한국농약과학회지 제2권 제1호(1998)
The Korean Journal of Pesticide Science
Vol. 2, No. 1, pp. 40~45(1998)

Characterization of an antifungal compound isolated from an antagonistic fungus *Aspergillus terreus* against phytopathogenic fungi

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Abstract : An antagonistic fungus inhibiting growth of various phytopathogenic fungi was isolated from greenhouse soils and identified. Mophological features of fruiting structures on potato dextrose agar and colorless globose to ovate heavy walled hyaline cells from the vegetative mycelium grown on MY20 agar indicate that this antagonist is *Aspergillus terreus*. The antagonistic activity is due to the production of antifungal compounds. An antifungal compound was purified from its culture filtrate using chloroform extraction, column chromatography, and thin layer chromatography. The purified antibiotic was effective to various phytopathogenic fungi and identified as butyrolactone I. ED_{50} values measured by petri-plate assay through effective dosage(ED)-probit analysis were 9.7, 13.7, 23.3, 42.6 and 102.7 ppm on *Botrytis cinerea*, *Rhizoctonia solani*, *Pythium ultimum*, *Phytophthora capsici*, and *Fusarium oxysporum*, respectively.(Received December 5, 1997, accepted February 27, 1998)

Key words : antagonistic fungus, *Aspergillus terreus*, butyrolactone I, ED-probit analysis, antifungal compound.

Introduction

Greenhouse horticulture to extend the cropping season of vegetables, fruits, and flowers, and to protect them from adverse environmental conditions is cost- and labor-intensive agriculture. Most of horticultural greenhouse technology is designed primarily to maximize production potential. The greenhouse environment is generally warm and humid; conducive to disease epidemics unless wise manipulation of its environment to reduce them. Little of this technology has been aimed for the reduction of losses from pests, but it is possible to manipulate crop and its environment for that purpose(Airhart, 1984).

The acreage of greenhouse cultivation has been expanded over 15% every year for 5 years and the total acreage amounts 44,780 ha in Korea. Eighty percent of that is composed of vinyl covered house cultivation (Seol *et al.*, 1996). This capital-intensive management enforces farmers to continue monoculture cropping system, that in turn causes serious pest problems on several cash crops grown under the greenhouse(Song *et al.*, 1996). Heavy dosage of pesticides have been applied in some cases to maintain the stable production of the crops and could bring harmful side effects, such as health harzards in the closed environment, and resurgence of pests due to resistance to the pesticides.

Reduction of pests can be used to minimize losses in yield and quality by integrating pest management strategies such as cultural practices, use of pesticides, and host plant resistance(Jarvis, 1992). Biological control of diseases is one of the means to reduce the potentially harmful fungicide use(Baker, 1992). However, there have been a few reports on either biological control of phytopathogens or integrated disease management to reduce fungicide use in

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greenhouse horticulture in Korea.

This study is aimed to isolate and identify biocontrol agents from greenhouse soils. An antifungal compound was purified and its biological activity against several plant pathogens was measured.

Materials and Methods

Isolation of antifungal fungi

Antagonistic fungi were isolated from greenhouse soils. Appropriate serial dilutions of soil suspension in sterile H_2O were plated on rose-bengal streptomycin agar(Martin, 1950) plates, and the plates were incubated at 28℃ for 7 days. Single colonies inhibiting growth of microflora nearby were isolated and screened for antifungal activity using the petri plate assay.

Examination of antifungal spectrum of the isolated fungi

The isolated fungus(AF2) was initially screened for the ability to inhibit growth of several plant pathogens on 1/5 strength of PDA(Difco) plates. A culture disk(5 mm diam.) of the isolated fungus was placed at the center of a plate, and pathogenic fungi were placed at distance around perimeter of the plate. The plates were incubated for 1-6 days at 28℃ depending on the growth rate of plant pathogens tested and examined for growth inhibition by the antagonistic fungi. The plant pathogenic fungi tested were *Pythium ultimum, Rhizoctonia solani, Phytophthora capsici, Botrytis cinerea*, and *Fusarium oxysporum*.

Identification of the isolated fungus

The fungus was identified according to the morphological keys and species descriptions by Raper *et al.*(1973), especially conidial morphology, foot cells on PDA and globose to ovate heavy hyaline cells of mycelium on an MY20 medium.

Isolation and purification of an antifungal compound

An antifungal compound was isolated from the culture broth of the antagonistic fungus(AF2) grown in a modified Czapek-dox medium(CDM), substituting asparagine for KNO_3, after growing at 28℃ in a shaking incubator for 6 days. The culture broth was first filtered through 4 layers of cheese cloth, and the filtrate was then extracted with 500 ml of chloroform/L of the filtrate. The chloroform extract was combined and concentrated *in vacuo*, and the concentrate was applied on a silica gel column(10 x 200 mm), and eluted with chloroform - acetone step gradients from

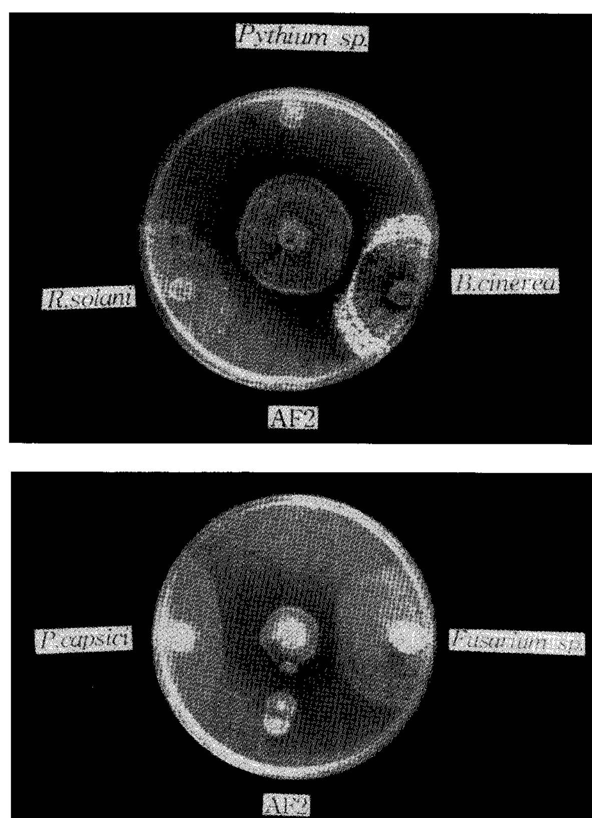

Fig. 1. Petri-plate assay for screening of antagonistic fungi. An antagonistic fungus(AF2) and five phytopathogenic fungi, *Botrytis cinerea*, *Phytophthora capsici*, *Pythium ultimum*, *Rhizoctonia solani*, and *Fusarium oxysporum* were inoculated at center or perimeter of a plate, respectively.

100 : 1, 50 : 1, 10 : 1, 1 : 1 to 0 : 100. Fractions with fifteen-milliliter were collected, and 10 μl aliquots from each fraction were spotted onto silicagel thin-layer chromatography plates(Merck 60 GF_{254}), and developed with cyclohexane : ether : acetone : methanol(2:6:1.5:0.5). The *in situ* bioassay of antifungal activity on the developed TLC plates was used for a preliminary detection of inhibitory compounds for 1st column chromatography. A spore suspension *Botrytis cinerea* in 1% PDA was sprayed on top of the TLC plate developed earlier by the solvent. Inhibition zones were observed after 4-5 day incubation at 28℃ in a humidified box. Fractions containing inhibitory activity were combined and taken to dryness *in vacuo*. The residues were loaded onto 2nd silica gel thin-layer chromatography plates and developed with chloroform : acetone : methanol (50:10:1).

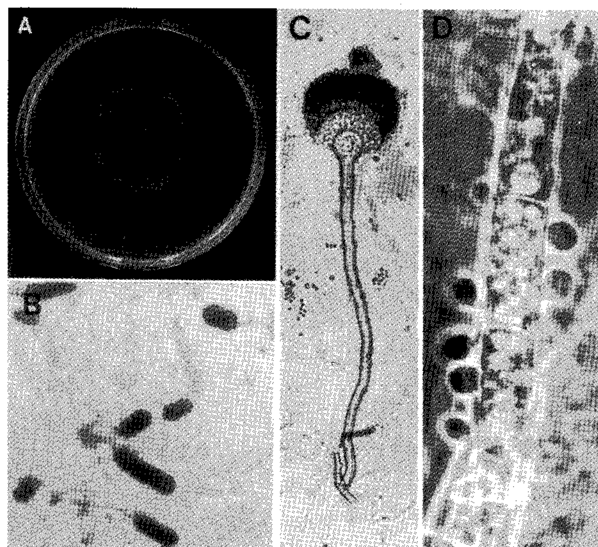

Fig. 2. Morphological characteristics of the antagonistic fungus, *Aspergillus terreus*. A: Colony mophology of antagonistic fungus grown on PDA, B: Columnar conidial heads, C: Biseriated conidiophore erected from a foot cell, D: Globose to ovate hyaline cells produced on the vegetative mycelium grown on MY20 agar.

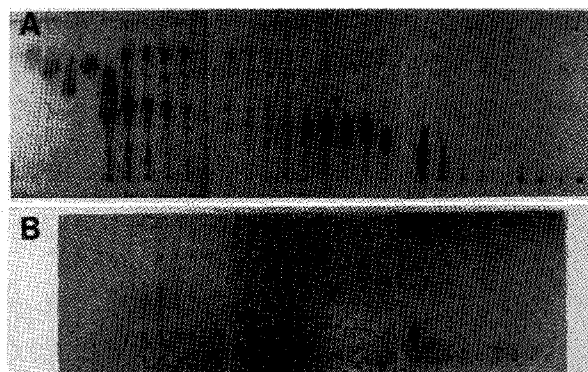

Fig. 3. Thin layer chromatogram of step gradient eluants after a column chromatography. A: Each column eluant fraction was developed by TLC. B: Direct inhibition assay on a TLC plate using conidial suspension of *Botrytis cinerea*. Cleared inhibition zones area indicated by the arrow.

Determination of antifungal spectrum and activity of the antibiotic.

The serially diluted the antibiotic was ammended in 1/5 strength of PDA plates as 0 to 200 ppm and a 5mm agar plug of five phytopathogenic fungi were placed onto a center of the plates. The diameters of mycelial

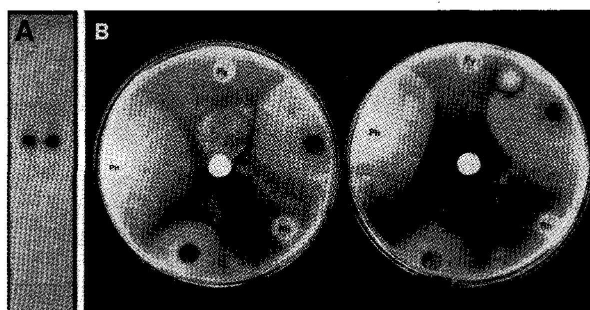

Fig. 4. The purified antifungal compound and its biological activity in a petri-plate assay. A: Purified antifungal compound identified on a TLC plate. B: Biological activity of purified antifungal compound on phytopathogenic fungi(20㎍/disc was applied: Py; *Pythium ultimum*, Ph; *Phytophthora capsici*, Rh; *Rhizoc-tonia solani*, Bo; *Botrytis cinerea*, Fu; *Fusarium oxysporum*). Left: Untreated control, Right: Butyrolactone I treated.

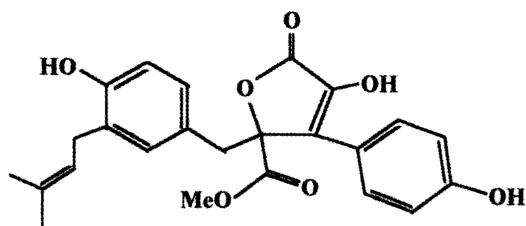

Fig. 5. Chemical structure of butyrolactone I, an antifungal compound from *Aspergillus terreus*.

growth were measured from 3 replicates of each treatment. The ED_{50} values of the mycelial growth inhibition were calculated by a probit analysis.

Results

Identification of antagonistic fungi

Several antagonistic fungi isolated from the greenhouse soil were screened by the petri-plate assay against five phytopathogenic fungi. One antagonistic fungus(AF2) showing a strong inhibition zone was selected for identification and purification of its antifungal compounds(Fig. 1). The fungus was identified to *Aspergillus terreus* based on morphological characterstics (Fig. 2). Conidial heads were compactly columnar in buff, cinnamon to orange brown shades and the sterigmata were biseriated. Conidio-phores were erected from foot cells. The white, smooth, and colorless globose to ovate or truncate heavy-walled hyaline cells were produced from the vegetative mycelium on MY20 agar.

Isolation and purification of an antifungal compound

Two separate cleared inhibition zones were observed at *Rf* 0.56 and 0.42(Fig. 3). One of the fractions(*Rf* 0.42) was further purified twice through column chromatography and TLC. A single spot on a TLC plate of the purified antifungal compound was shown in Fig. 4 and its antifungal activity on the pathogenic fungi in petri-plate assay was confirmed. Inhibition patterns on the pathogens by either the antagonistic fungus or the purified compound were very similar. That indicates this compound may play a major role in antagonistic activity. The chemical structure of a purified antifungal compound was identified as butryrolactone I(Fig. 5) based on elemental analysis, UV absorption spectrum, NMR, and GC/MS(Kim *et al.*, 1977).

Antifungal spectrum and activity of the antibiotic

This antibiotic showed strong activities on several plant pathogenic fungal species. ED_{50} values of the purified antifungal compound measured from degree of growth inhibition on PDA plates against *B. cinerea, R. solani, P. ultimum, P. capsici, and F. oxysporum* were 9.7, 13.7, 23.3, 42.6 and 102.7 ppm, respectively (Table 1). The slope of the probit regression line indicated that *P. ultimum*, exhibited higher sensitivity to the increasing concentration of the antibiotic.

Table 1. Inhibition of growth of phytopathogenic fungi by butyrolactone I

	ED_{50} (ppm)	Probit regression line		95% Limits	
		Slope	Intercept		
Pythium ultimum	23.29	3.71	0.94	10.89	53.76
Phytophthora capsici	42.63	2.71	1.41	26.71	71.03
Rhizoctonia solani	13.69	2.68	2.04	7.88	23.76
Botrytis cinerea	9.74	1.31	3.73	6.29	16.71
Fusarium oxysporum	102.66	2.96	0.94	87.15	261.14

Discussion

Recently, biological control measures have been deve-loped to reduce disease incidence(Baker, K. 1987; Baker, R. 1986; Baker and Cook, 1974; Cook and Baker, 1983; Fravel, 1988). Several antagonistic fungi(Papavizas, 1985) and bacteria(Weller, 1988) have been isolated and their efficacies on various plant pathogens have been tested.

Antagonistic effect of *A. terreus* has been observed on *R. solani* causing sheath blight of rice in India(Bhuyan *et al.*, 1994, Robin *et al.*, 1996). In this study, the isolated antagonistic fungus from greenhouse soils was identified as *Aspergillus terreus*. Its antifungal compound, butyrolactone I, had ED_{50} value 13.7 ppm on *R. solani*. *A. terreus* can be used as a biological control agent to reduce disease development caused by *B. cinerea*, *R. solani*, *P.ultimum* and *P. capsici* based on ED_{50} values measured if stable establishiment of the antagonist in natural soil conditions were made. This antagonistic fungus produced a copious amount of butyrolactone I in a CDM medium, estimated as 16 mg/10L. *A. terreus* is a typical soil inhabiting organism and hence, is abundant in soil upon decaying vegetation (Raper *et al.* 1973). This fungus produces abundant conidia and can survive in warm arable greenhouse soils where it originated. This species is known to produce butyrolactone derivatives(Kiriyama *et al.* 1977).

The biosynthesis and biological functions of butyro-lactone I have been investigated (Kiriyama *et al.* 1977; Nitta *et al.* 1983; Kitagawa *et al.* 1994). But there has been no report on antibiotic activities of this compound on plant pathogenic fungi even though efficacy on *R. solani* was observed.

Acknowledgments

This study is supported by the 1995 academic research fund of Ministry of Education, Republic of Korea.

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식물병원균 생육을 저해하는 *Aspergillus terreus*로부터 분리한 항균물질의 특성

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요약 : 온실 토양으로부터 주요 작물 병원균의 생육을 억제하는 길항곰팡이를 분리, 동정하였고, 그 균이 분비하는 항균물질을 분리하여 활성을 petriplate assay 로 검정했다. 길항균의 foot cell, 분생포자 및 MY20 배지상의 균사 등 형태학적 특징에 근거하여 이 균을 *Aspergillus terreus*로 동정하였다. *A.terreus* 의 배양여액을 chloroform으로 추출하고, 그 추출물을 column chromatography와 thin layer chromatography로 항균물질을 순수분리하였으며, 화학구조는 butyrolactone I이었다. 이 물질의 주요 작물병원균에 대한 ED_{50}은 *Botrytis cinerea*, *Phytophthora capsici*, *Pythium ultimum*, *Rhizoctonia solani*, 그리고 *Fusarium oxysporum*에 각각 9.7, 42.6, 23.3, 13.7, 102.7 ppm으로 나타났다.

*연락저자