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Screening of endophytic fungi with anti-phytopathogen activities from *Heptacodium miconioides*

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Abstract: [**Objective**] To find anti-phytopathogen compounds from endophytic fungi associated with the endangered species *Heptacodium miconioides*. [**Methods**] Fungi from *H. miconioides* with antifungal activities were isolated according to the plate growth inhibition method. The fungus with preferable antifungal activities was identified by morphological identification and 5.8S rRNA sequence analysis. The bioactive metabolites were isolated and purified by chromatographic methods; the structures were determined by spectroscopic analysis. [**Results**] *Alternaria solani* QZH 10 showed better antifungal activity against *Rhizoctorzia solani* and *Valsa mali* with the inhibition rates of 89.1% and 67.9%, respectively. The ethyl acetate crude extract of QZH 10 had strong antifungal activity against *Magnaporthe oryzae* with the rate of 100.0% under the concentration of 100 μg/mL. Two antifungal metabolites altersolanol A and 6-*O*-methylalaternin were isolated and determined from QZH 10. Altersolanol A possessed strong activity against *M. oryzae* with the inhibition rate of more than 85%, 6-*O*-methylalaternin had the mightily activity against *V. mali* with the inhibition rate of 100.0% under the concentration of 100 μg/mL. [**Conclusion**] Altersolanol A and 6-*O*-methylalaternin are potential fungicides originated from microorganisms.

Keywords: *Heptacodium miconioides*, *Alternaria solani*, endophytic fungi, antifungal activity, natural product
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Phytopathogen has always been a big problem in agriculture, weakening the quality of products and affecting the crop yield. Currently, the main measure to resist the phytopathogen is chemical pesticides. However, long-term use of chemical pesticides has brought some problems, such as phytopathogen resistance, food security, and environmental pollution[1]. Therefore, the development of new fungicides is imminent. Microbial control of phytopathogen has many potential advantages, and it is an important research direction of the new fungicides.

Endophytes can incubate in their host plants for a

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prolonged period or display disease symptoms when their hosts are growing under adverse environmental conditions[2], which play a major role in providing hosts resistance against different biotic and abiotic stresses[3-4]. Recent studies have demonstrated that the endophytic fungi communities living within plant tissues produce a wide range of metabolites with different bioactivities that may be used as scaffolds for the development of new drugs[5-7].

Heptacodium miconioides is a rare and endangered plant of China, which has a high economic and scientific study value. The endangered plant *H. miconioides* has some antifungal activities, it seemed possible that its tissues might be colonized by endophytic fungi which can produce antifungal compounds[8]. The objectives of the present studies were to separate the endophytic fungi from *H. miconioides*, screen the antifungal activities against several phytopathogen and isolate the active compounds, which expect to lay a foundation to find new natural antifungal metabolites for the development of microbial fungicides.

1 Materials and methods

1.1 Isolation of endophytic fungi

The fresh, healthy stems, leaves, bark samples of *Heptacodium miconioides* were collected and processed within 24 h of collection. They were washed in running water to remove soil particles. The leaves were sterilized by sequential immersion in 75% ethanol for 1 min, followed by 3% NaClO for 1 min and then rinsed 5 times in sterilized distilled water for 5 s each. The stems and bark were in 75% ethanol for 3 min, 3% NaClO for 5 min followed by rinsing 5 times in sterilized distilled water, and then dried on the sterile filter paper. The sterilized tissues were cut into 5 cm pieces and deposited on a Petri dish containing MEA medium (20 g of malt, 20 g of sucrose, 1 g of peptone and 20 g of agar in 1 L of distilled water). The material was incubated at 28℃ for 3 – 4 days and hyphal tips of the fungi protruding from the inner segments on the plates were further purified and transferred to slants.

1.2 Microbial fermentation

The fresh mycelium grown on MEA medium at 28℃ was inoculated into 250 mL Erlenmeyer flasks containing 100 mL of ME liquid medium (20 g of malt, 20 g of sucrose, 1 g of peptone in 1 L of distilled water), followed by shaking (200 r/min) continuously for 7 days at 28℃. The broth culture was filtered to separate the culture broth and mycelium. The culture broth was refiltered with a 0.45 μm bacterial filter to obtain the sterile fermentation broth, subsequently, which was kept at 4℃.

1.3 Efficacy of fermentation broth of endophytic fungi against phytopathogen *in vitro*

The fermentation broth bioactivities against *Rhizoctorzia solani* and *Valsa mali* were tested according to the plate growth inhibition method[9]. 1.0 mL fermentation broth of each endophytic fungus was mixed with 9.0 mL MEA medium (50 – 60℃) to the concentration of 10 fold dilution, 10.0 mL MEA medium was used as the blank control. After inoculating the 5 mm diameter phytopathogen mycelial disks onto the solid medium, the dishes were incubated in the dark at 28℃ for 3 – 4 days. The formula for counting the percentage of growth inhibition (GI) was shown as follows: $GI = \{(A-B)/A - 5\} \times 100\%$, where A was the diameter of the growth zone in the control dish (mm), B was the diameter of the growth zone in the experimental dish (mm)[10].

1.4 Antifungal activities of different polar solvent crude extracts of QZH 10

To discover the antifungal activities of different polar solvent crude extracts of the strain QZH 10, the fermentation broth was extracted three times with petroleum ether, ethyl acetate, 1-butanol in turns at room temperature, the remaining one was aqueous phase. Then, the different solvent was dried by a rotary evaporator to yield crude extract, respectively. The antifungal activities of the different crude extracts against *R. solani* and *V. mali* were tested by the method described above.

1.5 Antifungal spectrum of the ethyl acetate crude extract of QZH 10

The antifungal spectrum of the ethyl acetate crude extract was initially tested against six kinds of fungi, including *Fusarium graminearum*, *Dothiorella gregaria*, *Alternaria solani*, *Magnaporthe oryzae*, *F. oxysporum* f. sp. *momordicae*, *F. oxysporum* f. sp. *cucumerinum*.

1.6 Identification of QZH 10

QZH 10 was identified by comparing the morphological character and 5.8S rRNA sequence to those of standard record. The genomic DNA was extracted according to the procedures of Power kit, DNA isolation kit, and ITS1 (5′-TCCGTAGGTG AACCTGCGG-3′)-ITS4 (5′-TCCTCCGCTTATTGATA TGC-3′) primers set was chosen to amplify a major part of the 5.8S rRNA, resulting the intense PCR product of approximately 500 – 750 bp in size. Polymerase chain reactions were carried out by using 50 μL reaction volumes which containing approximately 1.0 μL of template DNA, 39.0 μL ddH_2O, 4.0 μL 10 × PCR Buffer, 4.0 μL dNTPs, 1.0 μL of each primer, 0.2 μL Taq DNA polymerase. Cycling parameters were: (1) 94℃ for 2 min; (2) 35 cycles of 94℃ for 1 min, 55℃ for 1 min and 72℃ for 2 min; (3) 72℃ for 5 min. A BLAST search was used to search for the closest matched sequences in the GenBank database[11]. The sequences obtained were compared with nucleotide sequences deposited in GenBank.

1.7 Isolation and identification of active compounds

The QZH 10 fermentation broth (30 L) was extracted with ethyl acetate (5 × 30 L) at room temperature. Evaporation of menstruum *in vacuo* gave a tan oily residue, which was subjected to chromatography over a silica-gel column eluting with CH_2Cl_2/MeOH mixtures of a stably growing polarity to give active compounds. Structural identifications of the bioactive metabolites were based on the spectroscopic analyses. The electrospray ionization mass spectrometry (ESI-MS) spectra were collected on a Mariner Mass 5304 instrument. The ^1H and ^{13}C nuclear magnetic resonance (NMR) data were acquired on a Bruker AVANCE-400 (Bruker Switzerland) spectrometer at 400 MHz[12].

1.8 Antifungal spectrums of the active compounds

The antifungal spectrums of active compounds were tested against the fungi mentioned above except the *D. gregaria*. Cycloheximide was used as the positive control.

2 Results

2.1 Fermentation broth of endophytic fungi against phytopathogen *in vitro*

Antifungal activities of all the endophytic fungi isolated from *H. Miconioides* against *R. solani* and *V. Mali* were tested by the plate growth inhibition method (Table 1). The endophytic fungi QZH 10, QZH 12, QZH 20 and QZH Ⅱ 06 displayed potent antifungal activities against the two phytopathogen with the inhibition rate of more than 55% under the concentration of 10 fold dilution of the fermentation broth. Especially, the QZH 10 had strong inhibition active against *R. solani* with the inhibition rate of 89.1% (Figure 1). However, the other strains presented the low inhibition rate against the tested phytopathogen.

2.2 Antifungal activities of different polar solvent crude extracts of QZH 10

As demonstrated in Table 2, the petroleum ether and ethyl acetate phase exhibited considerable activities against *R. solani* with the inhibition rate of more than 60%, and the ethyl acetate phase showed 96.2% radial growth inhibition rate against *V. mali* under the concentration of 100 μg/mL (Figure 2). Therefore, the main antifungal ingredients of fermentation broth may contain in the ethyl acetate fraction.

Table 1. Inhibition rate of 10 fold dilution of the fermentation broth from the endophytic fungi of *H. miconioides* against *R. solani* and *V. mali*

Strains No.	*R. solani*		*V. mali*		Strains No.	*R. solani*		*V. mali*	
	Colony diameter	Inhibition rate/%	Colony diameter	Inhibition rate/%		Colony diameter	Inhibition rate/%	Colony diameter	Inhibition rate/%
QZH 01	26.5 ± 3.2	25.2 ± 8.3	17.4 ± 2.4	51.7 ± 8.7	QZH 26	36.9 ± 1.4	0.0 ± 0.0	15.5 ± 3.8	58.9 ± 9.4
QZH 02	25.0 ± 1.7	30.2 ± 4.0	28.1 ± 1.3	9.6 ± 4.5	QZH Ⅱ 01	27.0 ± 0.9	23.3 ± 2.8	39.7 ± 3.1	0.0 ± 0.0
QZH 03	23.3 ± 2.4	36.2 ± 5.5	19.2 ± 0.8	44.5 ± 2.9	QZH Ⅱ 03	25.2 ± 2.6	29.7 ± 8.0	30.9 ± 2.7	0.0 ± 0.0
QZH 04	23.8 ± 2.7	34.5 ± 6.4	17.0 ± 1.4	53.0 ± 4.9	QZH Ⅱ 04	27.8 ± 9.9	20.6 ± 3.1	17.5 ± 2.5	51.0 ± 6.0
QZH 05	19.6 ± 1.2	49.1 ± 2.8	20.2 ± 2.1	40.8 ± 7.6	QZH Ⅱ 05	20.3 ± 1.8	46.7 ± 5.4	20.0 ± 1.4	41.5 ± 4.2
QZH 06	30.2 ± 0.9	12.0 ± 4.7	30.2 ± 0.8	1.4 ± 4.6	QZH Ⅱ 06	9.6 ± 0.3	84.1 ± 1.0	16.0 ± 1.6	57.0 ± 5.7
QZH 07	37.0 ± 2.7	0.0 ± 0.0	29.3 ± 3.9	5.0 ± 9.5	QZH Ⅱ 07	22.8 ± 1.2	38.0 ± 3.6	20.2 ± 0.6	40.5 ± 1.9
QZH 08	35.6 ± 0.9	0.0 ± 0.0	22.2 ± 1.6	32.7 ± 5.8	QZH Ⅱ 08	26.7 ± 2.0	24.3 ± 6.0	40.8 ± 2.3	0.0 ± 0.0
QZH 09	36.3 ± 1.7	0.0 ± 0.0	15.9 ± 2.0	57.6 ± 5.9	QZH Ⅱ 09	26.0 ± 2.3	26.8 ± 7.0	23.4 ± 2.0	28.1 ± 7.2
QZH 10	8.1 ± 2.6	89.1 ± 6.0	13.2 ± 1.9	67.9 ± 6.7	QZH Ⅱ 10	24.0 ± 1.1	33.9 ± 3.4	36.9 ± 3.1	0.0 ± 0.0
QZH 11	30.0 ± 2.8	12.8 ± 6.4	30.4 ± 1.2	0.7 ± 4.2	QZH Ⅱ 11	27.3 ± 1.7	22.2 ± 5.3	18.8 ± 2.5	45.9 ± 7.4
QZH 12	8.7 ± 1.0	87.0 ± 2.4	12.3 ± 0.9	71.5 ± 3.1	QZH Ⅱ 12	26.4 ± 0.9	25.4 ± 2.8	15.2 ± 2.1	60.3 ± 6.1
QZH 13	8.1 ± 1.8	89.1 ± 4.3	24.7 ± 2.7	22.9 ± 9.7	QZH Ⅱ 13	27.1 ± 1.6	23.1 ± 4.9	13.9 ± 1.9	65.3 ± 5.7
QZH 14	22.4 ± 3.5	39.5 ± 8.1	18.5 ± 2.6	47.4 ± 9.1	QZH Ⅱ 14	27.7 ± 1.5	21.0 ± 4.7	30.1 ± 1.4	1.8 ± 4.2
QZH 15	31.3 ± 3.9	8.3 ± 9.1	22.2 ± 1.5	32.9 ± 5.2	QZH Ⅱ 15	22.6 ± 2.1	38.8 ± 6.5	23.0 ± 3.2	29.6 ± 9.5
QZH 16	7.1 ± 0.5	92.8 ± 1.1	19.3 ± 2.3	44.1 ± 8.3	QZH Ⅱ 16	15.4 ± 0.8	63.8 ± 2.3	28.1 ± 2.0	9.7 ± 6.0
QZH 17	23.5 ± 2.4	35.5 ± 7.6	31.5 ± 4.0	0.0 ± 0.0	QZH Ⅱ 17	27.9 ± 1.9	20.3 ± 5.9	34.2 ± 1.5	0.0 ± 0.0
QZH 18	21.5 ± 2.0	42.3 ± 6.2	6.7 ± 0.6	93.3 ± 1.5	QZH Ⅱ 18	11.4 ± 1.1	77.6 ± 3.4	22.6 ± 1.0	31.2 ± 3.7
QZH 19	36.0 ± 1.8	0.0 ± 0.0	7.0 ± 1.1	92.2 ± 2.7	QZH Ⅱ 19	24.4 ± 2.6	32.6 ± 7.9	26.3 ± 2.4	16.7 ± 8.4
QZH 20	17.5 ± 1.9	56.5 ± 6.1	9.7 ± 1.3	81.8 ± 3.2	QZH Ⅱ 21	13.1 ± 0.6	71.8 ± 1.9	36.9 ± 1.3	0.0 ± 0.0
QZH 21	34.3 ± 2.2	0.0 ± 0.0	19.2 ± 2.4	44.5 ± 5.9	QZH Ⅱ 22	28.4 ± 2.6	18.5 ± 8.1	26.8 ± 1.2	15.0 ± 4.4
QZH 22	20.5 ± 2.3	46.0 ± 5.0	30.2 ± 1.6	1.4 ± 9.8	QZH Ⅱ 23	19.9 ± 3.6	48.0 ± 8.0	9.0 ± 0.9	75.6 ± 5.6
QZH 23	35.7 ± 3.3	0.0 ± 0.0	12.1 ± 3.0	72.3 ± 7.3	QZH Ⅱ 24	11.9 ± 1.1	76.1 ± 3.2	27.0 ± 2.8	14.0 ± 9.8
QZH 24	30.3 ± 2.4	11.9 ± 7.4	17.7 ± 3.4	50.3 ± 8.4	QZH Ⅱ 25	19.3 ± 2.0	50.1 ± 6.2	24.3 ± 2.7	24.6 ± 6.7
QZH 25	39.1 ± 1.8	0.0 ± 0.0	23.7 ± 3.0	27.0 ± 7.5	CK	33.7 ± 2.0		30.6 ± 2.5	

Unit of colony diameter: mm. All the values were mean values ± standard deviation of three determinations.

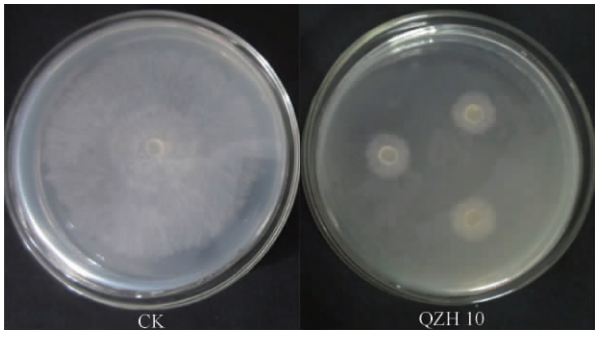

Figure 1. Inhibition rate of 10 fold dilution of QZH 10 fermentation broth against *R. solani*.

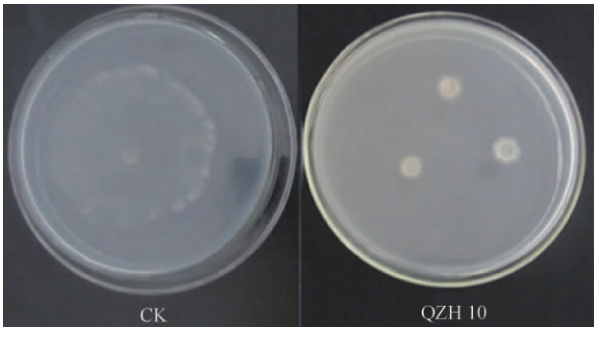

Figure 2. Antifungal activity of the ethyl acetate phase of QZH 10 against *V. mali*.

Table 2. Inhibition rate of antifungal activities of different polar solvent crude extracts of QZH 10 against *R. solani* and *V. mali* (%)

Solvent phase	*R. solani*	*V. mali*
Petroleum ether phase	61.8 ± 1.1	39.4 ± 8.9
Ethyl acetate phase	60.0 ± 2.3	96.2 ± 2.8
Butanol phase	12.1 ± 5.2	49.4 ± 9.3
Aqueous phase	5.0 ± 5.6	46.9 ± 2.3

All the values were mean values ± standard deviation of three determinations. The concentration of crude extract was 100 μg/mL.

2.3 Antifungal spectrum test of QZH 10 ethyl acetate crude extract

The antifungal spectrum of QZH 10 ethyl acetate crude extract against 6 phytopathogen was performed under the concentration of 100 μg/mL (Table 3). The crude extract showed strong inhibition activity against *M. oryzae* with the inhibition rate of 100.0%. However, the crude extract presented weak activities against other tested phytopathogen with the inhibition rate of less than 31%.

Table 3. Inhibition activity of antifungal activities of the ethyl acetate crude extract of QZH 10 against 6 phytopathogen

Phytopathogen	Colony diameter/mm	Control diameter/mm	Inhibition rate/%
F. graminearum	28.0 ± 1.5	32.8 ± 1.6	17.3 ± 5.6
D. gregaria	12.8 ± 0.5	12.9 ± 0.9	0.8 ± 6.3
A. solani	14.0 ± 0.6	17.9 ± 1.0	30.6 ± 4.7
M. oryzae	5.0 ± 0.0	20.8 ± 1.0	100.0 ± 0.0
F. oxysporum f. sp. *momordicae*	23.4 ± 0.4	25.3 ± 1.5	9.5 ± 1.9
F. oxysporum f. sp. *cucumerinum*	22.7 ± 1.5	22.1 ± 0.8	0.0 ± 0.0

All the values were mean values ± standard deviation of three determinations. The concentration of crude extract was 100 μg/mL.

2.4 Identification of QZH 10

QZH 10 was identified based on microscopical examination and 5.8S rRNA sequence analysis. The colonies of strain QZH 10 were dry and produced yellow pigment on the MEA agar medium. The aerial mycelia were flourishing and combined with the medium closely. The sporal chains were dark and the spores were dark, elongated and separated. The phylogenetic tree (Figure 3) indicated that the title strain was closely related to *Alternaria solani* (JF491194), with the 5.8S rRNA gene sequence similarity of 98%. Therefore, we treated this strain as *Alternaria solani*.

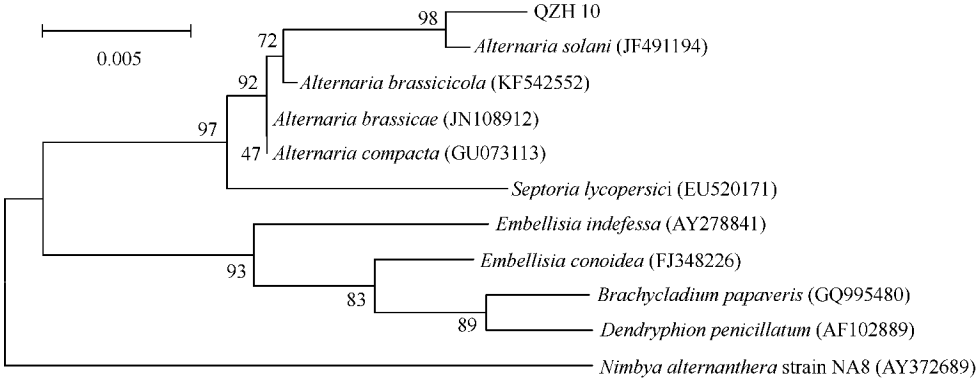

Figure 3. Phylogenetic tree based on the 5.8S rRNA sequences of the fungus QZH 10 from *H. miconioides*.

2.5 Isolation and identification of the active compounds of QZH 10

Bioassay-guided separation crude extract of QZH 10 (115.83 g) afforded two bioactive compounds **1** (10 mg) and **2** (11 mg). Both compounds were characterized by spectroscopic analyses and comparison with the literatures[13-14]. Both compounds were identified as altersolanol A (**1**) and 6-*O*-methylalaternin (**2**) based on the following data (Figure 4), respectively.

Altersolanol A (**1**): ESI-HRMS (*m/z* [2M + Na]$^+$ 695.1588, calculated for C_{32} H_{32} O_{16} Na 695.1588). ^{1}H-NMR (Acetone-d_6) δ: 1.47 (3H, s), 3.79 (1H, s), 3.92 (1H, d, *J* = 5.9 Hz), 4.00 (2H, s), 4.19 (1H, s), 4.26 (1H, s), 4.59 (1H, d, *J* = 5.0 Hz), 4.77 (1H, s), 4.88 (1H, d, *J* = 5.0 Hz), 6.77 (1H, s), 7.12 (1H, s). ^{13}C-NMR (Acetone-d_6) δ: 18.8, 21.8, 55.9, 69.1, 70.0, 100.0, 105.6, 107.4, 109.7, 133.7, 142.7, 164.5, 166.4, 166.8, 183.7, 189.9.

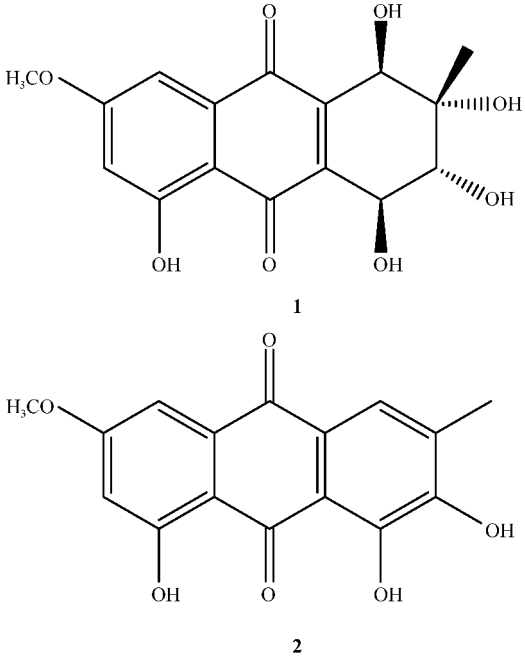

Figure 4. Molecular structures of 1 and 2.

6-*O*-methylalaternin (**2**): ESI-HRMS (*m/z* [M-H]$^-$ 299.0556, calculated for $C_{16}H_{12}O_6$ 299.0556). ^{1}H-NMR (DMSO-d_6) δ: 2.25 (3H, s), 3.91 (3H, s), 6.79 (1H, s), 7.11 (1H, s), 7.50 (1H, s), 10.37 (1H, s), 12.11 (1H, s).

2.6 Antifungal spectrums of 1 and 2

Under the concentration of 100 μg/mL, compound 1 had the potent antifungal activity against *M. oryzae* with the inhibition rate of more than 85% (Table 4), and compound 2 had the strong antifungal activity against *V. mali* with the inhibition rate of 100.0% (Figure 5), which was comparable to that of the positive cycloheximide.

Table 4. Inhibition rate of antifungal activities of monomer compounds to phytopathogen (%)

Phytopathogen	1		2		Cycloheximide[a]	
	50 μg/mL	100 μg/mL	50 μg/mL	100 μg/mL	50 μg/mL	100 μg/mL
R. solani	42.4 ± 3.8	60.9 ± 3.2	18.5 ± 2.4	39.7 ± 3.3	100.0 ± 0.0	100.0 ± 0.0
F. graminearum	46.1 ± 5.9	74.5 ± 3.8	32.0 ± 3.5	40.3 ± 6.5	91.5 ± 1.6	100.0 ± 0.0
A. solani	4.1 ± 4.2	0.0 ± 0.0	0.0 ± 0.0	1.9 ± 6.5	79.4 ± 9.6	89.7 ± 8.2
V. mali	8.5 ± 3.0	39.7 ± 3.1	3.0 ± 1.4	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
M. oryzae	N/T[b]	86.8 ± 3.7	55.3 ± 2.9	N/T	90.4 ± 7.7	100.0 ± 0.0
F. oxysporum f. sp. *cucumerinum*	30.2 ± 2.9	37.2 ± 6.4	9.3 ± 8.9	28.7 ± 3.8	0.0 ± 0.0	12.4 ± 6.8
F. oxysporum f. sp. *momordicae*	21.9 ± 9.7	34.4 ± 7.4	0.0 ± 0.0	0.0 ± 0.0	70.3 ± 3.9	76.1 ± 1.5

[a] Cycloheximide was co-assayed as a positive control. [b] N/T: not tested. All the values were mean values ± standard deviation of three determinations.

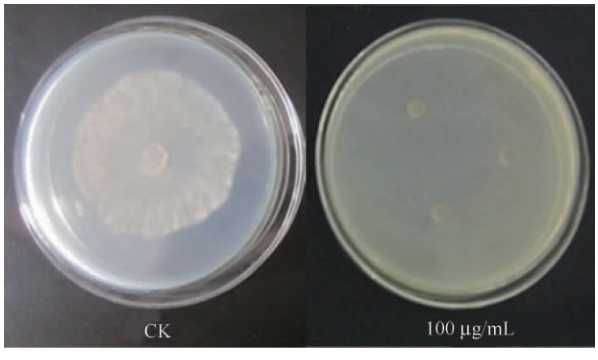

Figure 5. Antifungal activity of compound 2 against *V. mali* under the concentration of 100 μg/ mL.

3 Discussion and Conclusion

In summary, we performed the anti-phytopathogen screening of endophytic fungi associated with the endangered species *H. miconioides* and identified two bioactive compounds from the fungus *A. solani* QZH 10. Altersolanol A possessed strong activity against *M. oryzae*, and 6-*O*-methylalaternin had the potent antifungal activity against *V. mali*. These results suggested that altersolanol A and 6-*O*-methylalaternin had potential to be used as agricultural fungicides. Further studies will be carried out to better understand the mechanism of action associated with antifungal effects. In addition, the discovery of our study provided additional evidence that the endophytes related with endangered plant species, may inspire the discovery of useful metabolites with interesting bioactivities.

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七子花内生真菌的抗菌活性筛选及其活性先导化合物的发现

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摘要:【目的】从濒危植物七子花中分离出具有抗植物致病菌活性的内生真菌,并在其代谢产物中寻找具有抗菌活性的先导化合物。【方法】采用生长速率法测定内生真菌对植物病原菌的抑制活性。通过形态学和5.8S rRNA 序列法对具有较好抗菌活性的内生真菌进行鉴定。运用多种色谱方法对发酵产物进行分离、纯化,利用质谱和核磁共振谱分析鉴定出化合物的结构。【结果】茄交链孢 QZH 10 对水稻纹枯病菌和苹果树腐烂病菌的抑制活性较好,抑制率分别为 89.1% 和 67.9%。在供试浓度为 100 μg/mL 时,QZH 10 乙酸乙酯粗提物对稻瘟病菌具有强烈的抑制效果,抑制率为 100.0%。从 QZH 10 中分离到 2 个单体化合物并分别被鉴定为 altersolanol A 和 6-*O*-methylalaternin。在供试浓度为 100 μg/mL 时,altersolanol A 对稻瘟病菌具有较强的抑制效果,抑制率大于 85%;6-*O*-methylalaternin 对苹果树腐烂病菌具有强烈的抑制效果,抑制率为 100.0%。【结论】当前结果表明 Altersolanol A 和 6-*O*-methylalaternin 具有开发成微生物源杀菌剂的潜力。

关键词:七子花,茄交链孢,内生真菌,抗菌活性,天然产物

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