We thank G. Vali for constructive comments and suggestions, which are highly appreciated and have been taken into account upon revision of our manuscript. Detailed responses are given below.

G. Vali: In this work, the problem manifests itself in the pro quality of information that can be derived from Figs. 2, 3 and 4. Each of the lines shown in these figures have one point on left side that is lower but the rest
of the points are practically indistinguishable in magnitude along the ordinate scale.
With the large number of different samples to test, the measurement of ice nucleating ability had to be simple and modest. Nonetheless, perhaps the authors can find better representations of the data than what is seen in Figs. 2, 3 and 4.

Response: We optimized the figures and added binomial confidence intervals (as suggested by referee 1). We think that the number of IN per gram mycelium as a function of the temperature is the information the reader is looking for when a manuscript is about an ice nucleation active fungus.

G. Vali: It is mentioned in the paper that three replicate soil samples were takes from each location in the field. It is regrettable that no data are presented to show how much scatter was detected for the replicates.

Response: As described in the manuscript (page 12701, line 5) the three replicates were bulked together on site. For clarification we changed the text into: "…were mixed together".

We thank C. Morris for constructive comments and suggestions, which are highly appreciated and have been taken into account upon revision of our manuscript. Detailed responses are given below.

C. Morris: The interest of these results could be enhanced if more information were provided about the abundance of Mortierella alpina in the soils analyzed here. For example, the data presented in Table 2 represent crude counts. This would be more informative if the authors presented estimates of the number of CFU of Mortierella alpina per gram of soil - and as a fraction of the total microbial load of the soil. This type of information is important for future estimates of the mass of organic matter contributed by these fungi to organic soil dust.

Response: We agree that the total number of CFU and of ice nucleation active *M. alpina* per gram of soil is informative. We calculated the values and included them in table 2.

Specific comments, C.Morris:

P 4, L 22: To better understand the procedure used, the title of this section should be "Initial screening for ice nucleation activity". Somewhere in the text the authors should mention that this initial screening introduced a bias relative to the ability of the fungi to grow to sufficient densities in the liquid medium. If the fungal isolate did not produce sufficient mass to yield at least 1 ice nucleus per 50 l aliquot tested for INA, then the isolate was discarded as negative – right?

Response: The title of the section was changed as suggested. The initial screening was done twice. After the first aliquot was tested, fresh medium was added and after incubation the cultures were tested again (page 12701, line 16). For the initial screening we used aliquots of each culture containing visible mycelia (page 12702, line1). We added the following information in the text: "Out of 489 picked CFU 474 showed growth in the liquid medium and were thus tested for ice nucleation activity."

P 5, L 2: Please indicate the full species name of the Fusarium used in this work.

Response: We added the full species name (*F. acuminatum*).

P 6, L 25 to P 7, L 5: Here the authors indicate that they calculated the number of IN per mass of fungal mycelium. They did not indicate how the mass of the mycelium was determined (use of a precision balance to simply weight the tubes into which the mycelium was place?). It would also be interesting for the reader to have an idea of the total mass of mycelia that was recovered for these tests (mg? g?, etc.).

Response: As suggested, we added the information of how the mass of the mycelium was determined as well as the range of the total masses used for the tests: "…of a fungal culture was harvested by scraping it off the PDA agar surface and transferred it into a sterile 15 mL tube which was weighed before and after harvesting. Depending on the individual isolates between 0.1 g and 1.3 g mycelium could be harvested."

P 7, L 6: It would help the reader if the title of this section indicated that the objective was to determine the mass of the ice nucleation active material. It is confusing because of the mention of mycelial mass in the previous section.

Response: We changed the title into: „Size and mass determination of the IN"

Discussion section: Other points that could be discussed concern propositions for follow-up work to better understand the ecological context of Mortierella alpine. Molecular markers could be developed based on the strain collection the authors have established to enlarge the survey beyond the soils studied here so as to map the occurrence of these fungi. Their presence in soil could then be compared with the INA of the associated organic soil dust in the search of correlations to explain the origin of the organic INA material in soil dust.

Response: We added the following statement in the text: „ 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. “

P 13, L 12: The work presented here does not offer any support to this sentence (“The effect of biogenic IN might: : :”). It would be more appropriate to say that the pool of biological and biogenic IN might be larger than currently estimated.

Response: We changed the text as suggested.

Table 1a: It would be useful if the names of the sampling locations were indicated.

Response: We added the names of the sampling locations in table 1a.

Table 4 and Figure 2: Somewhere in the document, and best in this table and figure, information about the behavior of the reference fungi (Fusarium spp.) should be presented.

Response: As written in the manuscript (section 2.3.) the reference fungi were used as positive controls for the initial screening. *Fusarium* spp. are known to possess ice nucleation activity and several studies characterizing the *Fusarium* IN have already been published (e.g. Hasagawa et al., 1994; Tsumiki 1995). Thus, we decided not to perform the characterization experiments on *Fusarium*. However, we added the information about the number of IN per gram for *Fusarium acuminatum* in the text. “Aliquots of uninoculated DPY broth were used as negative controls. Ice nucleation active *Fusarium acuminatum* cultures (provided courtesy of Linda Hanson, Michigan State University, $\approx 10^9$ IN g^{-1} mycelium) were used as positive controls.“

We thank the anonymous referee #1 for constructive comments and suggestions, which are highly appreciated and have been taken into account upon revision of our manuscript. Detailed responses are given below.

Referee #1

Referee #1: The phrase ice nucleation active (INA) is used at numerous points throughout paper, beginning at the abstract (page 12698, line 6). However, the successful experimental observation of heterogeneous ice nucleation in the immersion mode is dependent on the concentrations of the nucleating material employed, and the temperature range which can be probed, as subject to instrumental limitations. While I would prefer the use of more concise language (e.g. "efficient ice nucleators" rather than "ice nucleation active"), as this phrase appears so widely throughout the paper, a concise definition of what is meant by INA from its first occurrence would be easier than having to reword throughout.

Response: As suggested we added a definition of what is meant by INA in the text: "ice nucleation active (INA) = inducing ice formation in the probed range of temperature and concentration"

Referee #1: One of the key results highlighted by the authors is that the ice nucleating particles produced by the fungus seem to be < 300 kDa in size. However, there is very little discussion on the centrifuge ultrafilters used in the study (e.g. section 2.6). For instance, can the authors provide information on how wide the pore size distributions on these filters are? A discussion of this, perhaps as part of the experimental section, would be useful to give an idea as to how constrained this estimate on the protein size is.

Response: We used Viviaspin filter tubes with a molecular weight cut-off of 300 kDa and 100kDa (see section 2.6.). As the ice nucleating particles could be filtered through a 300 kDa device but with a few exceptions not through a 100 kDa filter tube we concluded that the ice nucleating particles are smaller than 300kDa (table 4, page 12708 lines 3-8).

Referee #1: Page 12702 line 6: For those not familiar with the experimental setup, can you describe what is meant by the "head" in this sentence? At what point in the temperature ramp was the temperature variation measured? Does the value of ±0.2°C for the temperature variation across the "head" translate into a droplet-to-droplet temperature uncertainty of ±0.2°C?

Response: For clarification we changed this sentence into „ Temperature variation across the cooling block..."
The temperature variation was measured using a Thermistor verification probe, supplied by the manufacturer to verify functioning of thermal cyclers. The verification measurement was performed at each temperature of the ramp. The variation of ±0.2°C translates to a droplet-to-droplet temperature uncertainty of ±0.2°C, comparable to the uncertainties of similar immersion freezing measurement instruments as summarized in Hiranuma et al., 2014.

Hiranuma, N., Augustin-Bauditz, S., Bingemer, H., Budke, C., Curtius, J., Danielczok, A., Diehl, K., Dreischmeier, K., Ebert, M., Frank, F., Hoffmann, N., Kandler, K., Kiselev, A., Koop, T., Leisner, T., Möhler, O., Nillius, B., Peckhaus, A., Rose, D., Weinbruch, S., Wex, H., Boose, Y., DeMott, P. J., Hader, J. D., Hill, T. C. J., Kanji, Z. A., Kulkarni, G., Levin, E. J. T., McCluskey, C. S., Murakami, M., Murray, B. J., Niedermeier, D., Petters, M. D.,

O'Sullivan, D., Saito, A., Schill, G. P., Tajiri, T., Tolbert, M. A., Welti, A., Whale, T. F., Wright, T. P., and Yamashita, K.: A comprehensive laboratory study on the immersion freezing behavior of illite NX particles: a comparison of seventeen ice nucleation measurement techniques, Atmos. Chem. Phys. Discuss., 14, 22045-22116, doi:10.5194/acpd-14-22045-2014, 2014.

Referee #1: Figure 2 and Figure 3: Error bars in both temperature and the concentration of active ice nuclei should be shown. A discussion of the main uncertainties in the analysis used to produce this graph would also be useful in the main text.

Response: For the concentration of IN we added binomial confidence intervals (95%) derived by the formula 2 from Agresti and Coull (1998). We added this information in the text and figure captions. For the temperature we do not think that error bars would be beneficial (indeed, they may even be misleading) as the cooling was done in 0.5 -1°C steps which took 12 min for each step and included 5 min dwelling time at each T. We recorded the number of frozen droplets at these defined temperatures (like in other studies) but the freezing of the droplets already starts during the cooling to the next T due to the nature of the IN.

Referee #1: Page 12710 line 21: I'm unclear on exactly how the authors reach the conclusion that M. alpina seem to form only a "single activity class"? Could the authors elaborate?

Response: As written in the manuscript the statement that the M. alpina IN seem to form only a single activity class comes from the comparison to bacterial IN which are known to contain different classes of IN active at different temperatures due to different-sized aggregates. This can be seen by several strong increases of the number of IN at different temperatures as the IN active at lower temperatures are typically more abundant than the IN active at higher temperatures. We see no increases in the number of IN, over and above the initial onset of activity, within the tested temperature range and thus our samples might have contained IN proteins of only one single class.

Other comments/typos

Referee #1: Page 12708 line 14: Typo in the word significantly here.

Response: The typo was corrected.

Referee #1: Page 12704, line 19: I suggest it is worth spelling out for the reader why a 0.1 μm filter was used here.

Response: Pore sizes of 0.2 or 0.1 μm are used for sterile filtration to remove possible contamination with bacteria or other particles. To make it more clear we changed the text into …" sterile filtered deionized water".

Referee #1: Page 12709 line 9: Can the authors explain for readers interested in the study, but not necessarily possessing a background in biology, what "arbuscular mycorrhizal fungi" is?

Response: Arbuscular mycorrhizal fungi are fungi that form a symbiotic association (called mycorrhiza) with the roots of plants. They are found in more than 80% of the plant families. For clarification we changed the text into: "…but based on their ability to solubilize phosphorus, they can also form interactions with arbuscular mycorrhizal fungi, which are plant root symbionts."

We thank Referee #2 for constructive comments and suggestions, which are highly appreciated and have been taken into account upon revision of our manuscript. Detailed responses are given below.

Referee #2: The only thing that I am missing is a bit more effort in searching possible evidence for such INP in previous studies. Given the novelty of reported discoveries, no previous study is likely to be found where M. alpine and INP have been investigated together. However, the characteristics of its INP provide clues for signs to look for. As described in the manuscript, they catalyse ice formation within a narrow temperature range, mostly between -5 and -6° C, pass through a 0.1 micron filter, but are larger than 100 kDa, withstand heating to 60°C, but are deactivated by heating to 98°C. This fits the characteristics of leaf-derived INP studied by Schnell and Vali (1973). Leaf material from temperate regions carried only around 100 INP active at -6°C per gram, whereas leaves from microthermal regions had INP numbers that where 4 to 5 orders of magnitude larger, suggesting the relevance of INP derived from M. alpina, or other fungi producing the same kind of INP, might be limited to microthermal environments, i.e. the continental climates of Eurasia and North America.

Response: The referee is right. We did not explicitly mention the leaf-derived INP as we used always “biological residues” or “biogenic IN” which of course include leaf-derived INP. We compared the characteristics of M. alpina INP with the INP of Fusarium, lichen, pollen and bacterial INP. We now added the leaf-derived INP into this comparison. For the leaf-derived INP it was found that bacteria (P. syringae) are involved in the production (Maki et al., 1974). As the degradation of plant litter is also mediated by fungi, fungi might also contribute to the leaf-derived INP, as suggested for Fusarium by Pouleur et al. (1992). Indeed, M. alpina could be a major source of the prodigious populations of leaf-derived INP recorded in litters of humid microthermal D-type (ie, >50° N latitude) forests by Schnell and Vali (1976).

Maki, L. R., Galyan, E. L., Chang-Chien, M.-M., & Caldwell, D. R. (1974). Ice Nucleation Induced by Pseudomonas syringae. Applied Microbiology, 28(3), 456–459.

Pouleur, S., Richard, C., Martin, J.-G., & Antoun, H. (1992). Ice Nucleation Activity in Fusarium acuminatum and Fusarium avenaceum. Applied and Environmental Microbiology, 58(9), 2960–2964.

R. C. Schnell and Gabor Vali, 1976: Biogenic ice nuclei: part i. terrestrial and marine sources. J. Atmos. Sci., 33, 1554–1564.
doi: http://dx.doi.org/10.1175/1520-0469(1976)033<1554:BINPIT>2.0.CO;2

Referee #2: Questions I would like to see addressed in the discussion section are:

a) Is there evidence for M. alpina (or alike) INP in the atmosphere or in precipitation (e.g. INP active between -5 and -6 degree C and passing through a 0.1 micron filter)?

Response: For M. alpina INP there is no evidence at the moment as the IN activity in M. alpina is a new finding, and several other sources of INP (eg, INA bacteria, INA Fusarium, K-feldspar) are also active at this temperature. We have first to identify the protein and its corresponding gene to be able to detect the protein or the gene in atmospheric and precipitation samples.

When looking for similar fungal INP, it is known that the INP from lichen forming fungi and Fusarium spp. pass through a 0.2 micron filter (see also page 12711, starting line 11 and references therein). In a former study, we have been able to isolate two more ice nucleation active fungal species that were not known as ice nucleation active before from atmospheric samples. Both produce INP that pass through a 0.1 micron filter and one of them is active at -5°C. The results are published in Huffman et al., 2013 and Pummer et al., 2014. However, even if we could detect these fungal INP in atmospheric samples we could not yet assign them to a certain fungal species. Interestingly, fungal or other biological INP that pass through a 0.1 micron filter seem to be ignored in a lot of studies as the cloud water, snow or hail stone samples are usually melted and then either filtered to collect particles >0.2 micron for the freezing assay (e.g., Christner et al. 2008; Hill et al., 2013) or measured without size segregation (e.g., Schnell and Vali, 1976; Garcia et al., 2012; Michaud et al., 2014). We further note that Vali (1966) observed that 25-50% of INP in hail were tiny (<0.01 um).

As it is already written in the manuscript (page 12712, line 25) that: " …further studies are necessary for the identification of the IN themselves and the detection and quantification of these fungi and their IN in soil and atmospheric samples.", we will add "precipitation samples" in the text.

Christner, B. C., Morris, C. E., Foreman, C. M., Cai, R., and Sands, D.C.; Ubiquity of Biological Ice Nucleators in Snowfall, Science, 2008: 319 (5867), 1214. [DOI:10.1126/science.1149757]

Garcia, E., T. C. J. Hill, A. J. Prenni, P. J. DeMott, G. D. Franc, and S. M. Kreidenweis (2012), Biogenic ice nuclei in boundary layer air over two U.S. High Plains agricultural regions, J. Geophys. Res., 117, D18209, doi:10.1029/2012JD018343.

Hill, T. C. J., B. F. Moffett, P. J. DeMott, D. G. Georgakopoulos, W. L. Stump, and G. D. Franc (2014), Measurement of ice nucleation-active bacteria on plants and in precipitation by quantitative PCR, Appl. Environ. Microbiol., 80(4), 1256–67, doi:10.1128/AEM.02967-13.

Huffman, J. A., Prenni, A. J., DeMott, P. J., Pöhlker, C., Mason, R. H., Robinson, N. H., Fröhlich-Nowoisky, J., Tobo, Y., Després, V. R., Garcia, E., Gochis, D. J., Harris, E., Müller-Germann, I., Ruzene, C., Schmer, B., Sinha, B., Day, D. A., Andreae, M. O., Jimenez, J. L., Gallagher, M., Kreidenweis, S. M., Bertram, A. K., and Pöschl, U.: High concentrations of biological aerosol particles and ice nuclei during and after rain, Atmos. Chem. Phys., 13, 6151-6164, doi:10.5194/acp-13-6151-2013, 2013.

Michaud, A. B., J. E. Dore, D. Leslie, W. B. Lyons, D. C. Sands, and, J. C. Priscu (2014), Biological ice nucleation initiates hailstone formation, J. Geophys. Res. Atmos., 119, 12,186–12,197, doi:10.1002/2014JD022004.

Pummer, B. G., Budke, C., Augustin-Bauditz, S., Niedermeier, D., Felgitsch, L., Kampf, C. J., Huber, R. G., Liedl, K. R., Loerting, T., Moschen, T., Schauperl, M., Tollinger, M., Morris, C. E., Wex, H., Grothe, H., Pöschl, U., Koop, T., and Fröhlich-Nowoisky, J.: Ice nucleation by water-soluble macromolecules, Atmos. Chem. Phys. Discuss., 14, 24273-24309, doi:10.5194/acpd-14-24273-2014, 2014.

Schnell, R.C. and G. Vali, World-wide source of leaf derived freezing nuclei. Nature, 236, 212-213, 1973.

Vali, G. 1966. Sizes of atmospheric ice nuclei. Nature, 212, 384-385.

b) If so, is this evidence restricted to microthermal environments?

Response: We have been able to isolate two ice nucleating fungal species from atmospheric samples collected in a semi-arid forest in Colorado (Isaria farinosa and Acremonium implicatum, see Huffman et al., 2013). They are not part of this study but we dont see a restriction as we don´t know the origin and how far the fungal spores traveled before we sampled them. Of course, further studies are necessary to establish relations with climatic zones. Pinus contorta and P. ponderosa forests are restricted to mesothermal or warmer environments, and so we did not test for M. alpina in any forests with a microthermal range. Fig. 8 in Schnell and Vali (1976) suggests that, in rain samples at least, onset of INP activity in the -5 to -6° C range was more common in Canada than in Colorado/Nebraska, and onset of INP activity seemed to be around -6 to -7°C in Colorado rain and hail (Vali 1978). However, INP active at -5 to -6° C were detected in hail and in 12/16 snow samples tested in Hill et al. (2014), and Christner et al. (2008) observed the onset of activity in most Louisiana rain samples to be -5 C to -6°C. So, there is some evidence of INP with warmer activity (ie, with a profile suggestive of M. alpina) in precipitation from microthermal environments, but it is not restricted to these regions.

Christner, B. C., Cai, R., Morris, C. E., McCarter, K. S., Foreman, C. M., Skidmore, M. L., Montross, S. N. and Sands, D. C. 2008. Geographic, seasonal, and precipitation chemistry influence on the abundance and activity of biological ice nucleators in rain and snow. Proceedings of the National Academy of Sciences, 105, 18854-18859.

Vali, G. 1978. Freezing nucleus content of hail and rain in NE Colorado. American Meterological Society Monograph, 38, 93-105.

c) Or, in the absence of other such evidence from soil, atmosphere or precipitation samples, is evidence from environments outside the microthermal regions lacking an indication for M. alpina derived INP?

Response: As we are not able to specifically detect M. alpina INP in soil, atmospheric or precipitation samples this cannot be answered at the moment.
We added the following statement in the text: „ 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. “

Ice Nucleation Activity in the Widespread Soil Fungus Mortierella alpina

J. Fröhlich-Nowoisky[1], T. C. J. Hill[2], B. G. Pummer[1], P. Yordanova[1], G. D. Franc[3†], and U. Pöschl[1]

[1]{Max Planck Institute for Chemistry, Multiphase Chemistry Department, Mainz, Germany}

[2]{Colorado State University, Department of Atmospheric Science, Fort Collins, USA}

[3]{University of Wyoming, Plant Sciences Department, Laramie, USA}

Correspondence to: J. Fröhlich (j.frohlich@mpic.de)

†Deceased.

Abstract

Biological residues in soil dust are a potentially strong source of atmospheric ice nucleators (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 south-east Wyoming, we found ice nucleation active (INA, i.e., inducing ice formation in the probed range of temperature and concentration) 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°C to -6°C, and belonged to a single zygomycotic species, *Mortierella alpina* (*Mortierellales, Mortierellomycotina*). By contrast, the handful of fungal species so far reported as INA all belong within the *Ascomycota* or *Basidiomycota* phyla. *M. alpina* is known to be saprobic, widespread in soil and 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 nucleators (IN), and biological IN can dominate the fraction active at warmer temperatures (Conen et al., 2011; O'Sullivan et al., 2013; Schnell and Vali, 1972, 1976). 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.

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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 (Creamean et al., 2013; Pratt et al., 2009). 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 (Bowers et al., 2009; Christner et al., 2008; Diehl et al., 2001; Georgakopoulos et al., 2009; Iannone et al., 2011; Kieft, 1988; Morris et al., 2004; Pouleur et al., 1992; Vali et al., 1976). 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; Humphreys et al., 2001; Tsumuki and Konno, 1994). Similarly, the sensitivity of lichen mycobiont IN (Kieft, 1988) to protein-degrading treatments and heating >70°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 (Goldstein and Nobel, 1991, 1994; Krog et al., 1979).

So far, only a few ascomycotic and basidiomycotic fungal species have been reported as being INA (Haga et al., 2013; Jayaweera and Flanagan, 1982; Kieft, 1988; Morris et al., 2013; Pouleur et al., 1992; Richard et al., 1996), 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 a half to a few percent of the soil organic matter (Fierer et al., 2009; Wardle, 1992; Zak et al., 1994), 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 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 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°C for less than a week before being thoroughly mixed immediately before soil dilution plating.

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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). 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), 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 more days of incubation. Out of 489 picked CFU 474 showed growth in the liquid medium and were thus tested for ice nucleation activity.

It was originally intended to grow the isolates on malt extract agar. However, since the available product was found to contain some IN (active at -12°C) an approximate equivalent using IN-free ingredients (tested to -18°C) was constructed. This DPY broth/solid medium contained 10 g L^{-1} dextrose, 3 g L^{-1} peptone (as detailed above) and 0.3 g L^{-1} yeast extract filtered through a 0.2-µm-pore-diameter filter (PES disposable filter units, Life Science Products). For the solid medium, 15 g L^{-1} agarose (Certified Molecular Biology Agarose, Bio-Rad) 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 plates.

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). 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 true temperature measured using a thermistor (VPT-0300, Bio-Rad). After a 5 min dwell time at each temperature, the number of frozen wells was counted and the temperature lowered to

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the next level. Once at -9°C, the tray was transferred to a 96-well aluminum incubation block (VWR) 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, ≈ 10^9 IN g^{-1} mycelium) were used as positive controls. 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.

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2.4 Identification and phylogenetic analysis

For identification and phylogenetic analyses, hyphae and spores were first picked using sterile pipette tips into 20 µL water and lysed at 95°C for 10 min. This lysate was used as 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), 0.2 mM each dNTP (Roth), 0.33 µM of each primer (Sigma-Aldrich), and 1.25 units of JumpStart™ REDTaq DNA polymerase (Sigma-Aldrich). 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) 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) following the supplier's instructions. Colonies containing inserts were identified by 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) and 0.25 µM of each primer (Sigma-Aldrich). 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 ten colony PCR products per isolate and gene were chosen.

DNA sequences were determined with ABI Prism 377, 3100, and 3730 sequencers (Applied Biosystems) using BigDye-terminator v3.1 chemistry at the Max Planck Genome Center of the Max Planck Institute for Plant Breeding Research, Cologne. 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) 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), which was then connected to a Julabo Presto A30 cooling bath operating with Thermal HL40 (Julabo) 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 minutes, 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 g^{-1} mycelium, the entire mass of mycelium (containing spores) of a fungal culture was harvested by scraping it off the PDA agar surface and transferred it into a sterile 15 mL tube which was weighed before and after harvesting. Depending on the individual isolates between 0.1 g and 1.3 g mycelium could be harvested. Ten milliliter of 0.1-µm-pore-diameter sterile filtered (Acrodisc, PES, Pall) 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) 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 mL was calculated using the formula of Vali (1971): $-\ln(f)\cdot V^{-1}$ where f is the proportion of droplets not frozen and V the volume of each aliquot. The number of IN per gram was then calculated by using the dilution factor and the mass of the mycelium. Binomial confidence intervals (95%) were derived by using the formula 2 as recommended by Agresti and Coull (1998).

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[1] nach oben verschoben: 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.

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) and Vivaspin® filter tubes (Sartorius) of different mass exclusion limits (100 kDa, 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 of the enzymes: (i) papain (AppliChem) at 60°C, (ii) pepsin (Sigma) at 37°C, pH 1.5, or (iii) lipase (AppliChem) at 37°C; (B) 1-2 h at room temperature with (i) 6 M guanidinium chloride (Promega) or (ii) 0.3 M boric acid (National Diagnostics); (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.

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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 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 south-east Wyoming, USA (see Table 1a/b for site and soil details). Soil dilution series were prepared and all 474 fungal colony forming units (CFU) 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°C 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).

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 94-100% (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 neighbor joining (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 (Ho and Chen, 2008; Kwaśna et al., 2006). 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, while 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 (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), 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 datasets, 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 g^{-1} 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 tested isolates from pasture, forest, sugar beet, grassland, and potato exhibited a consistency in possessing an intermediate range of between $\approx 10^8$ -10^9 IN g^{-1} 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 IN activity (Table 4), but after passage through a 100 kDa device IN activity was removed, 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, 0.3 M boric acid or 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 IN activity after 98°C treatment. Lipase and boric acid did not affect the IN 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 IN activity when digested with papain. Clade A was thus treated with another protease, pepsin, which also did not affect the IN activity.

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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, previously, all reported INA fungi belonged to the phyla *Ascomycota* and *Basidiomycota* (Haga et al., 2013; Henderson-Begg et al., 2009; Huffman et al., 2013; Iannone et al., 2011; Jayaweera and Flanagan, 1982; Kieft and Ahmadjian, 1989; 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 (Bokhary and Parvez, 1995; Hyland et al., 1953; Kwaasi et al., 1998; Pawsey and Heath, 1964; Turner, 1966). *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 (Degawa and Gams, 2004; Turner, 1963; Upadhyay et al., 1981) or are mycoparasites themselves (Willoughby, 1988).

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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 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).

As *M. alpina* is a non-pathogen but 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 (Frisvad, 2008; Weete and Gandhi, 1999; Zachariassen and Kristiansen, 2000).

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 eicosapentateonic 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.* (Frisvad, 2008; Weete and Gandhi, 1999). Arachidonic acid is a polyunsaturated fatty acid that can comprise up to 54% of the fatty acids in the mycelium (Ho and Chen, 2008; Lounds et al., 2007; Weete and Gandhi, 1999) 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 phosphorous. 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 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 (Govindarajan and Lindow, 1988; Phelps et al., 1986; Ruggles et al., 1993; Turner et al., 1990), 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 (Figure 2, Table 3) would correspond with 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 to 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 IN activity, however, the 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 (Morris et al., 2013; Pummer et al., 2012), evidence points to proteins as the source of INA of the known INA fungi (*Fusarium*, lichen mycobionts) (Hasegawa et al., 1994; Kieft and Ruscetti, 1990).

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 (Kieft and Ruscetti, 1990; Pouleur et al., 1992; Schnell and Vali, 1976). The IN of *M. alpina* are smaller than 100 nm in size, between 100-300 kDa in mass and can be readily released into the surrounding medium. The latter is also a characteristic of several INA *Fusarium* species (Hasegawa et al., 1994; Humphreys et al., 2001; Pouleur et al., 1992; Tsumuki and Konno, 1994), leaf-derived IN (Schnell and Vali, 1973) some INA bacteria (Kawahara et al., 1993; Phelps et al., 1986), 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., 2013; Tobo et al., 2014).

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To understand the role of the IN of *M. alpina* and other INA fungi in soil and in the atmosphere, further surveys for 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 soil-borne 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, 2009b). 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 (Brown and Hovmøller, 2002; DeLeon-Rodriguez et al., 2013; Elbert et al., 2007; Griffin, 2004; Hawksworth, 2001; Imshenetsky et al., 1978; Kellogg and Griffin, 2006; Pearce et al., 2009; Prospero et al., 2005). 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 (Morris et al., 2008; Sands et al., 1982). However, the release of small extracellular IN into the soil might, unintentionally, confer IN 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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Table 1a. Description of sampling sites.

Site	Sampling date (2011)	Lat	Long	Elevation (m)	Annual precipitation (mm)	Annual avg air T (°C)	Days with T < 0°C	Vegetation
				Crop soils				
Alfalfa[1]	2nd 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[1]	2nd Mar	42.13167	-104.39413	1270	336	9.3	181	Bare at sampling. Previous year was a mixed crop of dry beans.
Potato[1]	2nd Mar	42.13167	-104.39516	1270	336	9.3	181	Bare at sampling. Previous year was potato.
Sugar beet[1]	2nd 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[2]	24th 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, heartleaf arnica.
Grassland[2]	24th May	41.2881	-106.11124	2420	385	4.6	214	Bluebunch wheatgrass, Idaho fescue, western wheatgrass and threetip sagebrush.
Pasture[1]	2nd Mar	42.13243	-104.39428	1270	336	9.3	181	Smooth brome and downy brome.

[1] Lingle [2] Centennial

Table 1b. Characterization of soil samples.

Site	Soil type	% SOM[3]	% N	pH
	Crop soils			
Alfalfa	Haverson & McCook light brownish-gray floodplain loams.[1]	0.95	0.076	8.1
Bean	Haverson & McCook light brownish-gray floodplain loams.[1]	-	-	-
Potato	Haverson & McCook light brownish-gray floodplain loams.[1]	-	-	-
Sugar beet	Haverson & McCook light brownish-gray floodplain loams.[1]	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.[2]	3.7	0.27	6.45
Forest	Ansile-Granile gravelly sandy loam. 5 cm layer of needles and bark residue.[2]	100	2.05	5.9
Pasture	Haverson & McCook light brownish-gray floodplain loams.[1]	4.7	0.465	7.85

[1] Soil survey of Goshen County, south part, Wyoming. 1971. United States Department of Agriculture, Soil Conservation Service, 102 pp. [2] Soil survey of Albany County Area, Wyoming. 1998. United States Department of Agriculture, Natural Resources Conservation Service, U.S Government Printing Office, 540 pp. [3] Soil organic matter (SOM) contents obtained by multiplying percentage carbon by 1.724.

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

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

Site	ID no.	Clade	$T_{initial}$ (°C)	ITS		GLELO	
				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
	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)	93.31
	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

Table 4. Changes of number of IN in orders of magnitude after filtration (5 µm, 0.1 µm, 100 kDa, 300 kDa), thermal (60°C, 98°C), chemical (guanidinium chloride (G.Cl), 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. Colors are defined as follows: Dark green: 0.9 to -1, light green: -1 to -2, orange: -2 to -3, red: <-3, blue: not clear, gray: 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	-5,6	-0,5	-0,8	-4,4	--
37B	-0,3	0,0	-0,2	-3,2	-0,5	< -7	< -7	-0,7	-0,7	-2,6	--
38B	0,5	0,0	0,1	-4,8	-2,0	-6,8	< -7	--	-0,8	-4,4	--
40B	0,0	0,0	0,0	-4,4	-0,5	< -7	-6,2	-0,2	-0,3	-2,2	--
14C	0,1	0,0	0,0	< -3	-0,4	< -3	< -3	-0,2	0,0	-2,6	--
16C	0,0	0,0	0,0	-2,0	0,1	-3,4	< -3	--	0,0	< -3	--
17C	-0,3	0,0	-0,1	-4,2	-1,2	< -4	< -4	-0,6	-0,5	-3,6	--
22C	0,0	0,0	0,0	< -2	0,1	< -2	< -2	0,0	0,0	< -2	--
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	-0,4	< -3	< -3	--	--	--	--
33C	-0,2	0,0	-0,6	< -3	-1,6	< -3	-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	< -4	< -4	-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	-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	-3,6	0,0	-0,1	-2,9	--
19D	0,2	0,0	0,0	-4,9	-0,1	< -5	< -5	-0,1	-0,3	-3,0	--

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).

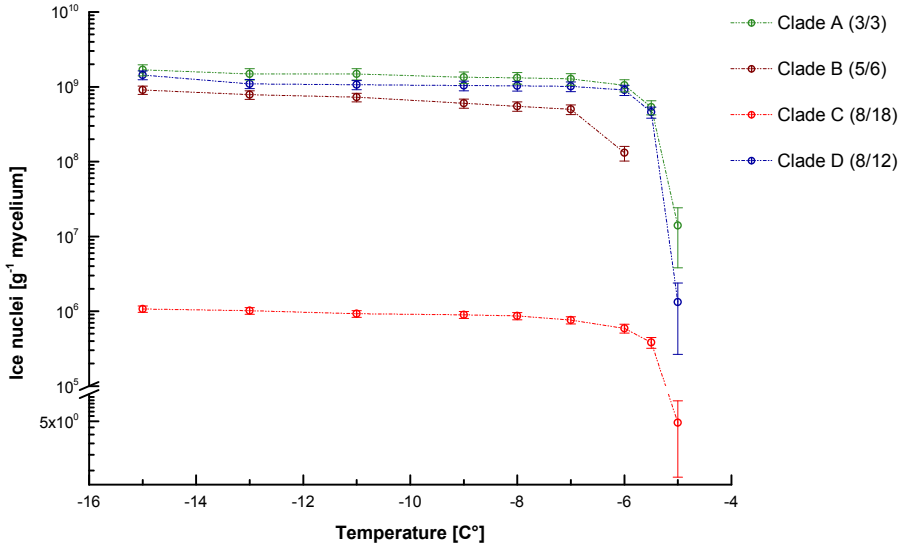

Figure 2. Average number of IN g^{-1} mycelium (fresh weight) for all clades. The clades are classified as A) predominantly uncultivated, B) forest, C) predominantly standard agricultural, and D) high organic matter input agricultural. The number in brackets represents the number of isolates tested out of total number of isolates from each clade. Error bars represent the 95% confidence intervals.

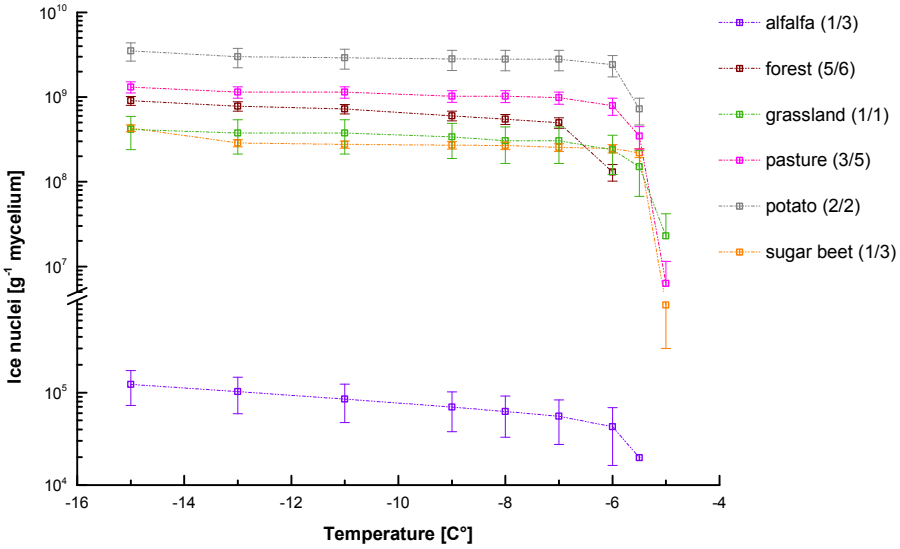

Figure 3. Average number of IN g^{-1} mycelium for the isolates of different soil types. The number in brackets represents the number of isolates tested out of the total number of isolates each sampling site. Error bars represent the 95% confidence intervals.

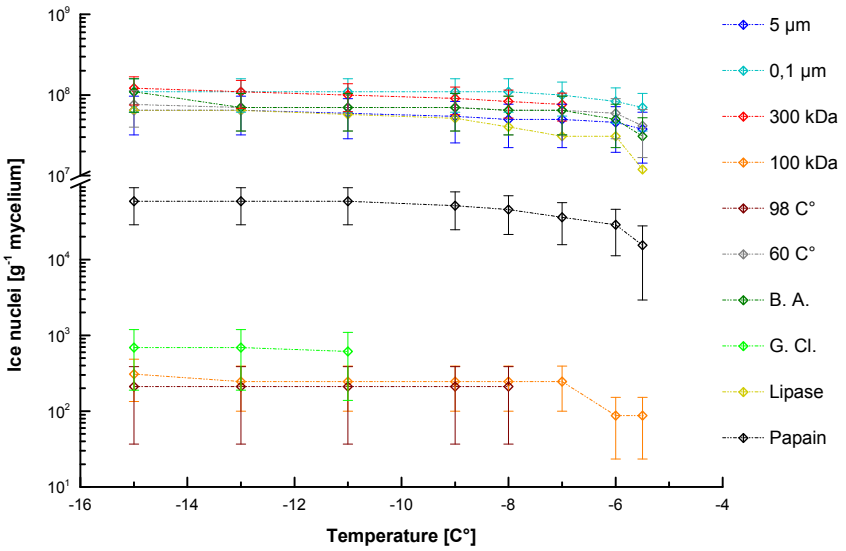

Figure 4. Number of IN g^{-1} mycelium for isolate ID6 after filtration, thermal, chemical, or enzymatic treatments. G.Cl stands for guanidinium chloride, B.A for boric acid. Error bars represent the 95% confidence intervals.