Response to Referee #1

This article presents investigations into lichen of the genus Peltigera as producers of ice nucleators (INs). It is well-conceived, methodologically sound, and enjoyable to read. The insights gained through these investigations are new and interesting, so they merit publication in Biogeosciences. There are a few minor issues I recommend the authors to consider in a revision:

Response: We thank the reviewer for carefully reading our manuscript. Below we addressed all comments, point-by-point.

1) Peltigera are mostly ground dwelling and have a compact morphology. Which process could dislocate particles small enough from them to escape the surface layer and reach higher altitudes? I would appreciate to a sentence or two on that issue in in the Conclusions.

Response: The reviewer makes a valid point that *Peltigera* are nearly exclusively ground-dwelling, but many lichens are epiphytic, or grow on rocks, and are often dominant in tundra and alpine habitats (Pojar and MacKinnon, 1994; Nash, 2008). Lichens are also common in cloud forests, including cyanolichens. *Peltigera* are found in all these habitats. In addition, asexual reproductive propagules of lichen are airborne. Therefore, lichen propagules are a likely source of lichen-derived INs at higher altitudes.

Action taken: We added additional text and references to the introduction and conclusions of the manuscript. Starting on line 68 of the Introduction, the text now reads, “Lichen-derived INs, specifically airborne asexual reproductive propagules (Marshall, 1996; Tormo et al., 2001), have been detected in the atmosphere, where they can contribute to cloud glaciation and trigger precipitation (Henderson-Begg et al., 2009: Moffett et al., 2015)”. Line 323 in the Conclusions now reads, “Given the presence of *Peltigera* in many world regions, the lichen INs may be prevalent in airborne fungal communities where they could remain active and exert a sustained influence on atmospheric processes (Henderson-Begg et al., 2009: Moffett et al., 2015: Womack et al., 2015)”.

2) Lines 31-33 and lines 330-333 state: "Our analysis suggests that the INs released from this fungus in culture are 1000 times more efficient than the most potent bacterial INs from Pseudomonas syringae." I find the term "efficient" problematic in this context because "efficient"

often refers to the activation temperature of INs, i.e., INs active > -10°C (e.g., Zhang et al., 2020, https://doi.org/10.1016/j.atmosres.2020.105129). You also define T50 as a measure of efficiency (lines 168-169).

Response: We thank the reviewer for these important points. We have updated our definitions and now define potency as the concentration required to induce ice nucleation at temperatures above the background freezing point (e.g., ~-23.5°C). We further agree that our current definition of T_{50} does not align with our use of the term "efficiency" in this manuscript. The T_{50} value is the temperature at which 50% of droplet freeze in the droplet-freezing assay and a standard measure of ice-nucleation activity, rather than a measure of efficiency.

Efficiency is now defined throughout the manuscript as the highest temperature at which an ice nucleator induces freezing, regardless of its concentration. By this definition, *P. syringae* is more efficient since it has the highest IN-activity, but L01-tf-B03 is 1000 times more potent, given that the total decay of *P. syringae* IN-activity occurs at a concentration of 1 ng mL^{-1} while L01-tf-B03 activity is not fully eliminated until 0.01 ng mL^{-1}.

Action taken: Based on the recommendation of the reviewer, we have made several changes within the manuscript and also altered the title of the paper. Specifically, we changed 'efficiency' to 'potency' on line 280, which now reads, "The potency of L01-tf-B03 is evident in comparison to the live bacteria from the strain *P. syringae* Cit7 (Renzer et al., 2024), which was subjected to the same dilution series as the lichen culture".

Specific definitions of potency and efficiency have been added on line 283, which now reads, "We define potency as the concentration of INs necessary to observe IN-activity at temperatures above the freezing point of background water (~ -23.5°C). Efficiency refers to the highest temperature at which an IN induces freezing, regardless of its concentration". In Section 3.4, the term 'efficiency' has been changed to 'potency' in every instance used to describe the IN-activity of L01-tf-B03. We also updated the wording in the Abstract and Conclusions. Line 32 now reads, "Our analysis suggests that the INs released from this fungus in culture are 1000 times more potent than the most IN-active bacterial INs from *P. syringae*". Line 331 has been updated to, "Our results show that the INs released from the fungal culture are nearly 1000 times more potent than the most IN-active bacterial cell-anchored INs classified to date".

We additionally edited the text on line 169 to read, “The freezing temperature of each droplet was identified based on the optical change in appearance that occurred with freezing and the temperature at which 50% of the droplets froze, T_{50}, was recorded.”

3) Line 113: What is meant with "bioavailability" here?

Response: We acknowledge that the use of ‘bioavailability’ may be unclear in this context. Therefore, we have changed the wording to clarify that samples were collected based on the availability of these lichens in nature.

Action taken: We updated the text on line 113 to read, “Lichen samples were collected based on their availability and accessibility in nature.”

4) Lines 168-169: "The temperature at which 50% of the droplets froze, T50, was recorded as a measure of the efficiency of the INs." I guess it means the same as "freezing efficiency", an expression first used in line 201? If so, please add (in brackets) this term to the end of the sentence in lines 168-169.

Response: We thank the reviewer for addressing the inconsistency in our definitions. We replaced the term “freezing efficiency” with “IN-activity” to improve clarity. As addressed above in point #2, we also updated the definition of T_{50}.

Action taken: The text on line 201 has been updated and now reads, “Notably, the two most IN-active lichens, the trimembered *P. britannica* JNU22 and bimembered *P. austroamericana* 34529, show only a 0.7°C difference in IN-activity.” Additionally, line 169 now reads, “…the temperature at which 50% of the droplets froze, T_{50}, was recorded.”

5) Line 170: The T50 value of water is relatively high (-11°C). Were values of Peltigera samples corrected for that and, if so, how?

Response: We thank the reviewer for raising this point. We recognize that this T_{50} value, in the Vali-type ice nucleation assay set-up, is relatively high. However, because all the *Peltigera* samples froze at T_{50} values well above -11°C, the background freezing temperature of water was not corrected for. In addition, inactivated *P. syringae* (Snomax) was consistently measured as an internal control and froze at ~ -3.5°C to ensure that the freezing was due to the presence of an ice

nucleator and not due to the water or environmental conditions in the lab. Most importantly, the Vali-type assay is only used for a prescreening of the crude estimates of IN-activity prior to more statistically rigorous TINA measurements.

Action taken: We added text on line 172 specifying that the Vali-type assay is used for crude, initial measurements. This line now reads, “Although the Vali-type apparatus was sufficient for crude initial tests of IN-activity, more statistically rigorous measurements were needed for quantitative analysis of the extracted INs.”

6) Line 173: Consider replacing "robust" with "precise".

Response: We thank the reviewer for the suggested improvement. The text has been revised to clarify that TINA measurements are more rigorous with higher statistics than the Vali-type assays.

Action taken: The line now reads, “Although the Vali-type apparatus was sufficient for crude initial tests of IN-activity, more rigorous measurements with higher statistics were needed for quantitative analysis of the extracted INs.”

7) Lines 212-216: The 96 droplets in TINA experiments may be large enough a number to derive differential IN-spectra from (see Vali, 2019, https://doi.org/10.5194/amt-12-1219-2019). Differential spectra afford clearer interpretation than cumulative spectra, especially in the context of your study.

Response: We agree that differential spectra offer valuable insights into the underlying number of IN subpopulations. However, because the primary focus of the manuscript is on the frequency and efficiency of *Peltigera* IN-activity, rather than specific characterization of the ice nucleators, we did not include the differential freezing spectra in the main manuscript. Given the additional interpretation of the cumulative spectra that the differential spectra offers, we have revised the text and added a differential freezing spectra of *P. britannica* JNU22 in the Supplement.

Action taken: We added text on line 219 that reads, “The differential freezing spectra of *P. britannica* JNU22 further confirms the presence of both classes”. The Supplement has been updated to include the differential freezing spectra of *P. britannica* JNU22 as determined by the heterogeneous underlying-based (HUB) stochastic optimization analysis (de Almeida Riberio et al., 2023).

8) Table 1: Isn't it surprising that the warmest T50 was found in a species collected in the tropics? Could this be taken as an indication for IN production in Peltigera being similarly incidental as it seems to be in pollen (Kinney et al., 2024, doi.org/10.5194/egusphere-2023-2705)?

Response: We thank the reviewer for providing this insight. While it is possible that ice nucleation in lichens is an incidental property, we believe it is premature to dismiss the idea that the INs serve a specific biological purpose. Lichens are freeze-tolerant organisms, in contrast to many of the plants that produce pollen, and the freeze tolerance suggests that the ability to nucleate ice could provide ecological or physiological benefits to lichens.

Action taken: We added text starting on line 206 that reads, "Given the range of environments in which IN-active *Peltigera* lichens grow, it is possible that ice nucleation ability is an incidental property (Lundheim, 2002; Moffett et al., 2015). However, considering the high IN-activity and frost tolerance of lichens, we cannot dismiss that the INs provide a specific physiological benefit."

9) There appears to be a contradiction in lines 263-265: "Based on the fast growth rate and presence of mycelial-like growth, we classified L01-tf-B03 as a lichen-associated fungus. It is notoriously difficult to isolate mycobionts (Cornejo et al., 2015), which are very slow growing, ..." Why should the fast growth rate seen in L01-tf-B03 support your classification when mycobionts are very slow growing?

Response: We thank the reviewer for bringing our attention to this point. Lichen-forming fungi grow very slowly compared to most fungi. Because the L01-tf-B03 culture grew more quickly than expected for the *Peltigera* mycobiont, we classified the culture as a lichen-associated fungus, i.e., not *Peltigera*. We have revised the text to clarify this point.

Action taken: We have added references and revised the text on line 264 to read, "It is notoriously difficult to isolate lichen mycobionts in pure culture (Cornejo et al., 2015). Moreover, they are very slow growing compared to most fungi. As far as we know, *Peltigera* was never grown successfully in culture. However, *Peltigera* lichen thalli are well-known to host diverse communities of endolichenic fungi, which are frequently isolated in pure culture from *Peltigera* thalli (Arnold et al., 2009; U'Ren et al., 2010, U'Ren et al., 2012). Compared to lichen mycobionts, endolichenic fungi grow quickly. Endolichenic fungi are also referred to as lichen-associated

fungi, which encompass all fungi associated with lichen thalli other than the lichen mycobiont. For all these reasons, we classified L01-tf-B03 as a lichen-associated fungus."

10) Lines 279-280: The T50 value of -23.5°C indicated here is much lower than the one mentioned in line 170 (-11°C). Please clarify.

Response: The variation in freezing temperatures in due to the different systems used for freezing assays – in the Vali-type freezing assay, used for initial measures of IN-activity, water froze at a T_{50} of -11°C. In the TINA set-up, which we used for more robust IN-activity measurements, the water control froze at a T_{50} value of -23.5°C.

Action taken: We updated the text on line 280 to "TINA T_{50}-value ~ -23.5°C" to clarify which freezing assay was used for the measurements shown in Fig. 3.

11) Lines 284-286: " The large decrease of over 4°C in bacterial freezing efficiency is in striking contrast to L01-tf-B03, for which the IN-activity is reduced by less than 1°C at the same concentration." I think this finding merits an attempt at interpretation.

Response: Membrane-supported bacterial ice nucleators are grouped in classes A-C, where large aggregates associated with class A enable IN-activity close to 0°C and class C consists of smaller INPs active ~ -7.5°C. Our current understanding suggests that, unlike bacteria, lichen ice nucleators are cell-free secreted molecules, yet, similar to bacteria, lichens also contain two classes (referred to as class 1 and 2) which contribute to the initial and lower freezing events, respectively. We speculate that, like bacteria, the two freezing temperatures are dependent on functional aggregation. L01-tf-B03 appears to maintain class 1 INs even at exceptionally low concentrations, by which point *P. syringae* INPs have shifted to class C. Our findings may suggest that L01-tf-B03 possesses a non-membrane dependent mechanism to stabilize aggregates. However, future experiments are needed to adequately characterize the molecular basis of the two classes.

Action taken: We added text on line 286 that reads, "It appears that L01-tf-B03 maintains class 1 INs at this concentration, while *P. syringae* INs have already shifted to class C. We tentatively speculate that L01-tf-B03 possesses a mechanism to stabilize the highly IN-active aggregates".