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 2

COS-OGA: A novel oligosaccharidic elicitor that protects grapes and cucumbers against powdery mildew

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Abstract

Many plant protection products have been banned due to their toxicity or because pathogens have become resistant to their active substances. Plant resistance inducers are a promising class of compounds that will hopefully reduce dependence on harmful chemicals for plant protection. The efficacy of COS-OGA, a novel elicitor composed of a complex of oligochitosans and oligopectates as active substances, has been assessed in production conditions against powdery mildew on grapevines and on cucumbers. COS-OGA induced a large reduction in the severity of powdery mildew for both crops. At the end of vineyard trials, COS-OGA, at a spraying rate of 37.5 g ha^{-1}, achieved a considerable reduction in *Erysiphe necator* severity on bunches of grapes with 78% protection in France and 76% protection in Spain. Similarly, in the last evaluation performed in greenhouses to assess the protection of cucumber crops against *Sphaerotheca fuliginea*, COS-OGA at 25 g ha^{-1} LWA (leaf wall area) yielded a 72% reduction in leaf disease severity in Belgium and 69% in Spain. In some trials, the efficacy of the COS-OGA active substance was even higher than the conventional chemical reference.

1. Introduction

Environmental and health issues have placed enormous pressure on both the agro-food industry and on governments regarding the use of chemical pesticides. The European framework for the plant protection products market has considerably evolved over the past few decades. The previous European Directive 91/414/EC was replaced by Regulation (EC) 1107/2009, which defined new evaluation criteria for pesticide approval, and was completed by Directive 2009/128/EC, which forced member states to develop a policy of sustainable use for pesticides. This latter directive requires member states to establish national action plans based on integrated pest management to reduce their dependence on chemical control for plant protection (Hillocks, 2012; Skevas et al., 2013; Villaverde et al., 2014).

Plant defense stimulation that is based on products of biological origin, such as elicitors, is a promising alternative to chemical pesticides. Until recently only two active substances, laminarin and benzothiadiazole (BTH), were registered in Europe (Regulation (EC) No 1107/2009) as plant protection products that have a mode of action that is solely reliant on defense stimulation to protect plants against diseases. Laminarin is an algae extract used to protect apple trees from scab and fire blight, strawberries from powdery mildew and wheat from *Septoria* and powdery mildew (Copping and Duke, 2007). BTH is a functional analogue of salicylic acid (SA) that is mainly used to control diseases provoked by biotrophic plant pathogens (Rohilla et al., 2002). Biological control agents (BCAs) composed of bacteria, oomycetes or fungal microorganisms, also stimulate plant defenses as part of their mode of action. Only 14 genera of BCA are authorized for use as plant protection products in Europe following Regulation (EC) No 1107/2009 (Gerbore et al., 2013).

Elicitors offer many advantages because they are not toxic to pathogens; instead, they are recognized by plant membrane receptors and induce mobilization of an entire array of plant defenses through innate immunity stimulation (Boller and Felix, 2009). The protection conferred is not specific and can potentially protect against multiple pathogens (Sharathchandra et al., 2004). The complementary use of elicitors together with fungicide at reduced rates or as a replacement for certain chemical treatments within a seasonal program of plant protection has also been considered (Walters et al., 2005).

COS-OGA is a novel elicitor that is currently undergoing evaluation at the European level for registration as a plant protection product. The COS-OGA active substance consists of a complex of chitosan fragments (chitooligosaccharides, COS), which are compounds found in fungal cell walls and crustacean exoskeletons, that are associated with pectin fragments (oligogalacturonides, OGA) originating from plant cell walls. The positively charged COS fragments stabilize the negatively charged OGA fragments by forming an egg-box conformation that is induced by a suitable ratio of calcium to sodium ions in the solution (Cabrera *et al.*, 2010).

Nevertheless, the management of most plant diseases still relies on numerous fungicide applications. The *Erysiphaceae* fungi, as the causal agents of powdery mildew, are no exception, as they are responsible for the largest share of fungicide sales in Europe. They target a diverse range of important crops such as wheat, barley and grapevine, numerous vegetables, such as cucumbers, tomatoes, melons and zucchinis, as well as ornamentals, such as roses. The disease presents problems to outdoor as well as greenhouse crops (Elad et al., 1996; Kiss, 2003). In grapevines (*Vitis vinifera* L.), powdery mildew caused by *Erysiphe necator* (Schwein) Burrill is one of the most common diseases and is often controlled by more than seven fungicide treatments (Kast W. K. and Bleyer K., 2011). Powdery mildew can lead to total yield loss, and strobilurines-resistant populations of *E. necator* have been characterized. Taksonyi et al. (2013) and Calonnec et al. (2004) also suggest that powdery mildew can reduce wine quality with higher acidity and lower varietal aroma. Research is ongoing to reduce fungicide use in vineyards because such use also negatively affects beneficial arthropods (Pertot et al., 2008). Powdery mildew is also a problem for high value crops grown under greenhouse conditions because of the confined atmosphere with high temperature and humidity. Cucumbers (*Cucumis sativus* L.) grown under greenhouse conditions are subject to *Sphaerotheca fuliginea* Poll, also known as *Podosphaera xanthii*, and fungicide treatment is not always effective due to fungal resistance (Ren et al., 2009). Even if tolerant cultivars (CVs) exist, numerous fungicide treatments are often required to control the disease (Giotis et al., 2012). Indeed, CV resistance is linked to the fungal race (Nuñez-Palenius et al., 2006) and is influenced by environmental conditions (Itagaki et al., 2014; Sakata et al., 2006).

The control of powdery mildew by elicitors has often been reported; however, good results have mostly been obtained in the laboratory under strictly controlled conditions (Faoro et al., 2008; Jaulneau et al., 2011; Sharathchandra et al., 2004). Here, we report on the efficacy of COS-OGA tested under farming conditions against powdery mildew on grapes in vineyards and on cucumbers in greenhouses.

2. Methods

Four trials were conducted to assess the efficacy of a soluble liquid concentrate (SL) formulation known as FytoSave® (FytoFend, Isnes, Belgium), containing 12.5 g l^{-1} of the active substance COS-OGA, against powdery mildew on two different crops (grape and cucumber). Two efficacy trials against *E. necator* on grape were conducted in 2012 in Spain and France. In addition, two efficacy trials against *S. fuliginea* on cucumber were conducted in Belgium (2011) and Spain (2012). As elicitors primarily have a preventive function, the application sequences were designed with at least two sprayings before any potential outbreak of the disease occurred. The observations were focused on the incidence and severity of the disease on grapevine bunches and on the leaves of cucumber plants. Application and evaluation dates were expressed in BBCH (Biologische Bundesanstalt Bundessortenamt und CHemische Industrie), and they correspond to specific development stages of the plant (Meier, 2001). For example, in grapevines, BBCH 77 corresponds to bunch closure and BBCH 81 to the beginning of berry ripening. In the same manner, in cucurbits, BBCH 61 corresponds to the first opening flower and BBCH 71 is when the first fruit is mature for harvest. Trial design, test compound efficacy evaluations and phytotoxicity assessments were all performed according to the European and Mediterranean Plant Protection Organization (EPPO) guidelines, which define the standard procedures for the evaluation of plant protection products. Powdery mildew trials in grapevines and in cucumber plants were performed according to EPPO guidelines PP 1/4(4) and PP 1/57(3), respectively, and details are given hereunder for each trial. Phytotoxicity assessments were performed according to EPPO guideline PP 1/135 (3), which requires the recording of potential delays in growth stages, plant discoloration, necrosis and deformation following test compound applications.

2.1. Powdery mildew on bunches of grapes (France, 2012)

The first trial was conducted outdoors during 2012 by Promo Vert (Serres-Castet, France) on the disease-susceptible grapevine CV Carignan in a vineyard located in Pujaut (Rhone Valley, Gard, France). Eight COS-OGA sprayings at three different rates were performed: 1 l ha^{-1}, 1.5 l ha^{-1} and 3 l ha^{-1}. Sprayings were performed between 18 April and 18 July with a theoretical 14-day interval. Sprayings were performed every 11 to 16 days (depending on the weather and disease risk) with an airblast sprayer and a total volume of water of 300 l ha-1. An untreated control and a chemical reference were included in the experiment, which had a randomized complete block design with four repetitions per treatment and a plot size of 23 m^2 containing 10 vines. A maintenance treatment against downy mildew and black rot was also applied. The chemical fungicide consisted of sulfur applied at a rate of 10 kg ha^{-1}. The incidence and severity of powdery mildew caused by natural *E. necator* infection were assessed on 50 bunches per plot at BBCH 77 (on 11 July) and at BBCH 81 (on 25 July). The test products and application sequence are detailed in Table 2.1.

Table 2.1: Test compounds and application scheme at the Carignan vineyard treated in 2012 in Pujaut.
Applications were performed as follows: A on 18 April at BBCH 13, B on 3 May at BBCH 15, C on 18 May at BBCH 18, D on 1 June at BBCH 63, E on 13 June at BBCH 73, F on 25 June at BBCH 75, G on 6 July at BBCH 75 and H on 18 July at BBCH 79.

Objects	Amount of active substance per area (g ha^{-1})	Water volume (l ha^{-1})	Application timing
Untreated control	-	-	-
COS-OGA SL formulation 1 l ha^{-1}	12.5	300	A B C D E F G
COS-OGA SL formulation 1.5 l ha^{-1}	18.75	300	A B C D E F G
COS-OGA SL formulation 3 l ha^{-1}	37.5	300	A B C D E F G
Sulfur	10 000	300	A B C D E F G

2.2. Powdery mildew on bunches of grapes (Spain, 2012)

The second trial was conducted outdoors during 2012 by Trial Camp (Monserrat, Spain) on the disease-susceptible table grape Moscatel in a vineyard located in Novelda (Alicante, Spain). Six COS-OGA sprayings at 3 l ha^{-1} were performed between 10 May and 18 July, with a 14-day interval (Table 2.2). Sprayings were performed using a motorized knapsack sprayer with the volume of water fixed at 600 l ha^{-1} on the first application (A), 800 l ha^{-1} on the second application (B) and 1000 l ha^{-1} for the third to sixth applications (C, D, E and F, Table 2.2). An untreated control and a chemical reference were included in the experiment with a randomized complete block design containing four repetitions and plot size of 60 m^2 containing 10 vines each. The chemical sequence was a classical fungicide program for controlling both powdery and downy mildew (Table 2.2). The incidence and severity of powdery mildew caused by natural infection of *E. necator* were assessed on 100 randomly selected bunches per plot on 21 June (BBCH79), 5 July (BBCH 81), 18 July (BBCH 81), 25 July (BBCH 81), 1 August (BBCH 83) and 22 August (BBCH 85).

Table 2.2: Test compounds and application scheme at a Moscatel vineyard treated in 2012 in Alicante (Spain).
Applications were performed as follows: A on 10 May at BBCH 57, B on 24 May at BBCH 65, C on 7 June at BBCH 75, D on 21 June at BBCH 79, E on 5 July at BBCH 81 and F on 18 July at BBCH 81.

Objects	Amount of active substance per area (g ha^{-1})	Water volume (l ha^{-1})	Application timing
Untreated control	-	-	-
COS-OGA **SL formulation 3 l ha^{-1}**	37.5	600 800 1000	A B C D E F G
Standard chemical program			
Sulfur Mancozeb Metalaxyl-M	1920 960 60	600	A
Azoxystrobin	200	800	B
Tebuconazol Copper oxychloride	250 500	1000	C
Azoxystrobin	250	1000	D
Tebuconazol Copper oxychloride	250 500	1000	E
Azoxystrobin	250	1000	F

2.3. Powdery mildew on cucumber leaves (Belgium, 2011)

The first trial on cucumbers was conducted by Provinciaal Proefcentrum voor de Groenteteelt Oost-Vlaanderen (Kruishoutem, Belgium). The powdery mildew susceptible cucumber CV Sheila (Nunhems) was used and grown on mineral wool. One month after sowing, the seedlings were transplanted on 7 July 2011 in a greenhouse. The trial was set up in a randomized block design with four repetitions, with each repetition corresponding to one parallel rank. Ranks were further divided in plot areas of 5.09 m^2 containing twelve cucumber plants. The experimental design included four modalities: an untreated control, a chemical reference and COS-OGA at two different application rates per ha of leaf wall area (LWA), which was defined by Pergher and Petris (2008) (Table 2.3). The elicitor was tested at rates of 12.5 g of COS-OGA per ha of LWA and 25 g of COS-OGA per ha of LWA. The chemical reference program consisted of an initial application of the fungicide bitertanol followed by four applications of sulfur. A volume of 500 l ha^{-1} LWA of water was sprayed regardless of the modality. The test products are detailed in Table 2.3.

Table 2.3: Test compounds and application sequences for the cucumber trial (CV Sheila, Nunhems) performed in 2011 in Kruishoutem (Belgium).
Applications were performed as follows: A on 29 July at BBCH 72, B on 5 August at BBCH 73, C on 12 August at BBCH 74, D on 19 August at BBCH 78 and E on 2 September at BBCH 81.

Objects	Amount of active substance per area (g ha^{-1} LWA)	Water volume (l ha^{-1}LWA[a])	Application timing
Untreated control	-	-	-
COS-OGA 1 l ha^{-1} LWA	12.5	500	A B C D E
COS-OGA 2 l ha^{-1} LWA	25	500	A B C D E
COS-OGA 2 l ha^{-1} LWA	25	800	A B C D E
Standard chemical program			
Bitertanol	300	500	A
Sulfur	4000	500	B C D E

[a] Leaf wall area (LWA) = 10 000 * b^{-1} * h with b, in m, being the row spacing and h, in m, being the height interval of the canopy (Pergher and Petris, 2008).

The sprayings were performed with a backpack sprayer that delivered a spray volume of 500 l water per ha LWA at an operating pressure of 2.2 - 3.8 bars. The treatments began on 29 July 2011 (application A, Table 2.3) followed by three additional sprayings at seven-day intervals (applications B to D, Table 2.3). The last application E was performed 14 days after application D on 2 September (Table 2.3). Seven days after application D, there was a general treatment of the greenhouse with the chemical penconazole to lower the powdery mildew disease pressure. However, the infestation was too strong and the assessments had to be stopped. Observations of *S. fuliginea* symptoms were performed at BBCH 73 (4 August), BBCH 74 (10 August), BBCH 78 (18 August), BBCH 79 (25 August) and BBCH 81 (1 September). The severity and incidence of powdery mildew were assessed on five leaves from five randomly selected plants per plot.

2.4. Powdery mildew on cucumber leaves (Spain, 2012)

A trial on cucumber leaves was performed by GMW Bioscience (Torrellano, Alicante, Spain) between February and May 2012 using cucumbers (CV Black) grown under greenhouse conditions with fertilization, irrigation and cultivation methods according to local practice. The seedlings were transplanted on 25 February 2012. The trial was also set up in a randomized block design with four parallel ranks divided in a plot area of 2.2 m^2, each containing five plants. Each rank contained six modalities: an untreated control, four COS-OGA spraying rates and a chemical reference, both of which were applied using 500 l water ha^{-1} LWA. The test products are detailed in Table 2.4.

Table 2.4: Test compounds and application sequences for the cucumber trial (CV Black) performed in 2012 in Torrellano (Spain).
Applications were performed as follows: A on 12 April at BBCH 13, B on 19 April at BBCH 40, C on 26 April at BBCH 66, D on 2 May at BBCH 69, E on 9 May at BBCH 71 and F on 16 May at BBCH 75.

Objects	Amount of active substance per area (g ha^{-1} LWA)	Water volume (l ha^{-1} LWA)	Application timing
Untreated control	-	-	-
COS-OGA 1 l ha^{-1} LWA	12.5	500	A B C D E F
COS-OGA 2 l ha^{-1} LWA	25	500	A B C D E F
COS-OGA 3 l ha^{-1} LWA	37.5	500	A B C D E F
COS-OGA 2 l ha^{-1} LWA	25	500	A C E
Triadimenol	62.4	500	A C E

COS-OGA applied at 25 g ha^{-1} LWA was the reference elicitor dose, it was compared to 12.5 g ha^{-1} LWA (half dose) and 37.5 g ha^{-1} LWA (higher dose), and the chemical reference was the fungicide Triadimenol applied at 62.4 g ha^{-1} LWA. Product applications began on 12 April (application A) before any symptoms had occurred and were performed with a time interval of 7 days between sprayings (Table 2.4). COS-OGA at 25 g ha^{-1} was also tested with a 14-day interval; therefore, only three applications were performed instead of six (A, C and E, Table 2.4). The sprayings were conducted by using a backpack sprayer at 500 l water per ha LWA and with an operating pressure of five bars. On 2 May, 20 days after application A, no symptoms had developed, and an artificial inoculation was conducted by uniformly introducing leaves infected by *S. fuliginea* in the experimental design, with one infected leaf per cucumber plant. Three assessments were performed on 11 May (BBCH 71), 16 May (BBCH 75) and 23 May (BBCH 81). Five plants were randomly selected per plot, and the incidence and severity of powdery mildew were evaluated for all of the leaves.

2.5. Data analysis

For each assessment date, the effects of the different treatments on disease severity and incidence were analyzed according to an analysis of variance (ANOVA, $P \leq 0.05$). In case of significant differences, the means of the disease incidence and severity were compared using a statistical Student-Newman-Keuls (SNK) test or individual pairwise comparisons with a Tukey's honestly significant difference (HSD) test at $P \leq 0.05$. When equality of variance could not be obtained with variable transformation, the non-parametrical statistical Friedman test was applied at $P \leq 0.05$.The effects of the treatment on disease incidence and severity were converted into protection following the Abbott formula (Equation 1).

Protection (%) = [(C-T)/C] * 100 Equation 1

where:

C = mean attack level in the untreated control plots, and

T = mean attack level in the treated plots.

3. Results

3.1. Powdery mildew on grape bunches (France, 2012)

This trial was conducted in a vineyard in the Rhone Valley using the Carignan CV, which is representative of the area and susceptible to powdery mildew. The field was located close to the Rhone River, which is an area that favors humidity and thus disease development. The field where the trial was conducted had encountered several historical infestations of powdery mildew. In 2012, there was a late but severe disease attack, and by 11 July at BBCH 77 (berries begin to touch), the powdery mildew incidence on untreated plots had reached 100% (Fig. 2.1). The attack was severe, and the damages impacted both grape quality and yield. In such harsh conditions of infection, the higher dose of COS-OGA elicitor offered a significant reduction in disease incidence at the first evaluation at BBCH 77. The chemical reference was the only treatment that provided a significant reduction of disease incidence for the two evaluations; however, the protection conferred by sulfur at 10 kg ha^{-1} dropped from 78.5% at BBCH 77 to 49% at BBCH 81 (Fig. 2.1). For each treatment, the eighth and last application was performed between the two assessments on 18 July.

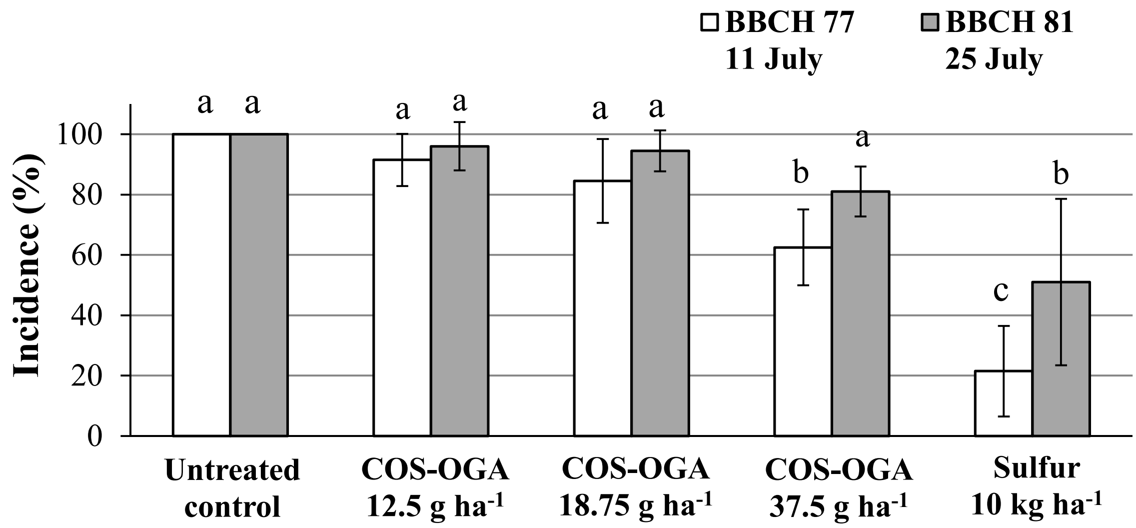

Fig. 2.1: Powdery mildew incidence (mean ± SD) on bunches of grapes of the CV Carignan in a vineyard in France (Pujaut, Rhone valley) in 2012.
Vines were sprayed eight times starting on 18 April at 11 to 16 days intervals with 1, 1.5 or 3 l ha^{-1} of a SL formulation containing 12.5 g l^{-1} COS-OGA or with a chemical reference (10 kg ha^{-1} of sulfur). Bars with different letters are significantly different for each individual date of evaluation (ANOVA and SNK test, $P < 0.05$).

The severity of powdery mildew symptoms in the untreated plot also reflected the high disease pressure, with 66% of the bunch surface affected at BBCH 77 and 94% at BBCH 81 (Fig. 2.2). The effect of COS-OGA on the percentage of bunch surface covered by the disease was clearly significant, even for the last evaluation. At BBCH 77, the disease severity was 33%, 26% and 12% for COS-OGA at 12.45 g ha^{-1}, 18.75 g ha^{-1} and 37.5 g ha^{-1}, respectively. Sulfur still offered the best protection with 3% severity on the bunch surface at BBCH 77; however, there was no statistically significant difference in disease severity between sulfur-treated berries and berries treated at the highest dose of COS-OGA (12% for 37.5 g ha^{-1}). A similar result was also observed at the beginning of ripening (BBCH 81), with a disease severity of 7% for the chemical reference and 21% for COS-OGA at 37.5 g ha^{-1} (Fig. 2.2). The elicitor effect on disease severity was clearly dose-dependent: 37.5 g ha^{-1} performed better than 18.75 g ha^{-1} or 12.5 g ha^{-1} (Fig. 2.2). The optimum bunch protection in terms of incidence achieved by COS-OGA (37.5 g ha^{-1}) was low and decreased from 38% at the first evaluation (BBCH 77) down to 19% at the second (BBCH 81). For severity, however, the protection remained high and constant, with 82% at the first assessment (BBCH 77) and 78% at the second (BBCH 81) (Fig. 2.2). Whatever the treatment, phytotoxicity was not observed.

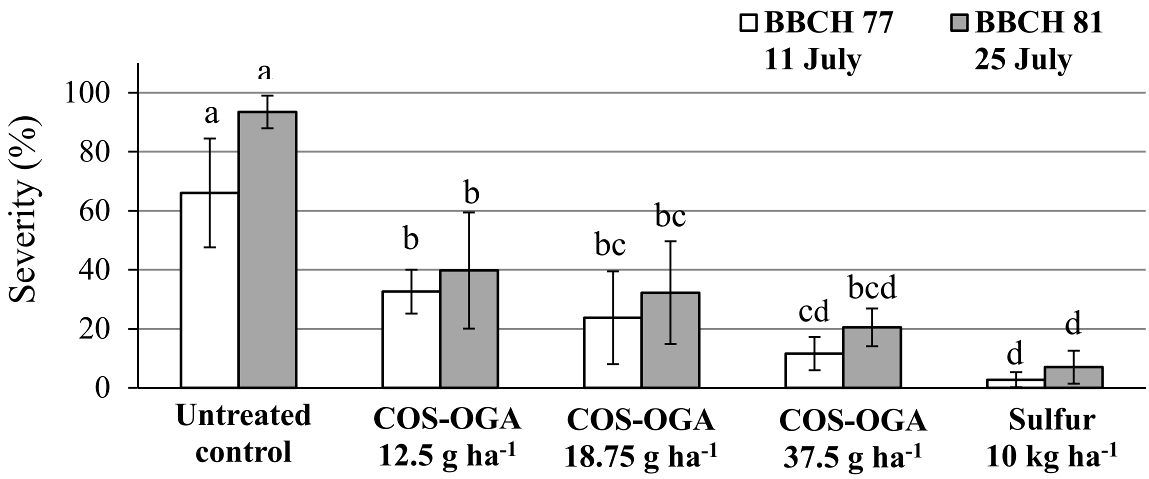

Fig. 2.2: Mean (± SD) of powdery mildew severity on bunches of grapes of the CV Carignan in a vineyard in France (Pujaut, Rhone Valley) in 2012.
Vines were sprayed eight times starting on 18 April at 11 to 16 days intervals with 1, 1.5 or 3 l ha^{-1} of a SL formulation containing 12.5 g l^{-1} COS-OGA or with a chemical reference (10 kg ha^{-1} sulfur). Bars with different letters are significantly different for each individual date of evaluation (ANOVA and SNK test, $P < 0.05$).

3.2. Powdery mildew on grape bunches (Spain, 2012)

At the first assessment during the vineyard trial in Spain, when the majority of the berries were touching (BBCH 79), the disease incidence on grape bunches in untreated plants was already 27% compared to 4% on plants treated with COS-OGA (Fig. 2.3). The disease spread dramatically until the third assessment (18 July), at which time up to 95% of untreated bunches were infected. COS-OGA maintained a stable disease incidence between 50% and 60% until the end of the trial. The disease incidence in the presence of the chemical reference program remained very low throughout the experiment.

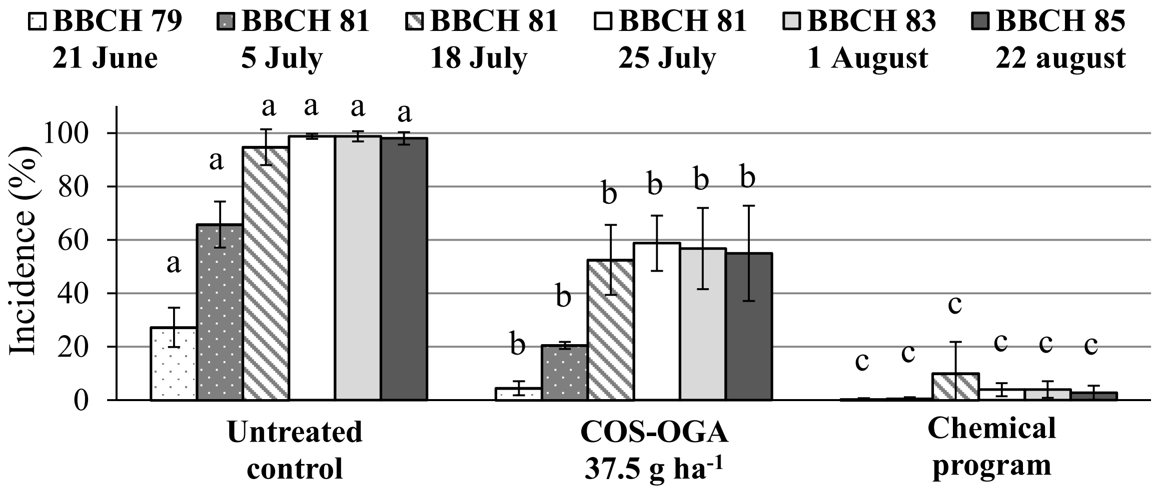

Fig. 2.3: Mean (± SD) of powdery mildew incidence on bunches of grapes in a vineyard in Spain (Alicante, 2012).
Vines were sprayed six times starting on 9 May at 14-day intervals with 3 l ha^{-1} of a SL formulation containing 12.5 g l^{-1} COS-OGA or with a chemical reference program in 600 to 1000 l ha^{-1}. Bars with different letters are significantly different for each evaluation date (ANOVA and SNK test, $P < 0.05$).

The disease severity on the bunches of grapes of untreated plants at the first evaluation (BBCH 79) was 3% and was low in the presence of both COS-OGA and the chemical reference (Fig. 2.4). The disease spread continuously until the end of the experiment at BBCH 85 on 22 August when it covered an area of 54% of the bunch in untreated plants. The severity increased to a much lower extent on COS-OGA-treated plants and reached a severity of 13% at the end of the experiment. It remained low in the presence of the chemical reference. The last application was performed on 18 July. More than one month later, the elicitor still showed good efficacy against powdery mildew in terms of severity.

The protection offered by COS-OGA to grape bunches regularly decreased in terms of incidence from 84% at first assessment down to 44% at the end of the experiment.

However, the protection in terms of severity remained high in the elicitor-treated plants over the course of the experiment; it reached 96% at the first assessment and decreased to 76% at the end of the experiment. The application of COS-OGA at 37.5 g ha^{-1} was selective to the table grapes, and there were no symptoms of phytotoxicity. However, the first treatment with a mixture of sulfur, mancozeb and metalaxyl-M sprayed on the chemical standard plot at application A caused brown spots on the grapes that were still present at harvest time.

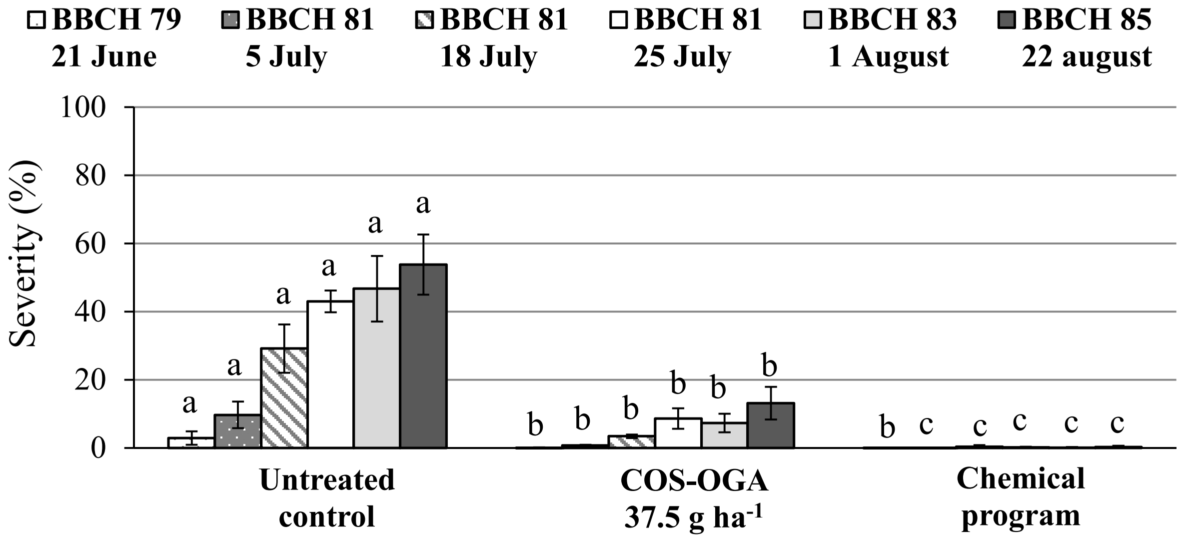

Fig. 2.4: Mean (± SD) of powdery mildew severity on bunches of grapes in a vineyard in Spain (Alicante) in 2012.
Vines were sprayed six times starting on 9 May at 14-day intervals with 3 l ha^{-1} of a SL formulation containing 12.5 g l^{-1} COS-OGA or with a chemical reference program. Bars with different letters are significantly different for each evaluation date (ANOVA and SNK test, $P < 0.05$).

3.3. Powdery mildew on cucumber leaves (Belgium, 2011)

This efficacy trial of COS-OGA against powdery mildew on cucumber started in July 2011, and the evolution of the disease incidence on leaves at each assessment is presented in Fig. 2.5. Once the disease was present in the greenhouse, it spread rapidly, and the untreated control exhibited a disease incidence of 76% on August 4 at BBCH 73, which was during fruit development, and it reached 100% two weeks later. At the first two assessments, the application of COS-OGA at 25 g ha^{-1} LWA in 500 l water provided a significant reduction in disease incidence. This treatment was even slightly more efficient than the complete chemical program at the first evaluation. Increasing the spray volume at a constant dose of COS-OGA did not further reduce the disease incidence. By the last observation, when the control had reached 100% disease incidence, only the chemical reference could significantly reduce the powdery mildew incidence.

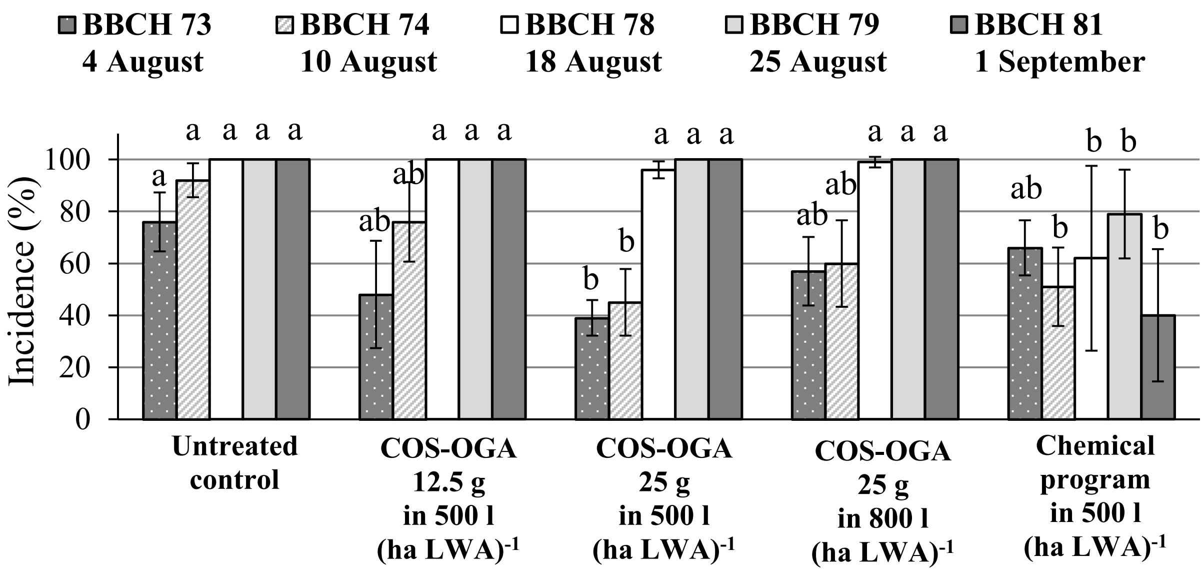

Fig. 2.5: Mean (± SD) of powdery mildew incidence evaluated on cucumber leaves in a greenhouse in 2011 (Kruishoutem, Belgium).
Cucumbers were sprayed five times starting on 29 July at 7-day intervals with 12.5 g ha^{-1} LWA or 25 g ha^{-1} LWA COS-OGA in 500 l or 800 l or with a chemical reference. Bars with different letters are significantly different for each evaluation date (ANOVA and Tukey's HSD or Friedman test, $P < 0.05$).

A severity assessment (Fig. 2.6) was possible at the end of fruit development (BBCH 78) when the pest severity was 19% in the untreated plot. The application of COS-OGA at 12.5 g ha^{-1} LWA reduced symptom severity by 72%, and at 25 g ha^{-1} COS-OGA in 500 L ha^{-1} LWA it reduced the severity by 85%. The application of 25 g ha^{-1} COS-OGA in 800 L ha^{-1} LWA was not as efficient as in 500 L ha^{-1} LWA (73% reduction in severity). At BBCH 79, the disease severity had dramatically increased to 70% in the untreated control, whereas at an application of 12.5 g ha^{-1}, COS-OGA limited the infection severity to 18%. The application of COS-OGA at 25 g ha^{-1} limited the severity to 12% and 19% for spray volumes of 500 and 800 L ha^{-1} LWA, respectively. Finally, at the beginning of ripening (BBCH 81), the disease severity was very high at 90% in the untreated plot; however, the chemical reference completely controlled the disease. COS-OGA reduced the symptom severity by 45% and 72% for 12.5 g ha^{-1} or 25 g ha^{-1} in 500 L ha^{-1}, respectively, and by 54% for 25 g ha^{-1} in 800 L ha^{-1} LWA. Thus in addition to the dose effect, a concentration effect also appeared, as an application of 25 g ha^{-1} in 500 L was always more efficient than the same amount in 800 L. All of the treatments were significantly different from the untreated control. No phytotoxicity was observed; however, the chemically treated cucumbers were not marketable as a result of numerous spots of sulfur on the fruits.

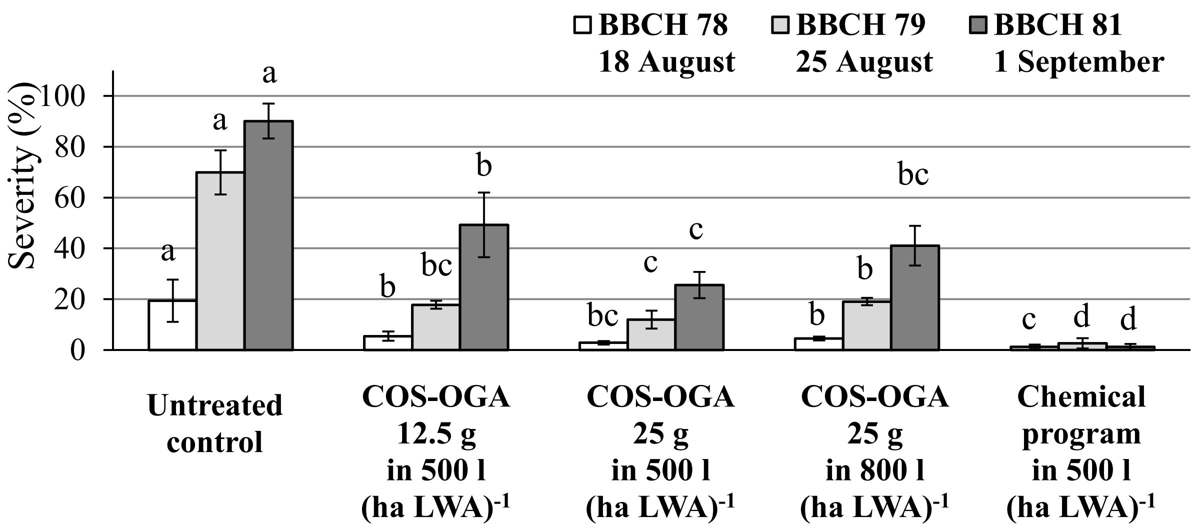

Fig. 2.6: Mean (± SD) of powdery mildew severity evaluated on cucumber leaves in a greenhouse in 2011 (Kruishoutem, Belgium).
Cucumbers were sprayed five times starting on 29 July at 7-day intervals with 12.5 g ha^{-1} LWA or 25 g ha^{-1} LWA COS-OGA in 500 l or 800 l and with a chemical reference. Bars with different letters are significantly different for each evaluation date (ANOVA and Tukey's HSD or Friedman test, $P < 0.05$).

3.4. Powdery mildew on cucumber leaves (Spain, 2012)

The aim of the second trial on cucumbers was to test both the dose and the time interval between treatments. At the first assessment at BBCH 71, which was during initial fruit development, the disease was newly established in the trial with a 24% incidence (Fig. 2.7) and 5% severity for the untreated plants (Fig. 2.8). Under these conditions, application of COS-OGA every 7 days at 12.5 g ha^{-1} resulted in protection in terms of both incidence and severity of 67% and 57%, respectively. Application of COS-OGA at 25 g ha^{-1} kept the disease under control with 70% protection in terms of incidence and 81% in terms of severity. Here, the higher dose of 37.5 g ha^{-1} did not significantly increase protection compared to a dose of 25 g ha^{-1} (71% protection in terms of incidence and 86% protection in terms of severity). Increasing the interval between sprayings (14 days instead of 7) reduced the protection for the same dose of 25 g ha^{-1}, but the difference was not significant (63% protection in terms of incidence and 71% protection in terms of severity). The efficacy of the chemical reference (64% protection against incidence and 83% protection against severity) reached the same level as that conferred by the application of COS-OGA at 25 g ha^{-1}. All of the tested products (COS-OGA and the chemical reference) were significantly different from the untreated control but were not significantly different from each other.

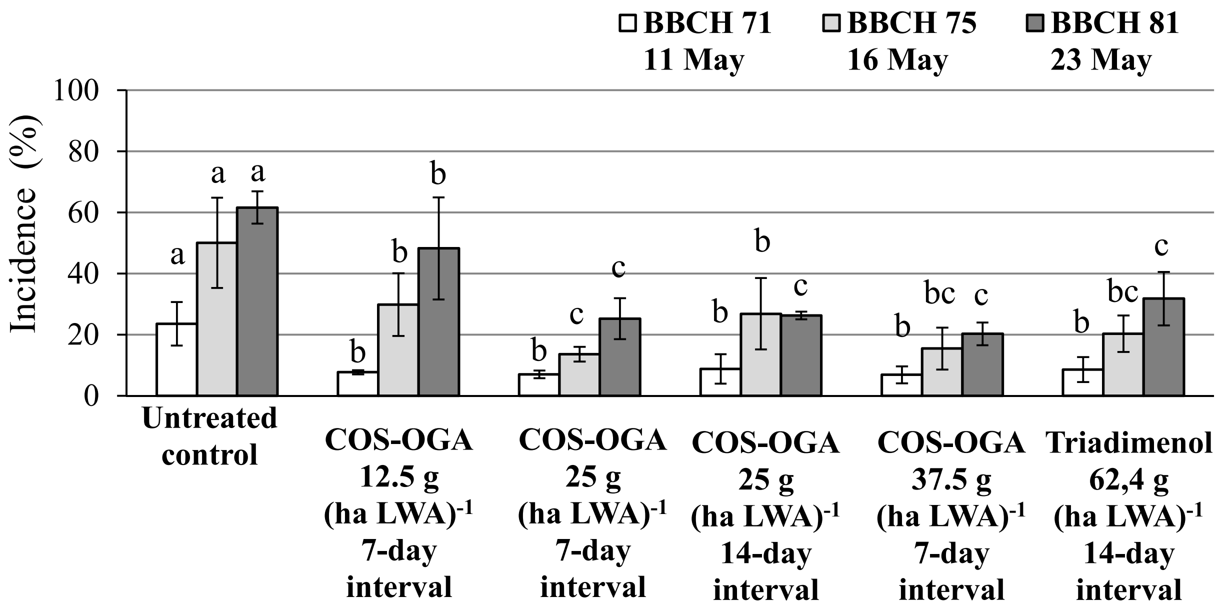

Fig. 2.7: Mean (± SD) of powdery mildew incidence on cucumber leaves under greenhouse in Spain (Torellano) in 2012.
Cucumbers were sprayed six or three times starting on 12 April at 7 or 14-day intervals with 1, 2 or 3 l ha^{-1} of a SL formulation containing 12.5 g l^{-1} COS-OGA or with a chemical reference. Bars with different letters are significantly different for each evaluation date (ANOVA and SNK test, $P < 0.05$).

At the second assessment at BBCH 75 (Fig. 2.7 and Fig. 2.8), performed prior to the last spraying, the disease incidence increased dramatically in the control plot at 50%, whereas the severity remained low at 9%. The protection conferred by application of COS-OGA at 12.5 g ha^{-1} resulted in a decrease in incidence (by 41%) and severity (by 43%). The application of COS-OGA at 25 g ha^{-1} still controlled the disease fairly well, with 73% protection in terms of incidence and 74% protection in terms of severity, and the protection conferred by the highest dose (COS-OGA 37.5 g ha^{-1}) remained high and stable (69% for incidence and 82% for severity). Surprisingly, a dose of COS-OGA at 25 g ha^{-1} (14-day interval) showed protection that was lower in terms of incidence (46%) and higher in terms of severity (78%) compared to the first assessment. The efficacy of the chemical reference remained stable compared to the first assessment (64% protection in terms of incidence and 83% in terms of severity). At this assessment, when applied at 7-day intervals, 25 g ha^{-1} COS-OGA had a statistically higher efficacy on disease incidence than 12.5 g ha^{-1}.

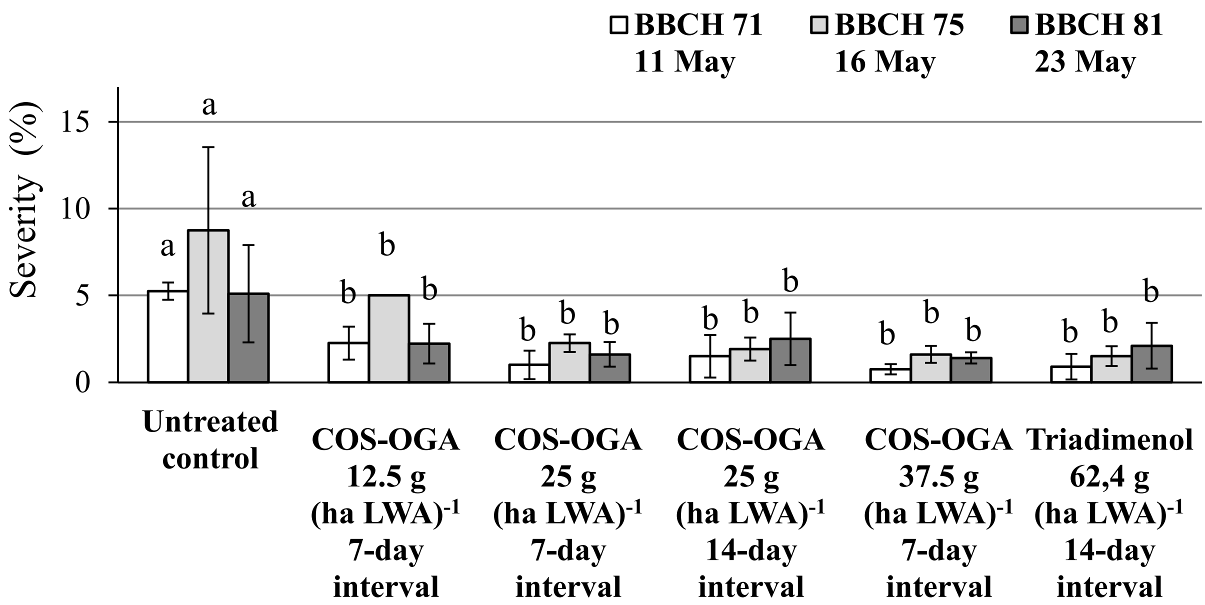

Fig. 2.8: Mean (±SD) of powdery mildew severity on cucumber leaves under greenhouse in Spain (Torrellano) in 2012.
Cucumbers were sprayed six or three times starting on 12 April at 7- or 14-day intervals with 1, 2 or 3 l ha^{-1} of a SL formulation containing 12.5 g l^{-1} COS-OGA or with a chemical reference. Bars with different letters are significantly different for each evaluation date (ANOVA and SNK test, $P < 0.05$).

At the third assessment at BBCH 81 (Fig. 2.7 and Fig. 2.8), the disease incidence continued to increase (62%) and the disease severity remained quite low (5%) for the untreated plot. The efficacy of COS-OGA at a dose of 12.5 g ha^{-1} COS-OGA was low (22% protection in terms of incidence and 56% protection in terms of severity); 25 g ha^{-1} fared better (59% protection in terms of incidence and 69% protection in terms of severity), whereas COS-OGA at 37.5 g ha^{-1} still conferred consistent protection (67% protection in terms of incidence and 73% in terms of severity). However, the protection achieved by COS-OGA at 37.5 g ha^{-1} was not significantly different from that of 25 g ha^{-1}. The difference in protection between COS-OGA at 25 g ha^{-1} (14-day interval) and COS-OGA at 25 g ha^{-1} (7-day interval) was unclear in terms of incidence but was more evident in terms of severity. Finally, the COS-OGA (25 or 37.5 g ha^{-1}) efficacy was numerically higher than that of the chemical reference (only 48% protection in terms of incidence and 59% in terms of severity).

4. Discussion

COS-OGA is a new stimulator of natural plant defenses that was tested in Spain, France and Belgium in 2011 and 2012 under commercial growing conditions. The protection conferred by preventive sprayings against powdery mildew on grapevines (*E. necator*) and cucumber plants (*S. fuliginea*) was assessed.

The COS-OGA elicitor was highly effective at reducing the severity of powdery mildew attack on grapevine bunches. In the first trial performed in France, three doses of COS-OGA were assessed: 12.5, 18.75 and 37.5 g ha^{-1} active substance. The powdery mildew infection was late but spread rapidly, and the control showed 100% incidence of disease at the first evaluation on 11 July (Fig. 2.1) and almost 100% severity two weeks later (Fig. 2.2). In such harsh conditions of disease pressure, the protection achieved by the most effective dose of COS-OGA (37.5 g ha^{-1}) was quite low in terms of incidence: 37% at the first evaluation and 19% at the second (Fig. 2.1). However, this dose of COS-OGA provided high protection in terms of severity, with a reduction of 75% at the first assessment and 78% at the second (Fig. 2.2). These levels of reduction in disease severity were not significantly different from those of the chemical reference sulfur.

In the second grape trial performed in Spain, only the dose of 37.5 g ha^{-1} was tested, and the results were similar. Despite a particularly dry season, the powdery mildew incidence on grape bunches reached approximately 100% in the untreated plots on 18 July at BBCH 81 (Fig. 2.3), and the severity was more than 50% 25 days later at the end of the trial (Fig. 2.4). The severity was well controlled by COS-OGA, which provided a 76% reduction in severity at the end of the experiment (Fig. 2.4).

These results also showed that the elicitor was better at reducing severity than incidence. Elicitors do not kill pathogens but precociously mobilize natural plant defenses that limit the subsequent spread of the disease, which is why disease incidence is less well controlled than severity. However, consumers do not tolerate rotten leaves on lettuce, for example, in which case protection in terms of incidence must be considered. A certain number of damaged berries is still acceptable for wine makers, which is why protection expressed in terms of severity is better adapted to evaluating treatment efficacy on berries.

Vinification and wine quality can also benefit from the replacement or reduction of chemically derived plant protection products by elicitors. Fungicide residues can still be present on grapes at harvest time and influence the selection and development of yeast strains during the fermentation process (Caboni and Cabras, 2010; Faoro *et al.*, 2008). However, the treatment of grapevines with elicitors can potentially increase secondary metabolite levels in grapes, specifically those of phytoalexins, such as stilbene derivatives (Ruggiero *et al.*, 2013; Steimetz *et al.*, 2012). The phytoalexin resveratrol is associated with positive effects on human health, such as reductions in heart disease and atherosclerosis (Donnez *et al.*, 2011).

Sawant *et al.* (2011) studied the field efficacy of the alternative product Milastin K, which is a formulation that contains *Bacillus subtilis* and acts against grapevine powdery mildew. Under low to moderate disease pressure, the efficacy of Milastin K at controlling powdery mildew was similar to that of sulfur, the chemical reference; however, under high disease pressure, the product tended to be less efficient than the reference. The integration of *B. subtilis* into an integrated pest management program controlled the disease while reducing fungicide use. Here, we showed that COS-OGA was similarly successful at protecting grapes, even when the disease severity exceeded 50% in the untreated control. Some elicitors have shown good efficacy in disease control but are responsible for negative side effects in the plants they treat. For example, Perazzolli *et al.* (2011) compared the effect of *Trichoderma harzianum* and benzothiadiazole (BTH) in downy mildew (*Plasmopara viticola*) control on grapevines and observed a similar efficacy for both products. Repetitive foliar applications of *T. harzianum* did not impair grapevine growth; however, BTH significantly reduced the number of leaves as well as the dry and fresh weight of the plants. BCAs are not always free from negative side effects, and Tamm *et al.* (2011) showed that Pen, an aqueous extract of the mycelium of *Penicillium chrysogenum,* was effective at reducing several diseases in vineyards, including powdery mildew; however, they reported phytotoxicity. From our experience, no metabolic load (detrimental effect on yield) has ever been observed with COS-OGA under normal conditions of use.

COS-OGA was also effective at reducing powdery mildew infestations in cucumbers, and the results were similar to those obtained for grapevines although with a higher degree of protection in terms of severity rather than incidence. Different doses, spray volumes and time intervals between applications were assessed. In the first trial in Belgium, both the dose and concentration of COS-OGA had an impact on protection. The severity reduction was higher for the highest dose and smaller spray volume (25 g ha^{-1} LWA COS-OGA in 500 L), with 85% protection at the first evaluation, which fell to 72% at the end of the assessments (Fig. 2.7). A similar level of protection was obtained in the second cucumber trial in Spain where the COS-OGA dose effect was clear up to 25 g ha^{-1}, even if the effect on severity was not statistically significant between the different doses and sprayings (Fig. 2.8). Increasing the spraying interval from one to two weeks did not affect the product's performance, which was as good as the chemical control.

Although the COS-OGA elicitor is not directly toxic to pathogens, it is detected by the plant, which then switches on signaling cascades that result in defense reactions against potential invaders. It has been shown by qRT-PCR that the COS-OGA complex triggers signal transduction through the SA pathway rather than through jasmonic acid (van Aubel *et al.*, unpublished results). Because SA is known to mediate defenses against biotrophic pathogens, it is not surprising that COS-OGA protects against biotrophic pathogens, such as powdery mildews (Rahman *et al.*, 2012). However, it is also well known that crosstalk exists between the SA and jasmonic acid pathways. Further investigations are required to determine the precise roles and signaling pathways of COS-OGA.

For fungicides that kill pathogens or inhibit their growth, proper control of the disease is linked to optimal coverage of the plant by the product. Optimal coverage depends on many factors (Siegfried *et al.*, 2007), including the dose of active substance, the time interval between applications, the volume sprayed, and the size and number of deposits. Proper plant defense stimulation likely relies on the same parameters. Although many laboratory experiment reports are available, little is known about the importance of spraying programs and techniques for using elicitors. Even if elicitors are well known triggers for systemic signaling, their primary target is likely membrane receptors, and the efficient stimulation of plant defenses primarily relies on the optimal spraying of leaves so that active molecules can penetrate cell walls, which mostly occurs through open stomata on the lower leaf surface (Zeng *et al.*, 2010).

To ensure that optimum protection with a hydrophilic molecule, such as COS-OGA, is reached, practical approaches that have been deduced from experience should be followed. Spraying equipment that is able to cover the lower faces of the leaves until run-off (with higher water volumes if necessary) should be used at an operating pressure of several bars. The current trend to reduce spraying volumes to avoid pesticide loss by drift or run-off is not applicable for plant defense inducers because they are usually not hazardous to the environment, and such a practice might compromise the treatment's effectiveness. A 7- to 14-day interval between sprayings also appears to be suitable for maintaining plants in a primed state. The persistence and cumulative effect of repeated COS-OGA applications were confirmed by laboratory bioassays (van Aubel *et al.*, unpublished results), which implies that at least two preventive sprayings must be performed before pathogen arrival. Under these conditions, good protection against powdery mildew was obtained for grapevines one month after the last application.

In addition to the protection offered by COS-OGA, which can help lower the amount of chemical plant protection products in conventional agriculture, the use of environmentally friendly plant protection products has other advantages. The pre-harvest interval (PHI) and re-entry interval are non-existent, whereas bitertanol in cucumbers, for example, requires a PHI of three days, and Triadimenol requires a PHI of three weeks. COS-OGA is also a promising alternative to sulfur in organic farming. Edwards-Jones and Howells (2001) evaluated a sulfur treatment that had "10 times the total environmental impact score than the least damaging integrated pest management strategy", which is a result of the sulfur treatment requiring high doses and many applications to be effective.

In conclusion, the COS-OGA elicitor provides adequate protection against powdery mildew under low to moderate disease pressure in grapevines and cucumber. In cases of higher disease pressure, fungicide applications may be scheduled within an integrated program based on COS-OGA. This approach reduces the application risks for operators and the environment, lowers the total amount of potential residues for consumers, increases the product's marketable value for growers, and offers a new mode of action that limits the risk of resistance build-up against fungicides. This work complements the current concerns of researchers who advocate elicitor inclusion in integrated pest management strategies to secure their efficacy (Walters *et al.*, 2013).

5. Acknowledgments

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