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Ability of *Pythium oligandrum* strains to protect *Vitis vinifera* L., by inducing plant resistance against *Phaeomoniella chlamydospora*, a pathogen involved in Esca, a grapevine trunk disease

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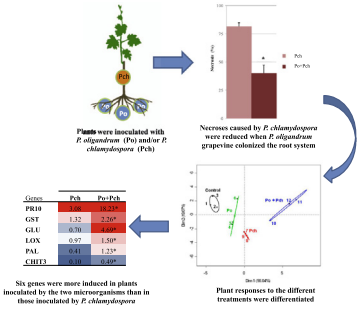
HIGHLIGHTS

- *P. oligandrum* inocula colonized the rhizosphere throughout the experimental period.
- Plant root system colonization by *P. oligandrum* reduced *P. chlamydospora* necroses.
- Plant molecular responses differed according to treatments.
- *P. oligandrum* enhanced certain gene expression in *P. chlamydospora* infected plants.

GRAPHICAL ABSTRACT



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ABSTRACT

Biological control of *Phaeomoniella chlamydospora*, a pathogen involved in Esca, a grapevine trunk wood disease, was performed using the oomycete, *Pythium oligandrum*. Three 4-month greenhouse assays showed that necrosis of *Vitis vinifera* L. cv. Cabernet Sauvignon cuttings caused by *P. chlamydospora* was significantly reduced (40–50%) when *P. oligandrum* colonized the plant root systems. The expression of a set of 22 grapevine defense genes was then quantified by real-time polymerase chain reaction to determine plant responses in the interaction between *P. oligandrum*/*V. vinifera* L./*P. chlamydospora*. In the trunk, specific grapevine responses to the different treatments (control, *P. oligandrum*, *P. chlamydospora* and *P. oligandrum* + *P. chlamydospora* treatments) were significantly differentiated. Expression levels of 6 genes associated with *P. chlamydospora* infection showed higher induction than when plants were pre-treated with *P. oligandrum*. These genes are involved in various pathways (PR proteins, phenylpropanoid pathways, oxylipin and oxydo-reduction systems).

1. Introduction

Over the last two decades, grapevine trunk diseases (GTDs) such as Esca, Eutypiosis and Botryosphaeriae diebacks, have become a subject of major concern for the wine industry worldwide (Abero et al., 2011; Ammad et al., 2014; Bertsch et al., 2013; Chebil

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et al., 2012; Correira et al., 2013; Diaz et al., 2012; Kaliternam and Milicevic, 2013; Mohammadi et al., 2013; Mondello et al., 2013; Rego et al., 2000; Urbez-Torres et al., 2009, 2012; Yan et al., 2011). In France, Grosman and Doublet (2012) reported that, in 2008, about 11% of French vineyards were unproductive, a figure that had increased to 13% in 2012. Moreover, Bruez et al. (2013) reported that in certain regions, such as Charentes or Jura, the proportion of unproductive grapevines due to GTDs affected plants in a vineyard reached 32.6% or 18.42%, respectively. Besides causing losses in grapevine yield, GTDs may also have a non-negligible effect on wines. Lorrain et al. (2012) reported a detectable loss of wine sensory quality, when only 5% of the grapes used to produce the wine were affected by Esca. Because Esca is the most frequent GTD in Europe, experiments have generally been focused on this disease. Esca is commonly thought to result from the pathogenic activity of fungal species, including *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & Mugnai) Crous & W. Gams (Chaetothyriales: Herpotrichiellaceae), *Phaeoacremonium aleophilum* W. Gams, Crous, M.J. Wingf. & Mugnai (Diaporthale: Togniniaceae) and *Fomitiporia mediterranea* M. Fisch. (Hymenochaetales: Hymenochaetaceae). However, *Eutypa lata* (Pers.) Tul. & C. Tul. (Xylariales: Diatrypaceae), *Stereum hirsutum* (Willd.) (Russulales: Stereaceae) and Botryosphaeriaceae species may also be involved in Esca (Bertsch et al., 2013). These fungi deconstruct the wood, causing various types of necroses: central necrosis, black punctuate necrosis, sectorial necrosis and a necrosis called white rot, or "amadou", typically associated with Esca (Maher et al., 2012). Attacks generally end in grapevine death a few years later (Guerin-Dubrana et al., 2013) but one form, apoplexy, is particularly severe, resulting in plant death within a few days only (Larignon et al., 2009).

Ever since the ban in 2001 on the use of sodium arsenate, the only pesticide registered to control GTDs, the use of alternative methods, such as biocontrol, has become essential. Various microorganisms have been tested to control the fungi involved in Esca. In the vineyard, treating fresh pruning wounds with *Trichoderma atroviride* P. Karst (Hypocreales: Hypocreaceae) strain USPP-T1 reduced the incidence of *P. chlamydospora* by 77% (Kotze et al., 2001). The authors showed that *Bacillus subtilis* Ehrenberg (Bacillales: Bacillaceae) is less effective than *T. atroviride* USPP-T1 in protecting grapevine against *P. chlamydospora* attack. The same study reported that *T. atroviride* strains were more effective at reducing pruning wound infection, because they had originally been isolated from grapevine and were, therefore, probably more adapted to the grapevine wood environment. In nurseries, it was shown that vascular streaking induced by *P. chlamydospora* was significantly reduced in *Trichoderma harzianum* Pers. (Hypocreales: Hypocreaceae)-inoculated shoots (Di Marco et al., 2004; Fourie and Halleen, 2006). Alfonzo et al. (2009) reported that antagonistic substances produced by a *B. subtilis* strain (AG1) could inhibit mycelial growth of *P. chlamydospora* and *P. aleophilum*.

One oomycete, *Pythium oligandrum* Dreschler (Pythiales: Pythiaceae), which naturally colonizes the rhizosphere of many plants, including grapevine (Gerbore et al., 2014), was assessed for the control of *P. chlamydospora*, a fungus involved in Esca. This oomycete was chosen for several reasons: (i) the interaction between pathogens/plants/*P. oligandrum* has already been described in numerous cases (Le Floch et al., 2005, 2009; Takenaka et al., 2003); (ii) *P. oligandrum* has several biocontrol agent properties that play an important role in the reduction of disease incidence. This oomycete can interact directly with the pathogens through mycoparasitism (Benhamou et al., 1997, 1999), antibiosis (Bradshaw-Smith et al., 1991; Benhamou et al., 1999), competition for nutrients (Martin and Hancock, 1987) or, indirectly, *via* the stimulation of plant defenses (Benhamou et al., 1997; Le Floch et al., 2003; Lherminier et al., 2003; Masunaka et al., 2010; Mohamed et al., 2007; Picard et al., 2000; Takenaka et al., 2003, 2006, 2008). *P. oligandrum* can reduce pathogenic attacks on many plants; this reduction varies from 15% to 100% (Gerbore et al., 2013). Among all its various direct and indirect modes of action, the stimulation of plant defenses and resistance has been the most frequently studied effect of *P. oligandrum* (Benhamou et al., 2012; Gerbore et al., 2013; Rey et al., 2008).

P. oligandrum produces three protein elicitors: oligandrin, POD-1 and POD-2, which activate the plant defense systems. Picard et al. (2000) have shown that *P. oligandrum* produces an elicitin-like protein with a molecular mass of 10 kDa, which is called oligandrin. This molecule induced resistance in tomato in order to control *Phytophthora parasitica* Breda de Haan (Peronosporales: Peronosporaceae). Mohamed et al. (2007) reported that when *Botrytis cinerea* Pers. (Helotiales: Sclerotiniaceae) was applied to leaves of oligandrin-pretreated plants, leaf invasion was limited and the protection level reached about 75%. On the other hand, Takenaka et al. (2003) extracted an elicitin from the cell wall protein fraction of *P. oligandrum* that contained two major proteins, POD-1 and POD-2. Foliar treatment of sugar beet, using cell wall protein fractions from *P. oligandrum*, induced defense-related genes, which were more rapidly expressed in cell wall protein-treated leaves than in control leaves treated with distilled water (Takenaka and Tamagake, 2009).

In the present study, the ability of various strains of *P. oligandrum*, whether isolated or not from the rhizosphere of grapevine to colonize and protect vine against *P. chlamydospora* was assessed. Accordingly, we determined whether *P. oligandrum* was able to reduce the symptoms, i.e. wood necrosis, caused by *P. chlamydospora*. The induced resistance in plants was then examined at trunk level (wood tissues) using a set of 22 genes involved in grapevine defenses as defined by Dufour et al. (2013).

2. Materials and methods

2.1. oligandrum strains

Three different inocula of *P. oligandrum* (Po1, Po2 and Po3) were evaluated. Each inoculum was a mixture of two strains: Po1 (Oth-2 and Oth-3), Po2 (Sto-1 and Oth-4) and Po3 (Sto-7 and Sto-11). Inocula (oospores-mycelium homogenate) were prepared by Biovitis (Saint Etienne Chomeil, France) and the concentration for each inoculum was adjusted to 2×10^4 oospores per mL.

The six different strains of *P. oligandrum* used in this study (Table 1) had already been genetically characterized by sequencing the Elicitin-like protein genes, i.e. genes that code for oligandrin (Oli-D1 or Oli-S1) and cell wall proteins (POD1-a, POD-1, POS-1) (Gerbore et al., 2014). Each strain had one gene encoding oligandrin and one gene encoding cell wall proteins. In this study, the amounts of oligandrin produced by each strain were evaluated using HPLC, as described by Gerbore et al. (2014).

Table 1
Elicitor genes (oligandrin and cell wall protein genes) and oligandrin production of *Pythium oligandrum* strains used in the 3 trials. At least 2 repetitions per strain were performed. Means values with different letters are significantly different at $P < 0.05$ (ANOVA and Tukey).

Strain	Inoculum	Oligandrin (mg/L) (±SE)	Cell wall protein genes	Oligandrin genes
Oth-2	Po1	61.08 ± 6.68ab	POD1-a	Oli-D1
Oth-3	Po1	100.07 ± 21.13b	POD-1	Oli-D1
Sto-1	Po2	81.10 ± 5.39ab	POD-1	Oli-D1
Oth-4	Po2	63.92 ± 6.49ab	POS-1	Oli-S1
Sto-7	Po3	77.66 ± 6.12a	POD-1	Oli-D1
Sto-11	Po3	65.85 ± 7.77ab	POS-1	Oli-S1

2.2. chlamydospora strain

P. chlamydospora strain SO37 (INRA-UMR SAVE collection, Bordeaux, France) was grown on malt agar medium at 25 °C in the dark. This strain is well known for its virulence and ability to induce necrosis in the wood (Laveau et al., 2009). The infection was performed using a 7-week-old culture of *P. chlamydospora*. The same strain of *P. chlamydospora* was used in all experiments.

2.3. Plant culture

For each experiment, grapevine plants (*V. vinifera* L. cv. Cabernet-Sauvignon) were propagated from 2-node wood cuttings in a greenhouse. The cuttings were rooted 2 months before infection and grown under controlled conditions. The temperature was maintained between 22 and 28 °C. Plants were watered for 2 min per day, *via* a drip system (2 l/h) and fertilized twice a week (nutrient solution N/P/K 20/20/20). On average, they received 16 h of light per day.

2.4. Experimental design

Three similar independent experiments trials, A, B and C, were carried out. The experimental design was conducted in a randomized complete block design with 9 plants per treatment for root and wood samplings, and 30 plants per treatment for necrosis assessment. Experimental conditions consisted of cuttings (i) inoculated on roots, with *P. oligandrum* inocula; (ii) infected by the pathogenic agent *P. chlamydospora*, at trunk level; (iii) inoculated on roots with *P. oligandrum* and then, one week later, infected with *P. chlamydospora* at trunk level; (iv) control cuttings with a hole (in order to mimic the infection procedure) used to represent mock control and (v) control cuttings not inoculated by *P. oligandrum*, nor infected by *P. chlamydospora.* Inoculum Po1 was evaluated in trial A, Po2 was evaluated in trial B, and Po3 was assessed in trial C.

2.5. Oomycete and fungal treatments of plants

At 7–8 leaf stage, rooted cuttings were inoculated twice with *P. oligandrum*, at the collar level of each plant: once with 50 mL of inoculum and with an additional 40 mL, three days later.

In order to infect plants with *P. chlamydospora*, the stem of each cutting was surface-sterilized with 95% ethanol and artificially wounded by drilling a hole (2 mm in diameter, 5 mm deep), 2 cm below the upper bud. The hole was filled with a *P. chlamydospora* mycelium plug cut off from the margin of each fresh culture on malt agar. The inoculation site was then immediately covered with paraffin wax. Cuttings inoculated with malt agar plugs were used as mock control.

2.6. Wood sampling

Wood samples were collected at two sampling times: 0 day-post-inoculation (dpi) = 2 h post infection with *P. chlamydospora* and 14 dpi with *P. chlamydospora*. Nine plants per treatment (3 plants and 3 replications) were collected each time and, for each plant, a part of the stem, (2 cm above and 2 cm below the wound-inoculation hole) was sampled. All samples were immediately frozen in liquid nitrogen, and stored at −80 °C for subsequent transcriptomic analyses. At the end of the experiment (120 dpi), 30 plants per treatment were collected for disease evaluation necrosis measurement.

2.7. Assessment of grapevine root colonization by P. oligandrum

Root colonization by *P. oligandrum* was monitored during the experiments at three time point, 14, 56 and 120 dpi, using plate-counting method. At each sampling time, for each plant, 20 root fragments were randomly collected, deposited on a selective medium CMA-PARP (Corn Meal Agar added with Pimaricin, Ampicillin, Rifampicin and Pentachloronitrobenzene) (Jeffers and Martin, 1986), and incubated at 25 °C in the dark for 10 days. For each root fragment, the presence of typical *P. oligandrum* echinulated-oospores (thick-walled spiny oospores, van der Plaats-Niterink, 1981) was recorded by optical microscope observation. The average frequency of *P. oligandrum* oospores was determined for each treatment.

The effects of the fixed factors "treatments" (2 levels) and "sampling time points" (3 levels), and of their interaction on the binomial response variable "Frequency of *P. oligandrum* root colinization", were tested with a two-way generalized linear model (GLM). As the interaction was significant ($P = 10^{-08}$), we then tested the effect of each factor alone, and when this effect was significant, we performed multiple comparison tests in order to determine whether the observed differences between treatments were significant. All the statistical analyses were done using software R. 3.1.1 and the library "multcomp" for analyses.

2.8. Assessment of plant protection induced by P. oligandrum against P. chlamydospora attack

At the end of each experiment, 120 dpi, 30 plants per treatment were collected. Then, the stem of each plant was sectioned longitudinally and the length of necrosis caused by *P. chlamydospora* was measured. The rate of necrosis was obtained by calculating the ratio between the length of necrosis and the total cutting length. Analysis of variance (ANOVA, R 3.1.2) was carried out to assess differences between treatments.

2.9. Plant total RNA extraction and reverse transcription

A fine powder was produced by grinding wood samples in liquid nitrogen, using a Tissue LyserII (Qiagen). For each treatment, 1.2 g of the three cuttings collected (400 mg per cutting) were weighed and combined into a "pool", which means that three "pools" of 3 cuttings (corresponding to 3 replicates per treatment) were obtained. The commercial kit (Qiagen) "RNeasy Plant Mini Kit" was used in accordance with the manufacturer's instructions with some modifications. Briefly, for each sample, 100 mg of powder were used twice, and 1% β-mercapthoethanol and 3% PVP40 were added to the lysis buffer (RLT). After homogenization of the mixtures, 200 µL of chloroform, isoamyl alcohol (24:1, v/v), were added to each tube. Solutions coming from the same sample were then pooled and transferred to the same spin column. A DNase treatment was performed (On-Column DNase I Digestion Set, using the protocol proposed by the supplier (Sigma–Aldrich)). Precipitation step was then performed in order to enhance the quality of RNA obtained: 0.1 volume of sodium acetate and 3 volumes of ethanol (99%) were added to the extracted RNA. Samples were then placed overnight at −20 °C. After 30 min of centrifugation at maximum speed, pellets were washed twice, using ethanol 70% and 99.9%, respectively. RNA yield and purity were estimated using a Nanodrop (ND-1000, ThermoScientific).

Reverse transcriptase assay was conducted following the MIQE guidelines (Bustin et al., 2009). For each material, about 0.5 µg of total RNA was reverse-transcribed in a total volume of 20 µL with M-MLV reverse transcriptase (Promega) following the manufacturer's instructions. The cDNA obtained were stored at −20 °C.

2.10. Quantitative polymerase chain reaction

The expression level of the 22 genes involved in the grapevine defense system was quantified, using real-time quantitative PCR (Table 2). Nineteen of the 22 studied genes have already been described by Dufour and co-workers (2013): 7 genes encoding PR proteins (*Pr1*, *Glu*, *Chit3*, *Chit4*, *Pin*, *Pr10* and *Pgip*); 3 genes involved in anthranilate pathway (*Chors*, *Ants*, *Chorm*); 6 genes involved in secondary metabolites biosynthesis, phenylpropanoid pathway (*Pal*, *Sts*, *Chi*, *Chs*, *Ban* and *Ldox*); one is a gene involved in the oxido-reduction system (*Gst*); *Acc* and *Lox* are respectively involved in the ethylene or oxylipin pathways. Three genes involved in wall thickness enhancement (*Cals*, *Cagt* and *Per*) were also analyzed (Dufour, 2011). Elongation factor 1 isoform γ (*Ef1 γ*, GenBank AF176496) and the gene for glyceraldehydes-3-phosphate dehydrogenase (*Gapdh*, GenBank CB973647) (Reid et al., 2006) were used as housekeeping genes to calculate transcript relative gene expression (Table 2). CFX 96 system thermocycler (Bio Rad) with SYBR®Green was used to assess the expression of genes. RT-qPCR was carried out as described by Dufour et al. (2013). Briefly, each reaction was performed in duplicate, using 1 µl of each primer at 1 µM, and 7 µl of 2 × Blue SYBR Green fluorescein mix and 5 µl of cDNAs. Relative expression was calculated using the $2^{-\Delta\Delta CT}$ method (Vandesompele et al., 2002). For each treatment, a fold change (FC) was obtained by calculating the relative gene expression between 0 and 14 dpi, and subsequently normalized by the relative gene expression between 0 dpi and 14 dpi of the corresponding control (mock control for *P. chlamydospora* and *P. oligandrum* + *P. chlamydospora* treatments or control for mock control and *P. oligandrum* treatments). The values of FCs between 0 and 1 correspond to repressions. The FCs obtained were studied by principal component analysis (PCA) with Analysis of variance (ANOVA, R 3.1.2) was carried out to assess differences between treatments.

Variables with cos2 > 0.5 on one of the first or second components (Dim1 or Dim2) were estimated as sufficiently well represented by the principal plan generated by this PCA.

Table 2
Genes involved in transcript profile analysis.

Family	Genes	Abbreviation
Housekeeping genes	Elongation factor 1-γ chain	*Ef1γ*
	Glyceraldehydes-3-phodphate dehydrogenase	*Gapdh*
PR-proteins	PR protein 1	*Pr1*
	PR protein 10	*Pr10*
	Chitinase class III	*Chit3*
	Chitinase class IV	*Chit4*
	Polygalacturonase inhibitor protein	*Pgip*
	β-1,3 glucanase	*Glu*
	Serine protease inhibitor	*Pin*
Phenylpropanoides	Phenylalanine ammonia lyase	*Pal*
	Stilbene synthase	*Sts*
	Chalcone isomerase	*Chi*
	Chalcone synthase	*Chs*
	Leucoanthocyanidin dioxygenase	*Ldox*
	Anthocyanidine reductase	*Ban*
Indoles	Antranilate synthase	*Ants*
	Chorismate mutase	*Chorm*
	Chorismate sythase	*Chors*
Wall thickness	Callose synthase	*Cals*
	Peroxidase	*Per*
	Coniferyl alcohol glucosyl transferase	*Cagt*
Others	Lipoxygenase 9	*Lox*
	Glutathione S-transferase	*Gst*
	1-Aminocyclopropane, 1-carboxylic acid oxidase	*Acc*

Expression levels of certain genes in plants treated by *P. oligandrum* and infected with *P. chlamydospora* were compared to plants infected with the pathogen only. A non-parametric test (Kurskal Wallis) was carried out to assess differences between treatments, using R 3.1.2).

3. Results

3.1. Oligandrin production by P. oligandrum strains

For the 6 *P. oligandrum* strains used in the study, the concentrations measured in the liquid medium by HPLC ranged from 61.08 mg L^{-1} (strain Oth-2) to 100,07 mg L^{-1} (strain Oth-3), and the average production was 74.94 mg L^{-1} (Table 1). The means of oligandrin production per inoculum were 72.51, 80.995 and 71.75 mg L^{-1} for Po1, Po2 and Po3, respectively. Oligandrin productions were significantly different between Sto7 (77.66 mg L^{-1}) and Oth-3 (100.07 mg L^{-1}) strains ($P < 0.05$).

3.2. Assessment of root colonization by P. oligandrum

P. oligandrum colonized the root systems of plants over the whole experimental period in the three trials (Fig. 1). *P. oligandrum* was not detected on non-inoculated plant roots. The lowest percentages of root colonization by the oomycete, obtained in experiment A, varied from 23% (14 dpi) to 6% (56 dpi) of the roots. In experiment B, 38% of roots at 14 dpi were colonized and 23% at 120 dpi. Higher values were obtained in experiment C, with root colonization percentages varying from 69% (14 dpi) to 32% (120 dpi). In all experiments, the percentage of root colonization by *P. oligandrum* was always significantly higher for plants inoculated by *P. chlamydospora* than for those inoculated exclusively by *P. oligandrum* at 120 dpi ($P < 0.05$).

Depending on the experiments and the treatments (roots inoculated exclusively with *P. oligandrum* vs *P. oligandrum* + *P. chlamydospora*), the percentage of root colonization by *P. oligandrum* (i) decreased over time, e.g. experiments A and B, for *P. oligandrum* treatment; (ii) increased over time, *e.g.* experiment B with both *P. oligandrum* and *P. chlamydospora* treatments; or (iii) stayed stable, e.g. experiment C for both *P. oligandrum* and *P. chlamydospora* treatments.

3.3. Protection of grapevine cuttings against P. chlamydospora attack

In order to test the resistance induced by *P. oligandrum* against *P. chlamydospora,* the length of necrosis in the grapevine wood was measured (Fig. 2). In the 3 experiments, plants treated at the root level by *P. oligandrum* displayed significant reduction in necrosis length compared with *P. oligandrum* non-inoculated plants ($P < 0.05$). Necroses caused in Cabernet Sauvignon cuttings by *P. chlamydospora* were estimated at around 80%. The necrosis reduction was estimated at 40% and 50% in experiments A and B, respectively, when the roots were colonized by *P. oligandrum*. In experiment C, necroses caused by the pathogen were about 50% and necrosis reduction was about 50% in plants inoculated by *P. oligandrum* at root level.

3.4. Assessment of grapevine-specific responses at trunk level by qRT-PCR

Plant response to the different treatments was evaluated in trunk at the transcriptional level by qRT-PCR, in the 3 trials. The expression level of 22 defense-related genes of grapevine was studied, and a PCA was performed to test possible separation between treatments according to their FCs (Fig. 3).

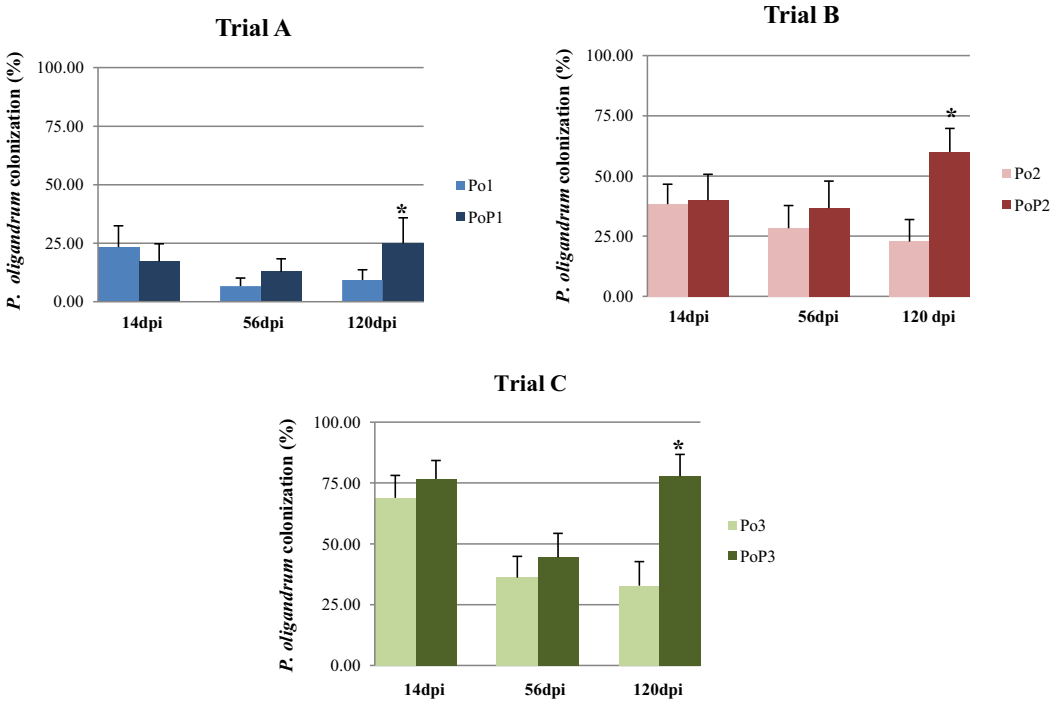

Fig. 1. Root colonization of vines by *P. oligandrum* assessed by plate counting in trials A, B and C. The values reported are means (±SE) of 9 samples collected in each treatment per sampling point. 14, 56 and 120 day-post-infection (dpi) with *P. chlamydospora* (*P. oligandrum* was inoculated 7 days before infection with the pathogen). Po1, Po2, and Po3 correspond to the 3 inocula of *P. oligandrum* (cf. Table 1), PoP1, PoP2 and PoP3: Po1 + *P. chlamydospora*, Po2 + *P. chlamydospora* and Po3 + *P. chlamydospora.* *Indicates means that are, within a trial, significantly different between the two treatments at $P < 0.05$ (generalized linear model).

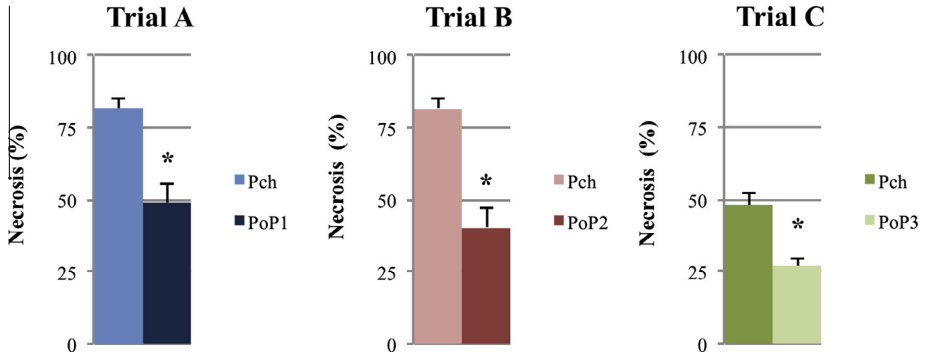

Fig. 2. Wood necrosis caused by *P. chlamydospora* in trunk cuttings treated or not with *P. oligandrum* at the root level, 120 days post infection. The values reported are means (±SE) of 30 samples collected in each treatment. Po1, Po2, and Po3 correspond to the 3 inocula of *P. oligandrum* (cf. Table 1), PoP1, PoP2 and PoP3: Po1 + *P. chlamydospora*, Po2 + *P. chlamydospora* and Po3 + *P. chlamydospora*, Pch: *P. chlamydospora*. *Indicates means that are, within a trial, significantly different between the two treatments at $P < 0.05$ (ANOVA).

For all trials A, B and C, PCA eigenvalues indicated that the first two principal components, Dim1 and Dim2, explained 68.92%, 75.11% and 70.01%, respectively, of total data variance. Results showed that grapevine responses differed significantly according to the treatment in the 3 trials.

Dim1, which represented 43.23%, 56.04% and 50.36%, respectively, in trials A, B and C, separated specific grapevine responses, *P. oligandrum* treatment and mock inoculation on one side, *P. chlamydospora* infection and *P. oligandrum* + *P. chlamydospora* treatment on the opposite side (A and B: negative *vs* positive coordinates; C: positive *vs* negative coordinates).

Dim2, which represented 25.69%, 19.07% and 21.34%, respectively, in trials A, B and C, separated grapevine responses to *P. chlamydospora* treatment and to mock inoculation (A and B: negative *vs* positive coordinates; C: positive vs negative coordinates). In trials A and C, *P. oligandrum* treatment and mock inoculation were also separated by Dim2 (A: positive *vs* negative coordinates; C: negative *vs* positive coordinates).

In order to characterize the effect of each treatment on grapevine defense responses, correlation circles were studied (Fig. 4) for the different trials, A, B and C. Only well represented genes were analyzed. In trials A and B, the studied genes were separated by Dim1 into two groups (negative *vs* positive coordinates) (Fig. 4a and b). The first group was composed of 5 genes: *Chs*, *Ban* and *Ldox* (phenylpropanoid pathway), *Chorm* (anthranilate pathway) and *Per* (wall thickness enhancement). Expression of these genes were more associated with grapevine response to mock inoculation. The second group of 11 genes belonged to different families involved in grapevine defenses: *Pr1*, *Pr10*, *Chit3*, *Pin* and *Glu* (PR Proteins family), *Sts*, *Pal* and *Ants*

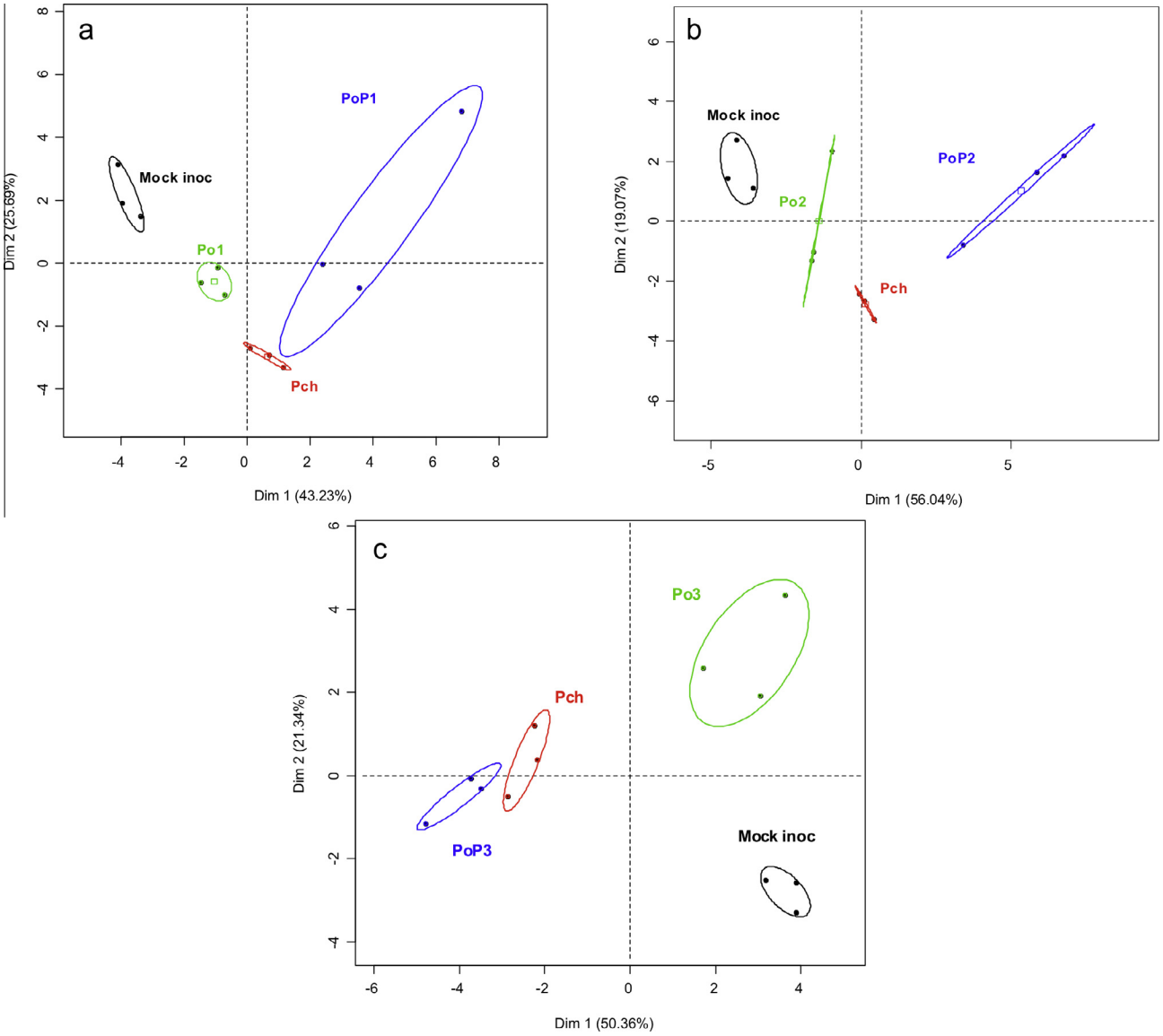

Fig. 3. Principal component analysis of specific plant responses (expression levels of 22 genes involved in plant defences) 14 days after pathogen infection at the trunk level: mock inoculation (black), *P. oligandrum* treatment (green), *P. chlamydospora* infection (red) and *P. oligandrum* + *P. chlamydospora* (blue). Ellipsoids represent the center of factors with 95% confidence. Po1, Po2, and Po3 correspond to the 3 inocula of *P. oligandrum* (cf. Table 1), PoP1, PoP2 and PoP3: Po1 + *P. chlamydospora*, Po2 + *P. chlamydospora* and Po3 + *P. chlamydospora*. Pch: *P. chlamydospora*. Mock inoc: mock inoculated. a, b and c for, respectively, trials A, B and C. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(phenylpropanoid pathway), *Lox* (oxylipin pathway) and *Gst* (oxido-reduction system). Their expressions were associated with grapevine responses to *P. chlamydospora* infection and *P. oligandrum* + *P. chlamydospora* treatment. According to the results obtained in Fig. 3a and b, the expression levels of this group of genes were higher in plants inoculated by the two microorganisms than in plants infected exclusively by the pathogenic agent.

For trial C, three groups of genes were differentiated (Fig. 4c). The first group contained genes encoding PR proteins (*Pgip*, *Chit3* and *Chit4*) and a gene involved in anthranilate pathway (*Ants*). The expression of these 4 genes was more associated with plant response to *P. oligandrum* treatment. Regarding plant response to mock inoculation, the expression of 8 genes was associated with: *Chi*, *Chs*, *Ldox* and *Ban* (phenylpropanoid pathway), *Chorm* (anthranilate pathway), *Pr1* and *Pr10* (PR Proteins) and *Cagt* (enhancing wall thickness). The genes of the third group had an expression associated with plant response to *P. chlamydospora* and *P. oligandrum* + *P. chlamydospora*. This group was composed of 4 genes: *Pal*, *Sts* (phenylpropanoid pathway), *Glu* (PR proteins) and *Gst* (oxido-reduction system).

As shown in Table 3, in all trials, six genes were more induced in plants inoculated with *P. oligandrum* and infected with *P. chlamydospora* than in those infected with *P. chlamydospora* alone (except the *Pal* gene in trial C).

Five genes were up-regulated in presence of *P. oligandrum* in all trials (except for the *Pal* gene in trial C). Two of these genes belong to the PR protein family (*Pr10* and *Glu*); another two genes, *Gst* and *Lox*, are involved, respectively, in oxydo-reduction system and oxylipin pathway. The fifth gene (*Pal*) is involved in phenylpropanoid pathways.

One gene, *Chit3* (PR protein family), was less repressed in plants inoculated with the two microorganisms than in those infected with the pathogen.

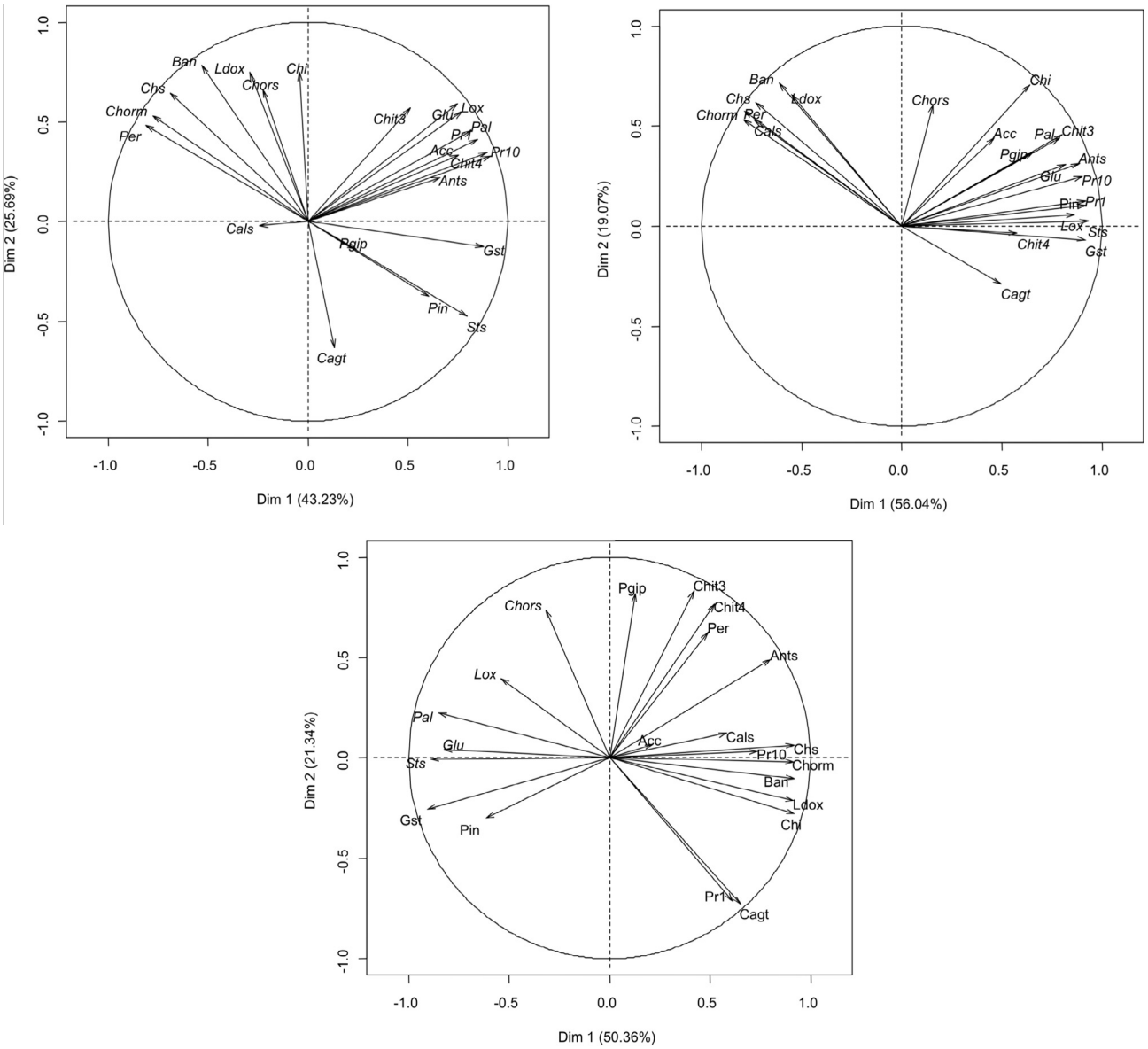

Fig. 4. Distribution into correlation circles of expression levels of 22 genes involved in plant defences 14 days after infection with the pathogen. a, b and c for, respectively, trials A, B and C. Pr1: PR protein 1, Pr10: PR protein 10, Chit3: chitinase class III, Chit4: chitinase class IV, Pgip: polygalacturonase inhibitor protein, Glu: β-1,3 glucanase, Pin: serine protease inhibitor, Pal: phenylalanine ammonia lyase, Sts: stilbene synthase, Chi: chalcone isomerase, Chs: chalcone synthase, Ldox: leucoanthocyanidin dioxygenase, Ban: anthocyanidine reductase, Ants: antranilate synthase, Chorm: chorismate mutase, Chors: chorismate sythase, Cals: callose synthase, Per: peroxidase, Cagt: coniferyl alcohol glucosyl transferase, Lox: lipoxygenase 9, Gst: glutathione S-transferase, Acc: 1-aminocyclopropane, 1-carboxylic acid oxidase.

4. Discussion

One well-described mode of action of *P. oligandrum* or of its elicitors (oligandrin, cell wall proteins) is their ability to stimulate plant system defenses (Mohamed et al., 2007; Takenaka et al., 2006; Takenaka and Tamagake, 2009). Oligandrin production could therefore be an important criterion for selecting strains of this oomycete. In our study, all 6 strains used to protect grapevine against *P. chlamydospora*, a pathogen involved in Esca, produced oligandrin. The quantities of oligandrin were relatively higher than those obtained by Picard et al. (2000). This result is in agreement with Gerbore et al. (2014) who showed that all these strains possess one gene encoding oligandrin, which suggests that production of oligandrin is a common trait among the *P. oligandrum* strains. In addition, each strain had also one gene encoding cell wall proteins, but these molecules are not secreted like oligandrin, they are located in the cell wall of *P. oligandrum*. Taken together, all this information and data on *P. oligandrum* indicates that all these strains have a great potential to induce resistance in plants.

In order to protect grapevine against infection by *P. chlamydospora*, it was shown that, for the 3 trials, *P. oligandrum* was always isolated in the rhizosphere during the 4-month experimental period. The same type of result was obtained by Le Floch et al. (2003) when they detected this oomycete on the roots of tomato grown in soilless culture 3 months after inoculation, using the same plate-counting method. They indicated that the highest level of *P. oligandrum* root colonization was detected 4 weeks after inoculation. In our study, the highest level of colonization was detected 21 days after *P. oligandrum* was inoculated on the grapevine roots, whatever the inoculum used. Surprisingly, comparison between plants treated with *P. oligandrum* only and those treated with both the oomycete and the pathogen, i.e. *P. oligandrum* and *P. chlamy-*

Table 3
Expression levels (Fold Changes) of 6 selected genes that were either more induced, or less repressed, in plants inoculated with *P. oligandrum* + *P. chlamydospora* than in those infected only with *P. chlamydospora*. The values reported are means of 3 Fold Changes (FCs). * Indicates means that are, within a trial, significantly different between the two treatments at $P < 0.05$ (Kurskal–Wallis test). Pch: *P. chlamydospora*; PoP1, PoP2 and PoP3: *P. oligandrum*1 + *P. chlamydospora*, *P. oligandrum*2 + *P. chlamydospora* and *P. oligandrum*3 + *P. chlamydospora*. *Pr10*: PR protein 10, *Chit3*: Chitinase class III, *Glu*: β-1,3 glucanase, *Pal*: Phenylalanine ammonia lyase, *Lox*: Lipoxygenase 9, *Gst*: Glutathione S-transferase.

	Trial A		Trial B		Trial C	
Genes	Pch	PoP1	Pch	PoP2	Pch	PoP3
Pr10	3.08	8.11*	3.08	18.23*	0.68	1.23*
Gst	1.32	2.10	1.32	2.26*	2.16	2.54
Glu	0.70	20.56*	0.70	4.69*	1.39	1.62
Lox	0.97	4.85*	0.97	1.50*	0.91	1.03*
Pal	0.41	0.81*	0.41	1.23*	2.05	1.43*
Chit3	0.10	0.22*	0.10	0.49*	0.62	1.07*

Scale 0.3 0.7 1 2 3

dospora, showed that *P. oligandrum* root colonization was higher in grapevine infected with *P. chlamydospora*. This trend was accentuated at the end of the experiment (4 months after inoculation). In order to explain this result, it could be hypothesized that the pathogen induced change in the plant physiology. This led to modifications in the emission and/or composition of root exudates which, in turn, had a positive influence on *P. oligandrum* colonization. Jones et al. (2004) suggested that the quantity and quality of root exudates both depend on the plant species, the age of individual plants and external biotic and abiotic factors.

Overall, the best level of grapevine root colonization by *P. oligandrum* was reached with the third inoculum (Po3), composed of the strains isolated from the rhizosphere of grapevine from the Bordeaux region (Gerbore et al., 2014). For the other two inocula evaluated (Po1 and Po2), at least one strain was obtained from the CBS collection. As hypothesized by Gerbore et al. (2014), vineyard strains may be more adapted to grapevine roots, thereby increasing the level of root colonization.

After investigating the colonization and persistence of *P. oligandrum* in the rhizosphere, the ability of the different oomycete inoculum (Po1, Po2, and Po3) to protect vine against *P. chlamydospora* was assessed. Whatever the *P. oligandrum*-inoculum used, the wood necroses of Cabernet Sauvignon cuttings caused by *P. chlamydospora* were significantly reduced when the oomycete strains colonized the root systems of young vines. To the best of our knowledge, this study shows, for the first time, the ability of *P. oligandrum* to protect vine against a fungus, *P. chlamydospora*, involved in grapevine trunk diseases. An earlier study by Mohamed et al. (2007), showed that *P. oligandrum*, by inducing plant defenses, protected vine against *B. cinerea* leaf infection.

In order to determine if *P. oligandrum* induces plant defense systems in our experiments, a set of 22 genes involved in grapevine defense mechanisms was used (Dufour, 2011; Dufour et al., 2013). PCA analyses indicated that, in all 3 trials, grapevine molecular responses to the different treatments were significantly differentiated. Moreover, plant responses to *P. oligandrum* treatment and mock inoculation were always separated from plant responses to *P. chlamydospora* infection and *P. oligandrum* + *P. chlamydospora* treatments.

Consequently, this difference in specific grapevine responses between treatments could be attributed to *P. chlamydospora* effect. Previous studies have shown that grapevine infection by *P. chlamydospora* induces plant defenses (Lorena et al., 2001; Bruno and Sparapano, 2006; Martin et al., 2009; Marta et al., 2011; Lambert et al., 2013). Martin et al. (2009) also pointed out that infection of young *V. vinifera* plants (cvs. Chardonnay, Touriga National and two clones of cv. Aragonez) with *P. chlamydospora* induced changes in phenolic compounds in wood tissues, i.e. increased accumulations of trans-resveratrol and ε-viniferin after infection with the pathogen.

In our experiment, certain genes were expressed when *P. chlamydospora* infection occurred. For example, the *Gst* gene involved in the oxydo-reduction system was activated after infection with *P. chlamydospora*. This result agrees with that obtained by Valtaud et al. (2009). They showed that the expression of *Gsts*, the extent of glutathione accumulation and the ratio of glutathione disulfide (GSSG) to total glutathione are early indicators of the presence of Esca disease in grapevine canes.

In all 3 trials, 6 genes were more expressed in plants inoculated with *P. oligandrum* + *P. chlamydospora* than in those infected only with *P. chlamydospora*. Accordingly, plant response to the pathogen attack is stronger in the presence of *P. oligandrum*. The oomycete triggered increases in certain PR proteins (*Pr10*, *Chit3* and *Glu*), secondary metabolite (*Pal*), *Gst* and *Lox* transcript levels, when the pathogen attack occurred.

Consequently, it can be assumed that *P. oligandrum* promotes a particular physiological condition called priming, in which the plant is able to mobilize its defense reactions more intensely in response to *P. chlamydospora* attack.

In our experiments, 3 different inocula of *P. oligandrum* were used. However, similar *P. oligandrum* root colonization and reduction of *P. chlamydospora* necroses were obtained for all 3 trials. Moreover, we could identify six genes that were more specifically expressed in plants inoculated by the two microorganisms than in those infected by the pathogen only. Regarding genetic characteristic of *P. oligandrum* strains, few differences were observed for the oligandrin and cell wall protein genes (Gerbore et al., 2014). Consequently, these similarities in elicitor genes between the different strains and inocula may explain that results obtained in the different trials were relatively similar.

In conclusion, it has been shown that various inocula of *P. oligandrum* are able to protect young vines against *P. chlamydopsora*, a pathogen involved in Esca. This study confirms the usefulness of *P. oligandrum* strains in inducing resistance and also that this is a common trait of many *P. oligandrum* strains. In each trial, specific grapevine responses were obtained according to the treatment applied to the vines, i.e. mock inoculation, *P. oligandrum* treatment, *P. chlamydospora* infection, *P. oligandrum* + *P. chlamydospora* treat-

ment. Certain genes associated with *P. chlamydospora* infection were more induced when plants were pre-treated with *P. oligandrum*. They will be used as markers of plant resistance against this trunk pathogen in the vineyards in further experiments.

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