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

We thank referee #3 for the review and positive assessment of our manuscript, and we are grateful for the detailed comments, which are very helpful for improving the manuscript. The comments and our answers are listed below (referee's comments marked with blue letters).

Referee comment: A main finding of this study is that filtration experiments suggest that the single cell-free Fusarium is smaller than 100 kDa. This is indeed very interesting and I wonder that the authors do not use the nomenclature of their own paper (Pummer et al., 2015) i.e. ice nucleating macromolecules (INM).
Author's response: We thank the referee for this remark and changed the nomenclature accordingly.

Referee comment: Indeed, water-soluble INMs have also been observed on many other primary biological aerosol particles (PABP) such as leaves, bark, pollen (Felgitsch et al., 2018), algae (Tesson et al., 2018), and bacteria (Failor et al., 2017). The sizes of these INM should be compared among each other, e.g. in a table.
Author's response: A precise comparison of the IN sizes in a table is rather difficult as most studies performed only a 0.2 µm filtration. A conclusion, which can be drawn upon these findings, is, that the IN are cell-free and stay active in solution.

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: The same is true for the chemical composition and for the stability against oxidation. Also for other PBAPs, proteins and polysaccharides have been found as main components of INM and their stability is extraordinary as well. I also wonder if the authors have carried out heating experiments in order to destroy the ice nucleation activity of the proteins. Eventually, the heating was not successful due to the stability of INMs which would be important information since many colleagues use heating experiments to prove or unprove the presence of PBAP- INPs.
Author's response: Many earlier studies already performed heat treatment experiments on different IN-active *Fusarium* species and strains, including strains of *F. acuminatum* and *F. avenaceum*, consistently showing a small reduction of ice nucleation activity after heating to 40 °C and a bigger loss after heating to 70 °C (Hasegawa et al. (1994), Pouleur et al. (1992), and Tsumuki and Konno (1994)). Thus, we expected similar results from strains and species of the genus *Fusarium* and we initially refrained from repeating these experiments. 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."

Comment:

Referee comment: The abbreviation "IN" has been used in a confusing way. In the text it means "ice nuclei" but also means "ice nucleation" and "ice nucleating". I recommend using "INP" for "ice nucleating particles" and write the full words in all other cases.

Author's response: We thank the referee for this comment. We clearly defined ice nuclei as IN and ice nucleation-active as IN-active in the abstract and the introduction. We used the abbreviation IN for ice nuclei in our former studies (Després et al., 2012, Fröhlich-Nowoisky et al., 2015, 2016, Kunert et al., 2018, Pummer et al., 2015), and for consistency reasons we

prefer to keep it this way. To avoid misunderstanding, we changed “IN activity” to “ice nucleation activity”.

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