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Ice nucleation activity in the widespread soil fungus *Mortierella alpina*

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Abstract. Biological residues in soil dust are a potentially strong source of atmospheric ice nuclei (IN). So far, however, the abundance, diversity, sources, seasonality, and role of biological – in particular, fungal – IN in soil dust have not been characterized. By analysis of the culturable fungi in topsoils, from a range of different land use and ecosystem types in southeast Wyoming, we found ice-nucleation-active (INA) fungi to be both widespread and abundant, particularly in soils with recent inputs of decomposable organic matter. Across all investigated soils, 8 % of fungal isolates were INA. All INA isolates initiated freezing at −5 to −6 °C, and belonged to a single zygomycotic species, *Mortierella alpina* (*Mortierellales*, *Mortierellomycotina*). To our knowledge this is the first report of ice nucleation activity in a zygomycotic fungi because the few known INA fungi all belong to the phyla *Ascomycota* and *Basidiomycota*. *M. alpina* is known to be saprobic and widespread in soil, and *Mortierella* spores are present in air and rain. Sequencing of the ITS region and the gene for γ-linolenic elongase revealed four distinct clades, affiliated to different soil types. The IN produced by *M. alpina* seem to be proteinaceous, < 300 kDa in size, and can be easily washed off the mycelium. Ice nucleating fungal mycelium will ramify topsoils and probably also release cell-free IN into it. If these IN survive decomposition or are adsorbed onto mineral surfaces, their contribution might accumulate over time, perhaps to be transported with soil dust and influencing its ice nucleating properties.

1 Introduction

Soil organic matter has long been proposed as a source of atmospheric ice nuclei (IN), and biological IN can dominate the fraction active at warmer temperatures (Schnell and Vali, 1972, 1976; Conen et al., 2011; O'Sullivan et al., 2014). When soils dry, small particles are liable to be aerosolized (Sing and Sing, 2010); soil dust emissions to the global atmosphere are estimated to be in the range of 500 to 5000 Tg a^{-1} (Goudie and Middleton, 2001). This makes large areas of the global landmass potentially strong sources of atmospheric biological IN, especially when the uplifting of dust by agricultural activities such as ploughing and harvesting is considered.

However, the sources and characteristics of biological IN produced and released by soils are poorly understood, and their contribution to the pool of the atmospheric IN remains unclear, even though their role in triggering glaciation and precipitation has recently been supported (Pratt et al., 2009; Creamean et al., 2013). Indeed, it has been suggested that most IN active at warmer than −15 °C in clouds could be biological particles (DeMott and Prenni, 2010).

Several diverse bioaerosol types, including bacteria, fungi, pollen, and lichen, have been identified as sources of biological IN, with some able to initiate the formation of ice at relatively high temperatures (Vali et al., 1976; Kieft, 1988; Pouleur et al., 1992; Diehl et al., 2001; Morris et al., 2004; Christner et al., 2008; Bowers et al., 2009; Georgakopoulos et al., 2009; Iannone et al., 2011). The best known are species of common plant-associated bacteria from the genera

Pseudomonas, *Pantoea*, and *Xanthomonas* (all within the γ-*Proteobacteria*). The ice nucleation activity of these bacteria is due to a protein embedded in the outer cell membrane, for which the corresponding gene has been identified and fully sequenced (Warren, 1995). In contrast, for ice-nucleation-active (INA; i.e., inducing ice formation in the probed range of temperature and concentration) eukaryotes, much less is known about the nature of their IN. For example, for some known species of INA fungi (Pouleur et al., 1992; Richard et al., 1996) – several species of *Fusarium* – there are indications that their IN are also proteinaceous (Hasegawa et al., 1994; Tsumuki and Konno, 1994: Humphreys et al., 2001). Similarly, the sensitivity of lichen mycobiont IN (Kieft, 1988) to protein-degrading treatments and heating $>70\,^{\circ}\mathrm{C}$ suggests that a similar molecular class is responsible (Kieft and Ahmadjian, 1989; Kieft and Ruscetti, 1990). However, other classes of molecules have also been shown to be INA. For example, an analysis of more than a dozen species of pollen showed that the IN are soluble macromolecules located on the grains, and that they show non-proteinaceous characteristics (Pummer et al., 2012). Furthermore, studies of IN in fluids of succulent plants point at saccharide compounds as being the INA sites (Krog et al., 1979; Goldstein and Nobel, 1991, 1994).

So far, only a few ascomycotic and basidiomycotic fungal species have been reported as being INA (Jayaweera and Flanagan, 1982; Kieft, 1988; Pouleur et al., 1992; Richard et al., 1996; Haga et al., 2013; Morris et al., 2013), but this is likely to rise significantly when systematic surveys of ice nucleation activity by soil or phylloplane fungi are undertaken. In soil, the typical decomposer community, which accounts for half a percent to a few percent of the soil organic matter (Wardle, 1992; Zak et al., 1994; Fierer et al., 2009), is often dominated by fungi; estimates of the average proportions of fungi in the total microbial biomass range from 35–75 % in arable/grassland soils, to 47–70 % in forest soils, and to 64–76 % in litters (Joergensen and Wichern, 2008). Ice nuclei produced by soil fungi may occur as living and recently dead hyphae, spores, cell-free IN, and even as a constituent of the soil organic matter if the biomolecules are more enduring than the fungal tissue or are adsorbed onto soil organic matter or clay.

Currently, little is known of the sources, abundance, spectra of IN activities, seasonality, and, ultimately, the overall contribution of fungal IN to the large pools of biological IN in most soils. By extension, we know even less about their influence in the atmosphere. Thus, the objective of this study is a regional investigation of the identity and relative abundances of culturable INA fungi in topsoils, an essential base for improving our understanding of the effects of microorganisms on climate and the hydrological cycle.

2 Material and methods

2.1 Sampling

In March 2011, five soil samples were collected from the University of Wyoming's Agricultural Experimental Station (SAREC) near Lingle, Wyoming, USA. Three samples were obtained from plots cropped to different broadleaf crops in an irrigated field, a fourth from a plot under fallow in an irrigated and organically managed field, and a fifth from a section of unmanaged roadside pasture. In May 2011, soil was sampled from native grassland and from beneath a lodgepole pine forest near Centennial, Wyoming (Table 1a, b).

At each plot or site, three replicate soil samples were obtained. Each was obtained from a separate 10×10 m area, and within each area three cores (5 cm depth and ≈ 10 cm in diameter) were retrieved and mixed together on site. Samples were stored at $4\,^{\circ}\mathrm{C}$ for less than a week before being thoroughly mixed immediately before soil dilution plating.

2.2 Cultivation

For cultivation of the soil fungi, dilution series were made using 0.45 µm pore diameter filtered 0.01 M PO_4 buffer (pH 7.0) and 0.1 % peptone (Difco Proteose Peptone No. 3; Becton, Dickinson and Company, USA). Two hundred and fifty microliters of dilutions 10^{-2}–10^{-6} were plated onto dextrose–peptone–yeast extract (DPY) solid medium (see below), and colonies were allowed to grow for 3–7 days at room temperature (RT, 22–24 °C) before being picked, using sterile pipette tips, into 100 µL aliquots of 0.2 µm pore diameter filtered DPY broth in sterile 96-well polypropylene PCR plates (VWR, USA), which were incubated at 16 °C for 7–10 days. After the first aliquot was tested, as described below, fresh DPY broth was added and the cultures were tested again after 20–30 days of incubation. Out of 489 picked colony forming units (CFUs), 474 showed growth in the liquid medium and were thus tested for ice nucleation activity.

We originally intended to grow the isolates on malt extract agar. However, since the available product was found to contain some IN (active at $-12\,^{\circ}\mathrm{C}$), an approximate equivalent using IN-free ingredients (tested to $-18\,^{\circ}\mathrm{C}$) was constructed. This DPY broth/solid medium contained 10 g L^{-1} dextrose (VWR, USA), 3 g L^{-1} peptone (as detailed above), and 0.3 g L^{-1} yeast extract (Merck, USA) filtered through a 0.2 µm pore diameter filter (PES disposable filter units, Life Science Products, USA). For the solid medium, 15 g L^{-1} agarose (Certified Molecular Biology Agarose, Bio-Rad, USA) was added, since standard agar was also found to contain IN. Broth and solid medium were sterilized by autoclaving at 121 °C for 20 min, then the agar was dispensed into 150 mm petri dishes (Greiner, USA).

Table 1a. Description of sampling sites.

Site	Sampling date (2011)	Lat	Long	Elevation (m)	Annual precipitation (mm)	Annual avg T (°C)	Days with air $T < 0$ °C	Vegetation
				Crop soils				
Alfalfa[a]	2 Mar	42.12266	−104.38585	1270	336	9.3	181	Dead material on surface from previous year's sowing of alfalfa, orchard grass, and meadow brome.
Bean[a]	2 Mar	42.13167	−104.39413	1270	336	9.3	181	Bare at sampling. Previous year was a mixed crop of dry beans.
Potato[a]	2 Mar	42.13167	−104.39516	1270	336	9.3	181	Bare at sampling. Previous year was potato.
Sugar beet[a]	2 Mar	42.12878	−104.39516	1270	336	9.3	181	Bare at sampling. Previous year was Roundup Ready sugar beet.
				Native and uncultivated soils				
Forest[b]	24 May	41.32436	−106.16007	2610	385	4.6	214	Lodgepole pine, with understory of elk sedge, low sedge, creeping juniper, Oregon grape, kinnikinnick, woods rose, and heartleaf arnica.
Grassland[b]	24 May	41.2881	−106.11124	2420	385	4.6	214	Bluebunch wheatgrass, Idaho fescue, western wheatgrass, and threetip sagebrush.
Pasture[a]	2 Mar	42.13243	−104.39428	1270	336	9.3	181	Smooth brome and downy brome.

[a] Lingle [b] Centennial.

Table 1b. Characterization of soil samples.

Site	Soil type	%SOM[c]	%N	pH
	Crop soils			
Alfalfa	Haverson & McCook light brownish-gray floodplain loams.[a]	0.95	0.076	8.1
Bean	Haverson & McCook light brownish-gray floodplain loams.[a]	–	–	–
Potato	Haverson & McCook light brownish-gray floodplain loams.[a]	–	–	–
Sugar beet	Haverson & McCook light brownish-gray floodplain loams.[a]	1.3	0.11	8.15
	Native and uncultivated soils			
Grassland	Greyback very cobbly sandy loam; outwash from alluvial fan. Surface layer grayish brown to brown very cobbly sandy loam.[b]	3.7	0.27	6.45
Forest	Ansile-Granile gravelly sandy loam. 5 cm layer of needles and bark residue.[b]	100	2.05	5.9
Pasture	Haverson & McCook light brownish-gray floodplain loams.[a]	4.7	0.465	7.85

[a] Soil survey of Goshen County, south part, Wyoming, 1971. United States Department of Agriculture, Soil Conservation Service, 102 pp. [b] Soil survey of Albany County Area, Wyoming, 1998. United States Department of Agriculture, Natural Resources Conservation Service, U.S Government Printing Office, 540 pp. [c] Soil organic matter (SOM) contents obtained by multiplying percentage carbon by 1.724.

2.3 Initial screening for ice nucleation activity

An aliquot of each culture containing visible mycelia was tested for its ice nucleation activity in a temperature range from −2 to −12 °C. Aliquots of 50 µL were transferred to wells of a fresh, sterile, 96-well PCR tray which was cooled in a thermal cycler (PTC-200, MJ Research, USA). The cycler was programmed to descend in 0.5 or 1 °C decrements from −2 to −9 °C (the limit of the machine). Temperature variation across the cooling block was ±0.2 °C of the real temperature measured using a thermistor (VPT-0300, Bio-Rad, USA). After a 5 min dwell time at each temperature, the number of frozen wells was counted and the temperature lowered to the next level. Once cooled to −9 °C, the tray was transferred to a 96-well aluminum incubation block (VWR, USA) which had been precooled to ≈ −12 °C inside a foam box in a freezer. The thermistor was inserted into a side well and after 10 min the block temperature and number of frozen wells was recorded. Aliquots of uninoculated DPY broth were used as negative controls. Ice-nucleation-active *Fusarium acuminatum* cultures (provided courtesy of Linda Hanson, Michigan State University) were used as positive controls ($\approx 10^9$ IN g^{-1} mycelium). Ice-nucleation-active isolates were then subcultured on DPY agar, incubated at RT for 3–7 days, and tested again (aerial mycelium picked and suspended in 50 µL fresh DPY broth) to confirm activity. To test for possible contaminants, microscopic investigations as well as qPCR on the bacterial *ina* gene following the protocol by Hill et al. (2014) were performed. Cultures which seemed to be mixed were subcultured by plating small pieces from the diffuse leading edge of growth to recover single isolates. Only pure cultures were used for further freezing tests and identification.

2.4 Identification and phylogenetic analysis

For identification and phylogenetic analyses, hyphae and spores were first picked using sterile pipette tips (Eppendorf, Germany) into 20 µL water and lysed at 95 °C for 10 min. This lysate was used as a PCR template. To amplify fungal DNA for sequencing, two PCRs, one of the internal transcribed spacer (ITS) and a second of a gene for γ-linolenic elongase (GLELO), were performed. Each 25 µL reaction mixture contained the template DNA (1 µL), 1 × PCR buffer (Sigma-Aldrich, Germany), 0.2 mM each dNTP (Roth, Germany), 0.33 µM of each primer (Sigma-Aldrich, Germany), and 1.25 units of JumpStart™ REDTaq DNA polymerase (Sigma-Aldrich, Germany). A negative control was included in all PCR runs.

PCR reactions were performed with the primer pairs GLELOfor/GLELOrev (Takeno et al., 2005) and ITS4/ITS5 (White et al., 1990). The thermal profile (DNA Engine, Bio-Rad Laboratories, Germany) was as follows: initial denaturing at 94 °C for 3 min; 35 cycles with denaturing at 94 °C for 30 s, annealing at 52.5 °C for 60 s (GLELO) or 54 °C for 30 s (ITS), elongation at 72 °C for 90 s (GLELO) or 45 s (ITS); and a final extension step at 72 °C for 5 min.

Amplification products for sequencing were cloned using the TOPO TA Cloning® Kit (Invitrogen, Germany) following the supplier's instructions. Colonies containing inserts were identified by means of blue–white selection and lysed in 20 µL H_2O for 10 min at 95 °C. The inserts of 6–12 colonies of each cloning reaction were amplified using 1.5 µL cell lysate in a 25 µL reaction. The PCR reaction mixture contained 1 × JumpStartREDTaq Ready Mix (Sigma-Aldrich, Germany) and 0.25 µM of each primer (Sigma-Aldrich, Germany). PCR reactions were performed with the primer pair M13F-40 and M13R, and the thermal profile was as follows: initial denaturing at 94 °C for 5 min; 40 cycles at 94 °C for 30 s, annealing at 55 °C for 1 min, elongation at 72 °C for 1 min; and a final extension step at 72 °C for 15 min. For sequencing, up to 10 colony PCR products per isolate and gene were chosen.

DNA sequences were determined with ABI Prism 377, 3100, and 3730 sequencers (Applied Biosystems, Germany) using BigDye Terminator v3.1 chemistry at the Max Planck Genome Centre of the Max Planck Institute for Plant Breeding Research, Cologne, Germany. The quality of all sequences was manually checked. For comparison with known sequences, databank queries using the Basic Local Alignment Search Tool (BLAST) were performed via the website of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/). Alignments were done using ClustalW within BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and manually checked. Phylogenetic trees were constructed using MEGA version 5 (Tamura et al., 2011). MEGA's model selection facility was used to choose the best models by employing the maximum likelihood method and optimizing a neighbor-joining (NJ) tree. DNA- and amino acid-derived trees were calculated using NJ with a 2000-replicate bootstrap analysis (Felsenstein, 1985).

2.5 Freezing spectra (number of IN)

After initial selection and identification, the fungi were subcultured on PDA (potato dextrose agar, VWR, Germany) plates, and further freezing experiments were performed to characterize their ice nucleation activity. To perform tests below −9 °C, another ice spectrometer for droplet arrays using 96-well PCR trays was constructed. Holes were drilled through the base of a 96-well aluminum block (VWR, Germany), which was then connected to a Julabo Presto A30 cooling bath operating with Thermal HL40 (Julabo, Germany) as cooling liquid. For accurate control and regulation of the block temperature, an additional PT100 temperature sensor was integrated within the aluminum block. The block, which was initially stabilized at −4 °C, was then cooled in 0.5 to 2 °C steps to −15 °C. Each transition took 12 min in order to allow time for the system to equilibrate and dwell at the new temperature for at least 5 min. The number of frozen wells was counted.

For the determination of the IN per gram of mycelium, the entire mass of mycelium (containing spores) of a fungal culture was harvested by scraping it off the PDA surface and transferred it into a sterile 15 mL Falcon tube (SPL Lifesciences, Korea) which was weighed before and after harvesting. Depending on the individual isolates, between 0.1 and 1.3 g of mycelium could be harvested. Ten milliliters of 0.1 µm pore diameter sterile, filtered (Acrodisc, PES, Pall, Germany), deionized water was added and the suspension shaken for 1 min on a vortex mixer. The solution was then filtered through a 5 µm pore diameter filter (Acrodisc, PES, Pall, Germany) and diluted up to 10^{-8} with 0.1 µm pore diameter filtered deionized water. From several of the dilutions, 24–88 (mostly 32) aliquots of 50 µL were then tested for freezing as described above. Aliquots of 0.1 µm pore diameter filtered deionized water were used as negative controls. The absence of IN on the PDA plates was confirmed as follows: a loop was scraped over the agar surface, as during mycelium harvest, and then dipped into 0.1 µm pore diameter filtered deionized water, which was tested. The concentration of IN per gram of mycelium was calculated using a variant of the formula of Vali (Eq. 1, Vali, 1971):

$$n_{\mathrm{m}}\left[g^{-1}\right] = -\ln(1 - f_{\mathrm{ice}}) \cdot \frac{V_{\mathrm{wash}}}{V_{\mathrm{drop}}} \cdot \frac{F_{\mathrm{dil}}}{m_{\mathrm{myc}}}, \quad (1)$$

where n_{m} is the number of IN per gram of mycelium, f_{ice} the fraction of frozen droplets, V_{wash} the volume of water added for washing (10 mL in this study), V_{drop} the droplet volume in the freezing assay (0.05 mL in this study), F_{dil} the dilution factor of the suspension, and m_{myc} the mass of the mycelium. Binomial confidence intervals ($CI_{95\,\%}$) were derived by using

Eq. (2) as recommended by Agresti and Coull (1998):

$$\mathrm{CI}_{95\,\%} = \left(f_{\mathrm{ice}} + \frac{1.96^2}{2n} \pm 1.96 \sqrt{\left[f_{\mathrm{ice}} \left(1 - f_{\mathrm{ice}}\right) + 1.96^2/4n \right]/n} \right) \quad (2)$$
$$/ \left(1 + 1.96^2/n\right),$$

where n is the total number of droplets.

2.6 Size and mass determination of the IN

The 5 µm filtrate was further filtered through 0.1 µm pore size filters (Acrodisc, PES, Pall, Germany) and Vivaspin® filter tubes (Sartorius, Germany) of different mass exclusion limits (100, 300 kDa). These filtrates were then tested for freezing activity as described above.

2.7 Enzymatic, chemical, and heat treatments

To further characterize the IN, the effects of protein- and lipid-degrading enzymes, protein- and carbohydrate-degrading chemicals, and heat were investigated. Aliquots of the 0.1 µm filtrates were treated as follows: (A) 1 h with 50 mg mL^{-1} of the enzymes: (i) papain (AppliChem, Germany) at 60 °C, (ii) pepsin (Sigma-Aldrich, Germany) at 37 °C, pH 1.5, or (iii) lipase (AppliChem, Germany) at 37 °C; (B) 1–2 h at room temperature with (i) 6 M guanidinium chloride (Promega, Germany) or (ii) 0.3 M boric acid (National Diagnostics, USA); and (C) 1 h at (i) 60 °C or (ii) 98 °C. Controls of enzyme or chemical solutions of the same concentration were included as reference measurements. The ice nucleation activity of the treated aliquots was tested after appropriate dilution as described above.

2.8 Nucleotide sequence accession numbers

The sequences from the isolates of the present study have been deposited in GenBank under accession numbers KJ469804-KJ469842 for internal transcribed spacer (ITS) sequences and KJ469843-KJ469875 for GLELO (γ-linolenic elongase) sequences.

3 Results

Soil samples were collected in spring 2011 at four cropped sites, one pasture, and from two areas of native vegetation in southeast Wyoming, USA (see Table 1a, b for site and soil details). Soil dilution series were prepared and all 474 fungal CFUs obtained were tested for ice nucleation activity to −15 °C. As shown in Tables 2 and 3, 8 % (39) of all CFUs from these seven soils showed freezing activity between −5 and −6 °C. The proportion of INA fungi varied for different soils, from 0 % in the bean plot to 25 % in an adjacent sugar beet plot (crops are the previous season's plantings, since plots were still bare at the time of sampling).

Table 2. Numbers and concentration of cultivable fungi and ice nucleating *M. alpina* in different soil types.

	Total CFU	INA *M. alpina* CFU		Fungi (CFU g^{-1})	INA *M. alpina* (CFU g^{-1})
Number	474	39	Mean	6.0×10^4	2.9×10^3
Crop soils					
Alfalfa	65	3		5.3×10^4	6.0×10^2
Bean	21	–		8.4×10^4	–
Potato	12	2		4.8×10^4	4.0×10^3
Sugar beet	88	22		6.4×10^4	8.0×10^3
Native and uncultivated soils					
Forest	36	6		4.3×10^4	4.8×10^3
Grassland	52	1		3.3×10^4	2.0×10^2
Pasture	200	5		9.7×10^4	2.8×10^3

All 39 INA isolates were identified as *Mortierella alpina* (*Mortierellales*; *Mucoromycotina/Mortierellomycotina*; Hibbett et al., 2007; Hoffmann et al., 2011) based on sequencing of both the ITS regions and the GLELO (γ-linolenic elongase) gene (Table 3). The identity of the sequences with the best matches in the GenBank database was 99–100 % (Table 3), although they showed a wider range of 95–100 % similarity when compared to each other, a reflection of the diversity within the group. Indeed, the identity level between the ITS regions of different *M. alpina* isolates ranges from 94 to 100 % (Ho and Chen, 2008), almost twice the value of 3.24 % suggested for intraspecific variability within the zygomycotic fungi by Nilsson and Kristiansson (2008). The phylogeny of *Mortierellales* is poorly understood and a new classification based on modern phylogenetic methods has been recommended (Petkovits et al., 2011).

For a better characterization of the *M. alpina* isolates, a NJ tree was constructed using a 515 bp sequence of the partial ITS1-5.8S-partial ITS2 region of all INA isolates. Included for comparison were the best match sequences obtained from a BLAST search (Table 3), as well as sequences from *M. humilis* (AJ878778.1), *M. gamsii* (AJ878508.1), and *M. macrocystis* (AJ878781.1), which were used as out-groups (Kwaśna et al., 2006; Ho and Chen, 2008). As shown in Fig. 1, four clades of *M. alpina* were formed, each supported with high bootstrap values. These were classified as (A) predominantly uncultivated, (B) forest, (C) predominantly standard agricultural, and (D) high organic matter input agricultural. The isolates from the forest site were restricted to clade B; the single native grassland isolate was placed in clade A; pasture and alfalfa isolates were mostly restricted to clade C; and isolates from the harvested and ploughed sugar beet field, which contained many broken and decaying pieces of sugar beet root, accounted for ≈ 90 % of group D, as well as being common in clade C.

In order to further characterize the populations, the GLELO gene was used; GLELO is responsible for the conversion of γ-linolenic acid to dihomo-γ-linolenic acid

Table 3. Characteristics of *M. alpina* isolates. Site, ID number, phylogenetic clade, highest observed initial freezing temperature (T_i), and closest GenBank matches and similarity for ITS and GLELO; n.s. stands for no sequence.

				ITS		GLELO	
Site	ID no.	Clade	$T_{initial}$ (°C)	Closest isolates (accession no.)	Similarity (%)	Closest isolates (accession no.)	Similarity (%)
				Crop soils			
Alfalfa	3	D	−5	*M. alpina* xsd08339 (EU918703)	99.4	*M. alpina* ATCC 32221 (AF206662)	96.6
	14	C	−5.5	*M. alpina* CBS 528.72 (AJ271629)	99.6	*M. alpina* ATCC 32221 (AF206662)	97.8
	34	C	−6	*M. alpina* CBS 528.72 (AJ271629)	99.4	*M. alpina* ATCC 32221 (AF206662)	97.6
Potato	12	D	−5.5	*M. alpina* xsd08339 (EU918703)	99.3	*M. alpina* ATCC 32221 (AF206662)	96.6
	13	A	−5.5	*M. alpina* ATT234 (HQ607903)	99.7	*M. alpina* (EU639657)	99.3
Sugar beet	5	D	−5	*M. alpina* xsd08339 (EU918703)	99.1	*M. alpina* ATCC 32221 (AF206662)	96.6
	6	D	−5.5	*M. alpina* xsd08339 (EU918703)	99.3	*M. alpina* ATCC 32221 (AF206662)	96.6
	7	D	−5.5	*M. alpina* xsd08339 (EU918703)	99.3	*M. alpina* ATCC 32221 (AF206662)	96.4
	8	D	−5	*M. alpina* xsd08339 (EU918703)	99.1	*M. alpina* ATCC 32221 (AF206662)	96.6
	9	D	−5.5	*M. alpina* xsd08339 (EU918703)	99.0	n.s	–
	10	D	−5	*M. alpina* xsd08339 (EU918703)	99.1	*M. alpina* ATCC 32221 (AF206662)	96.6
	11	D	−5.5	*M. alpina* xsd08339 (EU918703)	99.1	*M. alpina* ATCC 32221 (AF206662)	96.2
	15	D	−5.5	*M. alpina* xsd08339 (EU918703)	99.0	*M. alpina* ATCC 32221 (AF206662)	95.3
	16	C	−5.5	*M. alpina* CBS 528.72 (AJ271629)	99.3	*M. alpina* ATCC 32221 (AF206662)	97.5
	17	C	−5.5	*M. alpina* CBS 528.72 (AJ271629)	99.4	*M. alpina* ATCC 32221 (AF206662)	97.8
	18	D	−5.5	*M. alpina* xsd08339 (EU918703)	99.1	n.s	–
	19	D	−5	*M. alpina* xsd08339 (EU918703)	99.0	*M. alpina* ATCC 32221 (AF206662)	96.6
	20	D	−5	*M. alpina* xsd08339 (EU918703)	99.0	*M. alpina* ATCC 32221 (AF206662)	96.2
	21	D	−5.5	*M. alpina* xsd08339 (EU918703)	99.3	n.s	–
	22	C	−5.5	*M. alpina* CBS 528.72 (AJ271629)	99.6	*M. alpina* ATCC 32221 (AF206662)	97.8
	23	D	−5.5	*M. alpina* xsd08339 (EU918703)	99.1	*M. alpina* ATCC 32221 (AF206662)	96.6
	24	C	−5.5	*M. alpina* CBS 528.72 (AJ271629)	99.6	*M. alpina* ATCC 32221 (AF206662)	97.1
	25	D	−5.5	*M. alpina* xsd08339 (EU918703)	99.3	*M. alpina* ATCC 32221 (AF206662)	96.6

Table 3. Continued.

				ITS		GLELO	
Site	ID no.	Clade	$T_{initial}$ (°C)	Closest isolates (accession no.)	Similarity (%)	Closest isolates (accession no.)	Similarity (%)
				Crop soils			
	26	C	–6	*M. alpina* CBS 528.72 (AJ271629)	99.6	*M. alpina* ATCC 32221 (AF206662)	97.8
	27	D	−5.5	*M. alpina* xsd08339 (EU918703)	99.3	*M. alpina* ATCC 32221 (AF206662)	96.6
	28	D	−5.5	*M. alpina* xsd08339 (EU918703)	99.1	n.s	–
	42	C	−5.5	*M. alpina* CBS 528.72 (AJ271629)	99.0	*M. alpina* ATCC 32221 (AF206662)	97.8
				Native and uncultivated soils			
Forest	35	B	−5.5	Unc. *Mortierella* clone 1.12 (FN565294)	98.9	*M. alpina* CBS 608.70 (GU593327)	93.3
	36	B	−5.5	Unc. *Mortierella* clone 1.12 (FN565294)	99.0	n.s	–
	37	B	−6	Unc. *Mortierella* clone 1.12 (FN565294)	98.9	*M. alpina* CBS 608.70 (GU593327)	93.3
	38	B	−6	Unc. *Mortierella* clone 1.12 (FN565294)	98.7	*M. alpina* CBS 608.70 (GU593327)	93.3
	39	B	−6	Unc. *Mortierella* clone 1.12 (FN565294)	98.9	*M. alpina* CBS 608.70 (GU593327)	93.3
	40	B	−5.5	Unc. *Mortierella* clone 1.12 (FN565294)	98.9	n.s	–
Grassland	41	A	−5	*M. alpina* ATT234 (HQ607903)	99.9	*M. alpina* (EU639657)	100
Pasture	1	A	−5	*M. alpina* ATT234 (HQ607903)	99.7	*M. alpina* (EU639657)	99.3
	2	C	−5.5	*M. alpina* CBS 528.72 (AJ271629)	99.6	*M. alpina* ATCC 32221 (AF206662)	97.8
	31	C	−5.5	*M. alpina* CBS 528.72 (AJ271629)	99.0	*M. alpina* ATCC 32221 (AF206662)	97.8
	32	C	−5.5	*M. alpina* CBS 528.72 (AJ271629)	99.6	*M. alpina* ATCC 32221 (AF206662)	97.5
	33	C	−5.5	*M. alpina* CBS 528.72 (AJ271629)	99.1	*M. alpina* ATCC 32221 (AF206662)	97.5

(Takeno et al., 2005). GLELO DNA was successfully amplified from all four groups. A NJ tree was constructed by using a 447 bp sequence of the GLELO gene from 33 INA isolates and the closest matches obtained from BLAST (Fig. S1, Table 3). The tree again contained four clades with identical placement of the isolates in the clades A, B, C, and D as derived using ITS (Fig. 1). The variants of GLELO possessed sequence similarities of 88–96 % at the DNA level and 90–100 % at the protein level. Use of amino acid sequences to construct the tree led to branches C and D being grouped as a single clade (Fig. S2 in the Supplement), primarily due to the removal of codon degeneracies.

Recently, Wagner et al. (2013) studied the molecular phylogeny of the *Mortierellales* based on nuclear ribosomal DNA. They reported that the *M. alpina* complex formed a heterogeneous cluster, as also found in this study. To compare both data sets, a NJ tree was constructed including 22 of the *M. alpina* sequences from Wagner et al. (2013). The tree (Fig. S3) possessed six clades, with all isolates of this study distributed in four of the six clades.

For the characterization of the ice nucleation activity of *M. alpina*, freezing tests were performed from 24 randomly selected representatives from among the clades. The total number of IN per gram of mycelium (fresh weight) was in the range of $\approx 10^2$–10^9 (Fig. 2). Generally, clade C had distinctly lower numbers, namely $\approx 10^2$–$10^6\ g^{-1}$, while clade A and B had about 10^8–$10^9\ g^{-1}$, and clade D 10^6–$10^9\ g^{-1}$. When grouped according to different soil types (Fig. 3), the 23

Figure 1. Neighbor-joining tree based on ITS sequences. The evolutionary distances were computed using the Tamura 3-parameter method (Tamura, 1992); units are the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 0.25). Node support above 75 % is given. Note that the reference sequences named as *M. globalpina* and *M. amoeboidea* are also placed within the *M. alpina* complex as found by Wagner et al. (2013).

tested isolates from pasture, forest, sugar beet, grassland, and potato exhibited a consistency in possessing an intermediate range of between $\approx 10^8$ and 10^9 IN per gram of mycelium, whereas the single alfalfa field isolate had the lowest number of IN ($\approx 10^5 g^{-1}$), 3–4 orders of magnitude less than the isolates from the other soil types.

To estimate the size and mass range of the IN, the mycelium/spore suspensions were filtered through 0.1 µm pore diameter filters and Vivaspin® centrifugal concentrators with mass exclusion limits of 300 and 100 kDa. Filtrates of 0.1 µm pore diameter filters as well as 300 kDa spin columns retained activity (Table 4), but ice nucleation activity was removed after passage through a 100 kDa device, with a few exceptions. This equates to a minimum diameter range of 6.1–8.8 nm for the cell-free IN of most isolates, while for some it suggests the IN are < 6.1 nm (Erickson, 2009). To further characterize the IN, aliquots of the 0.1 µm filtrates were treated with different enzymes (papain, lipase), 6 M guanidinium chloride, or 0.3 M boric acid or were tested for heat stability at 60 °C and 98 °C (Fig. 4, Table 4). As shown in Table 4 the IN of most isolates were heat stable at 60 °C but lost ice nucleation activity after 98 °C treatment. Lipase and boric acid did not affect the ice nucleation activity significantly, whereas guanidinium chloride, a chemical that degrades proteins, had a strong effect. Treatment with the protein-degrading enzyme papain showed variable results: for clade A, papain had no effect, whereas clade B, C, and D showed a strong decrease in their ice nucleation activity when digested with papain. Clade A was thus treated with another protease, pepsin, which also did not affect the ice nucleation activity.

4 Discussion

To our knowledge, this is the first report of ice nucleation activity in the widespread soil fungus *M. alpina* (*Mortierellales*). Note that the placement of the order *Mortierellales* is currently under discussion: it is either placed within the subphyla *Mucoromycotina* or *Mortierellomycotina* (Hibbett et al., 2007; Hoffmann et al., 2011). However, this is also the first reported case of ice nucleation activity in a zygomycotic fungi, as all reported INA fungi previously belonged to the phyla *Ascomycota* and *Basidiomycota* (Jayaweera and Flanagan, 1982; Kieft and Ahmadjian, 1989; Henderson-Begg et al., 2009; Iannone et al., 2011; Haga et al., 2013; Huffman et al., 2013; Morris et al., 2013).

Mortierella (≈ 90 species) are widespread and prominent members of soil and compost communities (Anastasi et al., 2005; Buée et al., 2009; Christensen, 2001; Nagy et al., 2011; Wagner et al., 2013), but they have also been found in air, sand storm dust, and rain samples (Hyland et al., 1953; Pawsey and Heath, 1964; Turner, 1966; Bokhary and Parvez, 1995; Kwaasi et al., 1998). *Mortierella* spp. are saprobic organisms utilizing decaying organic matter (Wagner et al., 2013), but, based on their ability to solubilize phosphorus, they can also form interactions with arbuscular mycorrhizal fungi, which are plant root symbionts (Zhang et al., 2011). They are also known to be hosts for mycoparasites (Turner, 1963; Upadhyay et al., 1981; Degawa and Gams, 2004) or are mycoparasites themselves (Willoughby, 1988).

The ability to act as an IN may be incidental in *M. alpina*, but its high temperature of activity suggests it provides an ecological advantage. The known INA fungi and bacteria (e.g., *Pseudomonas syringae*, *Xanthomonas campestris*, *Fusarium avenaceum*, *Puccinia* spp.) are mostly plant pathogens. Possession of ice nucleation activity has

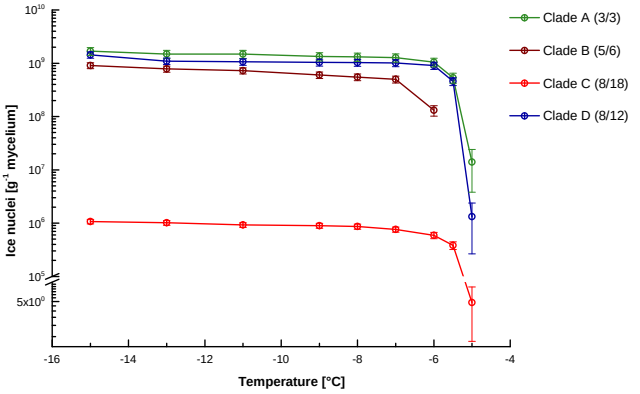

Figure 2. Average number of IN per gram of mycelium (fresh weight) for all clades. The clades are classified as **(a)** predominantly uncultivated, **(b)** forest, **(c)** predominantly standard agricultural, and **(d)** high organic matter input agricultural. The numbers in parentheses represent the number of isolates tested out of total number of isolates from each clade. Error bars represent the 95 % confidence intervals.

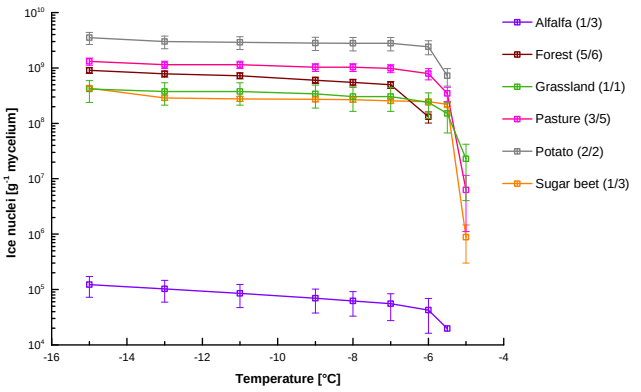

Figure 3. Average number of IN per gram of mycelium for the isolates of different soil types. The numbers in parentheses represent the number of isolates tested out of the total number of isolates each sampling site. Error bars represent the 95 % confidence intervals.

been correlated with aggressiveness (Morris et al., 2010), and it is hypothesized that the ice nucleation activity may have preceded the acquisition of virulence factors by both promoting precipitation to aid dissemination (Morris et al., 2008, 2010) and by helping to injure plant tissues to make nutrients available for establishment (Lindow, 1983; Morris et al., 2010).

M. alpina is not a pathogen, but it is a cold-adapted organism. The ice nucleation activity might be one aspect of its overwintering strategy, whereby physical damage can be avoided through protective extracellular freezing (Weete and Gandhi, 1999; Zachariassen and Kristiansen, 2000; Frisvad, 2008).

M. alpina is known to convert various carbon sources into lipids and to accumulate large amounts of fatty acids such as γ-linolenic, arachidonic, and eicosapentaenoic acid (Batrakov et al., 2002; Petkovits et al., 2011). The availability of much readily decomposable organic matter, due to the presence of many decaying fragments of sugar beet roots left behind after harvesting, may explain why *M. alpina* comprised 25 % of all fungal isolates from sugar beet, the highest of any soil sampled in this study. Fatty acids are known to play a protective role in psychrotolerant *Mortierella* spp. (Weete and Gandhi, 1999; Frisvad, 2008). Arachidonic acid is a polyunsaturated fatty acid that can comprise up to 54 % of the fatty acids in the mycelium (Weete and Gandhi, 1999; Lounds et al., 2007; Ho and Chen, 2008) and may help to regulate lipid fluidity, necessary for survival at low temperatures (Margesin and Schinner, 1994; Margesin et al., 2007). The ability of *Mortierella* to survive freezing was demonstrated by Morris et al. (1988), who obtained high recovery rates for *M. elongata* in cryo-preservation experiments using liquid nitrogen.

Other than that, the ice nucleation activity may play a role in mycoparasitism or even be a useful mechanism for cleaving soil aggregates or rock to expose new surfaces to facilitate the release of phosphorus. As suggested for *Fusarium* and lichens (Kieft and Ahmadjian, 1989; Pouleur et al., 1992), the ice nucleation activity in *M. alpina* may also be beneficial in attracting moisture and water in relatively dry soils, e.g., for germination.

In terms of number of IN per gram of mycelium (up to 10^9), the values obtained from *M. alpina* are similar to those obtained for *P. syringae* and *Fusarium acuminatum* (Pouleur et al., 1992). However, in contrast to bacterial IN, where different classes of IN are active at different temperatures due to different-sized aggregates (Phelps et al., 1986; Govindarajan and Lindow, 1988; Turner et al., 1990; Ruggles et al., 1993), the *M. alpina* IN seem to form only a single activity class within the tested temperature range. Interestingly, while the initial freezing temperature of -5 to -6 °C (Fig. 2, Table 3) would correspond to type 2 bacterial IN, i.e., the same as the glycoprotein structure (Kozloff et al., 1991; Ruggles et al., 1993), their < 300 kDa size is only about one-tenth of the corresponding bacterial type 2 IN (Govindarajan and Lindow, 1988).

For further characterization of the IN, chemical, enzymatic, and thermal treatments were performed. The sensitivity to guanidinium chloride, papain, and 98 °C heat treatment indicates that a protein is important in the activity of *M. alpina* IN. Interestingly, clade A IN are not affected by papain or pepsin, which might be explained by the specificity of the enzymes as clade A IN are also sensitive to guanidinium chloride, a chemical that degrades proteins. Thus, clade A IN seem to either differ in their amino acid sequence compared to the other clades, or might be protected by non-protein side chains. For all clades, lipids seem not to play any important role. Carbohydrate functionalization with boric acid showed no impact on the ice nucleation activity; however, the

Table 4. Changes of number of IN in orders of magnitude after filtration (5, 0.1 µm; 300, 100 kDa), thermal (60, 98 °C), chemical (guanidinium chloride (G.Cl) or boric acid (B.A)), or enzymatic (lipase, papain, pepsin) treatments at −11 °C relative to the activity of the 0.1 µm filtrate of selected *M. alpina* isolates; dashes mean not measured.

Isolate	5 µm	0.1 µm	300 kDa	100 kDa	60 °C	98 °C	G.Cl	B.A	Lip	Pap	Pep
01A	0.4	0.0	−0.4	−5.8	0.2	−4.2	−4.8	0.0	−0.2	0.0	−0.7
13A	0.1	0.0	0.2	−5.3	0.1	−4.8	−4.9	0.5	−0.1	0.1	−0.2
41A	0.3	0.0	0.1	−6.0	0.2	−4.6	−4.1	–	−0.4	−1.0	−0.1
35B	−0.1	0.0	−0.3	−5.2	−2.2	−6.2	−5.8	–	–	–	–
36B	0.2	0.0	−0.2	−5.4	0.0	< −7.0	−5.6	−0.5	−0.8	−4.4	–
37B	−0.3	0.0	−0.2	−3.2	−0.5	< −7.0	< −7.0	−0.7	−0.7	−2.6	–
38B	0.5	0.0	0.1	−4.8	−2.0	−6.8	< −7.0	–	−0.8	−4.4	–
40B	0.0	0.0	0.0	−4.4	−0.5	< −7.0	−6.2	−0.2	−0.3	−2.2	–
14C	0.1	0.0	0.0	< −3.0	−0.4	< −3.0	< −3.0	−0.2	0.0	−2.6	–
16C	0.0	0.0	0.0	−2.0	0.1	−3.4	< −3.0	–	0.0	< −3.0	–
17C	−0.3	0.0	−0.1	−4.2	−1.2	< −4.0	< −4.0	−0.6	−0.5	−3.6	–
22C	0.0	0.0	0.0	< −2.0	0.1	< −2.0	< −2.0	0.0	0.0	< −2.0	–
26C	0.2	0.0	0.0	< −0.5	< −0.5	< −0.5	< −0.5	–	−0.2	< −0.5	–
31C	0.8	0.0	0.2	< −3.0	−0.4	< −3.0	< −3.0	–	–	–	–
33C	−0.2	0.0	−0.6	< −3.0	−1.6	< −3.0	−3.1	−0.2	0.0	−2.5	–
34C	–	0.0	−0.3	< −0.5	−0.3	< −0.5	< −0.5	–	–	–	–
42C	0.1	0.0	−0.2	< −4.0	0.0	< −4.0	< −4.0	−0.9	0.1	−2.8	–
03D	−0.1	0.0	0.2	−4.5	0.0	−3.7	−5.9	0.0	−0.6	−2.6	–
05D	−0.3	0.0	−0.5	−5.1	−0.5	< −7.0	−4.6	–	–	–	–
06D	−0.3	0.0	0.0	−5.7	−0.2	−5.7	−5.3	−0.2	−0.3	−3.3	–
07D	0.2	0.0	0.3	−2.7	−0.1	−3.3	−4.0	0.2	0.2	−3.0	–
09D	0.1	0.0	−0.7	−6.8	−0.4	−5.4	−3.4	−0.9	−0.6	−3.4	–
12D	−0.2	0.0	−0.5	−4.6	−0.2	−4.5	−7.3	−0.4	−0.3	−3.2	–
15D	0.2	0.0	0.0	−4.6	−0.1	< −5.0	−3.6	0.0	−0.1	−2.9	–
19D	0.2	0.0	0.0	−4.9	−0.1	< −5.0	< −5.0	−0.1	−0.3	−3.0	–

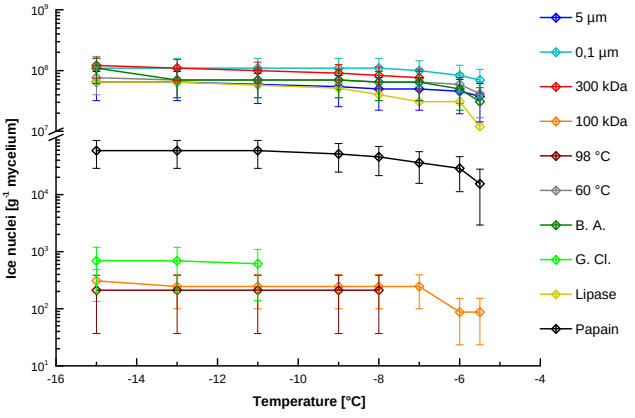

Figure 4. Number of IN per gram of mycelium for isolate ID6 after filtration, thermal, chemical, or enzymatic treatments. G. Cl stands for guanidinium chloride and B. A for boric acid. Error bars represent the 95 % confidence intervals.

possible role of carbohydrates cannot be fully ruled out based on this method. Apart from rust fungi and pollen IN, which are thought to be non-proteinaceous (Pummer et al., 2012; Morris et al., 2013), evidence points to proteins as the source of ice nucleation activity of the known INA fungi (*Fusarium*, lichen mycobionts) (Kieft and Ruscetti, 1990; Hasegawa et al., 1994).

The IN of *M. alpina* have more similarities to *Fusarium*, lichen, and leaf-derived IN as they are not only cell-free but are also heat stable at 60 °C (Schnell and Vali, 1976; Kieft and Ruscetti, 1990; Pouleur et al., 1992). The IN of *M. alpina* are smaller than 10 nm in size, between 100 and 300 kDa in mass, and can be readily released into the surrounding medium. The latter is also a characteristic of several INA *Fusarium* species (Pouleur et al., 1992; Hasegawa et al., 1994; Tsumuki and Konno, 1994; Humphreys et al., 2001), leaf-derived IN (Schnell and Vali, 1973), some INA bacteria (Phelps et al., 1986; Kawahara et al., 1993), and INA pollen (Pummer et al., 2012). In soil and decaying vegetation, these cell-free IN might contribute to the as-yet-unknown reservoir of biological residues which can enhance the ice nucleation activity of soil dust and boundary layer atmospheric aerosols (Conen et al., 2011; Garcia et al., 2012; O'Sullivan et al., 2014; Tobo et al., 2014).

To understand the role of the IN of *M. alpina* and other INA fungi in soil and in the atmosphere, further surveys of INA fungi of all phyla, and in particular soil fungi, are clearly necessary. Additionally, studies investigating the occurrence and the distribution of the INA fungi in aerosol samples,

samples of fugitive dust, and different agricultural and natural ecosystem soil types could help to estimate their contribution to the organic IN in soil and to establish relations to climatic zones. Recent studies have shown not only that the soilborne and airborne fungi are highly diverse (Buée et al., 2009; Fröhlich-Nowoisky et al., 2009; Schmidt et al., 2013) but also that their atmospheric transport leads to efficient exchange of species among ecosystems (Burrows et al., 2009a, b). The atmosphere serves as a primary medium for transport, and the global emissions of fungal spores are estimated to be 8–186 Tg a^{-1} (Després et al., 2012). Fungi have evolved several strategies for dispersal over long distances and at potentially high altitudes (Imshenetsky et al., 1978; Hawksworth, 2001; Brown and Hovmøller, 2002; Griffin, 2004; Prospero et al., 2005; Kellogg and Griffin, 2006; Elbert et al., 2007; Pearce et al., 2009; DeLeon-Rodriguez et al., 2013). Possession of ice nucleation activity that promotes the formation of precipitation would be a beneficial adaptation for airborne microbes since it aids their return to the land surface under favorable conditions (Sands et al., 1982; Morris et al., 2008). However, the release of small extracellular IN into the soil might unintentionally confer ice nucleation activity to a pool of small soil particles if the extracellular IN are embedded within or adsorbed. This population of fine dusts would occur at higher concentrations at cloud altitudes. Currently, this mechanism is not considered in models, which assume that fungal ice nucleation activity is restricted only to spores (Sesartic et al., 2013). Their potential contribution as IN in soil dusts depends critically upon whether or not they are rapidly decomposed by other soil microflora and whether they are de-activated or protected by adsorption onto soil organic matter and clays.

5 Conclusions

In this study we found ice nucleation activity in the widespread soil fungi *M. alpina*. Ice-nucleation-active isolates were obtained from six crop and native soils, with the highest abundance in soils with inputs of decomposable matter. The IN produced by *M. alpina* seem to be small extracellular proteins of 100–300 kDa which are not anchored in the fungal cell wall. These small, cell-free IN might contribute to the as-yet-uncharacterized pool of atmospheric IN released from soils as dusts, so that the pool of biogenic IN might be larger than currently estimated. As the atmospheric importance of different INA fungi, either directly or indirectly via their extracellular IN, depends not only on their relative contribution to the IN in soil dusts but also on their number concentrations at cloud altitudes, further investigations are necessary for the identification of the IN themselves and the detection and quantification of these fungi and their IN in soil, precipitation, and atmospheric samples.

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