

## Purification and Antifungal Activities of an Antibiotic Produced by *Gliocladium virens* G1 Against Plant Pathogens

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This study was undertaken to separate and identify antifungal substances produced by *Gliocladium virens* G1, a biocontrol agent used for the control of plant diseases caused by *Rhizoctonia solani*. The culture of *G. virens* G1 effectively inhibited the growth of *R. solani*, *Colletotrichum gloeosporioides*, and *Phytophthora capsici*, but less that of *Fusarium oxysporum*. The *n*-hexane extract of the *G. virens* culture, which was used for the purification of responsible substances, strongly inhibited *R. solani* and *C. gloeosporioides*, but not *P. capsici*, although the *n*-butanol extract was effective on all of the pathogens tested. An antifungal substance was purified using the *n*-hexane extract by Silica gel column chromatography and HPLC. The substance was examined for purity by HPLC and for nature by UV spectrometry, which differed from known antibiotic compounds such as gliotoxin, viridin and gliovirin. The antifungal substance was very lipophilic based on its solvent-solubility and *R<sub>f</sub>* values on TLC, and more inhibitory to *C. gloeosporioides* than other fungal pathogens tested.

**Keywords :** antifungal substance, biocontrol, *Gliocladium virens*.

*Gliocladium virens* was applied as a biocontrol agent for plant diseases especially soilborne fungi together with *Trichoderma* (Bae et al., 1995; Cambers and Scott, 1995) and *Pseudomonas* spp. (Chang et al., 1996; Lee and Kobayashi 1988; Park et al., 1993). An antifungal effect of the antagonist against major soilborne fungi such as *Rhizoctonia solani* (Pietro et al., 1993), *Pythium* spp. (Roberts and Lumsden, 1990) and *Phytophthora* spp. (Cambers and Scott, 1995) has been well documented.

In general, the antifungal activity of the soil inhabitant *G. virens* is known to be antibiosis (Roberts and Lumsden, 1990; Wilhite et al., 1994), hyperparasitism (Mukherjee et al., 1995), competition and lysis. Among these modes of action, the major antagonistic factor is derived from the secretion of antibiotic substances (Mukherjee et al., 1995;

Roberts and Lumsden, 1990; Wilhite et al., 1994).

Although many antifungal substances such as gliotoxin, gliovirin viridin, viridiol, heptelidic acid and valinotrocin, have already been reported to be produced by this fungus, gliotoxin and gliovirin were known as major antifungal substances against *Rhizoctonia solani* and *Pythium* species. In addition, these antifungal substance showed synergistic effect for disease control with intercellular enzymes produced by *G. virens* (Lumsden et al., 1992; Pietro et al., 1993; Roberts and Lumsden, 1990; Wilhite et al., 1994; Wilhite and Straney, 1996). Lumsden et al. (1992) reported that a variant with no gliotoxin production showed no control effect for seedling damping-off of zinnia caused by either *R. solani* or *P. ultimum*. Wilhite et al. (1994) found that the non-producing mutant decreased by 100% of control effect in the experiment. Therefore, it can be said that gliotoxin plays an important role in plant disease control.

Howell et al. (1983) reported that *P. ultimum* causing damping-off of cotton was suppressed by gliovirin, while *G. virens* deficient in gliovirin could not show the suppressive effect. In addition, a mutant producing increased gliovirin showed a strong suppressive action against *P. ultimum*, irrespective of somewhat decreased mycelial growth and parasitic activity of the antagonist. When gliovirin was treated together with synergistic materials such as endochitinase and polypeptide, a higher control effect was obtained (Pietro et al., 1993). Howell et al. (1993) pointed out that P group of *G. virens* producing gliovirin and heptalidic acid was inhibitory to *P. ultimum*, whereas, *R. solani* could be controlled only by Q group of the antagonist. The control efficiency may be dependent significantly upon *G. virens* strains.

*Gliocladium virens* produces several antifungal substances, and especially gliotoxin and gliovirin are important substances in terms of biocontrol of damping-off fungi. Therefore, this study was aimed to examine antifungal activities of *G. virens* G1 against *R. solani* and *Colletotrichum gloeosporioides* causing turfgrass *Rhizoctonia* blight and astragal stem rot, and plant anthracnose, respectively. Also its antifungal substances were purified and tested for the antifungal activity.

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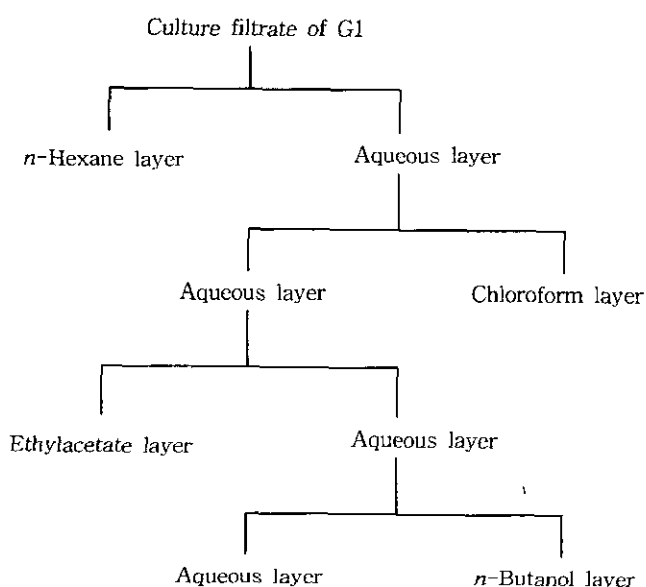
## Materials and Methods

**Antagonistic activities.** *Gliocladium virens* G1 (Chung and Chung, 1998; Kang et al., 1989) was used in this study, which has an antagonistic activity to Rhizoctonia blight of turfgrass and astragal stem rot (Youn, 1998). Antagonistic activities were investigated on four plant-pathogenic fungi including *R. solani*, *C. gloeosporioides*, *Phytophthora capsici* and *Fusarium oxysporum* f. sp. *cucumerinum*.

After the pathogenic fungi were cultured for 2 days in potato dextrose (PD) broth at 28°C, 1 ml of the macerated broth was poured into Petri dish and then mixed with warm potato dextrose agar (PDA). *G. virens* G1 was cultured on potato dextrose agar for 7 days and mycelial disks of the antagonist (5 mm in diameter) were placed on the pathogen-seeded medium, incubating for 2 days at 28°C. Mycelial growth and inhibition zone around the antagonistic fungus were examined 2 days later. Five replications were used for this experiment.

**Fermentation of *G. virens* and separation of antifungal substances by solvent fractioning.** For mass culture of the antagonistic fungi, PD broth was poured in a 30 l fermenter (Capaci Macro, Korea Fermentor, Inc.), adjusted to pH 5.5, and then heat-sterilized. *G. virens* G1 was inoculated to the culture medium and incubated for 2 days at 28°C. The culture was filtered twice with Toyo No. 1 filter paper to eliminate fungal mycelia. Prior to isolation of antifungal substances, antagonistic activity of the culture filtrate of *G. virens* G1 against four fungi including *R. solani*, *C. gloeosporioides*, *F. oxysporum*, *P. capsici* was examined using the paper disk method, in which the filtrate-soaked paper disks (8 mm in diameter, thick, Toyo Advantec Co, Japan) were placed on pathogen-seeded PDA to form an inhibition zone around the disk.

The initial separation of active substances from the culture filtrate follows the solvent fractioning procedured showin in Figure



**Fig. 1.** Intial separation procedures of antifungal compounds from the culture filtrate of *Gliocladium virens* G1.

1, using *n*-hexane, chloroform, ethyl acetate, *n*-butanol, and water as dual solvent systems. Briefly, the same amount of each solvent was equaled and mixed by shaking for 3 min in a separation funnel with 150 ml of the culture filtrate or aqueous layer, and separation was made between the solvent and aqueous layers. For antifungal activity test, paper disks (8 mm in diameter, thick, Toyo Advantec Co., Japan) were soaked with each solvent layer (70  $\mu$ l) and dried completely. The disks were then placed on PDA in which the test fungal pathogens including *R. solani* had been plated previously. Inhibition zones formed around paper disks were examined to compare the antifungal activity against the plant pathogens tested.

**Purification of the antifungal substance.** As the *n*-hexane layer showed a good antifungal activity, extraction and purification of antifungal substances were made using the *n*-hexane layer from the culture filtrate (18 l). The solvent layer was concentrated by a Macrorotar concentrator (Buchi Rotavapor-R152), and equaled to 10 ml volume of *n*-hexane. The *n*-hexane extract was subjected to column chromatography using G-60 silica gel column (particle size: 50-250  $\mu$ m, 220  $\times$  50 mm, Merck, Darmstadt, Germany), using benzene and acetone (9 : 1, v/v) as eluents. Separation speed was 10 ml per minute by gravity. Eleven fractions of 100 ml each were collected and tested for antifungal activity to the fungi by the paper disk method described as above. The fractions showing antifungal activity were combined and concentrated *in vacuo*, and the residue was dissolved in a small volume of methanol, which was subjected to LH-20 column chromatography. The column size was 1.2 cm  $\times$  25 mm and 80 g of LH-20 Lipophilic Sepadex (particle size: 25-100  $\mu$ m, Sigma Co.). The extracted residue was separated by eluting with each 3 ml of the eluents involving *n*-hexane : benzene : ethanol : H<sub>2</sub>O (5 : 1 : 3 : 1, v/v/v/v) at a rate of 0.5 ml per min, obtaining 43 fractions. Their antifungal activity was examined by the paper disk method described above using paper disks soaked with the eluates.

The most active fractions were combined and purified by HPLC (410 Series, Waters, USA) on a C-18 bondapak™ column (45 cm  $\times$  10 cm) in a acetonitrile : water (7 : 3, v/v) solvent system at a flow rate of 0.5 ml per min, obtaining one antifungal compound. This compound was subjected to HPLC again to confirm its purity by obtaining one absorption peak at the respective retention time by a Shimadzu photo diodemarray LC6A detector.

**Physical characters of the antifungal substance.** UV absorbance spectrum (200-300 nm) of the antifungal substance was also measured by the detector at the retention time of the active fraction during HPLC. Thin layer chromatography (TLC) was performed in various solvent systems. The compound spotted on the TLC plates was colored with 2% H<sub>2</sub>SO<sub>4</sub>.

## Results

**Antifungal activity of the *G. virens* G1 culture.** The antagonistic fungus, *G. virens* G1, was highly effective in inhibiting the mycelial growth of *R. solani*, *C. gloeosporioides* and *P. capsici* as a large inhibition zone was formed around the mycelial disk (Table 1). However, its antifungal

**Table 1.** Antifungal activities of the *Gliocladium virens* G1 against four plant pathogens<sup>a</sup>

Test pathogen	Inhibition zone (diameter, mm)
<i>Rhizoctonia solani</i>	35.0 ± 0.95 <sup>b</sup>
<i>Colletotrichum gloeosporioides</i>	35.0 ± 0.78
<i>Fusarium oxysporum</i>	10.0 ± 0.85
<i>Phytophthora capsici</i>	30.0 ± 0.76

<sup>a</sup>Mycelial disks of *G. virens* was placed on the pathogen-seeded potato dextrose agar, and inhibition zone formation around the mycelial disks were examined after 2 days of incubation.

<sup>b</sup>Averages and standard deviations of 5 replications.

**Table 2.** Antifungal activity of the culture filtrate of *Gliocladium virens* G1 separated by with several organic solvents<sup>a</sup>

Solvent	Inhibition zone (diameter, mm)			
	<i>R. solani</i>	<i>C. gloeosporioides</i>	<i>F. oxysporum</i>	<i>P. capsici</i>
<i>n</i> -Hexane	15	209	–	–
Chloroform	– <sup>b</sup>	20	–	–
Ethylacetate	–	–	–	–
<i>n</i> -Butanol	9	9	9	9
Water	–	–	–	–

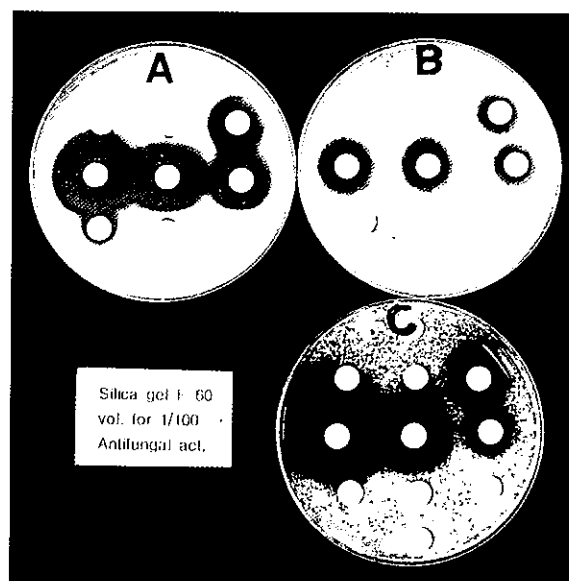
<sup>a</sup>Paper disks soaked with solvent and aqueous layers were placed on the pathogen-seeded potato dextrose agar, and inhibition zone formation around the disks were examined after 2 days of incubation.

<sup>b</sup>No antifungal activity detected.

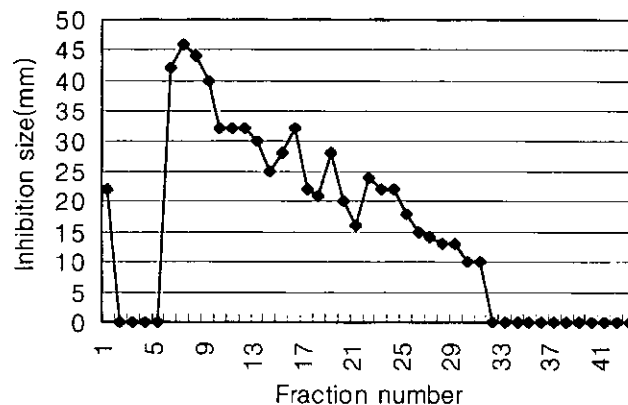
activity was less against *F. oxysporum* than against the other pathogens tested.

**Solvent fractioning of antifungal substances.** In the dual solvent systems for the separation of antifungal substances from the culture filtrate of *G. virens* G1, antifungal activity was found in *n*-hexane, chloroform, and *n*-butanol layers (Table 2). Among the solvent layers, the *n*-hexane layer contained larger amounts of antifungal substances or more active compounds than the others, because it showed the strongest antifungal activity to the pathogenic fungi except *P. capsici*. *P. capsici* was inhibited only by the *n*-butanol fraction. The chloroform fraction inhibited only *C. gloeosporioides*. The ethylacetate and aqueous fractions had no antifungal activity against the pathogens.

**Purification of the antifungal substance.** In silica gel column chromatography, 11 fractions were obtained, among which 4th through 7th ones in the eluting order showed higher antifungal activity against the fungi that was inhibited by the *n*-hexane layer of the culture filtrate of G1 (Fig. 2). The 5th fraction showed the highest antifungal activity, of which the 100-fold dilution formed an inhibition zone of 39 mm in diameter for *R. solani*, 28 mm for *C. gloeosporioides* and 20 mm for *F. oxysporum*. Based on the sizes of inhibition zone, *R. solani* was the most sensitive to the antifungal substance.



**Fig. 2.** Antifungal activities of 11 fractions from LH-20 column chromatography against *Colletotrichum gloeosporioides* (A), *Fusarium oxysporum* (B), and *Rhizoctonia solani* (C). Paper disks were soaked with each fraction, and placed on the pathogen-seeded potato dextrose agar, and inhibition zone formation was examined 2 days after treatment. Fraction numbers are in order of elution, and located from upper to lower and from left to right on the medium.



**Fig. 3.** Antifungal activities of 43 fractions of the culture filtrate of *Gliocladium virens* G1 separated by Sephadex SH-20 column chromatography. Paper disks were soaked with each fraction, and placed potato dextrose agar seeded with *Colletotrichum gloeosporioides*, and inhibition zone formation was examined 2 days after treatment. Fraction numbers are in order of elution.

The active fractions were combined and subjected to Sephadex LH-20 column chromatography, yielding 43 fractions. Among these fractions, the most active fractions were from 6th to 12th in order of elution (Fig. 3), which were combined, concentrated, and subjected to HPLC. A main compound was obtained, and its HPLC showed a single absorption peak at retention time of 10.11 min (Fig. 4), indicating approximately 99.7% purity. The purified com-

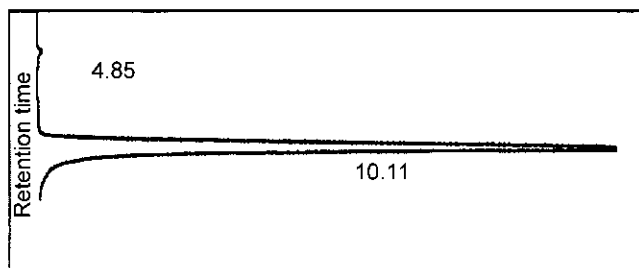


Fig. 4. HPLC chromatogram of the antifungal substance from the culture filtrate of *Gliocladium virens* G1.

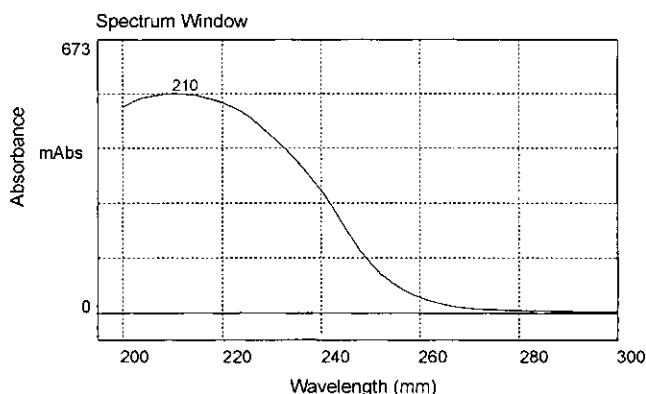


Fig. 5. UV spectrum of the antifungal substance from the culture filtrate of *Gliocladium virens* G1.

pond was again confirmed for antifungal activity in *in vitro* test using *C. gloeosporioides*, forming a large inhibition zone (Fig. 5).

**Physical characters of the antifungal substance.** The antifungal compound isolated from *G. virens* culture was a

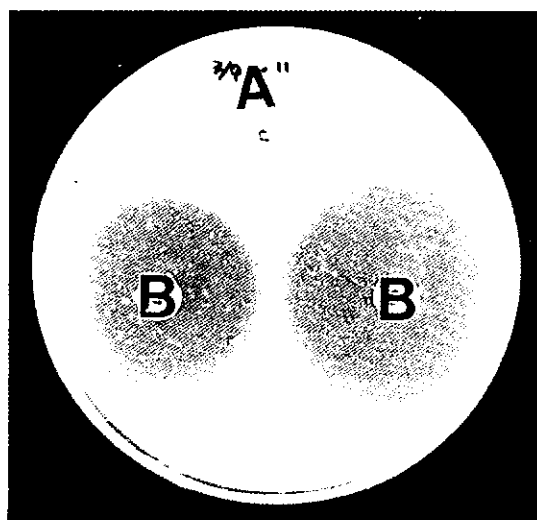


Fig. 6. Inhibition effect of the antifungal substance isolated from *Gliocladium virens* G1 against on *Colletotrichum gloeosporioides*. A: control, B: treatment.

**Table 3.** *R<sub>f</sub>* values of the antifungal compound produced by *Gliocladium virens* on TLC plates in various developing solvent systems

Developing solvent system	<i>R<sub>f</sub></i> value
Benzene + Acetone (9 : 1, v/v)	0.55
<i>n</i> -Hexane + Acetone (4 : 1, v/v)	0.75
<i>n</i> -Hexane + Benzene + Ethanol (5 : 2 : 3, v/v/v)	1.00
Benzene + Methanol (7 : 3, v/v)	0.92
Benzene + Acetone + Methanol (7 : 2 : 1, v/v/v)	0.90

colorless sticky oily substance. UV absorption maximum of the compound was 210 nm in our study (Fig. 6). In TLC of the compound, *R<sub>f</sub>* values were from 0.55 to 1.00 depending on the developing solvents (Table 3). Considering the *R<sub>f</sub>* values, the antifungal compound may be very hydrophobic.

## Discussion

Since *G. virens* is a promising antagonist for major soil-borne diseases, studies on its antifungal substances have extensively been made in order to find out the mode of antifungal action. In our study, the highest antifungal activity was found in the *n*-hexane layer separated from the culture filtrate of *G. virens* G1 in the dual solvent systems using solvents and water (Table 2). Using column chromatographies and HPLC, the compound was purified. It was an oily material with absorption maximum of 210 nm, and seemed to be hydrophobic. In the several tests for antifungal activity, the compound may have an inhibitory effect on the mycelial growth of *R. solani*, *C. gloeosporioides*, and *F. oxysporum*, although the antifungal activity was low against *F. oxysporum* and none to *P. capsici*. Considering that the *G. virens* G1 mycelial disk had as much fungal activity to *P. capsici* as to *R. solani* and *C. gloeosporioides*, antifungal substances responsible for the inhibition of *P. capsici* may be different from the purified compound in our study. As shown in Table 2, *P. capsici*-inhibiting substance may be isolated by *n*-butanol, and be more hydrophilic than the antibiotic compound.

With regard to UV spectra, the  $\lambda_{max}$  for the substance was 210 nm, whereas that for gliotoxin is 216 nm and 272 nm, for viridin, 242 nm and 300 nm, and for gliovirin, 205 and 301 nm. Thus, the antifungal substance of the fungus *G. virens* G1 in our study is different from the three known antibiotic compounds. The *R<sub>f</sub>* value of the antifungal substance was 0.55 in TLC (benzene : acetone = 9 : 1, v/v) and the retention time in HPLC was 10.11 min, also suggesting that it may not be one of the well known compounds in *G. virens*.

*Gliocladium virens* G1 was strongly suppressive to *R. solani*, *F. oxysporum* and *C. gloeosporioides*. The main responsible compound was also effective against the three

fungi. These aspects may give some prospectives in controlling diseases caused by the fungi by antibiotic materials. Living biocontrol agents have mostly been used so far in the biocontrol of the disease using *Trichoderma* and *Gliocladium*. Agronomic antibiotics have not been developed successfully from the antagonistic fungi. Therefore, chemical characteristics of the antifungal substance and its structural identification should be examined to develop a useful antifungal substance. Also for this, an application study on field performance of the compound with various formulations should be conducted in the near future.

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