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Frost related dieback in Estonian energy plantations of willows in relation to fertilisation and pathogenic bacteria

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Abstract

Two 9-year old Estonian *Salix* plantations suffering from dieback were studied: one situated on poor mineral soil and divided into fertilised and unfertilised plots (Saare plantation) and another growing on a well-decomposed and nitrogen-rich organic soil, without fertiliser application (Kambja plantation). Bacteria from internal tissues of visually damaged shoots from seven clones were isolated in spring and autumn. The strains were subsequently biochemically characterised and tested for ice nucleation activity and pathogenicity on *Salix*. Some strains were also analysed with 16S rRNA. High numbers of culturable bacteria were found, belonging mainly to *Erwinia, Sphingomonas, Pseudomonas* and *Xanthomonas* spp. Fertilised plots were significantly more colonised by bacteria than unfertilised plots and also more extensively damaged, showing a lower density of living plants after 7 years of culture. More ice nucleation active (INA) strains were found in Saare fertilised plots and at Kambja than in Saare unfertilised plots. Likewise, most pathogenic strains were isolated from Saare fertilised plots and from Kambja. For some of the willow clones studied, dieback appeared to be related to both clonal frost sensitivity and abundance of INA and pathogenic bacteria.

The plantations probably suffered from the presence of high amounts of pathogens and from frost related injuries aggravated by INA bacteria. Most probably the fertilisation at Saare and the nitrogen-rich soil at Kambja created a favourable environment for bacterial development and led to high dieback levels after the first harvest.

Keywords: *Salix*; Frost damage; Fertilisation; Ice nucleation; Pathogenicity

1. Introduction

Since the fall of the Soviet Union in 1991, Estonia has undergone a set of economical changes and more consideration has been given to renewable energy resources in an attempt to mitigate issues related to environment protection and land use and to find alternatives to fossil fuels [1]. In 1993, the Estonian Energy Forest Project was started in cooperation with the Swedish University of Agricultural Sciences and the first willow plantations of *Salix dasyclados* and *S. viminalis* were established in order to evaluate the potential of short rotation forestry as an energy source [2]. Three hectares of willows, spread over seven locations, were planted on different soil types [3,4] and have been used since then for different experimental purposes such as clonal selection for high productivity or vegetation filters for wastewater and sludge purification [2,5]. Fertilisation experiments were also carried out [6].

Dieback and stem damage were observed in the plantations since the first biomass harvest in 1998. The symptoms, typical bark necroses and discolourations have been previously reported at several places in Sweden and suggest a bacterial infection worsened by frost [7–9].

Pseudomonas syringae [7] and *Xanthomonas populi* [10] have been reported to be the two main pathogens responsible for willow dieback but recently, other species have also been found capable of causing damage to the plant [8,11].

Ice-nucleation active (INA) bacteria can nucleate ice from −1 °C downwards [12–14]. When a frost event occurs,

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these bacteria initiate the formation of ice crystals, causing plant cells membrane to rupture [15]. The released cell contents are subsequently exploited by the bacteria, leading to their multiplication [16]. Additionally, ice nucleation brings about wounds that allow pathogenic bacteria to penetrate ice-damaged tissues [15]. Therefore, the association of ice-nucleation and pathogenic properties with frost is an ideal combination for bacterial infection [11,15,17]. The most effective INA species known are *P. syringae* and *Erwinia herbicola* [17] but other species such as *P. viridiflava* [18], *E. ananas* [19], *X. campestris* [20] and some Gram positive species [8,11] also contain strains with a nucleation activity.

This study aims at characterising the culturable bacterial communities isolated endophytically, i.e. from tissues beneath the bark, from seven *Salix* clones grown in two different Estonian plantations. Examination of the combination of INA and pathogenic strains is of particular interest since the occurrence of INA bacteria has so far mostly been studied epiphytically whereas pathogens have been isolated endophytically. A number of studies have been carried out on the effect of fertilisation on e.g. development of plant frost resistance [21], biomass productivity [2,6] or plant disease [22,23]. This paper presents a comparison of fertilised and unfertilised plants with regard to their culturable bacterial communities, for plant damage of all clones appeared to be more pronounced in nutrient rich stands.

2. Material and methods

2.1. Plant material sampling

The studied *Salix* plantations are located in Saare and Kambja, Estonia. The former plantation grows on typical poor mineral soil, whereas the latter, on well-decomposed organic soil, rich in nitrogen. The plantation in Kambja is situated on a valley riverbank, therefore being particularly susceptible to spring frosts. Although surrounded by forests that protect it to some extent from short-term chilling, the Saare plantation can also be affected by spring frosts. A detailed description of both sites is given in a previous report [4].

In May 1993, the plantations were established with cuttings of six *S. viminalis* clones (78101; 82007; 78112; 78021; 78183 and 78195) and one *S. dasyclados* clone (81090) originating from Sweden. The clone numbers refer to the Swedish clone numbering system. The last two digits of these numbers will hereafter refer to the clones. Clones 83 and 12 are the most frost-resistant: they have a grade 1 on a scale from 0 to 4 (0—frost hardy and 4—frost sensitive). Clones 01 and 95 are frost sensitive (grade 3), clones 07, 21 and 90 are relatively frost resistant (grade 2) [24]. Cuttings of each *Salix* clone were planted manually in four randomly selected plots ($16 \times 16\,m^2$ or $8 \times 16\,m^2$) in double rows (distance between rows: 1.25 and 0.75 m). The planting density was 20 000 plants per hectare.

In May 1994, shoots were cut at 5 cm above ground to promote denser sprouting. At Saare, two randomly chosen plots per clone were fertilised with mineral fertiliser in June every year (between 60 and 170 kg N, 0 and 37 kg P, 0 and 70 kg K per year). Both plantations were harvested in March 1998, manually at Kambja and by harvester at Saare. In 1999, after the first winter of resprouting, numerous cracks were observed on the shoots bark at certain Saare plots. The most common symptoms of bacterial damage noted were: dead or damaged side shoots with necroses, bark cracks and colour changes of the bark (brown, yellow).

The influence of fertilisation on plant survival was studied: in autumn 2000, all living plants of each Saare plot were counted and mapped and the actual plant density was calculated for each clone. The survival rate of the seven clones was established by comparing the data obtained with the planted density (2 plants per m^2).

The samples for microbiological analyses were collected at Saare and Kambja in the end of March 2002. At Saare, seven clones grown on fertilised and unfertilised plots were sampled. Five clones were sampled at Kambja (07, 21, 83, 90, 95). After collection, the cuttings were stored for 11 weeks at $-4\,^{\circ}C$. Additional samplings, taken from the Saare plantation only, were cut in the end of August 2002. These cuttings were sampled from fertilised and unfertilised plots of clones 83, 90 and 95, and stored in plastic bags at $-4\,^{\circ}C$ for 2 weeks. These three clones were chosen because, among the clones sampled at both locations in spring, they had displayed the greatest Colony Forming Unit (per gram dry weight) differences between the fertilisation regimes. Besides, clone 83 is the standard reference clone in Swedish clone trials. On each plot, between one and three shoot cuttings from several trees were collected. If present, branches showing damages were preferentially sampled.

The experimental setup at Saare is a randomised two-factor design (fertilisation and clone as factors with, respectively 2 and 7 levels). At Kambja, it is a randomised one-factor design experiment (factor clone, 5 levels). Most, but not all factor combinations were sampled twice (each replicate corresponding to an experimental unit or block). The experiment is unbalanced since the number of observations is not equal for each treatment combination.

2.2. Bacterial isolation

The plant material was constantly kept at $-4\,^{\circ}C$ between field sampling and bacterial isolation, except during the 24 h-shipping from Estonia to Sweden when it was at ambient temperature. Once in Sweden, the cuttings were stored at $-4\,^{\circ}C$ for 1 or 2 days before the actual bacterial isolation.

Bacteria were isolated from the internal tissues of 36 cuttings in spring and 12 cuttings in autumn. The bark was thoroughly spray-sterilised with aqueous ethanol (70%), rinsed three times in sterile water and blot-dried on paper towels. The bark was then removed with a flame-sterilised