Characterization of the *ina*A gene and expression of ice nucleation phenotype in *Pantoea ananatis* isolates from Maize White Spot disease

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ABSTRACT. Maize White Spot (MWS), a foliar disease caused by *Pantoea ananatis*, could cause up to 60% yield loss. Some strains of *P*. *ananatis* harboring the ice nucleation gene *ina*A catalyze the formation of ice nuclei, causing tissue damage at temperatures slightly below freezing. Little is known about the relationship between the presence of the *ina* gene in this maize pathogen and its expression during the phenomenon of ice nucleus formation. Here, we attempted to verify the presence of the *ina*A gene and the expression of phenotype *in vitro*. The identity of the isolates and the presence of the *ina*A gene were determined by *P*. *ananatis* species-specific primers. The expression of the *ina*A gene was assessed *in vitro* by the visualization of ice-crystal formation in water at subzero temperatures.

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A total of ninety *P. ananatis* isolates from MWS lesions were characterized. The presence of the *ina*A gene was confirmed by gel electrophoresis of the 350-400-bp PCR products. The *ina*A primers did not lead to DNA fragment amplification in three isolates. The ice nucleation phenotype was expressed in 83.34% of the isolates carrying the *ina*A gene. Our study showed that the ice nucleation in *P. ananatis* isolated from MWS lesions was dependent on the presence of a functional *ina* gene in the genome. We also found evidence indicating that some *P. ananatis* strains have a mutated form of the *ina*A gene, producing a non-functional ice nucleation protein. This is the first report on *ina*A gene characterization in *P. ananatis* isolates from Maize White Spot.

Key words: Phytopathogenic bacteria; Foliar disease; Variability; INA phenotype

INTRODUCTION

Found in nearly all maize-producing areas, the Maize White Spot (MWS), caused by *Pantoea ananatis*, has emerged as one of the most important foliar diseases in maize culture in Brazil (Costa et al., 2013).

The onset of the disease is marked by a dark green water-soaked leaf spot, which later turns straw-colored and necrotic (Paccola-Meirelles et al., 2001). Moderate temperatures around 14°C and high relative humidity in the atmosphere associated with frequent rainfall favors the disease. The high incidence and severity of maize white spot disease has contributed to a significant reduction in corn yield in Brazil since the 1990s (Costa et al., 2013). The disease incidence has also increased significantly in many countries (Pérez-y-Terrón et al., 2009; Alippi and López, 2010; Krawczyk et al., 2010).

P. ananatis is a Gram-negative, non-sporulating, facultative anaerobic bacterium, which forms bright yellow mucoid colonies with regular borders. This species belongs to the family Enterobacteriaceae, phylum Gammaproteobacteria, and has a single circular chromosome and one plasmid (Choi et al., 2012; De Maayer et al., 2012). *P. ananatis* colonizes the leaf surface and maize straw (Sauer et al., 2015), and has been identified as the agent responsible for the formation of the foliar lesions in maize, in Brazil (Paccola-Meirelles et al., 2001), South Africa (Goszczynska et al., 2007), Mexico (Pérez-y-Terrón et al., 2009), Poland (Krawczyk et al., 2010), and Argentina (Alippi and López, 2010).

P. ananatis catalyzes the formation of ice nuclei or ice crystals at temperatures slightly below 0°C in the presence of water, similar to other species of the genus *Pantoea* (*Erwinia*), *Pseudomonas*, and *Xanthomonas* (Lindow et al., 1978a, 1982b; Lindow, 1983). This mechanism is known as ice nucleation (INA), and bacteria active in ice nucleation are phenotypically classified as INA^+ (Lindow et al., 1982a,b). According to Lindow et al. (1982a,b) and Lindow (1983), the INA^+ phenotype may be related to the virulence of *P. ananatis*. The ice formed by the bacterium in the intercellular spaces collapses the vegetal cell, leading to leakage of the cell contents, which in turn causes necrotic foliar spots, which are typical of MWS lesions (Lindow et al., 1982b; Sauer et al., 2014).

According to Kozloff et al. (1983) and Lindow (1983), the protein known as INP (ice nucleation protein) is responsible for the ice nucleation activity. This protein is 120-180 kDa in size, has a highly repetitive amino acid sequence (Green and Warren, 1985; Kawahara, 2002) and a similar

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primary structure in all INA$^+$ bacterial species, and is found inside vesicles localized in the bacterial cell wall (Lindow, 1983; Phelps et al., 1986; Kawahara, 2002).

Orser et al. (1983) demonstrated that the INA$^+$ phenotype is coded by a single gene localized in a 3.5- to 4.0-kb DNA region. The following genes were identified for different bacterial species: *ina*Z (Green and Warren, 1985), *ina*C (Lindow et al., 1989), and *ina*V (Warren, 1995) in *Pseudomonas syringae*; *ina*W (Warren et al., 1986) in *P. fluorescens*; *ina*E (Warren and Corotto, 1989) in *Erwinia herbicola*; *ina*A (Abe et al., 1989) in *Pantoea ananatis*; *ina*U (Michigami et al., 1994) in *Erwinia uredovora*; and *ina*X (Zhao and Orser, 1990) in *Xanthomonas campestris* pv. *translucens*.

The INA phenotype is unusually distributed among the bacterial species of different genera. Not all species within a genus, or all strains within a species, exhibit the INA$^+$ phenotype (Lindow, 1983). Moreover, not every cell in a population of an INA$^+$ strain bacterium actively performs ice nucleation (Lindow et al., 1982b; Lindow, 1983; Pearce, 2001). This phenotypic variability of INA suggests that the *ina* gene may have followed an unusual evolutionary pattern by horizontal transfer of bacterial *ina* genes (Edwards et al., 1994).

Little is known about the variability of the INA phenotype in populations of *P. ananatis* of MWS foliar lesions. In a recent study (Sauer et al., 2014), nine of fifteen *P. ananatis* isolates from MWS lesions from two different locations showed phenotypic variability for INA$^+$. However, the presence or absence of the *ina*A gene in those isolates was not tested using molecular biological methods. Therefore, the aim of this study was to characterize *P. ananatis* isolates from MWS lesions occurring in maize plants from different Brazilian locations, based on molecular detection of the *ina*A gene and its phenotypic expression *in vitro*.

MATERIAL AND METHODS

P. ananatis was isolated from MWS lesions using a method described by Paccola-Meirelles et al. (2001). The DNA of isolates was extracted as described by Ausubel et al. (1992). The internal transcribed space of the 16-23S rRNA genes of the isolates was amplified by polymerase chain reaction (PCR) with species-specific primers designed for *P. ananatis*: ANAF (forward), 5'-CGTGAAACTACCCGTGTCTGTTGC-3' and ANAR (reverse), 5'-TGCCAGGGCATCCACCGTG TACGCT-3', which amplifies approximately 360 or 388 bp of the *P. ananatis* DNA (Figueiredo and Paccola-Meirelles, 2012).

The PCR mixture was composed of 2.5 µL PCR 10X buffer (20 mM Tris-HCl, pH 8.4 and 50 mM KCl), 1.0 µL $MgCl_2$ (50 mM), 1.0 µL of each primer (3 mM); 2.5 µL dNTP mix (2.5 mM), 0.5 µL Taq DNA polymerase (5 U/µL), 1 µL DNA (~20 ng, quantified using a Scandrop® device (Analytik, Germany), and sterilized ultrapure water (final volume = 25 µL). The PCR was performed on a TX96 thermocycler (Amplitherm) under the following conditions: denaturation at 95°C for 3 min, 29 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s, and a final extension at 72°C for 10 min. After amplification, approximately 10 µL of each sample was submitted to horizontal electrophoresis on a 1% agarose gel 1X TAE (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) (8 V/cm^2) buffer. The gels were stained with ethidium bromide (0.5 µg/µL), visualized under ultraviolet light, and photographed with the L-Pix Ex Image photo documentation system (Loccus Biotecnologia, Brazil).

*ina*A gene expression was determined by PCR using the method described by Nejad et al. (2006b) utilizing species-specific primers (INA A upper primer 5'-AGG CTT TGA GAA CGG ACT AAC G-3' / INA A lower primer 5'-TTT CTG TCG GCT GCG TAC TG-3') for the *ina*A gene of *P. ananatis* (syn. *Erwinia ananas*), designed to amplify a DNA sequence of approximately 350-400

bp). The PCR mixture (20 μL) was composed of 2.5 μL 10X buffer, 2.5 μL 2.5 mM dNTP mix, 1 μL (50 mM) $MgCl_2$, 1 μL (5 μM) each primer, 0.5 μL (5 U/μL) Taq DNA polymerase, 1 μL DNA (50 ng), and 10.5 μL H_2O. The mixture was amplified in a thermocycler (Amplitherm TX96) at 94°C for 5 min, followed by thirty-five cycles of heating at 94°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 1 min, and a final extension at 72°C for 5 min. Ten microliters of each of the reaction samples were submitted to horizontal electrophoresis on a 1% agarose gel (with 1X TAE buffer) to confirm the amplification.

The expression of the *ina*A gene phenotype was confirmed by cultivating the isolates in tryptic soy broth (TSB) with 2.5% glycerol (v/v) (Lindow et al., 1978a), and incubating the cultures at 28°C for 24 h. The cultures were homogenized and the concentration of the bacterial suspension was adjusted to approximately 5 x 10^8 colony-forming units/mL in a spectrophotometer by ensuring an optical density at 600 nm (OD_{600}) of 0.3 (cell concentration). Next, 0.1 mL culture was added to assay tubes containing 1 mL sterilized ultrapure water (Milli-Q) and incubated in a -10°C ice bath for 2 min. The instantaneous ice formation in the tube upon addition of the bacterial solution revealed the positive INA phenotype (INA^+). The control consisted of TSB medium with 2.5% bacterium-free glycerol (v/v) in tubes containing sterile ultrapure water. Three replicates were prepared for each isolate.

RESULTS

Ninety *P. ananatis* isolates from MWS lesions were analyzed for the presence of the *ina*A gene, and its expression was characterized *in vitro* (Table 1). DNA fragments (approximately 350-400 bp in length) were obtained following the positive amplification of the *ina*A gene (Figure 1). PCR amplification with INA A primers did not yield any products in only three of the isolates (37, 84, and 87) (Table 1).

The ice nucleation phenotype was expressed in 83.34% of the *ina*A^+ isolates (Table 1). The results clearly indicated that the presence of the *ina*A^+ gene was in itself insufficient for the expression of the ice nucleation phenotype.

DISCUSSION

The INA phenotype is dependent on external factors, such as the age of the bacterium and culture conditions (Lindow et al., 1978b; Wysmierski et al., 2005; Nejad et al., 2006b; Sauer et al., 2014). Another study has also reported a relationship between the number of bacterial cells and ice-nucleation activity (Nejad et al., 2006a). As the size of INA bacterial populations varies among different plant species, sites, climates, and seasons, only a small percentage of cells in a population are effective as ice nucleators (Pearce, 2001). The presence of glycerol in the culture medium is crucial to increase the number of external vesicles in the cellular wall of *P. ananatis* (Sauer et al., 2014). These vesicles contain INP, which are responsible for the formation of ice nuclei (Phelps et al., 1986; Kawahara, 2002). Therefore, in our study, *P. ananatis* was grown on culture medium supplemented with glycerol, in order to induce ice nucleation activity.

The ice formation is not fully understood; moreover, the technique used to visualize the ice formation also has some limitations. In particular, the effects of solutes and mechanical pressure on the kinetics of liquid-to-solid phase transition of aqueous solutions to ice have remained unresolved (Koop et al., 2000).

Table 1. Identification and origin of the isolates, presence and phenotypic expression of the *ina*A gene responsible for the ice nucleation activity in *Pantoea ananatis* from MWS lesions.

Isolates	Origin	gene *ina*A	Phenotype INA$^+$	Isolates	Origin	gene *ina*A	Phenotype INA$^+$
1	Londrina/PR[1]	+	+	46	Warta/PR	+	-
2	Londrina/PR	+	+	47	Warta/PR	+	+
3	Londrina/PR	+	+	48	Warta/PR	+	+
4	Londrina/PR	+	+	49	Warta/PR	+	+
5	Londrina/PR	+	+	50	Rolândia/PR	+	+
6	Londrina/PR	+	+	51	Rolândia/PR	+	+
7	Londrina/PR	+	+	52	Rolândia/PR	+	+
8	Londrina/PR	+	+	53	Rolândia/PR	+	+
9	Londrina/PR	+	+	54	Cafelândia/PR	+	+
10	Londrina/PR	+	+	55	Cafelândia/PR	+	-
11	Londrina/PR	+	+	56	Cafelândia/PR	+	+
12	Londrina/PR	+	+	57	Cafelândia/PR	+	+
13	Londrina/PR	+	+	58	Sete Lagoas/MG[2]	+	+
14	Pinheirinho/PR	+	+	59	Sete Lagoas/MG	+	+
15	Pinheirinho/PR	+	+	60	Sete Lagoas/MG	+	+
16	Pinheirinho/PR	+	+	61	Sete Lagoas/MG	+	+
17	Pinheirinho/PR	+	+	62	Sete Lagoas/MG	+	-
18	Pinheirinho/PR	+	+	63	Sete Lagoas/MG	+	-
19	Pinheirinho/PR	+	+	64	Sete Lagoas/MG	+	+
20	Pinheirinho/PR	+	+	65	Uberlândia/MG	+	-
21	Campo Mourão/PR	+	+	66	Uberlândia/MG	+	-
22	Campo Mourão/PR	+	+	67	Ribeirão do Sul/SP[3]	+	+
23	Campo Mourão/PR	+	+	68	Ribeirão do Sul/SP	+	-
24	Campo Mourão/PR	+	+	69	Cândido Mota/SP	+	+
25	Campo Mourão/PR	+	+	70	Cândido Mota/SP	+	+
26	Campo Mourão/PR	+	+	71	Palmeirinha/PR	+	+
27	Campo Mourão/PR	+	+	72	Cascavel/PR	+	+
28	Campo Mourão/PR	+	+	73	Assis Chataubriand/PR	+	+
29	Campo Mourão/PR	+	+	74	Sertanópolis/PR	+	+
30	Campo Mourão/PR	+	+	75	Pitangueiras/PR	+	+
31	Campo Mourão/PR	+	+	76	Toledo/PR	+	+
32	Campo Mourão/PR	+	+	77	Juranda/PR	+	+
33	Campo Mourão/PR	+	+	78	Engenheiro Beltrão/PR	+	+
34	Campo Mourão/PR	+	+	79	São Miguel do Iguaçu/PR	+	+
35	Campo Mourão/PR	+	+	80	Peabiru/PR	+	-
36	Campo Mourão/PR	+	+	81	Ponta Grossa/PR	+	-
37	Campo Mourão/PR	-	-	82	Tamarana/PR	+	+
38	Campo Mourão/PR	+	+	83	II Centenário/PR	+	+
39	Campo Mourão/PR	+	-	84	Guaíra/PR	-	-
40	Campo Mourão/PR	+	-	85	Morrinhos/GO[4]	+	+
41	Campo Mourão/PR	+	+	86	Planaltinha de Goiás/GO	+	+
42	Campo Mourão/PR	+	+	87	São João da Aliança/GO	-	-
43	Campo Mourão/PR	+	-	88	Dourados/MT[5]	+	-
44	Campo Mourão/PR	+	+	89	Maracaju/MT	+	-
45	Campo Mourão/PR	+	+	90	Douradina/MT	+	-

[1]Paraná State, [2]Minas Gerais State, [3]São Paulo State, [4]Goiás, and [5]Mato Grosso State.

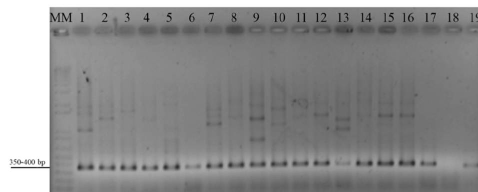

Figure 1. Agarose gel at 1% illustrating the product of genomic DNA amplification through polymerase chain reaction (PCR) with an *inaA* primer for *Pantoea ananatis* (*Pa*). *Lane MM* - Molecular marker of 1 kb plus DNA ladder Invitrogen. *Lanes 1* to *17* represent different *Pa* isolates of a total of 90 isolates and *lanes 18* and *19* are negative (mix) and positive control (WT2 isolate), respectively.

The ice nucleation proteins form aggregates of several sizes on the bacterial outer membrane. The larger the aggregate, the more efficient the ice nucleus (Hirano and Upper, 2000).

Ice nucleation has been used as a tool to differentiate strains among some pathovars of *P. syringe* (Jones et al., 1983; Hirano and Upper, 2000). Strains of *P. syringe* pv. *syringae* are generally INA$^+$, whereas *P. syringe* pv. *tomato* or pv. *morsprunorum* strains show no active ice nucleation. However, not all cells of INA$^+$ strains are active in the ice nucleation process at a specific time and temperature. Therefore, this feature displays considerable phenotypic variability (Lindow et al., 1982b; Lindow, 1983).

Edwards et al. (1994) also verified that the *ina* gene can be present in some strains of some species and absent in others, indicating dimorphism and uncommon evolution, probably caused by a horizontal gene transfer during the evolution of bacterial *ina* genes. Hybridization experiments utilizing DNA fragments flanking the *ina* gene indicated that the genotypic dimorphism of *ina* may be considered as an anomalous situation. One 800-bp fragment that flanked the final 3'-sequence of the *ina* gene hybridized the DNA of all *X. campestris* pathovars, even though the *ina* gene was present in only 50% of these pathovars. It is interesting to note that this flanking sequence in the final 3'-region was found in all the *ina*$^+$ gene, as well as in the *ina*$^-$ gene, isolates of *E. herbicola*, *P. syringae*, and *P. fluorescens* (Edwards et al., 1994). Based on these findings, we propose that the bacterial isolates used in this study may have undergone DNA mutation, resulting in alteration of gene expression or INP activity. Complete sequencing of the *ina*A gene of INA$^+$ and INA$^-$ phenotypes used in this study could help identify a solution to this problem.

The ice nucleation caused by bacteria increase the probability of lesion onset in plants. According to Sakai and Larcher (1987), the water inside the plant tissues does not undergo intra- or extra-cellular freezing, even at -12°C. However, in the presence of INA$^+$ bacteria, water freezes at temperatures slightly below 0°C (Lindow, 1987). The number of bacterial cells within an active population increases during ice nucleation (as the temperature decreases). According to Burke et al. (1976), the formation of intra- and extra-cellular ice crystals causes a mechanical rupture of cell membranes. Thus, tissue cryoinjuries would cause serious plant damage and yield loss.

As a majority of the cells within an INA$^+$ bacterial population are not active at specific times and temperatures, the probability of one or more cells becoming active at relatively high temperatures is highly dependent on the number of cells in the bacterial population. Therefore, the number of lesions caused at a specific temperature can be augmented by the size of the bacterial population in the phyllosphere (Hirano and Upper, 2000).

Our study showed that ice nucleation of *P. ananatis* isolated from MWS depends on the presence of the *ina* gene in the bacterial genome. A comparison of these results with those reported in the literature confirmed that phenotype expression is determined by the environmental and physiological factors such as the age of bacterial culture, temperature, composition of the medium during growth, and plant and bacterial genotypes, in addition to the presence of the *ina*A gene in the cell (Lindow et al., 1978b; Nejad et al., 2006b; Sauer et al., 2014).

Based on these findings, we propose that the bacterial isolates can have undergone DNA mutations resulting in alteration of gene expression or INP activity. Complete sequencing of the *ina*A gene of INA$^+$ and INA$^-$ phenotypes could help validate this theory.

Conflicts of interest

The authors declare no conflict of interest.

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REFERENCES

Abe K, Watabe S, Emori Y, Watanabe M, et al. (1989). An ice nucleation active gene of Erwinia ananas. Sequence similarity to those of Pseudomonas species and regions required for ice nucleation activity. *FEBS Lett.* 258: 297-300. http://dx.doi.org/10.1016/0014-5793(89)81678-3

Alippi AM and López AC (2010). First report of leaf spot disease of maize caused by *Pantoea ananatis* in Argentina. *Plant Dis.* 94: 487. http://dx.doi.org/10.1094/PDIS-94-4-0487A

Ausubel FM, Brent R, Kingston RE, Moore DD, et al. (1992). Current protocols in molecular biology. 2st edn. Greene Publishing Association and Wiley-Interscience, New York.

Burke MJ, Gusta LV, Quamme HA, Weiser CJ, et al. (1976). Freezing and injury in Plants. *Annu. Rev. Plant Physiol.* 27: 507-528. http://dx.doi.org/10.1146/annurev.pp.27.060176.002451

Choi O, Lim JY, Seo YS, Hwang I, et al. (2012). Complete genome sequence of the rice pathogen *Pantoea ananatis* strain PA13. *J. Bacteriol.* 194: 531. http://dx.doi.org/10.1128/JB.06450-11

Costa RV, Cota LV, Silva DD, Figueiredo JEF, et al. (2013). Patogenicidade de *Pantoea ananatis* e *Phaeosphaeria maydis* em plantas de milho. In: Boletim de Pesquisa e Desenvolvimento, Embrapa Milho e Sorgo, Sete Lagoas, 83: 26.

De Maayer P, Chan WY, Blom J, Venter SN, et al. (2012). The large universal Pantoea plasmid LPP-1 plays a major role in biological and ecological diversification. *BMC Genomics* 13: 625-637.

Edwards AR, Van den Bussche RA, Wichman HA and Orser CS (1994). Unusual pattern of bacterial ice nucleation gene evolution. *Mol. Biol. Evol.* 11: 911-920.

Figueiredo JEF and Paccola-Meirelles LD (2012). Simple, rapid and accurate PCR-based detection of *Pantoea ananatis* in maize, sorghum and *Digitaria* sp. *J. Plant Pathol.* 94: 663-667.

Goszczynska T, Botha WJ, Venter SN and Coutinho TA (2007). Isolation and identification of the causal agent of brown stalk rot, a new disease of maize in South Africa. *Plant Dis.* 91: 711-718. http://dx.doi.org/10.1094/PDIS-91-6-0711

Green RL and Warren GJ (1985). Physical and functional repetition in a bacterial ice nucleation gene. *Nature* 317: 645-648. http://dx.doi.org/10.1038/317645a0

Hirano SS and Upper CD (2000). Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae*-a pathogen, ice nucleus, and epiphyte. *Microbiol. Mol. Biol. Rev.* 64: 624-653. http://dx.doi.org/10.1128/MMBR.64.3.624-653.2000

Jones JB, Gitaitis RD and McCarter SM (1983). Evaluation of indirect immunofluorescence and ice nucleation activity as rapid tests for identifying foliar diseases of tomato transplants incited by fluorescent *Pseudomonas. Plant Dis.* 67: 684-687. http://dx.doi.org/10.1094/PD-67-684

Kawahara H (2002). The structures and functions of ice crystal-controlling proteins from bacteria. *J. Biosci. Bioeng.* 94: 492-496. http://dx.doi.org/10.1016/S1389-1723(02)80185-2

Koop T, Luo B, Tsias A and Peter T (2000). Water activity as the determinant for homogeneous ice nucleation in aqueous solutions. *Nature* 406: 611-614. http://dx.doi.org/10.1038/35020537

Kozloff LM, Schofield MA and Lute M (1983). Ice nucleating activity of *Pseudomonas syringae* and *Erwinia herbicola. J. Bacteriol.* 153: 222-231.

Krawczyk K, Kamasa J, Zwolinska A and Pospieszny H (2010). First report of *Pantoea ananatis* associated with leaf spot disease of maize in Poland. *J. Plant Pathol.* 92: 807-811.

Lindow SE (1983). The role of bacterial ice nucleation in frost injury to plants. *Annu. Rev. Phytopathol.* 21: 363-384. http://dx.doi.org/10.1146/annurev.py.21.090183.002051

Lindow SE (1987). Competitive exclusion of epiphytic bacteria by ice *Pseudomonas syringae* mutants. *Appl. Environ. Microbiol.* 53: 2520-2527.

Lindow SE, Arny DC and Upper CD (1978a). *Erwinia herbicola* - bacterial ice nucleus active in increasing frost injury to corn. *Phytopathology* 68: 523-527. http://dx.doi.org/10.1094/Phyto-68-523

Lindow SE, Arny DC, Upper CD and Barchet WR (1978b). The role of bacterial ice nuclei in frost injury to sensitive plants. In: Plant cold hardiness and freezing stress-mechanisms and crop implications (Li PH and Sakai A., eds.). Academic Press Inc, New York, 249-263.

Lindow SE, Arny DC and Upper CD (1982a). Bacterial ice nucleation: a factor in frost injury to plants. *Plant Physiol.* 70: 1084-1089. http://dx.doi.org/10.1104/pp.70.4.1084

Lindow SE, Hirano SS, Barchet WR, Arny DC, et al. (1982b). Relationship between ice nucleation frequency of bacteria and frost injury. *Plant Physiol.* 70: 1090-1093. http://dx.doi.org/10.1104/pp.70.4.1090

Lindow SE, Lahue E, Govindarajan AG, Panopoulos NJ, et al. (1989). Localization of ice nucleation activity and the iceC gene product in *Pseudomonas syringae* and *Escherichia coli. Mol. Plant Microbe Interact.* 2: 262-272. http://dx.doi.org/10.1094/MPMI-2-262

Michigami Y, Watabe S, Abe K, Obata H, et al. (1994). Cloning and sequencing of an ice nucleation active gene of *Erwinia uredovora. Biosci. Biotechnol. Biochem.* 58: 762-764. http://dx.doi.org/10.1271/bbb.58.762

Nejad P, Granhall U and Ramstedt M (2006a). Factors influencing pathogenic ice-nucleation active (INA) bacteria isolated from *Salix* plants, soil and litter. *J. Agric. Technol.* 1: 207-222.

Nejad P, Ramstedt M, Granhall U, Roos S, et al. (2006b). Biochemical characterization and identification of ice-nucleation-active (INA) willow pathogens by means of BIOLOG® MicroPlate, INA gene primers and PCR-based 16S rRNA-gene. *J. Plant Dis. Prot.* 113: 97-106.

Paccola-Meirelles LD, Ferreira AS, Meirelles WF, Marriel IE, et al. (2001). Detection of a bacterium associated with a leaf spot disease of maize in Brazil. *J. Phytopathol.* 149: 275-279. http://dx.doi.org/10.1046/j.1439-0434.2001.00614.x

Pearce RS (2001). Plant freezing and damage. *Ann. Bot. (Lond.)* 87: 417-424. http://dx.doi.org/10.1006/anbo.2000.1352

Pérez-y-Terrón R, Cuellan A, Muñoz-Rojas J, Castañeda-Lucio M, et al. (2009). Detection of *Pantoea ananatis*, causal agent of leaf spots disease of maize, in Mexico. *Australas. Plant Dis. Notes* 4: 96-99.

Phelps P, Giddings TH, Prochoda M and Fall R (1986). Release of cell-free ice nuclei by Erwinia herbicola. *J. Bacteriol.* 167: 496-502.

Orser C, Staskawicz BJ, Loper J, Panopoulos NJ, et al. (1983). Cloning of genes involved in bacterial ice nucleation and fluorescent pigment/siderophore production. In: Molecular Genetics of the Bacteria-Plant Interaction (Puhler A, eds.). Springer-Verlag, Berlin, 353-361.

Sakai A and Larcher W (1987). Frost survival of plants. Springer, Berlin.

Sauer AV, Rocha KR, Pedro ES, Meirelles, et al. (2014). Ice nucleation activity in *Pantoea ananatis* obtained from maize white spot lesions. *Semina Cienc. Agrar* 35: 1659-1666.

Sauer AV, Rocha KR, Gonçalves RM, Meirelles WF, et al. (2015). Survival of *Pantoea ananatis*, causal agent of maize white spot disease in crop debrids. *Agron. Sci. Biotech* 1: 21-24.

Warren G and Corotto L (1989). The consensus sequence of ice nucleation proteins from *Erwinia herbicola, Pseudomonas fluorescens* and *Pseudomonas syringae. Gene* 85: 239-242. http://dx.doi.org/10.1016/0378-1119(89)90488-5

Warren G, Corotto L and Wolber P (1986). Conserved repeats in diverged ice nucleation structural genes from two species of *Pseudomonas. Nucleic Acids Res.* 14: 8047-8060. http://dx.doi.org/10.1093/nar/14.20.8047

Warren GJ (1995). Identification and analysis of *ina* genes and proteins. In: Biological ice nucleation and its applications (Lee RE, Warren, GJ, Gusta LV, eds.). APS Press, St. Paul, 85-99.

Wysmierski PT, Escanferla ME and Paccola-Meirelles LD (2005). Atividade de nucleação de gelo e produção de exoenzimas em *Pantoea ananatis*, agente causal da Pinta Branca do Milho. In: Anais do 38º Congresso Brasileiro de Fitopatologia, Brasília.

Zhao JL and Orser CS (1990). Conserved repetition in the ice nucleation gene *ina*X from *Xanthomonas campestris* pv. *translucens. Mol. Gen. Genet.* 223: 163-166. http://dx.doi.org/10.1007/BF00315811