Pathogenic and Ice-Nucleation Active (INA) Bacteria causing Dieback of Willows in Short Rotation Forestry

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Abstract

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To find out whether bacteria isolated from diseased plant parts can be the main causal agent for the dieback appearing in Salix energy forestry plantations in Sweden during the last few years, and if the joint effects of bacteria and frost injury are synergistic, extensive sampling of shoots from diseased Salix plants was performed. We performed several laboratory and greenhouse investigations and used evaluation techniques on the functions of the Ice-Nucleation Active (INA) bacteria.

We carried out a comparison between spring and autumn bacterial communities isolated from within (endophytically) and surface (epiphytically) plant tissues of Salix viminalis. Seasonal variation of bacteria in willow clones with different levels of frost sensitivity and symptoms of bacterial damage was also investigated.

We further focussed on possible effect of fertilisation and nutrient availability on the bacterial community in relation to plant dieback in Estonian willow plantations.

The identification and detection of INA bacteria which cause damage in combination with frost to willow (Salix spp) plants in late fall, winter and spring was performed using BIOLOG® MicroPlate, biochemical tests, selective INA primers and 16S rDNA analysis. To distinguish the character for differentiation between these bacteria morphologically and with respect to growing ability different culture media were used.

We studied the temperature, at which ice nucleation occurred for individual bacteria, estimated the population of INA bacteria, effect of growth limiting factors, and evaluated the effect of chemical and physical agents for disruption and possible inhibition of INA among individual bacterial strains. The concentration of carbon, nitrogen and phosphorus on INA is discussed.

We demonstrate that among the bacterial isolates recovered from the willow plantations, there were many that were capable of ice nucleation at temperatures between –2°C and –10°C, many that were capable of inducing a hypersensitive reaction in tobacco, as well as causing necrotic symptoms on willows exposed to frost treatment.

The most frequently isolated types were found to be non-fluorescent P. fluorescens (biotype A, B, C, F, G) and/or Sphingomonas spp. Erwinia spp, P. fluorescens, Xanthomonas spp and P. syringae however, were considered to be the most important pathogens in the field.

We conclude that diseases caused by INA bacteria in relationship with frost are a limiting factor in willow and poplar plantations in Sweden and most likely also in other temperate regions in the world.

Key words: *Salix viminalis*, INA bacteria, frost damage, ina gene primer, BIOLOG®MicroPlate, nutrient starvation.

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Contents

Appendix

Papers I - V

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

Nejad, P., Ramstedt. M., and Granhall. U. 2004. Pathogenic ice-nucleation active bacteria in willows for short rotation forestry. Forest Pathology, 34:369-381.

Cambours, M., Nejad. P., Granhall. U and Ramstedt. M. 2005. 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, 28: 15-27

Cambours. M. A., Heinsoo. K., Granhall. U and Nejad. P. 2005. Frost related dieback in Estonian energy plantations of willow in relation to fertilisation and pathogenic bacteria. (submitted).

Nejad. P., Ramstedt. M., Granhall. U., Roos. S. and McIvor I. 2005. Biochemical characterization and identification of ice-nucleation active (INA) willow pathogens by means of BIOLOG Microplate, INA gene primers and PCR based 16S rRNA analyses. (submitted).

Nejad, P., Granhall. U. and Ramstedt. M., 2005. Factors influencing pathogenic and Ice-Nucleation Active (INA) bacteria isolated from *Salix* plants, soil and litter. (submitted)

Introduction

Bacterial damage of different kinds (de Kam, 1987; Hirano & Upper, 1990) is a serious problem for most kinds of cultivated crops that could lead to significant yield loss and economic problems for the growers.

A somewhat neglected problem, except for certain agricultural crops, is the damage caused by ice-nucleation active bacteria (INA) that in combination with frost not only aggravate the frost damage but also predispose the plants for pathogen attacks with a much more serious outcome (Lindow, 1982; Ramstedt *et al*., 1994; Nejad *et al*., 2002, 2004; Cambours *et al*., 2005). A limited damage by frost on a perennial plant could after bacterial infection give substantial dieback and yield loss.

In this thesis we focus on damage to woody plants caused by pathogenic INA-bacteria that in combination with frost seem to cause serious dieback in plantations of short rotation forestry. Reference are made mainly to conditions in different geographical areas of Sweden and Estonia.

The target plant in this study is basket willow (*Salix viminalis*), that is the most common species in energy forestry. It is fast-growing, easy to propagate vegetatively, and highly adaptable to a wide range of climatic and soil conditions. Traditionally used in forestry and integrated with agricultural systems. Willows are more recently being used as renewable energy sources and for soil remediation in contaminated sites (Ledin & Willebrand, 1995). These characteristics, combined with the wide range of wood, fibre, fuelwood and other forest products and services they provide, have led to the widespread use of poplars and willows around the world (IPC, IPS, FAO).

According to earlier investigation, this plant is considered disease resistant. We believe that their resistance, potential severity or susceptibility to diseases has not been thoroughly investigated. Our studies, contact with other researchers and growers, and through participating in international conferences proved to us that problem resulting from bacterial infection as a joint effect with frost has not yet been fully recognized or even noticed. During the last few years sudden and unexpected plant dieback has appeared in *Salix* energy forestry plantations in Sweden (Fig. 1), especially in older plantations with high production and seemingly healthy plants. The symptoms are reminiscent of frost damage but the appearance of necrotic tissues indicates bacterial infections, which seem to spread further in the tissue and is in agreement with the finding of Christersson *et al*. (1992), of similar damages occurring during the winter season of 1990-91 in Swedish energy forestry stands.

(a)

(b)

(c)

(d)

(e)

Fig. 1. Experimental sites in Uppsala (a, b) and Brunnby (c) showing damages of Salix plants by INA bacteria as necrotic infection of stems and drying up of side shoots (d), until outright death of entire plants (e).

Historical background

Early history

Long before the advent of mankind, the willow or *Salix* thrived throughout the world. In America most of the native *Salix* species are traceable to pre-ice age origin. *Salix viminalis* has been used in ancient crafts for thousands of years. Willow (*Salix* spp.) has been cultivated in Europe for more than two thousands years (Pohjonen, 1984; Samils, 2001). When basket making became important throughout the Europe in the 18th century *S. viminalis* L., the basket willow or common oiser, was introduced into Sweden (Larsson & Bremer, 1991).

In the1960s, a future deficiency of pulp and timber supply in Sweden was predicted to occur in the mid 1990s (von Fircks, 1994). To overcome this problem willow husbandary was resurrected and modernized by professor emeritus Gustav Sirén to become the modern concept of energy forestry. The intention was to use the high growth potential of young coppice shoots of willows, aspen and poplar species, which had previously been considered to be ”weeds” (von Fircks, 1994). After the oil crisis in 1973 the use of fast growing willows (*Salix* pp.) in short rotation coppice as an alternative source of energy was initiated in Sweden (Siren *et al*., 1984, Samils, 2001). In Sweden research for use of short-rotation forestry as energy source has been going on since 1976 (Hurtado, 2001).

Recent history

Currently, *Salix* plantations used for energy production cover 18000 hectares in the central and southern parts of Sweden, mainly established on surplus agricultural land, the area no longer required for food production (Samils, 2001).

Short rotation coppice (SRC) has also been introduced in other European countries, as for instance, in the United kingdom, Finland, Estonia, Germany, Poland, as well as spread widely on the other continents (such as Chile, New Zealand, United states, Canada and China). Energy forestry has an average yield of about 10 tonnes per hectare and year in practical plantations (Christersson, 1999). A much higher growth potential, i.e. 20-35 tonnes dry matter per hectare and year has been reported from several experimental studies in Sweden and other countries (Ericsson, 1984; Christersson, 1987; von Fircks, 2000).

SRC plantations

Cultivation and management

Salix viminalis is very tolerant to different kinds of growing conditions. The more desirable growing conditions are soil with a pH of 6.0-7.5 (slightly acidic, neutral or slightly alkaline), moist or water logged soils. In practice most arable soils and positions form suitable growing sites.

Planting is generally performed with un-rooted cuttings and can be carried out when the plant is dormant, usually with cuttings harvested during the winter the same year and stored at –4^{o}C. It is always beneficial to remove weed and grass plants from the planting area, these can either be dug out, or sprayed using a herbicide containing Glyphosate. Weeds compete for light, nutrients and water, and may impede the establishment of willow cuttings. Cuttings should be spaced between 75 cm and 105 cm apart, assuming that the resulting plants are to be coppiced or pollarded in the future. Initial fertilizing is not nesessary unless the soil is very poor. Application of general, commercial fertiliser every other growing season is advantageous, in particular if the rods are being harvested annually. Harvesting the rods is done when the crop is dormant, in late autumn or winter, but before end of winter when the sap is beginning to rise. Harvest will remove all of

the previous seasons growth, leaving only a small stump of wood to provide the shoots for next year.

Salix for multiple uses

Willow offers a great potential as a source of renewable energy and for bioremediation and polluted environments. Willow grows mainly in temperate and wet areas, and a few species are native to the area where they grow. Environmental questions are gaining more and more ground also in the agriculture. Pollution of water systems, decreasing quality of ground water, heavy metal accumulation in the ground and the increase of carbon dioxide in atmosphere set new challenges for agriculture. The importance of agriculture as a producer of renewable raw materials for energy production and industrial use is increasing.

As an energy crop, there are a number of reasons to use *Salix*, e.g:

• providing a lot of biomass in a short period and being among the fastest growing woody species in northern Europe;
• can be grown with low inputs of agro-chemicals;
• easily established from un-rooted cuttings;
• re-sprout vigorously after each harvest;
• provide sizeable potential for genetic improvement;
• bring into energy equilibrium in the region of 20:1 (i.e. the energy obtained can be 20 times as much as the energy used to grow the crop);
• can be used as a vegetation filter during "bio-remediation" of waste water or contaminated land.
• can be used for protection of water quality
• can be used for increasing soil content of organic material.

For more information about willows plantations and management under Swedish condition refer to Dalin (2004), and seek information from Swedish Energy Agency´s (STEM), annual reports and Agrobränsle AB:

(http://www.agrobransle.se).

Frost and ice-nucleation

Frost

In nature the condition in which temperatures fall below 0°C is named frost (Tsarouhas, 2002). Frost can be caused by movement of air masses in a horizontal direction and the resulting energy exchange with the surroundings is termed advection (Geiger, 1971; Tsarouhas, 2002). This type of frost is more drastic and is not influenced by the topography of the site concerned (Sakai & Larcher, 1987). Advection of large cold air masses from the north, particularly during spring and autumn, brings about drastic falls in temperature in large regions (Day & Peace, 1946; Biel, 1961; Christersson & von Fircks, 1990). Climatic factors such as

cloudiness, fog or mist will prevent the occurrence of advection frost (von Fircks,1994)

According to Christersson *et al*. (1992), frost is very common in Sweden also during the summer. Two kinds of summer frost exist; one is called advection frost that is caused by cold air coming down over the country from the north, and another is an inversion frost caused by long-wave radiation from the ground, taking place during calm, clear nights. In this way the air closest to the ground is cooled. Radiation frosts are often ground frosts, which rarely damage shoots above 1 to 2 m, while advection frosts, or interaction of radiation and advection frosts, may also damage growing shoot parts above 2 m.

Fast-growing shoots of *Salix* species has a long elongation zone and this explains why a fast-growing shoot is more severely damaged than a slow growing one at the same frost temperature. Two-year old or older shoots of different *Salix* species have never been damaged by summer frost because frosts do not reach the elongating zone. According to von Fircks (1994) frost damage occurs both during spring and autumn. In general, frost damage is restricted to the most distal shoot parts of the current year's growth. He explains that the apical meristem and top leaves show the first signs of frost damage. He also explains that frost during late summer and early autumn may cause dieback of distal stem parts, and defoliation, so that the leaves, which are the receptor sites that sense the photoperiod, may be lost. Ultimately leaf fall may hinder cold acclimation. Thereafter, immature stem parts (Fuchigami *et al.,* 1977) entering the winter dormancy phase will be killed by low temperatures. Frost damage mostly occurs in growing soft shoots in sites that are newly-planted or have newly been harvested (von Fircks, 1994).

It is clear that the interaction of the biological and physical environments of plants determines the extent of plant stress. Ice formed in or on frost sensitive plants spreads rapidly both intercellularly and intracellularly, causing mechanical disruption of cell membranes (Levitt, 1972; Burke *et al*., 1976). This disruption is usually manifested as a flaccidity and /or discoloration upon rewarming of the plant. Thus, most frost-sensitive plants have no significant mechanisms of frost tolerance and must avoid ice formation to avoid frost injury (Lindow, 1983). Since frost sensitive plants must avoid ice formation to avoid frost damage, frost injury to these plants might be considered a quantal response –either a plant part escapes ice formation or it does not. The extent of supercooling of plants in nature is known to be generally low (less than 2 to 3 degrees supercooling). Therefore, avoidance of ice formation is not concidered an important frost injury avoidance mechanism for plants under field conditions (Levitt, 1972).

Frost hardiness in plants

Frost hardiness in perennial plants varies widely during the year (Parker, 1955; Glerum, 1973; Sarvas, 1974; Fuchigami *et al*., 1982; Cannell & Sheppard, 1982; Koski, 1985; Christersson & von Fircks, 1990). According to Christersson *et al*. (1992) growing willow shoots are damaged at –2°C in spring and summer although, dormant shoots in winter tolerate temperatures of –80°C or lower (von

Fircks, 1992). Spruce, willow and birch shoots collected in the winter and frozen to –50, –84, –230 °C respectively (Sakai, 1956; Christensson & Krasavtsev, 1972; von Fircks, 1992), then thawed and placed in water, produced leaves or started to grow and did not exhibit any sign of damage. We therefore hypothesize that freezing alone is not the main cause of severe damage to such plants and if such damage or dieback occur there must be a synergistic effects between frost and bacterial infection (I, Fig. 2). Without bacteria involved, freezing damage on actively growing shoots is usually limited to the tip or outer branches, and the plant will recover.

The freezing process in plants

Ice crystal formation is supposed to take place inside plant tissues after supercooling to –2°C or to –3°C (Christersson & Sandstedt, 1978). In a tissue, ice nucleation can occur either in the cell inside the plasmalemma (intracellular ice crystal formation), or in the intercellular spaces between the cells. The latter process is called extracellular ice crystal formation (Levitt, 1980).

Intracellular ice crystal formation is supposed to be always lethal, probably due to mechanical destruction of biomembranes resulting from the rapid growth of ice crystals in the protoplast (Coger & Toner (1995) as reviewed by Fahy (1995). Christersson (1971) suggested that frost damage in plants when they are in a growing state could be caused by intracellular ice crystal formation since growing shoots loose their turgor completely, look very flaccid, and become totally dead, brown to black coloured, as soon as they are thawed after freezing. Most scientists believe that all ice crystal formation under natural condition occur as extracellular formation which takes place in the intercellulars (Sakai & Larcher, 1987). When this happens water is transported from the cell to the intercellular where it freezes. According to Pearce (2001) plants and plant parts freeze when they cannot avoid nucleation and cannot prevent the growth of ice (Levitt, 1980; Franks, 1985).

Laboratory tests often gives lower nucleation temperatures than those reported under natural conditions and can be unreliable indicators of behaviours in the field (Ashworth *et al*., 1985; Flinn & Ashworth, 1994). Ice can enter plants through stomata and hydathodes. Intrinsic nucleation of freezing can also occur (Pearce, 2001). Several of these causes may be due to the presence of Ice Nucleation Active (INA) bacteria (see below).

According to Christersson *et al*. (1992) there are no doubt that, except for the well known Water Mark disease caused by *Erwinia salicis*, most other bacterial diseases in willow are probably combined with frost. Consistent with his observation and our results there is a lower limit to freeze avoidance for plants and their organs and tissues when bacteria are present. Pearce (2001), in his investigation explains that even for single cells, if they have not frozen or been freeze-dehyderated at a higher temperaute, will freeze internally near –4°C (the homogeneous nucleation point being depressed by solutes). However, cells with highly viscous contents, such as any cells dehyderated by growth of extracellular ice are likely exceptions. These may form a glass (vitrify) rather than freeze

(Franks, 1985). Vitrification occurs in cells of deeply frozen poplar (Hirsh *et al*., 1985), and this may explain why some tree species can survive temperatures down to liquid nitrogen and liquid helium temperatures (Sakai & Larcher, 1987).

Nucleation sites determine the survival of frozen cells and defines slow and rapid cooling. Three possible fates can befall a cell during cooling to cryogenic temperature. Each fate is defined (Fig. 2). by the nature of the nucleation process involved. If nucleation is exclusively extracellular, the cell shrinks and tends to survive. If nucleation is intracellular, ice crystals form inside the cell and damage its internal structure usually leading to cell death. The third fate is that the nucleation fails to occur either intracellularly of extracellularly-instead of freezing, the cell and its surrounding medium pass into the glassy state i.e. they vitrify (Fahy *et al*., 1984). Very often vitrified cells survive on warming (Fahy, 1988). According to Mazur (1963) the cooling rate affects nucleation sites primarily for two reasons. First, the extracellular space is uniformly found to contain heterogenous nucleators of thus far undefined nature that are more effective than any putative intracellular nucleators may be (Franks *et al*., 1983; Rall *et al*., 1983). According to Rasmussen *et al*. (1975), consequently, in almost any biologically relevant situation with short or ultrarapid cooling, crystallization will always begin extracellularly. Second, the ability of the cell to respond osmotically to extracellular freezing is not instantaneous.

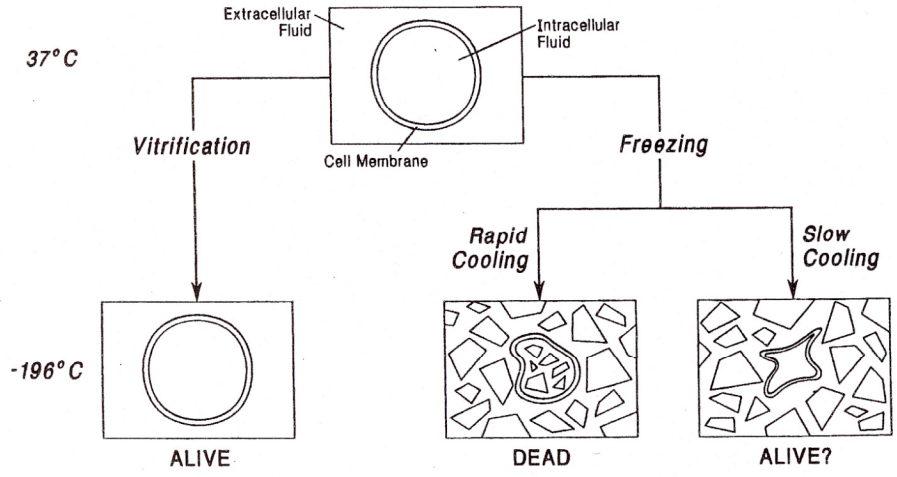


Fig. 2. Physiochemical process during cryopreservation of the cells. Freezing can occur exclusively in the extracellular space (right), it can occur in both the extracellular and the intracellular space (middle), or it may occur not at all (left). Adapted from Coger and Toner (1995) in Fahy (1995).

Freezing resistance

The freezing resistance include two main components, freezing avoidance and freezing tolerance. Freezing avoidance referes to the ability of any tissue of a plant

to prevent ice formation (von Fircks, 1994; Tsarouhas, 2002). Examples of freezing avoidance include: supercooling (cooling below the cells freezing point without immediate freezing), absence of free water and lowering of the freezing point by antifreeze substances. On the other hand, freezing tolerance is the ability of living tissues (cells) to resist the internal level and can be distinguished into two types: a, avoidance of freeze-induced dehydration and b, tolerance of freeze-induced cellular dehydration. (Tsarouhas, 2002; Lindow *et al*., 1978a,1982).

Plants may be arranged broadly into two groups with respect to plant cold hardiness, those that are frost hardy and those that are frost sensitive (Lindow, 1982). Many plants including conifers, many broad leaf perennials and some annual plants such as cereals grains and cabbage are generally considered to be frost hardy (Burke *et al*., 1976; Chandler, 1958; Levitt, 1972; Mazur; 1969; Olien, 1967). These plants can somehow restrict ice formation to the intercellular spaces. Water from the cell moves from the cytoplasm and freezes in equibrilium with ice in the intercellular spaces (Burke *et al*., 1976; Kaku, 1975). Intracellular ice formation is lethal to cells of these plants (Lindow, 1983b). Some plant parts of frost tolerant plants can be extensive and avoid damaging (Burke, 1976). Ice spreads intercellularly and intracellularly in these plants and cause mechanical disruption of cell membranes (Burke, 1976; Kaku, 1975). Extracellular ice formation occurs in the intercellular spaces (Levitt 1972; Mazur, 1969). The maximum degree of freezing resistance is different among plant species (Tsarouhas, 2002). Generally over-wintring crops can survive about –30°C and annual plants, for example *Arabidopsis thaliana*, can tolerate temperatures down to –10°C (Levitt , 1980; Gilmour *et al*.,1988; Wanner & Junttila, 1999), (this field was studied and reviewed by Tsarouhas, 2002). Woody plants can survive temperatures as low as –50°C after acclimation and some species like *Populus* and *Salix* can withstand the temperature of liquid nitrogen at their dormancy (Sakai, 1970).

Ice nucleation activity

Once the wet bulb temperature is known, there must be a way to predict whether water droplets will actually freeze at that temperature. Ice is the result of a liquid (water) becoming a solid (ice) by an event called nucleation (Fig. 3). In order to freeze, a water droplet must first reach its nucleation temperature. There are two types of nucleation, homogeneous nucleation and heterogeneous nucleation.

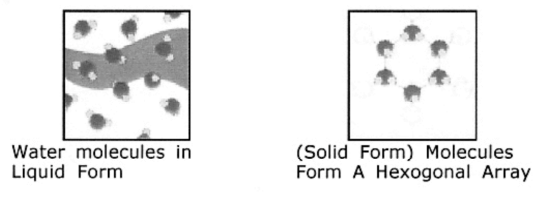


Fig. 3. Water –ice phase transition (snowYork).

Two general types of ice-nuclei exist. At warmer temperature, nonaqueous catalysts for ice formation known as heterogenous ice nuclei are required for the water–ice phase transition. Mineral particles are heterogenous, particularly silver iodide (Vonnegut, 1949). These mineral particles efficiently nucleate ice at temperatures lower than –8°C. Kaolinite is among the most active mineral ice nucleus sources. Silver iodide is used in weather modification studies as a cloud seeding agent. Crystals of several organic compounds also have ice nucleation activity (Lindow, 1983).

Catalysts for the water-ice phase transition are known as ice nuclei (Lindow, 1983). Once ice has formed the ice crystals accumulate moisture and grow in size and mass until they fall as either rain or snow depending on the temperature of the atmosphere near the ground. Water molecules come together to form a stable ice nucleus, either spontaneously (homogeneous nucleation) or when catalysed so to do by another substance (heterogeneous nucleation). Homogeneous nucleation is unlikely at temperature just below 0°C but, in contrast, in moist climate, heterogeneous nucleation is difficult to avoid (Pearce, 2001). To function, a potential heterogeneous nucleator must be in contact with water. Consequently, if the plant surface is dry, extrinsic nucleators will be ineffective. However, during radiation frosts in many climates, moisture will tend to condense onto plants surfaces so giving an opportunity for any heterogeneous nucleators present on the surface to function (Pearce, 2001).

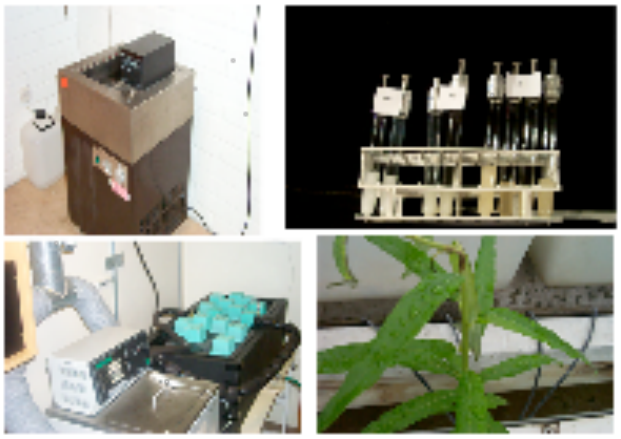
Ice nucleation test

The ice nucleation properties of isolated bacteria in this thesis were studied in a tube nucleation test (I, II, III & V). Deep frozen cultures of bacteria were revived on TSA for a period of 24-48 hrs, at room temperature (20-25°C) and recultured on TSA after checking purity. From a cell concentration of approx 10^9 colony forming units (cfu) /ml,10 to 50 µl of bacterial suspensions were transferred to test tubes (n=5) containing 9 ml sterile ice-nucleous free buffer (0.01 M KH_2PO_4, pH7.0) vortexed and incubated on a shaker for10 minutes before placing the tubes in a refrigerated bath (Hetofrig, Birkeröd, Denmark), filled with 96% alcohol. The temperature was lowered at a standard rate of 1°C for every half an hour and freezing was recorded from –1°C to –9°C or lower (–16°C).

For the freezing procedure (I, II, III) in *Salix* seedlings/cuttings (Fig. 4 a, b, c, d) the plants were placed in large test tubes in an upside-down position during the treatment to cool the plant material in dark, while the roots parts stayed in room temperature (Fig. 1c) (Ramstedt *et al*. 1994). When cuttings were used they were placed in an upright position in the test tubes. The plant material was then rewarmed to 4.0$\pm$ 1°C, remained in freezing bath for about 18-24 hrs in dark at 16°C and then acclimated for another few hours in dark before transferring them to greenhouse conditions.

Harvey (1918) was among the first to use refrigerators for testing plant materials. Since that time, increased technical precision has been achieved, Commercial programmable freeze chambers and cooling baths with high

performance are nowadays economically feasible. Most commercial air-cooled systems can reduce the temporal and spatial temperatures variation to within + 0.5°C (*e.g.* Arctest AB, Finland; Weiss Technik AB Germany) and liquid systems (ethanol) are able to maintain a test temperature within 0.1°C or better (e.g. Hetofrig, Danmark; Lauda Germany) (von Fircks, 1994), (Fig. 1d)



(a) (b)
(c) (d)

Fig .4. Shows the procedure for testing of ice-nucleation activity including subsequent pathogenicity tests of willow isolated bacteria: a, freezing bath for INA; b, ice nucleation test of isolated bacteria; c, subjecting inoculated plants to frost treatment using programable refrigerating bath; d, necrotic infection of Salix plant stem as a result of synergistic effects between bacteria and frost.

Ice-nucleating active (INA) bacteria and pathogenicity

Bacteria as ice-nucleators

Frost damage to crops is a serious problem which may cause major reduction in yield. For example, an early frost in august 1983 reduced the grain yields in parts of the Canadian central prairie provinces by 15 to 25%. Such damage may occur at temperatures only slightly below 0°C because of the presence of ice-nucleating bacteria (Kaneda, 1986). The presence of ice-nucleation bacteria may determine whether plants are severely damaged by periods of low tempeature (-2 to –10°C) which otherwise would not be harmful. According to Gross *et al.* (1984), the temperature at which ice formation occurs depends on the presence of active ice nuclei, and the temperature at which ice nuclei are active. Deciduous fruit trees are highly vulnerable to frost injury at temperatures between –2 and –5°C during bloom and post bloom stages of developments (Proebsting & Mills, 1978). The extent to which INA bacteria raise the temperature to which frost-sensitive flower bud tissue can supercool may increase tissues vulnerability to freeze injury. To establish such a role to INA bacteria, however, flower buds supercool to relatively low temperatures (*i.e.* –7°C) without injury in the absence of high populations of INA bacteria (Gross *et al.*, 1984).

Bacteria were first shown to act as ice nucleating agents by Maki *et al*. (1974). They found that as the concentration of *P. syringae* cells inceased, the temperatures at which freezing occurred became warmer. Arny *et al*. (1976), showed that susceptibility to frost damage in corn was increased after application of *P. syringae* to leaves. According to Lindow *et al*. (1982); Kaneda (1986), *P. syringae* and other epiphytic bacteria have been found to incite frost damage on numerous plants species and cultivated crops. A single ice nuclus is sufficient to initiate ice formation and subsequent frost injury to entire leaves, fruits, flowers, depending on the degree of restriction of ice propagation within the plant (Single & Olien, 1967).

INA bacteria commonly found on plants comprise strains of several species (I, II, III, IV, V) that produce a protein able to nucleate freezing at temperatures as high as –1°C. However, the presence of INA is not a universal explanation of nucleation of ice in plants. Sizes of INA bacterial populations (V) vary greatly between plant species, sites, climates and seasons (III), and only a small percentage of cells in a population are effective nucleators (Lindow, 1990). He argued that a very small population of INA bacteria could nucleate freezing throughout citrus trees since once nucleated freezing would spread rapidly. However, this argument would not carry to herbaceous plants, where each leaf (in grasses) or main shoot (in a dicot) would freeze separately because of the higher temperature of the crown compared to the leaves (Fuller & Wisniewski,1998; Pearce & Fuller, 2001).

The INA bacteria promote frost damages (I, II, III) in late fall, winter and spring, and if pathogenic they can initiate infestation after a massive increase in connection with plant tissue/bark cracking. Few studies have so far, however, actually shown that freezing injury in wooden perennials as willows and poplar (Ramstedt *et al*., 1994; Nejad *et al*., 2002, 2004; Cambours *et al*., 2004) can be caused by INA-bacteria or be part in the development of bacterial canker (Lansade, 1946; Sabet, 1953; Dong *et al*., 2001). Our earlier research in Sweden and other reports (Vali, 1971; Lindow , 1982; von Fircks & Verwijst, 1993; Ramstedt *et al*., 1994; Dong *et al*., 2001), support the hypothesis that pathogenic bacteria with INA properties could be an important factor causing frost related damage also in woody plants.

Effects of seasonal variation and geographical area on INA bacteria

All healthy plants are colonized by at least some bacteria and fungi (Leben, 1965). The diversity and magnitude of such microbial populations on plants differ, however, widely between plant species and geographical area (Lindow *et al*., 1978). The presence of relative concentrations of ice-nucleating –active (INA) bacteria varies in and between geographic locations and fluctuates over time (Gross *et al*., 1983; Lindow, 1982). According to Köppen (1954), the Earth is divided into six principal climatic zones depending on seasonal temperature and variation in precipitation pattern and physiography. In each case the ice nuclei content followed the climatic trend.

Epiphytic and endophytic bacteria

The old meaning of epiphyte is a plant that derives moisture and nutrients from the air and rain; usually grows on another plant but is not parasitic on it. The new meaning of epiphyte includes microorganisms that live on plant surfaces (Anonymous). The old meaning of endophyte is a plant that lives within a plant. The new broader meaning is an organism that lives within a plant. Endophytes are being found in leaves, stems, and roots (*e.g.* mycorrhiza). Endophytes are generally beneficial; organisms that are not beneficial are called pathogens. Many endophytes can be either/or, according to the situation, lacking clarity send confusing signals to many researchers. Many times the positive aspect of the endophyte can be failed to perceive when having active interest in looking for pathogens.

Epiphytic populations of INA-bacteria can constitute an inoculum source and be essential for the development of infection when temperatures reach slightly below zero (Hirano *et al*., 1982; Gaignard & Luisetti, 1993). Also, as stated earlier, endophytically appearing bacteria (Nejad & Johnson, 2000) 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 known from several other bacterial species.

The ice nucleation activity and ability for epiphytic and endophytic survival are important properties that are associated with these species. Epiphytic survival, expressed by a capacity to colonize aerial parts of plants or by a significant epiphytic multiplication characterizes most pathovars. High level of epiphytic populations can be recovered in the spring and also in the autumn on perennial plants. The epiphytic populations constitute as an inoculum source and are essential for the development of infection (Gaignard & Luisetti, 1993). Epiphytic communities are subjected to desiccation, cool temperatures and above all to a constantly fluctuating environment. On the opposite, endophytic bacteria are protected within the plant, from which they derive most (if not all) of their nutrients. For these reasons, epiphytic communities undergo much more frequent and drastic changes than endophytes. Consistent with results of Nejad & Johnson (2000), it has been shown by Wilson (1995) that there is possibility that to some extent some endophytes might have 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.

The biology and population dynamics of the plant pathogens are still unclear but endophytic opportunistic bacteria might reside latent in the plant tissue, act pathogenic and induce necrosis when temperature and other conditions are suitable. In many plants endophytic bacteria have been reported to have a unique ability to survive inside the plants with little or no microbial competition In our earlier research work (Nejad & Johnson, 2000) using endophytic bacteria as a biological weapon against soilborne fungal pathogens, we found that these bacteria

could create a barrier against pathogens. But our present work has also established that endophytic bacteria can develop pathogenic characteristics under certain environmental conditions such as frost. According to Christersson *et al.* (1992), latent infections by mycoplasma-like organisms (MLO) or endophytes could on the other hand be a more serious threat to future energy forest plantations especially since they could easily be distributed between countries, in cutting material and seedlings without being detected. Our investigations also support his hypothesis (I, II).

Bacteria that live on plant surfaces are considered to be distinct from other plant-associated bacteria and probably have acquired adaptation which allow them to tolerate physical and chemical barriers. Bacteria such as *E. coli* and *Rhizobium* spp. can achieve and maintain a large population size on leaves kept continuously moist under controlled conditions, but their population sizes greatly decrease upon drying of the leaf surface (Lindow, 1993). Our epiphytic INA bacterial species, such as *P. syringae* and *Sphingomonas* were shown to have large population sizes in most environmental conditions which make us believe that the ability of bacteria to survive under the harsh environment is not the only answer but that these INA bacteria may have special adaptations that only they posses. For example, they must be able to cope with rapid changes in water availability, high fluxes, UV light, and other ionizing and non-ionizing radiation, rapid changes in temperature and sometimes low nutrient concentrations (V).

Electrolyte leakage

Assessment of electrolyte leakage is a quantitative method which determines the amount of electrolytes leaking out of cells following exposure to heat or freeze induced stress (Palta *et al.*, 1977a; Rajashekar *et al.*, 1979; Sutinen *et al.*, 1992). An enhanced electrolyte leakage has been associated with increased cell injuries or death (Palta & Li, 1978). Results presented by Palta and co-workers (1977a, 1977b), made it clear that even freeze impaired cells which are alive show leakage of ions and sugars. The poplar bacterial canker disease resulting from ice–nucleation bacteria (Dong *et al.*, 2001). They showed that after the poplar trees were inoculated with INA bacteria, the moisture content of bark decreased but relative turgidity increased and electrolyte effusion rate increased and had a peak at temperatures of –4 and –5°C. Studying effect of winter stress on electrolyte leakage has become very popular (Fuchs *et al.*, 2000). Relationship between chilling injury and electrolyte leakage in cold storage and the conductivity of electrolyte leakage as a measure parameter of chilling injury has been studied by many researchers (Sukumaran & Weiser 1972; Zudo *et al.*, 1983). Chang *et al.* (2001), provided evidence that cold acclimation provide protection from chilling injury. Three days of acclimation at 10°C maximized mungbean seedling tolerance to chilling at 4°C. Acclimation significantly decreased the leakage of solutes and cations from the leaves of seedlings chilled at 4°C. According to Ma *et al.* (1990) and Chen *et al.* (1991), prolonged exposure to low temperatures increase the leakage of solutes, such as soluble sugars, free amino acids and cations, from the leaves. In willows, one effect of cold acclimation is the transformation of sugars into trehalose and sucrose (Tsarouhas, 2002).

In our preliminary study on effect of cold storage on INA bacteria followed by reisolation of bacteria from *Salix* plants which had been stored in cold storage (at –4°C) the INA of reisolated bacteria diminished after being stored (Nejad, unpublished). Furthermore, Maki *et al*. (1974) in his work explains that INA of harvested *P. syringae*, *P. fluorescens* and *E. herbicola* was not stable at 4°C even for 24 hrs. Here it deserves to point out that in our case cold storage (rather cold acclimation) reduced the INA, most probably because the electrolyte leakage was diminished, but the pathogenicity of the bacteria remained unchanged. Moreover, in our other study (II) the highest amount of INA pathogenic strains was found on clone Christina. Considering that this clone is very sensitive to frost, its INA community is likely to cause more serious damage by forming ice, i.e. to release more nutrients (electrolytes), giving pathogens the opportunity to attack and multiply (Dong *et al*., 2001; Granhall *et al*., unpublished). A high number of pathogenic strains were also isolated from clone 183 (II), this clone reportedly suffering from extensive bacterial damage. One explanation for this varying pattern of bacterial communities is probably a constitutive difference in electrolytical leakage of different *Salix* clones (Tsarouhas, 2002). Interestingly, as in Estonia (III), high bacterial damage was recorded on clones 1 and 90 also in Swedish plantations (Granhall *et al*., unpublished). In another preliminary experiment with inoculating bacteria on 15 *Salix* clones (5 frost susceptible, 5 medium and 5 frost resistant) we observed a high correlation of frost sensitivity and electrolytic leakage as well.

Chemical and molecular basis for ice nucleation

Several reports on the location and partial characterization of compound(s) responsible for ice nucleation activity have appeared (Lindow, 1983). Historically, the frequency of expression of the INA^+ phenotype in pure cultures of ice nucleation-active bacteria has been shown to be highly variable (Maki & Willoughby, 1987). The source of this variability in the frequency and level of INA^+ expression has been attributed to physiological responses of the INA^+ bacteria to nutritional and environmental signals. Hirano *et al*. (1985) showed that the ratio between the number of ice nuclei and the number of bacterial cells in a culture can vary with incubation temperature, growth medium composition, culture age, and genotype nucleation frequencies appear to be most dramatically impacted by temperature. As INA^+ bacteria progress through a growth cycle in culture, key growth-promoting nutrients are consumed at stoichiometric (ratio or quantities of substances) levels. Depletion of key nutrients signals the end of the active growth phase and progression into a stationary growth phase.

According to Fall & Wolber (1995), biochemical characterization of bacterial ice nuclei in INA bacteria has been hampered by a variety of factors for example: ice nuclei active at the warmest temperatures (i.e., –1.5 to –5°C) are destroyed by cell disruptions (V). The ice nucleation activity of *P. syringae*, *E. herbicola* and *P. fluorescence* is associated with the intact bacterium of these species and was not detected in extracellular by-products of these bacteria (Maki *et al*., 1974; Yankofsky, 1981). Several pieces of evidence indicate that the ice nucleating material in these species is membrane-bound and not a soluble cell component.

The work by Sprang & Lindow (1981) indicates that the ice nucleating material in *P. syringae* and *E. herbicola* is located in or on the outer cell membrane of these gram-negative bacteria. Ice nuclei active at temperature of –4°C or higher are eliminated by treatment of cells with respiratory inhibitors or with many reactive chemicals such as borate, urea, extreme pH, or by disruption of the cells by physical processes or phage lysis. However, the, same treatments do not affect the numbers of ice nuclei active at colder temperatures, e.g. (lower than -7°C). It appears that with a few possible exceptions, bacterial ice nuclei active at temperatures warmer than –4°C require a physically intact or physiologically normal cell for their expression (V), while those active only at colder temperature do not. Fall & Wolber (1995), also report that ice nucleation frequency is dramatically affected by growth conditions (V), (Maki *et al*., 1974; Yankofsky *et al*.,1981; Lindow, 1982). Whether ice nuclei active at different temperatures represent different substances or collections of compounds, or simply an alteration in their conformation (forma anpassa) environment and thus a different threshold ice nucleation ativity, is as yet unclear.

Localization of ice nucleating sites in or on the bacterial cells has become an important question. Lindow *et al*. (1989), has studied the localization of ice nuclei and reviewed the early evidence implicating cellular membranes in ice nucleus function. He found that ice nucleation is associated with the cell envelope and not the soluble cell contents and that ice nucleation of intact cells is sensitive to agents such as lipases, phospholipases, detergents and solvents that disrupt membranes. Phelps *et al*. (1986), demonstrated that natural bacterial ice nuclei are generally localized on the gram negative outer membrane but Wolber *et al*. (1986), demonstrated that active ice nuclei can also be assembled on the inner membrane.

The nucleating material in isolated membranes of both *P. syringae* and *E. herbicola* is sensitive to protease and protein denaturing agents, including sulfhydryl reagents (Kozloff *et al*., 1983), suggesting that an outer membrane protein determines or is involved in ice nucleation in these species. Genetically induced lipid changes in fatty acid auxotrophs of *P. syringae* also affected the ice nucleation activity of this bacterium. Some evidence exist from the study of antifreezing glycoproteins (Feeney & Yeh, 1978), that more than one substance, such as protein and carbohydrates or protein and lipids, may be required for ice nucleation. More studies of the genetic determinants of ice nucleation in *P. syringae* and *E. herbicola* should improve the understanding of this process.

The gene(s) for ice nucleation (Ice^+ phenotype) in strains of both *P. syringae* and *E.herbicola* as gram-negative bacteria have been cloned and expressed in *Escherichia coli*. The expression of ice nucleation in *E. coli* was largely similar quantitatively and qualitatively to that in the original DNA source strains. (Orser *et al*.,1983). The (Ice^+) phenotype, conferred by the ina gene, has been considered to be monogenic (Orser *et al*., 1983), but recent studies in our laboratory report some species to be likely to have several genes. Isolation and sequencing of ina genes from various bacteria has been carried out by researchers. The identification and analysis of ina genes and proteins are refered to in Tab. 1.

Tab. 1. *Isolated and sequenced ina genes from different bacterial species*

Species	Isolation	Sequencing	Gene
Erwinia ananas	Arai *et al*. (1989)	Abe *et al*. (1989)	*inaA*
E. herbicola	Orser *et al*. (1983)	Warren & Corotto (1989)	*ice E*
Pseudomonas fluorescens	Corotto *et al.* (1986)	Warren *et al*. (1986)	*inaW*
P. syringae	Orser *et al*. (1985)	Green & Warren (1985)	*inaZ*
Xanthomonas campestris	Zhao & Orser (1990)	Zhao & Orser (1990)	*inaX*

After Warren (1995).

People use ice^+ and ina^+ interchangeably. This is partially because ice nucleation genes have been called both ice and INA. The custom of calling the phenotype Ice^+ and Ina^+ thus comes from the convention used to describe proteins that are the product of single genes. Thus the protein encoded by the inaZ gene would be called the InaZ protein while that of the iceC gene would be the IceC protein. Protein names have the first letter capitalized and not italicized while gene names are small letters italicized. Thus it is probably technically correct to use either Ice^+ or Ina^+ to describe the ability of bacteria to make ice nuclei (since single genes are sufficient in all cases). (Lindow, pers. communication).

Ice nucleation inhibitors

A reduction of number of epiphytes in general and INA bacteria in particular with bacteriocides/antagonists in many cases has been shown to reduce frost injury in frost sensitive trees. This type of hindrances like heat treatment or spraying with water, are economically not worthy of being recommended or suggested methods in forest energy plantation. Up to now tests with natural occurring bacteria has not been conducted on *Salix* plants.

Lindow (1982), however, has shown that the control of frost injury with non-nucleation active bacteria is a good model system to study biological control processes for a number of reasons: 1. Frost injury is an important, worldwide problem; 2. The target microorganisms are well known and can be well quntified based on their phenotype of ice nuclation activity. INA populations have been successfully reduced by application of naturally occurring or genetically engineered (Ice^-) strains of *P. syringae*, *P. fluorescens*, and *E. 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 (Lindow, 1982; Lindow & Connell, 1984). They observed that antagonistic bacteria precluded INA bacteria from the leaves thus reducing their population density. Plants can thus be readily colonized by inoculated non-ice nucleation active bacteria under the right circumstances.

Plants grown in the absence of ice nucleation active bacteria could supercool and avoid damaging ice formation to – 5^{O}C or colder (Lindow *et al*., 1978; Lindow, 1982; Nejad *et al*., 2004). Because of this relationship any reduction in the number of ice nuclei would be expected to reduce plant frost injury under mild freezing conditions (above –5 ^{O}C) (Lindow, 1983b).

Non-ice nucleation active bacteria, such as *E. herbicola* or *Pseudomonas fluorescens* did not reduce the population size within target strains of *E. amylovora* or *P. syringae* substantially when co-inoculated on plant surfaces, but they greatly reduced the population sizes of the target strains when pre-inoculated onto plants prior to the target strain (Lidow *et al*., 1983a). This finding is consistent with our preliminary results (unpublished) when we co-inoculated bacteria as dual culture and observed reduction of pathogenicity (necrotic infection) on *Salix* plants. We carried out this experiment by co-inculating the bacteria both at the same time as target isolates and intervally, i.e. bacteria were preinoculated onto *Salix* plants prior to target strain as known pathogen. Probably the non-INA bacteria had higher growing rate than ice^{+} bacteria and consume all accessible nutrient sources prior to the inoculation of the ice $^{+}$ strains.

Studies utilizing mutants of *P. syringae* deficient in ice nucleation have provided direct evidence for competition for limiting environmental resources as a major mechanism determining the coexistence of bacteria on leaves. The answer is that bacterial strains of similar genotype and having similar ecological habit requirement would compete more directly for limiting environmental resources than dissimilar strains (Lindow, 1985c). Each isolate had been tested on plants individually before carrying out the mixed culture. With combination of pathogenic and INA^{+} bacteria as dual culture we wanted to verify the degree of aggresivity or pathogenic potentiality. With combination of pathogenic INA^{+} and non-pathogenic INA^{-} we wanted to find out the hampering effect of non-pathogenic INA^{-} on the pathogenic INA^{+}. The observation that single non-INA strains can represent a large percentage of epiphytic and endophytic bacterial community suggested that biological control of frost damage could be conferred by colonization of plants by certain non-INA strains. Conceptually, the presence of large population sizes of non-INA bacterial strains should prevent large population size of Ice^{+} bacterial strains from occurring on the same plant (Lindow, 1995). In our preliminary experiment (Nejad, unpublished) on the interaction between mixed bacterial populations (2 or 3 isolates) on plants we obtained such results but how such biological control can be implemented need to be understood.

Two major factors contribute to the ice formation and propagation in plants: 1) Minimum air temperature, and 2) duration of subfreezing temperatures. Hirano & Upper (1990) demonstrated that *P. syringae* populations can increase with a doubling time as short as two hours under field conditions. Field studies, however, are questionable because, as we discussed earlier the occurrence of freezing temperatures cannot be predicted accurately more than one day in advance (one such field study was conducted in Wisconsin, USA, Lindow, pers. comm).

Chemicals that quickly inactivate the ice nuclei (V) associated with ice nucleation active bacteria without necessarily killing bacterial cells have been termed" bacterial ice nucleation inhibitors" that retards or stops their nucleation activity Even though viable bacterial cells may remain on plants after treatment with bacterial ice nucleation inhibitors, the cells no longer catalyze ice formation and can not be responsible for initiating frost damage (Lindow, 1983). Also many chemicals can make bacterial ice nuclei inactive, many are though poisonous to plants, and all are water-soluble and possibly weather with rapid movement from plant surfaces and have destructive effects of such atmospheric conditions as high winds or heavy rains.

INA bacteria as backup equipment to make snow

Winter sports are big business, but the natural snowfall on which they depend is unpredictable in most places and so, in recent decades, snowmaking technology has become standard equipment at most ski resorts around the world. In recent years, a growing number of resorts have been using a protein (trade-named "Snowmax") derived from bacteria (*Pseudomonas syringae*) as a nucleating agent. This protein is added to the water and makes the droplets crystallize more readily, thus producing more snow for a given amount of water. This can reduce both power and energy requirement while shifting electrical loads without the refrigerant gases.

It is very important to introduce novel natural ice nucleators. Biological ice nucleators are utilized routinely as snowmaking additives in ski areas throughout North America , South America, Australia, New Zealand. LaDuca *et al*. (1995), explains that most naturally occurring ice nuclei in the atmosphere are not active at temperatures warmer than –10°C. For this reason there is a continued interest in searching for novel ice nuclei that can convert liquid water to ice crystals at the lowest degree of supercooling. In our work (I, V) we found numerous bacterial genera that nucleate ice at warmer temperatures that might be introduced commercially as biological ice nucleation-active products.

The application of *P. syringae* as an ice nucleator in the manufacture of artificial snow has previously raised questions concerning the release of microorganisms into alpine environments (Goodnow *et al*.,1990). The *P. syringae* cell, which is encapsulated in the ice crystals of man made snow, can contact soil and natural waters from snow melt runoff. Freeze-thaw cycles, sunlight and soil contact however, severely limit the survival of this microorganism (Goodnow *et al*.,1990). In our studies (Ganteil *et al*., unpublished) we also found that *P. syringae* seldom is present in soil samples. LaDuca *et al*. (1995), also found that naturally occurring strains of *P. syringae* are susceptible to lytic infection by bacteriophage. He further explains that cell lysis due to phage infection during fermentation is detrimental to production of ice nucleation activity and should be gaurded against in the production environment. Lytic effect in large–scale manufacturing of any microbial products including Ina^+ bacteria can be dealt with by isolating bacterial species which are insensitive to lytic infection. In this way it is necessary to find a

family of bacteriophage resistant Ina^+ producers, which are able to minimize the susceptibility of the commercial process to lytic fermentation failures.

Aim of the study

(i) To explore the presence of INA bacteria and study their potential pathogenic properties in *Salix* plantations that show frost related dieback. Sub-goals were to form a collection of a wide diversity of such organisms and to characterize the most common ones (I, II).

(ii) To set out a comparison between the spring and autumn bacterial communities present in different *Salix viminalis* clones, isolated from both endophytic and epiphytic parts (II).

(iii) To characterise the culturable bacterial communities isolated endophytically from *Salix* clones grown in two different Estonian plantations with different fertilizing levels (III).

(iv) To characterize and identify pathogenic and non pathogenic INA bacteria involved in causing diseases in *Salix* plants by means of BIOLOG® MicroPlate and molecular techniques in combination with growth characteristics on different culture media (IV).

(v) To describe methods for determining factors, e.g. temperature, population sizes, chemical and physical agents, for disruption and possible inhibition of INA among bacterial species. and of individual strains (V).

Results and discussion

Appearance of pathogenic INA bacteria in energy forest plantations (Paper I & II)

Extensive sampling of bacteria from within (I, II) and surface (II) of shoots of diseased *Salix* clones was performed in trials at two experimental sites in central Sweden. Field, greenhouse and laboratory studies were conducted to identify and characterize the plant-associated bacteria and determine their possible role in the severe dieback appearing in Short Rotation Forestry (SRF) plantations in Sweden during the last few years. Sampling of shoots for bacterial isolation from diseased *Salix* plants was performed from 14 *Salix* clones (I, Table. 2) in two different clonal trials near Uppsala and Brunnby (Västerås) in central Sweden. We describe the isolation and characterization of bacterial isolates associated with willow plants and dieback symptoms previously thought to be principally due to frost damage. This study ties ice-nucleation activity with bacterial disease development in willow plants, thus tackling the question, whether the dieback observed in willow plantations was caused by bacteria that were pathogenic and possibly active at ice nucleation.

We demonstrate that among the bacterial isolates recovered from the willow plantations, there were in fact many that were capable of ice nucleation at temperatures between –2°C and –10°C, many that were capable of inducing a hypersensitive reaction in tobacco (potential pathogens), and many that were capable of causing necrotic symptoms on willows exposed to frost treatment.

The description of the bacterial colony selection also included the goal to form a collection that represented the most common organisms as well as a wide diversity of organisms (thus the criterion of looking morphologically different). This collection was thus useful for looking for putative ice nucleating pathogens, as was done. The collection was not generated to be representative for all of the organisms present on the clones, but rather than just being random outcomes mostly demonstrates organisms that were selected from each clone.

The most frequently isolated types were found to be non-fluorescent *P. fluorescens* (biotype A, B, C, F and G) or *Sphingomonas* spp. However, *Erwinia* spp, *P. fluorescens*, *Xanthomonas* spp and *P. syringae* (I) were considered to be the most important pathogens in the field. Most of these pathogens which caused high level of damages to plants showed ice nucleation activity at temperatures between –2.5° C and –5° C. Our results support the hypothesis that pathogenic bacteria with INA properties could be an important factor causing frost related damage also in woody plants.

We observed clonal differences among collected *Salix* plants concerning isolated bacteria. On the reference clone 78183 nine of the twelve bacterial groups could be found (Tab. 2) while e.g. four other clones only harboured two bacterial groups. Certain bacteria appeared on almost all clones while others were found on only 2-3 clones. Group 12, 8, 10, and 11 in that order, which in our preliminary grouping represent *Erwinia* spp, *Pseudomonas / Sphingomonas* spp, *Xanthomonas* spp, and *Pseudomonas syringae* respectively, were the most common ones and appeared on most (77%) of the clones (Tab. 2).

The maximum bacterial population size on different plant species varies greatly by plant species but is characteristic for a given species. Population size could be correlated to disease occurrence as e.g. the *Salix*-clone “Christina” both was found to have the highest rate of dieback as well as the highest number of INA-bacteria (II). The correlation between frost susceptibility and electrolytic leakage (Tsarouhas, 2002) indicates that one possible reason for “frost sensitive clones” is that high leakage favour bacterial growth, which in turn could lead to higher disease occurrence. This was also considered the cause for bacterial canker in poplar (Dong *et al*., 2001).

A reason for finding the bacterial populations of clones 78118, Rapp, Eva and Jorunn (I; Tab. 2) less diverse than other clones might be the level of resistance among the clones to bacterial infection (unpublished), or dependence on other factors like properties of the bark which may play a role and determine the fitness for the growth of bacteria. An additional reason for obtaining less number of different bacteria of some clones comparing to other clones can be the limited

amount of samples that decrease the chance to find the less frequent strains. Factors like rainfall, humidity, time of sampling and plant density and genetic variability are important for creation of suitable conditions for the bacterial population and thus influence the most suitable times for samplings.

When *Salix* plants were inoculated without frost treatment (I; Fig. 2), the disease incidence was also rather low in most cases. The most severe effect (*Erwinia rhapontici*) was still only about 20% of maximum disease index. An exception was the control isolate 3426 (*Xanthomonas populi* subsp. *salicis*) that is reported as a *Salix*-pathogen in Holland (de Kam, 1978). This strain caused severe damage (71%) without frost treatment but instead lost activity when subjected to frost (I; Fig. 2).

Thus overall, most of the isolates sampled in the field caused significantly more damage (necrotic reactions) to *Salix* plants while exposed to frost treatment (I; Fig. 1b) than without frost stress.

Result variations can be used to define three classes of bacterial ice nuclei, Ice nucleation activity at temperature between –2°C and –5°C, –5 and –7°C and below –7°C. We grouped INA bacteria according to temperaure at which nucleation occurred (V). Those bacteria which nucleated below –7 caused minor damage to Salix plants but in few cases we observed that even those bacteria which froze below –7°C showed weak pathogenic activity, probably those bacteria which live inside the plant tissues are saprophytes or beneficial endophytic bacteria which protect the plants against pathogens.

The results clearly indicate that INA bacteria play a significant role in frost-related damages to plants and constitute a potential problem in energy forestry plantations. Dieback, that in many SRF-plantations is considered to be frost damage is, according to these results, instead very likely to be a result of bacterial infections, aggravated by frost. In our greenhouse experiments we found that presence of INA bacteria and freezing alone (or that neither freezing nor bacteria by itself) are sufficient to cause typical necrotic symptoms (I; Fig. 2). Frost does not kill or cause severe necrotic dieback directly to the plants in absence of bacteria (water or buffer).

Tab. 2. *Appearance of different bacterial groups in short rotation willow clones (Salix viminalis) isolated from field experiments*

Clone	Group
78118	2, 9
78183	1, 2, 4, 6, 7, 8,10,11,12
78198	2, 8,10
Anki	2, 8,10,12
Astrid	3, 4, 6, 8,10,11, 12
Bowle´s Hybrid	2, 6,7, 8, 9,11,12
Eva	3, 6
Gustaf	2, 4, 6, 7, 8, 10,11, 12
Hanna	8,10,11,12
Jorunn	2, 6
Marie	6, 8, 9,10,12
Orm	4, 7, 9,11,12
Rapp	1, 7
Ulv	2, 6, 8, 9,12

(*cf.* Nejad *et al*., 2004)

Seasonal variation and comparisons between epiphytic and endophytic bacterial populations (Paper II)

Four willow clones with different frost sensitivity levels showing visible symptoms of bacterial damage including partial dieback were investigated. Seasonal variation and differences in bacterial epiphytic and endophytic communities, particularly their share of INA and pathogenic strains, were studied.

Salix plant samples were collected in April and October 2001, in an experimental plantation of mainly *Salix viminalis* at Brunnby near Västerås, central Sweden. Bacteria were isolated both from surface (epiphytically) and within (endophytically) diseased part of the *Salix* stems. The surface texture, chemical composition, and availability of water varies greatly with species and climate. We could not take these variabilities or complications into account because there is little knowledge about the microbiology of stems. Differences also exist between *Salix* clones regarding frost sensitivity, geographical origin (Christersson, 1982), productivity (Koppel, 1996; Heinsoo *et al*., 2002) or uptake capacity (Yazdani, 1992). In our studies we mainly concentrated on clonal differences due to frost sensitivity.

As opposed to summer and winter, spring and autumn are periods when epiphytic populations of Ice Nucleation Active (INA) bacteria are generally high. All clones showed about the same number of epiphytic strains in both seasons. Clonal differences were, however, greater for endophytes. Endophytically isolated communities were generally more stable than epiphytes, both in number of isolates and type of bacteria. More bacterial types were found in autumn than in spring the same year, although the total number of strains isolated was rather constant. In contrast, more strains (and a higher percentage of the total community) expressed ice nucleating activity in spring than in autumn. The overall number of pathogenic

strains remained stable but their proportion among the community tested on plants increased. A close relationship was observed between the dieback rates in the field and the percentage of pathogenic strains found in the different clones. The dominating bacterial type isolated, *Sphingomonas* spp., also contained the highest percentage of ice nucleation active pathogenic strains.

Epiphytic strains were isolated in higher numbers than endophytic ones in both seasons (II; Tab. 2), i.e. more colonies were obtained after culture at 21°C. Epiphytes were significantly more abundant than endophytes for the clone Marie in April, and for 183 and Christina in October. However, as underlined above, no difference in the distribution of profiles was observed either in April or in October between all epiphytes and endophytes.

The seasonal comparison showed that the October community was more diverse (II; Fig. 1b), (though not displaying significantly higher numbers than the April one) but the *Sphingomonas* type was dominant at both periods. In October, the INA community seemed to be smaller and less diverse (II; Fig. 2). It displayed a higher ratio of *Sphingomonas* than the total community. Pathogenic strains were mostly of *Sphingomonas* and *Xanthomonas* types.

A larger proportion of pathogenic strains was found in autumn, in a smaller INA community. The fact that two bacterial types (*Sphingomonas* and *Xanthomonas*) contained high proportions of both INA and pathogenic strains in both spring and autumn may indicate that ice formation is a predisposing factor to bacterial infection and development of stem necroses, as has been previously reported.

Moreover, the highest amount of INA pathogenic strains was found on clone Christina (II; Tab. 6). Considering that this clone is very sensitive to frost, its INA community is likely to cause more serious damage by forming ice, i.e. to release more nutrients (electrolytes), probably giving pathogens the opportunity to attack and multiply (Süle & Seemüller, 1987; Tsarouhas, 2002; Granhall *et al*., unpublished).

Effects of mineral nutrient supply/ fertilisation on INA pathogenic bacteria and plant survival (Paper III)

Mineral nutrient supply is known to affect frost resistance and cold acclimation in *Salix* plants. Nutrient requirements for plant growth have to be considered with regard to the effects of plant nutrient status in relation to frost resistance (von Fircks, 1994) and frost damage to plants in relation to bacterial ice nuclei (V). In this study we focussed on the possible effect of fertilisation and soil nutrient availability on the bacterial community in relation to plant dieback. The influence of fertilisation on plant survival was studied as well.

Fertilisation with high concentrations of nitrogen, potassium, and/or phosphorus (N-P-K) stimulates rapid initial plant uptake of trace minerals (also known as trace elements) without providing for their replacement. It is common knowledge that

excessive nitrogen reduces the valuable calcium available to the plant. Calcium is a "cation", together with magnesium, potassium, and sodium. A reduction in the available calcium, always leads to an imbalance in the "cation exchange". If cations are out of balance, anions will also not work properly, nor will several important trace elements (Anonymous). 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 (Agrios, 1997 reviewed by Cambours, 2004).

In this study the effect of fertilisation on both bacterial population and plant density is most persuasive as fertilised plots sustained both higher plant mortality and higher bacterial colonisation (III; Fig. 2). More convincing 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. We have also observed that plant density was significantly lower in fertilised plots (Saare) than in unfertilised ones (Kambja) with an average of 1.66 and 1.85 plants per m^2 respectively (III; Fig. 1). Fertilisation leads to enhanced plant development, this might increase competition, and most likely also contribute to higher dieback rates. Furthermore, the spreading of INA bacteria is enhanced by elevated air humidity and small physical distance between plants and clone stands (Constantinidou *et al*.,1990).

Our study showed that beyond affecting plant susceptibility, fertilisation also led to higher pathogen and INA colonisation. Considering our results, it seems 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 (Heinsoo *et al*. (2002). Increased wood dry matter yield but also high stool mortality of *S. dasyclados* (clone 90) was also associated with fertiliser application. In 1996, Koppel reported that the higher productivity observed at Kambja (compared to Saare unfertilised) due partly to higher soil fertility decreased after three years, maybe because of nutrient imbalances. The higher plant mortality observed on the organic soil at Kambja can be due to the high availability of mineralised nitrogen, which is known to hinder the plants from going into dormancy thus leading to frost damage and dieback (Cambours, 2004).

Although we report differences in bacterial numbers between plantations and fertilisation regimes (either concerning total, INA or pathogen amounts), similar bacterial profiles were found in all 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 dominant by far (II; Fig. 5). Ice nucleation-active and pathogenic strains were predominantly *Erwinia*-like but the above-mentioned profiles were also represented (II; Tab. 3, 4, 5).

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 most likely sustain more frost injuries. These in turn enable higher pathogenic colonisation, leading to severe plant damage.

For attainment of unimpaired growth not only addition of all mineral nutrients essential for growth but also balanced fertilizers are necessary (Ingestad, 1979; Ericsson, 1981; McDonald *et al*., 1991). Heavy fertilization with phosphorus, leading to a high P-content in the plant, was shown to prolong the growth of Sitka spruce (*Picea sitchensis*) and causing subsequent winter damage to plants (Malcolm & Freezaillah, 1975). Our investigation provides evidence that nutrient imbalances, competition, climatic changes and pathogens could be causes for dieback as well.

With respect to seasonal variation in Estonia, clones were sampled in both late March and late August 2002 at Saare. *Erwinia* and *Sphingomonas* profiles were found dominant in both seasons. The number of INA strains as well as the average nucleation temperature increased between the two sampling times. 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 (Dubrovsky *et al*., 1989, Nejad *et al*., unpublished), which could partly explain the differences observed (ice nucleation activity decreased with longer cold storage).

This study showed that, *Erwinia* and *Sphingomonas* types were dominant as endophytes, both in spring and autumn. These two types thus infected *Salix* to the greatest extent in both Sweden and Estonia. During spring periods, the same bacterial types were found pathogenic in Estonia and Sweden i.e. *Erwinia*, *Sphingomonas, Xanthomonas*, *P. fluorescens* and *Clavibacter* (or *Corynebacterium*). In the autumn, *Sphingomonas* was the most common pathogenic type in both Sweden and Estonia. However, no similarities between the two regions were found regarding the INA communities. The explanation for this may be due to differences in gene expression due to temporal variations in climates rather than true differences in bacterial populations.

A number of studies of willows in energy forestry stands have been carried out on the effect of fertilisation on *e.g*. development of frost resistance (von Fircks, 1996), dry matter productivity (Heinsoo *et al*., 2002), or plant disease (Gallegly & Walker, 1949; Huber & Watson, 1974). All of them 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 is for example 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

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. For these reasons, the amount (and composition) of sludge or wastewater deposited on *Salix* plantations for purification should also be carefully monitored.

Identification methods (Paper IV)

Classical identification methods based on phenotypic traits (morphology, metabolism and physiological characteristics) can be used to group bacteria. In recent years the classification of bacterial genera has been improved using molecular techniques (Woese *et al*., 1983; Borrel *et al*., 1997; Koike, 2001; Kozinska *et al*., 2002; Nejad *et al*., 2003). For identification purposes and taxonomic relationships between bacterial groups and species 16S rRNA (Felsentein, 1993; Thompson *et al*.,1994; Page, 1996), as well as specifically designed primers, e.g. Oligo 4 software (http://www.oligo.net), are suitable tools.

The classification of bacteria is not sufficient to meet the need. A large number of bacterial species are not sufficiently described or classified in an adequate standard for identification purpose. Furthermore, not all strains within a given species may be positive for a common distinguishing feature and the same strain may display biochemical variability, small modifications of the test therefore may give false results. Failing or lack of material based on characterization of bacteria by facts can be the main reason for insufficiencies in classification, phylogeny of individual species, groups and subgroups. A basic source of phylogenetic structure for grouping of organisms into categories requires the knowledge of the organisms and structural relationship between species. The more we find out about the organism, the more precise and hard-and-fast rules for the term species becomes. Most probably it may advance and develop our knowledge of the organism. The term “species” become more complicated if we acquire more knowledge about the organism and most probably it advances our perception and learning of the organism.

Our hypothesis was to find out: what is the minimum testing required based on this work for a researcher to correctly identify their biotype to genus level or to species level? Can it be done by morphology alone using different media? Can it be done by biochemical testing alone? If so, just using BIOLOG or what additional tests? Can it be done using 16S rRNA alone? Where are the likely problem areas because of inconsistent responses from the same biotype / species?

Microbiological tests are conventional taxonomy methods used for identification of bacteria. This include gram-negative, gram-positive classification and others (I, II III, IV). We used a rapid method for gram differentiation of bacteria (Suslow & Schroth, 1982), although gram staining was performed when results were doutful (Dowson, 1957; Schaad, 1988). The gram staining is an important differential staining procedure widely used in bacteriology.

In this work the phenotypic analysis played an important role and made it possible to differentiate and identify most of the bacterial strains. But we believe that we have to involve also molecular taxonomy and multivariance data analysis to analyze large quantities of data and find an efficient way of identifying the unknown strains of bacteria. It was difficult to distinguish *Erwinia* from *Xanthomonas* species even using INA primers. In a few cases we found band pattern for *Xanthomonas* spp. while using *Erwinia* primer (IV; Fig. 4) and viceversa. To overcome this problem we carried out tests with biochemical characters, morphological properties and completed by growing bacterial on different culture media. The colony colour and growth morphology shown in paper IV (Tab. 2) are based on visual observation of growth on the various media. The need to more evidence for identification in our work is consistent with results of Lelliott & Dickey (1984), which e.g. demonstrated that only two species of *Erwinia* (*E. rhapontici* and *E. rubrifaciens*) have pink diffusible pigment. He describes that pigment production by *E. rhapontici* is more consistent on media containing 5% sucrose nutrient agar (SNA), which is true in our case. Recent work in the Salmond Group (University of Cambridge) BSPP News Number 41 Spring 2002) showed that a strain of *Erwinia* spp that develops orange coloured colonies but at very low levels, so appear to be white. Comparison of aerobic and anaerobic growth, together with the catalase (Nejad *et al*., 2004) and potato rot and other comparative biochemical test on bacterial genera (IV; Tab. 1) presumptively helped us for differentiation of certain genera especially *Xanthomonas*, *Erwinia*, *Sphingomonas* and *Sphingobacterium* spp.

In order to find out which types of microorganisms are present at different levels of pathogenicity in the same plants the most effective methods must be applied. This emphasizes the importance to carry out and combine several methods because one method, or two profiles, are not compatible for some species. Some of the molecular taxonomy methods, though highly effective, need special and expensive equipment. We suggest that for identification purposes and taxonomic relationships between INA bacterial groups and species 16S rRNA (Felsentein, 1993; Page, 1996; Thompson, 1994) and specific designed INA primers (IV) should be used in conjunction with other tests (i.e., an observable organismal attribute for defining the species or patovar based on their characters). In summary, most types of INA-bacteria that seem to be involved in frost related damage to cultivated willows (*Salix* spp.) in Sweden and Estonia were satisfactory identified.

In this study we report, for the first time occurrence of damage to willows associated with *Erwinia rhapontici*, *Pedobacter* sp., *Pseudomonas* spp., *P. brennerri*, *P. fluorescens, P. frederikbergensis, Sphingobacterium* spp, *Sphingomonas* spp, *Stenotrophomonas* sp. and *Xanthomonas* spp, which all had ice-nucleation active strains. We also found *Frigoribacterium faeni*, *Clavibacter* and *Bacillus* spp. among gram-positive bacteria that were shown to be pathogenic on willow. It also records new strains of known species; for example, *P. fluorescens* Biotype A, B, C, F and G which were shown to have INA-properties, cause disease to *Salix* plants and found to be plant residents.

Factors influencing pathogenic INA bacteria (Paper V)

Little is known about the environmental and physiological factors that regulate expression of Ina gene and assembly of ice-nucleation proteins (Govindarajan & Lindow, 1988). Expression of ice-nuclei of bacterial strains can be induced by nutrient limitation and low temperatures (Nemecek-Marshall *et al*., 1993). We also provided evidence, however, that growth limiting factors influences the bacterial strains differently.

In this study we investigated the temperature, at which ice nucleation occurred in individual bacteria, estimated the population of INA bacteria, effect of growth limiting factors, and additionally also evaluated the effect of chemical and physical agents for disruption and possible inhibition of INA among individual bacterial strains.

Our result shows that expression of ice-nuclei in bacterial strains depend on growing temperature and nutritional conditions, which are major factors that contribute to the ice formation and propagation most probably also in plants. The ambient temperature and duration of subfreezing temperatures can play a key role as well. We found that the ratio between the number of ice nuclei and the number of bacterial cells in culture can vary with growth medium composition (V; tab. 4).

Ice nucleation in intact cells is sensitive to agents such as lipases, phospholipases, detergents and solvents that disrupt the cell membrane (Lindow *et al*., 1989). Maki *et al*. (1974) have described a number of characteristics properties of the ice nucleating site of such bacteria. They have also reported that INA was sensitive to heating in the presence of heavy metal ions.

Our laboratory experiments may not substitute for fields scale experiments in forest nutrition management, but it gives us an indication that support the interpretation of several key differences in the effects of nutrient compositions and other factors like temperature, site, seasonal variation, physical and chemicals on INA bacteria.

In this experiment the growing ability of each individual bacterium under different temperature ranges and incubation time was checked regularly. For example, some bacterial genera did not grow within certain incubation periods and additional time per temperature interval was required. We also recorded the optimal growing temperature for INA bacterial species. We observed variability in INA among isolated bacteria from soil, litter and plant of the same bacterial groups.

In our experiment regarding the effect of nutrient starvation on INA bacteria N limitation played a key role and very strongly affected the tested bacteria and decreased their nucleation activity. In this sense our laboratory results are in agreement with the finding that higher numbers of pathogenic INA bacteria harboured in fertilized plot (Kambja) in Estonia under field conditions (III), i.e. availability of N triggers INA.

Different bacterial species also required certain temperatures to express the ice gene. The reason for varying ice-nucleating activity among bacterial species can be that the INA frequency vary among individual bacteria. We also believe that not all bacterial cells in a culture have an ice-nucleating site (cf. Maki *et al*., 1974). Hirano *et al*. (1985), suggested that culturing of different *P. syringae* strains under identical conditions resulted in ice nucleation frequencies at given temperature, such as –5°C, that vary from one in 100 cells to as few as one in a million cells.

Lindow *et al*. (1982) found that expression of ice nuclei can be enhanced when cultures are grown at 20°C to 24°C on nutrient agar containing glycerol, while Pooley & Brown (1991), observed increasing ice nucleation frequencies and elevated threshold ice nucleation temperatures for culture of *P. syringae* while grown on solid versus liquid culture. They further suggested that components within the complex medium might have inhibitory effects on the expression of ice nuclei that is maximal late in the growth cycle. We also attest the variability of ice nucleation activity (higher or lower) on different growth media.

Chemicals that quickly inactivate the ice nuclei associated with INA bacteria without necessarily killing bacterial cells have been termed " bacterial ice nucleation inhibitors". Even though viable bacterial cells may remain on plants after treatment with bacterial ice nucleation inhibitors, the cells no longer catalyze ice formation and cannot be responsible for initiating frost damage (Kozloff *et al*., 1983). Also many chemicals can inactivate bacterial ice nuclei, many are though phytotoxic for use on plants, and all are water-soluble and may weather rapidly from plant surfaces.

Consistent with our finding Maki & Willoughby (1978), Hirano *et al*. (1985), (Kozloff *et al*. (1983) explain that the frequency of expression of the INA bacteria has been shown to be highly variable and the source of variability in the frequency and level of INA^+expression has been attributed to physiological responses of the INA^+ bacteria to nutritional and environmental signals. Ice nucleation activity can be 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).

Govindarajan & 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, thus have been so far more difficult to isolate (Yankofsky *et al*., 1981; Lindow *et al*., 1989). According to Watanabe *et al*. (1990), nuclei active at lower temperatures are more stable but assemble more slowly.

In this study we concentrated on bacterial isolates, especially *P. syringae*, which were isolated from *Salix* plants. Tab. 5 (V) demonstrate the disruptions of membranes and decreasing INA of *P. syringae* (S 229) isolated within internal tissue of *Salix* stems. In the physical experiment we observed that heat treatment at

65°C in 10 min (V; Fig. 3) destroyed the INA at warmer temperature, number of cells decreased but their INA remained in colder temperature. Additionally we provide evidence that centrifugation of bacterial suspension for 15 min at 15000 X g and filtration of cultures through a 0.20 μm membrane filter separated the activity from the supernatant i.e the ice nucleation activity remained associated with the cells. Number of cells was reduced at least between 3 to 10% by some chemicals when added to cell concentrations (data not presented). Highest reduction of cell number was achieved by streptomycin (16%), penicillin (30%), methylen blue (46%) and heat treatment (36%), (data not shown). Heat treatment and methylen blue not only reduced the number of bacterial cells but also reduced INA significantly (V).

As to streptomycin treatments, one such study was conducted in the field in Wisconsin. Different batches of corn plants were sprayed with streptomycin at intervals starting 20 days before freezing and 1 day before a mild radiative frost with a minimum air temperature of about – 3°C. Interestingly, the incidence of freezing damage to leaves was hardly reduced on plants that received streptomycin spray more than about 10 days, or less than about 5 days, prior to exposure to subfreezing temperatures (Lindow, unpublished). The incidence of freezing damage was most reduced on plants that were sprayed 6 days prior to freezing event. These data were interpreted to indicate that ice nucleation sites in cells exposed to streptomycin less than 5 days earlier had insufficient time to be degraded before freezing temperatures were encountered. Plants that received eradicative applications of streptomycin more than 10 days prior to freezing presumably had been recolonized by Ice^+ bacteria after streptomycin residue diminished. More experiments of this kind are needed in order to formulate strategies for bactericide applications.

Conclusions

• Most epiphytic and endophytic bacteria are not harmful to the plant on or in which they reside, but in some cases, they can either be beneficial or detrimental . One case in point are those bacteria that promote the formation of ice crystals, such ice nuleation sites serve as loci for ice formation on plants at temperatures well above normal freezing. These bacteria can establish and develop pathogenic characteristics under certain environmental conditions such as frost.

• Diseases caused by INA bacteria in relationship with frost are a limiting factor in willow and poplar plantations in Sweden and most likely also in other temperate regions in the world (e.g., Baltic countries, China and Southern South America).

• Whether the dieback observed in the investigated willow plantations in Sweden and Estonia was caused mainly by bacteria that were invasive pathogens and possibly active at ice nucleation can be positively answered as we demonstrate that among the bacterial isolates recovered from the willow plantations, there were in fact many that were capable of ice nucleation at temperatures between –2°C and –10°C, many that were capable of inducing a hypersensitive reaction in tobacco,

and many that were capable of inducing necrotic symptoms in willows particularly when plants were exposed to frost treatment. Among those bacterial types that we found the ones that caused the strongest necrotic reactions on *Salix* plants had the highest level of ice-nucleating activity. There is also a close relationship betweeen dieback rates in the field and the percentage of pathogenic strains that could be isolated from the plants.

• Presence of INA bacteria or freezing alone are not sufficient to cause typical necrotic symptoms in willows. Frost thus do not kill the plants in absence of bacteria. When plants get stressed potentially pathogenic bacteria are favoured by ice formation prior to disease development. The joint effects of frost damage and bacterial pathogens in *Salix* plants, however, seem to be the main cause of plant dieback.

• Freezing injury and onset of bacterial infection could now be interpreted as causal not consequential.

• Number of INA and bacterial profiles are more stable in endophytic isolates than among epiphytes. With respect to the INA expression, not much similarities between the two regions (Sweden and Estonia) were found whereas many pathogens (potentially INA) were the same.

• Fertilisation indeed affected both bacterial population and plant density as fertilised plots sustained both higher mortality and higher bacterial colonisation. 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 parallelled the higher dieback figures recorded at Kambja and Saare fertilised.

• We confirm the existence of different INA bacteria both from plants, soil and litter using various diagnostic methods. Identification tools enabled us to demonstrate that dieback in these cultivated willows has been associated with *Bacillus*, *Erwinia*, *Pseudomonas*, *Sphingomonas*, *Stenotrophomonas*, *Xanthomonas* spp., and other related genera.

• For distingushing bacterial genera/species on the basis of genotypic and phenotypic characters combining different methods of identification are powerful tools because one method or two profiles are not compatible for some species.

• Several key differences (N, P, and K) and other factors like physical and chemicals has effects on INA bacteria. Growth nutrients are important for the level of ice nuclei. Lack of nutrients can cause premature or delay in INA. There is thus possibility to regulate the expression of ice nuclei by nutritional factors.

• The nucleation frequency of particular strains grown under specific conditions mostly increased with decreasing temperature.

• Detection and enumeration of INA bacteria from natural sources may be influenced by the probability that each individual cell will express an active ice nucleus at any particular time and temperature.

• We provide evidence that the gene(s) for ice nucleation among certain ice INA genera (*Xanthomonas* and *Erwinia*) are sometimes similar.

• Ice nuclei active at higher temperatures are eliminated by treatment of cells with many chemicals, or by disruption of the cells by physical processes or phage lysis. However the same treatments do not seem to affect the numbers of ice nuclei active at colder temperatures. Treatment of cells with antibiotics or chemicals does not cause rapid abolition of warm temperature ice nucleation activity. It appears that with a few possible exceptions, bacterial ice nuclei active at temperatures warmer than –5°C require a physically intact or physiologically normal cell for their expression, while those active only at colder temperatures do not.

Future aspects

Continuation of planting non-pathogen resistant clones can lead to development of even more aggressive pathogens. Monoculturing is itself a very well documented risk as well. In some cases storage defects of *Salix* cuttings at –4°C has been observed. We believe that it could originate from occurrence of INA bacteria (Nejad, unpublished). For cold storage of *Salix* cuttings and their sanitary measurements (bacteriocides or heat treatments) steps must be taken and there is a good reason for such investigations. Consequently we believe that it is about time to find out their correlation by identifying important environmental factors which affect the occurrence, spreading, and virulence among INA bacteria in the field. And at the same time to modify the frost hardiness tests to study the resistance aspects of INA-frost phytopathogens by using isolated and well characterized species in bioassays.

Large plantation areas need to be sampled in relatively short time. Both biased and systematically sampling from fields are necessary. Because of their occurrence in soil, a sampling strategy for bacterial species on the forest floor may be easier to develop, providing estimates of total and relative dominance and frequency, by species, for the sample area. For systematically collecting the plant samples and assessment of bacterial diseases in the field early field observations must be done. A strategy must be developed for an all taxa inventory for bacterial diversity, a logical approach is to sample for the major bacterial groups thus sampling the major substrates and functions within the ecosystem. In general, major taxonomic groups reflect ecological functions. Broad spectral molecular tools, e.g. T-FRLP should be used to characterize all dominant organisms in diseased plants, including non-culturable strains.

Factors favoring establishment of pathogens must be emphasised. Field evaluations are necessary in order to avoid selecting *Salix* clones in an area conducive to infection and subsequent disease development. Certain plant species/clones may develop new bacterial species. There is also the possibility that these bacteria may prefer certain plant parts. All collected clones must be tested for pathogenity and INA. More emphasis must be laid on endophytically isolated bacteria and tracking main bacterial species responsible for pathogenicity. Mechanistic studies must be paid more attention. If we know more about the background of a bacterium, then we can find out which kind of substances they

produce as those substances maybe involved in ice formation or pathogenicity as well.

Endophytic bacteria can be used for improving phytoremediation i.e. bacteria that live inside plants could make vegetation more effective in decontaminating polluted soils. There is a possibility to construct endophytic bacterial strains for degradation of organic pollutants by natural gene transfer and strain engineering. For example in our pilot study certain endophytic bacteria in *Salix viminalis*, (clone 183) made plants relatively resistant to pathogens under environmental stress, *i.e* high exposure to PAH:s (Granhall *et al*., unpublished). Dissimilarities in clones resistance and susceptibility to bacteria will be studied and eventually their correlation to frost resistance in the field will be investigated with electrolyte leakage. Studies utilizing mutants of bacteria deficient in ice nucleation will provide direct evidence for competition for limiting environmental resources as a major mechanism determining the coexistence of bacteria on plants. It has been hypothesized that bacterial strains of a similar genotype (and therefore having similar ecological habitat requirements) would compete more directly for limiting environmental resources than dissimilar strains. Care must though be taken that preconceived notions of the most important mechanisms involved in antagonism are not used to determine which antagonists are tested for biological control of frost injury, However, more information on the interactions that occur between mixed bacterial populations on plants is needed to understand how non-pathogenic INA-bacteria could influence disease development. Further knowledge about survival mechanisms throughout the different seasons is necessary.

To fully understand and advance our knowledge about the dynamics of frost damages with bacterial participation several aspects thus have to be taken into account: seasonal variation of bacterial populations, factors increasing (temp, rainfall, humidity, plant density) or limiting bacterial population (nutrients: Carbon source, N and P), diversity, variability and level of dominancy among bacterial species in different regions, the role of non-INA bacteria in plants (if they can protect the plants against pathogens, initiate pathogen infestation or are opportunistic).

Mechanism of frost injury / their mode of action that cause damages to frost-sensitive plants has not been paid much attention. Quorum Sensing (QS) is a system known to be active in several regulatory mechanisms in many bacteria, e.g. pathogenicity, but its possible role in INA regulation is yet to be known. With this in mind increased understanding of the contribution of QS to plant pathogen interactions could make biological control of frost mediated injury, by means of antagonistic / non-nucleation active bacteria an attractive possibility or by discovering different strains of INA bacteria (ice-plus) to find a mutant non-INA (ice-minus) by a recombinant DNA to protect the plants.

More important, however, is to find methods to include bacterial resistance in the breeding program for improvement of new and more productive clones for Short Rotation Forestry.

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