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Phytotoxin of Rice Aggregate Sheath Spot Pathogen *Rhizoctonia oryzae-sativae* and Its Biological Activities

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1 **Phytotoxin of Rice Aggregate Sheath Spot Pathogen *Rhizoctonia oryzae-sativae* and Its**
2 **Biological Activities**

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18 **ABSTRACT**

19 Rice aggregate sheath spot disease occurs in many countries, causing serious yield losses. In
20 China, the disease-causing fungus *Rhizoctonia oryzae-sativae* was reported in 1985, and since
21 then, it has rarely been reported in major rice-growing areas after almost 30 years. Compared with
22 *Rhizoctonia solani*, *R. oryzae-sativae* has a significantly different physiological morphology and
23 growth status, although both fungi affect rice leaves in very similar ways. The optimum
24 temperature for the suitable growth of *R. oryzae-sativae* is 31 °C, which is consistent with
25 previous reports. We extracted phytotoxins from *R. oryzae-sativae* and analyzed its biological

activity via the detached leaf and radicle inhibition methods. *Rhizoctonia solani* and *R. oryzae-sativae* exhibit differences in terms of pathogenicity and toxins activity, which indicates that these fungi may produce different toxins components. Based on gas chromatography–mass spectrometry data, esters, phenols, and other components were present in the crude toxins extract of *R. oryzae-sativae*. Our research provides a new method for studying the phytotoxins of *R. oryzae-sativae*. However, further studies are needed to elucidate the pathogenic mechanisms responsible for the aggregate sheath spot disease on rice.

Keywords Aggregate sheath spot; Phytotoxins; Gas chromatography–mass spectrometry; Biological activity.

Introduction

Rhizoctonia oryzae-sativae (teleomorphs are *Thanetophorus cucumeris*, *Waitea circinata* and *Ceratobasidium oryzae-sativae* respectively) is the fungus that causes rice aggregate sheath spot, which has been reported in many countries, such as India, Japan, Iran, Vietnam, Thailand, China, Italy, Chile, Brazil, Colombia, Venezuela, Australia, and the United States (Chaijuckam *et al.*, 2010; Lanoiselet *et al.*, 2007; Pereira *et al.*, 2017; Chavarro Mesa *et al.*, 2020). *R. oryzae-sativae* also infects corn, wheat, sorghum, oats, barley, and pea (Lanoiselet *et al.*, 2007). Based on morphological observations, the young hyphae colorless while older mycelium of *R. oryzae-sativae* is brown on potato dextrose agar (PDA) medium. Sclerotia may form inside infected plants and can be observed on the surface of the lesion. Aggregate sheath spot can cause high yield losses, ranging from as low as 4% in Uruguay to as high as 20% in Australia. In China,

1 although aggregate sheath spot was reported by Ou *et al.* In 1985, it has been rarely reported
2 causing yield losses in the major rice-cropping areas. One aspect of the reported low incidence of
3 the aggregate sheath spot is the difficulty in diagnostics of this disease amongst the
4 sheath-associated diseases (Pereira *et al.*, 2017). Regardless of the reason, the pathogen's
5 supposedly relative low importance in agricultural fields might explain the scarce research about
6 aspects of the biology and epidemiology of the aggregate sheath spot disease (Lanoiselet *et al.*,
7 2007).

8 Pathogenicity mechanisms of *R. solani* have been reported, besides a lot of cell wall
9 degrading enzymes, the role of phytotoxins is important. But the contents of phytotoxins from *R.*
10 *solani* have different results. Many researchers reported that *R. solani* produced phenylacetic acid
11 (PAA) and its derivatives in rice (Lakshman *et al.*, 2006; Mandava *et al.*, 1980; Aoki *et al.*, 1963).
12 Other reports indicated the phytotoxins were composed of phenylacetic acid,
13 hetero-hydroxyphenylacetic acid, ortho-hydroxyphenylacetic acid and para-hydroxyphenylacetic
14 acid (Ramalingam *et al.*, 2006). Vidhyasekaran *et al.* suggested that the phytotoxins from *R. solani*
15 were carbohydrates, including glucose, maltose, N-acetylgalactose and N-acetylglucose
16 (Vidhyasekaran *et al.*, 1997). Report about the phytotoxins of *R. oryzae-sativae* is hardly known.

17 In this study, we isolated *R. oryzae-sativae* from rice-growing areas in Haerbin, China, and
18 analyzed its phytotoxins. Gas-chromatography-mass spectrometry (GC-MS) analysis was
19 performed to investigate the chemical diversity of the metabolites obtained in the crude fungal
20 phytotoxins extract and their bioactivities to rice seeds germination.

21 **Materials and Methods**

22 **Pathogen collection**

The rice leaf and stem with typical symptoms of fungal disease were collected from three rice-growing areas in Haerbin, Qiqihaer, and Taixing, China, from 2016 to 2017 (Fig. 1). The leaves were cut, their surfaces were sterilized. Then, they were plated on potato dextrose agar (PDA). 24 *R. oryzae-sativae*-like cultures were obtained, purified, and incubated for 7 days.

Determination of fungal growth rate

Experiment of fungal growth rate was carried out. 10 *R. oryzae-sativae* isolates and 14 *R. solani* isolates were plated on PDA and incubated at different temperatures (25 °C–31 °C) for 2 days. Colony diameter were measured and data analysis with Data Processing System (DPS) software (Jia *et al.*, 2013). Analysis of Duncan's new multiple range test for difference significance.

Identification of isolates

All isolates were identified based on morphological characteristics. Furthermore, the identity of 10 isolates was determined via the analyses of internal transcribed spacer (ITS) sequences: ITS1, 5'TCCGTAGGTGAACCTGCGG, and ITS4, 5'TCCTCCGCTTATTGATATGC (White *et al.*, 1990). DNA was extracted from isolates. DNA samples were amplified under the following conditions: initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 72°C during 30 seconds; a final extension phase was performed at 72°C during 10 minutes. Reactions were carried out in 25 µl reaction volumes. The amplified products were purified using a PCR Purification Kit (TIANGEN Ltd., Beijing, China) according to the manufacturer's instructions. The purified PCR products were sequenced with both primers by Zoonbio Biotechnology Co., Ltd, Nanjing, China. DNA sequences were analyzed using the BLASTN online service in the NCBI database.

Construction of phylogenetic tree

All isolates and downloaded ITS sequences were aligned using the Clustal Omega tool (Sievers *et al.*, 2011). Phylogenetic trees based on maximum likelihood, illustrating the relationship among the ITS sequences of *R. solani*, *R. oryzae-sativae*. The maximum likelihood (ML) phylogenetic tree was generated using MEGA. For ML analyses, the default parameters were used. *Rhizoctonia zeae* and *Rhizoctonia oryzae III* were used to outgroup (Table 1).

Extraction of the crude phytotoxins

The culture medium (500 mL) was filtered with a sterile gauze and extracted with methanol and ether. The filtrate was shaken for 20 min and centrifuged at 3500 g for 15 min at 4 °C to remove the precipitate. Methanol was removed by evaporating to dryness at 60 °C, and ether was added to the aqueous layer and extract. The procedure was repeated for three times and evaporated to dryness at 45 °C. Afterward, a brown crude extract was obtained.

Inhibitory activity assay

The inhibitory activity of the radicle elongation of rice seeds was determined according to the method of Aliferis *et al* (2010b) with minor modifications. Jingang30 was used as the test material. The brown crude extracts from DBWK1, DBWK2, JSWK1, and JSWK2 isolate were dissolved in methanol (the concentration of the stock solution is 1g mL⁻¹) and then diluted into different concentrations using distilled water. Each crude extract was assayed at 2.5 and 5.0 mg mL⁻¹, respectively. Five seeds were added to a 24-well plate, and each well contained 200 µL of the crude extract. Three replicates were used for each treatment. The plates with the germinated seeds were stored in a chamber at 25 °C for 48 h in darkness. The radicle length of each seed was measured. The elongation inhibitory rate of the radicle was determined by using the method of Xu

et al (Xu *et al.*, 2015). The experiment has been replicated twice. Data analysis with DPS software. Analysis of Duncan's new multiple range test for difference significance ($p < 0.05$).

Chemical diversity of the metabolites from the crude phytotoxins

The crude extract was analyzed via gas chromatography–mass spectrometry (GC-MS) using capillary column TG-5 ms Phenyl Methyl Siloxane (30 m $\times$ 250 μ m $\times$ 0.25 μ m) in Agilent 6890N. Helium gas with flow rate of 1.0 mL/min was used as a carrier, and the mass detector was used in the split mode. The oven temperature was initially kept at 32 °C for 1 min and then ramped to 280 °C at 10 °C/min.

Results

Isolates collection and colony characteristics of isolates

The 34 fungal isolates obtained from the three rice-growing areas in Haerbin, Qiqihaer, and Taixing, China, were plated on PDA and incubated at 25 °C for 7 days. But 10 isolates grow slowly than other isolates. We compared these colony characteristics of isolates, identified that these fungal isolates were *R. oryzae-sativae*.

R. oryzae-sativae colonies were grown on PDA-based medium for 7 days. The vegetative mycelium, which was colorless when young, became pale brown as it grew and matured (Fig. 1). Sclerotia from each isolate are white when young but become brown upon maturity. Their diameter ranged from 0.25 mm to more than 2 mm due to aggregation.

Determination of fungal growth rate

Experiment of fungal growth rate was carried out. 10 *R. oryzae-sativae* isolates and 14 *R. solani* isolates were plated on PDA and incubated at different temperatures (25 °C–31 °C) for 2

days. Results showed that the optimum temperature for 9/10 fungal isolates obtained from Haerbin hyphal growth is 31 °C (Table. 2), and that for 9/14 fungal isolates obtained from Qiqihaer and Taixing hyphal growth is 29 °C.

Phylogenetic trees

The 10 fungal isolates obtained from Haerbin were amplified with ITS primers and sequenced. BLASTN results showed that these fungal isolates were *R. oryzae-sativae*. These results explained the different growth rate between fungal isolates obtained from Jiangsu area and Northeast China area. Phylogenetic inferences were based on rDNA ITS sequences of isolates from the two areas (Fig. 2). The phylogenetic tree confirmed the close relationship of *R. solani* JSWK1 to AG-1 subgroup IA. The 10 fungal isolates obtained from Haerbin (DBWK1-3, 5, 8, 10, 12-15) were found to cluster with *Ceratobasidium oryzae-sativae* (*Rhizoctonia oryzae-sativae*).

In vitro phytotoxic activity

In the radicle elongation inhibitory activity assay, in vitro phytotoxic activity was evaluated with Jingang30. Table 3 shows that compounds from DBWK1, DBWK2, JSWK1, and JSWK2 had remarkable toxicity at a range of 5.0 mg mL⁻¹. Compared with the inhibition on radicle elongation of rice seeds, DBWK1 and DBWK2 (*R. oryzae-sativae*) are less aggressive and damaging pathogens on rice seeds than JSWK1 and JSWK2 (*R. solani*) (Table 3).

GC-MS analysis of the phytotoxins from *R. oryzae-sativae*

The crude compounds were isolated and purified from rice sheath blight pathogen *R. solani* and rice aggregate sheath spot pathogen *R. oryzae-sativae*, and were analyzed by using GC-MS. A

total of 10 bioactive compounds were detected (Table 4) in *R. oryzae-sativae*. By comparing the mass spectra of the constituent compounds with information from the NIST library, 11 peaks were obtained (Fig. 3). Among the 11 compounds, 1 compound was the constituent of aromatic compounds, 2,4-bis(1,1-dimethylethyl)-phenol ($C_{14}H_{22}O$), 1 compound was the constituent of organic acid (Tetradecanoic acid), while others were derivatives of different hydrocarbons. The following several compounds were identified: 2,3-Butanediol ($C_4H_{10}O_2$) with retention time of 5.07; 2-methyl eicosane ($C_{21}H_{44}$) with retention time of 21.83; and 2-Hexyl-1-decanol ($C_{16}H_{34}O$) with a retention time of 27.25 (Table 4). 2,3-Butanediol ($C_4H_{10}O_2$) showed the highest percentage peak area in the crude extract. GC-MS analysis data indicated that this compound was the most abundant in the *R. oryzae-sativae* extract.

Discussion

In this study, rice sheath blight strains were collected from Jiangsu and Northeast China. The fungal strains showed significant differences in terms of physiological morphology and growth status although they manifest very similar symptoms in rice leaves. Diagnosis of the disease that occurs in the field is difficult. As the sequence analysis of ITS can substantially reflect the differences in base pairs between family, genus, and species and the ITS sequence fragments are small, the ITS sequence is easy to analyze and identify. Therefore, the ITS sequences of fungi from the two regions were analyzed. Results showed that the isolates from Jiangsu are *R. solani*, and the ones from Northeast China are *R. oryzae-sativae*. The optimum temperatures for the suitable growth of *R. solani* and *R. oryzae-sativae* are 29 °C and 31 °C, respectively, which are consistent with previous reports. Most of the rice-producing areas in Jiangsu have high temperature and high humidity. However, the rice-producing areas in the northeast have a high

1 temperature during the day and have a large temperature difference between day and night. *R.*
2 *oryzae-sativae* is common in California whose climatic conditions are similar to those in Northeast
3 China. California has a high temperature during the day but has a large temperature difference
4 between day and night. Therefore, whether the occurrence of the disease is directly related to the
5 geographical climate of the two regions still requires further research.

6 We analyzed the biological activity of the phytotoxins from *R. oryzae-sativae* via the
7 detached leaf and radicle inhibition methods. The results showed that the phytotoxins from *R.*
8 *oryzae-sativae* caused necrotrophic symptoms. However, the symptoms were not serious
9 compared with those of *R. solani*. The extracted toxins from *R. oryzae-sativae* had a stronger
10 inhibitory effect on rice radicle (Table 3). Studies indicate that the biological ability of toxins from
11 different strains was significantly different (Ren *et al.*, 2017; Xu *et al.*, 2015). The pathogenicity
12 of strains is positively correlated with the activity of toxins, and the sensitivity of different hosts to
13 toxins are also different. *R. solani* and *R. oryzae-sativae* showed differences in terms of
14 pathogenicity and toxins activity. This result indicated that these fungi may produce different
15 toxins components. By using GC-MS, the special components of the strain were detected from
16 different fungi. The component 2,4-bis(1,1-dimethylethyl)-phenol was detected from *R.*
17 *oryzae-sativae* obtained from Northeast China. The high concentration of phenol solution reduced
18 the nitrate reductase activity and chlorophyll content of rice leaves and increased the membrane
19 permeability of root cells. The critical concentration of phenol causing water pollution is 0.5
20 mmol/L; above this concentration, rice will be harmed (Brooks., 2007; Chen *et al.*, 2011).

21 The pathogenic activity of these toxin components is unclear. Such components and their
22 pathogenicity must be determined in subsequent studies to make a suitable evaluation.

The pathogen can secrete toxins into the PDA liquid medium. The toxins were separated from the culture filtrate via organic solvent extraction or activated carbon adsorption. Different methods obtained different toxin components. Brooks (2007) suggested that the toxins extracted using different methods and the toxin produced by different fusion groups or subgroups may be different. Vidhyasekaran *et al* (1997) isolated a host-specific toxin from *R. solani*, which was purified and analyzed as a carbohydrate containing mannose, glucose, N-acetylgalactosamine, and N-acetylglucose. Aoki *et al* (1963) isolated two strains of *R. solani* from beet, which was purified, and detected five components, namely, PAA, *m*-hydroxyphenylacetic acid, β -furoic acid, succinic acid, and lactic acid. In this study, the toxins were not dissolved with *n*-hexane, indicating that it were incompatible. Therefore, the crude toxins were dissolved with diethyl ether prior to GC-MS analysis. We detected esters, phenols, and other components, which may be the causative factors behind the rice disease, but the detailed mechanism of the toxin needs further research.

Our research is the first to extract the crude toxins using diethyl ether and obtain components that were not previously isolated. The present study provides a new method for studying the phytotoxins of *R. oryzae-sativae*. The purification of the isolated toxin components and the identification of their function may aid in the understanding of the pathogenic mechanism, thereby providing a method to control the plant disease.

Acknowledgements

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1 Table 1. Source of ITS sequences and isolates used in this study
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Isolate	Accession	AG/sub-group		Reference
1	AB000001.1	AG-3		Kuninaga et al., 1997
2	AB000003	AG7		Kuninaga et al., 1997
3	AB000006	AG4	HGII	Kuninaga et al., 1997
4	AB000007	AG4	HGI	Kuninaga et al., 1997
5	AB000010	AG1	IA	Kuninaga et al., 1997
6	AB000011	AG8		Kuninaga et al., 1997
7	AB000013	AG2-2		Kuninaga et al., 1997
8	AB000014	AG2-2	IV	Kuninaga et al., 1997
9	AB000019	AG6	HG1	Kuninaga et al., 1997
10	AB000021	AG5		Kuninaga et al., 1997
11	AB000022	AG6	GV	Kuninaga et al., 1997
12	AB000025	AG1	IB	Kuninaga et al., 1997
13	AB000029	AG1	IC	Kuninaga et al., 1997
14	AB000037	AG9		Kuninaga et al., 1997
15	AB000044	AGBI		Kuninaga et al., 1997
16	AB000046	AG9	TP	Kuninaga et al., 1997
17	AB196641.2	AGBa		GENBANK
18	AF222793	<i>R. cerealis</i>		Hsiang, T. and Dean, J. D. 2001
19	AF222799	<i>Rhizoctonia_zeae</i>		Hsiang, T. and Dean, J. D. 2002
20	DQ279013.1	AG-E		GENBANK
21	DQ279014.1	AG-F		GENBANK
22	DQ279052.1	AG-A		GENBANK
23	DQ279058.1	AG-Bb		GENBANK
24	DQ356417.1	<i>Rhizoctonia_oryz ae_III</i>		Okubaraet al., 2008
25	FJ231397.1	<i>Ceratorhiza_oryz ae-sativae</i>		GENBANK
26	KP171639.1	AG-D		GENBANK
27	KX674519.1	AG1	IA	GENBANK
28	KX674520.1	AG1	IA	GENBANK
29	KX674525.1	AG1	IA	GENBANK
30	MG397055.1	AG1	IA	GENBANK
31	MG397056.1	AG1	IA	GENBANK
32	MG397062.1	AG1	IA	GENBANK
33	MH861282.1	AG1	IA	GENBANK

JSWK1	MT587788	AG1	IA	current study
JSWK9	MT587794.1	AG1	IA	current study
JSWK14	MT620736.1	AG1	IA	current study
DBWK1	MT620754.1	<i>Ceratorhiza_oryz</i> <i>ae-sativae</i>		current study
DBWK2	MT620755.1	<i>Ceratorhiza_oryz</i> <i>ae-sativae</i>		current study
DBWK3	MT620756.1	<i>Ceratorhiza_oryz</i> <i>ae-sativae</i>		current study
DBWK5	MT620757.1	<i>Ceratorhiza_oryz</i> <i>ae-sativae</i>		current study
DBWK7	MT620758.1	<i>Ceratorhiza_oryz</i> <i>ae-sativae</i>		current study
DBWK8	MT620759.1	<i>Ceratorhiza_oryz</i> <i>ae-sativae</i>		current study
DBWK10	MT620760.1	<i>Ceratorhiza_oryz</i> <i>ae-sativae</i>		current study
DBWK13	MT620762.1	<i>Ceratorhiza_oryz</i> <i>ae-sativae</i>		current study
DBWK14	MT620763.1	<i>Ceratorhiza_oryz</i> <i>ae-sativae</i>		current study
DBWK15	MT620764.1	<i>Ceratorhiza_oryz</i> <i>ae-sativae</i>		current study

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1 Table 2. Colony diameter of *R. solani* in Jiangsu and *R. oryzae-sativae* in Northeast China at
2 different temperatures.
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Isolate	Average of three replicates of colony diameter (cm)/ Difference significance (p<0.01)				
	25 °C	27 °C	28 °C	29 °C	31 °C
JSWK1	58ABCD	60EF	66DE	69CDE	64CDE
JSWK2	61ABC	67CDEF	77ABCD	77ABCD	68BC
JSWK4	65ABC	70BCDE	77ABCD	76ABCD	71ABC
JSWK5	68AB	76ABC	69CDE	73ABCD	68BC
JSWK6	50CDE	61EF	79ABC	81AB	71ABC
JSWK7	69A	80AB	88A	79ABC	77A
JSWK8	71A	83A	79ABC	83A	69BC
JSWK9	46DEF	56F	60E	62E	57EFG
JSWK10	61ABCD	76ABC	70BCDE	79ABC	71ABC
JSWK11	68AB	75ABCD	78ABCD	80AB	74AB
JSWK12	59ABCD	64DEF	66DE	72BCD	60DEF
JSWK13	61ABC	75ABCD	81AB	75ABCD	68BC
JSWK14	54BCD	66CDEF	81AB	76ABCD	64CDE
JSWK15	57ABCD	61EF	68CDE	68DE	65CD
DBWK1	39EF	41G	42F	45F	47HIJ
DBWK2	39EF	42G	47F	45F	50HIJ
DBWK3	37EF	42G	44F	44F	52GH
DBWK5	37EF	40G	42F	45F	50HI
DBWK8	35F	38G	42F	43F	44IJ
DBWK10	39EF	42G	39F	42F	46HIJ
DBWK12	34F	37G	38F	40F	43IJ
DBWK13	36EF	36G	40F	41F	45HIJ
DBWK14	38EF	42G	44F	49F	48HIJ
DBWK15	36EF	38G	38F	39F	42J

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Table 3. Inhibitory activity of the compounds on the radicle elongation of rice seeds¹⁾.

Crude phytoxins concentration (mg mL ⁻¹)		Control	JSWK1	JSWK2	DBWK1	DBWK2
Inhibitory rate of the radicle elongation (%)	2.5	0	39a	38a	31b	34b
	5.0	0	87a	88a	75b	77b

¹⁾ The negative control (2% methanol) did not inhibit the radicle elongation of rice seeds.

Table 4 Compounds identified in the ether extract of *R. oryzae-sativae* DBWK1 via GC-MS.

Number	Retention time	Compound name	Formula	MP
1	5.07	2,3-Butanediol	C ₄ H ₁₀ O ₂	90
2	7.13	Methane, nitroso-	CH ₃ NO	45
3	12.9	Tetradecane, 1-iodo-	C ₁₄ H ₂₉ I	324
4	14.66	Dodecane, 2,7,10-trimethyl-	C ₁₅ H ₃₂	212
5	15.1	Sulfurous acid, 2-ethylhexyl hexyl ester	C ₁₄ H ₃₀ O ₃ S	278
6	18.24	Tetradecanoic acid	C ₁₄ H ₂₈ O ₂	228
7	19.82	Nonadecane, 2-methyl	C ₂₀ H ₄₂	282
8	20.26	<i>n</i> -Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	256
9	21.83	Eicosane, 2-methyl-	C ₂₁ H ₄₄	296
10	27.25	1-Decanol, 2-hexyl-	C ₁₆ H ₃₄ O	242
11	15.43	Phenol, 2,4-bis(1,1-dimethylethyl)-	C ₁₄ H ₂₂ O	206

Fig. 1. Comparative images showing the colonies and sclerotia of *R. oryzae-sativae* (a) and *R. solani* (b) on PDA.

Fig. 2. Phylogenetic trees based on maximum likelihood, illustrating the relationship among the ITS sequences of *R. solani*, *R. oryzae-sativae*. The maximum likelihood (ML) phylogenetic tree was generated using MEGA. For ML analyses, the default parameters were used. *Rhizoctonia zeae* and *Rhizoctonia oryzae III* were used to outgroup. Red color indicated the fungal isolates obtained from Haerbin area (DBWK1) and Jiangsu area (JSWK1).

Fig. 3. GC-MS analysis of *R. oryzae-sativae* phytotoxins. The crude compounds were isolated and purified from rice sheath blight pathogen *R. solani* and rice aggregate sheath spot pathogen *R. oryzae-sativae*. The crude extract was analyzed via gas chromatography–mass spectrometry (GC-MS) using capillary column TG-5 ms Phenyl Methyl Siloxane (30 m × 250 µm × 0.25 µm) in Agilent 6890N. Helium gas with flow rate of 1.0 mL/min was used as a carrier, and the mass detector was used in the split mode. The oven temperature was initially kept at 32 °C for 1 min and then ramped to 280 °C at 10 °C/min.

Fig. 1. Comparative images showing the colonies and sclerotia of *R. oryzae-sativae* (a) and *R. solani* (b) on PDA.

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