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Inhibition of growth of *Sclerotinia minor* and other pathogens by citrinin in the filtrate of *Penicillium citrinum*

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Abstract

Penicillium citrinum Thom, isolated from the sclerotia of *Sclerotinia minor*, was cultured in a broth of Czapek-Dox for 4 to 8 weeks. The filtrate obtained was incorporated into potato dextrose agar or Czapek-Dox agar at different concentrations (v/v). The amended media were tested for mycelial growth of *S. minor* and other pathogens. Mycelial growth of *S. minor* was completely inhibited on media amended with 20% (v/v) filtrate of *P. citrinum*, and considerable inhibition of *S. minor* occurred at 10 and 15% concentrations. Mycelial growth of *S. major, Sclerotium rolfsii, Rhizoctonia solani* (AG-4) was inhibited by similar concentrations of filtrate of *P. citrinum*. Inhibitor(s) in the filtrate were extracted with ethyl acetate and tentatively identified as citrinin. Citrinin was shown to be an active component in the filtrate against mycelial growth of *S. minor, S. major* and *Sclerotium rolfsii*.

Introduction

Penicillium citrinum has been reported to produce mycotoxin(s) that inhibit the mycelial growth of *Sclerotinia minor* and other pathogens [8]. Mycotoxins are produced by several *Penicillium* species commonly isolated from grains and other sources [3, 4, 6]. The mycotoxin, citrinin, is a toxic secondary metabolite of several fungal species belonging to the genera *Penicillium* and *Aspergillus* [2].

Citrinin was first isolated from the fungus *Penicillium citrinum* Thom by Hetherington & Raistrick [5]. Although other species of *Penicillium* also produce citrinin [2, 8, 10, 11], *P. citrinum* remains the primary source of citrinin. Rodig *et al.* [9] found that yield of citrinin from *P. citrinum* reached a maximum after 35–45 days in broth culture. They also discovered that the efficiency of citrinin produced varied somewhat with different generations of *P. citrinum* cultures used. Decomposition studies of citrinin revealed that citrinin undergoes only limited decomposition in heat or light [9].

In this study, filtrate of *P. citrinum* at different concentrations (v/v) was tested for possible effects on the mycelial growth of some plant pathogens, and attempts were made to extract the inhibitor(s), and test their activity on the mycelia of *S. minor*. All tests were repeated twice and similar results were recorded each time.

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Materials and methods

Culture of Penicillium citrinum

Penicillium citrinum was isolated from the sclerotia of *Sclerotinia minor* from a field planted to peanuts and maintained on potato dextrose agar (PDA) slants [7]. Fifty ml of Czapek-Dox broth in 300 ml bottles were autoclaved for 20 min. After cooling they were inoculated with two 5 mm diameter mycelial discs of *P. citrinum*, grown on Czapek-Dox agar (CDA). The broth cultures were incubated at 25 ± 2 °C, under fluorescent light (800 lux) for six to eight weeks. The bottles were hand-agitated daily for 5 sec. to facilitate conidial dispersal and rapid growth. The fungal biomass was separated from the broth by filtration through a glass fiber filter paper (Reeve Angel, Whatman, Inc., Clifton, New Jersey) in a 9 cm-diameter Buchner funnel.

Incorporation of filtrate into potato dextrose agar and mycelial growth tests

Filtrate of *P. citrinum* culture was incorporated into molten PDA, at 50 °C, at concentrations of 5, 10, 15 and 20% (v/v). The media amended with filtrate at varying concentrations had pH ranging from 6.5 to 6.7. PDA (pH 6.9) with no filtrate amendment was used as controls. Forty petri dishes (15 × 100 mm), containing 15 ml of media each, were made for each treatment. The pathogens tested for mycelial growth inhibition were: *Sclerotinia minor, Sclerotinia major, Sclerotium rolfsii*, and *Rhizoctonia solani* (AG-4). Discs (6 mm diameter) of each of the pathogens from three-day old cultures were inoculated into the middle of ten petri dishes at each concentration, and incubated in darkness at 25 ± 2 °C. After 48 h of incubation, areas of mycelial growth were traced with a marker and transferred to paper. Using a Li-Cor model 3100 area meter (Lambda Instruments Corporation, Lincoln, NE 68504), the mycelial growth area (cm^2) in each dish was determined. Another test was conducted to determine the rate of growth of mycelia of *S. minor, S. major* and *Sclerotium rolfsii* at different filtrate concentrations between 24 and 42 h of incubation.

Sclerotial germination and mycelial growth on potato dextrose agar amended with filtrate

Sclerotia of *S. minor* were produced on oat seeds, by incubating inoculated oats in flasks for 5 weeks at 25 ± 2 °C. Sclerotia were sieve separated from the oat seeds after spreading to dry on a laboratory bench for one week. The sclerotia were washed with deionized H_2O and surface sterilized with a 0.5% sodium hypochlorite solution for 4 min. They were then rinsed twice with sterile deionized water and spread to dry. Forty petri dishes (10 of each concentration) of PDA amended with filtrate of *P. citrinum* at concentrations of 5, 10, 15 and 20% (v/v), were inoculated with the sclerotia. One sclerotium was placed in the middle of each of ten petri dishes at each concentration. The dishes were incubated at 25 ± 2 °C. Following germination and growth of sclerotia, mycelial growth areas were traced and measured with the area meter.

Extraction of inhibitor(s) with ethyl acetate, and mycelial growth tests

Thirty ml of filtrate from 6 week-old Czapek-Dox broth cultures were placed in a 250 ml separatory funnel, 30 ml of ethyl acetate was added, and the mixture was vigorously shaken for 1 min. Solvent layers were then allowed to separate for 5 min. The lower aqueous layer and the upper ethyl acetate layer were collected. The aqueous layer was returned to the funnel and extracted three more times each with 30 ml of ethyl acetate. The total ethyl acetate fraction was flash evaporated under reduced pressure at 40 °C to near dryness. The ethyl acetate fraction yielded a thin layer of a yellowish solid material. The aqueous fraction was placed in the flash evaporator to remove traces of ethyl acetate. Residue of the ethyl acetate fraction was suspended in 30 ml of deionized water. Ten and 15 ml portions were incorporated into molten CDA at 50 °C to give concentrations of 10 and 15%, respectively. The amended media were autoclaved for 20 min, then 15 ml were dispensed in each of 10 petri dishes (15 × 100). Corresponding concentrations of the aqueous fraction were incorporated into CDA. Dishes were inoculated with

6 mm discs of *S. minor* taken from the leading edge of a 3-day-old culture grown on CDA. Mycelial growth areas in each dish was marked after 2 days of inoculation at 25 ± 2 °C in darkness and the area of growth was determined. Crude filtrate of *P. citrinum* at concentrations of 10 and 15% (v/v) were used for comparison with the extract portions.

The ethyl acetate fraction was separated by thin layer chromatography using a procedure described by Hald & Krogh for the analysis and confirmation of citrinin in barley [1].

Incorporation of Crystalline Citrinin into Czapek-Dox agar and mycelial growth tests

Ten mg of citrinin (Sigman Chemical, St. Louis, MO) were dissolved in 1 ml of ethyl acetate, then incorporated into one liter of CDA at 50 °C. Medium was occasionally swirled and kept for about 1 h at this temperature to allow the evaporation of the ethyl acetate. Medium in which ethyl acetate was added and treated similarly, served as control. Twenty ml of medium containing citrinin at 10 μg/ml and control medium (without citrinin) were dispensed in each petri dish, inoculated in the middle with mycelial discs from a 3-day-old culture of *S. minor*, and incubated for mycelial growth determination as previously described.

Results and discussion

When Czapek-Dox broth was inoculated with *Penicillium citrinum*, a progressive color change occurred in the broth during incubation. The clear medium turned yellow, then light brown, and finally dark brown, by the 8th week of incubation. Determination of the levels of inhibitors in the filtrate at different culture ages revealed that maximum inhibitors were present in filtrates from six week old cultures of *P. citrinum*, as shown by the inhibition of mycelial growth of *S. minor* (Table 1). Considerable growth occurred at 5%, but growth was greatly reduced at both 10 and 15% concentration levels (Table 1). At 10 and 15% concentrations, the pattern of the limited mycelia growth was very irregular. Also, the color of *S. minor* mycelia changed from tan-white to yellow after three days of incubation at these concentrations. Sclerotia were not formed from the discolored mycelia even after prolonged incubation. When filtrate from six week old cultures of *P. citrinum* was incorporated into PDA at a concentration of 20% (v/v), there was complete inhibition of the mycelial growth of *S. minor*. PDA amended at similar concentrations with filtrate of *P. citrinum* and tested for mycelial growth inhibition of *S. major*, *Sclerotium rolfsii* and *Rhizoctonia solani*, showed inhibition of growth of mycelia as filtrate concentration increased (Table 2). Inhibition of mycelial growth on media amended with 15 and 20% filtrate

Table 1. Mycelial growth of *S. minor* on potato dextrose agar (PDA), amended with different ages of filtrate of *P. citrinum* at different concentrations.

Age of filtrate (weeks)	Concentration of filtrate (v/v) in PDA				
	0	5	10	15	20
	cm^2				
2	28.62	24.30	21.52	21.10	18.30
3	27.57	21.62	20.41	18.74	13.36
4	29.37	19.41	16.37	11.46	10.28
5	26.82	12.89	7.24	3.13	1.47
6	27.34	7.21	2.42	0.23	0.00
8	28.41	9.48	3.26	1.27	0.29

Area of growth (cm^2) was measured after 42 hrs. Averages were determined from 10 replications representing each treatment.

Table 2. Mycelial growth of four pathogens on potato dextrose agar (PDA), amended with the filtrate of *Penicillium citrinum* at different concentrations.

Pathogen	Concentration of filtrate (v/v) in PDA				
	0	5	10	15	20
	cm^2				
S. minor	35.54	8.14	2.89	0.63	0.00
S. major	44.08	11.34	1.64	1.14	0.51
S. rolfsii	18.57	4.50	1.69	1.09	0.42
R. solani	29.25	18.37	13.58	10.64	8.33

Area of growth (cm^2) was measured after 48 hrs. Averages were determined from 10 replications representing each treatment.

of *P. citrinum* was greatest for *S. minor* and the least for *R. solani*. Mycelial growth of *S. rolfsii* and *S. major* was inhibited more than *R. solani*, but not as great as *S. minor*. The negative linear correlation values obtained when regressing the area of mycelial growth of the four pathogens and the filtrate concentrations give the approximate rates of the mycelial inhibition of the four pathogens as the filtrate concentration increases (Fig. 1).

Comparison of the rate of mycelial growth between 24 and 42 h of incubation, of *S. minor*, *S. major*, and *S. rolfsii*, in PDA amended with 0, 5, 10,

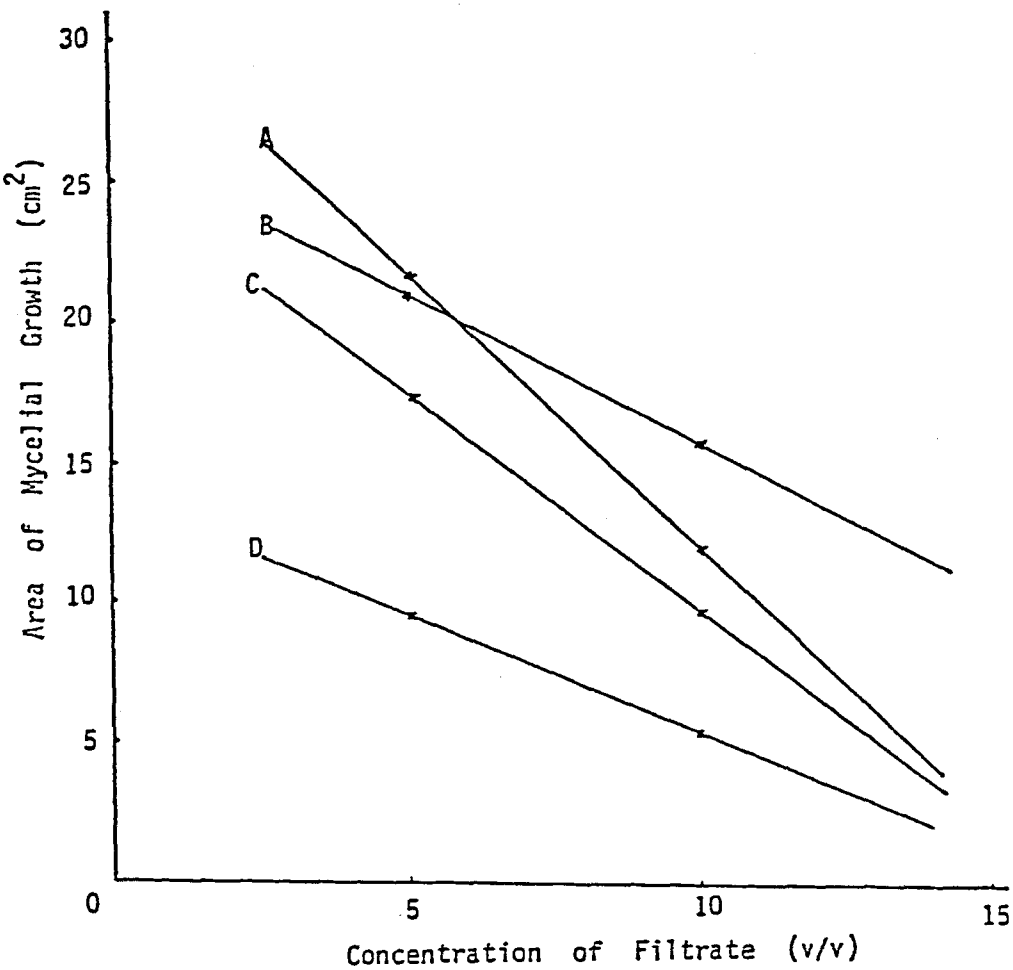

Fig. 1. Linear regression between the filtrate concentrations of *Penicillium citrinum* in potato dextrose agar and mycelial growth of four soilborne pathogens.

Table 3. Rate of mycelial growth of three pathogens on potato dextrose agar (PDA) amended with filtrate from six-wk-old cultures of *P. citrinum*.

Concentration (%) of filtrate (v/v) in PDA	Rate of growth (cm^2/h) for[1]		
	S. minor	*S. major*	*S. rolfsii*
0	1.36	1.86	0.31
5	0.36	0.77	0.16
10	0.07	0.25	0.07
15	0.01	0.03	0.05
20	0.00	0.02	0.03

[1]Determined from 10 replications for each treatment. Rate was determined from growth measurements at 24 and 42 h of incubation at 25 ± 20 in darkness.

15 and 20% filtrate indicated that the greatest reduction of rate of growth occurred in *S. minor* as compared to the other pathogens (Table 3).

Sclerotia of *S. minor* germinated at the 0, 5 and 10% concentration levels three days after incubation, and by the fifth day there was considerable sclerotial germination at all concentrations tested (Table 4). Sclerotia just initiated germination at the 20% filtrate concentration level, and the mycelia never extended to any appreciable area. Close examination of germinating sclerotia in the plates amended with filtrate at the 15 and 20% levels, showed a yellowish discoloration of the limited mycelia sprouting from the sclerotia. Growth of the mycelia was uneven, sparse and restricted around the germinating sclerotia. Further growth of the mycelia did not occur after the yellowish discoloration was observed. The limited growth areas were marked out and measured.

Extraction of inhibitors from the filtrate of *P. citrinum*, using ethyl acetate, produced a yellow residue that was tentatively identified with thin layer chromatography as citrinin. Information in the literature reveal this compound to be the main product of *P. citrinum* [5, 9].

Incorporation of purified citrinin at 10 μg/ml into CDA, showed 95% inhibition of mycelial growth of *S. minor* similar to that observed from incorporating the ethyl acetate fraction of the culture filtrate of *P. citrinum* into CDA (Table 5). Thin layer chromatography of the ethyl acetate fraction using a procedure described by Hald & Krogh [1], produced a yellow spot with similar Rf value of citrinin.

Incorporation of citrinin extracted with ethyl acetate from filtrate of *P. citrinum* into CDA at 10 and 15% (v/v), produced 98 to 99.5% inhibition of the mycelial growth of *S. minor*. Ninety two to 95% inhibition of mycelial growth occurred at similar con-

Table 4. Mycelial growth of *Sclerotinia minor* from sclerotia and mycelia on potato dextrose agar (PDA) amended with *penicillium citrinum* filtrate.

Concentration of filtrate (v/v) in PDA	Mycelial[1] discs (cm^2)	Sclerotia[2] (cm^2)
0	35.54a	41.13a
5	8.14b	8.91b
10	2.89c	5.09c
15	0.63d	4.21c
20	0.00e	0.43d

[1]Area of mycelial growth was measured after 48 hours of incubation. Average of 10 replications; [2]Mycelial growth was measured after 5 days in incubation to allow for sclerotial germination and growth. Average of 10 replications; [3]Means within columns followed by the same letter are not significantly different (P = 0.05) level.

Table 5. Mycelial growth of *S. minor* on Czapek-Dox agar (CDA) amended with filtrate, ethyl acetate and aqueous layer fractions of *P. citrinum* at different concentrations (v/v) as compared with medium containing 10 μg/ml of crystalline citrinin.

Concentration of fraction layer (v/v) in CDA	Fraction			
	P. citrinum filtrate	Ethyl Acetate fraction	Aqueous fraction	Crystalline citrinin[1]
	cm^2			
0	18.74[2, 3]	18.74	18.74	14.91
10	3.06	0.36	1.54	–
15	0.80	0.11	0.84	–
	–	–	–	0.79

[1]Crystalline citrinin was incorporated in the medium at 10 μg/ml; [2]Averages were determined from 10 replications at each concentration per treatment; [3]Mycelial growth (cm^2) was measured after 48 hrs.

centrations with the aqueous layer fraction (Table 5). This fraction was expected to have residual amounts of the inhibitor(s). It is likely that our extraction procedure did not completely extract all the citrinin in the filtrate. Or perhaps, and even likely, there are other inhibitor(s) besides citrinin, produced by *P. citrinum* in Czapek-Dox broth culture that still need to be determined.

In summary, *P. citrinum* produces a compound which was tentatively identified as citrinin. Citrinin extracted from culture filtrate of *P. citrinum* showed almost complete inhibition of mycelial growth of *S. minor*. Investigation of the persistence of filtrate extracts in soil will determine the possible effects of other soil microflora on its activity.

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