and 17 WAP (17 weeks after planting).

Chapter 6

Synthesis

6.1 Main findings

The scientific question of this PhD project is how does soil fumigation impact soil phosphorus (P) availability under different agroecosystems, and more specifically, under real farmlands with different fumigation histories, and soils with combined application of fumigants and fungicides. Ginger was chosen as the model plant in this thesis due to the serious soil-borne diseases affecting ginger growth and the wide-spread use of soil fumigants during ginger cultivation. The main findings of this thesis are shown in **Fig.6.1**.

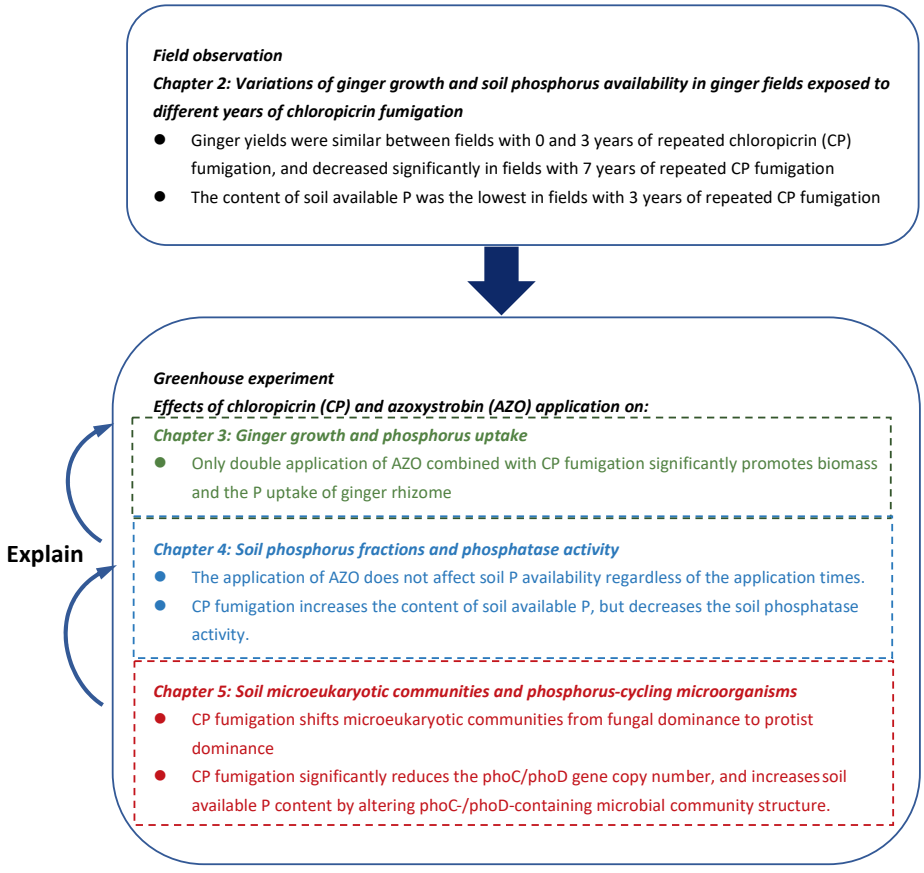


Fig. 6.1 An outline of the main findings in this thesis. AZO: azoxystrobin; CP: chloropicrin; P: phosphorus.

In **Chapter 2**, we first conducted field observations from ginger fields exposed to 0, 3 and 7 years of annual CP fumigation (F0, F3 and F7) to study ginger yield and soil P availability under the real farmland conditions. We found that the average ginger rhizome yields in F0 and F3 were almost the same at 70 t ha^{-1}, while the ginger rhizome yield of F7 was the lowest at 37.5 t ha^{-1}. The proportions of soil labile P fractions (Resin-P+$NaHCO_3$-P+NaOH-P) to total P was the lowest in F3, which may become an important

limiting factor for ginger yield. After 7 years of continuous CP fumigation, plant mortality became the main reason for the low ginger yields and the recovery of soil labile P fractions content.

In ginger fields of Chapter 2, farmers also use fungicides during the growing season. Therefore, in **Chapter 3**, we conducted a greenhouse experiment to study the effects of chloropicrin (CP) fumigant in combination with the application of azoxystrobin (AZO) fungicide on ginger growth and P uptake. The results showed that in soils without CP fumigation, AZO had no effect on ginger growth or P uptake regardless of the application times. Only CP fumigation combined with double applications of AZO significantly increased the biomass and P uptake of ginger rhizome. The physiological P use efficiency of ginger decreased with the application of the CP fumigant and AZO fungicide, indicating that, although ginger could uptake more P after CP and AZO application, the absorbed P was used mainly for shoot growth instead of rhizome production.

Changes in ginger growth and P uptake may be determined by the P cycling processes in the soil system. Therefore, in **Chapter 4**, we went deeper into the soil system to explore the variations of soil P availability including soil P fractions and acid and alkaline phosphatase activity. Results showed that the contents of total P were from 1.1 to 2.0 g kg^{-1} and no significant difference was observed between different treatments. CP fumigation increased the proportions of soil labile P (especially the organic P fraction $NaHCO_3$-Po) to total P by 2.9% ~ 15.5% as compared to untreated soils. AZO application did not affect soil phosphatase activity until 17 weeks after planting, regardless of whether the soil was CP fumigated or not, while the phosphatase activity of untreated soil was 1.1 ~ 1.5 times higher than that of CP fumigated soils. The increases in the proportions of organic P may have been due to the decreases in soil phosphatase activity, while the non-significant difference in the proportions of soil Resin-P may have been caused by the increases in the P uptake of ginger in the CP fumigated soils. The higher contents of soil labile inorganic P in CP-fumigated soils may have also been attributed to the lower soil pH values there, which will be explained later.

The production and activity of acid and alkaline phosphatases are mainly regulated by the *phoC* and *phoD* genes in soil bacteria. Therefore, in **Chapter 5**, we further analyzed the abundances of *phoC* and *phoD* genes and the *phoC*-/*phoD*-containing bacterial community structures. Soil microeukaryotic community structure was also considered since it plays a key role in plant health. The results showed that, similar to ginger growth and soil P availability, AZO application did not change the soil microbial communities regardless of the application times and whether the soil was CP fumigated or not. CP fumigation significantly reduced the *phoC*/*phoD* gene copy number. The *phoC* gene copy number was significantly positively correlated with the acid phosphatase activity, and the *phoD* gene copy number was significantly positively correlated with the alkaline phosphatase activity, indicating that CP fumigation reduced soil phosphatase activity by reducing the *phoC*/*phoD* gene copy number. CP fumigation significantly altered the *phoC*-/*phoD*-containing bacterial community structure and finally increased soil labile P content. The results showed that the soil microeukaryotic community shifted from fungal dominance to protist dominance, suggesting that CP fumigation improves ginger health by killing harmful pathogens and promoting pathogen predators.

Overall, field observations indicate that CP fumigation loses its efficiency between 3 and 7 years of continuous applications. However, the results of greenhouse experiment showed that in soils that had

never been planted with ginger, CP fumigation increased soil labile P content and reduced harmful soil fungal pathogens, but there was no significant difference in ginger yield. Evidence from this PhD project shows further insight into the side effects of soil fumigation on soil health in agroecosystems. Based on plant health and soil P availability, this study can provide basic support for integrated agricultural management practices to optimize soil-borne disease control and soil fertilization.

6.2 General discussion

Chemical fumigants and fungicides are widely used to protect crops from soil-borne diseases caused by harmful soil pathogens. However, due to their broad-spectrum activity, fumigants can also adversely affect beneficial non-target microorganisms in soil systems, which may have detrimental effects on soil functions such as nutrient cycling processes. Soil phosphorus (P) is one of the most important nutrients for plants, and its cycling processes are controlled by soil P solubilizing microorganisms that could also be influenced by fumigants. To date, the side effects of soil fumigation on the soil P cycling processes remain largely unclear. Therefore, this thesis assessed the impact of soil fumigation on soil P availability, filling knowledge gaps concerning the side effects of repeated fumigation and multiple pesticide application on soil P cycling. Unlike most previous studies that were conducted only in bare soil systems, our experiments were performed in soil-plant systems, where plant-soil-microorganisms interact and influence each other (**Fig 6.2**).

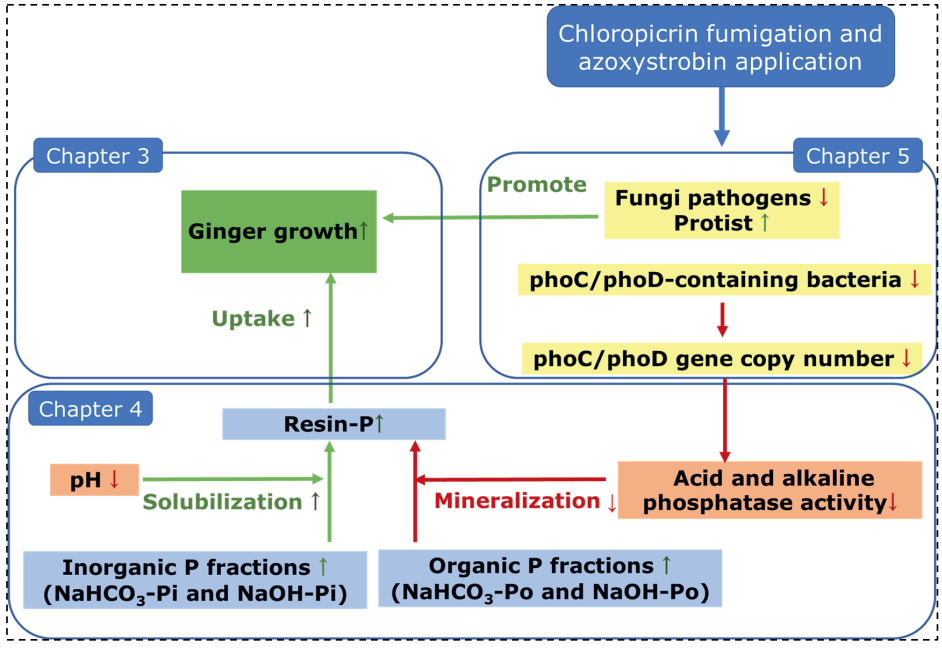


Fig 6.2 Changes in soil phosphorus (P) transformation processes and soil microbial communities in soils with chloropicrin fumigation and azoxystrobin application, and their effects on ginger growth.

Effects of soil fumigation on ginger growth and the underlying processes

The purpose of soil fumigation is to improve crop health and yield. We hence first observed the growth of ginger in field observation and greenhouse experiments. From the field observation in Chapter 2, we found no significant difference in the ginger yield among fields with 0 and 3 years of continuous CP fumigation (F0 and F3), while the ginger yields decreased significantly in fields with 7 years of continuous CP fumigation (F7). Our result is in line with a study conducted by Zhang et al. (2020b), who found that the strawberry yield increment varied with different consecutive years of CP fumigation with a maximum of 2.0 kg m^{-2} after 2 years, then decreased to 0.9 kg m^{-2} after 5 years. In our study, the lowest proportion of soil labile P in F3 may have been the main limiting factor for getting a higher ginger yield in F3. However, the high mortality caused by severe soil-borne diseases was the main reason for the lowest ginger yield in F7, suggesting that the inhibitory effect of CP fumigation on soil-borne diseases was greatly reduced between 3 to 7 years of continuous CP fumigation.

In Chapter 3, greenhouse experiment results showed that the application of AZO had no significant effect on ginger growth regardless of the application times and whether the soil was CP fumigated or not. This may be due to the low levels of AZO and the fast degradation time of AZO in our experiment. Unlike previous studies conducted in a lab, our greenhouse experiment was exposed to higher temperatures, more sunlight during the summer season and more phytoremediation by ginger plants, resulting in an AZO half-life of only 7 days. In addition, the AZO fungicide is a kind of preventative fungicide that should be used before symptoms of soil-borne diseases appear. Liu et al. (2021) found that a single foliar spray of 167 g ai. hm^{-2} of AZO 14 days after planting did not prevent the damping-off of sugar beet seedlings.

The height of ginger plants was significantly higher in soils with CP fumigation, while the biomass and P uptake by ginger rhizome were significantly increased only when CP fumigation was combined with double applications of AZO. The physiological P use efficiency of ginger decreased after CP fumigation and AZO application (Chapter 3). Changes in ginger growth and P use efficiency may be determined by soil microbial community structure and soil P cycling processes, which were altered by CP fumigation and AZO application.

Firstly, CP fumigation and AZO application improved ginger health and growth by killing soil pathogens. In this thesis, we found that CP fumigation significantly decreased the relative abundance of fungi such as *Ascomycota* and *Basidiomycota* that are pathogenic, saprophytic and endophytic microbes which can cause serious plant diseases. On the contrary, the relative abundance of some soil protists, especially *Ciliophora* and *Cercozoa*, increased significantly in CP-fumigated soils (Chapter 5). *Ciliophora* and *Cercozoa* are two important phagocytic protists (consumers) that can control soil-borne diseases by preying on pathogenic bacteria or fungi, or by secreting bacteriostatic metabolites (Wu et al., 2022a). Therefore, after CP fumigation, the soil microeukaryotic community changed from fungal dominance to protist dominance, suggesting that soil pathogen pressure was reduced by the decreases in the pathogenic fungi and increases in the pathogenic fungi consumers and ultimately increasing ginger production (Chapter 5).

The inhibitory effect of CP fumigation on pathogens is quiet shortly after CP fumigation. For example,

in Chapter 5, we found that the relative abundances of pathogenic fungi such as *Phymatotrichopsis* and *Phythium* were significantly lower in control samples (*Phymatotrichopsis*: 13.4%; *Phythium*: 8.9%) than in CP-fumigated soils (*Phymatotrichopsis*: 0.4%; *Phythium*: 1.0%) at the beginning of the experiment. 17 weeks after planting, the relative abundance of *Phymtotrichopsis* increased to 10.9% ~ 12.4%, while the relative abundance of *Phythium* increased to 4.5% ~ 6.1% in CP-fumigated soils. This result was confirmed by many previous studies that also found that pathogen populations rose again as the soil microbiome recovered. For example, Zhu et al. (2021) found that more than 95% of *Fusarium Oxysporum* were killed immediately after CP fumigation at 40 mg kg^{-1}, while the inhibition rate decreased to 78% at 40 days after CP fumigation. However, in the early seedling stage, the ginger shoot and leaves are dominated, while ginger rhizome is in the later rhizome vigorous growth stage (Nair, 2019). Therefore, with the recovery of soil-borne pathogens, CP fumigation may be more beneficial to the early ginger shoot growth than the later rhizome growth.

Apart from killing soil-borne pathogens, CP fumigation can also increase the proportions of soil labile P, which may provide more available P for ginger uptake (Chapter 4, as explained later). However, the available P released at the early stage was mainly used for shoot growth rather than later rhizome growth, resulting in lower physiological P use efficiency (PPUE) of ginger in CP fumigated soils (Chapter 3). In addition, the lower PPUE in CP-fumigated soils may also be attributed to the possible reduction of AMF that is responsible for the mycorrhizal uptake pathway that transfers soil P to ginger (Ipsilantis et al., 2012; Smith et al., 2011). However, the details of this mechanism need further study. A previous study suggested a reduction in the amount of P fertilizers used during the early growing stages after CP fumigation (<30 days) (Huang et al., 2020a). However, the reduction of P fertilizers at the ginger seedling stage may further aggravate the soil P deficiency during the growth stage of the ginger rhizome.

The insignificant difference in ginger biomass caused by CP fumigation may also be due to the initial health of the soils we used. In the greenhouse experiment, we selected fields that had never been planted with ginger before, and that did not have many soil-borne pathogens that could cause ginger diseases. Therefore, the differences in the number of soil-borne pathogens may not be so great that they could significantly change ginger yields. However, other studies conducted in soils heavily infected with soil-borne diseases found that CP fumigation significantly increased crop yields. Mao et al. (2014) found that CP fumigation at 50 g m^{-2} significantly increased ginger yields by 4.77 kg m^{-2} compared to untreated soils. Zhang et al. (2019b) found that CP fumigation at 30 g m^{-2} increased strawberry yield by 1.5-2.0 kg m^{-2}.

To summary, CP fumigation is effective in the early stage, but loses it efficiency after continuous application for several years. The results emphasized the need to find more comprehensive agricultural management practices other than CP fumigation alone in order to control soil-borne diseases in ginger cultivation.

Effects of soil fumigation on soil P availability

Soil P is the only source of P for plants (Achary et al., 2017), indicating that the changes in P content in ginger and the P use efficiency of ginger may be determined by soil P cycling processes. Therefore, in order to explain the variations in ginger P uptake, we went down into the soil system to examine the

changes in soil P availability. In Chapter 2, field observation found that the proportions of plant labile P fractions (Resin-P+$NaHCO_3$-P+NaOH-P) were the lowest in the fields continuously applied with CP fumigation for 3 years. The labile P levels in fields that had been fumigated for 7 years recovered to the similar levels in fields that had never been fumigated before. The phosphatase (especially acid phosphatase) activity decreased with the increase of the CP fumigation history. Changes in soil P availability are determined by soil properties, plant uptake and other environmental factors. To explore the effect of CP fumigation on soil P availability and its mechanism, a greenhouse experiment was conducted after field observation.

From the greenhouse experiment, we found that the application of AZO at the field recommended rate did not affect the composition of soil P fractions regardless of the application times and whether the soil was CP fumigated or not. Soil phosphatase activity was not influenced by AZO application until 17 weeks after planting (Chapter 4). The results may be due to the low amount of AZO applied (47.1 mg m^{-2}) every time and the short time between application and sampling. This result was in line with a previous study which found that the application of AZO at a rate of 2.0 mg kg^{-1} had no significant effect on the phosphatase activity on the 7th day of incubation in Spodosols (Wang et al., 2019).

However, CP fumigation significantly increased the proportions of easily labile P (Resin-P) and labile P ($NaHCO_3$-P) and decreased the proportions of unavailable P (Occluded-P), which means that soil P became more available for ginger utilization after CP fumigation (Chapter 4). The variations of different soil P fractions may be the result of changes in the soil microbiome (Chapter 5) and ginger P uptake (Chapter 3). CP fumigation can improve the amount of soil available P by killing microorganisms and breaking down microbial cells, releasing the organic P inside microbial cells into soils (Huang et al., 2020a). Organic P in microbial cells is composed of 50%-75% nucleic acid (e.g., DNA, RNA), 20% P monoesters and 5% phospholipids (Weihrauch et al., 2018). The released nucleic acid could be extracted by a 0.5 M $NaHCO_3$ solution ($NaHCO_3$-Po) while the released monoesters could be extracted by a 0.1 M NaOH solution (NaOH-Po). $NaHCO_3$-Po is unstable after being released into the soil and is easily mineralized by phosphatase (such as acid and alkaline phosphatases). However, phosphatase activity was significantly reduced in CP-fumigated soils, which resulted in the accumulation of soil organic P fractions, especially $NaHCO_3$-Po.

The production and activity of acid and alkaline phosphatases were regulated by the *phoC* and *phoD* genes, respectively. Therefore, the decreases in phosphatase activity may be due to the reduced amount of *phoC* and *phoD* genes that occurred together with soil microbial death. In order to verify our hypothesis, in Chapter 5, we quantified the gene copy number of *phoC* and *phoD* and found that CP fumigation significantly decreased *phoC* and *phoD* gene copy number. We also found that there were significantly positive correlations between acid phosphatase activity and *phoC* gene copy number, as well as between *phoD* gene copy number. The results confirmed that the decrease in *phoC* and *phoD* gene numbers was the main reason for the decrease in acid and alkaline phosphatase activity. The findings are also consistent with a previous study that found CP fumigation at 53 mg kg^{-1} and 106 mg kg^{-1} significantly reduced *phoD* gene copy number, even after 77 days of incubation (Huang et al., 2020a).

Then we sequenced the *phoC*-/*phoD*-containing bacteria (Chapter 5). Different soil bacteria showed different responses to CP fumigation and AZO application based on their different tolerances to

pesticide toxicity and their abilities to degrade pesticides (Huang et al., 2020a). The relative abundance of some *phoC*-containing bacteria that can form spores to resist environmental stress (Castellano-Hinojosa et al., 2021) such as *Firmicutes*, or that is highly resistant to antibiotics and toxic compounds (Guillaume, 2018) such as *Abditibacterium,* increased significantly in CP-fumigated soils. The relative abundance of some *phoD*-containing bacteria, such as *Streptomyces*, *Ralstonia* and *Sinorhizobium*, also increased significantly after CP fumigation, which may be due to their ability to degrade CP and use CP as a nutrient source (Apel et al., 2007). *Sinorhizobium* can produce organic acids, especially indole-3-acetic acid (IAA), to dissolve phosphate rock into available phosphorus (Bianco et al., 2010). The heatmap analysis also found that the increase of *Sinorhizobium* and *Streptomyces* positively contributed to the increase of the soil labile P proportions, especially $NaHCO_3$-Po, in CP-fumigated soils. In addition, *Streptomyces* are biocontrol agents used against soil-borne pathogens to increase crop yields (Wang et al., 2022; Zhang et al., 2021a). Therefore, CP fumigation could improve soil P availability and reduce ginger diseases by increasing the relative abundance of these P solubilizing microorganisms.

Soil chemical properties, especially soil pH, also determine soil P cycling processes. The optimum pH for the highest soil P availability is 6.0 to 6.5 (Weihrauch et al., 2018). CP fumigation could lower soil pH by breaking down microbial cells and releasing internal organic acid. The increased H^+ in CP-fumigated soils reacts with and dissolves the Ca-P-minerals, and those released organic acids could also promote the dissolution of Fe/Al-P-minerals through ligand exchange (Weihrauch et al., 2018). In our greenhouse experiment, soil pH was significantly lower in CP-fumigated soils (6.6) than in CK (6.9) 9 weeks after planting. The redundancy analysis in Chapter 2 showed that the lowest proportion of plant available P in F3 was attributed to the highest pH values there, while the random analysis in Chapter 4 showed that pH was the most important factor affecting the proportions of $NaHCO_3$-Pi and NaOH-Pi. Therefore, the increased proportions of inorganic P fractions in CP-fumigated soils may be attributed to the decreased soil pH after CP fumigation.

In addition to soil microbiome and soil chemical properties, ginger plants also affect the soil P availability, as plant uptake is one of the most important outputs of soil P in agricultural systems. Plant uptake and harvesting take away about 20-50 kg P hm^{-2} per year from agricultural soil (Kruse et al., 2015). Therefore, effects of soil fumigation on soil P cycling processes could be modified by plant P uptake. For example, in our greenhouse experiment, there was no significant difference in the absolute content of Resin-Pi and $NaHCO_3$-Pi among different treatments during the whole experiment (**Fig. S4.1**), which was different from the results of incubation experiments conducted by Huang et al. (2020a), where CP fumigation significantly increased the amount of available soil P. These different findings may be due to the presence of ginger plants in our experiment. While CP fumigation increased the amount of soil labile P, higher ginger P uptake in CP-fumigated soils indicated that more soil available P was taken away from the soil system, which resulted in no significant difference in the residual available P in the soils with and without CP fumigation.

In our greenhouse experiment, irrigation during ginger cultivation may have been another factor influencing soil P availability. In our experiment, the increased soil available P in the CP-fumigated soils was not absorbed by ginger and would leach out of the pot along with the irrigation water, especially in the early growing stage when the P present in rhizome seeds was sufficient enough to meet the needs of the ginger plants (Chapter 3). Huang et al. (2020a) also found that CP fumigation increased P leaching

from the soil column. In our field observation, significant reductions in ginger yield and P uptake in F7 resulted in more labile P fractions being stored in the soil (Chapter 2), which could lead to severe environmental problems such as water eutrophication caused by surface runoff or P leaching into the groundwater (Zhang et al., 2021b).

6.3 Implications

This thesis focused on the knowledge gaps in the effects of soil fumigation on soil P availability, which can provide practical information on the use of pesticides and fertilizers to avoid the overuse of pesticides and reduce unnecessary costs for farmers. For example, field observations found that CP fumigation lost its effectiveness between 3-7 years of continuous application, suggesting that continuous fumigation in the ginger fields is unreasonable. After a few years (3 - 7 years), ginger fields need to be rotated or fallow for a period of time to restored the productivity. In addition, if farmers want to grow ginger in new fields where ginger has never been grown before, soil fumigation is not required for the first year, as there was no significant increase in ginger yield in CP-fumigated soils in our greenhouse experiment. Our research could reduce farmers' reliance on pesticide retailer recommendations and the overuse of soil fumigation.

This study may also be of interest to other stakeholders such as researchers, pesticide producer and policymaker. For example, environmental science researchers concerned with the side effects of pesticides on soil health. Researchers who want to focus on crop protection may be interested in our study since it can offer practical information on how to improve ginger health and yield by applying fumigants and fungicides as well as providing information on how to adjust fertilizer applications. This project could also provide pesticide producers with feedback on actual pesticide behavior to help them improve the efficiency of future pesticides to better suppress soil-borne diseases. This project is crucial for the development of more comprehensive polices and recommendations for the prevention and control of soil-borne diseases in ginger cultivation. For ginger growers, the excessive and unnecessary use of pesticides and fertilizers could be avoided, economic losses could be reduced, and profits could be increased. The side effects of using pesticides are often overwhelming to the soil system and policy makers should make more sustainable rules controlling the use of different fumigants and fungicides.

6.4 Recommendations

Although soil fumigation kills nearly all soil microorganisms, it remains one of the most efficient and economical ways for farmers to boost ginger production (Gupta et al., 2017; Meenu et al., 2017). Therefore, how to improve the effectiveness of soil fumigation on crop production and reduce the adverse impact on the surrounding environment should be further concerned.

Organic fertilizers (such as humic acid and animal manure) or microbial fertilizers containing beneficial microorganisms could be applied to fumigated soils (Cheng et al., 2020) to mitigate the side effects of fumigants and promote the recovery of soil beneficial microorganisms. Our field observations found that CP fumigation lost its effectiveness on ginger production between 3-7 years of continuous application, therefore, crop rotation could be used during 3 - 7 years of continuous soil fumigation to preventing the accumulation of pathogens due to ginger monocultures. However, further research is

needed on the rotation sequence of crops and the rotation length for ginger cultivation to maximize the ginger yield and minimize the presence of soil-borne diseases. Biological agents consisting of plant growth-promoting microorganisms (such as *Bacillus* and *Trichoderma* species), or plant-related compounds with antimicrobial activities (such as root exudates and plant extracts) are gradually replacing chemical fungicides and fumigants (Huang et al., 2021; Kalhoro et al., 2022). While biocontrol appears to be a kind of sustainable agricultural management practice, the high targeting specificity of these biocontrol agents may lead to different combinations of agents, while the low efficiency, high vulnerability to the soil environment, and high price may also become major barriers to farmer acceptance.

In China, there are more and more large-scale farms owned by big food companies, supermarkets or single farmers, which have been the pioneers of modern agriculture practices such as organic and precision farming. Organic farming aims to eliminate the side effects of agrochemicals on soil ecosystems and improve food health by reducing inputs of pesticides and synthetic chemical fertilizers, which could lead to lower crop yields (Knapp et al., 2018). This lower yield could be offset by lowering the cost of agrochemicals and increasing food quality and price. Higher prices may then become another barrier that prevents consumers from choosing organic food. Therefore, a holistic policy based on all parts of the food chain should be developed to overcome the conflicts between environmental protection and economic promotion (Möhring et al., 2020). Precision farming with a decision-supporting system can strategize the application of pesticides or fertilizers based on the needs of crop growth, rather than calendar applications, thereby reducing unnecessary use of agrochemicals without affecting crop yields (Lázaro et al., 2021).

However, 70% of arable land is still cultivated by smallholder farmers in China (Jiao et al., 2019), which makes the wide application of these modern agricultural methods extremely difficult. Farmers normally have to prioritize economic income as they need to survive, which for them is more important than protecting the environment. Protecting the environment by reducing the use of chemical fertilizers or pesticides sometimes means lower crop yields, thus sacrificing farmers' economic interests. At this time, the government and factories should have the responsibility of protecting the environment and ensuring farmers' incomes. For example, governments can provide financial subsides to pesticide producers to use new technologies and methods based on the scientific research to produce cleaner and more efficient pesticides, but not more expensive ones.

Pesticide producers should make product labels on the products clearer to make sure that farmers know how to apply the pesticides efficiently and in an environmentally friendly manner. Local farmers, especially those in developing countries, are often poorly educated and take advice from pesticide retailers who are always profit-oriented. During our sampling, ginger farmers told us that once soil fumigation started, the fields were destroyed, and no other crops could be grown in those fields. Unfortunately, soil fumigation does not work after several years of continuous fumigation. On the other hand, farmers can't stop fumigating soil because if they don't fumigate the soil, the ginger diseases will become overwhelming. However, if fumigation is carried out before planting, there may be some hope. Farmers became really anxious when the ginger plants got sick and the retailers just told them to use more pesticides. The farmers didn't know how the pesticides worked exactly and they just blindly threw different pesticides at the problem. The professional advice provided by experts and given directly to

farmers, as well as the government regulation of pesticide producers and retailers, needs to be strengthened to avoid overuse or misuse of agrochemicals.

Although effective in controlling soil-borne disease, CP has been banned in the EU due to its high toxicity to the surrounding environment. China is also considering banning CP. However, in order to meet the needs of a growing population and ensure food safety, pesticides cannot be fully banned. The most important thing our scientists can do is to find more effective and cleaner pesticides. To achieve this goal, interdisciplinary cooperation should be strengthened. Nowadays, crop scientists focus only on how to improve crop yield through the use of different kinds of agricultural practices, such as new pesticides. When the crop yields are as expected, they say it is a good method and product. However, they are usually less concerned about the side effects of these new products on the surrounding environment. Environmental scientists focus only on risk assessments for these emerging products, often recommending banning these agrochemicals because of their adverse effects on the environment. This may be why we cannot always find an efficient and environmentally friendly way to produce crops. In addition, it is more difficult to scale new products and agricultural management practices developed in the laboratory to actual production situations. All stakeholders in agricultural production, such as pesticide producers, retailers, farmers, consumers and policymakers, should collectively consider the trade-off between environmental protection and economic benefits.

6.5 Research challenges and future studies

This thesis revealed the side effects of soil fumigation on ginger growth and soil phosphorus availability. However, after developing a better understanding of the side effects, we are still unable to give specific advice on how to better use soil fumigation or adjust P fertilizers accordingly, nor can we suggest a better alternative to soil fumigation in this thesis. Based on our project and thesis, in order to find an environmentally friendly way to maximum crop yields, several studies need to be carried out:

1) We now know the changes that happen in soil P availability after soil fumigation, but we need to discover how to adjust P fertilizer application accordingly. From this thesis, we know the soil P solubilizing microbial community composition, but the molecular mechanisms that alter soil P availability remain unclear. In addition, we need to discover how to use keystone species to improve soil P availability and reduce ginger diseases.

2) Soil-borne diseases may alter the uptake, translocation, distribution and utilization of nutrients by crops. Conversely, changes in soil nutrient availability induced by fertilization may also affect plant disease severity by altering soil-borne pathogens or plant disease resistance. The effects of fertilization on plant diseases vary with plant species so further research should be conducted to develop specific fertilization menus for each crop to meet plant nutritional and disease suppression needs.

3) Integrated agroecosystem management practices combined with agricultural management practices, biological control, chemical control and genetic tools should be developed to promote soil health and maximize crop production.

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English summary

Ginger is an important cash crop in China with high economic, edible, and medicinal values. Due to the intensive monoculture, ginger plants are seriously affected by various soil-borne diseases such as root rot and leaf wilt, leading to serious death of ginger and economic losses to farmers. Soil pretreatment with chemical fumigants is one of the most effective and popular methods to prevent soil-borne diseases of ginger, promote ginger health and increase the income of ginger farmers.

Soil fumigation reduces ginger soil-borne diseases by killing harmful soil pathogens. However, due to their broad spectrum of killing, fumigants may also have detrimental effects on soil beneficial microorganisms that play critical roles in soil functions such as soil nutrient cycling. Soil phosphorus (P) is one of the most important nutrient elements for plant growth, and its cycling process is controlled by soil P solubilizing microorganisms that may be affected by soil fumigation. However, the effects of soil fumigation on soil P cycling processes remain largely unclear. Understanding the transformation of soil P after soil fumigation is important for the risk assessment of fumigants, and can provide a theoretical basis for optimizing fertilization. Therefore, this thesis aimed to investigate the effect of soil fumigation on soil P availability under different agroecosystems including real farmlands with different fumigation histories, and soils where fumigants and fungicides are applied in combination.

To explore changes in ginger growth and soil P availability with different soil fumigation histories, in **Chapter 2**, field observations were conducted in ginger fields that were continuously fumigated with chloropicrin (CP) for 0, 3 and 7 years (F0, F3 and F7). The results showed that the average ginger rhizome yield of F0 and F3 was not significantly different at 70 t ha^{-1}, while the ginger rhizome yield of F7 significantly decreased to 37.5 ha^{-1}. However, the proportions of soil available P fractions (Resin-P+$NaHCO_3$+NaOH-P) was the lowest in F3, probably due to the highest pH values there. The highest mortality in F7 was the main reason for the lowest yield of ginger.

The differences in soil P availability observed in the fields might be determined by the changes in the soil P cycling processes and the activities of soil P solubilizing microorganisms caused by different CP fumigation histories. Therefore, after field observations, we further conducted a greenhouse experiment under control conditions to estimate the side effects of CP fumigation on ginger growth, soil P availability and its microbial mechanisms. In addition, azoxystrobin (AZO), a widely used fungicide in ginger fields, was also added in the greenhouse experiment to further analyze the combined effects of CP fumigant and AZO fungicide on soil P availability.

The purpose of soil fumigation is to promote ginger growth. Therefore, in **Chapter 3**, we first explored the ginger growth condition and P uptake under chloropicrin (CP) fumigation and azoxystrobin (AZO) application in the greenhouse experiment. The results showed that CP fumigation had no effect on the degradation of AZO in soil. A single application of AZO did not alter ginger biomass and P uptake regardless of whether the soil was CP fumigated or not, which may be due to the low application rate and fast degradation rate of AZO (half-life of 7 days) in our experiment. CP fumigation significantly increased ginger height, while only CP fumigation combined with two times application of AZO significantly increased ginger rhizome yield and P uptake. The ginger physiological P use efficiency

decreased after the application of CP and AZO, indicating that the absorbed P was more used for ginger shoot growth rather rhizome production.

The changes in ginger P uptake and P use efficiency may be due to changes in soil P cycling processes. Therefore, in **Chapter 4**, in order to explain the variation of ginger P uptake and P use efficiency, we went down into the soil system to investigate soil P availability under CP fumigation and AZO application in the same greenhouse experiment. Soil P availability was quantified by different soil P fractions extracted by Hedley's sequential extraction methods, as well as two phosphatase (acid and alkaline phosphatase, AiP and AlP) activity. The results showed that the application of AZO had no significant effect on soil P availability. The proportions of soil labile P fractions (Resin-P+$NaHCO_3$-Pi+$NaHCO_3$-Po) to total P in the CP-fumigated soil were 2.9% ~ 15.5% higher compared with the untreated soils. The accumulation of organic P fractions ($NaHCO_3$-Po) after CP fumigation and AZO application may be due to decreased soil phosphatase activity. In soils not subjected to CP fumigation, AiP and AlP activities were significantly reduced by AZO application 17 weeks after planting, while AiP and AlP activities were 1.1 ~ 1.5 times higher in untreated soils than in CP-fumigated soils throughout the experiment. In CP-fumigated soils, the main reason for the high content of soil available inorganic P ($NaHCO_3$-Pi and NaOH-Pi) was the lower soil pH value, while the lack of significant differences in the proportion of Resin-P may be due to the increased P uptake by ginger.

The production and activity of acid and alkaline phosphatase are controlled by bacterial *phoC* and *phoD* genes. Therefore, to explore the microbial mechanisms underlying soil P transformation and changes in soil phosphatase activity, in **Chapter 5**, we first quantified the gene copy numbers of *phoC* and *phoD* using real-time quantitative polymerase chain reaction (qPCR). Similar to ginger growth and soil P availability, the results showed that AZO application at the recommended amount had no significant effect on soil microbial community structure. We found that the gene copy numbers of *phoC* and *phoD* were significantly reduced by CP fumigation. The *phoC* gene copy number was significantly positively correlated with AiP activity, and the *phoD* gene copy number was significantly positively correlated with AlP activity, suggesting that the decrease in the number of *phoC* and *phoD* genes was the main reason for the decrease in AiP and AlP activity. The *phoC*-/*phoD*-containing bacteria were then sequenced using high-throughput sequencing method. The results showed that CP fumigation significantly increased the relative abundance of *Sinorhizobium* and *Streptomyces* that have significant positive effects on soil available P compositions, suggesting that CP fumigation could alter the *phoC*-/*phoD*-containing bacterial community structure, thereby determining soil available P content. We also sequenced soil microeukaryotic community structure using 18s rRNA high-throughput sequencing and found that CP fumigation decreased the relative abundance of harmful fungal pathogens but increased the relative abundance of protists that are fungal consumers. s

In conclusion, CP fumigation increased the soil available P content in the short term, while the addition of AZO fungicide did not show a significant synergetic effect. However, CP fumigation is not a sustainable method to increase soil P availability because of its inhibitory effect on soil phosphatase activity, as evidenced by the lowest soil P availability in fields with 3 years of continuous CP fumigation, which may become an important growth limiting factor for higher ginger yields. However, after 7 years of continuous CP fumigation, ginger mortality became the main growth limiting factor, so finding an integrated agricultural management practice rather than only CP fumigation to control soil-borne

diseases should be considered first.

This thesis focused on knowledge gaps regarding the side effects of soil fumigation on soil phosphorus availability in agroecosystem. The outcome of this thesis can provide theoretical support for optimizing soil-borne disease control and fertilization regulation. However, how to adjust fertilization after soil fumigation, or the effect of soil fertilization on soil-borne diseases, and how to adjust soil fumigation accordingly still need further research. Integrated agroecosystem management practices combined with agricultural management practices, biological control, chemical control and genetic tools should also be developed to promote soil health and maximize crop production.

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About the author

Yan Wang was born on October 21, 1990, in Shandong, China. In 2010, she started to study Environmental Engineering at Shandong University in Jinan, the capital city of Shandong province. After four years' bachelor study, she started the MSc programme in Environmental Engineering at Ocean University of China in Qingdao. In September 2017, she joined the National Engineering Laboratory for Improving Quality of Arable Land group of Chinese Academy of Agricultural Sciences to started her PhD study under the project "Wageningen University-CAAS joint PhD programme". In February 2018, she came to the Soil Physics and Land Management group at Wageningen University and became a SLMer. During her PhD, she worked on the effects of soil fumigation on soil phosphorus availability in ginger cultivation.

Publications

1. **Wang Y**, Yang X, Xu M, Geissen V (2022) Effects of chloropicrin fumigation and azoxystrobin application on ginger growth and phosphorus uptake. Ecotoxicol Environ Saf 232: 113246. doi: 10.1016/j.ecoenv.2022.113246.

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born on 21st of October 1990 in Shandong, China

has successfully fulfilled all requirements of the
educational PhD programme of SENSE.

Wageningen, 2nd November 2022

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SENSE PhD Courses

- Environmental research in context (2018)
- Research in context activity: 'Organise a workshop with local farmers on soil fumigation and fertilization in the ginger production' (2022)

Other PhD and Advanced MSc Courses

- Effective Academic Development Including Academic Writing and Presenting in English, WUR & CAAS (2017)
- Applied multivariate analysis: data mining, Liege university & Chinese Academy of Agricultural Sciences (2017)
- Guide to writing scientific papers, Liege university & Chinese Academy of Agricultural Sciences (2017)
- Scientific Writing, Wageningen Graduate Schools (2018)
- Information Literacy for PhD candidates including EndNote Introduction (2018)
- Course Design of Experiments, WIAS and PE&RC (2018)
- Basic Statistic, PE&RC and WIMEK (2018)
- Soil Ecology, PE&RC and WIMEK Netherlands (2022)

Oral and Poster Presentations

- *Effects of chloropicrin fumigation and azoxystrobin application on ginger growth and soil phosphorus availability*. EnvChem2022, 14-15 July 2022, York, United Kingdom
- *Response of Ginger Growth and Soil Phosphorus Availability to the Different Years of Chloropicrin Fumigation* & *Response of Soil Phosphorus Availability and Ginger P Uptake to the combined Application of Chloropicrin and Azoxystrobin*. SETAC Europe 32nd Annual Meeting, 15-19 May 2022, Copenhagen, Denmark

SENSE coordinator PhD education

Dr. ir. Peter Vermeulen

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