MS bg-2019-276, Kunert et al.: Highly active and stable fungal ice nuclei are widespread among Fusarium species

We thank referee #2 for his/her constructive comments and suggestions, which are highly appreciated and have been taken into account upon revision of our manuscript. The comments and our answers are listed below (referee's comments marked with blue letters).

Specific comments:

Abstract:

Referee comment: Indicate the biological relevance of Fusarium and its ice nucleation activity. This is discussed well in the introduction but will help to bridge the first few sentences of the abstract.
Author's response: We thank the referee for this suggestion and included the following sentences in the abstract: "Ice nucleation activity in fungi was first discovered in the cosmopolitan genus *Fusarium*, which is widespread in soil and plants, has been found in atmospheric aerosol and cloud water samples, and can be regarded as the best studied IN-active fungus."

Moreover, we modified the following sentences: "The frequency and distribution of ice nucleation activity within *Fusarium*, however, remains elusive. Here, we tested more than 100 strains from 65 different *Fusarium* species for ice nucleation activity."

Methods 2.1:

Referee comment: How were the initial samples obtained? Could their original environment (crop vs. airborne, etc.) shed light on IN frequency?
Author's response: Samples from the USDA-ARS/Michigan State University were collected from crop tissue (sugar beet), and samples from the Schmale Laboratory at Virginia Tech were collected with unmanned aircraft systems. There is no detailed information available for the sources of the strains for the Kansas State University Teaching collection. We found IN activity in isolates from crop and air samples. For the air samples we cannot draw any conclusions from their original environment. A controlled comparison of IN frequency from samples collected in the air versus crop plants (and maybe even different types of crop plants) would be important, now that more IN-active species are known.

However, we added the following paragraph to section 2.1: "The strains from the USDA-ARS/Michigan State University were collected from crop tissue (sugar beet). All isolates were from field-grown beets and were obtained by hyphal tip transfer. The strains from the Schmale Laboratory at Virginia Tech were collected with unmanned aircraft systems (UASs or drones) equipped with remotely-operated sampling devices containing a *Fusarium* selective medium (e.g., Lin et al., 2013, 2014). All of the Schmale Laboratory strains were collected 100 m above ground level at the Kentland Farm in Blacksburg, Virginia, USA. Detailed information is not available for the sources of the strains for the Kansas State University Teaching collection. However, some of these strains are holotype strains referenced in Leslie and Summerell (2006)."

We extended Table S1 and provided additional information about sampling location and date.

Referee comment: Line 21: Additional, more recent, studies have contributed to this understanding of IN as well. (Failor et. al. 2017, Hanlon et al. 2017, Stopelli et al. 2017, 2015, Joly et al. 2014).
Author's response: We thank the referee for this remark and added the references to our manuscript.

Referee comment: Line 24-6: Failor et al. (2017) further expanded on known gammaproteobacteria IN.
Author's response: We changed the sentences as follows: "The best characterized biological IN are common plant-associated bacteria of the genera *Pseudomonas*, *Pantoea*, and *Xanthomonas* (Garnham et al., 2011; Govindarajan and Lindow, 1988; Graether and Jia, 2001; Green and Warren, 1985; Hill et al., 2014; Kim et al., 1987; Ling et al., 2018; Schmid et al., 1997; Wolber et al., 1986), and recently, an ice nucleation-active (IN-active) *Lysinibacillus* was found (Failor et al., 2017). The first identified IN-active fungi were strains of the genus *Fusarium* (Hasegawa et al., 1994, Pouleur et al., 1992, Richard et al., 1996, Tsumuki et al., 1992)."

Referee comment: Line 118: Was the range of incubation times necessary to reach a specified optical density? If so, that indication would be useful. If not, elaborate of reasoning for the times.
Author's response: Here, we did not mean that we tested these different incubation times. The sentence was meant to indicate the procedure considering all of the different replications that we used. For clarification, we changed "incubated" to "equilibrated".

Referee comment: Line 119: Be specific for the 0.5°C freezing point depression. Is it 0.5°C or 0.5±x °C.
Author's response: We added the calculations to the supplementary information.

We modified the sentence: "Note, that the aqueous extracts were prepared in 0.9 % NaCl solution, which could reduce the freezing temperatures by 0.5 °C based on theoretical calculations."

Results 3.1:

Referee comment: This would be an interesting point to note the original sampling locations for the various strains and could further demonstrate the cosmopolitan nature of these IN-active species should any tends be identified.
Author's response: We thank the referee for this comment, but as described before, we had only a few different sampling locations for both, the USDA-ARS/Michigan State University and samples from the Schmale Laboratory at Virginia Tech. For samples from the Kansas State University, we cannot specify the original sampling locations further as we obtained these samples from a culture collection.

Referee comment: Lines 154-5: This is a risky assumption to make. Prior to the Failor et al. study, all bacterial IN were thought to be proteinaceous. Exposing a selection of the species to high heat could support this claim.
Author's response: As many earlier studies already performed experiments with heat treatment of *Fusarium* IN, we initially refrained from repeating these experiments. The studies of Hasegawa et al. (1994), Pouleur et al. (1992), and Tsumuki and Konno (1994) only investigated some species of the genus *Fusarium*, and we agree with the referee that it is risky to generalize these findings to the newly found IN-active *Fusarium* species. Based on the suggestion of

referee #2 and #3, we performed additional heat treatment experiments with four different *Fusarium* species: *F. acuminatum*, *F. armeniacum*, *F. avenaceum*, and *F. langsethiae*.

We added a new Figure 4, and renumbered the other figures.

We included the following sentence in the abstract: "Heat treatment at 40 °C to 98 °C, however, strongly reduced the observed IN concentrations, confirming earlier hypotheses that the INM in *Fusarium* largely consists of a proteinaceous compound."

We modified the following sentence in the introduction: "Furthermore, the stability of *Fusarium* IN upon exposure to ozone and nitrogen dioxide, under high and low or quickly changing temperatures, and after short- and long-term storage under various conditions was investigated."

We modified the following sentence in section 2.1: "For quantitative analysis, exposure experiments, heat treatments, freeze-thaw cycles, as well as short- and long-term storage tests a selection of IN-active tested strains was grown on full-strength potato dextrose agar (VWR International GmbH, Darmstadt, Germany) first at room temperature for four to six days and then at 6 °C for about four weeks."

We included the following sentences in section 2.2: "For heat treatment experiments, aliquots of aqueous extracts of *F. acuminatum* 3-68, *F. armeniacum* 20970, *F. avenaceum* 2-106, and *F. langsethiae* 19084 were incubated at 40 °C, 70 °C, and 98 °C, respectively, for one hour. The IN concentration was determined using TINA."

We changed the following sentences in section 3.3: "They can be exposed to chemically modifying agents like ozone and nitrogen dioxide, and physical stressors like high and low or quickly changing temperatures. To investigate the stability of *Fusarium* IN, we performed exposure experiments, heat treatments, freeze-thaw cycles, and long-term storage tests."

We included a new paragraph in section 3.3: "The stability of the INM in *Fusarium* was investigated in heat treatment experiments. The ice nucleation activity was reduced significantly at a 40 °C treatment (Fig. 4). Between 40 % and 90 % of IN were lost at this temperature depending on the species, which supports the hypothesis that the INM in *Fusarium* consists of a proteinaceous compound. A heat treatment at 70 °C reduced the ice nucleation activity to less than 0.01 % compared to the initial level. Moreover, the initial freezing temperature was shifted to lower temperatures indicating a breakdown of the large protein aggregates. After a 98 °C treatment, we still found ice nucleation activity for all investigated species except for *F.avenaceum* 2-106. The results are in agreement with previous studies, which also reported a reduction in ice nucleation activity with increasing temperature in heat treatment experiments (Hasegawa et al., 1994; Pouleur et al., 1992; Tsumuki and Konno, 1994). The remaining activity after the 98 °C treatment, however, could indicate that post-translational modifications like glycosylation and therefore polysaccharides could play a role in the ice nucleation activity of *Fusarium*. Further systematic and chemical analysis studies are needed for elucidation."

We included the following sentences in the conclusion: "A heat treatment of 40 °C reduced the IN concentration significantly, supporting the hypothesis that the INM in *Fusarium* largely consists of a proteinaceous compound. An involvement of polysaccharides, however, cannot be excluded."

Referee comment: Lines 184-6: With the drastic decrease in activity after the 300,000 MWCO filter and then again after 100,000, could the protein not be larger, but when damaged or broken still retains some ice nucleation activity?

Author's response: If the INM in *Fusarium* is a single large protein, which breaks into small parts upon filtration, we would expect based on Govindarajan and Lindow (1988) and Pummer et al. (2015) a much lower initial freezing temperature of the filtrate than the temperature, which we obtained in our experiments. The only small shift in the initial freezing temperature after filtration suggests that small IN reassemble again to larger aggregates with similar activity than before filtration. It is unlikely that a damaged or broken IN protein would show a similar activity even if the broken parts would aggregate.

Referee comment: Lines 195-6: Why would single proteins in the atmosphere be unlikely? Please elaborate on this statement.

Author's response: As hypothesized in Lines 184-185, the proteins tend to agglomerate, which make it unlikely that individual proteins will enter the atmosphere. However; if an individual protein would enter the atmosphere it would be in the nucleation mode size range of ~ 6 nm. These particles tend to grow by condensation of gaseous compounds (e.g., semi volatile organic compounds, sulfates, water) and grow to particles in the Aitken mode size range. In this size range further condensation and coagulation takes place and larger agglomerates are formed.

We included the following sentence to our manuscript: "Individual proteins with a diameter of ~ 6 nm which may enter the atmosphere would be in the nucleation mode size range, where particles tend to uptake gaseous compounds and grow to Aitken mode particles, which themselves tend to coagulate to larger agglomerates (Seinfeld and Pandis, 1998)."

Referee comment: Line 216: Change ". . .and the fungus could safe energy." to ". . .and the fungus could save energy.".

Author's response: Changed as suggested.

Referee comment: Figure 1. Inclusion of the positive control SnoMax curve would be beneficial here. Any incidence of spontaneous freezing of the negative control should also be noted (if any occurred with the methods you used).

Author's response: As the focus of this study is on fungal IN of *Fusarium*, we did not use Snomax in any of the TINA experiments. The *Fusarium* strains themselves served as positive controls based on the results of the initial screening (Table S1). Moreover, the correct functionality of TINA including a Snomax curve is presented in Kunert et al. (2018).

For freezing tests, however, a negative control is essential. We added the information about the negative controls and included the following sentences in the manuscript:

For the thermal cycler: "Aliquots of uninoculated DPY broth were used as negative controls, which did not freeze in the investigated temperature interval."

For LINDA experiments: "As a negative control, a 0.9 % NaCl solution was added to three uninoculated agar plates, and the freezing started below -14 °C."

For TINA experiments: "Pure water samples (0.1 µm filtered) served as a negative control for each experiment. These did not freeze in the observed temperature interval."

Pseudomonas syringae CC94 was used as positive control for the initial screening using LINDA as droplet freezing assay. We included the following sentence in section 2.3: "The freezing temperatures ranged from -3.46 °C to -4.58 °C."

Referee comment: Figure 3. You note in the text that SnoMax has been shown to decrease after exposure. Did you see this same result, or did you not use SnoMax because of this interaction?
Author's response: We showed in a previous study that the IN activity of Snomax decreased after exposure to O_3 and NO_2 (Kunert et al. 2018). As this manuscript is focused on the IN activity of *Fusarium*, we refrained from repeating the experiments.

References:

Govindarajan, A. G. and Lindow, S. E.: Size of bacterial ice-nucleation sites measured *in situ* by radiation inactivation analysis, Proceedings of the National Academy of Sciences of the United States of America, 85, 1334–1338, 1988.

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Pummer, B. G., Budke, C., 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, Atmospheric Chemistry and Physics, 15, 4077–4091, 2015.

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