Bacteria and fungal spores as ice nuclei from *Coffea arabical* L.

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Abstract

Airborne microorganisms from bacterial and fungal species are able to act as ice nuclei and can affect sensible crops to frost such as coffee trees. Consequently, frost is one of the major problems in South-Southeastern coffee crops in Brazil. In this research, it was found three categories of organisms with ice nuclei activity (INA) in coffee leaves, basing in the mean freezing point of saline solution, around -17°C. The first category, with strong INA, it was found the *Pseudomonas syringae var. garceae*, a coffee tree pathogenic, as INA+. *Pseudomonas syringae var. syringae* behaves with soft less INA+ efficiency, comparing to the var. *garceae*. This last variety also causes aureolar spot disease. The second category presents a partial ice nuclei activity, including two other bacteria, *Pantoea agglomerans* (that is known as ice nuclei), and *Corynebacterium*, with mean freezing point from -7°C to -10°C. And the third category presents non-ice nuclei activity (INA-), with freezing point below -11°C, including all other bacteria and fungi. Additionally, *H.vastatrix*, acoffee rust disease, which already causes lots of prejudice to the crops, can be associated with an INA+ bacterium, causing frost. That result deserves a refined research, trying to elucidate how this association should be done.

Therefore, two rust coffee diseases and aureole spot, as well as the presence of *Pantoea agglomerans,* can be directly or indirectly associated to ice nuclei activity, demanding a higher bio-control, particularly during wintertime, due the possibility of frost damage.

Resumo

Microrganismos na atmosfera de origem bacteriológica e fúngica têm a habilidade de atuar como nucleadores de gelo e afetar plantações sensíveis à geada como o café. Consequentemente, a geada é um dos maiores problemas para essa cultura no Sul-Sudeste do Brasil. Neste estudo, foram encontradas três categorias de organismos com atividade de nucleação de gelo (INA) em folhas de café, tendo por base o ponto de congelamento da solução salina, cerca de -17°C. Na primeira categoria, com forte atividade INA, foi encontrada a bactéria *Pseudomonas syringae var. garceae*, um patógeno de folhas de café, *Pseudomonas syringae var syringae* comportou-se com menor eficiência de INA comparando-a à var. *garceae*. Esta última variedade também causa a doença denominada "mancha aureolada". A segunda categoria apresentou uma atividade parcial de nucleação de gelo, bem como duas outras bactérias: *Pantoea agglomerans* (reconhecida na literatura como nucleante de gelo) e *Corynebacterium*, ambas com pontos de congelamento entre -7°C e -10°C. A terceira categoria não apresentou atividade nucleadora (INA-), com pontos de congelamento abaixo de -11°C, incluindo-se todas as demais bactérias e fungos. Adicionalmente, *Hemileia vastatrix*, o fungo responsável pela ferrugem de café, o qual já causa enormes prejuízos aos agricultores, pode estar associado a bactérias INA+, gerando geada. Esses resultados necessitam de mais estudos, visando a esclarecer como esta associação poderia ocorrer. Portanto, duas doenças de plantações de café, a ferrugem e a mancha aureolada, e a presença da bactéria *Pantoea agglomerans,* podem estar diretamente e indiretamente associadas à atividade de nucleação de gelo, demandando maior controle biológico, particularmente durante o inverno, devido à possibilidade de danos por geada.

Palavras-chave: atividade de nucleação de gelo, plantações de café, fungos, bactérias, *Pseudomonas syringae*.

1. Introduction

Airborne microorganisms were found in the atmosphere for the first time in the XIX century. Since then, many studies on airborne fungi have been carried out to investigate atmospheric concentrations and compositions and their impact on the environment such as involvement in cloud physic processes. Airborne bacteria can act as cloud condensation nuclei and some airborne bacterial and fungal species are able to act as ice nuclei and therefore induce rainfall in moderate climates and cause frost on crops (Schnell & Vali, 1973).

According to Lovelock (1988), if organisms seed clouds there could be biofeedback that would lead them to further evolve this propensity – as he proposed in the Gaia processes – but this idea still remains controversial. Based upon the fact of organisms disperse, and even if they consequently arrive in a new habitat that is not more advantageous than the previous habitat (Hamilton & Lenton, 1998), it can be suggested that some microbes have evolved to seed cloud formation to create local dispersal vehicles for themselves (Morris et al., 2005). An epiphytic bacterium, *Pseudomonas syringae* v. *syringae*, scattered from decayed leaves, was the first bacterium discovered to have an ice nucleation activity (O'Brien & Lindow, 1988 and 1989 and Schnell & Vali, 1973). Therefore, Schnell & Vali (1973, 1976) came to the conclusion that *P. syringae* is active as an ice nucleus because these bacteria produce a protein on their outer membrane that is one of the most active of the naturally-occurring ice nuclei (IN), which has compounds capable of catalyzing the freezing of water, and because freezing of cloud water is a critical step for rainfall over major parts of the earth (Sattler *et al.* 2001; Ariya & Amyot 2004; Diehl *et al.* 2000 and, Hamilton & Lenton, 1998; Blondeaux *et al.* (1999) and, Hazra *et al.*, 2004). These bacteria are widely distributed across the planet, survive airborne dissemination up to the clouds and fall out with precipitation. On the other hand, the ice nucleus activity (INA) of fungi has received little attention. Pouleur et al. (1992) reported that the species *Fusarium* can freeze water at around -1.0°C and -2.5°C. The role that these fungi and bacteria can play in catalyzing the formation of precipitation is under investigation in view of applications for drought mitigation.

Recently, according to Amato *et al.* (2005), the total bacterial count in clouds reached about $3x10^4$ cells.m^{-3} of cloud volume ($1x10^5$ cells L^{-1} of cloud water). Most of the isolated micro-organisms, including 12 fungal

and 17 bacterial strains, were described for the first time in atmospheric water. Amato *et al.* (2007) found bacteria mainly in the genera *Pseudomonas, Sphingomonas, Staphylococcus, Streptomyces, and Arthrobacter* and fungi in the genera *Cladosporium* or *Trametes*.

Also according to Morris *et al.* (2005), the body of information on biological ice nuclei, meteorology and atmospheric microphysics suggests that vegetation patterns, and agricultural practices in general, have consequences on cloud and precipitation processes. So, this leads them to ask: Might the preference of crops and the selection and breeding of their varieties have been different if we had taken into account the number of ice nucleation bacteria on plant varieties? These questions could be expanded toward those related to agricultural policies in general, also taking into account crop susceptibility to frost damage. There are many plant crops that are sensitive to frost damage associated to the presence of IN bacteria (Lindow, 1983; O´Brien & Lindow, 1988 and 1989).

Coffee tree (*Coffea arabica)* is a species of coffee indigenous to Ethiopia and Yemen, in Africa, usually cultivated between 1,300 and 1,500 m altitude. However there are plantations as low as sea level and as high as 2,800 m. The plant can tolerate low temperatures, but not frost, and it does best when the temperature hovers around 20°C (68°F). The Brazilian Southern region, where there are many coffee plantations, suffers frost damage from time to time. Therefore, frost is one of the major problems in South-Southeastern coffee crops in Brazil where there have been many frost events, particularly in 1975 when a large number of trees were destroyed by what has been called the "black frost".

Additionally, there are some articles that take into account epiphytic and endophytic fungi and bacteria in *Coffea arabica* L such as Vega et al. (2005), which found eighty-seven cultures endophytic bacterial isolates from 19 genera collected in Colombia ($n = 67$), Hawaii ($n = 17$), and Mexico ($n = 3$), where the genera *Pantoea* and *Pseudomonas* were among them. On the other hand, Santamaría and Bayman (2005) found some epiphytic and endophytic microorganisms of coffee for which the ecosystem interactions are poorly understood.

Consequently, studies of frost mechanisms in coffee leaves are very important for the protection of this crop. Therefore, the aim of this article is to evaluate the ice nuclei activity (INA+) of microbiota (endophytic and epiphytic fungi and bacteria) of coffe e

leaves, either decaying or still attached to the plant.

2. Materials and methods

2.1 *Coffea arabica* leaf sampling

In order to extract the microbiota (epiphytic and endophytic fungi and bacteria) from coffee leaves, they were sampled from trees (*Coffea Arabica*) and from the debris in commercial plantations. We chose mature leaves as well as decaying leaves. The material was collected from the *Nossa Senhora* farm, near Pinhal City, at about 800 m altitude and 150 km from São Paulo Capital of São Paulo State (see map of Figure 1).

Figure 1. Southern Brazil with São Paulo State showing Garça and Pinhal Cities as well as São Paulo Capital.

The leaves were divided in two different treatments. The first group, for endophytic bacteria and fungi, was cut and cleaned in a 25% sodium hypochlorite bath during 15 minutes and after that, in three water baths for rinsing. After cleaning, they were cut in peaces of 1 cm^2 and placed in potato-dextrose-agar (PDA) media, and incubated 3 to 4 days at 27°C. The PDA media used cooked potato water, 15 g of Agar (Difco) and 15 g of dextrose. After incubation, all fungi and bacterial colonies were isolated. The second group was only cut and placed at PDA media without cleansing in order to analyze epiphytic bacteria and fungi.

2.2 Isolation of bacteria and fungi

2.2.1 Bacteria

The colonies found on PDA media were divided in **F** categories, plus two found bacteria from apical leaves. They were identified according to Romeiro (1995), based on general colony appearance. After isolation, the bacterial colonies were placed in five media: a) nutrient agar; b) King's Medium B (for *Pseudomonas fluorescens,* according to King et al., 1954); c) Yeast Dextrose-Carbonate (to grow yeast); d) to identify *Erwinia herbicola* and; e) to identify *Corynebacterium*. These four media are described as it follows. a) Nutritive agar: meat extract (8.0 g), soya peptone (8.0 g), NaCl (5.0 g), Difco agar (15.0 g) and water (1 l); b) *B. de King* (pH=7.4): peptone (20.0 g), glycerin (10.0 g), K_2HPO_4 (15.0 g), $MgSO_4$ 7 (H_2O) (1.5 g), Difco agar (15.0 g) and water (1 l);c) Y.D.C (Yeast-Dextrose-Carbonate: glucose (20.0 g), yeast extract (10.0 g), $CaCO_3$ (15.0 g), Difco agar (15.0 g) and water (1 l); d) to identify *Erwinia herbicola:*-saccharose (10.0 g), arabinose (10.0 g), hydrolyzed acid casein (5.0 g), LiCl (7.0 g), NaCl (5.0 g), glycine (3.0 g), $MgSO_4$ 7(H_2O) (0.3 g), Na_2HPO_4 (0.1 g), dodecil sodium sulfate (0.05 g), bromotimol blue (0.06 g), acid fucsine (0.1 g), Difco agar 95.0 g) and water (1 l); e) to identify *Corynebacterium:* glucose (10.0 g), hydrolyzed casein (4.0 g), yeast (2.0g), NH_4Cl(1.0 g), $MgSO_4$ 7(H_2O) (0.3 g), LiCl (5.0 g), sodic azide (0.002 g), Li sulfite (1.2 g), Difco agar (15.0 g) and water (1 l).

All isolated bacteria were suspended in a sterile saline solution 0.85% and plated on nutrient agar at 27°C.

Two additional strains of bacteria from culture collections were also evaluated: *Pseudomonas syringae* pv. *syringae* and *Pseudomonas syringae* pv. *garceae*. The first one is well known as being INA+. The latter was

found in coffee leaves in Garça City, in 1955, Center-West of São Paulo State (see Figure 1). This bacterium causes also a disease in coffee leaves, called "aureolar spot". The two strains were classified as SDPABO-IB/281 (*P. syringae* pv. *syringae*) and SDPABO-I/158 (*P. syringae* pv. *garceae*), obtained from the *Instituto Biologico Data Bank*. Both bacteria were grown in nutrient agar, around 27°C, and afterwards suspended in saline 0.85%, as described above.

The suspended concentration in saline solution (0.85%) was 1.5×10^8 cells /ml for all bacteria.

2.2.2 Fungi

The isolation methodology for fungi was based on Schnell & Vali (1973). These authors sampled fungi from intact leaves as well as decaying leaves. PDA was used for isolation and identification as described above.

An additional test was also performed using urediospores of coffee rust: *Hemileia vastatrix* Berk. & Broome, as described in Section 2.3.2. This fungus causes "rust" disease over coffee leaves, generating a great damage in coffee crop production. They were sampled from intact infected leaves from the Nossa Senhora farm where they were scraped with a sterile scalpel from sporulating clusters under coffee leaves.

All fungi were suspended in saline solution 0.85%, from 18.0 ± 0.05 mg to 20.0 ± 0.05 mg / ml.

2.3 Freezing tests

2.3.1 Lyophilizer tests

Freezing tests were performed using a lyophilizer (Schnittzeichnungen, Delta 1-A, type 336 Osteröde/harz) in order to freeze the water (saline) droplets with suspended fungi and bacteria. Therefore, both types of microorganisms were analyzed with the purpose of identifying INA+ as follows: 30 droplets of 20 ìl of bacterial and fungal suspensions were subjected to freezing in the lyophilizer. The freezing point was observed for each droplet. Two tests were performed for a total of 60 droplets. A curve of droplet freezing distribution was calculated for each strain. Tests on sterile saline solution 0.85%, saturated NaCl and glucose (0.48 M) were also performed as controls. Temperatures were obtained from the mean of two thermometers, one placed beside the droplets and the other one from the lyophilizer freezer indicator.

2.3.2 *Freezing test for* H.vastatrix

Urediospores of Coffee rust (*Hemileia vastatrix*), previously submitted to the tests above (Section 2.3.1), was also sent frozen to the INRA Research Center in Montfavet, France in Oct 2008, in order to analyze their INA. At the time, the samples had 40% viability. These spores were suspended in sterile distilled water, rinsed and filtered across polycarbonate filters (8 μ m pore diameter) and then re-suspended in sterile distilled water free of ice nuclei at -9° C. The concentration of the suspension was 4 x 10^4 spores /ml as determined under the microscope with a haemocytometer.

Their INA evaluation is described as it follows: the capacity of the spores to induce freezing of water at temperatures from -2° C to -9° C was determined for droplets of the spore suspensions placed on a metal surface floated on a cooling bath as described in Morris *et al.* (2008).

3. Results

3.1 Bacteria and fungi isolation results

Fifteen bacterial strains (F1 – F13, M14 and M15) were isolated. F4 and F5 colonies were identified as *Corynebacterium* and M14 as *Pantoea agglomerans (*former *Erwinia herbicola)*. The others could not be correctly classified and strains F3 and M15 were not used herein.

Seven fungi strains were isolated and identified as: *Mucor sp., Cephalosporium sp., Chlamidomyces sp., Streptomyces sp., Chladophyarola sp., Melanospora sp.* and *Penicillium sp.*

Tables 1 and 2 as well as Figure 2 to 17 present all results as it follows: 3.2.1- blank tests; 3.2.2. Bacteria tests; 3.2.3. Fungi tests and 3.2.4 *H.vastatrix* test. Figures 2 to 4 show the tests performed with saline water 0.85% (Figure 2), saturated NaCl (Figure 3) and glucose 0.48 M (Figure 4).

First of all, the overall temperature distribution shows a reasonable Gaussian distribution. The freezing droplet temperature means from all Figures (2 to 4) present temperatures below -10°C, as expected, which saturated NaCl presenting the coldest mean temperature (-17.9°C). The overall results are also shown in Table 1 and 2.

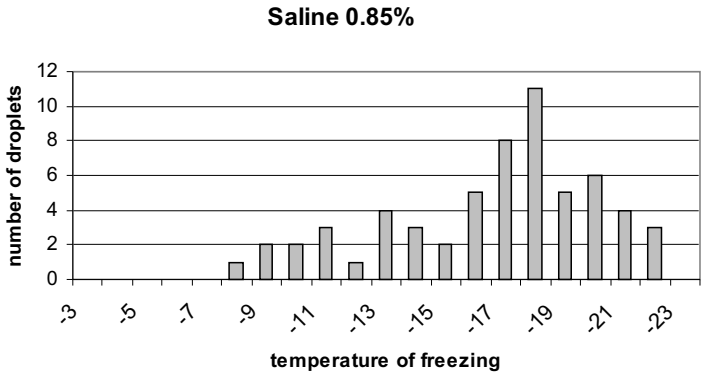

Figure 2. Tests with saline solution 0.85%, showing their freezing points

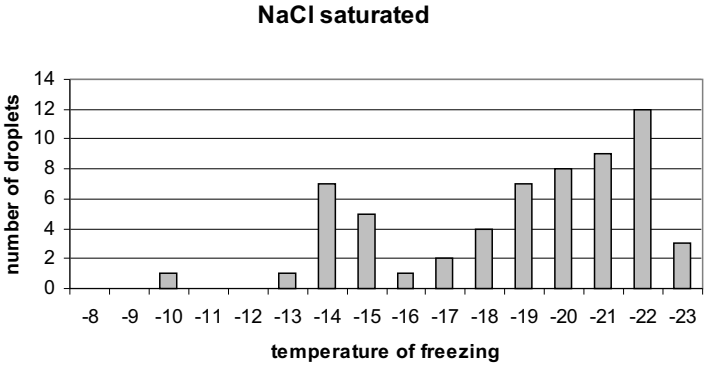

Figure 3. Tests with saturated NaCl, showing their freezing points.

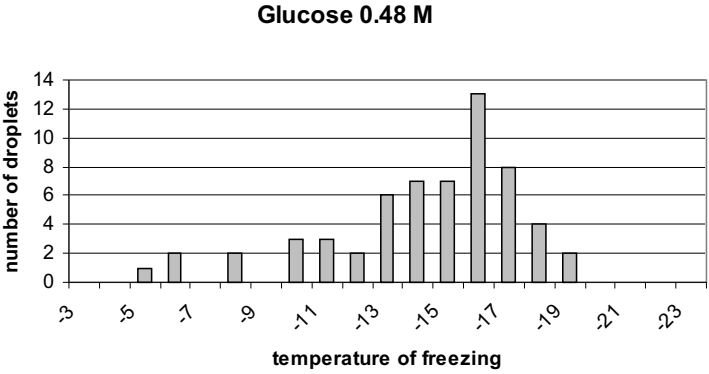

Figure 4. Tests with glucose 0.48M, showing their freezing points.

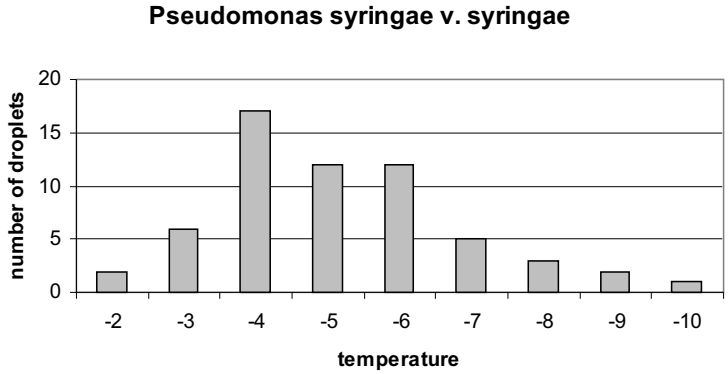

Figure 5. Tests with Pseudomonas syringae var. syringae, showing their freezing points.

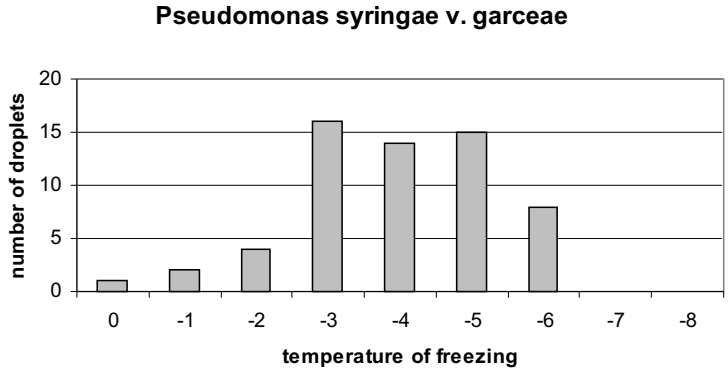

Figure 6. Tests with Pseudomonas syringae var. garceae, showing their freezing points.

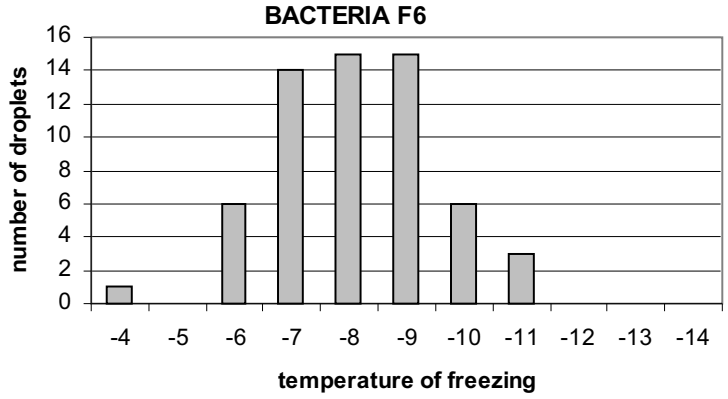

Figure 7. Tests with non-identified Bacteria F6, showing their freezing points.

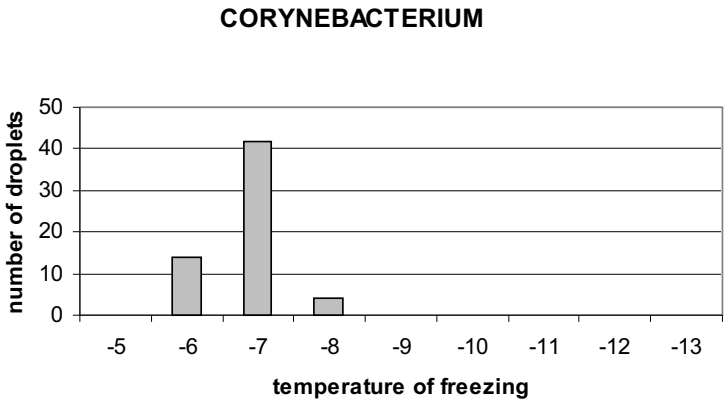

Figure 8. Tests with Corynebacterium (Figure 8), showing their freezing points.

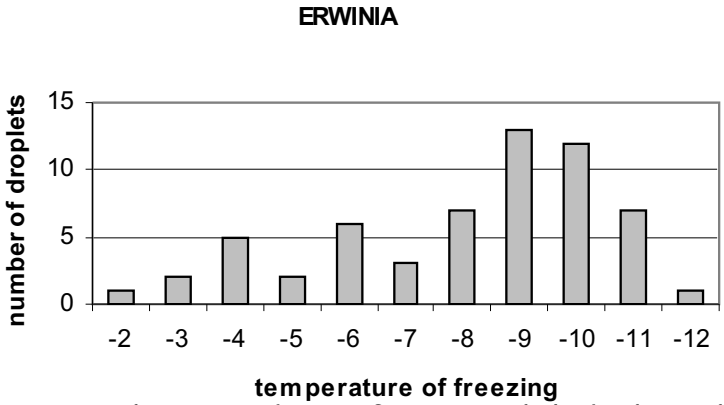

Figure 9. Tests with Pantoea agglomerans (former Erwinia herbicola), showing their freezing points.

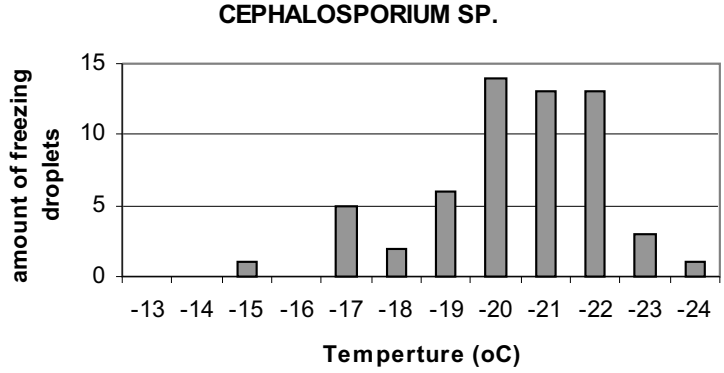

Figure 10. Tests with Cephalosporium sp., showing their freezing points.

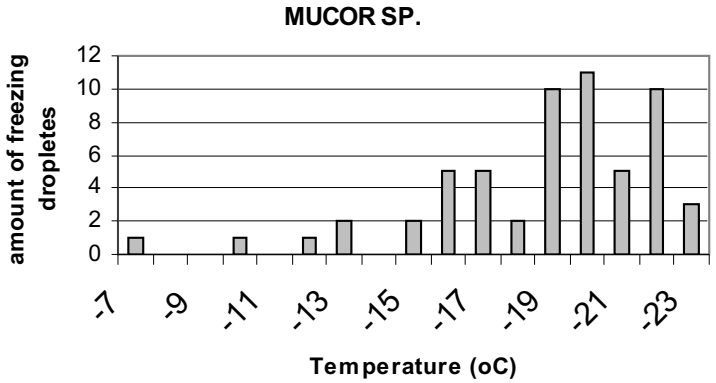

Figure 11. Tests with Mucor sp., showing their freezing points.

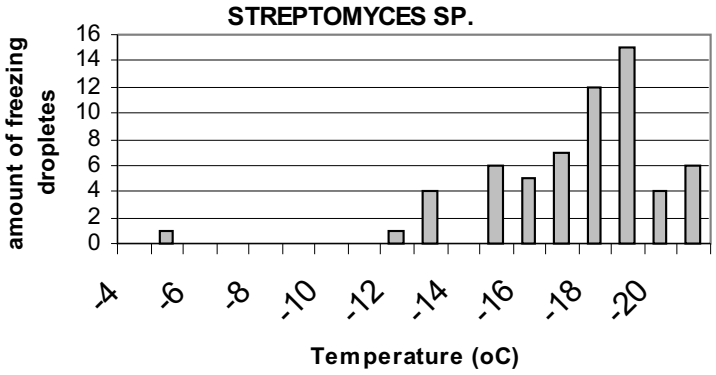

Figure 12. Tests with Streptomyces sp., showing their freezing points.

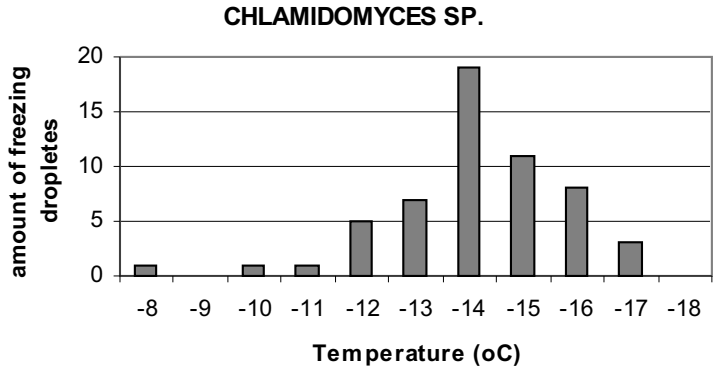

Figure 13. Tests with Chlamidomyces sp., showing their freezing points.

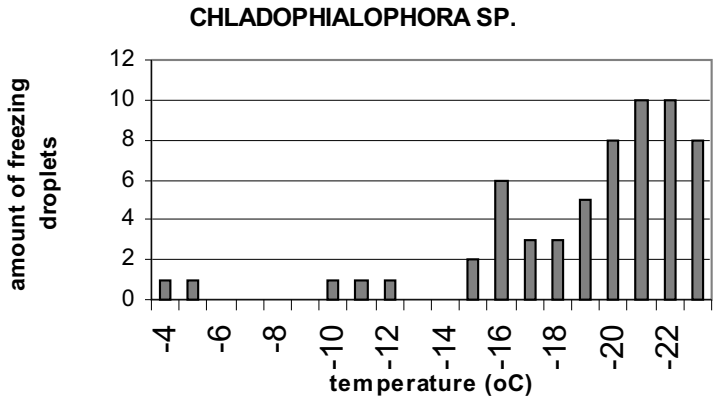

Figure 14. Tests with Chladophyarola sp., showing their freezing points.

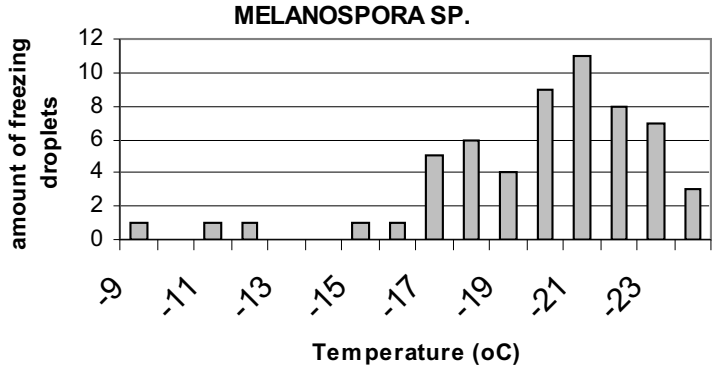

Figure 15. Tests with Melanospora sp., showing their freezing points.

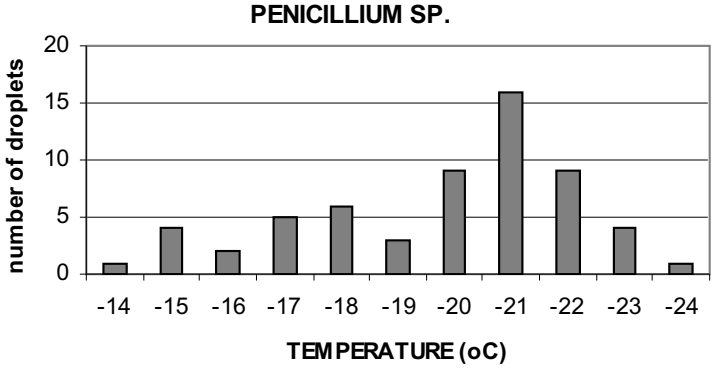

Figure 16. Tests with Penicillium sp., showing their freezing points.

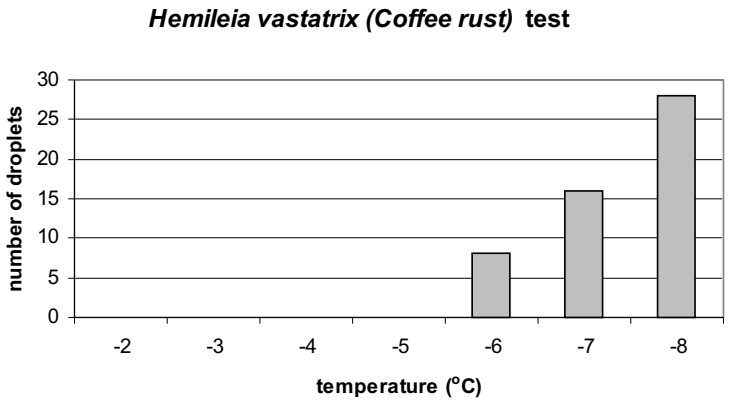

Figure 17. Tests with Hemileia vastratrix, showing their freezing points.

First of all, the overall temperature distribution shows a reasonable Gaussian distribution. The freezing droplet temperature means from all Figures (2 to 4) present temperatures below -10°C, as expected, which saturated NaCl presenting the coldest mean temperature (-17.9°C). The overall results are also shown in Table 1 and 2.

Figures 5 to 9 show the tests with *Pseudomonas syringae* pv. *syringae* (Figure 5); with *Pseudomonas syringae* pv. *garceae* (Figure 6); with F4 and F5, which were identified as *Corynebacterium* in Figure 8; and with M14, identified as *Pantoea agglomerans* in Figure 9. The overall results are also shown in Table 1 with all bacteria. Figure 7, for non-identified bacterium F6, clearly shows intermediate ice nucleation activity. F7 shows the same behavior as F6, and it is shown only in Table 1.

From those figures, it is clearly seen that both *Pseudomonas* strains present a higher (warmer) freezing point, and are clearly ice nucleation active bacteria (INA+), with activity far warmer than the other two bacteria and blank tests and the temperature freezing points are also in accordance with the literature, around -4° C and -5° C. Particularly pv. *garceae* presents the warmest freezing point mean, even compared to var. *syringae*.

Additionally, the other two bacteria, including *Pantoea agglomerans*, present also warmer freezing point means than the blank tests, around -8° C (see Table 1) as expected. The presence of *Pantoea agglomerans,* with known ice nuclei activity, is rather important to coffee crops, due to frost damage as discussed below.

Table 1. Blank and bacteria overall test results, including number of droplets and T (90), temperature which 90% of droplets are frozen. Obs. F3 is not considered. The suspended concentration in saline solution (0.85%) was 1.5 x 10^8 cells per milliliter for all bacteria.

Tests	Number of droplets	T90(° C)	Mean freezing temperatures (° C)
Saline water 0.85%	60	-19.0	-16.6 ± 6.2
NaCl saturated	60	-22.0	-17.9 ± 4.9
Glucose 0.48 M	60	-16.0	-13.1 ± 6.3
Pseudomonas syringae vr.garceae	60	-6.0	-4.0 ± 5.0
Pseudomonas syringae vr. syringae	60	-8.0	-5.2 ± 4.4
F1-bacteria	60	-12.0	-9.8 ± 5.0
F2- bacteria	60	-12.0	-8.1 ± 4.7
Corynebacterium (F4/F5)	60	-7.0	-7.0 ± 5.5
F6- bacteria	60	-10.0	-8.1 ± 4.9
F7- bacteria	60	-10.0	-7.1 ± 4.2
F8- bacteria	60	-18.0	-15.6 ± 5.7
F9- bacteria	60	-19.0	-16.2 ± 5.5
F10- bacteria	60	-19.0	-15.6 ± 5.1
F11- bacteria	60	-18.0	-15.2 ± 5.5
F12- bacteria	60	-21.5	-17.6 ± 5.3
F13- bacteria	60	-21.0	-17.9 ± 5.5
Pantoea agglomerans (M14)	60	-11.0	-8.1 ± 4.1

Table 2.Fungi test results, including number of droplets and T (90). All fungi were suspended saline solution 0.85%, from 18.0 ± 0.05 mg to 20.0 ± 0.05 mg per ml.

Tests	Number of droplets	T 90(° C)	Mean freezing temperatures (° C)
Hemileia vastatrix.(original)	52	-8.0	-7.4 ± 1.1
Penicillium sp.	61	-22.5	-19.8 ± 2.5
Chladophyarola sp.	60	-23.0	-19.1 ± 6.1
Cephalosporium sp.	58	-22.0	-20.5 ± 2.5
Chlamidomyces sp.,	56	-17.0	-13.6 ± 2.9
Streptomyces sp.	61	-19.5	-17.4 ± 4.9
Mucor sp.	58	-21.5	-18.5 ± 4.6
Melanospora sp.	58	-20.0	-19.6 ± 4.3

Figures 10 to 16 present the tests with *Mucor sp.* (Figure 10), *Cephalosporium sp.* (Figure 11), *Chlamidomyces sp.* (Figure 12), *Streptomyces sp.* (Figure 13), *Chladophyarola sp.* (Figure 14), *Melanospora sp.* (Figure 15) and, *Penicillium sp.* (Figure 16). Table 2 shows the overall results for fungi.

From those figures, it is clearly seen that none of the fungi act as ice nuclei. On the other hand, *Chlamidomyces* is the only fungus whose spores present a result similar to that of the glucose (0.48 M) freezing point mean, around -14° C. The others fungi present results similar to saturated NaCl, with freezing points below -17° C.

Figure 17 presents the test results with rust spores, using the methodology described in Section 2.3.2. Preliminarily, the tests with the previous methodology using urediospores of *H.vastatrix* clearly show INA+ behavior, with similar freezing point mean, around -4° C, as both *Pseudomonas* strains. No results for INA of obligate parasitic fungi have been presented previously in the literature. Figure 18 shows the spore of *H.vastatrix*.

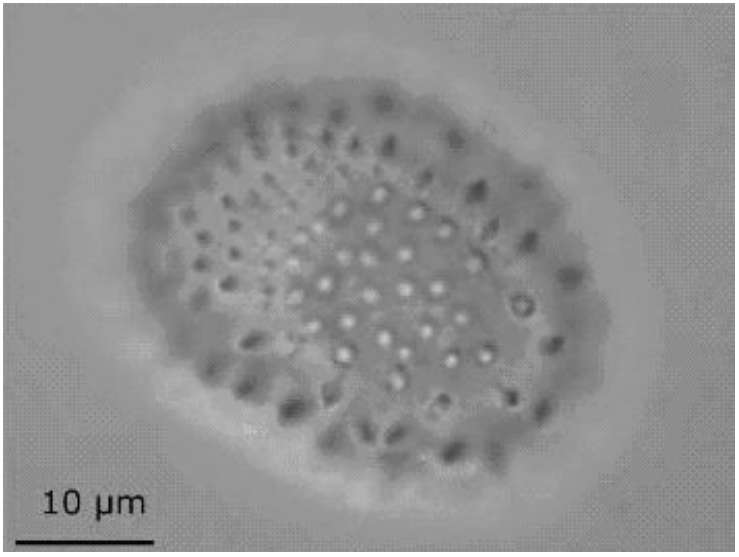

Figure 18. Hemileia vastatrix urediospore (extracted from http://www.scielo.br/revistas/fb/iinstruc.htm).

The results suggest that rust spores not only contribute to the diseases but might also be involved in frost damage to coffee.

4. Discussion

The overall results are shown in Table 1 and Table 2 with the freezing point mean and T 90 (temperature which 90% of droplets are frozen.) From both tables, it is possible to classify ice nucleation behavior in three different categories: a) **truly ice nucleation active or INA+ strains** which includes both *Pseudomonas* strains, with freezing point means between -4° C and -5° C; b) **partially ice nucleation active strains** with freezing point means around -7° C and -10° C, including a group of non-identified bacteria (F1, F2, F6 and F7), *Pantoea agglomerans* (*Erwinia herbicola)* and *Corynebacterium*, as well as, *H.vastatrix*,; and c) **non-ice nuclei active (INA-) strains**, with freezing points below -11° C, which includes all other fungi and bacteria, as well as saline, salt and glucose solutions. This group includes bacteria (F8 to F13) and all fungi (*Mucor sp., Cephalosporium sp., Streptomyces sp., Chladophyarola sp., Melanospora sp.* and, *Penicillium sp.*) with freezing point means around NaCl satured, at -18° C or below, except by *Chlamidomyces*, around -14° C . Saline solution 0.85% and glucose 0.48 M present freezing points around -17° C and -13° C, respectively.

The T 90 index shows similar distributions, but in two categories: presenting INA+, warmer or equal to -11° C including both *Pseudomonas* strains, *Pantogea agglomerans*, *Corynebacterium* and two non-identified bacteria (F6 and F7). Bacteria F1 and F2 could also be classified here (T 90 = -12° C), all warmer than saline solution (T 90= -19° C) or glucose (T 90 = -16° C). The INA- category includes all other bacteria and fungi with T 90 below -17° C, reaching -23° C with *Chladophyarola sp.*

5. Conclusions

In this research, we found three categories of INA among coffee leave microbiota. The first group, truly INA+, included *Pseudomonas syringae* pv. *garceae*, a pathovar of coffee leaves. That result is a possible new,

because this pathovar is not cited as INA+ in the literature. It must be notified that this pathovar also causes aureolar spot disease. The second category presents a partial ice nucleation activity, including some bacteria such as *Pantoea agglomerans,* which is known in the literature as INA+, and *Corynebacterium*. And the third category presents non-ice nucleation active (INA-) strains and includes all other bacteria (F8 to F13) and fungi (*Mucor sp., Cephalosporium sp., Streptomyces sp., Chladophyarola sp., Melanospora sp.* and *Penicillium sp.*).

Additionally, *Hemileia vastatrix*, the coffee rust, which already causes lots of prejudice to these crops, seems to be INA. But our spores were isolated directly from diseases leaves and might be contaminated with other micro-organisms. In any case, it suggests an additional problem for farmers. That result deserves a refined research, trying to elucidate how this ice nucleation activity is possible.

Therefore, two coffee pathogens - rust and *P. syringae* pv. *garceae* - and in addition to the presence of *Pantoea agglomerans,* are directly or indirectly associated with ice nucleation activity on the coffee leaves, demanding a higher effort for control by the coffee farmers, particularly during wintertime, due the frost damage.

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