Int. J. Indust. Entomol. Vol. 10, No. 2, 2005, pp. 119~123

International Journal of *Industrial Entomology*

Isolation and Characterization of Benomyl-Resistant Mutants in an Entomopathogenic Fungus, *Metarhizium anisopliae*

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(Received 28 January 2005; Accepted 10 May 2005)

Benomyl-resistant mutants of entomopathogenic fungus, *Metarhizium anisopliae* were isolated and their physiological characteristics were investigated. These mutants were obtained spontaneously or by UV irradiation in benomyl-treated media. Four spontaneous (S-2, S-11, S-18, S-19) and four UV-induced (UV-4, UV-5, UV-19, UV-24) mutants, which grow stably and normally were selected. No significant differences in conidia or hyphal shape, conidia viability, mycelial biomass, or virulent to the diamondback moth were observed between the wild type and their mutants. But differently from the mycelial growth of other benomyl-resistant mutants which was slower than that of the wild type on a modified Czapek-Dox, SDAY, 4% chitin, or 1% skim milk medium, that in the spontaneous mutants, S-18 and S-19, did not show any difference from the wild type. Especially, S-18 and S-19 grew well at benomyl concentrations up to 50 times or higher than that which inhibits wild type proliferation. These results suggested that S-18 and S-19 could potentially be used with the fungicide, benomyl.

Key words: Benomyl, Entomopathogenic fungus, *Metarhizium anisopliae*, Resistant mutant

Introduction

Metarhizium anisopliae is a well-known, wide-host-range insect pathogen, which has been used in experimental systems for the biological control of several insect pests (Rombach *et al.*, 1987; Zimmermann, 1992, 1993). However, the full potential of this fungus as a mycoinsecticide is not yet exploited, mainly because the biological and ecological basis of its pathogenesis in insects is not fully understood (Samson *et al.*, 1988). There are many problems in the development of effective products including probability of mass production, stability in storage, and compatibility with synthetic fungicides. Especially, as an adverse environmental condition, fungicides are major problems against the successful use of mycoinsecticides (Moore and Prior, 1993).

The synthetic fungicides are used as plant protections and applied prior to the appearance of the fungus and development of symptoms (Griffin, 1994). The benzimidazoles including benomyl, nocodazole, and thiabendozole are common fungicides and applied topically as dusts or sprays to inhibit spore germination and hyphal growth on the surface of the plant. Among them, benomyl is an important agricultural antifungal agent that is effective apparently because it inhibits the assembly of the microtubules of phytopathogenic fungi, while having little effect on the plants on which they grow (Davidse and Flach, 1977; Davidse, 1986; Deacon, 1997). However, most entomopathogenic fungi are very susceptible to benomyl, and consequently can not be used as plant mycoinsecticides on which the fungicide is applied.

In this study, therefore, we have isolated benomyl-resistant mutants of *M. anisopliae,* which can live on benomyl medium of high concentrations, and investigated their biological and physiological characteristics.

Materials and Methods

Fungal strain and the insect

Metarhizium anisopliae var. *anisopliae* KACC 40029

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(ATCC 20500) was obtained from Korean Agricultural Culture Collection, National Institute of Agricultural Biotechnology, Rural Development Administration, Suwon, Korea. The fungus was maintained on SDAY medium (Sabouraud dextrose agar + 0.2% yeast extract) at 28°C and subcultured periodically. Conidia were directly harvested from the surface of 3-week-old sporulating cultures by scraping, spread with a loop onto 90-mm petri dishes and then incubated for 3 days at 28°C. The culture was used for radial growth studies (Vidal *et al.*, 1997). The diamondback moth, *Plutella xylostella* pupae were also obtained from the National Institute of Agricultural Science and Technology, Rural Development Administration, Suwon, Korea.

Mutagenesis experiment

Fifteen ml conidium suspension (1.8×10^8 spores/ml) in a petri dish was irradiated with UV light ($\lambda = 254$ nm) and 0.1 ml samples (1.8×10^7 spores) were taken at several short intervals (30 sec, 60 sec, 90 sec, 120 sec, and 180 sec). The samples were plated on SDAY medium containing 250 ug/ml benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate, Aldrich Chemical Co., USA]. The irradiation was done by placing the petri dish covered with the suspension under a prewarmed 40 W UV tube at a distance of 40 cm (at a dose rate of 15 erg/mm^2/sec). Spore suspensions (1.8×10^7 conidia) were spread on SDAY medium containing 250 ug/ml benomyl to obtain benomyl-resistant mutants by spontaneous mutation. Spontaneous and UV-induced mutants were transferred and subcultured on SDAY medium. The mutants were subcultured several times, and all of them maintained their resistance to benomyl during their successive culture.

Mycelial growth experiments

A modified Czapek-Dox agar medium containing starch (30 g/L) and peptone (2 g/L) and SDAY medium with or without benomyl were used. The plates were all centrally inoculated with a 6-mm agar plug of the mutants taken from 3-days-old. The petri dishes were placed upside down and incubated for 16 days in dark chambers. Surface radial growth was recorded through two orthogonal axes, using a cardinal diameter.

Dry weight study

Conidium suspensions (5×10^6 spores) were inoculated into a 200 ml flask containing 40 ml Sabouraud dextrose broth (SDB). The flasks were incubated for 5 days in a dark shaking incubator (190 rpm). The liquid cultures were filtrated using filter papers and dried at 80°C for 24 hrs. The dry weight was measured.

Enzyme activity

Two media were indirectly used to test the enzyme activity of the mutants. A medium for chitinase activity contained (per liter): 0.4% colloidal chitin, 0.05% $MgSO_4 \cdot H_2O$, 0.03% KH_2PO_4, 0.07% K_2HPO_4, 0.001% $FeSO_4 \cdot 7H_2O$, 0.0001% $ZnSO_4 \cdot 7H_2O$, 0.0001% $MnCl_2$, and 1.5% agar. A medium for protease activity contained (per liter): 1% dextrose, 1% skim milk (35% protein, Seoul Milk Co., Korea), and 1.5% agar. Autoclaved paper discs (ADVANTEC, 8 mm, thin, Toyo Co., Japan) were singly transferred to the center of 90-mm petri dishes. The treatments were inoculated on the discs with 20 ul liquid culture of the mutants taken from the submerged culture. Each plate inoculated was incubated for 6 days in a dark chamber and then the colony diameters were measured.

Pathogenicity to pupae of the diamondback moth, *P. xylostella*

Conidia obtained from 21-day-old SDAY plates were suspended in 0.05% Tween 80 solution. The concentration of conidia was adjusted to 1×10^8 spores/ml by direct counting in a haemocytometer and a dilution method. *P. xylostella* pupae were inoculated by dipping into the spore suspensions for 20 sec and placed in 90-mm petri dishes containing a moistened filter paper. For one treatment 15 pupae were used. Each test was repeated at least four times. Mortality caused by mycoses was evaluated after 15 days.

Data analysis

Data were subjected to analysis of variance (PROC ANOVA; SAS, 1995) and means were compared using Duncan's multiple range test at the 5% level.

Results

Four spontaneous (S-2, S-11, S-18, S-19) and UV-induced (UV-4, UV-5, UV-19, UV-24) mutants each were selected on the basis of their growth pattern. The mutants grew better and sporulated on the media containing high concentration of benomyl. A notable difference between wild *M. anisopliae* and its mutants was neither detected in the conidium nor in mycelium shape. Generally, the mycelial growth of all the mutants was slower than that of the wild type on a modified Czapek-Dox (Fig. 1) or SDAY medium (Fig. 2). The fungal growth rate of spontaneous mutants, S-2 and S-11, and UV-induced mutants, UV-4, UV-5 and UV-19, was slower on a modified Czapek-Dox medium, but S-18, S-19 and UV-24 grew relatively well on the same medium.

The mycelial linear growth between the wild type strain

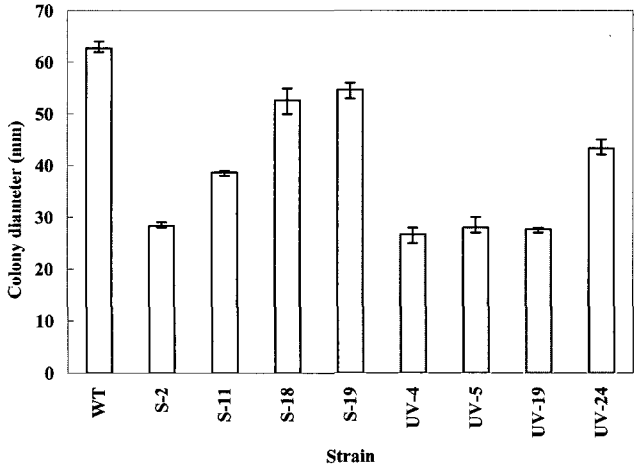

Fig. 1. Comparison of mycelial linear growth among *M. anisopliae* and its 8 mutants on a modified Czapek-Dox medium after 16 days of incubation (P = 0.05; Duncan's multiple range test).

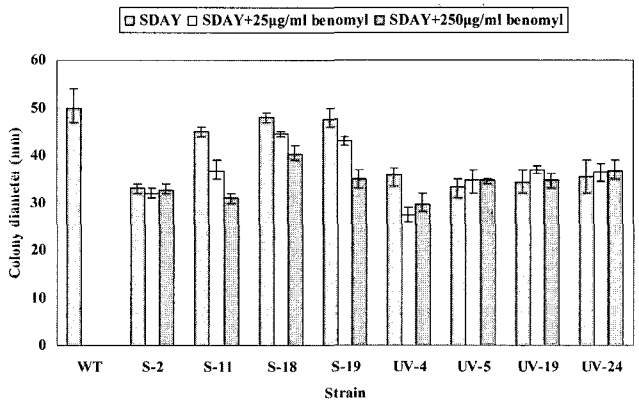

Fig. 2. Comparison of mycelial linear growth among *M. anisopliae* (WT) and its 8 mutants on the plate medium with or without benomyl for 16 days of incubation (P = 0.05; Duncan's multiple range test).

and its mutants was compared on SDAY medium containing 25 ug/ml or 250 ug/ml benomyl (Fig. 2). Fungal growth pattern was similar to that on a modified Czapek-Dox medium. Mutants did not show much difference in growth pattern at different concentrations of benomyl.

Mycelial biomass of the mutants cultured on the SDB medium was compared by dry weight (Fig. 3). But the dry weight of four mutants studied was similar to that of the wild type strain in the medium with 25 ug/ml benomyl or without benomyl.

Chitinase and protease activity were indirectly compared on 4% chitin and 1% skim milk medium by measuring their colony diameter (Fig. 4). The fungal growth of S-2, UV-4, UV-19 and UV-24 on 4% chitin medium was slower, but that of S-11, S-18, S-19 and UV-24 were

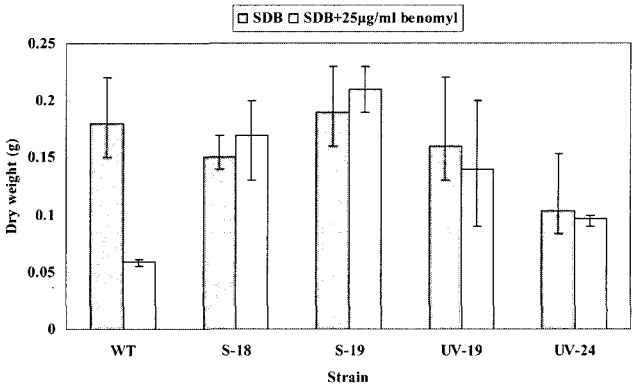

Fig. 3. Dry weight of liquid mycelial filtrates of *M anisopliae* and its 4 mutants after 5 days of incubation with or without benomyl in SDB medium (P = 0.05; Duncan's multiple range test).

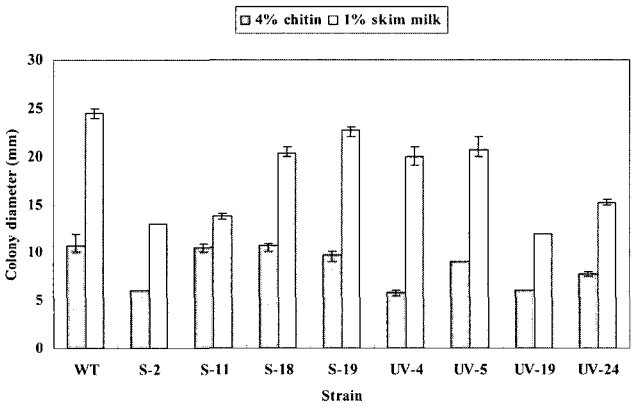

Fig. 4. Comparison of mycelial growth among the wild type and 8 mutants of *M. anisopliae* on chitin and skim milk plate media after 6 days of incubation (P = 0.05; Duncan's multiple range test).

similar to that of the wild type strain. The mycelial growth of S-18, S-19, UV-4 and Uv-5 was better than that of S-2, S-11, UV-19 and UV-24 on 1% skim milk medium and also similar to that of the wild type strain. Especially, the mycelial growth of S-18 and S-19 did not show any difference from that of the wild type on 4% chitin medium as well as 1% skim milk medium.

To study the survival percentage of conidia on the plate medium containing high concentration benomyl, conidium suspensions (1×10^8 spores) were spread on SDAY medium containing 250 ug/ml benomyl. All mutants had 100% conidium viability (unpublished data). To investigate the pathogenicity of the mutants, *Plutella xylostella* pupae were dipped in conidium suspensions (1×10^8 spores/ml) (Table 1). After 15 days of inoculation, infectivity of the mutants was similar to that of the wild type strain.

Table 1. Pathogenicity of *M. anisopliae* and its 8 mutants against *Plutella xylostella* pupae

Strain		Mortality (%)
Wild type		77.7 ± 4.0a
Spontaneous mutant	S - 2	80.0 ± 0a
	S - 11	77.7 ± 4.0a
	S - 18	80.0 ± 7.0a
	S - 19	71.3 ± 4.0a
UV-induced mutant	UV - 4	71.3 ± 7.5a
	UV - 5	75.3 ± 4.0a
	UV - 19	75.3 ± 4.0a
	UV - 24	71.0 ± 3.5a

Pathogenicity was determined at 15-days post inoculation (P=0.05; Duncan's multiple range test). Pupae were dipped in a suspension containing 1×10^8 spores/ml. Control pupae were treated with only 0.02% Tween 80 solution to result in 0% pupal mortality (data not shown).

Discussion

One strategy for the biocontrol of insects is to develop strains of fungal pathogens resistant to common fungicides. The resistant or transgenic strains can be used to control insect pests at the same time that fungicides are applied to control fungal pathogens of plants. To develop transgenic strains, *M. anisopliae* was transformed to benomyl resistance from *A. nidulans* (Goettel *et al.*, 1990). Transformants grew at benomyl concentrations up to ten times which inhibited the wild type and were mitotically stable on either selective or non-selective media or insect tissues. The transformants were pathogenic to *Manduca sexta* in the presence of 50 ug/ml of benomyl. This study suggested that transgenic entomopathogenic fungi could potentially be used with fungicides. However, because benomyl have been applied at concentrations of 250 ug/ml or more in fields, strains with higher resistance will have to be selected.

The objective of this study is to determine whether benomyl-resistant strains of *M. anisopliae* can be used as mycoinsecticides concurrently with an antifungal agent benomyl instead of the wild type strain.

To obtain predominant mutant strains having highly resistant to benomyl, the mutagenesis was induced spontaneously or by UV irradiation on SDAY media containing 25 ~ 250 ug/ml of benomyl. Eight of the mutants were selected; four spontaneous mutants (S-2, S-11, S-18, S-19) and four UV-induced mutants (UV-4, UV-5, UV-19, UV-24).

The mutants were mitotically stable after 5 successive transfers on selective or non-selective media and retained the ability to infect and kill the diamondback moth. These mutants also grew well at benomyl concentrations greater than the concentration that inhibits the growth on a modified Czapek-Dox or SDAY media containing benomyl and showed lower dry weight of liquid mycelial filtrates. But no significant differences in conidia formation, fungal morphology, spore viability, and pathogenicity were observed between mutant strains and the wild type. Especially, mycelial growth and biomass of the two spontaneous mutants, S-18 and S-19, were similar to those of the wild type. Most of them also showed less chitinase and protease activity on 4% chitin and 1% skim milk media when indirectly measured by colony diameter. But the two spontaneous mutants, S-18 and S-19, were not different from the wild type. These studies suggested that the two spontaneous mutants, S-18 and S-19, could potentially be used with the fungicide, benomyl.

Acknowledgement

This work was supported by Specific Research-promoting Joint Projects, RDA, Republic of Korea and By the Brain Korea 21 project.

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