Plant Pathol. J. 24(1) : 24-30 (2008)

The Plant Pathology Journal

High Throughput Screening of Antifungal Metabolites Against *Colletotrichum gloeosporioides*

Il-Pyung Ahn[1]*, Soonok Kim[2] and Yong-Hwan Lee[2]

[1]*National Institute of Agricultural Biotechnology, RDA, Suwon 441-100, Korea*

[2]*Department of Agricultural Biotechnology, Center for Fungal Genetic Resources, and Center for Agricultural Biomaterials, Seoul National University, Seoul 151-742, Korea*

(Received on July 10, 2007; Accepted on August 28, 2007)

***Colletotrichum gloeosporioides* forms an appressorium, a specialized infection structure, to infect its hosts. Among 400 and 600 culture filtrates from fungi and class Actinomycetes, six methanol extracts (A5005, A5314, A5387, A5560, A5597, and A5598) from the class Actinomycetes significantly inhibited appressorium formation in *C. gloeosporioides* infecting pepper fruits in a dose-dependent manner, while conidial germination was slightly enhanced. Two (A5005 and A5560) of them also exhibited distinctive inhibitory effect on the disease progress of pepper anthracnose. Water fractions of both culture filtrates also specifically inhibited appressorium formation in *C. gloeosporioides* and pepper anthracnose disease. Inhibition of appressorium formation by culture filtrate of A5005 was partially restored by the exogenous calcium. This results suggests that chemicals within A5005 extents its biological activity through disturbance of intracellular Ca^{2+} regulation during prepenetration morphogenesis by *C. gloeosporioides*. Together, cell-based and target-oriented screening system used in this study should be applicable for other plant pathogenic fungi prerequisite appressorium formation to infect their hosts.**

Keywords **:** *Capsicum annuum*, *Glomerella cingulata*, High throughput screening

Colletotrichum gloeosporioides (Penz.) Penz. & Sacc., is the most devastating causal agent of hot pepper (*Capsicum annuum* L.) anthracnose in Korea (Kim et al., 2007; Park and Kim, 1992). Intervention of this disease has been largely dependent on the synthetic fungicides inhibiting vegetative growth of the pathogen, however, the development of resistance to the fungicides and toxicity of the chemicals have given impetus searching for novel and bio-rational plant protectants.

For the infection to take place, a series of penetration-related morphological changes are required, initiated by spore adhesion to the host surface, spore germination, germ tube elongation, and culminated in appressorium formation (Kim, 1998). The developments must precede prior to the direct infection into the host cells. Among these prepenetration developments, appressorium formation of fungal conidia is regarded as the essential step for the successful infection of host cells (Delp, 1980). Previous studies with *C. gloeosporioides* and *C. trifolii* have shown that prepenetration morphogenesis, particularly appressorium formation, is induced by environmental cues involved with thigmo-signals of the contact surface such as hydrophobicity and rigidity (Uhm et al., 2003; Warwar and Dickman, 1996), and chemicals such as host surface wax and ethylene (Kolattukudy et al., 1995). The environmental signals, usually recognized by membrane-spanning receptors, are transferred into cells to express the appropriate cellular responses. Relaying the signal is referred as signal transduction, which is mediated by second messengers including cyclic nucleotides (Takano et al., 2001) and calcium (Ahn et al., 2004; Kim et al., 1998; Uhm et al., 2003).

Treatment of exogenous chemicals disturbs signal transduction culminated in appressorium formation and disrupts fungal genes participated in this specific cell development, resulting in the loss of pathogenicity and/or virulence of pathogens (Choi and Dean, 1997; Lee and Dean, 1993). Especially, inhibitory effects of microbial metabolites and plant extracts on spore differentiation have been investigated due to the bio-rational and environment safety. Numerous fungal secondary metabolites were reported to inhibit the specific infection stages of *M. grisea* and other plant pathogenic fungi without accompanying toxicity against mycelial growth (Thines et al., 2004). Inhibition of spore adhesion by zosteric acid resulted in the significant decrease of the development of rice blast disease (Stanley et al., 2002). Compounds effectively control ling rice blast caused by *Magnaporthe grisea* inhibited appressorium formation (Inoue et al., 1987) and its melanization (Froyd et al., 1976). To our knowledge, however, chemicals directly interfering prepenetration mechanisms of *C. gloeosporioides* infecting red hot pepper have not been reported.

*Corresponding author.
Phone) +82-31-299-1744 , FAX) +82-31-299-1722
E-mail) aip87@hanmail.net

We previously reported that culture filtrates and their fractions from the class Actinomycetes specifically inhibited appressorium formation of *M. grisea* and also controlled rice blast disease (Oh and Lee, 2000). In this study, identical screening system was used to select pepper anthracnose disease-inhibiting materials from 400 fungal and 600 Actinomycete culture filtrates. Comparative studies with *in vivo* tests were also conducted to determine reliability of this screening method. Our results presented here demonstrate that cell-based and target-oriented screening system used in this study can provide high throughput platform and be applicable for controlling other plant pathogenic fungi, those require appressorium formation to infect their hosts.

Materials and Methods

Fungal strain and culture conditions. *Colletotrichum gloeosporioides*, pathogenic on hot pepper was obtained from the National Institute of Agricultural Science and Technology, Rural Development Administration, Korea. Conidia were harvested from one week-old cultures grown on potato dextrose agar (PDA; Difco Lab., Detroit, USA) at 25°C in the dark. Fungal stock was maintained as conidial suspension amended with 20% glycerol at −70°C until use.

Preparation of methanol extracts and their fractions from culture filtrates of Actinomycetes and fungi. A total 1,000 methanol extracts from culture filtrates of 600 members of the class Actinomycetes and 400 fungal isolates from soil, were provided by S. U. Kim, the Korean Research Institute of Bioscience and Biotechnology (KRIBB), Taejon, Korea. Their methanol extracts were prepared from culture filtrates as described previously (Oh and Lee, 2000). Briefly, each isolate was grown in 5 ml of E-medium composed of 2% starch, 0.4% Bacto-soytone (Difco Lab., Detroit, USA), 0.3% Pharmamedia (Southern Cotton Oil Company, Memphis, USA), 0.2% polypeptone, 0.1% yeast extract, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.2% NaCl, and 0.3% $CaCO_3$ at 26°C for 4 days with constant shaking (150 rpm). After mixing the each culture filtrate with an equal volume of methanol for 30 min vigorously, the cell debris was removed by centrifugation. To trace the effective chemical components, methanol extracts were further separated into ethyl acetate and water fractions. The freeze-dried fractions were dissolved with 500 μl methanol. Methanol extracts and the two fractions were tested for their inhibitory effect on conidial germination and appressorium formation in *C. gloeosporioides*.

Conidial adhesion and germination, appressorium formation, and mycelial growth. To induce conidial adhesion, conidia from PDA were washed twice with sterile distilled water by centrifugation. Nine μl of conidial suspension (2×10^4 conidia/ml) placed on the hydrophobic surface of GelBond (FMC Corp., Rockland, USA) were mixed with one μl of each methanol extracts or methanol only (mock). Conidial adhesion was measured 1 h after incubation at room temperature with 100% relative humidity. After counting the total numbers of conidia, GelBond was submerged in water and shaken for 10 min, and the remaining conidia were counted again under a microscope. Conidial germination and appressorium formation assays were performed as previously described (Uhm et al., 2003). Forty-five microliter of conidial suspension (3×10^4 conidia/ml) was placed on the hydrophobic surface of GelBond and 5 μl of testing chemical solutions or fractions were added. Conidial germination and appressorium formation were measured after 16 h of incubation in the identical conditions described above. The percentages of germinated and germinating conidia, which developed to appressoria, were determined from direct microscopic examination on at least 100 conidia per replicate, composed of at least five experiments with three replicates per a treatment. To reveal the inhibition mechanism on appressorium formation by the methanol extracts, complementation tests were performed by the addition of $CaCl_2$, cAMP, 1,16-hexadecanediol (diol, a cutin monomer), and 3-isobutyl-1-methylxanthine (IBMX; an inhibitor of phosphodiesterase) in a final concentration of 1 mM, 10 mM, 2.5 mM, and 1 μM, respectively. To investigate the effects of selected methanol extracts on mycelial growth, mycelial disks (5 mm in diameter) from the margin of actively growing colonies on PDA plates were placed 3 cm apart from agar blocks and inoculated on fresh PDA plates and three paper disks (8 mm in diameter). Forty μl of 10% (v/v) methanol extracts were added on the paper disks every 24 h until mycelial in control plates reached the paper disks. The experiments were performed three times with three replicates.

***In vivo* tests.** Mature green hot pepper fruits were used for the *in vivo* inoculation assay. After washing fruit surface with a commercial detergent to remove any possible contamination of fungicide residue, surface disinfestation was performed with 10% sodium hypochloride for 5 min, and washed twice with sterile distilled water. Pepper fruits were sprayed with 10% (v/v) methanol extracts from A5005, A5314, A5387, A5560, A5597, and A5598 and with 10% (v/v) water-soluble fractions from A5005 and A5560, and dried for 24 h. Five drops from 30 μl conidial suspension of *C. gloeosporioides* (3×10^4 conidia/ml) with 250 ppm Tween 80 were inoculated on each treated pepper fruit in a humid plastic box. Symptomatic area on five pepper fruits was recorded 7 days after inoculation. The experiments were repeated more than three times.

Results

Methanol extracts of culture filtrates specifically inhibited appressorium formation in *C. gloeosporioides*. Primary screening with 10% final concentration (v/v) of 1,000 tested methanol extracts was done with two independent experiments composed of three replicates within a week. Among the methanol extracts, six extracts from A5005, A5314, A5387, A5560, A5597, and A5598, specifically diminished appressorium formation by *C. gloeosporioides* virulent on pepper in a dose-dependent manner (Figs. 1B and 2) meanwhile 10% methanol (mock) did not affect appressorium formation (Fig. 1A). All six extracts inhibited appressorium formation more than 58% (A5005) and further, extracts from A5314 and A5560 nearly completely abolished this prepenetration morphogenesis at a 1% (v/v) concentration (Fig. 2). Especially, methanol extracts of culture filtrates from A5005 and A5560 inhibited appressorium formation by about 39% and 33% at 0.5% concentration. Although conidial germination was slightly increased by the addition of methanol extract of each culture filtrate, that from A5005 inhibited conidial germination by about 29% at 10% concentration. In addition, all six extract frequently induced abnormal elongation and/or irregular branching of germ tubes (data not shown).

Effects of water-soluble fractions from methanol extract of A5005 and A5560 culture filtrates. Treatment of selected six methanol extracts on pepper fruits and leaves did not cause any obvious phytotoxic symptoms when examined for up to 7 days. In addition, pretreatment of two methanol

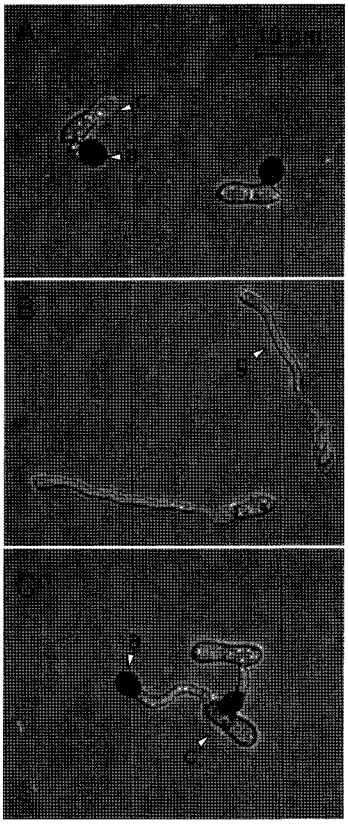

Fig. 1. Appressorium formation of *Colletotrichum gloeosporioides* infecting pepper fruits in the presence of 10% methanol (mock) (**A**), 10% (v/v) methanol extract from A5005 culture filtrate (**B**), and 10% (v/v) methanol extract from A5005 culture filtrate and 1 mM $CaCl_2$ (**C**). Conidial suspension with methanol extract and/or $CaCl_2$ was placed on the hydrophobic surface of GelBond and incubated in a humid plastic box at 25°C for 16 h. a, appressorium; c, conidium; g, germ tube.

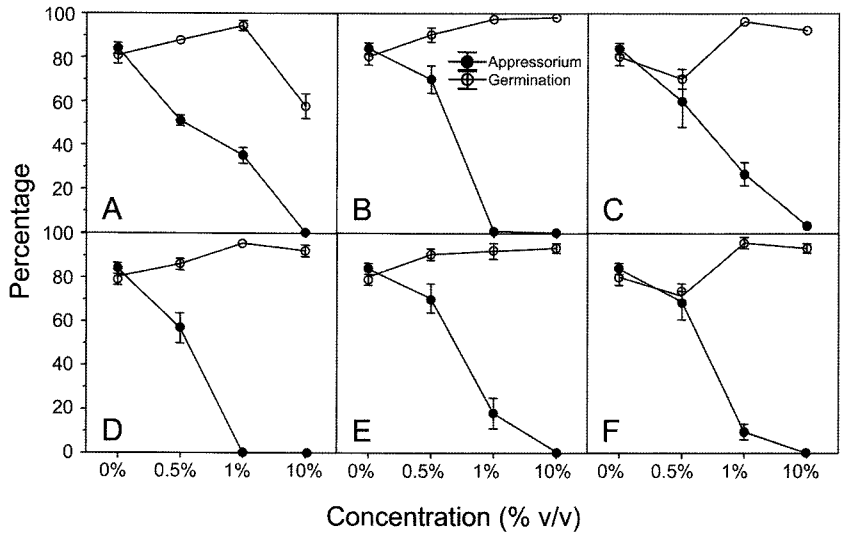

Fig. 2. Conidial germination and appressorium formation by *Colletotrichum gloeosporioides* infecting pepper fruits in the presence of methanol extracts from culture filtrates of A5005 (**A**), A5314 (**B**), A5387 (**C**), A5560 (**D**), A5597 (**E**), and A5598 (**F**). Conidial germination and appressorium formation was evaluated microscopically as described in Table 2. Error bars represent the standard deviation for the results of three replicate determinations. Representative results among 5 independent experiments were presented.

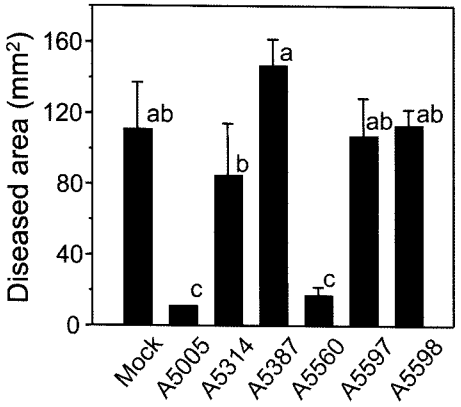

Fig. 3. Control of pepper anthracnose by methanol extracts from culture filtrates of 6 members of the class Actinomycetes. After methanol extracts (10%, v/v) sprayed, inoculation was performed by placing spore suspension (3×10^4 conidia/ml) of *Colletotrichum gloeosporioides* on the surface of pepper fruits. Symptomatic area was measured 7 days after inoculation. Error bar represents the standard deviation for the results of three replicate determinations. The same letters above bars are not significantly different at $P<0.05$, using the Duncan's multiple range tests.

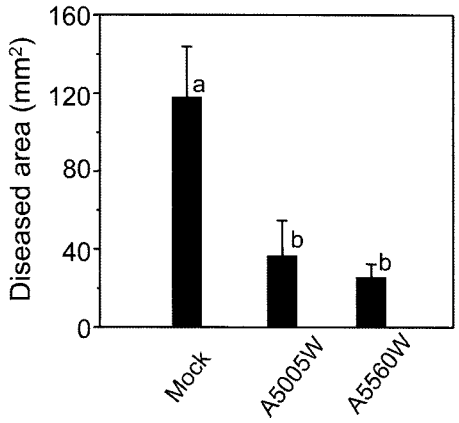

Fig. 5. Control of pepper anthracnose by water-soluble fractions of methanol extracts from A5005 and A5560 culture filtrates. After fractions (10%, v/v) sprayed, inoculation was performed by placing spore suspension (3×10^4 conidia/ml) of *Colletotrichum gloeosporioides* on the surface of pepper fruits. Error bars represent the standard deviation for the results of three replicate determinations. The same letters above bars are not significantly different at $P<0.05$, using the Duncan's multiple range tests.

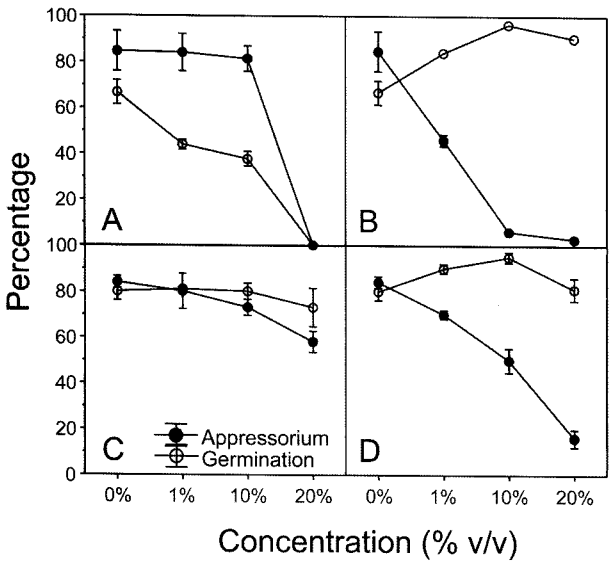

Fig. 4. Conidial germination and appressorium formation in *Colletotrichum gloeosporioides* infecting pepper fruits in the presence of substances in water-insoluble fraction of A5005 (**A**), water-soluble fraction of A5005 (**B**), water-insoluble fraction of A5560 (**C**), and water-soluble fraction of A5560 (**D**). Conidial germination and appressorium formation was evaluated microscopically as described in Materials and Methods. Error bars represent the standard deviation for the results of three replicate determinations. Representative results among 5 independent experiments were presented.

extracts from culture filtrates of A5005 and A5560 one day prior to inoculation significantly perturbed disease progress of pepper anthracnose by about 90% and 84%, respectively (Fig. 3). Although some small, brown lesions were began to appear 4-5 days after inoculation at inoculated sites, sporulation and further lesion enlargement were not observed thereafter. On the contrary, water-soaked lesions began to form at the inoculated sites on mock (10% methanol, v/v)-treated pepper 3 days after inoculation and massive sporulation of pathogen was observed 7 days after inoculation in the center of lesions. Remaining methanol extracts did not show distinctive disease inhibition and further, A5387 pretreatment slightly increased the lesion size.

To identify effective substances, both methanol extracts were further fractionated with ethyl acetate into water-soluble and water-insoluble components. Water-soluble fractions of A5005 and A5560 specifically inhibited appressorium formation by about 93% at 10% concentration and 81% at 20% concentration, respectively (Fig. 4). Same treatment slightly increased conidial germination, however, this was not significant. Water-insoluble fraction of A5005 inhibited conidial germination by about 34% at 1% concentration and appressorium formation was not affected. Conidial germination and appressorium formation in *C. gloeosporioides* was not affected significantly by the water-insoluble fraction of A5560. Treatment of water-soluble fractions from methanol extracts of A5005 and A5560 culture filtrates significantly inhibited pepper anthracnose by about 71% and 79% at 10% concentration, respectively (Fig. 5). Similar with the results from in vivo assays using methanol extracts, water-soluble fractions also inhibited lesion enlargement and sporulation of pathogens.

Partial restoration of appressorium formation in *C. gloeosporioides* reduced by methanol extracts of culture

filtrate. Inhibition of appressorium formation by methanol extracts of culture filtrates from A5005 was partly restored by $CaCl_2$ by about 52% (Fig. 1C, Table 2). However, 1,16-hexadecadiol and IBMX did not show any significant effects on the appressorium formation-inhibiting ability of this methanol extract. cAMP fortified inhibition of appressorium formation induced by methanol extracts of A5005 culture filtrate. In addition, no effector chemicals restored appressorium formation inhibited by methanol extracts of A5560 culture filtrate.

Discussion

Site-specific screening system for antifungal compounds on appressorium formation by *C. gloeosporioides* was used to identify pepper anthracnose-inhibiting substances. Primary screening based on the inhibition of appressorium formation was completed within relatively short time and this cell-based system is very reproducible. In addition, this technique requires relatively small amount of labor and space. Selected substances exhibited their activity through this system also inhibited anthracnose disease. As suggested previously (Oh and Lee, 2000), this system should be applicable for high throughput screening of substances or chemicals against *C. gloeosporioides* as well as another plant-pathogenic fungi requiring appressorium formation prior to host infection. Similar systems were also adopted in the screening of chemicals effectively inhibiting adhesion of *Candida albicans* on artificial surfaces such as polystyrene (Chandra et al., 2001; Imbert et al., 2002).

Among 1,000 methanol extracts of culture filtrates from members of the class Actinomycetes and fungal isolates from soil, six from Actinomycetes preferentially inhibited appressorium formation in *C. gloeosporioides* and conidial germination remained unaffected. To evaluate the relationship between the inhibitory effects on appressorium formation on an artificial surface and the control efficacy of pepper anthracnose, in vivo assays were conducted with above methanol extracts of culture filtrates prior to perform further experiments. Results here evidently showed that methanol extracts of A5005 and A5560 culture filtrates significantly inhibited anthracnose disease compared to mock (10% methanol with 250 ppm Tween 80)-treated control pepper fruits. However, remaining four methanol extracts did not exhibit disease control efficacy, although it had a differential inhibitory effect on appressorium formation on inducible artificial surface. It may be possible that the active components in four methanol extracts were volatile or were unstable and, thus, at the time point of inoculation, 24 h later, biological activity had been lost. Similar discrepancies were previously described in the comparison of in vitro and in vivo results (Oh and Lee, 2000). Together, selected substances exhibited significant control efficacy or pepper anthracnose in vivo proved the reliability of the cell-based screening system.

Given the agreement of *in vitro*, inhibition of appressorium formation and *in vivo*, disease progress-controlling activity, another possible inhibitory effects of methanol

Table 1. Effects of culture filtrates from selected members of the class Actinomycetes on conidial adhesion and mycelial growth of *Colletotrichum gloeosporioides* infecting red pepper

Culture filtrates	Concentrations (% v/v)	% Conidial adhesion[a] (Mean±SE)	Mycelial growth[b] (mm)
A5005	2	93.0±4.6	27.3±0.6
	10	90.0±6.6	27.7±0.6
A5560	2	91.3±5.3	26.7±0.6
	10	93.3±1.6	26.7±0.6
10% MeOH		91.3±1.6	28.0±0.0

[a]Conidial adhesion was measured on the hydrophobic surface of GelBond at 1 h after incubation at room temperature in a moistened plastic box as described in Materials and Methods.

[b]Three paper disks (8 mm in diameter) were placed 2 cm apart from inoculum, and 40 ml of methanol mixture at a fivefold dilution was added every 24 h until mycelial margins in control plates reached the paper disks. The experiment was repeated three times with three replicates.

Table 2. Effects of exogenous Ca^{2+}, cAMP, 1,16-hexadecanediol, and IBMX on the conidial germination and appressorium formation by *C. gloeosporioides* reduced by culture filtrates of the class Actinomycetes[a]

Treatments	% Conidial germination (±SD)	% Appressorium formation (±SD)
A5005 (10%)	97.7 ± 0.6a[b]	27.7 ± 3.5b
A5005 (10%)+$CaCl_2$ (1 mM)	95.7 ± 2.5a	54.3 ± 5.5c
A5005 (10%)+cAMP (10 mM)	97.7 ± 0.0a	17.7 ± 2.4ab
A5005 (10%)+diol (1 mM)	98.0 ± 0.0a	22.2 ± 10.7b
A5005 (10%)+IBMX (2.5 mM)	97.3 ± 2.1a	34.4 ± 9.7b
A5560 (10%)	97.0 ± 1.0a	1.0 ± 1.0a
A5560 (10%)+$CaCl_2$ (1 mM)	97.7 ± 0.6a	0.3 ± 0.6a
A5560 (10%)+cAMP (10 mM)	98.3 ± 0.6a	2.7 ± 1.5a
A5560 (10%)+diol (1 mM)	97.7 ± 0.6a	2.3 ± 0.6a
A5560 (10%)+IBMX (2.5 mM)	97.3 ± 0.6a	1.7 ± 1.2a
Distilled water	72.0 ± 1.7b	84.0 ± 2.0d
MeOH (10%)	75.0 ± 6.1b	83.7 ± 3.5d

[a]Thirty-two microliter of conidial suspension (3×10^4 conidia/ml) was dropped and additional 4 ml culture filtrates and 4 ml effector chemical solutions to be tested were added. Conidial germination and appressorium formation were measured on the hydrophobic surface of GelBond at 16 h after incubation at room temperature in a moistened plastic box, and incubated as described in Materials and Methods.

[b]Values in the column that are followed by the same letter are not significantly different ($P<0.05$), as determined by Duncan's multiple range test.

extracts from A5005 and A5560 culture filtrates were further confirmed in the respect of conidial adhesion and mycelial growth (Table 1). Both methanol extracts did not show any significant inhibitory effects on either event even at 10% (v/v) final concentration, therefore, disease progress curtailment should be due to the inhibition of appressorium formation, rather than other mechanisms. Although perturbation of pepper anthracnose by these methanol extracts was not induced by inhibition of conidial adhesion, this prepenetration step is another target site. For example, zosteric acid nearly completely curtailed plant diseases caused by *M. grisea* and *Colletotrichum lindemuthianum* through inhibition of conidial adhesion (Stanley et al., 2002). Interestingly, previous report about the effects of the same tested materials on the appressorium formation of rice blast fungus, *M. grisea*, indicated that methanol extracts of A5005 and A5314 culture filtrates inhibited *M. grisea* appressorium formation as well as rice blast disease progress at the same time, while that from A5560 culture filtrate did not show any significant effects on both events (Oh and Lee, 2000). On the contrary, methanol extracts of A5314 culture filtrate did not show any inhibitory effects on both events by *C. gloeosporioides* infecting red pepper. These results also suggest that screening system in this study could be used to screen anthracnose-inhibiting chemicals from previously discarded compounds that had no inhibitory effects on mycelial growth as well as on appressorium formation by other plant pathogenic fungi. Together, applying this system should enhance the selection probability of chemicals effective for diverse fungal species.

Inhibition of appressorium formation by methanol extracts from A5005 and A5560 culture filtrates was not completely recovered by Ca^{2+}, cAMP, 1,16-hexadecanediol (cutin monomer), and IBMX, known effector chemicals for appressorium formation, suggesting existence of more than one active chemicals within methanol extracts or novel chemicals inhibiting unknown target sites culminating in appressorium formation. Only Ca^{2+} partially restored appressorium formation in *C. gloeosporioides* inhibited by methanol extract of A5005 culture filtrate (Fig. 1C, Table 2). Although restoration was not complete, repeated experiments suggest that chemical(s) within methanol extracts of A5005 culture filtrate extents their inhibitory activity through inducing disturbance of intracellular Ca^{2+} regulation in *C. gloeosporioides* during prepenetration morphogenesis. Role of calcium/calmodulin-dependent signaling pathway on the prepenetration morphogenesis have been previously described in *C. gloeosporioides* virulent on avocado (Kim et al., 1998), *C. trifolii* (Warwar and Dickman, 1996), and *C. gloeosporioides* infecting red pepper (Ahn et al., 2004; Uhm et al., 2003). Similar phenomenon has been observed for calcium and polyamines. Exogenous Ca^{2+} nullifies the inhibitory effect of polyamines on appressorium formation in *C. gloeosporioides* infecting pepper fruits (Ahn et al., 2003). None of the effector chemicals nullified the appressorium formation-inhibiting activity of methanol extract from A5560 culture filtrate. Therefore, metabolites within this extract should act on novel signal transduction pathway(s) other than those mediated by cAMP and Ca^{2+}.

To identify effective components, methanol extracts of A5005 and A5560 culture filtrates were fractionated with ethyl acetate into water-soluble and water-insoluble components and effects of each fraction on the prepenetration morphogenesis of *C. gloeosporioides* were evaluated. Cell-based screening demonstrated that chemicals within water-soluble fractions of both substances were responsible for appressorium formation and inhibition of pepper anthracnose. In the previous study, water-insoluble fraction of methanol extracts from A5005 culture filtrate was responsible for inhibition of rice blast disease as well as reduction of appressorium formation by *M. grisea* and this inhibitory effect was nullified by addition of exogenous cAMP (Oh and Lee, 2000). These differences coincided with fungal species-specific inhibitory effects of tested substances described above and implied that more than one effective chemicals should exist within a substance from A5005.

Taken together, results presented in this study also confirm that cell-based system is effective for high throughput screening of potent candidates reducing pepper anthracnose disease as well as other plant diseases caused by plant pathogenic fungi forming appressoria for the prepenetration morphogenesis in the presence of artificial inductive surface. Multiple screening should improve the probability of candidate selection for diverse plant diseases. In addition, biochemical results from recovery tests of appressorium formation with known effector chemicals provide some biochemical information for the development of novel disease control strategies.

Acknowledgement

This research was supported by a grant from the National Institute of Agricultural Biotechnology.

References

Ahn, I. P., Kim, S., Choi, W.-B. and Lee, Y. H. 2003. Calcium restores prepenetration morphogenesis abolished by polyamines in *Colletotrichum gloeosporioides* infecting red pepper. *FEMS Microbiol. Lett.* 227:237-241.

Ahn, I. P., Uhm, K. H., Kim, S. and Lee, Y.-H. 2004. Signaling pathways involved in preinfection development of *Colletotrichum gloeosporioides*, *C. coccodes*, and *C. dematium* pathogenic on red pepper. *Physiol. Mol. Plant Pathol.* 63:281-289.

Chandra, J., Kuhn, D. M., Mukherjee, P. K., Hoyer, L. L., McCormick, T. and Ghannoum, M. A. 2001. Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. *J. Bacteriol.* 183:5385-5394.

Choi, W. and Dean, R. A. 1997. The adenylate cyclase gene *MAC1* of *Magnaporthe grisea* controls appressorium formation and other aspects of growth and development. *Plant Cell* 9:1973-1983.

Delp, C. J. 1980. Coping with resistance to plant disease control agents. *Plant Dis.* 64:652-657.

Froyd, J. D., Paget, C. J., Guse, L. R., Dreikorn, B. A. and Pafford, J. L. 1976. Tricyclazole: a new systemic fungicide for control of *Pyricularia oryzae* (blast disease) on rice. *Phytopathology* 66:1135-1139.

Imbert, C., Rodier, M. H., Daniault, G. and Jacquemin, J. L. 2002. Influence of sub-inhibitory concentrations of conventional antifungals on metabolism of *Candida albicans* and on its adherence to polystyrene and extracellular matrix proteins. *Med. Mycol.* 40:123-129.

Inoue, S., Kato, T. and Jordan, V. W. L. 1987. Inhibition of appressorial adhesion of *Pyricularia oryzae* to barley leaves by funcigides. *Pest. Sci.* 19:145-152.

Kim, Y. K., Li, D. and Kolattukudy, P. E. 1998. Induction of Ca^{2+}-calmodulin signaling by hard-surface contact primes *Colletotrichum gloeosporioides* conidia to germinate and form appressoria. *J. Bacteriol.* 180:5144-5150.

Kim, Y. S., Min, J. Y., Kang, B. K., Bach, N. V., Choi, W. B., Choi, W. B., Parkk, E. W. and Kim, H. T. 2007. Analyses of the less benzimidazole-sensitivity of the isolates of *Colletotrichum* spp. causing the anthracnose in pepper and strawberry. *Plant Pathol. J.* 23:187-192.

Kolattukudy, P. E., Rogers, L. M., Li, D., Hwang, C. S. and Flaishman, M. A. 1995. Surface signaling in pathogenesis. *Proc. Natl. Acad. Sci. USA* 92:4080-4087.

Lee, Y. H. and Dean, R. A. 1993. cAMP regulates infection structure formation in the plant pathogenic fungus *Magnaporthe grisea*. *Plant Cell* 5:693-700.

Oh, H.-S. and Lee, Y.-H. 2000. A target-site-specific screening system for antifungal compounds on appressorium formation in *Magnaporthe grisea*. *Phytopathology* 90:1162-1168.

Park, K.-S. and Kim, C.-H. 1992. Identification, distribution, and etiological characteristics of anthracnose fungi of red peper in Korea. *Kor. J. Plant Pathol.* 8:61-69.

Stanley, M. S., Callow, M. E., Perry, R., Alberte, R. S., Smith, R. and Callow, J. A. 2002. Inhibition of fungal spore adhesion by zosteric acid as the basis for a novel, nontoxic crop protection technology. *Phytopathology* 92:378-383.

Takano, Y., Komeda, K., Kojima, K. and Okuno, T. 2001. Proper regulation of cyclic AMP-dependent protein kinase is required for growth, conidiation, and appressorium function in the anthracnose fungus *Colletotrichum lagenarium*. *Mol. Plant-Microbe Interact.* 14:1149-1157.

Thines, E., Anke, H. and Weber, R. W. 2004. Fungal secondary metabolites as inhibitors of infection-related morphogenesis in phytopathogenic fungi. *Mycol. Res.* 108:14-25.

Uhm, K.-H., Ahn, I.-P., Kim, S. and Lee, Y.-H. 2003. Calcium/calmodulin-dependent signaling for prepenetration development in *Colletotrichum gloeosporioides*. *Phytopathology* 93:82-87.

Warwar, V. and Dickman, M. B. 1996. Effects of calcium and calmodulin on spore germination and appressorium development in *Colletotrichum trifolii*. *Appl. Environ. Microbiol.* 62:74-79.