Degradation of oxalic acid by *Coniothyrium minitans* and its role in suppression of *Sclerotinia sclerotiorum*

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Abstract

Degradation of oxalic acid (OA) by the mycoparasitic fungus *Coniothyrium minitans* (CM), and the effects of OA degradation on production and activity of β-(1, 3)-glucanase of CM were investigated. Over 85% of OA was degraded by CM grown at 20°C for 15 days in modified potato dextrose broth (PDB) containing OA at 0.8 to 28 mM. Meanwhile, pH of these cultures increased from 3.0-4.8 to 8.3-8.6. Capability of CM to degrade OA was evident in the bioassay experiment, in which toxicity of filtrates of 15-day-old CM cultures grown in PDB containing OA at 8, 16 or 24 mM to seedlings of oilseed rape was significantly reduced in comparison with controls. In dual cultures grown on potato dextrose agar, spread of CM onto colonies of *Sclerotinia sclerotiorum* (SS) resulted in elevation of pH from 2.9 to 6.6 and accumulation of β-(1, 3)-glucanase. Increase of pH on flower petals of oilseed rape inoculated with CM and SS was observed after incubation on water agar for 6 days. Inoculation of petals with CM and SS reduced petal-mediated infection of leaves of oilseed rape by SS. CM grown in PDB amended with OA at 16 mM for 9, 12 and 15 days increased production of β-(1, 3)-glucanase by 60.7, 22.8 and 51.6%, respectively, compared to untreated control (PDB alone). The activity of β-(1, 3)-glucanase produced by CM was enhanced by OA at 2 mM which conditioned pH at 6.0, but inhibited by OA at 4-32 mM which lowered pH to 3.5-1.5, whereas the pH of the control treatment (without OA) was 7.0. On the other hand, sodium oxalate at 2 to 32 mM, which conditioned pHs at 5.9-6.1, did not inhibit activity of this enzyme. These results suggest that degradation of OA by CM can nullify the pH effect caused by OA and may improve mycoparasitism of CM on SS by stimulating its production of β-(1, 3)-glucanase and/or activity of this enzyme.

Key words: *Coniothyrium minitans*, *Sclerotinia sclerotiorum*, oxalic acid, β-(1, 3)-glucanase, mycoparasitism, biological control

1. Introduction

Oxalic acid (OA) is toxic to many plants, animals and microorganisms. For instance, *Sclerotinia sclerotiorum* (Lib.) de Bary, causal agent of *sclerotinia* stem rot of oilseed rape (*Brassica napus* L. and *B. campestris* L.), produces OA in cultures or in infected plant tissues 41). Godoy et al. (18) studied OA-deficient mutants of *S. sclerotiorum* and concluded that OA plays a determinant role in pathogenesis of this pathogen. Mechanisms proposed for OA functions are: (i) acidifying ambient environment, which is favorable for activity of some pathogenesis-related enzymes such as polygalacturonase and acidic protease in *S. sclerotiorum* (3, 8, 40) and *Sclerotium rolfsii* Sacc. (3); (ii) capturing and chelating Ca^{2+} from plant cell walls (3); (iii) interrupting programmed opening and closure of stomata on plant leaves, resulting in leaf wilt of plants (19); and (iv) inhibiting defence-related enzymes such as *o*-diphenol oxidase (16) and polyphenoloxidase (40), and oxidative burst of host tissues (7). Previous studies revealed that a few species of oxalate-degrading bacteria could effectively suppress infection of plant tissues by *S. sclerotiorum* (10, 11, 49). Meanwhile, genes encoding oxalate-degrading enzymes including oxalate oxidase (EC 1.2.3.4) and oxalate decarboxylase (EC 4.1.1.2) in plants can confer resistance or tolerance of plants to infection by *S. sclerotiorum* (9, 12, 32).

Coniothyrium minitans Campbell is a mycoparasite (6), causing destruction of hyphae (26) and sclerotia (6, 25) of *S. sclerotiorum*. The extra-cellular enzyme β-(1, 3)-glucanase appears an important enzyme involved in the mycoparasitism of *C. minitans* on *S. sclerotiorum*, as the expression of the enzyme-encoding gene *cmg1* increases during the infection of sclerotia of *S. sclerotiorum* by *C. minitans* (17). Another extra-cellular enzyme chitinase of *C. minitans* may be less important than β-(1, 3)-glucanase in infection of *S. sclerotiorum*, as the yield of this enzyme in cultures of *C. minitans* and in infected sclerotia of *S. sclerotiorum* was negligible (22).

Numerous reports indicate that *C. minitans* is an effective agent for control of plant diseases caused by *S. sclerotiorum*, including *sclerotinia* wilt of sunflower (24), white mold of bean (28), blossom blight of alfalfa (35) and leaf blight/stem rot of oilseed rape (36). The fact that hyphae of *C. minitans* can grow over colonies of *S. sclerotiorum* in dual cultures (26) and colonize *S. sclerotiorum*-infected plant tissues (23,36) implies that *C. minitans* may have a mechanism to overcome the toxic effects caused by OA produced by *S. sclerotiorum*. Wei et al. (50) reported that *C. minitans* could tolerate the toxicity of OA at 1.2 mM for conidial germination and at 20 mM for mycelial growth on agar media (50). However, the mechanism for OA tolerance by *C. minitans* and the effects of OA on production and activity of β-(1, 3)-glucanase of *C. minitans* remain

unknown. The objectives of this study were to determine the efficiency of OA degradation by *C. minitans*, and the impacts of OA-degrading process on production and activity of β-(1, 3)-glucanase of *C. minitans*.

2. Materials and methods

2.1 Fungal strains, culture media and inoculum preparation

C. minitans strain Chy-1 and *S. sclerotiorum* strain Rap-1 were used in this study. The strain Chy-1 was isolated from a soil sample collected from Changyang County of Hubei Province, China (34) and the strain Rap-1 was isolated from a sclerotium of *S. sclerotiorum* collected from a diseased rapeseed plant near Wuhan of Hubei. Three cultural media used in this study were potato dextrose agar (PDA) (15), modified potato dextrose broth (PDB), and water agar (1.5%). Both PDA and PDB were made of fresh potato. PDA contained water extract of 200 g of peeled potato, 20 g of glucose, 20 g of agar, and 1,000 ml of distilled water (15). PDB was modified to contain water extract of 200 g of peeled potato, 5 g of dextrose, 1 g of KNO_3, and l, 000 ml of distilled water. Conidia of *C. minitans* were collected from 15-day-old PDA cultures and the concentration of conidial suspensions was determined using a haemocytometer under a compound microscope. The mycelial inoculum of *S. sclerotiorum* was prepared with 3-day-old PDA cultures.

2.2 Quantitative determination of degradation of oxalic acid by C. minitans

Oxalic acid (OA, Xilong Chemical Company, Guangzhou, China) was dissolved in PDB in 250-ml Erlenmeyer flasks with the final OA concentrations adjusted to 0 (control), 0.8, 4, 8, 12, 16, 20, 24, 28, 32, 40 or 80 mM. There were six flasks of PDB for each treatment, 50 ml/flask. Three flasks of each treatment were individually inoculated with conidia of *C. minitans* at 1×10^6 conidia per flask. The remaining three flasks of each treatment were used as controls without inoculation with *C. minitans*. The flasks were mounted on a rotary shaker (HQL150B, the Instrument Company of the Chinese Academy, Wuhan, China) and incubated at 200 rpm and 20°C for 15 days. The mycelial biomass, pH, content of OA and content of ammonia in the cultural filtrates of each flask were determined using the procedures described below. The experiment was repeated.

2.3 Determination of mycelial biomass, pH, and content of oxalic acid and ammonia

Cultural filtrates of *C. minitans* in each flask were separated from mycelial mats using a piece of pre-weighed filter paper (9 cm diam) (# 9, Hangzhou Xinhua Paper Manufacturing Company Ltd., Hangzhou, China). Mycelial mats were dried at 60°C for 48 h and weighed. pH of the filtrate from each flask was measured using a pH meter (Model pHS-3C, Shanghai Hongyi Instrument Company Ltd., Shanghai, China). A 25-ml cultural filtrate sample from each flask was used to estimate OA content. OA in each sample was purified by precipitation using 5 ml of 10% (w/v) $CaCl_2$ solution and centrifugation at 10,000 rpm for 5 min. The supernatant in each tube was discarded and 10 ml of H_2SO_4 solution (2 mM) was added to dissolve the precipitate. OA content was determined by the titration method using $KMnO_4$ solution (2.5 mM) as described by Bateman and Beer (3). The concentration of ammonia in the cultural filtrates was determined using Nessler's reagent with $(NH_4)_2SO_4$ as the standard for calibration (29).

2.4 Bioassay of oxalic acid degradation by C. minitans

Coniothyrium minitans was grown in PDB containing 0, 8, 16, 24 or 32 mM of OA at 20°C on a shaker for 15 days. Cultural filtrates were collected from each flask by filtering as mentioned above. PDB containing 0, 8, 16, 24 or 32 mM of OA without inoculation with *C. minitans* was used as controls. For OA toxicity tests, seedlings of oilseed rape (*Brassica napus* cv. Zhongyou Hybrid No.4) at 4-5 true-leaf stage were pulled out from soil and washed under running tap water for removal of soil remains. Roots of seedlings were place in each 50-ml bottle containing 25 ml of the cultural filtrate from each flask or 25 ml of the control PDB amended with different concentrations of OA, 5 seedlings per bottle. There were three replicates (bottles) for each treatment. The bottles were individually plugged with cotton swabs to prevent water evaporation and placed on a table at room temperature (20-25°C) for 3 days. The treated seedlings of oilseed rape were individually examined for wilt symptoms, using a scale of 0 to 4, where 0=healthy; 1=one leaf turned yellow or wilted, the stem and roots appeared healthy and firm; 2=two leaves turned yellow or wilted, the stem and roots were slightly flaccid; 3=three leaves turned yellow or wilted, the stem and roots were moderately flaccid; 4=four or five leaves turned yellow or wilted, the stem and roots were severely flaccid. The severity of OA toxicity for each replicate was expressed by toxicity index (TI), which was calculated by the following formula (Fang, 1998): $TI=(P_0\times0+P_1\times1+P_2\times2+P_3\times3+P_4\times4)/4$, where P_0, P_1, P_2, P_3 and P_4 are the number of seedlings of oilseed rape corresponding to each toxicity rating for each replicate.

2.5 Interaction of C. minitans and S. sclerotiorum in dual cultures

This difference in change of pH between *C. minitans* and *S. sclerotiorum* was used to test the effect of *C. minitans* on *S. sclerotiorum* in dual cultures. An agar plug (6 mm diam.) containing mycelia of *C. minitans* was removed from an 8-day-old PDA culture and inoculated on PDA amended with 0.1% (w/v) of bromophenol blue at 0.5 cm from the wall of a Petri dish (9 cm diam.). After incubation at 20°C for 5 days, The dishes were inoculated with *S. sclerotiorum* by placing mycelial plugs (6 mm diam.) from a 3-day-old PDA culture on the agar surface at 7 cm away from the inoculation point of *C. minitans*, one plug per dish. All dishes were incubated at 20°C and examined every two days for change of the color on the colonies and their growth substrate. Media colonized by *S. sclerotiorum* turned from blue to yellow, whereas the medium colonized by *C. minitans* remained blue. There were 10 dishes (replicates) in this experiment.

Twenty-day-old dual cultures of *C. minitans* and *S. sclerotiorum* on PDA without amendment of bromophenol blue were

used for determination of β-(1, 3)-glucanase in the agar medium colonized by *S. sclerotiorum* (Ss), *C. minitans* (Cm) or the two fungi (Ss + Cm). Agar media colonized by Ss, Cm or Ss + Cm in each of the 10 dual-culture dishes were removed using a sterilized dissecting knife and combined as a sample. Cold acetate buffer (0.2 M, pH 5.0) was added to each agar sample at a ratio of 1:2 (agar/buffer, w/v). The mixture was ground to fine powder in liquid nitrogen and kept at 4°C overnight. The slurry was centrifuged at 4,000 rpm at 4°C for 5 min. The activity of β-(1, 3)-glucanase in the supernatant of each sample was determined using the method described by Giczey et al. (17) with laminarin (Sigma Chemical Company, St Louis, MO, USA) as the substrate for the enzymatic reaction. There were three samples (replicates) for each treatment.

2.6 Interaction between C. minitans and S. sclerotiorum on flower petals of oilseed rape

Senescent flower petals play a major role in epidemics of *sclerotinia* stem rot of oilseed rape (*Brassica napus* L.), as they provide exogenous nutrients for germination of ascospores of *S. sclerotiorum*. Colonization of flower petals by *S. sclerotiorum* results in decrease of ambient pH due to the secretion of OA by this pathogen. The ambient pH can be increased if OA on flower petals is degraded by *C. minitans*.

Sclerotia of *S. sclerotiorum* were collected from 30-day-old cultures grown on autoclaved carrot slices (15) and used for production of apothecia using the method of Huang and Kozub (1989). Mature apothecia were ground to slurry using a sterilized pestle and mortar. Sterile distilled water was added to the slurry at a ratio of 3:2 (apothecial slurry: water; v/v), and the mixtures were filtered through a double-layered cheesecloth. The concentration of the resulting ascospore suspension was determined using a haemocytometer under a compound microscope. The viability of ascospores of *S. sclerotiorum* was tested on PDA and the suspensions with higher than 95% of germinated ascospores were used in this study.

Seeds of oilseed rape (cv. Zhongyou Hybride No.4) were sown in the early October of 2004 in a field near Wuhan, China. In the spring of 2005, flower petals of newly-opened florets were collected from this field, surface-sterilized in 70% ethanol for 30 sec, washed twice in sterile distilled water, blot-dried on sterilized paper towel and placed on water agar amended with 0.1% (w/v) bromophenol blue in Petri dishes (9 cm diam), 6 petals/dish. There were three treatments for the experiment, Cm + Ss, Ss alone and water alone. For the treatment of Cm + Ss, aliquots of 20 μl of conidial suspension of *C. minitans* (2.7×10^6 conidia/ml) were pipetted onto the centre of each petal, and the dish was uncovered under a laminar flow hood for 2 h to allow evaporation of the excess water on petals. Then, 20 μl of the ascospore suspension of *S. sclerotiorum* (2.7×10^5 ascospores/ml) was inoculated onto each petal. For the treatment of Ss, the petal was first treated with 20 μl of sterile distilled water, air-died for 2 h and inoculated with the ascospore suspension of *S. sclerotiorum* at 20 μl per petal. Petals treated with water alone were used as a control. After incubation at 20°C for 6 days, the color on the surface of each petal and the surrounding media was recorded. There were 5 dishes for each treatment.

The petal-mediated infection technique (Bremer et al., 2000; Li et al., 2006) was used to study the effect of colonization of flower petals of oilseed rape by *C. minitans* on suppression of leaf lesions of oilseed rape caused by *S. sclerotiorum*. Seeds of oilseed rape (*B. napus* cv. Westar) were sown in Cornell Peat-Lite Mix (4) in plastic pots (15 cm diam.) and kept in a greenhouse (15-20°C) until flowering. Petals of newly opened florets were removed, washed twice in 30 ml of distilled water containing 0.01% (v/v) of Tween 20 (Sinopharm Chemical Reagent Co. Ltd, Shanghai, China) and placed on leaves of 30-day-old plants of oilseed rape (cv Westar), 3 plants per pot, 4 leaves per plant and 1 petal per leaf. There were five treatments in the experiment: one treatment for Ss, three treatments for Cm + Ss, and the water treatment (control). For the treatments of Cm + Ss, aliquots of 20 μl of the conidial suspension of *C. minitans* at the concentrations of 7.5×10^5, 7.5×10^6, or 7.5×10^7 conidia/ml were pipetted onto the centre of each petal. They were air-dried for 2 h and then inoculated with the ascospore suspension of *S. sclerotiorum* containing 2.1×10^5 ascospores/ml at 20 μl per petal. For the treatments of Ss and water, petals of oilseed rape were treated first with water at 20 μl per petal, air-dried for 2 h and inoculated with Ss and water, respectively, at 20 μl per petal. There were four replicates (pots) for each treatment. The plants in each pot were covered with a clear plastic bag, kept in a growth room at 20°C under fluorescent light (12 h light per day) for 6 days. Infection of leaf tissues by Ss was recorded by measuring the diameter of each leaf lesion developed around each petal. The experiment was repeated.

2.7 Effect of oxalic acid on production of β-(1, 3)-glucanase by C. minitans

An aliquot of 100 μl of the conidial suspension of *C. minitans* (1×10^7 conidia/ml) was inoculated in 50 ml PDB or PDB amended with 16 mM of OA in a 250 ml-flask. The cultures were incubated at 20°C on a shaker (200 rpm). Three flasks of cultures for each treatment were sampled at 3-day intervals for 15 days at each sampling date, and determined the mycelial biomass, pH and OA content in the cultural filtrate from each flask using the methods described previously. The activity of β-(1, 3)-glucanase in the filtrates was assayed by the procedure described by Giczey et al. (17). One unit (U) of β-(1, 3)-glucanase was defined as microgram glucose released per minute by 1 ml of cultural filtrate. The yield of the enzyme produced by *C. minitans* was expressed as U/mg dry mycelia. The experiment was repeated.

2.8 Effect of oxalic acid on activity of β-(1, 3)-glucanase produced by C. minitans

Three experiments were conducted to investigate the effect of OA on the activity of β-(1, 3)-glucanase of *C. minitans*. Cultural filtrates of *C. minitans* were collected from PDB cultures on a shaker (200 rpm) at 20°C. The first experiment was to investigate the effect of OA on the activity of β-(1, 3)-glucanase of *C. minitans*. Ammonia sulphate was added to precipitate the β-(1, 3)-glucanase in the cultural filtrate with a ratio of 70% (w/v). Crude enzyme solution (CES) was prepared by dialysing the protein precipitate dissolved in acetate buffer (0.1 M, pH 5.0) against the same buffer at 4°C overnight. An

enzymatic reaction mixture was set up by mixing 0.1 ml of the CES, 0.5 ml of 1% (w/v) laminarin solution (dissolved in 0.1 M acetate buffer) and different concentrations of OA in a 5 ml-plastic tube. The volume of each reaction mixture was adjusted to 1 ml by addition of distilled water. The final concentration of OA was adjusted to 0 (control), 2, 4, 6, 8, 10, 16, 24 or 32 mM. pH of each reaction mixture was determined using pH indicator stripes (San Ai Si Chemical Company Ltd, Shanghai, China). The activity of β-(1, 3)-glucanase in each reaction mixture was determined by the procedure described by Giczey et al. (17).

The second experiment was to investigate the effect of sodium oxalate on activity of β-(1, 3)-glucanase of *C. minitans*. Crude enzyme solution (CES) was prepared by the same procedures described above. Sodium oxalate was used to replace OA to set up enzymatic reaction mixtures. The final concentration of sodium oxalate was 0 (control), 2, 4, 6, 8, 10, 16, 24 or 32mM. Performance of enzymatic reactions and detection of the activity of β-(1, 3)- glucanase were conducted by the procedures described by Giczey et al. (17).

The third experiment was to investigate the effect of pH on the activity of β-(1, 3)-glucanase of *C. minitans*. The enzymatic reaction mixtures were set up by mixing 0.1 ml of CES, 0.1 ml laminarin solution (5%, w/v, dissolved in distilled water), 0.4 ml of distilled water and 0.4 ml of different buffers to maintain pHs at 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10. Four different buffers were used, including the HCl-KCl buffer (0.2 M) for maintaining pH at 1 to 2, the citric acid-phosphoric acid buffer (0.1 M) for maintaining pH at 3 to 7, the Tris-HCl buffer (0.2 M) for maintaining pH at 8 to 9, and the disodium hydrogen carbonate buffer (0.05 M) for maintaining pH at 10 (Jeffery et al., 1996). The procedures described previously were used to perform all the reactions for determining the activity of β-(1, 3)-glucanase under different pHs.

2.9 Data analyses

Data were analyzed using the analysis-of-variance (ANOVA) program in SAS Software (SAS Institute, Cary, NC, USA, version 8.1). Data for the same treatment, but collected from different repeats, were pooled when they were not significantly different (P>0.05) in the F-test. The percentage data on the OA degradation and petal-mediated lesions of *S. sclerotiorum* on leaves of oilseed rape were arcsine-transformed prior to analysis. The means were back-transformed to percentage values after analysis. Means for different treatments in each experiment were compared and separated using Duncan's Multiple Range Test at P=0.05 level.

3. Results

3.1 Degradation of OA by C. minitans

Mycelial growth of *C. minitans* was related to the concentration of OA in culture media, vigorous growth in PDB amended with OA at 0.8 to 28 mM, but poor in PDB amended with OA at 32 or 40 mM of OA and no growth in PDB amended with OA at 80 mM (Table 1). The treatments with the highest mycelial biomass of *C. minitans* were the cultures grown in PDB containing 12 or 16 mM of OA, which yielded 190.4 and 193.2 mg per flask, respectively. The biomass of *C. minitans* in the control was 133.6 mg per flask.

The initial pH (day 0) in OA-amended PDB were low, ranging from 2.7 in the treatment of OA at 80 mM to 6.3 in the treatment of OA at 0.8 mM, compared to 7.2 in the PDB control (Table 1). After incubation for 15 days, pH in the cultural filtrates of *C. minitans* of PDB or PDB amended with OA at 0.8 to 28 mM increased to be higher than 8.0. Meanwhile, increased accumulation of ammonia from 2.7-4.9 to 5.8-6.4 μmol/ml was observed in these treatments (Table 1). In the treatments of OA at 32 to 80 mM, pH of the cultural filtrates of *C. minitans* did not change before (day 0) and after (day 15) incubation, and the amounts of ammonia accumulation in these treatments were significantly (P<0.05) lower than that in the treatments of OA at 0.8 to 28 mM (Table 1).

The percentage of the OA degradation in the filtrates of 15-day-old cultures of *C. minitans* was 85.9-91.6% for the treatments of OA at 0.8 to 28 mM, whereas no OA degradation was detected in PDB containing OA at 32, 40 or 80 mM (Table 1).

Table 1. Mycelial dry weight (DW), pH value, ammonia production and degradation of oxalic acid (OA) by *Coniothyrium minitans* in modified potato dextrose broth amended with OA

OA (mM)	Mycelial DW (mg)	pH value		NH_3 (μmol/ml)		OA degradation (%)[2]
		0 d	15 d	0 d	15 d	
0	133.6 bc	7.2 a	8.1 c	4.9 a	5.8 bc	_
0.8	139.4 bc	6.3 b	8.1 c	Nd[4]	nd	91.7 a
4	138.7 bc	4.8 c	8.5 ab	4.6 ab	6.4 a	90.7 ab
8	136.6 bc	4.4 d	8.6 a	3.8 b	6.1 ab	91.6 a
12	190.4 a	3.7 e	8.3 bc	2.9 c	6.0 bc	85.9 c
16	193.2 a	3.5 f	8.5 ab	2.9 c	6.0 bc	88.2 bc
20	149.0 b	3.3 g	8.5 ab	2.8 cd	5.9 bc	88.5 bc
24	153.5 b	3.1 h	8.3 bc	2.7 cd	6.0 bc	86.7 c
28	124.3 c	3.0 i	8.4 ab	2.7 cd	6.0 bc	90.2 ab
32	27.7 d	3.0 ij	3.1 d	2.3 cd	4.1 d	7.9 d
40	18.0 e	2.9 j	2.9 e	2.3 cd	4.0 d	0.0 e
80	0.0 e	2.7 k	2.6 f	2.1 d	3.3 e	0.0 e
LSD	20.5	0.1	0.3	0.7	0.3	2.7

3.2 Bioassay of oxalic acid degradation by C. minitans

When roots of oilseed rape seedlings were soaked in fresh PDB or in filtrates of *C. minitans* from 15-day-old PDB cultures, slight toxic symptoms of leaf yellowing on seedlings of oilseed rape were observed and the toxicity index was low, 0.6 for the fresh PDB and 1.1 for the cultural filtrate of *C. minitans* (Table 2). Severe toxic symptoms of leaf yellowing, leaf wilting, stem withering and/or seedling collapsing occurred on seedlings of oilseed rape treated with PDB containing various concentrations of OA (control) and the toxicity index was 1.7, 3.3, 3.5, 3.8 and 4.0 for the treatments of OA at 8, 16, 24, 32 and 40 mM, respectively (Table 2). In contrast, when PDB containing OA at 8, 16, or 24 mM were inoculated with *C. minitans* and incubated for 15 days, severe toxic symptoms were not observed on seedlings of oilseed rape treated with the 15-day-old cultural filtrates and the toxicity index was 1.2, 1.6 and 1.5, respectively, for these treatments (Table 2). When PDB was amended with OA at 32 or 40 mM, inoculated with *C. minitans* and incubated for 15 days, no OA degradation due to the complete inhibition of mycelial growth by *C. minitans* was observed in these media (Table 1). Consequently, the cultural filtrates from the two treatments remained highly toxic to seedlings of oilseed rape with the toxicity index being 3.8 and 4.0, respectively (Table 2).

Table 2. Toxicity of culture filtrates of *Coniothyrium minitans* grown in potato dextrose broth (PDB) amended with oxalic acid to seedlings of oilseed rape

OA in PDB (mM)	Treatment	Toxicity index (0-4)
0	Control	0.6 e[3]
	C. minitans	1.1 d
8	Control	1.7 c
	C. minitans	1.2 d
16	Control	3.3 b
	C. minitans	1.6 c
24	Control	3.5 b
	C. minitans	1.5 c
32	Control	3.8 a
	C. minitans	3.8 a
40	Control	4.0 a
	C. minitans	4.0 a
$LSD_{0.05}$		0.2

3.3 Interaction between C. minitans and S. sclerotiorum in dual culture

Results of the dual-cultural test on PDA amended with bromophenol blue (BB) showed that the color of medium colonized by *C. minitans* remained blue with pH of 6.6, whereas the color of the medium colonized by *S. sclerotiorum* turned yellow with pH ranging from 2.9 to 3.4. There was no antagonistic interaction between *C. minitans* and *S. sclerotiorum* in dual cultures. As *C. minitans* continued to grow and spread over the colonies of *S. sclerotiorum*, the area invaded by *C. minitans* changed from yellow to blue. The radius of the blue-colored area from the inoculation plug of *C. minitans* (Y) was positively proportional to the co-incubation days (X), $Y=0.1248X + 1.0856$ ($R^2=0.9826$, $P<0.01$). After the dual cultures were incubated for 33 days, the color of all dual cultures turned blue due to colonization of the entire dish by *C. minitans*. Meanwhile, sclerotia produced by *S. sclerotiorum* were parasitized by *C. minitans* with the formation of pycnidia on the surface of infected sclerotia. pH in the *C. minitans*-*S. sclerotiorum* interaction zone was 5.7 after co-incubation for 8 days and 6.6 after 33 days.

After incubation for 20 days, the amount of β-(1, 3)-glucanase was 7.9 U in the agar sample of *S. sclerotiorum* alone, which was significantly lower ($P<0.05$) than the values of 59.8 U in the interaction zone *S. sclerotiorum* + *C. minitans* in dual cultures and 56.8 U in the area colonized by *C. minitans* alone. No significant difference ($P>0.05$) in the amount of β-(1, 3)-glucanase was detected between the samples from the interaction zone of Ss + Cm and colony of *C. minitans* alone.

3.4 Interaction of C. minitans and S. sclerotiorum on flower petals of oilseed rape

Mycelial growth was profuse on flower petals of oilseed rape inoculated with ascospores of *S. sclerotiorum* and incubated on water agar for 6 days. The color of *S. sclerotiorum*- inoculated flower petals was yellow. In contrast, mycelial growth were sparse on flower petals inoculated with *C. minitans* + *S. sclerotiorum* and the color of these petals was blue, indicating the elevation of the ambient pH on the petals.

Results of the *in vivo* inoculation test showed that 92.5% of petals inoculated with ascospores of *S. sclerotiorum* alone developed lesions on leaves of oilseed rape with an average lesion diameter of 6.0 cm (Table 3). For the treatments of petals treated with water (control), all of the leaves of oilseed rape remained healthy without lesion developement around the petals. For the treatments of Cm (1.5×10^5 conidia/petal) + Ss, Cm (1.5×10^4 conidia/petal) + Ss, and Cm (1.5×10^3 conidia/petal) + Ss, the percentage of petals induced lesions on leaves of oilseed rape were 5.2, 6.3, and 46.5%, respectively, significantly ($P<0.05$) lower than the treatment of *S. sclerotiorum* alone (Table 3). The average lesion diameter was 0.3, 0.2 and 2.1 cm, respectively, for these treatments, significantly ($P<0.05$) smaller than the treatment of *S. sclerotiorum* alone (Table 3).

3.5 Effect of OA degradation on production of β-(1, 3)-glucanase by C. minitans

Degradation of OA in PDB amended with OA at 16 mM by *C. minitans* was detected after incubation for 3 to 15 days (Fig. 1A). The degradation efficiency was 13.0% after 3 days, increased rapidly to 77.2% after 6 days, and followed by steady increase to 90.0% after 9 days and to 93.6% after 15 days (Fig. 1A).

After incubation for 6 days, production of β-(1, 3)-glucanase by *C. minitans* was detected in filtrates of cultures grown in PDB alone or PDB amended with OA (16 mM). The difference between the two treatments was not significant (P>0.05) (Fig. 1B). From day 9 to day 15, the yield of β-(1, 3)-glucanase secreted by *C. minitans* in OA-amended PDB was significantly higher (P<0.05) than that in PDB alone. For example, after incubation for 12 days, the yield of β-(1, 3)-glucanase was 79.2 U/mg dry mycelia in PDB alone, but was 97.3 U/mg dry mycelia and in OA-amended PDB. The increased rates of β-(1, 3)-glucanase in OA-amended PDB were 60.7, 22.8 and 51.6%, respectively, after incubation for 9, 12 and 15 days, compared to that in PDB controls.

Table 3. Effect of *Coniothyrium minitans* (Cm) on colonization of flower petals by ascospores of *Sclerotinia sclerotiorum* (Ss) and subsequent infection of leaves of oilseed rape

Treatment	Petal-mediated lesions caused by Ss on leaves of oilseed rape (%)	Lesion diameter (cm)
Ss (4.2×10^3 ascospores/petal)	92.5 a	6.0 a
Cm (1.5×10^5 conidia/petal) + Ss	5.2 c	0.3 c
Cm (1.5×10^4 conidia/petal) + Ss	6.3 c	0.2 c
Cm (1.5×10^3 conidia/petal) + Ss	46.5 b	2.1 b
Water	0.0 d	0.0 d
$LSD_{0.05}$	5.4	0.2

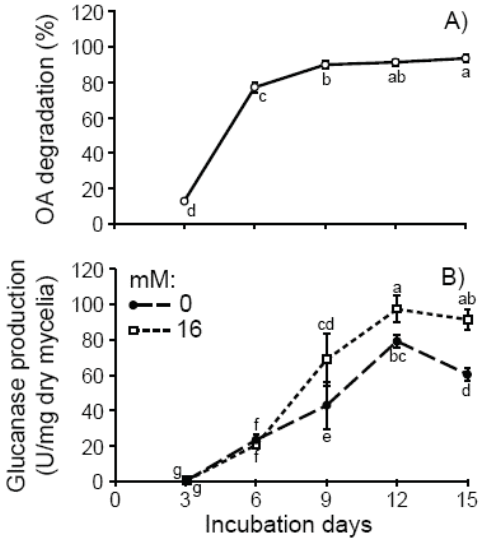

Fig. 1. Time-course of OA degradation (A) and β-(1, 3)-glucanase production (B) in potato dextrose broth (PDB) and PDB amended with 16 mM of OA.

3.6 Effect of oxalic acid and sodium oxalate on activity of β-(1, 3)-glucanase of C. minitans

In the first experiment with OA, the ambient pH in the reaction mixture without OA (control) was about 7.0 (Fig. 2A). When OA was added to reaction mixtures to the final concentrations ranging from 2 to 32 mM, the ambient pH of the reaction mixtures was decreased and the level of decrease in pH in reaction mixtures was related with the concentrations of OA. For example, the pH was 6.0 when the concentration of OA was adjusted to 2 mM, but was decreased to 1.5 when the concentration of OA was increased to 32 mM (Fig. 2A). On the contrary, in the second experiment with sodium oxalate, the pH value was 5.9 in the reaction mixture without amendment of sodium oxalate (control). When sodium oxalate was added, the pH in the reaction mixtures changed slightly (Fig. 2B).

In the first experiment, the activity of β-(1, 3)-glucanase of *C. minitans* was 621.8 μg glucose/ml/min in the control. Presence of OA in the reaction mixtures significantly affected the activity of the enzyme. When the concentration of OA was adjusted to 2 mM, the activity of β-(1, 3)-glucanase was 652.0 μg glucose/ml/min, significantly higher (P<0.05) than that in the control (Fig. 2C). With the increase in the concentration of OA from 4 to 32 mM, the activity of β-(1, 3)-glucanase decreased and the degree of decrease was related with the concentration of OA. For example, the activity of β-(1, 3)-glucanase was 503.5 μg glucose/ml/min when the concentration of OA was 4 mM, but was decreased to 155.9 μg glucose/ml/min when the concentration of OA was increased to 32 mM. On the contrary, in the second experiment, the activity of β-(1, 3)-glucanase was 474.6 μg glucose/ml/min in the control. When sodium oxalate was added to the reaction mixtures at 2 to 32 mM, a slight increase in the activity of β-(1, 3)-glucanase (487.3-500.8 μg glucose/ml/min) was observed (Fig. 2D).

The results in the third experiment showed that ambient pH significantly affected activity of β-(1, 3)-glucanase of *C. minitans*. With the increase of pH from 1-4, the activity of β-(1, 3)-glucanase increased from 90.1 to 854.4 μg glucose/ml/min. The optimum pH was 4 to 6 and the enzymatic activity ranged from 826.6 to 854.4 μg glucose /ml/ min. With the further increase of pH to 7-10, the activity of β-(1, 3)-glucanase decreased to 569.4-184.5 μg glucose/ml/min.

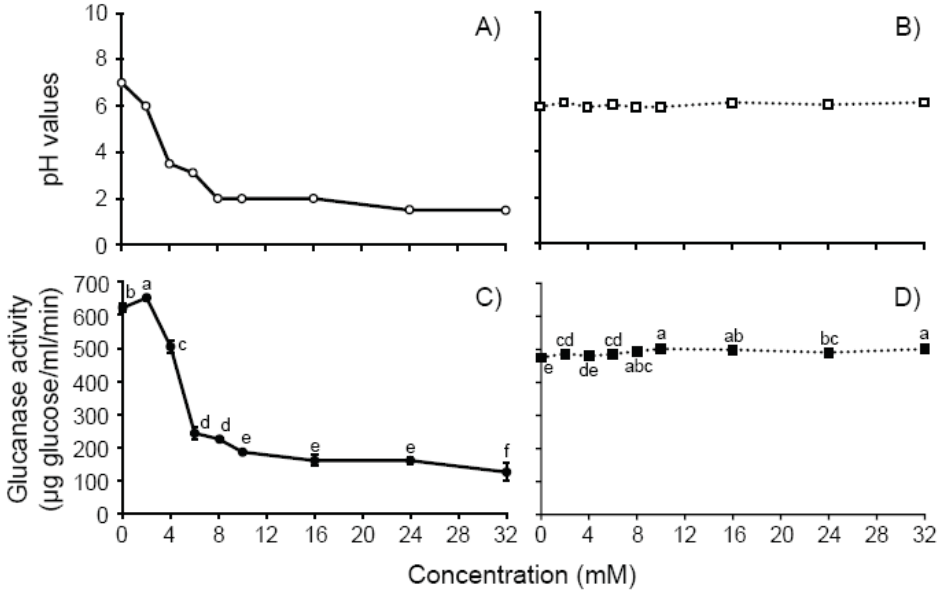

Fig. 2. Effect of concentration of oxalic acid (Fig. 2, A, C) and sodium oxalate (Fig. 2, B, D) on pHs in enzymatic reaction mixtures (Fig. 2, A, B) and the activity of the β-(1, 3)-glucanase produced by *Coniothyrium minitans* (Fig. 2, C, D).

4. Discussion

Previous studies revealed that in the process of pathogenesis of *S. sclerotiorum*, a large amount of OA was released to the ambient environment to assist its infection of plant tissues (18, 41) and to induce sclerotial formation (20). This phytotoxin can inhibit the germination of conidia and the growth of mycelia of *C. minitans* (50). The present study indicates that the mycoparasitic fungus *C. minitans* is capable of degradation of OA. In the dual-cultural experiment, colonization of the colonies of *S. sclerotiorum* by *C. minitans* resulted in the elevation of the ambient pH, thereby nullifying the harmful effect of acidic pH on other organisms including *C. minitans* itself. This result suggests that degradation of OA may occur in the mycelial interaction between *C. minitans* and *S. sclerotiorum*. Application of *C. minitans* on flower petals of oilseed rape is effective in reducing severity of petal-mediated infections and lesion development on leaves of oilseed rape. One of the mechanisms involved in this effect may be elimination of the toxic effect of OA released by *S. sclerotiorum* on tissues of oilseed rape, as elevation of the ambient pH was observed on flower petals inoculated with *C. minitans* + *S. sclerotiorum*, compared to the treatment of *S. sclerotiorum* alone. Therefore, OA degradation by *C. minitans* may be one of the mechanisms involved in the control of *sclerotinia* blight of oilseed rape by this mycoparasite.

S. sclerotiorum produces sclerotia serving as dormant structures for oversummering and overwintering. Small amounts of OA (2) and D-erthroascorbic acid, the precursor of OA (31), were readily detected in sclerotia of *S. sclerotiorum*. The present study indicates that degradation of OA by *C. minitans* can occur in PDB containing lower concentration (0.8 mM) or higher concentration (28 mM) of OA (Table 1). This result suggests that degradation of OA by *C. minitans* may occur during infection of sclerotia of *S. sclerotiorum* by this mycoparasite.

Degradation of OA has been reported in other fungi, including *Agaricus bisporus* Sing (30), *Aspergillus niger* Tiegh (14), *Ceriporiopsis subvermispora* Gilb. & Ryvarden (1), *Collybia* (=*Flammulina*) *velutipes* Staude (32), *Coriolus versicolor* Fries (13), and *S. sclerotiorum* (39). Oxalate decarboxylase was responsible for OA degradation in most of the fungi investigated (13, 14, 30, 32, 39). This enzyme catalyses the reaction of OA decarboxylation to produce formic acid (non-toxic to plant tissues) and carbon dioxide (32). In some fungi such as *Ceriporiopsis subvermispora*, oxalate oxidase was detected (1). It catalyzes the reaction of oxidizing OA to carbon dioxide and hydrogen peroxide (1). The gene encoding for oxalate decarboxylase in *Collybia velutipes* (30), or *Flammulina* sp. (9) has been successfully transferred to plants, including tobacco (30), tomato (30) and lettuce (9). In *C. minitans*, the molecular mechanism for degradation of OA remains unknown and warrants further investigations.

Previous reports indicated that the ambient pH significantly affected virulence of numerous species of fungi, including plant pathogens such as *S. sclerotiorum* (3, 8, 39), *Colletotrichum* spp. (44), *Alternaria alternata* Keisser and *Penicillium* spp. (45), insect pathogens such as *Metarhizium anisopliae* Sorokin (46, 47) and human pathogens such as *Candida* spp. (42). For example, in *S. sclerotiorum*, the acidic pH (mainly created by OA) stimulated the expression of pathogenesis-related genes encoding polygalacturonase (*pg*) (46) and non-aspartyl acid protease (*acp1*) (43). St. Leger et al. (46) found that enzymes responsible for degradation of the insect cuticle were synthesized by *M. anisopliae* only at pH values at which they function effectively. This study showed that the synthesis of β-(1, 3)-glucanase by *C. minitans* was enhanced in PDB media containing OA (16 mM). Increase in ambient pH values resulting from degradation of OA or/and accumulation of ammonia by *C. minitans* may be responsible for the increase of the transcription of the gene encoding β-(1, 3)-glucanase in *C. minitans* based on the theory proposed by St. Leger et al. (46), as the optimum pH for the activity of β-(1, 3)-glucanase of *C. minitans* was 4-6 according to this study.

OA has different effects on the activity of enzymes secreted by pathogens and host plants (20). The acidic pH conditioned by OA stimulates the activity of polygalacturonase (8, 40) and protease (43) secreted by *S. sclerotiorum* for assisting maceration of plant tissues (40). Chelation of manganese by OA can stimulate the activity of manganese peroxidase secreted by the wood-decaying fungus *Phanerochaete chrysosporium* Burdsall for assisting lignin degradation (33). On the other hand, OA produced by *S. sclerotiorum* inhibits resistance-related enzymes such as polyphenol oxidase in tomato (38), *o*-diphenol oxidase in apples and bean pods (16), polyphenol oxidase and phenylalanine ammonialyase in oilseed rape (37). This study reveals that OA significantly inhibits the activity of β-(1, 3)-glucanase produced by *C. minitans* when the concentration of OA reached 4 mM. The result that oxalate (2-32 mM) can not inhibit the activity of β-(1, 3)-glucanase (Fig. 4) suggests that acidic pH conditioned by OA is the major factor affecting the activity of β-(1, 3)-glucanase. Therefore, degradation of OA by *C. minitans* may create a pH condition favourable for the activity of β-(1, 3)-glucanase during infection of *S. sclerotiorum* by *C. minitans*. Thus, degradation of OA by *C. minitans* may improve its mycoparasitism on *S. sclerotiorum* by stimulating production of β-(1, 3)-glucanase and/or enhancing the activity of this enzyme.

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References

1. Aguilar, C., Urzúa, U., Koenig, C., Vicuña, R. 1999. Arch. Biochem. Biophy. 366, 275-282.
2. Anonymous. 1979. *Sclerotinia* Stem Rot of Oilseed Rape. Agriculture of China Press, Beijing (In Chinese).
3. Bateman, D. F., Beer, S.V. 1965. Phytopathology 55, 204-211.
4. Boodley, J. W., Sheldrake JR. R. 1977. N. Y. State Coll. Agric. And Life Sci., Inform. Bull. 43. 8 pp.
5. Bremer, E., Huang, H. C., Selinger, L. J., Davis, J. S. 2000. Plant Pathol. Bull. 9, 69-74.
6. Campbell, W. A. 1947. Mycologia 39, 190-195.
7. Cessna, S. G., Sears, V. E., Dickman, M. B., Low, P. S. 2000. Plant Cell 12, 2191-2200.
8. Cotton, P., Kasza, Z., Bruel, C., Rascle, C., Fèvre, M. 2003. FEMS Microbiol. Lett. 227, 163-169.
9. Dias, B. B. A., Cunha, W. G., Morais, L. S., Vianna, G. R., Rech, E. L., Capdeville, G., Aragão, F. J. L. 2006. Plant Pathol. 55, 187-193.
10. Dickman, M. B., Chet, I. 1998. Soil Biol. Biochem. 30, 1195-1197.
11. Dickman, M. B., Mitra, A. 1992. Physiol. Mol. Plant Pathol. 41, 255-263.
12. Donaldson, P. A., Anderson, T., Lane, B. G., Davidson, A. L., Simmonds, D. H. 2001. Physiol. Mol. Plant Pathol.59, 297-307.
13. Dutton, M. V., Kathiara, M., Gallagher, I. M., Evans, C. S. 1994. FEMS Microbiol. Lett. 116, 321-326.
14. Emiliani, E. Bekes, P. 1964. Arch. Biochem. Biophy. 105, 488-493.
15. Fang, Z. D. 1998. Research Methodology for Plant Diseases (3rd ed.). Chinese Agriculture Press, Beijing, pp. 8-12.
16. Ferrar, P. H., Walker, J. R. L. 1993. Physiol. Mol. Plant Pathol. 43, 415-442.
17. Giczey, G., Kerényi, Z., Fülöp, L., Hornok, L. 2001. App. Environ. Microbiol. 67, 865-871.
18. Godoy, G., Steadman, J. R., Dickman, M. B., Dam, R. 1990. Physiol. Mol. Plant Pathol. 37, 179-191.
19. Guimarães, R. L., Stotz, H. U. 2004. Plant Physiol. 136, 3703-3711.
20. Hegedus, D. D., Rimmer, S. R. 2005. FEMS Microbiol. Lett. 251, 177-184.
21. Hu, X., Dennis, L. B., Nasser, Y., Jonathan, P. D., Oswald, C., Otto, F., Lu, G. H. 2003. Plant Physiol. 133, 170-181.
22. Hu, Y. M. 2004. Master thesis, Department of Plant Protection, Huazhong Agricultural University 58p. (In Chinese with English abstract).
23. Huang, H.C. 1977. Can. J. Bot. 55, 289-295
24. Huang, H. C. 1980. Can. J. Plant Pathol. 2, 26-32.
25. Huang, H. C., Kokko, E. G. 1987. Can.J. of Bot. 65, 2483-2489.
26. Huang, H. C., Kokko, E. G. 1988. J. Phytopathol. 123, 133-139.
27. Huang, H. C., Kozub, G. C. 1989. Plant Prot. Bull. 31, 333–345.
28. Huang, H. C., Bremer, E., Hynes, R. K., Erickson, R. S. 2000. Biol Cont. 18, 270-276.
29. Jeffery, G. H., Bassett, J., Mendham, J., Denney R.C. 1996. Vogel's Text Book of Quantitative Chemical Analysis (5th Ed.). ELBS, Longman, Essex, UK. p679.
30. Kathiara, M., Wood, D. A., Evans, C. S. 2000. Mycol. Res. 104, 345-350.
31. Keates, S. E., Loewus, F. A., Helms, G. L., Zink, D. L. 1998. Phytochemistry 49, 2397-2401.
32. Kesarwani, M., Azam, M., Natarajan, K., Mehta, A., Datta, A. 2000. J. Biol. Chem. 275, 7230-7238.
33. Kuan, I.-C., Tien, M. 1993. Proceedings of National Academy of Sciences, USA, 90, 1242-1246.
34. Li, G. Q., Wang, D. B., Zhang, S. H., Dan, H. H. 1995. Journal of Huazhong Agricultural University 14, 125-129.
35. Li, G. Q., Huang, H. C., Acharya, S. N., Erickson, R. S. 2005. Plant Pathol. 54, 204-211.
36. Li, G. Q., Huang, H. C., Miao, H. J., Erickson, R. S. Jiang, D. H., Xiao, Y. N. 2006. Eur. J. Plant Pathol. 114, 345-355.
37. Liu, S.-Y., Zhou, B.-W., Pan, J.-R. 1998. Acta Phytopathologica Sinica, 28, 33-37 (in Chinese with English abstract).
38. Magro, P., Marciano, P., di Lenna, P. 1984. FEMS Microbiol. Lett. 24, 9-12.
39. Magro, P., Marciano, P., di Lenna, P. 1988. FEMS Microbiol. Lett. 49, 49-52.
40. Marciano, P., di Lenna, P., Margo, P. 1983. Physiol. Plant Pathol. 22, 339-345.
41. Maxwell, D. P., Lumsden, R. D. 1970. Phytopathology 60, 1395-1398.
42. Peñalva, M. A., Arst, H. N. Jr. 2002. Microbiology and Molecular Biology Reviews 66, 426-446.
43. Poussereau, N., Creton, S., Billon-Grand, G., Rascle, C., Fevre, M. 2001. Microbiology, 147, 717–726
44. Prusky, D., McEvoy, J. L., Leverentz, B., Conway, W. S. 2001. Mol. Plant-Microbe Interact. 14, 1105-1113.
45. Prusky, D, McEvoy, J. L., Saftner, R, Conway, W. S., Jones, R. 2004. Phytopathology 94, 44-51.
46. Rollins, J. A., Dickman, M. B. 2001. App. Environ. Microbiol. 67, 75-81.
47. St. Leger, R. J., Joshi, L., Roberts, D. 1998. App. Environ. Microbiol. 64: 709-713.
48. St. Leger, R. J., Nelson, J. O., Screen, S. E. 1999. Microbiology 145, 2691-2699.
49. Wang, T., Wu, J. S., Wang, J. S. 2001. J. Nanjing Agri. Univ. 24, 29-32.
50. Wei, S. J., Li, G. Q., Jiang, D. H., Wang, D. B. 2004. Acta Phytopathologica Sinica 34, 199-203.