Relationship between Development of Benomyl-Resistant Strains and Hyphal Fusion in *Botrytis cinerea*

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Abstract

A mycelial disk of benomyl-resistant strain (IHES-3) of *Botrytis cinerea* was contact-cultured in the center of colony of benomyl-sensitive strain (IPCR-1). Mycelial disks, which were collected from IPCR-1 colony contacted with IHES-3, were placed on PSA media containing benomyl. IPCR-1 mycelial disk (PH-1) which was 1 cm distant from IHES-3 disk showed resistance to benomyl. Benomyl showed MIC of 1,000 ppm to PH-1 which was a medial value between MIC to IPCR-1 (<3 ppm) and that to IHES-3 ($>3,000$ ppm). Colonies and hyphae of PH-1 were morphologically similar to those of IPCR-1, but the growth was more or less poor in contrast to IPCR-1. In hyphae of PH-1, nuclei which were similar to those of IHES-3 were found in addition to nuclei that resemble those of IPCR-1. Pathogenicity of PH-1 to cucumber showed nearly middle grades of the two strains. PH-1 was stable in the above properties, even though the subculture was repeated more than ten times on PSA media. Hyphal fusion was observed between hyphae of IPCR-1 and IHES-3 on PSA medium. Also, in conidial coculture of the two strains on glass slides hyphal fusions of 6 types were observed, and the frequency was about 2.5% between hyphae of IPCR-1 and IHES-3. The light microscopy showed transfer of nuclei between the two strains by hyphal fusion, but did not whether karyogamy was followed or not.

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Key words: benomyl-resistance, *Botrytis cinerea*, hyphal fusion.

Introduction

Resistant strains of *Botrytis cinerea* to benomyl were first reported to develop in 1971⁶⁾, and was subsequently isolated from fields and greenhouses extensively^{9,17,22,29,31)}. Recently, failure of control of naturally occurring disease has been reported, and especially, effective control of disease of greenhouse vegetables caused by *B. cinerea* has been confounded by the development of resistant strains.

In *B. cinerea*, numerous resistant strains that differ in degrees of resistance to benomyl were isolated from fields. These strains also showed difference in growth pattern *in vitro* and *in vivo*^{1,15)}, and pathogenicity to host plants²⁾. Density of the resistant strains will be decreased by suspension of spraying of benzimidazole-fungicides²¹⁾. In the fields, however, the density of resistant strains was reported not to be decreased by the suspension of spraying⁹⁾. Furthermore, the resistant strains were isolated in the fields where the fungicides were not sprayed²⁴⁾.

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In plant pathogens except for reports on *Venturia nashicola*^{12,13)}, resistance mechanisms to benzimidazole fungicides were hardly studied, although in non-plant pathogens, *i. e.* *Aspergillus nidulans*, the resistance mechanisms have been studied genetically^{27,28)} and biochemically^{7,8)}.

The present study was made to elucidate the mechanism of the development of benomyl-resistant strains of *B. cinerea*, regardless of the spraying of benomyl.

Materials and Methods

Test strains. A benomyl-sensitive strain (IPCR-1) of *Botrytis cinerea* Persoon was furnished by The Institute of Physical and Chemical Research. A benomyl-resistant strain (IHES-3) was supplied from Ibaraki Horticultural Experimental Station. Minimum inhibitory concentration (MIC) of benomyl to IPCR-1 and IHES-3 strains *in vitro* are values less than 3 ppm, and more than 3,000 ppm, respectively¹⁵⁾. The strains were preserved on potato-sucrose agar (PSA) at -40°C . Conidia of the two strains were collected by the method previously described¹⁴⁾.

Contact-culture of two strains on PSA media. Contact-culture of IPCR-1 and

Fig. 1. Procedure for contact-culture of IPCR-1 and IHES-3 strains of *Botrytis cinerea*. Numbers of PP and PH showed distances(cm) from mycelial disks of IPCR-1 or IHES-3. c, colony; md, mycelial disk(diameter of 6mm).

IHES-3 strains was carried out by the procedure shown in Fig. 1. Mycelial disks were collected from colonies of IPCR-1 contacted with mycelial disks of IHES-3, and the resistance to benomyl of the mycelial disks (PH-1, -2, -3 and -4) was examined on PSA media containing 5 or 10 ppm benomyl, respectively.

MIC of benomyl and three dicarboximide fungicides to PH-1. PH-1 was cultured and preserved by the same method of IPCR-1 and IHES-3. Mycelial disks of PH-1 were placed on PSA media containing benomyl of concentrations from 10 ppm to 2,000 ppm, and incubated at 20 C in the dark. Similarly, mycelial disks of PH-1 were placed on PSA media containing iprodione, vinclozolin or procymidone of concentrations from 5 ppm to 50 ppm, respectively.

Pathogenicity of PH-1 to cucumber. Pathogenicity of PH-1 was examined by cucumber leaf disk method of Ko *et al.*¹⁴⁾.

Growth and nuclear behavior in hyphal tip cells of PH-1 on glass slides.

A 15 μ l droplet of conidial suspension (1×10^4 conidia/ml) containing 2.5% glucose was placed on each glass slide. The glass slides were placed on moist filter paper in sterile plastic boxes, and incubated at 20 C in the dark. The glass slides were taken from the incubator 24 hr after the onset of incubation. Hyphae on the glass slides were fixed in Carnoy's solution for 30 min. Nuclei in the hyphal cells were stained by HCl-Giemsa method described previously⁴⁾.

Fusion between IPCR-1 and IHES-3.

Hyphal fusion between the two strains was carried out by the procedure shown in Fig. 2. The glass slides were taken from the incubator 48 hr after the onset of incubation. Hyphae on the glass slides were stained by HCl-Giemsa method. From morphological difference reported previously¹⁾, nuclei of IPCR-1 and IHES-3 were easily distinguished. Frequency of fusion between IPCR-1 and IHES-3 was examined under light microscope.

Fig. 2. Procedure of light microscopy for hyphal fusion between IPCR-1 and IHES-3 strains of *Botrytis cinerea*.

Results

1. Development of a new strain by contact-culture of IPCR-1 and IHES-3 strains

After 72 hr of contact-culture of the two strains, mycelial disks of IPCR-1, which were 1, 2, 3 and 4 cm apart from mycelial disks of IHES-3, were taken from the colony. These mycelial disks were placed on PSA media containing 5 or 10 ppm benomyl, respectively. A mycelial disk of IPCR-1 (PH-1), that is 1 cm distant from IHES-3 disk, showed remarkable growth on PSA media containing benomyl (Table 1 and 2, and Plate I 1).

A single cell of hypha was isolated from PH-1 colony on PSA medium containing benomyl, and subcultured on PSA media. Growth speed of mycelia and form of colony of PH-1 *in vitro*, and degree of resistance to benomyl hardly changed, even though PH-1 was repeatedly subcultured on PSA media more than ten times.

Table 1. Sensitivity to benomyl of four mycelial disks obtained from the colony of IPCR-1 strain which was contacted with IHES-3 strain of *Botrytis cinerea*^{a)}.

Concentration of benomyl(ppm)	Mycelial disks of IPCR-1 ^{b)}				IPCR-1	IHES-3
	PH-1	PH-2	PH-3	PH-4		
0	+	+	+	+	+	+
5	+	—	—	—	—	+
10	+	—	—	—	—	+

a) The fungal growth was examined 7 days after the onset of incubation.

b) PH-1, -2, -3 and -4 are the mycelial disks 1, 2, 3 and 4 cm apart from the point of contact, respectively.

Table 2. Sensitivity to benomyl of four mycelial disks obtained from the colony of IPCR-1 strain which was contacted with IPCR-1 strain of *Botrytis cinerea*^{a)}.

Concentration of benomyl(ppm)	Mycelial disks of IPCR-1 ^{b)}				IPCR-1	IHES-3
	PP-1	PP-2	PP-3	PP-4		
0	+	+	+	+	+	+
5	—	—	—	—	—	+
10	—	—	—	—	—	+

a) The fungal growth was examined 7 days after the onset of incubation.

b) PP-1, -2, -3 and -4 are the mycelial disks 1, 2, 3 and 4 cm apart from the point of contact, respectively.

2. Properties of PH-1

2.1 MIC of benomyl and three dicarboximide fungicides

Mycelial disks of PH-1 were placed on PSA media containing various concentrations of benomyl. A little growth of PH-1 was found on PSA media containing 1,000 ppm

benomyl, but not at 1,500 ppm (Table 3). On PSA media with benomyl from 0 to 50 ppm, mycelial growth of PH-1 was similar to that of IPCR-1 on the media without benomyl, but in the presence of more than 75 ppm benomyl, the growth was similar to that of IHES-3 (Fig. 3).

MIC of three dicarboximide fungicides to PH-1 was much the same to those to

Table 3. Sensitivity of three strains of *Botrytis cinerea* to benomyl and three dicarboximide fungicides^{a)}

Strains	MIC of four fungicides(ppm)			
	Benomyl	Iprodione	Procymidone	Vinclozolin
IPCR-1	<3	<5	10	<5
IHES-3	>3000	25	50	50
PH-1	1000	25	>50	>50

a) The strains were incubated at 20 C on PSA media containing the fungicides, and the fungal growth was examined 5 days after the onset of incubation.

Fig. 3. Mycelial growth of three strains of *Botrytis cinerea* on PSA media containing benomyl. a, Without benomyl; b, 10 ppm; c, 75 ppm; d, 1500 ppm. ●, IPCR-1; ▲, IHES-3; ■, PH-1.

IHES-3 (Table 3).

2.2 Pathogenicity to cucumber

Leaf disks of cucumber were inoculated with mycelial disks of PH-1, IPCR-1 or IHES-3, respectively, and formation of lesions was examined 4 days after inoculation. Disease incidence rate with PH-1 was 72%, nearly a medial value between those with IPCR-1 and IHES-3 (Table 4).

Table 4. Pathogenicity of three strains of *Botrytis cinerea* to cucumber leaves^{a)}

Strains	Disease incidence rate(%) ^{b)}
IPCR-1	90
IHES-3	44
PH-1	72

a) Disease of cucumber leaves was examined 4 days after inoculation with the mycelial disks.

b) Disease incidence rate(%) was calculated by the modified method of Ko *et al.*

2.3 Hyphal growth from conidia and nuclear behavior in hyphal tip cells

In the presence of 2.5% glucose solution, hyphae of PH-1 linearly elongated from conidia, and the hyphal tips showed wide-shaped form like IPCR-1 (Plate I 2-a).

In hyphae of PH-1 on glass slides, the hyphal cells where nuclei distributed more or less uniformly like those of IPCR-1, or the cells where nuclei of various size or shape were distributed irregularly like those of IHES-3, were observed to be confused (Plate I 2, arrow).

3. Hyphal fusion between IPCR-1 and IHES-3

In contact-culture of two strains on PSA media, hyphal fusion between the two strains was observed, though very scarcely. To examine hyphal fusion between the two strains in detail, their conidia were cocultured on glass slides in the presence of 2.5% glucose solution. Between IPCR-1 and IHES-3, 6 types of hyphal fusion were observed as follows: conidium-to-conidium (type 1), conidium-to-germ tube (type 2), germ tube-to-germ tube (type 3), germ tube-to-hypha (type 4) and hypha-to-hypha by connective hypha (type 5), and direct fusion of hypha-to-hypha (type 6) (Plate II 1-7 and Fig. 4). Fusion of type 5 was found most frequently, and type 1 and 6 barely, frequency of the

Table 5. Frequency of hyphal fusion between sensitive strain (IPCR-1) and resistant strain (IHES-3) of *Botrytis cinerea*^{a)}

Combination	Frequency of fusion(%)
IPCR-1-IHES-3	2.5 ^{b)}
IPCR-1-IPCR-1	5.1 ^{c)}
IHES-3-IHES-3	1.1 ^{d)}

a) Experimental procedure was shown in Fig. 3.

b) Ratio of number of fusion to total incubated conidia of two strains.

c) Ratio of number of fusion to total incubated conidia of IPCR-1.

d) Ratio of number of fusion to total incubated conidia of IHES-3.

Fig. 4. Diagram of various types of fusion between IPCR-1 and IHES-3 strains of *Botrytis cinerea*. 1. Conidium-to-conidium type; 2. Conidium-to-germ tube type; 3. Germ tube-to-germ tube type; 4. Germ tube-to-hypha type; 5. Hypha-to-hypha type; 6. Direct fusion between hyphae type. a, appressorium; c, conidium; ch, connective hypha; gt, germ tube; h, hypha.

hyphal fusion was about 2.5% between IPCR-1 and IHES-1 (Table 5), and hardly changed at times of contact-culture from 48 to 96 hr.

Observation of hyphal fusion by light microscope exhibited presence of nuclei in the connective hyphae (Plate II 2-4 and 6), transfer of nuclei between hyphae of the two strains through the connective hyphae (Plate II 3 and 6, arrows) and elongation of hyphae from the connective hyphae (Plate II 5, arrow). Nuclei in hyphal cells of IHES-3, which fused with hyphae of IPCR-1, changed in both shape and arrangement, and were similar to those in hyphae of IPCR-1 (Plate II 3, 4, 6 and 7).

Discussion

PH-1 of *Botrytis cinerea*, which was taken from IPCR-1 colony contact-cultured with IHES-3 mycelial disk, was different from the two strains in respect to resistance to benomyl, growth speed of mycelia, nuclear behavior and pathogenicity to cucumber. Also, these characteristics of PH-1 were stable in even the repeated subcultures of more than ten times. The above results may suggest that PH-1 is treated as a new benomyl-resistant strain. The similar results were obtained from experiments with the conidia formed on PH-1 colony.

No mycelial disks, which were taken from IPCR-1 colony contact-cultured with IPCR-1 mycelial disk, exhibited resistance to benomyl. *In vitro*, resistance of IPCR-1 to benomyl was not induced by treatment with the fungicide. These suggested that PH-1 did not develop from IPCR-1 by inductive effects of benomyl. PH-1 mediated

roughly between the two strains in some properties. While, hyphal fusion was observed between hyphae of IPCR-1 and IHES-3 on PSA media. In conidial coculture of the two strains, the hyphal fusion occurred at 2.5% of frequency, and was found in a short period of the culture. The above facts suggested that the hyphal fusion was concerned in development of new benomyl-resistant strain, PH-1. By this time, it has not been reported that hyphal fusion takes part in development of fungicide-resistant strains in plant pathogens. In some plant pathogens^{3,10,16,18}, however, the hyphal fusion has been reported to occur frequently, and to change various properties, *e. g.* pathogenicity. The hyphal fusion seemed to be one of methods that gave rise to transformation in fungi.

The morphological studies showed that IPCR-1 and IHES-3 were remarkably different in form of hyphae and behavior of nuclei in the hyphal cells^{1,5,15}. Hyphae of PH-1 were morphologically similar to IPCR-1. In the hyphal cells, however, nuclei that are similar to those in hyphae of IHES-3 were observed in addition to nuclei of IPCR-1. Considering that PH-1 was taken from the colony of IPCR-1, the above results suggested that a part of nuclei of IHES-3 were transferred to hyphae of IPCR-1 by hyphal fusion. Transfer of nuclei by hyphal fusion was reported in other plant pathogenic fungi^{16,32,33}. From the present study, it could not be confirmed whether karyogamy occurred or not.

Genetic studies on fungicide-resistance have been reported in some plant pathogens which the presence of sexual stage or parasexual stage was confirmed in the life cycle^{20,25,26}. In *B. cinerea*, however, the sexual or parasexual stages have not been discovered, and so the genetic studies have never been made except for reports on relationship between heterokaryosis and acquisition of fungicide-resistance^{19,30}. In the future, experiments using hyphal fusion may be a help to advance genetic studies on benomyl-resistance of *B. cinerea*, till culture-methods of the sexual generation would be established.

IHES-3 strain was remarkably poor in both growth and pathogenicity. In coculture of IPCR-1 and IHES-3 on glass slides without benomyl, hyphae, which differed from the hyphal form in monoculture of the each strain, were frequently found with the lapse of incubation time (unpublished data). In this contact-culture of benomyl-sensitive and resistant strains, a new resistant strain (PH-1), which surpassed IHES-3 in activities of both growth and pathogenicity, developed under condition without benomyl. These facts provide clues to solve the following questions on development of the resistant strains in *B. cinerea*. Firstly, in the fields, many benomyl-resistant strains, which differ in the degree of resistance, have developed^{1,15}. Secondly, density of the resistant strains occasionally did not deteriorate after suspension of benomyl-spraying in the fields⁹. The resistant strains were easily isolated on diseased plants with *B. cinerea*, but it was difficult to acquire them on media²³.

As the mechanism of development of resistant strains in *B. cinerea*¹¹, mutation, induction by fungicides, selection of the preexisting resistant strain by fungicides, *etc.* have been considered, but there are many unknown respects. The present result may suggest that hyphal fusion may be one of the mechanism of development of resistant strains in *B. cinerea*. At present, they are examining that isolation and culture of hyphal cells of IPCR-1 fused with hyphae of IHES-3, and relationship between develop-

ment of the benomyl-resistant strain and hyphal fusion of the two strains *in vivo*.

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和 文 摘 要

阿久津克己・入野達之・塚本貴敬・奥山 哲：灰色かび病菌 (*Botrytis cinerea*) のベノミル耐性菌出現と菌糸融合との関係

灰色かび病菌 (*Botrytis cinerea*) のベノミル感性菌・IPCR-1 (MIC<3 ppm) を PSA 培地で予め培養し、その菌叢の中心部にベノミル耐性菌・IHES-3 (MIC>3,000 ppm) 菌糸体片を接触・培養した後、IPCR-1 菌叢より菌糸体片を採取し、ベノミル添加培地上で耐性を検定した。ベノミル耐性を示す菌糸体片 (PH-1) が検出された。PH-1 は、ベノミル耐性 (MIC=1,000 ppm)、菌叢の形状、生育速度、菌糸の形状、核の配列様式、キュウリ葉に対する病原性が、IPCR-1、IHES-3 菌株と異なり、ほぼ両菌株の間に位置した。PH-1 の性質は、継代培養に因って変化はみられず、安定していた。次に IPCR-1 と IHES-3 の菌叢接触部を光顕観察した結果、菌糸融合が観察された。両菌株の菌糸融合についてスライドグラス上で詳細に調べた結果、6 タイプの融合 (接合菌糸による胞子間、胞子・発芽管間、発芽管間、発芽管・菌糸間、菌糸間の融合と菌糸間の直接融合) が認められ、その頻度は 2.5% であった。融合した菌糸細胞で、核の移行は観察されたが、核融合の有無は確認できなかった。

Explanation of plates

Plate I

1. Colonies of three strains of *Botrytis cinerea* on PSA media 5 days after the onset of incubation. PH-1: benomyl-resistant strain, IHES-3: benomyl-resistant strain, IPCR-1: benomyl-sensitive strain.
2. Nuclei in hyphal tip cells of three strains of *Botrytis cinerea* on glass slides in the presence of glucose. a) PH-1 strain. Note arrangement of nuclei (arrow). b) IPCR-1 strain. c) IHES-3 strain.

Plate II Hyphal fusion between IPCR-1(P) and IHES-3(H) strains of *Botrytis cinerea* on glass slides in the presence of glucose.

1. Conidium-to-conidium fusion by connective hypha(type 1).
2. Conidium-to-germ tube fusion by connective hypha(type 2).
3. Germ tube-to-germ tube fusion by connective hypha(type 3). Note transfer of nuclei to the connective hypha(arrow).
4. Germ tube-to-hypha fusion by connective hypha(type 4).
5. Hypha-to-hypha fusion by connective hypha(type 5). Note hyphal elongation from the connective hypha(arrow).
6. Hypha-to-hypha fusion by long connective hypha(type 6). Note transfer of nuclei to the connective hypha.
7. Direct fusion of hypha-to-hypha(type 7, arrow). (All scales: 10 μ m)

Abbreviations: c, conidium; ch, connective hypha; gt, germ tube; h, hypha; n, nucleus.

Plate I

Plate II