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Urediospores of rust fungi are ice nucleation active at $> -10\,^{\circ}C$ and harbor ice nucleation active bacteria

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Abstract. Various features of the biology of the rust fungi and of the epidemiology of the plant diseases they cause illustrate the important role of rainfall in their life history. Based on this insight we have characterized the ice nucleation activity (INA) of the aerially disseminated spores (urediospores) of this group of fungi. Urediospores of this obligate plant parasite were collected from natural infections of 7 species of weeds in France, from coffee in Brazil and from field and greenhouse-grown wheat in France, the USA, Turkey and Syria. Immersion freezing was used to determine freezing onset temperatures and the abundance of ice nuclei in suspensions of washed spores. Microbiological analyses of spores from France, the USA and Brazil, and subsequent tests of the ice nucleation activity of the bacteria associated with spores were deployed to quantify the contribution of bacteria to the ice nucleation activity of the spores. All samples of spores were ice nucleation active, having freezing onset temperatures as high as $-4\,^{\circ}C$. Spores in most of the samples carried cells of ice nucleation-active strains of the bacterium *Pseudomonas syringae* (at rates of less than 1 bacterial cell per 100 urediospores), but bacterial INA accounted for only a small fraction of the INA observed in spore suspensions. Changes in the INA of spore suspensions after treatment with lysozyme suggest that the INA of urediospores involves a polysaccharide. Based on data from the literature, we have estimated the concentrations of urediospores in air at cloud height and in rainfall. These quantities are very similar to those reported for other biological ice nucleators in these same substrates. However, at cloud level convective activity leads to widely varying concentrations of particles of surface origin, so that mean concentrations can underestimate their possible effects on clouds. We propose that spatial and temporal concentrations of biological ice nucleators active at temperatures $> -10\,^{\circ}C$ and the specific conditions under which they can influence cloud glaciation need to be further evaluated so as to understand how evolutionary processes could have positively selected for INA.

1 Introduction

Over the past few years there has been a renewed interest in biological particles that could be active as ice nuclei in the atmosphere at temperatures warmer than $-10\,^{\circ}C$. By catalyzing ice formation at these relatively high temperatures, ice nucleation active spores, pollen and bacterial cells transported into clouds could initiate the process of raindrop formation at temperatures where mineral particles are incapable of having an effect (Möhler et al., 2007). Particles of biological origin are among the only particles naturally present in the atmosphere known to be effective ice nucleators at temperatures $> -10\,^{\circ}C$. Hence, they have the potential to incite processes needed for rainfall that would otherwise not occur in mid- and high-latitude clouds at such temperatures. The potential importance of this environmental function has stimulated renewed research to discover, characterize and

quantify biological ice nucleators in the atmosphere for subsequent parameterization of cloud physics models that will contribute to estimating the impact of biological ice nucleators on rainfall (Morris et al., 2011).

The most well-described biological ice nucleator in the atmosphere is the bacterium *Pseudomonas syringae*. This bacterium, also known for its pathogenicity to plants, is disseminated via aerosols to clouds, in snow and rainfall, and via snowmelt, rain runoff and flowing streams between terrestrial and freshwater habitats mostly in temperate regions (Monteil et al., 2012; Morris et al., 2008). In spite of the biological and ecological data available for this bacterium, it is unclear if there are sufficient numbers of ice nucleation active cells in the atmosphere to have an effect on the quantity or frequency of rainfall (Hoose et al., 2010; Phillips et al., 2009). Hence, in addition to the effort to accumulate supplementary data on atmospheric concentrations of *P. syringae*, there has been a search for other biological ice nucleators potentially more abundant in the atmosphere. One approach in this direction has been to assess the ice nucleation activity (INA) of one of the most abundant and ubiquitous microorganisms in the atmosphere, *Cladosporium* spp. However, this fungus has not proven to be ice nucleation active at temperatures $> -15\,^{\circ}\mathrm{C}$ (Iannone et al., 2011). Likewise, data for INA of air and snow samples suggest that biological ice nucleators active at $> -10\,^{\circ}\mathrm{C}$ constitute only minor components of the microbial populations in these samples (Bowers et al., 2009; Mortazavi et al., 2008).

It has been suggested that one of the major roles of INA in the adaptation of *P. syringae* to its habitats is in fostering fallout from clouds with raindrops or aggregates of ice formed from the catalysis of glaciation (Morris et al., 2008). Without a force to precipitate the ultra-light cells of bacteria from the atmosphere, they could remain suspended in the atmosphere indefinitely. Another potential role for INA for this bacterium is to enhance access to host tissue by creating wounds due to frost damage. This led to one of the key field observations linked to the discovery of the INA of *P. syringae*: the accrued frost damage to crops in its presence compared to when it was absent (Arny et al., 1976). This same observation has also been made for the various other plant pathogenic bacteria that are ice nucleation active (Azad and Schaad, 1988; Lindow et al., 1978; Zeng et al., 1999). If we use these properties – fallout with rain and enhanced frost damage to plants – to search for other microorganisms that might benefit from INA, there is evidence from the epidemiology of diseases referred to as rusts that suggests that this trait would be very useful for this group of plant pathogens.

Rusts are fungi in the Pucciniales order (formerly called Uredinales). They have complex life stages that include the production of sexual spores (basidiospores) and up to four types of asexual spores that assure dissemination and winter survival. Among these, urediospores assure long distance dissemination. They are produced in massive quantities and are aerodynamically ultra-light. Since the early 1900s it has been demonstrated repeatedly that rusts are transported to altitudes of clouds and can travel for 100s to nearly 1000 km before returning to the Earth's surface (Stakman et al., 1923; Wolfenbarger, 1946; Nagarajan and Singh, 1990). The initiation of rust epidemics depends critically on rainfall as a source of primary inoculum that effectively deposits spores traveling these long distances (Nagarajan and Singh, 1990); dry deposition accounts for only a very small fraction of spores deposited from the atmosphere (Li et al., 2009; Rowell and Romig, 1966). Furthermore, the viability of urediospores of rusts is greatly reduced by temperatures below about $-5\,^{\circ}\mathrm{C}$ (Eversmeyer and Kramer, 1995; Gladders et al., 2007; Jurick II et al., 2008; Pfender and Vollmer, 1999). This strongly influences their winter survival rate, thereby re-enforcing the importance of new incoming primary inoculum in the spring to initiate epidemics. It also suggests that survival during aerial transport would be severely limited if the fungus remained airborne at very cold temperatures. Furthermore, rusts are among the very few diseases for which there are several reports of enhanced frost sensitivity of plants when they are present (McAinsh et al., 1990; Kessler, 1970; Paul and Ayres, 1991).

Apart from a report by one of the authors of this work of the preliminary characterization of coffee rust (Gonçalves and Massambani, 2011), the INA of rusts has not been investigated. Rusts are obligate parasites and this entrains two obstacles in particular to the measurement of their INA. Firstly, as they cannot be grown on microbiological culture media, they must be collected from plant material. This can be accomplished with greenhouse-grown plants, or by direct collection in the environment from wild or cultivated plants. Secondly, by culturing microorganisms in the laboratory, it is possible to obtain pure cultures that contain only a single strain of the target microorganism. In absence of such purification, it is likely that rusts harbor "contaminants" such as bacteria. Bacteria are well-known to be naturally associated with rust spores. Bacteria can be attached to spore surfaces and some of them can parasitize rust spores (Doherty and Preece, 1978; French et al., 1964; Perelló et al., 2002). Furthermore, contamination of rust fungal DNA with bacterial DNA can be a significant problem for sequencing the genome of obligate parasites such as rust fungi (Barnes and Szabo, 2008). Therefore, the presence of bacteria on rust spores could influence their INA, and in particular if the bacteria themselves were ice nucleation active. The objectives of this work were (1) to demonstrate that ice nucleation activity at temperatures $> -10\,^{\circ}\mathrm{C}$ is widespread among the urediospores of rust fungi, (2) to determine if this activity is due to the spores themselves or to the bacteria associated with the spores, and (3) to quantify the frequency of ice nuclei per urediospore in order to provide information useful for parameterizing atmospheric models, for comparing activity among biological ice nucleators and for enhancing understanding of the life history of the rust fungi.

Table 1. Sources of rust urediospores characterized for ice nucleation activity (INA) and bacterial contamination.

Plant host	Rust spp.	Location	Date	Log (spores mL^{-1})[1]	INA onset (°C)[2]	Log (spores per ice nucleus)[3]
Field samples						
Chenopodium album Lambsquarters	*Puccinia aristidae*[4]	St. Saturnin-les-Avignon, France	Apr 2009	5.68	−8	4.96
Senecio vulgaris Common groundsel	*P. lagenophorae*[4]	St. Saturnin-les-Avignon, France	Mar 2009	5.50	−8	4.78
		Montfavet, France	Jan 2010	5.53	−6	4.68
Festuca sp. Perennial tufted grass	*Puccinia* sp.[4]	St. Saturnin-les-Avignon, France	Apr 2010	5.45	−8	4.03
			Jun 2010	5.37	−6	5.17
Avena fatua Wild oats	*Puccinia* sp.[4]	St. Saturnin-les-Avignon, France	Jun 2010	5.55	−4	3.62
Dactylus glomerata Orchard grass	*Puccinia* sp.[4]	St. Saturnin-les-Avignon, France	Jun 2010	5.98	−6	5.28
Allium ampeloprasum Wild leeks	*P. allii*[4]	Montfavet, France	Jan 2010	5.36	−6	5.16
		St. Saturnin-les-Avignon, France	Apr 2010	5.40	−8	4.79
Allium schoenoprasum Wild chives	*P. allii*[4]	Montfavet, France	Apr 2010	5.45	−7	4.62
Triticum aestivum wheat, local land race	*P. striiformis*	Gaziantep, Turkey	Apr 2010	4.24	−7	3.88
T. aestvum cv. McNeal, wheat	*P. graminis* f. sp. *tritici*[5]	Bozeman, Montana, USA	Aug 2010	5.78	−5	5.27
			Sep 2011	5.26	−4	3.94
Coffea arabica Coffee	*Hemileia vastratrix*[4]	Pinhal City, Brazil	Jul 2008	6.04	−6	5.10
Rusts maintained on wheat in greenhouses						
T. aestivum cv. Michigan amber, wheat	*P. triticina*[6]	Versailles, France	May 2010	5.50	−9	5.30
				5.02	−6	4.51
T. aestivum cv. Morocco, wheat	*P. graminis* f. sp. *tritici*[6]	Aleppo, Syria	Apr 2010	4.38	−7	4.83
	P. striiformis[6]	Aleppo, Syria	Apr 2010	4.37	−7	4.82
	P. triticina[6]	Aleppo, Syria	Apr 2010	3.74	−7	3.53

[1] Concentration of washed spores used for determining INA and bacterial contamination rates. [2] Warmest temperature at which INA of the spore suspension was detected. [3] Number of spores necessary to detect an ice nucleus active at the threshold temperature. [4] Putative identification of rust species based on the known rusts of the plant species sampled here. [5] Rust identity was determined based on disease symptoms and spore morphology. [6] Rust cultures of known identity maintained in culture on greenhouse-cultivated plants.

2 Materials and methods

2.1 Collection of urediospores and associated bacteria

Urediospores of rusts were collected from naturally infected plants in the field and from greenhouse-grown host plants inoculated with spores of fungal cultures from laboratory collections (Table 1). Spores were collected with a suction device that allowed them to be taken up through sterile pipette tips and impacted onto polycarbonate filters (pore diameter, 8 µm). To remove bacteria that were not firmly bound to the spores, the spores were washed twice by passing 10 mL of sterile distilled water across the filter for each wash. Spores were then scraped from the filter surface and suspended in sterile distilled water to a concentration of about 10^5 spores mL^{-1}, depending on the total number of spores that were obtained (indicated in Table 1). The concentration of spores was determined by direct microscopic counts for 2 to 3 aliquots per sample. It was difficult to harvest enough spores to obtain a sufficient volume of spore suspensions at a concentration of greater than 10^5 spores mL^{-1}. Furthermore, the total volume of spore suspension that could be prepared varied among samples and had consequences on the detection levels and the range of tests that could be performed for each sample.

To determine the quantity of bacteria on the urediospores after washing, aliquots of the suspensions used for microscopic counts were dilution plated on two culture media: on King's medium B (KB) (for detection of fluorescent pseudomonads) (King et al., 1954) and 10 % tryptic soy agar (for total culturable mesophilic bacteria). Two to three replicates of each dilution were plated on each medium. Plates were incubated at 22–25 °C and colonies were counted at 2 and 5 days after incubation. Fluorescent colonies on KB were counted under UV light (312 nm). Population sizes of putative *P. syringae* were determined as previously described (Morris et al., 2008) by verifying the absence of cytochrome C oxidase on pure cultures made from 10 to 20 randomly selected fluorescent colonies from the same dilution of each sample. About 90 strains of putative *P. syringae* were put into storage at −80 °C in 40 % glycerol for later characterization of their INA. When fluorescent pseudomonads were not detected in samples, colonies of the dominant morphological types were purified and characterized for their INA as described below.

2.2 Quantification of ice nucleation activity

The capacity of the spores to induce freezing of water at temperatures from −2 to −10 °C was determined for droplets of

the spore suspension placed on a metal surface floated on a cooling bath, as described previously (Morris et al., 2008). The objective of these tests was to estimate the number of ice nuclei per spore according to the calculation of Vali (1971):

$$K_T = [\ln(N_{\text{total}}) - \ln(N_{\text{unfrozen}})]/d \quad (1)$$

where N_{total} is the total number of drops, N_{unfrozen} is the number of drops not frozen (liquid) at the each temperature, and d is the quantity of spores per drop. Depending on the total volume of spore suspension that was obtained for each sample, from 20 to 50 drops of 20 µL of the original suspension were tested. To determine if the INA of spore suspensions was due to bacteria, an aliquot of the suspension was incubated with lysozyme (final concentration of 3 mg mL^{-1}) for 72 h at 4 °C, as previously described (Christner et al., 2008a). To verify that INA was sensitive to boiling and therefore likely to be proteinaceous, an aliquot of the suspension was boiled for 10 min. For the lysozyme-treated suspension, 20 droplets were tested and 10 droplets were tested for the boiled suspension. For all tests, strain CC0094 of *P. syringae* (Morris et al., 2008) was used as a positive control and sterile distilled water was used as the negative control.

The INA of *P. syringae*-like and non-pseudomonad strains was quantified in a similar manner as for spores. Suspensions of 48 h cultures on KB medium were prepared in sterile distilled water, adjusted to 10^7 bacteria mL^{-1} and stored at 4 °C for about 1 h to foster optimal conditions for formation of the ice nucleation protein (Nemecek-Marshall et al., 1993). For each of 5 ten-fold dilutions of the bacterial suspension (10^3 to 10^7 bacteria mL^{-1}), 32 drops at 20 µL $drop^{-1}$ were tested at −2 to −9 °C. Data obtained under identical conditions for strains representing the range of INA observed in a large collection of *P. syringae* established during several studies (Morris et al., 2000, 2008, 2010) were used for comparison.

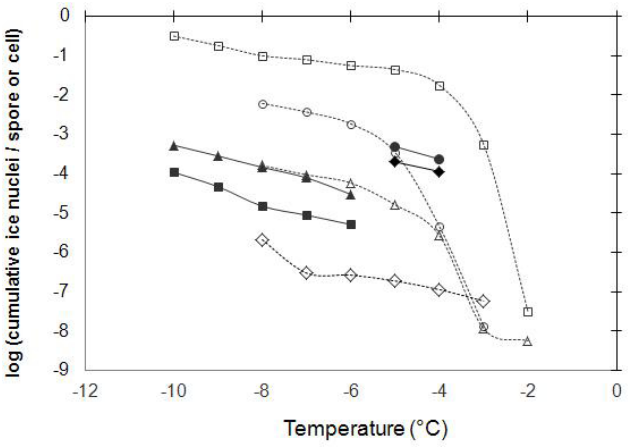


Fig. 1. Cumulative ice nucleation activity of urediospores of rusts (solid lines) compared to that of cells of *Pseudomonas syringae* (dashed lines). Examples are presented to represent the range of activities observed among the rust samples from this study and among strains of *P. syringae* from our collection and reported in the literature (Cit7 as reported in Fig. 4 of Orser and colleagues, Orser et al., 1985). Rusts whose activity is illustrated here were collected from *Dactylus* Orchard grass (■) (*Puccina* sp.), greenhouse-grown wheat (▲) (*P. triticina*), field wheat (♦) (*P. graminis* f. sp. *tritici*) and wild oats (●) (*Puccinia* sp.). Strains of *P. syringae* that illustrate the range of the activity of this bacterium include Cit7 (□) from a citrus orchard, CC0229 (◯) and CC0094 (△) from epidemics of cantaloupe blight, and CC0670 from a wild plant of the *Primula* species (◊). Curves for cumulative freezing spectra of rusts are not as complete as those for *P. syringae* because of the lack of sufficient spore quantities to prepare ample volumes for tests across a range of spore concentrations.

3 Results

3.1 Ice nucleation activity of rust spore suspensions

INA at temperatures > −10 °C was observed for all suspensions of rust spores characterized in this study. The temperature of onset of ice nucleation was between −4 °C and −9 °C for all samples (Table 1). For the sample with the coldest temperature of onset of freezing (−9 °C for wheat rust on cv. Michigan Amber plants cultivated in the greenhouse), the onset temperature increased to −6 °C after 2 additional weeks of incubation of the plants in the greenhouse. The frequency of ice nuclei per spore was in the range of the frequency of ice nuclei produced per bacterial cell of reference strains of *P. syringae* (Fig. 1). The rates of ice nuclei presented in Fig. 1 represent examples of the range of activities for the rust spores characterized in this study and for strains of *P. syringae* from plants among the many strains we have characterized in our collection and those reported in the literature (Orser et al., 1985). All of the values presented in Table 1 for rates of INA are also within the range of INA of the *P. syringae* strains presented in Fig. 1. These frequencies of INA are also in the same range observed for strains of *P. syringae* and related species isolated from cloud droplets (Attard et al., 2012).

3.2 Abundance of bacteria on washed rust spores

The size of the bacterial population carried on rust spores, after washing, was evaluated for 15 of the samples (those samples with sufficient spores to obtain working suspensions of at least 10^5 spores mL^{-1}; see Table 1). All spores carried detectable densities of total mesophilic bacteria at rates of 8×10^{-4} to 2.1×10^3 bacterial cells $spore^{-1}$ with 10 of the 15 samples having less than 1 bacterial cell $spore^{-1}$. *P. syringae* was detected on 11 of the 15 samples at rates of 8.4×10^{-5} to 0.18 bacterial cells $spore^{-1}$. In general, there was not a correlation between the abundance of bacteria on spores and the onset temperature of freezing. However, the spores with the warmest onset temperature carried the greatest amounts of total bacteria and among the greatest amounts of *P. syringae* (Fig. 2).

Table 2. Frequency of ice nuclei production required for bacteria to account for the ice nucleation activity observed in suspensions of rust spores.

Plant host	INA onset (°C)[1]	Log (ice nuclei mL^{-1})[2]	Log (total bacteria mL^{-1})[3]	Log (*P. syringae* mL^{-1})[3]	Log (ice nuclei *P. syringae* $cell^{-1}$)[4]	Log (ice nuclei mL^{-1} due to *P. syringae*)[5]	Minimum log (ice nuclei $cell^{-1}$) to account for freezing onset[6]	
							P. syringae	Total bacteria
Avena fatua	−4	1.92	7.20	3.95	−4.61	−0.66	−2.03	−5.28
Triticum aestivum cv. McNeal	−4	1.32	8.59	4.18	−4.08	0.09	−2.86	−7.27
T. aesitvum cv. McNeal	−5	0.48	3.29	2.61	−5.09	−2.52	−2.13	−2.81
T. aestivum cv. Michigan amber	−6	0.48	5.67	nd[7]				−5.19
Senecio vulgaris	−6	0.93	4.94	4.00	−3.1	0.90	−3.07	−4.01
Allium ampeloprasum	−6	0.18	5.36	3.48	−3.1	0.38	−3.30	−5.18
Festuca sp.	−6	0.18	5.69	4.64	−5.06	−0.42	−4.46	−5.51
Dactylus glomerata	−6	0.70	5.12	nd				−4.42
Coffea arabica	−6	0.94	5.30	nd				−4.36
Allium schoenoprasum	−7	0.81	3.64	1.98	−4.35	−2.40	−1.17	−2.83
Festuca sp.	−8	1.41	5.30	3.46	−3.38	0.08	−2.05	−3.89
Allium ampeloprasum	−8	0.71	3.20	3.85	< -8.0		−3.14	−2.49
Senecio vulgaris	−8	0.72	5.51	nd				−4.63
Chenopodium album	−8	0.74	2.59	3.32	NC[8]		−2.58	−1.85
T. aestivum cv. Michigan amber	−9	0.18	4.58	1.43	−1.76	−0.33	−1.25	−4.40

[1] Warmest temperature at which INA of the spore suspension was detected. [2] Concentration of ice nuclei, active at the freezing onset temperature, in the spore suspension. [3] Concentration of total bacteria and *P. syringae* in the suspension of washed spores. These are bacterial cells that were most likely adherent to the spores. [4] Maximum frequency of ice nuclei per cell of *P. syringae* among the strains isolated from the rust spore suspension and tested for INA under laboratory conditions. The frequency reported here is for the freezing onset temperature of the spore suspension. [5] Estimation of the concentration of ice nuclei in the spore suspension based on the amount of *P. syringae* cells in the suspension and the maximum rate of ice nuclei per cell observed among the characterized strains. This concentration is for the freezing onset temperature. [6] Frequency of ice nuclei per cell that would be required for the bacteria associated with the rust spores to account for the onset freezing temperature observed for the suspensions of rust spores. [7] *P. syringae* was not detected. The detection threshold was 10 bacteria mL^{-1}. [8] Bacterial strains were not collected from this sample.

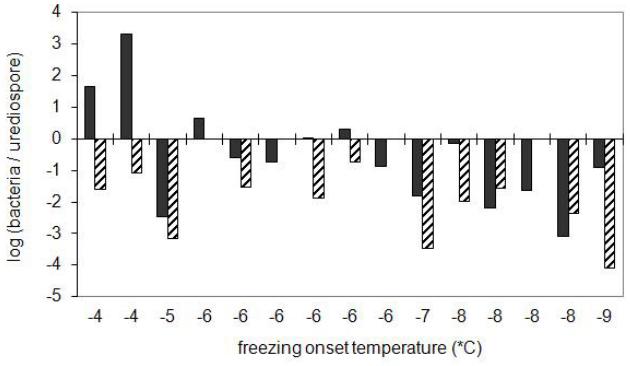


Fig. 2. Frequencies of urediospores carrying total bacteria and *Pseudomonas syringae*. Frequencies are presented as the log of total bacteria (solid bars) or *P. syringae* (hatched bars) per urediospore for samples ranked in the order of decreasing freezing onset temperature.

3.3 Evaluation of the hypothesis that ice nucleation activity of rust spores is attributable to bacteria on the spores

In Sect. 3.1 and in Table 1 we present calculated frequencies of INA based solely on the number of rust spores in the suspensions. But the spore suspensions we used also contained bacteria because they are naturally present on the spores when they are harvested from plants and cannot all be eliminated by washing. Microbiological analyses of spore suspensions allowed for us to calculate the concentration of total bacteria and *P. syringae* in these suspensions (Table 2). Based on these bacterial concentrations we were able to estimate the potential contribution of bacteria to the ice nuclei in the suspension, in this case by assuming that the bacteria – and not the rust spores – were the source of the ice nuclei. If we assume that total bacteria are responsible for INA, the frequency of ice nuclei per bacterial cell varied widely from 5×10^{-8} to 3×10^{-3}. Compared to the frequency of ice nuclei per spore, which ranged from 5×10^{-6} to 3×10^{-4}, this was erratic, representing a 100-fold greater to 1000-fold lower difference in frequencies for bacteria than for spores (Fig. 3). If we assume that only *P. syringae* is responsible for INA, frequencies of ice nuclei per bacterial cell were estimated to be 10-fold to 10 000-fold greater than the frequencies calculated for spores. However, for four samples, INA of spores could not be attributed to *P. syringae* because the bacterium was not detected in these samples. For two other samples, if *P. syringae* were responsible for the INA then it would be due to the presence of exceptionally active strains (10^{-2} nuclei $cell^{-1}$ at $-4\,°C$ and 10^{-1} nuclei $cell^{-1}$ at $-7\,°C$) having an ice nucleation rate equal to that of strain Cit7 under the conditions reported by Orser and colleagues (Orser et al., 1985) (Fig. 1). Overall, the frequency of ice nuclei per spore among the samples was significantly less variable than the frequency of ice nuclei calculated per total bacteria or per *P. syringae* cell in the suspensions (Bartlett's test for homogeneity of variance, $p = 0.026$).

To further evaluate the contribution of bacteria to the INA of the spore suspensions, we tested the INA of bacteria isolated from the spores. Eighty-eight strains of *P. syringae* associated with rust spores were isolated and tested for INA.

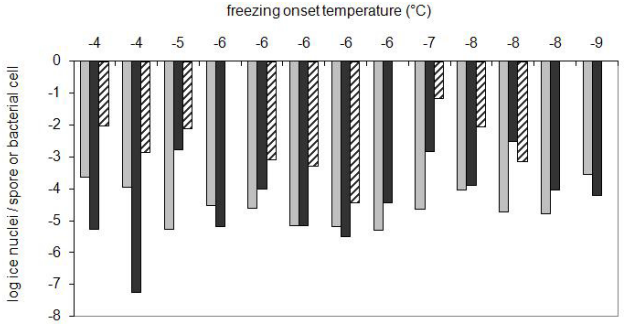


Fig. 3. Numbers of ic nuclei per particle in spore suspensions. Numbers were calculated according to three hypotheses about the origin of the ice nuclei in the suspensions: that the ice nuclei are associated only with (i) the urediospores or the cells (grey bars) of (ii) the total bacteria (black bars) or (iii) *Pseudomonas syringae* (hatched bars) in the spore suspensions. The abundance of ice nuclei per spore or bacterial cells are presented in the order of decreasing freezing onset temperature of the spore suspensions.

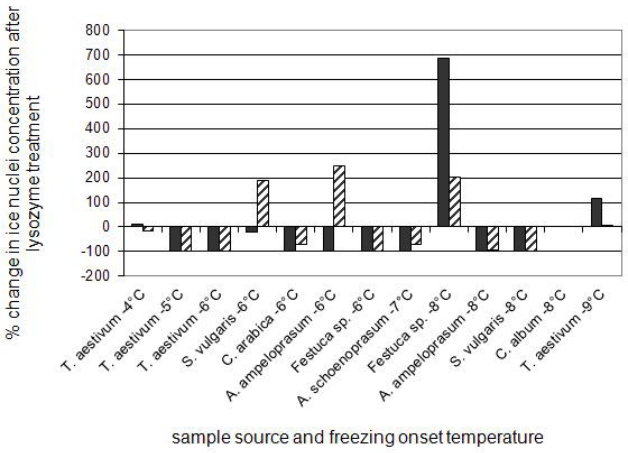


Fig. 4. Effect of lysozyme treatment on the abundance of ice nuclei in spore suspensions. The effect is presented as the percent of change in the number of ice nuclei per mL of spore suspension at the freezing onset temperature (black bars) and at one degree below the freezing onset temperature (hatched bars) for each sample. There was 0 % change in freezing behavior for spores from *C. album* for both temperatures.

For each strain, 32 drops of 20 µL of each 10-fold dilution from 10^8 to 10^4 cells mL^{-1} were tested from -2 to -9 °C. Hence, at any given temperature, the maximum frequency of ice nuclei that could be detected was 1.7×10^{-2} nuclei $cell^{-1}$ (31 drops frozen for the suspension at 10^4 cells mL^{-1}) and the minimum frequency detectable was 1.6×10^{-8} ice nuclei $cell^{-1}$ (1 drop frozen for the suspension at 10^8 cells mL^{-1}). The maximum frequency of INA at the corresponding onset freezing temperature of the sample for the strains from each sample (reported as log (ice nuclei *P. syringae* $cell^{-1}$) in Table 2) ranged from the minimum to the maximum detectable. We then used these values to estimate the maximum number of ice nuclei in each sample that could have been contributed by *P. syringae* at the onset freezing temperature. This was accomplished by multiplying the abundance of *P. syringae* in the spore suspension by the maximum frequency of INA of this bacterium observed among the strains of each sample. These values of potential abundance of ice nuclei from *P. syringae* (reported as log (ice nuclei mL^{-1} due to *P. syringae*) in Table 2) were compared to the total number of ice nuclei detected in the sample (reported as log (ice nuclei mL^{-1}) in Table 2). There were 10 samples in which *P. syringae* was detected and for which strains were isolated and checked for INA. In five of these there were from 17 to 1600 times more ice nuclei in the spore suspension than those that could have been contributed by *P. syringae*, and in three of them there were 3 to 4 × more ice nuclei in the suspension than what could have been contributed by *P. syringae*; in two samples *P. syringae* ice nuclei could have accounted for all of the ice nuclei detected. Likewise, the frequency of ice nucleation activity that *P. syringae* would have needed to account for the ice nuclei in the spore suspension was very high and in some cases much greater than what we have observed among the numerous strains we have characterized, as depicted in Fig. 1. The INA of strains other than *P. syringae* was not systematically characterized in this study. However, the dominant colony types were collected and characterized from a few samples and in particular from samples in which *P. syringae* was not detected. None of these strains were ice nucleation-active. As an additional means to evaluate if the ice nuclei in the spore suspensions were due to bacteria, we calculated the rates of ice nuclei production that would have been necessary among the total bacteria to account for the INA observed in spore suspensions. The total bacterial population would have needed to produce ice nuclei at similar or higher rates than observed among the *P. syringae* strains collected here.

3.4 Effect of lysozyme and heat treatments on ice nucleation activity of rust spore suspensions

Boiling eliminated all detectable ice nuclei at the freezing onset temperature and at one degree below this temperature for all samples. Lysozyme, on the other hand, had a more erratic effect on ice nuclei. Lysozyme treatment eliminated all detectable ice nuclei at the freezing onset temperature for 8 samples (Fig. 4) and at one degree below the freezing onset temperature for 5 of these samples (all samples showing −100 % change in ice nuclei concentration). For 2 samples, lysozyme treatment had little or no perceptible effect on the abundance of ice nuclei in the sample (samples showing near 0 % change in ice nuclei concentration). Surprisingly, lysozyme treatment caused a 150 % to a 700 % increase in the abundance of ice nuclei in four samples. But this increase

was not always observed at all the temperatures tested for the sample.

4 Discussion

All rust spores collected in the field in this work had onset freezing temperatures in immersion freezing tests at $-9\,^{\circ}C$ or warmer. This suggests that INA at temperatures $> -10\,^{\circ}C$ is a wide-spread trait among urediospores of rusts. Urediospores harbor bacteria including strains that are ice nucleation active. However, quantitative analyses of bacterial abundance and the rates of INA of the associated bacteria suggest that the rust spores themselves have inherent INA under the conditions of the tests here. Many of the bacteria associated with spores at the time of their harvest from leaves were removed by the washing step used in this study. Here, spores were washed in an attempt to reduce as much as possible the influence of bacteria on the INA of the spores to test the hypothesis that the urediospores themselves were INA. However, in their native state on leaves and during their dry release into the atmosphere, rust spores would most probably carry the bacteria that we removed during washing. We quantified the bacteria removed during washing (data not shown) and this allowed for us to estimate that spores carried several orders of magnitude more bacteria (both total and *P. syringae* populations) before than after washing, with several samples having up to equal numbers of urediospores as bacteria. In this light, it is likely that on spores liberated into the atmosphere directly from leaves under dry conditions, bacteria could account for much more of the INA of spores than observed here.

Although the results of the microbiological analyses suggest that bacteria do not account for the INA of washed rust spores, it should be noted that the analyses conducted here were based on the capacity of bacteria to grow on culture media. Dead or non-culturable bacteria would not have been quantified via these methods. Dead cells of *P. syringae*, for example, can maintain INA if the cells remain intact after death, as illustrated by the INA of Snomax®. To account for the INA of rust spores, dead and non-culturable cells would have needed to be at least 10 times more frequent on spores than culturable cells based on the rates of INA described for these bacteria. We observed rust spores under scanning electron microscopy (data not shown). Spores that had surface contaminants that could have been bacterial cells were rare (less than 1 in about 50 spores). Alternatively, bacteria might have made a greater contribution to urediospore INA if the INA of bacteria adhering to the spores were in fact one or more orders of magnitude greater than what was measured in cell suspensions grown in the laboratory. For many samples, lysozyme markedly reduced INA. This result is compatible with the hypothesis that the INA is due to bacteria. However, in some cases lysozyme treatment enhanced INA, a phenomenon that we have never observed for the INA of pure culture suspensions of bacteria.

Lysozyme is a glycoside hydrolase. It hydrolyses the 1,4-β-linkages between the glycoside residues in the peptidoglycan of bacterial cell walls and those between the N-acetyl-D-glucosamine residues in chitodextrins (Strynadka and James, 1991). In other words, it can react with the oligosaccharides from the hydrolysis of chitin, and the chain length of these oligosaccharides determines the rate of their hydrolysis by lysozyme (Rupley, 1964). Chitin is the principal cell wall constituent of the Eumycota. Along with hemicellulose and glucomannan it is the major component of the outer layer of urediospores (Ehrlich and Ehrlich, 1969; Staples and Wynn, 1965). The chemistry of the urediospore surface is highly dynamic as the spore matures, as its spines form and elongate, and as it prepares for germination. Urediospores are highly hydrophilic. When in contact with free water or under high relative humidity, the under layers of the spore wall absorb water in preparation for germination. This sets off chitin synthesis, degradation of the surface mucilage of urediospores and other enzymatic processes that lead to increased surface quantities of soluble carbohydrates (Staples and Wynn, 1965). It is plausible that variations in amounts and configurations of chitin oligosaccharides occur during these processes, and that these oligosaccharides were substrates for the lysozyme used in our treatments. Perhaps the building blocks of their derivatives are INA, but this has not been demonstrated. However, there is evidence for the INA of certain plant polysaccharides (Pummer et al., 2012, and citations within). The chemical nature of the INA of rust spores will need to be further explored.

Rust fungi sporulate prolifically. A hectare of slightly rusted wheat, for example, can harbor over 10^{15} urediospores, and a heavily rusted field would produce several times as many (Stakman and Christensen, 1946). These spores can be liberated into the atmosphere via wind and other mechanical agitation of the plants. This is highly accentuated during harvest: 1 h of combining (about 5 ha of wheat) can liberate about 10^{13} urediospores (Friesen et al., 2001). Likewise, for stem rust of perennial ryegrass it has been estimated that 10^{14} spores can be liberated from a 50 ha field in 24 h (Pfender et al., 2006). Spore release can result in near-ground (2 m) urediospore concentrations of 10^3 to $10^6\,m^{-3}$ (Gregory, 1978). Since the early 1920s it has been known that these rust spores can be transported up to several km into the troposphere (Stakman et al., 1923). Data collected during extensive sampling with manned aircraft indicate an overall logarithmic decrease in concentration with height, and at 1 to 2 km altitude the urediospore concentrations detected are on the order of 10 or fewer spores m^{-3} (Gregory, 1978; Hirst et al., 1967). If these concentrations of urediospores were associated with typical clouds containing about $1\,mL\,m^{-3}$ distributed into about 10^8 droplets m^{-3} (Pruppacher and Klett, 1997), then this would likely be insufficient to initiate rainfall via primary glaciation where about 1 ice nucleus per 10^5 or 10^6 (Pruppacher and Klett, 1997) or in some cases per 10^3 (Young, 1993) drops is required.

Nevertheless, the evidence for the importance of rainfall in dissemination of rusts is compelling. Initiating processes that lead to the formation of raindrops would be a useful adaptation for rusts, which are obligate biotrophs, to assure their deposition back to the Earth's surface, as has been suggested for *P. syringae* (Morris et al., 2008). Rain fallout of long distance disseminated spores has been the source of primary inoculum in initiating numerous rust epidemics such as in the first appearance of coffee rust in Brazil in 1966 – most likely resulting from the transport of spores with trade winds from regions of coffee production in Angola that coincided with the seasonal heavy rain in Brazil (Bowden et al., 1971; Nagarajan and Singh, 1990). Soybean rust, an emerging disease in North America, is spreading across the eastern US with rain (Barnes et al., 2009). Long distance dissemination of wheat rust across India is another example of the role of rainfall deposition of urediospores in the spread of this pathogen (Nagarajan et al., 1976). The presence of urediospores in rainfall has been demonstrated by collection and direct observation of rainwater (Rowell and Romig, 1966). Observations were made during periods prior to the start of wheat rust epidemics at experimental farms when local sources of urediospores were absent. Therefore the spores collected in the rain were not accumulated by scrubbing of the air as the drops fell. The authors reported the number of spores that fell per ft^2 of field and the total amount of rainfall (in inches) for each of 25 rain events in the spring of two wheat growing seasons. This allowed for us to calculate that there were between 0.01 and 1.2 urediospores of *Puccinia recondita* and *P. graminis* per mL of rainfall when these fungi were detected in rain. Interestingly, these values, as well as those for the concentrations of *P. syringae* in rain and snow (1–10 bacteria mL^{-1}, Morris et al., 2008) and for total biological ice nucleators in snow active at $-7\,^{\circ}C$ (0.001–0.23 ice nuclei mL^{-1}, Christner et al., 2008a, b), are all well below what would be expected for the ratios of ice nuclei : droplets proposed above for precipitating clouds. The coherence of these observations incites us to wonder in what way the available data on spore concentrations at cloud altitudes is representative of conditions conducive to precipitation.

We suggest that there are several factors that could explain why the currently available data on atmospheric concentrations of biological ice nucleators at cloud height are misleading relative to the processes considered to be involved in rainfall formation. Firstly, sampling from manned aircraft is generally conducted under stable atmospheric conditions with flight times that are very short relative to the volume of air for which estimates of particle concentrations will be made. Under these conditions, the data might not represent conditions most favorable for particle uplift and the turbulence favorable for precipitation. Microorganisms released from plant surfaces can be uplifted in thermal plumes. These plumes are not uniformly spaced across a landscape and can lift "packages" of air that are not markedly diluted as they rise, thereby preserving the near-surface particle concentrations. These packages of convected air will butt up against inversion layers where they can accumulate until the inversion layer degrades. Hirst and colleagues noted a trace of this phenomena in the uplift of urediospores in a case where concentrations increased with altitude up to 1 km (Hirst et al., 1967). Hence, it is likely that current data on microbial concentrations in the air at cloud height do not reflect the spatial and temporal variability of these concentrations. Secondly, cumulus clouds entrain dry air from their edges (Pruppacher and Klett, 1997), thereby ensuring that ice nuclei in dry aerosols can act as contact nuclei. The values for ice nucleation that we report here are for immersion freezing, and there is some discrepancy about which of these two modes of freezing (contact and immersion) is more efficient (Levin and Yankofsky, 1983; Möhler et al., 2007). Thirdly, in light of the temperature range of INA of urediospores, the freezing that they initiate could lead to ice multiplication via the Hallet–Mossop process which is restricted to the -3 to $-8\,^{\circ}C$ temperature range and can result in the production of several orders of magnitude more ice crystals than ice nuclei (Hallett and Mossop, 1974). Equipment to permit sampling that is more representative of the conditions under which clouds form and to describe the spatial and temporal variability in airborne concentrations of INA micro-organisms would make an important contribution to elucidating the conditions under which these microorganisms can influence rainfall.

Whatever the real concentrations of rusts and other INA microorganisms in the atmosphere, it seems that they are nevertheless minor components of airborne microbial populations. In atmospheric samples, ice nucleators active at $> -10\,^{\circ}C$ were no more frequent than 91 in 6.6×10^6 total microbial particles (Bowers et al., 2009). In fresh snowfall, biological ice nucleators were much less than 1 % of the total DNA-containing particles in the size range of microorganisms (Christner et al., 2008a). Likewise, urediospores detected at 1–2 km altitude were in background fungal populations that were generally 10 to 100 times more dense (Gregory, 1961; Hirst et al., 1967). This leads us to wonder about what to consider in determining the concentrations of these and other biological ice nucleators that can initiate cloud glaciation in such a way that it leads to rainfall. Excessive ice nuclei in clouds can inhibit the formation of large precipitable ice particles because the competition among crystals for water reduces the ultimate size of the ice crystals (Young, 1993). If ice that is induced by biological ice nucleators multiplies via the Hallet–Mossop process, then could this inhibit growth of cloud drops due to the competition among crystals? Our observations also highlight novel notions about the significance of the association of *P. syringae* and rust spores for their dissemination and survival. Cells of *P. syringae* could be protected in the mucilaginous matrix that covers rust spores, and this bacterium might also use rust spores as a food base. Urediospores are likely to be liberated more readily from plant surfaces than are bacterial cells and hence they could serve as dissemination vectors for bacteria.

The spores in turn could benefit from the INA of the bacteria when spores had low levels of activity. *P. syringae* and many of the rusts are favored by similar environmental conditions (cool, moist climates) and hence it is not surprising to find them together on plants; it could even be expected that they have been subjected to similar selective pressures of the physical environment.

Human activities clearly have had an impact on the abundance of rust spores in Earth's atmosphere. In North America, for example, cultivation of winter wheat spread widely across the Great Plains in the 1900s after American plant breeders successfully obtained cold-tolerant wheat varieties. This created a vast belt of wheat production from Mexico to Canada throughout the months of March to July that led to the emergence of severe and continuous epidemics of rust caused by *Puccinia graminis* f. sp. *tritici* as the spores of this highly specific fungus flew northward throughout the artificially prolonged period of availability of the host plant. Did this have any impact on cloud processes involving glaciation at temperatures $> -10\,^{\circ}\mathrm{C}$? It would be fascinating to explore the regional patterns of rainfall in light of wheat rust epidemics and in particular with regard to the Dust Bowl that devastated the Great Plains in the late 1920s. Wheat and the accompanying rusts spread across several continents in the early 1900s and were accompanied by intense disease monitoring. Meteorological records could be mined in this light.

5 Conclusions

Urediospores of several species of rust fungi are ice nucleators capable of initiating ice formation in immersion freezing tests at temperatures as high as $-4\,^{\circ}\mathrm{C}$ for suspensions at 10^5 spores mL^{-1}. Spores in most of the samples carried cells of ice nucleation-active strains of *P. syringae* (at rates of less than 1 bacterial cell per 100 urediospores), but the INA of the bacteria accounted for only a fraction of the ice nuclei in the spore suspensions. INA of urediospores was heatlabile. Changes in the INA of urediospores after treatment with lysozyme, which is a glycoside hydrolase attacking the 1,4-β-linkages between the glycoside residues in the peptidoglycan of bacterial cell walls and those between the N-acetyl-D-glucosamine residues in chitodextrins, suggest that the basis for INA of urediospores involves a polysaccharide. The INA of rusts was studied because of the compelling evidence for the importance of rainfall in the life history of this plant pathogen. The data in the literature on the abundance of rust spores in the atmosphere indicate that they are present at concentrations similar to those of INA bacteria – concentrations which have fostered the belief that they are in insufficient quantities to have an impact on cloud glaciation processes that would lead to precipitation. In light of the potential role of ice multiplication in clouds and in light of the difficulty of air sampling under conditions favorable for precipitation, our results lead us to suggest that we need further precision about the specific conditions of microbial interactions with cloud droplets under conditions that could lead to rainfall. This is important to delimit the circumstances under which they might have critical impacts on rainfall (either to enhance or to inhibit) and to understand the selection processes that could have favored the evolution of ice nucleation activity.

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