University of Namur

DOCTOR OF SCIENCES

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

van Aubel, Géraldine

Award date:
2017

Awarding institution:
University of Namur

Link to publication

CHAPTER 6

General conclusions and perspectives

There is an urgent need to develop new efficient solutions for integrated crop protection with favorable tox- and ecotoxicological profiles. Firstly, the legislative framework for pesticide registration in Europe becomes more stringent, which is positive for human health and environmental safety but which will likely contribute to decrease the number of available AIs for farmers. The reduction of the AI pool can lead to a decreased efficacy of plant disease control, because the reliance on a reduced range of modes of action can favor the emergence of resistant pathogens. Secondly, consumers become more and more concerned by pesticide residues in food products. An increasing proportion of the population turns to organic food and as a consequence many farmers also move to that mode of production. This trend is well illustrated at our regional level: the figures show that agricultural surfaces allotted to organic farming are steadily increasing in the Walloon Region (Hillocks, 2012; Lamichhane *et al.*, 2015; Statistics Belgium, 2016).

A first substitute for pesticides in disease control is the use of plant varieties bearing resistance genes, but monogenic characters are easily circumvented by pathogens that evolved effectors able to hijack resistance genes. Obtaining resistant varieties by other means than genetic modification is a very long process and even cisgenesis is not an option for the organic sector (Lammerts van Bueren *et al.*, 2008). In this context, the stimulation of plant immunity or PTI by PAMPs appears to be a promising alternative or at least a complementary strategy to the use of fungicides in plant disease management. PTI triggers a horizontal resistance mechanism based on polygenic traits and is thus supposed to be more durable. The defense reaction is not specific and the protection conferred can be potentially effective against multiple pathogens (Michelmore *et al.*, 2013).

The oligosaccharide complex COS-OGA is a PTI inducer made up of chitosan- and pectin-derived fragments, which renders it fully compatible with the current organic practices. COS-OGA mimics plant interaction with fungi and constitutes a combined signal that informs plant cells on both pathogen presence and cell wall degradation. Both polymers are safe for the human health and the environment and are abundantly available in nature at an affordable price. This is often an unmentioned problem when dealing with plant defense inducers: new elicitors are regularly described but their availability even at the kilogram scale for field trials is a problem (Meena and Kanwar, 2015). The second often referred problem is a lack of efficacy because lots of compounds show very promising results in controlled conditions, but once applied in the field, they are not so effective in controlling diseases (Alexandersson *et al.*, 2016).

At the beginning of this work, the first data available on COS-OGA were limited to *A. thaliana* cell suspension cultures, an excellent laboratory model but far from the field (Cabrera *et al.*, 2010). The aim of this work was thus to better understand the mode of action and the activity spectrum of COS-OGA, as well as the critical factors conditioning its field efficacy. In this work, two compositions were evaluated: FytoSave® and FytoSol supplied by FytoFend SA, both comprising two forms of COS-OGA at 12.5 g/l.

The first commercially available composition FytoSave® was evaluated on grapevine against powdery mildew provoked by *E. necator* and on greenhouse cucumbers against *S. fuliginea*. COS-OGA at 37.5 g ha^{-1} on grapevine and 25 g ha^{-1} LWA on cucumber conferred more than 70% protection against powdery mildew, which in some trials was even better than the conventional chemical reference. In all these trials we sprayed COS-OGA preventively, starting at the beginning of the season before any disease symptom and at a maximal 7- to 14-day interval between sprayings. We also prescribed a spraying equipment with high operating pressure to reach stomata on lower leaf surfaces. Since COS-OGA is a high molecular weight polar compound, its diffusion through plant hydrophobic cuticles is unlikely. Open stomata constitute the potential site of penetration and perception of COS-OGA. PAMP-induced stomatal closure is indeed an early mechanism of defense (Melotto *et al.*, 2008) and detached epidermal peel assays showed that chitosan and OGA individually are able to induce stomatal closure (Lee *et al.*, 1999). We performed similar experiments with COS-OGA and we also observed induction of stomatal closure (Thunissen C., 2011).

Recently, penetration of fluorochrome-derivatized laminarin in leaves through stomata was confirmed and penetration of laminarin was shown to be substantially increased by the use of appropriate surfactants (Paris *et al.*, 2016). As laminarin is also an oligosaccharide compound, COS-OGA should behave similarly and such labelling of reducing ends of COS and/or OGAs with fluorescein could also be considered. This would give information on the fraction of COS-OGA making its way inside the leaf and its persistence and degradation rate by plant enzymes or microflora. Anyway, in our trials performed in controlled conditions, we sprayed the elicitors on both leaf sides. We also adopted a high humidity level (90% HR) on the day of application to promote stomata opening. FytoFend also advises farmers to spray early in the morning when leaves are still moist from the dew and to avoid applications under full sun at the beginning of the afternoon, which could induce midday stomatal closure (Tenhunen *et al.*, 1984).

The FytoSave® intrinsic effect was first investigated on tomato, under controlled conditions and in absence of pathogen. COS-OGA was evaluated at 50 ppm which is close to the concentrations used in vineyards and greenhouse trials. Leaf proteomic study performed after two FytoSave® sprayings revealed an accumulation of PR proteins, especially subtilisin-like proteases, often associated with the SA pathway. Transcript quantification confirmed the upregulation of PR-proteins and SA-related genes while expression of JA and ET-associated genes was not modified. SA concentration and class III peroxidase activity were then quantified 24 h after each single application, and revealed cumulative increases dependent on the number of elicitor sprayings. This fact highlights the importance of repeated COS-OGA applications and perhaps explains the high efficacy observed in trials under commercial conditions. It would be interesting to see if these observations can be generalized to other elicitors, which would help designing efficient field strategies for other plant defense inducers.

These results also suggested that FytoSave® activated SAR in tomato, a signaling pathway often characterized by an increase in SA level in the plant (Pieterse *et al.*, 2014). This is compatible with the current model inferred from studies on *Arabidopsis* which states that SA-related defenses are more efficient against bio- and hemibiotrophic plant pathogens while JA/ET-linked pathways are involved in protection against necrotrophic pathogens. Powdery mildews mentioned above are biotrophic plant pathogens as well as the tomato powdery mildew *L. taurica* that was also quite well kept in check by FytoSave®.

As SA-related defenses were reported to be efficient in basal potato defense against the hemibiotrophic pathogen *P. infestans* (Halim *et al.*, 2007), we evaluated the efficacy of FytoSave® against late blight, an especially difficult disease (Fry *et al.*, 2015). FytoSave® was evaluated at a 1:200 dilution corresponding to 62.5 ppm COS-OGA sprayed on plants. To be on the safe side, we performed three sprayings at seven, three and one day before the inoculation of *P. infestans*, but we could only achieve 30% protection. The effect was significant but completely unsatisfactory for field application. Therefore, FytoFend provided us with a second composition with code name FytoSol that contained a modified COS-OGA composition, also at 12.5 g/l. After evaluation, it turned out to perfectly control potato late blight under the same controlled conditions.

A comparative study of both oligosaccharide elicitors on potato defense induction was performed, focusing on the effect of the number of applications on SA levels, on peroxidase activity and on the stimulation of SA, JA and ET pathways. The samplings were done at two specific time points, 24 and 72 HPI when *P. infestans* was in its biotrophic and necrotrophic growth stages, respectively. Here also, the results on potato indicated that FytoSave® strongly triggered the SA pathway by increasing SA levels in a cumulative way with the number of applications and it induced expression of SA-related genes. FytoSave® also increased the expression of several genes linked to the ET and JA pathways but without increasing the levels of JA and JA-derived compounds. Such a major induction of SA-related genes was also observed by Clinckemaillie *et al.* (2016) following FytoSave® treatment on potato, together with an upregulation of a proteinase inhibitor related to the ET and JA pathways. From the literature, it seems that in potato and rice which both contain high basal levels of SA, JA- and SA-related defenses do not act antagonistically but are likely components of the same pathways (Halim *et al.*, 2009; Tamaoki *et al.*, 2013; Thaler *et al.*, 2012; Yu *et al.*, 1997).

In a completely opposite way, the highly efficient FytoSol tended to decrease SA in potato leaves without affecting JA and JA-derived compounds, but a cumulative effect of FytoSol application was still observed on peroxidase activity levels. Anyway, FytoSol still succeeded in upregulating some genes associated with the SA and the JA pathways, notably the PR genes. *P. infestans* alone massively increased SA-related gene expression in its necrotrophic stage, at the point that no more difference could be observed between FytoSave® and water-treated plants at 72 HPI. At this stage, FytoSol for its part repressed the expression of SA-related genes. This observation indicates that *P. infestans* not only perfectly copes with SA-related defenses but it even hijacks them for its own benefit (Du *et al.*, 2015).

This is confirmed by the fact that the SA analog BTH was also reported to be inefficient against *P. infestans* in potato (Si-Ammour *et al.*, 2003).

Potato hormonal marker genes developed by Wiesel *et al.* (2015) were tested and confirmed the differential behavior of both oligosaccharide compositions on potato defense stimulation and showed that the hormonal regulation induced by both compositions did not rely on SA, JA and ET only, and probably involved other pathways. Concerning FytoSol, our marker genes confirmed the repression of SA-related defenses at 6 HPI and highlighted a strong early stimulation of the ABA marker at 1 HPT. The study of the interaction between potato plants with *P. infestans* and COS-OGA elicitors has been performed much later (24 and 72 HPI). Considering the early response of hormonal marker genes, the study of the kinetics of defense pathways stimulated by COS-OGA in presence of *P. infestans* should include time points earlier than 24 HPI.

The persistence and the cumulative effect of FytoSol protection against late blight were also investigated. The protection against late blight conferred by three FytoSol applications already decreased significantly when the inoculation was performed one week after the last spraying instead of one day before. FytoSol protection against late blight also increased with the number of applications and required three sprayings to be fully effective. In other words, FytoSol protection against late blight is a cumulative process with low persistence: three applications with the last spraying short before the inoculation were necessary to secure a complete efficacy against late blight. This is not incompatible with present potato late blight fungicide programs that often rely on weekly applications guided by decision support systems that predict infection peaks (Taylor *et al.*, 2003).

Another aspect of plant defense stimulation by COS-OGA that have been studied but not presented in this manuscript because of space limitation is systemicity. Tomato plant leaves or roots were treated with COS-OGA and measurements of peroxidase activity (van Aubel, 2012, unpublished results) and quantification of *PR1* and *PR2* expression (Clinckemaillie, 2017, unpublished results) confirmed that there was a clear bidirectional systemic signaling (upward and downward) following the elicitor application.

From the molecular point of view, both FytoSave® and FytoSol increased the peroxidase activities with the number of applications. The low persistence effect could also be suspected from PR gene expression with decreased for both compositions between 24 and 72 HPI. This last timing corresponds to four days after the last treatment with FytoSol. Similarly in absence of pathogen inoculation Clinckemaillie *et al.* (2016) showed that the upregulation of *PR1* in *PR2* in potato following potato treatment with FytoSave® strongly decreased at 72 HPI also four days after the last spraying. If the pattern of defense stimulation appeared different between the two oligosaccharides, the cumulative effect of the application and low persistence property seemed well conserved. To our knowledge, it is the first time in literature that the low persistence and cumulative effect of elicitor application is demonstrated which reinforced the necessity of specific preventive timed application program to obtain the maximal protective effect of an elicitor and provide a partial explanation why elicitor do not work in field especially if they are applied as certain fungicides with a single application performed for curative purposes.

The strong difference in mode of action between FytoSave® and FytoSol also stresses the need for a deeper investigation of the relationship between the structure of COS-OGA and its biological activity. This could be easily done on *A. thaliana* cell suspensions that allow fast multiple comparisons by following simple PTI parameters such as medium pH shift, K^+ release and ROS production. This would also allow to determine whether there is any receptor desensitization to COS-OGA and how long this desensitized state would last.

Finally, a RNA-seq study was performed to better understand the mode of action of FytoSol against late blight. Potato leaves were pretreated three times with FytoSol and collected 24 h after inoculation with *P. infestans*. Results from cDNA sequencing were analyzed using the MapMan ontology (Thimm *et al.*, 2004) with specific focus on biotic stress-related genes. The first observation was that *P. infestans* mainly downregulated genes involved in biotic stress processes while FytoSol treatment led to an enhanced transcription of those genes. The most contrasted effect was obtained between FytoSol-treated and inoculated leaves on the one hand and water-treated plants inoculated with *P. infestans* on the other hand.

The direct mode of action of FytoSol against *P. infestans* involved the transcription of downstream plant defense genes. FytoSol led to a massive overexpression of PR protein genes that encode pathogen wall-degrading enzymes (β-glucanases and chitinases), proteins involved in impairing pathogen membrane stability (osmotin and PR1) or directly toxic for oomycetes (hevein-like proteins). FytoSol also upregulated genes involved in terpene synthesis and long known to be toxic for *P. infestans* (Bostock *et al.*, 1981; Kumar *et al.*, 2016; Stotz *et al.*, 2013; van Loon *et al.*, 2006b).

About hormone signaling, FytoSol induced a major downregulation of the BRs and auxin pathways while ABA, ET and JA pathways were enhanced up to variable levels. The most regulated hormonal pathway was ET and the least regulated one was SA, consistent with the absence of SA accumulation after treatment. The only genes regulated in the JA pathway were not associated with JA synthesis but related to oxylipins production and encompassed several 9-LOXs as well as α-DOX 1. Oxylipins synthesis competes with JA synthesis as they both rely on the same substrate, which is consistent with the absence of JA accumulation after FytoSol treatment.

BABA is described as an efficient PTI inducer against *P. infestans* in potato and SA seems involved in its mode of action (Eschen-Lippold *et al.*, 2010). However, a transcriptomic study of BABA effect on potato leaves inoculated with *P. infestans* revealed that the defense stimulation involved ABA as well as JA and ET more than SA (Bengtsson *et al.*, 2014). Another RNA-seq study comparing the susceptible potato CV Russet Burbank with its transgene expressing a very efficient late blight R-gene also concluded that the ET rather than the SA pathway was involved in the resistance. The resistance mechanism was based on R-gene activation but the signaling components and downstream responses were the same as in PTI (Thomma *et al.*, 2011).

It is therefore quite possible that an efficient defense of potato against *P. infestans* requires other pathways than SA. This is at variance with the *Arabidopsis* model in which SA defenses are most efficient against biotrophic and hemibiotrophic pathogens (Caarls *et al.*, 2015; Glazebrook, 2005; Pieterse *et al.*, 2012). Validation of gene regulation by ET, ABA and oxylipin pathways should be performed on control and FytoSol-treated leaves harvested at different time points after inoculation with *P. infestans*. ABA should also be quantified to confirm its involvement in response to FytoSol while bearing in mind that the ABA concentration is subject to feedback regulation in the plant (Finkelstein, 2013).

However, the involvement of a SAR-like mechanism cannot completely be excluded from FytoSol signaling because transcripts involved in redox balance such as peroxidases, GSTs, glutaredoxin and thioredoxin were upregulated. This could help to establish a reducing environment necessary for NPR1 monomerization (Frederickson Matika and Loake, 2014) leading to transcriptional activation of the downstream SA-responsive genes in absence of SA accumulation. The FytoSol-induced SAR could therefore be redox-dependent rather than SA-induced. *Arabidopsis* spraying with GSH is indeed sufficient to mimic SA leading to *PR1* expression. The phenomenon appears to be dependent on NPR1 reduction and relocation to the nucleus (Foyer and Noctor, 2011).

If SA is well known to play a pivotal role in SAR establishment, its systemic role is still debated. New emerging systemic signaling molecules involved in SAR regulation have been recently discovered, notably pipecolic acid which is now shown to regulate SAR via SA-dependent and SA-independent pathways (Bernsdorff *et al.*, 2016; Shah and Zeier, 2013). Other signals than SA could be involved in the FytoSol-triggered signaling. Among them, pipecolic acid could constitute a good track to follow considering the obvious FytoSol independence from SA accumulation in potato.

A. Clinckemaillie (2017, unpublished results) investigated the protection conferred by FytoSave® and FytoSol against *Alternaria solani* on tomato, a necrotrophic plant pathogen. FytoSave®-treated plants similarly showed some early blight control while FytoSol treatment was much more efficient. Studies performed on the tomato *nahG* mutant unable to accumulate SA and the *Def 1* mutant unable to produce the JA precursor OPDA showed that both JA and SA pathways were involved in tomato protection against early blight (Audenaert *et al.*, 2002). Both mutants exhibited lower but non-zero protection, which suggests the contribution of another signaling pathway. This potential involvement of SA against a necrotrophic pathogen challenges another dogma of plant pathology, *i.e.* the widely-accepted antagonism between SA and JA defenses (Caarls *et al.*, 2015).

FytoSol treatment combined to *P. infestans* inoculation also caused the overexpression of several PRR receptors among which several homologs of the *Arabidopsis* pectin receptor WAK2 (Kohorn *et al.*, 2009). The WAK2 gene upregulated here could indeed constitute a potential candidate for COS-OGA perception. Otherwise, it is not surprising that the FytoSol – *P. infestans* combination triggered a significant increase in proteins involved in cell wall turnover. More particularly, the most upregulated genes in this category encoded a PMEI, a PAE and a pectate lyase and the two most downregulated ones encoded a PME and a cellulose synthase-like protein. PME downregulation and PMEI upregulation is consistent in the context of plant defense stimulation in which demethylesterified pectin is more susceptible to enzymatic degradation by pathogenic PGs (Pogorelko *et al.*, 2013a). If WAK1 is the *Arabidopsis* receptor of OGAs under egg box conformation (Brutus *et al.*, 2010; Decreux and Messiaen, 2005), no COS receptor has ever been discovered (Iriti and Faoro, 2009). Beside a direct interaction with PRRs, the oligosaccharide complex could also interact with cell wall pectin, leading to indirect activation of wall integrity sensors (Wolf *et al.*, 2012). Anyway, it will be interesting to study cell wall modifications provoked by FytoSol.

In conclusion, both FytoSave® and FytoSol were shown to be plant defense inducers. FytoSave® was highly effective against the biotrophic powdery mildews and its mode of action was shown to rely on SAR and SA accumulation in tomato as well as in potato leaves. FytoSol did not increase SA content in potato but was very efficient in controlled conditions against the hemibiotrophic pathogen *P. infestans*. While *P. infestans* in its biotrophic stage largely repressed potato gene expression, FytoSol strongly reversed the situation, although the exact signaling pathway followed is still obscure. The present work contributes to the knowledge of the COS-OGA class of elicitors and paves the way to a deeper understanding of the mechanisms involved, in parallel to the technological transfer of knowhow to the industrial partners and finally to the end users, the farmers.