Reassessment of Mycotoxin Productivity of *Microdochium nivale* in Japan

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Abstract

The reports about negation of mycotoxin productivity of *Microdochium nivale* (syn. *Fusarium nivale*) led us to reinvestigate Japanese isolates of *M. nivale*. Thirty isolates were collected from different district of Japan. These were from 5 species of gramineous plants in 1988-1993 and analyzed on their mycotoxin productivity within 2 years. Trials were conducted for combinations of medium (rice and wheat grain) with culture temperature (0°C and 20°C). In addition, we examined samples inoculated artificially on cut-spikes of winter wheat and field samples of orchardgrass infested with *M. nivale*. As a result, we did not detect the known mycotoxins (deoxynivalenol, 3-acetyldeoxynivalenol, nivalenol, fusarenon-X, T-2 toxin, neosolaniol, zearalenone) in all trials. Mycelial growth temperature of *Fusarium* sp. strain Fn-2B, from which nivalenol was firstly isolated, was higher than that of *M. nivale*. Fn-2B was not pathogenic to winter wheat under snow.

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Key words : *Microdochium nivale*, *Fusarium nivale*, mycotoxin, nivalenol.

INTRODUCTION

It is well documented in text books that *Fusarium nivale* (Fr.) Ces. is typical producer of trichothecenes mycotoxin. Nivalenol was first isolated from *F. nivale* isolate Fn-2B recovered from wheat head blight in Kumamoto Prefecture, Japan²¹⁾. Fusarenon and fusarenon-X were detected in the same isolate^{20,23,24)}. Marasas *et al.* reexamined toxigenic strains of *F. nivale* in International Toxic *Fusarium* Reference Collection and reported that Fn-2B should be identified as *Fusarium sporotrichioides* Sherb. (W&R, G, B, J)^{10,11)}. They excluded trichothecene toxin formation from *F. nivale* with 1 exception of isolate NRRL3289, which produced a low level of deoxynivalenol (1 µg/g). However, Logrieco *et al.* could not confirm deoxynivalenol production of NRRL 3289⁹⁾. Classification of *F. nivale* isolate Fn-2B was controversial because of mutations due to periodic transfers over 25 years. Recently, the isolate have been noted as *Fusarium* sp. strain Fn-2B²⁰⁾.

An additional confusion is the change of nomenclature of the genus. *F. nivale* is now considered to be *Microdochium nivale* (Ces. ex Berl. & Vogl.) Samuels & Hallett and its teleomorph is *Monographella nivalis* (Schaffnit) E. Müller^{2,3)}.

Chelkowski *et al.*¹⁾ and Ichinoe *et al.*⁴⁾ reported that *M. nivale* did not produce mycotoxin in Poland and Japan, respectively. However, these previous studies

examined only a few isolates and less attention had been paid to medium and culture conditions for mycotoxin productivity. Logrieco *et al.*⁹⁾ pointed out that it would be important to reevaluate strains of *M. nivale* for ability to produce mycotoxins because this species occurs commonly on wheat and grasses⁹⁾.

The objects of this study were to reexamine mycotoxin in productivity of Japanese isolates of *M. nivale*, taking account of source of isolates and culture conditions. Parts of this work have been reported previously in abstract¹⁵⁾.

MATERIALS AND METHODS

Collection of Microdochium nivale in Japan.

Thirty isolates of *M. nivale* were collected from different district of Japan (Table 1). The fungi were isolated from 5 species of gramineous plants in 1988-1993. Isolation sites were head blight, pink snow mold, foot rot, seed, and perithecia filling life cycle of *M. nivale*. The isolates were analyzed for mycotoxins within 2 years after isolation. *Fusarium* sp. Fn-2B and *Fusarium graminearum* R2118, which were provided from Science University of Tokyo, were used as positive controls of nivalenol^{20,23,24)} and deoxynivalenol⁵⁾ production, respectively.

Medium and culture conditions. The following 6 trials were conducted using combinations of media and incubation temperature. In trials 1 and 2, 300 ml flask,

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containing 30 g polished rice grains which were previously immersed in 15 ml deionized water for 3 hours, was autoclaved at 120°C for 20 min. We used *Fusarium*-free wheat grain instead of rice in trials 3 and 4. All isolates were incubated at 20°C for 14 days in trial 1, at 0°C for 50 days in trial 2, at 20°C for 14 days in trial 3 and 0°C for 50 days in trial 4. Uninoculated media treated in the same way were used as negative controls. In trial 5, 5 isolates of PSM-1, Me-1, 92-P-1, NK-93-8 and ARC2146 were inoculated artificially on cut-spikes of winter wheat at anthesis¹⁸⁾ and incubated at 20°C for 4 days in a moist chamber¹²⁾. In addition, we examined 3 field samples of orchardgrass (*Dactylis glomerata* L.) infested with pink snow mold collected in Iwate Prefecture.

Analysis for mycotoxins. Mycotoxins were examined by the method of Tanaka¹⁹⁾. Briefly, the samples were homogenized with acetonitrile-water (3:1, v/v) and shaken for 30 min by an automatic shaker, and the aqueous acetonitrile extract was filtered. The filtrate was partitioned with n-hexane. The upper n-hexane layer was discarded. The ethanol was added to the aqueous acetonitrile and the mixture was evaporated to dryness under vacuum. The toxins were purified by column chromatography on Florisil. After derivatization by trimethylsilylating reagent, the amounts of seven

mycotoxins (nivalenol, deoxynivalenol, 3-acetyl-deoxynivalenol, fusarenon-X, T-2 toxin, neosolaniol, zearalenone) were estimated by gas-liquid chromatography (GLC). Operating conditions of the GLC analysis were similar to the method of Ueda *et al.*²²⁾ with fused silica capillary column (Shimadzu HiCap CBP 1 column, 0.33 mm id×25 m). The detection limits were ranged from 0.1 to 0.2 µg/g.

Mycelial growth. The mycelial growth rates were determined by placing each 9 mm mycelial disc on PDA (Potato Dextrose Agar, Difco) plate and incubating at 0–35°C. The measurements of the radial mycelial growth lengths were taken every 24 hr and the results were shown by the averages of 5 isolates in *M. nivale* and by the averages of 3 replications in *Fusarium* sp. Fn-2B.

Pathogenicity under snow. *Fusarium* sp. Fn-2B and 9 isolates of *M. nivale* recovered from wheat head blight (Table 1) were tested for pathogenicity to winter wheat (*Triticum aestivum* L.) under snow. Seeds of cultivar Kitakamikomugi which is susceptible to pink snow mold¹³⁾ were sown in a plastic box (350×250×60 mm) at a density of 20 plants in 1 box on 12 October 1992. The experiment consisted of 30 boxes in a completely randomized design including 10 isolates and 3

Table 1. Isolates of *Microdochium nivale* collected in Japan

Isolate	Locality	Date	Source
SII-3	Shirataki, Hokkaido	May, 1990	Wheat, pink snow mold
PSM-5	Bihoro, Hokkaido	Apr., 1992	do.
PSM-1	Iwamizawa, Hokkaido	do.	do.
Kaw-2	Obihiro, Hokkaido	Jul., 1991	Wheat, head blight
Me-1	Memambetu, Hokkaido	do.	do.
Cn8937	Kunneppu, Hokkaido	Jul., 1989	Wheat, perithecia on lower stem
FK-1	Fukaura, Aomori	Jul., 1988	Wheat, head blight
TWY-6	Towada, Aomori	May, 1991	Orchardgrass, pink snow mold
TWY-10	do.	do.	Perennial ryegrass, pink snow mold
TAK-91-1	Takko, Aomori	do.	do.
TAK-91-2	do.	do.	do.
OM-3	Ichinohe, Iwate	Apr., 1991	Wheat, pink snow mold
OM-5	do.	do.	Orchardgrass, pink snow mold
91-E4-2	Morioka, Iwate	Jun., 1991	Wheat, perithecia on lower stem
91-E4-5	do.	do.	do.
91-E4-6	do.	do.	do.
92-P-1	do.	Jul., 1992	Wheat, head blight
92-P-5	do.	do.	do.
92-P-8	do.	do.	do.
92-S-1	do.	Oct., 1992	Wheat, seed
92-S-2	do.	do.	do.
92-S-3	do.	do.	do.
NK-93-8	Kashiwazaki, Niigata	Apr., 1993	Barley, pink snow mold
NK-93-2	do.	do.	do.
MAFF101050	Toyama	1988	Barley, head blight
MAFF236681	Nagano	1992	do.
T9149-1	Chiba, Chiba	Dec., 1991	Bentgrass, pink snow mold
MAFF235733	Ibaraki	1991	do.
ARC2158	Shimotsuma, Ibaraki	May 1990	Barley, foot rot
ARC2164	Tsukuba, Ibaraki	do.	Wheat, head blight

Fig. 1. Gas liquid chromatograms of metabolites by *Microdochium nivale* 92-P-1 produced in rice culture 14 days after inoculation at 20°C. Retention times of standard mycotoxins are represented by ×: deoxynivalenol, ▼: 3-acetyldeoxynivalenol, ∇: nivalenol, ◆: fusarenon-X, ○: T-2 toxin, ●: neosolaniol, and ◇: zearalenone.

Table 2. Pathogenicity to winter wheat, cv. Kitakami-komugi, under snow of *Fusarium* sp. strain Fn-2B and isolates of *Microdochium nivale* recovered from wheat head blight

Isolate	Plant survival ^{a)}	Disease index ^{b)}
Fn-2B	100.0 c	0.0 c
Kaw-2	0.0 a	3.0 a
Me-1	10.0 a	2.5 a
FK-1	15.0 a	2.3 b
92-P-1	0.0 a	3.0 a
92-P-5	33.3 b	2.1 b
92-P-8	40.0 b	1.8 b
MAFF101050	25.0 ab	2.2 b
MAFF236681	33.3 b	1.9 b
ARC2164	10.0 a	2.8 a

- a) Following 70 days incubation under snow, the percent survival of plant and disease index were recorded.
 b) Disease index is based on a 0-3 scale where 0, no visible injury; 1, 50% of leaves green; 2, only stems green; and 3, 100% necrosis.
 c) Values within a column followed by the same letter are not significantly different at $p=0.05$ as determined by Tukey-Kramer's multiple-range test.

replications. The boxes were set outdoors immediately after sowing till inoculation. Ten weeks after seeding (December 21), the naturally hardened plants with 2 to 3 tillers were artificially inoculated and covered with 50 cm snow. Inoculum of wheat bran-vermiculite medium¹³⁾ was spread on the soil surface at a level of 0.06 g/cm². The boxes were taken out after 90 days incubation under snow and transferred to a green house for recovery. After 2 weeks, we assessed visually degree of necrosis with a scale of 0-3 where 0, no visible injury; 1, 50% of leaves green; 2, only stems green; and 3, 100% necrosis¹³⁾.

Fig. 2. Difference in mycelial growth rate at different temperatures between *Fusarium* sp. strain Fn-2B (●) and *Microdochium nivale* (Δ).

RESULTS

Mycotoxin productivity of *Microdochium nivale* collected in Japan

One example of gas liquid chromatograms of extract from rice culture by *M. nivale* 92-P-1 incubated at 20°C is shown in Fig. 1. Retention times of 7 standard mycotoxins (deoxynivalenol, 3-acetyldeoxynivalenol, nivalenol, fusarenon-X, T-2 toxin, neosolaniol, zearalenone) were found in over 30 min. No peaks in over 30 min were observed in all isolates under all trials which combine medium and culture conditions. We did not detect any mycotoxins in samples inoculated artificially on cut-spikes of winter wheat and field samples of orchardgrass infested with *M. nivale*. On the contrary, we confirmed production of nivalenol, deoxynivalenol, 3-acetyldeoxynivalenol, fusarenon-X in positive controls.

Mycelial growth and pathogenicity under snow of *Fusarium* sp. strain Fn-2B

Fusarium sp. strain Fn-2B grew at temperatures rang-

ing from 5 to 35°C (Fig. 2). The optimum growth temperature was 25°C, little higher than that of *M. nivale* isolates. *M. nivale* could grow at 0°C, but not over 30°C and its growth rates under 15°C were much higher than that of the *Fusarium* sp. strain Fn-2B.

All isolates of *M. nivale* recovered from head blight were highly pathogenic to wheat under snow (Table 2). However, *Fusarium* sp. strain Fn-2B did not kill wheat plants nor cause any lesions under snow.

DISCUSSION

In previous reports^{1,4,9-11)} dealing with mycotoxin productivity of *M. nivale*, a few and old isolates have been tested. Rice medium, peptone-supplemented Czapek medium and incubation at 25-27°C were reported as good environment factors affecting mycotoxin production by using *Fusarium* sp. strain Fn-2B²³⁾. These culture conditions were unfavorable for real *M. nivale*. Therefore, we set up six trials for medium and culture conditions on the basis of ecological aspects of *M. nivale*. Optimum temperature of *M. nivale* for infection causing wheat head blight is 20°C¹⁴⁾ and pink snow mold prevails under snow with temperature of near 0°C⁶⁾. However, we could not detect any trichothecene mycotoxin and zearalenone reported in *Fusarium* spp. by other researchers in all trials.

M. nivale is a cryophilic fungus and a causal pathogen of pink snow mold in winter wheat⁶⁾. In previous articles^{1,4,9-11)} on mycotoxins, pathogenicity of *Fusarium* sp. strain Fn-2B under snow has not been studied. We confirmed that all isolates of *M. nivale* recovered from head blight of wheat and barley have pathogenicity to wheat plants under snow^{8,16)}, whereas *Fusarium* sp. strain Fn-2B was not pathogenic under snow and temperature response of mycelial growth of Fn-2B was different from that of *M. nivale*. Recently, Marasas *et al.*^{10,11)} reported that Fn-2B should be identified as *F. sporotrichioides*, while Ichinoe⁴⁾ noted Fn-2B was like *F. tricinctum* for his morphological observation. Our findings only supports that *Fusarium* sp. strain Fn-2B differs from *M. nivale*. Neither conidia nor any another spores were formed in our studies (Nakajima, unpublished). Correct identification might be impossible because the isolate was not maintained under optimal condition and periodical transfers over 25 years.

We conclude from this study that *M. nivale* in Japan does not produce any of the known mycotoxins. In northern Japan, the Hokkaido and the Tohoku district, *M. nivale* predominates when the summer is cool and wet^{7,8,16,25)}. However, other fusaria of *F. graminearum*, *F. sporotrichioides*, *F. poae*, *etc.*, affect wheat head in normal climate year^{4,17)}. We should continue to pay attention to mycotoxin contamination even in northern Japan.

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和 文 摘 要

中島 隆・内藤繁男：日本産 *Microdochium nivale* のマイコトキシン生産能の再評価

Microdochium nivale はマイコトキシン生産菌として知られていたが、nivalenol が初めて単離された Fn-2B 菌株の同定の誤りが指摘され、本来の *M. nivale* のマイコトキシン生産能が疑問視されているため日本の菌株について再検討した。5 種の植物から伝染環に基づいて収集した分離後 2 年以内の 30 菌株を供試し米培地で 20°C・2 週間および 0°C・50 日間、コムギ粒培地で 20°C・2 週間および 0°C・50 日間それぞれ培養した。また、コムギ穂に接種後 20°C・4 日間加湿した接種箱に静置したサンプル、圃場から採取したオーチャードグラス紅色雪腐病の被害茎葉も分析に供試した。その結果、全ての菌株・培養法で既知のマイコトキシン (deoxynivalenol, 3-acetyldeoxynivalenol, nivalenol, fusarenon-X, T-2 toxin, neosolaniol, zearalenone) は検出されなかった。Fn-2B 菌株の菌糸生長温度は *M. nivale* より高温性で、Fn-2B 菌株は積雪下でコムギに対する病原性はなかった。