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Pythium oligandrum induces grapevine defence mechanisms against the trunk pathogen *Neofusicoccum parvum*

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Summary. Grapevine trunk diseases (GTDs) are increasing in vineyards in many grape production regions. Among the pathogens causing these diseases, *Neofusicoccum parvum*, is one of the most frequent and virulent. To control GTDs, biocontrol is being developed using plant beneficial microorganisms. Strains of the oomycete *Pythium oligandrum* have been shown to naturally colonize grapevine roots in vineyards in several countries in Europe. This study examined the ability of the root-coloniser *P. oligandrum* to induce grapevine resistance against *N. parvum*, by deciphering the gene expression changes in a set of 62 genes involved in different grapevine defence pathways. Two greenhouse assays showed that the wood necrosis of vine cuttings caused by *N. parvum* was reduced by 65% when *P. oligandrum* colonized root systems of the plants. The relative expression levels of selected genes in the host trunks were studied by real-time PCR. Plant responses were assessed after inoculation by *P. oligandrum* and/or *N. parvum*, at three different sampling time points (0, 14 and 150 d after *N. parvum* inoculation). Sampling time influenced gene expressions for the different inoculation treatments. At each sampling time, specific host responses to the different treatments were also detected, for controls, and for inoculations with *P. oligandrum*, *N. parvum* or *P. oligandrum* + *N. parvum*. When *P. oligandrum* colonized grapevine root systems, inoculation with the pathogen was associated with increased up-regulation and over-expression of particular genes, including those regulating Pathogen-Related proteins, cell wall reinforcement proteins and hormone signalling pathways. A priming effect of the grapevine defence system was induced in roots colonized by *P. oligandrum*.

Keywords. Biocontrol, induced resistance, gene expression.

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INTRODUCTION

Grapevine Trunk Diseases (GTDs), mainly Esca, have become major concerns for the wine industry. GTDs have deleterious effects on vineyards,

associated with decreased harvest quality and quantity (Lorrain *et al.,* 2012; Bertsch *et al*., 2013; Gramaje *et al*., 2018; Mondello *et al.,* 2018). GTDs affect the wood of grapevines, in trunks, cordons and rootstocks. They complex pathosystems for research, mainly due to the long periods before wood necroses develop and leaf symptoms appear (Maher *et al.,* 2012). The three main GTDs are Esca, Botryosphaeria dieback and Eutypa dieback. In France, from 2012 to 2017, the proportion of unproductive vineyard area was approx. 12% due to these diseases (Doublet and Grosman, 2018). The resulting production losses in France were estimated to be worth approx. €1 billion (Lorch, 2014).

Botryosphaeria dothidea, *Diplodia seriata*, *Neofusicoccum parvum, N. australe*, *N. luteum* and *Lasiodiplodia theobromae* are among the most important pathogens associated with *Botryosphaeria* dieback (Úrbez-Torres and Gubler, 2011; Billones-Baaijens and Savocchia, 2019). These fungi are cosmopolitan and polyphagous. They have been isolated from different plant species and can cause large amounts of decay in host plants (Slippers and Wingfield, 2007). On grapevine, wood symptoms of the disease consist of sectoral and longitudinal brown wood streaking, and perennial cankers (Lecomte *et al.,* 2012). *Botryosphaeriaceae* species are known for differences in pathogenicity. *Neofusicoccum parvum* is one of the most virulent species, as shown by the extent of necroses it causes (Laveau *et al.,* 2009; Pitt *et al.,* 2013; Chen *et al.,* 2014; Bellée *et al.,* 2017).

Given the absence of chemical treatments against GTDs, some assessments of potential naturally-occurring biocontrol microorganisms have been carried out in nurseries and vineyards. Some studies have shown the ability of microorganisms to reduce infections caused by *Botryosphaeraceae* species. Bacteria, including *Bacillus subtilis* strains, *Pantoea agglomerans*, and *Enterobacter* sp., reduced necroses caused by *N. parvum* or *L. theobromae* (Kotze *et al.,* 2011; Haidar *et al.,* 2016a; Rezgui *et al.,* 2016). Additionally, the potential of *B. subtilis*, *B. pumilus*, *Paenibacillus sp*. and some actinobacteria to inhibit *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum* infections has also been demonstrated *in vitro* and *in planta* (Alfonzo *et al.,* 2009; Kotze *et al.,* 2011; Compant *et al*., 2013; Haidar *et al*., 2016b; Alvarez-Pérez *et al.,* 2017).

Trichoderma spp. have also been assessed as potential biocontrol agents against GTD pathogens (Fourie *et al*., 2001; Di Marco *et al.,* 2005; Kotze *et al.,* 2011; Mutawila *et al.,* 2011, 2015, 2016; Pertot *et al.,* 2016). Many formulations based on *Trichoderma* spp. strains, including Esquive WP®, Remedier®, Trichoflow-T®, Trichodex® and Vintec®, have been assessed for protection of grapevines against Esca. In France, two biofungicides based on different strains of *T. atroviride*, Esquive® WP (I-1237 strain) and Vintec® (TASCA strain), have been registered to control this disease. Mounier *et al*. (2016) applied Esquive® WP during 2 years on pruning wounds of mature grapevines, and these treatments reduced the expression of foliar symptoms of Esca by 50% and reduced plant mortality. The ability of certain other fungi such as: *Aureobasidium pullulans, Epicoccum layuense, Fusarium lateritium*, or *Cladosporium herbarum* to protect grapevine pruning wounds (John *et al.,* 2005; Rolshausen and Gubler, 2005), and to reduce necrosis in GTD pathogen-infected cuttings, has been shown (Gramaje *et al.,* 2018; Pinto *et al.,* 2018; Del Frari *et al.,* 2019). Most of these studies were made by directly applying fungi, bacteria or their metabolites onto pruning wounds. However, recent studies have shown that particular microorganisms, applied to host roots, can induce grapevine defences against GTD pathogens (Haidar et *al.,* 2016b; Yacoub *et al*., 2016; Daraignes *et al*., 2018; Trotel-Aziz et *al.,* 2019).

One of these microorganisms is the oomycete *Pythium oligandrum,* which is known as a biocontrol agent against many plant pathogens (Rey *et al*., 2008; Benhamou *et al*., 2012; Gerbore *et al*., 2014a). *Pythium oligandrum* strains produce different types of elicitor molecules (oligandrin and cell wall proteins) which induce plant resistance (Gerbore *et al*., 2014a). This oomycete naturally colonizes grapevine roots, and Gerbore *et al.* (2014b) showed that the isolated strains produced high amounts of oligandrin. A previous study showed that grapevine root treatments with *P. oligandrum* reduced by half the size of necroses caused by *P. chlamydospora* on young vines (Yacoub *et al.,* 2016). Moreover, Daraignes *et al.* (2018) observed that necroses caused by *N. parvum* and *P. chlamydospora* on young grafted grapevines were reduced, following plant treatment with *P. oligandrum*, either alone or in combination with beneficial bacteria.

The aim of the present study was to understand the mechanisms contributing to the biocontrol effects of the root biocontrol agent *P. oligandrum* against the grapevine trunk pathogen *N. parvum*. The ability of *P. oligandrum* to enhance grapevine resistance against *N. parvum* infection and induce the expression of genes involved in different pathways of plant defences, were investigated. High-throughput gene expression quantification was measured by microfluidic dynamic array (Fluidigm) technology as defined by Dufour *et al.* (2016). The genes analyzed were associated with Pathogen-Related (PR) proteins (18); secondary metabolite biosynthesis (13); cell wall reinforcement (11); indole pathway (5); Redox status regulation (3); oxylipin pathway (3); and hormone signalling pathway (9).

MATERIALS AND METHODS

Plant material

Two similar independent experiments were carried out in 2017 and 2018. Each experiment was conducted with rooted 'Cabernet Sauvignon' clone 191 (*Vitis vinifera* L.) cuttings, provided from the INRAE experimental vineyards near Bordeaux, France. The cuttings were grown for 2 months before inoculation, and grown under controlled conditions, as described by Laveau *et al.* (2009).

Oomycete and fungus inoculations

Inoculum of *P. oligandrum* strain Po37 was prepared by Biovitis SA Company (Saint Etienne Chomeil, France). At the five to six leaf stage, 50 mL of *P. oligandrum* inoculum was applied at the collar level of each plant. The inoculum concentration was adjusted to 3×10^4 mL^{-1}. Seven days after root inoculation with *P. oligandrum*, selected plants were inoculated with *N. parvum* strain COU02 (GenBank accession number is KT306957; INRAE-UMR SAVE collection, Bordeaux, France). This strain was originally obtained in 2008 from a 'Cabernet Sauvignon' cultivar Bordeaux, and was characterized as highly aggressive (Laveau *et al.,* 2009). The strain was subcultured on malt agar (MA) (15 g L^{-1} of malt (Biokar Diagnostics), 20 g L^{-1} agar (Setaxam®), and was incubated at 22°C (12 h light/12 h dark) for 1 week before plant inoculation.

The pathogen inoculations were carried out as described by Laveau *et al.* (2009). Briefly, each grapevine cutting was drilled at stem level, and then inoculated with 4 mm diam. agar plug from a *N. parvum* culture. The inoculation sites were then each covered with a protective film (Cellofrais®) to prevent external contamination.

Experimental layout

For each experiment, 250 plants were used and distributed between five treatments. The experimental design a randomized complete block with 50 plants per treatment. The different treatments were: (i) plants inoculated at root level with *P. oligandrum*; (ii) plants inoculated only with *N. parvum* at trunk level; (iii) plants inoculated on roots with *P. oligandrum* and then, 1 week later, with *N. parvum* at trunk level; (iv) plants not inoculated with microorganisms (experimental control); and (v) plants inoculated with sterile agar plugs (mock control).

Plant tissue samplings and evaluation of wood necrosis

Wood samples were collected at the end of each experiment (five months after the pathogen inoculations). For each treatment, 30 plants were collected, the stem of each plant was cut longitudinally and the length of wood necrosis was measured. The extent of necrosis was determined by calculating the ratio between the length of necrosis and the total length of the stem. Analysis of Variance (ANOVA, at $P \leq 0.05$) followed by pairwise comparisons of means using Tukey's *post hoc* test was performed to assess differences between treatments, using software R.3.1.2.

For the gene expression investigation, samples from the first experiment (2017) were analysed. Wood samples (1 cm above and 1 cm below the inoculation site on each plant) were collected at three time intervals after treatments were applied: 0 (five h), 14 or 150 d after pathogen inoculation (dpi). All samples were immediately frozen in liquid nitrogen, and stored at -80°C for gene expression analyses.

RNA extraction and cDNA preparation

For each sampling interval, the wood parts of six plants per treatment were sampled. The six samples were randomly grouped to obtain three biological repetitions. After crushing in liquid nitrogen, 100 mg of powder per sample was weighed into a 1.5 mL capacity tube pre-chilled with liquid nitrogen. Then, 1 mL of an extraction buffer (300 mM Tris HCl, pH 8.0; 25 mM EDTA; 2 mM NaCl; 2% CTAB; 2% polyvinylpolypyrrolidone; 0.05% spermidine trihydrochloride; and 2% β-mercaptoethanol, added extemporaneously), preheated to 56°C, was added to the wood powder. The mixture was stirred vigorously and incubated in a water bath at 56°C for 10 min under regular stirring. An equal volume of chloroform/isoamyl alcohol (24:1, v/v) was added, and the solution was centrifuged at 3500g for 15 min. The following steps were conducted, with RNA extracted with a MagMAX™-96 Total RNA Isolation Kit (ThermoFisher Scientific) following the manufacturer's instructions. RNA concentrations were determined with a Qubit3 fluorimeter (Invitrogen). The total amount of RNA obtained from each sample was reverse-transcribed using 2 µM oligo-$d(T)_{15}$, ribonuclease inhibitor and M-MLV reverse transcriptase (Invitrogen), following the manufacturer's instructions.

High-throughput gene expression quantification was carried out using microfluidic dynamic array (Fluidigm Corporation, California, USA) technology. The relative gene expression of 67 of the "NeoViGen96" chip was

quantified. The primers used were designed by Dufour *et al.* (2016). Among these genes, 62 are involved in different grapevine pathway defences (Figure 1 and Table S1), and five (*EF1γ*, *GAPDH*, *TIP41*, *TUA*, and *THIORYLS8*) are used as housekeeping genes.

Before analyses using qPCR Fluidogm technology, cDNA were preamplified as described by Dufour *et al.* (2016). Briefly, a reaction mixture containing all the pairs of primers (primers pool, 50 mM) and the TaqMan PreAmp Master Mix (1:2, Applied Biosystems) was added to cDNAs. The preamplification programme was as follows: 14 cycles of 95°C for 15 s followed by 60°C for 4 min. The cDNAs were then used for qPCR analyses in a reaction mixture containing TaqMan Gene Expression Master Mix (Applied Biosystems), DNA Binding Dye Sample Loading Reagent (Fluidigm Corporation, California, USA), and EvaGreen (Interchim). The preamplified cDNAs were stored at -20°C before being sent, in dry ice, to the GeT platform (Toulouse, France) for subsequent qPCR analyses. Real-time qPCR was carried out using a BioMark HD system (Fluidigm Corporation, California, USA). The 96.96 dynamic array was used for qPCR, following the manufacturer's protocol (http://www.fluidigm.com/ user-documents).

Data analyses

The fold changes (FCs) of gene expression were calculated using the 2 -ΔΔCT method (Vandesompele *et al.*, 2002), based on multiple gene normalization. The geometric mean of the five reference genes was used as a normalization factor. For each treatment, ER Relative expression gene level was calculated according to the corresponding control (control for *P. oligandrum* treatment, and the mock control for *N. parvum* and *P. oligandrum* + *N. parvum* treatments). FC values between 0 and 1 indicated a low gene expression level in treated samples compared to control samples. In this case, FC were considered biologically significant when 0.5 × < FC or FC > 2 ×, as in Spagnolo *et al.* (2012). The FCs obtained were studied using Principal Component Analysis (PCA) to assess differ-

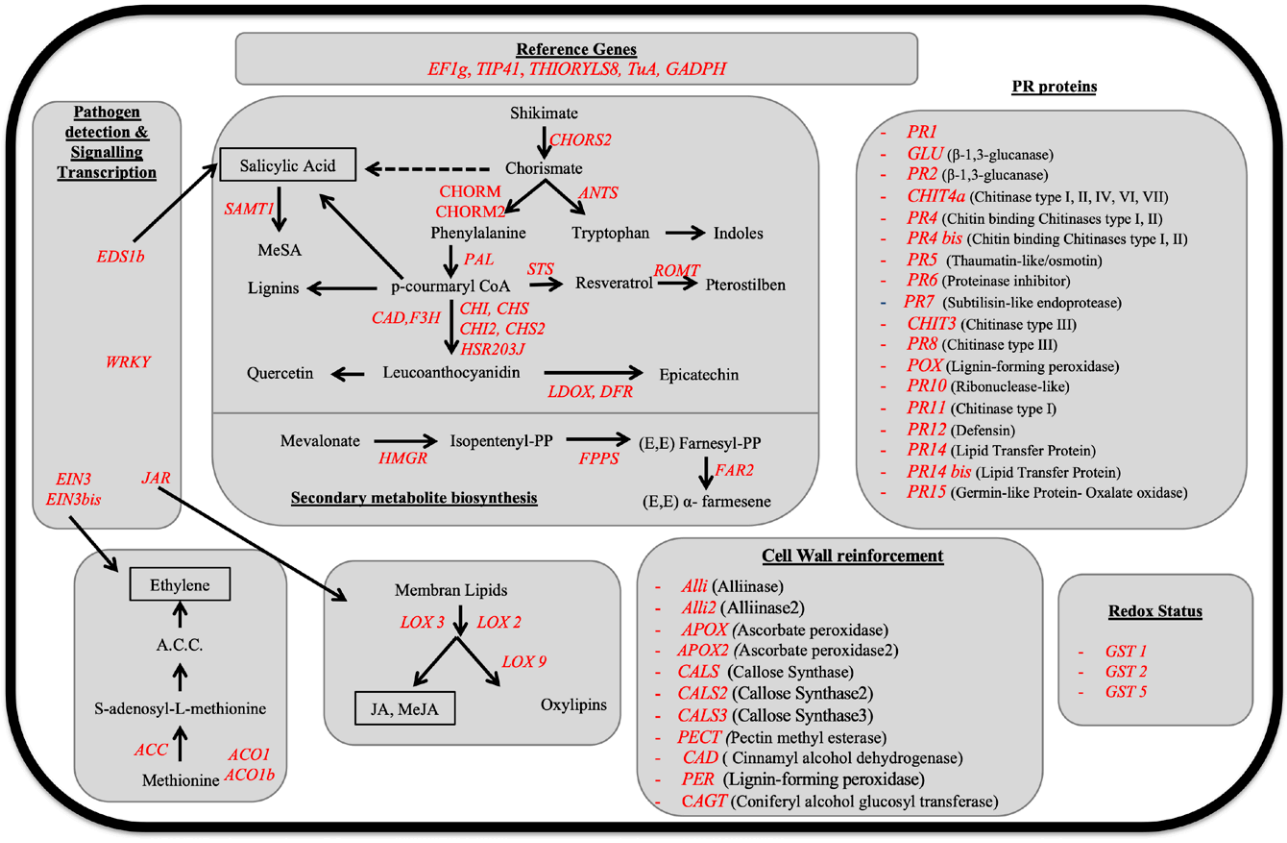

Figure 1. Sixty-two selected genes of "the NeoVigen96" chip are involved in different pathways of the grapevine defence system (Dufour *et al.*, 2016). Housekeeping (five genes), PR proteins (18), secondary metabolite biosynthesis (13), cell wall reinforcement (11), indole pathway (five), redox status regulation (three), oxylipin pathway (three) and hormone signalling pathway (nine genes).

ences between treatments. PCA was carried out using the RCMD package (version 2.6-2) and the plug-in FactoMiner (version 1.7) of R statistical software (version R 3.1.2). For each gene, differential gene expression was then subjected to statistical analysis, using nonparametric Kruskal Wallis tests (at $P \leq 0.05$), and statistically significant differences were determined compared to untreated controls.

To assess the evolution of gene expression, the FC ratios for each treatment were determined by calculating relative gene expressions, first between 0 and 14 dpi, then between 0 and 150 dpi (data not shown).

RESULTS

Assessment of wood necrosis caused by Neofusicoccum parvum *with or without* Pythium oligandrum

The ratios of necroses were measured in the grapevine wood at the end of the two experiments (150 dpi), to evaluate effects of *P. oligandrum* on the necrosis size caused by *N. parvum*. It should be noted that numerous previous studies showed that *N. parvum* strain 'COU02' is able to induce internal necroses in grapevine wood (Laveau *et al.,* 2009; Haidar *et al.,* 2016a; Daraignes *et al.,* 2018). Furthermore, *N. parvum* 'COU02' strain was re-isolated from different tissues sampled from infected plants, Laveau *et al.* (2009). Wood necroses were not observed in control or *P. oligandrum* inoculated plants.

Our results showed that plants inoculated by *N. parvum* at trunk level showed about 65% and 82% of necrosis ratios, in 2017 and 2018, respectively. However, for plants treated at root level with *P. oligandrum* before *N. parvum* inoculation, the ratios of necroses were significantly reduced; by 25% in 2017 and by 36% in 2018 (Figures 2A and B). Overall, the amounts of necrosis reduction were estimated to be 62% in 2017 and 56% in 2018, when the roots were inoculated with *P. oligandrum*. The results also showed that the inoculation method (drilling a hole at trunk level) induced necroses which were smaller than those induced by inoculations with *N. parvum*.

Grapevine trunk-specific responses detected from qPCR analyses

Effects of *Pythium oligandrum* and inoculation method on grapevine responses

In order to explore the effects of *P. oligandrum* root inoculation and the inoculation method on grapevine defences, expression levels of 62 grapevine defence genes were assessed (Figure 3). The gene expression levels in control plants were used as references. PCA was performed to evaluate effects of *P. oligandrum* and inoculation method (mock control) on transcriptomic grapevine responses, at three different sampling time points (0, 14 and 150 dpi) (Figures 3A and B). PCA eigenvalues indicated that the first two principal components explained 47.04% of total data variance. For each treatment, confidence ellipses revealed three statistically significant clusters corresponding to sampling time points, which were separated on Dim 1 (30.25% of the variability) for

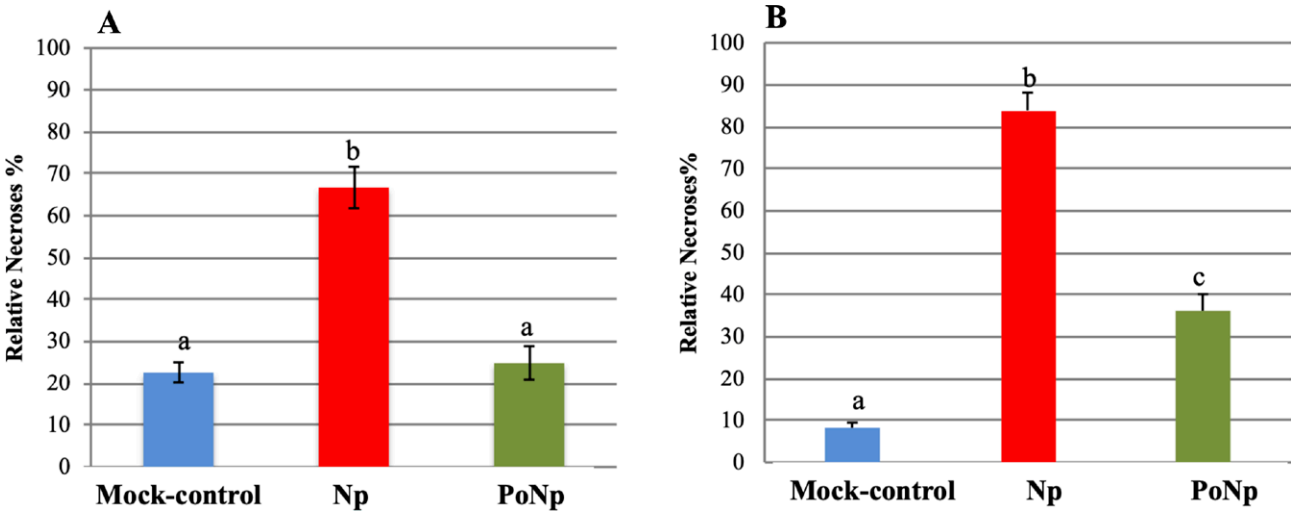

Figure 2. Wood necrosis resulting from inoculations with *Neofusicoccum parvum* in trunk cuttings with or without inoculation with *P. oligandrum* (Po) at root level, 150 days post-inoculation. The values reported are means (± SE) of 30 samples collected from each treatment. PoNp = Po + *N. parvum*, and Np = *N. parvum*. Different letters indicate differences ($P > 0.05$) between treatments.

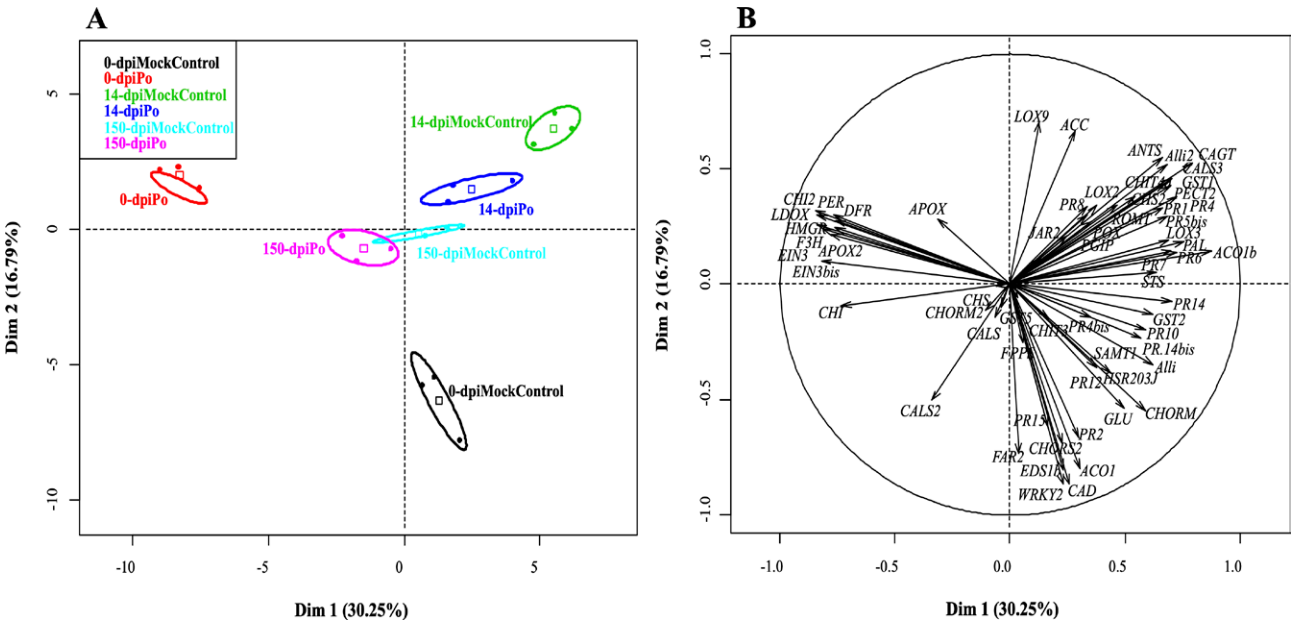

Figure 3. A. Principal component analysis of specific grapevine responses to *Pythium oligandrum* and mock-control treatments (relative expression levels of the 62 genes involved in plant defences) at 0, 14 or 150 d after treatments applied at trunk level. Gene expression of control plants was used as the reference to calculate relative expression. Ellipsoids represent the centres of factors with 95% confidence. The different groups are indicated by different colours. Po = *P. oligandrum*. B. Distribution into the correlation circles of the relative expression levels of the 62 genes studied.

P. oligandrum and Dim 2 (16.79% of the variability) for the mock-controls. Moreover, for each sampling time point, expression levels of the assessed genes were different according to the treatment, except at 150 dpi. Plant responses to *P. oligandrum* and mock-control treatments were greater at 0 dpi (5 hours after pathogen inoculation) than those assessed at 14 or 150 dpi.

In order to characterize the effects of *P. oligandrum* and mock-control treatments on grapevine defence responses, the corresponding correlation circles were examined (Figure 3B). Only well-represented genes are presented, and most of these genes correlated with grapevine responses to the mock-control treatment at 0 dpi. Eighteen genes involved in all the examined grapevine defence families (except the oxylipin family) were up-regulated following drilling of inoculation holes. Seven of these genes (*GLU*, *PR2*, *PR10*, *PR12*, *PR14*, *PR14bis* and *PR15*) belong to the PR protein family, , three genes (*HSR203J*, *CHORM* and *CHORS2*) are involved in the indole signalling pathway, and four genes (*EDS1*, *ACO1, SAMT1* and *WRKY2*) are involved in the hormone signalling pathway. Additionally, two (*CAD*, *Alli*) of the 18 genes induced after the hole drilling are in the cell wall reinforcement family, one (*FAR2*) is involved in phenylpropanoid synthesis and another (*GST2*) is involved in redox status regulation.. From the *P. oligandrum* treatment, grapevine responses were mostly associated with expression of six genes (*CHI2*, *CHI*, *LDOX*, *DFR*, *HMGR* and *F3H*) involved in secondary metabolite biosynthesis and three (*PER*, *APOX2* and *CHI*) involved in cell wall reinforcement, 7 days after the inoculation of *P. oligandrum* (0 dpi). The treatment with *P. oligandrum* is also correlated with up regulation of two genes (*EIN3* and *EIN3bis*) coding for a transcription factor acting as a positive regulator in the ethylene response pathway. At 14 and 150 dpi, grapevine responses to *P. oligandrum* and the mock-control were correlated with the same group of genes which are involved in different families of grapevine defences (Figure 3B).

Effects of *Neofusicoccum parvum* and *Pythium oligandrum* + *N. parvum* on grapevine responses

The biological system studied here included wounding in the pathogen infection process, and this induced strong plant responses. In order to subtract responses due to the inoculation method, and to evaluate effects of microorganism inoculations on grapevine responses, gene relative expression levels in *N. parvum* and *P. oligandrum* + *N. parvum* inoculated plants were calculated in relation to those measured in mock-control plants. The effects of *N. parvum* and *P. oligandrum* + *N. parvum* inoculation treatments on grapevine responses were

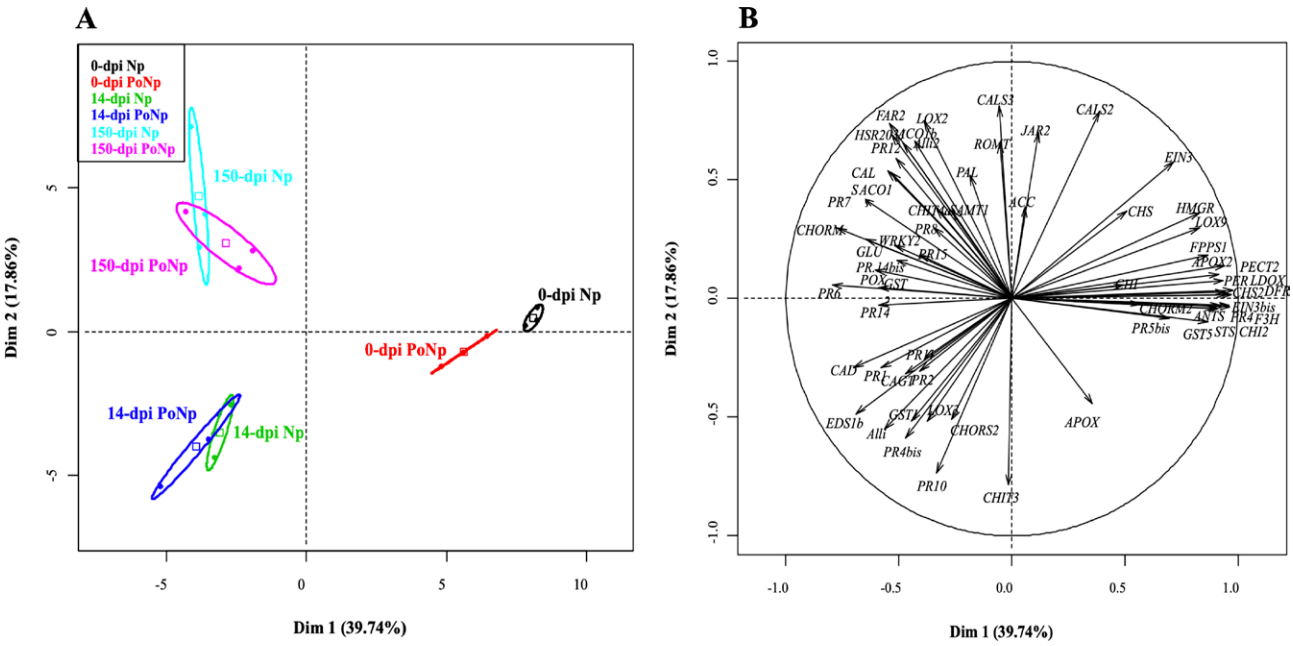

Figure 4. A. Principal component analysis of specific plant responses to *Neofusicoccum parvum* and *Pythium oligandrum* + *N. parvum* inoculation treatments (relative expression levels of the 62 genes involved in plant defences), at 0, 14 or 150 days after inoculations at trunk level.. Ellipsoids represent the centre of factors with 95% confidence. The different groups are indicated by different colours. Np = *N. parvum*, PoNp = *P. oligandrum* + *N. parvum*. B. Distribution into the correlation circles of the relative expression levels of the 62 genes studied.

then analysed with PCA, at 0,14 and 150 dpi (Figure 4A). PCA eigenvalues indicated that the first two principal components explained 57.6% of total data variance. Results showed that relative gene expression levels of the assessed genes involved in grapevine defences were more differentiated at the sampling time points than to the inoculation treatment factor. PCA showed that, at each sampling time point, the same genes were modulated following the *N. parvum* and *P. oligandrum* + *N. parvum* inoculation treatments. However, the amplitude of gene expressions differed according to the treatment.

The correlation circle corresponding to grapevine responses to *N. parvum* and *P. oligandrum* + *N. parvum* treatments showed that three different groups of genes were distinguished (Figure 4B). Each group of genes was associated with grapevine responses to the two inoculation treatments, at each sampling time point. The first group, correlated with grapevine responses at 0 dpi, included genes mostly involved in secondary metabolite biosynthesis (*CHS*, *HMGR*, *FPPS1*, *LDOX*, *CHS2*, *DFR*, *F3H*, *STS* and *CHI2*). However, grapevine responses to *N. parvum* and *P. oligandrum* + *N. parvum* treatments, at 14 or 150 dpi were more associated with the up-regulation of PR proteins.

Relative gene expression levels were separately compared between *N. parvum* and *P. oligandrum* + *N. parvum* treatments at each sampling time point, to focus on the effect of *P. oligandrum* on host responses to *N. parvum* inoculation (Figure 5). PCA eigenvalues indicated that the first principal components Dim1 and Dim2 explained 59.16% of the total data variance at 0 dpi, 63.11% at 14 dpi, and 51.35% at 150 dpi. Furthermore, at 0 dpi, Dim1, which represented 35.8% of total data variance, separated grapevine responses to *N. parvum* and *P. oligandrum* + *N. parvum* treatments (Figure 5A). At 14 dpi, host responses to the two inoculation treatments were separated by Dim 2, which represented 23.26 % of total data variance (Figure 5B). No statistically significant differences, were observed at 150 dpi, however, for responses between the two treatments (Figure 5C). Overall, at each sampling time point, *P. oligandrum* significantly modulated grapevine responses to *N. parvum* inoculation, except at the end of the experiment (150 dpi).

The distribution into a correlation circle of FC of the 62 genes involved in plant defences at 0 dpi, showed that two groups of genes were differentiated by Dim1 (Figure 5D). Most of the genes studied (approx. 70%) were associated with grapevine responses to infection by *N. parvum*. Only nine genes (*PR6*, *PR7*, *PR11*, *PR14bis*, *CHORS2*, *HSR203J*, *GST*, *CAGT* and *POX*) were associated with grapevine responses to the *P. oligandrum* + *N. parvum* inoculation treatment. However, at 14 dpi, after

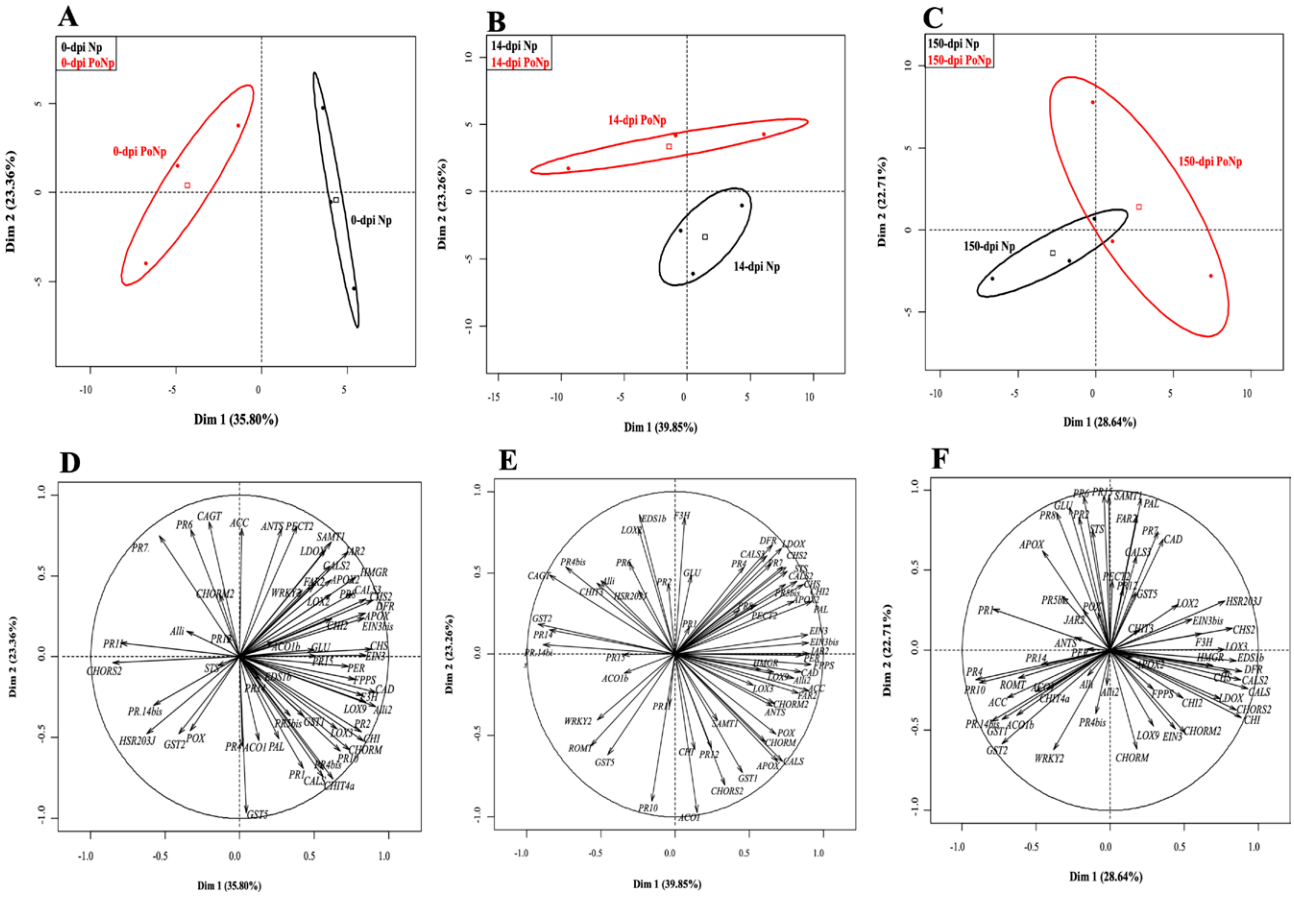

Figure 5. A, B and C. Principal component analyses of specific plant responses to *Neofusicoccum parvum* and *Pythium oligandrum* + *N. parvum* treatments (relative expression levels of the 62 genes involved in plant defences) at each trunk level sampling time (A for 0 days after pathogen inoculation (dpi), B for 14 dpi, and C for 150 dpi). Ellipsoids represent the centre of factors, with 95% confidence. The different groups are indicated by different colours. Np = *N. parvum*, PoNp = *P. oligandrum* + *N. parvum*. D, E and F. Distributions into correlation circles of the relative expression levels of the 62 genes studied (D for 0 dpi, E for 14 dpi and F 150 days dpi).

P. oligandrum inoculations to roots, more host genes (30) were associated with response to *N. parvum* inoculations than those in plants inoculated only with the pathogen (25 genes) (Figure 5E). At 150 dpi, the numbers of genes correlated with each treatment decreased (Figure 5F). Despite this decrease of over-expressed genes (32 genes), the results were similar to those observed at 14 dpi, with more correlated genes with responses to *P. oligandrum* + *N. parvum* inoculation (21 genes) than to *N. parvum* inoculation (11 genes).

In order to obtain more details about gene expression changes induced by *P. oligandrum* on host responses to *N. parvum* inoculation, relative expression levels of all the studied genes associated with *N. parvum* and *P. oligandrum* + *N. parvum* treatments was examined at the three different sampling time points (Figure 6). Overall, the host responses to *N. parvum* and *P. oligandrum* + *N. parvum* inoculation treatments were similar, at 0-dpi. For both treatments, all the assessed genes involved in phytoalexin biosynthesis were strongly up-regulated, except *FAR2* which was repressed by both treatments at 0 dpi. All the genes involved in the other assessed defence gene families were strongly repressed at 0 dpi, except for a few genes from each family. Overall, ten genes were significantly up-regulated by both treatments at 0 dpi. One of these genes, *PR4*, encoded PR proteins; four genes (*APOX2, PECT2, PER* and *CALS2*) are in the cell wall reinforcement family; two (*EIN3* and *EIN3bis*) are involved in the hormone signalling pathway; and *GST5*, *ANTS* and *LOX9* are involved, respectively, in redox status regulation, the indole pathway, and the oxylipin pathway. For all the over-expressed genes, grapevine responses to *N. parvum* inoculation were slightly reduced after *P. oligandrum* inoculation of the root system.

Fourteen days after *N. parvum* inoculation, particular genes, especially those encoding PR proteins includ-

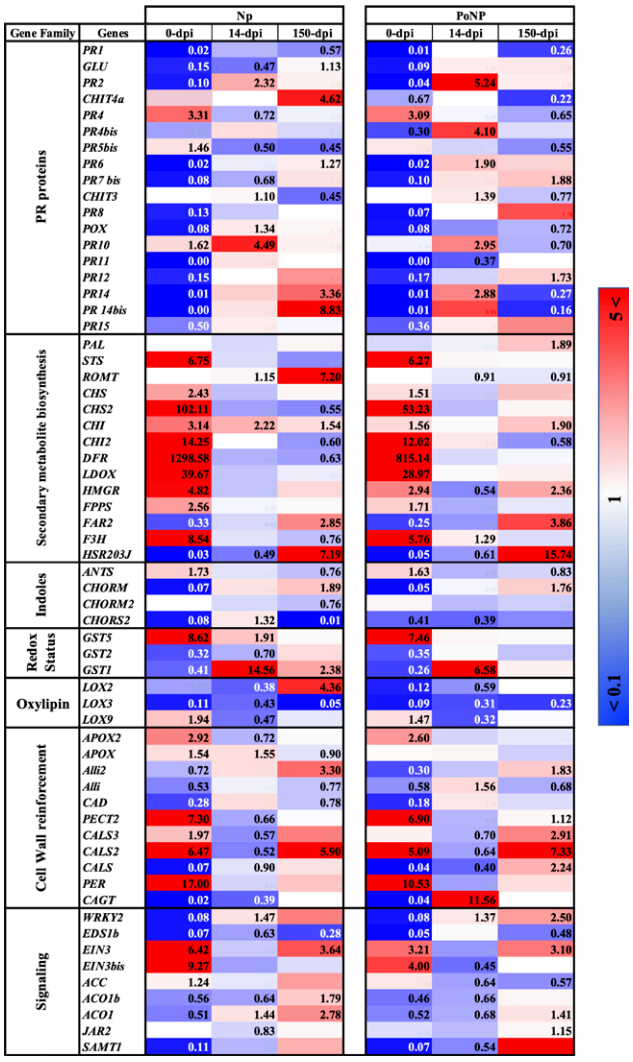

Figure 6. Relative expression levels of 62 defence genes in grapevine wood 0 ,14 or 150 days after inoculations. Gene expression of mock-control plants was used as reference to calculate the relative expression. Each column represents the time point after inoculation treatments (Np = *Neofusicoccum parvum* and PoNp = *Pythium oligandrum* + *N. parvum*), and each line corresponds to one gene, represented by a single row of boxes. The colour scale bars represent the ratio values corresponding to the mean of three independent samples. Up-regulated genes are shown in shades of red, with relative expression levels greater than 5 in bright red. Down-regulated genes are shown in shades of blue, with relative expression less than 0.1 in dark blue. Numbers in boxes represent the significant changes ($P < 0.05$; Kurskall-Wallis test) in gene expression compared to the mock-control. Np = *N. parvum*, PoNp = *P. oligandrum* + *N. parvum*.

ing *PR2*, *PR4bis, PR6*, *CHIT3*, *PR10* and *PR14*, were more highly expressed in plants inoculated with *P. oligandrum* + *N. parvum* than in those inoculated with *N. parvum* alone. One gene involved in the cell wall reinforcement pathway, i.e. *CAGT*, was strongly over-expressed after *P. oligandrum* inoculation of the roots.

At the end of the experiment, relative expression levels of most of the studied genes were similar for the two inoculation treatments, but some differences were detected. Three genes, *CALS2* involved in cell wall reinforcement, *FAR2* in phytoalexin synthesis and *HSR203J* in the indole pathway, were more up-regulated in plants inoculated with the two microorganisms than in those only inoculated with the pathogen. In contrast, eight genes, associated with PR proteins (*CHIT4*a, *PR14* and *PR14bis*), secondary metabolite synthesis (*ROMT*), Redox status (*GST1*), the oxylipin pathway (*LOX2*), cell wall reinforcement (*Alli2*) and hormone signalling (*ACO1*), were over-expressed in plants inoculated only with *N. parvum*.

To highlight effects of *P. oligandrum* on the evolution of grapevine relative gene expression levels, the ratios of relative gene expression were calculated, first between 0 and 14 dpi (Figure 7), and then between 0 and 150 dpi (data not shown). Eleven grapevine genes encoding PR proteins were considerably more over-expressed in plants inoculated with *P. oligandrum* and *N. parvum*, than in those only inoculated with *N. parvum*. In plants inoculated with *P. oligandrum*, the ratios of three PR protein genes (*GLU*, *PR2* and *PR4*) were approx. five times more expressed than in plants inoculated with *N. parvum*. The same trend occurred for three other genes, *LOX2* (oxylipin pathway), *CAGT* (cell wall reinforcement) and *EDS1b* (hormone signalling). However, after *P. oligandrum* inoculation of host roots, the *CAGT* gene was the most up-regulated between 0 dpi and 14 dpi after *N. parvum* inoculation. Expression of this gene was 19 times greater in plants pre-treated with *P. oligandrum* and then inoculated with *N. parvum* (ratio = 323.3) than in those only inoculated with *N. parvum* (ratio = 17.01).

DISCUSSION

Microorganisms have been used as Biocontrol Agents (BCAs) for control of GTDs. *Trichoderma* spp. and *B. subtilis* strains are known to have direct effects on GTD pathogens, through competition for nutriments, antibiosis and mycoparasitism. These organisms are frequently applied onto pruning wounds, or by dipping grapevine cuttings in the BCA solutions (Bertsch *et al.* 2013; Gramaje *et al.* 2018; Mondello *et al.*, 2018). In the present study, a root BCA, *P. oligandrum*, which naturally colonises grapevine roots (Gerbore *et al.*, 2014b), was studied to evaluate inoculation with this oomycete to induce grapevine responses against *N. parvum*. It is important

to underline that *P. oligandrum* is able to colonize grapevine plant roots during, at least, 4 months following its inoculation (Yacoub *et al.,* 2016; 2018).

The study has provided evidence that grapevine root treatment with *P. oligandrum* reduced wood necrosis (about 60%) resulting from *N. parvum* inoculation. This confirms the results obtained by Daraignes *et al.* (2018), who observed that wood necroses caused by *N. parvum* and *P. chlamydospora* were reduced following the application of *P. oligandrum*, either alone or in combination with particular beneficial bacteria, in young grafted grapevines. That study there was no contact between the root BCA and the trunk pathogen, so it was assumed that protection by *P. oligandrum* was due to the induction of the grapevine defence system, but this point was not investigated. Some previous studies have shown the capacity of root BCAs to induce systemic resistance against GTD pathogens. Yacoub *et al.* (2016) demonstrated that three different inocula of *P. oligandrum* applied to grapevine roots reduced wood necroses caused by *P. chlamydospora* in host trunks. Expression of 22 grapevine defence genes was differentiated for each combination in this tripartite interaction (i.e. control, *P. oligandrum*, *P. chlamydospora* and *P. oligandrum* + *P. chlamydospora* treatments). Trotel-Aziz *et al.* (2019) showed that grapevine root treatment with *B. subtilis* strain PTA-271 reduced, by 63–75%, cankers and stem lesions, caused by *N. parvum*, compared to non-bacteria pre-treated plants.

In the present study, following observation of reductions of wood necroses, and to decipher the grapevine responses in the plant/*P. oligandrum*/*N. parvum* interaction, high throughput gene expression quantification was carried out using microfluidic dynamic array (Fluidigm) technology. The relative expression levels of the 62 genes involved in grapevine defence mechanisms (Dufour *et al.,* 2016) were studied at 0,14 and 150 dpi, and effects of the inoculation method used (drilling holes in the plant stems) on host responses over time were evaluated.

Compared to plants inoculated only with *P. oligandrum* (not wounded), the inoculation method induced strong modulation of gene expression, especially a few hours (0 dpi) after plant wounding. The most modulated genes were mainly those affecting PR proteins (*PR2*, *PR10*, *PR12*, *PR14*, *PR14bis* and *PR15*) and those involved in the indole (*HSR203J*, *CHORM* and *CHORS2*) and hormone signalling pathways (*EDS1*, *ACO1, SAMT1* and *WRKY2*). PCA analyses indicated that grapevine molecular responses to mock-control and *P. oligandrum* inoculation were differentiated at 0 and 14 dpi, but not at 150 dpi. This indicated that the effects of the inoculation method on grapevine defences was transient. This conclusion is partly supported by the results of Pierron *et al.* (2016), who showed that plant internodes responded intensely to injuries 10 to 120 h following wounding. In the present study, six of the 11 selected genes (*PAL*, *PR10.3*, *TL*, *TLb*, *Vv17.3* and *STS*) were up-regulated, but expression of other genes, including *PIN*, was unaffected.

For responses to *P. oligandrum* inoculations 7 d after the oomycete inoculation (0 dpi), several genes involved in secondary metabolite biosynthesis, cell wall reinforcement and the ethylene response pathway were up-regulated. This result is similar to those obtained by Miotto-Vilanova *et al.* (2019), who showed that *Paraburkholderia phytofirmans* PsJN systemically induced overexpression of all genes implied in phenylpropanoid and flavonoid pathways. Activation of ethylene pathway genes after BCAs inoculation has also been demonstrated in previous studies (reviewed by Pieterse *et al.,* 2014).

As wounding caused significant host stress, a separate investigation of the grapevine trunk responses was required to evaluate BCA and pathogen effects on plant defences, so grapevine relative gene expression levels were calculated following microorganism inoculations. Mock-inoculated plants were used as references. Overall, sampling time point after inoculations had a major effect on relative gene expression levels, whatever the treatment. This result is similar to those in previous studies, showing that effects of GTD pathogens on the grapevine defence system, applied individually or in combinations with BCAs, differ according to periods post inoculation (Haidar *et al.,* 2016b; Pierron *et al.* 2016; Massonnet *et al.,* 2017; Mutawila *et al.,* 2017; Trotel-Aziz *et al.,* 2019; Zhang *et al.,* 2019). For effects of *N. parvum* and *P. oligandrum* + *N. parvum* treatments on grapevine gene expression, at each sampling time point, PCAs indicated that the relative expression levels of the 62 studied genes were different at 0 and 14 dpi. However, no significant differences were observed at the end of experiment (150 dpi). This indicates that *P. oligandrum* modulated grapevine responses only at early stages post inoculation.

After an initial analysis of grapevine responses to *N. parvum* and/or *P. oligandrum* inoculations, a specific analysis was carried out to evaluate the modulation, over time, of each gene expression after each treatment. Firstly, the heatmap analyses of relative gene expression levels indicated that a similar tendency was observed in plant responses to *N. parvum* and to *P. oligandrum* + *N. parvum* treatments, with strong repression of genes affecting PR proteins, and strong up-regulation of most of the genes involved in phenylpropanoid biosynthesis, 5 h after pathogen inoculation (0 dpi). These results are sim-

ilar to those of Yacoub *et al.* (2016), who reported that wood infections by *P. chlamydospora* induced repression of *PR10* and *GLU* genes affecting PR proteins, and over-expression of *PAL*, which embodies the phenylpropanoid pathway. Pierron *et al.* (2016) and Massonnet *et al.* (2017) also showed that *PAL* and *STS*, involved in Stilbene synthase, were up-regulated in grapevine trunks a few hours after GTD pathogen inoculations.

Fourteen d after pathogen inoculations, the trends observed at 0 dpi were reversed for *N. parvum* and *P. oligandrum* + *N. parvum* treatments. Most of the studied PR Protein genes were up-regulated, as were genes involved in secondary metabolite biosynthesis. This was similar to the results of Haidar *et al.* (2016b), who showed that *P. chlamydospora* infections in grapevine trunks gave over-expression of PR protein genes *PR10* and *CHIT3*, and repression of the gene encoding *PAL*, 2 weeks after pathogen inoculation.

To focus more on effects of *P. oligandrum* on grapevine responses to *N. parvum* infection, the evolution of the FC gene levels were calculated between 0 and 14 dpi (Figure 7). This showed that, after inoculations with *P. oligandrum* to plant roots, stronger over-expression of genes encoding PR proteins was detected than in plants inoculated with *N. parvum* alone. This confirmed the ability of *P. oligandrum* to induce amplification of the PR proteins genes *PR1*, *GLU*, *PR2*, *PR4bis*, *PR6*, *PR7*, *PR8*, *PR14* and *PR14bis*, which has also been observed in tomato leaves after infection with *Botrytis cinerea* (Le Floch *et al.,* 2003, 2009). In addition, plants pretreated with *P. oligandrum* and inoculated with *N. parvum* showed more rapid up-regulation of *LOX2* (oxylipin pathway) and *GST2* (glutathione-S-transferase genes) relative gene expression levels, than was measured in plants inoculated only *N. parvum*. This result is similar to those of Yacoub *et al.* (2016), who showed, in three experiments, that grapevine root treatment with *P. oligandrum* induced over-expression of *GST2* and *LOX2* in response to *P. chlamydospora* inoculation. Oxylipin pathway and glutathione-S-transferase genes are known to be induced in plants after elicitor application (Dufour *et al.,* 2013; Harel *et al.,* 2014; Bellée *et al.,* 2018).

For genes involved in cell wall reinforcement, *CAD* and *CAGT* were more induced in *P. oligandrum* + *N. parvum* inoculated plants than in those inoculated only with *N. parvum*. As the *CAD* and *CAGT* genes are involved in the lignin pathway, this result suggests that over-expression of these genes following *P. oligandrum* root colonisation enhanced the ability of the grapevine plants to reinforce cell walls via lignin accumulation.

Among the genes involved in pathogen detection signalling transcription, *P. oligandrum* induced

		FC 14-dpi/FC 0-dpi	
Gene Family	**Genes**	**Np**	**PoNp**
PR proteins	*PR1*	35.38	68.92
	GLU	3.22	14.10
	PR2	22.37	147.95
	CHIT4a	NA	NA
	PR4	0.22	0.31
	PR4bis	2.28	13.79
	PR5	0.34	0.62
	PR6	57.11	88.99
	PR7	8.01	14.01
	CHIT3	NA	NA
	PR8	6.30	15.33
	POX	16.51	7.06
	PR10	2.77	3.08
	PR11	1167.32	137.97
	PR12	6.52	4.81
	PR14	120.72	208.29
	PR 14bis	306.34	675.69
	PR15	2.56	3.52
Secondary metabolite biosynthesis	*PAL*	0.84	1.11
	STS	0.13	0.18
	ROMT	NA	NA
	CHS	0.31	0.53
	CHS2	0.01	0.01
	CHI	0.71	0.67
	CHI2	0.08	0.11
	DFR	0.00	0.00
	LDOX	0.02	0.04
	HMGR	0.16	0.18
	FPPS	0.38	0.40
	FAR2	2.61	2.40
	F3H	0.11	0.22
	HSR203J	16.89	11.67
Indoles	*ANTS*	0.51	0.43
	CHORM	19.99	19.16
	CHORM2	0.84	0.65
	CHORS2	16.43	0.97
Redox Status	*GST5*	0.22	0.15
	GST2	2.20	3.14
	GST1	35.95	24.87
Oxylipin	*LOX2*	0.59	4.71
	LOX3	3.91	3.30
	LOX9	0.24	0.22
Cell Wall reinforcement	*APOX2*	0.25	0.33
	APOX	1.01	1.10
	Alli2	2.05	2.61
	Alli	1.78	2.69
	CAD	5.41	7.36
	PECT2	0.09	0.10
	CALS3	0.29	0.58
	CALS2	0.08	0.13
	CALS	13.72	9.62
	PER	0.05	0.06
	CAGT	17.01	323.30
Signalling	*WRKY2*	17.74	16.94
	EDS1b	8.83	20.18
	EIN3	0.12	0.19
	EIN3bis	0.07	0.11
	ACC	0.73	0.46
	ACO1b	1.14	1.44
	ACO1	2.85	1.31
	JAR2	0.79	0.85
	SAMT1	6.80	7.52

Figure 7. Ratios of relative expression levels of the 62 defence genes in grapevine wood between 0 d post inoculation (dpi: 5 h after inoculations) and 14 dpi. Gene expression of mock-controls was used as the reference to calculate relative gene expression. Each column represents the time point after treatment (Np = *Neofusicoccum parvum* and PoNp = *Pythium oligandrum* + *N. parvum*), and each line corresponds to one gene represented by a single row of boxes. Color gradient from blank (low values) to dark green (high values) was used to indicate the magnitude of FC values.

genes involved in the Salicylic Acid (SA) pathway, with the SA-dependent (Enhanced Disease Suceptibility, *EDS1b*) gene, and that controlling SA-methyl transferase (*SAMT1).* This result indicates the involvement of the SA pathway in induction of systemic resistance. This result differs from those in previous studies, which have demonstrated the implication of Jasmonic Acid and Ethylene signalling pathways in other *P. oligandrum*/pathogen/plant interactions (Hase et *al.,* 2006, 2008; Wang *et al.,* 2011; Ouyang *et al.,* 2015). Other studies have also showed an over-expression of *EDS1* induced systemic host resistance against pathogens (Gao *et al.,* 2010; Tahir *et al.,* 2017; Fan *et al.,* 2018).

By comparing the evolution of relative gene expression levels between 0 and 14 dpi, the present study has shown that *P. oligandrum* induced strong over-expression of particular grapevine defence-related genes and activation of priming after pathogen inoculation. The oomycete root colonization induced enhanced expression of genes involved in three different categories. These were:

(i) PR proteins genes, including *PR1*, a marker of the SA pathway and antifungal activity, *GLU* and *PR2* encoding β-1,3-glucanase, *PR4bis* encoding chitinase and *PR14* involved in the defense signalling pathway;
(ii) cell wall reinforcement genes (*CAD* and *CAGT*); and
(iii) genes affecting the SA pathway (*SAMT1* and *EDS1*)

These genes were expressed in grapevine trunks, after *N. parvum* inoculations, indicating that priming was not restricted to JA signalling. A similar result was obtained by Song *et al.* (2015) in leaves of tomato plants colonized with *Funneliformis mosseae* upon *Alternaria solani* infections. The ability of BCAs to induce the 'priming state' has also been demonstrated in many host plants, and with different microorganisms (Perazzolli *et al.,* 2011; Spagnolo *et al.,* 2012, 2014; Gruau *et al.,* 2015; Magnin-Robert et *al.,* 2016). The present study has demonstrated that the root oomycete *P. oligandrum* also induced the priming state in the grapevine/*N. parvum* interaction.

The results of the present study provide evidence that grapevine root inoculation with the oomycete *P. oligandrum* reduced grapevine trunk necrosis caused by *N. parvum*. *Pythium oligandrum* induced plant systemic resistance as indicated by the strong priming of genes involved in the grapevine defence system. After the pathogen inoculations, the FC of genes involved PR proteins, redox status, oxylipin and SA signalling pathways were induced when plants were pre-treated with *P. oligandrum*. These genes could be used as markers of plant resistance, induced by *P. oligandrum* against *N. parvum* in future studies, involving experiments performed in the vineyards.

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