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## Factors influencing pathogenic Ice-Nucleation Active (INA) bacteria isolated from *Salix* plants, soil and litter

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This paper deals with bacterial strains from three independent geographical locations representing different bacterial groups of *Salix* pathogens such as: *Bacillus*, *Clavibacter*, *Erwinia*, *Pseudomonas fluorescens*, *P. syringae*, *Sphingomonas* and *Xanthomonas* species that were collected to investigate the role and effects of growing temperatures, cell numbers, growth-limiting factors (e.g. carbon, nitrogen, phosphorus), and physical or chemical agents on their ice nucleation activity (INA). Methods for measurement of the cell mass for INA involved both direct plate counting and optical density. Ice nucleation temperatures of individual bacterial strains were determined by tube nucleation tests. Mean populations of bacteria per ml needed to cause ice nucleation were observed. For a typical *P. syringae* strain  $10^2$  cells per ml gave a nucleation temperature of  $-4.5^\circ\text{C}$  while  $10^9$  cells per ml initiated freezing already at  $-2.5^\circ\text{C}$ . Mean cell density for a representative *P. fluorescens* were  $10^5$  cells per ml for freezing at  $-5.5^\circ\text{C}$  and  $10^9$  cells per ml for freezing at  $-3.5^\circ\text{C}$ . Expression of ice-nuclei in certain bacterial strains was clearly dependent on growing temperature and nutritional conditions e.g. growth limiting factors. Limitation of C affected nucleation activity of *P. fluorescens*, *Xanthomonas* spp. and to some extent *Erwinia* spp. Except for *P. fluorescens* and *P. syringae*, N-limitation very strongly affected all the tested bacteria and decreased their nucleation activity. Starvation for P affected INA of *Sphingomonas*, whereas *P. syringae* was only slightly affected. In *Bacillus*, P-limitation totally inhibited ice-nucleation activity. Chemical and physical agents decreased but did not directly or completely inhibit the ice-nucleation activity of harvested cells as freezing still occurred but at lower temperatures. From this we hypothesize that initiation of freezing at higher temperatures (e.g.  $-2^\circ\text{C}$ ) is closely connected with cells that are physically and functionally intact.

### Introduction

Freezing is a major environmental stress, imposing economical damage on crops and limiting the spread of both wild and crop species in sizeable parts of the world. Damage to woody plants resulting from ice nucleation of bacteria and frost injury has been reported from several places (de Kam, 1978;

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Ramstedt *et al.*, 1994; Pearce, 2001; Nejad *et al.*, 2002, 2004). Plants and plant parts freeze when they cannot avoid nucleation and prevent the growth of ice (Pearce, 2001). Substances in nature that can act as heterogeneous nucleators include not only Ice Nucleation-Active (INA) bacteria and other biological molecules and structures, but also inorganic debris (Pearce, 2001).

The population size of ice<sup>+</sup> bacteria on frost sensitive plants, and also their development as disease agents often varies dramatically with time (Lindow, 1982, 1995; Cambours *et al.*, 2005). Physical and environmental factors greatly influence the population size on leaf surfaces and generally population tends to increase with age of the leaf (Lindow, 1995). For *P. syringae* and *E. herbicola* less than 100 cells per gram of plant leaves could be found during summer in absence of rainfall, but populations might escalate by 100 to 1000-fold during winter and spring months (Lindow, 1982). Maximum populations coincide with the period of maximum likelihood of frost damage (Constantinidou *et al.*, 1991).

The incidence of frost damage to sensitive plant species and the supercooling potential of different plant parts is proportional to the logarithm of the population size of INA-bacteria on the plants at the time of freezing (Lindow, 1982; Lindow *et al.*, 1982a,b; Gross *et al.*, 1984; Hirano and Upper, 1986; Lindow, 1993). The mean supercooling point for bean leaves harbouring about 1000 *P. syringae* cells per gram was found to be about  $-3.5^{\circ}\text{C}$ , whereas leaves harbouring  $10^6$  cells per gram supercooled only to about  $-2^{\circ}\text{C}$  (Lindow, 1993).

As reported earlier most of the populations of INA bacteria found on plants (epiphytic) comprise strains of several species, for example *P. syringae*, (Lindow, 1982; Ramstedt *et al.*, 1994; Nejad *et al.*, 2002, 2004), or *E. herbicola* (Dye, 1957), and a wide range of INA bacteria have also been found as endophytic (within plant tissues) populations in *Salix* plants (Nejad *et al.*, 2002, 2004; Cambours *et al.*, 2005). The role of these INA bacteria as agents for frost injury of sensitive plants has been well documented (Arny *et al.*, 1976; Anderson *et al.*, 1982) and in a recent study we have reported that the presence of INA bacteria as *Bacillus*, *Erwinia*, *P. fluorescens*, *P. syringae*, *Sphingomonas*, *Xanthomonas* and other related species play a major role in frost damage on willows (Ramstedt *et al.*, 1994; Nejad *et al.*, 2002, 2004; Cambours *et al.*, 2005). Some strains of *P. fluorescens* biotypes A, C, B, G and F (Maki and Willoughby, 1978; Gross *et al.*, 1983; Nejad *et al.*, 2002, 2004), and *P. viridiflava* (Paulin and Luisetti, 1978; Nejad *et al.*, 2003; Cambours *et al.*, 2005) have also been shown to cause damage to plants in combination with frost.

The INA-properties of certain INA strains seem to be expressed only after cultivation at particular temperatures (Gross *et al.*, 1983). Another source of variability is according to Hirano *et al.* (1985) attributed to physiological responses of the ice nucleation active bacteria to nutritional and environmental signals, although the mechanisms behind this are unknown. According to Nemecek-Marshall *et al.* (1993) lack of nutrients can cause premature or delay in INA.

The objectives of this study were to determine the temperature, at which ice nucleation will occur by individual bacterial strains isolated from willow plantations (stems, soil and litter), estimate the population size of INA bacteria required, the effect of growth limiting factors, and chemical and physical agents for disruption or inhibition of INA among individual bacterial strains.

## **Materials and methods**

### ***Bacterial isolates***

The bacterial strains used in this study, were isolated from stems as well as soil and litter of diseased *Salix* plants in three different geographical locations i.e. at two experimental sites in central Sweden (Uppsala and Brunnby) and one at Saare in Estonia. Further description of the sites and grouping of the bacteria are given in Cambours (2004) and Cambours *et al.* (2005). Isolates were selected on basis of their group identity and ice nucleation activity at higher or lower temperatures. The bacteria have been identified on the basis of their bacteriological characteristics on different agar media, by BIOLOG<sup>®</sup> MicroPlate, and molecular techniques (Nejad *et al.*, 2003, 2004).

### ***INA-test***

Ice-nucleation activity of the isolates was recorded after incubation at different conditions regarding temperature, nutrients and possible inhibitors (cf. below). The sample buffer (Pb), MgSO<sub>4</sub> (Mg) and Lauria Broth (Lb) were used as controls regarding their ice nucleation temperature in all experiments. The ice nucleation activity for each isolate was determined by ice formation in test tubes with buffer as earlier described (Nejad *et al.*, 2004). A bacterial cell suspension ( $>10^8$  colony-forming units per ml (CFU/ml) was prepared and adjusted in sterile phosphate buffer, (Pb; pH 7, 0.1M) and ten-fold serial dilutions in sterilised liquid TSB were prepared from original bacterial suspensions to find the proper cell concentration for INA tests. In the study

reported, 100 µl of bacterial suspensions with a concentration of INA  $10^7$  CFU ml<sup>-1</sup> were added to 9 ml of the phosphate test-buffer and the freezing temperature was recorded.

#### ***Effect of growth temperature on Ice Nucleation Activity.***

Selected bacterial isolates (cf. above) were taken from deep frozen cultures and revived on TSA for a period of 24-48 hours at room temperature (25°C). The bacteria were routinely cultured on Tryptic Soy Agar (TSA; 10 gram Tryptic Soy Broth, Difco Ltd., and 12 gram Technical Agar, Oxoid Ltd in 1000 ml distilled water). A known reference culture (*P. syringae* pv. *syringae*, V1D1) from the Department of Plant Pathology and Biocontrol Unit, SLU and an isolate of *Xanthomonas populi* ssp. *salicis* (strain 3624 from NCPPB Central Science Labs UK) were also included. The 23 bacterial cultures were grown on TSA at the temperatures 2, 4, 10, 15, 20 and 25°C between 1 to 5 days depending on growth rate. The ability of bacterial strains to grow at the above-mentioned temperatures was checked regularly. Only clearly growing colonies were used for subsequent tests for INA and freezing temperature.

#### ***Effect of growth limiting factors on Ice Nucleation Activity***

Six different bacterial isolates (Table 3) were individually cultured from deep-frozen vials on Tryptic Soy Agar (TSA) at 25°C for 24-48 hours. The media used for growth limiting factors has been described by Sikyta *et al.* (2000). A single colony of each bacterial isolate was then cultured in a 100-ml Erlenmeyer flask, containing either 50 ml of TSB (control) or growth nutrient starvation media for C, N and P, and incubated in liquid culture on a shaker at 120 rpm at room temperature. Enumeration of viable bacterial cells was performed every 24 hours for 6 days, using a spectrophotometer to measure optical density (OD) at 600 nm. Results were verified by visible cell counts after plating on agar plates for 24-48 hours at 25°C. Bacterial samples for INA-test were taken after 72 hours of growth limiting treatment.

#### ***Effect of physical and chemical agents on Ice Nucleation Activity***

To determine the effect of physical agents on the INA activity, suspensions of a typical bacterium, isolated within internal tissue of *Salix* stems (*P. syringae*, strain 229), was subjected to heat treatment for 10 min at 65°C (water bath instrument, Grant Labassco) to disrupt the cells. Effect of chemical agents (antibiotics and dyes, Fig. 3) on the INA activity, were examined by

adding known concentrations of the agents to the bacterial cultures. Correlation of ice nucleation activity and presence of bacterial cells, were recorded after filtration of bacterial suspensions through membrane filters (filtropur SARSTEDT 0.20  $\mu\text{m}$  and 0.45  $\mu\text{m}$ ) or centrifugation at  $15000 \times g$  for 10 min in a Sorvall RC-5B centrifuge. Bacterial cell preparations and supernatants/filtrate were tested separately for INA.

## Results

### *Effect of growth temperature on Ice Nucleation Activity*

In general the growth temperature played a significant role for expression of ice nucleation activity (Tables 1, 2), but the effect was variable among bacterial groups. We also observed variability in INA and optimal growth temperature among isolated bacteria from soil, litter and plant of the same bacterial groups (Table 1).

For bacterial group 2 (Nejad *et al.*, 2004; Cambours *et al.*, 2005), representing *Bacillus* spp, a very high nucleation activity was expressed when grown at 15 and 20°C, while higher or lower incubation temperatures resulted in low or no INA at all. Similar results, with a narrow temperature range, were obtained for group 4 (*Bacillus*) and group 7 (*P. fluorescens*).

Group 8, which represent *Sphingomonas* spp., do not seem to be able to nucleate ice except at lower temperatures, but there are also nucleators active between  $-4$  and  $-6^\circ\text{C}$  after growth at 15 to 25°C (Table 1). For *P. syringae* (group 11) ice nuclei formation does not seem to be dependent on previous growth temperatures. Two of the *P. syringae* strains (S229 and V1D1) initiated ice formation at  $-2$  to  $-4^\circ\text{C}$  while another *P. syringae* strain (S217) was shown to express INA at a somewhat lower temperature ( $-4.5$  to  $-7^\circ\text{C}$ ) irrespective of growth temperature. Another strain (F202) isolated from *Salix* litter differed from the other group 11 members (*P. syringae*-types) in ability to express INA genes only after growth at 20°C. Similar results were obtained concerning the *Xanthomonas* group (group 10) i.e. one indifferent to temperature variations whilst the other three had very low INA or only expressed it after growth at a certain temperature (F67, Table 1). Also *Erwinia* spp (group 12) were indifferent to growth temperature showing INA between  $-3$  to  $-7.5^\circ\text{C}$  or  $-8$  to  $-10^\circ\text{C}$  as presented in Table 1.

The percentage of bacterial isolates for different bacterial groups showing INA at specific temperatures is presented in Table 2. Some bacterial genera did not grow within 1-2 days of incubation and additional time (up to 5 days) for INA, especially for lower temperatures, was sometimes required.



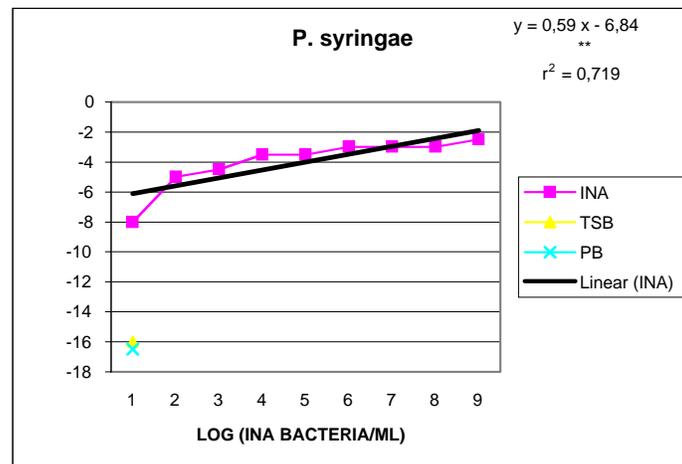
An ideal growing temperature for selected bacterial strains, to be able to freeze between  $-2$  to  $-5^{\circ}\text{C}$ , was mostly  $15^{\circ}\text{C}$  (48%) or  $20^{\circ}\text{C}$  (52%).

**Table 2.** Percentage of frozen bacterial strains (from Table 1) at different freezing temperature intervals.

Freezing temperature interval	Growth					
	2*	4*	10*	15	20	25
-2 to -5	21	25	25	48	52	22
<-5 to -7	4	0	0	13	4	12
<-7 to -16	34	37	30	17	27	61
Not frozen at -16	37	34	41	22	17	5

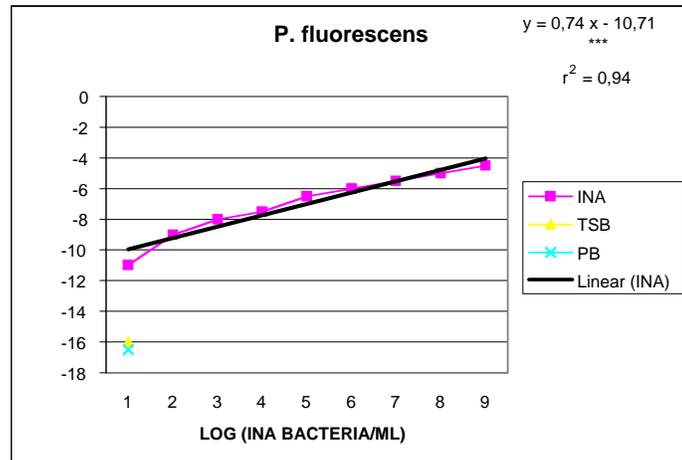
\*Four percent of the bacterial isolates did not grow on the TSA agar plate.

The relationships between bacterial population density and nucleation temperatures are demonstrated for two bacterial strains in Figs 1-2. Mean populations of INA bacteria per ml for *P. syringae* was  $10^2$  with nucleation temperature of  $-4.5^{\circ}\text{C}$  and  $10^9$  at  $-2.5^{\circ}\text{C}$  respectively and mean populations for *P. fluorescens* was  $10^5$  to freeze at  $-5.5^{\circ}\text{C}$  and  $>10^9$  to freeze at  $-3.5^{\circ}\text{C}$ . These results show that nucleation temperature is highly dependent on bacterial cell density. The cell density required for nucleation at  $-2.5$  and  $-5^{\circ}\text{C}$  for most other INA bacteria ranged from  $10^5$  to  $10^2$  CFU  $\text{ml}^{-1}$  as determined by measuring optical density.



**Fig. 1.** Dependence of Ice Nucleation Activity (INA) on cell concentration of *P. syringae* (S229) isolated from *Salix* plants. TSB = Tryptic Soy Broth, PB = Phosphate buffer.

Our results indicate that some bacteria have constitutive INA properties (certain *P. syringae*, *Xanthomonas* and *Erwinia spp.*) whereas others (*Bacillus*, *P. fluorescens* and *Sphingomonas spp.*) have inducible INA properties. Thus certain bacterial species require certain temperatures to express the ice gene.



**Fig. 2.** Dependence of ice nucleation activity on cell concentration of *P. fluorescens* isolated from *Salix* plants

### ***Effect of growth limiting factors on Ice Nucleation Activity***

Nutrient limitation (C, N and P) affected both the growth of bacteria and ice nucleation activity after 72 hours of incubation (Table 3) but the effect varied among bacterial genera. Carbon limitation lowered nucleation activity of *P. fluorescens*, *Xanthomonas spp.* and to some extent *Erwinia spp.* Nitrogen limitation affected all groups very strongly, and decreased their INA, except for *P. fluorescens* and *P. syringae* which were only slightly affected (Table 3). In *Bacillus* P limitation totally stopped ice nucleation activity. It had strong negative effect on *Sphingomonas*, whilst *P. syringae* was only slightly affected. In both *Erwinia* and *Xanthomonas* there were on the contrary a slight stimulation with P-limitation.

**Table 3.** Effect of growth limiting factors on growth and ice-nucleation activity of bacteria after 72 hours incubation in terms of optical density

Bacteria*	Strain	Medium	OD	INA (°C)
<i>Bacillus</i> sp.	(S294)	TSB	1.720	-9.5
		CL	0.830	-9.5
		NL	0.750	-12
		PL	1.270	nf
<i>Erwinia</i> sp.	(S113)	TSB	1.820	-7
		CL	0.412	-8.5
		NL	0.412	-13
		PL	1.060	-5.5
<i>P. fluorescens</i>	(M103)	TSB	1.600	-4.5
		CL	0.820	-7
		NL	0.660	-5
		PL	0.790	-5
<i>P. syringae</i>	(S229)	TSB	1.700	-2.5
		CL	0.500	-3
		NL	0.920	-3.5
		PL	1.255	-4
<i>Sphingomonas yanoikuyae</i>	(E200)	TSB	1.920	-6.5
		CL	0.500	-5.5
		NL	0.520	-13.5
		PL	1.450	-8
<i>Xanthomonas campestris</i>	(S117)	TSB	1.730	-5
		CL	0.480	-9
		NL	1.070	-7
		PL	1.250	-4

\* all strains are isolated from *Salix* stems

TSB as control froze at -16.5°C and phosphate buffer at -16°C

CL= carbon limitation, NL = nitrogen limitation, PL = phosphorus limitation.

nf = not frozen

Probably many INA bacteria (in our study 88%) under limiting conditions could regain growth after a period of stagnation, indicating an alteration in metabolism. For example, the OD values for *P. fluorescens* with N starvation after 24 and 48 hours were 0.835 and 0.920 respectively, but the day after the OD was 0.660, which indicates diminishing cell density. The subsequent days, however, it increased and at 144 hours the OD was found to be 1.250, a sign of increasing cell populations. These variations were not true for the bacterial isolates while grown in TSB without nutrient limitation.



***Effect of physical and chemical agents on Ice Nucleation Activity***

Fig. 3, demonstrates decreasing INA in *P. syringae* (S 229) after different physical and chemical treatments, which is likely to depend on the disruptions of membranes. In the physical experiment we observed that heat treatment at 65°C for 10 min (Fig. 3) destroyed the INA at higher temperatures, and that the number of cells decreased but that their INA at lower temperatures partly remained.

The number of cells was reduced between 3 to 10% by most chemicals when added to concentrated cells. Highest reduction of cell number was achieved by streptomycin (16%), penicillin (30%), methylene blue (46%) and heat treatment (36%). Heat treatment and methylene blue not only reduced the number of bacterial cells but also reduced INA most significantly (Fig. 3).

Additionally we found that centrifugation of bacterial suspensions for 15 min at 15000 × g or filtration of cultures through a 0.20 -µm membrane filter separated the activity from the supernatant. Thus most of the ice nucleation activity was associated with the cells (Table 4).

**Table 4.** Effect of cell filtration and centrifugation on ice-nucleating active bacterium (*P. syringae*, strain S229) from *Salix* plants

<b>Treatment</b>	<b>INA temperature (°C)</b>
Before centrifuging*	-2.5
Centrifuged (supernatant) <sup>a</sup>	-7.0
Centrifuged (pellet) <sup>a</sup>	-2.5
Before filtering*	-2.6
Filtrate <sup>b</sup>	-8.5
Resuspended cells	-2.8

<sup>a</sup>15000 x g, 10 min

<sup>b</sup>0.20µm membrane filter (Non Pyrogenci, Millipore).

\* Control

**Discussion**

According to Lindow (1982) bacterial ice nucleation frequency is dependent on the conditions under which the cells are grown, including not only temperature but also composition of the medium during growth. In our experiments any set of growth conditions changed the ice nucleation frequency for many bacterial strains. In conformity with Lindow (1982) and Hirano *et al.* (1985) we have also observed that there is a relationship between number of bacterial cells and ice-nucleation activity, i.e. a certain population density is required for INA to operate.

The INA expression has, however, been attributed not only to the population density but also to the ratio between the number of ice nuclei and the number of bacterial cells in culture (Hirano *et al.*, 1985), which can vary with incubation temperature and growth medium composition. It means that the ice-nucleation activity varies among individual bacteria, and that not all bacterial cells in a culture by necessity have an ice-nucleation site. For example, Maki *et al.* (1974) found that only 1 in 1000 *P. syringae* cells had active ice-nucleation sites and for other bacterial species it can be one in ten- to hundred.

The present study shows that many INA bacteria found on willows are dependent on temperature fluctuations to express INA. In field conditions a high temperature during daytime, stimulating growth, for some bacteria also activates INA-properties which could further damage the host when the night temperature falls below zero. The most serious pathogens on *Salix*, as e.g. *P. syringae*, were however independent of temperature for initiation and will express INA constitutively at any temperature. There is thus a probability that at any specific occasion and temperature, different individual cells of INA bacteria may express an active ice nucleus. Our results (Table 2) can also be used to define three classes of bacterial ice nuclei, in keeping with Yankofsky *et al.* (1981): a) Nuclei, active at temperatures between  $-2^{\circ}\text{C}$  and  $-5^{\circ}\text{C}$ , b) nuclei active between  $-5$  and  $-7^{\circ}\text{C}$  and c) nuclei, active below  $-7^{\circ}\text{C}$ .

Although our laboratory experiments may not replace field scale experiments with regard to forest nutrition management, it gives a denotation of how several important factors e.g. nutrient compositions, temperature, site, seasonal variation, physical and chemicals can affect INA bacteria. In this study we put emphasis on the importance of growth and nutrients for the regulation and expression of the INA gene.

Our results show that nutrient limitation in most cases partly or totally inhibited INA or in a few cases did not affect it at all. At C starvation growth was affected (compared to the control medium TSB), but the nucleation activity was not affected significantly, except for *P. fluorescens* and *Xanthomonas* where INA were less expressed. In *P. syringae* and *P. fluorescens* N limitation alone affected the INA only to a minor extent, but showed high impact on INA properties of *Erwinia*, *Xanthomonas*, *Sphingomonas* and *Bacillus* spp. where it lowered the nucleation temperature with 2 -  $7^{\circ}\text{C}$ . P limitation totally stopped INA activity of *Bacillus*, lowered the action of *Sphingomonas* but instead slightly improved nucleation activity of *Xanthomonas* and *Erwinia*.

We could also demonstrate that the presence of intact cells or cell walls from the bacteria were necessary to affect ice nucleation after activation of the

bacteria which is supported by Phelps *et al.* (1986) that detected cell-free ice nuclei in the growth medium after centrifuging at  $200,000 \times g$  (concentrating ice nuclei). According to Lindow *et al.* (1989), ice nucleation is sensitive to agents such as lipases, phospholipases, detergents and solvents that can disrupt membranes. Warren and Wolber (1991) denote a unique outer membrane protein with highly repetitive amino acid sequence to be responsible for the ice nucleation at relatively high temperatures. The nature of ice nuclei sites of such cells has been studied by Maki and co-workers (Maki *et al.*, 1974; Maki and Willoughby, 1978). They reported that much of the INA of *P. fluorescens* was lost upon sonic disruptions of the cells and that the remaining activity was associated with the cell wall. This is in keeping with our results with separation of cells or cell walls from the culture filtrate.

Treatment of cells with antibiotics or dyes did not generally seem to cause rapid termination of INA in the most active strains, a finding also supported by several other authors (Lindow *et al.*, 1982b; Anderson and Ashworth, 1986). Some chemicals are effective in reducing INA as we found in laboratory conditions but how they work in nature is yet to be clarified. While spectinomycin has been reported to cause rapid reduction in ice nucleation activity at higher temperatures, its effect may also be dependent on the environmental conditions during or after such treatment (Anderson and Ashworth, 1986; Lindow *et al.*, 1989).

To formulate proper usage of e.g. chemical applications to make damage caused by INA bacteria insignificant, more trials of this kind are required. Field studies are though difficult to perform, since the exact occurrence of freezing temperatures is hard to anticipate in advance. Farmers also often have such large acreage of vulnerable crops that are susceptible to frost attack that bactericides could not be applied in short time between frost prediction and frost event (Lindow *et al.*, 1989). This certainly applies to willows (*Salix* spp.) and poplars in energy forest plantations.

Plant damage in the field varies significantly between different *Salix* clones. This could be due to variation in resistance to bacteria, frost-tolerance or different levels of epiphytic bacterial populations. In preliminary tests we have shown that electrolytic leakage differs naturally between clones of willows (Tsarouhas, 2002; Granhall *et al.*, unpublished). Cellular leakage means that nutrients are secreted and could lead to increase of certain epiphytic bacterial populations and cell multiplication. This selective growth is likely to be one explanation for the significant variation in bacterial populations and damage registered on different *Salix* clones.

One alternative to chemical control of diseases are breeding or selection of plants that do not support the growth of INA bacteria, e.g. by favouring a

competing epiphytic flora (Lindow, 1982) or by low excretion of electrolytes (Tsarouhas, 2002; Granhall *et al.*, unpublished) keeping all bacterial populations at a low level. Epiphytic INA populations (Cambours *et al.*, 2005) can constitute an inoculum source and be essential for the development of infection when the conditions are suitable and temperature falls slightly below zero. Also, as stated in our earlier work (Nejad and Johnson, 2000; Nejad *et al.*, 2004), endophytically resident bacteria may strategically be at the right place without affecting the plant, building up its forces for a successful attack when the population density becomes high enough, maybe by involvement of quorum sensing (QS) known from several other bacterial species. Quorum Sensing is a system known to be active in several regulatory mechanisms in many bacteria, e.g. pathogenicity, but its possible role in INA regulation has yet to be elucidated. In our preliminary tests using three different methods 9 out of 20 isolates with nucleation activity and pathogenicity were found to communicate using QS signals.

## Conclusions

1. Ice-nucleation ability of certain bacterial strains is dependent both on nutritional conditions and temperature.
2. The growth temperature and cell density affect INA differently in different *Salix* pathogens.
3. Some bacteria have constitutive, some inducible INA.
4. C, N and P limitation affected INA strains to different extent, generally by lowering the activity.
5. The most active INA-bacterium, *P. syringae*, was most indifferent to nutritional conditions.
6. Heat treatment and methylene blue were the most effective treatments for inhibition of INA.
7. INA at higher temperatures (e.g. -2°C) is closely correlated with physically and functionally intact cells.

In our study we concentrated on bacteria isolated from frost damaged *Salix* plantations. Further studies should be performed to find out the variation in INA activity between isolates of the same bacterial species but of different host origin.

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## References

- Anderson, J.A. and Ashworth, E.N. (1986). The effects of streptomycin, desiccation, and UV radiation on ice-nucleation by *Pseudomonas viridiflava*. *Plant Physiology* 80: 956-960.
- Anderson, J.A., Buchanan, D.W., Stall, R.E. and Hall, C.B. (1982). Frost injury of tender plants increased by *Pseudomonas syringae* van Hall. *Journal of the American Society for Horticultural Science* 107: 123-125.
- Army, D.C., Lindow, S.E. and Upper, C.D. (1976). Frost sensitivity of *Zea mays* increased by application of *Pseudomonas syringae*. *Nature* 262: 282-284.
- Cambours, M.A. (2004). Ice nucleation-active and pathogenic bacteria in Swedish and Estonian *Salix* short-rotation forestry plantations suffering from frost-related dieback. Licentiate thesis, Swedish University of Agricultural Sciences, Uppsala, Sweden.
- Cambours, M.A., Nejad, P., Granhall, U. and Ramstedt, M. (2005). Frost related dieback of willows, comparison of epiphytic and endophytic bacteria of different *Salix* clones, with emphasis on ice- nucleation activity, pathogenic properties and seasonal variation. *Biomass and Bioenergy* 28: 15-27.
- Constantinidou, H.A., Hirano, S.S., Baker, L.S. and Upper, C.D. (1991). Atmospheric dispersal of ice nucleation-active bacteria: the role of rain. *Phytopathology* 80: 934-937.
- De Kam, M., (1978). *Xanthomonas populi* subs. *salicis*, cause of bacterial canker in *Salix dasyclada*. *European Journal of Forest Pathology* 8:, 334-337.
- Dye, D.W. (1957). The effect of temperature on infection by *Pseudomonas syringae*, van Hall. *New Zealand Journal of Scientific Technology* 38: 500-505.
- Gross, D.C., Cody, Y.S., Proebsting, E.L., Jr., Radamaker, J.K. and Spotts, R.A. (1983). Distribution, population dynamics of ice nucleation-active bacteria in deciduous fruit tree orchards. *Applied and Environmental Microbiology* 46: 1370-1379.
- Gross, D.C., Proebsting, E.L., Jr. and Andrews, P.K. (1984). The effects of ice nucleation-active bacteria on the temperatures of ice nucleation and low temperature susceptibilities of *Prunus* flower buds at various stages of development. *Journal of American Society* 109: 375-380.
- Hirano, S.S., Baker, L.S. and Upper, C.D. (1985). Ice nucleation temperature of individual leaves in relation to population sizes of ice nucleation active bacteria and frost injury. *Plant Physiology* 77: 259-265.
- Hirano, S.S. and Upper, C.D. (1986). Temporal, spatial and genetic variability of leaf associated bacterial populations. In: *Microbiology of the Phyllosphere* (eds. N.J. Follema and V.D. Del Heuve). Cambridge University Press, London: 235-251.
- Lindow, S.E., (1982). Population dynamics of epiphytic ice nucleation active bacteria on frost sensitive plants and frost control by means of antagonistic bacteria. In: *Plant Cold Hardiness and Freezing stress-Mechanisms and Crop implications* (eds. P.H. Li, and A. Sakai). Academic Press, New York: 395-416.
- Lindow, S.E., (1993). Novel method for identifying bacterial mutants with reduced epiphytic fitness. *Applied Environmental Microbiology* 59: 1586-1592.
- Lindow, S.E., (1995). Control of epiphytic ice nucleation active bacteria for management of plant frost injury. In: *Biological Ice Nucleation and its Application* (eds. R.E. Lee, G.J. Warren and L.V. Gusta): 239-256.
- Lindow, S.E., Army, D.C. and Upper, C.D. (1982a). Bacterial ice nucleation: A frost injury to plants. *Plant Physiology* 70: 1084-1089.
- Lindow, S.E., Army, D.C. and Upper, C.D. (1982b). The relationship between ice nucleation frequency of bacteria and frost injury. *Plant Physiology* 70: 1090-1093.

- Lindow, S.E., Lahue, E., Gocindarajan, A.G., Panopoulos, N.J. and Gites, D. (1989). Localization of ice nucleation activity and the ice gene product in *Pseudomonas syringae* and *Escherichia coli*. *Molecular Plant-Microbe Interactions* 2: 262-272.
- Maki, L.R., Galyon, E.L., Chang-Chien, M. and Galdwell, D.R. (1974). Ice nucleation induced by *Pseudomonas syringae*. *Applied Microbiology* 28: 456-459.
- Maki, L.R. and Willoughby, K.J. (1978). Bacteria as biogenic sources of freezing nuclei. *Journal of Applied Meteorology* 17: 1049-1053.
- Nejad, P., Ramstedt, M. and Granhall, U. (2002). Synergistic effect between frost damage and bacterial infection in *Salix* plants. Poster presented at International Poplar Symposium III, on Basic and Applied Aspects of Poplar and Willow Biology; held in Uppsala, Sweden, August 26-29, 2002. Access: <<http://www.forestresearch.co.nz/PDF/07.56Nejadetal.pdf>>.
- Nejad, P., Ramstedt, M., Granhall, U., Cambours, M.A. and Roos, S. (2003). Biochemical characterization and identification of Ice-Nucleation- Active (INA) willow pathogens by means of BIOLOG MicroPlates, INA gene primers and PCR based 16S rRNA analyses. Poster presented at 8<sup>th</sup> International Congress of Plant Pathology (ICPP) held in Christchurch, New Zealand. February 2-7, 2003. Access: <<http://www.forestresearch.co.nz/PDF/00.02Nejadetal.pdf>>.
- Nejad, P. and Johnson, P.A. (2000). Endophytic bacteria induce growth promotion and wilt disease suppression in oilseed rape and tomato. *Biological Control* 18: 208-215.
- Nejad, P., Ramstedt, M. and Granhall, U. (2004). Pathogenic ice-nucleation active bacteria in willows for short rotation forestry. *Forest Pathology* 34: 369-381.
- Nemecek-Marshall, M., Laduca, R. and Fall, R. (1993). High level expression of ice nuclei in *Pseudomonas syringae* strain is induced by nutrient limitation and low temperature. *Journal of Bacteriology* 175: 4062-4070.
- Paulin, J.P. and Luisetti, J. (1978). Ice nucleation activity among phytopathogenic bacteria. In: *Proceedings of the Fourth International Conference on Plant Pathogenic Bacteria, Vol 2*, Station de Pathologie Végétale et Phytobactériologie, Institute National Recherche Agronomique, Beaucauzé, France, 1978: 725-731.
- Pearce, R.S., (2001). Plant freezing and damage. *Annals of Botany* 87: 417-424.
- Ramstedt, M., Åström, B. and Von Fircks, H.A. (1994). Dieback of poplar and willow caused by *Pseudomonas syringae* in combination with freezing stress. *European Journal of Pathology* 24: 305-315.
- Phelps, P., Giddings, T.H., Prochoda, M. and Fall, R. (1986). Release of cell free ice nuclei by *Erwinia herbicola*. *Journal of Bacteriology* 167: 496-502.
- Sikyta, B., Spilkova, J. and Dusek, J. (2000). The influence of growth rate and growth limiting factors on the protection of ice nuclei in *Pseudomonas syringae* CCM 4073 in continuous culture. *Acta Biotechnology* 20: 369-372.
- Tsarouhas, V. (2002). Genome mapping of quantitative trait loci in *Salix* with an emphasis on freezing resistance. Doctoral thesis. Swedish University of Agricultural Sciences, Uppsala, Sweden. Agraria 327.
- Warren, G. and Wolber, P. (1991). Molecular aspects of microbial ice-nucleation. *Molecular Microbiology* 5: 239-243.
- Yankofsky, S.A., Levin, Z., Bertold, T. and Sandlerman, N. (1981). Some basic characteristics of bacterial freezing nuclei. *Journal of Applied Meteorology* 20: 1013-1019.

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