Microscopic Examination of the Suppressive Action of Antifungal Substances from *Pseudomonas aeruginosa* on Asexual Sporulation of Fungi

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Two fractions with unusual antifungal activity that suppress asexual sporulation of several fungi were obtained from culture filtrate of *Pseudomonas aeruginosa* and were partially purified through the repeated silicagel flash column chromatographies. The sporulation-suppressive actions of these fractions in *Aspergillus nidulans*, *Rhizopus stolonifer*, and *Coprinus cinereus*, were analyzed by light and electron microscopes. The germination ability of the spores produced in the presence of these fractions were also checked to determine the persistent effects of these antifungal substances on the next generation. Light microscopic observation of developing sporangia of *R. stolonifer* grown in the presence of both fractions revealed that the significant number of sporangia failed to reach maturity, and frequently, uncontrolled growths of hyphae and rhizoids from the sporangiophores were found. In *A. nidulans*, addition of these fractions appeared to cause different classes of morphological abnormality in conidia development, which included a- borted formation of conidiogenous cells from the apex of conidiophores and enhanced hyphal growths either at the tip or middle of the conidiophores. Germination abilities of spores obtained from the cultures grown in the presence of antifungal fractions were 40~60% in *Aspergillus*, 50~80% in *Coprinus* (thallic spores), and 30~40% in *Rhizopus* compared to those of normal spores.

Key words: Antifungal substances, asexual sporulation, *Aspergillus nidulans*, *Coprinus cinereus*, *Pseudomonas aeruginosa*, *Rhizopus stolonifer*, sporulation-suppression

Pseudomonads are bacteria widely distributed in the nature, performing variety of ecological roles in the soil by producing many biologically active secondary metabolites (5). Thus these organisms are frequently known to maintain a subtle balance along with various other microorganisms in the soil environment. Among the pseudomonods, fluorescent *Pseudomonas* that are named for their release of water-soluble yellow-green pigments produce over 60 secondary metabolites, some of which have antibiotic properties against a variety of microorganisms (3, 5). It has been known that several species of fluorescent *Pseudomonas* produce antifungal substances that may inhibit growth of soil-borne fungi, and recent studies showed that these bacteria actually could act as antagonistic organisms against plant pathogenic fungi in rhizospheres of crop plants (1, 5, 8, 11, 12, 13, 15). Several species of *Pseudomonas* have been extensively studied for potential biological controls of the plant pathogenic

fungi in soil (3, 9, 13, 16, 17, 18), notably for the control of wheat take-all disease (2, 3, 8, 23). Currently, several groups of antifungal compounds from the fluorescent *Pseudomonas*, such as phenazines (2, 8, 11, 22, 23, 26), pyroles (4, 11, 14, 19), and phloroglucinols (4, 5, 11, 18, 19, 20, 25) have been chemically characterized and the nature of their antifungal activities toward some soil-borne pathogens are well elucidated.

In the preliminary study (15), we reported that the culture filtrate of *Pseudomonas aeruginosa* obtained from the farm soil had a marked antifungal activty, and some of its fractions exhibited an unusual sporulation-suppressing activity against several taxonomically different groups of fungi. Most of the antifungal substances so far known, whether derived from microorganisms or chemically synthesized, unvariably suppress mycelial growth. Thus, the study of antifungal substances in relation with sporulation seems to be a different approach in the area of control of fungi and the elucidation of such compound(s) is undoubtedly worthwhile for basic research per se as well as for the development of antifungal compounds with new concept. Recently, we obtained

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further purified fractions retaining antifungal activity in the asexual sporulation and determination of their chemical structures is underway. In this paper, we reported the light and electron microscopic results of the investigations on the direct effects of these antifungal fractions in sporulation, and also tried to determine the nature of persistent effects of these antibiotics through the examination of germination in spores previously affected by the antibiotics, if there is any.

Materials and Methods

Bacterial strain for the production of antifungal substances

Pseudomonas aeruginosa KMCS1 was isolated from nearby locality (15). Bacteria were cultured in YEPD broth (Difco yeast extract, 10 g; Difco bactopeptone, 20 g; dextrose, 20 g; distilled water, 1 L) in shaking incubator at 120 rpm for 24 hrs at 37°C. For the production of antifungal substances, YEPD broth-cultured *Pseudomonas* were transferred to maltose minimal medium (KH_2PO_4, 1 g; $(NH_4)_2SO_4$, 3 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; $CaCl_2$, 0.1 g; maltose, 10 g; 5 mM L-proline; distilled water, 1 L, pH 6.0) then cultured at 37°C in a shaking incubator at 120 rpm for 72 hrs.

Fungal strains used for biological assay

Rhizopus stolonifer was isolated from the laboratory, and *Aspergillus nidulans* was donated from Dr. Choi in our department. *Coprinus cinereus* ATCC 18064 (monokaryon) was obtained from ATCC. These fungi were cultured on PDA (Difco) at full strength or at 10% dilution.

Fractionation of antifungal substances

Three day culture of *P. aeruginosa* was centrifuged at 10,000 g, and then filtered through 0.45 μm membrane filter. The filtrate was applied to Amberlite XAD-2 column chromatography (15) and washed with methanol (1). The organic solvent in the eluate was removed *in vacuo* and extracted with ethyl acetate 3 times. The combined ethyl acetate extracts were dried over sodium sulfate, filtered and then, evaporated to dryness. The crude extract was purified by silicagel flash column chromatography (silicagel 60, 230~400 mesh, 300×25 mm) with hexane-ethyl acetate (1:2, 1:3), by silicagel flash column chromatography (165×25 mm) with hexane-chloroform (1:10, 1:20), and then by silicagel flash column chromatography (135×25 mm) with toluene-ethyl acetate (1:1). The purified fractions 4 and 5 were concentrated *in vacuo* and dried for biological tests against fungal strains (15).

Microscopic observation of effects of antifungal fractions on the formation of asexual spores

To determine the nature of inhibition in formation of asexual spores in *R. stolonifer* and *A. nidulans*, morphological abnormalities of sporulating structure caused by the antifungal fractions were investigated with light and electron microscopes. In this microscopic examination, asexual structure of *Coprinus cinereus* was not included because this fungus produces the thallic spores which is an atypical asexual unit unlike the conidia or condial typed spores of *Aspergillus* and *Rhizopus*. Germination of normal spores in the presence of antifungal fractions was also examined microscopically. In addition, to test if there are any persistent effects of antifungal substances on the next generation, the germination ability of spores produced in the presence of antibifungal fractions was examined. The most prevailing and prominent morphological features were recorded as evidences for this paper.

Morphological abnormalities of sporulating structure caused by antifungal fraction 5

To examine the morphological abormality that may be associated with the reduced rate of spore production, spores of *A. nidulans* and *R. stolonifer* were inoculated to the thin slab of PDA (0.5 mm × 0.5 mm) containing antifungal fraction 5 of 12.5 μg/ml concentration, covered with cover slip, and allowed to grow at 25°C in a moisture chamber. After 48 hrs (*R. stolonifer*) and 72 hrs (*A. nidulans*), agar slabs with fully grown mycelia were fixed in 4% glutaraldehyde (in 0.1M cacodylate buffer, pH 7.2) for 2 hrs at room temperature, examined under the Polyvar Type A light microscope equipped with Nomarski differential interferrence contrast and conventional phase contrast devices, and photographed with Kodak Tmax 400 B/W films. For the examination of developing sporangia with electron microscope, *R. rhizopus* was cultured on the strips of cellophane (5 mm × 50 mm) coated with 0.5% locust bean gum laid on the YMG agar medium containing 12.5 μg/ml of antifungal fraction 5 and allowed to grow for 2 days at 25°C. Pieces of cellophane (approx. 5 mm × 5 mm) with developing sporangia were cut, fixed in 4% glutaraldehyde as above, and post-fixed in 2% osmium tetroxide for 2 hrs, dehydrated with acetone series. Dehydrated specimens were en-bloc stained in saturated uranyl acetate in acetone for 1 hr, embedded with epoxy resin, and polymerized at 60°C overnight. All the above procedures except polymerization of plastic blocks were conducted at room temperature. Hardened fungal specimens were thin-sectioned with diamond knife, stained with conventional lead citrate and uranyl acetate,

and observed with Zeiss-109 trans- mission electron microscope.

Effects of antifungal fractions on germination of normal spores of Rhizopus stolonifer

To test the direct effect of antifungal fraction 5 on the germination of normal spores, spores of *Rhizopus* were allowed to germinate on the diluted (1/10) YMG agar medium (Difco yeast extract, 4 g; malt extract, 10 g; glucose, 4 g; agar, 20 g; water, 1 L) containing antifungal fraction 5 at 12.5 µg/ml. Approximately 20~30 spores were added on the pieces of cellophane (0.5 mm × 0.5 mm) coated with locust bean gum laid on YMG agar medium. Cellophane pieces containing germinating spores were fixed in 4% glutaraldehyde and examined under the light microscope.

Persistent effects of antifungal fractions 4 and 5 on the germination ability of spores

In this experiment, the persistent effect of antifungal fractions in spore of *R. stolonifer*, *A. nidulans*, and *C. cinereus* were investigated. In order to determine these secondary effects of antifungal fractions, inflicted spores were collected from the cultures grown on PDA in the presence of fractions 4 and 5 at following concentrations: 50 µg, 25 µg, and 12.5 µg/ml medium for fraction 4; 25 µg, 12.5 µg, and 6.25 µg/ml for fraction 5. Approximately 1×10^4 spores/plate were sown on PDA medium, allowed to germinate for 14hrs (*Rhizopus*), 24hrs (*Aspergillus*), and 36hrs (*Coprinus*) at 25°C, and the number of colonies grown from a germinated spore were counted. Rate of inhibition in spore germination was calculated as follow.

Inhibition rate (%) =
$$\frac{\text{Percent of germination (control} - \text{treated spores)}}{\text{Percent of germination of control}}$$

Results

Morphological abnormalities in sporulation and germination elicited by antifungal fraction 5

In order to analyze the sporulation-suppression mechanism, *Aspergillus* and *Rhizopus* were grown with fraction 5, and their sporulating structures were examined with light and electron microscopes.

Rhizopus

Examination of *Rhizopus* revealed that morphological abnormalities appear to be mostly associated with sporangial development rather than mycelial growth. Abnormal hyphal growths or branchings associated with developing sporangia appeared to be
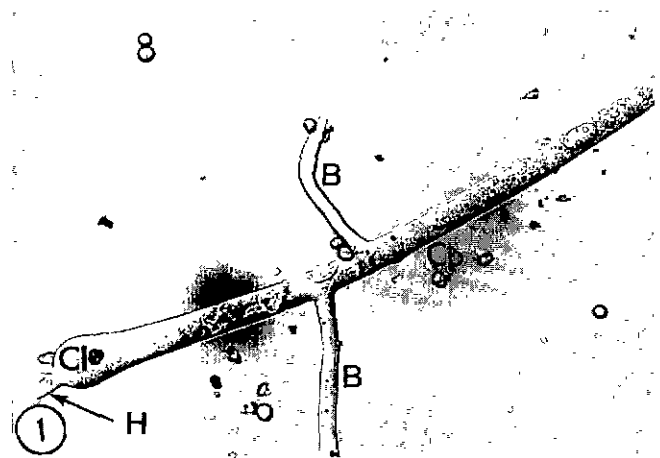

Fig. 1. Conidiophores from the 48 hr grown culture of *Rhizopus stolonifer* treated with 12.5 µg/ml of antifungal fraction 5. Tip of conidiophore failed to form a normal sporangium, instead there were abnormal hyphal growths from it. Also, it is noteworthy that several branchings formed in the mid-region of the conidiophore. (×100). B, branch; Cl, columella; Cp, conidiophore; H, growing hyphae; Rh, rhizoids; Sp, sporangium.

the most prominent abnormalities in this fungus (Figs. 1 and 2). Abnormal branchings from the mid-region of the sporangiophore were commonly observed (Fig. 1), and hyphal growth from the apex of the developing sporangia (Fig. 1) or incompletely developed sporangia (not shown) were frequently seen in the preparations. Rhizoid formation from the immature sporangium was another abnormal feature associated with sporulation in this fungus (Fig. 2).

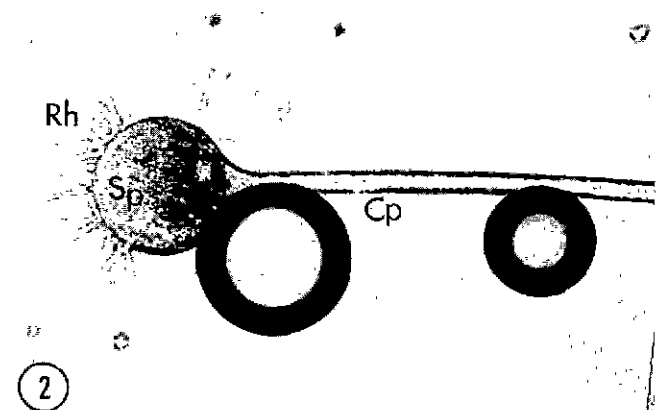In gross, the significantly reduced number of normally matured sporangia in treated culture seemed

Fig. 2. Conidiophores from the 48 hr grown culture of *Rhizopus stolonifer* treated with 12.5 µg/ml of antifungal fraction 5. In this micrograph, the sporangium seemed to reach its full size; however, there was a massive growth of rhizoids from the apical surface of the sporangium. (×85). Abbreviations for picture are same as those of Fig. 1.
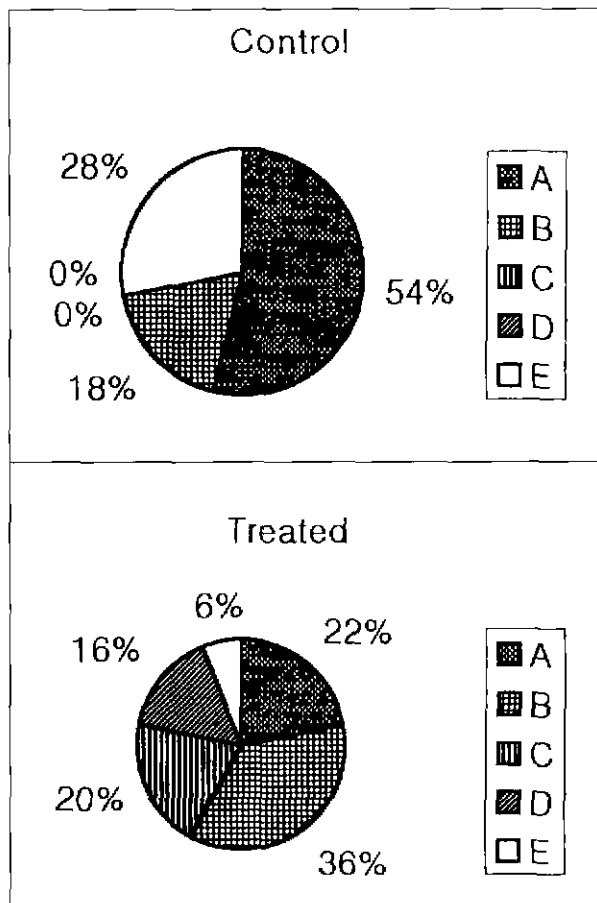
Control

28%

0%
0%

18%

54%

- ▦ A
- ▦ B
- ▥ C
- ▨ D
- ▢ E

Treated

6%

16%   22%

20%

36%

- ▦ A
- ▦ B
- ▥ C
- ▨ D
- ▢ E

Fig. 3. Gross comparison of patterns of sporogenesis in cultures of *Rhizopus stolonifer* after treatment with antifungal fraction 5 and control cultures without treatment. It is of interest that the number of both normally matured and spent sporangia in treated cultures were significantly lower than those of the control; however, the number of immature sporangia with full size was much higher in treated culture than that of control. A, number of the mature sporangia with full size; B, immmature sporangia with full size; C, cases of sporangia and sporangiophore with hyphal growths; D, sporangia with rhizoids; E, number of normally dehiscenced sporangia.

to directly reflect the deterred sporogenesis in this fungus by antifungal fraction 5. Fig. 3 shows the results of comparison between overall number of normal vs abnormal sporangia formed after treatment with fraction 5 at 12.5 μg/ml for 72 hrs at 25°C. With treatment, number of fully matured sporangia (undehiscenced) was only 22% compared with 54% of the control, while the percentage of total number of immature sporangia in treated cultures was twice (36%) as much as that of the control (18%). It is worthy to mention that the number of abnormally formed sporangia comprised almost 36% (20%+16%, bottom figure of Fig. 3) of all counted sporangia in the treated cultures which was virtually not seen in
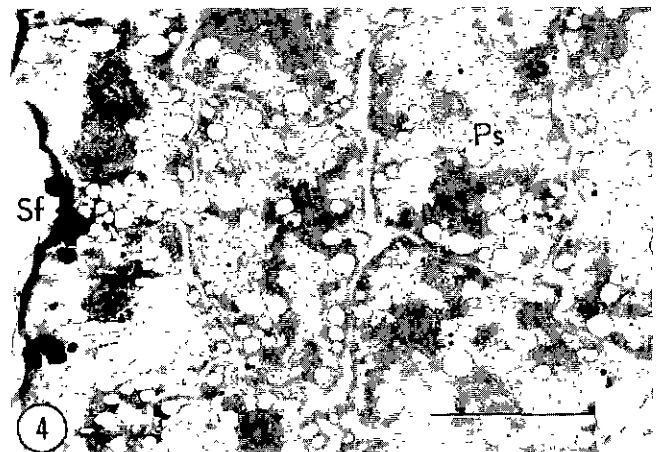

Fig. 4. Electron micrograph of near median sections of immature sporangia at the identical stage obtained from culture of *Rhizopus stolonifer* without addition of antifungal fraction 5. Bar=4 μm. In this control, immature spores were well packed within the sporangium. The margin on the left indicates the surface of the sporangium. Ps, prespore; Sf, surface of sporangium; Vc, vacuole.

the untreated control. Also the number of normally dehiscenced sporangia represented only 6% in treated cultures; however, it was as high as 28% for the untreated control. The observation of full sized, but immature sporangia of both treated and untreated control cultures with transmission electron microscope revealed that with treatment there was a significantly reduced number of prespores within the
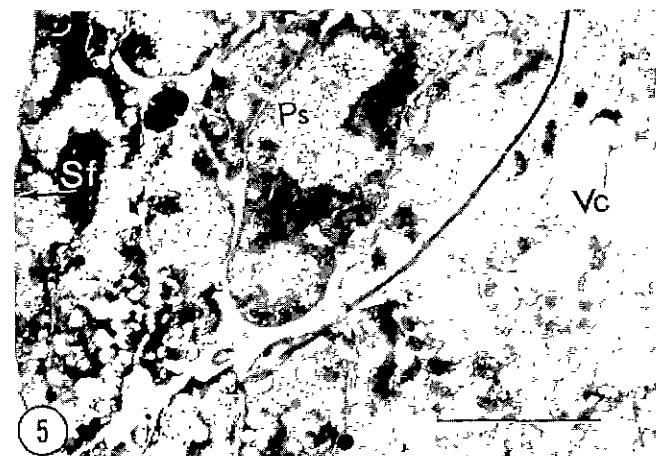

Fig. 5. Electron micrograph of near median sections of immature sporangia at the identical stage obtained from culture of *Rhizopus stolonifer* with addition of antifungal fraction 5. Bar=4 μm. In treated culture. number of immature spores were mainly located only near the surface of the sporangium (to the left side). Also, it is remarkable that heavily vacuolized area occupied a considerable part of the central sporangium (toward right). The margin on the left indicates the surface of the sporangium. Ps, prespore; Sf, surface of sporangium; Vc, vacuole.
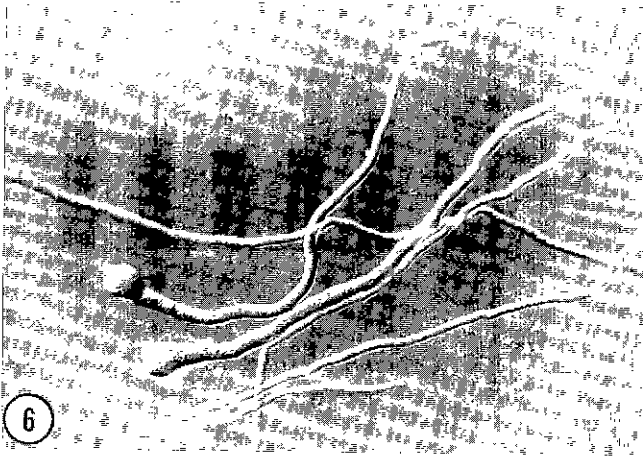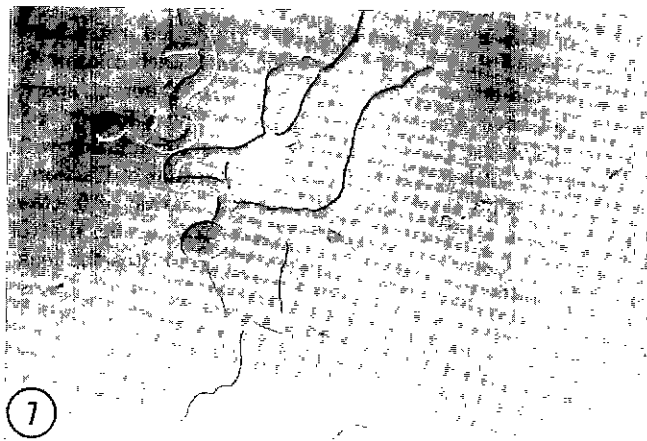
Fig. 6. Germinating normal spores of *Rhizopus stolonifer* treated without antifungal fraction 5. (×110).

developing sporangium compared to that of the control (Figs. 4 and 5). When normal spores of *Rhizopus* were germinated in the presence of antifungal fraction 5, there was an immediate and extensive branchings from the sprouting hypha from the germinating spore (Figs. 6 and 7).
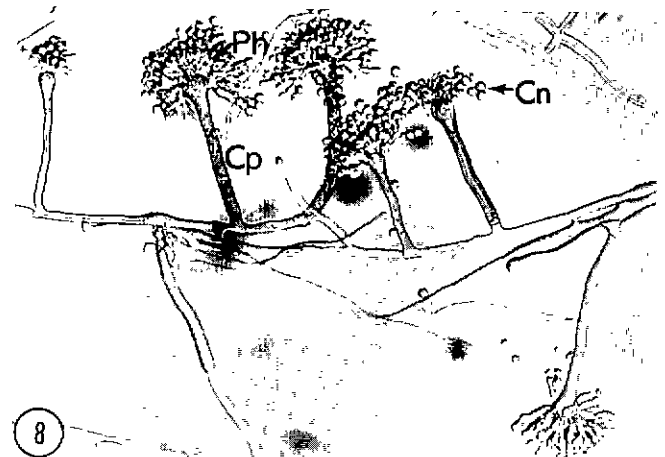
Aspergillus

Based upon microscopic examinations, it appeared that the inhibition in conidiogenesis of *Aspergillus* by the antifungal fraction 5 seemed to be directly associated with the incomplete development of conidiating structure, resulting in significantly reduced number of conidia production compared to that of the control (Figs. 8, 9, 10, and 11). The most prominent morphological changes by the antifungal fraction appeared to be the inability to from conidiogenous cells (phialides) at the vesicle of conidiophore (Figs. 9 and 10); howev-

Fig. 8. Conidiating structures from 72 hr grown culture of *Aspergillus nidulans* without antifungal fraction 5. In this preparation, normally developed conidial heads were numerous. (×150). Cp, conidiophore, Ph, phialide; Cn, conidia.

er, the formation of conidiophores seemed not to be affected (Figs. 9 and 10). In rare cases, a limited number of conidiogenous cells formed on the vesicles (Fig. 11). Scanning all types of deformations associated with the sporulating structures of treated *Aspergillus*, it was of particular interest to observe that there is an obvious tendency of enhanced and somewhat uncontrolled hyphal growths from vesicles or conidiophores in treated specimens (Figs. 9, 10, and 11).

Persistent effects of antifungal fractions on spore germination

To find out whether the spores produced in the re-

Fig. 7. Germinating normal spores of *Rhizopus stolonifer* treated without and with antifungal fraction 5. (×110). 7. In treated samples, sprouting hyphae of germlings were heavily branched.

Fig. 9. Conidiating structures from 72 hr grown culture of *Aspergillus nidulans* with antifungal fraction 5. In treated culture, morphologically abnormal conidial vesicle was remarkable. In this micrograph, the formation of phialides seemed aborted, instead, several hyphae were simultaneously sprouting from the vesicle (arrow). (×375). Cp, conidiophore; Ph, phialide; Cn, conidia.
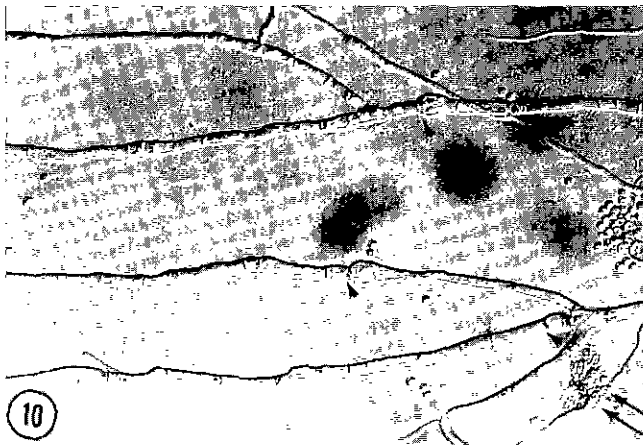
Fig. 10. Other abnormalities associated with the treament of fraction 5 in *Aspergillus nidulans*. Many conidiophores were obviously unsuccessful in the formation of phialides, instead, unusual hyphal growths occurred at the vesicle (arrow head). Normal conidial heads occasionally formed but they were somewhat reduced in size (double arrows). (×150).

gime of antifungal substances are still under the influence of these substances, the rate of germination of antibiotic-borne spores was analyzed. Since, in the earlier study (15), fraction 5 showed the higher degree of suppression in sporulation than fraction 4, fraction 5 at concentration lower than that of fraction 4 was applied in this study. Observation of germinating spores with treatment of fractions 5 and 4, revealed apparent inhibition in germination (Fig. 12). Rate of inhibition in spore germination, compared with that of unaffected spores, was in between 40 to 55% in *Aspergillus* and 45 to 75% in oidia of *Coprinus*; however,

Fig. 11. Other abnormalities associated with the treament of fraction 5 in *Aspergillus nidulans*. In this micrograph, it should be noted that a hypha in the center produced many vesicles in tandem without entering the successful phialide formations. Compare the poorly developed conidial head (arrow) with those of the normal culture in Fig. 8. (×300).
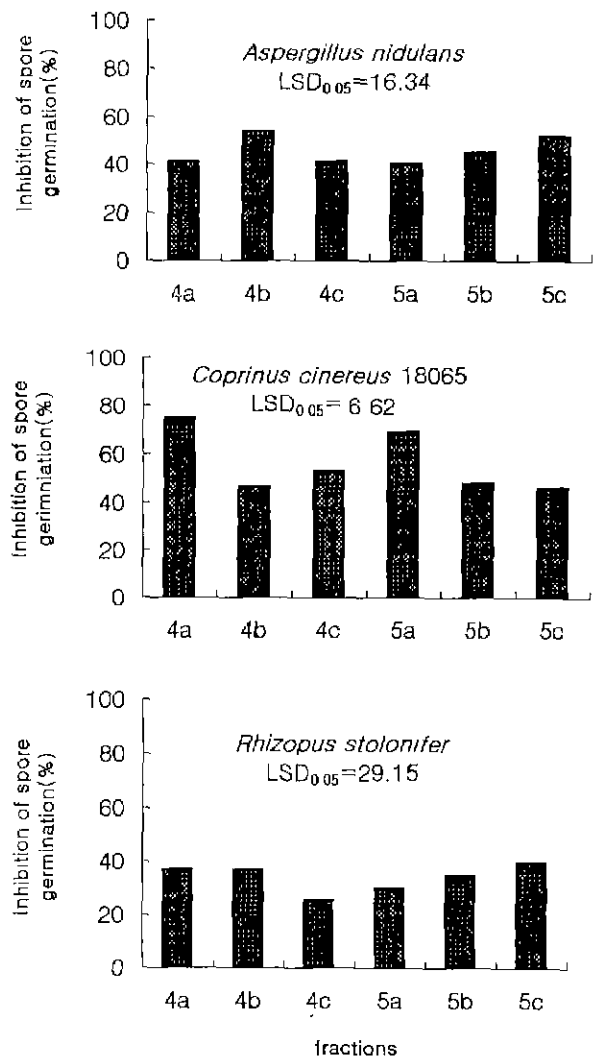

Fig. 12. Persistent effects of antifungal fractions 4 and 5 examined with germinating spores of *Aspergillus nidulans*, *Coprinus cinereus*, and *Rhizopus stolonifer*. Spores with antifungal treatment at the time of sporogenesis were harvested and were allowed to germinate on the antifungal fraction-free PDA medium, and their germination rates were compared with that of control (see the text). Each column denotes the inhibition rate in germination of different spores which had been formed in media containing different concentrations of fractions 4 and 5; 4a, spores germinating with a supplement of fraction 4 at 50 μg/ml; 4b, that of fraction 4 at 25 μg; 4c, that of fraction 4 at 12.5 μg; 5a, that of fraction 5 at 25 μg/ml; 5b that of fraction 5 at 12.5 μg; 5c, that of fraction 5 at 6.25 μg, respectively. LSD_{005} indicates the least significant difference at P=0.05.

in *Rhizopus*, the inhibition appreared somewhat lower than those of *Aspergillus* and *Coprinus* and was in between 30 to 40% (Fig. 12). However, the results showed that there were no noticeable differences in the degree of inhibition in germination by the different concentrations of antifungal fractions 4 and 5

in *Aspergillus and Rhizopus.*

Discussion

Inhibitory actions of antifungal fractions obtained from culture filtrate of *Pseudomonas aeruginosa* was preliminarily investigated on sporogenesis of *Aspergillus nidulans*, *Pyricularia oryzae*, and *Coprinus cinereus* (15). Through this report, we were able to confirm that certain fractions effectively inhibit the sporogenesis, while not significantly inhibiting mycelial growth. In this paper, we tried to look into insight how these antifungal fractions affect sporogenesis in *A. nidulans* and *Rhizopus stolonifer* at microscopic level.

Sporulation-suppressing antifungal fractions evidently restrict the normal development of sporulating structure in the tested fungi in several different ways, depending on the checked fungi. For example, they appeared to reduce the over-all number of normal conidiating structures in *Aspergillus*, and also seemed to markedly increase the frequency of the new hyphal growths from conidiophores or sporangiophores of both *Aspergillus* and *Rhizopus*, a feature normally not seen in untreated controls. It was especially interesting that in the treated *Aspergillus*, the formation of conidiogenous cells from the conidiophores was effectively blocked, thus there was a complete halt of spore formation. In *Rhizopus*, the over-all reduction in the number of mature sporangia seemed to be the possible direct effect of the treatment of the antifungal fractions to this fungus. It should be noted that, in both *Aspergillus* and *Rhizopus*, such deleterious effects of antifungal fractions in sporogenesis seem to be associated with enhanced secondary hyphal growths in these fungi. Also it is noteworthy that the spores formed in the presence of antifungal fractions produced extensive branchings when they germinated, and this phenomenon seemed to indicate that antifungal fractions may have unusual activities which specifically induce the hyphal growth in some fungi. Conventionally, antifungal activity of typical antibiotics designates the properties which inhibit 'fungal growth', like the mycelial growth (4, 7), unlike the antibiotic property of sporulation-suppressing fraction that we reported in this paper. A number of fluorescent *Pseudomonas* spp. are known to produce several antifungal substances in their culture filtrate. Among these, phenazines (2, 8, 11, 21, 23, 26), phloroglucinol families (4, 11, 25), and siderophores (8) are the best studied antifungal substances that inhibit the growth of plant pathogenic ascomycetous fungi. It is interesting that all of these substances have been dealt with fungal

growth but not with the sporulation. However, phosmidosine, a nucleotide antibiotic from *Streptomyces* is known to inhibit spore formation in *Botrytis cinerea*, an ascomycete (24). This indicates that the sporulation-suppressing antifungal substances reported in this paper may be a formerly unknown class of antifungal antibiotic that may act more or less specifically on asexual sporogenesis of fungi. Recently, we further purified some active fractions retaining anti-sporulation activity, and determination of their chemical structures is underway. Our preliminary analytical study indicated that the active fractions contain phthalates (methyl, ethyl, and butyl esters), several esters of high-carbon fatty acid, and bezenethanols (data not shown).

Another aspect of antifungal activity of fractions 4 and 5 which should be accounted is persistent effects of these substances in germination of affected spores in addition to direct cause for the reduced sporulation. It is believed that with such secondary effects in germination of affected spores, over-all efficiency of these antifungal fractions toward the control of fungal growth must be evaluated. Currently, fractions 4 and 5 are under further purification, and obtained fractions are being tested against several plant pathogenic fungi.

Acknowledgments

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