We would like to thank Dr. Hill for taking the time to review our article and for his helpful comments. Our replies are given in blue, while changes to the text are listed in *blue italic*.

The ability of organic INPs ("leaf-derived nuclei") to adsorb to and confer enhanced IN activity to minerals (esp. clays) was first proposed and demonstrated by Russ Schnell almost 40 years ago. Recent research has generated an awareness of the potential importance of this process, especially following recent studies showing that organic soil INPs are often very small (ie, would not be detected when bound to mineral particles). This paper is an important and overdue advancement of this topic. Its main new contribution is that IN proteins are readily adsorbed onto clays while maintaining their activity. Secondarily, it shows that ionic concentration (in the range found in soil water) and, to lesser extent, composition are important positive variables enhancing binding, but that paper pH is not. It is also useful and intriguing to be shown that the adsorption is reversible, which to me suggests that the binding process is relatively gentle and, hence, doesn't deform and potentially inactivate the proteins.

I have some suggestion for minor amendments.

P. 3 L. 18: "released" would be better than "lost".

Agreed- changed.

P. 3 L. 19: date missing for Pouleur.

Corrected

P. 3 L. 21: no comma.

Corrected

P. 4 L. 15: "much higher".

Corrected

P. 5 L. 4-14: Very nice background to this aspect.

Many thanks!

P. 6 L. 22: I would add "(collected in the filtrate)" for those who need reminding of their small size.

Added "*after collection in the filtrate*" here

P. 8, L. 22: Can you remind us what K is here?

Added "*(no. of ice-nucleating sites per ml of suspension, eq. 1)*"

P. 8 Clay-protein interactions in the absence of electrolytes section: In relation to Fig 2., I notice that in Fig. 3 the 1 mM NaCl treatment was also apparently unimpressive after 2 h, but given time was very effective. The same may have held true for the no electrolytes case. If you didn't test these ones for 48 h then you should acknowledge this even though it doesn't change the underlying story, and even though in a soil solution you would seldom encounter such a lack of ions. IN Fig. 2, it would also be nice to know what the underlying kaolinite INP profile was. I assume it's the log-linear line that would exist of the hump staring from -11 C and warmer was removed. Could this be mentioned?

Thanks for these points. The aim of this section was purely to show that regardless of pH, adsorption was not observed to occur within a contact time typical of when adsorption has been observed to

occur for a wide variety of other proteins on minerals. To add further context to this section, and justify why we chose the 2 hour contact time in the first place, we have added to section 3.1:

"As adsorption is a time-dependent process, it is possible that extending the contact time could result in uptake of the protein to the clay. Nevertheless, the results here show that on timescales characteristic of protein adsorption to clays (Yu et al., 2013), uptake of the P-INP was minor in the absence of electrolytes."

With relation to Fig. 2, the kink below -11 °C does not appear to be related to the presence of kaolinite, which is active at far lower temperatures. We also note that it is not related to the potato dextrose broth in which the fungus was grown. To illustrate this we have added a supplementary figure where we show the K values for PDB and kaolinite. We have also added the text in section 3.1:

"Measured K values for kaolinite on its own (with no adjustment to pH) are far below those of either the pellet or supernatant (Fig. S1)."

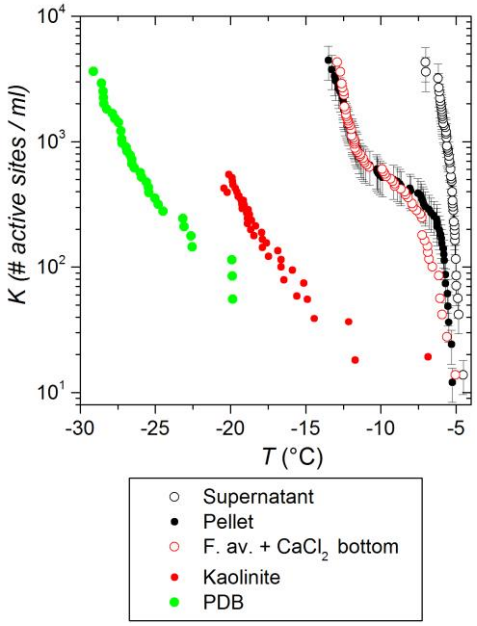

Fig. S1: Results of supernatant depletion test at pH 5.7 (as per Fig. 2, no added salt ions), compared to tests for ice nucleation by potato dextrose broth (PDB, diluted 1/10 from the original broth) and a 1 wt% kaolinite suspension (Whale et al. (2015)). Note that only a trace of PDB will adhere to the mycelium, and hence make it into the supernatant depletion suspensions; accordingly there is no significant contribution to the observed ice nucleation activity from PDB. Nucleation from kaolinite is many orders lower than *F. avenaceum*. Also shown is the bottom 0.1 mL fraction from a control supernatant depletion test performed on F. avenaceum in 1 mM $CaCl_2$, where no kaolinite was added. No enrichment of the protein in the bottom fraction can be seen, consistent with a lack of protein flocculation.

P. 9 L.7: Couldn't it simply be that the ionic strength as just so low that the tendency was for surface ions of any type to remain in the bulk phase, just as K+ is rapidly stripped off K-feldspar when that is put in DI?

On the contrary, it would be expected that the release of exchangeable surface cations in deionized water for kaolinite Kga 1-b would be diminishingly small, as there are no cations in solution to replace those lost from the surface to the solution. The equilibrium between ions at the surface and those solvated in solution will lie heavily towards those remaining at the surface, suggesting that ions being lost from the surface play at most only a very minor role in explaining the adsorption dynamics. In this case we prefer to keep the discussion around that of Tombácz and Szekeres, 2006 which is already cited in the paper.

P. 9 L.11: Surely it's not just the charge, but also the presence of ions in solution to replace any that leave the surface of the clay, in a dynamic equilibrium? It would be useful, too, to provide a typical range for ionic strengths in soil solutions. I see Edmeades et al (1985; Aust J of Soil Res., 23, 151) gives a range of 5-16 mM for NZ grassland soil, which is very relevant to your Fig. 4., supporting the case that under normal conditions the INPs would rapidly bind to the clays.

Agreed- we've added *"and promote cation exchange at the surface of the clays"* here.

With regards to typical ionic strengths of soil solutions, we have added further info for the interested reader: "Ionic strengths of 1 and 10 mM were chosen to approximate those found in soil solutions (Campbell et al., 1989;Griffin and Jurinak, 1973;Edmeades et al., 1985;Dolling and Ritchie, 1985)."

P. 9 L.17: Date for Yu et al.

Fixed.

P. 9 L.18: Fig. 3 is impressive.

We were also intrigued to see how strong an effect the addition of salts had!

P. 11 L. 9: Also, divalent cations may cause flocculation of both clay particles and proteins, producing clumps of these. The natural pH of the kaolinite soln (5.7) would promote this.

For solutions of *F. avenaceum* washing waters only, 1-10 mM ionic strength is a very low concentration in terms of that required to cause proteins to flocculate on their own. This was verified with control supernatant depletion experiments where kaolinite was omitted- no increases in the bottom 0.1 mL of the suspension were observed (see Fig. S1). When the clay is present, yes, this perhaps encourages the clay particles to stick together, but this does not affect the conclusion that the proteins preferentially adsorb to the clay. We have modified the pertinent line in the methods section, which now refers to the new Fig S1:

"*Control experiments were performed in the absence of kaolinite with Milli-Q® purified water alone, or with CaCl2 showing that there was no significant preferential sedimentation of the protein (see Fig S1).*"

P. 12. L.24: Maybe add the caveat that adsorption to kaolinite did not cause deactivation. During the process of drying, for example, the adsorption/binding may become stronger and so deform and affect the IN activity and propensity to be desorbed.

We have added the caveat:

"although further environmental processing, such as repeated wetting and drying of soils, could potentially perturb protein activity"

P. 13 L. 7: Nicely put.

P. 13. L. 14: I don't think all the efficient ones are proteins. Or, at least, it's premature to generalize?

Agreed- this statement was too strong. Changed to:

"Indeed, the characteristics of the organo-clay INPs examined in this study are similar to the efficient INPs which have been identified in topsoils (O'Sullivan et al., 2015;Conen et al., 2011), a substantial proportion of which were shown to be thermally labile and exhibit exceptional ice-nucleating abilities."

P. 16. L. 16: Fusarium are common in soils yes, but not the most common. They tend to be pathogens. And only a few species are IN active. This sentence is overstating their abundance I think. Since this work would apply equally, I assume, to other IN fungi, such as Mortierella alpina, and other as-yet undiscovered IN fungi (the most dominant species, in terms of vegetative biomass, tend to be the Basidiomycetes, which are notoriously difficult to grow in pure culture), this section could be broadened to include adsorption of IN proteins released by many soil organisms.

Thanks for drawing our attention to this, we had worded this poorly. This has been reworded as below.

With regards to this work applying equally to other sources of proteinaceous INPs, it is correct that the conclusion that P-INPs from other sources could also confer activity to soils, although this must be caveated by noting that the extent of adsorption may differ. Nonetheless, this is an important point, and to this end we have broadened our discussion. The section now reads:

"In addition to Fusaria, other organisms can also contribute to the reservoir of cell free P-INPs in soils, such as the fungus M. alpina (Fröhlich-Nowoisky et al., 2015), certain lichens (Kieft and Ruscetti, 1990), and even ice-nucleating bacteria (Phelps et al., 1986). For Fusaria, ice-nucleating activity has been observed in a number of species, and species such as Fusaria are widespread in soils, occurring across the globe wherever crops are grown (Pitt et al., 2009)"

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