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Bacterial density and diversity in the phylloplane of ice-nucleating citrus leaves from northeastern México is impacted by climatic change

Abstract

Citrus production in the northeast of México is affected by frosts that cause economic losses. A climatic problem, today, greenhouse gases from various sources, especially agricultural areas, have caused climate change in combination with bacterial agents from the phyllosphere of the foliage of these citrus fruits, which at temperatures close to freezing point and relative humidity greater than 80%, cause damage by freezing, with a negative economic impact in the citrus area of the state of Nuevo León, northeastern, México. There for the objectives of this work were: i) To analyze the density of native populations of ice-nucleus-activating bacteria from these citrus fruits and ii) to demonstrate the capacity of these populations to induce ice nuclei *in vitro* and iii) to biochemically identify the main bacterial groups with INAB in citrus leaves from the region of the state of Nuevo León, northeastern of México. For this purpose, healthy citrus leaves were collected from the municipalities of Monterrey: El Cercado, Villa de Santiago and Gral. Terán, N.L, northeastern of México. Each different bacterial isolate was subjected the ice nucleus activation test (INAT) and then identified based on the biochemical pattern. The results showed that the total populations of ice nuclei-activating bacteria ranged from $5.0X10^2$ to $6.1X10^6$ CFU/g of fresh citrus leaf. While 84% were positive in the genetic capacity of ice nuclei activation, in an average time of 20 - 30 minutes. The diversity biochemical identification supports that INAB genera and species were detected: *Alcaligenes faecalis, Citrobacter cloacae*, *Erwinia herbicola, Pseudomonas syringae* and *Xanthomonas campestris*, that exist as natural epiphytes on citrus leaves in N.L northeastern México. While the density and diversity of the natural ice nuclei-activating population depends on the geographic location of the orchards, the climate and the physiological state of the citrus trees and now from global warming in that sense, sustainable crops should be the way one to prevent freezing damage.

Keywords: citrus, global warming, epiphytes, ice nuclei activation induction, microbial population

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Introduction

The management of agricultural soil where citrus fruits are grown, the anaerobic decomposition of organic matter and the application of nitrogen fertilizers with oxygen such as NO_3 in the soil can cause the emission of greenhouse gases CH_4 or N_2O, are released into the atmosphere, influencing climate change, which leads to early or late frosts causing damage to agricultural production, especially because there is evidence that microorganisms in the phylloplane of plants have the ability to act as ice nucleators, or ice- nucleus-activating bacteria (INAB) that is, INAB have the ability to respond to low temperatures close to the freezing point and humidity above 70%.[1-7] So controlling greenhouse gas emissions not only prevents global warming can also prevent agriculture from being affected by preventing INAB populations that exist in the phylloplane of domestic plants such as citrus fruits from detonating,[8-12] harming citrus production in the northeastern of the state of Nuevo León, México. In the citrus zone of the state of Nuevo León, northeastern of México during the last 20 years due to the incidence of early and late frosts, the effects of climate change have been greater with winters that are variable in time and duration.[1] During leaf freezing, other parts of the tree suffer damage: foliage, buds, flower buds, fruit and even the wood depending on the intensity and duration of the frost in all those facts a biological process was suspected.[2-4] Considering reports published by different researchers related to the effect of low temperatures on regional citrus crops where some microorganisms were involved.[5] It has been reported for more than 60 years that there is a biological factor that influences the damage to citrus production by frost;[6-8] this factor refers to the sensitization of the leaves of agricultural crops by biological agents, saprophytic epiphytes or called population of ice-nucleus-activating bacteria that reproduce at certain times of the year,[9-11] such as the rainy season and at the beginning of the winter season, the forecast of early and late frosts depends on the population density are a key part to understand what is involved.[12-14] Ther for the objectives of this work were: i) to analyze the density of native populations of ice-nucleus-activating bacteria from these citrus fruits and ii) to demonstrate the capacity of these populations to induce ice nuclei *in vitro* and iii) to biochemically identify the main bacterial groups with INAB in citrus leaves from the region of the state of Nuevo León, northeastern of México.

Materials and methods

Collection of healthy citrus leaves from the state of Nuevo León (N.L), northeastern of México. Healthy citrus leaves were collected from the following areas: El Cercado, Villa de Santiago and General Terán, municipalities in the south of the state of N.L, México, during the months of March, April and May 2022. The leaves were kept in polyethylene bags under refrigeration (5°C) until the analysis of the population.[13,15]

Quantification of the population of ice nuclei-activating bacteria in the citrus areas of Nuevo León, Northeast of México.

From healthy and fresh citrus leaves, 0.5 g were weighed in 47.5 ml of sterilized 2.0 M phosphate buffer pH 7.0 and left in rotary

agitation at 250 rpm for 1-2 h, the suspension was diluted up to 10-4 and 0.2 ml was inoculated in quadruplicate on D-3 and D-4 agar, both were incubated at 28+°C/48 h (L), to determine the INAB density and colonies characteristic of *Pseudomonas syringae* were selected for the extracellular green pigment and of *Erwinia herbicola* for the intracellular orange pigment.[16] The isolates were assigned the codes: S-5, Q-5, S-567, X-5M, X-10A, X-9A, X-3, X-a, C-13N, C-8, X-7, C-4c1, T-13VL, C-4B, R-2b; for later identification of this other bacteria found (were re-seeded in 18x150 tubes with nutrient agar-glycerol and stored in refrigeration for preservation at 5°C.[5-9]

Isolation of total populations of INAB from the citrus zone of N.L. Northeast of México.

Leaves healthy sample from citrus plants were inoculated on Agar D-4 for *P. syringae* (g/L): gelatin peptone 6.0; yeast extract 3.0; beef extract 1.5; agar-agar 15.0; pH 7.2, 30 mg of SDS (Sodium dodecyl sulfate) and 1000 ml of distilled water.[2,3] Agar D-3 for *E. herbicola* (g/L); NaCl 5.0; $MgSO_4.H_2O$ 0.3; NH_4Cl 5.0; bromothymol blue 0.06 acid fuschina 0.1; sucrose 20.0 agar-agar 20.0. 1000 ml of distilled water.[8,10]

Demonstration of ice nucleation activity test (INAT) *in vitro*

Pseudomonas and *Erwinia* isolates were activated on nutrient agar for 48h/30°C, then a batch of these was taken and suspended in 13x100 tubes with 5 ml of sterilized supersaturated saline solution, used as negative control and another tube with only distilled water used as positive control: were placed in a container, that was kept in a freezer at -7 and -8°C. The time until the negative control tube froze was registered, the tubes with bacterial isolates that did not freeze during this period were considered negative for ice nuclei formation.[11] Macroscopic, microscopic and biochemical characterization of the main genera and species of ice nuclei-activating bacteria. INAB isolates were identified based on the scheme in the manual by Bergey.[10-13] All numerical results were analyzed by ANOVA-Tukey.

Results and discussion

Density of the ice-nucleus-activating bacterial population (INAB).

Table 1 shows the density of the native population of in the phyllosphere of healthy citrus leaves, collected in the municipalities of Monterrey, El Cercado, Villa de Santiago and Gral. Terán, N.L. in the northeastern of the Mexican Republic; that fluctuated from $5.0X10^2$ to $6.1X10^6$ UFC/g of fresh leaf, as reported by some research.[10] Test to determine the ice-nucleus-forming activity in native bacteria recovered from the citrus area of N.L. Northeast of México. In this test it was observed that a high percentage of the isolated bacteria 84% were positive for the ice-nucleus-forming activity, in an average time of 20 to 30 minutes. The highest percentage of test-positive isolates were recovered on D-4 Agar, as reported in similar agricultural crops in the United States,[14-16] as presented in Table 1.

Table 1 Quantification of the native population of ice nuclei-activating bacteria (INAB) on citrus leaves in the state of Nuevo León, northeastern México

No of samples	Municipality of the state of Nuevo Leon	*Log. UFCX10^6/g dry leaf	*Log UFCX10^6/g dry leaf	Percentage (%) of populations of epiphytic ice nuclei-activating bacteria from citrus leaves in the state of Nuevo Leon, northeastern of México.	
		Agar D-4	Hoja Fresca Agar D-3	Agar D-4	Agar D-3
1	Monterrey	0.5**	-	60	-
2	"	1.6^d	7.5^d	ND	ND
3	"	2.9^d	3.1^d	50	50
4	"	0.9^e	1.2^d	ND	ND
5	El Cercado	2.0^d	-	100	-
6	Villa de Santiago	1.8^d	2.2^d	50	30
7	"	1.7^d	6.5^d	50	50
8	"	0.5^e	1.1^d	10	50
9	"	8.0^d	2.0^d	50	50
10	"	3.1^d	5.5^d	ND	ND
11	"	4.0^d	8.5^d	50	50
12	"	3.0^d	812.0^b	50	10
13	"	34.0^c	17.0^c	50	50
14	"	3.5^d	15.0^c	50	50
15	"	-	8.0^d	-	100
16	"	0.6^e	-	ND	-
17	"	35.5^c	0.5^e	100	ND
18	"	210.0^b	145.0^b	30	ND
	Gral. Terán				
19	"	127.5^b	44.5^c	ND	100
20	"	185.0^b	60.0^c	50	50
21	"	97.5^c	119.0^b	100	50
22	"	545.0^b	765.0^b	100	ND
23	"	5150.0^a	1900.0^a	100	100
24	"	1550.0^a	300.0^b	100	100
25	"	6150.0^a	1335.0^a	100	100

ND, Not determined; (-): No growth. All values were the average of 6 replicates.

**Values with different letters were statistically different according to Tuke's ANOVA

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Specifically, in the leaves of citrus trees from orchards in General Terán, N. L. Northeastern of México a population density of INAB ranging from $4.4X10^4$ to $6.1X10^6$ CFU/g of fresh leaf was detected; in leaves of citrus trees from Cercado and Villa de Santiago, N. L., from $5.0X10^2$ to $3.5X10^4$ CFU/g of fresh leaf and the lowest population density was detected in the leaves of the vegetation of the orchards of the municipality of Monterrey, which fluctuated from $5.0X10^2$ to $7.0X10^3$ CFU/g of fresh leaf. These results on the density of the INAB population indicate its variability in the citrus zone of the state of Nuevo León, northeastern of the Mexican Republic or México, probably because the leaves analyzed were collected in four different geographic areas. The above agrees with what was reported by several research,[14-16] when investigating INAB in citrus leaves of a completely geographical distribution. In general, the density of epiphytic INAB in citrus is reported to be evidently influenced by the environmental condition prevailing in the habitat, to which this vegetation is adapted.[17-20]

In vitro ice nucleation activation and biochemical identification of INAB.

Table 2 shows the division of the isolates into four colonial morphological groups: yellow, orange, white, green, and a translucent green and lemon variant of a spore-forming Gram-positive rods of the genus *Lysinibacillus.* The microscopic morphology of the isolates was short, straight Gram-negative rods, with the exception of one isolate that tested positive for the Gram reaction. The isolates were composed of spore-bearing bacilli with no biochemical identification except for the spore and aerobic growth. The microscopic and colonial morphologies correspond to those reported in the literature.[18-20] Suspected INAB colonies were positive in 84% for the INAT, although this was also variable, indicating that there are different ecotypes of genera and bacterial species involved,[21] since each geographic area is different, as well as the citrus varieties as well as soil management and doses of nitrogen fertilizers that included soil amendments with organic matter.[15,16] It was observed that the selectivity of the culture media used was not a limitation for the detection of other genera and species of INAB,[18,21] such as: *Alcaligenes* and *Citrobacter* in D-3 Agar and *Xanthomonas* in D-4, in addition to four isolates that were not identified that were related to the sporulated Gram-positive rods tentatively recognized as *Lysinibacillus* as reported in other plant crops.[18,22,23] In this investigation, we expected to find only the widely reported INAB isolates: *P. syringae* and *E. herbicola*. According to numerous studies, these isolates are primarily responsible for the sensitization of citrus leaves in this area to early and late frosts, which are also associated with greenhouse gases due to nitrogen fertilization schemes, moisture management, and organic matter used as soil improvers.[18,20,21] INAB genera and species are common epiphytes on citrus leaves, as well as on native ornamental vegetation (data not shown). Some INAB isolates are reported for the first time in this area of the country,[17,19,20] but with climate change, their role is becoming more relevant due to the commercial importance of this citrus-growing region in northeastern México.[15] The detection of INAB different from that reported is explained based on the ambient temperature, the climate of the region and the types of citrus cultivated, although today they are also attributed to climate change due to greenhouse gases,[22,23] since INAB showed an evident capacity to adapt to these sites,[24,25] as well as its complex interaction with the leaves of the host citrus.[18,20] Although biological ice nucleation activity has been known in the United States since before 1974 and is reported to cause damage to agricultural crops,[23,26] the results of this research are part of the reports on the distribution and density of INAB in the citrus-growing area of northeastern México, which is correlated with frost damage to citrus crops in the region that is now linked to the agricultural production system that contributes to the generation of greenhouse gases, reasons for making changes in soil management and fertilization schemes to prevent further release of greenhouse gases associated with agriculture.[19-23,25]

Table 2 Microscopic and macroscopic morphology of genera and species of epiphytic ice-nucleus-activating bacteria (INAB) on citrus leaves in the state of Northeast of Nuevo León, México

Genera Species	Colony	Microscopy	No. of Isolates
Erwinia amylovora *Alcaligenes faecalis* *Citrobacter cloaceae*	Yellow, mucous, circular, convex colonies with continuous border, smooth surface.	Short, Gram-negative rods	15
Erwinia herbicola	Yellow, flattened, irregular with wavy edges. Raised, convex, punctate orange colonies with continuous edge.	short Gram negative rods	6
Xanthomonas campestris pv citri	White, mucous, punctate colonies, convex with continuous edge, smooth surface.	short, Gram negative rods	6
Pseudomonas syringae	Translucent green, irregular colonies with wavy edges. Extracellular green pigment.	short, Gram negative rods	6
Pseudomonas congelans	Translucent green colonies, circular, convex, continuous edge, smooth surface.	short, Gram negative rods	6
Pseudomona sp	Creamy lemon green colonies, circular, continuous edge, smooth surface.	short, Gram negative. Rods	6
Unidentified		large Gram positive rods	1

Table 3 presents the biochemical properties of INAB isolated on D-4 agar for *P. syringe* a strain donated by Dr. S. Lindow of the University of California, USA of which was compared to two released a green fluorescent extracellular pigment; the Q-5 isolate was oxidase positive, mobile, reduced nitrates, was catalase positive, used citrate as a carbon source and fermented glucose with acid formation. The isolate S-567 was oxidase positive, formed levan, hydrolyzed gelatin, produced hydrogen sulfide, was catalase positive, used citrate as a carbon source and reduced nitrates. An isolate was recovered that showed a biochemical response similar to isolate S-567 (with the exception of the weak oxidase) with a different colonial morphology, suggesting that these are genera and species of bacteria to INAB in agricultural crops other than citrus.[25,27-33]

Table 3 Biochemical identification of ice nucleation active bacteria from citrus leaves in the state of Nuevo León, Northeastern of México, associated with *Pseudomonas syringae*

	Pseudomonas syringae		
Biochemical test [a]	**Strain type[l]**	**Q-5**	**S-567**
Oxidase	+	+	+a
Levan	+	-	+
Gelatinase	-	-	+
Arg. dihydrolase	-	-	-
Amylase	-	-	-
Diffusible pigment	+	+	+
Mucoid growth Anaerobiosis	+	+	+
NO3 reduction	-	-	+
Catalase	+	+	+
Hydrogen sulfide	-	-	-
Indole	-	-	-
Mobility	-	-	-
Malonate	+	+	+
Citrates	+	+	+
MRVP	-/-	-/-	+/-
TSI (lactose/sucrose/glucose)	-/-/-	-/-/-	-/-/+
Yellow pigment on nutrient agar (30°C)	+	+	+
Caseinase	-	-	-
Growth in KCN broth	-	-	-

Symbology: (+) = Positive, (-) = Negative, (+a) = weak positive, all results are average of 4 replicates. Strain type[l]

Donated by Dr S Lindow of University of California, USA.

Table 4 shows those INAB detected in D-3 Agar; they showed characteristics similar to the genus *E. herbicola*,[20-22] these were the isolates designated as: X-10A, X-9A, X-3 and X-8A, negative to the oxidase test; four formed levan and yellow pigment at 30 °C; they grew anaerobically, used malonate as a carbon source, some hydrolyzed gelatin, but not casein or starch, this isolates were variable in the reduction of nitrates, as well as the MR-VP reaction, in the use of citrates, none grew in KCN and most fermented glucose, which represents a different group to the known INAB of the *P. syringae* type.[25,30-34]

Table 4 Biochemical identification of *Erwinia herbicola* ecotype as INAB native to citrus leaves from the Northeastern of state of Nuevo León, México

	***Erwinia herbicola* genotypes**			
Biochemical test [a]				
	X-9A	**X-10A**	**X-3**	**X-8[a]**
Oxidase	-	-	-	-
Levan	+	+	+	-
Gelatinase	-	-	+	+
Arg. dihydrolase	-	-	-	-
Diffusible pigment	-	-	-	-
Mucoid growth Anaerobiosis	+	+	+	-
NO3 reduction	-	-	+	-
Catalase	+	+	+	+
Hydrogen sulfide	-	-	-	+
Indole	-	-	-	-
Mobility	-	-	-	+
Malonate	+	+	+	+/-
Citrates	+	+	+	+
MRVP	-/-	-/-	+/-	-/-
TSI (lactose/sucrose/glucose)	-/-/-	-/-/-	-/-/+	+/+/+
Yellow pigment on nutrient agar (30°C)	+	+	+	+
Caseinase	-	-	-	-
Growth in KCN broth	-	-	-	-

Symbology: (+) = Positive, (-) = Negative, (+a) = weak positive, all results are average of 4 replicates.

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Table 5 shows the biochemical profile of the isolates representing the main groups of epiphytic INAB from the phylloplane of most citrus trees in the citrus-growing area of northeastern Nuevo León state. These isolates are affected by the way soil fertility is managed, especially by the type of nitrogen fertilizer such as NH4NO3 and the organic amendments recommended for this area. In Table 5 the response of isolates C-13N that showed is an *X. campestris*, while as *Citrobacter cloacae* registered as C-8 and X-7 had a biochemical response *Alcalingenes faecalis* had a biochemical response characteristically reported for genera and species of INAB: This isolated did not grow anaerobically, so they did not reduce NO_3, although this isolated used sodium citrate and malonate as the only sources of carbon and energy, consequently did not synthesize oxidase, nor acetyl methyl carbinol (Vogues-Proskauer reaction) due to the type of aerobic respiration.[2,5,9] Isolate C-8 does not synthesize levan, a sugar polymer that allows it to adhere to the surface of citrus leaves, it synthesized indoyl tryptophan, incapable of fermenting lactose and sucrose, although it oxidizes glucose.[35-37] The above supports that the environmental conditions of the area increase or reduce the diversity of INAB.[38-40]

Table 5 Biochemical identification of native ice nucleation active bacteria genera and species other than *Pseudomonas syringae* and *Erwinia herbicola* from leaves of the citrus zone of the state of Nuevo León, Northeastern of México.

Biochemical test	***Xanthomonas campestris***	***Citrobacer cloacae***	***Alcaligenes faecalis***
	(C-13N)	(C-8)	(X-7)
Oxidase	-	-	-
Levano	+	-	+
Gelatinase	+	+	+
Arg. hydrolase	-	-	-
Amylase	ND	ND	-
Yellow growth on Nutrient agar (30°C)	+	+	-
Mucoid growth	+	-	+
anaerobiosis	-	-	-
Hydrogen sulfide	-	+	-
Indole	+	+	-
Mobility	-	+	-
NO_3 reduction	+	+/-	+
Catalase	+	+	+
Malonate	+	+	+
Citrates	+	+	+
MRVP	-/-	+/-	-/-
TSI (lactose/sucrose/glucose)	-/-/-	-/-/+	-/-/
Caseinase	-	-	-
Growth in KCN broth	ND	ND	ND

Symbology: (+) = Positive, (-) = Negative, all results are average of 4 replicates.

Table 6 shows other genera and species of INAB recovered in the D-3 agar were: C-4cl, was mobile C-4B and R-2b different from *P. syringae* and *E. herbicola*. The isolate C-4cl formed levan, hydrolyzed gelatin, catalase and citrate positive and the rest of the biochemical tests were negative. The isolate C-4B was mobile, formed yellow pigment at 30 °C, grew anaerobically, reduced nitrates, was catalase positive and used malonate, the rest were negative.[3,5,8] The isolate R-2b was similar in some responses to the isolate C-4B, such as pigment formation at 30 °C, growth in anaerobiosis and nitrate reduction, but different in the MR test where it was positive and the VP negative, it fermented sugars in the TSI, the rest tests were negative.[9-11] The isolate T-13VL, in D-4, had a microscopic morphology of Gram-positive tetrad-arranged cocci and the following biochemical characteristics: catalase positive, oxidase negative, malonate slightly positive and fermented the three sugars of TSI, did not grow anaerobically, did not reduce nitrates, did not utilize citrates, was immobile, and did not produce levan.[1-5] This supports that this pattern does not correspond to *E. syringae* or *E. herbicola* and suggests that the INAB population is more diverse than reported.[40-43]

Table 6 Biochemical identification of *Xanthobacter* in citrus leaves from northeastern of the state of Nuevo León, México

Biochemical test	**Isolates**			
	C-4c1	**T-13VL**	**C-4B**	**R-2b**
Oxidase	-	-	-	+
Levano	+	-	-	+
Gelatinase	+	-	-	+
Arg. hydrolase	-	ND	ND	ND
Yellow growth on nutrient agar (30°C)	-	-	+	+
Mucoid growth anaerobiosis	+	-	-	+
Hydrogen sulfide	-	-	-	-

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Table 6 Continued....

Indole	+	-	+	-
Mobility	-	-	+	+
NO3 reduction	-	-	+	+
Catalase	+	+	+	+
Malonate	ND	+/-	+	-
Citrates	+	-	-	-
MRVP	-/-	-/-	-/-	+/-
TSI(lactose/sucrose/glucose)	-/-/-	+/+/+	-/-/-	+/+/+
Caseinase	-	-	-	-
KCN broth growth	ND	-	-	-

Symbology: (+) = Positive, (-) = Negative, (+/-) = Slightly positive, ND, Not determined; all values are the average 4 replicates

Conclusion

The density of INAB populations was influenced by the geographical location of the orchards in the citrus region, environmental conditions, the period of citrus growth as well as the management of the fertility of the orchards, especially the application of nitrogen fertilizer such as NH_4NO_3, responsible for the generation of greenhouse gases that impacted the density and biochemical variability of the epiphytic bacterial genera and species that activate ice nucleation in the phylloplane of citrus leaves in the northeastern of the state of Nuevo León. The detection of ice core activating activity in *P. syringae* and *E. herbicola*, as well as other bacterial genera not commonly reported with this ability to activate such as *A. faecalis, C. cloacae* and *X. campestris*., as well as Gram positive coccus and *Lysinibacillus* supports that this group is more complex especially due to climate change that favors the presence of early and late frosts, so it is important to apply environmental measures to avoid climate change that will cause the worsening of this situation in the northeast of the state of N. L. México.

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Declaration of conflicts of interest

The authors declare that there is no type of conflict of interest in its planning, execution and writing with the institutions involved, as well as those that financially supported this research.

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