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Native and sulfated oligoglucuronans as elicitors of defence-related responses inducing protection against *Botrytis cinerea* of *Vitis vinifera*

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ABSTRACT

During the past two decades, β-(1,4)-D-polyglucuronic acids (glucuronans) have been intensively described in literature for their physico-chemical and biological properties in different models. In this study, a bacterial glucuronan partially acetylated was degraded by a fungal glucuronan lyase to obtain unsaturated oligogluguronans, finally tested for their potential as elicitors of natural defences in *Vitis vinifera*. The objective was to correlate biological activities with specific structural features using oligoglucuronans varying in degree of polymerization, acetylation and sulfation. The results demonstrated that different defence reactions elicited by these anionic oligomers were dependent on degree of polymerization and nature of substituents (acetyl or sulfate groups). Acetylated oligoglucuronans with degrees of polymerization of 10 and 14 induced transient production of H_2O_2 and expression of some defence-related genes (*PAL*, *STS*, *Chit4c* and *PGIP*). The infection of grapevine leaves by *Botrytis cinerea* was reduced (23%) after treatment with acetylated oligoglucuronans having degree of polymerization of 14. Sulfated oligoglucuronans were not effective for production of H_2O_2 but induced strong expression of other defence-related *PIN*, *PGIP* genes. This work opens the way to new and valuable strategies to protect *V. vinifera* and other plants against pathogens.

1. Introduction

A wide range of pathogens attack grapevine (*Vitis vinifera* L.) causing yield losses and affecting wine quality. Intensive use of phytochemicals is necessary to reduce the impact of these pathogens in vineyards. However, because of the development of pesticide-resistant pathogen strains (Leroux, Chapeland, Desbrosses, & Gred, 1999) and in order to reduce the impact of pesticides on the environment, considerable efforts have been made to develop alternative protection strategies. One solution might be the induction of natural plant defences based on the use of elicitors. Downstream of elicitor perception, rapid signalling events such as the production of active oxygen species (AOS) occur (Alvarez et al., 1998; Jabs, Tschope, Colling, Hahlbrock, & Scheel, 1997) and activate defence-related genes expression leading to reinforcement of plant cell walls, accumulation of antimicrobial compounds such as phytoalexins, and synthesis of proteins with hydrolytic or inhibitory activities towards microbes (Kombrink & Somssich, 1995). Various types of molecules can act as elicitors, including oligosaccharides, proteins, glycoproteins, lipids and glycolipids (Boller, 1995; Côté, Ham, Hahn, & Bergmann, 1998; Varnier et al., 2009). Among them, oligosaccharides have been abundantly studied for their action on growth regulation, plant development, elicitation of defence systems against biotic, or abiotic stress in plants. Oligosaccharides frequently investigated were notably β-(1,3)-(1,6)-glucans, α-(1,4)-oligogalacturonic acids (OGA) and β-(1,4)-cellodextrins (Aziz, Heyraud, & Lambert, 2004; Aziz et al., 2007; Côté et al., 1998; Ferrari et al., 2007). In tobacco (Klarzynski et al., 2000), *Arabidopsis thaliana* (Ménard et al., 2004), rice (Inui, Yamaguchi, & Hirano, 1997), alfalfa (Kobayashi, Tai, Kanzaki, & Kawazu, 1993) and grapevine (Aziz et al., 2003), β-(1,3)-glucans were able to induce a variety of defence reactions conferring resistance to viral, bacterial and fungal pathogens. The biological activity of β-(1,3)-glucans were shown to be dependent on their degree of polymerization (DP), their substituents and on the plant considered (Côté et al., 1998; Darvill et al., 1992; Inui et al., 1997; Ménard et al., 2004). OGA oligomers with a DP higher than 9 have been shown to be active in several plants, including *Arabidopsis* (Moscatiello, Mariani, Sanders,

Abbreviations: AOS, active oxygen species; Chit, chitinase; DP, degree of polymerization; Gluc, glucanase; MAP, mitogen-activated protein; OGA, oligogalacturonides; PAL, phenylalanine ammonia-lyase; PGIP, polygalacturonase inhibiting protein; PIN, serine-protease inhibitor; STS, stilbene synthase; MW, microwave.

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& Maathuis, 2006) and grapevine (Aziz et al., 2004). However, in some reports small-size oligomers induced the accumulation of protease inhibitor proteins and ethylene synthesis in tomato, while larger oligomers were ineffective (Simpson, Ashford, Harvey, & Bowles, 1998).

The structure of oligosaccharides is also important for their biological activities. It is well established that sulfate groups of some oligosaccharides are implicated in biochemical and physiological processes occurring in algae and land plants. As an example, the pattern of sulfate substitution in carrageenans is crucial for recognition between *Chondrus cripus*, a marine red algae, and its green algal pathogenic endophyte, *Acrochaete operculata* (Bouarab, Potin, Correa, & Kloareg, 1999). In the same way, the sulfation of Nod factors is a determinant feature for host specificity in the *Sinorhizobium meliloti*-alfalfa interaction (Roche et al., 1991). In tobacco and *Arabidopsis*, laminarin (β-(1,3)-glucan) became, after chemical sulfation, an inducer of the salicylic acid (SA) signalling pathway leading to protection against tobacco mosaic virus (Ménard et al., 2004). So the development of strategies for structural modification of oligosaccharides appears as a good alternative in the research of new oligosaccharidic elicitors.

Over the past 25 years, microwave-assisted organic synthesis (MAOS) has taken an undeniable place in chemical laboratory practice as a very effective and eco-friendly process (Kappe, Dallinger, & Murphee, 2009; Loupy, 2006). Microwave (MW) heating under controlled conditions presents distinct advantages over conventional heating including shorter reaction times, higher yields, controlled selectivity, lower energy consumption, as well as the possibility to minimize or avoid organic solvents. Nowadays, this technique is considered as an important tool towards green chemistry. It proved to be a clean, cheap and convenient technique. MW technology has been extended to almost all fields of chemistry but little developments have been performed in carbohydrate chemistry (Corsaro, Chiaccho, Venerando, & Giovanni, 2004; Richel, Laurent, Wathelet, Wathelet, & Paquot, 2011). Previous works have reported the rapid access of esterified and/or glycosylated derivatives of D-glucuronic acid from its 6,1-lactone (Bosco et al., 2010; Rat, Mathiron, Michaud, Kovensky, & Wadouachi, 2007) and the synthesis of esterified-glycosylated oligoglucuronans of DP3 under microwave irradiation (Rat et al., 2011). Therefore, using MW irradiation, we have chemically modified (sulfation) oligoglucuronans obtained after enzymatic degradation of a β-(1,4)-D-polyglucuronic acid (glucuronan) excreted by the *S. meliloti* M5N1CS mutant strain (NCIMB 40472) (Delattre, Michaud, Lion, Courtois, & Courtois, 2005; Elboutachfaiti, Delattre, Petit, & Michaud, 2011). The ability to induce an oxidative burst and up-regulation of the expression of defence-related genes of deacetylated, acetylated and sulfated oligoglucuronans with different degree of substitution and DP was compared. Their ability to protect grapevine leaves against *Botrytis cinerea* was also investigated.

2. Materials and methods

2.1. Production of native, highly acetylated and deacetylated glucuronans

Highly acetylated and native glucuronans were produced by the *S. meliloti* M5N1CS mutant strain in bioreactor on RCS medium supplemented or not with Mg^{2+} as described previously (Michaud et al., 1995). Polysaccharides in the cell-free broth were precipitated by addition of 3 volumes of isopropanol and collected by centrifugation (33,900 × *g*, 20 min, 20 °C). Pellets were then freeze-dried. The dry glucuronan was dissolved (4 g/L) in water and isopropanol precipitation step was repeated twice.

Deacetylated glucuronan was obtained after incubating native glucuronan during 12 h at pH 11.8 (addition of KOH 2 M) and at 50 °C. The deacetylated glucuronan was purified by isopropanol precipitation as described above (80% yield).

2.2. Production of crude enzyme extract with glucuronan lyase activity

Trichoderma sp. GL2 was cultivated on 4 L of *Trichoderma* minimum medium supplemented with glucuronan (4 g/L) as sole carbon source (Delattre et al., 2005). The strain grew at 25 °C on a rotary shaker (120 rpm) for 90 h. The extracellular medium was collected by a step of vacuum filtration on fritted glass (150 μm), followed by filtration on a Millistak+ Mini C0CH cellulose ester capsule (Millipore, Bedford, USA). Afterwards, the extracellular medium was concentrated down to 400 mL using an ultrafiltration device (normal molecular weight cut off (NMWCO): 5 kDa, 0.1 m^2 from Sartorius, Goettingen, Germany). A final concentration step was realised on an Amicon stirred cell holding a 10 kDa membrane of polyethersulfone (Millipore, Bedford, USA) down to a final volume of 50 mL. Considering that one unit of activity, measured at 235 nm corresponds to the release of 1 μmol of dp 3 oligoglucuronan per minute (Delattre et al., 2006), 1800 U of glucuronan lyase (GL) activity were collected.

2.3. Enzymatic degradation of deacetylated and acetylated glucuronans

Deacetylated, native and highly acetylated glucuronans were degraded at room temperature in water under gentle stirring using 12 U of crude enzyme per gram of substrate. In order to obtain different oligoglucuronans mixes (60–80% yield), the reaction time reached from 30 min (deacetylated glucuronan) to 24 h (highly acetylated). Intermediate depolymerizations were carried out using native glucuronan as substrate to produce acetylated oligomers with degrees of polymerization between 7 and 19. All enzymatic reactions were stopped by heating the medium at 95 °C during 5 min and insoluble particles were removed by centrifugation during 15 min at 8000 × *g*. Supernatant was precipitated by addition of 7 volumes of isopropanol at 4 °C overnight. Pellets were recovered after a step of centrifugation at 8000 × *g* for 15 min and dissolved in water at approximately 5 g/L before being freeze-dried.

2.4. Microwave-assisted sulfation of oligoglucuronans

2.4.1. Method in solvent conditions

Oligoglucuronans (200 mg) and Amberlite IR-120 (H^+) resin in deionized water (20 mL) were stirred at room temperature for 30 min (final pH 1). After filtration, a solution of triethylamine (1 M in MeOH) was added to the filtrate (final pH 9), followed by solvent evaporation and freeze-drying. The residue was dried under vacuum for 1 h. A solution of the sample and SO_3·DMF (2.4 equiv./free OH) in dry DMF (20 mL) was irradiated (10 W) at 55 °C for 1 h, and the solvent was evaporated under reduced pressure.

2.4.2. Method in solvent-less conditions

Oligoglucuronans (200 mg) and the sulfating reagent (2.4 equiv/free OH) were separately impregnated on alumina (400 mg). The two powders were mixed, placed in the MW oven, and then irradiated (15 W) for 15 min at 60 °C. The powder was extracted with H_2O mQ (3 × 50 mL) and the aqueous solutions were combined and evaporated under reduced pressure.

2.4.3. Purification of sulfated oligoglucuronans

The crude sulfated product was dissolved in MeOH (20 mL), and sodium acetate (600 mg) was added at 0 °C. After stirring 30 min

at 0 °C, the precipitate of sulfated oligoglucuronans was separated by centrifugation, and redissolved in milliQ water (20 mL). A 1 M solution of $Sr(OAc)_2$ (500 μL) was added. After stirring for 30 min at 0 °C, the precipitate of sulfate was removed by centrifugation. Chelex 100 resin (250 mg) was then added to collect supernatant. After stirring for 30 min and filtration, the solution was concentrated to a syrup. Modified oligoglucuronans obtained were then size-fractionated to remove salts by preparative gel filtration on a low pressure column (5 cm × 90 cm) packed with a Superdex 30 phase equilibrated and eluted at 3.5 mL/min with 50 mM $NaHCO_3$. Detection was achieved with a refractometer (R401, Waters). After chromatography, the collected oligosaccharide fractions were stirred under reduced pressure in presence of Amberlite IR-120 (H^+) resin before being treated with Amberlite IR-120 (Na^+) resin. After filtration and drying, the sulfated oligoglucuronans were then obtained in a sodium salt form (56–71% for average DP4, 64–77% for average DP7, 71% for average DP10 and 43–57% for average DP14). The sulfate content was determined by conductimetric NaOH titration of sulfated oligomers transformed into acid form by ion exchange (Casu & Gennaro, 1975) and expressed as the number of sulfate per glucuronic acid unit.

2.5. NMR analysis

^1H NMR analysis was achieved at 80 °C with a Bruker Avance 300 spectrometer of 300 MHz equipped with $^{13}C/^1H$ dual probe. The NMR experiments were recorded with a spectral width of 3000 Hz, an acquisition time of 1.36 s, a pulse width of 7 μs, a relaxation time of 1 s and a number of 256 scans. The HOD signal was presaturated by a presaturation sequence. All samples were previously dissolved in D_2O (99.9% D) and freeze-dried to replace exchangeable protons with deuterium. The freeze-dried samples were then dissolved in D_2O at a 10–30 g/L concentration. The ratio between H-1 signal integration of β-Δ-(4,5)-glucuronic acid (H-1Δ) and all H-1 signal integrations (H-1Δ, H-1, H-1α and H-1β) allowed to access to the average polymerization degree. The *O*-acetyl-esterification proportion for all oligoglucuronan pools was analysed by ^1H NMR spectroscopy with integration of the signals from downfield, upfield and acetyl regions according to previous studies (Courtois et al., 1994; Dantas et al., 1994). They were expressed as the degree of acetylation (DA) which is the number of acetate per glucuronic acid unit.

2.6. Biological materials

Grapevine 41B (*V. vinifera* cv. Chasselas × *Vitis berlandieri*) cell suspensions and vitro-plantlets of grapevine (*V. Vinifera* cv. Chardonnay 75) were cultured as previously described (Bezier, Lambert, & Baillieul, 2002; Varnier et al., 2009).

B. cinerea strain 630 (kind gift of Dr C. Levis, INRA, Versailles, France) was grown on solid tomato/agar medium (tomato juice 25% (v/v), agar 2.5% (w/v)) at 22 °C.

2.7. Treatments

1-day-prior assays, cells were suspended at 0.1 g FW/mL into fresh medium and equilibrated overnight on a rotary shaker (110 rpm, 22 °C). Water-diluted solutions of oligoglucuronans were added directly to the cell suspensions at the indicated concentrations. Then, grapevine cells were used for measurements of H_2O_2 production and gene expression.

2.8. H_2O_2 production measurement

H_2O_2 production was determined using chemiluminescence of luminol. 200 μL aliquots of cells suspensions were added to 25 μL of 300 mM MES buffer (pH 7.0) containing 500 μM luminol. Chemiluminescence, measured within a 10 s period with a luminometer (Berthold Lumat LB9507), was integrated and expressed as μmol H_2O_2/L of extracellular medium, using a standard calibration curve obtained by H_2O_2 added to cell-suspensions aliquots.

2.9. RNA extraction and real-time quantitative RT-PCR

Aliquots of grapevine cell suspensions (1 mL) in the culture medium were filtered and subsequently frozen in liquid nitrogen. Total RNA isolation was obtained by adding 1 mL of trizol (Gibco-BRL) following the manufacturer's procedure.

Total RNAs (150 ng) were reverse-transcribed using the ABsolute MAX 2-step quantitative reverse-transcription (qRT)-PCR SYBR green kit (ABgene, Epsom, UK) with a blend of random hexamers and anchored oligo-dT 3:1 as primers.

RT-PCR was performed with Absolute qPCR SYBR Green Mix (ABgene) using the detection system Chromo4 Real-Time Detector (Biorad). Specific primers were designed using the Primer Express software (Applied Biosystems, Foster City, CA, USA) (Table 1). PCR reactions were carried out in duplicate in 96-well plates according to Aziz et al. (2007), except that EF1, encoding the elongation factor 1 alpha, was used as internal control.

2.10. Protection assays

Conidia of *B. cinerea* strain 630 were collected with 2 mL of potato dextrose liquid medium from a 14-day-old potato dextrose agar culture, filtered to remove mycelial debris and concentration was adjusted to 1×10^6 conidia/mL. For each treatment, leaves of 10-week-old *in vitro* grapevine plantlets were infiltrated during 10 min with the different solutions of oligoglucuronans applied at 200 μM. After 96 h, 30 leaves corresponding to 5 different plantlets (for each treatment) were excised and placed on wet filter paper in plastic petri dishes. 5 μL of a suspension of 1×10^6 conidia/mL was put on each leaf. Quantification of disease development in grapevine leaves after inoculation with *B. cinerea* was measured as average diameter of lesions formed during infection.

All results reported in text and figures are the mean (±SE) of independent determinations. Differences between the means in two groups were evaluated using Student's paired *T*-test; p values < 0.05 were considered significant.

3. Results and discussion

3.1. Production of the deacetylated, acetylated and chemically modified oligoglucuronans

Enzymatic degradation of glucuronan using fungal glucuronan lyase led to various mixes of oligomers with specific degree of polymerization. Higher degrees of polymerization have been obtained playing on the acetylation degree of substrate and incubation times with enzyme according to Section 2. Effectively, acetate substituents have been previously described as inhibitor of glucuronan degradation by glucuronan lyases. So highly acetylated substrates led to oligomers mixes with higher degrees of polymerization (Delattre et al., 2006; Elboutachfaiti et al., 2011). Oligomers generated by these treatments were analysed by ^1H NMR. Their average DP and DA were quantified as described in Section 2 (Table 2) and several families of unsaturated oligoglucuronans with DP between 4 and 19 and DA between 0 and 1.8 were then available for sulfation and biological tests. In order to study the impact of acetate substituents on results of biological tests, some acetylated oligomers were submitted to a soft alkaline treatment. For this, oligomers at a concentration of 5 g/L were incubated at pH 11.5 (KOH addition) during 12 h at 30 °C. These deacetylated oligoglucuronans

Table 1
Primers used for amplification of defence-related gene cDNAs.

Name	Identification[a]	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
EF1	EC959059	GAACTGGGTGCTTGATAGGC	AACCAAAATATCCGGAGTAAAAGA
STS	X76892	AGGAAGCAGCATTGAAGGCTC	TGCACCAGGCATTTCTACACC
PAL	X75967	TCCTCCCGGAAAACAGCTG	TCCTCCAAATGCCTCAAATCA
CHIT4C	AY137377	GCAACCGATGTTGACATATCA	CTCACTTGCTAGGGCGACG
GLUC	AF239617	ATGCTGCAGCAAGTTTGGTT	CAGCCACTCTCCGACAGCAC
PIN	AY156047	AGTTCAGGGAGAGGTTGCTG	CGTCGACCCAAACACGGACCCTAGTGC
PGIP	AF499451	CCTAGACAATCCCTACATTC	GACATTGGGGTCGAATCCTC

[a] NCBI accession number or TC TIGR number.

were treated by two alcoholic precipitations with 7 volumes of isopropanol and freeze-dried. A part of them were then submitted to a MW-assisted sulfation as literature data clearly notified that sulfated oligomers were generally good elicitors of plant defence reactions (Ménard et al., 2004; Trouvelot et al., 2008). Microwave (MW) technology has been investigated because MW activation leads to considerable acceleration of the chemical transformation compared to conventional thermal methods. Moreover, it offers simple, clean, efficient and economic process. While conventional conditions of sulfation require a solvent as DMF or pyridine and longer heating in an oil bath, MW short reaction times prevent decomposition of carbohydrates. Several mixes of oligoglucuronans with average DP between 4 and 14 were variably sulfated (Table 2). The low DP were directly sulfated in solvent-less conditions whereas higher DP (superior to 10) needed to be transformed into their ammonium salt and treated with the sulfating reagent in DMF to obtain satisfactory yields.

3.2. NMR Analysis of sulfated oligosaccharides

^{1}H and ^{13}C NMR spectra of oligoglucuronan mixtures sulfated or not (average DP 4) are shown in Figs. 1 and 2 as examples. Assignments could be performed using 2D sequences (COSY, HSQC) for non-sulfated and sulfated oligosaccharide samples. No NMR data concerning sulfated oligoglucuronans were reported in literature. However some signals could be attributed based on published ^{13}C and ^{1}H NMR spectra of glucans (Gao et al., 1997), heparans sulfate (Papy-Garcia et al., 2005), chitosans (Vikhoreva et al., 2005) and dermatan sulfate (Ludwig-Baxter, Liu, & Perlin, 1991) derivatives. From these published data, the following general observations can be summarized for ^{13}C NMR spectra: upon sulfation, a shielding of about 1–7 ppm of the resonance of the sulfated carbon atom (C′), a shielding of 0–2.5 ppm of the corresponding α carbon atom, and a deshielding of 0–2 ppm for the β carbon is produced. On the other hand, variable deshieldings highly dependent on structure were observed on ^{1}H NMR spectra, but no other general rules could be established from the literature. The differences observed on ^{1}H and ^{13}C NMR chemical shifts between sulfated and non-sulfated oligoglucuronans reported here are depicted in Table 3. In ^{13}C NMR spectra (Fig. 2), for a sulfate/monosaccharide rate of 0.9 (thus one sulfate at *O*-2 or *O*-3) a shielding of 3.2 ppm was observed for C3Δ (Δ = non reducing end) and C3Δ' (Δ' = sulfated non reducing end). For C1′ and C1″ a very small shielding (−0.6 ppm) suggested a major sulfation at *O*-3. However, the high number of signals indicated that the sulfation was not regioselective. For ^{1}H NMR spectra (Fig. 1), changes in chemical shifts showed a deshielding of sugar protons after sulfation, however no correlation with the position of the sulfate group could be clearly established (Fig. 1 and Table 3).

Table 2
Nomenclature and characteristics of tested oligoglucuronans.

Oligoglucuronans[a]	Average DP	Acetate/ GlcA ratio[b]	Sulfate/ GlcA ratio[c]
4D	4	0.0	0.0
4S1	4	0.0	0.9
4S2	4	0.0	2.0
7D	7	0.0	0.0
7A	7	0.4	0.0
7S2	7	0.0	2.0
10A	10	0.6	0.0
10S1	10	0.0	1.2
14D	14	0.0	0.0
14A	14	0.7	0.0
14S1	14	0.0	1.0
14S2	14	0.0	2.0
19D	19	0.0	0.0
19A	19	1.8	0.0

[a] Oligoglucuronans have been tested as mix of oligomers.
[b] Estimated by ^{1}H NMR.
[c] Determined by conductimetric titration.

3.3. Induction of oxidative burst

Production of AOS is an early event following the successful pathogen recognition but is also triggered by purified elicitors (Torres, Jones, & Dangl). The potential of deacetylated, acetylated, partially and persulfated oligoglucuronans with different DP to induce an extracellular oxidative burst after application on grapevine cells was investigated. Deacetylated (Fig. 3a) and sulfated oligoglucuronans (Fig. 3c and d) were unable to induce any significant oxidative burst whatever their degrees of polymerization.

Table 3
Influence of sulfation on ^{1}H and ^{13}C NMR chemical shifts.

Assignment	δ (ppm)	Changes observed on sulfation
H5′, H5″	4.12-4.15	Small deshielding and peak broadening (≈+0.1)
H4	3.89-3.72	Small deshielding and peak broadening (≈+0.1)
H4Δ	5.90	Small deshielding, peak at 6.06 (≈+0.2)
H3	3.73-3.55	Peak broadening
H3Δ	4.23-4.20	Small deshielding (≈+0.1)
H2	3.47-3.28	Peak broadening
H2Δ	3.89	Small deshielding and peak broadening (≈+0.2)
H1β, H1′, H1″	4.69-4.54	Peak broadening
H1α	5.25	Small deshielding (≈+0.3)
H1Δ	5.14	Deshielding and peak broadening (≈+0.3)
C6	175.1	No change
C5, C3, C2,	75.5-72.0	No determined
C5Δ	144.6	No change
C4	80.9-79.8	81.1 (+1.3 ppm)
C4Δ	106.8	107.8 (+1.1 ppm)
C3Δ	65.6	62.4 (−3.2 ppm)
C2Δ	69.3	Small shielding (−0.2 ppm)
C1′, C1″	102.6	102.0 (−0.6 ppm)
C1α, C1β	90.4 and 96.8	No determined
C1Δ	99.1	Small shielding (+0.4 ppm)

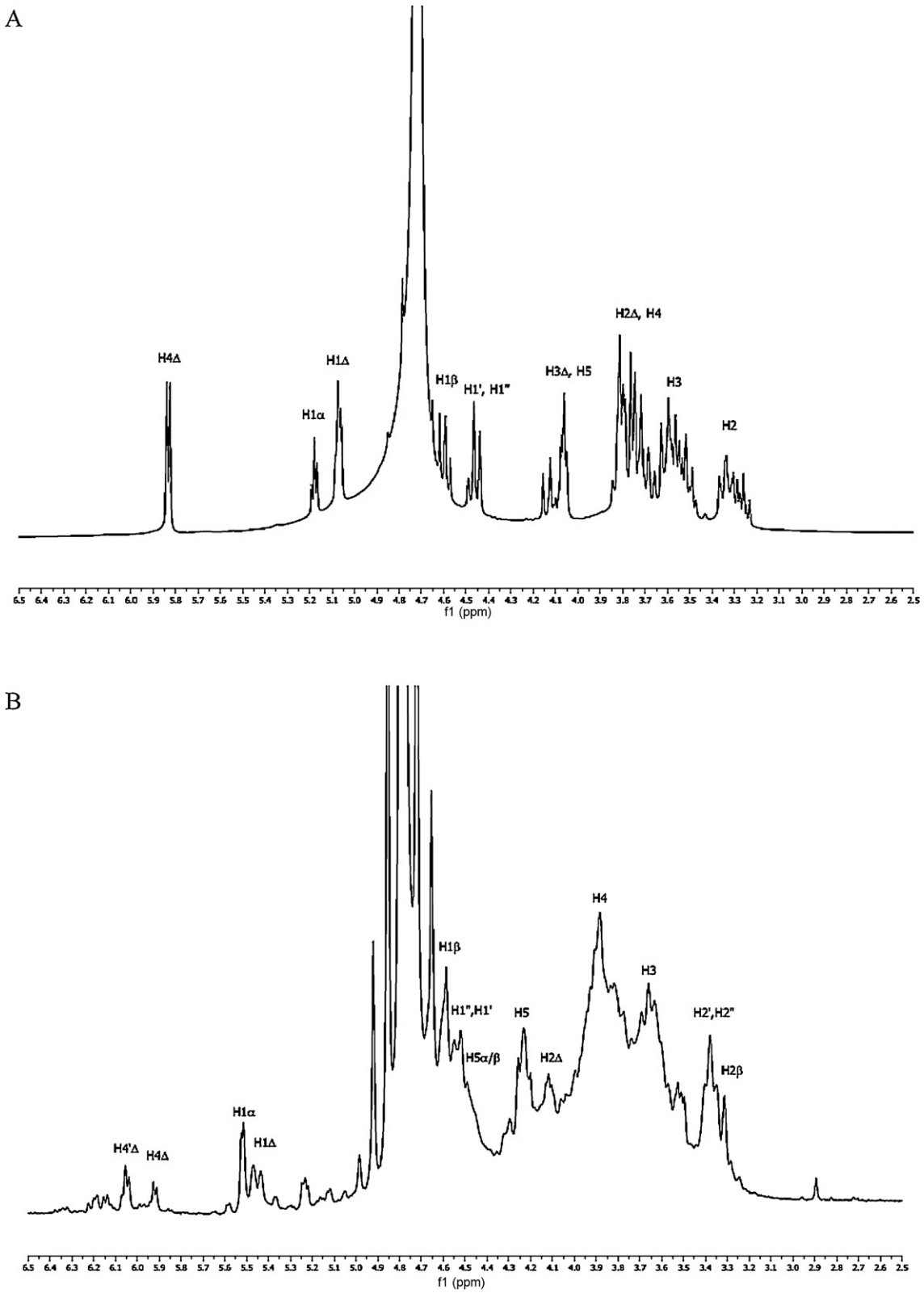

Fig. 1. ^{1}H NMR spectra of oligoglucuronans (DP 4) native (A) and sulfated (B).

Acetylated oligoglucuronans of DP 7 and 19 also failed to induce H_2O_2 production (Fig. 3b), but 10A and 14A induced the production of H_2O_2 that reached a maximum level at 40–50 min after elicitation. The oxidative burst induced by 14A was three times the one measured with 10A (Fig. 3b). For these two oligomers, the transient production of H_2O_2 was significant at 100 μM (data not shown). Concerning the other oligomers, no H_2O_2 production was induced even with treatments up to 1 mM (data not shown). Differential capacity to induce H_2O_2 production or defence responses according to the DP of the oligomers has been previously reported

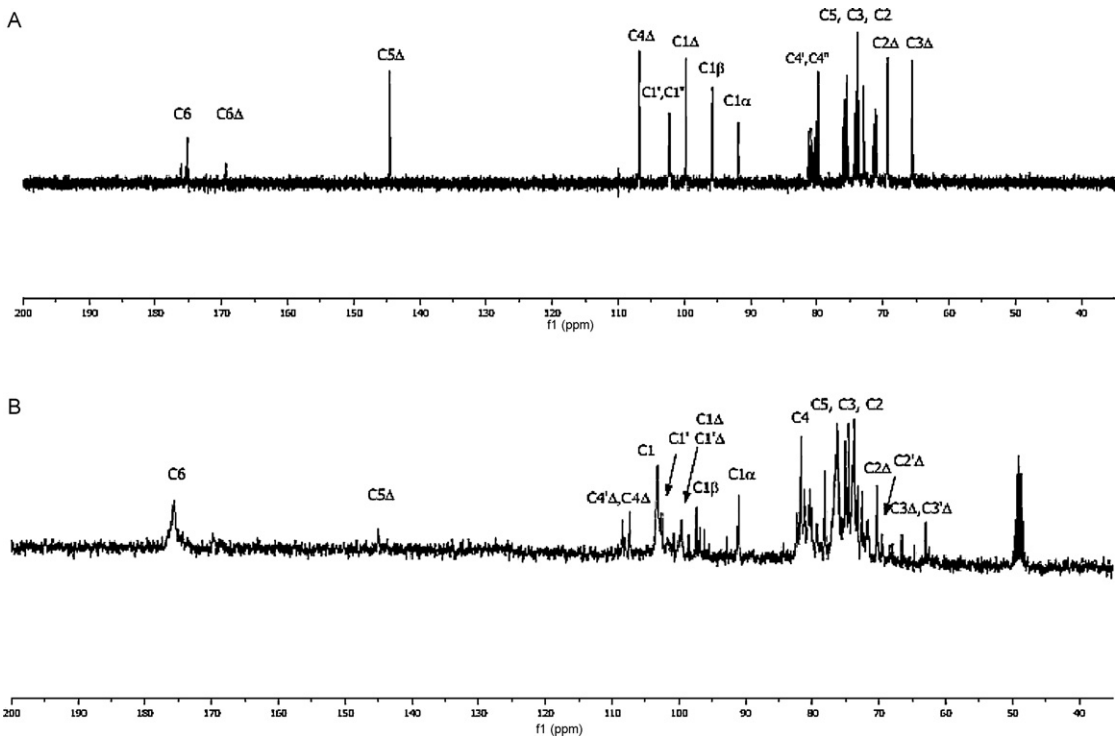

Fig. 2. ^{13}C NMR spectra of oligoglucuronans (DP 4) native (A) and sulfated (B).

in numerous plants (Hahn, 1996; Klarzynski et al., 2000) as well as in grapevine cells treated with oligogalacturonides, β-(1,3)-glucans and cellodextrins (Aziz et al., 2007). Some variations have also been noticed concerning the minimal DP required for efficient elicitation among plants (Hahn, 1996). Here, acetylated oligoglucuronans (DA between 0.6 and 0.7) with DP 10 and 14 were clearly identified as active for H_2O_2 production by grapevine cells while the deacetylated and sulfated forms of 14A (respectively 14D and 14S2) did not induce this H_2O_2 production. Furthermore it was interesting to note that smaller and longer oligoglucuronans (respectively 7A and 19A) which had a different DA, were also ineffective. These results highlight the importance of the DP but also the role of substituents, here acetates versus sulfate, in the oligoglucuronans biological activities.

3.4. Expression of defence related-genes in grapevine cells

Grapevine cells were treated with different oligoglucuronans at 200 μM and the expression of five genes known to be up-regulated in response to various elicitor treatments (Aziz et al., 2007) was analysed using real-time quantitative polymerase chain reaction (qPCR) and specific primers (Table 1). These genes encode phenylalanine ammonia-lyase (*PAL*), a key enzyme of the phenylpropanoid pathway; stilbene synthase (STS), downstream of PAL

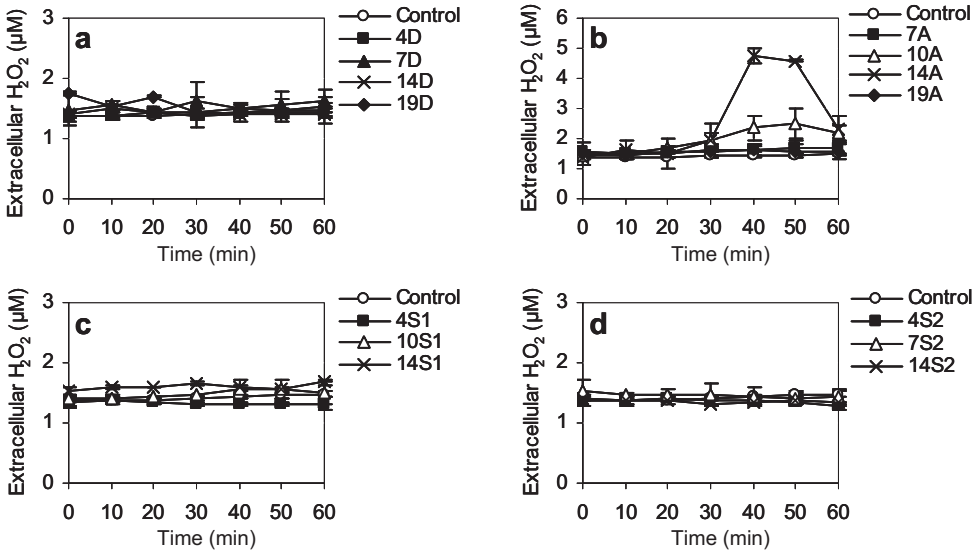

Fig. 3. Time-course of H_2O_2 production in grapevine cell suspensions in response to deacetylated (a), acetylated (b) or mono- and disulfated (c and d, respectively) oligoglucuronans fragments with different DP (4, 7, 10, 14 or 19) applied at 200 μM. The control consisted of grapevine cells treated with an equal volume of water. Data are means ± SE of two independent experiments.

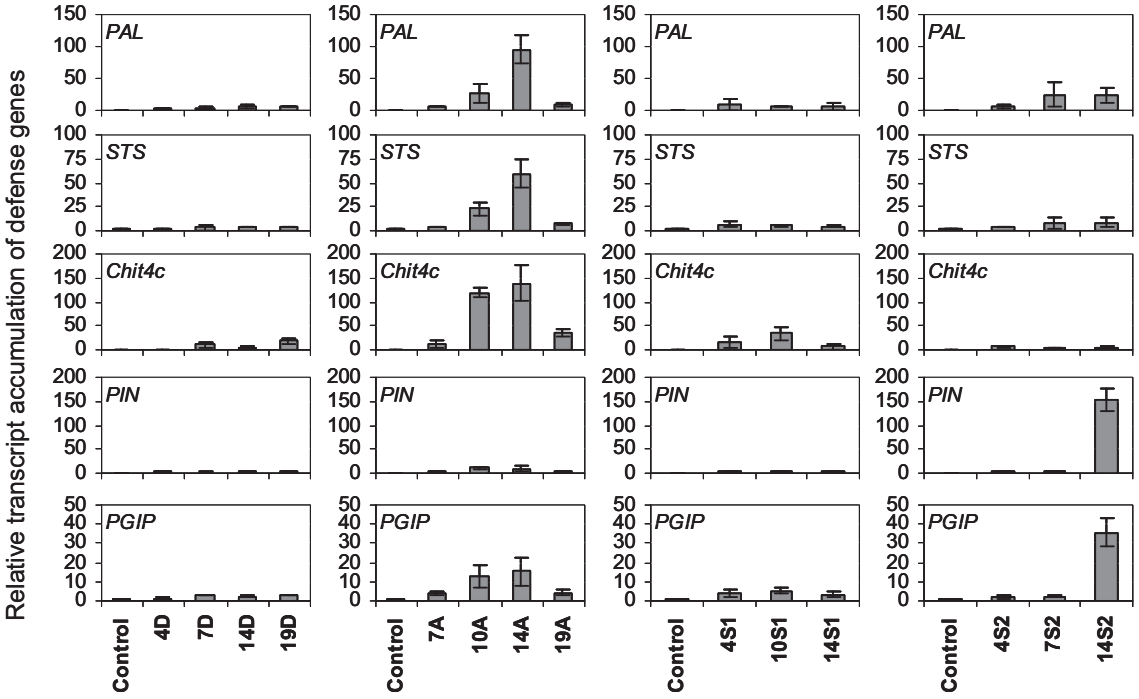

Fig. 4. Expression of defence-related gene transcripts in grapevine cells treated with deacetylated (D), acetylated (A) or mono- and disulfated (S1 or S2) oligoglucuronans fragments with different DP (4, 7, 10, 14 or 19). All oligoglucuronan fragments were applied at 200 μM. Cells were harvested after 3 h of treatment for analysis of phenylalanine ammonia-lyase (*PAL*), stilbene synthase (*STS*), and acidic class IV chitinase (*Chit4c*), at 9 h for serine-protease inhibitor (*PIN*) and polygalacturonase inhibiting protein (*PGIP*). Total RNA was isolated, and transcribed mRNAs were analysed by real-time quantitative RT-PCR. Results are expressed as the fold increase in transcript level compared with the control (water-treated) and are means ± SE of two independent experiments.

and responsible for the synthesis of resveratrol; an acidic class IV chitinase (*Chit4c*) of the PR-3 family, a protease inhibitor (*PIN*) of the PR-6 family and a polygalacturonase-inhibiting protein (*PGIP*). A time-course experiment was analysed (data not shown) and for each gene, the time corresponding to maximum expression was illustrated (Fig. 4).

Deacetylated and partially sulfated (S1) oligomers were the less active elicitors, whatever the oligomer DP and gene considered. Maximal increase was achieved for *Chit4c* with 10S1 (33-fold), 19D (18-fold) and 4S1 (15-fold). Transcript level of the other genes increased by less than 10-fold.

Treatments with acetylated oligomers induced gene expression dependent on the DP and DA. Strongest gene up-regulation was measured after treatment with oligomers 10A and 14A (DA between 0.6 and 0.7) whereas 7A and 19A (DA = 0.4 and DA = 1.8, respectively) remained less active. *PAL* and *STS* inductions were higher in response to 14A than to 10A, whereas *Chit4c*, *PIN* and *PGIP* expressions were similar in response to both oligomers. *PAL* and *STS* expressions were induced, respectively, 95-fold and 60-fold by 14A, and about 3-fold less by 10A. The highest gene expression was recorded for *Chit4c* (about 130-fold) and the lowest gene expression was measured for *PIN* and *PGIP* (about 10–15-fold).

A different pattern of gene expression was induced in response to persulfated (S2) oligomers. The strongest gene up-regulation was measured after treatment with oligomer 14S2 whereas 4S2 and 7S2 induced no or weak gene expression. The highest gene expression was recorded for *PIN* and *PGIP* (respectively 150- and 35-fold) and the lowest gene expression was measured for *Chit4c* (about 6-fold). *PIN* was only induced by persulfated oligomer 14S2 whereas this oligomer mix had no effect on production of H_2O_2. These results suggest that AOS production after elicitation is not necessary for induction of this gene. The observation, in grapevine, that DPI (diphenylene iodonium, inhibitor of AOS production) does not affect induction of *STS* and *PIN* (cells previous treated with OGA) confirmed previous results indicating that the oxidative burst could be independent of phytoalexin or other defence responses (Levine, Tenhaken, Dixon, & Lamb, 1994). Therefore, the role of AOS in defence responses appears to be plant- and gene-specific and elicitor-dependent.

3.5. Protection of grapevine leaves against B. cinerea

Leaves of plantlets were infiltrated with different oligoglucuronans applied at 200 μM. After 96 h, leaves were detached and inoculated with a conidial suspension of *B. cinerea* to evaluate the resistance. Under these conditions, the average diameter of the

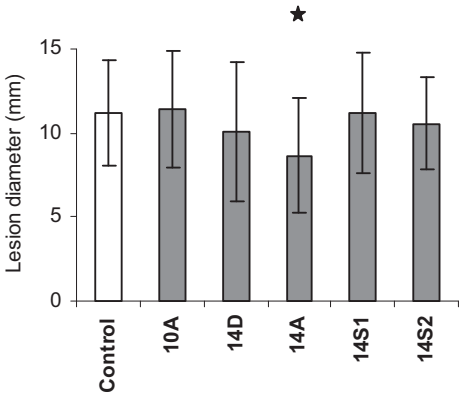

Fig. 5. Protection of grapevine leaves against *Botrytis cinerea* by 10A, 14D, 14A and 14S oligoglucuronans. Leaves of plantlets were infiltrated during 10 min with the different oligoglucuronans applied at 200 μM. After 96 h, detached leaves were inoculated with a conidial suspension (1×10^6 conidia/mL) of *B. cinerea*. Data are means ± SE of diameter of lesions 48 h post-inoculation from 30 different leaves of 10 plantlets. Results are from one representative experiment out of two. Asterisk indicates that values are significantly different ($p < 0.05$) according to the *T*-test.

necrotic lesion measured 48 h after inoculation was reduced (23%) only in 14A oligomer-treated leaves (Fig. 5). The other oligoglucuronans of DP 14 (14D, 14S1 and 14S2) as well as 10A had no significant effect. This result reinforced the elicitor properties of acetylated oligoglucuronans of DP 14.

4. Conclusion

In this study, the induction of differential defence responses in grapevine (*V. vinifera*), namely H_2O_2 production and induction of defence-related genes by linear β-(1,4)-D-oligoglucuronic acids according to their DP and the nature of their substituents was demonstrated. The results showed that treatment of grapevine with oligoglucuronans mobilizes various metabolic pathways in grapevine, including AOS production and induction of expression of different defence-related genes. Acetylated oligoglucuronans of DP10 and DP14 induced transient production of H_2O_2 and the same profile of gene expression (*PAL*, *STS*, *Chit4c* and *PGIP*). But the overall data indicate that elicitation was stronger with DP14 compared to DP10. 14S2 induced only a strong expression of *PIN* and *PGIP* and a slight one of *PAL*. All the other oligomers induced no oxidative burst, and no or weak gene expression. Irrespective of the DP, we also demonstrated that the substituent modified the elicitor properties of the oligoglucuronans. The infection by *B. cinerea* was only significantly reduced by 14A oligomer as with this oligosaccharide the development of the phytopathogenic fungi was reduced by approximately 23%. Oligomers with lower DP (10A) or with different substitutions (14D, 14S1 or 14S2) had no significant effect.

The main advantage of oligomers tested in this work is their availability in large scale and with specific acetylation degrees and sizes, notably with the recent developments of enzymatic reactors with immobilized glucuronan lyase (Tavernier, Michaud, Wadouachi, & Petit, 2009). More, the development of green methodologies of sulfation under microwave irradiation and solventless conditions opens the way to a large variety of these compounds to establish a link between their structural features and their biological properties.

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