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Ultrastructural changes produced in plantlet leaves and protoplasts of *Vitis vinifera* cv. Cabernet Sauvignon by eutypine, a toxin from *Eutypa lata*

by

CORINE DESWARTE[1]), P. ROUQUIER[1]), J. P. ROUSTAN[1]), R. DARGENT[2]) and J. FALLOT[1])

[1]) Ecole Nationale Supérieure Agronomique, Toulouse, France
[2]) Laboratoire de Cryptogamie, Mycologie cellulaire et Phytopathologie, Université Paul Sabatier, Toulouse, France

S u m m a r y : Eutypine is a toxin produced by *Eutypa lata* (Pers.: Fr.) Tul., the causal agent of dying-arm disease of the grapevine. Ultrastructural alterations induced by eutypine in leaf cells and protoplasts isolated from plantlets of *Vitis vinifera* cv. Cabernet Sauvignon, a very sensitive variety cultured *in vitro*, were observed for the first time by transmission electron microscopy. Eutypine caused early cytoplasm lysis and vesiculation followed by chloroplast swelling with thylakoid dilation. The eutypine-induced alterations of the cellular ultrastructure are similar to those previously described *in vivo* in the leaves of diseased grapevines. These results confirm that eutypine, synthesized by *E. lata* mycelium present in the trunk or arms, is involved in symptom expression of eutypiosis in the herbaceous parts of grapevine.

K e y w o r d s : eutypiosis, eutypine, toxin, Eutypa lata, plantlet leaf, protoplast, ultrastructure, Vitis vinifera.

Introduction

Eutypiosis, due to the ascomycete fungus *Eutypa lata* (Pers.: Fr.) Tul., affects a number of vineyards throughout in the world. In France, several quality *Vitis vinifera* cultivars such as Cabernet-Sauvignon are very sensitive. The parasitic fungus colonizes the trunk and arms of old grapevines and acts from a distance causing, in particular, foliar lesions. In June, dying arm disease symptoms in the vineyard are clearly visible. Leaves show weak development, yellowing, and, as soon as they unfold, small necrotic patches appear. The disease induces ultrastructural alterations of the leaf cells of the grapevine as observed by electron microscopy such as cytoplasmic lysis with plasma membrane detachment and complete chloroplast disorganization (PHILIPPE *et al.* 1992).

One compound has been isolated from the culture medium of *E. lata* which appears to be toxic to the grapevine (RENAUD 1985; TEY-RULH *et al.* 1988). This compound, hydroxy-4 (methyl-3 butene-3ynyl-1)-3 benzaldehyde, named eutypine, is always present in diseased grapevines, especially in the leaves, and is absent in healthy grapevines (TEY-RULH *et al.* 1991).

However, an *in vivo* study is difficult because of the 3-10 years delay between ascospore infection of the wood vessels and the appearance of symptoms. Moreover, symptom expression varies from year to year suggesting the dependence on eutypine synthesis for leaf damage effects. On the other hand, environmental conditions could interfere with toxic effects and lead to false observations. Bioassays, based on the treatment of leaves from plantlets cultured *in vitro* or protoplasts with eutypine, have previously been developed (FALLOT *et al.* 1990; TEY-RULH *et al.* 1991). They allow the application of a determined concentration of toxin in controlled environmental conditions.

The present study was initiated to determine the ultrastructural alterations caused by eutypine in excised Cabernet-Sauvignon plantlet leaves and protoplasts, and also to compare these alterations with those observed in leaves of severely diseased grapevines in the vineyard.

Materials and methods

P l a n t m a t e r i a l a n d i s o l a t i o n o f p r o t o p l a s t s : Plantlets of *V. vinifera* cv. Cabernet Sauvignon n°15 were cultured *in vitro* as described by MAURO *et al.* (1988). Protoplasts were isolated from leaves of two-month-old plantlets placed in the dark for 24 h, prior to protoplast isolation. The leaves were cut into 1 mm slices and the cell walls digested in the enzyme-containing medium (20 ml for 150 mg of mesophyll): 0.8 M mannitol, 1 mM $CaCl_2$, 0.03 % pectolyase Y23, 0.2 % caylase 345L, 25 mM MES, pH 5.6. After 19 h at 24 °C, the tissues were dissociated by stirring (120 rpm) for 20 min. The protoplasts released were purified by filtering through cheese cloth and washed three times at 100 *g* for 5 min in MURASHIGE and SKOOG medium (1962) to which had been added 0.8 M mannitol, 58 mM sucrose, 0.4 % polyethylene glycol and 25 mM MES (pH 6.0).

B i o a s s a y s : To obtain rapid symptom expression and in order to limit variability of the toxic response, chemically synthesized eutypine (DEFRANQ *et al.* 1993) was applied at various high concentrations. For the leaf bioassay, the leaves were excised from 2-month-old plantlets, cultured *in vitro*, between 3rd and the 5th node level. Each

Correspondence to: J. FALLOT, Ecole Nationale Supérieure Agronomique, 145 Avenue de Muret, F-31076 Toulouse Cedex, France.

leaf was submerged in 2.5 ml of eutypine solution (500 µM, 750 µM or 1 mM) for 48 hours at 25 °C under 100 µmol m^{-2} s^{-1} with a photoperiod of 16 h. The toxicity of eutypine was estimated using a scale quantifying the degree of necrosis (MAURO *et al.* 1988). For each treatment, 6 leaves were incubated.

Protoplast bioassay was developed by incubating 1 ml of protoplast suspension containing 4.5 x 10^5 protoplasts in the presence of 100 or 500 µM eutypine using the conditions described for plantlet leaves. The toxicity of eutypine was determined as the percentage of dead protoplasts estimated by observing the lack of cell fluorescence after staining with fluorescein diacetate (LARKIN 1976). For each treatment, 3 experiments were performed and approximatly 100 protoplasts were examined.

Processing for electron microscopy: Leaves were placed in a Petri dish containing 1 % glutaraldehyde in 0.1 M K-phosphate buffer pH 7.2, and the margin of the leaf was cut to facilitate fixative penetration. After 10 min under vacuum (400 mm Hg), samples were incubated at 4 °C for 50 min. Leaves were fixed at 4 °C for 90 min in 3 % glutaraldehyde, 0.1 M K-phosphate buffer, pH 7.2. Then 2 mm^2 fragments were cut from each leaf near the marginal and petiolar areas, outside the necrotic parts. Samples were rinsed 5 times (15 min) with 0.1 M K-phosphate buffer pH 7.2. The tissues were fixed in 1 % osmium tetroxide in 0.1 M cacodylate buffer at pH 7.3 for 90 min. They were then washed 4 times in the cacodylate buffer for 10 min, dehydrated in an ethanol series followed by propylene oxide twice for 30 min. The samples were embedded after 2 h in a propylene oxide/epon (1/1, v/v) mixture, 12 h at room temperature in Epon and 48 h at 60 °C for epon polymerisation. Thin sections were cut on a Reichter Om U_3 ultramicrotome with a diamond knife, mounted on copper grids, stained with uranyl acetate-lead citrate (REYNOLDS 1963) for 30 min at room temperature, and viewed in a Philips EM 301 electron microscope at an accelerating voltage of 80 kV. The protoplasts were fixed in 2.5 % glutaraldehyde, 0.1 M K-phosphate buffer, pH 7.2, at 4 °C for 4 h. They were washed 8 times for 15 min in the K-phosphate buffer. Fixation, dehydration and embedding were the same as described for plantlet leaves.

Results

Biological tests: After 24-h incubation in the presence of 500 µM, 750 µM and 1 mM eutypine, leaves from the cultured plantlets showed some necrosis located in the marginal area which later extended to the leafstalk (Fig. 1). The speed of symptom spread appeared to increase with eutypine concentration. After 48 h of incubation with 750 µM or 1 mM eutypine, the whole leaf area was discolored, whereas in the presence of 500 µM eutypine, less than the half leaf was injured.

The protoplasts were treated with 100 or 500 µM eutypine. Cell death appeared most quickly with the highest concentration of eutypine (Fig. 2). In the presence of 500 µM eutypine, all the cells were dead after 24 h of incubation, whereas after 48 h with 100 µM eutypine mortality only reached 20 %.

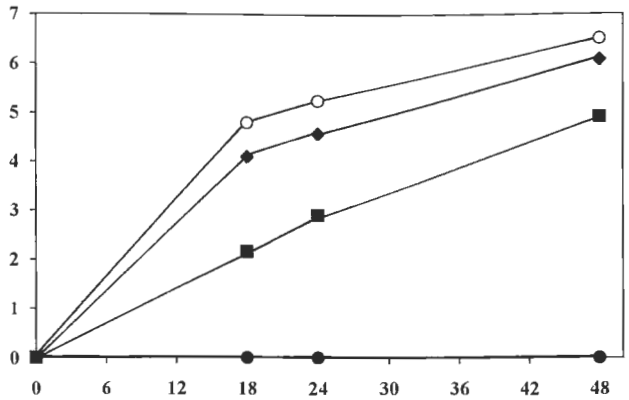

Fig. 1: Development of necrosis in excised leaves treated with eutypine; 0 (●), 500 (■), 750 (◆) or 1000 µM (○). Results are the mean of 6 experiments (SD < 1 %).
Scale of necrosis: 0: no necrosed areas, 1: end of a few serrations, 2: all serrations, 3: ≤ 1/2 leaf, 4: > 1/2 leaf, 5: the whole leaf brown-green colored, 6: the whole leaf green-yellow colored, 7: the whole leaf yellow colored.

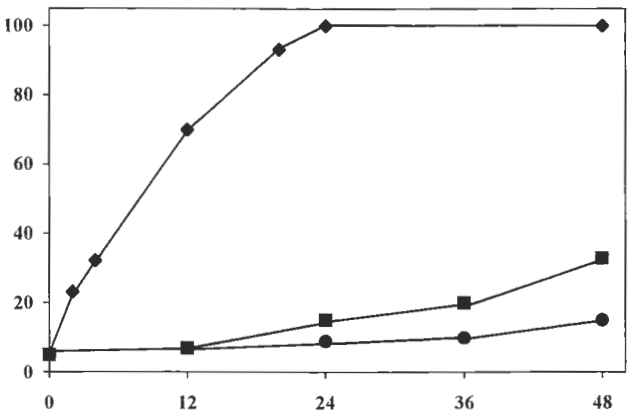

Fig. 2: Development of protoplast death with time of exposure to eutypine; 0 (●), 100 (■) or 500 µM (◆). Values represent the mean of 3 experiments (SD < 10 %).

Ultrastructure of untreated mesophyll cells and protoplasts: Grapevine mesophyll cells (Fig. 3-a) present a conventional structure. The cytoplasm appears in a relatively thin layer. Near a large vacuole, the nucleus can be observed. Chloroplasts (5.4 µm long, 2.5 µm wide), peripherally situated, are surrounded by the typical envelope composed of two membranes; they contain a tight lamellar network and some plastoglobuli in their stroma. Mitochondria often appear proximal to the chloroplasts. Similar good preservation of structure was obtained with protoplasts (Fig. 4-a).

Ultrastructure of mesophyll cells after eutypine treatment: Because of the small difference on the scale quantifying necrosis between 1 mM and 750 µM eutypine concentration, we chose to study the ultrastructural effects of toxin on plantlet leaves with 750 µM eutypine. Cell ultrastructural modifications were observed in marginal area where necrosis appeares first, and petiolar areas of leaves after a 6 or 24 h period of incubation.

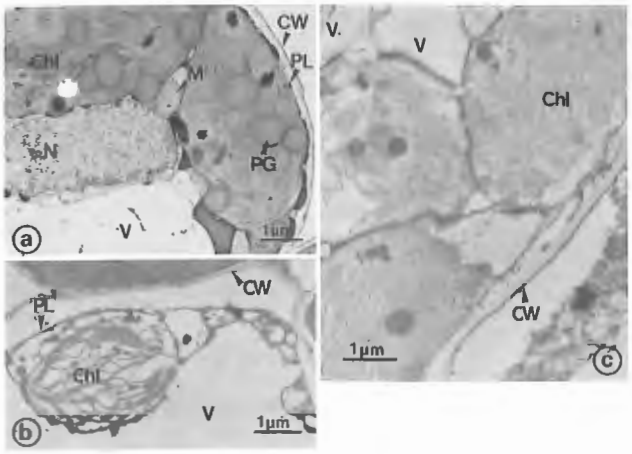

Fig. 3: Electron micrographs of plantlet leaves of *Vitis vinifera* cv. Cabernet Sauvignon. a- control; b- and c- 750 µM eutypine treated leaves, for 6 h in marginal and for 24 h in petiolar area respectively. Note total disorganization of treated cells with unstructured lamellar network of chloroplasts and cytoplasm vesiculization. Key abbreviations: Chl: chloroplast; CW: cell wall; M: mitochondria; N: nucleus; PG: plastoglobuli; PL: plasmalemma; V: vacuole.

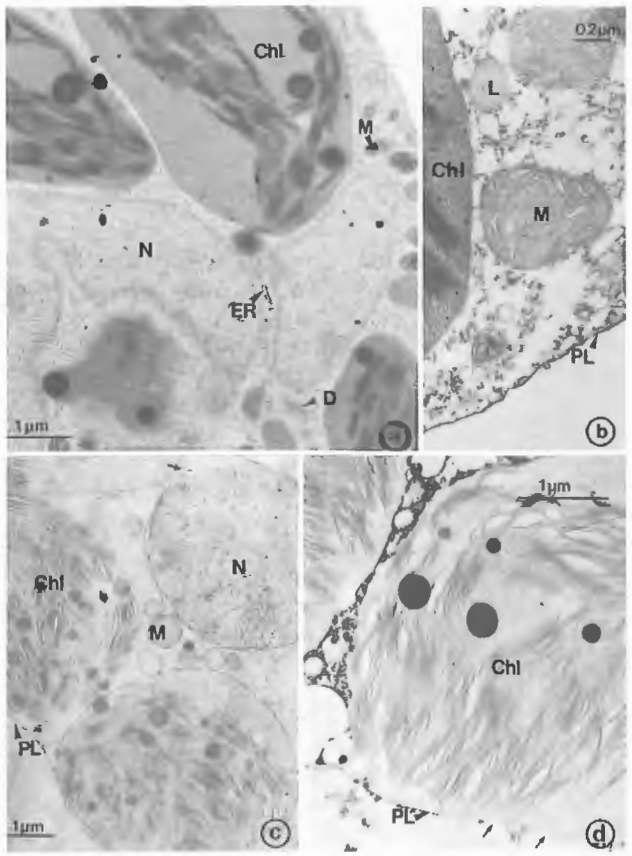

Fig. 4: Electron micrographs of protoplasts isolated from plantlet leaves of *Vitis vinifera* cv. Cabernet Sauvignon. a- control; b- 100 µM eutypine for 24 h: note cytoplasmic lightening and persistence of plasmalemma and organelles integrity; c- 500 µM eutypine treated protoplasts for 30 min: note chloroplast swelling and nuclear membrane dilation (arrow); d- 500 µM eutypine treatment for 1 h: total disorganization of chloroplast membranes is observed and plasmalemma shows many breakages (arrows). Key abbreviations: Chl: chloroplast; D: dictyosome; ER: endoplasmic reticulum; L: lipid droplet; M: mitochondria; N: nucleus; PL: plasmalemma.

After 6 h of treatment, in marginal leaf areas, some ultrastructural cell modifications were already very noticeable. Near intact cells, others presented total disruption with high cytoplasmic vesiculation, vacuolar fragmentation and, in chloroplasts, thylakoid vesiculation and stroma lightening (Fig. 3-b). In the petiolar area, 6 h after eutypine treatment, disorganization was not very marked and the cytoplasm was as dense as in the controls and the chloroplasts remained intact.

After 24 h of treatment, in marginal leaf areas, total disruption of cells was observed. In the petiolar area, the alterations were enhanced (Fig. 3-c). The volume of the chloroplasts increased (5.8 µm long, 4.1 µm wide) and the shape was changed. They presented an unstructured lamellar fretwork. Moreover, the cytoplasm lacked electron density and had developed small vesicles.

Ultrastructure of eutypine treated protoplasts: When the protoplasts were treated with 100 µM eutypine, the ultrastructure of protoplasts appeared intact, compared with the control, after a 2-h incubation period. The cytoplasm was electron-dense with a well developed endoplasmic reticulum, the plasma membrane was unaltered, as were the mitochondria and the chloroplasts (data not shown). After a 24-h incubation period, ultrastructural alterations were obvious (Fig. 4-b). The cytoplasm was clear and had lost its electron density and homogeneity and appeared highly vesiculated. Thylakoid arrangement seemed normal with grana stacks.

When treated with a high eutypine concentration (500 µM), protoplasts showed cytosolic alterations as early as 30 min with vesiculization and lightening of contents (Fig. 4-c). Sometimes the outer layer of the nuclear envelope was elongated. Chloroplasts were swollen and were nearly spherical (5.6 µm long, 4.3 µm wide). In some of them the envelope was broken and the network system of thylakoids was disorganized. Modifications became more extensive and universal as the time of toxin treatment was prolonged. After 2 h, the mitochondria swelled and lost their homogeneity. The chloroplastic lamellar swelling had increased and the chloroplast envelope had disappeared. The plasma membrane showed many points of breakage (Fig. 4-d)

Discussion

The results of the present study show that eutypine caused the rapid appearance of marked symptoms for the excised leaves from Cabernet Sauvignon plantlets cultured *in vitro* and provoked the death of protoplasts. Since these bioassays were performed in controlled conditions to avoid environmental artifacts, the symptoms observed must be attributed only to the action of eutypine. The intensity of the symptoms and the speed of their appearance were proportional to the eutypine concentration. However, the effects of eutypine appeared earlier on protoplasts than on the leaf tissues because of the more direct contact with the toxin. Used for the first time, such simplified experimental systems seem well adapted to study the ultrastructural alterations induced by eutypine.

The protoplast bioassay constitutes an efficient system to characterize a gradation of ultrastructural damage: early cytoplasm vesiculization and a slight swelling of chloroplasts, then lack of cytoplasm electron-density, and finally alterations of all organelles and breakage of the plasma membrane. The observed alterations are not restricted to the action of eutypine because other toxins can lead to similar disorganization. For example, treatment with cercosporin caused cytoplasmic vesiculization in beet leaves (STEINKAMP *et al.* 1979) and tabtoxin induced ultrastructural modifications in tobacco leaf chloroplasts (DURBIN 1971).

The ultrastructural alterations of leaf cells of plantlets caused *in vitro* by eutypine can be compared at those observed on leaves of grapevines affected by eutypiosis. Indeed, such comparisons have been made by other authors. For example, STEINKAMP *et al.* (1981) showed by comparing the ultrastructural effects of cercosporin, a toxin produced by *Cercospora beticola*, on *Beta vulgaris* leaves, with the fungus-induced damages, that cercosporin was involved in causing some disease symptoms even if others appeared only in infected plants. In our study, the ultrastructural alterations caused by eutypine under controlled conditions were similar to those noted by PHILIPPE *et al.* (1992) on leaves from grapevines affected by eutypiosis. Indeed, *in situ*, in the cells of slightly affected leaves, the enlarged chloroplasts (x 2.6) showed dilated thylakoids and contained large starch grains. In severely affected leaves, the cells were completely disorganized, with vesiculization of endomembrane systems and cytoplasm lysis. *In vitro*, in eutypine-treated plantlet leaves, lysis and vacuolisation of cytoplasm, and chloroplast swelling (x 1.6) with thylakoid spacing were observed early, followed by alteration of organelles and breaks in the plasma membrane. However, in the *in vitro* tissues the accumulation of starch grains into the chloroplasts was not observed probably because the photosynthetic activity of the *in vitro* excised leaves has been demonstrated to be very low. The similarity of ultrastructural alterations observed in diseased leaves and in eutypine-treated plantlet leaves and protoplasts confirms that eutypine is responsible for the ultrastructural damages observed in eutypiosis-affected leaves.

Ultrastructural effects observed with eutypine treatment can be correlated with biochemical events described in our laboratory. TEY-RULH (1988) noted rapid electrolyte leakage, particulary of potassium, probably involved in osmotic processes, after fungus filtrate had acted on excised plantlet leaves. Thus, eutypine could act by altering the selective permeability of the plasma membrane even if plasmalemma breakage appears to be a terminal event.

In conclusion, eutypine produced serious symptoms on the leaves of Cabernet Sauvignon in controlled environmental conditions. Furthermore, this toxin affected the cytoplasm early, then produced a disorganization of chloroplasts and finally induced breakage of the plasma membrane. These observations demonstrate the toxic role of eutypine on the leaf cells of the grapevine. On the other hand, the notable similarities between fungus-induced changes in leaves of diseased plants and eutypine-induced changes on excised plantlet leaves and on isolated protoplasts shows that eutypine is involved in foliar symptom expression. Our scheme to explain the action from a distance of *Eutypa lata* is that eutypine, synthetized by mycelium present in trunk or arms, is transported into the herbaceous parts and causes the characteristic symptoms of dying-arm disease. In order to develop ways to act against eutypiosis, the study of the mechanism of action of eutypine is now in progress.

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