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Oligosaccharides successfully thwart hijacking of the salicylic acid pathway by *Phytophthora infestans* in potato leaves

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Potato growing is severely threatened by the late blight agent *Phytophthora infestans*, which is usually controlled by massive amounts of fungicides. While variety resistance is often bypassed by the pathogen, the plant innate immunity opens the way to new biological plant protection tools, e.g. the COS-OGA elicitor. This oligosaccharide composition mimics the interaction between plants and fungal pathogens as it combines chitosan oligomers (COS) with pectin-derived oligogalacturonides (OGA). Two different COS-OGA elicitors were evaluated against potato late blight: FytoSave, mainly efficient against powdery mildews, and FytoSol, a new composition still under development. In addition, a comparative study of plant defence induction was performed, focusing on the effect of repeated sprayings as well as on the stimulation of salicylic acid (SA), jasmonic acid and ethylene-related pathways during the biotrophic and necrotrophic growth stages of the pathogen. The FytoSave elicitor strongly increased the SA content but failed to induce sufficient protection against late blight, while FytoSol maintained or even decreased the free SA content in the presence of *P. infestans* and was completely efficient. Surprisingly, the necrotrophic development of *P. infestans* occurred along with a strong leaf accumulation of free SA and SA-related transcripts. This may represent an attempt by *P. infestans* to divert plant defences for its own benefit. Preventive sprayings with FytoSol but not FytoSave completely impeded this hijacking. FytoSol seemed to keep the SA pathway under control, thereby preventing its diversion by *P. infestans*.

Keywords: COS-OGA, oligosaccharides, plant innate immunity, potato late blight, salicylic acid, *Solanum tuberosum*

Introduction

Potato late blight caused by the oomycete *Phytophthora infestans* is the most important potato disease worldwide, accounting for more than €5 billion losses per year. The control of the disease requires weekly fungicide applications. However, chemical control is under pressure as *P. infestans* strains become increasingly aggressive, bearing resistance to several control products. Even when fungicide choice and application timing are driven by decision support systems that foresee infection periods, a complete growing season usually requires about 15 sprayings to achieve an efficient disease control. 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 sufficient long-term protection against late blight. Current hopes to create a lasting resistance rely on multiple *R* gene stacking thanks to cisgenesis, a technique that produces genetically modified plants whose introduced resistance genes derive from crossable species. But beside regulatory issues with genetically modified plants, the deployment of potato cisgenes will probably require the use of a certain amount of fungicide, as numerous *P. infestans* strains are already able to bypass several *R* genes simultaneously thanks to multiple effectors known to impair ETI (Haesaert *et al.*, 2015).

A complementary strategy could be the stimulation by pathogen-associated molecular patterns (PAMPs) of the so-called PAMP-triggered immunity (PTI), which is an evolutionarily conserved mechanism relying on pattern recognition receptors localized in the membrane. It allows plants to recognize conserved molecules from pathogens (microbe-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 defences including cell wall reinforcement accompanied by accumulation of secondary metabolites, phytoalexins and pathogenesis-related (PR) proteins (Pieterse *et al.*, 2014).

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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 homologues NPR3 and NPR4, 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, including *Arabidopsis thaliana*, show that defences regulated by SA are mainly effective against biotrophic pathogens.

In contrast ISR, essentially stimulated by beneficial microbes, triggers jasmonic acid (JA)- and ethylene (ET)-dependent plant defences, being mainly active against necrotrophs and herbivorous insects (Pieterse *et al.*, 2014; Caarls *et al.*, 2015). 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. Jasmonoyl-isoleucine (JA–Ile) is the bioactive form originating from JA conversion 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), an F-box protein that targets JAZ proteins to the proteasome. JAZ degradation finally leads to expression of JA-responsive genes (Wasternack & 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 regulate EIN3 that activates the transcription of downstream ET-responsive factors (ERFs) (Saubeau *et al.*, 2016). SA and ET/JA pathways are often considered antagonistic, even if synergy can occur at low hormone concentrations. This antagonism 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 defence elicitor based on a MAMP/DAMP combination containing chitosan-derived chitooligosaccharides (COS) and pectin-derived oligogalacturonides (OGA). The commercial product, FytoSave (12.5 g L^{-1} COS-OGA) is effective against Erysiphaceae in Solanaceae, Cucurbitaceae and grapevines, all biotrophic plant pathogens. The mechanism involved probably relies on SAR, as FytoSave 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 defence 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 is not sufficient to control potato late blight in the open field (Clinckemaillie *et al.*, 2017).

Knowing that the degree of polymerization of oligosaccharides as well as the degree of OGA methylation and the degree and pattern of COS acetylation can strongly modify the way oligosaccharides stimulate PTI (Ridley *et al.*, 2001; Cabrera *et al.*, 2006), another COS-OGA composition called FytoSol was supplied by the company FytoFend for testing. This new composition was assessed against potato late blight and compared to FytoSave. The effect of both compositions 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 symptoms, while at 72 hpi the interaction is fully necrotrophic and the first necrosis becomes visible (Grenville-Briggs *et al.*, 2010). Hormones were also assayed and defence and hormone synthesis gene transcripts were quantified to shed some light on the mode of action of both oligosaccharide compositions on plant defence against potato late blight.

Materials and methods

Plant material and elicitor applications

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 into stem pieces including one node, and rooted in loam under saturated humidity for 3 weeks. After acclimation, potato plants were transplanted in 1 L containers and watered with FloraSeries nutrient solutions (GHE).

Six weeks after acclimation, potato plants were sprayed with a hand sprayer on both sides of the leaves either with water or with a 1:200 dilution of FytoSave (registered against powdery mildews) or FytoSol (composition under development), both containing 12.5 g L^{-1} COS-OGA oligosaccharides (FytoFend SA). The application volume was 25 mL per plant which corresponds to 750 L ha^{-1}, taking into account a planting density of 30 000 potato plants ha^{-1}. The day of the treatment, plants were watered and held at 20 °C and 90% relative humidity (RH) to maintain open stomata. For most bioassays, the classical spraying sequence comprised three preventive sprayings performed at 7, 3 and 1 days before inoculation (dbi) or before leaf harvest for the specific experiment without the pathogen. For the study of the persistence of elicitor treatment, three sprayings in 1 week were performed as usual but the last spraying was performed at either 1, 7, 14 or 21 dbi. For the investigation of the cumulative effect,

the sprayings were performed either once (at 1 dbi), twice (at 3 and 1 dbi) or three times (at 7, 3 and 1 dbi).

Pathogen culture and inoculum preparation

Phytophthora infestans (strain 10-022, mating type A2, isolated in Belgium in 2010, with a virulence profile bypassing *R1, R3, R4, R5, R7, R10* and *R11*, provided and characterized by CRA-W Gembloux, Belgium) was maintained in the dark on rye agar medium at 18 °C. Sporangia were collected by scraping *P. infestans* sporulation onto Petri dishes with distilled water containing 0.0025% Tween 20. The sporangial suspension was adjusted to 1.5×10^4 sporangia mL^{-1}, kept for 2 h in the dark at 4 °C, and used to droplet inoculate detached potato leaves. Leaves were incubated in sealed containers under saturated humidity at 20 °C with a 16 h/8 h day/night regime. Ten days later, *P. infestans* sporulation was collected from the leaves as described above and adjusted to 1.5×10^4 sporangia mL^{-1}.

Inoculation and disease assessments on whole plants

Seven-week-old potato plants were sprayed until run-off with a Venturi glass sprayer on both sides of the leaves with *P. infestans* at 1.5×10^4 sporangia mL^{-1}. Mock-inoculated plants were sprayed similarly with distilled water containing 0.0025% Tween 20. Plants were then 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 3 days. RH was then lowered down to 70% for the rest of the experiment. Disease severity was scored three times per week starting 3 days after inoculation on 10 leaves per plant, and eight plants per experimental condition, by estimating the percentage leaf surface covered by late blight on each plant. The area under the disease progression curve (AUDPC) was calculated from the disease severity (Clinckemaillie *et al.*, 2017) and used to calculate a percentage of protection using the formula: $(AUDPC_{control} - AUDPC_{treated})/(AUDPC_{control}) \times 100$.

Inoculation and disease assessments on detached leaves

In a first experiment, detached leaves collected from 6-week-old potato plants cv. Bintje were inoculated with 25 µL droplets (two droplets per leaflet, five leaflets per leaf) of the spore suspension 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 1 week before scoring each inoculation point (for scoring scale, see Fig. S1). In a second experiment, 6-week-old Bintje plants were sprayed at 7, 3 and 1 dbi with either water or 0.5% FytoSave or FytoSol. The day of inoculation, leaves were cut and directly inoculated or washed twice in 0.0025% (v/v) Tween 20 and gently dried with absorbent paper prior to inoculation. Leaves were inoculated with *P. infestans* at 1.5×10^4 sporangia mL^{-1}, incubated in sealed containers for 1 week and scored as described above. Leaf disks were punched around inoculation droplets from detached potato leaves and examined with Stereo Discovery V8 microscope equipped with Axiocam ICc1 (Carl Zeiss NV).

Peroxidase activity

Liquid nitrogen-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 a Pierce 660 nm protein assay (Thermo Fisher Scientific). Guaiacol peroxidase activity was measured at 420 nm for 5 min on 10 µL 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.

Phytohormone quantification

Extraction of free SA and its conjugated form glycosyl-SA (SAG) was performed on 0.5 g cryogenically ground leaf samples as previously described (Verberne *et al.*, 2002). SA was separated on an 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 prepurification from 50 mg cryogenically frozen leaf samples 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 (Balcke *et al.*, 2012).

Quantification of defence genes expression

Total RNA was extracted with RNeasy Plant Mini kit (QIAGEN) using 100 mg nitrogen-ground leaf samples and treated with DNase (Roche Diagnostics). RNA purity was determined by the $OD_{260\ nm}$:$OD_{280\ nm}$ 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). Primers (Table S1) for three stable housekeeping genes and for genes of interest were retrieved from literature or designed with VECTOR NTI ADVANCE v. 10 software (Thermo Fisher Scientific). The results were normalized using the formula previously described which takes into account three reference genes (encoding the elongation factor 1-α, actin and the ubiquitin-conjugating enzyme) by a geometrical averaging using the mean of threshold cycle value obtained on the control plants as calibrator (van Aubel *et al.*, 2016).

Statistical analysis

Results were analysed with statistical software MINITAB v. 17.3.1 using ANOVA followed by Tukey post hoc tests for multiple comparisons with $P < 0.05$. To counter the high error rate associated with multiple comparisons, Tukey's method adjusts the confidence level for each individual interval in order to obtain the 95% joint confidence level (Minitab Inc.). If variances equality could not be obtained even by data transformation, Kruskal–Wallis test ($P < 0.05$) was applied and followed by multiple comparisons with Student's *t*-test using a Bonferroni correction on the *P*-value.

Results

Protective effect of FytoSol and FytoSave

To assess the efficacy of oligosaccharide elicitors against late blight under controlled conditions, potato plants were preventively sprayed three times with water

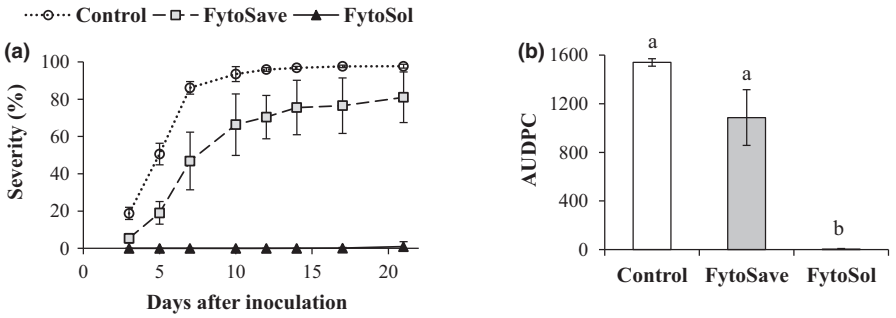

Figure 1 Effect of FytoSave and FytoSol on late blight progression. Potato plants were sprayed three times either with water (control) or with FytoSave or FytoSol, 7, 3 and 1 days before inoculation (dbi) with *Phytophthora infestans* at 1.5×10^4 sporangia mL^{-1}. Data presented are the means ± standard deviation on eight plants per experimental condition and values with different letters are significantly different (ANOVA and Tukey test, $P < 0.05$). (a) Disease severity progression curve of late blight over 3 weeks assessed on 10 leaves per plant; (b) area under the disease progression curve (AUDPC) calculated from disease severity progression curves.

(control) or either FytoSave or FytoSol at a dilution rate of 1:200 corresponding to 62.5 ppm COS-OGA. Disease severity was followed for 3 weeks (Fig. 1a) and by the end of the experiment, control plants reached an area under the disease progression curve (AUDPC) of 1540 while FytoSave- and FytoSol-treated plants reached an AUDPC of 1087 and 2, respectively (Fig. 1b). In other words, both FytoSave and FytoSol reduced late blight symptoms but FytoSave protection only reached 29% while FytoSol achieved almost 100% protection. The efficient foliage protection by FytoSol against late blight (Fig. S2a) also had a significant positive impact on tuber yield (Fig. S2b).

This striking difference raised concern that the higher protection obtained with FytoSol was due to direct toxicity against *P. infestans*. Experiments were thus performed on detached potato leaves inoculated on the abaxial face with droplets of *P. infestans* at 1.5×10^4 sporangia mL^{-1}. In a first experiment, sporangial suspensions were directly inoculated (controls) or mixed just before inoculation with either FytoSave or FytoSol to observe the direct effect of oligosaccharides on *P. infestans* infection. After 1 week, no significant difference could be seen between late blight lesion scores from control or from inoculum containing FytoSave or FytoSol (Fig. 2a).

In a second experiment, whole potato plants were sprayed three times preventively with water, FytoSave or FytoSol. On the day of inoculation, leaves were detached before droplet-infection with *P. infestans* sporangial suspension (elicited, Fig. 2b). Half of the collected leaves were washed just before inoculation (elicited + washed, Fig. 2b). Late blight lesions almost reached the maximum score in control and FytoSave-sprayed leaves, whether elicited only or elicited and then washed. FytoSol-treated leaves reached a score close to 1 (small necrosis) in both conditions and had significantly reduced lesion scores compared to control leaves.

First lesions on detached control leaves as well as on whole plant experiments appeared at 72 hpi. In leaves detached from FytoSol-treated plants, necroses with hypersensitive response (HR) characteristics started earlier at 24 hpi (Fig. S3) but the symptoms did not develop any further. These HR-like lesions were never observed on whole plants preventively treated with FytoSol, in which the inoculum was sprayed uniformly in very small droplets on both sides of the leaves and left to dry for about 1 day in a growth cabinet at 99% RH. Because the *in planta* defence responses were different compared to the detached leaves, all later experiments were performed on intact plants only.

FytoSol-induced protection against late blight

Considering the strong destructive potential of potato late blight and the polycyclic nature of the disease, farmers do not tolerate any disease spot in their fields, which means that the FytoSave composition has no value for this pathosystem. However, the high efficacy of FytoSol under controlled conditions was promising but it remained to be seen whether it also required multiple sprayings to keep the plant in a primed state (van Aubel *et al.*, 2016).

Therefore, plants were sprayed three times in 1 week, but the last spraying was performed at different times before the inoculation with *P. infestans* to quantify the persistence of the protection conferred by FytoSol. AUDPC increased with the interval between the last spraying and the inoculation and reached 898 for control plants and 0, 198, 457 and 787 for FytoSol treatments with the last spraying performed at 1, 7, 14 and 21 dbi, respectively (Fig. 3). The protection remained complete for plants treated with FytoSol with a last spraying on the day before inoculation, but decreased down to 78%, 49% and 12% for a last spraying performed 7, 14 and 21 days before inoculation, respectively. The persistence of the FytoSol effect was thus limited in time, and a last spraying close to the inoculation was necessary to secure 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). Two weeks after infection,

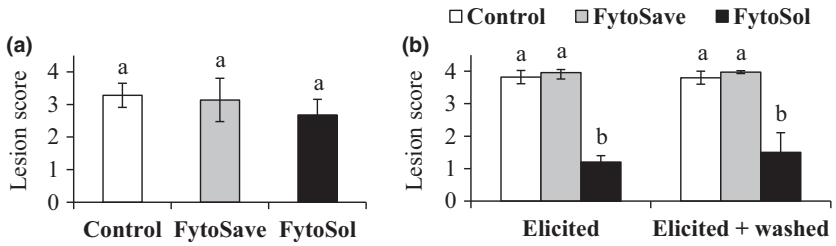

Figure 2 Late blight qualitative lesion score from detached leaf assay. *Phytophthora infestans* was droplet-inoculated at 1.5×10^4 sporangia mL^{-1}. On each leaf, 10 droplets were scored 7 days after inoculation and data presented are the means ± standard deviation on five leaves per experimental condition. Values with different letters are significantly different (ANOVA and Tukey test, $P < 0.05$). (a) *In planta* direct toxicity of the elicitors. *Phytophthora infestans* sporangial suspensions were directly inoculated (control) or mixed just before inoculation with FytoSave or with FytoSol. (b) Comparison between leaves from elicited plants and leaves from elicited plants washed just before inoculation. Plants were preventively sprayed three times with water (control) or with either FytoSave or FytoSol. Detached leaves were either directly inoculated (elicited) or rinsed and gently dried before inoculation (elicited + washed).

three FytoSol applications had prevented any late blight symptom development, with an AUDPC reaching 764 for control plants and 144, 24 and 0 for FytoSol applied one, two and three times before inoculation, respectively (Fig. 4). The disease progression curve for the persistence and the cumulative effect of FytoSol are shown in Figures S4 and S5.

Leaves were also collected from uninoculated potato plants sprayed one, two or three times, 24 h after each FytoSave or FytoSol application, to quantify peroxidase activity, free SA and SAG contents. The effect of FytoSave and FytoSol on peroxidase activity, a key component of PTI, was similar and two applications were required to observe a significant increase that was cumulative for both compositions (Fig. 5a). FytoSave significantly increased the free SA level in potato leaves after the second application, also in a cumulative way, while FytoSol did not affect it (Fig. 5b). Concerning SAG, the conjugated storage form of SA, it appeared to be 100 times more abundant in leaves than free SA. FytoSave dramatically increased SAG right from the first application, while FytoSol slightly increased it (Fig. 5c, log scale). Although FytoSave and FytoSol had similar effects on peroxidase levels, the two compositions clearly affected the SA balance in totally different ways. Repeated sprayings with FytoSave led to a clear accumulation of SA and SAG, while repeated sprayings with FytoSol only weakly affected SA and SAG levels in the leaves.

Induction of the salicylic acid pathway

To understand how the two oligosaccharide compositions and the pathogen affect potato defences, plants were sprayed three times with water (control) or with FytoSave or FytoSol and split into two groups: the first was mock-inoculated and the second was inoculated with *P. infestans*. Leaves were collected for analysis at 24 and 72 hpi, which corresponds to the biotrophic and necrotrophic stages of *P. infestans*, respectively (Grenville-Briggs *et al.*, 2010).

Leaf peroxidase activity was quantified and an increase was noted in leaves treated with FytoSave and FytoSol, but no difference between these two treatments could be observed at any time point, even after inoculation with *P. infestans*. Peroxidase activity also slightly increased in

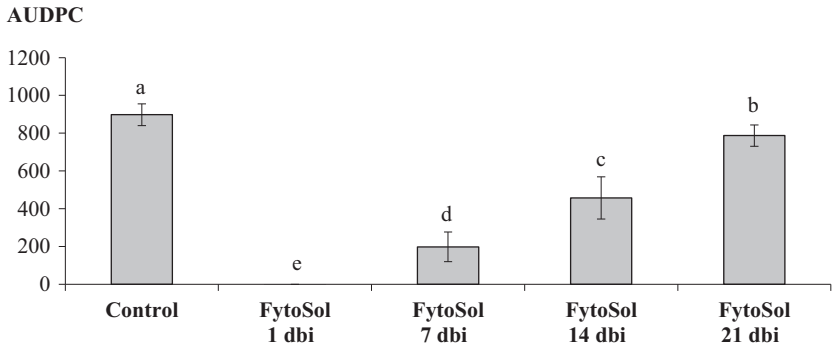

Figure 3 Persistence of the effect of FytoSol. Area under the disease progression curve (AUDPC) calculated from leaf disease severity followed over 2 weeks after the inoculation of potato plants with *Phytophthora infestans*. Control plants were untreated and FytoSol-treated plants were preventively sprayed three times per week with the last spraying performed at 1, 7, 14 or 21 dbi. Data presented are the means ± standard deviation on eight plants per experimental condition and values with different letters are significantly different (ANOVA and Tukey test, $P < 0.05$).

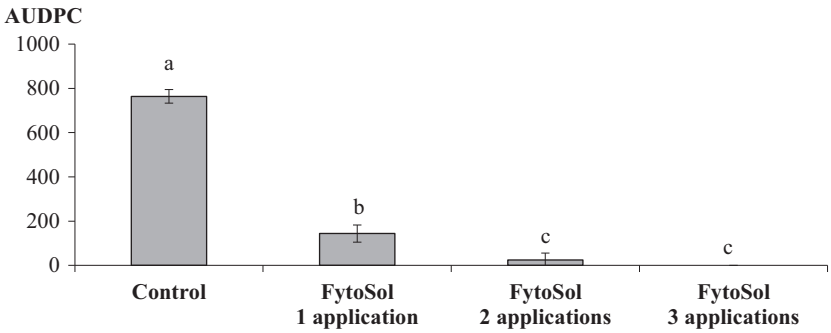

Figure 4 Cumulative effect of FytoSol applications. Area under the disease progression curve (AUDPC) calculated from leaf disease severity followed over 2 weeks after the inoculation of potato plants with *Phytophthora infestans*. Control plants were untreated and FytoSol-treated plants were preventively sprayed either once, twice or three times. Data presented are the means ± standard deviation on eight plants per experimental condition and values with different letters are significantly different (ANOVA and Tukey test, $P < 0.05$).

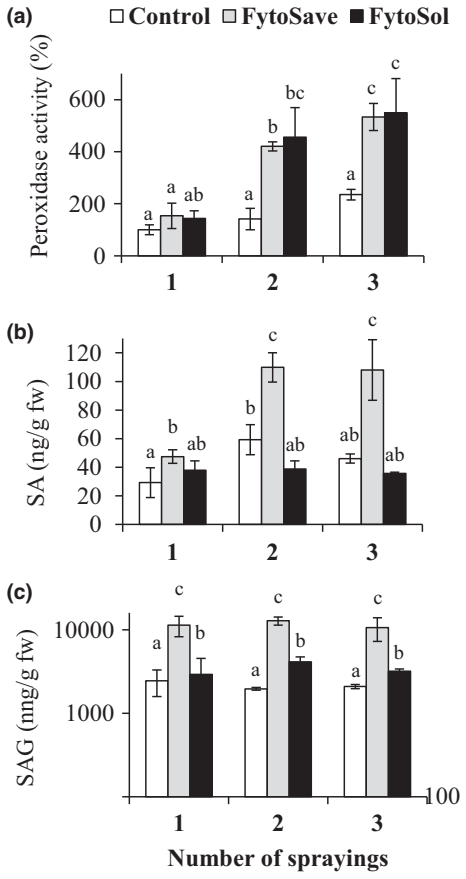

Figure 5 Cumulative effects of FytoSave and FytoSol sprayings in the absence of the pathogen on peroxidase activity (a), free salicylic acid (SA) (b) and glycosyl-SA (SAG) (c) contents in leaves of potato plants sprayed once, twice or three times with water (control), FytoSave or FytoSol. Data presented are the means ± standard deviation on three plants per experimental condition and values with different letters are significantly different (ANOVA and Tukey test, $P < 0.05$).

the inoculated control at 72 hpi (Fig. 6a). SA and SAG levels were expressed using a log scale (Fig. 6b,c). There was a significant increase of SA in FytoSave-treated leaves as well as a significant decrease in FytoSol-treated ones without the pathogen, while the 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 per g fresh weight (fw) in mock- and in pathogen-inoculated leaves at 24 hpi but it increased up to more than 400 ng per g fw at 72 hpi in the presence of *P. infestans*. Plants preventively sprayed with FytoSave reached a similarly high SA level at 72 hpi with *P. infestans*. In contrast, FytoSol treatment in the absence of the pathogen led to a slight reduction of free SA contents at both time points (Fig. 6b, log scale). After inoculation with *P. infestans*, FytoSol sharply decreased the free SA concentration down to the level of inoculated plants in the biotrophic stage (i.e. 24 h). 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. 6c, log scale).

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

The expression of genes linked to the SA pathway (Fig. 7, log scale) was investigated with *PAL1* involved in SA synthesis, and *ISC1* (Dempsey *et al.*, 2011). *PAL1* was slightly overexpressed by FytoSave treatment at 24 hpi in the presence or absence of *P. infestans*. *PAL1* transcripts dramatically increased in inoculated leaves at 72 hpi for control and FytoSave-treated plants, which is consistent with the high free SA increase observed in these leaves. *ISC1* transcripts did not accumulate after elicitation and were even reduced in the inoculated control and FytoSave-treated plants at 72 hpi with the pathogen, but were significantly increased at this time point in FytoSol-treated plants (Fig. S6).

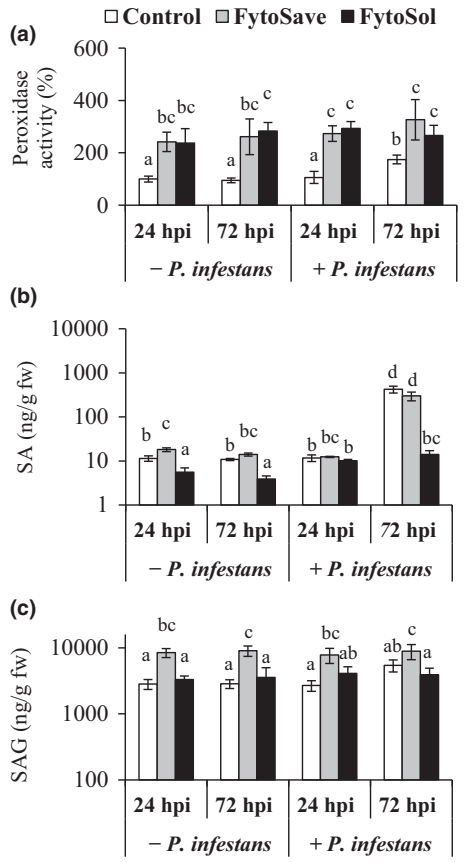

Figure 6 Peroxidase activity (a), free salicylic acid (SA) (b) and glycosyl-SA (SAG) (c) contents at 24 and 72 hpi in leaves of potato plants sprayed three times with water (control), FytoSave or FytoSol before mock-inoculation or inoculation with *Phytophthora infestans*. Data presented are the means ± standard deviation on three plants per experimental condition and values with different letters are significantly different (ANOVA and Tukey test, $P < 0.05$).

WRKY1, a SA-transduction pathway marker (Saubeau *et al.*, 2016), statistically responded only to inoculated control and FytoSave-treated plants at 72 hpi but not after FytoSol treatment. The expression of *PR1* encoding the basic pathogenesis-related protein PR1-1 (Gallou *et al.*, 2009), was similar but with an even higher level of expression in control and FytoSave-treated plants at 72 hpi and a complete absence of response in FytoSol-treated plants at the same time point. *PR1* expression is linked to SAR, as is *PR2b* that encodes the glucan endo-1,3-β-glucosidase isoform b (Ahmad *et al.*, 2014). Both FytoSol and FytoSave induced a significant transient increase of *PR2b* at 24 hpi but again the largest transcript accumulation occurred in control and FytoSave-treated plants at 72 hpi in the presence of *P. infestans*. Once again, FytoSol pretreatment prevented the overexpression of a salicylic acid-dependent gene at the necrotrophic stage of the infection. The same pattern of gene expression was also observed for *PR2a* (Fig. S6). For both oligosaccharide compositions in the absence of the pathogen, the gene expression of SA-related genes *WRKY1*, *PR1* and *PR2b* tended to peak at 24 hpi and decrease afterwards. An increase in *PAL1* and *PR2a* gene expression between 24 and 72 hpi was unexpectedly observed in noninoculated control plants.

The levels of transcripts of JA-related genes were also examined (Fig. 8), starting with those involved in JA and oxylipins synthesis: *LOX2* and *AOC*, encoding a 13-lipoxygenase and an allene oxide cyclase, respectively (Ahmad *et al.*, 2014). An increase of *LOX2* expression was observed at 72 hpi without *P. infestans* in FytoSave- and FytoSol-treated leaves compared to controls. But at 72 hpi, *LOX2* levels of inoculated plants were significantly higher for FytoSol-sprayed plants only. The same trend was also observed for *AOC*, both genes showing a completely opposite regulation to the SA-related genes at 72 hpi in the presence of the pathogen. *PR3*, a class II chitinase linked to the JA pathway (Ahmad *et al.*, 2014) was up-regulated in FytoSave- and FytoSol-treated leaves at 24 hpi with and without inoculation, and in *P. infestans*-infected control and FytoSave-treated plants at 72 hpi. Interestingly, the expression of *JAZ1*, encoding the pivotal JA-responsive gene repressor, was significantly increased in control and FytoSave-treated leaves at 72 hpi in the presence of *P. infestans* but was clearly repressed in FytoSol-treated leaves at the same time point. The *PR3*, *JAZ1* and *WRKY33* genes acting downstream to JA were all down-regulated in FytoSol-treated plants 72 h after inoculation by *P. infestans*.

As JA-dependent defence often acts in concert with ET-regulated genes, transcript levels of *ACO1* encoding the ACC oxidase 1 (Saubeau *et al.*, 2016) involved in ET synthesis were also determined (Fig. 9). *ACO1* transcripts also accumulated in the necrotrophic stage of *P. infestans* development (72 hpi) in control and FytoSave- but not FytoSol-treated plants. Concerning the ET-response factor *ERF3* (Robert-Seilaniantz *et al.*, 2011), a higher expression was only seen 72 hpi on inoculated control and FytoSave-treated plants. Just like with SA- and JA-regulated genes, the ones acting downstream to ET were strongly down-regulated in FytoSol-treated plants 72 h after inoculation by *P. infestans* in comparison to control and FytoSave-treated plants at the same time point.

As mentioned above, the main effect observed was a slight increase in the expression of most defence genes at 24 hpi induced by FytoSave and FytoSol treatments, but especially a large increase at 72 hpi in the presence of the pathogen for control and FytoSave-treated plants, while at the same time point defence gene expression decreased for FytoSol down to the level of the uninoculated control at 24 hpi. The only genes that showed an opposite trend were *LOX2* and *AOC*, related to the JA-pathway, as they were overexpressed at 72 hpi by FytoSol in the presence of *P. infestans*. Moreover *JAZ1*, the JA pathway repressor, was overexpressed at 72 hpi by *P. infestans* in control and FytoSave-related plant but repressed by FytoSol treatment.

Several JA-related compounds were therefore quantified using the same experimental design as mentioned

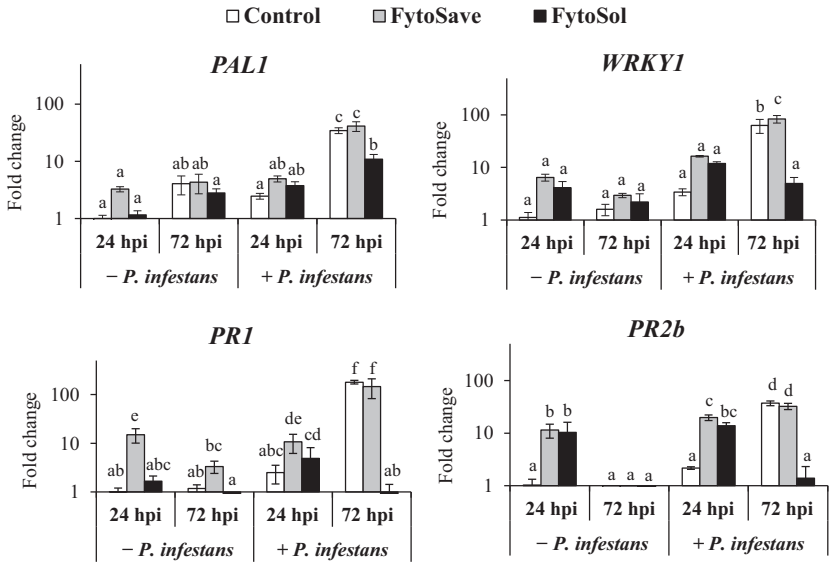

Figure 7 Accumulation of salicylic acid-related defence gene transcripts at 24 and 72 hpi in leaves of potato plants preventively sprayed three times with water (control), FytoSave or FytoSol before mock-inoculation or inoculation with *Phytophthora infestans*. *PAL1*, phenylalanine ammonia-lyase 1; *WRKY1*, WRKY transcription factor 1; *PR1*, PR protein-1; *PR2b*, glucan endo-1,3-β-glucosidase B. Bars with different letters are statistically different (ANOVA and Tukey test, $P < 0.05$). The standard deviation range was calculated from three biological replicates and takes into account two technical replicates.

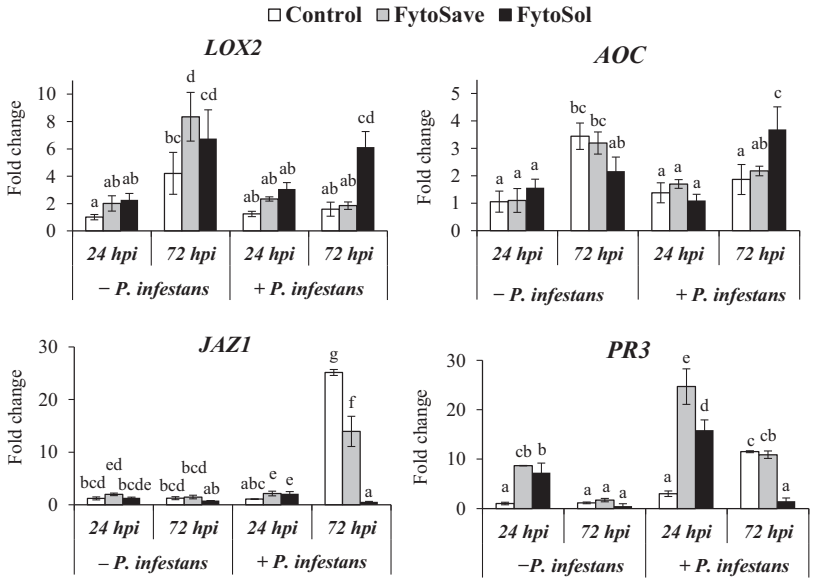

Figure 8 Accumulation of jasmonic acid-related defence gene transcripts at 24 and 72 hpi in leaves of potato plants preventively sprayed three times with water (control), FytoSave or FytoSol before mock-inoculation or inoculation with *Phytophthora infestans*. *LOX2*, 13-lipoxygenase; AOC, allene oxide cyclase; *JAZ1*, jasmonate ZIM-domain protein 1; *PR3*, basic class II chitinase. Bars with different letters are statistically different (ANOVA and Tukey test, $P < 0.05$). The standard deviation range was calculated from three biological replicates and takes into account two technical replicates.

above (Fig. S9): 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.

Discussion

This study investigated the effect of two oligosaccharide compositions, FytoSave and FytoSol, against *P. infestans* on potato. Both products comprise defined COS and OGA fractions that constitute a combined signal informing the plant on both pathogen presence and plant cell

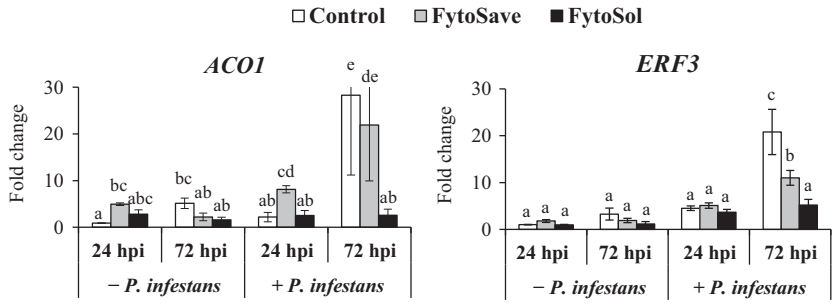

Figure 9 Accumulation of effector-triggered-related defence gene transcripts at 24 and 72 hpi in leaves of potato plants preventively sprayed three times with water (control), FytoSave or FytoSol before mock-inoculation or inoculation with *Phytophthora infestans*. *ACO1*, ACC oxidase 1; *ERF3*, ET-responsive factor 3. Bars with different letters are statistically different (ANOVA and Tukey test, $P < 0.05$). The standard deviation range was calculated from three biological replicates and takes into account two technical replicates.

wall degradation. In experiments involving the hemibiotrophic oomycete *P. infestans* inoculated on leaves of the susceptible cv. Bintje, FytoSave reduced late blight by only 29%, while FytoSol nearly perfectly controlled the disease. These results were similar to previous data for FytoSave, which provided almost 50% protection at a reduced inoculum concentration (Clinckemaillie *et al.*, 2017). Here it is shown that FytoSol needs three applications, with a last spraying close to the time of inoculation, to achieve complete control of *P. infestans*, and that FytoSol protection against late blight is a low persistence phenomenon with a cumulative effect of the number of sprayings.

FytoSol and FytoSave protection against late blight seems to depend on PTI because inoculation of *P. infestans* sporangial suspensions mixed with any of the elicitors resulted in full late blight symptoms on detached control leaves. Moreover, leaves detached from plants pretreated three times with FytoSol showed HR-like lesions as soon as 24 hpi. These HR-like lesions were not observed on whole plants, probably because detached leaves lack communication with the rest of the plant, which can be particularly important for hormonal signalling, among others. Liu *et al.* (2007) showed that symptoms of another hemibiotroph, *Colletotrichum* sp. on *Arabidopsis*, were more intense on detached leaves in which gene expression was more associated with senescence than with plant defence. The contrasting potato protection conferred by the two COS-OGA compositions offered an opportunity to help decipher potato defences against late blight. The initial study was on the effect of multiple sprayings in the absence of the pathogen. A cumulative stimulation of class III peroxidase activity, a well-known PTI marker, was observed by both FytoSol and FytoSave, something already shown with FytoSave on tomato (van Aubel *et al.*, 2016). These enzymes actively take part in plant defence by stiffening cell walls, but class III peroxidases can also produce $O_2^{\cdot -}$ that dismutates into H_2O_2, thereby modifying the apoplastic ROS level and the whole cell redox homeostasis, notably through the redox-sensitive protein NPR1 (Herrera-Vásquez *et al.*, 2015; Raggi *et al.*, 2015).

FytoSave greatly increased the free SA levels in leaves depending on the number of applications, while SAG content reached a plateau right from the first spraying. Free SA accumulation in tomato was also previously reported to depend on the number of FytoSave applications (van Aubel *et al.*, 2016). The two COS-OGA compositions had clearly different effects: FytoSave induced a dramatic free SA and SAG accumulation while FytoSol weakly affected SA levels and increased SAG only slightly. In other words, in the absence of the pathogen, FytoSol did not behave as a strict SAR inducer in potato but was clearly more efficient at controlling late blight than FytoSave, a typical SAR-inducer. It is often reported that SA-dependent defences play a critical role against (hemi)biotrophic pathogens that feed on living tissues while JA/ET is more effective against necrotrophic pathogens (Glazebrook, 2005). However, some authors assume that both JA and SA pathways are important for potato basal defence against *P. infestans* (Halim *et al.*, 2009), while for others defence does not rely on SA, JA and ET (Smart *et al.*, 2003).

Therefore, the comparison between FytoSave and FytoSol was investigated more thoroughly in the presence of *P. infestans*, with a focus on the SA and JA/ET pathways at two specific time points: 24 hpi when *P. infestans* was still in its biotrophic stage, and 72 hpi characterized by a fully necrotrophic development of the pathogen. Similar increases of peroxidase activity for both compositions as well as strong increases of SAG in FytoSave-treated plants only were again observed whether the plants were inoculated or not. However, at 72 hpi in the presence of the pathogen, a tremendous free SA accumulation was observed solely in control and FytoSave-treated plants; FytoSol pretreatment prevented inoculated plants from increasing their free SA content. This was also observed in Bintje 48 hpi with virulent *P. infestans* (Floryszak-Wieczorek & Arasimowicz-Jelonek, 2016), while a resistant cultivar, Bzura, maintained SA levels down to the uninoculated control, exactly as observed here after FytoSol pretreatment. Moreover, accumulation of an SA-related gene was observed in resistant Bzura as soon as 3 hpi with *P. infestans* but was delayed up to 24 hpi in susceptible Bintje. A very similar trend was observed for SA-related genes at 72 hpi: the inoculation with *P. infestans* of control and FytoSave-treated plants greatly increased the

expression of the SA genes *PAL1*, *WRKY1*, *PR1* and *PR2b* whereas FytoSol-treated plants had much lower transcript levels. In the absence of *P. infestans*, SA-related genes were more up-regulated by FytoSave than by FytoSol and the transcript levels tended to drop between 24 and 72 hpi for both compositions. The strong but inefficient increase in SA-related gene expression that occurred during the necrotrophic stage of the pathogen suggests that control and FytoSave- but not FytoSol-treated plants were misled by *P. infestans*. The ability of FytoSol to prevent the uncontrolled runaway of the SA pathway could be the key to late blight resistance, indirectly revealing the stratagem used by *P. infestans* that hijacked potato defence by manipulating its SA pathway. Such a strategy has been reported for the necrotroph *Botrytis cinerea* that induces the SA pathway to exploit its well-known antagonism towards the JA pathway and favour disease development on tomato leaves (Rahman *et al.*, 2012).

Here, *JAZ1*, encoding the repressor of JA-inducible genes, was highly overexpressed in the presence of *P. infestans* at 72 hpi in control and FytoSave-treated plants. In contrast, *JAZ1* was considerably repressed at the same time point in FytoSol-treated plants, which is a way to allow expression of JA-responsive genes without requiring elevated levels of JA-Ile. Indeed, none of the JA-related compounds was found to increase in FytoSave- or FytoSol-treated plants with or without *P. infestans*. Furthermore, in inoculated plants at 72 hpi, *LOX2* and *AOC* transcript levels were significantly higher only in FytoSol-sprayed plants, in complete opposition to the pattern of response of SA-related genes. Concerning ET-related genes, their regulation resembled the SA pathway, and only FytoSave induced a significant accumulation of the associated transcripts.

The potato mutant *NahG*, unable to accumulate SA, did not show any increased susceptibility to *P. infestans*, but had a reduced ability to induce SAR in response to application of the arachidonic acid elicitor (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, which could partly explain why FytoSol could induce SA-related genes to some extent without inducing any direct SA accumulation in potato leaves. Nevertheless, the main hormone regulated in potato in response to FytoSave was SA, while none of the three hormones SA, JA and ET appeared to be obvious regulators of the response to FytoSol. The latter rather repressed expression of downstream SA-, JA- and ET-related genes during the necrotrophic stage of *P. infestans*.

In conclusion, the data here show that the SA-stimulating elicitor FytoSave failed to induce a decent protection against late blight, while FytoSol did not induce SA accumulation and was completely effective under controlled conditions. An early and sustained PR induction such as the one observed after FytoSave spraying should have protected the plants against *P. infestans* development (Vleeshouwers *et al.*, 2000), but the pathogen in its necrotrophic stage accommodated perfectly with extremely high SA levels. Strong and late SA increase is clearly not enough to mount an efficient resistance against potato 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 behaviour probably involves a finely tuned temporal coordination of the type of effector secreted: at the beginning of infection *P. infestans* effectors suppress host plant immunity and HR, while at later times other effectors promote necrosis development (Lee & Rose, 2010). Which signalling pathway is induced by FytoSol to protect potato against late blight is still unknown. Earlier time points in the plant–pathogen interaction or 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 the potato–late blight interaction (Gao & Bradeen, 2016).

The present results open perspectives for the use of COS-OGA 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 do not need to be in opposition: 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 programme (Liljeroth *et al.*, 2016). Potato culture is a large pesticide consumer with about 15 sprayings a year, and even in some places systematic sprayings every 5–7 days. Halving fungicide use in potato fields would on its own 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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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. Scale for scoring late blight lesions caused by *Phytophthora infestans* on detached leaves of potato cv. Bintje.

Figure S2. Protective effects of FytoSave and FytoSol against late blight.

Figure S3. Representative leaf disks taken around an inoculation droplet on leaves detached from elicited plants at 24 and 72 h post-inoculation (hpi).

Figure S4. Persistence of the effect of FytoSol on potato plants.

Figure S5. Cumulative effect of FytoSol application on potato plants.

Figure S6. Accumulation of SA-related defence gene transcripts at 24 and 72 hpi in leaves of potato plants sprayed three times with water (control), FytoSave or FytoSol before mock-inoculation or inoculation with *Phytophthora infestans*.

Figure S7. Accumulation of JA-related defence gene transcripts at 24 and 72 hpi in leaves of potato plants sprayed three times with water (control), FytoSave or FytoSol before mock-inoculation or inoculation with *Phytophthora infestans*.

Figure S8. Accumulation of ET-related defence gene transcripts at 24 and 72 hpi in leaves of potato sprayed three times with water (control), FytoSave or FytoSol before mock-inoculation or inoculation with *Phytophthora infestans*.

Figure S9. OPDA (a), JA (b) and JA-Ile (c) content at 24 and 72 hpi in leaves of potato plants sprayed with water (control), FytoSave or FytoSol three times before mock-inoculation or inoculation with *Phytophthora infestans*.

Table S1. List of genes and corresponding primers used for qRT-PCR.