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FULL RESEARCH PAPER

Suppression of *Sclerotinia sclerotiorum* by antifungal substances produced by the mycoparasite *Coniothyrium minitans*

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Abstract A study was conducted to investigate production of antifungal substances (AFS) by *Coniothyrium minitans* (Cm), a mycoparasite of *Sclerotinia sclerotiorum* (Ss), in modified Czapek-Dox (MCD) broth and potato dextrose broth (PDB), and effects of AFS of Cm on mycelial growth and germination of sclerotia and ascospores of Ss and incidence of leaf blight of oilseed rape caused by Ss. Results showed that mycelial growth of Ss was reduced by 41.6 and 84.5% on 3 day-old cultures grown on potato dextrose agar (PDA) amended with 10% (v v^{-1}) of cultural filtrates of Cm grown in MCD (MCD_{cm}) after incubation for 6 and 15 days, respectively, and by 2.7 and 15.7% on PDA amended with 10% (v v^{-1}) of cultural filtrates of Cm grown in PDB for 6 and 15 days, respectively. In addition to retardation of mycelial growth, morphological abnormality of Ss such as hyphal swellings and cytoplasm granulation were also observed in colonies grown on PDA amended with cultural filtrates of MCD_{cm}. Sclerotia of Ss soaked in the filtrates of MCD_{cm} for 24 h remained viable, but their ability to undergo myceliogenic germination on PDA was delayed, compared to sclerotia treated with MCD. Germination of ascospores of Ss was unaffected on PDA amended with 10% of the filtrates of MCD_{cm}. However, germ tubes of Ss were shortened and deformed by the formation of hyphal swellings in the treatment of MCD_{cm}. Treatment of leaves of oilseed rape with cultural filtrates of MCD_{cm} reduced incidence of leaf blight caused by Ss, compared to the controls (water or MCD). This study suggests that AFS produced by Cm plays an important role in the suppression of mycelial growth and germ-tube development of ascospores of Ss and that there is potential for using AFS of Cm to control leaf blight of oilseed rape caused by ascospores of Ss.

Keywords Mycoparasite · *Coniothyrium minitans* · Antifungal substances · *Sclerotinia sclerotiorum* · Biological control

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Introduction

Coniothyrium minitans is a mycoparasite of *Sclerotinia* spp. (Campbell 1947; Whipps and

Gerlagh 1992). Numerous studies showed that *C. minitans* is an effective agent for control of diseases caused by *S. sclerotiorum* (Huang 1980; Bogdanova et al. 1986; McLaren et al. 1994; Budge et al. 1995; Gerlagh et al. 1999, 2003; Huang et al. 2000; Li et al. 2005, 2006), *S. minor* (Partridge et al. 2006; Rabeendran et al. 2006), *S. trifoliorum* (Turner and Tribe 1975) and *S. cepivorum* (Ahmed and Tribe 1977). Commercial products of *C. minitans* such as Contans® WG (Prophyta Biologischer Pflanzenschutz GmbH, Germany) have been registered for disease control in several European countries including Germany, Switzerland and Norway (de Vrije et al. 2001) and USA (Partridge et al. 2006).

Mycoparasitism is the well-recognized mechanism for *C. minitans* to parasitize *S. sclerotiorum* (Campbell 1947; Huang and Kokko 1987, 1988) and some other sclerotium-forming fungal species (Whipps and Gerlagh 1992). Thus, *C. minitans* can be applied to soils to control diseases caused by *S. sclerotiorum* on sunflower (Huang 1980) and lettuce (Budge et al. 1995; Jones et al. 2004), *S. minor* on peanut (Partridge et al. 2006; Rabeendran et al. 2006) and *S. cepivorum* on onion (Ahmed and Tribe 1977).

In most cases, *S. sclerotiorum* initiates infection of plant tissues by ascospores which are released from apothecia produced by carpogenically-germinated sclerotia of this pathogen. Exogenous nutrients from senescent plant tissues such as flower petals are usually required for ascospore germination, germ-tube development and formation of infection cushions of *S. sclerotiorum* (Huang and Kokko 1992; Hegedus and Rimmer 2005). *Coniothyrium minitans* inoculated on flower petals of bean (Bremer et al. 2000) or oilseed rape (Li et al. 2003, 2006) could effectively suppress colonization of the petals by *S. sclerotiorum*, thereby reduce the petal-mediated infection of leaves of bean or oilseed rape by this pathogen (Bremer et al. 2000; Li et al. 2003, 2005, 2006). These studies (Bremer et al. 2000; Li et al. 2003, 2005, 2006) suggested that some antibiotic mechanism might be involved in suppression of *S. sclerotiorum* by *C. minitans* on flower petals of bean or oilseed rape, as the spore germination and mycelial growth of *C. minitans* are much slower than the ascospore germination and the mycelial growth of *S. sclerotiorum*. Several reports indicated that the antagonistic effect of *C. minitans* on *S. sclerotiorum* in dual cultures on media such as potato dextrose agar (PDA) was negligible (Campbell 1947; Huang and Kokko 1988; Whipps and Gerlagh 1992; Li et al. 1995). However, McQuilken et al. (2002) reported that cell-free filtrates collected from cultures of a European strain of *C. minitans* could inhibit mycelial growth of *S. sclerotiorum*. One of the antifungal substances (AFS) produced by this strain was purified and identified as macrosphelide A (McQuilken et al. 2003).

Our previous study showed that the Chinese strain Chy-1 of *C. minitans* is an effective biocontrol agent for sclerotinia diseases of oilseed rape when applied as aerial spray on this crop (Li et al. 2006). Whether this strain can produce AFS remains unknown. Meanwhile, information on the inhibitory effects of AFS of *C. minitans* on germination of sclerotia or ascospores of *S. sclerotiorum*, and on incidence of sclerotinia diseases has not been documented so far.

The objectives of this study are: (1) to determine production of AFS by the Chinese strain Chy-1 of *C. minitans* in different media; (2) to detect the inhibitory effects of AFS of *C. minitans* on mycelial growth and germination of sclerotia and ascospores of *S. sclerotiorum*; and (3) to evaluate the efficacy of AFS of *C. minitans* in control of leaf blight of oilseed rape caused by *S. sclerotiorum*.

Materials and methods

Fungal strains and media

Fungal strains used in this study were *C. minitans* strain Chy-1 and *S. sclerotiorum* strain Rap-1. The strain Chy-1 was isolated from a soil sample collected from Chang Yang County, Hubei, China (Li et al. 1995). The strain Rap-1 was isolated from a sclerotium of *S. sclerotiorum* collected from a diseased plant of oilseed rape (*Brassica napus*) near Wuhan, Hubei. Cultural media used in this study were PDA, potato dextrose broth (PDB) and modified Czapek-Dox (MCD) (McQuilken et al. 2003). Both PDA and PDB were made of fresh potato using the procedures described by Fang (1998).

Preparation of cultural filtrates of *C. minitans*

An aliquot of 100 µl of the spore suspension of *C. minitans* (1×10^7 conidia ml^{-1}) collected from

15 day-old PDA cultures (Li et al. 2006) was inoculated in 50 ml of MCD in an Erlenmeyer flask (250 ml). The cultures, designated as MCD_{cm}, were incubated at 20°C on a shaker (200 rpm) for 6 and 15 days. The culture in each flask was filtered and the cultural filtrate was centrifuged at 4, 000 rpm at 4°C for 10 min to remove hyphal fragments. The cell-free supernatant was used for determination of pH using a pH meter (Model pHS-3C, Shanghai Hongyi Instrument Company Ltd., Shanghai, China) and for antifungal activity in the following bioassays. Mycelial mats of *C. minitans* harvested from each flask were dried at 50°C for 48 h and weighed to determine mycelial biomass. Using the same procedures, the cultural filtrates of *C. minitans* grown in PDB, designated as PDB_{cm}, were prepared, and mycelial biomass of *C. minitans* and pH of PDB_{cm} in each flask were determined.

Bioassay 1: Antifungal activity of filtrates of *C. minitans* in PDB and MCD cultures

The 6- or 15 day-old filtrate of MCD_{cm} or PDB_{cm} was incorporated into PDA at a concentration of 10% (v v^{-1}) and the mixture poured into Petri dishes (9 cm diam.), 20 ml per dish. MCD or PDB incorporated into PDA also at a concentration of 10% was used as controls, which were designated as MCD_{ck} or PDB_{ck}. A mycelial agar plug (6 mm diam.) of *S. sclerotiorum* removed from the margin of 3 day-old colonies was inoculated on the centre of each dish. There were nine dishes (replicates) for each treatment. After incubation at 20°C in the dark for 60 h, the colony diameter of *S. sclerotiorum* in each dish was measured. Inhibition of growth (IG) of *S. sclerotiorum* by filtrates of *C. minitans* was calculated using the formula: $IG\ (\%) = (AD_{ck} - D_{cm})/AD_{ck} \times 100$, where AD_{ck} represents the average colony diameter in the treatment of MCD_{ck} or PDB_{ck}, D_{cm} represents the colony diameter in the treatment of MCD_{cm} or PDB_{cm}. This bioassay was repeated.

Bioassay 2: Suppression of mycelial growth of *S. sclerotiorum* by AFS of *C. minitans*

The filtrate from the 15 day-old MCD_{cm} was incorporated into PDA at a concentration of 0, 0.1, 0.2, 0.3, 0.6, 1.3, 2.5, 5.0, or 10.0% (v v^{-1}), while MCD was added to PDA at 0, 0.1, 0.2, 0.3, 0.6, 1.3, 2.5, 5.0, or 10.0% as the controls. Mycelial plugs (6 mm diam.) of *S. sclerotiorum* removed from 5 old-day PDA cultures were inoculated on the centre of dishes, 1 plug per dish. There were nine dishes (replicates) for each treatment. The same procedures described in the previous bioassay were used for incubation of the cultures, measurement of colony diameter of *S. sclerotiorum* and calculation of inhibition of growth of this pathogen. The experiment was repeated.

Bioassay 3: Suppression of myceliogenic germination of sclerotia of *S. sclerotiorum* by AFS of *C. minitans*

Mycelial plugs of *S. sclerotiorum* were inoculated on PDA in Petri dishes (9 cm diam.) and the dishes were incubated at 20°C in dark. After 30 days, sclerotia of *S. sclerotiorum* formed in each dish were harvested and air-dried at room temperature (20–25°C). Four sclerotial samples, each containing 100 sclerotia, were used for the following two experiments. The size of the tested sclerotia of *S. sclerotiorum* was about 4.5 × 3.5 mm (L × W). The first experiment was to treat one of the sclerotial samples in 60 ml of the filtrate from the 15 day-old MCD_{cm} or in 60 ml of MCD at room temperature (20–25°C) for 24 h. The sclerotia in each treatment were collected, blotted dry on sterilized paper towel, surface-sterilized in 0.1% (w v^{-1}) $HgCl_2$ for 5 min, rinsed in sterile distilled water (SDW) three times, 1 min each time, and individually inoculated on PDA in Petri dishes (9 cm diam.), 5 sclerotia per dish. The sclerotial germination and the colony diameter of *S. sclerotiorum* around each sclerotium were recorded after incubation at 20°C in the dark for 2, 3 and 4 days. A sclerotium was considered to have germinated when white, cottony mycelia appeared on the sclerotial surface or on the agar medium.

The second experiment was to incorporate the filtrate of the 15 day-old MCD_{cm} or MCD into PDA in Petri dishes (9 cm diam.) at a concentration of 10% (v v^{-1}). Surface-sterilized sclerotia of *S. sclerotiorum* were individually inoculated on PDA in Petri dishes, four sclerotia per dish and 100 sclerotia per treatment. The sclerotial germination and the colony diameter of *S. sclerotiorum* were recorded after incubation at 20°C in dark for 2, 3 and 4 days.

Bioassay 4: Suppression of germination of ascospores and development of germ tubes of *S. sclerotiorum* by AFS of *C. minitans*

Sclerotia of *S. sclerotiorum* were collected from 30 day-old PDA cultures (20°C) and used for production of apothecia and ascospores by the method of Huang and Kozub (1989). Ascospore suspensions were prepared from mature apothecia using the procedures described by Li et al. (2005, 2006). The concentration of ascospores in the suspensions was determined using a haemocytometer under a compound light microscope. An aliquot of 100 µl of the ascospore suspension of *S. sclerotiorum* (1×10^6 ascopsores ml^{-1}) was pipetted onto PDA containing 10% (v v^{-1}) of the filtrate of the 15 day-old MCD_{cm} or MCD in a Petri dish (9 cm diam.) and spread evenly over the surface of the agar medium using a sterilized glass rod. There were five dishes for each treatment. After incubation at 20°C in the dark for 12 and 24 h, the frequency of ascospore germination of *S. sclerotiorum* was determined by randomly counting at least 150 ascospores in each dish under a compound light microscope. An ascospore was considered to have germinated when the length of the germ tube was equal to or greater than the diameter of that ascospore. After incubation for 48 h, mycelial growth of *S. sclerotiorum* in each dish was observed. The experiment was repeated.

Bioassay 5: Effect of AFS of *C. minitans* on incidence of leaf blight of oilseed rape caused by *S. sclerotiorum*

Seeds of oilseed rape cv. Zhong You Za No.4 were sown in soil in pots. The pots were kept in a greenhouse (20–25°C) and watered as required. After 45–55 days, leaves (10 × 8 cm, L × W) were excised from the central part of each plant. Eight detached leaves were randomly selected and placed in two rows (4 leaves per row) on moist towels in an enamelware tray (45 × 30 × 2.5 cm, L × W × H) for each the following treatments: *C. minitans* AFS (CM_{AFS}); CM_{AFS} + *C. minitans* conidia (CM_{AFS} + CM_C); MCD; MCD + CM_C; water; and water + CM_C. For the treatments of CM_{AFS} + CM_C, MCD + CM_C or water + CM_C, an aliquot of 1 ml of the spore suspension of *C. minitans* (1.6×10^8 conidia ml^{-1}) was added to 9 ml of the filtrate of the 15 day-old MCD_{cm}, MCD or water, and the resulting mixture for each of these treatments was spread evenly on the surface of eight leaves of oilseed rape in a tray using a wool brush, about 1 ml per leaf. For the treatments of CM_{AFS}, MCD or water, the filtrate of MCD_{cm}, MCD or water was directly spread on leaves of oilseed rape in a tray also at about 1 ml per leaf. The treated trays were left open on a laboratory bench at room temperature (20–25°C) for 3 h to allow the evaporation of the excess water from the surface of the leaves of oilseed rape. Inoculations of these treated leaves of oilseed rape with mycelial agar plugs of *S. sclerotiorum* were done using the method described by Li et al. (2006). There were two agar plugs for each leaf. The trays were individually sealed with a clear plastic film to maintain high moisture and incubated in a growth chamber at 20°C under fluorescent light (12 h per day). After 3 days, leaf lesion diameter developed from each inoculum plug was measured. The bioassay was repeated four times.

Data analyses

Analysis of variance (ANOVA) (SAS Institute, Cary, NC, USA, Version 8.0, 1999) was used to determine the statistical significance of differences among treatments in each bioassay. The % data on inhibition of growth of *S. sclerotiorum* by AFS of *C. minitans* in each replicate was arcsin-transformed to angular data prior to ANOVA. After each analysis, means were individually back-transformed to numerical values. Data of the same treatment, but collected from different repeats in Bioassay 1, Bioassay 2 and Bioassay 4 were pooled, as they were not significantly different in the *F*-test of ANOVA ($P > 0.05$). In Bioassay 3, each colony developed from each sclerotium of *S. sclerotiorum* was regarded as a replicate for each treatment. In Bioassay 5, data collected from each trial were independently analyzed, as the lesion diameters varied greatly among the four trials. Means for different treatments in each bioassay or trial were separated using the Least Significant Difference Test at $P = 0.05$ level.

Results

Bioassay 1: Production of AFS by *C. minitans* in different culture media

Results showed that MCD and PDB had different effects on production of mycelial biomass, ambient pH and production of antifungal substances (AFS) by the strain Chy-1 of *C. minitans*. After incubation for 6 days at 20°C, mycelial biomass of *C. minitans* in MCD cultures was 6.9 mg ml^{-1}, not significantly different ($P > 0.05$) from 5.7 mg ml^{-1} in PDB cultures; however, mycelial biomass of *C. minitans* was increased to 11.2 mg ml^{-1} after incubation for 15 days in MCD, significantly higher ($P < 0.05$) than 5.1 mg ml^{-1} in PDB (Table 1). The ambient pH was reduced from the initial 4.6 to 3.9 and 3.6 after incubation for 6 and 15 days, respectively, in MCD, but it was increased from the initial 5.9 to 7.2 and 6.0 after incubation for 6 and 15 days, respectively, in PDB (Table 1). On PDA amended with the 6 and 15 day-old cultural filtrates of MCD_{cm}, mycelial growth of *S. sclerotiorum* was reduced by 41.6 and 84.5%, respectively (Table 1), compared to the controls. However, on PDA amended with the 6 and 15 day-old cultural filtrates of PDB_{cm}, mycelial growth of *S. sclerotiorum* was reduced by 2.7 and 15.7%, respectively (Table 1), also compared to the controls.

Bioassay 2: Suppression of mycelial growth of *S. sclerotiorum* by AFS of *C. minitans*

On MCD-amended PDA, hyphae of *S. sclerotiorum* grew normally (Fig. 1A) and formed normal branches (Fig. 1B) with dense, evenly-distributed cytoplasm in the hyphal cells (Fig. 1A, B). After incubation for 5 days, the colony with dense mycelia covered the entire medium surface of the Petri dish (Fig. 1C). However, on MCD_{cm}-amended PDA, hyphae of *S. sclerotiorum* showed a degenerative appearance of hyphal tips (Fig. 1D) and formed swollen cells (Fig. 1E) with unevenly-distributed, granulated cytoplasm in the hyphal cells (Fig. 1D, E). After incubation for 5 days, the colony remained small due to poor hyphal growth and it was surrounded by a white halo zone (Fig. 1F). There were numerous crystal particles in this halo area, where the pH value was reduced to 3.0–4.0, compared to pH 5.0–6.0 in the agar medium outside the halo area (data not shown).

The efficacy (Y) of inhibition of growth of *S. sclerotiorum* by the cultural filtrates of MCD_{cm} was positively related to the concentration of the filtrates (X) incorporated in PDA, $Y = 18.268 \text{ Ln } (X) + 44.152$ ($R^2 = 0.9702$, $P < 0.01$). With the increase of the concentration of the MCD_{cm} filtrate from 0.1% to 2.5%, the % of inhibition of growth (IG) of *S. sclerotiorum* was increased rapidly from 0.9% to 68.2% (Fig. 2). When the concentration of the filtrate was increased to 5.0% and 10.0%, the IG values were slowly increased to 79.2% and 82.5%, respectively (Fig. 2).

Bioassay 3: Suppression of sclerotial germination of *S. sclerotiorum* by AFS of *C. minitans*

In the first experiment of this bioassay, sclerotia of *S. sclerotiorum* soaked in MCD_{cm} or MCD for 24 h could germinate myceliogenically. After incubation

Table 1 Production of antifungal substances by *Coniothyrium minitans* strain Chy-1 in modified Czapek-Dox (MCD) and potato dextrose broth (PDB) (Bioassay 1)

Medium	Mycelial biomass (mg ml^{-1})		pH[1]		Inhibition of growth (%)	
	6 d	15 d	6 d	15 d	6 d	15 d
MCD	6.9 a[2]	11.2 a	3.9 b	3.6 b	41.6 a	84.5 a
PDB	5.7 a	5.1 b	7.2 a	6.0 a	2.7 b	15.7 b
$LSD_{0.05}$ (df)	2.5 (10)	1.6 (10)	0.5 (10)	0.1 (10)	5.3 (34)	2.4[3] (34)

[1] The original pH was 4.6 for MCD and 5.9 for PDB

[2] Means followed by the same letters within each column are not significantly ($P > 0.05$) different according to the Least Significant Difference Test. df = degrees of freedom

[3] The value was calculated with arsine-transformed data

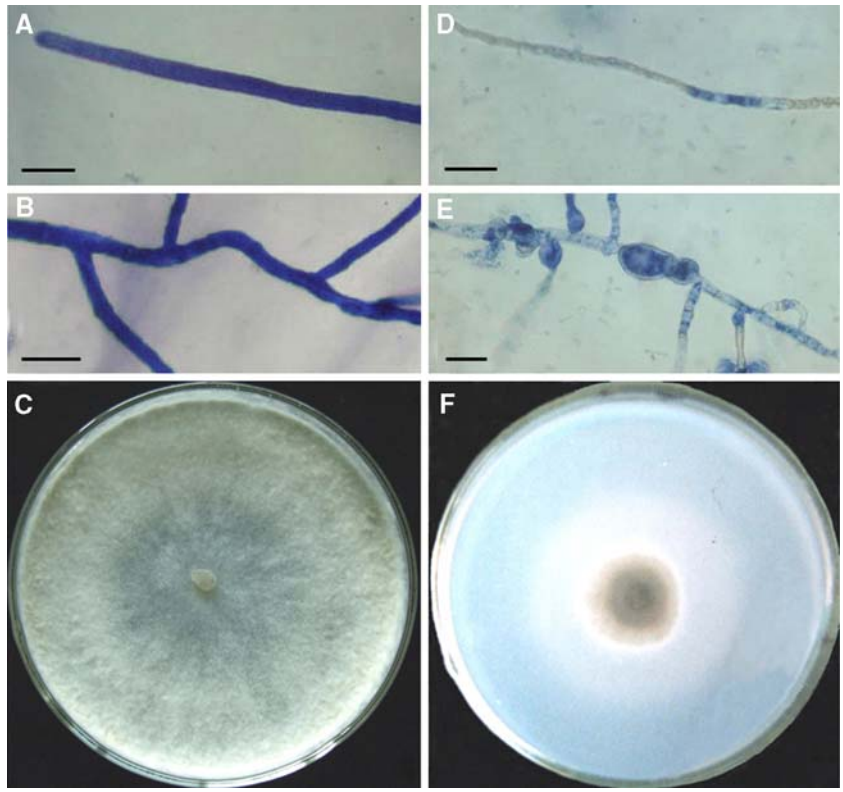

Fig. 1 Inhibition of mycelial growth of *Sclerotinia sclerotiorum* (Ss) by AFS produced by *Coniothyrium minitans* strain Chy-1 on PDA (Bioassay 2). In the MCD treatment, a hypha of Ss showed dense, evenly distributed cytoplasm in the hyphal tip (**A**) and branched hyphal cells (**B**). A well-developed colony of Ss was formed after incubation on PDA at 20°C for 5 days (**C**). However, in the MCD_{cm} treatment, a hyphal tip of Ss showed a degenerative appearance with granulated, unevenly-distributed cytoplasm (**D**) and swollen hyphal cells (**E**). A small colony of Ss was developed after 5 days with the formation of a white halo surrounding the colony (**F**). All bars represent 10 μm in length. Mycelia of Ss were stained with cotton blue in lactophenol

on PDA for 48 h, the germination rate was 24% and 93% for the treatments of MCD_{cm} and MCD, respectively (Table 2). After incubation for 72 h, the germination rate increased to 95% for MCD_{cm} with 74% of the germinated sclerotia developed into colonies, and to 99% for MCD with 80% of the germinated sclerotia developed into colonies. The average colony diameter of the 72 h-old cultures was 14.7 mm for the treatment of MCD_{cm}, significantly smaller ($P < 0.05$) than 25.8 mm for the treatment of MCD (Table 2). After incubation for 96 h, all of the sclerotia germinated in both MCD_{cm} and MCD treatments.

In the second experiment of this bioassay, sclerotia of *S. sclerotiorum* inoculated on PDA supplemented with 10% (v v^{-1}) of the cultural filtrates of MCD_{cm} or MCD could germinate myceliogenically. After incubation for 48 h, the germination rate was 56% for both treatments. After incubation for 72 h, the germination rate increased to 98% for the MCD treatment with 45% of the germinated sclerotia developed into colonies, and to 100% for MCD_{cm} treatment with 46% of the germinated sclerotia developed into colonies. The average colony diameter of the 72 h-old cultures was 9.4 mm for MCD_{cm}, significantly smaller ($P < 0.05$) than 20.9 mm for MCD (Table 2). After incubation for 96 h, all of the sclerotia in both treatments germinated (Table 2). Colonies of *S. sclerotiorum*, developed in some sclerotia treated with MCD, were intermingled, whereas colonies from sclerotia treated with MCD_{cm} were still suppressed for expansion (data not shown).

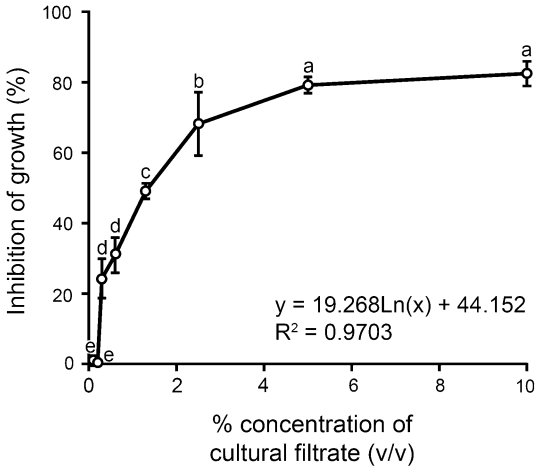

Fig. 2 Effect of the concentration of AFS-containing cultural filtrates of *Coniothyrium minitans* on inhibition of growth of *Sclerotinia sclerotiorum* (Bioassay 2). The filtrate of 15 day-old MCD cultures of *C. minitans* was incorporated at different concentrations into PDA for incubation of *S. sclerotiorum* at 20°C for 60 h. Each data point is the average of 10 replicates. Means with the same letter are not significantly ($P > 0.05$) different according to the Least Significant Different Test

Bioassay 4: Suppression of ascospore germination of *S. sclerotiorum* by AFS of *C. minitans*

For the treatment of MCD, ascospores of *S. sclerotiorum* germinated by 98.8% after incubation for 12 h. Germ tubes grew rapidly and hyphal branches developed after 24 h. Colonies were large with the formation of cottony, floccose aerial mycelia (Fig. 3). For the treatment of MCD_{cm}, ascospores of *S. sclerotiorum* germinated by 99.4% after 12 h. However, the germ tubes were retarded with the formation of swollen cells and multiple septae after 24 h. Colonies of *S. sclerotiorum* were small without formation of cottony aerial mycelia after 48 h (Fig. 3).

Table 2 Effect of antifungal substances produced by *Coniothyrium minitans* on myceliogenic germination of sclerotia of *Sclerotinia sclerotiorum* on PDA (Bioassay 3)

[1]Treatment	Number of myceliogenically-germinated sclerotia (n = 100) after incubation for			[2]Colony diameter (mm)
	48 h	72 h	96 h	
Sclerotia soaked in				
MCD	93	99	100	25.8 ± 5.2 a
MCD_{cm}	24	95	100	14.7 ± 3.1 b
$LSD_{0.05}$ (df)				1.4 (148)
PDA amended with				
MCD	56	98	100	20.9 ± 4.9 a
MCD_{cm}	56	100	100	9.4 ± 2.9 b
$LSD_{0.05}$ (df)				1.9 (89)

[1] MCD_{cm} represents the filtrates of *C. minitans* cultures grown in MCD at 20°C for 15 days. For soaking treatments, sclerotia of *S. sclerotiorum* were submerged in MCD or MCD_{cm} for 24 h, and then surface-sterilized and inoculated on PDA. For the PDA-amending treatments, MCD or MCD_{cm} was incorporated into PDA at 10% (v v^{-1}) and then surface-sterilized sclerotia of *S. sclerotiorum* were inoculated on PDA media

[2] Diameter of colonies of *S. sclerotiorum* around each germinated sclerotium was measured after incubation for 72 h. Means followed by the same letters within the column are not significantly different ($P > 0.05$) according to the Least Significant Difference Test. Results are expressed as mean ± SEM, df = degrees of freedom

Fig. 3 Germination of ascospores and development of germ tubes of *Sclerotinia sclerotiorum* on PDA amended with MCD (left dish) or the filtrates of MCD_{cm} (right dish) at a concentration of 10% (v v^{-1}). The dishes were incubated at 20°C for 48 h. Note cottony, floccose aerial mycelia formed on PDA amended with MCD, but did not form on PDA amended with MCD_{cm}

Bioassay 5: Effect of AFS of *C. minitans* on infection of leaves of oilseed rape by *S. sclerotiorum*

Results of the four trials showed that the incidence of leaf blight of oilseed rape caused by *S. sclerotiorum* was significantly ($P < 0.05$) reduced in the treatments of CM_{AFS} and CM_{AFS} + CM_C which had average lesion diameters (ALD) of 2.0–11.8 and 2.0–14.6 cm, respectively (Table 3), compared to the treatments of MCD, MCD + CM_C, water, and water + CM_C which ALD values of 16.0–21.3, 21.1–25.4, 22.6–29.0 and 17.8–22.6 cm, respectively. The synergistic effect of the combined treatment of CM_{AFS} + CM_C on reducing the size of leaf lesions of oilseed rape was observed only in Trial 4 with the ALD value being 2.0 mm for CM_{AFS} + CM_C, significantly smaller ($P < 0.05$) than that for CM_{AFS} alone (10.6 mm) and CM_C alone (17.4–22.5 cm). However, this effect was not observed in the other three trials. In Trial 1 and Trial 2, the ALD value for the treatment of CM_{AFS} + CM_C was not significantly ($P > 0.05$) different from that for the treatment of CM_{AFS} (Table 3). In Trial 3, the ALD value for CM_{AFS} + CM_C was significantly ($P < 0.05$) larger than that for CM_{AFS} (Table 3).

Discussion

This study revealed that cultural filtrates of the strain Chy-1 of *C. minitans* grown in modified Czapek-Dox (MCD) could effectively inhibit mycelial growth of *S. sclerotiorum*, suggesting that the MCD filtrates of *C. minitans* (MCD_{cm}) contained antifungal substances (AFS). This confirms the reports of McQuilken et al. (2002, 2003) that *C. minitans* produced antifungal metabolites in media including MCD. One of the antifungal metabolites produced by the European strain IMI134523 of *C. minitans* was identified as macrosphelide A (McQuilken et al. 2003). Whether macrosphlide A is produced by the Chinese strain Chy-1 of *C. minitans* remains unknown and thus warrants further investigations.

This study also indicates that production of AFS by *C. minitans* is affected by culture media. For example, the AFS activity was negligible in cultural filtrates of *C. minitans* in PDB, but it was high in cultural filtrates in MCD. Therefore, PDB is not a suitable cultural medium for production of AFS by *C. minitans*. This may be the reason for the lack of antagonism of *C. minitans* on *S. sclerotiorum* in dual cultures on PDA (Huang and Kokko 1988; Li et al. 1995). *Coniothyrium minitans* Chy-1 in PDB cultures was different from MCD cultures in mycelial biomass and ambient pH, especially in 15 day-old cultures (Table 1). Therefore, the study suggests that AFS production by *C. minitans* may be associated with nutritious factors such as medium type, the amount of mycelial biomass of *C. minitans* and the change of ambient pH values. Mycelial biomass may not be responsible for the negligible production of AFS by Chy-1 in PDB, as the mycelial biomass of 6 day-old

Table 3 Efficacy of cultural filtrates and/or conidia of *Coniothyrium minitans* in suppression of infection of leaves of oilseed rape caused by *Sclerotinia sclerotiorum* (Bioassay 5)

Treatment[1]	Lesion diameter (cm)			
	Trial 1[2]	Trial 2	Trial 3	Trial 4
Water	25.2 a	23.4 b	25.4 a	21.1 bc
Water + CM_C	16.4 c	21.3 b	16.0 b	17.4 c
MCD	22.6 ab	29.0 a	25.7 a	27.7 a
MCD + CM_C	17.8 bc	22.6 b	22.6 a	22.5 b
CM_{AFS}	6.8 d	11.8 c	2.0 d	10.6 d
CM_{AFS} + CM_C	4.2 d	14.6 c	6.6 c	2.0 e
$LSD_{0.05}$ (df)	5.2 (42)	4.7 (42)	4.5 (42)	4.7 (42)

[1] CM_C = Conidia of *C. minitans*, final spore concentration of *C. minitans* was 1.6×10^7 conidia ml^{-1}, CM_{AFS} = Cultural filtrates of MCD_{cm} containing AFS

[2] Means followed by the same letter within each column are not significantly ($P > 0.05$) different according to the Least Significant Difference Test

PDB cultures (5.7 mg ml^{-1}) was not significantly different ($P > 0.05$) from that of MCD cultures (6.9 mg ml^{-1}); however, the AFS activity of filtrates of Chy-1 in PDB cultures which had the IG value of 2.7% was lower than that of the MCD cultures which had the IG value of 41.6%. Additional studies are necessary to clarify effects of nutritional factors and ambient pH on AFS production by *C. minitans* and to determine the relationship between the two factors in regulation of AFS production by *C. minitans*.

The present study demonstrated that AFS of *C. minitans* is capable of the suppression of mycelial growth of *S. sclerotiorum*. On the other hand, sclerotia of *S. sclerotiorum* treated with AFS-containing filtrates of MCD cultures of *C. minitans* for 24 h were viable and capable of undergoing myceliogenic germination. Furthermore, ascospores of *S. sclerotiorum* could germinate on PDA containing AFS from cultural filtrates of *C. minitans*. Therefore, mycelial growth of *S. sclerotiorum* appears to be more sensitive than sclerotia or ascospores of this pathogen to AFS of *C. minitans.*

The four in vitro trials demonstrated that treatment of leaves of oilseed rape with AFS-containing cultural filtrates of *C. minitans* resulted in an effective suppression of the disease incidence of leaf blight caused by *S. sclerotiorum.* These results suggest that AFS of *C. minitans* has potential for control of diseases caused by ascospore infection of *S. sclerotiorum*. Further studies under field conditions are required to confirm the role and importance of AFS of *C. minitans* for control of sclerotinia blight of oilseed rape.

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