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Defense Responses in Grapevine Leaves Against *Botrytis cinerea* Induced by Application of a *Pythium oligandrum* Strain or Its Elicitin, Oligandrin, to Roots

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ABSTRACT

Mohamed, N., Lherminier, J., Farmer, M.-J., Fromentin, J., Béno, N., Houot, V., Milat, M.-L., and Blein, J.-P. 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.

Pythium oligandrum is known to display antagonistic activities against several species of pathogenic fungi. It also produces an elicitor of plant defense named oligandrin, which belongs to the elicitin family (10-kDa proteins synthesized by *Phytophthora* and *Pythium* species). Here, the potential of *P. oligandrum* or its purified elicitin to limit the progression of *B. cinerea* on grapevine leaf and the resulting plant–microorganism interactions are described. *P. oligandrum* or oligandrin were applied to roots, and changes in the ultrastructure and at the molecular level were examined. When *B. cinerea* was applied to leaves of pretreated plants, leaf invasion was limited and the protection level reached about 75%. On leaf tissues surrounding *B. cinerea* inoculation, modifications of cuticle thickness, accumulation of phenolic compounds, and cell wall apposition were observed, indicating that grapevine can be considered reactive to elicitins. No macroscopic hypersensitive reaction associated with the elicitation treatment was observed. At the molecular level, the expression of three defense-related genes (LTP-1, β-1,3-glucanase, and stilbene synthase) was studied. RNAs isolated from *B. cinerea*-infected leaves of grapevine challenged or not with *P. oligandrum* or oligandrin were analyzed by real-time reverse transcription-polymerase chain reaction. In grapevine leaves, LTP-1 gene expression was enhanced in response to oligandrin, and RNA transcript levels of β-1,3-glucanase and stilbene synthase increased in response to all treatments with different magnitude. Taken together, these results open new discussion on the concept of plant reactivity to elicitins, which has until now, been mainly based on plant hypersensitive responses.

Additional keywords: plant–pathogen interactions, *Vitis vinifera*.

Grapevine (*Vitis vinifera* L.), one of the most economically important fruit species grown around the world, is susceptible to many fungal pathogens, such as *Botrytis cinerea* which induces gray mold disease at various developmental stages and on different plant organs (22). Until now, the disease has been controlled via fungicide sprayings. However, due to the negative impact of phytochemicals on both the environment and the quality of food products, in addition to the development of fungal resistance (31), efforts are now focused on finding alternative methods of control. Such methods involve either biological control or the use of elicitors of plant defense. Originally, biological control agents included microorganisms that display antagonistic activities against soilborne pathogens leading to plant protection (12). Later, applications of these protective strains to control aerial pathogens were proposed. In the case of *B. cinerea*, bacteria (39), yeast (35,48) and fungi (20,25) have been shown to display antagonism or mycoparasitism. However, their efficiency in vineyards remains to be established since, when soilborne protective microorganisms are applied by spraying, it is difficult to ensure their survival on aerial plant organs. Some have been used as biocontrol agents, among them, *Trichoderma harzianum* has been shown to be effective against *B. cinerea* and formulated as Trichodex (16). Recently, Le Floch et al. (30) showed that *Pythium oligandrum* Drechsler inoculation of tomato roots can protect the plants against *B. cinerea*. In this case, the phenomenon responsible for protection cannot be a direct interaction between the antagonist and the pathogen, but rather a signal from *P. oligandrum* itself or from the plant–microorganism interaction. *P. oligandrum* is also known to secrete an elicitor of plant defense, oligandrin (41), which belongs to a family of proteinaceous elicitors, the elicitins (42). To date, the target plants used in protection studies have been mainly tobacco (*Nicotiana tabacum* var. Xanthi) and tomato (*Lycopersicon esculentum* Mill.), and protection against *Phytophthora parasitica* (41) and phytoplasma (32) has been shown. Chemicals or elicitors (other than elicitins) produced by microorganisms, resulting from plant–microorganism interactions such as laminarin or chitosan have been tested for their capacity to induce grapevine defense reactions against fungal pathogens (2,3,6). Relevant genes associated with the defense mechanisms of grapevine include those involved in the production of stilbenic phytoalexins, mainly stilbene synthase (1,9,27,44). The gene expression and activities of pathogenesis-related (PR) proteins such as chitinases, β-1,3-glucanases (7,10,46,47,51), and nonspecific lipid-transfer proteins (nsLTP) (18) also have been largely studied and related to defense responses.

In this present study, our first objective was to determine if inoculation with *P. oligandrum*, or treatment with its corresponding elicitin, oligandrin, could trigger defense reactions in grapevine and induce protection against the pathogen, *B. cinerea*. Changes in ultrastructure resulting from the plant–microorganism interactions were examined and some investigations at the molecular level were performed. The second objective was to compare these data with the previous data which concern elicitins and mainly tobacco.

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MATERIALS AND METHODS

Plant material. *V. vinifera* cv. Pinot noir (clone 113) grapevine shoots were provided by J.-M. Menant from BIVB (Bureau Interprofessionnel des Vins de Bourgogne), Beaune, France. Herbaceous cuttings (about 10 cm long) were planted in vermiculite in a growth chamber at 20°C (day) and 18°C (night) with a 16-h photoperiod (a 180 µE m^{-2} s^{-2} light irradiance) and a controlled hygrometry (60 ± 8%). Rooted cuttings were obtained and used for bioassay, when three to four expanded leaves were formed. At that time, the root system was composed of 10 to 15 roots, 10 ± 5 cm long.

Fungal inoculum preparation. *P. oligandrum*, strain Py 7, isolated from garlic by Dr. Mugnier (in the southwest of France) was provided by M. Ponchet from the IPMSV collection (IPMSV, [Interactions Plantes-Microorganismes et Santé Végétale] INRA, Antibes, France). It was maintained on malt-agar medium (1%, wt/vol) at 25°C in the dark. Inoculum for plant protection assays was obtained as follows: *P. oligandrum* was grown in petri dishes containing V8 vegetable juice, diluted five times with water, for 4 days. To obtain larger amounts of inoculum, the method described by McQuilken et al. (36,37) was used: the mycelium was transferred in Roux flasks containing 100 ml of glucose-asparagine medium to which cholesterol was added (30 mg/ml final concentration). After 21 days of culture at 25°C in the dark, mycelium was removed by filtration on a Whatman No. 1 filter paper, mycelial mats were rinsed three times with sterile water and ground with water in a Potter tube. For root inoculation, the density of the fungal inoculum was adjusted to 25×10^4 oospores in 25 ml (which corresponds to about 60 mg fresh weight of mycelium).

B. cinerea (strain Bc-3) (provided by B. Paul, IUVV [Institut Universitaire de la Vigne et du Vin]-Université de Bourgogne, Dijon, France) was maintained on malt-agar medium (1%, wt/vol) and subcultured every 3 weeks on the same medium. For grapevine inoculation, *B. cinerea* was grown on potato dextrose broth (PDB) (Sigma, St. Louis, MI) for 8 days. Both cultures were performed at 25°C in the dark.

Oligandrin. Oligandrin (from *P. oligandrum* strain Py 7), provided by M. Ponchet (IPMSV, INRA, Antibes, France), was purified as previously described by Lascombe et al. (28). It is the same protein as that previously purified from another strain of *P. oligandrum* (Po 1010) isolated from pea in Denmark and used in previous works (4,41) (their cDNAs are identical).

Grapevine protection bioassay. Three independent assays were performed with nine plants for each condition. For each assay, three groups of 18 plants were treated at the root level with either *P. oligandrum* inoculum, oligandrin, or water as the control. Each group was then divided into two series, with or without *B. cinerea* leaf inoculation (described below). The plants were maintained in a growth chamber (described previously) during the experiment except, after *B. cinerea* inoculation, the relative humidity was raised to 100%.

Grapevine root inoculation with *P. oligandrum*. *P. oligandrum* inoculation was performed by immersing the roots of 18 plants for 1 h in 25 ml (for each plant) of an oospore and mycelium suspension obtained as described above. Plants were then transferred into pots containing soil and the remaining *P. oligandrum* inoculum was poured onto the soil surface. The 18 control plants were treated the same way except that the inoculum was replaced by water.

Grapevine root treatment with oligandrin. For another group of 18 plants, the roots were rolled and dipped in tubes containing 5 µg of oligandrin dissolved in water (1 ml). Once the solution had been absorbed (3 to 6 h), the plants were transferred into pots containing soil.

Grapevine leaf inoculation with *B. cinerea.* Plants were infected with *B. cinerea* the same day, i.e., 7 days after *P. oligandrum* inoculation or 2 days after oligandrin treatment of the grapevine roots. *B. cinerea* mycelium disks of actively growing hyphae (0.5 cm in diameter) removed from 8-day-old cultures on PDB were placed on the upper surface of three leaves per grapevine plant (one disk per leaf). This constitutes 27 replicates per assay. For each experimental series, inoculation was performed on leaves that were carefully chosen (similar size and position of insertion). After 1, 2, 3, 5, and 7 days, the development of *B. cinerea* was estimated by measuring the areas of the lesions around the mycelial disks. *B. cinerea* leaf invasion is expressed as the percentage of damaged tissue of the total leaf areas and protection against *B. cinerea* as the percentage of the difference between the necrosed areas of the damaged tissues of nonelicited plants and that of the elicited ones, divided by that of nonelicited ones. The statistical analyses of data include an analysis of variance with a significance level of $P < 0.05$.

Analyses of grapevine–microorganism interaction by microscopy. For each experimental series, samples of leaf and root tissues were removed from three grapevine plants, 7 days after *B. cinerea* infection, i.e., 14 days after *P. oligandrum* inoculation and the corresponding control and 9 days after oligandrin treatment. From plants challenged with *B. cinerea*, samples of leaf tissues were removed 1 to 2 cm from the necrotic zone of infection. For noninfected leaves, samples were removed from corresponding areas. Morphological modifications of both plant tissues and microorganisms were analyzed.

From leaf tissues of each of the three series inoculated with *B. cinerea*, five embedded blocks were sectioned and for each block, three levels of sectioning were selected with the light microscope (for which at least two *B. cinerea* hyphae were visible on the leaf surface) before being examined with the transmission electron microscope. The same sampling strategy was used for *P. oligandrum* or oligandrin pretreated plants noninfected with *B. cinerea* although the three levels of sectioning in each block were randomly performed.

Sampling of tertiary lateral roots was randomly done on control plants and *P. oligandrum* or oligandrin pretreated plants. Three different embedded blocks were sectioned at three levels: one in root tip and two in root regions where vascular tissues were differentiated.

Assessment of grapevine root colonization by *P. oligandrum*. Root colonization by *P. oligandrum* was assessed using the trypan-blue staining method proposed by Phillips and Hayman (40). Root systems were removed, heated at 90°C for 30 min in 10% KOH (to clear the tissues), rinsed in tap water, and stained in 0.05% lactophenol trypan-blue solution for 30 min at 90°C. The roots were then examined by bright field microscopy (described below).

Analyses of grapevine–microorganism interaction by light and transmission electron microscopy. Plant tissues were excised in 0.1 M sodium phosphate buffer (pH 7.2) containing 3% (vol/vol) glutaraldehyde and 2% (wt/vol) paraformaldehyde. They were then immersed in the fixative solution under vacuum for 4 h at 4°C, post-fixed with 1% (wt/vol) osmium tetroxide in the same buffer for 1 h at 4°C, and embedded in Epon (Spi-Chem Neyco, Paris, France) according to the usual procedure (34). Thick and ultrathin sections were cut with a Reichert Ultracut E microtome (Leica, Rueil-Malmaison, France). Thick sections (0.5 µm) were mounted on glass slides and stained with 0.1% (wt/vol) toluidine blue, pH 11, prior to examination by bright field microscopy with a DMRB microscope (Leica, Rueil-Malmaison, France). Black and white images were recorded with a Hamamatsu Orca 100 camera coupled with image analysis Visilog software (Noesis, Les Ulis, France). Ultrathin sections collected on grids were counterstained with 3% (wt/vol) uranyl acetate in ethanol and lead citrate for conventional transmission electron microscopy. Sections were examined with a Hitachi 7500 (Hitachi Scientific Instruments Co., Tokyo, Japan) transmission electron microscope operating at 80 kV

and equipped with an AMT camera driven by AMT software (AMT Corp., Danvers, MA).

Cuticle structure and thickness were examined on transversal thin leaf sections. For each series, 15 micrographs, taken from three sections made on each of three blocks, were recorded and analyzed. In the case of *B. cinerea* infection, measurements of cuticle thickness were performed where *B. cinerea* hyphae were visible at the leaf surface. For all plants noninfected with *B. cinerea*, measurements were randomly done on examined sections.

Analyses of root tissues inoculated with *P. oligandrum* by scanning electron microscopy (SEM). Fully hydrated root samples were placed and maintained at –10°C in a variable pressure chamber of a Hitachi S-3000N SEM (Hitachi Scientific Instruments Co., Tokyo, Japan) equipped with an Environmental Secondary Electron Detector and operating at 15 kV with a pressure chamber of 110 Pa.

Reverse transcription-polymerase chain reaction quantification of defense-associated gene transcripts in grapevine leaf tissues: *RNA extraction.* Total RNA was isolated from grapevine leaves (*V. vinifera* cv. Pinot noir) using the method proposed by Chang et al. (11) and further purified using the Rneasy MinElute Cleanup kit (Qiagen GmbH Hilden, Germany).

Real-time reverse transcription-polymerase chain reaction analysis. RNAs (1 µg) from the different leaf samples were incubated in the presence of 1 unit of RQ1 RNase free DNase (Promega, Madison, WI) according to the manufacturer's instructions. Non-reverse-transcribed RNA samples were checked for the absence of contaminating genomic DNA by polymerase chain reaction (PCR) using primers for constitutively expressed actin (7). Samples were then reverse-transcribed using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA), and diluted to a final volume of 400 µl.

Reverse transcription (RT)-PCR analyses were carried out in duplicate in a Bio-Rad Icycler (Bio-Rad) in a final volume of 25 µl containing 5 µl of diluted cDNA, 200 nM of each primer, and 12.5 µl of iQ SYBR Green Supermix (Bio-Rad) for 50 cycles of 10 s at 95°C, 20 s at 59°C, and 30 s at 72°C. Two no-template (water) control wells and one non-reverse-transcribed sample were included as negative controls for each primer pair. Standard curves were generated for each primer pair by performing real-time PCR on serial dilutions (in duplicate) of a specific purified PCR product. Real-time PCR efficiencies were derived from standard curves constructed on the target gene, and were checked to be between 90 and 110%.

To establish the presence of a single PCR product and the absence of primer-dimer, melting analysis was done immediately after PCR by increasing the set point temperature, in 20 s, from 59 to 96°C by 0.4°C steps.

Primers used for actin, β-1,3-glucanase, and stilbene synthase gene expression are as published by Bonomelli et al. (9). Primers for grapevine LTP1 (VitLTP1-F1: GATGGTGATATGCATGGTGGTGG, VitLTP1-R1: CTRCAGCACCCAGSTGGCACTG) were designed on the basis of accessions AF465408.1, AF467945.1, AF467946.1 from GenBank database.

Relative gene expression was calculated using the $\Delta\Delta C_T$ method described by Livak and Schmittgen (33) and grapevine actin as a constitutive gene (7).

RESULTS

Grapevine protection against *B. cinerea* associated with *P. oligandrum* or oligandrin treatment. The development of *B. cinerea* on *V. vinifera* cv. Pinot noir was monitored for 7 days following its application on leaves of rooted cuttings. Grapevine plants subjected to either *P. oligandrum* inoculation or oligandrin pretreatment exhibited reduced leaf lesions compared with non-pretreated plants (Fig. 1). Under both conditions, the decrease of symptom intensities was very noticeable 3 days after inoculation with the foliar pathogen: the lesions caused by *B. cinerea* infection represent 16.5, 13, and 38.5% of the leaf areas for the plants treated with *P. oligandrum*, oligandrin, and the non-pretreated plants, respectively (Fig. 1). Thus, the degree of protection obtained with *P. oligandrum* or oligandrin could be estimated at 57 and 66% (compared with the necroses measured for plants infected by *B. cinerea* without pretreatment), respectively. Five and seven days after *B. cinerea* inoculation, the phenomenon was even more obvious. For the final measurements, in the non-pretreated plants, the spread of *B. cinerea* infection resulted in the rotting of infected leaves that finally fell from the stem, whereas in the other two cases, pathogen progression was slowed and protection could be estimated at 64% for *P. oligandrum* pretreatment and 73% for oligandrin pretreatment. Thus, protection was slightly more efficient with oligandrin pretreatment than with *P. oligandrum* preinoculation throughout the experiment (Fig. 1B). Neither oligandrin nor *P. oligandrum* induced macroscopic foliar necrotic features typically associated with hypersensitive reaction.

Colonization of grapevine roots by *P. oligandrum* and studies of the morphology of root tissues and hyphae. At the end of these experiments, i.e., 2 weeks after *P. oligandrum* inoculation, root branches were sampled and examined: roots (inoculated or not) displayed the same architecture and no necrotic lesions were visible on the *P. oligandrum*-inoculated roots. Oogonia (Fig. 2A) and germinated zoospores (Fig. 2B), which characterize the sexual and asexual life cycles of oomycetes, were regularly dispersed on the root surface of lateral tertiary roots in the mature region. Most of the hyphae emerging from these zoospores had established close contact via appressorium structures with the root surface, and appeared to penetrate tissues at a short distance from the germinated spore (Fig. 2B). In addition, we noticed that only a few hyphae had grown over the root surface, showing light network organization. The fungus was never detected in the apical region of the root. SEM revealed that the hyphae were able to penetrate within the root through intercellular spaces of the epidermis (Fig. 2C). Hyphae were mainly restricted to intercellular spaces between the epidermis and the first outer cortex cells (Fig. 2D). Attempts to penetrate host cell wall by *P. oligandrum* occurred, as seen in Figure 2E, but hyphae failed to penetrate or to disrupt the host cell wall. In

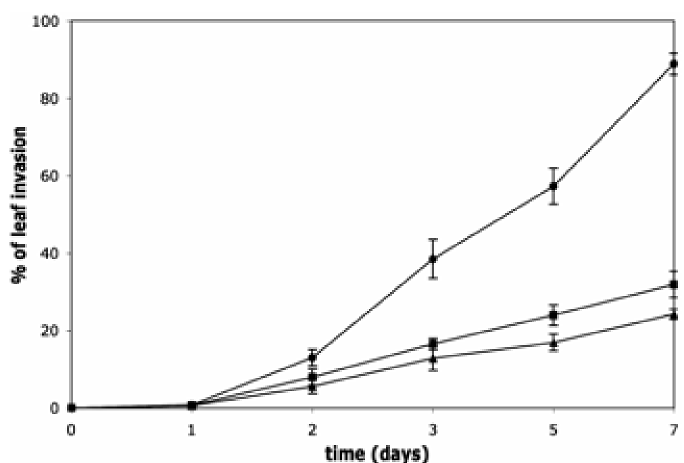

Fig. 1. Time-course development of gray mold (*Botrytis cinerea*) on Pinot noir rooted cuttings preinoculated with *Pythium oligandrum* or pretreated with oligandrin, both at the root level, 7 and 2 days, respectively, before *B. cinerea* inoculation, for *B. cinerea* without elicitation (●), with a *P. oligandrum* preinoculation (■), and with an oligandrin pretreatment (▲). The disease assessment was determined by measuring the average areas ± standard error of lesions formed 1, 2, 3, 5, and 7 days after inoculation (27 replicates) expressed as the percentage of the total leaf area. The experiment was repeated three times.

this grapevine–*P. oligandrum* interaction, host cell walls in contact with the fungus do not exhibit alterations commonly observed when enzymatic degradation occur in planta. The hyphae were highly vacuolated and exhibited altered cytoplasmic content. Cortical host cells also exhibited cytoplasmic content disorganization (Fig. 2D and E). However, no major morphological modifications were observed in *P. oligandrum*-inoculated plants either in leaf or in root tissues compared with the control plants.

Histological and ultrastructural features of oligandrin-treated grapevine root and leaf tissues. Compared with the roots of control plants (Fig. 3A), transverse sections of oligandrin-treated root tissues mostly revealed histological modifications in epidermal and endodermal cells (Fig. 3B). Toluidine-blue stained deposits, which also appeared as electron-dense bodies in transmission electron microscopy (Fig. 3C), had accumulated in the vacuoles. These deposits were previously commonly identified as phenolic compounds.

Observations of thin sections of leaves of oligandrin-treated plants revealed cytological changes suggesting plant defense responses. Palisade and spongy parenchyma cells exhibited electron-opaque structures resembling phenolic inclusions that had accumulated and bordered the tonoplast (Fig. 3D). These compounds also accumulated in vacuoles of phloem parenchyma cells (Fig. 3E). Moreover, occlusions of the lumen of mature sieve tubes of phloem bundles by phloem proteins (P proteins) were regularly observed (Fig. 3E).

Development of *B. cinerea* infection in symptomless leaf tissues of nonelicited grapevine plants. Some initial events of the *B. cinerea* colonization pattern were observed in nonnecrotic areas of infected grapevine leaves. Fungal cells from 7 to 10 µm in diameter, with dense cytoplasm and clearly visible organelles (Fig. 4A) established contact with the leaf surface. Infecting hyphae were also seen inside epidermal cells. The cytoplasm of host epidermal cells was also altered. Most of the hyphae seen on the leaf surface seemed to penetrate leaf tissues through natural

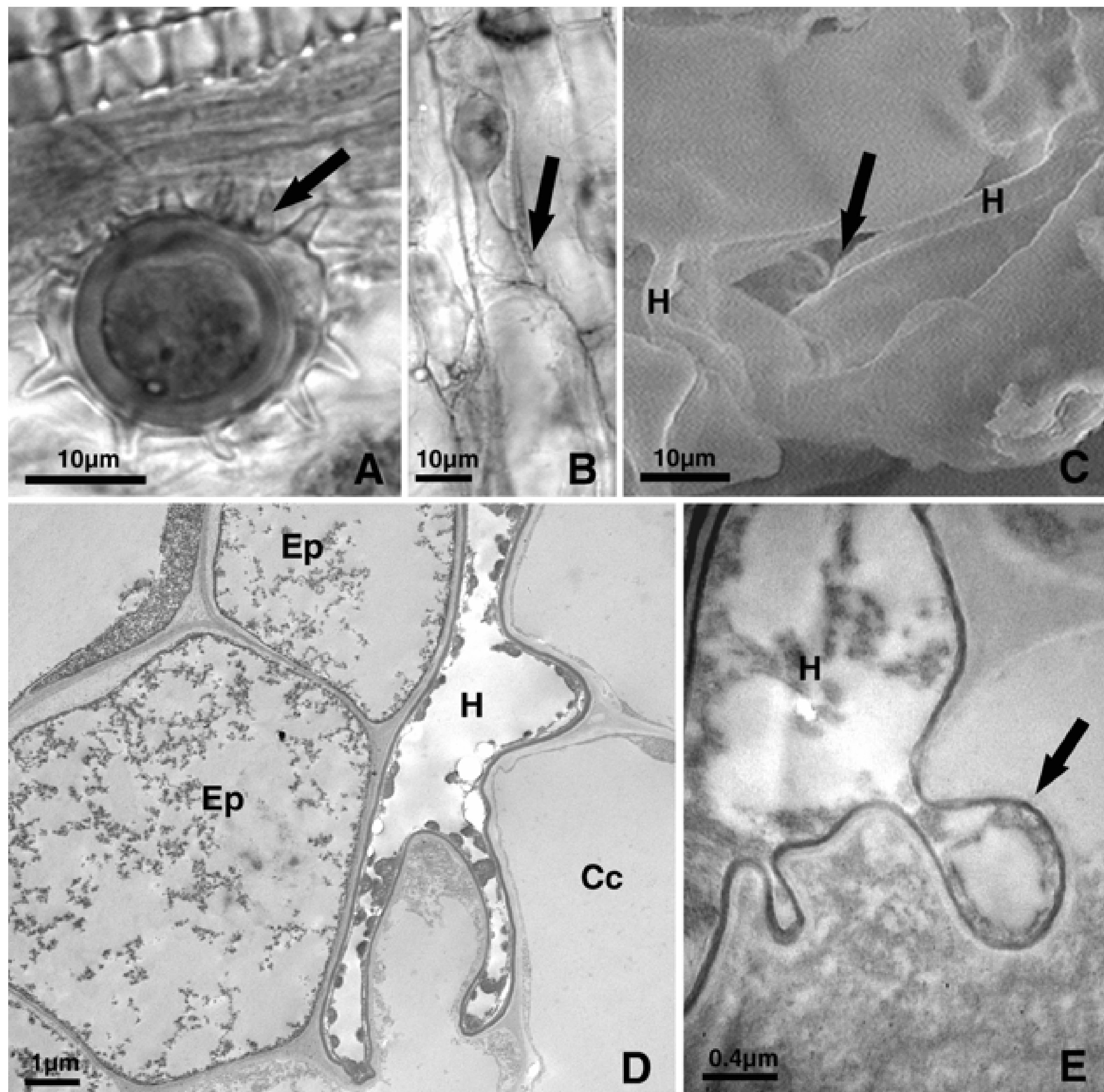

Fig. 2. *Pythium oligandrum* colonization of grapevine (Pinot noir) roots 14 days after inoculation. **A and B,** Light microscope observations of grapevine roots stained with lactophenol-trypan blue. **A,** Oogonium (arrow) and **B,** germinated zoospore with germ tube forming appressorium structures (arrow) visualized on the root surface. **C,** Scanning electron microscopy: presence of hyphae (H) on the grapevine root surface and evidence of penetration through intercellular spaces (arrow). **D and E,** Transmission electron micrographs of transverse section of a tertiary root. **D,** Hypha (H) in intercellular space between epidermis (Ep) and the first cortical cell (Cc) layer of the roots exhibiting high vacuolation and altered cytoplasmic content. **E,** Failure of host cell penetration attempts by *P. oligandrum* hypha (H) and plant cell wall folding without disruption (arrow).

openings such as stomata (Fig. 4B). These results were confirmed by direct light microscopy observations of fresh leaves that showed that *B. cinerea* penetration occurred mostly by stomata and involved hyphae or protoappressoria (data not shown). In the leaf area studied here, *B. cinerea* did not grow in other tissues. Fronting the pathogen hyphae, impregnation of the epidermal cell walls with electron-dense material and indentation of the cuticle were detected (Fig. 4C). The cuticle appeared less osmiophilic, as shown by the reduced electron density (Fig. 4D), than the wax cover of unchallenged leaves (Fig. 4E). Moreover, a significant reduction in the thickness of the cuticle of *B. cinerea*-infected leaves compared with the cuticle thickness of noninfected plants was noted (Table 1).

B. cinerea **interaction with** ***P. oligandrum*** **preinoculated grapevine plants.** Preinoculation of grapevine roots with *P. oligandrum* resulted in a reduction in leaf infection by the pathogen, *B. cinerea*. In the nonnecrotic leaf area, only a few pathogen hyphae were detected. About 50% of these hyphae exhibited marked changes including high vacuolation, lipid accumulation, and disorganization of the cytoplasm, in which mitochondria were no longer visible (Fig. 5A). Wall appositions were visualized at potential penetration sites (Fig. 5A and B). These appositions displayed a matrix impregnated with electron-dense material delimited by a layer of translucent material, probably consisting of callose. Some hyphae appeared altered or as empty shells (Fig. 5C). Electron-dense deposits, which could be phenolic compounds, occurred in the vacuoles of palisade cells (Fig. 5D). Cuticle fronting hyphae was jagged (Fig. 5E), but its thickness was similar to that of the control plants (Fig. 4E; Table 1), or that of *P. oligandrum*-inoculated plants unchallenged with *B. cinerea* (Fig. 5F; Table 1). However, compared with *B. cinerea* infection alone, a significant increase could be seen (0.25 ± 0.03 μm instead of 0.16 ± 0.02 μm).

B. cinerea **interaction with oligandrin pretreated grapevine plants.** In oligandrin pretreated grapevine plants, electron microscope studies of the colonization pattern of *B. cinerea* revealed that 80% of the fungal cells attached to the leaf surface were altered (Fig. 6A). Moreover, part of mycelium was completely devoid of cytoplasm content (Fig. 6B). Electron-dense cuticle layers, stained with osmium tetroxide, specific of lipid components (Fig. 6C), were always significantly thicker than that of the pretreated plants unchallenged with *B. cinerea* and the phenomenon was more pronounced than that observed with *P. oligandrum* preinoculation (Fig. 6D; Table 1). Some epidermis cell walls were impregnated with compounds possibly of phenolic nature (Fig. 6E and F). Strong plant defense responses also occurred locally in epidermis and parenchyma cells, such as reinforcement of cell walls with electron-dense material and translucent deposits along the primary cell wall (Fig. 6G). There was some evidence of plant cell death in the vicinity of dead hyphae (Fig. 6F). Surprisingly, electron-dense material was seen to fill the xylem vessel lumen, and reinforcement of the primary cell walls of vascular parenchyma cells with translucent layer was also visible (Fig. 6H).

RT-PCR quantification of defense-associated gene transcripts in grapevine leaf tissues. The expression of three defense-related genes, LTP-1, β-1,3-glucanase, and stilbene synthase, was analyzed in grapevine leaves by real-time RT-PCR in response to treatment by oligandrin or *P. oligandrum* and/or to infection with *B. cinerea*. The grapevine actin gene was selected as an internal control to normalize the different samples for differences in amounts of plant RNA. Gene expression was considered enhanced when induction was greater than five times that of the untreated control leaves. This technique gave reproducible results that were confirmed by northern blot analysis (data not shown). In control leaves, the transcript levels were low except in the case of stilbene synthase. This slight induction could be a result of slight stress related to the experimental conditions. Our experiments allowed us to compare each treatment with the control and each "double" treatment with its corresponding "single" treatment.

Transcript accumulation levels of LTP-1 in leaves at 36 or 72 h following infection with *B. cinerea*, in plants either preinoculated or not with *P. oligandrum* and/or infected with *B. cinerea* were similar to the control, indicating that LTP-1 expression was not enhanced in response to elicitation by *P. oligandrum* and/or inoculation with *B. cinerea* (Fig. 7A). In contrast, expression of LTP-1 increased in response to oligandrin compared with the control, with greater induction observed at 36 h. A similar level of induction of LTP-1 expression was observed when elicitation by oligandrin was associated with *B. cinerea* infection, indicating that oligandrin was responsible for enhancing LTP-1 expression.

Compared with the control, induction of β-1,3-glucanase was observed in plants treated with oligandrin, and when oligandrin treatment was followed by inoculation with *B. cinerea*. The slight gene induction observed in plants treated with *P. oligandrum* increased following inoculation with *B. cinerea*. High levels of transcripts were found from nonelicited plants 72 h following inoculation with *B. cinerea* (Fig. 7B). Induction was less important when inoculation of *B. cinerea* followed treatment with *P. oligandrum* or oligandrin than with *B. cinerea* alone. In all treatments involving *B. cinerea*, transcript accumulation levels observed after 72 h were greater than those observed after 36 h.

Stilbene synthase transcript levels in grapevine leaves increased in response to treatment with *P. oligandrum*, oligandrin, or *B. cinerea* at 36 and 72 h (Fig. 7C). When *B. cinerea* inoculation was associated with either *P. oligandrum* or oligandrin, induction levels were less important than for *B. cinerea* alone.

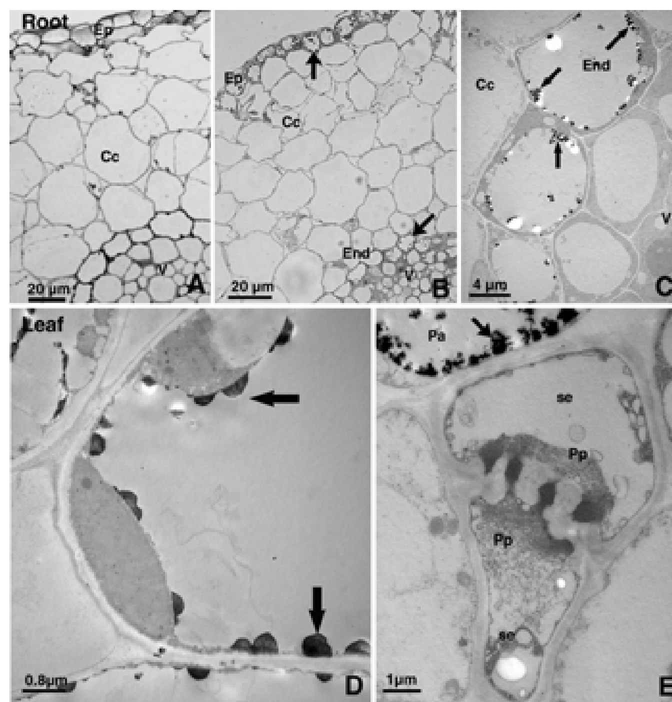

Fig. 3. Histological and cytological modifications induced by oligandrin (9 days after treatment) on grapevine plants in **A to C,** root and **D and E,** leaf tissues. **A and B,** Light micrograph of transverse section of grapevine root tissues stained with toluidine blue. **A,** control. **B,** Section of oligandrin-treated roots showing slight accumulation of densely stained droplets (arrows) in vacuoles of epidermis (Ep) and endodermis (End). Cc, cortical cells; V, vascular cylinder. **C,** Transmission electron micrograph observations of endodermal cells (End) with accumulation of material, likely phenolic compounds, in vacuoles (arrows). **D and E,** Thin leaf sections of oligandrin-treated grapevine plants. **D,** Similar material regularly dispersed along the tonoplast of palisade cells (arrow), **E,** also visualized in parenchyma cells of phloem bundles (Pa) (arrow). Occlusion of pore sites of sieve plate with phloem proteins (Pp) in the lumen of sieve elements (se).

DISCUSSION

The results presented in this paper describe the ability of either *P. oligandrum* or oligandrin to induce grapevine defense reactions against *B. cinerea* infection. The cytological investigations gave some information on the mechanisms by which protection against *B. cinerea* infection is conferred by *P. oligandrum* or oligandrin.

In symptomless leaf tissues of non-pretreated plants, the penetration and postpenetration processes of grapevine leaves by *B. cinerea* occurred as already described for other plants (13,17,23).

Very few studies have reported the ultrastructure features of elicitin-treated plant tissues. Benhamou et al. (4) briefly mentioned that exogenous applications of oligandrin on a tomato leaf petiole did not yield appreciable effects on the induction of plant defense reactions in root tissues. In addition, Lherminier et al. (32) previously reported that oligandrin, when applied onto the decapitated apex of stems or leaf petioles of tobacco, elicited in both, plant defense responses such as impregnation of cell walls with phenolic compounds, formation of calcium pectate gels in intercellular spaces, and accumulation of phloem proteins in phloem bundles (32). In direct line with these earlier results, in this work, some structural modifications were observed in grapevine leaves and roots after treatment of the roots with oligandrin: deposits, deemed to be phenolic compounds, appeared in the vacuoles of root endodermis cells and leaf parenchyma cells, and phloem proteins were observed in the lumen of leaf sieve tubes. Altogether, these results suggest that oligandrin elicits, in planta, typical defense responses that seem to be common to different plant species.

P. oligandrum and oligandrin pretreated plants appear to display resistance to *B. cinerea* infection by reinforcing protective barriers at the first steps of the infection process. They induce both cell wall appositions (papillae) and impregnation or coating of cell walls with electron-dense compounds, that are most probably phenolic compounds, which form efficient barriers in both epidermal and first parenchyma cells at potential penetration sites of the pathogen. These responses restrict progression and further penetration of *B. cinerea* into plant leaf tissues as previously reported (15,38). However, in oligandrin pretreated plants, these host reactions could lead to host cell death close to *B. cinerea* penetration sites. The cuticle also appeared to be an important barrier to *B. cinerea* penetration as already reported (15,19,45). In *P. oligandrum*-inoculated plants, the cuticle thickness was not significantly increased compared with that of the

TABLE 1. Cuticle thickness of noninfected versus infected grapevine leaves

Grapevine plant root system with	Cuticle thickness[a] ± SD (μm)	
	Noninfected leaves	*Botrytis cinerea*-infected leaves
Water pretreatment	0.21 ± 0.02 (100%)	0.16 ± 0.02 (76%)
Pythium oligandrum preinoculation	0.23 ± 0.03 (110%)	0.25 ± 0.03 (119%)
Oligandrin pretreatment	0.26 ± 0.03 (124%)	0.37 ± 0.03 (176%)

[a] For grapevine leaves challenged with *B. cinerea*, measurements were performed where hyphae were detected on transverse sections of leaves, at a microscope magnification of ×40,000. Expression of the changes in percent compared with the water treatment is indicated in parentheses.

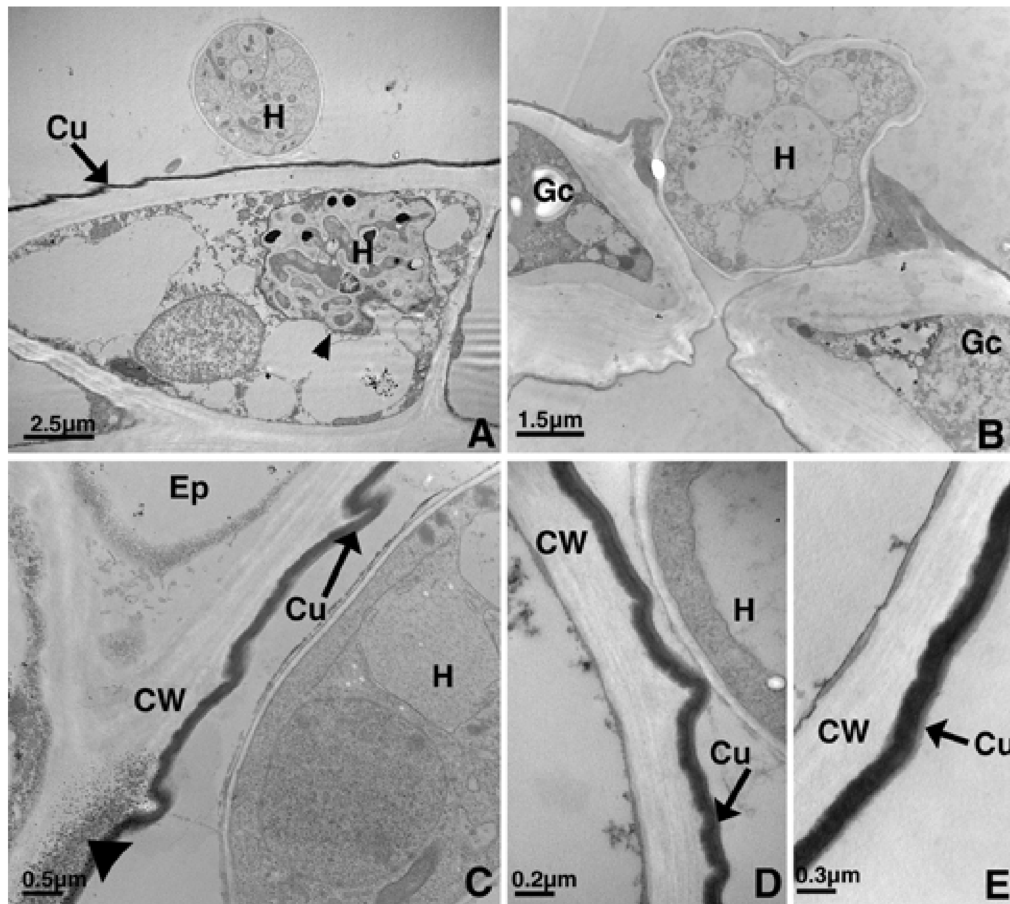

Fig. 4. Transmission electron micrographs of colonization pattern of *Botrytis cinerea* in symptomless area of an inoculated leaf (non-pre-treated plant). **A,** Jagged cuticle (Cu) fronting a hypha (H) (arrow). Intracellular colonization of epidermal cell with branched hyphae (H) surrounded by a membrane (arrowhead). **B,** Hypha (H) penetration through stomata. Gc, guard cell. **C,** Slight impregnation of the primary cell wall (CW) with electron-dense material corresponding to localized plant defense reaction (arrowhead). **D,** Cuticle thickness is significantly reduced (0.16 ± 0.02 μm) compared with **E,** cuticle thickness of noninoculated control grapevine leaf (0.21 ± 0.02 μm).

control. In contrast, in the presence of the pathogen hyphae, the preinoculation induced an increase in cuticle thickness compared with *B. cinerea* alone, which counteracted the degradation probably due to *B. cinerea* cutinases. In oligandrin-treated plants, cuticle was slightly thicker than that of the controls, and the increase following *B. cinerea* infection was more pronounced that with *P. oligandrum* preinoculation. Ultrastructural changes that involve the internal layering of the cuticle material occurred, suggesting an increase in fatty acid content of the cuticle membrane. Such structural modifications have already been reported in other plant–microbe interactions (26,53). The hypothesis that, among the responses associated with defense against *B. cinerea* infection of oligandrin-treated and to a lesser extent, *P. oligandrum*-inoculated grapevine plants, LTP genes could be induced was confirmed by real-time RT-PCR experiments, where the elicitin oligandrin is shown to be efficient in inducing expression of transcript levels of defense-related genes, LTP-1, β-1,3-glucanase, and stilbene synthase. LTPs in grapevine cell suspensions have been shown to be induced by defense-related signals such as ergosterol, or by a proteinaceous elicitor secreted by *B. cinerea* (18). During plant–pathogen interactions, nsLTPs might transport lipids (cutin monomers) to favor the synthesis of cutin at the outer surface of the epidermal cells (21,24,50). This contributes to enhance the physical properties of epidermal cell walls. Moreover, these nsLTP-cutin monomer complexes are known to trigger plant defense mechanisms leading to the death of hyphae (8). Neither *P. oligandrum* nor *B. cinerea* alone or together induced an LTP response in grapevine. This may reflect a dose effect. *P. oligandrum*, oligandrin, and *B. cinerea* triggered increases in β-1,3-glucanase and stilbene synthase transcript levels. Interestingly, in both cases, both β-1,3-glucanase and stilbene synthase appeared to be less induced when *B. cinerea* was associated with either oligandrin or *P. oligandrum*. This could be due to a different kinetics of accumulation, with a maximum reached earlier when the elicitation is applied. β-1,3-Glucanase is an important PR protein involved in protection against fungal attack. Stilbene synthase is a key enzyme in the production of phytoalexins, and is a well-characterized defense reaction in grapevine (49). Bézier et al. (7) reported stilbene synthase gene expression in grapevine leaves infected with *B. cinerea*. An accumulation of stilbene synthase transcripts also was detected during grapevine leaf and *Pseudomonas syringae* incompatible interactions with a maximum about 72 h after inoculation (46).

The ability of *P. oligandrum* to colonize grapevine roots is very poor and its ingress is restricted to the epidermis cell layer. No host defense reactions were seen either in roots or in aerial organs of these *P. oligandrum*-colonized grapevine plants. This restricted colonization was associated with minor damage to the root cells adjacent to *Pythium* hyphae, whereas structural alterations of invading *P. oligandrum* hyphae have been seen. This *P. oligandrum* root colonization pattern has previously been mentioned on rice (14) and cucumber roots (52). In contrast, it has been reported that *P. oligandrum* extensively colonizes tomato roots (5,29,43), and that this phenomenon is associated with host defense reactions such as the formation of papillae-like structures at sites of hypha penetration (5). Therefore, the interaction between *P. oligandrum* and plants seems to display some host specificities. Although *P. oligandrum* is largely distributed in vineyard soils, our experiments suggest that grapevine is a nonhost plant for this *Pythium* species. Nevertheless, despite its poor colonization ability, *P. oligandrum* can protect grapevine plants from *B. cinerea* attack. *P. oligandrum* is known to secrete oligandrin in vitro and it is probably able to secrete this protein in planta, but in an amount inferior to the one we applied. This could explain the slightly higher degree of protection with oligandrin treatment compared with *P. oligandrum* inoculation.

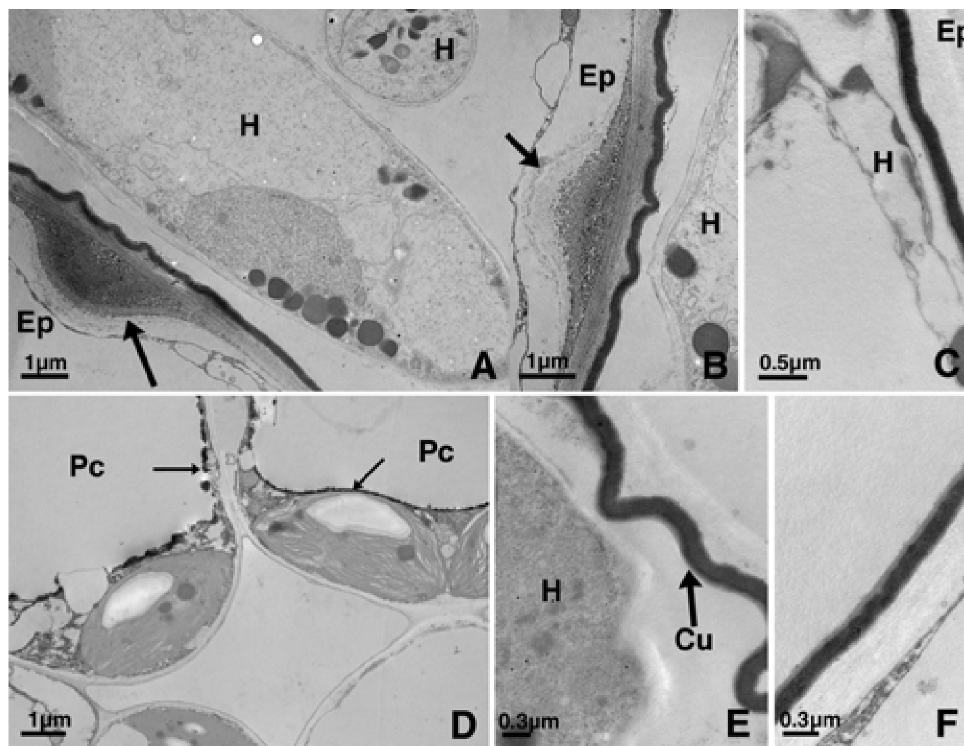

Fig. 5. Transmission electron micrographs of grapevine leaf tissues of plants preinoculated with *Pythium oligandrum* and **A to E,** challenged or **F,** unchallenged with *Botrytis cinerea.* **A and B,** Highly vacuolated hyphae (H) of *B. cinerea* at the leaf surface and a wall apposition formed at a potential pathogen penetration site in the epidermis cells (Ep) (arrows). **C,** Severely damaged hyphae at the leaf surface. **D,** Accumulation of electron-dense material in parenchyma palisade cells (Pc) due to *B. cinerea* infection (arrows). **E,** Jagged cuticle (Cu) fronting pathogen hypha (H). Cuticle ultrastructure and thickness (0.25 ± 0.03 µm) are comparable to that of noninoculated leaves of plants pretreated with *P. oligandrum* (0.23 ± 0.03 µm), **F** but cuticle seems to be restored compared with that of the leaf challenged with *B. cinerea* without pretreatment (0.16 ± 0.02 µm) (Fig. 4D).

Thus, oligandrin induces grapevine defense responses. When cryptogein, an elicitin from *Phytophthora cryptogea*, was assayed in the same experimental conditions, similar results were obtained. Grapevine plants were protected against *B. cinerea* infection and the molecular markers used as described above were expressed (data not shown). Grapevine response to elicitin is not associated with a hypersensitive reaction with either oligandrin or cryptogein. In the same way, in tobacco, oligandrin induced plant defense against pathogen infection and no necroses were observed (32,41). In contrast, cryptogein is known to trigger a hypersensitive-like reaction (42). In addition, and with the exception of oligandrin, when elicitins are infiltrated in tobacco leaves, the cells of the corresponding area are killed and form a necrotic zone. Determination of plant reactivity to elicitins has been based mainly on this criterion (42). Tobacco and only some members of the family *Brassicaceae* were found to develop necroses in a cultivar-specific manner (42).

In conclusion, grapevine can be protected against *B. cinerea* leaf infection by a *P. oligandrum* inoculation or an elicitin pretreatment applied at the root level of rooted cuttings with compar-

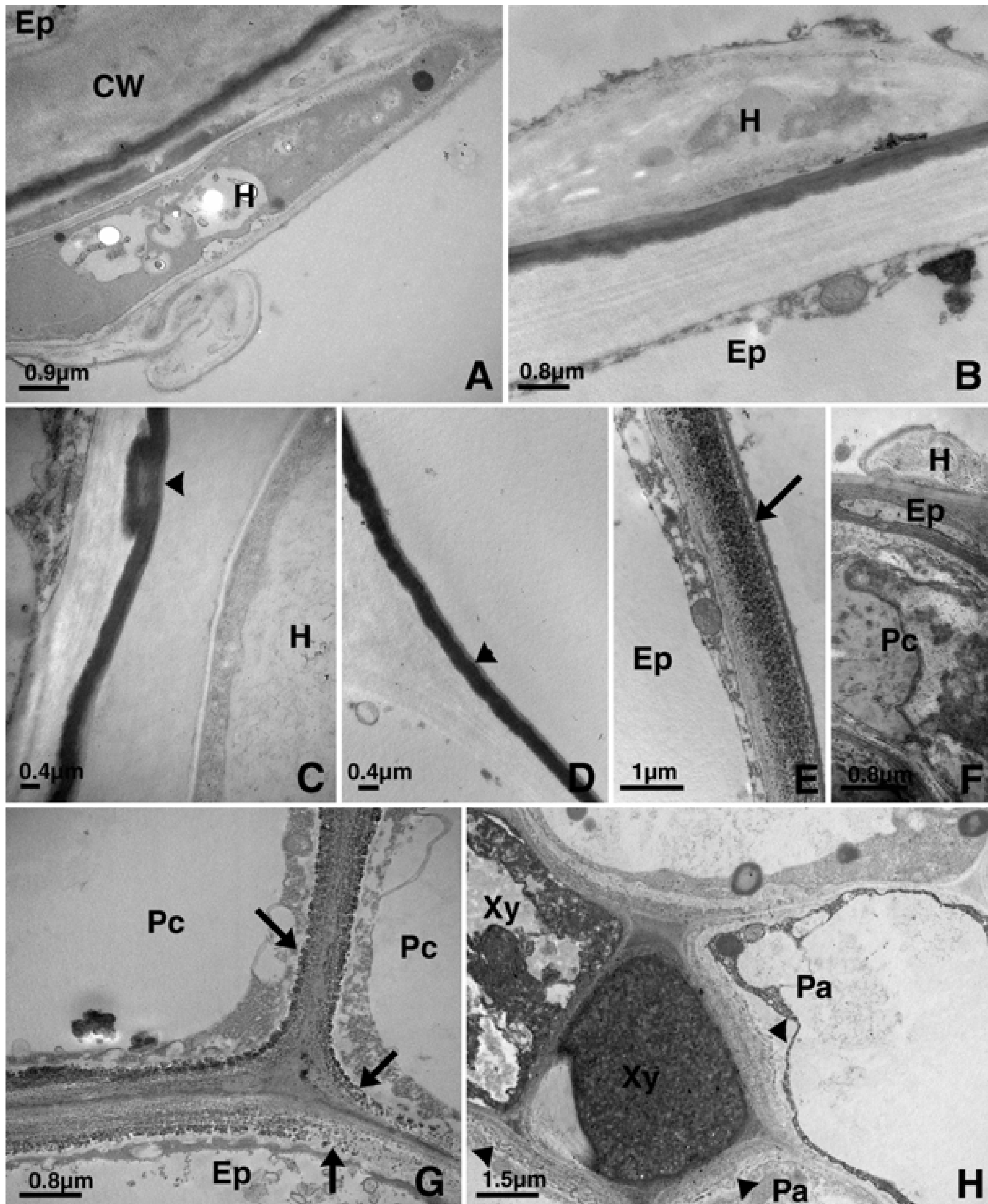

Fig. 6. Transmission electron micrographs of grapevine leaf tissues of plants pretreated with oligandrin and **A to C and E to H,** challenged or **D,** unchallenged with *Botrytis cinerea.* **A and B,** Dead hyphae (H) detected at the leaf surface. **C,** Modification of cuticle morphology and thickness (0.37 ± 0.03 µm) compared with **D,** oligandrin-treated plants unchallenged with the pathogen (0.26 ± 0.03 µm) and with the leaf challenged with *B. cinerea* without pretreatment (0.16 ± 0.02 µm) (Fig. 4D). **E,** Impregnation of epidermis (Ep) cell wall with electron-dense material. **F,** Plant cell death occurring in the vicinity of pathogen hyphae (H). Ep, epidermis; Pc, parenchyma palisade cell. **G,** Reinforcement of lateral epidermal cell (Ep) and primary wall of palisade cells (Pc) with electron-dense material and deposit of callose (arrows). **H,** Occlusion of xylem vessels (Xy) with dense material and wall apposition in parenchyma vascular cells (Pa) (arrowheads).

able efficiencies. The accumulation of defense-related gene transcripts differ. This could mean that in both cases oligandrin is involved but in the case of *P. oligandrum* preinoculation, other mechanisms may interfere with it. While results here indicate the induction of defense-related genes, an interesting complementation to the present work would be to study the kinetics of these inductions, to investigate enzymatic activity, immunodetection of gene products, and accumulation of phytoalexins. It also would be interesting to see if these two pretreatments could be efficient in reducing symptoms due to (i) *Plasmopara viticola* on leaves or (ii) *B. cinerea* in grapevine inflorescences or at different stages of berry maturation, which are major problems in viticulture.

This work also brings evidence that the hypersensitive reaction is not an obligatory mechanism in the general elicitin mode of action. It may occur at a microscopic level, which is not as easy as the macroscopic one to detect. Therefore, it will be necessary to reassess plant reactivity to elicitin, not only with symptom description but also at the molecular level and to evaluate stimulation of plant defense. This work opens new perspectives in the field of plant defense mechanisms using elicitins as tools in phytoprotection.

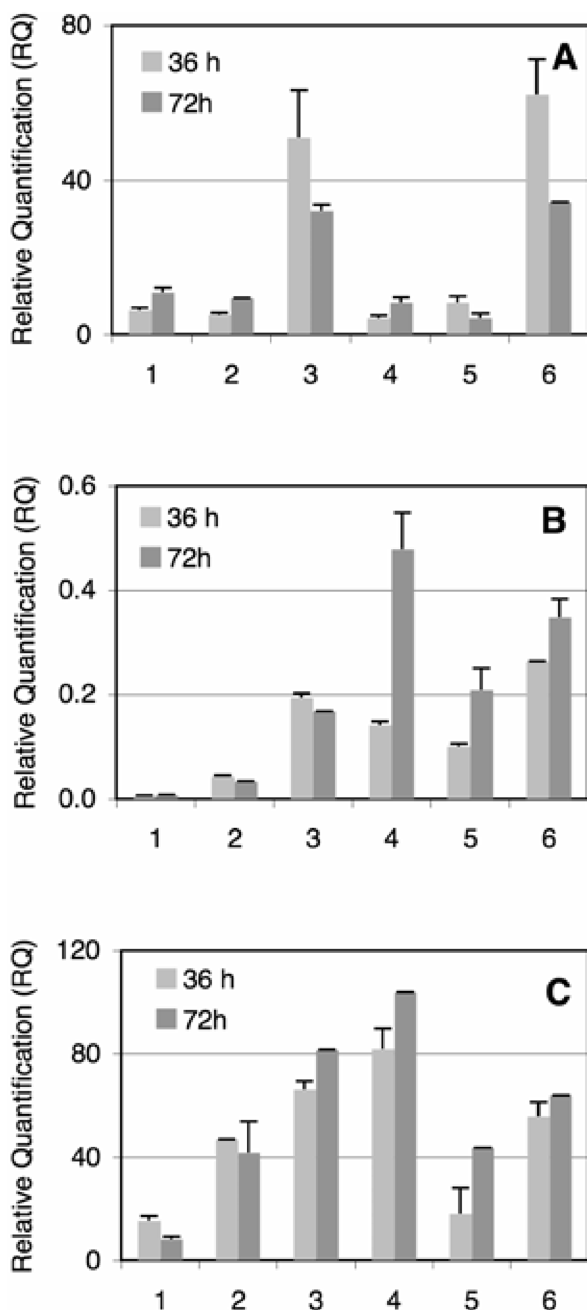

Fig. 7. Real-time quantitative reverse transcription (RT)-polymerase chain reaction analysis of the modification of some grapevine gene transcript accumulation. **A,** LTP-1, **B,** glucanase, and **C,** stilbene synthase. Equivalent cDNA samples were obtained from RT of RNA, from leaves of grapevine (cv. Pinot noir), following elicitation with *Pythium oligandrum*, oligandrin (5 µg per plant), and/or challenged or unchallenged with *B. cinerea* for 36 h (light gray bars) or for 72 h (dark gray bars). The transcript accumulation of three defense-related genes is expressed as the percentage of the amount of actin transcript. 1, control; 2, *P. oligandrum*; 3, oligandrin; 4, *B. cinerea*; 5, *B. cinerea* and *P. oligandrum*; and 6, *B. cinerea* and oligandrin.

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