Bioactivity of the Novel Fungicide SYP-14288 Against Plant Pathogens and the Study of its Mode of Action Based on Untargeted Metabolomics

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Abstract

Plant disease is a major threat to crop production, and fungicide application is one of the most effective methods to control plant disease. With emerging issues related to toxic residues and pathogen resistance, new fungicides with novel modes of action are urgently needed. SYP-14288 is a novel fungicide that could efficiently promote respiration and inhibit ATP biosynthesis in target organisms, but its bioactivity against various plant pathogens and exact mode of action are still unknown. In this study, we found that SYP-14288 is highly effective against 31 important plant pathogens belonging to a range of taxonomic groups. In addition, SYP-14288 has demonstrated excellent activity against all life stages of the important fungal plant pathogen *Magnaporthe oryzae* and is especially effective during the pathogen's high energy consumption stages. SYP-14288 showed good preventative control efficacy against pepper blight and rice blast in

the greenhouse and field, respectively. In an untargeted metabolomics assay designed to determine the exact mode of action of SYP-14288, significant changes occurred in 25 metabolites, with the accumulation of seven fatty acid metabolites and a decrease in 18 starch and sugar metabolites (e.g., from the tricarboxylic acid cycle). This suggests that SYP-14288 is an uncoupling agent similar to 2,4-dinitrophenol, which can allow for accumulation of various fatty acids after destroying oxidative phosphorylation coupling, thereby inhibiting the growth of the phytopathogen. These results indicate that the novel uncoupler SYP-14288 is a promising agrochemical in plant disease management.

Keywords: bioactivity, control efficacy, uncoupler, fatty acids, SYP-14288, untargeted metabolomics

Plant pathogens are among the many crop pests that significantly reduce crop yield and food quality. The principal method for plant pathogen control is fungicide application. The yields of most fruits and vegetables would be reduced by 50 to 95% without fungicide application, and fungicides are estimated to provide \$13 billion in annual income to the U.S. economy (Gianessi and Reigner 2006). In China, pesticide application has been increasing annually, and fungicides have become an integral part of efficient food production.

Many fungicides targeting different plant pathogens with various modes of action have been reported (Avenot and Michailides 2010; Bartlett et al. 2002; Becher and Wirsal 2012; Kumar et al. 2010); however, with emerging issues related to toxic residues, disease resurgence, and pathogen resistance (Bridges 1975; Clary and Ritz 2003; Fungicide Resistance Action Committee 2018; Gordon 2007; Hurley 1998), the use of many fungicides is restricted. Therefore, novel fungicides with low toxicity to nontarget organisms, high activity against target plant pathogens, and new modes of action are urgently needed.

SYP-14288, 2,4-dinitro-5-chloro-[1-(2,6-dichloro-4-nitroaniline)]-toluene (Supplementary Fig. S1), is a novel fungicide developed by

the China Shengyang Research Institute of Chemical Industry in 2012 to control microbial plant pathogens. SYP-14288 is undergoing the process of registration for the control of many plant diseases in China, and it effectively accelerates respiration and inhibits ATP biosynthesis in *Phytophthora capsici*. The chemical structure of SYP-14288 is similar to the standard uncoupler fluazinam; thus, it has been speculated to uncouple oxidative phosphorylation, but the exact mode of action of agrochemical uncouplers such as SYP-14288 is still poorly understood (Wang et al. 2018).

Uncouplers have played an important role in chemical control since the first uncoupler fungicide fluazinam was developed in the 1980s, and they are highly efficacious against a much broader spectrum of plant pathogens than any other type of fungicide (Liang et al. 2015). Fluazinam is the only oxidative phosphorylation uncoupling fungicide in common use today (Schepers et al. 2018). It demonstrates good control of a broad range of plant pathogens including fungi and oomycetes, and it has been registered on a wide range of plant diseases in China, such as potato blight, pepper anthracnose, and pepper blight (Shao et al. 2015). SYP-14288 has proven highly active against *Phytophthora capsici*, the causal agent of pepper blight (Wang et al. 2018). The antifungal activity of SYP-14288 against other plant-pathogenic fungi and oomycetes, however, is still unknown.

The metabolome can qualitatively and quantitatively describe the metabolic level of an organism when it is under biotic and abiotic stresses. The development of metabolomics provides a powerful analytical platform for the study of the mode of action of fungicides. Allen et al. (2004) used direct-injection mass spectrometry (MS) to analyze the metabolic footprints of ergosterol synthesis inhibitors, respiratory inhibitors, and oxidative phosphorylation uncouplers against *Saccharomyces cerevisiae*; the fungicides were correctly classified according to their mode of action by hierarchical cluster analysis. Gas chromatography/mass spectrometry (GC/MS)-based metabolic fingerprinting was used to cluster 14 fungicides, and most of them were assigned to the individual group that coincided with their modes of action according to Fungicide Resistance Action Committee code (Hu et al. 2019).

Metabolomics is a continually growing set of tools in the study of drug action modes, especially in natural antibiotics research. Yu et al.

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(2007) established a high-performance liquid chromatography-electrospray ionization/MS metabolic profiling method to determine the effect of *Tinospora capillipes* extract on nucleic acid synthesis. *Staphylococcus aureus* was treated with the extract of *T. capillipes* with an unknown mode of action and nine antibiotics with known modes of action. Metabolic profiles were used to analyze the mode of action of the extract, and it was found to be similar to that of rifampicin and norfloxacin, which interfere with nucleic acid synthesis (Yu et al. 2007). Birkenstock et al. (2012) used extracellular metabolomics and enzymology to elucidate the role of the pyruvate dehydrogenase complex of the antibiotic triphenylsulfonium dichloride.

In the current study, important fungal and oomycete plant pathogens were used as targets. The objectives of this study were to determine (i) the in vitro antifungal activity of SYP-14288 against various plant pathogens, (ii) the fungicidal activities of SYP-14288 on different developmental stages of *Magnaporthe oryzae*, (iii) the preventative and curative actions of SYP-14288 on downy mildew by leaf disk assay inoculated with *Pseudoperonospora cubensis*, (iv) the control efficacy of SYP-14288 on pepper blight and rice blast in the greenhouse or field, and (v) the mode of action of SYP-14288 by metabolomics. All of the results obtained in the current study could benefit the registration and application of the novel fungicide SYP-14288.

Materials and Methods

Media, fungicides, chemicals, and pathogens. Potato dextrose agar (PDA) medium (200 g of potato infusion, 15 g of glucose, and 12 g of agar in 1 liter of sterile deionized water) was used for routine culture of the fungi. V8 juice medium (100 ml of V8 juice, 1.5 g of CaCO₃, and 15 g of agar in 1 liter of sterile deionized water) was used for culture of oomycetes. Water agar (12 g of agar in 1 liter of sterile deionized water) was used for the spore germination assay.

The fungicide SYP-14288 (98% a.i.) was kindly provided by the China Shenyang Research Institute of Chemical Industry. The chemical was dissolved in dimethylsulfoxide (DMSO) and diluted to 10 mg/ml as a stock solution. Other fungicides sourced commercially were fluazinam (98.4% a.i.; Sigma-Aldrich Shanghai Trading Co., Ltd., Shanghai, China) and dimethomorph (95% a.i.; Frey Agrochemicals Ltd., Jiangsu, China). For field testing, SYP-14288 (5% a.i. SC) was provided by the China Shenyang Research Institute of Chemical Industry; fluazinam (50% a.i. SC), tricyclazole (75% a.i. WP), and isoprothiolane (40% a.i. EC) were purchased from Fuchuan Biology Co. Ltd., Jiangxi Shenghua Biological Pesticide Co. Ltd., and Jiangsu Jiangnan Chemical Co. Ltd., respectively. They were applied according to the instructions.

Analytical-grade *N,O*-bis(trimethylsilyl) acetamide (containing 1% TMS), methoxyamine hydrochloride, pyridine, and methyl alcohol were obtained from Sinopharm Chemical Reagent Co. Ltd. (Beijing, China), and salicin (used as an internal standard) was purchased from Sigma-Aldrich (St. Louis, MO). Ultrapure water was prepared using the Milli-Q Academic A10 water purification system from Millipore (Billerica, MA).

The activity of SYP-14288 was tested against 31 plant pathogens listed in Table 1. The plant pathogens were cultured in the dark at 25 or 18°C. For the metabolomic assay, *Phytophthora capsici* BYA5 was isolated from pepper in the field in Gansu, China. Before SYP-14288 treatment, BYA5 was placed on 10% V8 medium for 4 days and then exposed to light for 5 to 7 days for sporangia production. For release of zoospores, 10 ml of water was added to sporangia at 4°C for 30 min and then at room temperature for 40 min. The basal medium (Jee and Ko 1997) was used for maintenance of test organisms. One milliliter of 1×10^6 /ml spore suspension was added to 150 ml of basal medium, incubated at 120 rpm and 25°C for 48 h, then incubated with SYP-14288 for 1, 6, or 12 h.

Table 1. In vitro mycelium growth inhibition of plant pathogens by SYP-14288

Organism	Species	Equation	Correlation coefficient ^a	EC ₅₀ (μg/ml) ^b
Oomycetes	<i>Phytophthora infestans</i>	$Y = 4.9219 + 1.06X$	0.99	1.185
	<i>Phytophthora capsici</i>	$Y = 4.8049 + 1.08X$	0.99	1.198
	<i>Phytophthora litchii</i>	$Y = 5.1492 + 1.05X$	0.99	0.599
	<i>Phytophthora sojae</i>	$Y = 5.3682 + 1.22X$	0.99	0.573
	<i>Pythium ultimum</i>	$Y = 5.4153 + 0.79X$	0.99	1.681
	<i>Pythium aphanidermatum</i>	$Y = 5.0914 + 0.97X$	0.99	0.805
Zygomycota	<i>Rhizopus chinensis</i>	$Y = 5.6073 + 0.20X$	0.99	0.001
Basidiomycota	<i>Thanatephorus cucumeris</i>	$Y = 6.1498 + 0.44X$	0.99	0.031
Ascomycota	<i>Magnaporthe oryzae</i>	$Y = 6.2530 + 1.53X$	0.99	0.169
	<i>Valsa mali</i>	$Y = 6.4437 + 0.84X$	0.99	0.019
	<i>Ceratocystis fimbriata</i>	$Y = 6.1405 + 0.91X$	0.99	0.056
	<i>Gaeumannomyces graminis</i>	$Y = 6.3703 + 1.23X$	0.98	0.077
	<i>Setosphaeria turcica</i>	$Y = 5.6551 + 0.37X$	0.99	0.017
	<i>Sclerotinia homoeocarpa</i>	$Y = 6.8852 + 1.17X$	0.99	0.025
	<i>Sclerotinia sclerotiorum</i>	$Y = 6.7311 + 0.58X$	0.99	0.001
	<i>Botryosphaeria berengeriana</i>	$Y = 6.1900 + 1.10X$	0.99	0.006
	<i>Botrytis cinerea</i>	$Y = 5.3750 + 0.51X$	0.99	0.184
	<i>Monilinia fructicola</i>	$Y = 6.1111 + 0.38X$	0.99	0.001
	<i>Colletotrichum acutatum</i>	$Y = 5.6982 + 0.90X$	0.99	0.200
	<i>Colletotrichum capsici</i>	$Y = 5.5416 + 1.05X$	0.98	0.305
	<i>Colletotrichum gloeosporioides</i>	$Y = 5.6789 + 0.76X$	0.99	0.127
	<i>Fusarium subglutinans</i>	$Y = 6.5084 + 1.06X$	0.99	0.012
	<i>Fusarium graminearum</i>	$Y = 5.0376 + 1.07X$	0.99	0.736
	<i>Fusarium moniliforme</i>	$Y = 5.9910 + 0.42X$	0.99	0.004
Anamorphic fungi	<i>Fusarium poae</i>	$Y = 5.1241 + 0.93X$	0.98	0.736
	<i>Fusarium oxysporium</i>	$Y = 5.9037 + 0.58X$	0.99	0.056
	<i>Curvularia lunata</i>	$Y = 6.6449 + 0.65X$	0.99	0.003
	<i>Pestalotiopsis theae</i>	$Y = 6.6018 + 0.77X$	0.99	0.008
	<i>Alternaria solani</i>	$Y = 5.3047 + 0.27X$	0.99	0.074
	<i>Bipolaris maydis</i>	$Y = 5.2868 + 0.99X$	0.99	0.512
	<i>Corynespora cassiicola</i>	$Y = 5.7826 + 0.91X$	0.99	0.139

^a Correlation between the probability value transformed by the percentage of inhibition relative to the control (solvent only) and the concentration (log-transformed) of the fungicide.

^b EC₅₀, median effective concentration.

Inhibitory activity of SYP-14288 in vitro. For mycelium growth inhibition tests, the stock solution of SYP-14288 in DMSO was diluted serially in PDA medium to achieve final concentrations according to preliminary testing (*data not shown*). Control plates contained medium with corresponding concentrations of DMSO (0.1%). For each of the 31 test plant pathogens listed in Table 1, mycelium plugs (5 mm) were transferred from the leading edge of an actively growing colony to the control and fungicide-containing dishes, with percentage inhibition from 10 to 90%. The dishes were incubated at 25°C in the dark. When the colony in the control plates covered 75% of the agar surface, the diameter of all colonies was measured. The percentage of inhibition of radial growth was calculated. The tests were replicated three times for each microorganism.

For asexual spore germination inhibition assays, spores were obtained according to Moore-Landecker (2002). Different volumes of SYP-14288 stock solution were added to 1 ml of pathogen spores ($10^5/\text{ml}$) in sterile water to dilute to concentrations according to preliminary testing (*data not shown*). The tubes were incubated at 25°C in the dark for 24 to 72 h according to their growth rates. There were five tubes per fungicide concentration, and the experiment was performed three times. The germination rates of spores in each tube were investigated under microscopy, and inhibition rates were calculated according to fungicide treatments and controls.

For the above tests, the median effective concentration (EC_{50}) value was calculated by regressing the percentage of inhibition (probability value transformed) against the logarithm value of fungicide concentration (log-transformed). Regression analysis was performed with Microsoft Excel 2007.

Effect of SYP-14288 on different developmental stages of *M. oryzae*. Effects of SYP-14288 on the mycelial growth and spore germination of *M. oryzae* were measured as described above. Spore production was induced according to Wang et al. (2009) and measured under microscopy. To determine the inhibitory effect of SYP-14288 on melanin production, the colored colony diameters were measured on PDA and the proportion of darkened area was calculated compared with that on control plates. Three replicates were performed for all tests.

Preventive and curative activity of SYP-14288 on the downy mildew. *Pseudoperonospora cubensis* is an obligate parasite, which is an excellent model to study the preventative and curative efficacy of fungicide owing to its typical lesion on leaves, rapid growth rate, and the capacity of the high-throughput assay on leaf disks. The methods for determining the preventive and curative activity of *Pseudoperonospora cubensis* on cucumber leaf were similar to those described previously (Miao et al. 2018). Seeds of Jingchun variety cucumbers were purchased from a supermarket. Cucumber seedlings were grown in trays in a greenhouse, and the leaves were collected after 30 days and cut into 1-cm disks by hole punch. Sporangiums were collected from *Pseudoperonospora cubensis* isolate SG1 (isolated from Shangong, China) from infected fresh cucumber leaves by rinsing with double-distilled water. Each disk was inoculated with 10 μl of sporangium suspension ($10^8/\text{liter}$). SYP-14288 was applied either 24 h prior to inoculation (preventative treatment) or 24 h after inoculation (curative treatment). Three control treatments, including water spray, fluazinam, and dimethomorph, were performed. After 8 days of incubation at 18°C with a 16-h photoperiod, the percentage of diseased disks was recorded, and the relative control efficiency was calculated according to Mitani et al. (2002). The experiment was performed three times with six replicates per experiment. Each replicate consisted of 30 inoculations.

Control efficacy of SYP-14288 on pepper blight in the greenhouse and rice blast in the field. The control efficacy of SYP-14288 on pepper blight in the greenhouse was assayed similar to methods described previously (Miao et al. 2016). Seeds of the Xichengdaniujiao variety pepper were purchased from a supermarket, and two to four pepper seedlings were grown in each 3 $\times$ 3-cm hole of the growing tray (60 holes in each tray) in the greenhouse. For inoculation, 3 ml of zoospore suspension ($10^5/\text{ml}$) was poured into each hole. SYP-14288 was applied 24 h before the seedling was inoculated (preventative treatment) or 24 h after the seedling was

inoculated (curative treatment). Three control treatments, including sterile water, no inoculation, and fluazinam, were conducted. After 5 days, the length of lesions related to the height of seedlings was recorded, and the relative control efficiency was calculated according to Miao et al. (2016). The experiment was performed three times with three replicates per experiment. Each replicate consisted of 12 inoculations.

To assess the efficacy of SYP-14288 for control of rice blast in the field, the fungicides were applied to a rice field in Liaoning Province. Three concentrations of SYP-14288 (100, 200, and 400 g of a.i./ hm^2) were applied and compared with fluazinam, tricyclazole, and isoprothiolane applied at 200, 200, and 800 g of a.i./ hm^2 , respectively. For a control, plants were sprayed with water. Treatments were replicated four times in a randomized block with 100 m^2 per replication. All fungicides were applied by a sprayer. The disease index was recorded according to Liu et al. (2017), and the percentage of control efficacy was calculated as:

$$[(B - A)/B] \times 100$$

in which A is the disease index with fungicide treatment and B is the disease index with water application.

Metabolomic analysis. Hyphae samples were obtained by a vacuum pump filter device and then placed in liquid nitrogen to arrest enzymatic activity. Hyphae samples were shaken in a ball mill to produce a homogenized powder, after which 30 mg of powder was added to a 2-ml microcentrifuge tube. Metabolites were extracted from oomycetes according to an optimized, published method (Heuberger et al. 2014). Salicin (internal standard) was added to methanol/water (vol/vol, 4/1) to prepare a 10 mg/ml extraction solution. Metabolites were extracted from 30 mg of dry weight powder of oomycete hyphae. The extraction and derivatization of oomycete hyphae samples was based on existing methods (Dai et al. 2019). Metabolites of *Phytophthora capsici* BYA5 were identified and quantified using an Agilent 7890B/7200 GC-QTOF System (Agilent Technologies, Parc Technopolis-ZA Courtaboeuf, Les Ulis, France). A cross-linked 5% phenylmethylpolysiloxane (Optima 5-MS Accent; Macherey Nagel) column (30 $\times$ 0.25 mm, 0.25- μm film thickness) was employed. The oven temperature was programmed to increase from 60 to 325°C at 10°C/min, then hold at 325°C for 5 min. Accurate mass spectra were recorded across the mass-to-charge ratio range of 50 to 600 and 250°C for the ion source. Perfluorotributylamine was utilized for daily MS calibration. Qualitative Analysis B.07.00 (Agilent) was used to acquire mass spectrometric data. Compounds were identified by comparison of their time-of-flight mass spectra, accurate mass, and retention times with the Fiehn and NIST2014 mass spectrum library. Quantification of each compound involved extraction of specific, accurate fragment ions, peak integration, and determination of individual response factors using external standards.

Data processing and statistical analysis. For metabolic analysis, Agilent's MPP (Mass Profiler Professional; Agilent Technologies, Palo Alto, CA) was used for multivariate statistical analysis, which includes several sample class prediction algorithms for further processing. The internal standard selected for GC/MS analysis was applied for normalization of the peak intensity to account for the difference in the abundances of each compound. Stepwise reduction of dimensionality of entities was performed based on their presence across samples and parameter values (filter by flags), frequency of occurrence (filter by frequency), and abundance of the respective entities in classes (filter by sample variability). The quality control of samples was performed by principal component analysis (PCA). The *t* test and fold change were both employed for statistical analysis (*P* value < 0.05 and fold change > 1). Six replicates of samples were detected per treatment. For other experiments, differences among means was determined with Duncan's multiple range test at *P* = 0.05.

Results

In vitro activity of SYP-14288 against plant pathogens. SYP-14288 inhibited the in vitro mycelial growth and asexual spore germination of all 31 tested plant pathogens (including oomycetes,

zygomycota, basidiomycota, ascomycota, and anamorphic fungi), with EC₅₀ values of 0.001 to 1.681 and 0.02 to 0.09 µg/ml, respectively. SYP-14288 showed a greater inhibitory effect on most of the tested plant pathogens than fluazinam (Fig. 1; Tables 1 and 2).

Effect of SYP-14288 on the different developmental stages of *M. oryzae*. A previous study of SYP-14288 showed that it is highly active against various life stages of a representative plant pathogenic oomycete, *Phytophthora capsici* (Wang et al. 2018). Here, we showed that SYP-14288 substantially inhibited four important life stages of *M. oryzae* (Table 3), an important plant pathogenic fungus. The EC₅₀ values were 0.169, 0.247, 0.057, and 0.055 µg/ml for mycelia growth, melanin biosynthesis, spore production, and spore germination, respectively. The inhibition activity of SYP-14288 was greater for spore germination than for any other life stages, which

is a stage with heavy energy consumption. All of the inhibitory effects were similar to fluazinam, which was also showed good activity against different life stages of *M. oryzae*.

Preventative and curative activity of SYP-14288 on cucumber leaf disks against *Pseudoperonospora cubensis*. SYP-14288 provided much better control efficacy on *Pseudoperonospora cubensis* on cucumber leaf disks when applied 24 h before inoculation (preventative activity) than 24 h after inoculation (curative activity) (Table 4). The efficacy for preventative activity was as high as 96.30%, which was similar to the efficacy of fluazinam and dimethomorph at the same concentration.

Control efficacy of SYP-14288 on pepper blight in the greenhouse and rice blast in the field. For pepper blight, similar preventative control effects were observed in SYP-14288 (250 or 500 g of a.i./hm²) and fluazinam (500 g of a.i./hm²); all were above 82.11% (Fig. 2A). No curative control effect was shown for all treatment dosages of SYP-14288 and fluazinam, which further confirms that SYP-14288 has no curative effect on plant pathogens.

Significant differences among fungicide treatments were observed in rice, and all fungicide treatments were statistically superior to the (water) control ($P < 0.05$) except the lowest dose of SYP-14288 (Fig. 2B). The SYP-14288 treatment dose (400 g of a.i./hm²) controlled rice blast significantly better than lower concentrations of SYP-14288. Fluazinam, isoprothiolane, and SYP-14288 provided comparable rice blast control at similar doses, whereas the greatest efficacy was found in 75% tricyclazole (200 g of a.i./hm²).

Analysis of the untargeted metabolome and differential metabolite interpretation. Metabolomics provides new methods for the analysis of the mode of action of fungicide. In this study, approximately 300 peaks were extracted by spectral deconvolution of the total ion chromatogram (TIC). A representative TIC obtained from the wild-type *Phytophthora capsici* BYA5 inoculated with or without SYP-14288 for 1, 6, and 12 h is shown in Supplementary Figure S2. The areas of all peaks were normalized with the area of the reference peak. The PCA model clustered these metabolites, and the PCA plot shows that the control and treatment samples can be separated in space. The sum of the explained variability for three components is > 70% (Fig. 3C); because this plot shows a large portion of the information in the data, the relationships can be interpreted with a high degree of certainty (according to the guidance of Unscrambler X 10.3; CAMO, Trondheim, Norway). The differential metabolites were identified by *t* test and fold change. PCA suggested that there were differential metabolites that could be distinguished between control and treatment samples, and these differential metabolites may be related to the mode of action of SYP-14288. These differential metabolites include amino acids, organic acids, sugars, alcohols, and other metabolites exposed to SYP-14288 (Table 5). Significant changes occurred in 25 metabolites, with accumulation of seven fatty acid metabolites and a decrease in 18 metabolites, and as the treatment with SYP-14288 continued, the resulting differential metabolites also increased.

Fig. 1. SYP-14288 is similar to fluazinam in antifungal activity against various plant pathogenic microbes. **A.** Mycelium growth inhibition. **B.** Asexual spore germination inhibition. EC₅₀, median effective concentration.

Table 2. In vitro asexual spore germination inhibition of plant pathogens by SYP-14288

Organism	Species	Equation	Correlation coefficient ^a	EC ₅₀ (µg/ml) ^b
Oomycetes	<i>Phytophthora capsici</i>	$Y = 8.94701 + 2.66X$	0.88	0.06
Ascomycota	<i>Magnaporthe oryzae</i>	$Y = 10.4795 + 4.35X$	0.98	0.05
Zygomycota	<i>Colletotrichum gloeosporioides</i>	$Y = 18.5564 + 9.06X$	0.99	0.03
Anamorphic fungi	<i>Colletotrichum acutatum</i>	$Y = 13.423 + 7.38X$	0.97	0.07
	<i>Colletotrichum capsici</i>	$Y = 10.1972 + 4.22X$	0.95	0.09
	<i>Botrytis cinerea</i>	$Y = 9.93640 + 4.66X$	0.92	0.09
	<i>Rhizopus chinensis</i>	$Y = 8.27285 + 1.82X$	0.87	0.02
	<i>Monilinia fruticola</i>	$Y = 9.43480 + 4.55X$	0.99	0.02
	<i>Bipolaris maydis</i>	$Y = 5.49530 + 1.10X$	0.99	0.03
	<i>Alternaria solani</i>	$Y = 5.90915 + 1.20X$	0.99	0.05
	<i>Cladosporium cucumerinum</i>	$Y = 7.81123 + 2.01X$	0.94	0.04

^a Correlation between the probability value transformed by percentage of inhibition relative to the control (solvent only) and the concentration (log-transformed) of the fungicide.

^b EC₅₀, median effective concentration.

The influence of SYP-14288 on *Phytophthora capsici* could be shown through the metabolic profile. The change patterns of the metabolites were consistent for 1, 6, and 12 h, but the number of differential metabolites increased as the treatment period increased. Among the samples obtained at different time points, the sample treated for 12 h was the most representative because it showed the largest changes compared with the control. The heatmap indicated that the standard deviation can be roughly estimated based on the color, and the six replicates of the treatment group and control group

can be clustered into one group (Fig. 4). From the heatmap, we found that nine metabolites—5-aminovaleic acid, talose, lactulose, L-proline, N-acetyl-D-mannosamine, L-lysine, L-glutamine, palmitoleic acid, and alpha-linolenic acid—showed the greatest change among the differential metabolites. Based on the changed metabolites, the metabolic pathways of *Phytophthora capsici* disturbed by SYP-14288 treatment included fatty acid metabolism, starch and sugar metabolism, the tricarboxylic acid (TCA) cycle, glutathione metabolism, glycine aspartate, and glutamate metabolism.

Discussion

SYP-14288 is a potentially effective fungicide, as it shows good activity against various plant pathogens, including ascomycota, basidiomycota, and oomycota. It also has high efficiency on different plant-pathogen life stages and shows good preventative activity against pepper blight and rice blast. Metabolomics results indicate that SYP-14288 could efficiently affect the pathways related to energy production of *Phytophthora capsici*. All of these results demonstrate that SYP-14288 could be a suitable control agent against various plant pathogens.

SYP-14288 is a novel fungicide that shares a similar chemical structure with fluazinam, and it belongs to diarylamine compounds. SYP-14288 is a fungicide with a 50% lethal dose (LD_{50}) > 5,000 mg/kg for acute oral toxicity and LD_{50} > 2,000 mg/kg for acute percutaneous toxicity in rats, respectively, thus it is a promising fungicide with high bioactivity and is also environmentally friendly

Table 3. The in vitro effect of SYP-14288 on different developmental stages of *Magnaporthe oryzae*

State	SYP-14288			EC ₅₀ of fluazinam (μg/ml)
	Equation	Correlation coefficient	EC ₅₀ (μg/ml) ^a	
Mycelia growth	Y = 5.8645 + 1.91X	0.97	0.169	0.402
Melanin biosynthesis	Y = 7.0772 + 3.46X	0.97	0.247	0.782
Spore production	Y = 6.9306 + 1.58X	0.96	0.057	0.143
Spore germination	Y = 9.1937 + 2.78X	0.88	0.055	0.131

^a EC₅₀, median effective concentration.

Table 4. Preventative and curative effect of SYP-14288 evaluated by the control effect against cucumber downy mildew

Fungicide	Concentration (μg/ml)	Protective treatment			Relative efficacy of curative treatment (%)
		Relative efficacy (%)	Equation	EC ₉₀ (μg/ml) ^a	
SYP-14288	16	96.30	Y = 1.133 + 5.730X	8.147	0
	8	92.59			0
	4	29.63			0
	2	14.81			0
	1	0.00			0
Fluazinam	16	100.00	Y = 3.979 + 2.711X	7.067	0
	8	92.59			0
	4	66.67			0
	2	51.85			0
	1	7.41			0
Dimethomorph	16	96.30	Y = 2.437 + 3.575X	11.901	38.79
	8	81.48			15.67
	4	22.22			0
	2	22.22			0
	1	7.41			0

^a EC₅₀, median effective concentration.

Fig. 2. Efficacy of SYP-14288 and other fungicides for pepper blight control in the greenhouse and rice blast control in the field in Liaoning. **A**, Efficacy of SYP-14288 on pepper blight. **B**, Efficacy of SYP-14288 on rice blast. Different letters within a group means the data was significantly different ($P < 0.05$).

(C. Liu, unpublished data). Diarylamines are not commonly used in agrochemicals but are widely used in medicines. For example, diarylamine GDC-0449, a smoothened receptor inhibitor, can effectively inhibit the hedgehog pathway, which is ubiquitous in mammals (Robarge et al. 2009). Attributable in part to the absence of the hedgehog pathway in microbes (McMahon 2000), the potential protein target of SYP-14288 is still unknown.

SYP-14288 has potential for control of diseases caused by 31 plant pathogens, including ascomycota, basidiomycota, and oomycota. According to the in vitro experiment, the EC₅₀ values for SYP-14288 were < 1.2 µg/ml for mycelial growth and asexual spore germination of all tested plant pathogens except *Pythium ultimum*. Although the data collected under controlled conditions might not be the same as those in the field, they could still reflect the potential effectiveness of SYP-14288 as a fungicide with a very broad range of target plant pathogens.

High inhibition of all life stages of *M. oryzae* was observed, which implies that SYP-14288 targets a fundamental pathway in plant pathogens (Wang et al. 2018). Similar to the inhibition effect on *Phytophthora capsici*, SYP-14288 showed greater activity against high energy consumption stages including spore production and

germination (with lower EC₅₀ values than other stages), which indicates that SYP-14288 may be an energy disruptor (Wang et al. 2018). In general, respiration inhibitors such as azoxystrobin and oxidative phosphorylation inhibitors such as fluazinam are known as two major classes of energy disruptor. They are known to strongly inhibit spore germination rather than hyphal growth (Sisler and Ragsdale 1984), and they both showed a positive cross-resistance against the SYP-14288-resistant isolates of *Phytophthora capsici* (Wang et al. 2020), which implies that they share a similar mode of action. The behavior observed for energy disruptors in various life stages differs as a result of changing energy requirements (e.g., spore production and germination need more energy than hyphal growth) (Wang et al. 2018).

SYP-14288 exhibits significantly better preventative fungicidal activity than curative activity, which implies that SYP-14288 should only be applied before disease outbreak. It is strongly recommended to apply SYP-14288 before pathogen infection, such as during seed treatment to control plant disease during the whole growth period. SYP-14288 shows high efficacy in the greenhouse and field against pepper blight and rice blast, which are caused by important and ubiquitous plant pathogenic oomycete and fungi. *Phytophthora capsici*

Fig. 3. A-C, Three-dimensional principal component (PC) analysis plots of *Phytophthora capsici* BYA5 treated with SYP-14288.

can cause disease in hundreds of vegetable varieties (Lamour et al. 2012) and *M. oryzae* is a significant threat to rice production (Jia et al. 2009), making them both important plant pathogens that can cause substantial agricultural losses. Increasing fungicide resistance of these two pathogens has been discovered in recent years, including metalaxyl and dimethomorph in *Phytophthora capsici* (Keinath 2007; Qi et al. 2012) and isoprothiolane and tricyclazole in *M. oryzae* (Hu et al. 2014; Kunova et al. 2014). Thus, new fungicides with a novel mode of action are urgently needed. SYP-14288 does not demonstrate cross-resistance to any popular fungicides except fluazinam and azoxystrobin (Wang et al. 2020), which suggests that it could be an alternative method of controlling *Phytophthora capsici* and *M. oryzae* plant diseases.

Metabolomics has been widely used in drug toxicity and mechanism studies (Keun 2006; Lindon et al. 2005), microbiology and plant research (Rochfort 2005), disease diagnosis in animal and plant models (Gerszten and Wang 2008), and the exploration of gene function (Catchpole et al. 2005). In the current three-dimensional PCA plots, the proximity of the samples is positively correlated with the similarity, so this plot can be used to explain differences and similarities within and between groups.

Changes in metabolites are sensitive indicators of environmental stress and developmental stage (Aliferis and Chrysai-Tokousbalides 2006), allowing untargeted metabolomics to be utilized to investigate the mode of action of novel fungicides. In this study, we concluded that SYP-14288 induced a dramatic change in the metabolome of *Phytophthora capsici*, including fatty acid, sugars, and amino acids. After 12-h exposure, fatty acids were especially susceptible to upregulation, including palmitic acid (2.8-fold change), stearic acid (1.8-fold change), oleic acid (16.2-fold change), linoleic acid (8.8-fold change), alpha-linolenic acid (>100-fold change), eicosapentaenoic acid (2.9-fold change), and myristic acid (2.4-fold change). Two pathways are known to accumulate fatty acids, the acetyl-coenzyme A (CoA) biosynthetic pathway in glycolysis (Rawsthorne 2002), and phospholipase-mediated membrane breakdown (Chefurka

Fig. 4. Heatmap visualization of the differential metabolites of *Phytophthora capsici* BYA5 treated with or without SYP-14288 for 12 h. CK means BYA5 treated with dimethylsulfoxide only and S means BYA5 treated with SYP-14288, with six replicates per treatment. FC, fold change.

Table 5. Differential metabolites of BYA5 in response to SYP-14288 treatment

Category	Metabolite ^a	RT (min) ^b	<i>m/z</i>			Fold change ^c		
			1	2	3	1 h	6 h	12 h
Amino acids	L-proline	10.04	230	140	231			↓↓↓
	L-homoserine	12.07	146	103	130			↓↓
	L-cysteine	13.38	220	218	100		↓	↓↓
	L-glutamic acid	14.12	246	128	147	↓↓	↓↓	↓↓
	L-glutamine	15.84	156	155	245			↓↓↓
	L-lysine	17.42	175	317	156			↓↓↓
Organic acids	Malic acid	12.57	147	233	245	↓↓	↓↓	↓↓
	3-phosphoglyceric acid	16.30	299	147	357		↓	↓
	Myristic acid	16.49	117	132	285		↑	↑↑
	5-aminovaleric acid	16.65	174	318	147			↓↓↓
	Palmitoleic acid	18.24	311	117	129		↑↑↑	↑↑↑
	Palmitic acid	18.43	117	313	132		↑↑	↑↑
	Alpha-linolenic acid	19.79	75	335	350		↑↑↑	↑↑↑
	Linoleic acid	19.95	75	337	81		↑↑↑	↑↑
	Oleic acid	19.99	117	339	55		↑↑↑	
	Eicosapentaenoic acid	21.38	79	75	91		↑↑	↑↑
Sugars	3-alpha-mannobiose	17.86	204	217	319			↓↓
	Talose	18.58	204	191	217			↓↓↓
	Beta-D-fructopyranose	19.68	204	147	437			↓↓
	Lactulose	24.14	147	204	361			↓↓↓
	Maltose	24.53	204	191	361		↓	↓
	Sophorose	24.86	319	204	147		↓	↓
	Trehalose	24.91	204	191	147		↓↓	↓↓
	Beta-gentiobiose	25.16	204	191	103		↓↓	↓↓
	Isomaltose	25.34	361	204	217			↓↓
	N-acetyl-D-mannosamine	19.08	205	147	319			↓↓↓
Others	Adenosine	20.57	236	230	245			↓↓

^a The metabolite content changed significantly ($P < 0.05$) for BYA5 treated with SYP-14288 compared with those treated with dimethylsulfoxide by *t* test after 1, 6, and 12 h.

^b RT, retention time; *m/z*, mass-to-charge ratio.

^c Fold change < 2 is indicated by ↓ or ↑, 2 ≤ fold change < 5 is indicated by ↓↓ or ↑↑, and fold change ≥ 5 is indicated by ↓↓↓ or ↑↑↑.

Fig. 5. Metabolic pathway networks of significant different metabolites of *Phytophthora capsici* BYA5 in response to SYP-14288 for 12 h. CoA, coenzyme A; PEP, phosphoenolpyruvate.

and Dumas 1966). Among the differential metabolites (Fig. 5), except for those of fatty acids, the pathways involving acetyl-CoA synthesis, including carbohydrate metabolism and glycolysis, were all downregulated. Therefore, fatty acid accumulation could not have resulted from acetyl-CoA biosynthesis, but from breakdown of the membrane. It has been reported that well-known uncoupler 2,4-dinitrophenol can affect the function of mitochondria, promote the aging of mitochondria, and accumulate fatty acids in mitochondria, including palmitic acid, stearic acid, oleic acid, linoleic acid, and arachidonic acid (Chefurka and Dumas 1966). These results are similar to those of the current study, which indicated that SYP-14288 operates similarly to 2,4-dinitrophenol, and upregulation of fatty acids is the response of the oomycetes to the uncoupler.

Oxidative phosphorylation, which takes place in the inner membrane of mitochondria, is the most important process in ATP production. Uncoupling agents can break the complete functional coupling between the electron transport chain and ATP synthase, causing energy to be lost as thermal energy, and increase the breathing rate and oxygen consumption (Terada 1990). The acceleration of the electron transfer chain requires more intermediate compounds of the TCA cycle, and these substances in the treatment group should be lower than those in the control group. Indeed, the intermediate compounds of the TCA cycle and glycolysis such as malic acid (0.5-fold change) and 3-phosphoglyceric acid (0.6-fold change) were downregulated, which could subsequently lead to an amino acid decrease because they can form the amino acid carbon skeleton (e.g., a 0.45-fold change of L-glutamic acid, a 0.4-fold change of L-cysteine, and less than a 0.01-fold change of L-proline). Sugars, including maltose (0.5-fold change), trehalose (0.4-fold change), and isomaltose (0.5-fold change), were also downregulated. This downregulation could be caused by the decreased uptake of carbon sources and accelerated

consumption through glycolysis to supply compensatory energy as a result of the insufficient energy supply after SYP-14288 treatment. The results of the metabolome are consistent with the proteomic data of SYP-14288 (Cai et al. 2019).

In conclusion, SYP-14288 demonstrated substantial *in vitro* activity against a very broad spectrum of plant pathogens. The fungicide showed substantial activity through all life stages of the target organisms and high preventative efficiency against rice blast and pepper blight. Metabolomics study results showed that SYP-14288 is an uncoupling agent similar to 2,4-dinitrophenol, which can affect the synthesis of energy by destroying the coupling of oxidative phosphorylation, thereby inhibiting the growth of the phytopathogen. The novel fungicide SYP-14288 may be expected to be an effective agrochemical in plant disease management.

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