RESEARCH ARTICLE

Characterization of *Pythium oligandrum* populations that colonize the rhizosphere of vines from the Bordeaux region

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Abstract

This study focused on one oomycete, *Pythium oligandrum*, well-known for its plant protection abilities, which thrives in microbial environment where bacteria and fungal communities are also present. The genetic structures and dynamics of fungal and bacterial communities were studied in three Bordeaux subregions with various types of soil, using single-strand conformation polymorphism. The structure of the fungal communities colonizing the rhizosphere of vines planted in sandy–stony soils was markedly different from that those planted in silty and sandy soils; such differences were not observed for bacteria. In our 2-year experiment, the roots of all the vine samples were also colonized by echinulated oospore *Pythium* species, with *P. oligandrum* predominating. *Cytochrome oxidase I* and *tubulin* gene sequencings showed that *P. oligandrum* strains clustered into three groups. Based on *elicitin-like* genes coding for proteins able to induce plant resistance, six populations were identified. However, none of these groups was assigned to a particular subregion of Bordeaux vineyards, suggesting that these factors do not shape the genetic structure of *P. oligandrum* populations. Results showed that different types of rootstock and weeding management both influence root colonization by *P. oligandrum*. These results should prove particularly useful in improving the management of potentially plant-protective microorganisms.

Introduction

The vineyard region of Bordeaux, thanks to its prestigious wines, enjoys a worldwide reputation. It also has a long history of plant diseases that have left their mark. For instance, various epidemics, such as powdery and downy mildew, as well as phylloxera, broke out in the Bordeaux vineyards, causing severe losses to grapevine harvests or even to lifespan (Galet, 1982). Viticulture is now confronted with other upheavals, brought about by the need to control various diseases, while dealing with strong consumer demands for an environmentally friendly viticulture. The objective of the viticulture sector is thus to dramatically reduce the use of chemical pesticides to control pathogens, as for instance powdery mildew caused by *Erysiphe necator* that threatens all the vineyards. So, the development of environmentally friendly methods is currently necessary.

Managing microbiota that colonize the rhizosphere of grapevines to promote suppressive activity could be useful in providing new tools to achieve effective plant protection. In the vineyards, potentially beneficial microorganisms naturally colonize the roots of grapevines (Compant *et al.*, 2013), but their persistency over a long period is relatively unknown. Cultural practices such as weeding management (Whitelaw-Weckert *et al.*, 2007) and soil morphology (Steenwerth *et al.*, 2008) have an impact on the soil microbiota of vineyards. They could thus shape the population structures of beneficial microorganisms. In the present study, we have focused our attention on a common oomycete inhabitant of soils worldwide, *Pythium oligandrum*. It has been described as one of the most numerous species in several agricultural soils (Schmitthenner, 1962; Klemmer & Nakano, 1964; Vaartaja & Bumbieris, 1964; Kobayasi *et al.*, 1977; Plaats-Niterink,

1981; Ali-Shtayeh, 1985; Mulligan & Deacon, 1992; Ribeiro & Butler, 1992). Martin & Hancock (1986) have found high propagule densities of *P. oligandrum* in soil that is suppressive to the pathogen, *P. ultimum,* in cotton fields in California. However, although *P. oligandrum* is rhizospheric competent for many plants (McQuilken *et al.*, 1990; Al-Rawahi & Hancock, 1998; Le Floch *et al.*, 2003; Takenaka *et al.*, 2008), its ability to colonize the roots of grapevines is still poorly investigated, although Mohamed *et al.* (2007) suggested it is common in viticulture soils. The same authors showed that, after treatment of vine roots with this oomycete, plant protection against the gray mold agent, *Botrytis cinerea*, was obtained. Numerous plant diseases have been controlled by *P. oligandrum*, with a reduction in plant pathogenic attacks varying from 15% to 100% (Gerbore *et al.*, 2014). As recent review by Benhamou *et al.* (2012) and Gerbore *et al.* (2014), the plant protection provided by *P. oligandrum* is the result of a complex process that includes (1) direct and/or (2) indirect effects on the pathogens. Concerning the direct effect, features of mycoparasitism and/or antibiosis and/or nutrient elements competition are currently observed when *P. oligandrum* interacts with many fungi and oomycetes (Benhamou *et al.*, 1999; Picard *et al.*, 2000; Rey *et al.*, 2008). Vallance *et al.* (2012) have demonstrated that, after introduction of *P. oligandrum* in the root system of hydroponic tomatoes, transient perturbations in the rhizospheric indigenous bacterial communities were observed, suggesting that it may also have a potential antibacterial effect. However, Hase *et al.* (2006) reported that the ability of *P. oligandrum* to control tomato wilt caused by the bacterial pathogen, *Ralstonia solanacearum*, resulted from stimulation of the plant defenses, rather than from a direct antagonistic process. This particular point is worth being mentioned because, for many scientists, the main mode of action of *P. oligandrum* is the resistance it induces in numerous plants.

It should be noted that this induced resistance begins after the establishment of an atypical relationship between *P. oligandrum* and the plant: the hyphae penetrate quickly and deeply into the tomato root tissues without causing damage, but they rapidly degenerate and cannot stay alive *in planta* (Rey *et al.*, 1998 Le Floch *et al.*, 2005, 2009). After this unusual root colonization process, local and systemic induced-resistances occur in plants and, as a result, protection is provided against a broad range of pathogens (Benhamou *et al.*, 1997; Madsen & de Neergaard, 1999; Picard *et al.*, 2000; Le Floch *et al.*, 2003; Mohamed *et al.*, 2007; Rekanovic *et al.*, 2007; Takenaka *et al.*, 2008; Takenaka & Ishikawa, 2013).

Consequently, *P. oligandrum,* particularly the strains that produce the three elicitin-like proteins (ELPs), that is, oligandrin, POD1, and POD2, responsible for this induced resistance, has considerable potential in protecting vines from pathogenic diseases. Previous reports have already shown that *P. oligandrum* strains are useful on a range of crop plants in small-scale field, soil and soilless greenhouse trials, but enhanced protection should certainly come from the selection and use of elicitin-like producing strains. Because the three ELPs had not been isolated and described at the time, this point was not verified in many of the previous publications on *P. oligandrum*. Another issue is that the protection endowed with biocontrol agents (BCA) in the field experiments often fails because of inconsistency (Alabouvette *et al.*, 2006). This inconsistency is generally associated with numerous factors, including biotic and abiotic factors, which may have an influence on BCA activity. For instance, Edel-Hermann *et al.* (2009) demonstrated that the biocontrol agent, *Fusarium oxysporum* strain 47, was more susceptible to the biotic factor than to the physico-chemical properties of the soils. In addition, the ecological fitness of BCA is also worth being considered (Chacón *et al.*, 2007; Robertson *et al.*, 2008; Alabouvette *et al.*, 2009). So, selection of BCAs that can be active under various environmental factor, and also determination of their ecological fitness, are certainly prerequisites before being used in the field.

Accordingly, in this study, we aim to (1) determine whether *P. oligandrum* is able to naturally colonize the roots of grapevines planted in different types of soil from the Bordeaux vineyards; (2) study the rhizospheric microbiological environment (bacteria and fungi) in which *P. oligandrum* can grow; (3) assess the genetic variability of the strains by sequencing various phylogenic and elicitor genes; (4) evaluate the strains abilities to produce oligandrin and to induce disease resistance, this experiment have been carried out using a bioassay against powdery mildew.

Materials and methods

Root sampling in vineyards

Samples of roots were taken from grapevines grown in vineyards from the Bordeaux region (France). Twelve plots were selected: four in the Médoc subregion of Bordeaux, four in the Libournais subregion and four others in the Graves subregion (Fig. 1). Each subregion and plot is characterized by a typical soil composition given in Table 1, the percentages of silt, clay, sand, and stone were measured by the LCA Bordeaux Laboratory (Blanquefort, France).

The vineyards were planted with the same cultivar, Cabernet-Sauvignon (*Vitis vinifera* L, 20–24 years old),

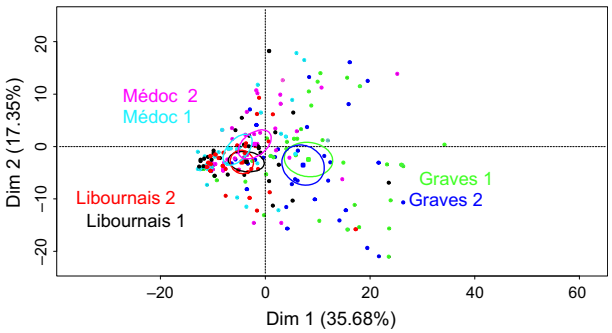

Fig. 1. Comparison of genetic structure of rhizospheric fungal communities from roots of grapevines grown in Graves, Libournais, and Médoc by PCA. The SSCP profiles of fungal communities are based on mitochondrial large-subunit rDNA gene. Ellipsoids draw the center of factors with 95% confidence. 1 = sampling in year 2010 and 2 = sampling in year 2011.

with three rootstocks (coded: no. 3309, no. 101-14 et SO4) and were trained using the Guyot double method. Depending on the vineyards, weeding management was made either by applying herbicides (two treatments per year on average) or by mechanical plowing between the rows of grapevines. Note that, 10–12 fungicide treatments on average were applied each year in these vineyards to control downy and powdery mildews.

Ten plants were randomly selected from healthy grapevines in the summers of 2010 and 2011 (July and August). For each plant, a minimum of 5 g roots was taken at a depth of 20 cm. The rhizosphere of the same grapevines was sampled in 2010 and 2011. The root samples were subsequently used for microbial and molecular analyses.

DNA extraction from root samples

The root samples were ground in liquid nitrogen with a one-ball mill of Dangoumau type. DNA was extracted from 70 mg of root tissue with the Indvisorb Spin Plant mini Kit (Eurobio, France) in accordance with the manufacturer's instructions. DNA extracts were then quantified with a Nano-drop (ND-1000, Thermoscientific, Labtech) and homogenized at a concentration of 10 ng μL^{-1}.

Analysis of the fungal and bacterial communities of the rhizosphere

Pairs of universal primers recognizing mitochondrial *large-subunit rDNA* (ML1/ML2) (White *et al.*, 1990) gene and the variable regions V5-V6 of the *16S rRNA* (799F/1115R) (Redford *et al.*, 2010) were used for capillary electrophoresis fingerprint technique (CE-SSCP) analysis of the fungal and bacterial communities, respectively. PCR was performed on DNA extracts from the 240 root samples collected. DNA was amplified by PCR in a PTC-100 thermocycler (MJ Research, Inc.) in a reaction mixture (25 μL final volume) consisting of 1 μL of DNA template, 0.2 mM concentration of each deoxynucleoside triphosphate, 2 ng μL^{-1} of each primer, 2.5 μL of 10× PFU Turbo buffer, and 0.05 U of PFU Turbo DNA polymerase (Stratagene, the Netherlands). The cycling conditions were as follows: enzyme activation at 95 °C for 2 min, 35 cycles of denaturation at 95 °C for 30 s, hybridization for 30 s at 58 °C for fungi and 61 °C for bacterial primers, extension at 72 °C for 30 s, and final extension at 72 °C for 10 min. The PCR products were visualized by ethidium bromide staining on agarose gel electrophoresis prior

Table 1. Characterization of the soils from 12 vineyards located in the Bordeaux region (France)

Vineyard ID	Subregion	Coordinates		Year of planting	Rootstock	Weeding management	Physical properties			
							Silt (%)	Clay (%)	Sand (%)	Stones (%)
1	Médoc	45°03′02″N	0°40′28.5″O	1988	3309	Mecha	16	22	62	14.75
2	Médoc	45°01′32″N	0°39′46″O	1987	101-14	Mecha	11	10	79	33.06
3	Médoc	44°56′36.5″N	0°37′27.8″O	1990	3309	Chem	8	9	83	18.86
4	Médoc	45°01′42.3″N	0°39′48.4″O	1990	101-14	Mecha	9	13	78	NA
5	Libournais	44°51′42″N	0°00′60″O	1987	101-14	Chem	52	21	27	1.39
6	Libournais	44°59′29.5″N	0°18′07″O	1987	3309	None	55	14	31	2.61
7	Libournais	44°55′24″N	0°02′18″O	1987	NA	Mecha	49	31	20	3.92
8	Libournais	44°54′37.5″N	0°11′43″O	1986	SO4	Chem	51	35	14	16.87
9	Graves	44°46′12.5″N	0°38′15.5″O	1988	NA	Mecha	14	6	80	38.98
10	Graves	44°40′26″N	0°27′53″O	1989	3309	Mecha	13	8	79	49.81
11	Graves	44°43′15″N	0°37′34.5″O	1988	101-14	Mecha	10	6	84	53.40
12	Graves	45°1′42.3″N	0°39′48.4″O	1988	NA	Chem	10	7	83	58.72

We sampled vineyards from three main geographical subdivisions of the Bordeaux region: Graves, Libournais and Médoc (four vineyards per subregion). Physical properties of soils: all the values are expressed as percentages. Weeding management: mechanical (mecha) via plowing, chemical (chem) via herbicide treatments (two on average per year).
NA, nonavailable.

to CE-SSCP analysis. The lengths of the fragments yielded by amplification of fungi DNAs were 250 bp for fungi and 350 bp for bacteria. CE-SSCP analyses were performed on an ABI Prism 3130 genetic analyzer (Applied Biosystems) using four 36-cm-long capillary. One microliter of a PCR product was mixed with 18.8 μL Hi-Di formamide (Applied Biosystems) and 0.2 μL of the internal standard DNA molecular weight marker Genescan 400 HD ROX (Applied Biosystems). The sample mixture was denatured at 95 °C for 5 min and immediately cooled on ice for 10 min before being loaded onto the instrument. The nondenaturing polymer consisted of 5% POP conformational analysis polymer (Applied Biosystems), 10% glycerol, EDTA buffer (10×), and water (Applied Biosystems). The migration time was set for 50 min, the voltage at 12 kV, and the temperature at 32 °C. CE-SSCP is based on the electrophoretic mobility of single-stranded DNA fragments. This mobility is different according to their three-dimensional conformation. The samples were then allowed to comigrate with the fluorescent size standard (GeneScan 400 ROX) to enable comparison of migration profiles between samples. Patterns were aligned with the StatFingerpints program (Michelland *et al.*, 2009) and studied by principal component analysis (PCA) with R software (version 2.15.2).

Structure and diversity analysis of microbial communities

The characterization of the rhizospheric microbial (fungi and bacteria) communities' structure and diversity was performed with profiles obtained by the CE-SSCP method, as previously described by Kimsé *et al.* (2012) and Michelland *et al.* (2009, 2010). All readable molecular fingerprint profiles were aligned with the internal ROX ladder and normalized, to produce relative abundance data with the R package Statfingerprints v1.3 software (Michelland *et al.*, 2009; Kimsé *et al.*, 2012). This yielded a matrix in which root samples were indicated in rows, and fluorescence values (1315 scans) in columns. A fluorescence profile may be seen as a quantitative descriptor of the microbial assemblage of a sample. Bigger differences in fluorescence scans between profiles indicate a greater dissimilarity in composition between samples (Michelland *et al.*, 2009; Kimsé *et al.*, 2012).

Rhizospheric microbial (fungi and bacteria) community diversity was evaluated using the Simpson diversity index. This diversity index was estimated by summarizing a complex community, represented in a molecular fingerprint profile, by a single value. This takes into account the number of species (number of peaks) and their relative abundance (area under each peak). The Simpson diversity index (D') was calculated with the R package Statfingerprints v1.3, as previously mentioned by Onaindia *et al.* (2004) and Michelland *et al.* (2010), respectively:

$$D' = -\log \Sigma pi^2 = \Sigma |ni(ni-1)/N(N-1)| \Sigma pi^2 = \Sigma [ni(ni-1)/N(N-1)]$$

where ni is the number of individuals in the i species and N is the total number of individuals. pi is the relative abundance of species i. According to this method of calculation, Simpson index values will be higher than 1 and will allow relative comparisons between the various data.

Fingerprint molecular profiles were studied using PCA in relation to environmental factors with R software (version 2.15.2, including FACTOMINER packages), and the Simpson index was subject to analysis of variance (ANOVA) and Tukey test comparisons.

Assessment of root colonization by *Pythium* spp. with echinulated oospores and purification of strains

For each of the 240 plants sampled, *Pythium* spp. with echinulated oospores rhizosphere colonization was assessed by setting 30 root fragments (5 mm length) on a selective medium for *Pythium*, made up of Corn Meal Agar supplemented with antibiotics, coded CMA-PARP (Jeffers & Martin, 1986). Five and ten days after incubation of Petri dishes in the dark at 25 °C, oomycete structures were observed with the optical microscope (Olympus BH-2, France). Using the Plaats-Niterink (1981) morphological descriptions of *Pythium* species, echinulated oospores could be identified. Hypha emerging from each root fragments were taken and cultured several times, until pure cultures were obtained for each isolate.

For each sample, the number of root fragments colonized by hyphae producing echinulated oospores was determined. The differences between vineyards were evaluated by ANOVA, after normalization (transformation in log $(x + 1)$ of the data) and validation of variance homogeneity (with Levene test) with R software version 2.15.2. The Tukey test was used to compare the means.

DNA extractions of *Pythium* spp. strains

For each purified *Pythium* strain, genomic DNA was extracted from young mycelium, scraped from the surface of a malt agar plate using a sterile tip. Samples were freeze-dried overnight (Alpha 1-4 LO plus, Bioblock Scientific) and ground with a Tissue Lyser II (Retsch, Qiagen) for 60 s at a frequency of 30 s^{-1}. Four hundred μL of CTAB (1×) was added to each sample. After incubation at 65 °C for 1 h, 400 μL of chloroform–isoamyl alcohol (24 : 1, v/v) was added, and the samples were

centrifuged for 30 min at 2250 ***g***. The aqueous phase was transferred into another tube, and 200 μL of cold isopropanol was added. Samples were then kept at −20 °C overnight for DNA precipitation. After 20 min centrifugation at 3700 r.p.m., the supernatant was discarded and 500 μL of ethanol 70% was added to wash the DNA. Once the ethanol was discarded, the pellets were air-dried and suspended in 70 μL of sterile distilled water (DW). DNA concentration was estimated using Nano-drop (ND-1000, Thermoscientific).

Molecular identification and characterization of strains by sequencing

Molecular identification of *P. oligandrum* isolates was performed by sequencing the rDNA repeat unit, that is, 3′ end of the *18S rRNA* gene, *ITS-1, 5.8S rRNA* gene, *ITS-2*, and 580 bp of the 5′ end of the *28S rRNA* (Bakkeren *et al.*, 2000). *P. oligandrum* elicitor detections were carried out by amplification of *oligandrin* gene (oligandrinF and oligandrinR primers) and *POD1* gene (T1C and T1F primers) as described by Masunaka *et al.* (2010). Strain genetic polymorphism was evaluated using *tubulin* (tub1f and tub2R primers, designed with PRIMERCLADE software, Gadberry *et al.*, 2005) and *cytochrome* region genes (COI-Levup[c] and FM85-mod primers, Bala *et al.*, 2010).

Polymerase chain reaction was carried out in a thermocycler (Mastercycler gradient Eppendorf AG 22331, Eppendorf, France) with 10 ng of template DNA. Six μL of the PCR product was used for electrophoresis on 2% agarose gel and then visualized using ethidium bromide staining to control PCR efficiency. The PCR products were sequenced, on two strands, by GATC Biotech AG (Konstanz, Germany), and consensus sequences were determined after alignment, using CODONCODE ALIGNER software (version 4.2.1). ITS sequences were assigned to species, based on 99% sequence similarity threshold, after blasting with GenBank database. Phylogenetic trees were constructed using the PHYML method on SEAVIEW software (version 4.4.0), based on multiple alignments (Gouy *et al.*, 2010).

Oligandrin production from culture filtrates of *P. oligandrum* strains

Liquid media of *P. oligandrum* strains were obtained from mycelium culture in a defined medium stimulating the production of elicitin (Bonnet *et al.*, 1996). The flasks were incubated in the dark for 10 days at 24 °C. Culture filtrates of *P. oligandrum* were recovered after mycelium removal on a GF/C filter (Whatman, Clifton, NJ) under vacuum, in a 10-fold concentration. Oligandrin production was quantitatively assessed by HPLC as described by Picard *et al.* (2000) with slight modifications. Briefly, HPLC (HP 1100 Series HPLC System, Agilent Technologies) was performed on a C8 column (Zobrax Eclipse XDB-C8, 5 μm, 4.6 × 150 mm, Agilent Technologies), and 10 μL of culture filtrate was loaded. Elution was carried out using the following solvents: A [20% CH_3CN, 10 mM $(NH_4)_2$ SO_4, and 20 mM HCOONa] and B (40% CH_3CN and 100 mM HCOONa) using a linear gradient: 100% A 3 100% B (10 min) and 100% B hold for 2 min. The flow rate was 1 mL min^{-1}.

Elution was monitored with an Agilent G1315A Diode Array Detector (200–400 nm, resolution: 4 nm). Integration at 280 nm, spectra, peak purity, and all calculations were obtained using HP CHEMSTATION software (version G1656B, Agilent Technologies). A purified extract of oligandrin provided by Dr Michel Ponchet (UMR 1301 Interactions Biotiques et Santé Végétale, INRA Sophia Antipolis, France) was used as a standard.

Leaf grapevine protection bioassay

In vitro inhibition of powdery mildew (*Erysiphe necator*) was assessed in a leaf grapevine bioassay test. After leaf disinfection (10 min in 3% hypochlorite, rinsed in sterile DW), leaf discs (24 mm diameter) were cut (seven replicates per treatment) and dipped for 4 h in (1) *P. oligandrum* culture filtrates (diluted at 1/50); (2) oligandrin (1.8 μg mL^{-1}); or (3) in sterile DW as control.

Discs were then removed and set, top side up, in Petri dishes containing agar (20 g L^{-1}) and benzimidazole (30 mg L^{-1}). Pathogen inoculation, *E. necator* strain GF 5.5.3, was carried out using an air pump to blow spores from 12-day-old sporulating leaves onto the upper surface of the discs. The density of deposited conidia, assessed with a hemacytometer, was 850 and 750 spores per cm^2, respectively, for the first and second experiment. Leaves were incubated in growth chamber at 22 °C. Disease was assessed at 12 days after inoculation. For each of the seven replicates, the disc area covered by the fungus mycelium was assessed visually and the number of spores counted, using a particle counter (Coulter Counter Multisizer 3; Beckman Coulter, Paris-Nord, France). The experiment was repeated twice and statistical analysis of variance (ANOVA) and Tukey comparisons were made using R software.

Results

Characterization of vineyards

Vineyard soils were characterized by their physical parameters as shown in Table 1, with all of these soils containing various quantities of clay, sand, silt, and stone. For instance, the vineyards of the Médoc subregion have the greatest quantities of sand (62–83%); but stone

(14–33%), silt (8–16%), and clay (9–20%) are also detected. Of the 12 vineyards, those of the Libournais subregion contained the highest quantities of silt (49–55%), but clay (14–35%) and sand (from 14% to 31%) were also relatively frequent. In the soil of the four vineyards from the Graves subregion, sand was predominant (79% to 84%) along with stone (from 38% to 58%), and the lowest values were for clay (6–8%).

Using these data, together with weeding management and rootstock as factors, PCA was generated (Supporting Information, Fig. S1). Eigenvalues of PCA indicated that the first two principal components, Dim1 and Dim2, covered 93.5% of the total data variance and gave a consistent description of the global database. The Graves (mainly sandy–stony texture) soils were clearly separated from Médoc (mainly sandy texture) and Libournais (mainly silty texture) soils by Dim1 (respectively, negative vs. positive coordinates).

Structure of the fungal and bacterial communities that colonize the rhizosphere of grapevines

For the fungal communities, PCA eigenvalues indicated that the first two principal components, Dim1 and Dim2, explained 53.1% of the total data variance (Fig. 1). The SSCP profiles of fungal communities (based on *mitochondrial large-subunit rDNA* gene) from roots of grapevines grown in Médoc and Libournais subregions were clearly separated from those of the Graves vineyards by Dim1 (negative vs. positive coordinates, respectively). Fungal communities from grapevines of Médoc tended to be separated from those of Libournais by Dim2 (positive vs. negative coordinates, respectively). It should be noted that the same results were obtained for the 2 years of experiments.

For the bacterial communities, PCA eigenvalues indicated that the first two principal components, Dim1 and Dim2, explained 84% of the total data variance (Fig. S2). The SSCP profiles of bacterial communities (based on *16S rRNA* gene) from grapevine roots grown in the three subregions, Graves, Médoc, and Libournais, cannot be separated by Dim1 or Dim2. The same results were obtained for the 2 years of experiment.

For the three regions, based on weeding management or rootstocks, the fungal and bacterial communities colonizing the roots of grapevines were not separated by PCA analysis (data not shown).

Diversity of fungal and bacterial communities colonizing the rhizosphere of grapevines

The Simpson index, which takes into account the relative abundance of each population, was calculated to assess the diversity of rhizospheric fungal and bacterial communities. This index is heavily weighted toward the most abundant species so that, when the index is high, diversity is low. In 2010, the Simpson index of fungal communities from Graves vineyards was statistically higher (6.38) than in the Libournais (5.00) and Médoc (4.73) vineyards (Table 2). In 2011, all these values increased but a similar trend was observed.

For the bacterial communities, the Simpson indexes were relatively similar for the three vineyard subregions, varying from 7.03 to 7.98 in 2010 and from 7.6 to 7.93 in 2011. For the 2 years, these values were not significantly different.

Assessment of echinulated oospores *Pythium* spp. root colonization in the various vineyards

Root colonization by *Pythium* spp. with echinulated oospores was assessed using plate counting (Fig. 2). *Pythium* spp. were isolated in 11 of 12 vineyards in 2010 and, in all the vineyards, in 2011. The highest value of grapevine roots colonization was obtained in vineyard no. 11, 22.67% in 2010, and the lowest, 0%, in vineyard no. 5 in 2010.

For the Graves subregion, the highest values, that is, over 15% of roots colonized by *Pythium* spp. with echinulated oospores, were obtained in 2 (vineyards no. 9 and 11) of the 4 vineyards, in both 2010 and 2011. A similar trend was observed for the four Libournais vineyards, with the highest values (over 8%) being obtained in vineyard no. 7 and 8. In the Médoc subregion, the lowest values (below 5%) were obtained in two vineyards (no. 1 and 4) of 4, although these same vineyards showed the highest values (over 15%) in 2011.

Nevertheless, as regards echinulated oospores *Pythium* spp. root colonization, no statistically significant differences were observed between the 2 years of sampling.

Table 2. Simpson diversity indexes of bacterial and fungal communities colonizing the rhizosphere of grapevines from the vineyards of the three subregions of the Bordeaux region

		Simpson index	
Year	Soil	Bacterial communities	Fungal communities
2010	Graves	7.98 ± 0.3 a	6.38 ± 0.23 a
	Libournais	7.03 ± 0.47 a	5 ± 0.26 b
	Médoc	7.93 ± 0.42 a	4.73 ± 0.39 b
2011	Graves	7.93 ± 0.3 a	6.48 ± 0.22 a
	Libournais	7.6 ± 0.36 a	5.57 ± 0.15 b
	Médoc	7.69 ± 0.32 a	5.54 ± 0.16 b

Mean values of Simpson indexes (± SE) with different letters are significantly different at $P < 0.05$ according to ANOVA and Tukey test.

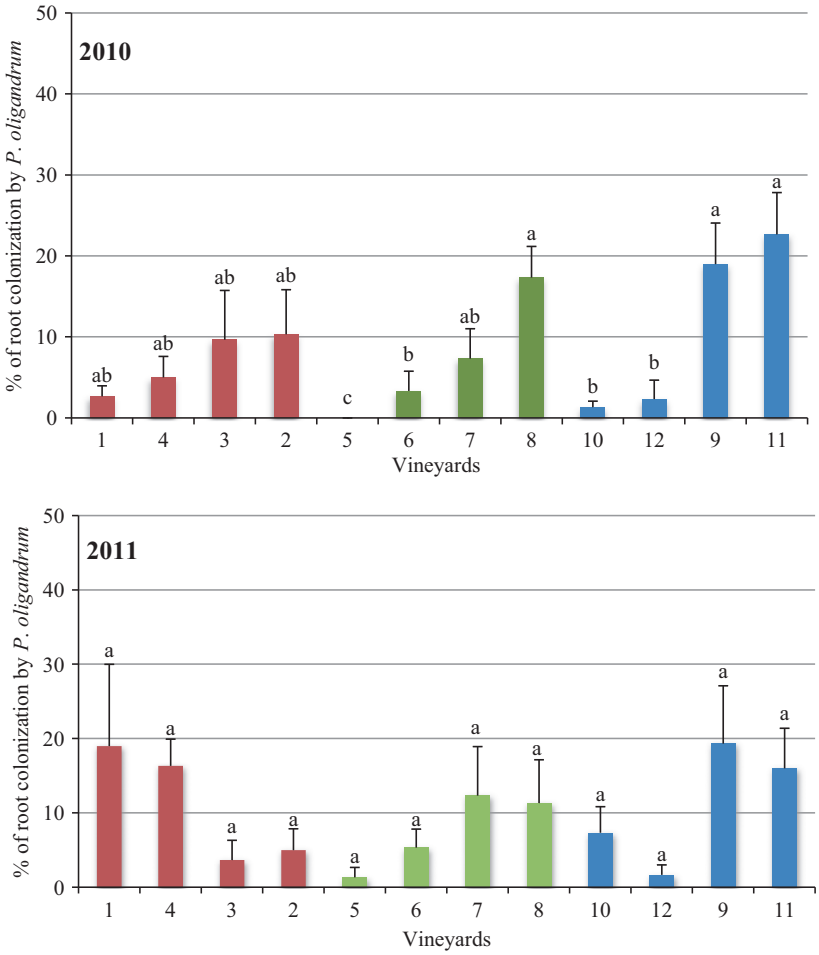

Fig. 2. Root colonization of grapevines by *Pythium* spp. with echinulated oospores assessed by plate counting in summer 2010 and 2011. The values reported are means of 10 samples collected in each vineyard. Means with different letters are significantly different at $P < 0.05$ (ANOVA and test of Tukey). Red for Med, Médoc; green for Lib, Libournais; blue for Gra, Graves; these colors indicate the origin of the *Pythium* spp. with echinulated oospores. The number refers to vineyards (see Table 1).

Influence of various factors on echinulated oospores *Pythium* spp. root colonization

For the 2 years of experiment, the impact of various factors, that is, region sampling sites, rootstocks, weeding management, on echinulated oospores *Pythium* spp. root colonization was assessed (Table 3).

Roots from plants growing in soil from the Graves vineyards tended to be more colonized in 2010, but the differences with the results obtained from the Libournais and Médoc vineyards were not significant. Similar results were obtained in 2011.

Pythium spp. with echinulated oospores were significantly more frequently isolated on SO4 rootstock (17.33%) in comparison with no. 101–14 (9.5%) and no. 3309 (4.33%) rootstocks in 2010. A similar trend was observed in 2011, although the differences were not statistically significant.

In 2011, *Pythium* spp. root colonization was significantly lower in vineyards with chemical weeding management than in vineyards with mechanical weeding. A similar trend was observed in 2010, although the differences were not statistically significant.

Molecular identification of *Pythium* spp. with echinulated oospores

A subset of 44 isolates from the 12 vineyards and 4 *P. oligandrum* strains from CBS were sequenced for identification on the ITS1&2 rDNA region. The PCR of the ITS1&2 rDNA region yielded a 1500 bp single product. Forty strains were identified as belonging to *P. oligandrum*, based on 99% BLAST sequence similarity with the ITS1&2 rDNA region sequences of the strain CBS 382.33 (AY598618.1). Four strains were identified as *P. acanthicum*, based on 99% BLAST sequence similarity with the ITS1&2 rDNA region sequences of the strain CBS 377.3 (AY598617.1). Only *P. oligandrum* strains have been used for the further analyses or experiments. The *P. oligandrum* sequences obtained showed six haplotypes which differed by only a very few mutations: the two most frequent haplotypes ($n = 32$) varied by only one

Table 3. Influence of sampling sites (i.e. the three subregions), rootstocks, and weeding managements on root colonization by *Pythium* spp. with echinulated oospores

	Years	
Abiotic factors	2010	2011
Localization of sampling sites		
Médoc (n = 40)	7.00 ± 2.14 a	11.00 ± 3.13 a
Libournais (n = 40)	7.00 ± 1.75 a	7.58 ± 2.33 a
Graves (n = 40)	11.33 ± 2.39 a	11.08 ± 2.69 a
Rootstock		
No. 3309 (n = 40)	4.33 ± 1.69 a	8.83 ± 3.06 a
No. 101-14 (n = 40)	9.50 ± 2.34 a	9.67 ± 2.03 a
S04 (n = 10)	17.33 ± 3.84 b	11.33 ± 5.82 a
Weeding management		
Chemical (n = 40)	7.33 ± 2.12 a	4.50 ± 1.73 a
Mechanical (n = 80)	9.81 ± 1.56 a	13.62 ± 2.24 b

The values are means (%) of root colonization by *Pythium* spp. with echinulated oospores. Mean values (± SE) with different letters are significantly different at $P < 0.05$ according to ANOVA and Tukey test.

nucleotidic change, and the four others (n = 12) were characterized by a number of mutations, ranging from 1 to 4, compared with the most distant haplotype (Fig. S3).

Comparing the strains isolated from vineyards with the CBS strain, two strains were identical to the *P. oligandrum* Dutch strains CBS 530.74, fifteen to the UK (382.34) and to the Australian (118746) strains. None of the isolated strains were similar to the Danish (109982) strain. The others strains (23) were distinct from the CBS strains.

Sequencing of genes coding for ELPs

Sequences were obtained from 44 strains for the ELPs, oligandrin and Cell Wall Proteins (CWPs). Each strain had at least one gene coding for oligandrin and one gene coding for a CWP, either POD or POS.

For the *oligandrin* gene, we found two sequences corresponding to Oli-S1 and Oli-D1 (AB474244 and AB474242 accession numbers, respectively), differing by only one silent mutation at position 165 (data not shown).

For the *CWP* genes, four different haplotypes were obtained out of *P. oligandrum* strains: two haplotypes corresponded to the previously described *POD-1* and *POS-1* genes (GenBank accession no. AB217820 and AB47424, respectively). The two other haplotypes, POD-1a and POD-1b, were closely related to POD-1. POD-1a protein shows two nonsynonymous mutations in position 114 and 116, with asparagine and glycine amino acids instead of lysine and aspartic acid amino acids, respectively. POD-1b protein shows one nonsynonymous and one synonymous mutation, respectively, in position 233 and 243 with alanine instead of aspartic acid amino acid in position 233 (Table S1). POD-1a and POD-1b are deposited in GenBank database (GenBank accession no. KJ125071 and KJ125072 respectively). POS-1 sequence was the most divergent compared with the three *POD1* genes, with eight nucleotide mutations corresponding to seven amino acids changes.

For each strain, we could establish an 'ELP' profile (Table 4). This led to the identification of six different ELP profiles. The most abundant profile observed, ELP-1, was made up of 17 strains possessing *POS-1* and *Oli-S1* genes. The second profile (ELP-2) was composed of three strains with *POD-1b*, *POS-1*, and *Oli-S1* genes. The third profile (ELP-3) was characterized by eight strains with four genes, *POD-1*, *POS-1*, *Oli-D1*, and *Oli-S1*. Six strains possessing *POS-1*, *Oli-D1*, and *Oli-S1* genes represented the ELP-4 profile, and seven strains with *POD-1* and *Oli-D1* genes, corresponded to the ELP-5 profile. The last profile, ELP-6, was composed of three strains possessing POD-1a and Oli-D1.

Out of the 44 strains analyzed, 27 had ELPs with only two genes, that is, groups 1, 5, and 6. Only eight strains had ELP with four genes.

No clear correlation was observed between vineyard subregions (i.e. Médoc, Graves, and Libournais) and ELP profiles. Nevertheless, ELP-6 was only found in Médoc soils. ELP-1 was dominant and isolated from the three regions. ELP-5 and 2 were observed in Graves and Libournais subregions, ELP-3 in Médoc and Graves subregions, and ELP-4 only in Médoc and Libournais subregions.

Phylogenetic analysis of *P. oligandrum* strains

Phylogenetic reconstruction was performed using the tubulin (GenBank accession no. from KJ944454 to KJ944486) and cytochrome c oxidase subunit 1 (COI) sequences (GenBank accession no. from KJ944421 to KJ944453, Table S2). The phylogenies obtained from the individual gene regions analyzed separately, and from the concatenated dataset, produced congruent topologies. The concatenated tree of 33 *P. oligandrum* strains grouped the strains into three clusters (Fig. 3). We found no association between soil type and phylogenetic clusters. Most cluster A strains had the *POD-1* and *Oli-D1* genes corresponding to a majority of ELP-5. Cluster B was mostly made up of strains with *POS-1* and *Oli-S1* genes corresponding to ELP-1. Thus, ELP-5 was dominant in cluster A that was also represented by ELP-3, 4.6 and 1. Cluster B was mainly composed of ELP-1 (11 strains), ELP-3 (2 strains), and ELP-4 (1 strain). ELP-2 and 4 were equally represented in cluster C, and one strain had an ELP-3 profile.

Table 4. ELP profiles of *Pythium oligandrum* strains based on sequencing of Cell Wall Protein and oligandrin genes

	Cell wall protein				Oligandrin	
	POD-1	*POD-1a*	*POD-1b*	*POS-1*	*Oli-D1*	*Oli-S1*
ELP-1						
Med-13	–	–	–	+	–	+
Med-14	–	–	–	+	–	+
Lib-2	–	–	–	+	–	+
Oth-4	–	–	–	+	–	+
Gra-11	–	–	–	+	–	+
Gra-12	–	–	–	+	–	+
Oth-1	–	–	–	+	–	+
Med-5	–	–	–	+	–	+
Lib-9	–	–	–	+	–	+
Lib-11	–	–	–	+	–	+
Lib-13	–	–	–	+	–	+
Lib-5	–	–	–	+	–	+
Gra-2	–	–	–	+	–	+
Gra-3	–	–	–	+	–	+
Gra-4	–	–	–	+	–	+
Gra-6	–	–	–	+	–	+
Gra-9	–	–	–	+	–	+
ELP-2						
Lib-8	–	–	+	+	–	+
Lib-14	–	–	+	+	–	+
Gra-10	–	–	+	+	–	+
ELP-3						
Med-12	+	–	–	+	+	+
Gra-5	+	–	–	+	+	+
Gra-8	+	–	–	+	+	+
Med-6	+	–	–	+	+	+
Med-7	+	–	–	+	+	+
Med-8	+	–	–	+	+	+
Med-10	+	–	–	+	+	+
Med-11	+	–	–	+	+	+
ELP-4						
Lib-12	–	–	–	+	+	+
Lib-1	–	–	–	+	+	+
Lib-10	–	–	–	+	+	+
Med-2	–	–	–	+	+	+
Med-3	–	–	–	+	+	+
Med-4	–	–	–	+	+	+
ELP-5						
Lib-3	+	–	–	–	+	–
Lib-4	+	–	–	–	+	–
Lib-6	+	–	–	–	+	–
Lib-7	+	–	–	–	+	–
Gra-1	+	–	–	–	+	–
Gra-7	+	–	–	–	+	–
Oth-3	+	–	–	–	+	–
ELP-6						
Med-1	–	+	–	–	+	–
Med-9	–	+	–	–	+	–
Oth-2	–	+	–	–	+	–

Lib, Libournais; Med, Médoc; Gra, Graves; Oth, CBS strains (Oth-1, CBS 382.34; Oth-2, CBS 530.74; Oth-3, CBS 109982; Oth-4, CBS 118746); these abbreviations indicate the origin of the *P. oligandrum* strains.

Oligandrin production by *P. oligandrum* strains

Production of the extracellular ELP named oligandrin was evaluated by HPLC for 19 *P. oligandrum* strains, with at least one strain for each previously described ELP profiles. All the strains produced oligandrin (Table S3). The mean production was of 68.95 mg L^{-1}, and the highest quantity was obtained with Lib-14 and Oth-3 with, respectively, 123.81 and 100.07 mg L^{-1}. The lowest production was for Med-1 (24.05 mg). Significant differences, depending on the strain, were observed. Lib-14, Oth-3, and Med-13 produced statistically more oligandrin than Lib-5, Med-11, Med-2, and Med-1. Regarding the genetic clade, A, B, or C of *P. oligandrum* strains, no significant differences were observed (data not shown). Nevertheless, elicitin production differed according to ELP profiles (Table 5). The highest quantity was produced by a strain with ELP-2 profile (123.8 mg mL^{-1}), and the lowest quantity measured for ELP-6 strains (34.6 mg mL^{-1}).

Control of *Erysiphe necator* on grapevine leaves

Grapevine leaves, subject to either *P. oligandrum* culture filtrate or oligandrin, reduced *E. necator* development compared with control treatment (DW) (Table 6). The pathogen leaf coverage and sporulation was significantly reduced, respectively, on average, by 48% and 44%, compared with control (DW). ELP-3 and ELP-2 strains were significantly more effective in controlling pathogen leaf coverage compared with ELP-4. Interestingly, no significant differences in efficacy were observed between culture filtrate from strains and oligandrin treatments.

Discussion

To our knowledge this study shows, for the first time, that *P. oligandrum* naturally colonizes the rhizosphere of grapevines. This colonization does not depend on the type of soil that characterizes three vineyard subregions from the Bordeaux region. Neither does it depend on the global microbial populations colonizing the rhizosphere. Rootstocks or weeding management are, however, associated with various level of root colonization by the oomycete. Based on the sequencing of various genes, various genetic structures of *P. oligandrum* strains were observed, but they were not related to the isolation site of the oomycete.

The soil types and the microbiota associated with the rhizosphere of grapevines provided relevant information on the abiotic–biotic environment in which *P. oligandrum* strains grow. Differences in material compositions, that

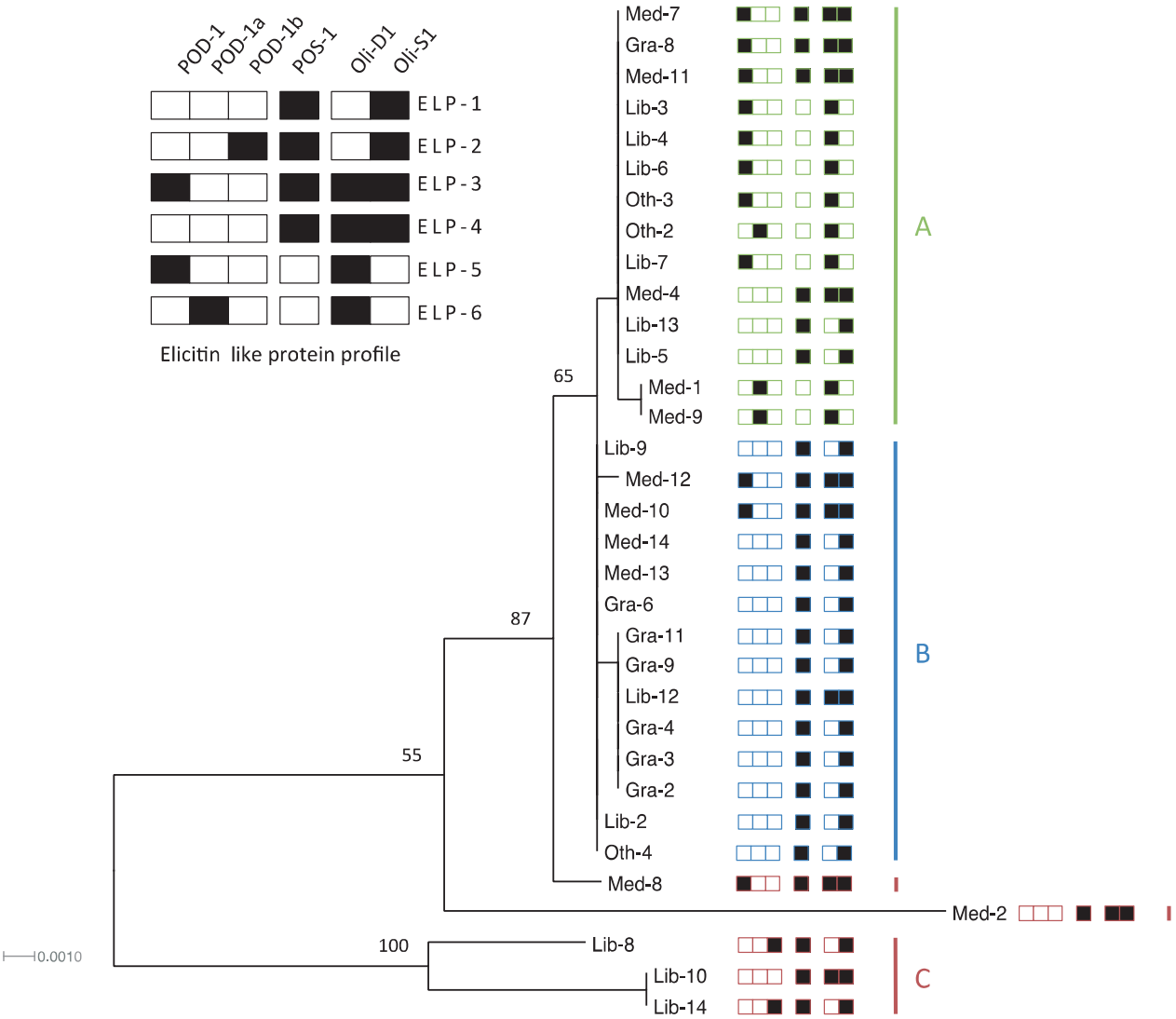

Fig. 3. PHYML tree based on sequencing of Tubulin and COI genes of *Pythium oligandrum* strains. Bootstrap values based on 1000 replications are indicated as percentages in the internode when replication frequencies exceed 50%. Shaded boxes indicate the ELP genes that are detected within the *P. oligandrum* strains names: POD-1, POD-1a, POD-1b, POS-1, Oli-D1, and Oli-S1. Note that the legend for shaded boxes corresponding to the 6 ELP profiles is shown in the inset. Lib, Libournais; Med, Médoc; Gra, Graves; Oth, CBS strains (Oth-1, CBS 382.34; Oth-2, CBS 530.74; Oth-3, CBS 109982; Oth-4, CBS 118746); these abbreviations indicate the origin of the *P. oligandrum* strains.

Table 5. Oligandrin production by *Pythium oligandrum* strains belonging to the 6 ELP profiles

Genetic profile	Oligandrin (mg L^{-1})
ELP-2	123.8 ± 20.5 a
ELP-5	73.8 ± 6.7 ab
ELP-1	75.3 ± 6.8 abc
ELP-3	67 ± 6.8 bcd
ELP-4	38.8 ± 15.9 cd
ELP-6	34.6 ± 7.4 d

At least two repetitions per genetic profile have been carried out. Mean values (± SE) with different letters are significantly different at $P < 0.05$ according to ANOVA and Tukey test.

is, sand, stones, silt, and clay, are the main drivers of distinctness between these soils. This point is worth being mentioned because several authors (Latour *et al.*, 1996; Marschner *et al.*, 2004; Viebahn *et al.*, 2005; Mougel *et al.*, 2006; Buée *et al.*, 2009) reported that soil type is one of the major abiotic factors affecting the structure of microbial communities in the rhizosphere. In our experiment, SSCP data indicate that the structure of rhizosphere fungal communities of plants growing in stony soils (Graves subregion) was markedly different from those in the silty (Libournais) and sandy–stony (Médoc) soils. This result is in accordance with Balestrini *et al.*

Table 6. Assessment of *Erysiphe necator* inhibition on leaves treated by *Pythium oligandrum* culture filtrates, oligandrin, or DW (control)

Genetic profile	Mean of leaf coverage (%)	Mean of pathogen sporulation
ELP-1	55 ± 9 ab	1118 ± 262 a
ELP-2	38 ± 6 b	774 ± 216 a
ELP-3	30 ± 4 b	827.7 ± 97 a
ELP-4	67 ± 6 a	1366 ± 149 a
ELP-5	47 ± 8 ab	1269 ± 167 a
ELP-6	42 ± 7 ab	677 ± 156 a
Control	96 ± 1 c	2276 ± 271 b
Oligandrin	53 ± 8 ab	1049 ± 140 a

The disease was determined by pathogen leaf coverage and spore production 12 days after inoculation (14 replicates per treatment, one strain per treatment), the experiment was repeated twice. Mean values (± SE) with different letters are significantly different at $P < 0.05$ according to ANOVA and Tukey test.

(2010) and Fujita *et al.* (2010) who showed that soil type is the main factor shaping fungal structure in vineyards. Kandeler *et al.* (2000) indicated that coarse sand size fractions (2000–250 μm) were colonized by fungi more than by clay (2–0.1 μm) and silt fractions (63–2 μm). However, our study showed there was less diversity in fungal communities of the rhizosphere from plants growing in the stony soils than in the two others, as calculated with the Simpson diversity index. As reported by Fujita *et al.* (2010), fungal communities structure was more sensitive than the bacterial communities to environmental factors. This is also the case here because a clear cutoff between bacterial communities of the three viticulture regions was not observed for the bacteria. Sessitsch *et al.* (2001) found that soil particles have an influence on the bacterial communities, with soils with coarse particles yielding lower microbial diversity than soils with small particles. The same observation on particle size was made by Badin *et al.* (2012). The factors that drive microbial colonization in soils, such as finer size particles, provide a protective habitat for microorganisms through pore size exclusion of predators (protozoa) (Elliot *et al.*, 1980; Postma & van Veen, 1990) or higher nutrient availability that cause higher bacterial diversities (Sessitsch *et al.*, 2001). However, here, we did not see such influence because our experiment was focused exclusively on the bacterial communities of the rhizosphere and not on those colonizing soil particles.

In our 2-year experiment, with the exception of one sampling of 24, *P. oligandrum* strains were always isolated for the rhizosphere, and clear differences between vineyards within the same subregion, or between the subthree regions, were not observed. These results differed from those obtained by Spies *et al.* (2011) who studied *Pythium* and *Phytophthora* species associated with grapevines in South Africa. They identified 22 *Pythium* species, but they did not detect *P. oligandrum*. Several reasons could be proposed to explain this: (1) the use of different resistant rootstocks, as it was obvious in our experiment that some of them could facilitate or not the colonization of roots by *P. oligandrum*. (2) The planting of cover crops, such as wheat between rows in South African vineyards, can impact *Pythium* species colonization of roots. (3) Weeding strategies through herbicide applications have an impact on *P. oligandrum* populations in Bordeaux region. The influence of fungicides in controlling powdery and downy mildews cannot be discarded either, although they did not prevent *P. oligandrum* root colonization of grapevines in the Bordeaux region. Spies *et al.* (2011) mentioned that a fungicide program has a significant effect on *Phytophthora* populations in South Africa and should have a significant influence on *P. oligandrum* in their specific environment, but further studies need to be performed to verify this point. All these factors, along with the different types of vineyard soil, climate and microbiota and more generally terroirs in South Africa and Bordeaux, could have contributed to inducing changes in the *Pythium* species diversity, which could explain why *P. oligandrum* was not detected by Spies and co-authors. The role of each factor still remains to be determined.

The abiotic or biotic factors we studied do not seem to display an influence on shaping the genetic diversity of *P. oligandrum*. This was observed after the sequencing of three genes, used for either identification or phylogeny, and six elicitin-like genes coding for elicitors. The phylogenetic analyses highlighted that most of the isolated *Pythium* strains with echinulated oospores were *P. oligandrum* (99% nucleotide similarity), but that among these strains, very few nucleotide variations were observed in ITS region. *P. oligandrum* strains originating from UK, Denmark, Australia, and Netherlands were genetically very close to those of the Bordeaux vineyards. The same kind of observation was made by Godfrey *et al.* (2003) when they used strains from various origins, suggesting to them that *P. oligandrum* is a species with worldwide distribution, whose strains are closely related. Besides low divergence in ITS gene, cytochrome oxidase I and tubulin gene sequencing showed that strains clustered into three groups, but no correlation was found between *P. oligandrum* genotypes and the subregion from which the strains were isolated.

Elicitin-like genes, whose proteins are key components in inducing systemic resistance in plants, at least one gene of oligandrin (two genes) and cell wall protein (four genes), were detected in all the *P. oligandrum* strains. Masunaka *et al.* (2010) also detected these genes in *P. oligandrum* strains isolated from Japanese soils, confirming that they are certainly frequent worldwide within this

oomycete. Note, however, that in our study, variations within this family of genes were observed. Two types of *oligandrin* genes corresponding to Oli-D1 and Oli-S1 previously described by Masunaka *et al.* (2010) were detected in all the *P. oligandrum* strains. It should be noted that the proteins deduced from the DNA sequences were similar and that the production of oligandrin quantities depended on the strains but not on their origin. As oligandrin induces disease resistance in many plants, including young vines, it can be hypothesized that strains producing great quantities of oligandrin have a major effect on the plant induction of resistance. CWP gene sequencing revealed the presence of two genes similar to POD-1 and POS-1 genes as described by Masunaka *et al.* (2010) and two new sequences named POD-1a and POD1-b. Interestingly, each deduced protein was distinct.

In our study, a great variability of elicitin-like genes among the *P. oligandrum* strains collected was observed. We create ELP profiles groups to determine the distribution of the elicitin-like sequencing genes within *P. oligandrum* strains. Six different ELP groups were obtained with a dominant one, ELP-1. Again, none of these six groups was assigned to a particular subregion of the Bordeaux vineyards. However, these results suggest that, depending on the *oligandrin* and *CWP* genes, *P. oligandrum* strains' ability to stimulate plant defense mechanism should be different. However, our bioassay test, aimed at controlling *E. necator* on vine leaves, did not show any differences among ELP groups, indicating that all the strains displayed the same ability in protecting plants. Further studies on the induction of resistance in grapevines should be carried out to determine which defense systems are preferably triggered or not *in planta* by the *P. oligandrum* strains of each ELP group.

In conclusion, the present study suggests that in vineyards cultivated in three subregions of the Bordeaux region, whose global fungal and bacterial communities may differ or not depending on the type of soil, and where the plants are regularly treated with pesticides, plant-beneficial microorganisms such as the rhizospheric-colonizer oomycete, *P. oligandrum*, can frequently colonize the roots. Whatever the conditions in which *P. oligandrum* strains grow, external factors do not seem to shape the genetic structure of the oomycete. These results suggest that managing potentially rhizospheric-beneficial microorganisms in the vineyards is possible, provided that the factors favoring root colonization are applied. For instance, these results suggest that through managing plants (rootstocks in our case because they have an influence on root colonization), weeding management (herbicide treatment or not), it is possible to promote the root colonization by *P. oligandrum* populations. Within that population, internal variations, particularly those observed in the elicitor genes, suggest that the induction of plant resistance is only linked to the type of *P. oligandrum* strains. Determining which factors favor specific genotypes could also be carried out. Based on the studies on this model microorganism, they should provide key information to manage other oomycete, fungi or bacteria that are useful for plant protection.

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References

Alabouvette C, Olivain C & Steinberg C (2006) Biological control of plant diseases: the European situation. *Eur J Plant Pathol* **114**: 329–341.

Alabouvette C, Olivain C, Migheli Q & Steinberg C (2009) Microbiological control of soil-borne phytopathogenic fungi with special emphasis on wilt-inducing *Fusarium oxysporum*. *New Phytol* **184**: 529–544.

Ali-Shtayeh MS (1985) *Pythium* populations in Middle Eastern soils relative to different cropping practices. *Trans Br Mycol Soc* **84**: 695–700.

Al-Rawahi AK & Hancock JG (1998) Parasitism and biological control of *Verticillium dahliae* by *Pythium oligandrum*. *Plant Dis* **82**: 1100–1106.

Badin AL, Mustafa T, Bertrand C, Monier A, Delolme C, Geremia RA & Bedell JP (2012) Microbial communities of urban stormwater sediments: the phylogenetic structure of bacterial communities varies with porosity. *FEMS Microbiol Ecol* **81**: 324–338.

Bakkeren G, Kronstad JW & Levesque CA (2000) Comparison of AFLP fingerprints and ITS sequences as phylogenetic markers in *Ustilaginomycetes*. *Mycologia* **92**: 510–521.

Bala K, Robideau GP, Désaulniers N, de Cock AWAM & Lévesque CA (2010) Taxonomy, DNA barcoding and phylogeny of three new species of *Pythium* from Canada. *Persoonia* **25**: 22–31.

Balestrini R, Magurno F, Walker C, Lumini E & Bianciotto V (2010) Cohorts of arbuscular mycorrhizal fungi (AMF) in *Vitis vinifera*, a typical Mediterranean fruit crop. *Environ Microbiol Rep* **2**: 594–604.

Benhamou N, Rey P, Cherif M, Hockenhull J & Tirilly Y (1997) Treatment with the mycoparasite *Pythium oligandrum* triggers induction of defense-related reactions in tomato roots when challenged with *Fusarium oxysporum* f. sp. *radicis-lycopersici*. *Phytopathology* **87**: 108–122.

Benhamou N, Rey P, Picard K & Tirilly Y (1999) Ultrastructural and cytochemical aspects of the interaction between the mycoparasite *Pythium oligandrum* and soilborne plant pathogens. *Phytopathology* **89**: 506–517.

Benhamou N, le Floch G, Vallance J, Gerbore J, Grizard D & Rey P (2012) *Pythium oligandrum*: an example of opportunistic success. *Microbiol-SGM* **158**: 2679–2694.

Bonnet P, Bourdon E, Ponchet M, Blein JP & Ricci P (1996) Acquired resistance triggered by elicitins in tobacco and other plants. *Eur J Plant Pathol* **102**: 181–192.

Buée M, De Boer W, Martin F, Van Overbeek L & Jurkevitch E (2009) The rhizosphere zoo: an overview of plant-associated communities of microorganisms, including phages, bacteria, archaea, and fungi, and of some of their structuring factors. *Plant Soil* **321**: 189–212.

Chacón MR, Rodríguez-Galán O, Benítez Fernández T, Sousa S, Rey M, Llobell A & Delgado-Jarana J (2007) Microscopic and transcriptome analyses of early colonization of tomato roots by "*Trichoderma harzianum*". *Int Microbiol* **10**: 19–27.

Compant S, Brader G, Muzammil S, Sessitsch A, Lebrihi A & Mathieu F (2013) Use of beneficial bacteria and their secondary metabolites to control grapevine pathogen diseases. *Biocontrol* **58**: 435–455.

Edel-Hermann V, Brenot S, Gautheron N, Aime S, Alabouvette C & Steinberg C (2009) Ecological fitness of the biocontrol agent *Fusarium oxysporum* Fo47 in soil and its impact on the soil microbial communities. *FEMS Microbiol Ecol* **68**: 37–45.

Elliot ET, Anderson RV, Coleman DC & Cole CV (1980) Habitable pore space and microbial trophic interactions. *Oikos* **35**: 327–335.

Fujita K, Furuya S, Kohno M, Suzuki S & Takayanagi T (2010) Analysis of microbial community in Japanese vineyard soils by culture-independent molecular approach. *Int J Wine Res* **2**: 75–104.

Gadberry MD, Malcomber ST, Doust AN & Kellogg EA (2005) Primaclade – a flexible tool to find primers across multiple species. *Bioinformatics* **21**: 1263–1264.

Galet P (1982) *Les Maladies et Parasites de la Vigne Tome 2*. Lavoisier, France.

Gerbore J, Benhamou N, Vallance J, Le Floch G, Grizard D, Regnault-Roger C & Rey P (2014) Biological control of plant pathogens: advantages and limitations seen through the case study of *Pythium oligandrum*. *Environ Sci Pollut Res* **21**: 4847–4860.

Godfrey SC, Monds RD, Lash DT & Marshall JW (2003) Identification of *Pythium oligandrum* using species-specific ITS rDNA PCR oligonucleotides. *Mycol Res* **107**: 790–796.

Gouy M, Guindon S & Gascuel O (2010) SEAVIEW version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol Biol Evol* **27**: 221–224.

Hase S, Shimizu A, Nakaho K, Takenaka S & Takahashi H (2006) Induction of transient ethylene and reduction in severity of tomato bacterial wilt by *Pythium oligandrum*. *Plant Pathol* **55**: 537–543.

Jeffers SN & Martin SB (1986) Comparison of two media selective for *Phytophthora* and *Pythium* species. *Plant Dis* **70**: 1038–1043.

Kandeler E, Tscherko D, Bruce KD, Stemmer M, Hobbs PJ, Bardgett RD & Amelung W (2000) Structure and function of the soil microbial community in microhabitats of a heavy metal polluted soil. *Biol Fert Soils* **32**: 390–400.

Kimsé M, Bayourthe C, Monteils V, Fortun-Lamothe L, Cauquil L, Combes S & Gidenne T (2012) Live yeast stability in rabbit digestive tract: Consequences on the caecal ecosystem, digestion, growth and digestive health. *Anim Feed Sci Technol* **173**: 235–243.

Klemmer HW & Nakano RY (1964) Distribution and pathogenicity of *Phytophthora* and *Pythium* in pineapple soils of Hawaii. *Plant Dis Rep* **48**: 848–852.

Kobayasi Y, Matsushima T, Takada M & Hagiwara H (1977) Reports of Japanese mycological expedition to Mts Ruwenzori, Central-Africa. *Trans Mycol Soc Jpn* **18**: 64–94.

Latour X, Corberand T, Laguerre G, Allard F & Lemanceau P (1996) The composition of fluorescent pseudomonad populations associated with roots is influenced by plant and soil type. *Appl Environ Microbiol* **62**: 2449–2456.

Le Floch G, Rey P, Déniel F, Benhamou N, Picard K & Tirilly Y (2003) Enhancement of development and induction of resistance in tomato plants by the antagonist, *Pythium oligandrum*. *Agron* **23**: 455–460.

Le Floch G, Benhamou N, Mamaca E, Salerno MI, Tirilly Y & Rey P (2005) Characterisation of the early events in atypical tomato root colonisation by a biocontrol agent, *Pythium oligandrum*. *Plant Physiol Biochem* **43**: 1–11.

Le Floch G, Vallance J, Benhamou N & Rey P (2009) Combining the oomycete *Pythium oligandrum* with two other antagonistic fungi: root relationships and tomato grey mold biocontrol. *Biol Control* **50**: 288–298.

Madsen AM & de Neergaard E (1999) Interactions between the mycoparasite *Pythium oligandrum* and sclerotia of the plant pathogen *Sclerotinia sclerotiorum*. *Eur J Plant Pathol* **105**: 761–768.

Marschner P, Crowley DE & Yang CH (2004) Development of specific rhizosphere bacterial communities in relation to plant species, nutrition and soil type. *Plant Soil* **261**: 199–208.

Martin FN & Hancock JG (1986) Association of chemical and biological factors in soils suppressive to *Pythium ultimum*. *Phytopathology* **76**: 1221–1231.

Masunaka A, Sekiguchi H, Takahashi H & Takenaka S (2010) Distribution and expression of elicitin-like protein genes of the biocontrol agent *Pythium oligandrum*. *J Phytopathol* **158**: 417–426.

McQuilken MP, Whipps JM & Cooke RC (1990) Control of damping-off in cress and sugar beet by commercial seed-coating with *Pythium oligandrum*. *Plant Pathol* **39**: 452–462.

Michelland RJ, Dejean S, Combes S, Fortun-Lamothe L & Cauquil L (2009) StatFingerprints: a friendly graphical

interface program for processing and analysis of microbial fingerprint profiles. *Mol Ecol Res* **9**: 1359–1363.
Michelland RJ, Combes S, Monteils V, Cauquil L, Gidenne T & Fortun-Lamothe L (2010) Molecular analysis of the bacterial community in digestive tract of rabbit. *Anaerobe* **16**: 61–65.
Mohamed N, Lherminier J, Farmer MJ, Fromentin J, Beno N, Houot V, Milat ML & Blein JP (2007) Defense responses in grapevine leaves against *Botrytis cinerea* induced by application of a *Pythium oligandrum* strain or its elicitin, oligandrin, to roots. *Phytopathology* **97**: 611–620.
Mougel C, Offre P, Ranjard L, Corberand T, Gamalero E, Robin C & Lemanceau P (2006) Dynamic of the genetic structure of bacterial and fungal communities at different development stages of *Medicago truncata* Geartn. Cv. Jemalong line J5. *New Phytol* **170**: 165–175.
Mulligan DFC & Deacon JW (1992) Detection of presumptive mycoparasites in soil placed on host-colonized agar plates. *Mycol Res* **96**: 605–608.
Onaindia M, Dominguez I, Albizu I, Garbisu C & Amezaga I (2004) Vegetation diversity and vertical structure as indicators of forest disturbance. *Forest Ecol Manag* **195**: 341–354.
Picard K, Ponchet M, Blein JP, Rey P, Tirilly Y & Benhamou N (2000) Oligandrin. A proteinaceous molecule produced by the mycoparasite *Pythium oligandrum* induces resistance to *Phytophthora parasitica* infection in tomato plants. *Plant Physiol* **124**: 379–395.
Plaats-Niterink AJVd (1981) *Monograph of the Genus Pythium*. Studies in Mycology. No. 21. Centraalbureau voor Schimmelcultures, Baarn, the Netherlands.
Postma J & van Veen JA (1990) Habitable pore space and survival of *Rhizobium leguminosarum* biovar *trifolii* introduced into soil. *Microb Ecol* **19**: 149–161.
Redford AJ, Bowers RM, Knight R, Linhart Y & Fierer N (2010) The ecology of the phyllosphere: geographic and phylogenetic variability in the distribution of bacteria on tree leaves. *Environ Microbiol* **12**: 2885–2893.
Rekanovic E, Milijasevic S, Todorovic B & Potocnik I (2007) Possibilities of biological and chemical control of *Verticillium* wilt in pepper. *Phytoparasitica* **35**: 436–441.
Rey P, Benhamou N, Wulff E & Tirilly Y (1998) Interactions between tomato (*Lycopersicon esculentum*) root tissues and the mycoparasite *Pythium oligandrum*. *Physiol Mol Plant Pathol* **53**: 105–122.
Rey P, Le Floch G, Benhamou N & Tirilly Y (2008) *Pythium oligandrum* biocontrol: its relationships with fungi and plants. *Plant–Microbe Interactions* (Ait Barka E & Clément C, eds), pp. 43–67. Research Signpost, Kerala, India.
Ribeiro WRC & Butler EE (1992) Isolation of mycoparasitic species of *Pythium* with spiny oogonia from soil in California. *Mycol Res* **96**: 857–862.
Robertson MP, Kriticos DJ & Zacharlades C (2008) Climate matching techniques to narrow the search for biological control agents. *Biol Control* **46**: 442–452.
Schmitthenner AF (1962) Isolation of *Pythium* from soil particles. *Phytopathology* **52**: 1133–1138.
Sessitsch A, Weilharter A, Gerzabek MH, Kirchmann H & Kandeler E (2001) Microbial population structures in soil particle size fractions of a long-term fertilizer field experiment. *Appl Environ Microbiol* **67**: 4215–4224.
Spies CFJ, Mazzola M & McLeod A (2011) Characterisation and detection of *Pythium* and *Phytophthora* species associated with grapevines in South Africa. *Eur J Plant Pathol* **131**: 103–119.
Steenwerth KL, Drenovsky RE, Lambert JJ, Kluepfel DA, Scow KM & Smart DR (2008) Soil morphology, depth and grapevine root frequency influence microbial communities in a Pinot noir vineyard. *Soil Biol Biochem* **40**: 1330–1340.
Takenaka S & Ishikawa S (2013) Biocontrol of sugar beet seedling and taproot diseases caused by *Aphanomyces cochlioides* by *Pythium oligandrum* treatments before transplanting. *Jpn Agric Res Quarter* **47**: 75–83.
Takenaka S, Sekiguchi H, Nakaho K, Tojo M, Masunaka A & Takahashi H (2008) Colonization of *Pythium oligandrum* in the tomato rhizosphere for biological control of bacterial wilt disease analyzed by real-time PCR and confocal laser-scanning microscopy. *Phytopathology* **98**: 187–195.
Vaartaja O & Bumbieris M (1964) Abundance of *Pythium* species in nursery soils in South Australia. *Aust J Biol Sci* **17**: 436–445.
Vallance J, Deniel F, Barbier G, Guerin-Dubrana L, Benhamou N & Rey P (2012) Influence of *Pythium oligandrum* on the bacterial communities that colonize the nutrient solutions and the rhizosphere of tomato plants. *Can J Microbiol* **58**: 1124–1134.
Viebahn M, Veenman C, Wernars K, van Loon LC, Smit E & Bakker PAHM (2005) Assessment of differences in ascomycete communities in the rhizosphere of field-grown wheat and potato. *FEMS Microbiol Ecol* **53**: 245–253.
White TJ, Bruns TD, Lee S & Taylor J (1990) Analysis of phylogenetic relationships by amplification and direct sequencing of ribosomal RNA genes. *PCR Protocols: A Guide to Methods and Applications* (Innis MA, Gelfand DH, Sninsky JJ & White TJ, eds), pp. 315–322. Academic Press, New York, NY.
Whitelaw-Weckert MA, Rahman L, Hutton RJ & Coombes N (2007) Permanent swards increase soil microbial counts in two Australian vineyards. *Appl Soil Ecol* **36**: 224–232.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Comparison of vineyards from three sub-regions of the Bordeaux vineyards (Graves, Libournais and Médoc) by Principal Component Analysis.
Fig. S2. Comparison of genetic structure of rhizospheric bacterial communities from roots of grapevines grown in

Graves, Libournais and Médoc sub-regions by Principal Component Analysis.

Fig. S3. PHYML tree based on sequencing of 18S, ITS1, 5.8S and ITS2 rDNA genes of *Pythium oligandrum* strains.

Table S1. Polymorphic sites of nucleic acid and deduced amino acid sequences of genes coding for Cell Wall Proteins of *Pythium oligandrum*.

Table S2. GenBank accession no. of cytochrome c oxidase subunit 1 and tubulin gene sequences obtained from the *Pythium oligandrum* strains.

Table S3. Oligandrin production by *Pythium oligandrum* strains.