

Ice Nucleation Activity in *Fusarium acuminatum* and *Fusarium avenaceum*†

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Twenty fungal genera, including 14 *Fusarium* species, were examined for ice nucleation activity at -5.0°C , and this activity was found only in *Fusarium acuminatum* and *Fusarium avenaceum*. This characteristic is unique to these two species. Ice nucleation activity of *F. avenaceum* was compared with ice nucleation activity of a *Pseudomonas* sp. strain. Cumulative nucleus spectra are similar for both microorganisms, while the maximum temperatures of ice nucleation were -2.5°C for *F. avenaceum* and -1.0°C for the bacteria. Ice nucleation activity of *F. avenaceum* was stable at pH levels from 1 to 13 and tolerated temperature treatments up to 60°C , suggesting that these ice nuclei are more similar to lichen ice nuclei than to bacterial ones. Ice nuclei of *F. avenaceum*, unlike bacterial ice nuclei, pass through a $0.22\text{-}\mu\text{m}$ -pore-size filter. Fusarial nuclei share some characteristics with the so-called leaf-derived nuclei with which they might be identified: they are cell free and stable up to 60°C , and they are found in the same kinds of environment. Highly stable ice nuclei produced by fast-growing microorganisms have potential applications in biotechnology. This is the first report of ice nucleation activity in free-living fungi.

Pure water can remain in a liquid phase at temperatures below 0°C in a subcooling state. Until now, only a few species of bacteria and a small number of lichen mycobionts were known to produce biological ice nuclei that initiate the crystallization of subcooled water at temperatures above -5.0°C (12, 18, 21). Ice nucleation-active bacteria are studied extensively since they increase frost damage to many frost-sensitive plants on which they live (19). The search for ice nucleation activity (INA) sources began with investigations to gain a better understanding of climatologic phenomena (6, 33). Now there is a great deal of interest in ice nucleation-active substances for industrial use (22) such as snow making, freeze texturing of foods, and controlling insects in stored grain (5). Very few studies have been done to find INA in fungi (6). Because some lichenized fungi produce ice nuclei (12) and freezing increases root infection by some *Fusarium* species (27, 32), we hypothesized that *Fusarium* spp. could produce ice nuclei. Here we report on INA in two *Fusarium* species and on the potential of using this characteristic in the identification of these species. *Fusarium* nuclei showed a high tolerance to heat and to a wide range of pH, indicating important similarities with the leaf-derived nuclei (LDN) described by Schnell and Vali (30) and with lichen ice nuclei (13).

MATERIALS AND METHODS

Search for fungal INA. Fungal strains of 20 genera from the fungal culture collection of the Agriculture Canada Sainte-Foy Research Station were investigated for INA at -5.0°C (Table 1). Fungal cultures were replicated on potato dextrose agar in petri dishes with a mycelium plug and grown at 23°C in the dark. After 28 days, the cultures were tested for INA by a modified tube nucleation test (26). About 2 cm^2 of

the mycelium mat was scraped off the agar surface and agitated vigorously in 1 ml of sterile 10 mM phosphate buffer (pH 7.0). Forty microliters of the mycelial suspension was transferred into 2 ml of sterile phosphate buffer subcooled at -5°C in a Lauda refrigerating circulator bath (model RC-6; Brinkmann Instrument Co., Rexdale, Ontario, Canada). Freezing of the tube contents at -5.0°C within 10 min was recorded as positive. The absence of ice nuclei in tubes and buffer used in all tests previously had always been observed in assays done at -10°C for 1 h (11). Each strain was tested in two replicates.

Characterization of INA. Fungal INA was further investigated with *Fusarium avenaceum* (strain SRSF 411) and was compared with bacterial INA by using an ice nucleation-active *Pseudomonas* sp. (strain SRSF DEK-1) isolated from the root xylem of alfalfa (*Medicago sativa* L.). A mycelium mat from a culture of *F. avenaceum* grown on potato dextrose agar was suspended in 10 ml of sterile distilled water and shaken vigorously on a vortex mixer. The suspension was plated on potato dextrose agar (1 ml per plate) and incubated in the dark at 23°C for 5 days. The mycelium was then scraped off the agar surface, put in sterile 10 mM phosphate buffer (pH 7.0) to get 10 mg ml^{-1} (fresh mass), and homogenized in a glass tissue grinder. The suspension was then diluted to obtain 1 mg of mycelium ml^{-1} . *Pseudomonas* sp. was grown for 48 h in the dark at 22°C on nutrient agar supplemented with glycerol (2.5% [vol/vol]). The bacterial growth was suspended in 10 mM phosphate buffer (pH 7.0), and the cell concentration was adjusted to 10^8 cells ml^{-1} by using a spectrophotometer.

INAs of bacterial and fungal suspensions were determined by the droplet freezing method (20, 35). Thirty $10\text{-}\mu\text{l}$ droplets of 10-fold dilutions were placed on an aluminum weighing pan (1) coated with paraffin and cooled at the rate of $0.3^{\circ}\text{C min}^{-1}$ to a temperature of -10.0°C on the surface of ethylene glycol-water (1:1 [vol/vol]) in the refrigerating circulator bath. The number of frozen droplets was noted at intervals of 0.5°C . Cumulative nucleus concentrations were calculated

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TABLE 1. Fungi investigated for INA at -5.0°C in 28-day-old cultures

Species	No. of strains tested
<i>Alternaria humicola</i>	1
<i>Alternaria solani</i>	1
<i>Alternaria tenuis</i>	1
<i>Aphanomyces euteiches</i>	1
<i>Ascochyta imperfecta</i>	1
<i>Aspergillus niger</i>	1
<i>Colletotrichum trifolii</i>	1
<i>Cylindrocarpon</i> sp.....	2
<i>Dreschlera phlei</i>	2
<i>Fusarium</i> spp. ^a	
<i>Gliocladium roseum</i>	2
<i>Leptosphaerulina briosiana</i>	2
<i>Mucor circinelloides</i>	1
<i>Mucor plumbeus</i>	1
<i>Penicillium citrinum</i>	1
<i>Phoma medicaginis</i>	2
<i>Phytophthora cryptogea</i>	1
<i>Phytophthora megasperma</i>	1
<i>Pythium irregulare</i>	1
<i>Pythium paroecandrum</i>	1
<i>Pythium ultimum</i>	1
<i>Rhizoctonia solani</i>	2
<i>Sclerotinia trifoliorum</i>	2
<i>Stemphylium botryosum</i>	2
<i>Trichoderma viride</i>	5
<i>Verticillium albo-atrum</i>	2
<i>Verticillium dahliae</i>	1

^a Species tested are listed in Table 2.

as described by Hirano and Upper (11) from the formula of Vali (35), i.e., $N(T) = -\ln(f)V^{-1}$, where $N(T)$ is the concentration of nuclei active at temperature T or warmer, f is the proportion of unfrozen droplets, and V is the volume of individual droplets. The number of nuclei per gram of bacteria was calculated by assuming that the fresh mass of a bacterial cell is 10^{-12} g.

The effects of heat, pH, and filtration on fungal and bacterial INAs were investigated. In each experiment, INA was estimated by the temperature at which 50% of 30 10- μ l droplets of treated suspensions froze (15). For the heat treatments, fungal and bacterial suspensions were subjected for 10 min (13) to various temperatures (from 24 to 90°C) and immediately cooled to 20°C in melting ice; INA was then estimated. To measure the effect of pH, initial suspensions of *F. avenaceum* and *Pseudomonas* sp. were made in sterile deionized distilled water and diluted in different buffers with pHs of 1 to 13 (37) to obtain a final suspension of 1 mg of mycelium ml⁻¹ or 10⁸ bacterial cells ml⁻¹. INA was estimated after 30 min of stabilization. To determine if nuclei were bound to the cell, INAs of fungal and bacterial suspensions were estimated before and after filtration through a 0.22- μ m-pore-size filter (Syrfil-MF; Nuclepore Corp., Pleasanton, Calif.). The experiments on the effects of pH and heat treatments were done twice. The mean and the standard error were calculated.

RESULTS AND DISCUSSION

Search for fungal INA. INA was detected only in the genus *Fusarium* (Table 1). The absence of INA in the genera *Alternaria*, *Mucor*, and *Penicillium* agrees with the results of Fresh (6). Of the 14 *Fusarium* species tested, only *Fusarium*

TABLE 2. Occurrence of INA at -5.0°C in some *Fusarium* species at 28 days

Section	Species ^a	No. of strains with INA/total no. of strains tested
Eupionnotes	<i>F. melanochlorum</i>	0/2
Arachnites	<i>F. nivale</i>	0/1
Sporotrichiella	<i>F. poae</i>	0/5
	<i>F. tricinctum</i>	0/1
Roseum	<i>F. avenaceum</i>	16/16
Arthrosporiella	<i>F. semitectum</i>	0/1
Gibbosum	<i>F. acuminatum</i>	5/5 ^b
	<i>F. equiseti</i>	0/10
Discolor	<i>F. culmorum</i>	0/2
	<i>F. graminearum</i>	0/9
	<i>F. sambucinum</i>	0/5
Liseola	<i>F. moniliforme</i>	0/2
Elegans	<i>F. oxysporum</i> ^c	0/30
Martiella	<i>F. solani</i>	0/17

^a *Fusarium* species were named by the method of Nelson et al. (24).

^b One strain was ice nucleation active at 35 days.

^c Including *F. oxysporum*, *F. oxysporum* f. sp. *medicaginis*, and *F. oxysporum* var. *redolens*.

acuminatum and *F. avenaceum* showed INA (Table 2). This characteristic seems consistent since all tested strains of these two *Fusarium* species were positive independent of their origin or their host (Table 3). Moreover, this characteristic seems stable over time since INA was found in *Fusarium* strains isolated more than 30 years ago (Table 3). Some physiological and biochemical criteria are used for *Fusarium* identification (34), but there is a need for inexpensive methods to give more rapid and reliable identification (3). Although further investigations with more *Fusarium* species and strains from all over the world are needed, we suggest the inclusion of this characteristic to validate the identification of *F. acuminatum* and *F. avenaceum* in the same manner as it is used for pathovar identification of *Pseudomonas* spp. (10). Some ice nucleation-active strains of *F. avenaceum* were previously identified as *Fusarium arthrosporioides* (Table 3) by the method of Wollenweber and Reinking as described by Gordon (8), but this latter species was recently included in *F. avenaceum* (24, 25). INA in the so-called *F. arthrosporioides* supports its placement in the ice nucleation-active species *F. avenaceum*.

The production of ice nucleation-active substances by some *Fusarium* species might also be useful in taxonomic studies as a nonmorphological approach since primary and secondary metabolites (such as mycotoxins) can be used to separate species (3, 34).

Characterization of INA. Cumulative nucleus spectra obtained for *F. avenaceum* and *Pseudomonas* sp. have the same shape, and the concentrations of ice nuclei active at -5.0°C are almost the same for both microorganisms (Fig. 1). The warmest temperature at which ice nucleation occurred for *F. avenaceum* was -2.5°C (Fig. 1), which is close to the highest temperature of lichen INA (-2°C) (13) and slightly lower than that of *Pseudomonas* INA (-1°C; Fig. 1). This indicates a lower efficiency of the fungal INA as compared with bacterial INA under the experimental conditions used.

Fungal INA decreased after exposure, for 10 min, to a temperature of 60°C or higher, whereas bacterial INA decreased in two steps, above 30°C and above 80°C (Fig. 2). Fungal INA was stable at pH levels from 1 to 13, whereas

TABLE 3. Description of ice nucleation-active strains of *F. acuminatum* and *F. avenaceum*

Species and SRSF ^a no.	Origin ^b	Year of isolation	Host
<i>F. acuminatum</i>			
116	Pennsylvania (K. T. Leath, no. 927 ^c)	1974	<i>Medicago sativa</i>
251	La Pocatière, Québec	1975	<i>Medicago sativa</i>
299	La Pocatière, Québec	1981	<i>Medicago sativa</i>
303	Saint-Boniface, Québec	1981	<i>Medicago sativa</i>
368	Sainte-Madeleine, Québec (A. Devaux ^d)	1986	<i>Pisum sativum</i>
<i>F. avenaceum</i>			
47	Guelph, Ontario (G. Pelletier, SRSF)	1970	<i>Daucus carota</i>
140	Alberta (DAOM 74741 ^e)	1960	<i>Trifolium</i> sp.
246 ^f	La Pocatière, Québec	1975	<i>Medicago sativa</i>
250 ^f	La Pocatière, Québec	1975	<i>Medicago sativa</i>
254	La Pocatière, Québec	1975	<i>Medicago sativa</i>
287	Sainte-Foy, Québec	1980	<i>Medicago sativa</i>
293	Joliette, Québec	1981	<i>Medicago sativa</i>
316	Deschambault, Québec	1983	<i>Medicago sativa</i>
345	Bathurst, Ontario (DAOM 170461 ^e)	1978	<i>Pisum sativum</i>
346	Bathurst, Ontario (DAOM 170462 ^e)	1978	<i>Pisum sativum</i>
372	La Présentation, Québec	1986	<i>Glycine max</i>
411	Matane, Québec	1988	<i>Medicago sativa</i>
523	Warwick, Québec (L. M. Bordeleau, SRSF ^g)	1991	<i>Lupinus albus</i>
525	Warwick, Québec (L. M. Bordeleau, SRSF ^g)	1991	<i>Lupinus albus</i>
531	Saint-Jean-sur-Richelieu, Québec	1991	<i>Brassica oleracea</i> var. <i>capitata</i>
532	Québec (J. Mercier, Université Laval ^h)	1990	<i>Brassica oleracea</i> var. <i>italica</i>

^a SRSF, Station de recherches de Sainte-Foy, Agriculture Canada.

^b All places of origin, except Pennsylvania, are in Canada.

^c Leath and Kendall (17).

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^e National Identification Service, Agriculture Canada, Economic Fungi, Centre for Land and Biological Resources Research, Ottawa, Ontario, Canada.

^f These two strains were previously identified as *F. arthrosporioides* sensu Wollenweber and Reinking (8).

^g Mercier et al. (23).

bacterial INA was drastically reduced at pH values below 6 and above 9 (Fig. 3). Part of the *Fusarium* ice nuclei do not seem to be bound to cells because the freezing temperature of 50% of the droplets of the suspension was nearly the same before (-2.3°C) and after (-2.4°C) filtration (data not shown). In contrast, the freezing temperature of 50% of the droplets of the bacterial suspensions was -0.9°C before filtration and below -10.0°C after filtration, confirming that active bacterial nuclei are attached to the cell (19). These results also indicate that fungal INA is more similar to lichen INA than to bacterial INA because lichen nuclei are stable up to 60°C, show a similar stability over the same broad pH range, and pass through a 0.22- μ m-pore-size filter (13). The teleomorph (*Gibberella* spp.) of the two ice nucleation-active *Fusarium* spp. belongs to Ascomycotina, to which the ice nucleation-active lichen mycobionts also belong. Therefore, lichen INA and *Fusarium* INA could have a common origin.

Ice nuclei of *F. acuminatum* and *F. avenaceum* could be a

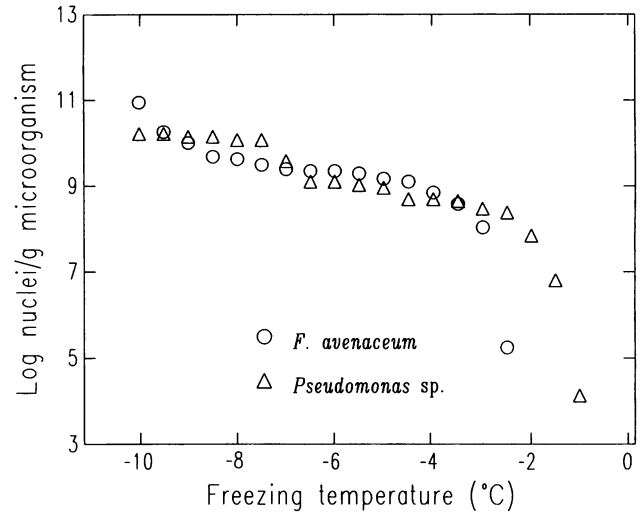


FIG. 1. INA spectra of *F. avenaceum* and *Pseudomonas* sp. The logarithm of the cumulative number of ice nuclei per gram of microorganisms is shown for decreasing temperatures.

source of the so-called LDN observed in decomposing leaf litter and described by Schnell and Vali (30). The nature of LDN is not known, but it was suggested that highly active and unstable bacterial nuclei are precursors of LDN, which are active at lower temperatures but are more stable (36). As for *Fusarium* ice nuclei, LDN tolerate heating up to 60°C and pass through a 0.22- μ m-pore-size filter (31). Another observation supports the hypothesis that *Fusarium* ice nuclei could be the source of LDN. *F. acuminatum* and *F. avenaceum* are both soilborne fungi and are more prevalent in temperate or cold climates (4, 14), where LDN concentrations are the highest (31). Schnell and Vali (30) observed that LDN are present in decomposing litter and that the abundance of nuclei is related to the climate prevailing where the samples came from (30). They suggested that the microflora

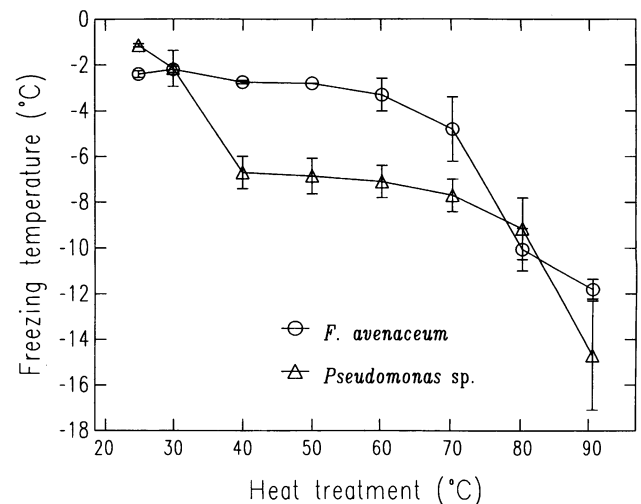


FIG. 2. Effect of 10-min heat treatment on INAs of *F. avenaceum* and *Pseudomonas* sp. INA is estimated by the freezing temperature of 50% of droplets containing the tested microorganisms. The vertical bars represent the standard errors of the means of the freezing temperatures.

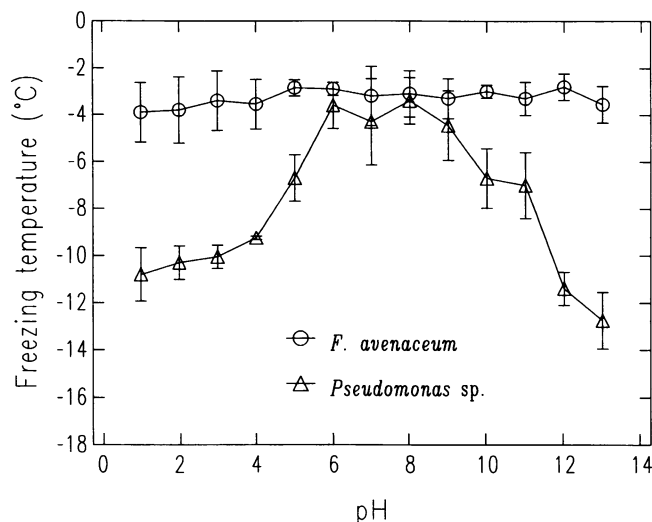


FIG. 3. Effect of pH on INAs of *F. avenaceum* and *Pseudomonas* sp. INA is estimated by the freezing temperature of 50% of droplets containing the tested microorganisms. The vertical bars represent the standard errors of the means of the freezing temperatures.

responsible for the decomposition of organic matter is influenced by the climate and could be a factor in LDN production (30).

F. acuminatum and *F. avenaceum* are widespread pathogens causing mainly cortical rot of the underground part of stressed plants (2). These fungi easily infect roots wounded by freeze stress (27, 28, 32). Moreover, *F. avenaceum* can grow at subzero temperatures (16) and is able to infect some vegetables during storage (7, 23). We observed that macroconidia (produced in culture on potato dextrose agar) of the two ice nucleation-active *Fusarium* species, free from any visible mycelium, were ice nucleation active. Ice nucleation-active spores might cause minute lesions and penetrate their host during light frost as do ice nucleation-active bacteria (19). The ability to produce nuclei may give an ecological advantage to *Fusarium* spp. as has been suggested for lichens (12). Nucleation of ice by spores is likely to attract free water and moisture from the surrounding environment during the freezing process, providing water for their germination when the ice melts.

Since there are *Fusarium* spores in the atmosphere up to 6 km in altitude (9), it is possible that they contribute to cloud formation as do other INA sources (29).

INA is a new characteristic that can be used in the identification of *Fusarium* species and in the study of their ecology. The production of stable ice nuclei by fast-growing microorganisms such as *F. acuminatum* and *F. avenaceum* may be useful in food industry, insect control, or other commercial applications. The study of this type of nuclei should help explain the biological nucleation phenomenon.

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