Journal of Chromatography B, 872 (2008) 83–89

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Identification of quorum sensing signal molecules and oligolignols associated with watermark disease in willow (*Salix* sp.)

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ARTICLE INFO

Article history:
Received 25 March 2008
Accepted 10 July 2008
Available online 17 July 2008

Keywords:
Watermark disease
Brenneria salicis
Quorum sensing
Willow
Oligolignols

ABSTRACT

The bacterium *Brenneria salicis* is the causal agent of watermark disease in willow. This work shows the importance of *in situ* studies and high-resolution separation of biological samples with ultrahigh performance liquid chromatography combined with ion trap mass spectrometry to unambiguously identify molecular compounds associated with this disease. Approximately 40 oligolignols accumulated in wood sap of watermark diseased willow, and are indicative for degradation of the xylem cell wall, of which 15 were structurally assigned based on an earlier study. Many bacteria are known to produce and release quorum sensing signal molecules that switch on the expression of specific, sometimes pathogenic functions. Two quorum sensing signal molecules, *N*-(3-oxohexanoyl)-L-homoserine lactone and *N*-(hexanoyl)-L-homoserine lactone, were present in 4/1 ratios in diseased wood and in high-density *in vitro* cultures of *B. salicis* at 0.13–1.2 μM concentrations, and absent in healthy wood and in low-density *in vitro* cultures of *B. salicis*. Although it is not a proof, it can be an indication for involvement of quorum sensing in *B. salicis* pathogenesis. Cyclic dipeptides were present at high concentrations in high-density *in vitro* cultures of *B. salicis*, but not *in situ*, and were found not to be involved in quorum sensing signaling, therefore, the attribution of quorum signal properties to cyclic dipeptides isolated from *in vitro* cultures of pathogenic bacteria should be reconsidered.

1. Introduction

The bacterium *Brenneria salicis* (Bs) [1] is the causal agent of watermark disease (WMD) in willow [2–4]. It affects the wood xylem resulting in a dysfunctional water transport system, with wetwood formation and wilting of the leaves on the diseased branches [5,6]. The pathogenesis of Bs is still not elucidated and extra, yet unknown, factors and conditions would be needed for disease expression [7,8]. A nutritional imbalance caused by nitrogen excess was correlated with the occurrence of WMD in white willow [9]. Enzyme profiles present in wood with WMD [10] indicated that cell wall degradation might be involved in the pathogenesis. The degradation products resulting from these enzyme activities have never been shown *in situ*.

During our study on WMD, we have found that Bs is widely spread, also in healthy willow trees. The xylem sap of WMD wood is typically colonized by a dense and almost pure culture of Bs (average 10^{11} cfu/ml of sap), while in healthy willows, Bs is present at much lower concentrations, in some periods even at the detection limit in PCR. Density-dependent pathogenesis exists in several bacterial systems, also in plant–pathogenic bacteria. This pathogenesis is frequently regulated by cell-to-cell communication known as quorum sensing (QS) that works through chemical signal molecules produced by the bacteria. QS enables the species to assess its local population density or physical confinement and to coordinate and synchronize gene expression [11]. QS is often associated with the onset of virulence factors [12–14].

We present data on the methodology used for identifying and quantifying QS signal molecules produced by Bs and oligolignols in WMD wood sap. Different bacterial strains have been used as biosensors to detect different ranges of *N*-acyl-homoserine lactones (AHLs) produced by other Gram-negative bacteria for their QS signalization [11,13,14]. In these biosensor strains, the autoproduction of QS-active compounds is disrupted, while the QS detection system still functions and induces production of a compound that can be visualized upon activation. We used the biosensor strains *Chromobacterium violaceum* CV026 [15,16], *Escherichia coli* JM109 pSB401 and *E. coli* JM109 pSB1075, harboring the plasmids pSB401

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doi:10.1016/j.jchromb.2008.07.011

and pSB1075, respectively [17,18]. Together, their QS sensitivity range covers AHLs with acyl side chains from C4 to C14. Various analysis procedures were used first to detect QS activity *in situ* in wood sap and compare QS concentrations and Bs cell densities with *in vitro* cultures of Bs. Ultra high performance liquid chromatography coupled to a photodiode array detector and an ion trap mass spectrometer (UPLC–PDA–MS/MS) were used for high-resolution analysis of the biological samples, enabling clear separation of the low abundant QS-active products from abundant contaminating compounds, chemical structure assignment, and quantitative measurements of the QS and other products typically present in WMD wood, such as oligolignols.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Bs was grown in tryptic soy medium enriched with sucrose (TSM), containing 15 g pancreatic digest of casein (Remel, San Juan, Puerto-Rico), 5 g papaic digest of soybean meal (Remel), 5 g sodium chloride, and 2.5% (w/v) sucrose per liter water. *C. violaceum* CV026 [15,16] and *E. coli* JM109 pSB401, containing the plasmid pSB401, were used as lux-based AHL-dependent biosensor strains, and *E. coli* JM109 pSB1075, containing the plasmid pSB1075 [17,18], as a *lasR*-based AHL-dependent biosensor strain. CV026 produces the purple pigment violacein in the presence of AHLs with C4–C8 acyl side chains and their 3-oxo derivates, whereas JM109 pSB401 and JM109 pSB1075 are bioluminescent when in contact with exogenous AHLs with C6–C10 and C10–C14 acyl side chains and their 3-oxo derivates, respectively. CV026, JM109 pSB401, and JM109 pSB1075 were grown in Luria broth (LB) (ForMedium, Norfolk, UK) supplemented with 20 μg/ml kanamycin, 20 μg/ml tetracycline, and 50 μg/ml ampicillin, respectively. In liquid medium, all strains were grown under shaking (160 rpm) at 28 °C for Bs and CV026 and 37 °C for the *E. coli* strains. Bs was cultivated 24 h and 48 h to obtain a density of 10^6 cfu/ml or 10^9 cfu/ml, respectively. Bs concentrations in biological and culture samples were determined by serial dilution plating on TSM plates.

2.2. Bs culture sample preparation

The procedure was based on Steidle et al. [19]. Bs was grown in 250 ml TSM until stationary phase (48 h, 28 °C). After centrifugation (30 min, 5000 × *g*, 4 °C), the supernatant was sterilized through a 0.2-μm filter and used in the plate assay. For TLC and UPLC analysis, the filtrate was extracted twice with dichloromethane (DCM) (supernatant/DCM, 25/10, v/v). Both extracts were pooled and anhydrous magnesium sulfate was added. After 4 h, the extract was filtered and stored in a dark-colored glass recipient at 4 °C. The appropriate volumes were vacuum-dried and dissolved in ethyl acetate to use in the TLC assay or in start buffer (see below) to load on UPLC. No volatiles were involved in this QS system, because plate assays with samples before and after drying show an identical halo.

2.3. Wood sap sample preparation

Branches (diameter 1.5–2 cm) from healthy and diseased trees were collected and cut in 17-cm-long segments. After external sterilization through ethanol spraying and flaming, the wood sap was collected by squeezing the branch segment with a mechanical press. After sterilization through 0.2-μm filtration, 50 μl of the sap was used in the plate assays. DCM extraction and preparation for TLC and UPLC were as described above.

2.4. QS and CDP reference compounds

The following AHL standard molecules were used: *N*-(3-oxohexanoyl)-L-homoserine lactone (L-OHHL) (K3007, Sigma–Aldrich, St. Louis, MO, USA), *N*-(3-oxohexanoyl)-DL-homoserine lactone (DL-OHHL) (K3255, Sigma–Aldrich), *N*-(hexanoyl)-L-homoserine lactone (L-HHL) (10007896, Cayman, Ann Arbor, MI, USA), *N*-(hexanoyl)-DL-homoserine lactone (DL-HHL) (09926, Sigma–Aldrich), *N*-(butyroyl)-DL-homoserine lactone (DL-BHL) (09945, Sigma–Aldrich) and *N*-(dodecanoyl)-DL-homoserine lactone (DL-DHL) (17247, Sigma–Aldrich). Standards were dissolved in ethyl acetate for TLC analysis. The following standards of cyclic dipeptides (CDP) were used: cyclo(L-Pro–L-Val), cyclo(L-Met–L-Pro), cyclo(L-Leu–L-Pro), cyclo(L-Phe–L-Pro), cyclo(L-Pro–L-Tyr), cyclo(L-His–L-Pro), and cyclo(L-Ala–L-Ala) (G-4730, G-4725, G-1750, G-4720, G-4715, G-1745, and G-1655, respectively; Bachem, Bubendorf, Switzerland). Serial dilutions from standard CDPs and AHLs were prepared freshly for plate assay with biosensors and calibration of LC–MS response [20]. For details, see below.

2.5. Growth plate assay with strain CV026

The growth plate assay was adapted from McClean et al. [16]. A liquid culture of CV026 was grown overnight; 50 μl of the culture was added to 5 ml of molten LB-soft agar (0.6% agar), and immediately poured over the surface of a preheated (28 °C) LB agar plate (1.5% agar). After solidification of the top agar, 5-mm wells were punched in the agar and filled with 50 μl sample. After incubation (24 h, 28 °C), samples containing an appropriate QS molecule formed a purple halo around the well. Sterile LB medium and HHL (10 μM) served as negative and positive controls, respectively.

2.6. TLC with strain CV026

The appropriate volume of sample was dried and dissolved in a minimal volume of ethyl acetate, spotted on a C18-reversed phase TLC plate (Merck, Darmstadt, Germany), and developed in methanol/water (60/40) with 1 μl of the standard solutions (20–40 μM) as reference. After drying, the analytical plate was overlaid with 2 ml of a CV026 culture, grown overnight in 200 ml LB medium, suspended in 0.8% agar (w/v). The plate was incubated for 24 h at 28 °C and the purple spots recorded [21].

2.7. Bioluminescence assay with E. coli strains JM109 pSB401 and JM109 pSB1075

In the wells of a 96-well plate, 100 μl of sample, appropriate standard, or control solution and 100 μl of a 1/10 dilution of an overnight grown biosensor culture were mixed [18] and incubated while shaking (28 °C, 150 rpm, 24 h). Bioluminescence of *E. coli* JM109 pSB401 and *E. coli* JM109 pSB1075 was recorded with a camera (UVP-BioImaging Systems, Upland, CA, USA).

2.8. Conditions for liquid chromatography–mass spectrometry

LC–MS analysis was done by coupling an Acquity UPLC system with a PDA detector (Waters, Milford, MA, USA) to a ThermoQuest 'LCQ Classic' ion trap mass spectrometer (Thermo, San Jose, CA, USA). Samples were analyzed [22–24] on an Acquity UPLC BEH C18 column (Waters) (1.7 μm, 150 mm × 2.1 mm) with 0.1% aqueous acetic acid (solvent A) and methanol–acetonitrile (25/75, v/v) acidified with 0.1% acetic acid (solvent B). The gradient elution conditions were: time = 0 min/5% B; time = 0.5 min/5%

B; time = 30.5 min/100% B; flow rate = 0.2 ml/min; temperature = 40 °C; injection loop 20 μl. All solvents were UPLC grade purity (Biosolve, Valkenswaard, The Netherlands). UV–vis absorption spectra were recorded between 190 and 450 nm, with sample rate = 10 Hz and resolution = 1.2 nm; data were collected with Empower software (Waters) and chromatograms as maxplots between 190 and 450 nm. Full MS and MS/MS spectra of the eluting compounds were obtained with electrospray ionization (ESI) operated in positive mode under the following conditions: capillary temperature 185 °C, spray voltage 4.5 kV, sheath gas 53, auxiliary gas 50. During separation, the most abundant ion in each full MS scan was fragmented in the next dependent MS/MS scan; collision energy was set at 35%.

UPLC–MS profiling of oligolignols was obtained under the same UPLC conditions as described above. Atmospheric pressure chemical ionization (APCI)–MS was done in the negative mode as previously reported [24].

2.9. Conditions for UPLC fractionation and analysis

The equivalent of 200 μl supernatant of a Bs *in vitro* culture was loaded on UPLC. Fractions of 0.25 min were collected in 96-well plates starting from 0 to 24 min, with a BioFrac fraction collector (Bio-Rad, Hercules, CA, USA). UPLC conditions were as described above. Fractions were tested for QS activity by developing 2–4 μl on analytical TLC plates with reporter strain CV026.

2.10. Identification of AHLs, CDPs, and oligolignols in wood sap samples

AHLs and CDPs were identified by comparing the UPLC–PDA–MS/MS data with those of purchased standards. Oligolignols were identified by comparing retention time, UV–vis spectra, and MS/MS spectra to previously identified and structurally assigned oligolignols [24].

2.11. Determination of QS and CDP component concentrations

A series of dilutions was prepared with standard products, bacterial culture, and wood sap extracts. A semi-quantitative measure of the biological activity was determined according to the violacein plate assay in analogy to Blosser and Gray [25]. LC quantifications were based on external calibration. L-HHL (from 0.123 mM to 0.48 μM) and L-*O*HHL (from 0.121 mM to 0.47 μM) were quantified by using peak areas of the corresponding extracted ion chromatogram (XIC) MS responses [26]. CDP concentrations were obtained from the peak height of the UV response at 193 nm (from 1.0 mM to 1.0 μM external calibration).

3. Results and discussion

3.1. Production of QS signals by Bs

Wood sap of willows with active development of WMD contained Bs concentrations of approximately 10^{11} cfu/ml and produced violacein in the CV026 growth plate assays (Fig. 1b). The wood sap of healthy willow trees did not induce violacein production (Fig. 1a), also not with a 40-fold concentrated sap sample. When Bs was grown *in vitro* to saturation, a concentration of approximately 3×10^9 cfu/ml was reached and the culture supernatant induced a violacein halo on the plate (Fig. 1c). The active samples also induced weak bioluminescence in the *E. coli* strain JM109 pSB401, but not JM109 pSB1075, indicating that the active AHL compound had an acyl side chain length between C4 and C10. No QS signals were detected in Bs cultures with concentrations below 10^6 cfu/ml nor in the 100–400-fold concentrated 10^6 cfu/ml culture supernatant.

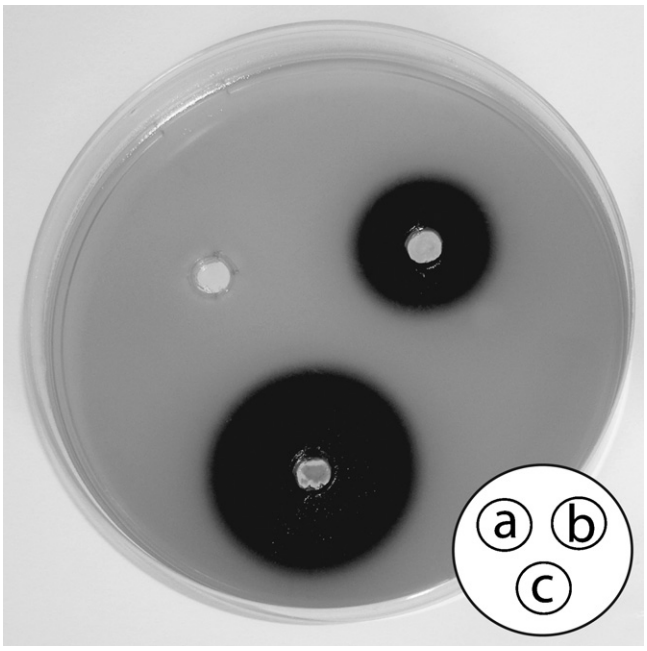

Fig. 1. Plate assay with *Chromobacterium violaceum* CV026 for detection of AHLs with C4–C8 acyl side chains and their 3-oxo derivates by violacein production. The wells contained equal volumes filter-sterilized wood sap of a healthy branch (a), a diseased branch (b), and the supernatant of an *in vitro* grown Bs culture (c).

3.2. Identification of the QS signal molecules of Bs

CV026 was further used for elucidation of the QS-active compounds. Wood sap and culture samples were extracted with DCM. TLC analysis confirmed the presence of QS molecules in the extracts of diseased wood and high-density Bs cultures by the production of two separate spots (Fig. 2, inlay). Controls were DCM extracts from sterile TSM growth medium and healthy wood, and references AHL standards. A 1/3 excess of healthy sap was spotted on the thin layer, corresponding originally to 187.5 μL healthy sap relative to 125 μL of diseased sap (Fig. 2, inlay). Additional TLC was run with more concentrated extracts from healthy sap and from bacterial supernatants with growth below 10^6 cfu/ml. None showed any violet spots on the TLC plates (data not shown).

QS-active products of Bs co-migrated with the standards *O*HHL and HHL (Fig. 2, inlay) and not with the other AHL standards (data not shown). Wood and Bs culture extracts were analyzed through UPLC–PDA–MS/MS, while 0.25-min fractions were collected and tested for QS activity on TLC. The QS-active fractions eluted at 8.5 min and 15.8 min and were found both in diseased wood and in high-density Bs culture extracts (Fig. 2; Fig. 3, and TLC inlay). The first compound at 8.5 min had a molecular mass of 213 Da and a MS/MS fragmentation pattern corresponding to the MS/MS spectrum of *O*HHL (Fig. 4, top). The second compound at 15.8 min had a molecular mass of 199 Da and a MS/MS fragmentation pattern identical to that of HHL (Fig. 4, bottom). When spiked to the Bs culture and the wood sap sample, the *O*HHL and HHL standards co-eluted with their corresponding natural products. In UPLC–MS analysis of diseased wood sap, the *m/z* 102 daughter ion, diagnostic for all AHLs [20,23], was highly present at retention times 8.5 min and 15.8 min for *O*HHL and HHL, respectively, and not at any other retention time. The *m/z* 102 was not detected in concentrated healthy wood sap. By XIC monitoring, a very clear *m/z*

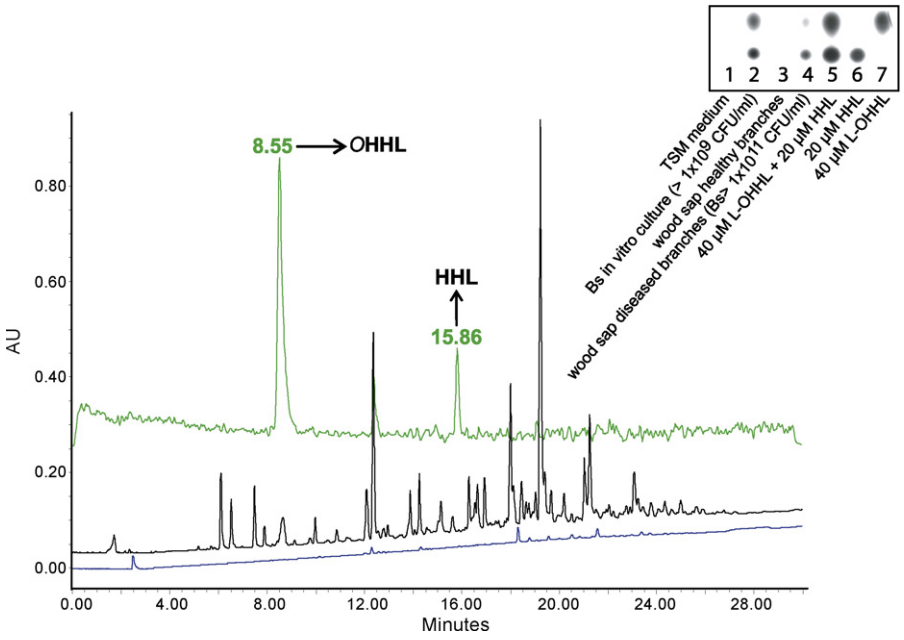

Fig. 2. Chromatograms of DCM extracts of sap from healthy (bottom, UV–vis maxplot 190–450 nm) and diseased (middle, UV–vis maxplot 190–450 nm) willow wood. The top line represents the $[M+H]^+$ XIC from DCM extract of diseased willow of HHL (*m/z* 200) and *O*HHL (*m/z* 214), confirming the presence of HHL and *O*HHL in the DCM extract of the sap of a diseased willow. The inlay is a TLC of representative samples and reference products, developed with the violacein-producing reporter strain CV026.

102 signal was present with any AHL standard spiked to samples. So, we could conclude that no other AHLs were present in the samples. *O*HHL and HHL with 3-oxohexanoyl and hexanoyl (C6) acyl side chains are the most commonly known QS molecules [11] described in many other plant–pathogenic bacteria, such as *Pectobacterium carotovora* [27], *Pantoea stewartii* [28], *Erwinia amylovora* [29], and *Pseudomonas syringae* pv. *tabaci* [30]. Within 300 isolates representing 5 genera and 17 species of soil-borne and plant-associated bacteria, including pathogenic and nonpathogenic strains, approximately 17% were reported to produce AHLs [31]. In *Erwinia carotovora*, *O*HHL controls a range of virulence factors, including the production of cell wall-degrading enzymes [32,33]. HHL is produced by the pathogens *Ralstonia solanacearum* [34], *P. syringae* [35], *Erwinia chrysanthemi* [36], and *Pseudomonas aureofaciens* [37]. In *Serratia plymuthica* [38] and *Pseudomonas chlororaphis* [39], *O*HHL plays a role in the production of antibiotics. These and more aspects of bacterial QS have recently been reviewed [40]. Compared with the high numbers of AHLs found in other Enterobacteriaceae, *E. chrysanthemi* [36], and especially *Yersinia pseudotuberculosis*, which were identified through LC–MS/MS [20], it is remarkable that HHL and *O*HHL were the only two AHLs found in Bs.

By comparing the bioactivity of the enantiomer mixtures (the DL-AHLs) with the pure L-forms of these molecules in the CV026 growth plate assay, identical halos were obtained with 5 μM DL-*O*HHL and 2.5 μM L-*O*HHL, and with 0.5 μM DL-HHL and 0.25 μM L-HHL. The HHL was approximately 10-fold more active in CV026 than *O*HHL and in the L-forms 2-fold as active as the DL mixtures according to the violacein halo response. Of course, a different dose–response for these compounds can exist between Bs and the biosensor CV026. Based on these results and the amounts determined by UPLC–MS/MS in natural samples and their violacein halos, the QS molecules from Bs were conclusively L-enantiomers, which is in accordance with previous results [41–43], and the AHL D-forms had no quorum quenching activity. The importance of stereospecificity has recently been described for a QS autoinducer analog from *Pseudomonas aeruginosa* to probe the regulator binding site [43].

L-*O*HHL and L-HHL concentrations in diseased wood sap were approximately 60% of those in the bacterial culture supernatant, but present in the same 4/1 ratios and in concentration ranges: for L-*O*HHL, 0.60 ± 0.05 μM and 1.11 ± 0.09 μM, and for L-HHL, 0.14 ± 0.01 μM and 0.23 ± 0.02 μM, respectively. Quantitative determination of the AHLs is worth addressing because the high cell densities found for Bs *in situ* (10^{11} cfu/ml) did not produce higher AHL concentrations than those found at the quorum size of Bs (10^7–10^9 cfu/ml) in cultures *in vitro*. In other words, *in situ*, the AHL molecules are not overproduced in function of cell density, but maintain their bioactive concentration at a μM range, involving a subtle regulation of synthesis.

3.3. Involvement of cyclic dipeptides in QS activity

DCM extracts of *in vitro* grown Bs cultures contained a highly diverse family of CDPs, also called diketopiperazines, in concentrations of approximately 20 μg/ml or 0.1 mM. Because of their abundance and large diversity, they co-eluted on conventional C18-reversed phase TLC and HPLC–MS. Following spiking with CDP standards (0.1 mM) on HPLC–MS, the MS signal of the AHLs (0.13–1.2 μM) was suppressed. CDPs were present in sterilized TSM growth medium, but absent in the extracts of healthy and diseased wood. No QS activity was found for any CDP isolated with UPLC from the medium with biosensors CV026, JM109 pSB401, or JM109 pSB1075, nor for the synthetic CDP standards. UPLC–MS resulted in reliable separation of the CDPs and AHLs in bacterial culture extracts and delivered clear-cut CDP–MS and AHL–MS spectra identical to the standards. In the biosensor tests, QS activity coincided with AHL- and not with CDP-containing fractions. The series of CDPs present in sterilized [44] liquid culture medium (Fig. 3), were identified through UPLC–MS/MS (Table 1). Some CDPs (b, c, and e) show multiple retention times, diastereoisomers [44,45], for which the conformation (D,L) was not assigned here. Eventually, a role for CDPs in QS has to be clarified, because several bacterial strains have been reported with CDPs as QS molecules [11,13,18,46–49], and reconsidered for the abundant CDPs found in *Pseudomonas putida* cultures [50]. AHL QS molecules had mostly been isolated through

Table 1
MS/MS data of CDPs

CDP[a]	Retention time	Molecular weight	MS/MS ions[b]
cPro-Val (a)	8.1	**196**	69.8 (9%), 71.8 (19%), 123.9 (10%), 140.9 (18%), 169.0 (100%), 180.0 (14%)
cPro-Leu (b)	11.5/12.0	**210**	69.9 (31%), 85.9 (11%), 126.9 (5%), 138.1 (5%), 154.9 (10%), 166.9 (5%), 183.0 (100%), 194.1 (21%)
cPro-Met (c)	8.6/9.3	**228**	181.0 (100%)
cPro-His (n.f.)		**234**	69.7 (6%), 110.0 (54%), 137.7 (19%), 162.1 (49%), 165.9 (100%), 189.9 (32%), 207.0 (44%)
cPro-Tyr (d)	7.8	**260**	106.8 (5%), 118.9 (3%), 136.0 (100%), 147.0 (15%), 154.9 (15%), 205(6%), 215(6%), 233(50%), 244(8%)
cPro-Phe (e)	13.3/14.3/16.3/16.6	**244**	69.8 (9%), 120.0 (100%), 217.0 (44%)
cAla-Ala (n.f.)		**142**	43.7 (44%), 69.9 (5%), 97.2 (6%), 97.9 (100%), 114.9 (26%)

[a] (a)–(e) refer to the peaks in the mass chromatogram (Fig. 3A); n.f. not found in the medium.
[b] Intensity of daughter ions relative to the base peak is indicated between parentheses.

TLC and HPLC, and, as shown, these techniques suffer from insufficient resolution. Indeed, active AHL components, present in minor amounts, are sometimes masked by co-elution of products at high concentrations [23]. Here, we show that under the described UPLC conditions, the CDPs from the growth medium are clearly separated from the AHLs and not QS–active compounds and that minor products and multiple CDPs have clear-cut molecular ions and MS/MS spectra in UPLC–MS.

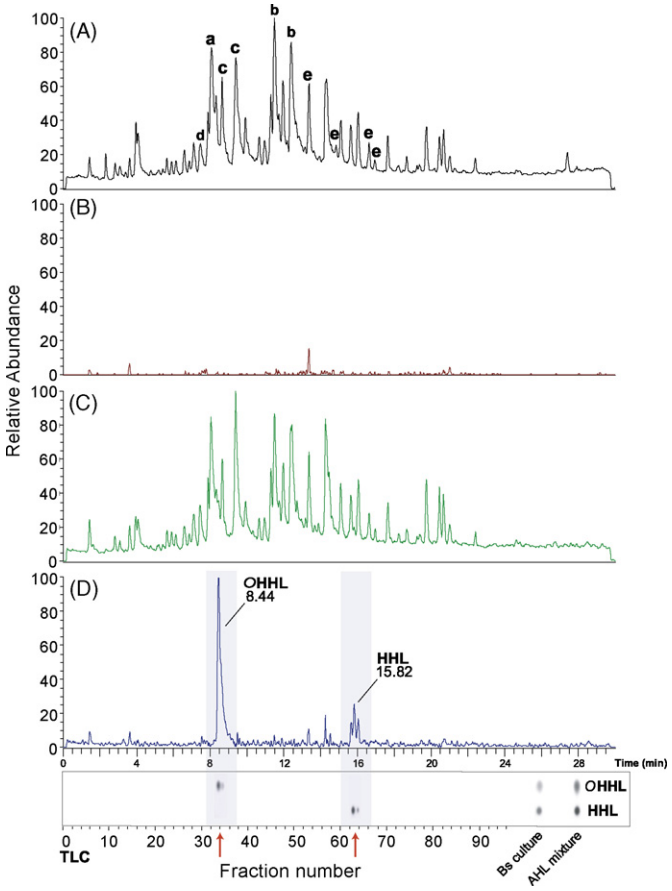

Fig. 3. Mass chromatograms. (A) and (C) Total ion current mass chromatograms of DCM extracts from sterile growth medium and Bs culture in growth medium, respectively; (B) and (D) XIC for *m/z* 200 and 214 $[M+H]^+$ corresponding to the upper chromatograms (A) and (C), respectively. Different CDPs and their isomers present in the medium are referred (a–e) in chromatogram (A) at their corresponding retention times and MS/MS ions are shown in Table 1. For chromatogram (C and D), the corresponding TLC analysis for QS activity in the UPLC fractions collected (from 1 to 96; each fraction 0.25 min) is shown. Fractions 34, 35, 63 and 64 contain QS-active products (arrows), also corresponding to the retention times on UPLC of standard HHL and *O*HHL on TLC. On the right, an extract of 10 μl *in vitro* Bs-grown culture (>10^9 cfu/ml) and a 1-μl mixture of AHLs (40 μM *O*HHL + 20 μM HHL) were spotted as reference compounds.

3.4. Identification of oligolignols in WMD wood

Sap samples from WMD wood produced a complex profile in UPLC–MS. Besides the AHLs, 40 peaks could be attributed to oligolignols by their UV and MS/MS spectra. The spectra of several olignolignols were known from a previous study on lignifying tissue in poplar in which material was collected from scraped xylem homogenized in liquid nitrogen [24]. By comparing retention time, UV, and MS/MS spectra, 15 oligolignols (dimers, trimers, and tetramers) were unambiguously identified in sap of WMD-affected willow wood (Table 2; Fig. 5). None of these oligomers occurred in healthy wood sap. In the adopted shorthand notation, bold **G**, **S**, **G′**, and **S′** are used for guaiacyl (coniferyl alcohol) and syringyl (sinapyl alcohol) units, and units derived from their corresponding hydroxycinnamaldehydes, respectively, coupled by a

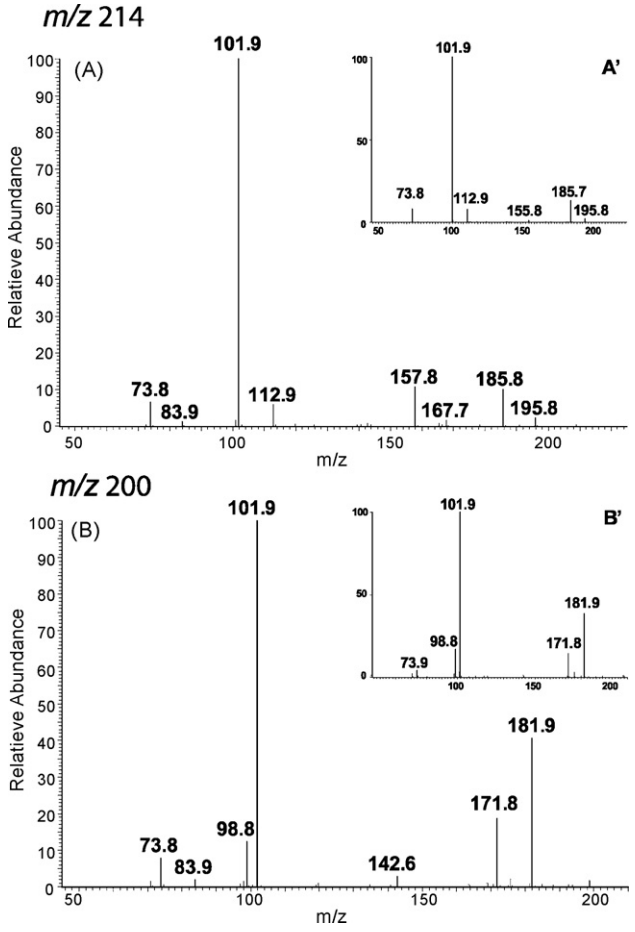

Fig. 4. MS fragmentation spectra of natural *O*HHL (A) and HHL (B) and of standard *O*HHL (A′) and HHL (B′), *m/z* is the mass-to-charge ratio.

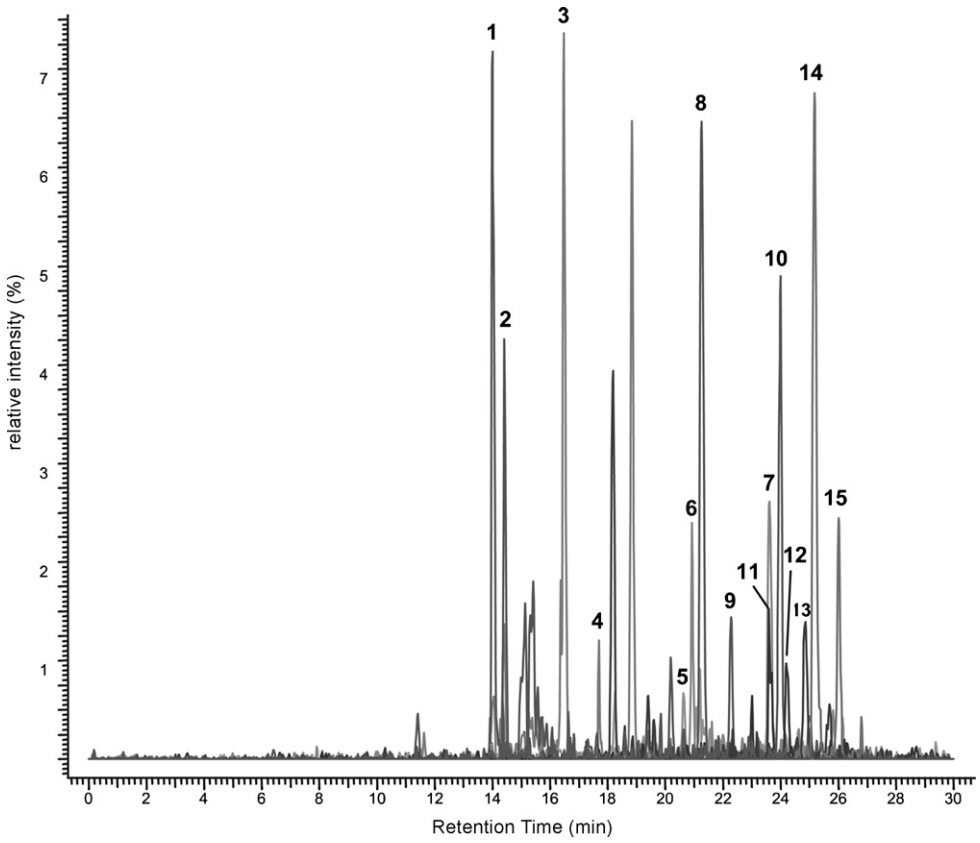

Fig. 5. Oligolignols profiles. Extracted ion chromatograms in the negative mode of DCM extract of wood sap from WMD willows are shown, $[M–H]^-$ XIC for *m/z* 375 (1, 2); *m/z* 435 (3, 4); *m/z* 629 (5); *m/z* 613 (6, 7); *m/z* 583 (8, 9, 10); *m/z* 851 (11, 12); *m/z* 839 (13); *m/z* 809 (14, 15). The numbers refer to the identified oligolignols as listed in Table 2.

8-O-4, 8-5 or 8-8 linkage [24]. Previous research indicated that the wood inside the watermark symptom is affected without signs of delignification [51]. The presence of a huge family of oligolignols in WMD wood proved that the xylem cell wall is degraded. Oligolignols are normally found embedded in the secondary cell wall of lignifying xylem cells and have been identified as precursors in the formation of lignin and involved in wood quality [24]. Their presence in WMD wood is a marker for the collapse of the xylem secondary cell wall and release of these lignin precursors in the wood sap. Different enzymes have been associated with WMD-affected wood [10]. When enzymes that could be involved in degradation of xylem vessel walls were tested, we detected cellulase but not pectinase nor polygalacturonase activity associated with WMD wood and high-density *in vitro* cultures of Bs, indicating cellulase as an important pathogenicity factor. Hypothetically, cellulase destabilizes the maturing cell wall by digestion of the cellulose embedded in the pectin layer and induces the cell collapse. It cannot be excluded that more oligolignols are formed by uncontrolled radical reactions initiated upon damage of the membranes and cytoplasmic radical scavenging environment. Among the WMD-associated enzymes [10], peroxidase and polyphenoloxidase are responsible for more oligolignol polymer formation from the monomers coniferyl and sinapyl alcohols. The observed β-glucosidase [10] might provide supplementary monomers from cytoplasmic (and/or vacuolar) coniferin and synapin hydrolysis. In a third scenario, one of these oxidases could be involved in lignin degradation into oligolignols and function as lignase.

Table 2
Identified oligolignols in DCM extracts of the wood sap of WMD trees

Peak no.	Retention time	Molecular weight	Oligolignol
1	14.01	376	**G**(*t*8-O-4)**G**
2	14.41	376	**G**(*e*8-O-4)**G**
3	16.44	436	**S**(*t*8-O-4)**S**
4	17.7	436	**S**(*e*8-O-4)**S**
5	20.68	630	**G**(*t*8-O-4)**S**(8-8)**S′**/**G**(*t*8-O-4)**S′**(8-8)**S**
6	20.95	614	**G**(*t*8-O-4)**S**(8-5)**S**
7	23.62	614	**G**(*t*8-O-4)**S**(8-8)**S**
8	21.26	584	**G**(*t*8-O-4)**S**(8-5)**G**
9	22.26	584	**G**(*e*8-O-4)**S**(8-5)**G**
10	23.95	584	**G**(*t*8-O-4)**S**(8-8)**G**
11	23.6	582	**G**(*t*8-O-4)**S**(8-5)**G′**
12	24.22	582	**G**(*e*8-O-4)**S**(8-5)**G′**
13	24.9	840	**S**(8-O-4)**S**(8-8)**S**(8-O-4)**G**
14	25.2	810	**G**(8-O-4)**S**(8-8)**S**(8-O-4)**G**
15	26.01	810	**G**(8-O-4)**G**(8-O-4)**S**(8-8)**S**

Peak number refers to Fig. 5. **G** and **S**, guaiacyl (coniferyl alcohol) and syringyl (sinapyl alcohol) units and their aldehydes **G′** and **S′**; 8-O-4′, 8-5′ or 8-8′ linkages; *t* and *e*, threo- and erythro-isomers, respectively.

3.5. Conclusion

We showed that UPLC–MS/MS analysis is a suitable methodology to analyze products in complex biological samples. Two product types could be correlated with WMD in the wood sap of willow, first an accumulation of oligolignols, and second, the presence of QS-active compounds. Production of the AHL–QS molecules was associated with high Bs concentrations and both coincided in wood with WMD development. This does not imply involvement of QS in Bs pathogenesis. However, Bs is widely spread as an endophyte in willow and the trigger for pathogenicity is not elucidated. A correlation between WMD occurrence and the nutritional status of the tree has been found linked to the specific nutritional conditions of the soil on the growth site [9]. Possibly tree nutrition might be

a factor influencing the composition of the wood sap as a growth medium for Bs, but also the impact of other environmental factors must be envisaged.

Acknowledgments

The authors wish to thank Prof. W. Verstraete for providing the *C. violaceum* CV026 strain, Prof. P. Williams for providing the plasmids pSB401 and pSB1075, Martine De Cock and Kris Morreel for critical reading of the manuscript and M.-A. Van Bost for technical assistance. This work was supported partly by the "Agentschap voor Natuur en Bos" (B&G/21/2001).

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