Plant Physiol. Biochem. 39 (2001) 681–698

S0981942801012839/FLA

Oligandrin, the elicitin-like protein produced by the mycoparasite *Pythium oligandrum*, induces systemic resistance to *Fusarium* crown and root rot in tomato plants

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Received 20 January 2001; accepted 4 April 2001

Abstract – Oligandrin, the elicitin-like protein produced by the mycoparasite *Pythium oligandrum*, crab shell chitosan and crude glucans, isolated from *P. oligandrum* cell walls were applied to decapitated tomato plants and evaluated for their potential to induce defence mechanisms in root tissues infected by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. A significant decrease in disease incidence was monitored in oligandrin- and chitosan-treated plants as compared to water-treated plants whereas glucans from *P. oligandrum* cell walls failed to induce a resistance response. Ultrastructural investigations of the infected root tissues from water-treated (control) plants showed a rapid colonization of all tissues including the vascular stele. In root tissues from oligandrin-treated plants, restriction of fungal growth to the outer root tissues, decrease in pathogen viability and formation of aggregated deposits, which often accumulated at the surface of invading hyphae, were the most striking features of the reaction. In chitosan-treated plants, the main response was the formation of enlarged wall appositions at sites of attempted penetration. These wall appositions were found to vary greatly in their appearance from multi-textured to multi-layered structures and to contain large amounts of callose. The use of the WGA/ovomucoid-gold complex provided evidence that the wall-bound chitin component in *Fusarium* cells colonizing roots of oligandrin-treated tomato plants was not substantially altered even over cell walls of hyphae showing obvious signs of degradation. Evidence is provided in this study that oligandrin has the ability to induce systemic resistance in tomato. Exogenous, foliar applications of the fungal protein sensitize susceptible tomato plants to react more rapidly and more efficiently to *F. o.* f. sp. *radicis-lycopersici* attack, mainly through the massive accumulation of fungitoxic compounds at sites of attempted pathogen penetration. Although cell wall modifications do not represent the central core of the oligandrin-mediated host response in tomato, they are part of the multicomponent defence system elaborated to fend off *Fusarium* invasion. © 2001 Éditions scientifiques et médicales Elsevier SAS

biological control / chitosan / elicitors / host-pathogen interactions / oligandrin / phenolic compounds / wall appositions

FORL, *Fusarium oxysporum* f. sp. *radicis-lycopersici* / SAR, systemic acquired resistance / WGA, wheat germ agglutinin

1. INTRODUCTION

In recent years, the concept of systemic acquired resistance (SAR), initially restricted to enhanced protection against plant pathogens by prior inoculation with necrotizing agents [24], has expanded to include resistance induced by an array of biotic and abiotic agents [36, 37]. Several lines of evidence have shown that activation of the natural plant defence system could occur upon exogenous applications of chitin and chitosan oligosaccharides [3], natural products such as salicylic acid [25] and certain chemicals including 2,6-dichloroisonicotinic acid (INA), β-aminobutyric acid [18] and benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) [4].

Another class of resistance elicitors that is attracting much attention concerns the fungal-derived proteinaceous molecules grouped under the generic name 'elicitins' [39]. The rationale for such an interest in

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elucidating the biological properties of these molecules is that not only do elicitins have specific mechanisms of action on gene expression in plants [16, 20, 27], but also their simple nature offers the best prospects for the production of synthetic analogues that can be introduced as new biocontrol strategies for crop protection [15]. Since the first identification of proteinaceous elicitors in the culture filtrate of *Phytophthora cryptogea* [14], molecules with similar characteristics including size (98 amino acids), amino acid composition, UV spectrum and three-dimensional structure have been isolated from a large number of *Phytophthora* species [30]. Later on, evidence was provided that production of such peptides was not restricted to the *Phytophthora* genus but occurred also within some *Pythium* isolates [28].

In spite of the increasing amount of research devoted to the antagonistic activity of *P. oligandrum* [11, 19], the exact mechanisms responsible for the observed reduction of disease incidence following soil treatment with *P. oligandrum* are still unknown [26]. While control of the pathogen population density through direct antimicrobial activity may be associated, at least partly, with the enhanced plant protection described by several authors, it is only recently that the ability of *P. oligandrum* to trigger indirect effects by stimulating the plant defence machinery has been reported [10]. In direct line with these observations, attempts were recently made to identify the trigger involved in *P. oligandrum*-mediated induced resistance. The isolation of a proteinaceous metabolite bearing the 'elicitin signature', as shown by the amino acid composition of its N-terminal end and by its migration profile within the plant tissues [30], led to the inclusion of this peptide, termed oligandrin, into the elicitin family [29]. While treatment of tomato plants with the oligandrin failed to provoke the HR-associated necrotic response, a reaction consistently found to occur in tobacco plants treated with true elicitins [30, 32], a substantial level of protection against the oomycete fungus, *Phytophthora parasitica*, was noticed thus substantiating the concept that oligandrin could be considered as a resistance elicitor.

To determine whether the signalling role of oligandrin as an inducer of resistance against a foliar pathogen [29] was operational against a soilborne pathogen, we investigated the effectiveness of a stem treatment with oligandrin in inducing systemic resistance in tomato plants against the root pathogen, *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL). The objectives of the present research were to investigate ultrastructurally the outcome of the tomato-*Fusarium* interaction upon oligandrin treatment and to compare the cytologically visible consequences of the induced response to those triggered by other biotic elicitors including chitosan [5] and *P. oligandrum* cell wall glucan extracts. The rationale for the choice of these compounds was based on the concept that chitosan was already known to be a strong inducer of structural defence-related response in FORL-infected tomato plants [5–7, 21] and that microbial oligosaccharides including β-linked glucans could stimulate a general resistance response in plants [3, 5]. Data are presented indicating that tomato root cells undergo marked ultrastructural modifications upon oligandrin treatment that correlate with restriction of pathogen ingress towards the vascular stele. The nature and spectrum of these changes appear, however, to depend on the nature of the elicitor, thus suggesting that elicitation of metabolic changes do not follow a general pathway upon signal recognition.

2. RESULTS

2.1. Symptomatology

Non-inoculated, decapitated tomato plants treated with sterile water instead of elicitor solutions did not apparently suffer from wounding damage as judged by the absence of visible symptoms such as root lesions and leaf chlorosis. By contrast, typical root symptoms, characterized by severe rotting or even loss of the primary seminal root and occurrence of numerous brown lesions along the lateral roots were easily noticed on decapitated tomato plants that were challenged with FORL (*table I*). When the outer layers of crown and lower stem were sliced off, a chocolate-brown vascular discoloration was visible along the vascular stele (not shown).

Application of either chitosan or oligandrin to the apex of decapitated tomato plants substantially reduced symptom severity of *Fusarium* wilt as compared with controls (*table I*). By day 5 after inoculation, the treated plants were free of visible symptoms such as wilting and exhibited a markedly reduced number of root lesions (*table I*). When present, the lesions were much smaller in size than those observed in untreated plants and did not spread through the root system. No difference in terms of plant viability was observed among treated plants whether they received chitosan or oligandrin.

Table I. Effect of chitosan, oligandrin and glucans from *Pythium oligandrum* cell walls on the number of root lesions induced by *Fusarium oxysporum* f. sp. *radicis-lycopersici* in tomato roots. [a] The number of root lesions was determined from observations of ten main roots per day after inoculation with *Pythium ultimum*; [b] values are standard errors of the mean.

Treatment	Number of root lesions (4 d after inoculation)
Control (water)	12 ± 2.0
Chitosan	1[a] ± 0.50[b]
Oligandrin	2 ± 0.50
Cell wall glucans	10 ± 1.5

Treatment of tomato plants with a crude glucan preparation from *P. oligandrum* cell walls did not prevent disease establishment as judged by the development of leaf and root symptoms similar to those observed in inoculated, untreated plants (*table I*). In the absence of pathogen inoculation, elicitor-treated plants exhibited an appearance similar to that of elicitor-free, non-inoculated plants.

2.2. Histological observations of root tissues from decapitated tomato plants

In the absence of pathogen challenge, treatment of tomato plants with the elicitors failed to stimulate visible cellular changes as judged by the absence of typical wall appositions or intercellular space occlusions (not shown).

Root tissues from water-treated tomato plants (controls) were massively colonized by hyphae of FORL, 5–6 d after inoculation (*figure 1A*). Pathogen ingress towards the vascular stele was accompanied by severe host cell alterations including cell wall breakdown as evidenced by the reduced density of wall staining with toluidine blue in some areas (*figure 1A*, arrow).

Pre-treatment of tomato plants with chitosan prior to inoculation with FORL did not halt pathogen penetration into the root epidermis (*figure 1B*). However, striking differences in the rate and extent of fungal colonization were observed as compared to controls. Pathogen growth was restricted to the outermost root tissues including the epidermis and first cell layers of the outer cortex. Such a restriction of fungal growth was found to correlate with the elaboration of structural changes mainly characterized by an increase in staining density of the host cell wall (*figure 1B*, arrow) and by the formation of elongated wall appositions at sites of potential pathogen penetration (*figure 1C, D*). Other typical modifications included the deposition of an amorphous material in some invaded cells and intercellular spaces. Fungal cells, trapped in this material, appeared as empty hyphal shells (*figure 1D*).

Treatment of tomato plants with oligandrin prior to challenge with FORL was also associated with restricted fungal growth and with marked host metabolic changes (*figure 1E*). One of the most prominent responses to oligandrin treatment was the accumulation of a densely stained material along the primary walls of several infected host cells (*figure 1F*, arrows). This material extended usually towards the inside to form either droplets that frequently surrounded hyphae of the pathogen (*figure 1F*, arrowheads) or an amorphous matrix in which fungal cells were embedded (*figure 1H*) . A large number of intercellular spaces in the invaded outermost root tissues were also filled with a granular material that stained densely with toluidine blue (*figure 1G*). Although less frequently encountered than in chitosan-elicited plants, wall appositions were occasionally seen in areas neighbouring invaded intercellular spaces (*figure 1I*).

2.3. Ultrastructural features of root tissues from elicitor-free tomato plants

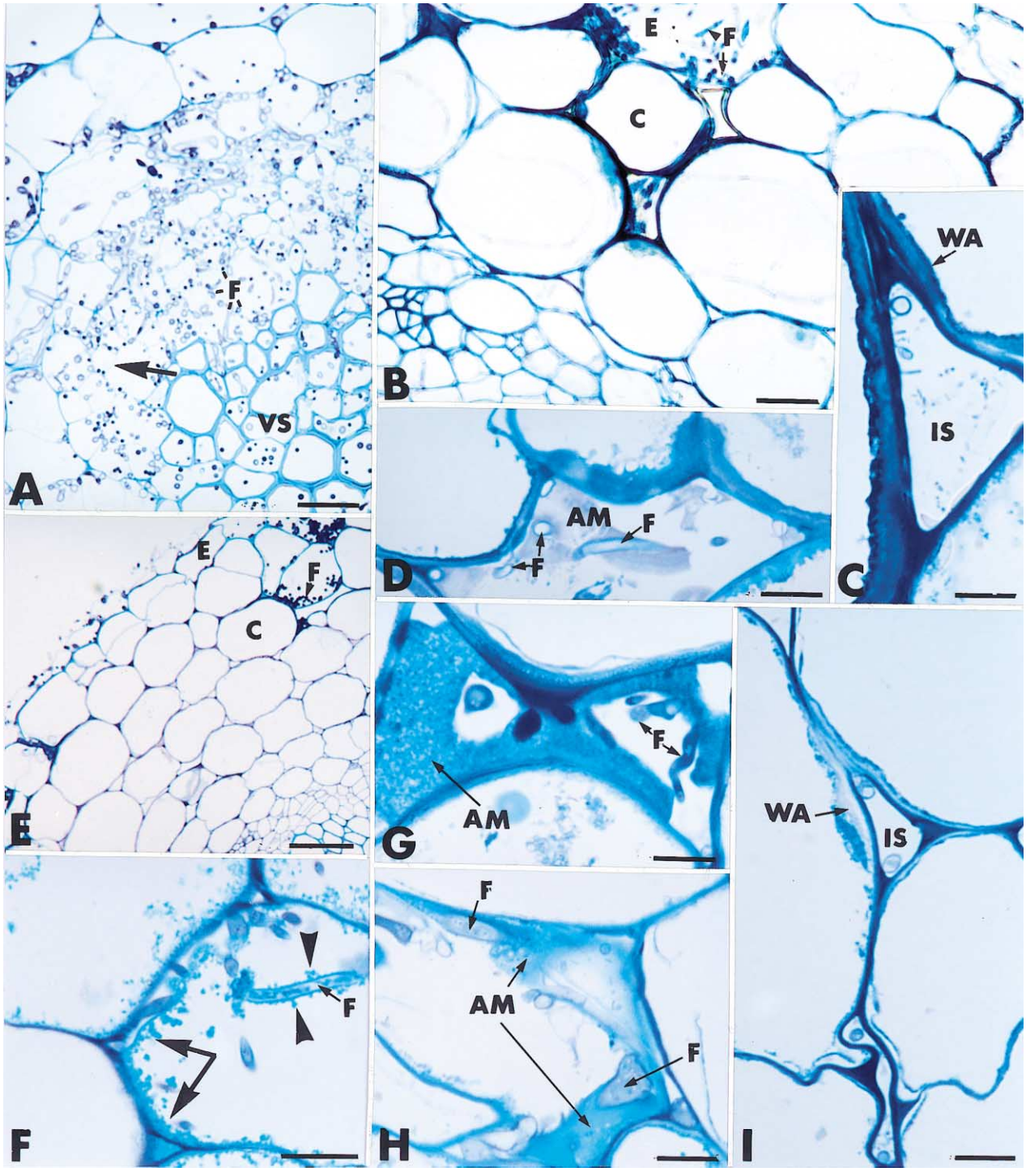
2.3.1. Non-inoculated plants

Root samples from non-inoculated tomato plants that were treated with water at the decapitated apex showed an ultrastructure similar to that known to occur in healthy, entire plants. Cellular damage as well as cell wall and organelle alterations were not observed. Typical host cell defence reactions such as wall appositions and intercellular space occlusion were not detected (not shown).

2.3.2. Inoculated plants

Examination of root samples, collected near the infection sites 4 d after inoculation with FORL, revealed a pattern of fungal colonization identical to that previously described with entire tomato plants [7]. The pathogen ramified in all tissues, including the vascular stele, causing severe cell damage including cytoplasm disorganization and organelle disintegration (*figure 2*). Primary cell walls were markedly altered as judged by their reduced electron density and their shredded aspect (*figure 2A, B*). In most cases, cell walls were reduced to strands of disorganized fibrils (*figure 2A, B*, arrows). Direct cell wall penetration by means of constricted hyphae was frequently seen (*figure 2C*). Colonization of the vascular stele occurred by day 4

after inoculation and proceeded via the infection of the paratracheal parenchyma cells. Pathogen penetration from one xylem vessel to another occurred through the pit membranes (*figure 2D*). This massive invasion of the vascular stele correlated with the brownish discoloration seen in longitudinally sectioned tomato roots.



2.4. Ultrastructural and cytochemical features of root tissues from elicitor-treated tomato plants

2.4.1. Non-inoculated plants

Transmission electron microscope examination of ultrathin sections from roots of chitosan- and oligandrin-treated plants showed that treatment with the two elicitors did not yield appreciable effect on the induction of plant defence reactions, except for the occasional presence of electron-opaque deposits in some cells (not shown).

2.4.2. Inoculated plants

The root system of plants treated with chitosan or oligandrin prior to being challenged with FORL appeared much more vigorous than that of non-treated inoculated plants. In all cases, the crown and main root as well as the secondary roots were free of apparent symptoms, except for the formation of localized lesions at sites of fungal penetration. By contrast, a substantial number of root lesions was detected along the root system of glucan-treated plants.

2.4.2.1. Effect of glucan treatment on the cytology of infection

Exogenous applications of crude glucans from *P. oligandrum* cell walls did not halt or delay pathogen colonization of the root tissues. The pattern of fungal invasion and host cell degradation was, indeed, similar to that observed in control plants. Host defence reactions were seldom detected (not shown).

2.4.2.2. Effect of chitosan treatment on the cytology of infection

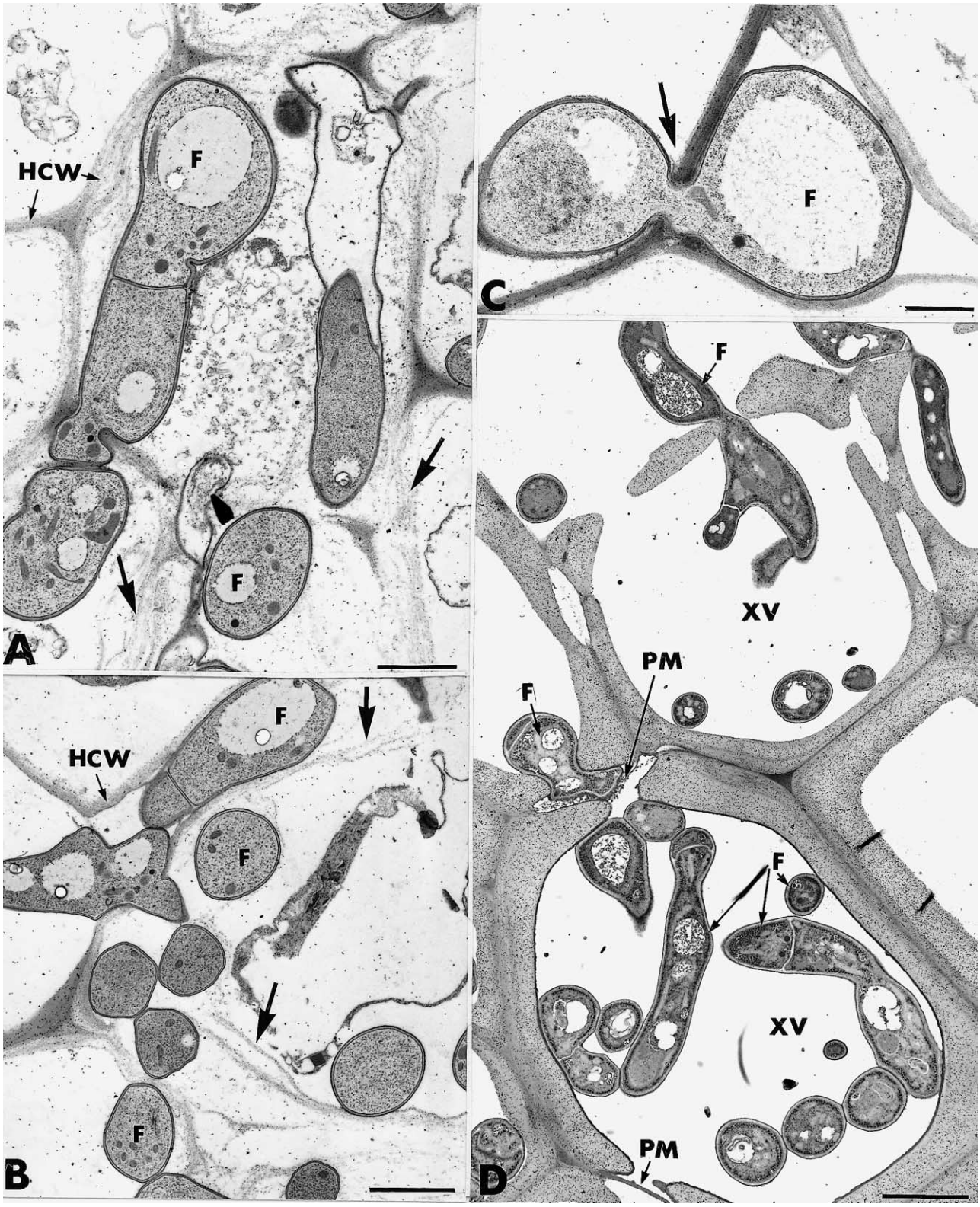
Examination of root samples, collected from chitosan-treated plants 4–5 d after inoculation with FORL, confirmed that fungal colonization was restricted to the epidermis and the first cortical cell layers. This considerable decrease in fungal biomass, as compared to the pattern of colonization seen in root tissues of elicitor-free plants, was always associated with marked alterations of the invading hyphae (*figure 3*). Such cytological disorders included plasmalemma retraction, increased vacuolation (*figure 3B*) and, in some cases, complete depletion of the protoplasm (*figure 3C*).

As previously reported [5], formation of structural barriers at sites of attempted fungal penetration was an important feature of the host reactions induced by chitosan treatment. The most commonly encountered barriers were heterogeneous wall appositions which formed in the invaded epidermis and outer cortex (*figure 3*). The wall appositions elaborated in the reacting host cells were found to vary in their appearance from hemispherical protuberances resembling papillae (*figure 3A*) to elongated structures along a large portion of the host cell wall (*figure 3B–D*). The host cell wall itself displayed a higher electron density than normal, thus indicating the probable infiltration of structural molecules (*figure 3B, C*). A typical host reaction was the deposition of electron-dense globules in most infected intercellular spaces (*figure 3C*). The wall of the invading hyphae was frequently coated by this osmiophilic material (*figure 3C*, arrow). Upon incubation of sections with the tobacco β-1,3-glucanase, a considerable number of gold particles were detected over all wall appositions, regardless of their size, shape, texture and architecture while only a few scattered gold particles occurred over the host cell walls (*figure 3D*). Control tests including incubation of the enzyme-gold complex with laminarin prior to section labelling yielded negative results (not shown).

2.4.2.3. Effect of oligandrin treatment on the cytology of infection

Extensive growth of FORL was seen at the root surface (*figure 4A*). Pathogen ingress in the root epidermis occurred often through direct wall penetration but could also occur through localized cell wall disruptions (not shown). Pathogen growth towards the inner root tissues was apparently prevented by the

Figure 1. Light microscope photographs of tomato root tissues infected by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL). **A**, Control plants. Hyphae of the pathogen (F) multiply in the epidermis, the cortex and reach the vascular stele (VS) by centripetal growth. Host cell alterations including cell wall dissolution are visible (arrow). Bar = 20 µm. **B**, **C** and **D**, Treatment with chitosan prior to inoculation with FORL. **B**, Areas of fungal (F) colonization are restricted to the epidermis (E) and the outer cortex (C). An increased staining density of host cell wall is noticed (arrows). **C**, Restriction of fungal growth correlates with marked changes characterized by the formation of elongated wall appositions (WA) along the host cell walls surrounding an intercellular space (IS). **D**, An amorphous material (AM) in which invading fungal cells (F) are trapped is deposited in some invaded cells. Bars = 10 µm. **E–I**, Treatment with oligandrin prior to inoculation with FORL. **E**, Fungal (F) growth is mainly restricted to the epidermis (E) and the outer root cortex (C). Bar = 20 µm. **F**, A densely stained material accumulates along the primary walls of infected host cells (arrows) and forms droplets that surround hyphae of the pathogen (F) (arrowheads). **G** and **H**, An amorphous material (AM) in which fungal cells (F) are embedded accumulates in some invaded host cells. **I**, Wall appositions (WA) are occasionally seen in areas neighbouring invaded intercellular spaces (IS). Bars = 10 µm.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formation, at the epidermis level, of wall appositions varying in size, shape and texture (*figure 4A*). In spite of this structural defensive line in the epidermis, a few fungal cells could penetrate and colonize some cells and intercellular spaces in the cortical area (*figure 4B–D*).

In these colonized root areas, a large number of cortical cells and intercellular spaces (about 80 %) were filled with aggregated deposits (*figure 4B, D*) which often accumulated at the cell surface of invading hyphae (*figure 4C*). Hyphae trapped in this osmiophilic material were usually distorted and exhibited marked changes including increased vacuolation and densification of the cytoplasm associated with the formation of enlarged, osmiophilic inclusions which extended in most of the space initially occupied by the cytoplasm (*figure 4D, E*). In such fungal cells, typical organelles such as nuclei and mitochondria were no longer visible (*figure 4E*).

Beside the accumulation of these small aggregated deposits, treatment with oligandrin triggered the elaboration of other host reactions mainly characterized by the formation of fibrillo-granular networks in about 30 % of the infected host cells (*figure 5A–C*). The networks varied greatly in their appearance from granular and of high electron density (*figure 5A*) to more compact and composed of variously densified zones containing numerous inclusions (*figure 5C*). Occasionally, the network would be made up of intermingled fibrils which encased invading fungal cells (*figure 5B*). In all cases, *Fusarium* hyphae embedded in the newly-formed matrices suffered from severe damage as evidenced by their appearance of either empty shells (*figure 5C*) or aggregated, polymorphic structures (*figure 5B*).

Finally, another feature, occasionally seen in reacting cortical cells, was the formation of wall appositions at sites of potential fungal penetration (*figure 5D*). These appositions, which could greatly vary in size and shape, were usually found to be made up of an amorphous matrix which was impregnated by osmiophilic substances and was surrounded by a layer of aggregated cytoplasmic material (*figure 5D*).

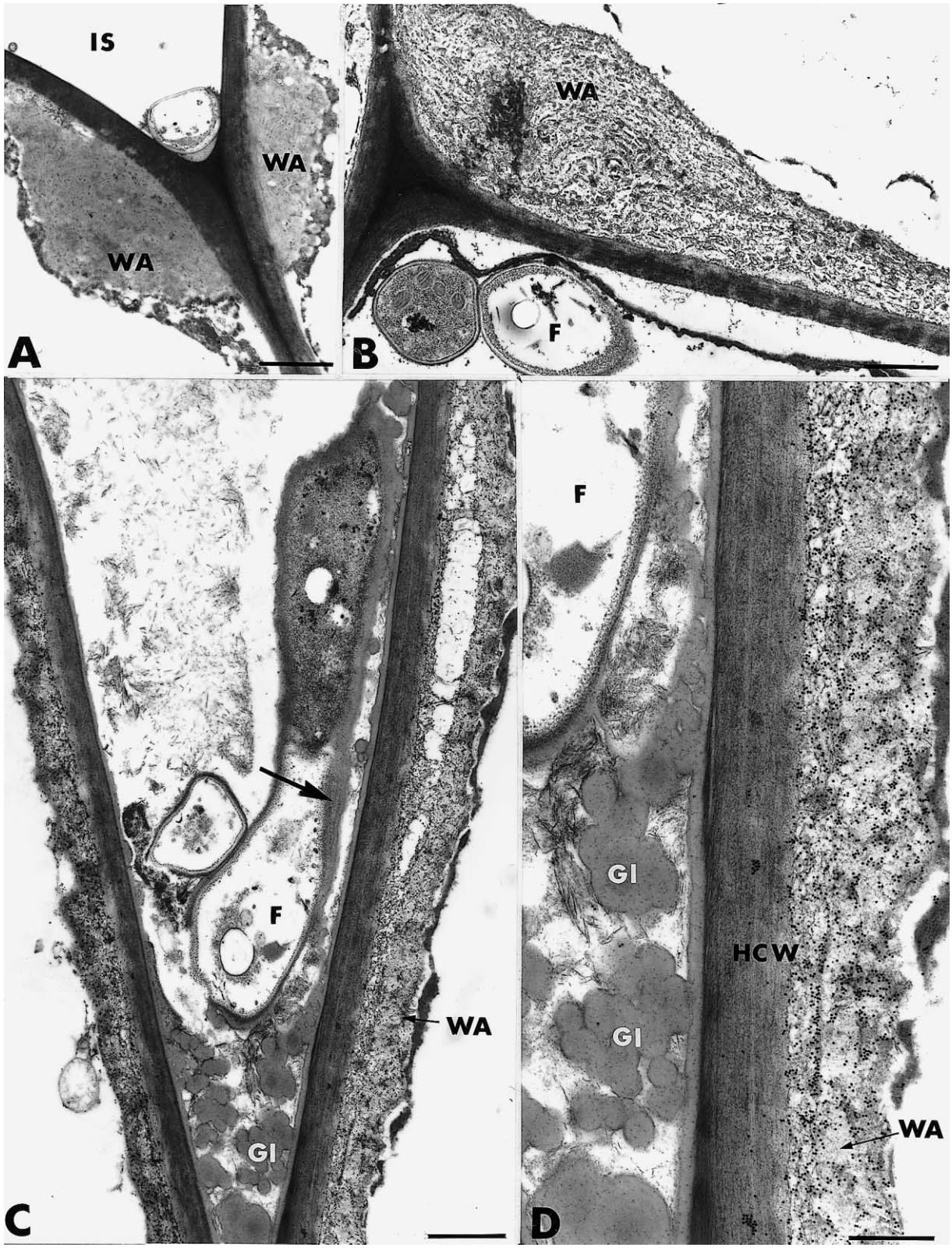
When the WGA/ovomucoid-gold complex was applied to root sections of oligandrin-treated tomato plants, variations in the pattern of fungal wall labelling was noticed (*figure 6*). Gold labelling was indeed evenly distributed over the walls of invading hyphae even when those were substantially altered (*figure 6B*) whereas it was nearly absent over large wall portions of empty fungal shells (*figure 6C*, arrows). All control tests, including previous adsorption of the WGA with N,N',N''-triacetychitotriose yielded negative results (not shown).

3. DISCUSSION

In recent years, the process of plant 'immunization' or induced resistance to disease has been the focus of considerable interest and has been abundantly documented [22, 23]. The discovery that non-pathogenic, mycoparasitic fungi such as *P. oligandrum* [10] and *Trichoderma harzianum* [38] could sensitize host plants to respond more rapidly and efficiently to pathogen attack has opened new research avenues in the field of microbial-mediated induced resistance. In an attempt to identify the molecule which could be responsible for the activation of a set of defence genes leading to the formation of structural and biochemical barriers in tomato plants challenged by *P. oligandrum* [10], we isolated a water soluble protein, exhibiting strong sequence homology with the elicitins from *Phytophthora* sp. [29]. Results of the present study provide the first evidence that this small protein, termed oligandrin, has the potential to induce a systemic resistance in susceptible tomato plants infected by a root pathogen and bring strong support to the concept that this elicitin-like molecule may become a potential disease control agent in tomato.

Our observations demonstrate that the beneficial effect of oligandrin in reducing the extent of fungal colonization in the root tissues correlates with a massive accumulation of antifungal compounds in infected cells and intercellular spaces. Interestingly, this response differs from that observed in chitosan-treated tomato plants where, as expected from earlier studies [5, 7], the formation of structural barriers at sites of attempted pathogen penetration was the main feature of the reaction. Although electron-dense deposits were also seen in the root tissues as a result of chitosan elicitation, their level never reached that monitored in oligandrin-treated plants. While it is clear that both elicitors share the ability to protect tomato

Figure 2. Transmission electron micrographs of samples from tomato root tissues infected by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (control plants). **A** and **B**, Hyphae of the pathogen (F) colonize abundantly the root tissues. Host cell walls (HCW) are markedly altered and reduced to fine strands of fibrillar material (arrows). Bars = 2 µm. **C**, Cell invasion through direct host wall penetration is observed (arrow). Bar = 1 µm. **D**, Pathogen (F) invasion of the xylem vessels (XV) occurs through the pit membranes (PM). Bar = 2 µm.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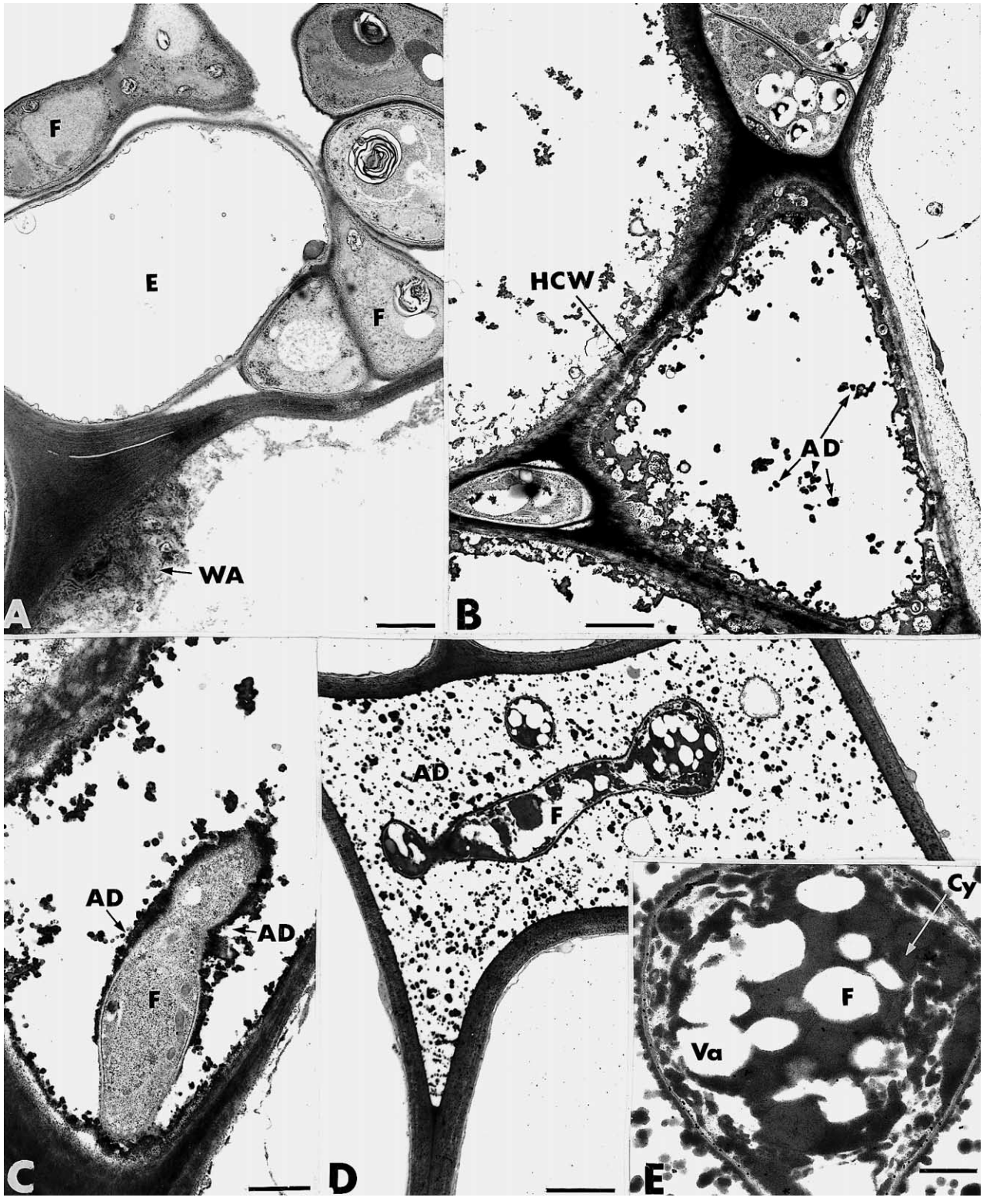
root tissues from massive pathogen invasion, they induce a differential response. In line with these observations, Bottin et al. [16] reported differential responses of tobacco cells to elicitors from two *Phytophthora* sp. The authors showed that although both elicitors could induce the common accumulation of some defence molecules such as proteinase inhibitors, they differed in their ability to trigger the production of active oxygen species, known to be involved in the regulation of specific defence-related responses [34]. Several lines of evidence have shown that callose deposition in structural barriers was directly modulated by the intracellular concentration of free Ca^{2+} which is known to control the activity of one of the key structural enzymes, β-1,3-glucan synthase [21]. Considering the polycationic properties of chitosan, chitosan-elicited membrane alterations in the epidermal cells may have promoted internal osmotic imbalances, resulting in a huge electrolyte leakage and in an accelerated Ca^{2+} entrance into the cytoplasm due to the opening of non-specific and/or ion-specific channels [21]. Recent investigations of the effect of fungal elicitins on the host cell responses have shown that these proteinaceous molecules triggered the same early effects (e.g. Ca^{2+} influx, H_2O_2 production) with, however, some differences in terms of Ca^{2+} uptake intensity [12, 31]. The extent of the Ca^{2+} influx did not correlate with the basic or acidic characters of the elicitins [17] but rather could be associated with the activation of distinct or additional signalling pathways leading to differential cell responses [12]. However, the possibility that different intracellular Ca^{2+} concentrations may stimulate the same pathway but with differential modulation cannot be ruled out and further studies are required to provide more details on the role of Ca^{2+} in the complex network of cell signalling that generate the second messengers and trigger the inducible host response [13]. Whether different levels of Ca^{2+} influx in oligandrin or chitosan-treated tomato plants stimulate different signalling pathways resulting in distinct cellular changes remains to further investigated. Whatever the reasons why oligandrin failed to massively induce callose deposition, our observations suggest that these defence-related structural modifications are not major components of the oligandrin-mediated induced response in tomato.

Although the exact mechanisms by which oligandrin operates to reduce *Fusarium* wilt incidence in tomato roots are not fully elucidated, our cytological results indicate that its effect in protecting tomato plants against FORL attack is associated with an increased resistance to pathogen colonization. The restricted fungal development often coincided with a marked decrease in pathogen viability, suggesting the presence of a fungitoxic environment in the invaded areas. Surprisingly, glucan extracts from *P. oligandrum* cell walls failed to induce a similar defence response. Although absence of plant reactions to such compounds may reflect some problems in the extraction procedure, the confirmation that, as previously reported [5], induction of a host response occurred following treatment with crude glucans from *Phytophthora megasperma* f. sp. *glycinea* cell walls (not shown) supports the view that the chemical composition of *P. oligandrum* cell walls differs from that of other oomycetes. In that context, one may suggest that glucan elicitors in *P. oligandrum* cell walls are not exposed at the cell surface and, therefore, not directly accessible to plant cell membrane receptors. In this fungus, the low amount of β-1,3-glucans, probably present in the innermost wall layers, may be buried in considerable amounts of cellulosic β-1,4-glucans which are not known to be molecules with active eliciting properties.

While in control water-treated tomato plants, rapid necrotrophic colonization of all root tissues occurred in association with major host cell disorganization, an abnormal accumulation of electron-dense substances was seen in the colonized root areas of oligandrin-treated plants. Examination of the spatial distribution of these host reactions revealed that both the intensity and the magnitude of the response decreased at the cortical level to become hardly discernible in the endodermis. Because FORL is a vascular pathogen which colonizes the xylem vessels (see *figure 2D*), one may expect that the resistance process involves the formation of structural and chemical barriers in the outer tissues to prevent further spread of the pathogen towards the vascular bundle. The observation that *Fusarium* hyphae coated by the electron-dense material were markedly damaged at a time when the chitin component of their cell walls was preserved supports

Figure 4. Transmission electron micrographs of root tissue from decapitated tomato plants treated with oligandrin and infected by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. **A**, Extensive growth of *Fusarium* (F) hyphae was seen at the root surface. A small wall apposition (WA) is seen in the epidermis (E). Bar = 1 µm. **B**, Host cells in the cortical area are filled with aggregated deposits (AD) which accumulate along the host cell wall (HCW) as well as in the cell lumen. Bar = 2 µm. **C**, The electron-dense, aggregated deposits (AD) accumulate at the cell surface of an invading fungal cell (F). Bar = 1 µm. **D** and **E**, Fungal cells (F), surrounded by the aggregated deposits (AD), are severely damaged and exhibit marked changes including increased vacuolation (Va), and densification of the cytoplasm (Cy). **D**, Bar = 2 µm; **E**, Bar = 0.5 µm.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the concept that the newly-formed material contains phenolic compounds with fungitoxic properties. The possibility that these polymerized substances constitute a potential source of phenolic compounds appears realistic, considering not only the hyphal alterations but also their texture and osmiophilic properties as previously reported by Scalet et al. [33]. Although impregnation of phenolic compounds in the cell walls, as evidenced by their enhanced electron density, may indirectly contribute to disease resistance by reinforcing the mechanical strength of these barriers, the main role played by the phenolic-enriched material appears to rely on a direct antifungal activity.

In an attempt to determine whether fungal wall hydrolysis was associated with the frequent disorganization of fungal hyphae colonizing the outer root tissues in oligandrin-treated plants, chitin was ultrastructurally localized by using the WGA/ovomucoid-gold complex. Analysis of the labelling pattern over invading fungal cells clearly revealed that chitin was altered in severely damaged hyphae. This was taken as an indication that the plant cells were signalled to produce chitinases that accumulated at invaded sites. However, the finding that chitin molecules were still present over cell walls of hyphae showing obvious signs of degradation leads to suggest that production of hydrolytic enzymes such as chitinases is not an early event in the expression of oligandrin-mediated induced resistance. A similar conclusion was reached by Benhamou and Thériault [7] and Benhamou et al. [9] who observed the same phenomenon in plants treated with either chitosan or endophytic bacteria. According to our observations, it is more likely that synthesis of toxic substances (i.e. phenolics) with direct incidence on the pathogen precedes the production of chitinases which, probably, contribute to a more complete disintegration of the fungal cells.

In conclusion, evidence is presented in this study that pre-treatment of tomato plants with oligandrin reduces disease incidence caused by FORL by sensitizing the plants to elaborate an efficient defence strategy. Since the rhizosphere provides the first line of defence for roots against attack by pathogens and because plants have sophisticated defence mechanisms that can be naturally activated by environmental factors and microorganisms, the possibility of enriching the rhizosphere with adapted microorganisms has become a challenging priority for plant pathologists. An improved, integrated protection programme implies the availability of highly efficient biofungicides of which *P. oligandrum* offers an excellent prospect for root disease management. Because of its antimicrobial activity and its ability to produce large amounts of oligandrin, the incorporation of selected strains of *P. oligandrum* into the arsenal of strategies currently developed for controlling diseases caused by soilborne fungi is a promising step towards elaborating integrated pest management programmes which will allow greenhouse producers to reduce yield losses due to root rot diseases while participating in the current trend toward reducing the use of chemical pesticides.

4. METHODS

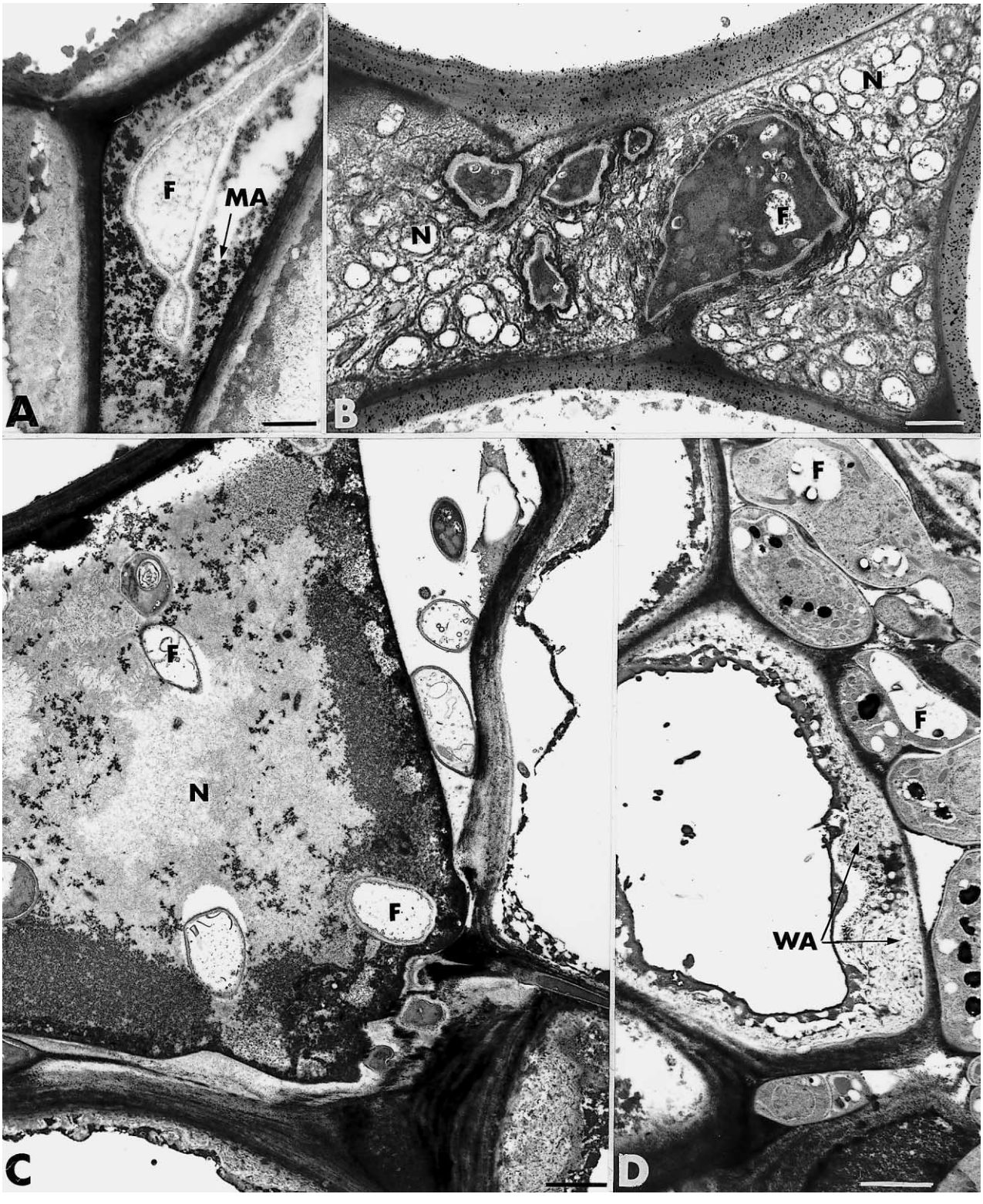
4.1. Fungal cultures and growth conditions

A tomato isolate of *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL) (kindly provided by P.O. Thibodeau, MAPAQ, Quebec, Canada) was grown on potato dextrose agar (PDA, Difco Laboratories, Detroit, MI, USA) at 24 °C and subcultured every week. It was periodically inoculated and re-isolated from ripe tomato fruits. *Pythium oligandrum* Drechsler, strain 1010, was isolated from pea roots in Denmark. It was kindly provided by Dr J. Hockenhull, The Royal Veterinary and Agricultural University, Copenhagen, Denmark. For plant inoculation, the fungus was cultivated on PDA medium at 25 °C in the dark. For oligandrin purification, *P. oligandrum* was grown in a defined medium known for its potential for stimulating elicitin production [14]. The flasks were incubated in the dark for 8 d at 24 °C. Culture filtrates of *P. oligandrum* were recovered after mycelium removal on GF/C filter (Whatman) under vacuum. For glucan extraction, *P. oligandrum* was grown for 7 d at 24 °C in V8-agar (Campbell Soup Company Ltd, Toronto, Ontario, Canada) containing for 1 L of deionized water: V8-juice 200 mL; $CaCO_3$, 2.5 g; β-sitosterol, 0.1 g; agar, 15 g; and deionized water, 800 mL.

4.2. Plant material

Tomato seeds (*Lycopersicon esculentum* Mill. cv. Bonny Best, susceptible to FORL) were sterilized by

Figure 5. Transmission electron micrographs of root tissue from decapitated tomato plants treated with oligandrin and infected by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. **A**, A fungal cell (F) is trapped in a granular matrix (Ma) of high electron density. Bar = 1 µm. **B**, A network (N), made up of intermingled fibrils, encases invading fungal cells (F). Bar = 1 µm. **C**, Fungal cells (F) are trapped in a compact network (N) composed of variously densified zones containing numerous inclusions. Bar = 2 µm. **D**, An elongated wall apposition (WA) is seen in a host cell neighbouring an invaded area. Bar = 2 µm.


A
F
MA
B
N
F
N
C
F
N
F
D
F
F
WA

immersion in 1 % (v/v) sodium hypochlorite for 30 min and sown in a mixture of peat-perlite-vermiculite (2:1:1) at a density of four seeds per 6-cm pot. Plants were propagated in a glasshouse at 22–24 °C with a 14-h photoperiod and were fertilized twice a week with a commercial plant nutrient solution. Experiments were performed with 2-month-old plants harbouring five or six fully expanded leaves.

4.3. Elicitor preparation

Oligandrin, purified crab shell chitosan, and cell wall crude glucans from *P. oligandrum* were used as elicitors.

4.3.1. Oligandrin purification

Oligandrin was purified from the culture filtrate of *P. oligandrum* according to the procedure described by Picard et al. [29]. Briefly, culture filtrates of *P. oligandrum* were concentrated by evaporation under vacuum at 35 °C and dialysed against several baths of deionized water for 24 h at 4 °C. After addition of 0.34 M Na-acetate to the concentrated filtrate, the pH of the resulting solution was adjusted to 3.5 with 10 % (v/v) aqueous trifluoroacetic acid (TFA). The concentrated filtrate was loaded on a 20-mL cationic exchange Macroprep sulfopropyl High S column (BioRad, France) previously equilibrated with 10 mM Na acetate (pH 3.5). The retained fraction was eluted with 10 mM Na-acetate containing 0.25 M NaCl (pH 3.5) and adjusted to pH 7.0 before being subjected to reverse phase liquid chromatography (RPLC) using a Synchroprep C4 column (30 µm, 300 Å, Synchrom Inc.) which was pre-equilibrated with 10 mM Na-acetate containing 0.25 M NaCl (pH 7.0). Elution was carried out at room temperature using a gradient of acetonitrile (CH_3CN) (20, 30, 40 %, v/v) in 50 mM aqueous Na-formate (HCOONa). The purified protein, termed oligandrin, was recovered from the 40 % CH_3CN fraction. Each chromatographic step was qualitatively assayed by HPLC (Waters 625 LC system solvent delivery). Integration at 280 nm, spectra, peak purity and all calculation were achieved by Millenium software (Waters). For the last step, a peak was visualized from the 40 % CH_3CN fraction. After removal of CH_3CN under vacuum, the pure protein was extensively dialysed against ultra-pure Millipore H_2O and freeze-dried. Purity of the protein was further assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 15.4 % polyacrylamide-SDS gels (20 $mA \cdot gel^{-1}$ in 0.25 mM Tris, 1.92 M glycine, 0.1 % SDS) [27].

4.3.2. Chitosan preparation

Chitosan (Sigma Chemical Co., St Louis, MO, USA) was prepared according to a previously described procedure [7]. Briefly, it was ground to a powder by extensive grinding, washed repeatedly in distilled water, pelleted by low-speed centrifugation and air-dried. Sheets of chitosan were solubilized by stirring in 0.25 N HCl, centrifuged at $10\,000 \times g$ for 10 min to remove the insoluble material, and precipitated by neutralization with 2.5 N NaOH. The chitosan pellets, recovered by centrifugation at $25\,000 \times g$ for 15 min, were thoroughly washed with deionized water to remove salts and freeze-dried. For experimental use, purified chitosan was dissolved in 0.05 N HCl under continuous stirring to obtain a final concentration of 1 $mg \cdot mL^{-1}$. The pH of the resulting solution was adjusted to 5.6 using 1 N NaOH. The amount of NaCl formed in the chitosan solution was estimated to be less than 0.05 M.

4.3.3. Glucan preparation

Glucans from *P. oligandrum* were obtained according to the method described by Sharp et al. [35]. Two grams lyophilized fungal walls were suspended in 200 mL 2 N trifluoroacetic acid (TFA) for 3 h at 80 °C under shaking in a water bath and centrifuged at $10\,000 \times g$ for 15 min to remove insoluble residues. The supernatant was filtered through a GF/C filter and residual TFA was eliminated by retroevaporation at 30 °C. The suspension was suspended in 20 mL ice cold double distilled water, neutralized with 1 N NaOH, and brought to a final volume of 50 mL. Two-millilitre fractions, recovered after gel filtration on a Bio-Gel P_2 column (Bio-Rad, CA, USA), were diluted in sterile distilled water (1 $mg \cdot mL^{-1}$ glucose equivalent) and used for tomato plant treatment.

4.4. Plant elicitation and challenge inoculation

Two-month-old tomato plants were decapitated above the third fully expanded leaf [30] just prior to applying 30 µL of each elicitor solution (1 $mg \cdot mL^{-1}$) onto the fresh wound which was immediately covered with Parafilm. Control plants were decapitated and treated with sterile water or with 0.05 M (w/v) aqueous NaCl. One day after treatment, tomato plants were inoculated by introducing a plug of actively growing mycelium of FORL as close as possible to the root system. Control plants were treated similarly but with fungus-free agar plugs. Root samples were collected, 4 to 6 d after fungal inoculation, from six plants for each elicitor treatment in two replicate experiments.

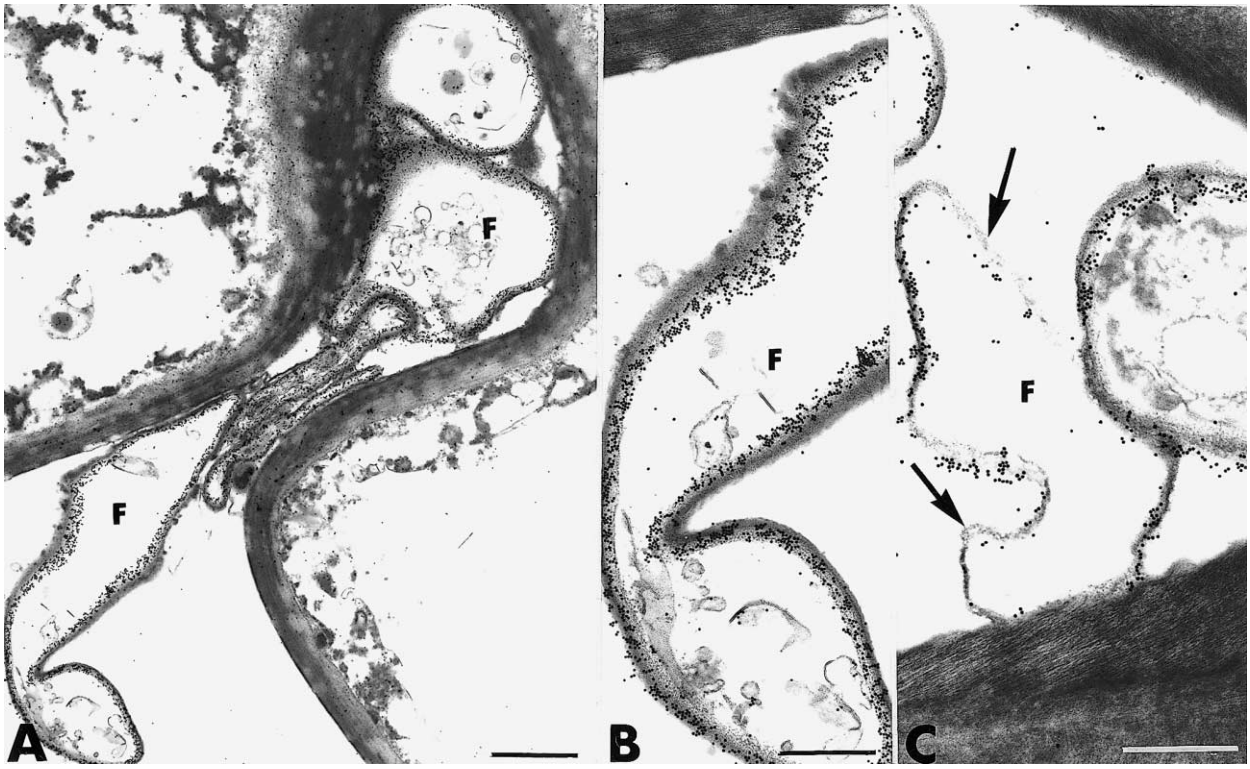


Figure 6. Transmission electron micrographs of root tissue from decapitated tomato plants treated with oligandrin and infected by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. **A–C**, Labelling of chitin with the WGA/ovomucoid-gold complex. Gold labelling is quite evenly distributed over the walls of an invading hypha (F) showing substantial alteration (**A** and **B**). By contrast, labelling is nearly absent over large wall portions of empty fungal shells (**C**, arrows). **A**, Bar = 1 μm; **B** and **C**, bars = 0.5 μm.

4.5. Tissue processing for electron microscope studies

Samples (2 mm^3), collected from the crown and the main root at the sites of fungal entry (root lesions), were fixed by immersion in a mixture of 3 % (v/v) glutaraldehyde and 3 % (w/v) paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 2 h at room temperature and post-fixed with 1 % (w/v) osmium tetroxide in the same buffer for 1 h at 4 °C. Root samples were dehydrated in a graded ethanol series and embedded in Epon 812 (JBEM Chemical Co., Pointe-Claire, Quebec, Canada). Ultrathin sections were either contrasted with uranyl acetate and lead citrate or further processed for cytochemical labelling.

4.6. Cytochemical labelling

The colloidal gold suspension, with particles averaging 15 nm in diameter (BDH Chemicals, Montreal, Canada) was prepared as described by Benhamou [1]. For the localization of cellulosic β-1,4-glucans, an exoglucanase (β-1,4-D-glucan cellobiohydrolase, EC 3.2.1.21), purified from a cellulase produced by the fungus *Trichoderma harzianum* was kindly provided by Dr C. Breuil, Forintek, Canada. It was complexed to colloidal gold at pH 9.0 and used in a one-step procedure [8]. Ultrathin sections were first incubated on a drop of phosphate buffered saline (PBS), pH 6.0, containing 0.02 % (w/v) of polyethylene glycol 20 000 (PEG 20,000, Fisher Scientific, Nepean, Ontario, Canada) for 5 min at room temperature. They were, thereafter, transferred to a drop of the gold-complexed exoglucanase for 30 min at room temperature in a moist chamber. After washing with PBS and rinsing with distilled water, grids were contrasted as described above. Specificity of the labelling was assessed by incubating the sections either with the gold-complexed exoglucanase to which were previously added β-1,4-glucans from barley (1 $mg \cdot mL^{-1}$ in PBS) or with stabilized or an unstabilized gold suspension. For the localization of callose, a β-1,3-glucan polymer, a

β-1,3-glucanase, purified from tobacco reacting hypersensitively to tobacco mosaic virus (TMV), was used according to a previously described method [2]. The enzyme was conjugated to colloidal gold at pH 5.5 and incubation of sections was carried out as described for the gold-complexed exoglucanase. Control tests included incubation of the sections with the gold-complexed β-1,3-glucanase to which was previously added laminarin (1 mg·mL^{-1} in PBS). Wheat germ agglutinin (WGA), a lectin with N-acetylglucosamine binding specificity was used for localizing N-acetylglucosamine residues (chitin) [1]. Because of its low molecular mass, the lectin could not be directly complexed to colloidal gold. It was used in a two-step procedure, using ovomucoid, conjugated to gold at pH 5.4, as a second step reagent. For the labelling of N-acetylglucosamine residues, sections were first floated on a drop of PBS, pH 7.4, for 5 min, then transferred to a drop of WGA (12 µg·mL^{-1} in PBS, pH 7.4) for 60 min at room temperature in a moist chamber. After washing with PBS, pH 7.4, sections were incubated on a drop of the ovomucoid-gold complex (1:30 in PBS-PEG, pH 6.0) for 30 min at room temperature. Sections were washed with PBS, rinsed with distilled water and contrasted as described above. Controls included incubation with the WGA to which was previously added an excess of N-N'-N''-triacetylchitotriose (1 mg·mL^{-1} in PBS) and incubation with the WGA, followed by unlabelled ovomucoid and finally by ovomucoid-gold complex.

Acknowledgments. The authors wish to thank Alain Goulet and Chantal Garand for excellent technical assistance. This research was supported by grants from the 'Fonds Québécois pour la Formation de Chercheurs et l'Aide à la Recherche' (FCAR) and the Natural Sciences and Engineering Council of Canada (NSERC).

REFERENCES

[1] Benhamou N., Preparation and applications of lectin-gold complexes, in: Hayat M.A. (Ed.), Colloidal Gold: Principles, Methods and Applications, vol. 1, Academic Press, New York, 1989, pp. 95–143.

[2] Benhamou N., Ultrastructural detection of β-1,3-glucans in tobacco root tissues infected by *Phytophthora parasitica* var. *nicotianae* using a gold-complexed tobacco β-1,3-glucanase, Physiol. Mol. Plant Pathol. 41 (1992) 351–370.

[3] Benhamou N., Elicitor-induced plant defense pathways, Trends Plant Sci. 1 (1996) 233–240.

[4] Benhamou N., Bélanger R.R., Induction of systemic resistance to *Pythium* damping-off in cucumber plants by benzothiadiazole: ultrastructure and cytochemistry of the host response, Plant J. 14 (1998) 13–21.

[5] Benhamou N., Lafontaine P.J., Ultrastructural and cytochemical characterization of elicitor-induced responses in tomato root tissues infected by *Fusarium oxysporum* f. sp. *radicis-lycopersici*, Planta 197 (1995) 89–102.

[6] Benhamou N., Nicole M., Cell biology of plant immunization against microbial infection: the potential of induced resistance in controlling plant diseases, Plant Physiol. Biochem. 37 (1999) 703–719.

[7] Benhamou N., Thériault G., Treatment with chitosan enhances resistance of tomato plants to the crown and root rot pathogen, *Fusarium oxysporum* f. sp. *radicis-lycopersici*, Physiol. Mol. Plant Pathol. 41 (1992) 33–52.

[8] Benhamou N., Chamberland H., Ouellette G.B., Pauze F.J., Ultrastructural localization of β-1,4-D-glucans in two pathogenic fungi and in their host tissues by means of an exoglucanase-gold complex, Can. J. Microbiol. 33 (1987) 405–417.

[9] Benhamou N., Kloepper J.W., Quadt-Hallmann A., Tuzun S., Induction of defense-related ultrastructural modifications in pea root tissues inoculated with endophytic bacteria, Plant Physiol. 112 (1996) 919–929.

[10] Benhamou N., Rey P., Chérif M., Hockenhull J., Tirilly Y., Treatment with the mycoparasite, *Pythium oligandrum*, triggers the induction of defense-related reactions in tomato roots upon challenge with *Fusarium oxysporum* f. sp. *radicis-lycopersici*, Phytopathology 87 (1997) 108–122.

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

[12] Binet M.N., Bourque S., Lebrun-Garcia A., Chiltz A., Pugin A., Comparison of the effects of cryptogein and oligogalacturonides on tobacco cells and evidence of different forms of desensitization induced by these elicitors, Plant Sci. 137 (1998) 33–41.

[13] Binet M.N., Humbert C., Lecourieux D., Vantard M., Pugin A., Disruption of microtubular cytoskeleton induced by cryptogein, an elicitor of hypersensitive response in tobacco cells, Plant Physiol. 125 (2001) 564–572.

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

[15] Bonnet P., Poupet A., Bruneteau M., Toxicité vis-à-vis du tabac des fractions purifiées d'un filtrat de culture de *Phytophthora cryptogea* Pethyb. & Laff, Agronomie 5 (1985) 275–282.

[16] Bottin A., Véronési C., Pontier D., Esquerré-Tugayé M.T., Blein J.P., Rusterucci C., et al., Differential responses of tobacco cells to elicitors from two

Phytophthora species, Plant Physiol. Biochem. 32 (1994) 373–378.
[17] Bourque S., Ponchet M., Binet M.N., Ricci P., Pugin A., Lebrun-Garcia A., Comparison of binding properties and early biological effects of elicitins in tobacco cells, Plant Physiol. 118 (1998) 1317–1326.
[18] Cohen Y., 3-Aminobutyric acid induces systemic resistance against *Peronospora tabacina*, Physiol. Mol. Plant Pathol. 45 (1994) 441–456.
[19] Deacon J.W., Studies on *Pythium oligandrum*, an aggressive parasite of other fungi, Trans. Brit. Mycol. Soc. 66 (1976) 383–391.
[20] Keller H., Czernic P., Ponchet M., Ducrot P.H., Back K., Chappell J., Ricci P., Marco Y., Sesquiterpenoide cyclase is not a determining factor for elicitor- and pathogen-induced capsidiol accumulation in tobacco, Planta 205 (1998) 467–476.
[21] Köhle H., Jeblick W., Poten F., Blaschek W., Kauss H., Chitosan-elicited callose synthesis in soybean cells as a Ca^{2+} dependent process, Plant Physiol. 77 (1985) 544–551.
[22] Kùc J., Plant immunization and its applicability for disease control, in: Chet I. (Ed.), Innovative Approaches to Plant Disease Control, John Wiley & Sons, New York, 1987, pp. 255–274.
[23] Lyon G.D., Reglinski T., Newton A.C., Novel disease control compounds: the potential to immunize plants against infection, Plant Pathol. 44 (1995) 407–427.
[24] Madamanchi N.R., Kuc J., Induced systemic resistance in plants, in: Cole G.T., Hoch T.A. (Eds.), Fungal Spores and Disease Initiation in Plants and Animals, Plenum Publishers, New York, 1991, pp. 347–362.
[25] Malamy J., Klessig D.F., Salicylic acid and plant disease resistance, Plant J. 2 (1992) 643–654.
[26] Martin F.N., Hancock J.G., The use of *Pythium oligandrum* for biological control of preemergence damping-off caused by *P. ultimum*, Phytopathology 77 (1987) 1013–1020.
[27] Mikes V., Milat M.L., Ponchet M., Panabières F., Ricci P., Blein J.P., Elicitins excreted by *Phytophthora* are a new class of sterol carrier proteins, Biochem. Biophys. Res. Comm. 245 (1998) 133–139.
[28] Panabières F., Ponchet M., Allasia V., Cardin L., Ricci P., Characterization of border species among Pythiaceae: several *Pythium* isolates produce elicitins, typical proteins from *Phytophthora* spp., Mycol. Res. 101 (1997) 1450–1468.
[29] Picard K., Ponchet M., Blein J.P., Tirilly Y., Benhamou N., Oligandrin: A proteinaceous molecule produced by the mycoparasite, *Pythium oligandrum*, induces resistance to *Phytophthora parasitica* infection in tomato plants, Plant Physiol. 124 (2000) 379–395.
[30] Ponchet M., Panabières F., Milat M.L., Mikes V., Montillet J.L., Suty L., Triantaphylides C., Tirilly Y., Blein J.P., Are elicitins cryptograms in plant-oomycete communications?, Cell Mol. Life Sci. 56 (1999) 1020–1047.
[31] Pugin A., Frachisse J.M., Tavernier F., Bligny R., Gout E., Douce R., et al., Early events induced by the elicitor cryptogein in tobacco cells: involvement of a plasma membrane NADPH oxidase and activation of glycolysis and the pentose phosphate pathway, Plant Cell 9 (1997) 2077–2091.
[32] Ricci P., Induction of the hypersensitive response and systemic acquired resistance by fungal proteins: the case of elicitins, in: Stacey G., Keen N.T. (Eds.), Plant-Microbe Interactions, Chapman & Hall, New York, 1997, pp. 53–75.
[33] Scalet M., Crivaletto E., Mallardi F., Demonstration of phenolic compounds in plant tissues by an osmium-iodide post-fixation procedure, Stain Technol. 64 (1989) 273–290.
[34] Schreck R., Rieber P., Baeulerle P.A., Reactive oxygen intermediates are apparently widely used messengers in the activation of the NF-kB transcription factor and HIV-1, EMBO J. 10 (1991) 2247–2258.
[35] Sharp J.K., Valent B., Albersheim P., Purification and partial characterization of a β-glucan fragment that elicits phytoalexin accumulation in soybean, J. Biol. Chem. 259 (1984) 11312–11320.
[36] Sticher L., Mauch-Mani B., Métraux J.P., Systemic acquired resistance, Annu. Rev. Phytopathol. 35 (1997) 235–270.
[37] Uknes S.J., Mauch-Mani B., Moyer M., Potter S., Williams S., Dincher S.S., Chandler D., Slusarenko A., Ward E.R., Ryals J.A., Acquired resistance in *Arabidopsis*, Plant Cell 4 (1992) 645–656.
[38] Yedidia I., Benhamou N., Chet I., Induction of defense responses in cucumber by the biocontrol agent *Trichoderma harzianum*, Appl. Environ. Microbiol. 65 (1999) 1061–1070.
[39] Yu L.M., Elicitins from *Phytophthora* and basic resistance in tobacco, Proc. Natl. Acad. Sci. USA 92 (1995) 4088–4094.