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Spores of most common airborne fungi reveal no ice nucleation activity

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Abstract

Fungal spores are ubiquitous biological aerosols, which are considered to show ice nucleation (IN) activity. In this study the respective IN activity was tested in oil emulsion in the immersion freezing mode. The focus was laid on species of economical, ecological or sanitary significance. For the first time, not only common moulds, but also edible mushrooms (Basidiomycota, Agaricomycetes) were investigated, as they contribute massively to the total amount of fungal spores in the atmosphere. Only *Fusarium avenaceum* showed freezing events at low subzero-temperatures, while the other investigated fungal spores showed no significant IN activity.

Furthermore, we selected a set of fungal strains from different sites and exposed them to occasional freezing stress during cultivation. Although the total protein expression was altered by this treatment, it had no significant impact on the IN activity.

1 Introduction

Ice nucleation (IN) induced by primary biological aerosol particles, which include pollen, bacteria, and spores of fungi, is a current topic of intense debate. Several bacterial species (*Pseudomonas syringae*, *Pseudomonas putida*, *Erwinia herbicola*) were already found to catalyse ice formation at temperatures as warm as about 271 K, which is much higher than for most organic or inorganic substances (e.g. Gurian-Sherman and Lindow, 1993; Morris et al., 2004; Vali et al., 1976; Warren and Wolber, 1991). Microorganisms, which inhabit plant surfaces, are able to initiate the ice formation and cause frost injury to frost-sensitive plants (Gurian-Sherman and Lindow, 1993) what resolves in a negative impact on agricultural crops. However, ice nuclei are useful in ice nucleation-limited processes such as artificial snow production, the freezing of some food products, and possibly in future weather modification schemes (Gurian-Sherman and Lindow, 1993). In the past, several researchers studied non-bacterial ice nucleation and found high potential ice nuclei in some lichen (Kieft, 1988; Kieft and Ahmad-

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jian, 1989; Kieft and Ruscetti, 1990) and some species of a plant pathogenic fungus *Fusarium* (Pouleur et al., 1992; Hasegawa et al., 1994; Tsumuki and Konno, 1994; Tsumuki et al., 1995). It was proposed that these ice nuclei are proteins, but have little in common with the well-known bacterial ice nucleation proteins. For example, they are considered to be purely proteinaceous, while the bacterial IN are lipoglycoprotein-complexes. Furthermore, they are more tolerant towards heat and acid treatment. Finally, they are not anchored in the wall, but can be easily washed off (Kieft and Ruscetti, 1990; Hasegawa et al., 1994; Tsumuki and Konno, 1994). Only very recently, IN activity was found in other fungal species, namely *Isaria farinosa* and *Acremonium implicatum* (Huffman et al., 2013). The characteristics of these new found ice nuclei have not been resolved yet.

Nevertheless, there are still open questions and discords about ice nucleation caused by fungi. For example, the mycelia of many fungal species have been investigated, but only in rare cases the fungal spores (Jayaweera and Flanagan, 1982; Pouleur et al., 1992; Iannone et al., 2011), which are of higher importance for atmospheric implications. Another point of discussion is the divergence between results of former studies. Although other workgroups already investigated several different fungal species (Kieft, 1988; Kieft and Ruscetti, 1989; Pouleur et al., 1992), some common moulds, which are of economical, ecological or sanitary importance, like most *Aspergillus*, *Penicillium* and *Trichoderma* species as well as the whole group of mushrooms (Agaricomycetes, Basidiomycota, Dikarya) – certainly all available in the planetary boundary layer – were not studied before. Most relevant among the Basidiomycota is the Agaricomycetes class, to which most of the edible mushrooms belong. According to recent studies, the spores of Agaricomycetes could comprise about 56 % of all fungal spores in the atmosphere (Fröhlich-Nowoiski et al., 2009). Because of of the high presence of fungal spores on the one hand (Bauer et al., 2002) and the scarcity of experimental data about their IN behaviour on the other, estimations about the atmospheric impact of fungal spores are difficult to be made. So it is still an open question, whether spores of fungi are of no importance for atmospheric ice nucleation at all, if

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they carry a huge potential that simply has not been detected up to now in scientific studies, or if the truth lies in between.

At last, the atmospheric concentration of fungal IN is still debated. On the one hand, they appear in far lower numbers than bacteria or mineral dust particles (Hoose et al., 2010; Sesartic et al., 2013). According to current climate models, the global impact of fungal spores is negligible (Sesartic et al., 2013). On the other hand, local events, for example rainfall in a forest area, can boost biological IN concentrations to significant levels (Huffman et al., 2013).

In this study we measured a broad spectrum of fungi in order to close the most obvious gap and to gain a greater knowledge of some important species, which have not been studied up to now, as well as *Fusarium avenaceum*, of which the IN activity is already known.

2 Materials and methods

2.1 Species, cultivation and sampling design

We chose the set of samples depending on four criteria: (1) species which had not been analysed concerning their IN activity before, (2) two well-studied *Fusarium* species in order to be able to compare our results with other published studies, (3) species which are abundant in the atmosphere, such as Agaricomycetes (Fröhlich-Nowoiski et al., 2009), *Cladosporium* (Schüller, 2008; Ariya et al., 2009) and typical food moulds (*Aspergillus* and *Penicillium*), (4) species that have been found beyond the tropopause, like *Engyodontium album*, *A. niger* and *P. chrysogenum* (Imshenetsky et al., 1978; Pearce et al., 2009). Strains used in this study and their origin are presented in Table 1.

The spores of the Basidiomycota, namely *Agaricus bisporus albidus* (white button mushroom), *Agaricus bisporus aveallaneous* (creme button mushroom), *Lentinula edodes* (shiitake), *Pleurotus ostreatus* (oyster mushroom) and *Psilocybe cubensis* (magic mushroom) were purchased from the Mushroom Research Centre of Austria.

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Furthermore, cultures of *A. fumigatus*, *A. niger*, *A. oryzae*, *C. cladosporioides*, *C. herbarum*, *E. album*, *F. avenaceum*, *F. oxysporum*, *P. chrysogenum*, *P. digitatum, P. glabrum*, *T. atroviride*, *T. virens*, *T. longibrachiatum* and *T. reesei* were obtained from the University of Natural Resources and Life Sciences in Vienna (Austria) and TU Collection of Industrial Microorganisms (TUCIM; Vienna University of Technology, Vienna, Austria). Additionally we sampled *A. niger*, *P. chrysogenum* and *P. citrinum* spores from decomposed food products (bread, carrot, lemon). The glycerine cultures of the strains are stored at 193 K in the TUCIM.

The obtained strains were cultivated on plates with different nutrient media in order to determine the optimum growth conditions. We applied malt extract agar (Sifin), potato dextrose agar (Sifin and Difco), synthetic nutrient-poor agar (Niernberg et al., 1979) and overboiled rice (Pathre and Mirocha, 1978) as nutrient media. We achieved the greatest success in sample augmentation by cultivation on 3 % potato dextrose agar (PDA, Difco) and incubation for seven days at 298 K in 12 h illumination cycles. Spores were then harvested using two different approaches. First we removed the fungal spores by gently beating a turned-around plate and collected them on a clean sterile Petri dish cover. The advantage of this method is that the sample is not in contact with any equipment except for the collecting dish, so the risk of contamination is negligible. However, the efficiency of this technique is rather low and inappropriate for species which produce fewer or tighter bound spores. In the second approach about 10 mL ethanol was poured on the culture. The spores were then scratched off with a Drigalski speedle, and the suspension was filtered through glass wool, to separate the spores from the mycelia. The sustained spore suspension was then centrifuged, the supernatant is decanted, and finally the spore pellet was air dried.

To investigate the effect of occasional freezing, a set of fungal strains (Table 2) was cultivated on four plates each: two of plates were grown as before at 298 K, while the two other were exposed to occasional freezing events, meaning that they were left in the freezer for 12 h. Then one plate of both procedures was harvested. The acquired spores were then tested for their IN activity.

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2.2 Ice nucleation measurements

We applied a cryo-microscopic setup, as described in Pummer et al. (2012), to determine the IN activity of the spores. Therefore a water-in-oil emulsion consisting of 440 mg g^{-1} paraffin, 110 mg g^{-1} lanolin (water-free grade) and 450 mg g^{-1} MilliQ® water was spiked with fungal spores. A spore concentration of about 20 mg mL^{-1} emulsion was chosen, so that all droplets contained several spores, and to make the data comparable with former bioaerosol measurements (Pummer et al., 2012). The so-prepared sample was placed in a cryo cell, which was cooled by a Peltier stage at a cooling rate of about 2 K min^{-1}, where it was observed with a microscope at 200 × total magnification. The number of frozen droplets was counted and plotted against temperature to generate a nucleation spectrum. The temperature with 50 % of all droplets frozen, known as the median freezing temperature T_{50}, was chosen to compare different samples with a numeric value.

Additionally, the surface morphology of spores was investigated by scanning electron microscopy with a FEI Quanta™ 200 FEGSEM.

2.3 Protein quantification assay

The plates left from Sect. 2.1 were harvested analogously, but in water as solvent instead of ethanol. The suspensions were then investigated with the Bradford method (Bradford, 1976) to determine the total protein content. Therefore, spore suspensions were diluted to a given concentration by measuring the light transmission. A vial of pure water (100 % transmission) was prepared, and spores suspensions were slowly added, until the transmission dropped to 66–68 %. Then 1 mL of the suspension was mixed with 0.5 mL of Bradford solution, which was prepared by diluting commercially available BioRad Protein Assay Dye Reagent Concentrate to the fivefold volume. For each sample and the blank, three vials were prepared and measured. We measured the absorbance at 565 nm to quantify the total protein content. The correlation between total protein content and T_{50} from Sect. 2.2 is plotted in Fig. 2.

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3 Results and discussion

3.1 General part

Our measurements affirmed that fungal IN activity seems to be limited to only a few species. All but one strain of fungi tested in this study showed no or negligible IN activity, which is far less than found for several other bioaerosol species (Pummer et al., 2012). *Fusarium avenaceum* shows high activity in our measurements, which is comparable to Snomax™ activity. *Lentinula edodes* shows mediocre IN activity, comparable to that of average pollen IN and below that of the relatively active mineral dusts kaolinite and ATD (Pummer et al., 2012). However, as it is one individual sample that was measured in this study, it is questionable, if *Lentinula edodes* species is generally a better ice nucleus than most other species. All other fungal spores can be considered IN-inactive, since an increase in nucleation temperature by some Kelvin can be caused by many kinds of heterogeneous surfaces.

We applied spore concentrations of 20 mg mL^{-1} in our study. In our previous study (Pummer et al., 2012) we had pollen concentrations of 50 mg mL^{-1}. As average fungal spores diameters are about a tenth of pollen diameters, the volume ratio is about a factor of 1000. Assuming a similar density of all our cellular samples, one would expect that a spore concentration of 0.05 mg mL^{-1} would be comparable. However, due to the high hydrophobia of fungal spores, many of them are lost into the oil phase of the emulsion, so far higher concentrations are necessary in our experiment. Furthermore, since most fungal spores did not show IN activity at all, an overdosing is of less concern than an underdosing. In the second case, the IN inactivity could be the result of low concentrations, while IN inactivity in the first case is only explicable by the absence of IN on the fungal spores.

The basic problem of fungal IN measurements is the fact that the same species can show different behavior in different studies, as they can lose their IN activity after in vitro maintenance as a result of changes in gene regulation (Tsumuki et al., 1995), what makes comparability with other studies difficult. For example, we could reproduce

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the loss of IN activity of *Fusarium avenaceum* culture within time. Tsumuki et al. (1995) determined the loss of IN activity in the second (*F. moniliforme*) to fifth (*F. avenaceum*) subculture when growing them in liquid media. Our *F. avenaceum* cultures, which were grown on agar plates, stayed active for more than ten generations. Transcription regulation, like the shutting on and off of a gene, is an important, but very complex tool of evolution to generate biological diversity (Levine and Tjian, 2003). Furthermore the setup parameter themselves, like the IN content per droplet, have an impact on nucleation temperature. Therefore the results of a study have to be compared to well-known reference points, like the median freezing temperature of Snomax™ at given concentration or sample-free water.

3.2 IN activity of fungal spores does not depend on protein concentration

The Bradford assay demonstrated the impact of freezing stress during sporulation on the total protein content of spores. While there was a reduction in protein content of *Fusarium* spores as a response to stress, the *T. atroviride* strains were not affected by it. The Antarctic *T. longibrachiatum* species even increased its protein content in response to cryo-stress. It might be that this certain strain is adapted to dwell in cold environments.

As a whole, no correlation between total protein content and IN activity is visible. It has to be considered that the T_{50}-axis in Fig. 2 shows only a very narrow temperature range, so in fact all species show more or less the same median freezing temperature.

It can be seen in the SEM captures that despite the enormous differences in surface topology, the differences in IN activity are negligible. This way we can exclude that ice nucleation can be caused by topology alone.

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4 Conclusions

It seems that fungal ice nucleation is an exclusive property restricted to a few closely related species of fungi. Nevertheless, further investigation with more species might be necessary to state this claim undoubtedly. The purpose of fungal IN expression could be either a cryoprotective mechanism like in lichen (Kieft and Ahmadjian, 1989), or a tool of causing frost injury to plants, thus making nutrients more available for phytopathogenic moulds like *Fusarium* species (Richard et al., 1996). The same process has already been observed for IN-active bacteria (Lindow, 1983). As *Fusarium* and lichen species typically grow under cold, harsh outdoor conditions, adaptation by IN expression seems a sensible strategy. On the other hand, the typical moulds affecting food storages, like *Aspergillus* and *Penicillium*, are exposed to far more convenient life conditions, what could explain IN inactivity.

Iannone et al. (2011) suggested that the poor ice nucleation ability of *Cladosporium* spores may be attributed to the surface which is coated with hydrophobins. These are small (about 10 kDa) cysteine-rich amphiphilic proteins unique for filamentous fungi (Wösten et al., 1995; Frischmann et al., 2013). As they are much smaller than the known ice nucleation proteins, their activity principally has to be negligible, since an IN active center is the more effective, the more active surface it facilitates (Warren and Wolber, 1991). The four *Trichoderma* species investigated in this study (*T. virens*, *T. atroviride*, *T. reesei* and T. *longibrachiatum*) have the profound expansion of hydrophobin genes in their genomes compared to the other species, including members of the *Fusarium* genus (Kubicek et al., 2011). Moreover the transcriptomic study on genes involved in conidiation in these fungi confirmed the high level of hydrophobins expression asssociated with the conidiation (Metz et al., 2011). As none of the four species in this study showed any considerable IN activity in our study, hydrophobins might be correlated with poor ice nucleation activity.

As *F. avenaceum* has an outstanding IN activity in comparison to other species investigated in this study, it is most probable that fungal IN activity is caused by only a few

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specific proteins or other macromolecular compounds. Bacterial IN can be derived from one protein that only slightly varies among IN-positive species (Lindow, 1995). Furthermore, this IN protein is only expressed by a small fraction of known bacterial strains. As the situation seems to be analogous in fungi, their IN activity could be correlated with one defined sequence on the genome. Unfortunately, the genome of *F. avenaceum* is not fully sequenced, so for further progress on this topic a full sequencing is of crucial importance.

The occurrence of freezing events had no impact on the fungal IN activity. This could mean either that other parameters are more important for expressing of IN (e.g. starvation), or that a combination of factors is necessary. Furthermore, one might increase IN activity by overexpression of suitable proteins in a fungal isolate.

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Table 1. Origin and properties of studied *Ascomycota*.

Species	StrainNo.	Origin	Source of isolation	Date
Fusarium avenaceum	MA 1220	Austria	paprika rhizoplane	Nov 97
F. oxysporum	MA 1085	Austria	–	–
	TUCIM 4301			
F. sp.	TUCIM 4237	Austria	–	
	TUCIM 4682	–	rye	
	TUCIM 4686	–	rye	
Aspergillus oryzae	MA 71	–	–	Aug 93
A. fumigatus	MA 753	Tansania	lodge	Apr 97
A. niger	MA 1239	Austria	marzipan praline	Jan 98
	IMI 041874	United States	–	–
	–	Austria	bread	Sep 11
	–	Austria	carrot	Sep 11
Penicillium chrysogenum	MA 265	–	–	Nov 94
	–	Austria	bread	Sep 11
P. citrinum	–	Austria	lemon	Mar 12
P. digitatum	MA 602	Austria	–	–
P. glabrum	TUCIM 3351	–	–	–
Trichoderma atroviride	TUCIM 626	Ethiopia	–	–
	TUCIM 1680	Slovenia	soil	–
	TUCIM 3001	Israel	marine	–
T. longibrachiatum	TUCIM 1301	Antarctica	–	–
T. reesei	QM 6a	Salomon Islands	cotton duck shelter	–
	TUCIM 917			
T. virens	TUCIM A134	–	soil	–
Cladosporium cladosporioides	MA 890	Austria	gypsum	–
	TUCIM 4298			
C. herbarum	MA 1065	Austria	–	–
Engyodontium album	MA 2848	Austria	limestone	–

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Table 2. A list of T_{50} values for different ice nuclei. Values of references are taken from Pummer et al. (2012). The column labeled with *N* marks the data of naturally grown fungal samples. *d* is the longitudinal diameter of a single spore, *C* is the total protein concentration. Columns marked with an asterisk show results of cultures that were exposed to freezing stress during cultivation. No error bars were given for *C*, because it was only 1–2 µg mL^{-1} with exception of strain number 0626, where it was 4–5 µg mL^{-1}.

Group	Species	TUCIM #	*d* [µm]	T_{50} [K]	T_{50} [K] *	*C* [µg mL^{-1}]	*C* [µg mL^{-1}] *	T_{50} [K] N
Ascomycota	*F. avenaceum*		7	264				
	F. oxysporum		7	238				
	F. sp	4237	–	237	238	173	144	
	F. sp	4682	–	237	237	153	124	
	F. sp	4686	–	238	237	145	113	
	A. oryzae		3	242				
	A. fumigatus		3	239				
	A. niger		3	237				239
	P. chrysogenum		3	236				239
	P. citrinum		3					237
	P. digitatum		3	240				
	P. glabrum		3	237				
	T. atroviride	626	4	239	237	99	96	
	T. atroviride	1680	–	238	238	102	99	
	T. atroviride	3001	–	240	239	79	93	
	T. longibrachiatum		–	240	238	77	110	
	T. reesei		3	237				
	T. virens		4	237				
	C. cladosporioides		6	237				
	C. herbarum		6	237				
	E. album		3	237				
Basidiomycota	*Lentinula edodes*		4	247				
	Agaricus bisporus avellaneous		6	243				
	Agaricus bisporus albidus		6	241				
	Pleurotus ostreatus		5	240				
	Psilocybe cubensis		9	238				
references	Snomax™			268				
	Betula pendula			254				
	Blank			237				

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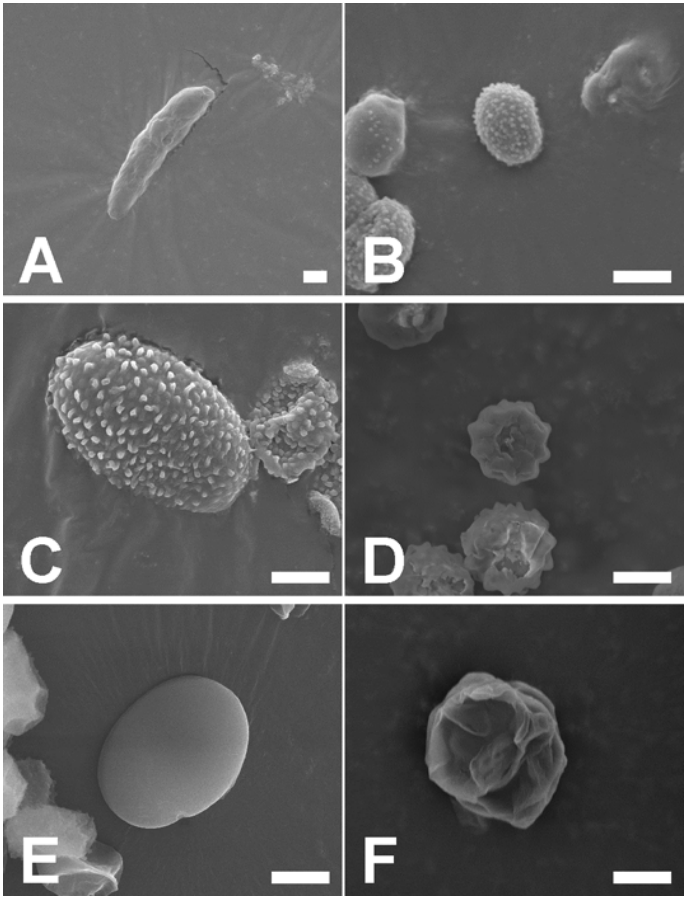

Fig. 1. Scanning electron microscopy images of some fungal spores: *Fusarium avenaceum* **(A)**, *Trichoderma atroviride* **(B)**, *Cladosporium herbarum* **(C)**, *Aspergillus niger* **(D)**, *Agaricus bisporus* **(E)** and *Lentinula edodes* **(F)**. The white bar represents a length of 2 µm and is valid for all pictures.

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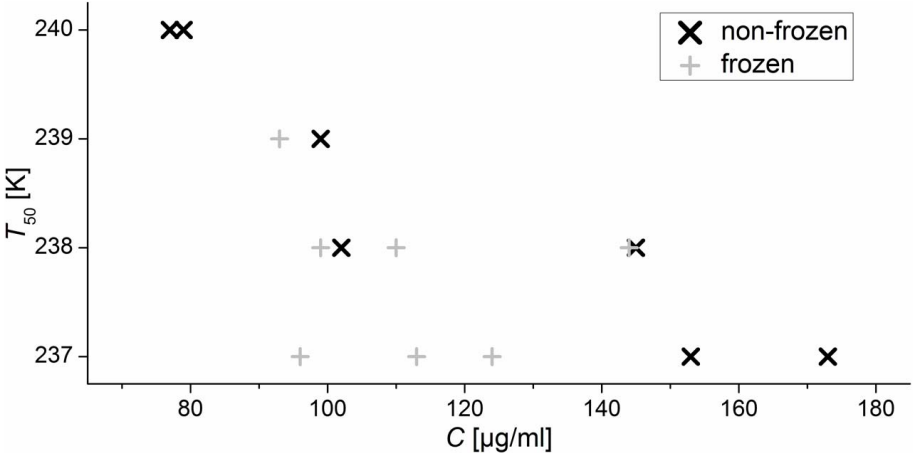

Fig. 2. Correlation between total protein content C and median freezing temperature T_{50}. Note the narrow range of the y-axis, which shows that the impact of protein concentration is negligible.

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