## Responses to RC2

We thank Professor Grothe for his thoughtful comments and suggestions for improving the manuscript. The specific comments made (blue text) are each addressed below (black text) with corresponding changes to the manuscript text included (red text).

## Major comments

When Pummer 2012 recognized that soluble macromolecules can trigger heterogeneous ice nucleation, they assumed polysaccharides being responsible. The same conclusion was later also drawn by other authors, e.g. Dreischmeier 2017 and Gute 2020. The reason is that in the FTIR spectra the bands of polysaccharides are so intense that they overlay all other signals. However, Pummer 2013 already detected protein signals in their detailed study by Raman and FTIR spectroscopy. Only recently Burkart 2021 found evidence that proteins are present and are the responsible INMs. The fact that proteins and polysaccharides are present in the same solution might account for inherent mixtures of both or even for glycoproteins as the important INMs. In contrast to FTIR spectroscopy, fluorescence spectroscopy can clearly differentiate the proteins. Therefore, I strongly recommend to add fluorescence-excitation-emission-maps to figure 5, in order to have more meaningful results.

Burkart et al. (2021) revealed the strength of protein signal in birch pollen washing water decreased with decreasing solution concentration, correlating with a reduction in IN activity. At present, despite this observed correlation, the behaviour of pollen solutions in response to physical and chemical tests supports that the IN activity of these solutions is not, or at least not exclusively, proteinaceous in nature. The IN activity of pollen solutions has been shown to be stable in response to high temperatures (Dreischmeier et al., 2017; Daily et al., 2022) and exposure to protein denaturants and digesting enzymes, including guanidinium chloride (Pummer et al., 2012) and trypsin (Pummer et al., 2015), contrasting sharply with the response of known ice nucleating proteins. More recently, the possibility that proteins play some role in pollens' activity has been considered and we acknowledge that this is not resolved. However, we argue that the current evidence for the role of proteins does not justify carrying out additional measurements aimed at protein detection to this study, where the focus is not on exploring the structure of these ice nucleators.

The distortion of fluorescence signals in birch pollen solution, discussed by Seifried et al. (2022), also suggests that fluorescence spectroscopy cannot be used straightforwardly to compare protein content in pollen solutions which comprise complex mixtures of biomolecules. Seifried et al. (2022) attribute shifts in the wavelength of protein signals for a single pollen solution to concentration-dependant quenching or filter effects; indicating that the capacity for fluorescence measurements without additional analyses to compare between different pollen solutions, where the concentration of components is unknown and likely to vary, is severely limited.

We recognise that IR spectroscopy does not provide a comprehensive analysis of the content of pollen solutions. The structure of the INMs responsible for the IN activity of pollen remains ambiguous and undoubtedly warrants further investigation. The aims of this study were to test the IN activity of pollen from a broad range of species and to identify trends in the measured activity against various plant and pollen features; while we include some discussion of the INMs and have included FTIR spectra for three pollen solutions, detailed analyses of composition is ultimately out of the scope of this study, in particular because limits on available pollen quantities for some samples meant measurements had to be focussed in order to

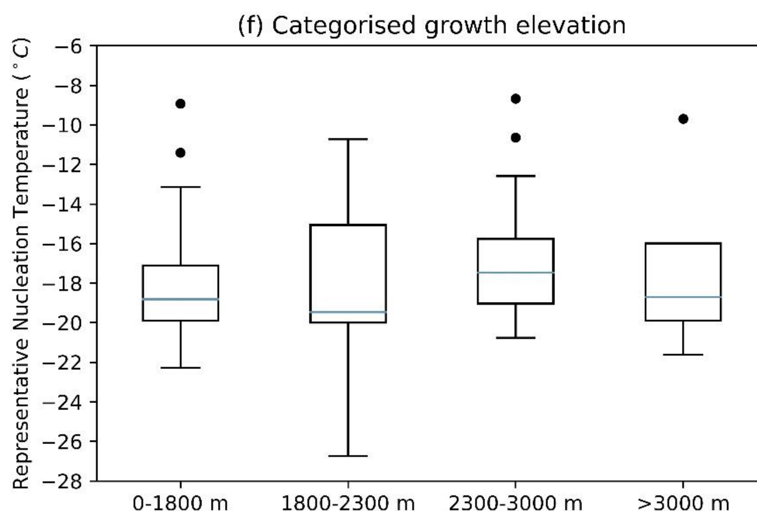
achieve such a large comparison and identifying the active component of these solutions remains a difficult problem in its own right.

In figure 3 the authors have correlated the representative nucleation temperature with biological and geographical parameters and show that the impacts of these are not significant. However, when comparing fig 4 with the results in figure 3f, it becomes obvious that the growth elevation is only categorized in three classes, the selection of which is not clear. In literature, at least 4 categories are known, i.e. mountain zones 0-1800m, 1800-2300m, 2300-3000m and 3000-xm. When applying these mountain zone categories then a difference might become visible showing the change from alpine to snow zone being related to a significant increase of the nucleation temperature. In general, I appreciate such correlation boxplots. However, I wonder that the authors did only correlate representative nucleation temperature but did not also correlate other important parameters such as extractable amount of INMs, average mass of the INMs, size of the INMs, sizes of the aggregates of the INMs or even the intensity of the fluorescence signal (related to the protein concentration). This would significantly enhance the information value of the paper.

For Figure 3, species were divided into three plant growth elevation categories, described in Section 2.7 (L 219-223). It is important to highlight that the data available for plant growth elevations was limited, particularly for some rare species tested in this study. Nonetheless, limits for upper and lower possible growth elevations were assigned as shown in Table S1 based on the information available.

In contrast to the boxplot categorisation based primarily on the upper limit of plant growth elevation, Figure 4 shows the mean representative nucleation temperature for the full range of possible growth elevations, which have been binned into groups for every 50 m. For example, for the 1000 m bin, the representative nucleation temperature of all species which have a minimum elevation less than 1000 m and a maximum elevation greater than 1000 m are used in the calculation of the mean (and standard error). It must therefore be noted that some species fall into multiple bins. This figure shows mean representative nucleation temperature for elevations up to 3000 m is consistent. After 3000 m there seems to be a positive relationship however due to the small number of species in this range this is likely biased by an outlier.

As far as we are aware there is no standard categorisation for growth elevation based on upper and lower elevation limits – we could not find by searching the reference for the four mountain zones as defined by the reviewer. Using the values for upper elevation listed in Table S1 to categorise species into the four ranges suggested, gives the following figure:




Critically, echoing the analysis of Figure 4 in L 233-237, the number of data points in the highest elevation category (upper growth elevation limit >3000 m) is too small to include in a statistical test. The ANOVA test failed to reject that there is a significant difference in mean representative nucleation temperature between 0-1800 m, 1800-2300 m, 2300-3000 m elevation categories ($F = 0.655$, $p = 0.525$), therefore we cannot determine a relationship between IN activity and these categories. This is not to say that there isn't a relationship between IN activity and plant growth elevation in nature but rather that from our data, even using these new categories, a relationship cannot be determined.

We agree that it would be valuable to consider the relationships between the selected plant features and additional parameters, such as the quantity and size distribution of soluble components. Unfortunately, we were limited by sample volumes. We prioritised nucleation temperature measurements for this study which allowed for a broad comparison of pollen from diverse plant species.

**Minor comments**

The term "INMs" has been coined by Pummer 2012 as "ice nucleating macromolecules". Please add "macro" when referring to this definition.

We appreciate the importance of keeping definitions clear and consistent in the literature and will change this in the text as advised.

INM $mg^{-1}$ extracted pollen was the general value. Unfortunately, this is not a very precise value since pollen have different amounts of extractable material on their surface (see Burkart 2021). More precise would be to determine the amount of soluble material in the solution subsequently to the extraction process by evaporating the water (or at least to show that the difference between $mg^{-1}$ Pollen and $mg^{-1}$ solute is neglectable).

We agree that determining the mass of soluble material in the pollen solutions would be of interest, particularly to compare between structurally diverse pollen. Unfortunately, due to limits on pollen volume it was not possible to measure this for the range of species in this study.

To reflect on this more clearly in the text, as well as related points made by Prof. Morris, we intend to add the following section to Section 2.10:

"Animal-pollinated plants can afford to allocate a lesser proportion of their resources to pollen production than wind-pollinated plants; plant-pollinator relationships ensure a higher likelihood that an individual pollen grain will reach the stigma of another plant and result in fertilisation (Herrera and Pellmyr, 2002). Therefore, it is challenging to collect large quantities of pollen from many animal-pollinated species without extensive harvesting of flowers. In this study, we prioritised nucleation temperature measurements which allowed a broad comparison of pollen from diverse plant species to be made. Limits on pollen quantities for some species restricted possibilities for further analysis, such as evaluating the quantity and composition of soluble material in each of the pollen solutions.

We have controlled for the mass of pollen added to a volume of water and time-in-suspension in an attempt to ensure consistency across the pollen samples tested, but this is not a direct measure of the concentration of INMs in each solution. Murray et al. (2022) compared the mass of dried soluble material from solutions of *Carpinus betulus* and *Betula pendula* pollen solutions, finding that for both types approximately 0.7% of the total mass of the samples was soluble material from pollen. However, even the quantity of soluble material is a proxy for the quantity of the INM component, which is estimated to be far smaller (Murray et al., 2022). That the activity of *Pinus mugo* pollen collected from plants in different locations, in different years, was so similar would seem to indicate the measurements carried out are in some capacity representative of the true pollen activity. Ultimately, these ambiguities highlight, in our view, the importance of work towards identifying the structure of the molecules responsible for the IN activity in pollen."

INMs have not only be found on pollen but also on leaf, bark, stem and branches (see Felgitsch 2018).

We acknowledge this point and intend to add this reference to the Introduction (L 29-33):

"The identification of ice nucleators associated with various organisms, from bacteria (Lindow et al., 1982; Maki et al., 1974; Lukas et al., 2022) to lichens (Kieft, 1988; Eufemio et al., 2023) and plants (Gross et al., 1988; Brush et al., 1994; Felgitsch et al., 2018), is of biological and atmospheric interest."

As well as adding the following sentence to L 42:

"Other plant parts including wood and leaves have also been found to host INMs (Felgitsch et al., 2018)."

## References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