Identification of Ice-nucleating Active Fungus Isolated from the Gut of the Rice Stem Borer, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae) and a Search for Ice-nucleating Active *Fusarium* Species

Hisaaki TSUMUKI*, Hideki YANAI* and Takayuki AOKI**

Abstract

Ice-nucleating active fungus isolated from the gut of larvae of the rice stem borer, *Chilo suppressalis* Walker was identified as *Fusarium moniliforme* Sheldon var. *subglutinans* Wollenweber et Reinking. Ice-nucleating activity (INA) around -5°C was detected in the mycelial suspension and cell-free culture medium of the fungus. INA was also detected in two strains of *F. moniliforme*, two of *F. avenaceum* and one of *F. tricinctum* among the 37 *Fusarium* strains tested. The activity of these five strains was similar to that of *F. moniliforme* var. *subglutinans*. However, *F. moniliforme* and *F. avenaceum* ceased producing ice nuclei within a short time after subculture in liquid medium.

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Key words: ice-nucleating active *Fusarium*, *Fusarium moniliforme* var. *subglutinans*, *Chilo suppressalis*, insect gut.

INTRODUCTION

Several epiphytic bacteria serve as ice nuclei⁷⁾. Ice-nucleating active lichen fungi have also been reported^{4,5)}. In the previous paper, we isolated an ice-nucleating active fungus, which was tentatively identified as *Fusarium* sp., but the specific name has remained unknown, from the gut of larvae of the rice stem borer (*Chilo suppressalis* Walker)¹⁶⁾. Ice-nucleating activity (INA) was detected in the mycelial suspension and culture filtrate of the fungus. It is the first report that species of *Fusarium* sheds ice nuclei into the culture medium during mycelial growth. Pouleur *et al.*¹⁴⁾ have also found ice-nucleating active *Fusarium acuminatum* and *F. avenaceum* in 14 *Fusarium* species. Furthermore, they suggested the presence of another ice-nucleating active *Fusarium* species.

In this study the strain of the ice-nucleating active *Fusarium* isolated from the gut of larvae of the rice stem borer was reidentified to the rank of species. Furthermore, we searched for ice-nucleating active *Fusarium* species and compared the INA among them.

MATERIALS AND METHODS

Observation of morphology of the gut strain.

Strain of *Fusarium*, reported previously to have INA¹⁶⁾, was observed morphologically. Potato sucrose agar (PSA) and potato dextrose agar (PDA)¹⁾ were used for examination of growth rate and cultural coloration. Carnation-leaf agar (CLA)⁹⁾ and SNA¹⁰⁾ were applied for measurement of micromorphology. Cultivation was made at 25°C under 12 hr light and 12 hr dark condition.

Search for ice-nucleating active *Fusarium* species. All *Fusarium* strains tested are listed in Table 1. The 37 *Fusarium* strains were supplied from the MAFF collection of microorganisms (National Institute of Agrobiological Resources, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Ibaraki, Japan). Fungal cultures were replicated on PSA in test tubes and grown at 25°C for seven days in the dark¹⁶⁾. About 0.25 cm² of the mycelial mat was then inoculated into 10 ml of liquid medium (20 g of galactose, 2 g of L-asparagine, 1 g of K₂HPO₄, 0.5 g of KCl and 10 mg of Fe-EDTA in 1 liter of distilled water at pH 5.6)⁶⁾ and cultured at 25°C with reciprocal shaking¹⁶⁾. Thereafter for 14 or 21 days, the culture medium was centrifuged at $10,000\times g$ for 20 min. The supernatants and mycelial suspensions were tested for INA. Crystallization temperatures above -10°C were recorded as INA positive.

The activity of the ice nuclei produced by *Fusarium* species. After cultivation in the liquid medium at 25°C for 12 days¹⁶⁾, the mycelial pellets were separated

* Research Institute for Bioresources, Okayama University, Chuo, Kurashiki, Okayama 710, Japan 岡山大学資源生物科学研究所

** National Institute of Agrobiological Resources, Kannondai, Tsukuba, Ibaraki 305, Japan 農業生物資源研究所

from the culture medium by filtration through a nitrocellulose filter (0.45 μm pore size). The filtrate was concentrated using an Amicon ultrafiltration device (PM-10 membrane with 10 kDa cut-off; Amicon Corp., Lexington, MA, USA), and then dialyzed at 4°C overnight against 100 mM phosphate buffer (pH 7.4). The protein concentration in the solution was determined using bovine serum albumin as the standard⁸⁾. The solution was diluted with distilled water and the crystallization temperatures of each diluted solution were measured.

The effect of subculture on the production of ice nuclei. Subcultures were routinely passed by inoculating 10 ml of suspensions from the 12–14 day-old culture of the fungus into 250 ml of new liquid medium. The 12–14 day-culture suspensions of every subculture were centrifuged at $10,000\times g$ for 15 min. INA in the supernatants and in the mycelial suspensions was measured.

Measurement of INA. The crystallization temperatures of the mycelial suspensions, culture filtrates or supernatants were measured by a multichannel temperature recorder as described in the previous papers^{15,16)}

RESULTS AND DISCUSSION.

Morphological characterization and identification of the strain of Fusarium isolated from the rice stem borer

An ice-nucleating active fungus isolated from the gut of the borer was tentatively identified as *Fusarium* sp.¹⁶⁾ The strain was re-identified morphologically by comparison with given descriptions and with the strains deposited in the MAFF collection of microorganisms: *i.e.* *Gibberella fujikuroi* var. *moniliforme* MAFF 235820 (=

ATCC 38932) and MAFF 235821 (=ATCC 38933), *Gibberella fujikuroi* var. *subglutinans* MAFF 235822 (=ATCC 38943) and MAFF 235823 (=ATCC 38936), *Gibberella fujikuroi* var. *fujikuroi* MAFF 235824 (=ATCC 38940) and MAFF 235825 (=ATCC 38938). The strain was proven to be *F. moniliforme* var. *subglutinans* (anamorphosis of *Gibberella fujikuroi* var. *subglutinans*)¹⁾.

The description of the strain is given as follows;

F. moniliforme Sheldon var. *subglutinans* Wollenweber et Reinking (Fig. 1, A–C).

Growth: on PSA and PDA initially colorless, rapid (4.3 cm diam. after 4 days). **Cultures:** from below orange grey, reddish grey to purplish grey. **Aerial mycelium:** abundant, floccose, white to greyish white at the margin, purplish grey in central zone. **Conidiophores:** aerial or submerged, sometimes sporodochial, consisting of simple lateral phialides to of branched and proliferating complex conidiophores. **Microconidia:** formed mostly from aerial conidiophores, oval, ellipsoid to obclavate, often truncated at base, mostly aseptate, some 1 to 2-septate, $6\text{--}12\times 2.0\text{--}2.5\ \mu\text{m}$. **Macroconidia:** formed from aerial or submerged-sporodochial conidiophores, thin-walled, falcate, rather straight, slender, often with a hamate apical cell and a distinct foot cell, up to 6-septate (mostly 3–5 septate) and measured:

- 1 septate $20\text{--}25\times 2.5\text{--}3.0\ \mu\text{m}$
- 2–3 septate $23\text{--}45\times 2.5\text{--}3.5\ \mu\text{m}$
- 4–5 septate $43\text{--}52\times 2.5\text{--}3.5\ \mu\text{m}$
- 6 septate $50\text{--}84\times 3.0\text{--}3.5\ \mu\text{m}$

Transitional form of conidia between macro- and micro-conidia: present, septate with truncate base. **Chlamydospores:** absent.

Taxonomy and species concepts of *F. moniliforme*

Fig. 1. Morphology of the fungus isolated from the gut of *Chilo suppressalis* larvae. A: Conidia, B: Conidial drops (false heads) (dry mount), C: Branched and sympodially proliferated polyphialides (sensu Booth).

Table 1. Strains of *Fusarium* investigated for ice-nucleating activity

Species Strain No. ^{a)}	Location	Source of isolation	Year of isolation	Presence of INA ^{b)}
<i>F. acuminatum</i>				
MAFF 235736	Chiba	<i>Abutilon avicennae</i>	1990	N
MAFF 235830	Oita	<i>Zoysia tenuifolia</i>	1989	N
MAFF 236464	Hiroshima	<i>Triticum aestivum</i>	1991	N
<i>F. avenaceum</i>				
MAFF 101040	Hokkaido	<i>Triticum aestivum</i>	1983	A
MAFF 101041	Hokkaido	<i>Triticum aestivum</i>	1983	A
<i>F. equiseti</i>				
MAFF 236434	Hokkaido	<i>Triticum aestivum</i>	1991	N
MAFF 236475	Ibaraki	<i>Triticum aestivum</i>	1990	N
MAFF 236480	Ibaraki	Soil (wheat field)	1990	N
<i>F. graminearum</i>				
MAFF 101031	Kagawa	<i>Triticum aestivum</i>	1979	N
MAFF 101033	Okayama	Soil (wheat field)	1981	N
MAFF 101035	Hokkaido	<i>Triticum aestivum</i>	1983	N
MAFF 235980	Hokkaido	<i>Triticum aestivum</i>	1988	N
<i>F. lateritium</i>				
MAFF 840045	Shiga	<i>Morus</i> sp.	1972	N
<i>F. moniliforme</i>				
MAFF 235949	Ibaraki	<i>Oryza sativa</i>	1991	N
MAFF 236408	Ibaraki	<i>Oryza sativa</i>	1992	N
MAFF 425089	Tottori	<i>Pinus densiflora</i>	1984	A
MAFF 425090	Ibaraki	<i>Cryptomeria japonica</i>	1986	A
<i>F. moniliforme</i> var. <i>subglutinans</i>				
MAFF 236397	Kagoshima	<i>Pinus luchuensis</i>	1992	N
MAFF 236399	Okinawa	<i>Pinus luchuensis</i>	1992	N
<i>F. nivale</i>				
MAFF 101050	Toyama	<i>Hordeum vulgare</i>	1986	N
MAFF 511030	Yamagata	<i>Lolium multiflorum</i>	1976	N
<i>F. oxysporum</i> f. sp. <i>fragariae</i>				
MAFF 305557	Shizuoka	<i>Fragaria grandiflora</i>	1978	N
MAFF 744009	Fukuoka	<i>Fragaria grandiflora</i>	1978	N
<i>F. pallidoroseum</i>				
MAFF 236386	Okinawa	<i>Bambusa</i> sp.	1991	N
MAFF 236521	Ibaraki	<i>Triticum aestivum</i>	1990	N
MAFF 236660	Ibaraki	<i>Oryza sativa</i>	1992	N
MAFF 840048	Tokushima	<i>Morus alba</i>	1973	N
<i>F. poae</i>				
MAFF 236648	Hokkaido	<i>Triticum aestivum</i>	1991	N
MAFF 305947	Shizuoka	<i>Dianthus caryophyllus</i>	1987	N
<i>F. solani</i>				
MAFF 235975	Hokkaido	<i>Solanum tuberosum</i>	1987	N
MAFF 425096	Tokyo	<i>Prunus jamasakura</i>	1985	N
<i>F. solani</i> f. sp. <i>phaseoli</i>				
MAFF 305607	—	<i>Phaseolus vulgaris</i>	—	N
<i>F. solani</i> f. sp. <i>pisi</i>				
MAFF 305125	—	<i>Lathyrus odoratus</i>	—	N
<i>F. sporotrichioides</i>				
MAFF 236639	Hokkaido	<i>Triticum aestivum</i>	1992	N
MAFF 236641	Hokkaido	<i>Triticum aestivum</i>	1991	N
<i>F. tricinctum</i>				
MAFF 235551	Ibaraki	<i>Triticum aestivum</i>	1990	N
MAFF 236637	Hiroshima	<i>Triticum aestivum</i>	1991	A

a) MAFF No: Registered code number in catalogue of microorganisms, 2nd edition (1993), National Institute of Agrobiological Resources, Ministry of Agriculture, Forestry and Fisheries, Japan.

b) Presence of INA

A: Crystallization temperature was above -10°C .

N: Crystallization temperature was below -10°C .

and allied species are presently under discussion. The gut strain formed septate falcate macroconidia and also aseptate obclavate microconidia (Fig. 1-A). These microconidia were formed abundantly in false heads but not in chains (Fig. 1-B). Branched conidiophores and sympodially proliferated polyphialides (sensu Booth) were also produced by the strain (Fig. 1-C). Pascoe^{12,13} has suggested that sympodial mesoconidiophores, forming mesoconidia are observed in *F. subglutinans* (Wollenweber et Reinking) Nelson, Toussoun & Marasas ($\equiv$ *F. moniliforme* var. *subglutinans*) and several other species of *Fusarium*, instead of polyphialides by Booth¹¹. The mesoconidia, mostly septate, were defined to be produced singly and holoblastically from conidiogenous cells, which proliferate sympodially in the aerial mycelium. Then the conidial base becomes truncate without obvious pedicellate morphology. Nirenberg¹⁰, Gerlach & Nirenberg³) and Nelson *et al.*⁹) have also given similar figures for the conidiogenous structure and conidia of the same fungus but used the term "polyphialides". Taxonomically, Gams²) and the German school (Nirenberg¹⁰) and Gerlach & Nirenberg³) have proposed the name, *F. sacchari* (Butl.) W. Gams for the fungus, by a recombination of a species placed formerly in the genus *Cephalosporium*. The German school has recognized further three varieties under the species rank, based on the microdimensional differences: *i.e.* *F. sacchari* var. *sacchari*, var. *subglutinans* (Wollenweber et Reinking) Nirenberg, and var. *elongatum* Nirenberg. On the other hand, American researchers have proposed another name, *F. subglutinans* by the elevation of taxonomical rank⁹). The polyphialidic characters of conidiophores, formation of microconidia in false heads and absence of chlamydospores were still used as very important distinguishing characters for the fungus (species) in both the taxonomical systems, as well as in the work by Booth¹¹. Although the name of the fungus, terminology, and definition of the conidiophore characters are, therefore, still far from the conclusion¹¹), we identified our strain as *F. moniliforme* var. *subglutinans* by following Booth's concept¹¹. Morphological characteristics mentioned above, and cultural features of the strain agreed very well with the description given for

the fungus and with strains preserved in MAFF (235822 and 235823). Microdimension of our strain was somewhat narrower in conidial width, which was approaching the values for *F. sacchari* var. *sacchari* in spite of difference in other cultural features¹⁰).

Search for ice-nucleating active *Fusarium* species

INA was found in two *F. avenaceum* strains, two *F. moniliforme* strains and one *F. tricinctum* strain among the 37 *Fusarium* strains tested (Table 1). INA around -6°C was detected in the mycelial suspensions and supernatants of these five strains after cultivation for 14 days, whereas the absence of INA below -15°C was detected in 32 other *Fusarium* strains after cultivation for 14 or 21 days. Recently, Pouleur *et al.*¹⁴) have also found INA in *F. avenaceum* and *F. acuminatum*. In this study, INA was not detected in *F. acuminatum*, showing that the activity was dependent on the strains even in the same *Fusarium* species. Although *F. moniliforme* var. *subglutinans* isolated from the borer produced ice nuclei, two strains in the same species from the MAFF collections of microorganisms did not. The same facts were also observed in *F. moniliforme* and *F. tricinctum* strains.

Comparison of the activity of ice nuclei produced by *Fusarium* species

INA was compared among *F. avenaceum* (MAFF 101041), *F. moniliforme* (MAFF 425090), *F. tricinctum* (MAFF 236637) and *F. moniliforme* var. *subglutinans* from the borer. INA was detected in the mycelial suspension of all four strains tested after 4 days at 25°C . Furthermore, these *Fusarium* species also produced cell free ice nuclei in the culture filtrate. Ice nuclei produced by *Fusarium* species are composed of protein^{14,17}) and INAs were enhanced with mycelial growth¹⁶). INA per unit protein was measured in the culture filtrates of these four strains (Table 2). The warmest crystallization temperature at which ice nucleation occurred in the culture filtrate of *F. moniliforme* var. *subglutinans* was around -5°C , which was close to the highest temperature of another three *Fusarium* species. Ice nucleation above -10°C was detected when the filtrates were diluted to a protein concentration of $5\text{ }\mu\text{g/ml}$. Furthermore, the crystallization temperatures per each protein con-

Table 2. Effect of protein concentration on crystallization temperatures in culture filtrate of four *Fusarium* species

Species (No.)	Protein concentration ($\mu\text{g/ml}$)				
	0	5	10	40	200
<i>F. moniliforme</i> var. <i>subglutinans</i> ^{a)}	-19.7 ± 0.1	-6.3 ± 0.6	-5.8 ± 0.3	-5.1 ± 0.1	-5.1 ± 0.2
<i>F. avenaceum</i> (MAFF 101041)		$-9.1 \pm 0.9^*$	-6.6 ± 0.4	-5.8 ± 0.6	-5.6 ± 0.5
<i>F. moniliforme</i> (MAFF 425090)		-8.4 ± 0.4	-7.0 ± 0.4	-5.9 ± 0.3	-5.7 ± 0.2
<i>F. tricinctum</i> (MAFF 236637)		-7.6 ± 0.4	-7.0 ± 0.4	-6.4 ± 0.3	-5.8 ± 0.2

a) Strain isolated from the gut of the rice stem borer. Each value is the mean (mean $\pm$ SE) of five replicate samples.

* Significantly different (Student *t* test) from the value of *F. moniliforme* var. *subglutinans* on the same column, $p < 0.05$.

Fig. 2. Effect of subculture on the crystallization temperatures in the supernatant of culture medium. ○: *F. moniliforme* var. *subglutinans*, △: *F. avenaceum* (101041), ●: *F. moniliforme* (425090), ▲: *F. tricinctum* (236637). Bars indicate standard errors ($n=5$).

centration were similar among these four species. However, *F. moniliforme* var. *subglutinans* from the borer grew faster and shedded higher protein in culture filtrate (data not shown), showing that the ability to produce ice nuclei by the fungus is higher than that by another three *Fusarium* species.

The effect of subculture on the production of ice nuclei

Although there was no significant difference in the warmest crystallization temperatures of the mycelial suspensions and supernatants in the four ice-nucleating active *Fusarium* species, differences in the ability to produce ice nuclei during subculture were observed among these *Fusarium* species (Fig. 2). *F. moniliforme* var. *subglutinans* from the borer and *F. tricinctum* (MAFF 236637) maintained the ability to produce ice nuclei after several subcultures. But *F. moniliforme* (MAFF 425090) and *F. avenaceum* (MAFF 101041) reduced or lost the activity during several subcultures. Since INA was found in the later two *Fusarium* species isolated more than 7 years ago (Table 1), their abilities to produce ice nuclei seemed to be stable for a long period. However, they were in fact unstable and disappeared at the second and fifth subcultures, respectively, when they were cultured in liquid medium. Although the mycelia of these species that ceased producing ice nuclei were incubated at 10 or 15°C, which was optimum temperature for the production of bacterial ice nuclei⁷⁾, the characteristic was not recovered. We are cloning the ice nucleation gene in these species. The cause of the rapid reduction of the characteristics during subculture may be revealed hereafter.

In this study, the ice-nucleating active fungus isolated from the gut of the rice stem borer was identified as *Fusarium moniliforme* var. *subglutinans* by morphological comparison with the strains deposited in the MAFF collection of microorganisms. Furthermore, INA was detected in two strains of *F. moniliforme*, two of *F.*

avenaceum and one of *F. tricinctum* among 37 *Fusarium* strains tested. However, *F. moniliforme* and *F. tricinctum* ceased producing ice nuclei within a short period after subculture in liquid medium.

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和 文 摘 要

積木久明・梁井秀樹・青木孝之：ニカメイガ幼虫の消化管から分離された氷核糸状菌の同定と氷核活性 *Fusarium* 属菌の探索
先に著者らは、ニカメイガ幼虫の消化管から氷核活性を有す

る糸状菌 (*Fusarium* 属菌) を初めて分離する事に成功した。しかし、種名までは明らかにできなかった。本実験において、その種名を同定するとともに、農業生物資源研究所が保有する *Fusarium* 属菌の中から氷核活性を有する菌株を探索した。ニカメイガより分離された氷核糸状菌は、*Fusarium moniliforme* var. *subglutinans* と同定された。本糸状菌の菌体懸濁液と無細胞培養液に氷核活性 (凍結温度: -5°C 付近) がみられた。調べた *Fusarium* 属 14 種 37 菌株中、*F. moniliforme* 2 菌株、*F. avenaceum* 2 菌株、*F. tricinctum* 1 菌株に活性がみられた。これらの 5 菌株の氷核活性はニカメイガ由来の菌株とよく似ていた。しかし、*F. moniliforme* と *F. avenaceum* の各 1 菌株は液体培地で継代培養すると短期間で氷核を生産しなくなった。この原因については現在研究中である。