Fungicide SYP-14288 Inducing Multidrug Resistance in *Rhizoctonia solani*

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Abstract

Rhizoctonia solani is a widely distributed soilborne plant pathogen, and can cause significant economic losses to crop production. In chemical controls, SYP-14288 is highly effective against plant pathogens, including *R. solani*. To examine the sensitivity to SYP-14288, 112 *R. solani* isolates were collected from infected rice plants. An established baseline sensitivity showed that values of effective concentration for 50% growth inhibition (EC₅₀) ranged from 0.0003 to 0.0138 µg/ml, with an average of 0.0055 ± 0.0030 µg/ml. The frequency distribution of the EC₅₀ was unimodal and the range of variation factor (the ratio of maximal over minimal EC₅₀) was 46.03, indicating that all wild-type strains were sensitive to SYP-14288. To examine the risk of fungicide resistance, 20 SYP-14288-resistant mutants were generated on agar plates amended with SYP-14288. Eighteen mutants

remained resistant after 10 transfers, and their fitness was significantly different from the parental strain. All of the mutants grew more slowly but showed high virulence to rice plants, though lower than the parental strain. A cross-resistance assay demonstrated that there was a positive correlation between SYP-14288 and fungicides having or not having the same mode of action with SYP-14288, including fluazinam, fentin chloride, fludioxonil, difenconazole, cyazofamid, chlorothalonil, and 2,4-dinitrophen. This result showed a multidrug resistance induced by SYP-14288, which could be a concern in increasing the spectrum of resistance in *R. solani* to commonly used fungicides.

Keywords: multidrug resistance, *Rhizoctonia solani*, resistance risk, cross resistance

Rhizoctonia solani is a ubiquitous soilborne plant pathogen, and inflicts damages on a wide range of economically important crops, including wheat, rice, maize, soybean, potato, and others (Ajayi-Oyetunde and Bradley 2018; Anderson et al. 2016; Zheng et al. 2013). Due to its importance, relevant prevention and control measures are imperative. Conventional resistance breeding strategies are highly expected but hindered by the rareness of tractable genetic resistance in any crop host (Mahoney et al. 2016; Oladzad et al. 2019). Although pesticides may pose risks to humans and the environment, they are still the main strategies at present (Bartholomäus et al. 2017; Zhang et al. 2018).

In chemical controls, the development of resistance to fungicides is a major concern. It is believed that *R. solani* has a low risk of developing fungicide resistance, possibly because of the lack of spore production (Ajayi-Oyetunde et al. 2017). However, studies of population genetics of *R. solani* have revealed remarkable genotypic diversity among different anastomosis groups, indicating a potential of *R. solani* for developing fungicide resistance (Ogoshi 1987). In fact, quinone outside inhibitor fungicides have lost their efficacies in controlling *R. solani* due to the development of fungicide resistance (Olaya et al. 2012). In addition, resistance of *R. solani* to succinate dehydrogenase inhibitors has also been documented (Mu et al. 2017; Zhao et al. 2019). Therefore, there is an urgent need to find

alternative fungicides with different modes of action (MOA) to overcome the resistance developed in *R. solani*.

SYP-14288 is a novel fungicide developed by Shenyang Sinochem Agrochemicals R&D Co., Ltd. in China. It belongs to the diarylamine group and shares similar chemical structures and MOA with fluazinam (Fig. 1), which exhibits a strong fungitoxicity against various phytopathogens (Z. Wang et al. 2018). The MOA involves interrupting the production of cellular energy of fungi with an uncoupling of mitochondrial oxidative phosphorylation (Hou et al. 2019; Qu et al. 2018; X. H. Wang et al. 2018). In comparison with fluazinam, SYP-14288 provides better control efficacy on plant pathogens, especially on *R. solani*, *Phytophthora capsici*, *Colletotrichum orbiculare*, and *Pseudoperonospora cubensis* (Lan et al. 2012). Therefore, SYP-14288 has broad application prospects as a promising candidate for plant disease management (Z. Wang et al. 2018).

Although fluazinam has been used for more than 30 years, resistance to this fungicide is rare, probably because it lacks a specific target protein or binding site in the pathogen. Therefore, the Fungicide Resistance Action Committee (<https://www.frac.info/>) has classified fluazinam as low risk for resistance. To date, there is one case of field resistance to fluazinam found in *Botrytis cinerea* in Japan (Tamura 2000), and one with reduced efficacy of fluazinam against *Phytophthora infestans* is reported in The Netherlands (Scheepers et al. 2018). For research purposes, fluazinam-resistant strains of target fungi have been frequently induced in laboratory conditions such as mutants in *B. cinerea* (Shao et al. 2015b), *Sclerotinia sclerotiorum* (Mao et al. 2018), and *Ustilago maydis* (Vitoratos 2014).

Multidrug resistance (MDR) has become a concern in recent years in both human medical treatments and plant pathogens (Moye-Rowley 2019; Nishikawa et al. 2016). The emergence of MDR caused by increased efflux activity has created a significant threat to both human health and agricultural production (Healey et al. 2016; Sang et al. 2015). MDR field populations have been described in *Penicillium digitatum*, *Mycosphaerella graminicola*, and *B. cinerea* (Mernke et al. 2011; Nakaune et al. 2002; Roohparvar et al. 2008). MDR has been extensively studied in molecular mechanisms in *B. cinerea* (Hahn 2014). There are no reports on MDR induced by uncouplers. It is unknown whether MDR could be induced by

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uncoupler fungicides. We would like to investigate the possibility of *R. solani* developing resistance to SYP-14288 because *R. solani* is polynuclear filamentous and possesses various anastomosis groups. The objectives of this study were to (i) establish a baseline sensitivity of *R. solani* to SYP-14288, (ii) assess the fitness of the resistant mutants, and (iii) analyze cross-resistance relationships between SYP-14288 and other groups of fungicides.

Materials and Methods

Fungicides and pathogens. All fungicides used in this study were of pure technical grades (Supplementary Table S1). Fungicides were individually dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions ($1 \times 10^5 \mu\text{g ml}^{-1}$), which were stored at 4°C in the dark to preserve antifungal activity. Potato dextrose agar (PDA), consisting of 200 g of potato, 20 g of dextrose, and 16 g of agar per liter of distilled water, was prepared for regular growth of *R. solani*. Wild-type strains of *R. solani* used in the study were collected from rice fields in different regions of China and maintained in our laboratory at China Agricultural University (Mu et al. 2017). Background information on the isolates is provided in Supplementary Table S2. Neither SYP-14288 nor fluazinam had been previously sprayed in any of the locations where *R. solani* was isolated.

Baseline sensitivity of *R. solani* strains to SYP-14288. To establish a baseline sensitivity to SYP-14288, 112 *R. solani* strains were previously collected from infected rice plants on which diamine fungicides had not been used. Agar plugs (5 mm in diameter) cut from the periphery of a 3-day-old colony of each strain were placed on a series of PDA plates amended with SYP-14288 to obtain final concentrations of 0.0001, 0.0005, 0.001, 0.005, 0.01, 0.05, and $0.5 \mu\text{g/ml}$. The concentration of DMSO in the medium was adjusted to 0.1%. Plates amended with only DMSO at the final concentration of 0.1% in volume were used as a control. Each isolate was incubated at 25°C in the dark for 2 days and radial mycelial growth was measured by measuring colony diameter perpendicularly. Mean colony diameter (minus the diameter of the inoculation plug [5 mm]) was measured and expressed as a percentage of growth inhibition (the percentage of growth inhibition determined relative to the fungicide-free check). Effective concentration for 50% inhibition (EC_{50}) of mycelial growth were determined by probit-transformed inhibition data against the logarithmic value of SYP-14288

concentrations (Hou et al. 2018). There were three replicates for each treatment and the experiment was performed twice.

Induction of SYP-14288-resistant *R. solani* mutants. SYP-14288-resistant mutants were generated by exposing *R. solani* cultures to SYP-14288. Specifically, mycelial plugs (5 mm in diameter) were excised from 3-day-old colonies of wild-type isolates and transferred onto PDA plates containing SYP-14288 at $0.05 \mu\text{g/ml}$. Fast-growing mycelial tips were selected and transferred to a new PDA plate containing SYP-14288 at $0.05 \mu\text{g/ml}$. Then, cultures from the plugs were transferred to PDA containing progressively higher SYP-14288 concentrations (0.5, 5, 10, 20, 50, 100, 200, 300, 400, 500, 600, and $700 \mu\text{g/ml}$), successively (Mu et al. 2017). During this process, the fastest-growing colonies at each concentration were picked as potential mutants and transferred to a fungicide-free PDA plate. The EC_{50} of each mutant was then measured, and mutants were judged to be SYP-14288 resistant if the EC_{50} value was 10 times the baseline sensitive.

Resistance level and stability of SYP-14288-resistant *R. solani* mutants. EC_{50} values of both SYP-14288-resistant mutants

Table 1. Concentrations of fungicides used to determine cross-resistance of SYP-14288-resistant mutants of *Rhizoctonia solani*

Fungicide	Fungicide concentrations ($\mu\text{g/ml}$)	
	SYP-14288-sensitive isolates	SYP-14288-resistant mutants
SYP-14288	0, 0.001, 0.0025, 0.005, 0.01, 0.05, 0.1	0, 0.05, 0.1, 0.5, 1, 5, 10
Fluazinam	0, 0.01, 0.05, 0.1, 0.5, 1	0, 0.05, 0.1, 0.5, 1, 5, 10
Carbendazim	0, 0.1, 0.25, 0.5, 0.75, 1	0, 0.1, 0.25, 0.5, 0.75, 1, 10
Azoxystrobin ^z	0, 0.001, 0.01, 0.05, 0.1, 1, 10	0, 0.01, 0.1, 1, 10, 50, 100
Thiophanate-methyl	0, 0.01, 0.05, 0.1, 0.25, 0.5	0, 0.01, 0.05, 0.1, 0.5, 1
Difenoconazole	0, 0.01, 0.1, 0.25, 0.5, 1	0, 0.01, 0.1, 0.5, 1, 5, 10, 50
Fludioxonil	0, 0.01, 0.025, 0.05, 0.075, 0.1	0, 0.01, 0.05, 0.075, 0.1, 0.5
Chlorothalonil	0, 0.1, 0.5, 1, 5, 10	0, 0.1, 0.5, 1, 5, 10, 50
Cyazofamid	0, 1, 5, 10, 25, 50	0, 1, 5, 10, 25, 50, 100
Fentin chloride	0, 0.1, 0.5, 1, 5, 10	0, 0.1, 1, 5, 10, 50
2,4-Dinitrophenol	0, 1, 5, 10, 25, 50	0, 1, 5, 10, 25, 50, 100

^z To determine azoxystrobin toxicity, salicylhydroxamic acid at $100 \mu\text{g/ml}$ was added to completely inhibit the alternative oxidase pathway.

Fig. 1. Chemical structure of SYP-14288.

Fig. 2. Frequency distribution of effective concentration for 50% of growth inhibition (EC_{50}) of *Rhizoctonia solani* in SYP-14288. In total, 112 isolates of *R. solani* were examined by mycelial growth assay on potato dextrose agar.

and their wild-type parents were derived as previously described. To measure the level of resistance, resistance factor (RF) was calculated as $RF = EC_{50}$ of mutant/ EC_{50} of parental strain. To assess the stability of the resistant mutants, mycelial plugs from the margins of actively growing colonies were subjected to 10 successive transfers on fresh and fungicide-free PDA plates. Mutants were cultured for 2 weeks at 25°C in darkness for each transfer, and EC_{50} values were measured after the 1st and 10th transfers. The stability of resistance was represented by factor of sensitivity change (FSC), where $FSC =$ the ratio of RF values at the 1st to 10th transfers (Miao et al. 2016). Each mutant was evaluated in triplicate, and the experiment was conducted twice.

Cross-resistance. In all, 20 SYP-14288-resistant mutants, their parental strain, and 5 randomly selected wild-type strains were used to test the sensitivity of *R. solani* to fungicides having different MOAs, as described previously (Bi et al. 2011). The selected fungicides included fluzazinam, carbendazim, azoxystrobin, thifluzamide, difenoconazole, fludioxonil, chlorothalonil, 2,4-dinitrophenol, fentin chloride, and cyazofamid. EC_{50} values for mycelial growth were calculated as described above (Table 1). Each combination of strain and concentration was represented by three replicated plates, and the likelihood of cross-resistance between SYP-14288 and other fungicides was examined. The experiment was conducted twice.

Mycelial growth rate. At the edge of colonies of the parental strain and resistant mutants, 5-mm-diameter mycelial plugs were cut and transferred onto fungicide-free PDA plates and cultured in the dark at 25°C. Colony diameter was measured perpendicularly at 12, 24, 36, 48, 72, and 96 h. For calculating mycelial growth, the measured diameter was subtracted from the diameter of the mycelial plug (5 mm). The linear regression equation was established to reflect the relationship between mycelial growth and time, and the slope was used to represent the growth rate. There were four replicated plates for each culture, and growth rate of the resistant mutant was compared between mutants and their parental wild-type strain.

Mycelial growth under different temperatures. SYP-14288-resistant mutants and corresponding parental isolates were incubated on PDA plates at 4, 14, 25, 30, and 37°C. Colony diameters were measured in two perpendicular directions after 72 h of incubation in the dark. Each combination of isolate and temperature was represented by four replicated plates.

Virulence of SYP-14288-resistant mutants in rice infection. Rice cultivar Beidao 7, which is susceptible to *R. solani*, was inoculated with SYP-14288-resistant mutants and their parental isolate according to a previously described method (Ghosh et al. 2014), with minor modifications. Before sowing, rice seed of similar size and shape was surface sterilized with 1% NaClO, then washed with sterile distilled water three times. The seed was sown in greenhouse pots containing autoclaved soil and nursery substrates and incubated in a greenhouse at 28°C and 80% relative humidity, with supplemental lighting. At the four- to six-leaf stage, mycelial plugs (5 mm in diameter) of each strain were inoculated on the second-topmost collar of the tiller, just below the leaf collar on the inner side of sheath; then, the sheath was wrapped with aluminum foil to maintain high humidity, which is conducive for sheath blight disease. Each experiment was replicated four times with 20 tillers of each cultivar per isolate. The same number of uninoculated tillers was used as a control. Appearance of disease symptoms was routinely monitored, and percentage of the diseased area was determined at 7 days after inoculation. Disease scores were converted using the evaluation standard as previously described (Jia et al. 2013).

Data analysis. Data were analyzed using SPSS 22 software (IBM Corp., Armonk, NY, U.S.A.). Treatment effects were examined using analyses of variance. If the *F* test was significant ($P \leq 0.05$), treatment means were compared using Fisher's least significant difference test. For examining cross resistance between SYP-14288 and other fungicides, Spearman's rank correlation tests were performed.

Results

Baseline sensitivity of *R. solani* to SYP-14288. In total, 112 *R. solani* field strains were used to determine their sensitivity to SYP-

14288 based on inhibition of mycelial growth on PDA plates. EC_{50} values for SYP-14288 ranged from 0.0003 to 0.01381 µg/ml and the average was 0.0055 ± 0.0030 µg/ml (Fig. 2). The baseline sensitivity showed a frequency distribution of the EC_{50} being unimodal and the range of variation factor (the ratio of maximal and minimal EC_{50} values) was 46.03, indicating the absence of SYP-14288-resistant strains among the field populations.

Generation of SYP-14288-resistant mutants of *R. solani*. With repeated exposure to SYP-14288, 20 mutants (X19-01 to X19-20) were obtained from parental strain X19 on PDA plates with SYP-14288 at 600 and 700 µg/ml.

Resistance level and stability of *R. solani* mutants. As a component of fitness, resistance level and stability of 20 resistant mutants were examined (Table 2). Initial EC_{50} values for the SYP-14288-resistant mutants ranged from 0.0588 to 0.6218 µg/ml, and the corresponding RF values ranged from 11.3 to 119.4. After 10 transfers on fungicide-free PDA, RFs of some mutants had changed somewhat as indicated by FSC values. RF values of the mutants ranged from 1.1 to 125.7. Judged by the resistance criteria of 10 times of baseline sensitivity, mutants X19-13 and X19-15 almost lost their resistance; however, the other 18 mutants kept their resistance to SYP-14288. RFs of mutants X19-03, X19-04, X19-13, and X19-14 decreased after 10 transfers; however, mutants X19-02, X19-18, and X19-20 had high stability of resistance, indicated by unchanged RFs after 10 transfers; and mutants X19-07 and X19-17 even had higher RFs, indicating an increased level of resistance.

Cross-resistance. EC_{50} values of SYP-14288 for the test isolates were highly correlated with the resistance to fluzazinam and fentin chloride, with ρ values being 0.798 and 0.750, respectively, and $P < 0.001$ (Fig. 3A and I). The correlation was also positive and significant between SYP-14288 and either fludioxonil, difenoconazole, chlorothalonil, or cyazofamid, with ρ values were 0.705, 0.610, 0.603 and 0.654, respectively, and $P < 0.001$ (Fig. 3B, C, F, and

Table 2. Resistance factors (RFs) and stability of SYP-14288-resistant mutants of *Rhizoctonia solani*^w

Isolate	Phenotype ^x	EC_{50} (µg/ml)		RF ^y		FSC ^z
		1st transfer	10th transfer	1st transfer	10th transfer	
X19	S	0.0052	0.0043	–	–	–
X19-01	R	0.6218	0.3234	119.4	74.9*	0.63
X19-02	R	0.6218	0.5428	119.4	125.7	1.05
X19-03	R	0.5769	0.1056	110.8	24.5*	0.22
X19-04	R	0.5769	0.1424	110.8	33.0*	0.30
X19-05	R	0.0623	0.0804	12.0	18.6	1.56
X19-06	R	0.1227	0.1912	23.6	44.3*	1.88
X19-07	R	0.0621	0.2449	11.9	56.7*	4.76
X19-08	R	0.0621	0.0746	11.9	17.3	1.45
X19-09	R	0.0621	0.0944	11.9	21.9	1.83
X19-10	R	0.1084	0.2299	20.8	53.3*	2.56
X19-11	R	0.1084	0.0471	20.8	10.9	0.52
X19-12	R	0.0588	0.0268	11.3	6.2	0.55
X19-13	R	0.6085	0.0130	116.9	3.0*	0.03
X19-14	R	0.6085	0.0912	116.9	21.1*	0.18
X19-15	R	0.0788	0.0049	15.1	1.1*	0.07
X19-16	R	0.0788	0.0397	15.1	9.2	0.61
X19-17	R	0.0889	0.3362	17.1	77.9*	4.56
X19-18	R	0.0889	0.0930	17.1	21.5	1.26
X19-19	R	0.0889	0.1097	17.1	25.4	1.49
X19-20	R	0.0621	0.0630	11.9	14.6	1.22

^w Symbols: – indicates no data were available and * indicates a significant difference of RFs between 1st and 10th transfers.

^x Mutants were obtained by mass selection amended with SYP-14288 in media. S represents sensitive to SYP-14288 and R represents resistant to SYP-14288.

^y RF = effective concentration for 50% growth inhibition (EC_{50}) of the fungicide-resistant mutant/ EC_{50} of the parental isolate.

^z FSC = factor of sensitivity change, calculated as the ratio of RF values at the 1st over the 10th transfers.

G). A relatively weak correlation was observed between SYP-14288 and 2,4-dinitrophen ($P < 0.05$ and $\rho = 0.379$; Fig. 3J). No cross-resistance was detected between SYP-14288 and the other fungicides such as carbendazim ($\rho = 0.285$, $P > 0.05$), thifluzamide ($\rho = 0.222$, $P > 0.05$), and azoxystrobin ($\rho = 0.191$, $P > 0.05$) (Fig. 3D, E, and H). However, the sensitivity of most SYP-14288-resistant mutants decreased in response to the three fungicides (Fig. 4). For example,

EC₅₀ of resistant strains to carbendazim increased up to 6.29 times higher compared with parental isolate X19 (Fig. 4A). The sensitivity of mutants to thifluzamide and azoxystrobin showed a similarly decreasing pattern, with the maximum change ratios in EC₅₀ being 8.43 and 262.12, respectively (Fig. 4B and C).

Mycelial growth rate. To examine the fitness of mutants, mycelial growth was observed. Mycelial growth rate of all SYP-14288-

Fig. 3. Cross-resistance of *Rhizoctonia solani* in response to SYP-14288 and one of the following fungicides: **A**, fluzinam; **B**, fludioxonil; **C**, difenoconazole; **D**, carbendazim; **E**, thifluzamide; **F**, chlorothalonil; **G**, cyazofamid; **H**, azoxystrobin; **I**, fentin chloride; and **J**, 2,4-dinitrophen analyzed by Spearman rank correlation. Effective concentrations for 50% mycelial growth inhibition ($\log \text{EC}_{50}$) were logarithmically transformed.

resistant mutants was lower than the parental isolate X19 (Table 3). Mycelia of X19 grew faster and were close to covering PDA plates after 72 h, whereas most of resistant mutants had a colony diameter less than 30 mm. Mutants X19-09, X19-14, X19-16, and X19-17 had significantly higher mycelial growth rates than other strains, though still lower than X19. The resistance to SYP-14288 was negatively associated with mycelial growth.

Mycelial growth under different temperatures. Mycelial growth was examined to determine the fitness of mutants. The parental isolate X19 had a faster growth rate than all of the SYP-14288-resistant mutants regardless of temperatures. The optimal temperature for mycelial growth of all strains was 25°C, and none of the resistant mutants or parental isolate of *R. solani* grew on PDA at 4°C (Table 4). Resistant mutants did not grow or barely grew at 37°C, except mutants X19-09, X19-14, X19-16, and X19-17, whose mycelial growth was slightly lower than X19 ($P < 0.05$). Mycelial growth increased gradually as the temperature increased from 14 to 25°C, and mycelial growth of strains of X19, X19-09, X19-14, X19-16, and X19-17 sharply increased at 18°C compared with 14°C. Growth rate of the resistant mutants at 30°C was slightly less than at 25°C, when the mycelial growth of all strains reached the highest level; however, there was no significant difference between parental isolate X19 and its corresponding resistant mutants. In summary, the response of the SYP-14288-resistant mutants to different temperatures varied, indicating that resistance is independent from growth characteristics at various temperatures.

Pathogenicity of resistant mutants on rice sheaths. To examine the change of pathogenicity of the mutants, fungal cultural plugs were inoculated on rice plants at the basal stem. Both parental isolate X19 and SYP-14288-resistant mutants were virulent and caused typical sheath blight disease symptoms (Fig. 5A). Resistant mutants showed a high level of virulence but were significantly less virulent than the parental isolate (Fig. 5B; $P < 0.05$).

Discussion

We have demonstrated that natural populations of *R. solani* were all sensitive to SYP-14288 based on the established baseline sensitivity. Resistant mutants derived from a wild-type isolate had low to high resistance levels depending on the individual mutant. We have found that some mutants had a high fitting potential, which was indicated by a stable resistance, and unreduced growth characteristics and pathogenicity. More importantly, the SYP-14288 resistance was associated with MDR, which should be a concern in disease management.

Because SYP-14288 is highly similar to fluazinam in structure and MOA, we can refer to fluazinam data for interpreting our results (Lan et al. 2012). Resistance of fungal isolates to fluazinam in fields has

rarely been observed, although this fungicide has been used for many years (Chen et al. 2018; Lehner et al. 2017; Ruano-Rosa et al. 2018). The mechanism of fluazinam resistance is not well understood. Mitogen-activated protein kinases and histidine kinases in osmotic signal transduction are believed to be involved in fluazinam resistance in *S. sclerotiorum* (Mao et al. 2018).

It is generally known that cross-resistance occurs between fungicides that share the same MOA (Popko et al. 2018). However, cross-resistance was detected not only between SYP-14288 and the uncouplers fluazinam and 2,4-dinitrophen but also between SYP-14288 and either fludioxonil (osmotic signal transduction), cyazofamid (quinone inside inhibitor), difenoconazole (demethylation inhibitor), chlorothalonil (multisite), or fentin chloride (inhibitor of ATP synthase). The latter group have different MOAs. Our results confirmed that MDR in *R. solani* populations was induced by SYP-14288. As supporting evidence, positive cross-resistance has been reported between fluazinam and fungicides such as procymidone and fludioxonil in *B. cinerea* (Shao et al. 2015a), suggesting uncouplers involved in MDR.

MDR possibly induced by the uncoupler SYP-14288 can be a challenge to managing fungicide resistance in fields because it can

Table 3. Mycelial growth rate of SYP-14288-resistant mutants (X19-1 to X19-20) and their parental isolate X19 on potato dextrose agar at 25°C

Isolates	Mycelial growth rate ^a
X19	18.3 ± 0.19 a
X19-1	5.0 ± 0.14 ef
X19-2	3.9 ± 0.05 ij
X19-3	3.5 ± 0.16 jk
X19-4	4.6 ± 0.14 fgh
X19-5	3.2 ± 0.27 k
X19-6	4.4 ± 0.03 ghi
X19-7	7.3 ± 0.41 c
X19-8	5.3 ± 0.08 e
X19-9	14.4 ± 0.25 b
X19-10	4.9 ± 0.10 efg
X19-11	6.9 ± 0.13 c
X19-12	5.9 ± 0.41 d
X19-14	14.5 ± 0.19 b
X19-16	14.5 ± 0.04 b
X19-17	14.0 ± 0.09 b
X19-18	4.6 ± 0.13 fgh
X19-19	4.1 ± 0.22 hi
X19-20	4.5 ± 0.18 fghi

^a Means in a column followed by different letters were significantly different according to Fisher's least significant difference test ($P < 0.05$).

Fig. 4. Fold change of sensitivity (effective concentration for 50% growth inhibition) of SYP-14288-resistant mutants to **A**, carbendazim; **B**, thifluzamide; and **C**, azoxystrobin compared with parental isolate X19.

cause the loss of multiple fungicides simultaneously (Mernke et al. 2011). This may replace the common knowledge that fungicide resistance can be overcome by using fungicides having different MOAs.

In terms of resistance mechanisms, target-site modifications, overexpression of target genes, and increase of fungicide efflux are the most commonly reported for site-specific fungicides (Hellin et al. 2018; Mohd-Assaad et al. 2016; Sang et al. 2017). It is not clear whether such a mechanism exists in *R. solani* because we do not know if uncouplers have a specific target protein or binding site. In response to uncoupling fungicides, resistant mutants may enhance the coupling activity and even improve the ability of respiratory metabolism by mutation or overexpression of the target gene. Consequently, the target gene expresses resistance to fungicides having different MOAs. Cross-resistance between SYP-14288 and fungicides of different MOAs may also indicate the possibility of multitarget site mutation or overexpression simultaneously.

By referring to MDR in *B. cinerea*, we may consider other resistance mechanisms involving the MDR induced by uncoupler SYP-14288. MDR phenotypes in *B. cinerea* are associated with increased drug efflux activity and overexpression of two efflux transporters: ATP-binding cassette (ABC) transporters and major facilitator superfamily transporters (Kretschmer et al. 2009).

Leroux (2004) has proposed possible resistance mechanisms involving detoxification of multisite fungicides and fluazinam. In insects and weeds, MDR has been conferred by detoxification of chemical pesticides through enzymatic metabolism involving glutathione S-transferases, cytochrome P450s, hydrolases, or esterases, which constitutes a resistance mechanism and confers resistance (Délye et al. 2013; French-Constant 2013); however, this mechanism has rarely been observed in fungicide resistance in phytopathogenic fungi. MDR has been proposed to go through the xenobiotic detoxification pathway in filamentous fungi (Sang et al. 2018). MDR phenotypes of *S.*

Table 4. Mycelial growth, measured in diameter of colony, of *Rhizoctonia solani* isolates on potato dextrose agar under different temperatures^z

Isolates	Diameter of colony (mm)					
	4°C	14°C	18°C	25°C	30°C	37°C
X19	–	32.0 ± 1.5 a	68.5 ± 1.2 a	85.0 ± 0.3 a	85.3 ± 0.2 a	23.7 ± 0.7 a
X19-1	–	9.5 ± 0.4 ghij	12.8 ± 0.9 ijk	23.3 ± 0.8 hij	23.8 ± 0.6 hi	6.5 ± 0.4 efg
X19-2	–	9.8 ± 0.5 ghi	15.7 ± 0.8 gh	23.7 ± 0.5 hi	22.0 ± 0.6 ijk	6.2 ± 0.3 fghi
X19-3	–	7.3 ± 0.3 j	12.7 ± 0.5 jk	22.2 ± 0.5 ij	20.8 ± 0.9 k	5.2 ± 0.2 ij
X19-4	–	11.7 ± 0.6 fg	16.3 ± 0.8 fg	24.0 ± 0.7 hi	23.7 ± 0.5 hi	6.3 ± 0.2 efgh
X19-5	–	8.2 ± 0.5 ij	13.7 ± 0.7 hijk	21.5 ± 0.6 j	21.7 ± 0.4 jk	–
X19-6	–	12.5 ± 0.9 ef	18.3 ± 0.8 ef	25.3 ± 0.4 h	24.0 ± 0.4 h	6.8 ± 0.3 ef
X19-7	–	9.8 ± 0.6 ghi	19.2 ± 0.9 e	32.8 ± 0.6 ef	31.3 ± 0.7 f	7.2 ± 0.3 ef
X19-8	–	8.2 ± 0.6 ij	15.3 ± 0.7 ghi	29.2 ± 1.2 g	28.5 ± 0.6 g	5.3 ± 0.2 hij
X19-9	–	17.7 ± 1.1 c	40.3 ± 0.8 d	63.3 ± 0.8 d	64.5 ± 1.1 c	16.5 ± 0.8 b
X19-10	–	9.3 ± 0.6 hij	14.7 ± 0.5 ghij	31.2 ± 0.7 fg	27.5 ± 1.5 g	5.5 ± 0.2 ghij
X19-11	–	16.7 ± 1.0 cd	19.3 ± 1.1 e	33.5 ± 0.8 e	34.0 ± 0.6 e	9.0 ± 0.6 d
X19-12	–	14.5 ± 0.8 de	20.7 ± 1.0 e	34.0 ± 1.1 e	32.8 ± 0.8 ef	7.3 ± 0.2 e
X19-14	–	21.8 ± 1.1 b	46.2 ± 1.1 b	72.5 ± 0.7 b	66.7 ± 0.9 b	16.8 ± 0.5 b
X19-16	–	18.2 ± 1.1 c	44.2 ± 1.7 bc	69.7 ± 1.0 c	63.0 ± 0.6 cd	14.3 ± 0.3 c
X19-17	–	18.0 ± 1.0 c	42.8 ± 1.2 cd	64.2 ± 0.8 d	61.2 ± 0.6 d	15.2 ± 0.5 c
X19-18	–	8.8 ± 0.7 hij	11.5 ± 0.4 k	24.3 ± 0.5 h	23.5 ± 0.4 hij	–
X19-19	–	10.7 ± 0.5 fgh	13.7 ± 1.3 hijk	22.2 ± 0.5 ij	23.2 ± 0.3 hij	5.2 ± 0.2 ij
X19-20	–	9.2 ± 0.6 hij	11.2 ± 0.7 k	23.7 ± 0.3 hi	23.0 ± 0.8 hij	–

^z Means in a column followed by different letters are significantly different according to Fisher's least significant difference test ($P < 0.05$); – indicates that the isolates did not grow.

Fig. 5. Pathogenesis of SYP-14288-resistant mutants and their parental isolate on rice. **A**, Sheath blight symptoms on rice between parental isolate X19 and its derived mutant X19-16 and **B**, disease severity of inoculated rice sheath. Data are means of four biological replicates with 20 infected tillers. Means followed by different letters are significantly different ($P < 0.05$) according to Fisher's least significant difference test.

homoeocarpa were identified by the overexpression of cytochrome P450s and ABC efflux transporters, which are regulated by a gain-of-function mutation in the fungus-specific transcription factor (Sang et al. 2018). Interestingly, fungicides showing cross-resistance with SYP-14288 share a similar molecular group, which contains reducible groups such as multiple halogenated groups or functional groups of $-NO_2$ (Clarke et al. 1998; Green et al. 2018; Souza et al. 2019). This may signify a possible detoxification mechanism in *R. solani* leading to the occurrence of resistance; however, this remains to be determined.

In conclusion, the natural population of *R. solani* has a potential to develop resistance to SYP-14288, which may also induce MDR that could affect multiple fungicides having different MOAs. This work provides a significant reference for understanding the resistance mechanisms and possible risk in the field.

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