DOCTOR OF SCIENCES

Study of the mode of action of COS-OGA, a new class of elicitors of plant innate immunity

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CHAPTER 4

COS-OGA oligosaccharides differentially induce defenses in potato leaves and protect against *Phytophthora infestans*

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Abstract

Potato culture is severely threatened by *Phytophthora infestans*, the late blight agent which is controlled by massive amounts of fungicides. Resistant varieties are often bypassed by the pathogen but the plant innate imunity opens the way to new biological plant protection tools among which COS-OGA. This new active substance mimics the interaction between plants and fungal pathogens as it combines chitosan oligomers (COS) with pectin-derived oligogalacturonides (OGA). Two different compositions comprising COS-OGA were evaluated against potato late blight: FytoSave®, already registered in Europe against powdery mildews and FytoSol which is still under development. Next to the evaluation of their protective effect, a comparative study of plant defense induction was performed focusing on the stimulation of salicylic acid, jasmonic acid and ethylene-related pathways. Our data show that the elicitor FytoSave® strongly triggered the SA pathway but failed to induce a sufficient protection against late blight while FytoSol repressed the SA pathway and was completely efficient. Both oligosaccharide products induced an early accumulation of pathogenesis-related protein transcripts but marker genes suggested that the hormonal regulation induced by both compositions did not rely on SA, JA and ET only and probably included other pathways.

1. Introduction

Potato late blight caused by the oomycete *P. infestans* is the most important potato disease worldwide, accounting for more than 5 billions euros cost per year. The control of the disease requires weekly fungicides applications. However, chemical control is under pressure as *P. infestans* strains become increasingly aggressive, bearing resistance as well as tolerance towards several control products. Even if fungicide choice and application timing are driven by decision support systems that foresee infection periods, a complete growing season usually requires about fifteen sprayings to achieve an efficient disease control (Haesaert et al., 2015). On the other hand, there is a general trend towards reducing the use of chemical pesticides because of environmental concerns and following consumer demand. Potato growers can use varieties bearing a resistance (R) gene that allows protein-mediated activation of the effector-triggered immunity (ETI) but no single R gene offers a sufficient long-term protection against late blight, leading to the selection of "resistance-breaking-strains" (Fry, 2008; Liu *et al.*, 2016a). Current hopes to create a lasting resistance rely on multiple R genes stacking thanks to cisgenesis, a technique that produces GM plants whose introduced resistance genes derive from crossable species. A main advantage of the genetic engineering approach is the speed of the process that allows modifying an existing variety keeping its processing properties, cultivation techniques and consumer's familiarity assets (Jo *et al.*, 2014). But beside regulatory issues with GM plants, the deployment of potato cisgenes will probably require the use of a certain amount of fungicides as numerous *P. infestans* strains are already able to bypass several R genes simultaneously.

A complementary strategy could be the stimulation by pathogen-associated molecular patterns (PAMPs) of the so-called PAMP-triggered immunity (PTI) pathway. PTI is an evolutionary conserved mechanism relying on pattern recognition receptors localized to the membrane. It allows plants to recognize conserved molecules from pathogens (molecular-associated molecular patterns, MAMPs) or deriving from their activity (damage-associated molecular patterns, DAMPs). ETI and PTI show a certain overlap in the downstream expressed defenses including cell wall reinforcement accompanied by accumulation of secondary metabolites, phytoalexins and pathogenesis-related proteins (PR) (Pieterse *et al.*, 2009). So far, two main mechanisms have been characterized: the systemic acquired resistance (SAR) that depends on the plant hormone salicylic acid (SA) and the SA-independent induced systemic resistance (ISR). SAR can be induced by both PTI and ETI and

is associated with local and often systemic accumulation of SA (Pieterse *et al.*, 2014). In plants, SA originates from chorismate and can be obtained by two distinct pathways characterized by their respective main enzyme: the isochorismate synthase 1 (ICS1) and the phenylalanine ammonia-lyase (PAL) (Dempsey *et al.*, 2011). NPR1 (non-expressor of PR genes 1) is a redox-sensitive protein and in combination with its homologs NPR3 and 4, they act as SA receptors and activators of SA-responsive genes such as *pathogenesis-related protein (PR) 1* and other *PR* genes, as well as several WRKY transcription factors. Results obtained with various mutants in model species among which *Arabidopsis thaliana* show that defenses regulated by SA are mainly effective against biotrophic pathogens (Caarls *et al.*, 2015; Pieterse *et al.*, 2009).
On the contrary, ISR which is essentially stimulated by beneficial microbes such as plant growth-promoting rhizobacteria, activates jasmonic acid (JA)- and ethylene (ET)-dependent plant defenses, mainly against necrotrophs and herbivorous insects (Pieterse *et al.*, 2014).

JA derives from lipid oxidation in the chloroplast membrane where linolenic acid is modified by several successive enzymes including 13-lipoxygenase, allene oxide synthase, and allene oxide cyclase (AOC). The main intermediate 12-oxo-phytodienoic acid (OPDA) is then reduced by OPDA reductase 3 followed by three rounds of β-oxidation in the peroxisome leading to JA. JA can be converted in its bioactive form, jasmonoyl-isoleucine (JA–Ile) by jasmonate resistant 1 protein (JAR1) in the cytosol. Rising levels of JA-Ile are sensed by repressor proteins jasmonate ZIM-domain (JAZ) that interact upon binding of JA-Ile with coronatine insensitive 1 (COI1), a F-box protein that targets JAZ proteins to the proteasome. JAZ degradation finally leads to expression of JA-responsive genes (Wasternack and Hause, 2013). ET which often acts in concert with JA, is a gaseous hormone obtained from S-adenosyl-methionine conversion into 1-1 aminocyclopropane carboxylic acid (ACC) by ACC synthase, followed by the action of ACC oxidase (ACO). Rising levels of ET positively regulates EIN3 that activates the transcription of downstream ethylene responsive factors (ERF) (Saubeau *et al.*, 2016). SA and ET/JA pathways have been reported to be mainly antagonistic, even if synergy can occur at low hormone concentrations. This antagonistic process seems to be regulated by reactive oxygen species (ROS), mitogen-activated protein kinases and transcription factors including WRKY, NPR1, JAZ, and TGA (Pieterse *et al.*, 2012).

COS-OGA is a plant defense elicitor based on a MAMP/DAMP combination containing chitosan-derived chitooligosaccharides (COS) and pectin-derived oligogalacturonides (OGA). The commercial product, FytoSave® contains 12.5 g/l COS-OGA and is effective against *Erysiphaceae* in *Solanaceae*, *Cucurbitaceae* and grapevines, all biotrophic plant pathogens (van Aubel *et al.*, 2014). The mechanism involved likely relies on SAR as COS-OGA sprayings induce a cumulative accumulation of SA as well as an induction of SA-related genes and proteins in tomato leaves (van Aubel *et al.*, 2016). SA-mediated defense seems important for basal resistance in potato against *P. infestans* (Halim *et al.*, 2007). Recent results show that FytoSave® is able to reduce late blight severity in potato CV Bintje and that disease control involves the accumulation of *PR1* and *PR2* transcripts. However, the protection obtained is far from complete and the use of FytoSave® alone shall not be sufficient to control potato late blight in open field (Clinckemaillie *et al.*, 2016). Knowing that the degree of polymerization of oligosaccharides as well as the degree of OGA methylation, the degree of acetylation and the pattern of acetylation of COS can strongly modify the way oligosaccharides stimulate PTI (Cabrera *et al.*, 2006; Madhuprakash *et al.*, 2015; Ridley *et al.*, 2001), another COS-OGA composition called FytoSol was supplied for testing by the company FytoFend. This new composition was assessed against potato late blight and compared to FytoSave®. The effect of FytoSol treatments was investigated at two specific time points: 24 h post inoculation (HPI) and 72 HPI. Indeed, *P. infestans* is a hemibiotrophic plant pathogen and at 24 HPI, the pathogen is in its biotrophic stage and the disease is latent with no symptom while at 72 HPI the interaction is fully necrotrophic and the first necrosis become visible (Grenville-Briggs *et al.*, 2010). Hormones were assayed and defense and hormone synthesis gene transcripts were quantified to shed some light on the mode of action of both oligosaccharide compositions on plant defense against potato late blight.

2. Materials and Methods

2.1. Plant material and growth conditions

The late blight-susceptible potato CV Bintje obtained from CRA-W Gembloux, Belgium was maintained in vitro on MS medium in a growth room at 24°C with a 16 h/8 h day/night regime. Three week-old *in vitro* plantlets were cut in stem pieces including one node and rooted in loam under saturated humidity during three weeks. After acclimation, potato plants were transplanted in 1 L containers and watered with FloraSeries nutrient solutions (GHE).

2.2. Oligosaccharide elicitor application

Six week-old potato plants (starting from acclimation) were sprayed with a hand sprayer (Comfort Pump Sprayer 0.5 l, Gardena) on both sides of the leaves either with water or with 1:200 dilution of FytoSave® (FytoFend SA) containing 12.5 g/l COS-OGA or with 1:200 dilution of FytoSol (FytoFend SA) containing 12.5 g/l oligosaccharides. The day of the treatment, plants were well watered and held at 20°C and 90% relative humidity (RH) to maintain stomata open.

2.3. Pathogen culture and inoculation

Ten days before inoculation, a pathogenicity restauration process was started consisting in harvesting sporangia from rye agar plates of 4-week old cultures of *P. infestans* (strain 10-022, mating type A2, CRA-W Gembloux, Belgium) by physical scraping with distilled water containing 0.0025% Tween 20. Droplets of 25 µl of inoculum adjusted at 1.5*104 sporangia/ml kept for 2 h in the dark at 4°C were used to inoculate detached potato leaves maintained in sealed container to ensure saturated humidity at 20°C with a 16 h/8 h day/night regime. The day of the inoculation *P. infestans* sporulation was scraped from the leaves with distilled water containing 0.0025% Tween 20 and adjusted at $1.5*10^4$ sporangia/ml and kept for 2 h at 4°C before inoculation. Mock-inoculated plants were sprayed with distilled water containing 0.0025% Tween 20 on both sides of the leaves till run off with a Venturi glass sprayer. *P. infestans*-inoculated plants were sprayed similarly with the same solution containing *P. infestans* at $1.5*10^4$ sporangia/ml. Following inoculation, plants were kept in a growth cabinet GC-1000 (LabCompanion) at 20°C, 99% RH and a 16 h/8 h day/night regime for the first three days. RH was then lowered down to 70% for the rest of the trial.

2.4. Disease assessments on whole plants

Disease severity was scored three times per week starting 3 days after inoculation on 10 leaves per plant according to a modified visual James scale (James, 1971) to estimate the leaf surface covered by late blight (0 - 1 - 5 - 10 - 25 - 50 - 75 - 95 - 100%). The area under the disease progression curve (AUDPC) was calculated from disease severity as described by Campbell and Madden (1990) and used to calculate a percentage of protection using the formula: (AUDPC control – AUDPC treated) / (AUDPC control) * 100.

2.5. *In vitro* and *in planta* direct toxicity

To assess the *in vitro* direct toxicity of the oligosaccharides against *P. infestans* (strain 10-022), agar discs from 10 mm diameter were taken from the periphery of an actively growing V8 culture and placed face side down in the center of Petri dishes containing V8 supplemented with 0, 0.1, 0.2, 0.5, 1 and 2% (v/v) of FytoSave® or FytoSol. To avoid thermal modifications, oligosaccharides were directly added in the hot medium cooled at 50-60°C just before pouring into sterile Petri dishes. The growth diameters of the colonies were measured after two-weeks of incubation in darkness at 18°C. To assess the oligosaccharides direct toxicity *in planta*, two experiments were conducted starting from fresh sporangium solution at 1.5 * 10^4 sporangia/ml scrapped from rye agar plates of 4-week old cultures of *P. infestans* (strain 10-022) with 0.0025% (v/v) Tween 20. In a first experiment, detached leaves collected from 6-week old potato plants CV Bintje were inoculated with droplets of 25 µl (two droplets per leaflet, five leaflets per leaf) of the sporangium solution kept for 2 h in the dark at 4°C alone or in combination with 0.5% FytoSave® or FytoSol. Leaves were then incubated in sealed containers for one week before scoring each inoculation point (Fig. 4.1). In a second experiment, 6-week old Bintje plants were sprayed at 7, 3 and 1 DBI either with water or with FytoSave® or FytoSol, both at 0.5%. The day of the inoculation, leaves were cut and directly inoculated or washed twice in 0.0025% (v/v) Tween 20 and gently dried with an absorbent paper prior to inoculation. Leaves were inoculated with *P. infestans* at 1.5 * 10^4 sporangia/ml, incubated in sealed container for one week and scored as described above. Leaf disks were punched around inoculation droplets from detached potato leaves and examined with Stereo Discovery.V8 microscope (Carl Zeiss NV) equipped with Axiocam ICc1 (Carl Zeiss NV).

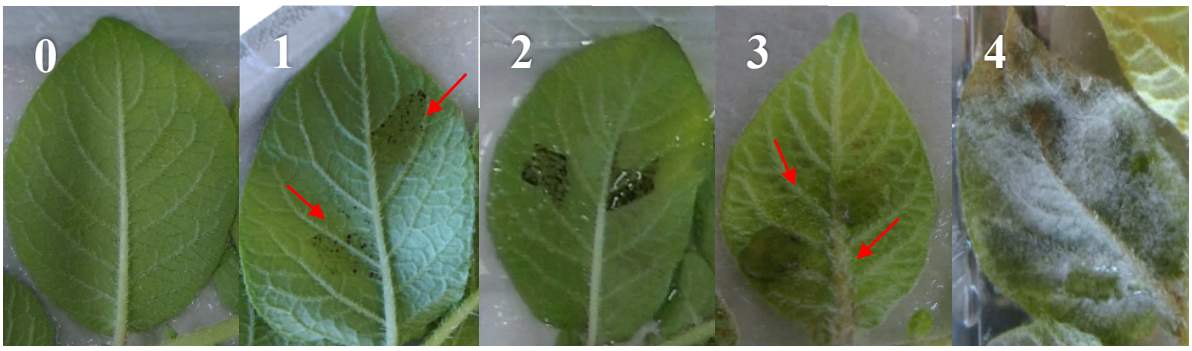

Fig. 4.1: Scale for scoring late blight lesions caused by *P. infestans* on detached leaves of potato CV Bintje.
0, no symptom; 1, small necrosis; 2, evolving necrosis; 3, weak sporulation; 4, intense sporulation.

2.6. Peroxidase activity

Cryogenically-frozen leaf samples (0.5 g) were homogenized in 2 ml 50 mM sodium acetate, 1 M NaCl, 5 mM EDTA, pH 5.2. After centrifugation at 17,000 g, the supernatant was collected and protein content determined with Pierce 660 nm Protein Assay (Thermo Fisher Scientific). Guaiacol peroxidase activity measurement was followed at 420 nm for 5 min on 10 µl of the protein extracts mixed with 0.2 M H_2O_2, 0.2 M guaiacol in 100 mM phosphate buffer, pH 5.8. Guaiacol peroxidase activity was then expressed as percentage of the control average value.

2.7. Phytohormone quantification

Free SA and its conjugated form glycosyl-SA (SAG) extraction were performed on 0.5 g of cryogenically-ground leaf samples as previously described (Verberne *et al.*, 2002). SA was separated on Alltima C18-HL column (2.1 mm × 150 mm, 3 m, Grace). The mobile phase was 0.2 M sodium acetate buffer pH 5.5/methanol (90/10). SA was detected with an Ultimate 3000RS Fluorescence Detector (Thermo Fisher Scientific) with an excitation wavelength of 305 nm and an emission wavelength of 407 nm. Results were expressed in ng SA or ng SAG per g fresh weight.

JA, OPDA and JA-Ile were quantified by LC-MS/MS after extraction and pre-purification from 50 mg of cryogenically frozen leaf sample as described by Balcke *et al.* (2012) using $^2H_5$-OPDA, $^2H_6$-JA, and $^2H_2$-JA-Ile as internal standards. Results were expressed in pmol OPDA, JA or JA-Ile per g fresh weight.

2.8. Measurement of transcript accumulation by qRT-PCR

Total RNA was extracted with RNeasy Plant Mini Kit (Qiagen) using 100 mg of nitrogen-ground leaf samples and treated with DNAse (Roche Diagnostics). RNA purity was determined by the 260/280 nm ratio, quantified with Nanodrop (Thermo Fisher Scientific) and integrity-checked by electrophoresis. Reverse transcriptions were performed on 2 µg RNA with Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics). Quantitative PCR (qPCR) was performed with GoTaq®qPCR Master Mix reagents (Promega) as described by van Aubel *et al.* (2014). Primers for three stable housekeeping genes and for genes of interest were retrieved from literature or designed with Vector NTI Advance 10 software (Thermo Fisher Scientific) (Table 4.1). The results were normalized using the formula previously described (Hellemans *et al.*, 2007) which takes into account three reference genes by a geometrical averaging using the mean of threshold cycle value obtained on the control plants as calibrator. Expression analysis of marker genes *StHB20*, *StOsmotin2*, *StSAUR2*, *StmRNA*, and *StJas* were performed using FastStart Universal Probe master (Rox) reagents and Universal ProbeLibrary probes (Roche Diagnostics) as described by Wiesel *et al.* (2015) also using geometrical means of two stable housekeeping genes (*StNuclear* and *St40S*).

2.9. Statistical analysis

Results were analyzed with Minitab® statistical software, version 17.3.1 (Minitab Inc.) using ANOVA followed by post hoc test for multiple comparisons with $P < 0.05$.

Table 4.1: List of genes and corresponding primers used for qRT-PCR.

Gene	Gene function	Primer sequence (5'-3')	Accession[1]	Reference[2]
EF1	Elongation factor 1-alpha	CCAGATTGGAAACGGATATGC TCCTTACCTGAACGCCTGTCA	SGN-U277726	Nicot et al. (2005)
ACT	Actin	GCTATCCAGGCTGTGCTTTC CAGTAAGGTCACGACCAGCA	SGN-U268955	This study
UBC	Ubiquitin conjugating enzyme	TGATGGTTACCCATTTGAGCC ACTGGTCCTTCAGGATGTC	SGN-U271188	Gallou et al. (2009)
PGK	Phosphoglycerate kinase	TCTACAAGGCCCAAGGTTATG GCAGCAAACTTGTCCGCAATC	SGN-U578082	Ghareeb et al. (2011)
PAL1	Phenylalanine ammonia-lyase 1	GCTTCAAGGCTACTCTGGCATTAG CCTGAGGCAGTGACCGTTCC	SGN-U268525	Saubeau et al. (2016)
ISC1	Isochorismate synthase 1	GTTTGACCGAGGAATGTATGCTG GCACCAAGACCCTTTTCAACC	SGN-U284423	This study
PR1	PR protein-1	GGTGCAGGAGAGAACCTT GGTACCATAGTTGTAGTTTGGCT	SGN-U269202	Gallou et al. (2009)
PR2a	Glucan endo-1,3-β-glucosidase A	AATCAAGATCTTGAAGCCCTAGCCA GATCAACTTCGTTTCCAACGGCTAT	SGN-U269731	van Aubel et al. (2016)
PR2b	Glucan endo-1,3-β-glucosidase B	TGCACCTTTGCTCGTTAACA CCATCGCAGCATAAACAGAA	SGN-U269323	This study
WRKY1	WRKY transcription factor 1	TCAGCATCATCGTCGTCATC TGCCATTAGTCCCAAGAACC	SGN-U273670	Saubeau et al. (2016)
LOX2	13-lipoxygenase	ACTTTTGTGGATTGTTGCTGCTGAG TGTCACCATCCACATCTCGTCCA	SGN-U269397	This study
AOC	Allene oxide cyclase	CGGTAGAGCCAACACCTGAA CAGAACGACCCCAACAAAAGT	SGN-U271935	This study
OPR3	OPDA reductase 3	CAATCCCCTTTTCTCTCCTTACA CGTCGCTCTCTGCTCGTAGT	SGN-U273630	This study
JAZ1	Jasmonate ZIM domain protein 1	TTCATCATCGTCATCGTCGT GGGGTTTTGTTTGTTGGCTA	SGN-U271565	Saubeau et al. (2015)
WRKY33	WRKY transcription factor 33	ATGACCTAAGAACGGGGAACG CAAAGGAAATCCGAACCCA	SGN-U291112	Gong et al. (2015)
PR3	Basic class II chitinase	GCGGTGTCATCTCAAATTCC CAGCACTGATGAAGGCATTG	SGN-U268804	This study
ACO1	1-aminocyclopropene 1-carboxylate oxidase 1	AACAGACGGGACACGAATG ATCTTGGCTCTTTGGCTTGA	SGN-U271918	Saubeau et al. (2016)
EIN3	EIN3-binding F-Box Protein 1	CGACTCTGCTGCTACCGATGG GGTTCTTTCACTCTCAGGTTGCTC	SGN-U269240	Saubeau et al. (2016)
ERF1	Ethylene responsive factor 1	AGGGAGGAGATTGAGCCAGT CGTAAGTTCCAAGCCAAACC	SGN-U292301	Saubeau et al. (2016)
ERF3	Ethylene responsive factor 3	AGGAGGAAGATCTGAAATTCCC AACCATAACCGGACTCGAC	SGN-U278906	Gallou et al. (2011)
PXD	Peroxidase	CCCTCCGTGAAATGGTTGCG TCAGAATCAGTAAGCGTGGCGG	SGN-U285556	This study
SOD	Superoxide dismutase	GCGGGTGACCTGGGAAACAT CCACAAGTGCTCGTCCAACAAC	SGN-U271071	Song et al. (2011)

[1] Accession numbers from Sol Genomics Network, https://solgenomics.net/

[2] Primers either designed in this study or retrieved from literature

3. Results

3.1. FytoSol better protects potato through PTI against late blight compared to FytoSave®

To assess the efficacy of oligosaccharide elicitors against late blight in controlled conditions, potato plants were sprayed three times at seven, three and one day before inoculation (DBI) with water (control) or either FytoSave® or FytoSol at a dilution rate of 1:200 which corresponds to 0.5% (v/v). Sprayings were repeated because previous results obtained on tomato indicated that plant defense stimulation by FytoSave® is a cumulative process that increases with the number of applications (van Aubel *et al.*, 2016). The inoculation was performed by spraying whole plants till run off with about 50 ml per plant of a sporangia suspension of *P. infestans* at $1.5 * 10^4$ sporangia/ml. Disease severity was followed during three weeks (Fig. 4.2) and by the end of the experiment, control plants reached an area under the disease progression curve (AUDPC) of 1,540 while FytoSave®- and FytoSol-treated plants reached an AUDPC of 1,087 and 2, respectively. In other words, both FytoSave® and FytoSol protected potato leaves against late blight but FytoSave® protection only reached 29% protection while FytoSol reached almost 100%. The particularly efficient foliage protection of FytoSol against late blight (Fig. 4.2C) also had a significant positive impact on tuber yield (Fig. 4.2B).

This striking difference raised concern that the higher protection obtained with FytoSol was due to direct toxicity against *P. infestans* since fungicidal activity of chitosan derivatives is well known on several plant pathogens (El Hadrami *et al.*, 2010). FytoSave® and FytoSol were therefore included at different concentrations in V8 agar medium and the growth diameter of *P. infestans* mycelium was measured after two weeks to assess the *in vitro* direct toxicity of both compositions (Fig. 4.3). FytoSave® decreased *P. infestans* growth diameter at the highest dilution tested (0.1%). *P. infestans* growth decreased with increasing concentrations of FytoSave® in the medium but was never completely inhibited in the range tested. FytoSol showed a higher reduction of *P. infestans* growth and the inhibition was complete at 0.5% which is the same concentration as the one used in whole plant efficacy trials and corresponds to 62.5 ppm oligosaccharides in the growth medium. The EC50 values calculated by linear interpolation for FytoSave® and FytoSol were 0.37% (46 ppm) and 0.08% (10 ppm), respectively.

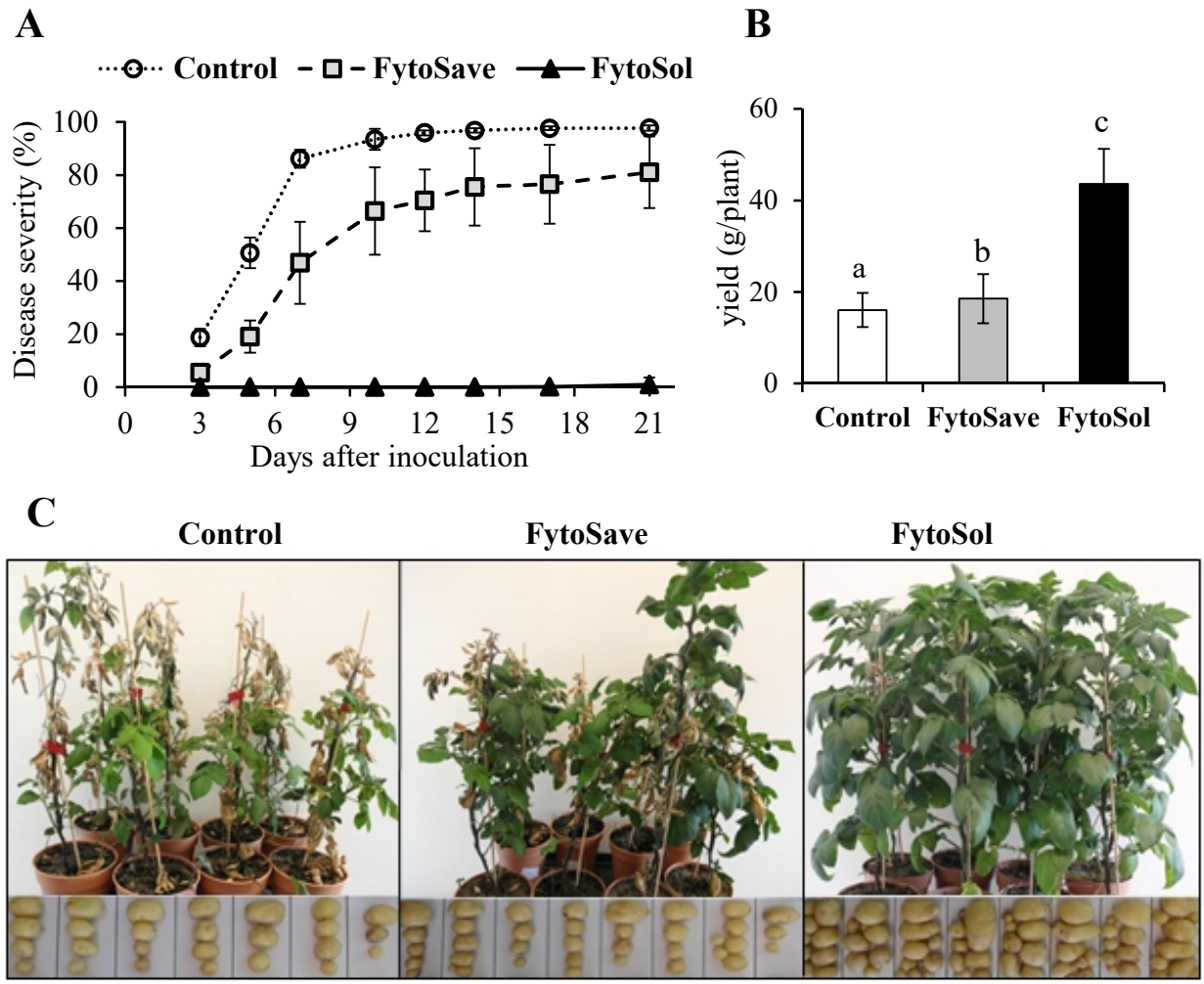

Fig. 4.2: Protective effects of FytoSave® and FytoSol against late blight.
Potato plants (CV Bintje) were sprayed either with water (control) or with FytoSave® at 0.5% or FytoSol at 0.5%, seven, three and one DBI with *P. infestans* at 1.5 * 10^4 sporangia/ml.
A, Development of late blight disease severity during three weeks on ten leaves per plant. Data presented are mean ± SD (n = 8).
B, Mean ± SD (n = 8) of tuber yield (in g/plant) three weeks after inoculation. Bars with different letters are statistically different (ANOVA and Student t test, $P < 0.05$).
C, Pictures of plants and tubers of each treatment at the end of the experiment.

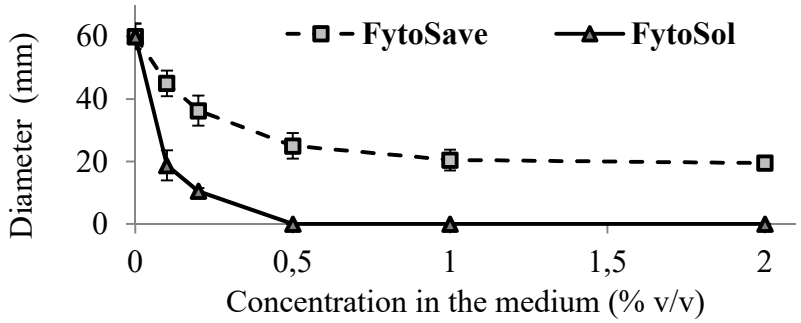

Fig. 4.3: *In vitro* direct toxicity of the oligosaccharide elicitors FytoSave® and FytoSol. Both FytoSave® and FytoSol contain 12.5 g/l COS-OGA. Results are mean ± SD (n = 4) of growth diameter of *P. infestans* mycelium after 14 days on V8 medium in Petri dishes including FytoSave® or FytoSol at various concentrations.

To complete the data on direct toxicity, experiments were performed *in planta* on detached potato leaves inoculated on the abaxial face with ten droplets of 25 µl (two droplets/leaflet, five leaflets/leaf) of *P. infestans* at 1.5 * 10^4 sporangia/ml. Leaves were then incubated in sealed containers for one week before scoring each inoculation point to assess late blight development. In a first experiment, *P. infestans* sporangia suspensions were mixed just before inoculation with either Tween 20 (0.0025%, control), FytoSave® or FytoSol at 0.5%. After one week, no statistically significant difference could be seen between lesion scores in the control, in FytoSave®- or FytoSol-treated plants (Fig. 4.4A).

In a second experiment, whole potato plants were sprayed preventively with the usual sequence at 7, 3 and 1 DBI with water, FytoSave® or FytoSol, each at 0.5%. On the day of inoculation, leaves were detached before droplet-inoculation with *P. infestans* sporangia suspension as described above (elicited, Fig. 4.4B). Part of the collected leaves were washed twice and gently dried with absorbent paper just before inoculation (elicited + washed, Fig. 4.4B). Late blight lesions almost reached the maximum score in control and FytoSave®-sprayed leaves, whether elicited only or elicited and washed. FytoSol-treated leaves reached a score close to 1 in both conditions and had significantly reduced lesion scores compared to control leaves. These data suggest that even if FytoSol showed a certain direct toxicity *in vitro*, the *in planta* effect relied on PTI stimulation and not on any direct effect on the pathogen.

Detached leaves from plants preventively sprayed with FytoSol developed small necroses with hypersensitive response (HR) characteristics. Indeed, first lesions on detached control leaves as well as on whole plant experiments appeared at 72 HPI. First necroses in leaves detached from FytoSol-treated plants started earlier at 24 HPI (Fig. 4.4C) but the symptoms did not develop any further and 7 days later they were still scored as small necrosis (Fig. 4.4B). We never observed these HR-like lesions on whole plants preventively treated with FytoSol in which the inoculum was sprayed uniformly in very small droplets on both side of the leaves and left to dry in about one day in a growth cabinet at 99% RH. Since the defense responses were different in the detached leaf assays, probably because among others systemic signals were lost, all later experiments were performed on whole plants only.

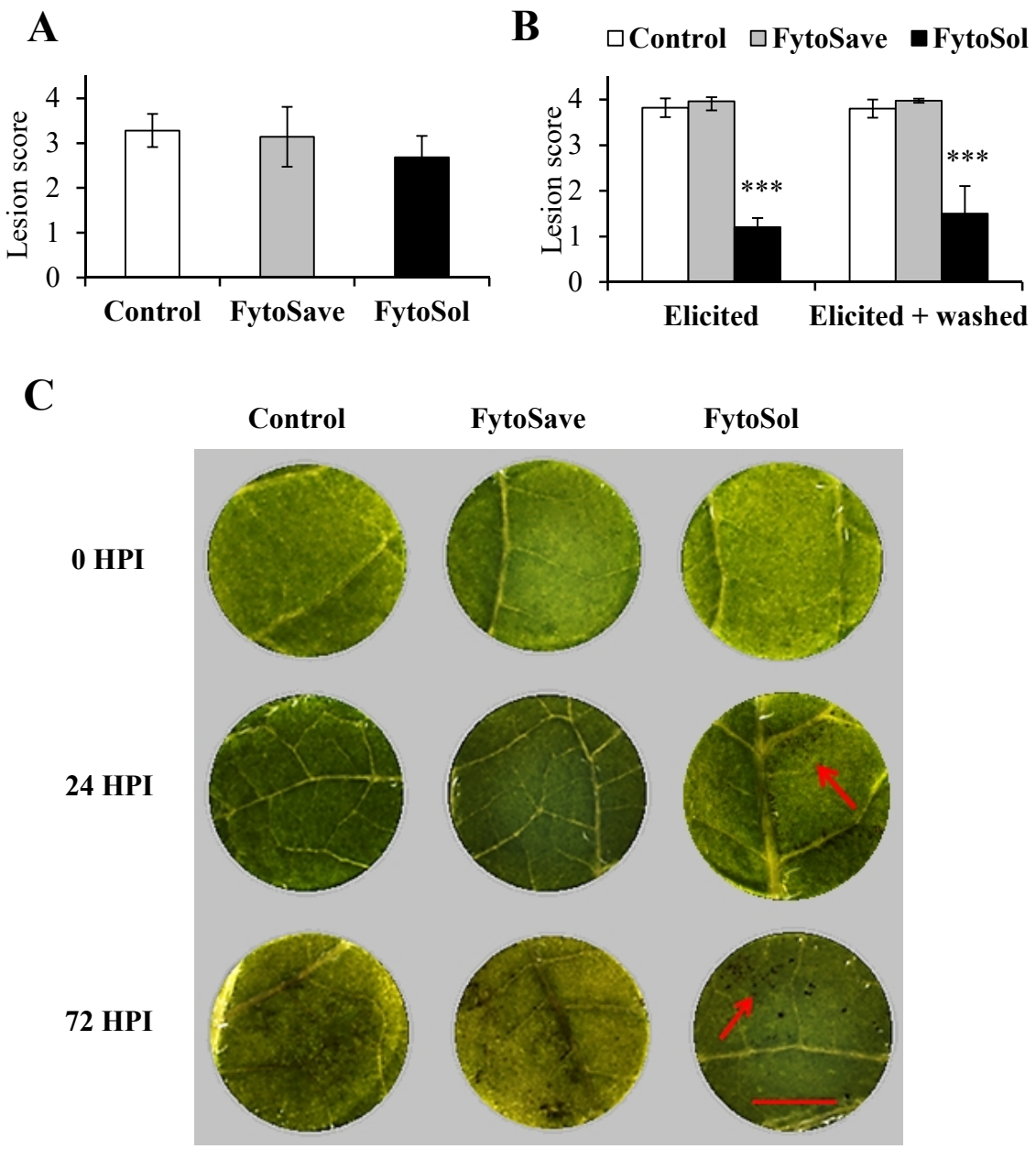

Fig. 4.4: Detached leaf assay on potato (CV Bintje) inoculated with ten 25 µl droplets per leaf of *P. infestans* at 1.5 * 10^4 sporangia/ml. Results obtained seven days after inoculation are presented as mean ± SD (5 leaves, 10 inoculation point/leaf) of late blight lesion development score. Significant differences with the control are indicated by asterisks (ANOVA and Dunnett with *** for $P < 0.001$).
A, ***In Planta* direct toxicity of the elicitors.** *P. infestans* was inoculated on untreated leaves in combination with Tween (0.0025%, control) or with FytoSave® (0.5%) or with FytoSol (0.5%).
B, **Comparison of late blight lesion score between leaves from elicited plants and leaves from elicited plants washed just before inoculation.** Sprayings were performed at seven, three and one DBI with water (control) or either with FytoSave® (0.5%) or with FytoSol (0.5%). Washed leaves were rinsed twice with 0.0025% (v/v) Tween 20 and dried. Leaves were then detached and inoculated with *P. infestans*.
C, **Representative leaf disk punched over an inoculation droplet from detached leaves originating from elicited plants.** Red arrows indicate small necrosis observed in leaves from FytoSol-treated plants. Red scale bar represents 5 mm.

3.2. FytoSol-induced protection against potato late blight is a cumulative process with low persistence

Considering the destructive potential of potato late blight and the polycyclic nature of the disease, farmers do not tolerate any disease spot in their field, which means that the commercial product FytoSave® has no value for this pathosystem. However the high efficacy of FytoSol presents a potential interest for field application but it remained to be determined whether FytoSol has the same behavior as FytoSave® that requires multiple sprayings to keep the plant in a primed state (van Aubel *et al.*, 2014).

Therefore, plants were sprayed three times in one week, but the last spraying was performed at different times before inoculation with *P. infestans*, *i.e.* at 1, 7, 14 and 21 DBI to examine the persistence of the protection conferred by FytoSol. Disease severity was followed during two weeks after inoculation and compared to an untreated control (Fig. 4.5). Disease severity at the end of the experiment increased with the interval between the last spraying and the inoculation. AUDPC reached 898 for control plants and 0, 198, 457 and 787 for FytoSol with a third spraying performed at 1, 7, 14 and 21 DBI, respectively. This means that the protection remained complete for plants sprayed with FytoSol the day before inoculation but decreased down to 78%, 49% and 12% for a last spraying performed one, two and three weeks before inoculation, respectively. Persistence of FytoSol effect was thus limited in time and a last spraying close to the inoculation was necessary to secure a complete protection against late blight.

The cumulative effect of FytoSol treatments was then assessed on potato plants sprayed once, twice or three times before inoculation with *P. infestans* (Fig. 4.6). Two weeks after inoculation, three FytoSol applications had prevented any late blight symptom development while the disease reached 4% severity on plants sprayed twice and 27% after only one application. AUDPC reached 764 for control plants and 144, 24 and 0 for FytoSol applied 1, 2 and 3 times before inoculation, respectively.

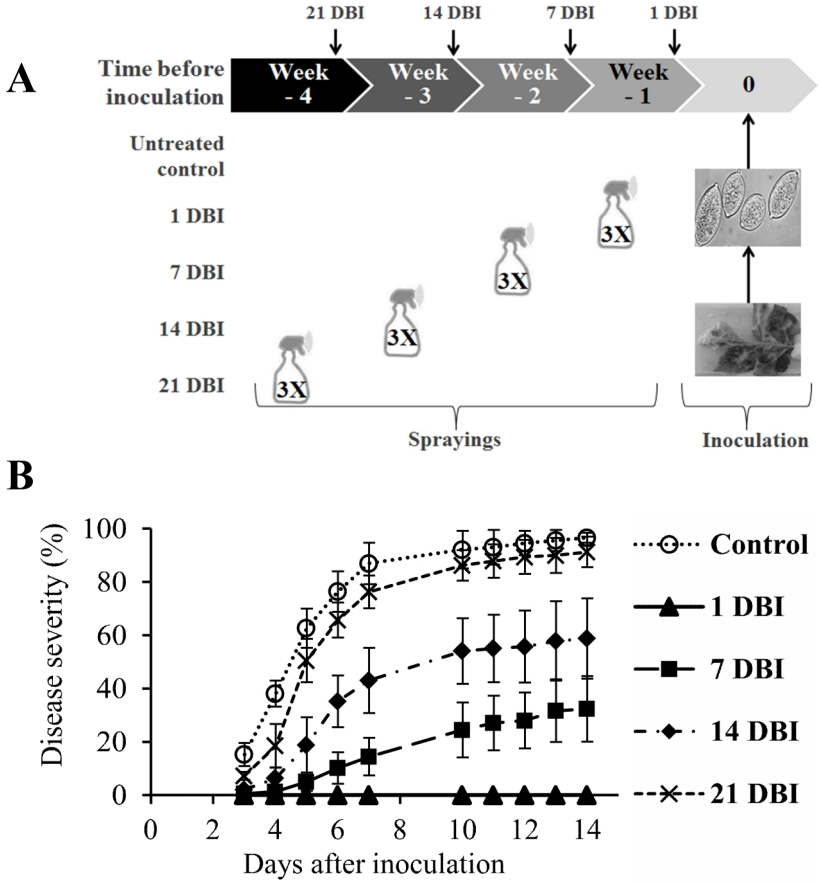

Fig. 4.5: Persistence of the effect of FytoSol on potato plants (CV Bintje).
A, Detail of the application sequence. Control plants were not treated and FytoSol (0.5%) treated plants were sprayed three times per week, seven, three and one day before the last spraying performed at 1 DBI, 7 DBI, 14 DBI or 21 DBI.
B, Development of leaf disease severity was monitored during two weeks on ten leaves per plant inoculated *with P. infestans* ($1.5 * 10^4$ sporangia/ml). Data presented are mean ± SD (n = 8).

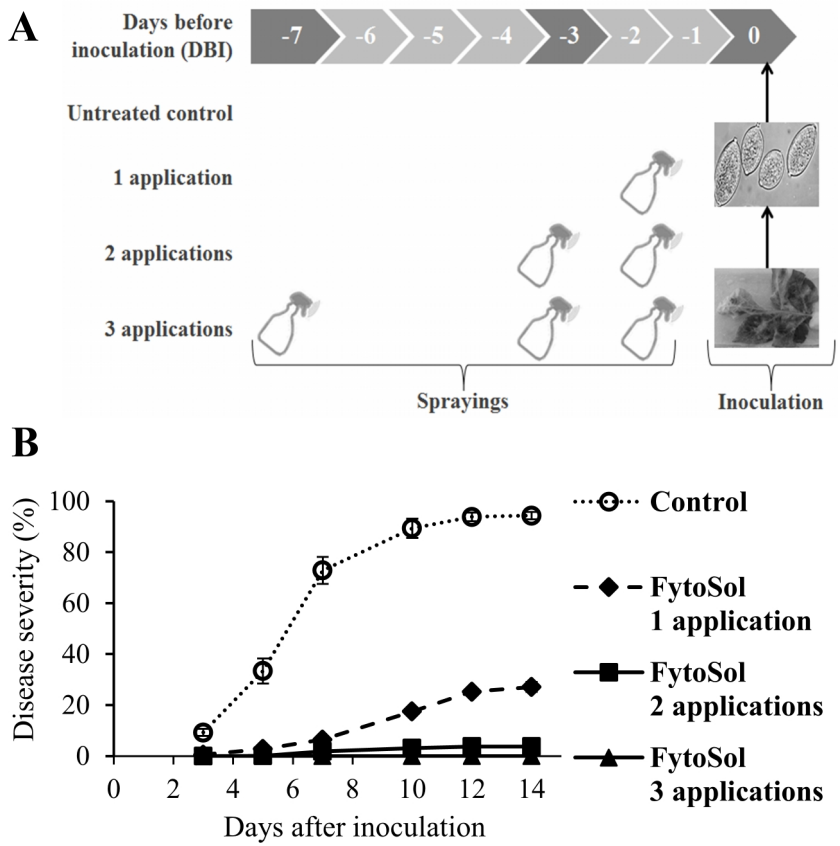

Fig. 4.6: Cumulative effect of FytoSol application on potato plants (CV Bintje).
A, Detail of the application sequence. Control plants were untreated and FytoSol- (0.5%) treated plants were sprayed either once at one DBI, twice at three and one DBI or three times at seven, three and one DBI.
B, Development of leaf disease severity was monitored during two weeks on ten leaves per plant inoculated with *P. infestans* at $1.5 * 10^4$ sporangia/ml. Data presented are mean ± SD (n = 8).

Leaves were also collected from uninoculated potato plants sprayed once, twice or three times, 24 h after each FytoSave® or FytoSol application to quantify peroxidase activity, SA, and glycosyl-SA contents, compared to control plants sprayed with water. Activity of class III peroxidases, key components of PTI involved in H_2O_2 metabolism as well as in synthesis of phytoalexins, suberin and lignin, was previously shown in tomato plants to increase in a cumulative way with the number of FytoSave® sprayings (van Aubel *et al.*, 2016). Here, the effect of FytoSave® and FytoSol on peroxidase activity was similar and two applications were required to observe a significant increase that was cumulative for both compositions (Fig. 4.7A). FytoSave® significantly increased the SA level in potato leaves after the second application while FytoSol showed an opposite trend (Fig. 4.7B). However, while an ANOVA test on all results did not reveal any significant difference, a pairwise Student t test showed that free SA decreased after the second ($P = 0.041$) and the third ($P = 0.006$) FytoSol application. Concerning SAG, the conjugated storage form of SA, it appeared to be 100 times more concentrated in leaves than free SA. Again, FytoSave® increased SAG right from the first application while FytoSol effect was slight and non-significant according to ANOVA tests (Fig. 4.7C), but a pairwise comparison revealed that SAG significantly increased after the second ($P = 0.004$) and the third ($P = 0.001$) application (Fig. 4.7C). Although FytoSave® and FytoSol had similar effects on peroxidase levels, the two compositions clearly affected SA balance in a totally different way. Repeated sprayings with FytoSave® led to a clear accumulation of SA and SAG while repeated sprayings with FytoSol tended to decrease SA and slightly increase SAG.

3.3. FytoSave® but not FytoSol mainly stimulates SAR in potatoes challenged by *P. infestans*

To study the mode of action of the two compositions in presence of *P. infestans,* plants were sprayed three times with the usual sequence at 7, 3 and 1 DBI either with water (control) or with FytoSave® or FytoSol at 0.5%. Potato plants were then split in two groups: the first was mock-inoculated by whole plant spraying (Tween 20, 0.0025%) and the second was inoculated with the same solution containing *P. infestans* at $1.5 * 10^4$ sporangia/ml. Leaves were collected for analysis at 24 and 72 HPI, which corresponds to the biotrophic and the necrotrophic stages of *P. infestans*, respectively (Grenville-Briggs *et al.*, 2010).

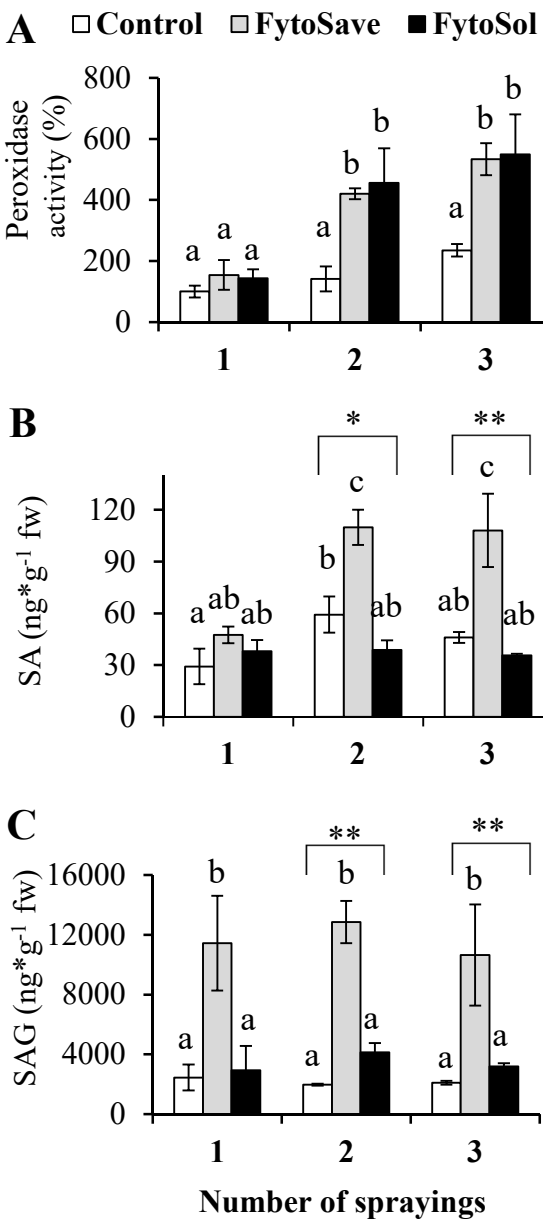

Fig. 4.7: Cumulative effects of FytoSave® and FytoSol sprayings on peroxidase activity (A), free SA (B) and SAG (C) contents of potato leaves CV Bintje sprayed once at one day before harvest (DBH), twice at three and one DBH or three times at seven, three and one DBH with water (control), FytoSave® (0.5%) or FytoSol (0.5%). Results are expressed as mean ± standard deviation (n = 3). Bars with different letters are statistically different (ANOVA and Student-Newman and Keuls test, $P < 0.05$). Significant differences with the controls are indicated by asterisks (ANOVA and Student t test with * for $P < 0.05$, ** for $P < 0.01$).

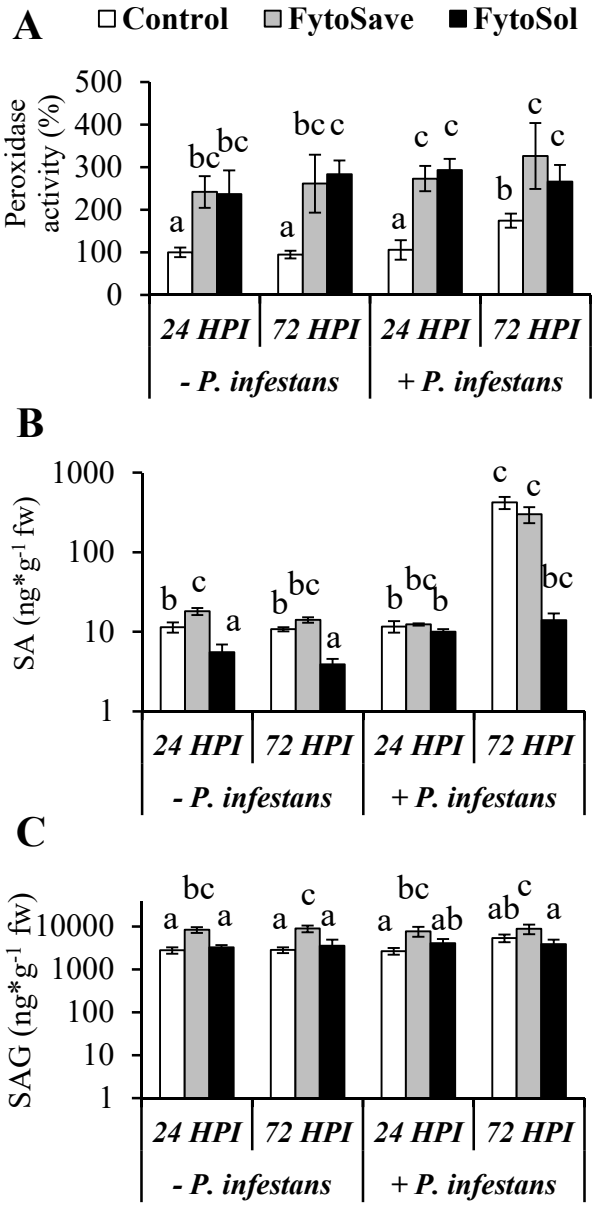

Fig. 4.8: Leaf peroxidase activity (A), free SA (B) and SAG (C) contents of potato plants CV Bintje sprayed with water (control), FytoSave® (0.5%) or FytoSol (0.5%), three times on seven, three and one DBI with *P. infestans* at $1.5*10^4$ sporangia/ml or mock inoculation with Tween 20 (0.0025%). Samples were harvested at 24 and 72 HPI, which corresponds to 48 and 96 hours after the last spraying, respectively. Results are expressed as mean ± standard deviation (n = 4). Bars with different letters are statistically different (ANOVA and Tukey test, $P < 0.05$).

Leaf peroxidase activity was quantified and an increase was noted in leaves treated with FytoSave® and FytoSol but no difference between treatments could be observed at any time point, even after inoculation with *P. infestans*. Peroxidase activity also increased in the inoculated control at 72 HPI (Fig. 4.8A). SA and SAG levels were expressed using a log scale in Fig. 4.8B and Fig. 4.8C.

There was a significant increase of SA in FytoSave®-treated leaves as well as a significant decrease in FytoSol-treated ones, and inoculation with *P. infestans* led to a dramatic accumulation of SA at 72 HPI in both control and FytoSave®-treated plants. The SA content was slightly above 10 ng g^{-1} fresh weight (fw) in mock- and in pathogen-inoculated leaves at 24 HPI but it increased up to more than 400 ng g^{-1} fw at 72 HPI in presence of *P. infestans*. Plants preventively sprayed with FytoSave® reached a similarly high SA level at 72 HPI with *P. infestans*. Plants sprayed with FytoSol in absence of pathogen had significantly reduced free SA contents (Fig. 4.8B) at both time points. Concerning SAG, leaves from plants sprayed with FytoSave® showed significant accumulation at both time points whether inoculated or not while FytoSol had no effect (Fig. 4.8C). After inoculation with *P. infestans*, FytoSol sharply decreased the free SA concentration down to the control level of uninoculated plants.

In the same experiment, transcript levels of defense-related genes were studied by qPCR to assess the effects of both oligosaccharide compositions at 24 and 72 HPI, with or without *P. infestans* inoculation. The genes studied were sorted into SA-, JA- or ET-related genes and three types of genes were considered: genes involved in hormone synthesis, upstream genes coding for transcription factors and downstream hormone-responsive genes such as *PR* or *ERF*.

The expression of genes linked to SA pathway (Fig. 4.9) was investigated starting with *PAL1* involved in SA synthesis as well as *ISC1* which also takes part in phylloquinone synthesis (Dempsey *et al.*, 2011; Garcion *et al.*, 2008). *PAL1* was slightly but significantly overexpressed by FytoSave® treatment at 24 HPI in presence or absence of *P. infestans*, which is consistent with the free SA accumulation observed in sprayed leaves. *PAL1* transcripts dramatically increased in inoculated leaves at 72 HPI for control and FytoSave®-treated plants, which is also consistent with the high free SA increase observed in these leaves.

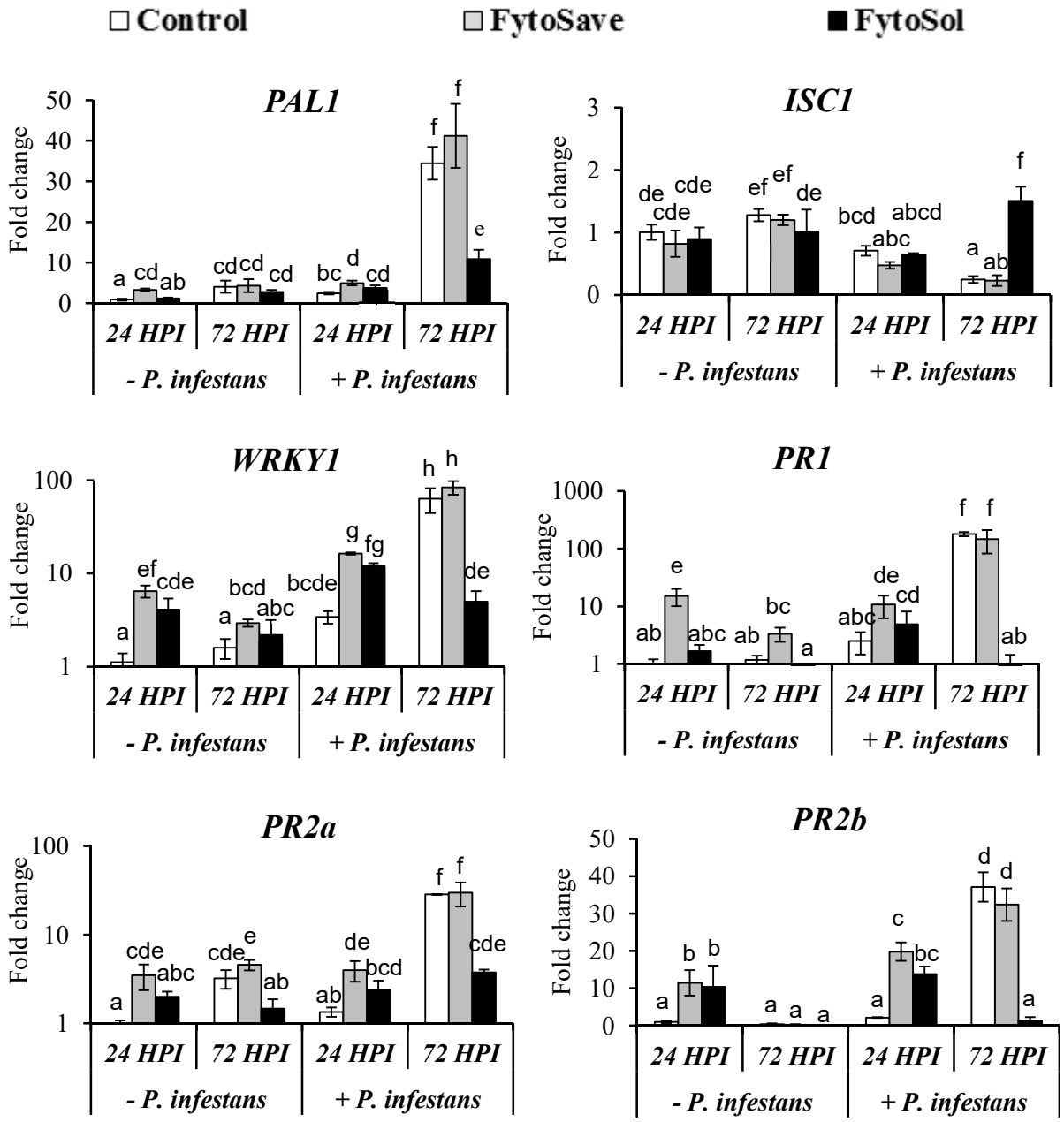

Fig. 4.9: Fold change in transcript accumulation of SA-related defense genes of potato leaves CV Bintje sprayed with water (control), FytoSave® (0.5%) or FytoSol (0.5%), three times, seven, three and one DBI with *P. infestans* at $1.5*10^4$ sporangia/ml or mock inoculated. Samples were harvested at 24 and 72 HPI which corresponds to 48 and 96 hours after the last spraying, respectively. *PAL1* encodes the phenylalanine ammonia-lyase 1; *ISC1* the isochorismate synthase 1; *WRKY1*, the WRKY transcription factor 1; *PR1*, the PR protein-1; *PR2a*, the glucan endo-1,3-β-glucosidase A and *PR2b*, the glucan endo-1,3-β-glucosidase B. Results were normalized using three housekeeping genes *EF1* (Elongation factor 1-alpha), *ACT* (actin) and *UBC* (ubiquitin conjugating enzyme) and were expressed as mean ± standard deviation (n = 3). Bars with different letters are statistically different (ANOVA and Tukey test, $P < 0.05$).

Concerning *ISC1*, controls and treatments did not differ, except at 72 HPI with *P. infestans* where *ISC1* expression dropped for control and FytoSave®-treated plants but was slightly increased for FytoSol treatments. *WRKY1*, a SA-transduction pathway marker (Saubeau *et al.*, 2016) was overexpressed by both elicitor compositions at 24 HPI with and without pathogen. *WRKY1* transcripts also increased with pathogen development at 72 HPI for control and FytoSave® but not for FytoSol. The expression of *PR1* encoding the basic pathogenesis related protein PR1-1 (Gallou *et al.*, 2009), was induced by FytoSave® but not by FytoSol in absence of *P. infestans*. A similar observation could be done after pathogen inoculation but the level of expression was highly increased in control plants at 72 HPI up to a level comparable to FytoSave®-treated plants. *PR1* expression is linked to SAR as is the case for *PR2a* and *PR2b* that encode glucan endo-1,3-β-glucosidase isoforms a and b (Ahmad *et al.*, 2014). The pattern of expression of *PR2a* and *b* was largely similar to the one of *PR1* for control and FytoSave®-treated plants. The expression of both *PR2* genes increased in control and FytoSave® treatments at 72 HPI. FytoSol induced a slight increase of *PR2a* expression in presence of *P. infestans* and a significant transient increase of *PR2b* at 24 HPI. For both oligosaccharide compositions, without pathogen inoculation the gene expression of SA-related genes *WRKY1*, *PR1* and *PR2b* tended to drop from 24 to 72 HPI. Except for *ISC1*, the most conspicuous effect was the strong down-regulation of downstream SA-related genes, particularly evident at 72 HPI with *P. infestans,* after treatment by FytoSol, but not by FytoSave®.

The levels of transcripts of JA-related genes were examined (Fig. 4.10), starting with those involved in JA and oxylipins synthesis: *LOX2*, *AOC*, *OPR3* encoding a 13-lipoxygenase, an allene oxide cyclase and the OPDA reductase 3, respectively (Wasternack and Hause, 2013). An increase of *LOX2* expression was observed at 72 HPI without *P. infestans* in FytoSave®- or FytoSol-treated leaves compared to controls. This increase was not observed for *AOC* but in the presence of *P. infestans*, the levels of *LOX2* and *AOC* transcripts were significantly higher in FytoSol-sprayed leaves. On the contrary there was no significant increase of *OPR3* transcripts in FytoSol-treated leaves. *OPR3* expression was slightly higher after inoculation with *P. infestans* at 24 HPI for FytoSave® and at 72 HPI for control and FytoSave®-treated plants. *PR3*, a class II chitinase linked to the JA pathway (Ahmad *et al.*, 2014) was upregulated in FytoSave®- and FytoSol-treated leaves at 24 HPI w/without inoculation and in inoculated control and FytoSave®-treated plants at 72 HPI.

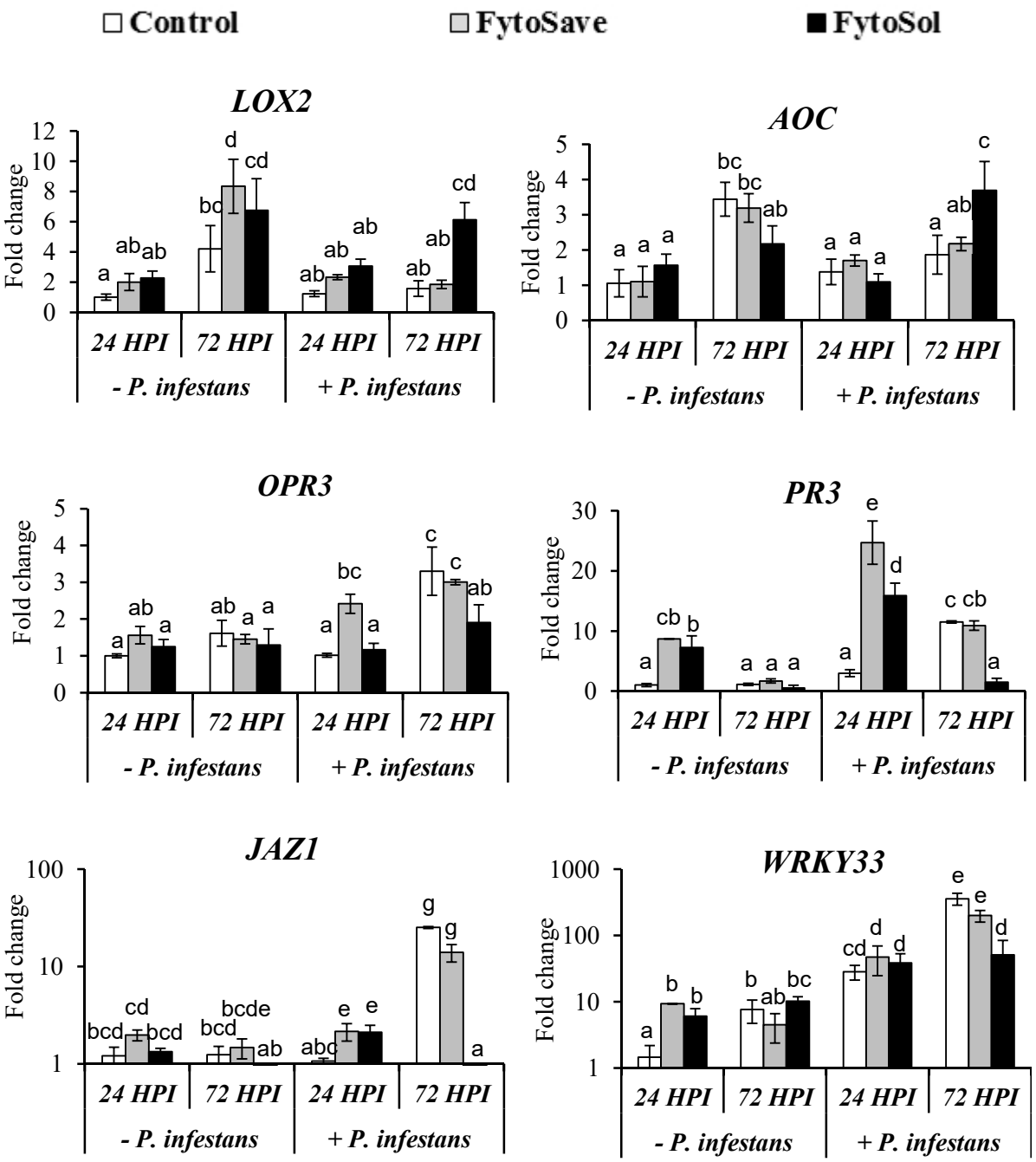

Fig. 4.10: Fold change in transcript accumulation of JA-related defense genes of potato leaves CV Bintje sprayed with water (control), FytoSave® (0.5%) or FytoSol (0.5%), three times, seven, three and one DBI with *P. infestans* at $1.5*10^4$ sporangia/ml or mock inoculated. Samples were harvested at 24 and 72 HPI which corresponds to 48 and 96 hours after the last spraying, respectively. *LOX2* encodes the 13-lipoxygenase; *AOC,* the allene oxide cyclase, *OPR3*, the OPDA reductase 3; *PR3*, the basic class II chitinase; *JAZ1*, the jasmonate ZIM-domain protein 1 and *WRKY33*, the WRKY transcription factor 33. Results were normalized using the three housekeeping genes *EF1*, *ACT* and *UBC* and were expressed as mean ± standard deviation (n = 3). Bars with different letters are statistically different (ANOVA and Tukey test, $P < 0.05$).

The expression of *JAZ1*, encoding the pivotal JA-responsive gene repressor, was significantly increased at 24 HPI without pathogen in FytoSave®-treated plants. *JAZ1* transcripts accumulated in control and FytoSave®-treated plants at 72 HPI in presence of *P. infestans* but were highly repressed after FytoSol treatments. *WRKY33* transcripts are required for resistance against necrotrophic fungal pathogens and act as positive regulators of JA-responsive genes (Saubeau *et al.*, 2016; Zheng *et al.*, 2006). The gene was overexpressed at 24 HPI after treatment by both oligosaccharide compositions in absence of *P. infestans*. Its transcripts also accumulated in all plants after pathogen challenge at both time points but the increase was more important for control and FytoSave® at 72 HPI. *PR3*, *JAZ1* and *WRKY33* are genes acting downstream to JA and they were all strongly down-regulated in FytoSol-treated plants 72 h after inoculation by *P. infestans*.

As JA-dependent defense often acts in concert with ET-regulated genes, we also determined transcript levels of *ACO1* encoding the ACC oxidase 1 (Saubeau *et al.*, 2016) involved in ET synthesis (Fig. 4.11). *ACO1* transcripts also accumulated in the necrotrophic stage of *P. infestans* development (72 HPI) in control and FytoSave®- but not FytoSol-treated plants. Both oligosaccharide complexes induced a non-significant accumulation of *EIN3* at 24 HPI w/without *P. infestans*. *EIN3* encodes a transcription factor that accumulates after ET increase and regulates the expression of many defense-related genes such as *ERF1* and *ERF3* (Robert-Seilaniantz *et al.*, 2011). The expression of *ERF1* was slightly but not significantly increased at 24 and 72 HPI without the pathogen in FytoSol-treated leaves. *P. infestans* inoculation seemed to increase *ERF1* expression but it was only significant for FytoSave®-treated plants at 72 HPI. Concerning *ERF3*, a higher expression was only seen after inoculation at 72 HPI for control and FytoSave®-treated plants. Just like with SA- and JA-regulated genes, genes acting downstream to ET were strongly down-regulated in FytoSol-treated plants 72 h after inoculation by *P. infestans*.

We have also studied two genes involved in ROS regulation (data not shown). FytoSol treatment followed by pathogen inoculation led to transcript accumulation for both genes encoding peroxidase and superoxide dismutase, while FytoSave® led to accumulation of peroxidase transcripts only.

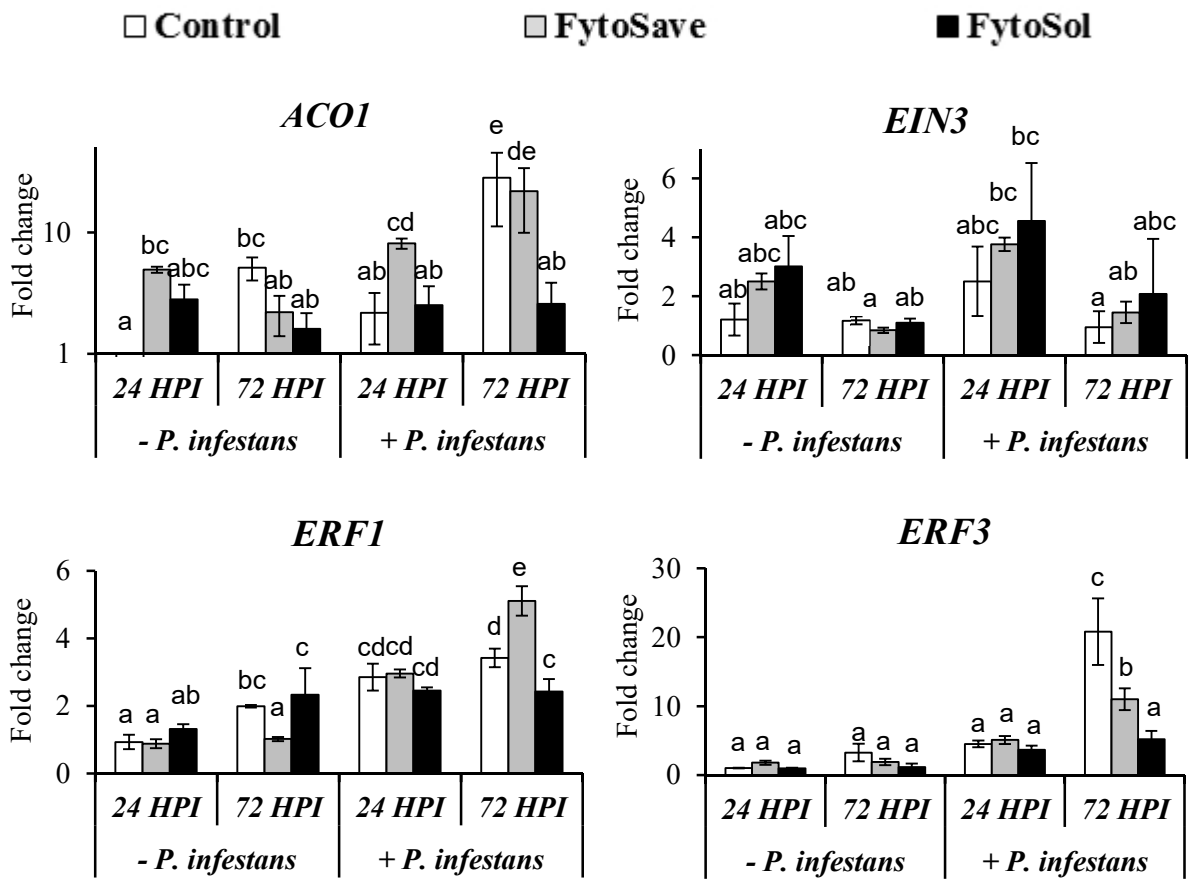

Fig. 4.11: Fold change in transcript accumulation of ET-related defense genes of potato leaves CV Bintje sprayed with water (control), FytoSave® (0.5%) or FytoSol (0.5%), three times, seven, three and one DBI with *P. infestans* at $1.5*10^4$ sporangia/ml or mock inoculated. Samples were harvested at 24 and 72 HPI which corresponds to 48 and 96 hours after the last spraying, respectively. *ACO1* encodes the ACC oxidase 1; *EIN3,* the EIN3-binding F-Box protein 1; *ERF1*, the ET responsive factor 1 and *ERF3*, the ET responsive factor 3. Results were normalized using the three housekeeping genes *EF1*, *ACT* and *UBC* and were expressed as mean ± standard deviation (n = 3). Bars with different letters are statistically different (ANOVA and Tukey test, $P < 0.05$).

As mentioned above, FytoSave® induced a higher expression of the JA-linked *PR3* gene. Therefore, several jasmonate-related compounds were quantified using the same experimental design as mentioned above (Fig. 4.12): the JA precursor OPDA, JA and its biologically active form JA-Ile did not accumulate at any time in potato leaves pretreated with either FytoSave® or FytoSol. The inoculation with *P. infestans* also did not alter the levels of all three compounds in leaves of control plants or plants treated with the two oligosaccharide compositions under study.

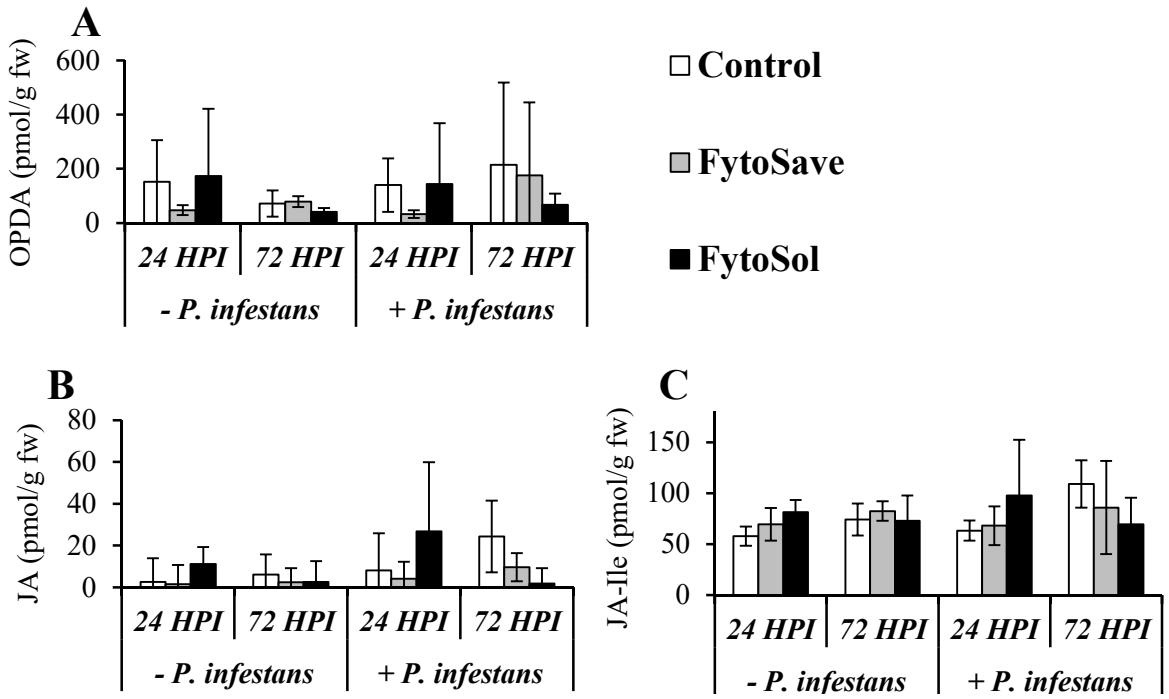

Fig. 4.12: OPDA (A), JA (B) and JA-Ile (C) content in potato leaves, CV Bintje sprayed with water (control), FytoSave® (0.5%) or FytoSol (0.5%), three times, seven, three and one DBI with *P. infestans* at $1.5*10^4$ sporangia/ml. Samples were harvested at 24 and 72 HPI, which corresponds to 48 and 96 hours after the last spraying, respectively. Results are expressed as mean ± standard deviation (n = 4). No statistical difference could be observed (ANOVA, $P < 0.05$).

Since FytoSave® and FytoSol could potentially induce the stimulation of other hormonal pathways, we tested recently validated defense marker genes obtained in potato by Wiesel *et al.* (2015). Using microarrays, these authors identified regulated transcripts at 1 and 6 hours after application of pure hormones (HPT) and they specifically designed primer pairs coupled with TaqMan® probes for detection of hormone-regulated transcripts. We harvested potato leaves at 1 and 6 h after one spraying with water (control), with FytoSave® or FytoSol, both at 0.5% to quantify marker genes expression with the specific TaqMan® probes (Fig. 4.13). *StHB20*, the abscisic acid marker was significantly upregulated by FytoSol at 1 HPT but not at 6 HPT, and by FytoSave® at 6 HPT. *StOsmotin2*, the ET marker was only upregulated by FytoSave® treatment at 6 HPT. The expression of *StSAUR2*, the brassinosteroid (BR) marker was halved by both elicitors at 1 and 6 HPT. Interestingly, *StmRNA*, the SA marker was upregulated 4-times by both elicitors at 1 HPT but almost completely repressed by FytoSol at 6 HPT with an expression level more than 10-times lower than the control. Concerning the JA marker *StJas*, a similar upregulation of 4-times was observed at 1 HPT but at 6 HPT the only significant upregulation was observed in FytoSave®-treated leaves.

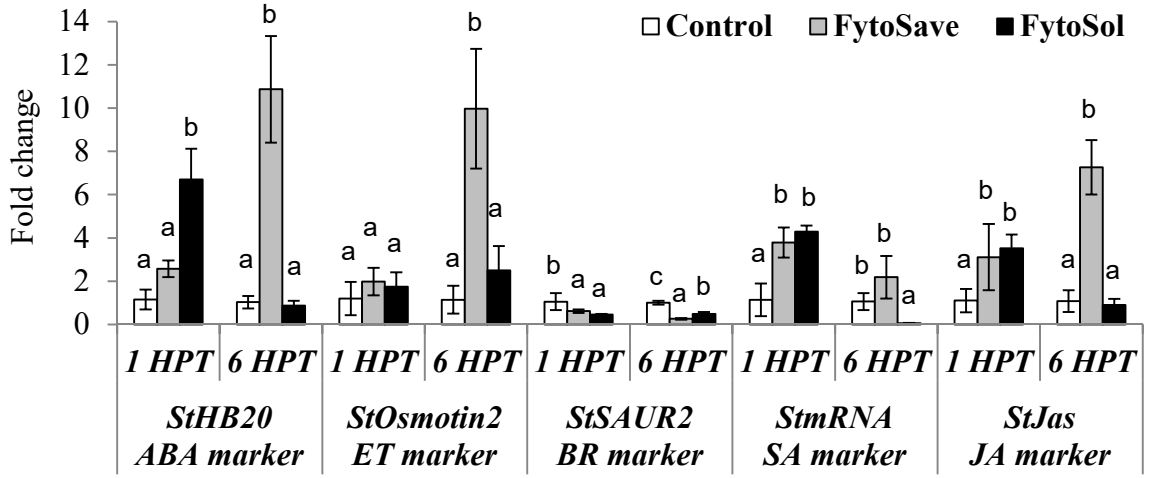

Fig. 4.13: Fold changes in transcript accumulation at one and six HPT for marker genes assessed with TaqMan in potato plant leaves CV Bintje sprayed once with water (control), FytoSave® (0.5%) or FytoSol (0.5%). *StHB20* encodes the homeodomain 20 transcription factor used as ABA marker; *StOsmotin2,* the Osmotin OSML 15 used as ET marker; *StSAUR2*, the auxin-induced SAUR used as BR marker, *StmRNA* , a probable WRKY40 transcription factor used as SA marker and *StJas*, the jasmonate ZIM-domain protein 1 used as JA marker. Results were normalized using the two housekeeping genes *StNuclear* (Small nuclear ribonucleoprotein G) and *St40S* (40S ribosomal protein S8) and were expressed as mean ± standard deviation (n = 4). For each gene and time point, bars with different letters are statistically different (ANOVA and Tukey test, $P < 0.05$).

4. Discussion

We investigated the effect of two oligosaccharide compositions, FytoSave® and FytoSol against *P. infestans* on potato. Both products comprise defined COS and OGA fractions representing MAMPs and DAMPs respectively that constitute a combined signal informing the plant on both pathogen presence and cell wall degradation. In our experiments involving the hemibiotrophic oomycete *P. infestans* inoculated on potato leaves CV Bintje in controlled conditions, FytoSave® reduced late blight symptoms by 29% only while FytoSol nearly perfectly controlled the disease. These results were similar to those obtained by Clinckemaillie *et al.* (2016) for FytoSave® which provided almost 50% protection at a slightly reduced inoculum concentration.

FytoSol had a higher *in vitro* direct toxicity than FytoSave® but the inoculation of *P. infestans* combined with FytoSol in the sporangia suspension did not reduce late blight symptoms on plants while leaves detached from plants pretreated three times with FytoSol showed HR-like lesions as soon as 24 HPI. By comparison, the EC50 of the conventional fungicide Mandipropamid on mycelial growth of *Phytophthora parasitica* is about 0.011 ppm which is about one thousand times lower than the EC50 concentration calculated for FytoSol on *P. infestans* (Wang et al., 2013). Since mycelial growth inhibition by MAMPs such as chitosan and rhamnolipids is often reported (Varnier *et al.*, 2009), it is not surprising that FytoSol had some negative effect *in vitro* on mycelium development. But direct toxicity studies *in planta* could be further refined to determine if certain growth stages of *P. infestans* are more sensitive by evaluating sporangia germination, conidia viability and mycelium sporulation in presence of FytoSol.

FytoSol showed a cumulative effect with the number of sprayings: to achieve a complete control of *P. infestans*, it had to be applied three times with a last spraying close before the inoculation. The effect of FytoSol was therefore due to PTI stimulation, although FytoSol had probably also a certain antimicrobial effect against *P. infestans.* The cumulative stimulation of PTI by FytoSol and FytoSave® was also observed with class III peroxidase activity, something that had already been demonstrated with FytoSave® on tomato. Changes in ROS levels are an early conserved event of PTI acting upstream of hormonal modulation. Class III peroxidases take actively part in plant defense by consuming H_2O_2 to synthetize phenolic compounds, lignin polymers and to crosslink proteins and ferulic acid with pectin. The result is a major cell wall stiffening that ensures reinforcement of plant's pre-invasive barriers to infection. Class III peroxidases have a dual role as they can also produce $O_2^{\cdot-}$ that dismutates into H_2O_2, a systemic signal (Pastor *et al.*, 2013; Raggi *et al.*, 2015). Both compositions increased peroxidase transcript levels but only FytoSol led to superoxide dismutase transcripts accumulation. These enzymes can modulate either positively or negatively apoplastic ROS that indirectly act as PTI modulators, notably through redox-sensitive proteins among which NPR1 (Herrera-Vásquez *et al.*, 2015). We also examined the cumulative effect of FytoSave® and FytoSol on SA. On potato leaves, FytoSave® greatly increased free SA and SAG levels, depending on the number of applications for free SA while SAG content reached a plateau right from the first spraying. SA accumulation was previously reported in tomato to depend on the number of FytoSave® application (van Aubel *et al.*, 2016). Here, FytoSol did not increase neither free SA nor SAG. The two COS-OGA compositions had clearly antagonistic effects on potato plants: FytoSave® induced SA accumulation whereas FytoSol decreased it.

The mode of action of the FytoSol composition seems thus different from the one of FytoSave® and does certainly not rely on SA accumulation. In other words, without pathogen FytoSol does not behave as a strict SAR inducer in potato.

P. infestans is an hemibiotroph and it is often reported that SA-dependent defenses play a critical role against biotrophic pathogens that feed on living tissues while JA/ET is more effective against necrotrophic pathogens (Glazebrook, 2005), but concerning *P. infestans* the situation is still unclear. Some authors assume that both JA and SA pathways are important for potato basal defense against *P. infestans* (Halim *et al.*, 2009) when others pretend that defense does not rely on SA, JA and ET (Smart *et al.*, 2003). It could also be that potato defense against *P. infestans* requires all those signaling pathways but that they are not sufficient to ensure an effective control of late blight. Here we studied the mode of action of FytoSave® and FytoSol, focusing on the SA and JA/ET pathways at two specific time points: 24 HPI when *P. infestans* is still in its biotrophic stage and 72 HPI which is characterized by a fully necrotrophic development of the pathogen in control plants.

Results show that SA-related genes were more strongly induced by FytoSave® than by FytoSol. In absence of the pathogen, the levels of transcripts of these sets of genes tended to drop between 24 and 72 HPI for both oligosaccharide treatments, which probably explains the low persistence effect observed for FytoSol and the timed application procedure recommended for FytoSave® (van Aubel *et al.*, 2014). The inoculation of control plants with *P. infestans* greatly increased the expression of genes involved in the SA pathway up to a level similar to the one reached by FytoSave®-treated plants at 72 HPI. It is noteworthy that the increase of expression of SA-related genes at that timing occurred when the first symptoms of late blight appeared on control and FytoSave®-treated plants. This was probably linked to the necrotrophic development of *P. infestans* because FytoSol-treated plants did not exhibit any late blight symptom at 72 HPI and the gene expression remained either at a similar level or considerably declined compared to 24 HPI. Increase in SA-related gene expression triggered by *P. infestans* in its necrotrophic stage looks similar to the strategy used by the necrotroph *Botrytis cinerea* that manipulates the SA pathway to promote its development on tomato leaves (Rahman *et al.*, 2012). Here, a strong increase in SA was observed for both FytoSave® and control plants at 72 HPI, caused by the presence of *P. infestans*. Potato leaves have high basal SA levels, which increase at 48 HPI upon infection by virulent *P. infestans* (Floryszak-Wieczorek and Arasimowicz-Jelonek (2016).

However, the potato mutant *NahG* unable to accumulate SA did not show any increased susceptibility to *P. infestans*, but showed a reduced ability to induce SAR in response to arachidonic acid elicitor application (Yu *et al.*, 1997). This led to the conclusion that SAR in potato plants might rely on increased sensitivity to SA rather than on increased SA levels and could partly explain, why FytoSol induced SA-related genes to some extent without inducing any direct SA accumulation in potato leaves. It is also well known that the SA pathway is linked to HR which is effective against biotrophs but increases disease susceptibility towards necrotrophs (Glazebrook, 2005). Interestingly, we observed early small non-progressive necrotic lesions similar to HR in detached potato leaf assays using plants sprayed with FytoSol, something we did not observe on whole plants. Detached leaves lack communication with the rest of the plants which can be particularly important for hormones and/or their precursors synthetized in the roots and transported to the shoot such as cytokinins (Ghanem *et al.*, 2011), ABA (Jiang and Hartung, 2008), gibberellin and ACC, the ET precursor (Van de Poel and Van Der Straeten, 2014).

At certain time points, FytoSave® led to increased transcript levels of JA-related genes, such as *LOX2*, *OPR3*, *PR3*, *JAZ1* and *WRKY33*, and of ET-related genes, such as *ACO1* and *ERF1*. In contrast, FytoSol induced the early expression of JA-related genes, especially *PR3*, *JAZ1* and *WRKY33* while transcript levels of *LOX2* and *AOC* were enhanced at 72 HPI. Interestingly *JAZ1*, encoding the repressor of JA-inducible genes, was first induced by FytoSol and then highly repressed at 72 HPI, which mirrored the effect FytoSol had on most SA-related genes.

Repression of *JAZ1* is a possible way to allow expression of JA-responsive genes without requiring elevated levels of JA-Ile necessary to target the repressor to ubiquitin/26S proteasome-dependent proteolytic pathway.

None of the JA-related compounds was found to be increased in FytoSol-treated plants with or without *P. infestans*. Similarly, no accumulation or decrease of JA or of JA derivatives was observed after FytoSave® treatment, which highlights the fact that SA- and JA/ET-linked defense pathways do not always act antagonistically, as already observed for potato plants (Halim *et al.* (2009).

To summarize, it seems that FytoSave® was more prone to induce early expression of SA-related genes in absence of pathogen, while FytoSol strongly repressed expression of downstream SA-, JA- and ET-related genes in the necrotrophic phase. Concerning ET-related genes, only FytoSave® induced a significant accumulation of the associated transcripts.

The master hormonal regulator identified in potato response to FytoSave® was thus SA which seemed to act synergistically with the downstream components of the ET and the JA pathways. In the case of FytoSol, none of these three hormones appeared to be the obvious regulator of the potato response to the elicitor. We therefore used the hormone-dependent defense marker genes published by Wiesel *et al.* (2015). Gene expression was quantified at one and 6 h after a single elicitor treatment without pathogen inoculation. Beside the repression of the BR marker gene, there was a clear effect of both FytoSave® and FytoSol showing opposite behavior: transcript levels mostly increased with time after FytoSave® treatment while they peaked at one HPT with FytoSol and then decreased. The gene with the highest level of transcript was the ABA marker at one HPT for FytoSol, while FytoSave® had the greater effect on ET and ABA marker at 6 HPT. The ABA, SA and JA marker genes were indeed repressed at 6 HPT with FytoSol, particularly the SA marker gene *StmRNA*, underlining once again the opposite behaviors of these two COS-OGA elicitors.

In conclusion, our data show that the SA-stimulating elicitor FytoSave® failed to induce a decent protection against late blight, while the SA-independent FytoSol was completely effective in controlled conditions. Early and sustained PR induction observed after FytoSave® spraying should have protected the plants against *P. infestans* development, as previously shown by Vleeshouwers *et al.* (2000a), but the pathogen in its necrotrophic stage accommodated perfectly with extremely high SA levels. SA-related responses are clearly not the ones necessary to ensure potato resistance against late blight and it is even possible that the pathogen exacerbates the SA response pathway and diverts it to its own advantage. Indeed *P. infestans* possesses a broad arsenal of virulence factors and its hemibiotrophic behavior likely involves a finely tuned temporal coordination of the type of effector secreted. It seems that at the beginning of infection *P. infestans* effectors suppress host plant immunity and HR while at latter timing other effectors promote necrosis development (Kelley *et al.*, 2010; Lee and Rose, 2010). Which signaling pathway is induced by FytoSol to protect potato against late blight is still unknown: other regulators than SA, JA, and ET will need to be investigated with the help of mutants but the development of next generation sequencing techniques like RNA-seq should be addressed as a priority, considering recent work in the context of potato-late blight interaction (Gao and Bradeen, 2016; Rotter *et al.*, 2007).

Nevertheless, the present results open perspectives for the use of FytoSol-type elicitors in potato protection, but late blight is a polycyclic disease with such devastating effects that farmers do not tolerate any trace of disease in their fields, which is presently only achievable with the help of chemical fungicides. However, organic and conventional farming strategies must not always be opposed: elicitor use should not be considered as an alternative to chemical pesticides and resistant varieties, but as part of an integrated pest management strategy. There are already practical examples of this complementary approach: The combination of partly resistant potato varieties with a half dose fungicide and with phosphites applied weekly was as efficient as a complete fungicide program (Liljeroth *et al.* (2016). Potato culture is a big pesticide consumer with about 15 sprayings a year and even in some places systematic sprayings every 5 to 7 days. Halving fungicide use in potato fields would already have a great environmental impact. The challenge ahead will be to include such new biopesticides in an integrated pest management system aimed at protecting potato efficiently against *P. infestans*.

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