Monitoring of Endophytic *Brenneria salicis* in Willow and its Relation to Watermark Disease

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Abstract

B. salicis was monitored in willow stands, sampling trees with and without watermark disease. The study focused on *Salix alba* and *S. fragilis*. *B. salicis* – presence was shown not to be restricted to diseased trees. With a *B. salicis*-specific PCR, *B. salicis* was frequently detected in the wood of healthy willows. This phenomenon was further studied with PCR-DGGE of endophytic bacterial populations in healthy and diseased willow. *B. salicis* concentrations were fluctuating during the year, synchronized with tree activity. Water stagnation in the wood as in winter was associated with a high *B. salicis* concentration, while the sap stream in active trees drained *B. salicis* from the wood vessels towards the leaves. We concluded that disease risk can not be assessed by testing the presence of *B. salicis*.

Keywords: *Brenneria salicis*; watermark disease; willow; endophyte; PCR-DGGE

INTRODUCTION

Watermark disease is one of the most serious bacterial diseases of willows. The first symptoms are sudden wilting of leaves and branches. Within a few years big sections of the tree or the whole tree can die. Already decades ago a correlation was made between the disease and the presence of the bacterium *Brenneria salicis*. Cuts of affected branches show a typical watery reddish-brown zone from which a concentrated suspension of *B. salicis* oozes in case of severe disease progression. Literature mentions that *B. salicis* is only pathogenic for the genus *Salix* (DE KAM 1984), but with important differences in disease susceptibility within the genus. Extended disease is noticed in *S. alba* and *S. fragilis* and their hybrids.

It is yet unknown how watermark disease spreads in nature and different pathways, as through epiphytical dissemination with wind and rain (VAN DER ZWEEP & DE KAM 1982), or through contaminated cuttings (DOWSON 1937; DE KAM 1988) have been investigated but not confirmed. *B. salicis* is a wood-inhabing bacterium. Wood pathogens often have complex biological adaptations to this specific environment and are able to survive for long periods and with a slow disease progression. This makes phytopathological research difficult. Artificial inoculation tests have a low and unpredictable success rate and the general condition of the tree seems to have an impact on its susceptibility.

To investigate possibilities for breeding and selection of willow with resistance towards watermark disease it is important to have knowledge on *B. salicis*, on its geographical distribution and endophytic behaviour and pathogenesis, and on the relation between *B. salicis* – presence and the disease. We present data indicating that misconceptions in the epidemiology of *B. salicis* can now be pointed responsible for unsuccessful disease control.

MATERIALS AND METHODS

DNA tests. For detection of *B. salicis*, 5 μl of extract was analyzed in a PCR with primers specifically designed for *B. salicis* (HAUBEN *et al.* 1998). To obtain

Supported by the Ministry of the Flemish Community, Departement Leefmilieu en Landbouw.

PCR specificity in wood extract samples, the annealing temperature was raised to 66°C. The expected amplified fragment is 554 bp long and was visualized on a 1.5% agarose gel with ethidium bromide.

PCR-DGGE profiles of the bacterial communities in the extracts were based on the sequence variability present in the V3-16SrRNA sequences of bacteria and were produced as described by MUYZER *et al.* (1998). The bands produced on the acrylamide gels were silver stained (Plus One Silver Staining Kit, Amersham Biosciences). The gels were photographed and processed with the Kodak 1D Imaging Analysis software.

Tree sampling for B. salicis detection in willow with PCR. From each tree, three 2-year-old branches were sampled and the wood sap was squeezed out with a mechanical press. Every sap sample was separately tested in the *B. salicis*-specific PCR. From one positive PCR result on, the tree was scored positive for *B. salicis* presence.

Tree sampling for PCR-DGGE monitoring of endophytes. Three branches with a diameter of about 2.5 cm were cut from one tree and inspected for external and internal symptoms of watermark disease. The material was immediately covered with plastic and transported to the lab for extraction. A 15 cm segment was taken from the middle of each branch and externally disinfected with ethanol. The xylem fluid was squeezed out in a mechanical press. After recuperation of the sap, the segments were further cut in 0.5 cm pieces and extracted for 2.5 h in a 120mM phosphate buffer with addition of NaPP (0.1%), PVPP (25 g/l) and Tween 20 (0.1%). These extracts were pooled with the respective sap preparations. Extracts and sap preparations from the branches of the same tree were pooled. The bacteria were concentrated by centrifugation and bead beated. DNA was prepared with the Puregene kit (GENTRA), and extra purified with one phenol/chloroform extraction and ethanol precipitation. DNA was resuspended in a TE buffer (pH 8) and the concentration was estimated on agarose gel.

RESULTS

Geographical distribution. Around 520 willow trees were sampled. One part was from the nursery of the Institute of Forestry and Game Management, containing autochthonous and allochthonous willow clones. None of these willows had disease symptoms. The collection of autochthonous material is composed of *S. alba, S. fragilis and S.* × *rubens* clones. In PCR 34% of these clones was positive for *B. salicis*. Sixty-one different willow clones originated from other European countries were also tested. In this collection 13 different willow species and 3 hybrids are represented. In 49% of the tested plants *B. salicis* was detected.

Upon *B. salicis* monitoring in natural willow stands in Flanders, more than 80% of the trees were without symptoms. Disease was only observed in the *S. alba* – *S. fragilis* complex and most diseased willows were found in 3 of the 6 Flemish provinces. In 25% of the 254 sampled trees *B. salicis* was detected with PCR. These positive trees were found throughout Flanders where *S. alba* and *S. fragilis* clones grow.

Evolution of endophytic B. salicis populations. Two willow stands were selected. In both *B. salicis* had been detected with the *B. salicis*-specific PCR, although one was with and the other without watermark disease. Two trees were sampled on the diseased site; one with extended symptoms, the other still without symptoms at the start, but evolving to disease during the project. One big healthy tree was sampled on the healthy spot. The three willows were sampled during 1.5 year. The endophytic bacterial populations in the branches were monitored in the different seasons, starting in winter 1999–2000. PCR-DGGE profiles were run in parallel, and with the PCR-DGGE products of *B. salicis* strains and of other bacteria that had been isolated from willow wood as references. In winter *B. salicis* was shown to be dominantly present in healthy and diseased wood, although *B. salicis* concentrations were more accentuated in diseased (Figure 1). Upon leaf come out and reactivation of the trees in spring, these high *B. salicis* concentrations disappeared from the wood, making other wood-inhabiting bacteria visible in DGGE. Depending on the tree different bacterial profiles could be observed during summer. In case of less efficient or no reactivation due to watermark disease, the water content of the wood and the endophytic *B. salicis* concentrations stayed high (Figure 2). The winter situation, with high *B. salicis* concentrations in all three willows, was again installed in November 2000 and stayed during the winter 2000–2001, after which this circular event was repeated.

DISCUSSION

In this work the spread of watermark disease in willow was not evaluated on the basis of visual external or internal symptoms on the tree, but by monitoring of the causal bacterial agent *B. salicis*. The bacteria were detected in wood extracts with PCR and not by culture plating. This overcomes the problems of false

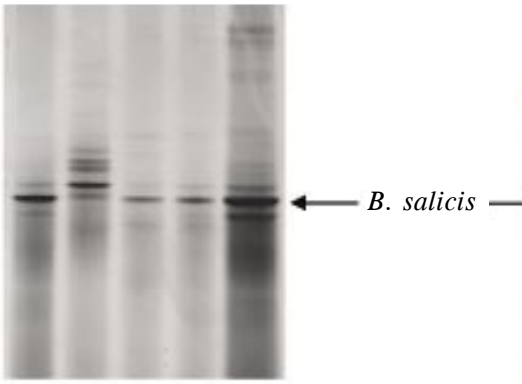

Figure 1. PCR-DGGE profiles of bacterial populations in branches of willows with (D) and without (H) watermark disease and sampled during the winter period (22/02/2000). The profiles of a typical *Brenneria salicis* (Ref) and of another bacterium isolated from willow wood (*End1*) were run as references

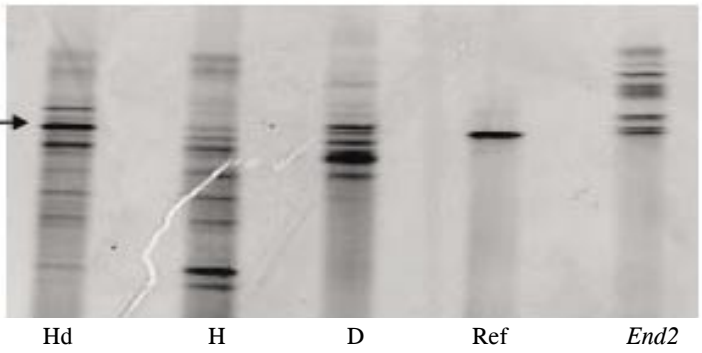

Figure 2. PCR-DGGE profiles of bacterial populations in branches of willows differing in respect to watermark disease and sampled during summer (20/07/2000). Willow with partial and slow disease progression (D), willow without disease (H), and willow that evoluated from healthy to disease during this study (Hd) and in which high *B. salicis* concentrations were also observed during partial activation of the tree in summer. The profiles of a typical *Brenneria salicis* (Ref) and of another endophyt of willow wood (*End2*) were loaded as references

negative detection caused by unculturable stages or outcompeted *B. salicis* growth.

B. salicis was shown to be widely spread in the nursery, although none of the trees had disease symptoms. *B. salicis* presence was not restricted to a specific clone or origin. Clones derived from countries where watermark disease has never been described were also harbouring this bacterium. It can be argued that *B. salicis* persists in this nursery where different *B. salicis* infection experiments have been performed in the past. But also in natural willow stands *B. salicis* was detected in healthy trees. At the same time we showed that the *B. salicis* concentrations fluctuate considerably during the year. Since this *B. salicis* screening with PCR was not repeated different times in different periods, we can even expects that the detection results underestimate the real situation.

The results clearly suggest that *B. salicis* can live in willow in a non-pathogenic form that is widely spread in the nursery and in the field. Presence and spread of *B. salicis* is not sufficient for disease induction in willow and other, yet unknown factors have a role in directing the plant-endophyte balance towards pathogenesis. It seems that certain conditions in some regions and on specific spots are disease-inducive. The link with the specific environment will be further studied.

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