EFFECTS OF NON-GLYCOSYLATED AND GLYCOSYLATED POLYPEPTIDES SECRETED BY THE GRAPEVINE PATHOGEN EUTYPA LATA, ON THE STRUCTURAL FEATURES AND MEMBRANE PROCESSES IN GRAPEVINE CELLS

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SUMMARY

Eutypa dieback is a devastating disease of Vitis vinifera L. caused by the fungal pathogen Eutypa lata (Pers.: Fr.) Tul. et C. Tul., which colonizes the vascular tissues of the trunk. Symptoms observed in developing shoots and in the foliage indicate that a necrotic signal spreads at a distance from the infected area. Previous studies have shown that toxic polypeptides isolated from E. lata cultures are able to induce some modifications typical of the disease observed in grapevines. The main aim of this study was to investigate the biological effects of polypeptide sub-fractions (glycosylated and non-glycosylated) for determining the mode and site of action of these toxic compounds. Compounds in the non-glycosylated fraction induced leaf necrosis, increased vacuolar tannin synthesis, modified mitochondria, but induced only a partial damage of the cell wall. They hindered the H+ flux and nutrient uptake as a result of the modification of H+ gradient, as seen on plasma membrane vesicles, without affecting the H+-ATPase activity. Eutypine induced the same effects (i.e. on mitochondria and protonophoric action). Polypeptides of the glycosylated fraction increased anthocyanin synthesis in isolated leaves, promoted the decrease of the starch content and caused heavy damage to the cell wall. They also modified H+ flux and nutrient uptake but, in this case, these processes may be the consequence of a direct effect on the H+-ATPase activity. Our results show that the different toxic compounds secreted by E. lata present a complex frame of action on various plant cellular sites. These effects impair plant metabolism and damage cell structure thus favouring host invasion by the fungus.

Key words: Eutypa dieback, Eutypa lata, polypeptide toxins, proton pump, Vitis vinifera, ultrastructural damage.

INTRODUCTION

The ascomycete Eutypa lata (Pers.: Fr) Tul. et C. Tul. was identified by Moller and Kasimatis (1978) as the causal agent of Eutypa dieback, a vascular disease affecting grapevines around the world (Carter et al., 1983). Ascospores infect the xylem tissue through pruning wounds and then colonize the vascular tissues of the branches and trunk, inducing a dark and wedge-shaped necrosis of the wood. After an incubation period of several years (Carter, 1978), particular symptoms appear in the canopy of the vines, consisting of dwarfed and withered shoots, marginal necrosis of the leaves and dryness of inflorescences. A difficulty in identifying disease evolution lies in the fact that the extent of foliar symptoms varies from one year to another, depending, in particular, on climatic conditions (Dumot et al., 2004). Several years after symptom appearance, the infected branches or the whole plant may die. As a consequence, the production life of the vineyard is shortened, resulting in serious economic losses (Munkvold et al., 1994; Molyneux et al., 2002).

Intriguingly, no mycelium is found in the annual canes or in the leaves of infected plants, suggesting that a necrotic signal spreads from the hyphae localized in the trunk towards the distal part of the canopy. At least two types of toxins produced by the fungus may be responsible for symptom production. First, eutypine and related compounds have been isolated (Renaud et al., 1989; Tey-Rulh et al., 1991; Mahoney et al., 2003) and shown to act at various levels in the cells (Deswarte et al., 1996; Amborabé et al., 2001; Kim et al., 2004). However, we have previously observed that eutypine may explain some, but not all, modifications induced in the vascular tissues at a distance from the colonisation site (Rudelle et al., 2005). Secondly, polypeptide compounds were recovered from E. lata culture filtrates, which induced ultrastructural changes in the cell walls of xylem elements of infected plants (Octave et al., 2006a), possibly caused by extracellular hydrolytic enzymes secreted by the fungus (Schmidt et al., 1999).

The aim of this work was to continue investigations on the effects of the polypeptides secreted by the pathogen on some key cellular processes, to secure in-
formation on the way pursued by the fungus for spreading the disease within affected vines. To this aim, we did not try to analyze the action of individual compounds but, rather, we observed the effect of global fractions, possibly synthesized by the fungus in vivo, thus acting as a whole in the host plant. In particular, the attention was focused on specific polypeptide sub-fractions, (glycosylated and non-glycosylated) for assessing tentatively the nature and the cellular site of action of this type of toxins secreted by the pathogen. Indeed, toxic properties have been ascribed to glycosylated polypeptides in other fungal diseases (Wevelsiep et al., 1991; Baillieul et al., 1995; Fogliano et al., 1998).

Experiments, carried out on two models, i.e. cultured grapevine cells and mesophyll cells in isolated leaves, first addressed the ultrastructural damage induced by a treatment with polypeptide fractions and, secondly, the effects on the plasma membrane, which is a primary site of action of many toxic compounds. Changes induced by polypeptides were compared with those triggered by eutypine.

MATERIALS AND METHODS

Fungal growth conditions, isolation of polypeptides and gel electrophoresis. The strain of Eutypa lata BI 1 was grown on yeast nitrogen base minimal medium (YNBm, Difco, USA) at 1.7 g l\(^{-1}\) supplemented with glucose (10 g l\(^{-1}\)) and proline (5 g l\(^{-1}\)) according to Ambórabé et al. (2005). Culture conditions and isolation of polypeptides were as previously described (Octave et al., 2006a). Briefly, fungal liquid cultures in 250 ml flasks were kept in the dark at 21±1°C on a rotary shaker (125 rpm). Mycelium was removed from the culture after 30 days by filtration through Whatman No. 1 filter paper. The filtrate was centrifuged (30,000 g, 30 min, 4°C) and the supernatant was filtered through 0.22-µm Whatman filters and desiccated. The pellet was resuspended in water and desalted by elution through a sephadex G 25 column (PD 10, Amersham Biosciences, UK). Non-glycosylated polypeptides (NF) were recovered first and glycosylated polypeptides (GF) comparatively to eutypine (gift of J.P. Roustan, ENSAT, Toulouse, France). For LM and TEM observations standard. Electrophoresis of protein extracts was according to Laemmli (1970), under a constant current of 18 mA. The PlusOne Silver staining kit (Amersham Biosciences, UK) was generally used, however the kit Pro-Q TM Fuchsia gel stain (Molecular Probes, The Netherlands) was used specifically to detect the glycosylated polypeptides.

Plant materials. Grapevine 41BT cells were obtained from a Vitis vinifera × Vitis berlanderi hybrid grown in Murashige and Skoog medium (MS) (Duchefa M 0230), pH 5.8, with macro- and microelements, vitamins and NH\(_4\) diluted by half. The medium was complemented with sucrose (30 g l\(^{-1}\)), auxin (0.05 mg l\(^{-1}\)) and benzylaminopurine (2 mg ml\(^{-1}\)) and sterilized by autoclaving for 15 min at 0.5 bar. Cells were inoculated and grown at 26°C in the dark under constant agitation (170 rpm). The cell culture was renewed every 10 days.

Grapevines (Vitis vinifera L. cv. Cabernet sauvignon) were grown in pots containing organic compost supplemented weekly with Snyder solution and placed in a greenhouse under natural day-light conditions. Mature leaves were excised under water from canes grown on 2-years-old cuttings from vines which did not show disease symptoms. Petioles were dipped for 5 days in Eppendorf vials containing 2 ml of the assayed fungal extract. In this experimental model, the distance of solute transport is reduced and the problem of the dilution of active compounds that occurs in the case of isolated canes is overcome.

Microscopy. Light microscopy (LM) was used to observe the toxic effect of TF on 41BT cells in in vitro culture. TF was added at the desired concentration after filtration through 0.22 µm pore sized filters in 10 ml Erlenmeyers containing 5-day-old cultures. At various times, aliquots of cells were harvested, put in a Malassez counting cell and stained with Evans blue at 10\(^{-5}\) M. After 5 min staining, the ratio stained (dead) cells/live cells was calculated. In these 41BT cells, transmission electron microscopy (TEM) was used to determine which cell compartments were affected by the treatments. LM and TEM were used to observe the structural and ultrastructural modifications induced in mesophyll cells of isolated leaves by the polypeptide fractions (TF, NF and GF) comparatively to eutypine (gift of J.P. Roustan, ENSAT, Toulouse, France). For LM and TEM observations the same previously described procedure (Rudelle et al., 2005) was used. Briefly, the samples (in vitro- grown cells and leaf blade fragments) were fixed at room temperature (20-25°C) for 45 min in 2% paraformalde-
hyde-0.5% glutaraldehyde adjusted at pH 7.3 with 0.1 M Sörensen buffer. Washing was in the same buffer supplemented with sucrose and postfixation in 1% osmium tetroxide. Dehydration was by graded ethanol dilutions (20, 50, 70, 95, and 100%, 10 min each) and embedding in London resin white polymerized overnight at 60°C. Semi-thin sections (1 µm thick) were stained with toluidine blue in borax for examination with a Zeiss Axioskop light microscope and ultrathin sections (70 nm) were collected on gold grids and stained with uranyl acetate and lead citrate prior to examination with a Jeol JEM 1010 electron microscope.

Measurement of pH variations. pH variations were measured in the incubation medium of 5-day-old cell cultures. The cells, washed two times in medium M composed of 175 mM sorbitol, 5 mM CaCl2, 0.5 mM K2SO4, 0.25 mM MgSO4 and 1 mM sucrose, were recovered on a filter. Four g of cells were resuspended in 10 ml of medium M. pH variations in the incubation medium were read with a Beckman expandomatic SS2 pH meter provided with a combination electrode and linked to a potentiometric recorder. The incubation medium was aerated with a rod stirrer (Metrohm E 622, France). Compounds were added as indicated in Fig. 9 A, B.

Proton pumping and H⁺-ATPase activity of plasma membrane vesicles. In order to obtain purified plasma membrane vesicles (PMVs), 30 g grapevine cells were frozen in liquid nitrogen and ground in 200 ml of a medium composed of 100 mM Tris/4-morpholineethanesulfonic acid (MES), 20 mM ethylenediaminetetraacetic acid, 500 mM saccharose, 10 mM dithiothreitol (DTT) and 1 mM phenylmethanesulfonyl fluoride and supplemented with polyvinylpolypyrrolidone (0.6% W/V). After filtration through three layers of cheesecloth, the filtrate was centrifuged for 15 min at 7,500 g. The supernatant was centrifuged for 35 min at 10,000 g. The pellet containing the microsomal fraction was resuspended in 2 ml of the following medium B: 5 mM potassium phosphate, 0.25 mM sucrose and 1 mM DTT, pH 7.8. PMVs were prepared by two phases partitioning according to a method adapted from Lemoine et al. (1991). The microsomal fraction was added to a 15-g partition system of polyethylene glycol/dextran 6.4% prepared in medium B. A final centrifugation was carried out for 1 h at 100,000 g. The pellet containing PMVs was resuspended in medium B supplemented with glycerol (20% W/V) and 1 mM ATP. PMV were stored at -80°C. Proton pumping of PMVs was followed by the decrease of 9-aminoacridine absorbance at 495 nm, and vanadate-sensitive ATPase activity of the PMV was measured as described in Noubahni et al. (1996).

Uptake of radilabelled compounds. The uptake capacity of cells was studied by assays adapted from Roblin et al. (1998). Briefly, cells obtained as above were incubated in medium N (300 mM mannitol, 0.5 mM CaCl2, 0.25 mM MgCl2) buffered with 20 mM MES (pH 5.5) containing 1 mM [U14C]-sucrose (11.0 kBq ml⁻¹), 1 mM [3H]-valine (9.25 kBq ml⁻¹) or 8 µM [3H]-tetraphenylphosphonium bromide (18.5 kBq ml⁻¹) (TPP). After treatment with the assayed inhibitors for various durations and incubation with the labelled substrates, cells were recovered at regular intervals on glass microfibre filters and washed three times with cold medium N to remove apoplastic labelling. Filters and cells were dipped in 4 ml ecolyte and radioactivity was measured by liquid scintillation spectroscopy.

RESULTS

Polypeptide toxins of E. lata acted at various levels on grapevine cells in culture. First, a preliminary experimental step concerned the verification that the model of 41BT cells in culture responded similarly to other models used previously after a treatment with TF (Octave et al., 2006b). To this purpose, effect of TF was assayed on three different processes: cellular death, structural cell modifications and membrane potential changes.

In cells treated with TF at various concentrations (5, 10, 15 µg ml⁻¹), the rate of cell mortality was measured under the light microscope by quantifying the dead cells stained with Evans Blue. As seen in Fig. 1, the number of dead cells decreased in control sets (from 14 to 8% after 72 h) as a consequence of cell multiplication. By contrast, the number of dead cells in treated sets increased earlier when the applied TF dose increased. After 24 h treatment, the percentage of dead cells reached 90% at 10 and 15 µg ml⁻¹ TF concentration.

![Fig. 1. Evolution of the mortality of grapevine 41BT cells treated with increasing concentrations of the polypeptide fraction (TF) secreted by E. lata in its culture medium. Data represent mean ± SD; n = 3.](image-url)
Fig. 2. Comparative effect of the polypeptide fraction (10 µg ml⁻¹) secreted by *E. lata* and eutypine (10⁻⁴ M) on the structure of grapevine 41BT cells in culture. **A**. Phase contrast view of 41BT cell profiles associated by groups. Large central vacuole (va) surrounded by a narrow cytoplasm (white arrow), thin wall (W), nucleus (N). Bar = 5 µm. **B**. TEM micrographs of a control cell showing a lobate nucleus (N), a large nucleolus (nu), numerous proplastids (P) containing starch grains (s), mitochondria (m) with small cristae, rough endoplasmic reticulum cisternae (er), dictyosome saccules (G) and a thin wall (W). **C**. Detail of the three-layered (L1, L2, L3) wall (W) associated with fibrils (f) along the intercellular space (Is). The periplasmic space (ps) was bordered by irregular plasmalemma profiles (open arrow). **D**. Opaque middle lamella (ml) between contiguous cells, dense cytoplasm containing long and dilated cisternae of endoplasmic reticulum (er), stacks of dictyosome saccules (G) and mitochondria (m) with small cristae (m). **E**. Cells treated with TF: detail of plastid (P) with dense matrix, mitochondria with dilated cristae (m) and nucleus (N) with clear matrix. **F**. External part of the wall (L1) in cells treated with TF bordered by a network of opaque fibrils (f) that also occurs in intercellular space (Is), median and internal wall parts. L2, L3 show a loose structure, small aggregates of ribosomes (thin arrow in particular nearby the plasma membrane (open arrow). **G**. Damaged wall between contiguous cells treated with TF with remaining parts of the opaque middle lamella (arrow) and large caveolae (cav) containing membrane residues, mitochondria (m) with dilated cristae. **H**. Detail of organelles in cells treated with eutypine: mitochondria with large cristae, lobate nucleus (N). **I**. Three-layered wall and damaged vacuolar (va) membrane (thin arrow). Note the thin network of fibrils (f) decorated with polysomes in the cytoplasm. Paraformaldehyde/ glutaraldehyde/0.6% LRWhite, ultrathin sections, uranyl/lead. Bars (B-I) = 1 µm.

As shown in Fig. 2A, grapevine 41BT cells in culture grew in groups of 3-5. They were elongated, 30-50 µm wide and surrounded with a narrow pectocellulosic wall. These cells were highly vacuolated (about 90% of the cell volume). Ultrastructural observation (Fig. 2B) showed a multilobate nucleus with a voluminous nucleolus, numerous mitochondria, plastids containing large starch granules, abundant rough endoplasmic reticulum and many dictyosomes. The pectocellulosic wall was distinctly formed by the 3 characteristic layers (L1, L2, L3), the most external being surrounded with a fibrillar layer (Fig. 2C). The contact area between two cells showed wall layers of uniform density (Fig. 2D).

A 24 h treatment with TF at 10 µg ml⁻¹ induced dramatic cellular modifications. The nucleus contained a rather electron lucent nucleoplasm, mitochondria had dilated cristae and plastids had no starch (Fig. 2E). The cell wall was particularly altered since the L layers were no longer distinguishable (Fig. 2F) and the middle lamella appeared disrupted in certain places (Fig. 2G). Compared with controls, cells treated with eutypine had a homogenous cytoplasmic matrix, a normally appearing nucleus and mitochondria with large cristae (Fig. 2H). By contrast to TF, the treatment did not modify the cell wall structure, in particular the L layers were clearly visible (Fig. 2I). These data show that, compared to other types of toxins possibly present in the *Eutypa* dieback disease, structural features of grapevine cells were specifically altered by the fungal polypeptides.

Previous works have shown that the early action of microbial toxins affected the plasma membrane (Dixon *et al.*, 1994), resulting in particular of the electrical potential of treated cells. Electrical membrane potential variation that could be induced by molecules of TF was measured by TPP uptake in 41BT cells. Indeed, TPP has been used in various experiments as a probe to estimate transmembrane potential in different cell models (Lin, 1985; Komor and Tanner, 1976). As seen in Fig. 3, TPP was differently absorbed according to the assayed product. Thus, compared with controls, TPP was found in higher amount after 20 min in the cells treated with eutypine and FC (by 26 and 100%, respectively), whereas absorption was inhibited by 47% in TF-treated cells. This data indicate that TF depolarized the membrane, as confirmed by the result obtained with FC, which is known to hyperpolarize plant cell membrane. The induced modification of membrane potential implicated that some ions were differently distributed and, noteworthy, that TF and eutypine acted at this level in an opposite way.

Taking into account these results as a whole, it should be stressed that polypeptide toxins induced in cultured cells modifications similar to those observed previously in other plant models by decreasing cell viability, inducing characteristic structural damage, and modifying membrane processes. Therefore, 41BT cells in culture appear as a convenient experimental model suitable for subsequent studies on particular physiological processes induced by the various *E. lata* toxins.
Glycosylated and non-glycosylated polypeptides did not act by the same mechanism. The separation of polypeptides secreted by *E. lata* in two distinct pools was done since the presence of glycosylated proteins showing toxic activities had been already recorded in other fungi such as *Rhynchosporium secalis* (Wevelsiep et al., 1991), *Phytophthora megasperma* (Baillieul et al., 1995) and *Phoma tracheiphila* (Fogliano et al., 1998).

Affinity chromatography on concanavalin A column allowed the separation of GF and NF from TF. Quantitatively, polypeptide isolated in GF corresponded to about a third of the total protein content. Subsequent electrophoretic analysis showed that glycosylated peptides (at least 10 bands visualized) were secreted in the fungal culture medium, the molecular mass of which ranged from 36 to 150 kDa. It should be stressed that, contrary to NF fraction, polypeptides with low apparent molecular mass (< 36 kD) were not found in GF (Fig. 4A). This was corroborated by staining with the kit Pro-Q™ fuchsia stain (Fig. 4B). Superposition of GF and NF patterns allowed to recover the TF pattern.

Experiments conducted on excised leaves have the advantage that the distance of substrate transport is reduced, so that the problem relative to dilution of the active compound that may occur in the case of leaves grown on isolated treated canes is overcome (Octave et al., 2006a). The three polypeptide fractions (TF, NF, GF) and eutypine induced different macroscopic modifications on isolated leaves. Thus, compared to control (Fig. 5A), TF induced necrosis along the veins and at the tips of leaf denticles (Fig. 5B), NF induced a dramatic necrosis of leaf tissues during the short time lapse of the experiment, leading to a total withering of the leaf (Fig. 5C), and GF triggered less deleterious effects exemplified by red areas along the veins of the redberried cv. Cabernet sauvignon due probably to the syn-

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**Fig. 4.** SDS-PAGE patterns of polypeptides secreted by *E. lata* after 30 days in culture. TF: total polypeptide fraction, GF: glycosylated polypeptides, NF: non-glycosylated polypeptides. A. Acrylamide 12 %; 1.4 µg protein per lane; silver nitrate staining. B. Acrylamide 10 %; 5 µg protein; Pro-Q™ fuchsia staining. kDa: molecular mass.

**Fig. 5.** Comparative effect of polypeptide fractions applied at 10 µg ml⁻¹ (B) TF, (C) NF, (D) GF, extracted from *E. lata* culture medium and (E) 10⁻⁴ M eutypine on *Vitis vinifera* isolated leaves (cv. Cabernet sauvignon). Petioles of the leaves were dipped for 5 days in the medium containing the products. A. Control in water. Ne = necrosis; an = anthocyanins.
thesis of anthocyanins (Fig. 5D). On the leaves of the white-berried cv. Ugni-Blanc similarly treated, symptoms were chlorotic areas appearing in the same areas (not shown). The effect of $10^{-4}$ M eutypine was low (Fig. 5E) and detected by a faint chlorosis at leaf tip spreading on the whole lamina in the days that followed the treatment.

Treatments with the various fractions did not modify the general leaf structure but induced macroscopic changes at the tissue level as observed in light microscopy (Fig. 6). Cross section in a control leaf showed that palisade parenchyma contained tannins in the central vacuole and a large amount of starch in the plastids (Fig. 6A). After treatment with TF, the tannin content increased in the vacuoles and starch granules decreased in volume (Fig. 6B). NF induced a characteristic high
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development of tannins in palisade and spongy mesophyll and acted also on plastids, although some small starch grains remained (Fig. 6C).

The application of GF induced the formation of some tannins globules and a dramatic loss of starch (Fig. 6D). Under etupine treatment, the loss of starch was lower than under TF treatment but the presence of tannins was also noted (Fig. 6E). Thus, it is plausible to conclude that the polypeptide fraction and etupine acted on two particular metabolic steps, namely tannin synthesis and plastid metabolism, as shown by the regulation of starch content.

Observations at the ultrastructural level corroborated the preceding remarks and detailed the effect of the various treatments on the different cell organelles in leaf parenchyma cells. Control cells had a nucleus with chromatin clusters, vacuoles contained few tannin and mitochondria had small cristae (Fig. 7A, C). Plastids contained very large starch grains and large globules with amorphous content (Fig. 7A, B). Peroxisomes possessed a loose matrix (Fig. 7C).

Treatment with TF induced the formation of irregularly shaped nuclei with dense chromatin content. The synthesis of tannins, lining the tonoplast and forming internal deposits was enhanced (Fig. 7D). Mitochondria showed clear areas containing electron-opaque deposits (Fig. 7E). Starch grains persisted in the plastids, together with large plastoglobules that protruded from the organelle’s body, thus altering its shape. The grana were disorganized and thylakoids were dilated (Fig. 7F). Peroxisomes were also affected as shown by their dense matrix (Fig. 7G).

Treatment with NF led to modifications of various extent. Nuclei contained a matrix with a low density., large amount of tannins occurred in the vacuoles (Fig. 7H) and mitochondria showed dilated cristae (Fig. 7I). Long thylakoids and small grana were observed in the plastids but very few starch grains were present (Fig. 7J). Peroxisomes contained a dense matrix (Fig. 7I).

After GF treatment, nuclei had a very fine granular content, tannins were abundant mainly along the tonoplast (Fig. 7K), mitochondria had very small cristae and showed clear areas in the matrix (Fig. 7L). Characteristically, a high decrease in starch content occurred in the plastids (Fig. 7K, M), some grana persisted, but the thylakoids occupied the majority of the plastid volume and some plastoglobules were also present (Fig. 7M). Peroxisomes were voluminous and exhibited a dense matrix (Fig. 7N).

Nuclei of etupine-treated cells showed a nucleoplasm with a washed out appearance and chromatin condensed in small patches along the nuclear membrane (Fig. 7O). Tannins occurred along the tonoplast and as spherical inclusions in the vacuoles. Many large cristae were observed in mitochondria (Fig. 7P). In plastids, starch grains were still present but were less voluminous than in control cells, thylakoids were strongly dilated and the periplastidial reticulum was abundant in the dense stroma (Fig. 7Q). As in controls, peroxisomes had a loose matrix sometimes containing an opaque inclusion (Fig. 7Q).

Particular attention was paid to the modifications of the cell walls since previous observations on cane tissues had shown that this cell site was typically damaged by TF treatment. In control cells, the wall frame was uniform, the middle lamella was dense between adjacent cells and the external border along intercellular spaces was also denser than the median part of the wall (Fig. 8A). Treatment with the TF led to a degradation of the wall. In its external part, groups of fibrils formed a dense granular border and were located in the intercellular space where long parietal fragments also occurred (Fig. 8B). The presence of debris stuck to the wall was also frequently observed (Fig. 8C) as in the leaves from isolated treated canes (Octave et al., 2006a). The treatment with NF induced damages in the internal border of the wall in which many opaque granules were formed but the middle lamina still persisted (Fig. 8D). The treatment with GF resulted in the formation of opaque granules in the whole thickness of the wall and in the loosening of the middle lamella (Fig. 8E). In contrast with the above treat-
ments, eutypine did not modify the wall structure which appeared identical to that of controls (Fig. 8F).

Considering the previous results on the modification of membrane potential, a particular attention was paid to the plasma membrane H+-ATPase playing a major role in the regulation of cytoplasmic pH and electrical potential gradients (Sze, 1984). To investigate this issue, our study was first addressed to the effect of the various polypeptide fractions on the proton fluxes. Fig. 9A shows the time-course of pH variations recorded in the bathing medium of grapevine cells after different treatments. It was observed that cells induced a spontaneous acidification of the incubation medium, decreasing the pH from 6.2 to a steady value of 4.1 in nearly 4 h. A transitory

Fig. 8. Effects of polypeptide TF, NF, GF at 10 µg.mL⁻¹ and 10⁻³M eutypine on the wall structure in palisade parenchyma cells of isolated leaves. A. In control leaves, cell wall (W) is constituted by a regular network of fibrils; ml = middle lamella. B. In leaves treated with TF, damage occurs in the cell wall whose fibril network is loosened particularly in ml and partly released in the intercellular space (IS). C. Detail showing the granulous aspect of the internal border (asterisk) of the wall and a dense opaque material (white cross) lining its external border. D. NF induced damage on the internal edge of the wall that contains opaque granules (asterisk). E. GF modifies the wall that becomes granular (asterisk) in its whole thickness and fibrils (arrow) occur in the loosen middle lamella. F. In leaves treated with eutypine, the cell wall looks like that in control. Paraformaldehyde/glutaraldehyde/os04, LRWhite, ultrathin sections, uranyl/lead. Bars = 1 µm.

Fig. 9. Comparative effect of polypeptide fractions excreted by E. lata, 10⁻⁴ M eutypine and 10⁻⁵ M FC on the time course of pH variations in the bathing medium of grapevine 41BT cells in culture. C: control. A. Effect of TF applied at various concentrations (2.5, 5, 20 and 30 µg ml⁻¹), the heat-inactivated TF (In), eutypine (Eu) and fusicoccin (FC). B. Effects of TF, GF and NF applied at 10 µg ml⁻¹. Polypeptide fractions were added at the first arrow and 10⁻⁵ M fusicoccin (FC) at second arrow. Experiment was carried out 3 times with similar result.
dose-dependent pH rise was induced following addition of the TF solution at polypeptide concentrations higher than 2.5 µg ml\(^{-1}\). At 5 µg ml\(^{-1}\), a slowing down of the proton efflux was only observed after a latency period of 10.4±0.9 min (n = 3). At 10 µg ml\(^{-1}\), the pH rise (0.30±0.06 unit; n = 7) began after 2.2±0.3 min and reached a peak at 28.6±1.4 min. At 30 µg ml\(^{-1}\), the processes were amplified as the latency period only lasted 10-30 sec, the amplitude reached 0.68±0.11 unit (n = 3) and the maximum was observed at 21.7±1.8 min. This effect was truly due to the polypeptide nature of the toxic components since treatment with protease (not shown) or with a denatured TF stock solution (boiled for 5 min) abolished pH variation (Fig. 9A). By comparison, eutypine at a relatively high concentration (10\(^{-4}\) M) only triggered a transitory pH rise of small amplitude. By contrast, fusicoccin increased the rate and amplitude of pH acidification (Fig. 9A). The three fractions (TF, GF, NF) modified the H\(^{+}\) fluxes but with a different efficiency (Fig. 9B). Thus, by comparing the various recordings, at the same polypeptide concentration, GF triggered a lower pH rise than NF (0.13±0.03 versus 0.50±0.05 pH unit) with an increased latency period (4.1±0.3 min).

The opened question was to determine whether these modifications in H\(^{+}\) fluxes resulted from a direct effect on the proton pump ATPase of the plasma membrane. A part of the response was given by experiments carried out on PMV. As seen in Fig. 10A, the three fractions acted on the PMV proton conductance in a dose-dependent manner up to 30 µg ml\(^{-1}\). At this final concentration, TF and NF inhibited nearly completely the H\(^{+}\) influx in the inside-out PMV whereas GF only inhibited the process by 60%. In every case, the threshold concentration was at 2.5 mg ml\(^{-1}\). The inhibition of H\(^{+}\)-ATPase activity (Fig. 10B) was only detected at 10 µg ml\(^{-1}\). At a final concentration of 30 µg ml\(^{-1}\), an inhibition was induced following treatment with TF (45%) and GF (40%). By contrast, NF only inhibited by 10% the enzyme activity. Thus, the present data suggest that GF acts directly on the proton pump activity whereas NF merely modified H\(^{+}\) fluxes through a protonophoric activity.

![Fig. 10](image1.jpg)

**Fig. 10.** Effects of TF, NF and GF secreted by *E. lata*, applied at various concentrations on (A) the proton pumping and (B) the H\(^{+}\)-ATPase activity of PMV purified from grapevine 41BT cells in culture. Data represent mean ± SD; n = 9. Different letters indicated data significantly different at the 5% probability level by Student-Fisher t-test.

![Fig. 11](image2.jpg)

**Fig. 11.** Effects of TF, NF and GF applied at 10 µg mL\(^{-1}\) on the time course of (A) 1 mM sucrose uptake and (B) 1 mM valine uptake by grapevine 41BT cells in culture. Data are mean ± SD; n = 9. C: control. Data obtained with TF, NF, GF are significantly different from those in control at the 5% probability level by Student-Fisher t-test.
As a consequence of the modifications described above, concerning proton gradients and membrane potential, it may be expected that the membrane transport activities would be affected. In particular, the uptake of nutrients driven by $H^+$-coupled co-transport systems should be hindered (Rheinhold and Kaplan, 1984; Serrano, 1989). As expected, the uptake of valine and sucrose was greatly decreased following treatment with the three polypeptide fractions (Fig. 11A, B). In all cases, the inhibition of transport was significant as soon as 10 min after the beginning of the treatment with the active fractions. After 30 min treatment with GF, NF and TF, sucrose uptake was inhibited by 36, 47 and 53% respectively. In the same conditions, valine uptake was also inhibited in the same range (38% with GF, 43% with NF and 48% with TF).

**DISCUSSION**

Eutypa dieback is a severe disease of grapevines the evolution of which is still poorly understood. In particular, the molecular agents that induce the observed symptoms are not fully known. Until recently, symptoms have been attributed to the production of small acetylenic molecules, namely eutypine (Renaud et al., 1989; Tey-Ruhl et al., 1991) and related compounds (Mahoney et al., 2003) synthesized by E. lata. We have previously shown that eutypine alone cannot explain ultrastructural modifications observed in xylem cells (Rudelle et al., 2005). We recently showed that polypeptide compounds isolated from fungal culture are transported over long distance in canes and induce changes in leaf cells consisting of damage to plastids, mitochondria, peroxysomes and cell walls. These modifications were induced both in mesophyll cells and vessel-associated cells (Octave et al., 2006a). Phytotoxic peptides have been shown to intervene in other diseases induced by several pathogenic fungi belonging to various fungal families, e.g. Ceratocystis ulmi (Strobel et al., 1978), Pyrenophora tritici-repentis (Ballance et al., 1989), Rhynchosporium secalis (Wevelsiep et al., 1991), Cladosporium fulvum (Scholtens-Toma and De Wit, 1988), Phoma tracheiphila (Fogliano et al., 1998) and many Phytophthora species (Capasso et al., 1999). In some cases, these active peptides are glycosylated.

The major aim of this work was therefore to discriminate the mode of action of the two polypeptide pools (i.e. glycosylated and non-glycosylated) secreted by E. lata on the grapevine cell metabolism, in particular, in the early stages of their action at their arrival on the plasmalemma. In this attempt, which did not aim at the isolation of particular compounds, we hypothesized that the compounds secreted in the fungal culture medium might be the active agents acting as a whole in the host plants infected by the fungus. The investigations carried out at various levels have shown that non-glycosylated and glycosylated components act by different mechanisms and that their effects differ from those induced by eutypine. This conclusion derives, first from the macroscopic damage observed on excised leaves treated with the various solutions and, secondly, from the observations at the tissue level, that showed a modulation of parenchyma cell metabolism in excised leaves. In particular, it should be stressed that GF induced a characteristic synthesis of tannins. Another typical feature observed at the tissue level was the modification of the starch content, which tallies with the results obtained with leaves from isolated canes (Octave et al., 2006a). However, a difference was observed with starch metabolism in the two models, since the isolation of leaves led to starch accumulation in untreated mesophyll cells.

The above observation are also supported by molecular data. It has been shown that early effects of tested polypeptides interfere with ionic exchanges at the plasmalemma level. The transitory dose-dependent $H^+$ flux modification and the induced membrane depolarization can be causally linked since a $H^+$ entry in cells corresponds with a membrane depolarization. In most cases, such proton influx results from inhibition of membrane $H^+$-ATPase (Hageendoorn et al., 1991). Experiments carried out with PMV, allowing avoidance of interference with metabolism, showed that inhibition of $H^+$-ATPase was restricted to glycosylated fraction, as inferred from the decrease observed in catalytic activity and the increase in proton conductance. By contrast, the non-glycosylated pool may only possess a protonophoric activity without acting directly on the $H^+$-ATPase (Fig. 10). In this way, the active compounds in this fraction may act as eutypine which is known to affect mitochondria and plasma membrane functioning (Deswarte et al., 1996; Amborabé et al., 2001). The effects on the $H^+$-ATPase activity and on the plasma membrane proton conductance that destroy the ionic gradients are expected to lead to a dramatic impairment of the energetics of the cell. Consequently, a decrease in the assimilate uptake linked to the proton motive force may bring about a metabolic starvation. This assumption is sustained by the data obtained on the uptake of sucrose and valine (Fig. 9).

A second major point to consider is that, beside the early action on the plasma membrane discussed above, some polypeptides possess a more long lasting effect as shown by the induced cell wall damage observed in planta (Octave et al., 2006a). The data on cells in culture and mesophyll cells in excised leaves corroborated this observation. This result indicates that some polypeptides within the culture medium possess enzyme activity. This result was specific for the polypeptide fraction since no modification was observed after the eutypine treatment. More precisely, GF induced heavier wall damage. Enzyme activities have already been detected in extracts of
**E. lata** in culture, namely, cellulase, xylanase and 1,3-β-glucanase activities (Schmidt et al., 1999).

The major conclusion is that in complex diseases such as those associated with wood degradation, pathogenic fungi may act through several types of chemical weapons acting at various levels of cell functioning. Indeed, we showed here that fungal action occurs at least at three steps by means of four types of compounds. The first signal, brought by the glycoprotein fraction, is directed against the normal plasmalemma function by inhibition of H+-ATPase, a key enzyme of cell energetics. Two other actions, resulting from the protonophoric properties of eutypine-like compounds and some non-glycosylated polypeptides, led to a disturbance of the ionic transmembrane gradients in the plasma membrane, mitochondria and chloroplasts. A third level corresponds to the degradation of the cell wall structure and subsequently of the cell integrity by proteins with enzyme activity. In *vivo*, these various toxic compounds may have a cooperative action explaining why this disease is so difficult to perceive in its whole symptomatology.

Many points remain to be elucidated and future works will need to integrate the present data in the knowledge of the evolution of the disease. A major point is to demonstrate the occurrence of these toxic polypeptide compounds in *vivo* and their link with symptomatology which, remarkably, appears only 3 to 10 years after infection. This delayed effect might be related to the early isolation of hyphae in *vivo* by means of physical and chemical barriers built up by the plant in reaction to fungal colonization. It is therefore probable that the mycelium needs to overcome a barrier to develop and allow symptom expression.

A second crucial point is to determine the pattern of polypeptide secretion during a yearly cycle in order to understand the variability observed in the expression of symptoms as a function of the season: i.e. symptoms are observed in spring. This observation has to be made on an extended period since symptoms vary from one year to another.

A final point is the isolation and characterization of the active compounds entering each type of physiological action described in this work. Future data in these directions might increase our understanding of the insidious character of the disease.

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**REFERENCES**


Pathogenetic effects of *Eutypa lata* to the grapevine


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