Frost-Related Dieback of Swedish and Estonian *Salix* Plantations due to Pathogenic and Ice Nucleation-Active Bacteria

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Abstract

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During the past decade, important dieback has been observed in short-rotation forestry plantations of *Salix viminalis* and *S. dasyclados* in Sweden and Estonia, plantations from which the isolation of ice nucleation-active (INA) and pathogenic bacteria has also been reported. This thesis investigates the connection between bacterial infection and frost as a possible cause for such damage, and the role played by internal and external factors (*e.g*. plant frost sensitivity, fertilisation) in the dieback observed.

Bacterial floras isolated from ten *Salix* clones growing on fertilised/unfertilised mineral soil or nitrogen-rich organic soil, were studied. Culturable bacterial communities present both in internal necrotic tissues and on the plant surface (*i.e*. epiphytes) were isolated on two occasions (spring and autumn). The strains were biochemically characterised (with gram, oxidase and fluorescence tests), and tested for ice nucleation-activity. Their pathogenic properties were studied with and without association to a freezing stress. Certain strains were eventually identified with BIOLOG plates and 16S rRNA analysis.

A high number of culturable bacterial strains was found in the plant samplings, belonging mainly to *Erwinia* and *Sphingomonas* spp.; pathogenic and INA communities being mostly *Erwinia*-, *Sphingomonas*- and *Xanthomonas*–like. The generally higher plant dieback noted in the field on nutrient-rich soils and for frost sensitive clones was found connected to higher numbers of pathogenic and INA bacteria in the plants. We thus confirm *Salix* dieback to be related to a synergistic effect of frost and bacterial infection, possibly aggravated by fertilisation.

Keywords: willow, freezing damage, ice formation, pathogenicity, frost resistance, fertiliser, *Sphingomonas*, *Erwinia*.

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Contents

Appendix

Papers I-II

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

I. Cambours M-A., Nejad P., Granhall U. & Ramstedt M. 2004. Frost-related dieback of willows. Comparison of epiphytically and endophytically isolated bacteria from different *Salix* clones, with emphasis on ice nucleation activity, pathogenic properties and seasonal variation. *Biomass and bioenergy* (In press).

II. Cambours M-A., Heinsoo K., Granhall U., & Nejad P. Frost-related dieback in Estonian energy plantations of willows in relation to fertilisation and pathogenic bacteria. (Submitted).

Background

1. *Salix*: purposes of cultivation

1.1 Short-rotation forestry and biomass production

Salix has been cultivated since ancient times for basket making (Makkonen, 1968) but growing for biomass production started only in the middle of the 1970's (Christersson, 1996). The genus *Salix*, family *Salicaceae*, is made up of more than 300 species (Larsson & Bremer, 1991) that usually grow on riverbanks and in meadows close to water. They are also able to grow on very poor or degraded soils (*e.g.* peat lands, bogs, wet lands, areas subjected to flooding, sandy soils) where traditional agriculture and silviculture are impossible. In Sweden, willow plantations were first aimed at energy production to decrease the petrol imports after the oil crisis. Systematic breeding of *Salix viminalis*, *S. dasyclados* and *S. schwerinii* for short-rotation forestry (*i.e.* systematic and rational cultivation of fast-growing clones and species on lands unsuitable for traditional agriculture) started at SLU (Swedish University of Agricultural Sciences) in the early 80's (Tsarouhas, 2002). The former two species are the most suitable for cultivation under conditions in Sweden (Sennerby-Forsse, 1994), with the highest biomass production capacity per unit area and time (von Fircks, 1994). *Salix viminalis* is suitable for culture on mineral soils while *S. dasyclados* grows better on wetter and more organic soils (*Salix* kloninformation, 1994). Differences also exist between *Salix* clones regarding frost sensitivity (*cf.* Table 2), thought to be related to clone geographical origin (Christersson, 1982), productivity (Koppel, 1996; Heinsoo, Sild & Koppel, 2002) or heavy metal uptake capacity (Yazdani, 1992). Willow plantations in Sweden nowadays occupy around 18000 ha (Aronsson *et al.*, 2002) and the production of woody biomass averages 10 to 12 tonnes of dry matter per ha (Christersson, 1996), the equivalent of 4 to 5 m3 of oil. The plants are harvested in winter every 2 to 5 years, when the stools are dormant, the harvest during dormancy securing a root pool of nutrients for resprouting the following year (Sennerby-Forsse, 1994). After cutting into chips, the harvest is used as fuel in heating plants. Around 20% of the total energy use in Sweden is covered by bioenergy from conventional and energy forests. Such energy constitutes a CO_2-neutral and less polluting alternative to fossil fuels (Perttu, 1996).

Since the fall of the Soviet Union in 1991, the use of renewable sources of energy has also increased in Estonia and the establishment of five experimental willow plantations in 1993 set the start to the Estonian Energy Forest Project, in collaboration with Sweden. To date, seven plantations cover about 3 ha (Koppel, 1998) and wastewater purification is studied on two of them. However, before large-scale plantations can be implemented there, several issues need to be addressed, *e.g.* the necessary cooperation between farmers since only large plantations are economically profitable.

1.2 Phytoremediation, wastewater and sludge purification

Salix has other major uses which have proved beneficial for environment conservation. Some *Salix* clones are able to take up heavy metals (*e.g.* cadmium) or contaminants (ammonia, inorganic and organic compounds, pesticides and radionuclides) from soil and accumulate them into their shoots. After harvest, the plants are burned and the metals can be separated from the ashes. High biomass production and high translocation to the shoots as well as high uptake efficiency and specificity are required for an efficient removal. As hyperaccumulators, willows and poplars are the most commonly used species for phytoremediation of polluted sites due to their rapid growth, deep root system and high uptake of water (Isebrands *et al*., 2002). Owing to its well-developed root system, *Salix* is also used for sewage sludge decontamination and wastewater purification (*e.g.* nitrate polluted water). Sludge or water application on the plantation provides fertilisation, contributing to high biomass production, which in turn leads to a better removal. This method also reduces the amount of waste deposited on landfills (Christersson, 1996, 1998).

2. Freezing stress in plants

2.1 Mechanisms of ice formation and injuries caused

Plants have the ability to supercool *i.e.* even though the temperature drops below 0°C (freezing point of water), ice formation does not occur and temperatures as low as *ca*. –40 °C can be tolerated before any injury is sustained (Lindow, 1983; Chen, Burke & Gusta, 1995). However, plants will only handle such low temperatures in absence of ice nuclei. Even in dispersed phases like water, aggregates of the condensed phase exist and when the temperature reaches –40 °C, their random aggregation enables the formation of an ice nucleus (*i.e*. nucleation) (Vali, 1995). If water molecules only are present, this is called a homogeneous nucleation. However, most of the time, nucleation involves foreign substances and becomes heterogeneous. Plants harbour many potential substrates for nucleation like bacteria, fungi or insects, and ice formation occurs at a much lower supercooling degree than in a case of homogeneous nucleation.

Extensive supercooling is a mechanism that enables the plant to survive very low temperatures (Bervaes, Ketchie & Kuiper, 1977) but it can become a disadvantage if a sudden freezing eventually occurs. Indeed, the more a plant supercools before freezing, the more injuries it will sustain (Rajashekar, Li & Carter, 1983; Andrews, Proebsting & Gross, 1986) because the ice growth will then be too fast for an equilibrium to be reached (Olien, 1964) and ice will often form intracellularly (Chen, Burke & Gusta, 1995). A single ice nucleus can be sufficient to initiate ice formation and cause frost injury in entire leaves or even groups of leaves, depending on the degree of restriction for ice propagation (Single & Olien, 1967). After the first nucleation event, ice spreads rapidly extra and intracellularly, disrupting cell membranes, which lose their semipermeability (Levitt, 1980). Levitt (1980) divided frost damages into two groups: (1) primary injuries due to intracellular freezing, leading to membrane disruption and cellular death and (2) secondary injuries induced by extracellular ice formation (*i.e.* on the

surface of the cell or between the protoplasm and the cell wall). Intracellular ice formation mostly occurs after rapid freezing (Siminovitch, Singh & de la Roche, 1978), spontaneously only below –10 °C (Mazur, 1977). Extracellular freezing occurs at low cooling rates and is promoted by the presence of ice nucleators like bacteria. It leads to the diffusion of intracellular water towards the extracellular ice, resulting in cell dehydration and contraction (Palta & Weiss, 1983) and to the eventual collapse of the contracted cells. This constitutes the main cause of freezing injury in higher plants. If the cells have not been injured by ice formation, they can reabsorb the water upon rewarming and regain turgor. Extracellular freezing is also considered as a plant adaptation to frost since as opposed to intracellular freezing ice formation outside the cell is not always fatal (Levitt, 1980).

The most common external symptoms of frost damage in *Salix* are dead shoot tips and necrotic bark tissues in the basal part of the shoot that will eventually decrease its supply of water and nutrients, causing dieback (von Fircks, 1994). Other symptoms described by Tsarouhas (2002) comprise *e.g.* discolorations and tissue shrinking.

2.2 Cold acclimation and freezing resistance

The two main types of freezing resistance are avoidance and tolerance (Levitt, 1980). The former mechanism involves a tissue capacity to prevent ice formation. Accumulation of antifreeze solutes, absence of freezable water and moderate supercooling are three ways by which plants can partially avoid freezing. Extensive supercooling is also a frost avoidance mechanism although, as mentioned above, it can come very harmful to cells. It is believed to have played a major role in defining the northern limit of distribution of many species of hardwood (George *et al*., 1974). Freezing tolerance is based on the ability of cells to withstand extracellular ice formation without sustaining any injury, either by avoiding or by tolerating the freeze-induced dehydration.

Freezing resistance increases upon cold acclimation. The ability to acclimate to cold and the rate of acclimation will determine plant survival to frost and low temperatures (Chen, Burke & Gusta, 1995). In woody plants, the acclimation process is mainly initiated by short days and low night temperatures (Chen & Li, 1978). Glerum (1985) suggested it to be a three-stage process. The first phase, in early autumn, is associated with growth cessation and leaf coloration. During the second phase, as temperatures continue to fall, the tree hardening response increases. The third stage is induced by temperatures between –15 and –50 °C; it is attained only by very hardy species (Sakai, 1965). Degree of frost hardiness also depends on species and clones (Heinsoo, Sild & Koppel, 2002).

During acclimation, cryoprotective solutes (*e.g*. carbohydrates, proteins) accumulate in the cytosol. The type of carbohydrate stored, mostly sucrose and raffinose in *Salix* (Ögren, 1999), depends on the plant metabolism. Sugars alter osmotic potential and therefore reduce water losses from the cell, limiting freeze-induced dehydration. They also act as cryoprotectants for cell constituents and membranes during freeze-thaw cycles (Sakai & Larcher, 1987). Protein

accumulation has mainly been reported in herbaceous species and includes both structural proteins and enzymes (Chen, Burke & Gusta, 1995).

Since the plasma membrane is generally the primary site of frost injury, its protection is of fundamental importance. Changes in its lipid and protein composition during acclimation have been reported (*e.g.* increase in unsaturated fatty acids and phospholipids) (Chen, Burke & Gusta, 1995). Increases of the cellulose and extensine contents of the cell wall have also been associated with an increase of freezing tolerance (Weiser, Wallner & Waddell, 1990). Cell wall rigidity and strength are indeed of major importance to limit the cell reduction caused by freeze-induced dehydration (Rajashekar & Burke, 1982) and to resist extracellular ice pressure, thus preventing fatal intracellular freezing (Levitt, 1980).

As a result of hardening, dormant shoots of *Salix* can tolerate winter temperatures as low as –85 °C. Fertilisation studies have shown that nitrogen supply could delay cold acclimation and make the trees more sensitive to frost (von Fircks, 1994). The nutrient balance (mostly N, P and K) as well as the timing and rate of nutrient supply may also affect the development of frost resistance. From a molecular point of view, development of freezing tolerance is accompanied by synthesis of new mRNA and polypeptides. To date, several cold-regulated genes associated with cold hardiness have been sequenced (Chen, Burke & Gusta, 1995). With the development of the genetic approach, we may be able to decipher the process of hardening and increase freezing tolerance of plants to reduce frost-related dieback.

3. Diseases and dieback in *Salix*

3.1 Abiotic factors

Abiotic stresses like drought, rain or frost constitute an important cause of production losses in agriculture and forestry as they can bring about dramatic decrease in crop quality and output. The average life-cycle of a willow-stand ranges from 20 to 30 years and plant survival is especially important for production during the first cutting cycle. Local conditions (*e.g.* soil texture, water availability) and management (*e.g.* nutrient supply) are essential factors for the successful establishment and subsequent productivity of a plantation (Christersson, 1996). In Sweden, with a semi-humid climate and light conditions almost optimal during the growing season, the decisive factor for good plant development is the temperature, and the number of night frosts during the growing period has to be limited (Ledin, 1996). Very low temperatures usually occurring during plant dormancy and unseasonal frosts are the two main frost-related stresses. A frost event during the growing season leads to dieback of distal shoot parts, defoliation or deformation of leaves and stems (von Fircks, 1994). Plant growing parts can be frost damaged already around –3 °C (*Salix* kloninformation, 1994).

Frost constitutes the main obstacle to the expansion of the use of *Salix* for short rotation forestry in northern countries (von Fircks, 1985). *Salix* cultivation, at first

limited to southern Sweden, has been gradually moved northwards, and production losses have been observed because of the over long vegetative periods, which are not adapted to the colder conditions (Verwijst *et al*., 1996). Today, many clones cannot be grown in northern and even central parts of Sweden because of frost (Larsson, 1998). During the past years, breeding programmes have led to the cultivation of clones with extended vegetative periods at the expense of early growth cessation. However, the more productive a clone, the longer its vegetative period and the higher the risk of frost damage during late spring and early autumn (von Fircks, 1996).

3.2 Biotic factors: Salix *bacterial pathogens*

3.2.1 Ice Nucleation-Active (INA) bacteria

Species concerned

INA bacteria limit plant supercooling by catalysing ice formation within the tissues already around –1 °C (Maki *et al*., 1974; Lindow, Arny & Upper, 1978a). They are very effective ice nucleators and can retain their activity up to two weeks after cell death providing the temperature is low enough (Lindow, 1982). INA bacteria are ubiquitous colonisers of plant surfaces and can be found on a large number of species (Lindow, Arny & Upper, 1978b). At least ten bacterial species are known to be INA (Anderson *et al*., 1982; Kaneda, 1986) (Table 1). The most commonly found on plants are *Pseudomonas syringae*, *P. fluorescens* and *Erwinia herbicola*; *P. syringae* and *E. herbicola* being the most active. At least 50 % of the pathovars of *P. syringae* are INA (Lindow, 1983). Other species, newly found to include INA strains, are reported in this thesis (**I**, **II**) and in Nejad, Ramstedt & Granhall (2004).

Table 1. *Known species of ice nucleation-active bacteria (found on and in plants)*

Species	Selected references
Pseudomonas syringae	Maki *et al*., 1974; Ramstedt, Åström & von Fircks, 1994
P. fluorescens	Kaneda, 1986
P. viridiflava	Paulin & Luisetti, 1978
P. chlororaphis	Schnell *et al*., 1991
Erwinia herbicola	Lindow, Arny & Upper, 1978b
E.ananas	Paulin & Luisetti, 1978
E. uredovora	Newton & Hayward, 1986
Xanthomonas campestris pv. *translucens*	Kim *et al*., 1987
X. campestris	Goto *et al*., 1988
Unidentified pseudomonads	Yankofsky, Levin & Moshe, 1981

Population dynamics and injury mechanism

INA bacteria increase plant frost susceptibility (Anderson *et al*., 1982). Several bacterial species have been found which are both necessary and sufficient to account for the frost sensitivity of all frost sensitive plants examined to date (Lindow, 1983). The freezing temperature of plant tissues is a function of both the population size of INA bacteria and their nucleation frequency (Hirano, Baker & Upper, 1985). However, the injury sustained at a given temperature is more directly related to the number of actual ice nuclei at the time of freezing than to the

size of the INA population (Lindow, 1982). Since (almost) all ice nuclei present on leaf surfaces above –5 °C are of bacterial origin (Lindow *et al*., 1976, 1978), plant supercooling between 0 and –5 °C is mainly limited by INA bacteria (Hirano & Upper, 2000). Therefore, only above –5 °C is the frost injury proportional to the population size of (Ice^+) bacteria (Lindow, 1995).

The population dynamics of phyllosphere INA bacteria has been studied on a number of plant species. The number of bacteria generally increases after germination or bud break, a time that coincides with the maximum frost hazard period, and decreases in autumn and winter (Lindow, 1982). As a rule, the population increases under mild and wet conditions and decreases under hot and dry weather. Infection and spread are also easier in spring and autumn due to the ambient humidity and favourable canopy proximity (Hirano & Upper, 2000).

Injured tissues and leaves show a greater carrying capacity for INA bacteria, due to the release of nutrients triggered by freezing. Indeed, Kaneda (1986) reported an increase of the INA population on bean leaves after the first frost event, supposedly due to the increase of available nutrients. It is believed that plants harbouring higher epiphytic levels of INA bacteria also exude more nutrients onto the leaf surface (Lindow, 1995). In willow, extensive electrolyte leakage during acclimation has been connected to higher frost sensitivity (Tsarouhas, 2002) and increased frost-related bacterial damage (Granhall *et al*. unpublished). Frost injuries have often been associated with higher pathogenic infections (Klement *et al*., 1984; Süle & Seemüller, 1987). Enhanced access to damaged tissue might be a greater advantage for weak pathogens than for aggressive ones, better able to colonise the plant without INA injuries (Hirano & Upper, 1995). Although ice nucleation-activity may constitute a competitive advantage under mild frosts (Buttner & Amy, 1989), the selective pressure mechanisms for the INA phenotype, as well as for the association between INA and pathogenic characteristics, remain unclear since tissue destruction due to INA is unfavourable for pathogen colonisation. Therefore, other mechanisms, as yet unidentified, are thought to play a role for selection of this phenotype (Hirano & Upper 1995).

Factors influencing INA *in vitro* and *in planta*

Not every cell expresses the ice nucleation phenotype at a given time (Lindow *et al*., 1982). Blondeaux & Cochet (1994) found that *in vitro* INA was expressed at best during late log phase or beginning of the stationary phase. *In vitro* ice nucleation-frequency (*i.e*. ratio between number of ice nuclei and number of bacterial cells in a culture) depends on the bacterial species (Lindow, 1982), the temperature (Gurian-Sherman & Lindow, 1995) and the medium composition (Fall & Fall, 1998). Nemecek-Marshall, LaDuca & Fall (1993) have proposed a model for INA expression in *Pseudomonas syringae* in which the *ina* gene is most expressed when cells are exposed to low temperatures and nutrient limitations. In *Erwinia herbicola*, phosphate starvation coupled with low temperature had the greatest effect on INA but it was also affected by carbon, nitrogen and iron starvation. This gives an insight into the mechanism by which INA bacteria present on plant surfaces, and often submitted to low temperatures and starvation, trigger ice formation, releasing nutrients from plant cells (Fall & Fall, 1998). Medium water activity also affects INA. High water activity leads to high

membrane fluidity, which in turn leads to the disaggregation of nucleating sites and depresses INA. On the contrary, a low water activity is associated with enhanced INA (Blondeaux *et al*., 1999). INA bacteria can also undergo low-temperature conditioning. Rogers, Stall & Burke (1987) reported that shifting (Ice^+) bacteria grown at relatively warm temperatures (*e.g.* 30 °C) to a lower temperature (*e.g.* 5°C) triggered the appearance of new ice nuclei active above –5°C.

This is a non-exhaustive list of the factors influencing INA. As more studies are carried out, more is discovered about what governs this property *in vitro*. However, it remains difficult to correlate the laboratory results to the field situation because of the impact of environmental conditions on INA. O'Brien & Lindow (1988) observed that the expression of ice nucleation on plants was highly variable, depending on strains, plant species, humidity, light intensity, plant nutrition and found that ice nucleation-frequency was generally lower in culture than after growth on plants. More work is needed to assess, for example, the actual effect of fertilisation on INA *in planta*.

Molecular aspects of INA

The (Ice^+) phenotype, conferred by the *ina* gene, is monogenic (Orser *et al*., 1983). The gene has been cloned and sequenced from *Pseudomonas syringae* (*ina*Z) (Green & Warren, 1985), *P. fluorescens* (*ina*W) (Warren, Corotto & Wolber, 1986), *Xanthomonas campestris* pv. *translucens* (*ina*X) (Zhao & Orser, 1990), *Erwinia ananas* (*ina*A) (Abe *et al*., 1989) and *E. herbicola* (*ice*E) (Warren & Corotto, 1989). The open-reading frame of the gene appears highly conserved for these 5 species (Edwards *et al*., 1994). Based on the similarities observed in nucleotide sequences among *ina* genes from different species, Wolber & Warren (1991) proposed that the gene had once evolved by natural selection, and then had been horizontally transferred by conjugation between phyllosphere-inhabiting INA species. This hypothesis is supported by the phylogenetic analysis of the *ina* genes (Edwards *et al*., 1994). The (Ice^+) phenotype is expressed rather infrequently by the cells carrying the *ina* gene: Maki *et al*. (1974) found that only one cell of *P. syringae* out of 1000 had an active nucleating site.

Lindow *et al*. (1989) found ice nucleation activity to be associated with the cell envelope, with the Ina protein probably located on the outer membrane (Fig. 1). However, biochemical analysis has been hindered by a low copy number of Ina proteins per cell (Deininger, Mueller & Wolber, 1988) associated with only a few nucleation sites (Fall & Wolber, 1995). The Ina protein acts as a nucleation center. Its amino acid sequence is rather hydrophilic (Wolber, 1993) with a hydrophobic part possibly being the membrane anchor (Warren, 1987). The internal repetitive domain may provide the template structure organizing water (Fall & Wolber, 1995). Kozloff *et al*. (1983) suggested that *E. herbicola* cells have at least two separate ice nucleating sites while *P. syringae* may have four or more.

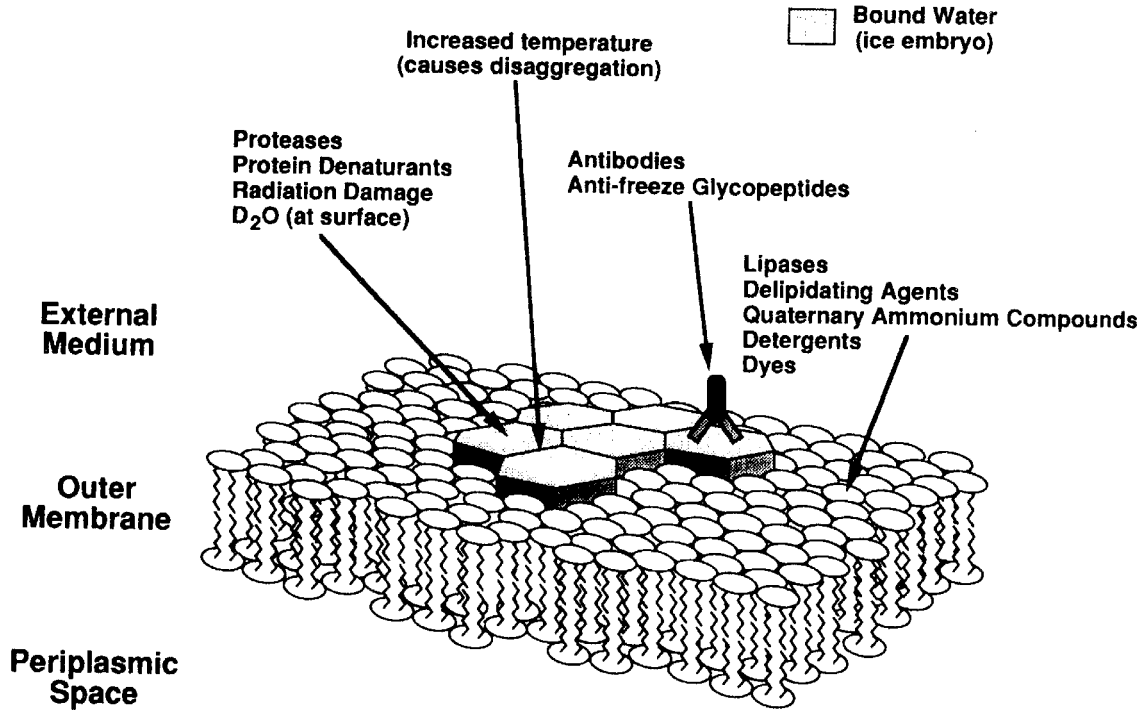

Fig. 1. A model of the quarternary structure of bacterial ice nuclei in the outer cellular membrane. The hexagonal prisms represent copies of the ice nucleation protein. The proposed sites of action of various treatments degrading bacterial ice nuclei are emphasized by the arrows (from Fall & Wolber 1995).

Yankofsky *et al.* (1981) defined three classes of ice nuclei according to their temperatures of nucleation: Type 1, active between -2 and –5 °C, Type 2, active between -5 and –7 °C and Type 3, active below –7 °C. Govindajaran & Lindow (1988) suggested that the nucleation temperature depended on the aggregate size of the *ina* gene product: the larger the aggregate, the warmer the nucleation temperature. Nuclei active at warmer temperatures also require an intact or physiologically normal cell and have therefore been more difficult to isolate so far (Yankofsky *et al.*, 1981, Lindow *et al.*, 1989). Nuclei active at lower temperatures are more stable but assembled more slowly (Watanabe *et al.*, 1990). Ice nucleation activity is inhibited by *e.g.* heat, heavy metals or antifreeze glycopeptides. Compounds reacting with proteins, membrane lipids and carbohydrates also affect INA of intact cells, either by direct reaction with the Ina protein or by alteration of the cell membrane (Fall & Wolber, 1995).

Control of INA bacteria

Reduction of INA bacterial numbers or activity helps to decrease plant frost sensitivity. Because of bacterial high multiplication rates, preventive treatment is preferable to eradication of established populations. To date, only copper compounds and streptomycin are registered for INA bacteria control on crop

plants. Copper products, chemically stable, have a broad toxicity, but are easily removed from plants by rainfall and wind. Streptomycin is a photolabile antibiotic, its concentration quickly decreases after light exposure. Apart from the rapid concentration drop below the lethal threshold and the high application frequency necessary to insure the compound presence on all tissues, an increasing issue is the resistance developed by bacterial strains to bactericide treatments (numerous streptomycin- or copper-resistant *P. syringae* populations have been reported) (Lindow, 1995). Taking also into consideration the high cost of such products, bactericides cannot be regarded as a sustainable solution. Inhibitors of ice formation, like urea and sodium carbonate, which affect the expression of ice nucleation activity and reduce the numbers of nuclei active at –5 °C can also be used (Lindow & Connell, 1984).

A more promising technique is the biological control of plant frost injury. INA populations have been successfully reduced by application of naturally occurring or genetically engineered (Ice^-) strains of *Pseudomonas syringae*, *P. fluorescens*, and *Erwinia herbicola*. The non-INA strains, competing on the plant surface for nutrients and space with the indigenous INA community, were found effective in limiting or preventing colonisation by INA bacteria, though not in eliminating already established populations (Lindow & Connell, 1984). Lindow (1982) observed that antagonistic bacteria precluded INA bacteria from the leaves, thus reducing their population density. However, the decrease was not as large as that observed when the initial INA population was low. Therefore, a preventive application seems more effective than an exterminative one to achieve significant reduction of frost injury. Naturally occurring (Ice^-) strains are isolated from the dominant flora of healthy plants. It is important that they have the same ecological habitat requirements as the target strain to enable them to grow as well on plant surfaces and exclude them effectively. The control of frost injury by engineered non-INA strains has been found as efficient as that by naturally occurring ones (Lindow, 1995). Finally, improving plant resistance to bacteria constitutes another option (*e.g.* via the selection for plants exuding less nutrients onto their surface).

3.2.2 Pathogenic bacteria

Pathogenicity is defined by the ability to cause disease. Few species are able to invade and multiply in healthy plant tissue, leading to plant deterioration. These belong to five major taxonomic groups: *Erwinia*, *Pseudomonas*, *Xanthomonas*, *Agrobacterium* and coryneform bacteria. Except for coryneforms, Gram-positive bacteria have no significant pathogenicity. The *P. syringae* species comprises most plant pathogenic bacteria. Green-fluorescent epiphytes, they can infect a very large number of plants, including *Salix* (Ramstedt, Åström & von Fircks, 1994; Nejad *et al*., 2003), each pathovar being host specific (Anderson *et al*., 1982). Another well-known plant pathogen is *X. campestris*. This species causes many plant diseases, not always easy to distinguish from one another (de Kam, 1984). *X. populi* subsp. *salicis* is responsible for bacterial canker on *Salix dasyclados* giving bark tissue a glassy appearance and leading to eventual necrosis and girdling of the branch (de Kam, 1978). In this thesis (**I**, **II**), strains belonging to *P. fluorescens* species, *Erwinia*, *Sphingomonas*, *Clavibacter* and *Bacillus* genera are also reported pathogenic to *Salix*.

Pathogenic bacteria cause disease in susceptible hosts by altering cell membranes. The release of water and nutrients, evidenced by water-soaked tissues, leads to eventual cell death. In necrotic diseases, as is the case here, the major part of bacterial division and growth takes place between living cells before the release of nutrients due to bacterial toxins or cell wall degrading enzymes (*e.g*. cellulases, proteases). Secondary invaders only survive in tissue after these pathogenic or INA strains have triggered the release of substrates, making their growth and plant colonisation possible (Sigee, 1993). Hirano & Upper (2000) noted that the only known advantage of lesions (and frost injuries) is to provide a place of survival for the bacteria during unfavourable conditions. In this regard, lesion formation (*i.e*. habitat destruction) would be an unfortunate accident of overpopulation due to successful colonisation, unlikely to benefit the bacteria. Epiphytic bacterial populations of *P. syringae* are believed to be the source of inoculum for infection of stems and branches leading to canker development (Crosse, 1959). This observation could probably be generalised to all bacterial species if more pathogen-host systems had been studied, and for which a quantitative relationship had been found between epiphytic population sizes and subsequent disease development.

Two sets of genes play a role in plant disease reaction: *hrp* (hypersensitive reaction and pathogenicity) and virulence (*vir*) genes. *hrp* genes trigger the hypersensitive reaction on non hosts (*e.g*. tobacco) and resistant cultivars/plants. They are also necessary to elicit a disease reaction in host plants. *hrp* genes occur as cluster and are highly conserved. They are believed to code for *e.g*. membrane proteins and enable for example the alteration of the plant cell membrane to release nutrients. *vir* genes are involved in disease development. They speed up plant colonisation by *e.g*. controlling the production of toxins, extracellular enzymes or plant hormones. At least some *vir* genes are conserved (Sigee, 1993).

3.3 Combination of frost and bacteria

The interaction of physical and biological factors determines the extent of plant stress. In the present case, a frost event is believed to interact with a bacterial infection. INA bacteria bring about ice formation at temperatures when no freezing would have normally occurred, and aggravate frost injuries, providing a route for bacterial infection (Ramstedt, Åström & von Fircks, 1994). INA bacteria can themselves be pathogenic or let other pathogenic bacteria (INA or not) penetrate the plant. It must be noted that pathogenic bacteria can also get into the plant via a frost event unrelated to INA bacteria. Freezing injury has been found to help, for example, the development of bacterial canker in poplar (Sabet, 1953) and the infection of sour cherry leaves (Süle & Seemüller, 1987) by *Pseudomonas syringae* pv. *syringae*. de Kam (1982) also observed that *P. syringae* increased plant tissue damage due to freezing, providing further evidence for interaction between frost and bacterial infection.

Symptoms of bacterial infection in *Salix* tend to remind of those of frost damage. However, the presence of extensive necroses and discolorations of the bark that spread further in underlying tissues, associated with a soaked and glassy tissue appearance, confirm bacterial infection, possibly associated with frost

damage. The symptoms observed are primarily due to bacterial pathogenicity and not only the result of their ice nucleation activity (Ramstedt, Åström & von Fircks, 1994).

Aims of the study

In the beginning of the 1990's, stem damage presenting the symptoms of a frost-associated bacterial infection was reported, together with plant dieback, in willow plantations of southern and central Sweden. Similar damage was observed in Estonian *Salix* plantations in the end of the 1990's. Investigation of the dieback causes led to the isolation of INA strains of *Pseudomonas syringae* from the plants internal tissues (Ramstedt, Åström & von Fircks, 1994). The present thesis studies bacterial communities sampled from Swedish (**I**) and Estonian (**II**) *Salix* plantations on two occasions (spring and autumn), both from epiphytic (**I**) and diseased internal (**I**, **II**) parts. Between four and seven clones were sampled depending on time and location.

The objectives were to:

1. Determine the dominant bacterial species present, their ice nucleation activity and their pathogenicity on *Salix* (**I**, **II**).
2. Evaluate their seasonal dynamics (**I**).
3. Compare diseased and epiphytic parts in terms of community composition, and pathogenic and INA characteristics (**I**).
4. Investigate possible bacterial community differences between clones and, should this be the case, to study if they were related to the frost resistance degree of the clones (**I**, **II**).
5. Determine the influence of fertilisation regime and soil composition on bacterial community (**II**).

Material and methods

1. The plantations

The Swedish plantation, established in 1990 on 0.5 ha of clay soil, is located in central Sweden, at Brunnby, Västerås (59°35N 16°40E, 10m above sea level). Twelve *Salix* spp. clones were planted in four randomised blocks at a density of 20000 cuttings/ha or 2 cuttings/m^2, and fertilised. The climate is semi-humid (Ledin, 1996) and the frequency of frosts during the growing season is low (von Fircks, 1994).

The Estonian plantations studied were established in 1993 at Saare and Kambja, eastern Estonia, near Tartu (58°22N 26°43E). The same seven clones were planted at both sites at a density of 20000 cuttings/ha, in four randomised blocks. The

Kambja plantation (0.3 ha) is situated at the bottom of a valley, on the banks of a river on a well-decomposed organic soil (deep Eutric histosol), very rich in nitrogen but poor in available potassium and phosphorus. In autumn 1995, 10% of the plantation was destroyed by beavers, which totally severed plants on several blocks (Koppel *et al*., 1996). This plantation has now been abandoned. The Saare plantation (0.61 ha) is situated on the plane of the ancient lake Peipsi on a mineral soil (gleyic podzoluvisol) with low availability in nitrogen and potassium but rather good phosphorus availability. Poor aeration can occur in autumn and after rainy periods because of the two-layered soil structure. The plantation was fertilised on half the blocks in 1994 and 1995 with ammonium nitrate, superphosphate and potassium chloride (Koppel, Perttu & Ross, 1996). The influence of fertilisation on plant survival was studied in two ways. First, shoot survival was determined for one fertilised and one unfertilised plot of clone 83 at Saare. In spring 1999, all living shoots in a six by six-plant area were marked and diameter at 55 cm above the ground was measured. In spring 2000 and 2001, the shoots were counted to determine their survival rate. Furthermore, in autumn 2000, all living plants of each Saare plot were counted and mapped and the actual plant density was calculated for each clone. Saare and Kambja soil types are the two most available for large-scaled energy forestry in Estonia (Koppel, 1996). Estonian climate is similar to that of the southern part of Sweden. In Tartu, the average precipitation during the growing season is 420 mm (Heinsoo, Sild & Koppel, 2002). The growing season lasts about 6 months (Kuusemets & Mauring, 1996).

Plant samplings were collected on two occasions, both in Sweden and Estonia. In Sweden, cuttings from four clones were taken in April and October 2001 (Table 2). In Estonia, the first sampling was made in March 2002 for the seven clones at Saare and five clones at Kambja (Table 2). The second sampling, in August 2002, was only performed on three clones (78183, 81090 and 78195) at Saare. Hereafter, the clones will be referred to only by the last two digits of their number (*e.g*. 21 for clone 78021) or by their name.

Table 2. *Descriptive summary of the Salix clones sampled in Sweden and Estonia*

Clone No./name	Species	Frost sensitivity [a]	Sampling [b]		
			Brunnby	Saare	Kambja
78021	*S. viminalis*	2		+	+
78101	*S. viminalis*	3		+	
78112	*S. viminalis*	1		+	
78183	*S. viminalis*	1	+	+	+
78195	*S. viminalis*	3		+	+
81090	*S. dasyclados*	2		+	+
82007	*S. viminalis*	2		+	+
Christina (821629)	*S. viminalis*	3	+		
Marie (832501)	*S. viminalis*	2	+		
Anki (832502)	*S. viminalis*	2	+		

Notes: [a] Scale 0-4: 0=resistant, 4=sensitive (the figures are relative rather than exact)
[b] sampled clones are indicated with +
(source: *Salix* kloninformation, 1994)

2. Bacterial isolation

The Swedish study is based on the isolation of bacterial communities from epiphytic and internal diseased parts of plants while the Estonian study focuses on strains from internal tissues only. The technique for bacterial isolation was somewhat upgraded between the two studies. In paper (**II**), serial dilution platings of the bacterial suspensions were performed, providing precise estimations of the numbers of bacteria per gram dry weight of plant material.

Endophytic bacteria colonise internal tissues of healthy plants without causing symptoms of disease (Wilson, 1995). Some of them have been reported effective in promoting plant growth and suppressing wilt disease in *e.g*. potato and tomato (Kempe & Sequira, 1983; Nejad & Johnson, 2000). Endophytes are found in numerous plant species and most of them belong to *Pseudomonas* and *Bacillus* genera (Chanway, 1996). A certain number of the strains we isolated from internal tissues were INA or/and pathogenic and consequently, cannot be regarded as true endophytes. However, for the sake of simplicity in this thesis and paper (**I**), they are referred to as endophytic bacteria (as opposed to epiphytes). Epiphytic bacteria are associated with plant surfaces (root or aerial regions). They play a role in frost damage or biological control of plant pathogens. Many of them are saprophytes, some are parasites, spending part of their life on the plant and part in the plant tissue (Sigee, 1993). Epiphytes were considered only in the Swedish study.

3. Biochemical and molecular tests

The bacterial strains selected for further studies were submitted to gram, oxidase and fluorescence tests. According to the tests results, they were classified into 12 profiles (**I**, **II**; Table 1). The major bacterial types isolated belonged to:

Bacillus genus (profiles 1, 2 and 4): gram positive, oxidase positive, non-fluorescent strains (a few were found oxidase weak positive or fluorescent).

Clavibacter genus (profile 6): gram positive, oxidase negative, non-fluorescent strains.

Fluorescent pseudomonads (profiles 7, 9 and 11, comprising *P. fluorescens*, *P.viridiflava* and *P. syringae*): gram negative, oxidase negative (*P. syringae*), positive (*P. fluorescens*) or weak positive (*P.viridiflava*), yellow-green fluorescent strains.

Non-fluorescent pseudomonads (*Pseudomonas* spp.) and *Sphingomonas* spp. (profile 8): gram negative, oxidase positive, non-fluorescent strains.

Xanthomonas genus (profile 10): gram negative, oxidase weak positive, non-fluorescent strains.

Erwinia genus (profile 12): gram negative, oxidase negative, non-fluorescent strains.

Four additional tests were performed to distinguish between the strains placed in *Erwinia* and *Xanthomonas* genera: growth on Yeast Dextrose Calcium carbonate (YDC) and Nutrient Agar (NA), levan production on Sucrose Nutrient Agar (SNA) and anaerobic growth ability. For more accurate classification, BIOLOG and 16S rRNA analyses of a few strains were performed.

4. Hypersensitivity test on tobacco

The hypersensitivity test on tobacco determines if a strain has a pathogenic potential. Non-pathogenic strains do not induce the hypersensitive reaction, a localised resistant plant response overcoming the entry of a wide range of potential pathogens. This test is used for bacteria causing vascular wilts, necroses and some soft rots but is not effective to screen for opportunistic pathogens. Tobacco is usually used because it shows a clear rapid reaction with most incompatible plant pathogens, except *Xanthomonas* (Klement, 1990). Environmental factors (*e.g*. temperature, bacteria metabolic state, inoculum level) influence the induction and expression of the hypersensitive reaction (HR).

5. INA and pathogenicity tests

Bacterial ice nucleation can be measured by the drop freezing technique, a cumulative method estimating the number of nuclei active at or above each temperature tested (Lindow, Arny & Upper, 1982). This technique presents several limitations, the main ones being the risk of cross contamination between the droplets and the impossibility to preserve them for repeated tests due to their evaporation. INA can also be monitored *in situ*, *i.e*. on leaves or stems (Hirano, Baker & Upper 1985; Ramstedt, Åström & von Fircks, 1994). We used a technique derived from Ramstedt, Åström & von Fircks (1994) and monitored the freezing temperature of bacterial suspensions submitted to a gradual temperature decrease.

The pathogenicity tests were performed on cuttings of *Salix viminalis*. Bacterial suspensions were inoculated and the cuttings were submitted to below zero temperatures (-2 °C) for several hours. They were checked for apparition of brown to black necroses on the bark and wood up to 12 days later. In the Estonian study, half of the cuttings were also inoculated without any following freezing treatment. In the disease (or compatible) reaction, the loss of electrolytes is much slower than during the HR and this delay in apparition of adverse conditions (*i.e*. plant cell death) enables the bacteria to multiply and spread beyond the site of infection. Tissue collapse and appearance of necroses occur at a later stage than during the hypersensitive reaction (Sigee, 1993).

In his postulates, Koch stated the four steps that must be fulfilled for an organism to be regarded as the cause of a disease:

1. The organism must be consistently associated with the diseased tissue.
2. The organism must be isolated and grown in pure culture.
3. The organism must be inoculated into healthy plants of the same species and produce the same disease.
4. The organism must be reisolated and reinoculated into healthy plants to produce the same disease.

These requirements were verified for the Estonian but not for the Swedish study, where the postulate #4 was not fulfilled. Due to the difficulties met when attempting to satisfy Koch's postulates for canker and dieback diseases, Klement (1990) suggested the alternative use of simpler tests to confirm the pathogenicity of selected bacterial strains. Most of them were included here, namely HR test,

INA test and cutting inoculation. However, fulfilment of all Koch's postulates will be required before considering the strains as fully pathogenic.

Discussion of results

1. Influence of fertilisation and soil type

Mineral nutrient supply is known to affect frost resistance and cold acclimation in *Salix*. The study presented in paper (**II**) focused on investigating the possible effect of fertilisation and soil nutrient availability on the bacterial community in relation to plant dieback.

After seven years of cultivation, the plant density was significantly lower in Saare fertilised plots than in those unfertilised with an average of 1.66 versus 1.85 plants per m^2 (**II**; Fig. 1). The average density at Kambja was even lower (1.58 plants per m^2). However, this was partly due to beaver damage, which also made taking all the plots into account impossible. A well-known cause of higher mortality rates in well-growing stands is plant competition (Verwijst, 1996). Fertilisation leads to enhanced plant development, thus increased competition, and very probably higher dieback rates. However, our field observations excluded competition as the only cause of dieback. As shown in Table 3, the percentage of living shoots in clone 83 after two years was twice as high for unfertilised plots as for fertilised plots. While dieback on unfertilised plots mainly concerned shoots of less than 8 cm diameter, even thicker shoots (*i.e*. main stems) that usually survive competition died on a large scale in fertilised plots (dieback was recorded up to 18 cm diameter).

Table 3. *Comparison of the number of living shoots present on clone 83 at Saare in spring 1999, 2000 and 2001 for fertilised and unfertilised plots. Percent shoot dieback between 1999 and 2000 or 2001*

	Year	83 unfertilised	83 fertilised
Number of	1999	819	988
Living	2000	553	341
Shoots	2001	330	191
% dieback	2000	33	66
	2001	60	80

A higher culturable bacterial community was found in fertilised trees than in unfertilised ones (**II**; Table 2). A multivariate analysis of variance (MANOVA) indicated that fertilisation indeed significantly affected both CFU and plant density with fertilised plots sustaining both higher mortality and higher bacterial colonisation (**II**; Fig. 2). More compelling evidence was given by the higher percentages of INA and pathogenic bacteria isolated from Saare fertilised and Kambja plots compared to Saare unfertilised, which paralleled the higher dieback figures recorded at Kambja and Saare fertilised plots.

Nitrogen abundance results in the production of young, succulent growth, prolonged vegetative period and delayed maturity of the plant. All these effects make the plant more susceptible to pathogens that normally attack such tissues, and for longer periods (Agrios, 1997). Our study showed that beyond affecting plant susceptibility, fertilisation also led to higher pathogen and INA colonisation, the exact extent of which remains to be quantified. However, we can already confirm, from our results, that fertilisation (and nitrogen abundance at Kambja) participated in increasing *Salix* bacterial related dieback. Previous studies of these two plantations had already stressed the impact of fertilisation and soil nutrient availability on willow mortality. In Heinsoo, Sild & Koppel (2002), increased wood dry matter yield but also high stool mortality of *S. dasyclados* (clone 90) was associated to fertiliser application. In 1996, Koppel reported that the higher productivity observed at Kambja (compared to Saare unfertilised) due to higher soil fertility decreased after 3 years, maybe because of nutrient imbalances (von Fircks, 1994). The higher plant mortality observed on the organic soil at Kambja could be due to the high availability of mineralised nitrogen, which is known to hinder plants from going into dormancy, thus leading to frost damage and dieback.

Although we report differences in bacterial numbers between plantations and fertilisation regimes (either concerning total, INA or pathogen amounts), similar bacterial profiles were found at the three sites. *Erwinia* was the dominant bacterial profile found in the tissues, followed by *Sphingomonas*, *P. fluorescens* and *Xanthomonas*. The strains profile distribution was more even at Kambja than at Saare, where *Erwinia* was very dominant (**II**; Fig. 5). Ice nucleation-active and pathogenic strains were predominantly *Erwinia*-like but the above-mentioned profiles were also represented (**II**; Tables 3, 4, 5).

2. Clone differences

In Estonia (**II**), all fertilised clones sustained a higher dieback than their unfertilised counterparts. The plant density after seven years was especially low for clones 1, 90, 95 and 83-fertilised at Saare (**II**; Fig. 1), and for clones 1, 90 and 95 at Kambja. The highest percentage of INA bacteria was found in clone 95, followed by 83, 1 and 90 (**II**; Table 6). Clones 83, 90 and 95 also harboured the highest ratios of pathogenic strains. In other words, the most frost sensitive clones, namely clones 1 and 95, were the most damaged at both locations, along with clone 90, which has an intermediate frost resistance degree. Only one frost resistant clone (83), sustained high dieback, though only on fertilised plots. Besides, on all these clones, high percentages of INA and pathogenic strains were isolated, some of the strains also having both properties. The results obtained from the Swedish study were similar. The highest INA ratio was found on the most frost sensitive clone, Christina (**I**; Table 6). Pathogenic strains were also present on this clone at high percentages at both seasons, especially endophytically, coinciding with the clone high dieback rate (57%) (Nordh 2001, personal communication). A high number of pathogenic strains was also isolated from clone 83 (**I**; Table 6), this clone reportedly suffering from extensive bacterial damage. Interestingly, as in Estonia, high bacterial damage was also recorded on clones 1 and 90 in Swedish plantations (Granhall *et al*., unpublished).

From these two studies, it appears that a high frost sensitivity degree associated with significant INA and pathogenic plant colonisation is likely to lead to extensive field dieback. A possible explanation is that a frost-sensitive clone harbouring a high INA population will also be most likely to sustain more frost injuries. These in turn enable higher pathogenic colonisation, leading to severe dieback. Why certain frost resistant clones were nevertheless quite susceptible to bacterial infection (*e.g.* clone 83) remains to be clarified.

3. Seasonal dynamics

In Estonia, clones 83, 90 and 95 were sampled in both late March and late August at Saare. *Erwinia* and *Sphingomonas* profiles were found dominant in both seasons on the three clones. The number of INA strains as well as the average nucleation temperature increased between the two sampling times while the percentage of pathogenic strains remained stable at around 50% (of the strains tested). Seasonal comparison of the bacterial communities is made difficult by different cold-storage durations for the spring (11 weeks) and autumn (2 weeks) materials. Storage conditions have been reported to alter bacterial characteristics, *e.g.* ice nucleation activity (Dubrovský *et al*., 1989, Nejad *et al*. unpublished), which could explain the differences observed. More experiments are needed to determine whether the INA increase observed is due to an actual seasonal change, an expanding bacterial infection or only a result of different storage durations.

In Sweden, the autumn community was more diverse than the spring community (both epi- and endophytically), *i.e*. more bacterial profiles were isolated (**I**; Fig. 1a, 1b). In April, *Sphingomonas* and *Erwinia* types represented 50% of the total community while in October, although *Sphingomonas* remained the dominant profile, it was closely followed by *Bacillus*, *Erwinia* and *Xanthomonas* types. As opposed to Estonia, the number of INA strains decreased between spring and autumn. The ratio of pathogenic strains increased from 30% of the strains tested in spring to 49% in autumn. At both sampling occasions, *Sphingomonas* and *Xanthomonas* profiles showed high percentages of INA and pathogenic bacteria (**I**; Table 4). This study provides a starting point for the understanding of bacterial seasonal dynamics in *Salix*; more studies must be carried out to determine bacterial communities annual general variations.

4. Epiphytes *vs*. endophytes

While the Estonian study concerned only bacteria isolated from internal tissues, the Swedish study compared both the communities isolated from bark and internal tissues (*i.e*. epiphytes and "endophytes"). No differences between epiphytic and endophytic communities were found in terms of bacterial types. However, both INA and pathogenic endophytic communities appeared more stable than their epiphytic counterparts. While *Sphingomonas* and *Xanthomonas* profiles were dominant among INA and pathogenic endophytes in both seasons, the predominant profile among epiphytes shifted from *Erwinia* in April to *Sphingomonas* in October (**I**; Table 5). Moreover, the number of ice nucleation-

active strains decreased among epiphytes but remained stable among endophytes. The percentage of pathogenic strains increased among both endophytes and epiphytes.

Epiphytic communities are subjected to desiccation, cool temperatures and above all to a constantly fluctuating environment. On the other hand, endophytic bacteria are protected within the plant, from which they derive most (if not all) their nutrients. For these reasons, epiphytic communities undergo much more frequent and drastic changes than endophytes. Wilson (1995) suggested that some endophytes might have, at some point, a pathogenic association with their host. They might, for example, reside latent within plant tissue and only act as pathogen when the conditions are favourable (*e.g.* at low temperature). In this regard, endophytes and pathogens might not be completely opposed and the two terms not totally incompatible.

Conclusions and future perspectives

1. Final discussion

1.1 Comparison of the two studies

Apart from clone 83, the two studies presented in this thesis focused on different *Salix* clones. The techniques used (*e.g.* for bacterial isolation), as well as the aspects investigated (*e.g.* epiphytes *vs.* endophytes in **I**, fertilisation in **II**) were sometimes different. However, as several dissimilarities appear, some major common conclusions can also be drawn. First, plant density after seven years of cultivation is the same at Saare fertilised and Brunnby (*ca*. 1.65 plants/m^2, Nordh unpublished), where the trees also grow on fertilised mineral soil. *Sphingomonas* and *Erwinia* profiles were the dominant bacterial types isolated in both studies, even if not in exactly the same proportions. The main difference concerns the seasonal change in INA strains total number, which increased in Estonia but decreased in Sweden between spring and autumn. However, it must be noted that besides a possible influence of the storage duration in Estonia, the decrease observed in Sweden was mainly due to epiphytes, endophytes remaining stable. Pathogenic and INA communities were mainly *Erwinia*-like in Estonia; *Sphingomonas*- and *Xanthomonas*–like in Sweden.

Most importantly, results of both studies were in accordance with the field observations made on dieback differences between clones and fertilisation regimes. In other words, higher numbers of INA and pathogenic bacteria were found (1) in fertilised and in Kambja plots, which also had the highest mortality rates, and (2) on clones most dieback-affected in both countries. What is more, frost sensitivity, INA community and dieback rate were found to be interconnected at the clone level. These results, along with the fact that a large majority of our pathogenic strains were also able to nucleate ice, strongly indicate that the cause for *Salix* plantations dieback in both countries is an interaction between frost and bacterial infection.

1.2 Fertilisation issues

A number of studies have been carried out on the effect of fertilisation on development of frost resistance (von Fircks, 1996), dry matter productivity (Heinsoo, Sild & Koppel, 2002), or plant disease (Gallegly & Walker, 1949; Huber & Watson, 1974). All have emphasized the high response variability depending on the plant species, the nutrient applied, or even the plant part considered. Results from different studies are therefore not easily comparable and generalisations must be made very carefully. Nitrogen, for example, is essential for plant growth but has also often been reported to increase disease. However, its effect depends on the plant-pathogen association and in this regard, each disease must be considered separately (Huber & Watson, 1974). In poplar, nitrogen fertilisation has been found to lead to partial defoliation (Wait, 2002) and to affect plant-herbivores interactions (Glynn *et al*., 2002). For these reasons, the amount (and composition) of sludge or wastewater deposited on *Salix* plantations for purification should also be carefully monitored. In our case, a fundamental question that needs addressing is the threshold above which the dieback due to the increase of pathogenic and INA strains will outweigh the beneficial effect of fertilisation on productivity.

2. Importance of *Salix* and short-rotation forestry

At a time when global warming appears as the major issue humanity will have to deal with in the coming decades and with our environment becoming daily more contaminated by pollution, activities like biomass production, phytoremediation, wastewater purification or sludge decontamination appear not only attractive but also vital. The short-rotation cultivation of *Salix* has proved efficient in all these areas. Solving the dieback problems by understanding plant pathology is therefore of the utmost importance. Improvement of the plant frost resistance in order to limit yield losses has lately received great attention (Tsarouhas, 2002) but unless it is paralleled by the search for clones resistant to bacterial pathogens, the results could well be limited.

3. Applications of bacterial ice nucleation activity

Ice nucleation-active bacteria are much more than a source of plant injury. In fact, they are used in agriculture and industry for many purposes. During the past decade, considerable research has been devoted to the use of INA bacteria for biological control of insect pests (Lee, Costanzo & Lee, 1998; Castrillo *et al*., 2000). Indeed, the bacteria can elevate the insects' freezing point, thus reducing their ability to supercool and therefore their winter survival. To date, the main challenges have been to develop an efficient way of delivering the bacteria to the insects (spraying, feeding), have them retain their nucleating activity until low temperatures occur, and enhance their persistence in the target organism.

Industrial applications of INA bacteria include food processing, *e.g.* freeze concentration of milk and fruit juice where the bacteria are used to minimise the

number of nucleation events and form larger ice crystals (Watanabe & Arai, 1995). In artificial snow production, they reduce the amount of energy and water required and allow snow production at warmer temperatures.

4. Future investigations

Further research is needed to improve our understanding of the synergistic effect of frost with INA and pathogenic bacteria. The exact role of several factors affecting this interaction remains to be completely elucidated (*e.g*. fertilisation), along with the influence of long-term cold storage on the bacterial communities. The molecular and biochemical identification presented in this thesis, needs to be refined, especially for pathogenic and INA strains, in order to delimit precisely which species are responsible for the dieback. Moreover, the study of quorum sensing mechanisms may provide a better understanding of the regulation of *ina* genes *in planta* and explain how the (Ice^{+}) phenotype can constitute a competitive advantage for plant colonisation.

Via genome mapping of Quantitative Trait Loci (QTL), Tsarouhas (2002) demonstrated that freezing resistance and phenological traits in *Salix* were, at least in part, controlled by a common set of genes. On the contrary, acclimatised and non-acclimatised freezing resistance were reported to be, at least in part, controlled by different genes. Another report by Florin (2002) suggested that cold acclimation of *Salix* clones influenced their resistance to pathogenic bacteria. Should a link be established between these two studies, *e.g*. a relationship to be found between the QTL for freezing resistance on the one hand and pathogenic resistance on the other hand, this could lead the way to development of clones that are both frost and bacteria resistant.

References

Abe, K., Watabe, S., Emori, Y., Watanabe, M. & Arai, S. 1989. An ice nucleation active gene of *Erwinia ananas*. *FEBS letters* 258, 297-300.

Agrios, G.N. 1997. *Plant pathology*. 4th edition. Academic Press. 635 pp.

Anderson, J.A., Buchanan, D.W., Stall, R.E. & 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.

Andrews, P.K., Proebsting, E.L. Jr. & Gross, D.C. 1986. Ice nucleation and supercooling in freezing-sensitive peach and sweet cherry tissues. *Journal of the American society for horticultural science* 111, 232-236.

Aronsson, P., Heinsoo, K., Perttu, K. & Hasselgren, K. 2002. Spatial variation in above-ground growth in unevenly wastewater-irrigated willow *Salix viminalis* plantations. *Ecological engineering* 19, 281-287.

Bervaes, J.C.A.M., Ketchie, D.O. & Kuiper, J.C. 1977. Kinetics of freezing damage in apple bark and pine needles. *Physiologia plantarum* 40, 35-38.

Blondeaux, A. & Cochet, N. 1994. High-level expression of the ice-nucleation activity of *Pseudomonas syringae* in relation to its growth characteristics. *Applied microbiology and biotechnology* 42, 116-120.

Blondeaux, A., Hamel, J.F., Widehem, P. & Cochet, N. 1999. Influence of water activity on the ice-nucleating activity of *Pseudomonas syringae*. *Journal of industrial microbiology and biotechnology* 23, 514-519.

Buttner, M.P. & Amy, P.S. 1989. Survival of ice nucleation-active and genetically engineered non-ice-nucleating *Pseudomonas syringae* strains after freezing. *Applied and environmental microbiology* 55, 1690-1694.

Castrillo, L.A., Lee, R.E., Lee, M.R. & Rutherford, S.T. 2000. Identification of ice-nucleating active *Pseudomonas fluorescens* strains for biological control of overwintering Colorado potato beetles. *Journal of economic entomology* 93, 226-233.

Chanway, C.P. 1996. Endophytes: they're not just fungi! *Canadian journal of botany* 74, 321-322.

Chen, T.H.H., Burke M.J. & Gusta L.V. 1995. Freezing tolerance in plants: an overview. In: *Biological ice nucleation and its applications*. Lee, R.E., Warren, G.J. & Gusta, L.V. (eds). APS Press, pp. 115-130.

Chen, H.H. & Li, P.H. 1978. Interaction of low temperature, water stress and short days in the induction of stem frost hardiness in red osier dogwood. *Plant physiology* 62, 833-835.

Christersson, L. 1982. Energy forestry and frost hardiness. In: *Plant cold hardiness and freezing stress: mechanisms and crop implications*. Academic Press, pp. 605-613.

Christersson, L. 1996. Sustainable cultivation of broadleaved trees in a recycling community. *Swedish University of Agricultural Sciences, Department of Short Rotation Forestry, Report 57*. 7-13. ISSN 0282-6267.

Christersson, L. 1998. Theoretical background to and practical utilization of short-rotation forestry. *Swedish University of Agricultural Sciences, Departement of Short Rotation Forestry, Report 64*. 5-19. ISSN 0282-6267.

Crosse, J.E. 1959. Bacterial canker of stone-fruits. IV. Investigation of a method for measuring the inoculum potential of cherry trees. *Annals of applied biology* 47, 306-317.

de Kam, M. 1978. *Xanthomonas populi* subsp. *salicis*, cause of bacterial canker in *Salix dasyclada*. *European journal of forest pathology* 8, 334-337.

de Kam, M. 1982. Damage to poplar caused by *Pseudomonas syringae* in combination with frost and fluctuating temperatures. *European journal of forest pathology* 12, 203-209.

de Kam, M. 1984. *Xanthomonas campestris* pv. *populi*, the causal agent of bark necrosis in poplar. *Netherlands journal of plant pathology* 90, 13-22.

Deininger, C.A., Mueller, G.M. & Wolber, P.K. 1988. Immunological characterization of ice nucleation proteins from *Pseudomonas syringae*, *Pseudomonas fluorescens*, and *Erwinia herbicola*. *Journal of bacteriology* 170, 669-675.

Dubrovský, M., Petera, V., Sikyta, B. & Hegerova, H. 1989. Measurement of the ice nucleation activity of *Pseudomonas syringae* CCM 4073. *Biotechnology techniques* 3, 173-178.

Edwards, A.R., Van Den Bussche, R.A., Wichman, H.A. & Orser, C.S. 1994. Unusual pattern of bacterial ice nucleation gene evolution. *Molecular biology and evolution* 11, 911-920.

Fall, A.L. & Fall, R. 1998. High-level expression of ice nuclei in *Erwinia herbicola* is induced by phosphate starvation and low temperature. *Current microbiology* 36, 370-376.

Fall, R. & Wolber, P.K. 1995. Biochemistry of bacterial ice nuclei. In: *Biological ice nucleation and its applications*. Lee, R.E., Warren, G.J. & Gusta, L.V. (eds). APS Press, pp. 63-81.

Florin, L. 2002. Resistance and susceptibility of *Salix* clones to pathogenic INA bacteria. *Swedish University of Agricultural Sciences, Plant Pathology and Biocontrol Unit*.

Gallegly, M.E. Jr. & Walker, J.C. 1949. Plant nutrition in relation to disease development. V. Bacterial wilt of tomato. *American journal of botany* 36, 613-623.

George, M.F., Burke, M.J., Pellet, H.M. & Johnson, A.G. 1974. Low temperature exotherms and woody plant distribution. *HortScience* 9, 519-522.

Glerum, C. 1985. Frost hardiness of coniferous seedlings: principles and applications. In: *Evaluating seedlings quality: principles, procedures, and predictive abilities of major tests*. Duryea, M.L. (ed). Forest Research Laboratory, Oregon State University, Corvallis, pp. 107-123.

Glynn, C., Herms, D.A., Egawa, M., Hansen, R. & Mattson, W.J. 2002. Effects of nutrient availability on constitutive and rapid-induced herbivore resistance in poplar: a test of the growth/differentiation balance hypothesis (GDBH). In: *Abstracts: 3rd international poplar symposium on basic and applied aspects of poplar and willow biology, Uppsala, Sweden, August 26-29*. p. 126.

Goto, M., Huang, B.L., Makino, T., Goto, T. & Inaba, T. 1988. A taxonomic study on ice nucleation-active bacteria isolated from gemmisphere of tea (*Thea sinensis* L.), phyllosphere of vegetables, and flowers of *Magnolia denudare* Desr. *Annals of the phytopathological society of Japan* 54, 189-197.

Govindajaran, A.G. & Lindow, S.E. 1988. Size of bacterial ice-nucleation sites measured *in situ* by radiation inactivation analysis. *Proceedings of the national academy of sciences of the United States of America* 85, 1334-1338.

Green, R.L. & Warren, G.L. 1985. Physical and functional repetition in a bacterial ice nucleation gene. *Nature* 317, 645-648.

Gurian-Sherman, D. & Lindow, S.E. 1995. Differential effects of growth temperature on ice nuclei active at different temperatures that are produced by cells of *Pseudomonas syringae*. *Cryobiology* 32, 129-138.

Heinsoo, K., Sild, E. & Koppel A. 2002. Estimation of shoot biomass productivity in Estonian *Salix* plantations. *Forest ecology and management* 170, 67-74.

Hirano, S.S., Baker, L.S. & Upper, C.D. 1985. Ice nucleation temperatures of individual leaves in relation to population sizes of ice nucleation active bacteria and frost injury. *Plant physiology* 77, 259-265.

Hirano, S.S. & Upper, C.D. 1995. Ecology of Ice Nucleation-Active bacteria. In: *Biological ice nucleation and its applications*. Lee, R.E., Warren, G.J. & Gusta, L.V. (eds). APS Press, pp. 41-61.

Hirano, S.S. & Upper, C.D. 2000. Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae* – a pathogen, ice nucleus, and epiphyte. *Microbiology and molecular biology reviews* 64, 624-653.

Huber, D.M. & Watson, R.D. 1974. Nitrogen form and plant disease. *Annual review of phytopathology* 12, 139-165.

Isebrands, J.G., McDonald, E.P., LeBlanc, C. & Westphal, L.M. 2002. A phased phytoremediation strategy with *Populus* and *Salix* for Indian ridge marsh in the Calumet region of Chicago, Illinois, USA. In: *Abstracts: 3rd international poplar symposium on basic and applied aspects of poplar and willow biology, Uppsala, Sweden, August 26-29*. p. 172.

Kaneda, T. 1986. Seasonal population changes and characterization of ice-nucleating bacteria in farm fields of Central Alberta. *Applied and environmental microbiology* 52, 173-178.
Kempe, J. & Sequeira, L. 1983. Biological control of bacterial wilt of potatoes: attempts to induce resistance by treating tubers with bacteria. *Plant disease* 67, 499-503.
Kim, H.K., Orser, C., Lindow, S.E. & Sands, D.C. 1987. *Xanthomonas campestris* pv. *translucens* strains active in ice nucleation. *Plant disease* 71, 994-997.
Klement, Z. 1990. Inoculation of plant tissues. In: *Methods in phytobacteriology*. Klement, Z., Rudolph, K. & Sands, D.C. (eds). Akadémiai Kiadó, Budapest, pp. 104-106.
Klement, Z., Rozsnyay, Z.D., Balo, E., Panczel, M. & Prileszky, Gy. 1984. The effect of cold on development of bacterial canker in apricot trees infected with *Pseudomonas syringae* pv. *syringae*. *Physiological plant pathology* 24, 237-246.
Koppel, A. 1996. Above-ground productivity in Estonian energy forest plantations. *Swedish University of Agricultural Sciences, Department of Short Rotation Forestry, Report 57*. 81-86. ISSN 0282-6267.
Koppel, A. 1998. Short rotation crops in Estonia. *Swedish University of Agricultural Sciences, Departement of Short Rotation Forestry, Report 64*. 41-46. ISSN 0282-6267.
Koppel, A., Perttu, K. & Ross, J. 1996. Estonian energy forest plantations - General information. *Swedish University of Agricultural Sciences, Department of Short Rotation Forestry, Report 57*. 15-24. ISSN 0282-6267.
Kozloff, L.M., Schofield, M.A. & Lute, M. 1983. Ice nucleating activity of *Pseudomonas syringae* and *Erwinia herbicola*. *Journal of bacteriology* 153, 222-231.
Kuusemets, V. & Mauring, T. 1996. Wastewater purification in a willow plantation. The case study at Aarike. *Swedish University of Agricultural Sciences, Department of Short Rotation Forestry, Report 57*. 159-165. ISSN 0282-6267.
Larsson, S. 1998. Genetic improvement of willow for short-rotation coppice. *Biomass and bioenergy* 15, 23-26.
Larsson, G. & Bremer, B. 1991. Korgviden-nyttoväxter förr och nu. *Svensk botanisk tidskrift* 85, 185-200.
Ledin, S. Soil characteristic and production potential of willow on farmland. *Swedish University of Agricultural Sciences, Department of Short Rotation Forestry, Report 57*. 25-31. ISSN 0282-6267.
Lee, R.E., Costanzo, J.P. & Lee, M.R. 1998. Reducing cold-hardiness of insect pests using ice-nucleating active microbes. In: *Temperature sensitivity in insects and application in integrated pest management*. Hallman, G.J. & Denlinger, D.L. (eds). Westview, Boulder, CO, pp. 97-124.
Levitt, J. 1980. *Responses of plants to environmental stresses. I. Chilling, freezing and high temperature stresses*. 2nd edition. Academic Press, New York. 497 pp.
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, vol. 2*. Li, P.H. & Sakai, A. (eds). Academic Press, New York, pp. 395-416.
Lindow, S.E. 1983. The role of bacterial ice nucleation in frost injury to plants. *Annual review of phytopathology* 21, 363-384.
Lindow, S.E. 1990. Importance of phytopathogenic bacteria in biotechnology. In: *Methods in phytobacteriology*. Klement, Z., Rudolph, K. & Sands, D.C. (eds). Akadémiai Kiadó, Budapest, pp. 535-540.
Lindow, S.E. 1995. Control of epiphytic ice-nucleation active bacteria for management of plant frost injury. In: *Biological ice nucleation and its applications*. Lee, R.E., Warren, G.J. & Gusta, L.V. (eds). APS Press, pp. 239-253.
Lindow, S.E., Arny, D.C., Barchet, W.R. & Upper, C.D. 1976. Bacterial ice nuclei as incitants of warm temperature frost damage. *Proceedings of the American phytopathological society* 3, 224.
Lindow, S.E., Arny, D.C., Barchet, W.R. & Upper, C.D. 1978. The role of bacterial ice nuclei in frost injury to sensitive plants. In: *Plant cold hardiness and freezing stress*. Li, P.H. (ed). Academic Press, New York, pp. 249-263.

Lindow, S.E., Arny, D.C. & Upper, C.D. 1978a. *Erwinia herbicola*: an active ice nucleus incites frost damage to Maize. *Phytopathology* 68, 523-527.
Lindow, S.E., Arny, D.C. & Upper, C.D. 1978b. Distribution of ice-nucleation active bacteria on plant in nature. *Applied and environmental microbiology* 36, 831-838.
Lindow, S.E., Arny, D.C. & Upper, C.D. 1982. Bacterial ice nucleation: a factor of frost injury to plants. *Plant physiology* 70, 1084-1089.
Lindow, S.E. & Connell, J.H. 1984. Reduction of frost injury to almond by control of ice nucleation active bacteria. *Journal of the American society for horticultural science* 109, 48-53.
Lindow, S.E., Hirano, S.S., Barchet, W.R., Arny, D.C. & Upper, C.D. 1982. Relationship between ice nucleation frequency of bacteria and frost injury. *Plant physiology* 70, 1090-1093.
Lindow, S.E., Lahue, E., Govindarajan, A.G., Panopoulos, N.J. & Gies, D. 1989. Localization of ice nucleation activity and the *ice*C gene product in *Pseudomonas syringae* and *Escherichia coli*. *Molecular plant-microbe interaction* 2, 262-272.
Maki, L.R., Galyan, E.L., Chang-Chien, M.M. & Caldwell, D.R. 1974. Ice nucleation induced by *Pseudomonas syringae*. *Applied microbiology* 28, 456-459.
Makkonen, O. 1968. Roomalaisten taimitarhat. Summary: The nurseries of the Ancient Romans. *Silvae fennica* 2, 126-132.
Mazur, P. 1977. The role of intracellular freezing in the death of cells cooled at supraoptimal rates. *Cryobiology* 14, 251-272.
Nejad, P. & 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. & Granhall, U. 2004. Pathogenic ice-nucleating active bacteria in willows for short rotation forestry. *Swedish University of Agricultural Sciences, Plant Pathology and Biocontrol Unit*.
Nejad, P., Ramstedt, M., Granhall, U., Cambours, M.A. & Roos, S. 2003. Characterization and identification of endophytic ice-nucleation active (INA) bacteria in *Salix* plants. *Poster presented at the 8th international congress of plant pathology (ICPP) held in Christchurch, New Zealand, February 2-7* (www.forestresearch.co.nz/PDF/00.02Nejadetal.pdf; 10-08-2004).
Nemecek-Marshall, M., LaDuca, R. & Fall, R. 1993. High-level expression of ice nuclei in a *Pseudomonas syringae* strain is induced by nutrient limitation and low temperature. *Journal of bacteriology* 175, 4062-4070.
Newton, D. & Hayward, A.C. 1986. Ice nucleation activity of some reference cultures of *Pseudomonas syringae* and field isolates of bacteria from wheat and barley in Queensland. *Australasian plant pathology* 15, 71-73.
O'Brien, R.D. & Lindow, S.E. 1988. Effect of plant species and environmental conditions on ice nucleation activity of *Pseudomonas syringae* on leaves. *Applied and environmental microbiology* 54, 2281-2286.
Ögren, E. 1999. Fall frost resistance in willows used for biomass production. II. Predictive relationships with sugar concentration and dry matter content. *Tree physiology* 19, 749-754.
Olien, C.R 1964. Freezing processes in the crown of 'Hudson' barley (*Hordeum vulgare* L.). *Crop science* 4, 91-95.
Orser, C., Staskawicz, B.J., Loper, J., Panopoulos, N.J., Dahlbeck, D., Lindow, S.E. & Schroth, M.N. 1983. Cloning of genes involved in bacterial ice nucleation and fluorescent pigment/siderophore production. In: *Molecular genetics of the bacteria-plant interaction*. Pühler, A. (ed). Springer-Verlag, Berlin, Germany, pp. 353-361.
Palta, J. & Weiss, L.S. 1993. Ice formation and freezing injury: an overview on the survival mechanisms and molecular aspects of injury and cold acclimation in herbaceous plants. In: *Advances in plant cold hardiness*. Li, P.H. & Christersson, L. (eds). CRC Press, Boca Raton, Florida, USA, pp. 143-176.
Paulin, J.-P. & Luisetti, J. 1978. Ice nucleation activity among phytopathogenic bacteria. In: *Proceedings of the international conference on plant pathogenic bacteria, 4th*. Institut National de la Recherche Agronomique, Beaucouzé, France.

Perttu, K. 1996. Willow vegetation filters: Principles, results and economy. *Swedish University of Agricultural Sciences, Department of Short Rotation Forestry, Report 57*. 149-157. ISSN 0282-6267.
Rajashekar, C.B. & Burke, M.J. 1982. Liquid water during slow freezing based on cell water relations and limited experimental testing. In: *Plant cold hardiness and freezing stress – Mechanisms and crop implications, vol. 2*. Li, P.H. & Sakai, A. (eds). Academic Press, New York, pp. 211–220.
Rajashekar, C., Li, P.H. & Carter, J.V. 1983. Frost injury and heterogeneous ice nucleation in leaves of tuber-bearing *Solanum* species. *Plant physiology* 71, 749-755.
Ramstedt, M., Åström, B. & von Fircks, H.A. 1994. Dieback of poplar and willow caused by *Pseudomonas syringae* in combination with freezing stress. *European journal of forest pathology* 24, 305-315.
Rogers, J.S., Stall, R.E. & Burke, M.J. 1987. Low-temperature conditioning of the ice nucleation active bacterium, *Erwinia herbicola*. *Cryobiology* 24, 270-279.
Sabet, K.A. 1953. Studies on the bacterial die-back and canker disease of poplar III. Freezing in relation to the disease. *Annals of applied biology* 40, 645-650.
Sakai, A. 1965. Survival of plant tissue at super-low temperatures III. Relation between effective prefreezing temperatures and the degree of frost hardiness. *Plant physiology* 40, 882-887.
Sakai, A. & Larcher, W. 1987. Frost survival of plants: response and adaptation to freezing stress. In: *Ecological studies 62*. Springer-Verlag, Berlin. 321pp.
Salix kloninformation. 1994. SLU info, Håkan Johansson, Box 7072, S-75007 Uppsala, Sweden.
Schnell, R.C., Fall, R., Nemecek-Marshall, M., Sweeting, K. & LaDuca, R. 1991. A new INA bacterium from high altitude equatorial vegetation. In: *International conference on biological ice nucleation, 5th*. Madison, WI.
Sennerby-Forsse, L. 1994. The Swedish energy forestry programme. *Swedish University of Agricultural Sciences, Department of Ecology and Environmental Research, Report 50.* 19-21. ISSN 0282-6267.
Sigee, D.C. 1993. *Bacterial plant pathology: cell and molecular aspects*. Cambridge University Press. 325 pp.
Siminovitch, D., Singh, J. & de la Roche, I.A. 1978. Freezing behavior of free protoplasts of winter rye. *Cryobiology* 15, 205-213.
Single, W.V. & Olien, C.R. 1967. Freezing processes in wheat stems. *Australian journal of biological sciences* 20, 1025-1028.
Süle, S. & Seemüller, E. 1987. The role of ice formation in the infection of sour cherry leaves by *Pseudomonas syringae* pv. *syringae*. *Physiology and biochemistry* 77, 173-177.
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*. ISSN 1401-6249.
Vali, G. 1995. Principles of ice nucleation. In: *Biological ice nucleation and its applications*. Lee, R.E., Warren, G.J. & Gusta, L.V. (eds). APS Press, pp. 1-28
Verwijst T. 1996. Competition in short rotation forestry. *Swedish University of Agricultural Sciences, Department of Short Rotation Forestry, Report 57*. 71-79. ISSN 0282-6267.
Verwijst, T., Elowson, S., Li, X. & Leng, G. 1996. Production losses due to a summer frost in a *Salix viminalis* short-rotation forest in southern Sweden. *Scandinavian journal of forest research* 11, 104-110.
von Fircks, H.A. 1985. Frost hardiness of fast-growing *Salix* species. In: *Plant production in the North*. Kaurin, Å., Junttila, O. & Nilsen, J. (eds). Norwegian University Press, Oslo, pp. 199-204.
von Fircks, H.A. 1994. Frost resistance in *Salix*. *Doctoral thesis, Swedish University of Agricultural Sciences, Uppsala, Sweden*. ISSN 1401-6249.
von Fircks H.A. 1996. Impact of abiotic factors on frost resistance and cold acclimation in *Salix* species and clones. *Swedish University of Agricultural Sciences, Department of Short Rotation Forestry, Report 57*. 61-69. ISSN 0282-6267.
Wait, D.A. 2002. Nitrogen fertilization, growth rate trajectories and leaf developmental age tracking by folivores: predicting food availability for folivores and their impacts on

Populus growth. In: *Abstracts: 3rd international poplar symposium on basic and applied aspects of poplar and willow biology, Uppsala, Sweden, August 26-29*. p. 164.
Warren, G.J. 1987. Bacterial ice nucleation: molecular biology and applications. *Biotechnology and genetic engineering reviews* 5, 107-135.
Warren, G. & Corotto, L. 1989. The consensus sequence of ice nucleation proteins from *Erwinia herbicola*, *Pseudomonas fluorescens*, and *Pseudomonas syringae*. *Gene* 85, 239-243.
Warren, G.L., Corotto, L. & Wolber, P. 1986. Conserved repeats in diverged ice nucleation structural genes from two species of *Pseudomonas*. *Nucleic acid research* 20, 8047-8060.
Watanabe, M. & Arai, S. 1995. Applications of bacterial ice nucleation activity in food processing. In*: Biological ice nucleation and its applications*. Lee, R.E., Warren, G.J. & Gusta, L.V. (eds). APS Press, pp. 299-313.
Watanabe, N.M., Southworth, M.W., Warren, G.J. & Wolber, P.K. 1990. Rates of assembly and degradation of bacterial ice nuclei. *Molecular microbiology* 4, 1871-1879.
Weiser, R.L., Wallner, S.J. & Waddell, J.W. 1990. Cell-wall and extensin messenger RNA changes during cold acclimation of pea-seedlings. *Plant physiology* 93, 1021-1026.
Wilson, D. 1995. Endophyte: the evolution of a term, and clarification of its use and definition. *Oikos* 73, 274-276.
Wolber, P.K. 1993. Bacterial ice nucleation. *Advances in microbial physiology* 34, 203-237.
Wolber, P.F. & Warren, G.J. 1991. Evolutionary perspective on the ice nucleation gene-encoded membrane protein. In: *Microbial Ecology of Leaves.* Andrews, J.H. & Hirano, S.S. (eds). Springer-Verlag, New York, pp. 315-330.
Yankofsky, S.A, Levin, Z., Bertold, T. & Sandlerman, N. 1981. Some basics characteristics of bacterial freezing nuclei. *Journal of applied meteorology* 20, 1013-1019.
Yankofsky, S.A., Levin, Z. & Moshe, A. 1981. Association with citrus of ice-nucleating bacteria and their possible role as causative agents of frost damage. *Current microbiology* 5: 213-217.
Yazdani, R. 1992. Genetic variation and inheritance of metal and heavy metal tolerance in *Salix viminalis*. *Swedish University of Agricultural Sciences, Department of Short Rotation Forestry, Report 47.* 59-67. ISSN 0282-6267.
Zhao, J.L. & Orser, C.S. 1990. Conserved repetition in the ice nucleation gene *ina*X from *Xanthomonas campestris* pv. *translucens*. *Molecular and general genetic*s 223, 163-166.

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