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

We thank referee #1 for his/her comments, questions, and suggestions, which 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).

1. Title:

Referee comment: The title is misleading: the authors' conclusion is that 16% of the tested strains were ice active above -14 °C. I would argue that this percentage does not equate to "widespread". The authors also did substantial work with physical and chemical processing of their material which is not reflected in the title, but could be. For example, something along the lines of "Ice nucleation ability of 65 different Fusarium species: Effects of storage, size and chemical processing"

Author's response: We changed the title to: "Macromolecular fungal ice nuclei in *Fusarium*: Effects of physical and chemical processing" and modified the corresponding parts in the manuscript accordingly.

2. Abstract:

Referee comment: Why did the authors choose -14 °C as their threshold? This discussion should be added here in the abstract (and in the text as well).

Author's response: We thank the referee for pointing this out. We actually meant -12 °C, and we realized that we had a typing error here, which we corrected now.

As *Fusarium* nucleates in a broad temperature range between -1 and -9 °C (Hasegawa et al., 1994; Humphreys et al., 2001; Pouleur et al., 1992; Richard et al., 1996; Tsumuki et al., 1992; Tsumuki and Konno, 1994), and as the water background of LINDA started to freeze at -14 °C, we set the threshold to -12 °C.

Referee comment: The relevance of Fusarium should be explained in 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."

3. Introduction:

Referee comment: Lines 16-17: more recent references should be added, especially because of the mention of macromolecules. Also see review by (Knopf et al., 2018).

Author's response: We thank the referee for this comment and included further references in our manuscript.

Referee comment: Lines 18-20: it would be important to mention nonetheless that recent work has made contributions to our understanding of IN and precipitation references by (Petters and Wright, 2015; Stopelli et al., 2015, 2017).

Author's response: We thank the referee for this remark and added the references to our manuscript.

Referee comment: Lines 21-23: the 3+9 references could be better represented by explaining what each one has observed in one or two sentences each. This added discussion could help set the stage for the relevance of the work under review.

Author's response: The discussion of each reference in one or two sentences each would result in a very long introduction with a review character, especially as referee #2 suggested to add additional references here. Adding a detailed discussion of the different types of biological ice nuclei at this point goes beyond the scope and focus of this manuscript and could lead to confusion of the readers. Instead, some of the references are discussed in more detail in the results and discussion section.

Referee comment: Lines 24-26: same comment as above in addition to this reference (Šantl-Temkiv et al., 2015)

Author's response: We included the suggested reference as well as the reference, which was suggested by referee #2 (Failor et al., 2017), but we prefer to not extend the bacterial IN part of the introduction as the focus of the manuscript should be on fungi, particularly *Fusarium*.

We modified the following sentences: "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: Lines 28-30: when temperatures are reported, what fraction does it represented? The onset? 1%? Temperature when 50% of the droplets are frozen- T50? See (Vali, 2019)

Author's response: As mentioned in Line 28, the temperatures are reported as initial freezing temperatures, which corresponds to the onset freezing temperature: "To date, a few more fungal genera with varying initial freezing temperatures such as *Isaria farinosa* (~ -4 °C), *Mortierella alpina* (~ -5 °C), *Puccinia species* (-4 °C to -8 °C), and *Sarocladium* (formerly named *Acremonium*) *implicatum* (~ -9 °C) have been identified as IN-active (Fröhlich-Nowoisky et al., 2015; Huffman et al., 2013; Morris et al., 2013; Richard et al., 1996)".

Referee comment: Line 39: define the positive selective pressure for IN activity

Author's response: We thank the referee for pointing out the ambiguity of our statement. For clarification, we modified the sentence: "While the factors for a positive selective pressure for ice nucleation activity in *Fusarium* and other fungi have not been directly identified, an ecological advantage of initiating ice formation is easily conceivable." For example, the bioprecipitation feedback cycle can be such a factor, which is discussed in more detail later (Lines 47-49).

Referee comment: It would be useful for the authors to discuss the mode of freezing investigated and why immersion freezing was used and what is its relevance.

Author's response: The droplet freezing assays, which were used in this study, all measure ice nucleation activity in the immersion freezing mode, where the IN is contained inside a liquid droplet when initiating freezing. Biological IN are often proteins, which are surrounded by a hydration shell, so the immersion freezing mode is suitable for biological IN. Thus, the most common techniques to study biological IN are droplet freezing assays (Després et al., 2012; Hoose and Möhler, 2012).

To avoid misunderstanding, we modified the sentence: "Ice nuclei of selected *Fusarium* species were further analyzed in immersion freezing mode using the high-throughput Twin-plate Ice Nucleation Assay (TINA) (Kunert et al., 2018)."

Referee comment: Good overview of bioprecipitation. Great description of the evolutionary reasons for fungal species to be good ice nuclei.
Author's response: We thank the referee for this comment.

4. Materials and Methods
Referee comment: In general, controls and filter blanks are missing from the data description and analysis and the authors are encouraged to show this data (perhaps in supplementary information) and to discuss this data. For example, what was the IN activity of the water background? What was the activity of the filter background? How did the backgrounds differ from LINDA to TINA?
Author's response: 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."

Referee comment: It is clear that the authors used two techniques for their experiments, yet their discussion does not include any comparison plots or discussing the differences in the two instruments. Each figure (and Table S1) should also state which instrument was used to acquire the data.
Author's response: As described in Lines 104-105, the initial screening was performed with two independent droplet freezing assays in two laboratories. Strains of the USDA-ARS/Michigan State University were screened with a thermal cycler as described in Fröhlich-Nowoisky et al. (2015) (Lines 106-108). Strains from the Schmale laboratory at Virginia Tech and strains from the Kansas State University Teaching Collection were screened with LINDA (Lines 111-113). Table S1 provides a summary of all tested strains, the strain collection they originate from, and the results of the screening. Table 1 shows the mean freezing temperatures for the positively tested species. All further analyses were performed with TINA.

Referee comment: Line 115: could the authors show the positive control data?
Author's response: We included the following sentence in section 2.3: "The freezing temperatures ranged from -3.46 °C to -4.58 °C."

Referee comment: Lines 119: clarification: can the authors show their calculations here and are the data presented corrected for the freezing point depression or is the 0.5 C part of the overall uncertainty?
Author's response: We added the calculations to the supplementary information.

The data presented here were not corrected for the freezing point depression as highly concentrated *Fusarium* extracts were used for the initial screening. Thus, we cannot exclude that the high concentration of *Fusarium* IN compensates the effect of NaCl on the freezing

temperature. We added this information in the manuscript: "We cannot exclude, however, that the high concentration of IN compensates the effect of NaCl on the freezing temperature. This is supported by the investigations of Stopelli et al. (2014), who did not find a systematic suppression of freezing at this salt concentration in LINDA experiments."

Referee comment: Additional experiment: dilution series of an active strain to see if the behaviour of the IN active material in solution is linear. I would argue that this experiment would be important to help support the seemingly accurate high freezing temperature data observed for certain strains, for example in Figures 3 and 4 and S1.

Author's response: All samples, which were analyzed with TINA, were measured in a dilution series. We described this in Lines 121-123: "The aqueous extracts were serially diluted 10-fold with pure water by a liquid handling station (epMotion ep5073, Eppendorf, Hamburg, Germany), and 96 droplets (3 µL) were tested per dilution with a continuous cooling rate of 1 °C min^{-1} from 0 °C to -20 °C."

For clarification, we optimized the sentences: "The aqueous extract was serially diluted 10-fold with pure water by a liquid handling station (epMotion ep5073, Eppendorf, Hamburg, Germany) to a dilution where droplets remained liquid in the investigated temperature interval. Of each dilution, 96 droplets (3 µL) were tested with a continuous cooling rate of 1 °C min^{-1} from 0 °C to -20 °C."

5. Results and Discussion

Referee comment: It is necessary for the authors to define their reported freezing temperatures. Are they the onset, the equivalent of one well freezing? If so, how do the authors address the recommendations of not using the onset addressed in (Polen et al., 2018)? Reporting freezing temperatures as T10 and T50 would be additionally helpful.

Author's response: Except for the initial screening, we always report the initial freezing temperatures (T_i) for our measurements, which is equivalent to the onset. We first reported the freezing temperatures for the initial screening as initial freezing temperatures, but we actually meant mean freezing temperatures.

We replaced "initial" by "mean" several times in the text, where we talk about the initial screening.

Referee comment: Lines 141-144: could the authors offer a hypothesis to this lack of verifiability?

Author's response: The fungal culture plates, which were used for the initial screening, could not be used for the measurements with TINA, as different laboratories were involved in this study. Moreover, it is well known that some *Fusarium* species can reduce or lose their IN activity after several subcultures (Pummer et al., 2013; Tsumuki et al., 1995). We discussed this in Lines 156-159 in the manuscript: "It is known that *Fusarium* can regulate the gene expression for IN production depending on environmental conditions such as nutrient availability (Richard et al., 1996), and some *Fusarium* species reduce or lose their IN activity after several subcultures (Pummer et al., 2013; Tsumuki et al., 1995)."

Referee comment: The hypothesis of proteinaceous material acting as IN is valid. What about polysaccharides? (Dreischmeier et al., 2017)

Author's response: We cannot exclude that polysaccharides are involved in the ice nucleation of *Fusarium*. To our knowledge, however, there is no published study showing that polysaccharides are involved in the ice nucleation activity of *Fusarium*.

We discussed a potential role in section 3.3: "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 studies including chemical analyses are needed for elucidation."

We included the following sentence in the conclusion: "An involvement of polysaccharides, however, cannot be excluded."

Referee comment: Line 166: was there any hypothesis associated with the selection of the strains presented in this section?

Author's response: Not all *Fusarium* strains were available for the experiments with TINA, as the initial screening was performed in different laboratories. But we tried to cover as many different species as possible and selected species, which were long known for ice nucleation activity (*F. acuminatum*, *F. avenaceum*) as well as all the newly identified species.

For clarification, we included this information in section 2.3: "Ice nuclei of selected *Fusarium* species, which were long known for ice nucleation activity (*F. acuminatum*, *F. avenaceum*) as well as all the newly identified species, were further analyzed in immersion freezing mode using the high-throughput Twin-plate Ice Nucleation Assay (TINA) (Kunert et al., 2018)."

Referee comment: Size experiments should be compared to (Irish et al., 2019; Wilson et al., 2015) for example. In addition, the Wilson et al., Nature 2015 paper has a nm parameterization that the authors should include in their discussion of their values.

Author's response: We included the following sentence: "Moreover, biological INMs smaller than 200 nm were also found in various organisms e.g., other fungi (Fröhlich-Nowoisky et al., 2015; Pummer et al., 2015), leaves, bark, and pollen from birch trees (*Betula* spp.) (Felgitsch et al.,2018; Pummer et al., 2012), leaf litter (Schnell and Vali, 1973), some microalgae (Tesson and Šantl-Temkiv, 2018), strains of *Lysinibacillus* (Failor et al., 2017), and biological particles in the sea surface microlayer (Irish et al., 2019; Wilson et al., 2015)."

Referee comment: Lines 184-185: I do not understand how the authors arrived at this conclusion. According to figure 2, the majority of the IN activity was lost between 300 and 100 kDa. I would have concluded that the best IN are within that size, not smaller than 100 kDa. I agree with the authors nonetheless that there are still IN active material below 100 kDa, but not the most active.

Author's response: As IN were found in all size fractions, we concluded that *Fusarium* IN are likely single proteins smaller than 100 kDa, which can agglomerate to large protein complexes in solution. We did not claim that the single proteins smaller than 100 kDa are the most active ones. Lines 184-185: "We hypothesize that *Fusarium* IN are single proteins smaller than 100 kDa, which agglomerate to large protein complexes in solution."

As explained in Lines 177-178, filtration through a 300 000 MWCO filter unit decreased the cumulative number of IN per gram of mycelium about 50 % to 75 %. Further filtration through a 100 000 MWCO filter unit reduced the IN number to less than 1 % of the initial concentration (Lines 180-181). So, the majority was lost upon 300 000 MWCO filtration, which were the most efficient IN nucleating at the highest temperatures.

Referee comment: For the discussion to flow, it would be important to explain in line 189 why Erickson came to that conclusion.

Author's response: We changed the sentence to: "Erickson (2009) determined the size of proteins based on theoretical calculations. As the interior of proteins is closely packed with no

substantial holes and almost no water molecules inside, proteins are rigid structures with approximately the same density (~1.37 g cm^{-1}). Assuming the protein as a smooth spherical particle, the minimum diameter of the INM would be smaller than 6.1 nm".

Referee comment: The null effect of chemical processing with O3 and NO2 was somewhat surprising. Based on (Borduas-Dedekind et al., 2019; Gute and Abbatt, 2018; Kunert et al., 2018), I would have expected to see oxidation of the proteinaceous material and thus decrease in IN ability. A discussion involving a hypothesis to the resistance of the strains to oxidation is warranted in light of these studies. Did the authors attempt to extend the exposure to longer times to force a reaction? On a pedantic note, I would argue that ozone exposure of 1 ppm over 4h is not equivalent to 200 ppb over 20h. The experiment was done while bubbling ozone into extracts and there are concentration effects to consider as well as the diffusion of the ozone could affect the chemistry. I would simply omit this sentence and just state the concentration with no mention of equivalence.

Author's response: Based on our results, we cannot exclude that post-translational modifications of the *Fusarium* IN protein occurred during oxidation. These potential modifications do not seem to influence the ice nucleation activity of the protein. For example, they could be in parts of the protein, which are not involved in the nucleation process. We agree with the referee that further investigations are necessary, and we will consider these experiments for future studies.

Moreover, we included the suggested references in the manuscript and extended the following sentence: "This is in contrast to other biological IN e.g., bacterial IN (Snomax®) (Kunert et al., 2018), birch and alder pollen (Gute and Abbatt, 2018), and dissolved organic matter (Borduas-Dedekind et al., 2019), where exposure to oxidizing agents reduced the IN activity."

We deleted the statement and modified the following sentence: "Briefly, a mixture of 1 ppm O_3 and 1 ppm NO_2 was bubbled through 1 mL aliquots of aqueous extract for 4 h, and the IN concentration was determined using TINA."

Referee comment: Null results are difficult to present. To further substantiate the authors' conclusion, I would recommend that the authors show material that indeed reacted under their O3 and NO2 conditions. The authors did do a positive control (Lines 205-206) and showing that data would help further support their claim.

Author's response: As the focus of this study is on fungal IN of *Fusarium*, we did not use Snomax in any of the experiments. As described in the manuscript (Lines 205-206), we found a reduction of IN activity upon exposure to O_3 and NO_2 for Snomax in a previous study (Kunert et al., 2018).

Referee comment: Finally, the storage effects were also null results, but did the authors also do a positive control? In any case, these results are very useful for the community.

Author's response: We could not include a positive control in our storage tests as a suitable control for such experiments was not available. We agree that further IN should be tested for effects of storage.

Referee comment: Figure S1 arguably belongs in the text. The reproducibility between fungal culture plates is remarkably the largest change observed compared to other treatments such as O3 and NO2 exposure. A discussion relating this uncertainty to the other analyses would be important.

Author's response: The data in Figure S1 were obtained from three different fungal culture plates, whereas the exposure experiments were performed with the same aqueous extract of the

particular fungal species. The variability of measurement with individual fungal culture plates is higher than measurements of the same aqueous extract, as the differences did not result from the measurements themselves but rather from the fact that we investigated biological samples.

Referee comment: Report the weights of the mycelium measured gravimetrically (for example in Table S1).

Author's response: Table S1 shows the results of the initial screening, which was performed with two different droplet freezing assays, first a thermal cycler and second the LINDA instrument (section 2.3). For the thermal cycler, mycelium was picked and directly transferred into 96-well PCR plates (Lines 108-110), and for LINDA, 0.9 % NaCl solution was added to the fungal culture plates, which were scraped afterwards to obtain a suspension of mycelium and spores (Lines 80-82). As the initial screening was only a yes or no test, it was not deemed necessary to determine the weight of the mycelium.

Referee comment: Is there value in considering the work in the context of food science and cryogenic food storage? Is it more likely that these strains be found in food or in the atmosphere?

Author's response: *Fusarium* species are frequently associated with plant material (Leslie and Summerell, 2006), including many food types, and some of the strains used in the current study were initially isolated from plants. Thus, IN from such fungi could be important in food response to freezing temperatures, which could be worth future investigation. Considering the work in the context of food science and cryogenic food storage, however, would be outside the scope of this manuscript, in which we focus on atmospheric aspects of ice nucleation activity in *Fusarium*.

Referee comment: Table S1 should present quantitative details. The authors should specify what their criteria is for "IN-active" strains. 1/96 wells? Onset? Temperature range? It would also be useful to add a fourth column with the freezing temperatures (T10 or T50 or T90). Did the authors consider making a parameterization with their data as an upper limit of IN activity of Fusarium species?

Author's response: For the initial screening using the thermal cycler, up to seven droplets were investigated for each sample. If the sample was IN-active, all droplets froze in the investigated temperature interval. We included the following sentence: "Up to seven droplets were measured for each sample, and the mean freezing temperature was calculated."

For the initial screening with LINDA, three droplets were investigated for each sample, which was described in the manuscript in Lines 113-114: "Aliquots of 200 µL of each aqueous extract were transferred to three separate 500 µL tubes and placed on ice for 1 h prior to the LINDA experiments." If the sample was IN-active, all droplets froze in the investigated temperature interval. For clarification, we included the following sentence: "The mean freezing temperature for three droplets was calculated."

The suggested fourth column would correspond to Table 1, which already provides more details about the mean freezing temperatures of the initial screening.

We thank the referee for this suggestion, and we will consider a parameterization in a future study.

6. Conclusion

Referee comment: I would revise the statement on line 226 to say that the most IN-active components were actually between 300-100 kDa, but that IN activity still remained smaller than 100 kDa.

Author's response: As described above, the most IN-active components were larger than 300 kDa, and we hypothesize that these are aggregates consisting of individual proteins smaller than 100 kDa.

Technical comments

Referee comment: The authors use upper case Nm which is arguably inconsistent with the literature using lower case nm. See Wex et al., ACP, 2015 - Line 14: "impact" should be replaced by "implication", since the authors did not quantify the water cycle or the climate in their experiments. - The short summary is very good indeed! (although I would recommend changing the statement to 300 kDa, rather than 100 kDa.)

Author's response: We thank the referee for this comment. For consistency reasons with our former studies, we prefer to keep upper case Nm.

As suggested by the reviewer, we changed "impact" to "implication".

References:

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