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Taxonomic Characteristics of Six Species of Entomopathogenic Fungi Isolated from the Silkworm, *Bombyx mori*

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Six entomopathogenic fungus isolates, *Beauveria bassiana* J57A, *Nomuraea rileyi* J125A, *Paecilomyces farinosus* J3A, *Paecilomyces fumosoroseus* J50A, *Metarhizium anisopliae* J88, *Aspergillus* sp. J64A, causing muscardine disease and aspergillosis in the silkworm, *Bombyx mori* were investigated for their cultural and morphological characteristics (on PDA culture media within 14 days at 24°C). The results showed that they differ each other from the features of cultural characteristics (colony elevation, colony color, colony growth rate) or morphological characteristics (conidiogenous cell structure, phialides, conidia size and shape). Among cultural characteristics, colony color is the easiest recognizable character between isolates. The morphological characteristics of each fungal isolate correspond to the descriptions of current system of classification.

Key words: Muscardine disease, *Beauveria, Nomuraea, Paecilomyces, Metarhizium, Aspergillus*

Introduction

Fungal diseases are caused due to fungi parasitizing the body of larvae, pupae, and adult silkworms. Since the infected dead larvae appear hard, these conditions are called muscardine or calcino. There are many kinds of muscardines, which are classified according to the color of the spores on the dead worm (*i.e.*, white muscardine, green muscardine, yellow muscardine, black muscardine, red muscardine, and aspergillosis). These diseases are widely prevalent in all sericultural countries (Aoki and Yanase, 1970; UN, ESCAP, 1990). The more common fungal diseases are, however, white muscardine, green muscardine and aspergillosis. Each kind of muscardine diseases has its own typical features, but some of them have similar color at initial stage such as white color of white muscardine, yellow muscardine and green muscardine or at advanced stage of pathogenesis such as green color of green muscardine and black muscardine. These similarities sometimes cause confusion in the diagnosis of fungal diseases. Although there were few studies done on muscardine and aspergillosis diseases in the silkworm but most of them concentrated on the germination, penetration or invasion into silkworm larvae of white muscardine (Kumar *et al.*, 1999) and green muscardine (Vineet *et al.*, 1997), or control measures (Javaregowda, 1994; Kumar *et al.*, 2003), but so far there havent been any systematic studies on morphological characteristics of these fungi. Therefore some problems of nomenclature still exist. In this paper we investigated the cultural and morphological characteristics of six entomopathogenic fungi, which cause muscardine diseases and aspergillosis. The results could serve as basis for identification and classification of these fungi as well as contribution in control management of fungal diseases in the silkworm, *B. mori* L.

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Materials and Methods

Fungus isolates

Six isolates were used in the study, *Beauveria bassiana* J57A, *Nomuraea rileyi* J125A, *Paecilomyces farinosus*

J3A, *Paecilomyces fumosoroseus* J50A, *Metarhizium anisopliae* J88, *Aspergillus* sp. J64A. These isolates, that caused the fungus diseases in the silkworm named as white-, green-, yellow-, red-, black muscardine and aspergillosis, respectively, were isolated from infected silkworm larvae in Korea. They are preserved in the entomopathogenic fungus collection of Division of Sericulture and Honeybee of The National Institute of Agricultural Science and Technology (NIAST, RDA), Suwon, Korea.

Cultivation

Fungi were grown on Potato Dextrose Agar (PDA) media containing 24 g potato dextrose and 15 g agar in 1 litre of distilled water. Agar media were sterilized at 121°C for 20 min and molten media were poured into 9 cm petri plates and allowed to cool. The petri plates were inoculated with 3 mm agar plug of the species taken from the growing margin of the colony, and the single plug was placed upside down in the central of the petri plate. The plates were sealed by parafilm and incubated at 24°C in darkness for 14 days, each species 3 replicates. The colonies were measured for their diameters at 14th day as the measurement of the mycelial growth, and the characteristics of colonies on agar plates also were observed.

SEM preparations

After cultivation on PDA media for 14 days, the sample of each fungus was prepared for Scanning Electron Microscopy (SEM) by cutting squares of 1 cm^2 colony, and fixed in Karnovskys fixative solution prepared in 0.1 M dimethyl arsenic acid at pH 7.2 and stored overnight at 4°C. After fixing, the specimens were washed 3 times (30 min each) in 0.05 M cacodylate buffer (pH 7.2) and 3 times (15 min each) in distilled water. They were dehydrated through 50, 75, 90, 95, 100% ethanol (30 min in each stage) and 2 changes in 100% ethanol at room temperature. The species were then transferred 2 times (30 min each) to amyl acetate 100%. After that, the samples were dried and coated by gold and examined under Leo440 electron microscope.

Results

Morphological characteristics of six entomopathogenic fungi

Characteristics of colonies on media and microscopic features of conidiophores and conidia of six species are as showed in Table 1. Each species has its own typical feature, and they are different each other in some features from culture characteristics such as colony form, colony

Table 1. Morphological characteristics of the entomopathogenic fungi after 14 days of cultivation on PDA media at 24°C

Characteristics	*B. bassiana* J57A	*N. rileyi* J125A	*P. farinosus* J3A	*P. fumosoroseus* J50A	*M. anisopliae* J88	*A.* sp. J64A
Colony form	circular	circular	circular	circular	circular	circular
Surface elevation	flat	flat	raise	raise	flat	flat
Microscopic						
Appearance: - edge	entire	undulate	entire	entire	entire	entire
- internal	granule	granule	filamentous	filamentous	granule	granule
Colony diameter (mm)	44.0 ± 7.21	20.3 ± 2.52	32.7 ± 2.52	56.3 ± 1.53	30.3 ± 2.31	79.3 ± 1.15
Mycelial density	compact	compact	compact	compact	compact	compact
Mycelial color:						
- surface	white	grass green	yellowish white	gray yellowish pink	black green	moderate deep yellow green
- reverse side	pale yellow	grayish yellow	strong yellow	light yellow	greenish yellow	tea green
Hyphal width (μm)	0.93 – 1.7	2.6 – 3	1.2 – 1.54	1.4 – 2.2	1.8 – 2.5	8.5
Conidiophore width (μm)	1.3 – 2.1	2.1 – 2.5	0.9 – 2	0.7 – 2.3	1.5 – 2	6 – 7
Conidial shape	globose to ellipsoidal	oval	oval	oval	fusiform	subglobose
Conidial size (μm)	2.1 – 1.7	3.2 – 1.6	2.4 – 1.4	2.8 – 1.4	10.6 – 2.3	5.1 – -4.8
No. of phialides	-*	3 – 4	3 – 4	3 – 5	1 – 3	Many
Phialide size (μm)	-*	4.0 – 2.3	3.6 – 1.5	4.7 – 1.9	9.8 – 1.8	9.6 – 4.3

Note: Conidial shape is followed to the "Dictionary of the fungi 9th edition, 2001". Mycelial color is followed to the "Concise manual of color names" Korea color research Institute, 1991. Conidial size value is average of n = 100.
Conidiophores structures were observed and measured on SEM photographs. * : *B. bassiana* has no phialides.

elevation, colony margins, colony color or from the size and shape of conidia and conidiogenous cells. The features of each species are as follows:

White muscardine, *Beauveria bassiana* J57A

The classification systems presently used for *Beauveria* are based on morphology and many researchers around the world are investigating the validity of the species currently recognized (Glare and Inwood, 1998). Isolate of *B. bassiana* J57A showed typical characteristics of those described elsewhere (De Hoog, 1972; Kim, 1999). Colonies on PDA grow moderately fast, and attain a diameter of 44 mm within 14 days of cultivation at 24°C. Mycelial density is thick, and mycelial color is white (Fig. 1A). Reverse side of colonies is pale yellow (Fig. 1B). Vegetative hyphae are smooth-walled, hyaline, 0.93 – 1.7 μm wide. Conidiophores are single, 1.3 – 2.1 μm wide. Conidiogenous cells form in tightly clustered groups. *B. bassiana* is characterized by the toothed zig-zag appearance of the conidiogenous rachis as showed in Fig. 1C. Conidia are hyaline, globose to ellipsoidal, 2.1 – 1.7 μm (Fig. 1D).

Green muscardine, *Nomuraea rileyi* J125A

Colonies on PDA grow slowly, attain a diameter of 20 mm within 14 days of cultivation at 24°C. Colonies consist of a basal felt from which erect conidiophores arise. Mycelial density is thick and mycelial color is grass green (Fig. 2A). Reverse side of colonies is grayish yellow (Fig. 2B). Vegetative hyphae are smooth-walled septate, hyaline, 2.6 – 3 μm wide. Conidiophores are erect, septate, 2.1 – 2.5 μm wide and form dense clusters of branches. Each branch bears 3 or 4 compacted phialides. Phialide is 4 – 2.3 μm in size, and has a short neck of 1 μm in width (Fig. 2C). Conidia are in dry divergent chains, oval, smooth-walled, 3.2 – 1.6 μm (Fig. 2D).

Yellow muscardine, *Paecilomyces farinosus* J3A

Colonies on PDA grow moderately fast, attain a diameter of 32 mm within 14 days of cultivation at 24°C. Colonies consist of a basal felt from which numerous conidiophores arise. Mycelial density is thick, and mycelial color is yellowish white (Fig. 3A). Reverse side of colonies is strong yellow (Fig. 3B). Vegetative hyphae are smooth-walled, hyaline, 1.2 – 1.5 μm wide. Conidiophores are 0.9 – 2 μm wide, and consist of verticillate branches with whorls of 3 to 4 phialides. Phialide is 3.6 – 1.5 μm in size, and has a swollen basal portion tapering into a distinct nect of 0.46 μm in width (Fig. 3C). Conidia are oval, smooth-walled,

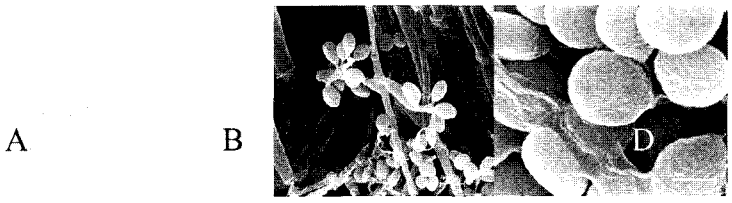

Fig. 1. *B. bassiana* J57A. A: Surface of colonies on PDA after 14 days of cultivation at 24°C, B: Reverse side of colonies, C: Conidiophores with the toothed zig-zag appearance of the conidiogenous rachis (bar = 3 μm), D: Conidia (bar = 1 μm).

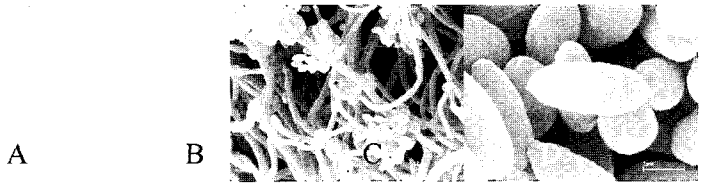

Fig. 2. *N. rileyi* J125A. A: Surface of colonies on PDA after 14 days of cultivation at 24°C, B: Reverse side of colonies, C: Conidiophores and phialides with a short neck (bar = 10 μm), D: Conidia (bar = 1 μm).

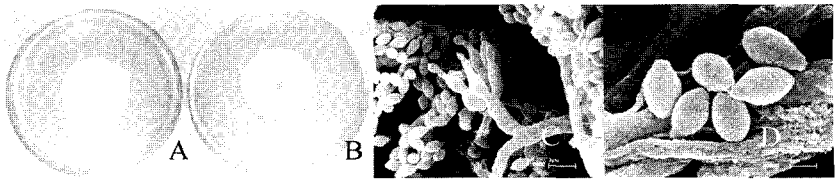

Fig. 3. *P. farinosus* J3A. A: Surface of colonies on PDA after 14 days of cultivation at 24°C, B: Reverse side of colonies, C: Conidiophores and phialides with swollen bases and prominent necks (bar = 3 μm), D: Conidia (bar = 1 μm).

hyaline, 2.4 – 1.4 μm (Fig. 3D).

Red muscardine, Paecilomyces fumosoroseus J50A

Colonies on PDA grow moderately fast, attain a diameter of 56 mm within 14 days at 24°C. Colonies consist of a basal felt with raised floccose overgrowth. Mycelial density is thick, and mycelial color is gray yellowish pink (Fig. 4A). Reverse side of colonies is light yellow (Fig. 4B). Vegetative hyphae are smooth-walled hyphae, 1.4 – 2.2 μm wide. Conidiophores consist of verticillate branches bearing whorls of 3 to 5 phialides. Phialide is 4.7 – 1.9 μm in size, and has an oval basal portion, which tapers into a long distinct neck of 0.5 μm in width (Fig. 4C). Conidia are oval and smooth-walled, 2.8 – 1.4 μm (Fig. 4D).

Black muscardine, Metarhizium anisopliae J88

M. anisopliae was isolated from the beetle *Anisoplia austriaca* by Metchnikoff (1879). The entomopathogenic fungus *M. anisopliae* has been reported to infect more than one hundred species of insects belonging to a variety of insect orders (McCoy *et al.*, 1988). Colonies on PDA grow moderately fast, attain a diameter of 30 mm within 14 days of cultivation at 24°C. Mycelial density is thick, and mycelial color is black green (Fig. 5A). Reverse side of colonies is moderate greenish yellow (Fig. 5B). Vegetative hyphae are 1.8 – 2.5 μm wide. Conidiophores are in compact patches. Individual conidiophores are broadly branched, densely intertwined, and are 1.5 – 2 μm wide. Phialide is 9.8 – 1.8 μm in size. Phialide neck is 0.9 μm wide (Fig. 5C). Conidia are fusiform, slightly narrowed in the middle, 10.6 – 2.3μm (Fig. 5D).

***Aspergillus* sp. J64A**

Aspergillosis, an important disease in the young age silkworm, *Bombyx mori* L., is caused by several species of *Aspergillus* fungi, which can grow saprophitically in the silkworm rearing environment like on soil surface and rearing appliances (Ayuzawa *et al.*, 1972). Colonies on PDA grow fast and attain a diameter of 79 mm within 14 days of cultivation at 24°C. Mycelial density is thick, and mycelial color is deep yellow green (Fig. 6A). Reverse side of colonies is tea green (Fig. 6B). Vegetative hyphal is 8.5 μm wide. Conidiophores are thick, 6 – 7 μm wide, and the distal end of conidiophore expands into a globular structure bearing phialides. Phialide is hyaline, 9.8 – 1.8 μm. Phialide neck is 2.3 μm wide (Fig. 6C). Conidia are subglobose, echinulate, 5.1 – 4.8 μm (Fig. 6D).

Fig. 4. *P. fumosoroseus* J50A. A: Surface of colonies on PDA after 14 days of cultivation at 24°C, B: Reverse side of colonies, C: Conidiophores and phialides with swollen bases and prominent necks (bar = 5 μm), D: Conidia (bar = 1 μm).

Fig. 5. *M. anisopliae* J88. A: Surface of colonies on PDA after 14 days of cultivation at 24°C, B: Reverse side of colonies, C: branched conidiophores and phialides (bar = 10 μm), D: Fusiform conidia (bar = 2 μm).

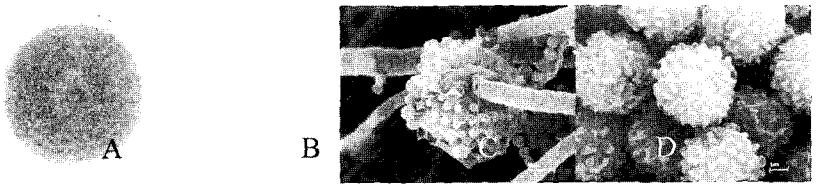

Fig. 6. *Aspergillus* sp. J64A. A: Surface of colonies on PDA after 14 days of cultivation at 24°C, B: Reverse side of colonies, C: Conidiophores and phialides (bar = 10 μm), D: Echinulate conidia (bar = 1 μm).

Discussion

Morphological characteristics of fungi causing muscardine diseases in the silkworm are different from each other in the form and color of colonies, conidiophores structures, conidia size and shape etc. For primary diagnosis, one of the most typical characteristics, which were named for muscardine diseases as white-, green-, yellow-, red-, black-muscardine, is the color of fungus colonies. Although some of them in the early stage of development on the media are very similar, and these similarities are able to cause confusion in diagnosis on the color. As the white color of white muscardine, *B. bassiana* and yellow muscardine, *P. farinosus* or the green color of green muscardine, *Nomuraea rileyi* and black muscardine, *M. anisopliae*. But when the fungus colony develops in the late stage, the character color of each isolate will appear typically, such as white, green, yellowish white, pink, black green and deep yellow green color of white-, green-, yellow-, red-, black- muscardine and aspergillosis, respectively. Furthermore, for an exact checking, the other microscopic characteristic features will give exact evidences of each fungus genus. Species in nearly every genus of entomopathogenic Hyphomycetes are distinguished by the morphologies of their conidia, conidiogenous cells, and the identity of their hosts (Humber, 1997). *Beauveria* species are classified by the shape of their conidia and the placement of conidia on the conidiogenous apparatus (Glare, 1998). They are characterized by having conidiophores consisting of whorls and dense clusters of sympodial, short and globose or flask-shaped conidiogenous cells with apical denticulate rachi (giving a distinct zig-zag appearance), and one-celled conidia (De Hoog, 1972; Samson *et al.*, 1988; Tanada and Kaya, 1993; Humber, 1997). Hoogs (1972) authoritative monograph of *Beauveria* recognized three species. Two of which are entomogenous. *B. bassiana* is nearly globose conidia, 1.5 $5 \times 1.5 - 3$ μm, and *B. brongniartii* is ellipsoidal conidia, 2 $6 \times 1.5 - 3$ μm. Because cultural characters were highly variable and could not be used suitable for species determination, conidial form was the most useful criterion to distinguish between species. Genus of *Nomuraea* was retained as distinct from the genus of *Paecilomyces* by a green color of colony (Fig. 2A) and by whorls of short phialides without distinct necks (Fig. 2C). Genus of *Metarhizium* is highly distinct and not likely to be confused with any other fungi, which affect insects. Black muscardine, *M. anisopliae* is sometimes called by another name, as green muscardine in some countries. Probably that is due to its greenish black color on infected dead larvae or on colony, but actually green muscardine caused by *N. rileyi* and black muscardine caused by *M. anisopliae* are different each other not only from the color of colonies (green color of green muscardine vs black green color of black muscardine) but also from the size or shape of conidia and conidiophores as showed above. *M. anisopliae* is recognized by conidiophores branching repeatedly at broad angles. Conidia borne in parallel chains and usually have green in mass (Humber, 1997). The *M. anisopliae* isolates obtained from the mulberry field soil were divided into three forms according to their conidial size and color of colony (Kawakami, 1979). The feature of *Aspergillus* sp. J64A used in this study is globose and echinulate conidia, and conidia born on phialides with a short neck as showed in Fig. 6C. Our study showed the taxonomical characteristics of six entomopathogenic fungi which cause muscardine and aspergillus diseases in the silkworm. The morphological characters of importance for identification of genus or species used in this study are consistent with the published books or monographs. *Beauveria* corresponds to descriptions of De Hoog (1972), *Nomuraea* and *Paecilomyces* to Samson (1974), *Metarhizium* to Samson *et al.* (1988), and *Aspergillus* to Samson (1992). For further study on the taxonomy of these fungi, molecular techniques will be very useful. The level of confidence in taxonomic conclusion will be increased when both morphological and molecular techniques support the same conclusions.

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