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Effects of cerato-platanin, a toxin from *Ceratocystis fimbriata* f. sp. *platani*, on *Platanus acerifolia* leaves: an ultrastructural study

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Abstract — Cerato-platanin (CP), a protein previously isolated from the ascomycete *Ceratocystis fimbriata* f.sp. *platani* (*Cfp*), the causal agent of plane canker stain, has been studied with regard its phytotoxic effect in plane leaf cells by transmission electron microscopy.
When infiltrated in plane leaf mesophyllum, pure cerato-platanin induced, 24 and 48 h after treatment, several negative responses in palisade and spongy tissues.
One of the most important responses consisted, already after 24 h, in a great production of primary starch in these tissues. Moreover, the ultrastructural observations evidenced numerous other symptoms, such as strong alterations in chloroplasts, nuclei and in the cytoplasm. These changes were particularly serious in spongy parenchyma cells. In addition, these cells appeared strongly squashed with a reduced and completely disorganized protoplast.

Key words: canker stain, cell ultrastructure, cerato-platanin, plane-tree, toxicity.

INTRODUCTION

The London plane, *Platanus acerifolia* (Ait.) Willd., is the most common ornamental tree in Southern Mediterranean countries. A serious problem of this plant involves many fungal disease and, among these, in particular, the ascomycete *Ceratocystis fimbriata* (Ell. and Halst.) Davidson f. sp. *platani* Walter (*Cfp*), which is responsible for canker stain disease (El Modafar *et al.* 1996; Panconesi 1999). *Cfp* enters through wounds in the roots and branches, and causes in adult trees foliar withering and trunk canker. The trees die four or five years after the appeareance of canker (El Modafar *et al*. 1993). The use of chemical treatments is ineffective. Prevention is the only method to reduce the incidence of canker stain and prevent its transmission (Panconesi 1999). Another method is to obtain resistant clones, but, at moment, has not been achieved. Only an hybrid plane, recently obtained between *P. occidentalis* L., a species naturally resistant to *Cfp*, and *P. acerifolia* showes canker stain resistance (Vigoruoux and Olivier 2004).

In this context, the isolation and purification of a protein from the culture filtrate of *Cfp* constituted a result of great interest. This protein, named cerato-platanin (CP), has been characterized from the chemical and molecular point of view. Its complete aminoacid sequence was determined as well as the molecular weight (Pazzagli *et al.* 1999). This protein showed the N-terminal region very similar to that of hydrophobin protein family (Ebbole 1997) and, in particular, the cerato-ulmin, responsible of Dutch elm disease (Del Sorbo *et al.* 2002). However, the most noteworthy property of CP was a biotoxic effect inducing either cell necrosis in tobacco leaves or the production of phytoalexins in *P. acerifolia* leaves and soybean cotyledons (Pazzagli *et al.* 1999; Scala *et al.* 2004). Moreover, Boddi *et al.* (2004) demonstrated that CP was located in the *Cfp* hyphae, conidia and spore cell walls.

In an our previous research CP effect was tested in plane leaves using two different experimental methods (Bennici *et al.* 2005). The histological observations revealed symptoms induced by CP, mainly a great starch synthesis, an increase of phenolic compounds in palisade cells and a certain degree of spongy tissue disorganization. Similar, but less evident effects than those of CP, were induced by *Cfp* treatment, confirming a toxic effect of this protein (Bennici *et al.* 2005).

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These observations induced us to carry out in the subsequent year further investigation at ultrastructural level to better characterize the *P. acerifolia* leaf tissue response to CP. Moreover, in this new research a different experimental procedure regarding CP treatment has been used.

MATERIALS AND METHODS

The purification procedure of CP from the virulent strain Cf AF 100 of *Ceratocystis fimbriata* f. sp. *platani*, isolated and cultured on a sterile Sigma potato dextrose broth (PDB), has been described by PAZZAGLI *et al.* (1999).

Contrarily to the our previous investigation (BENNICI *et al.* 2005), that reported the application of CP solution as droplets simply placed on the plane leaf surface, or by dipping the leaf petiole in the protein solution, in the present work the CP solution (and water as control) was infiltrated within plane leaf mesophyllum by means of a hypodermic syringe with a subtle needle.

Also in this investigation newly matured leaves, removed in May (2005) from two *P. acerifolia* trees (the same of the previous research), were used. Sixteen leaves (8 per tree) of the same age were cut from two branches of each tree (4 leaves per branch). The leaves, carefully washed with distilled water and dried on both sides with filter paper, were placed in previously sterilized moist Whatman filter paper sheets in Petri dishes (1 leaf per dish). The treatment consisted of ten 10 μl injections of water each containing 1.0 nm of pure CP (the same dose previously used) applied to the right half of the plane leaf lower side. As control, sterile distilled water was infiltred to the left half part of the same leaf, and in the same quantity. The treatments were evenly distributed within the leaf. These leaves were maintained in Petri dishes at 25° C under continuous light (Sylvania Daylight fluorescent tubes F36W/154-ST, 35 μmol m^{-2} s^{-1}) for 24 and 48 h.

For TEM examination, at the end of each of these two periods, leaf fragments from treated and control parts were excised under a stereomicroscope. These fragments, corresponding in number to all the leaf regions where the protein was introduced, consisted of pieces including an area (0.5 cm wide) surrounding the injection point. Treated and control leaf fragments were prefixed overnight in a solution of 2.5% glutaraldehyde and 4% paraformaldheyde, adjusted to pH 7.2 with 0.1 M phosophate buffer, then washed with the same buffer and fixed in 2% osmium tetroxide. After dehydration through a gradual alcohol series from 40 to 100%, these materials were embedded in SPURR's (1969) epoxy resin. Ultrathin sections obtained with a Reichert ultramicrotome (OM U3-S) were stained with uranyl acetate and lead citrate, and examined under a Philips TEM 300.

RESULTS

The zone immediately adjacent to the treated leaf point (about 250-300 μm wide) was characterized by evident cell damages due to mechanical injures and, therefore, excluded from the observations. The most interested area to CP treatment was comprised between 300-350 and 500-550 μm, starting from the injection point.

In the leaf treated with water, 24 and 48 h after the treatment, the palisade cells appeared completely normal in all their organelles and cytoplasm structure, with a prominent nucleus containing chromatin aggregates. A central vacuole(s) occupied the most part of the cells; it was filled of very electrondense material, (tannins and phenolic compounds). The chloroplasts, in particular, exhibited a well developed thylakoidal system and primary starch grains (Fig. 1, A, B, C). Also the spongy parenchyma cells showed a well organized protoplast with dense cytoplasm, large vacuole(s), nuclei, organelles, membranes, and a moderate content of starch material (Fig. 1, D, E). At 48 h starch reserves were not observed in palisade cells and, especially, in spongy tissue (Fig. 1, B, C, D, E).

Cyto-histological responses to CP application within the leaves were clearly visible already 24 h after treatment. The cells of the palisade tissue contained more starch than the respective controls; these starch grains were very conspicuous in size. The chloroplasts showed abnormal forms with irregular or indistinct thylakoids. Moreover, the cytoplasm appeared less organised in comparison with palisade control cells; in some points the protoplast seemed lightly detached from the cell wall. The nucleus in the most part of these cells was not distinguishable from the surrounding cytoplasm because both showed the same opaque appearance or electrondensity. The vacuoles were very rich in electreondense material (Fig. 2, A, B). This cell behaviour was particularly emphasized in the spongy tissue cells. Also these cells were very rich in starch grains. Plastids were completely disorganised; thylakoids were not discernible. The cytoplasm was amorphous, densely stained with

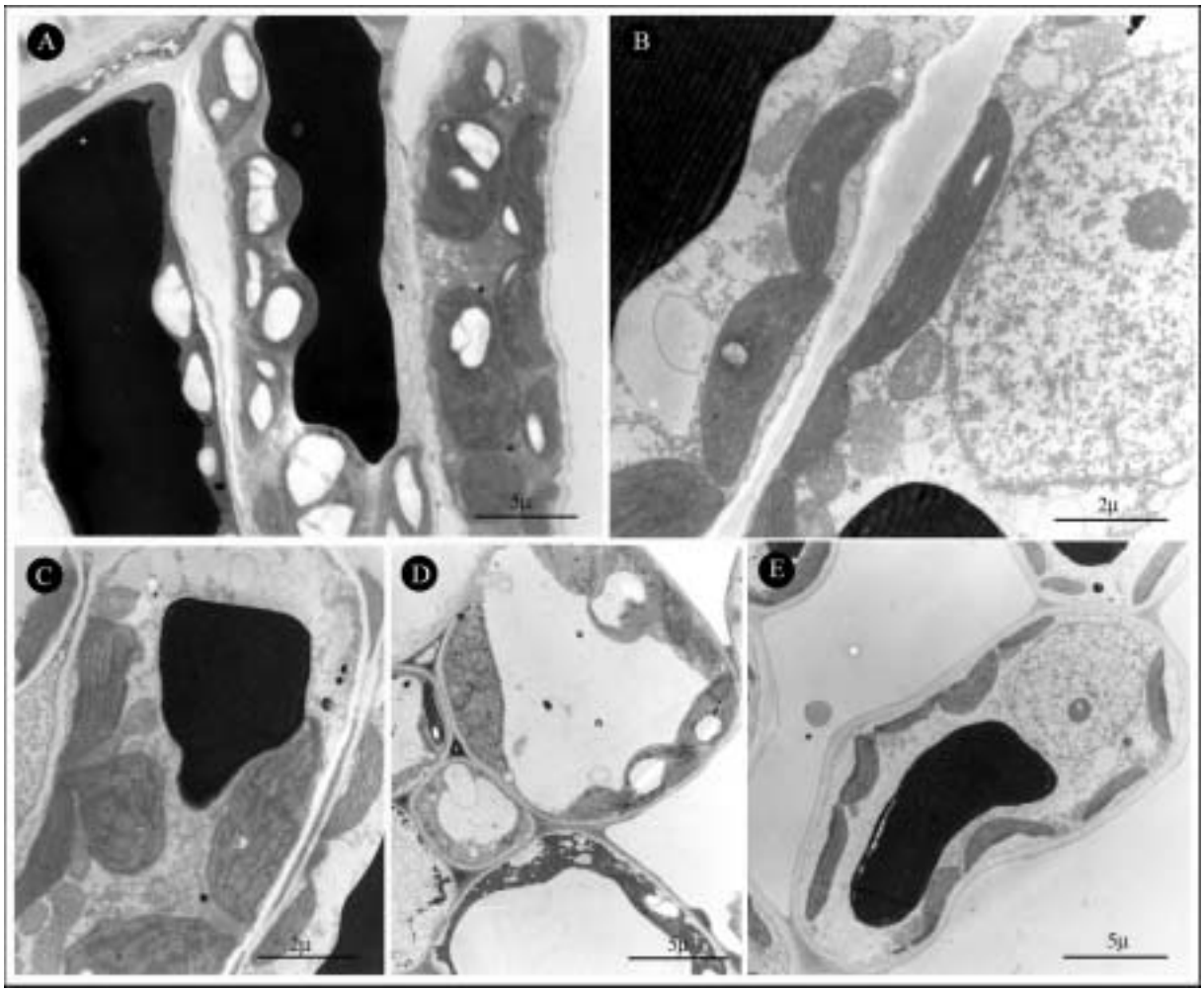

Fig. 1 — Control leaf cells. (A) Palisade cells at 24 h: the cells are normal with large starch grains in the chloroplasts. (B, C) The same cell at 48 h after treatment showing well developed chloroplasts, a large nucleus, mitochondria; note the absence of starch reserves. (D) Spongy cells at 24 h with a dense cytoplasm, a great vacuole lacking electrondense material and presence of starch grains. (E) Spongy cells at 48 h: starch is not present in the chloroplasts which are well structured, the vacuole appears filled of electrondense material.

the nucleus not evident. Also the other organelles could hardly be distinguished. The more drastic effect observed was, however, the occurrence of many spongy cells crushed and with a reduced cytoplasm which in many cases appeared completely degenerated or seemed very condensed; the plasma membrane was often separated from the walls. These cells exhibited walls strongly coarctate and irregularly thickened (Fig. 2, C, D).

Similar cellular responses were also found after the subsequent 24 h. In fact, the cell symptoms or damages previously described remained practically the same. Although in some palisade cells chloroplasts exhibited almost normal thylakoids (Fig. 3, A), most of these resulted very abnormal in their structure (Fig. 3, B). Starch grains were absent or appeared in a hydrolysis phase (Fig. 3, C). Plasmalemma and the other endomembranes were indistinct. The nucleus, in particular, continued to be negatively interested by CP effects. Moreover, within the cell walls more osmiophilic zones were observed. These walls displayed also an irregular profile (Fig. 3, C). Spongy squashed cells were still present with the cytoplasm strongly altered and densely stained (Fig. 3, D, E).

DISCUSSION

Although in our previous work (Bennici *et al.* 2005) a number of significant responses to CP treatments were found, the quantitative penetration of CP through the epidermatic tissue barrier or absorbed through the petiole remained an im-

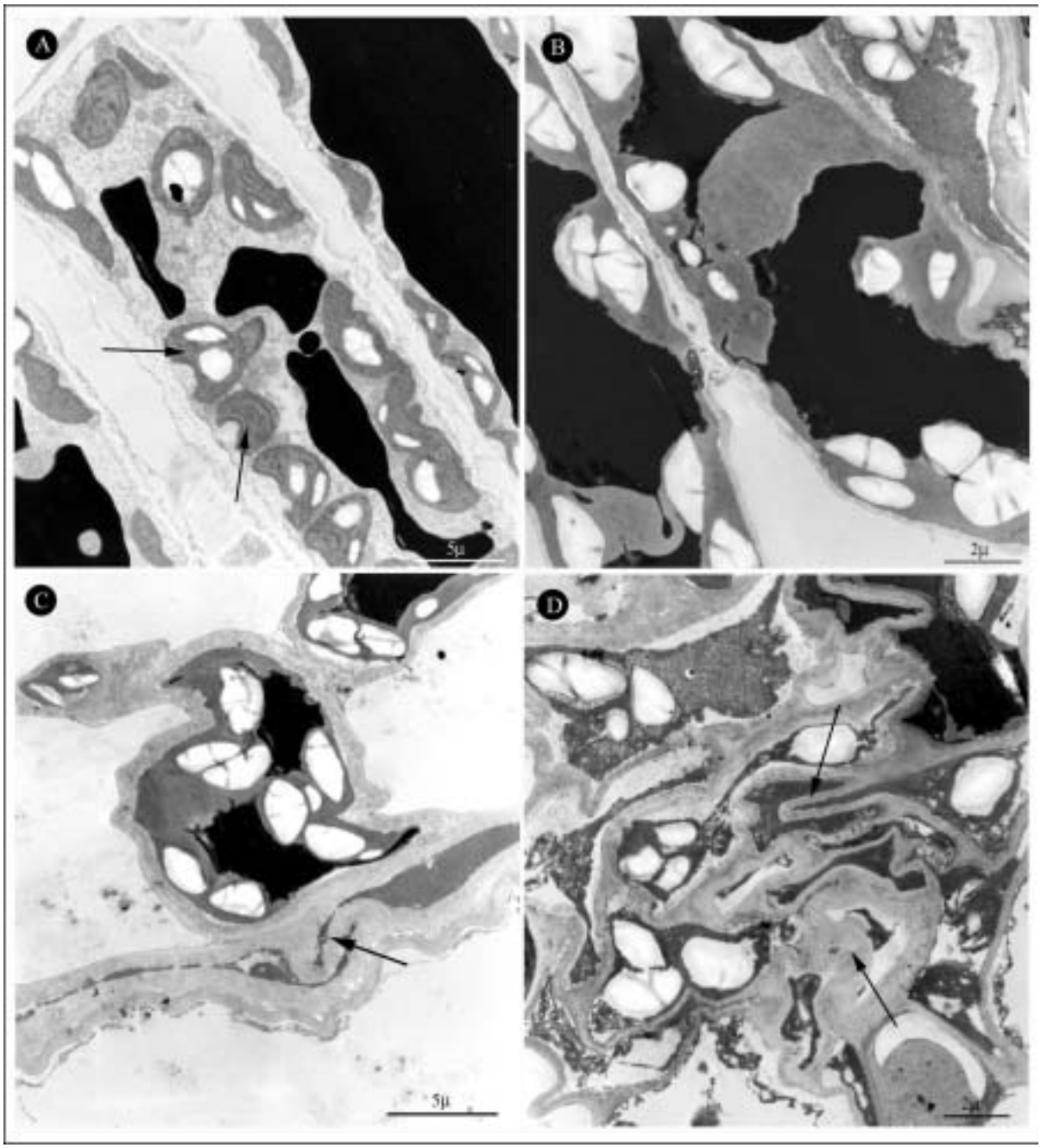

Fig. 2 — CP treated plane leaf cells 24 h after the treatment. (A, B) Palisade cells showing abnormal chloroplasts (arrows); numerous starch grains are visible. Organelles are not well distinct and, in particular, the nucleus appears often of the same electrondensity of the cytoplasm. The vacuoles contain electrondense material. (C, D) Spongy cells: the starch reserves are very abundant; the cytoplasm appears opaque and with not well discernible organelles. Particularly evident are crushed cells with coarctate walls in which the thickness is variable (arrows).

portant question. To overcome, at least in part, this problem, the CP solution has been introduced directly within the leaf tissue.

Undoubtedly, the pathogen interactions with plants (or plant cells) are complex and dynamic. Invasion by the pathogen or its toxin(s) triggers recognition and response in the plant leading to signalling cascade and expression of genes involved in this interaction that may cause the defence against the pathogen itself. The photosynthesis is the basic metabolic process of plants. Therefore, a primary effect of an infection may interfere with this process causing related symptoms. In fact, one major change was, already 24 h

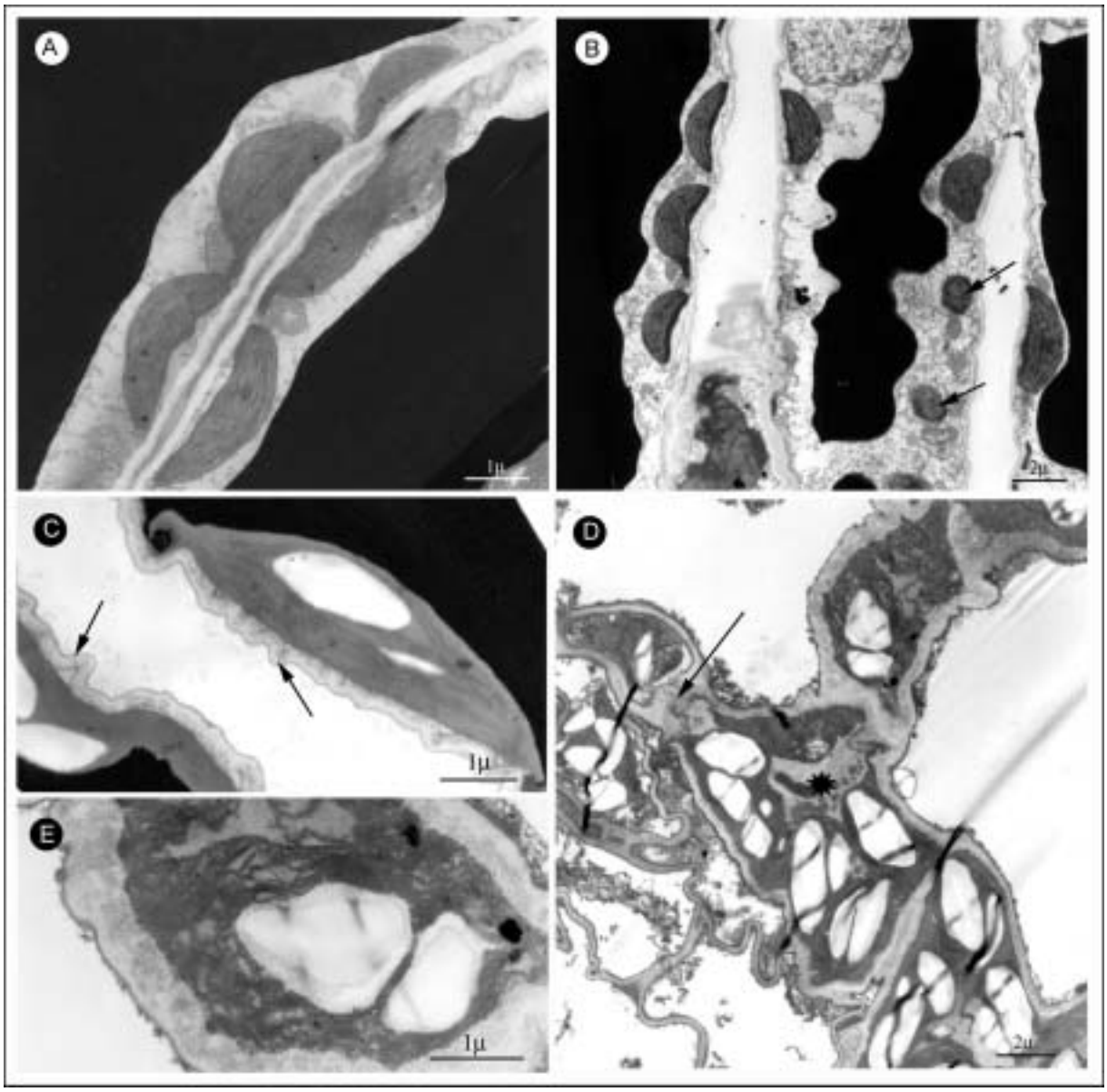

Fig. 3 — CP treated plane leaf cells 48 h after the treatment. (A, B) Some of palisade cells contain altered chloroplasts (arrows); the cytoplasm shows a disorganized structure, starch are not present. (C) Particular of palisade cells showing an irregular wall in which more electrondense zones appear (arrows). In the chloroplasts starch grains seem to be partially hydrolysed. (D, E) Spongy cells in great part squashed with walls of different thickness (arrow, asterisk). The cytoplasm is degenerated. (E) Particular of a spongy cell: note a different electrondensity within the wall and the cytoplasm.

after CP treatment, an alteration of chloroplast structure and a strong accumulation of primary starch reserves either in palisade or spongy tissue cells. Larger and more numerous starch grains were observed in the treated leaf zones than in the controls. An increase of starch synthesis in leaves or other plant parts are reported to be induced by many biotic stresses. In fact, responses of this type are reported by MAC DONALDS and STROBEL (1970) in rust-infected wheat leaves, GROSS *et al.* (2002) in leguminous infected with *Rhizobium* spp., ABBAS *et al.* (2001) in the frond cells of duckweed (*Lemna pausicostata* L.) following treatment with apicidin, a toxin produced by *Fusarium*. Also abiotic stress induced strong starch formation (DE GROOT *et al.* 2003; KIM and WETZSTEIN 2003; KIVIMAENPAA *et al.* 2003; WIDODO *et al.* 2003; LIU *et al.* 2004).

Therefore, the greater quantity of starch content in the CP treated leaves, in comparison with

the controls, can be caused by CP that could act as a primary signal in the plane leaf cells causing a rise in photosynthesis level in a wider metabolic context, and/or a greater activity of enzymes involved in the starch biosynthesis or in a reduction of starch hydrolysis. These events may be related with biochemical reactions leading to the formation of some plant defence compounds or their precursors (BENNICI *et al.* 2005). The partial starch hydrolysis observed after 24 h of treatment strengthens this hypothesis. Phenolic substances such as scopoletin and umbelliferone, two phytoalexins with well known antifungal action (LUSSO and KUC 1999), were produced in plane leaves infected by *Ceratocystis fimbriata* (EL MODAFAR *et al.* 1993; 1995; 1996). Also treatment of cells in suspension cultures of *P. acerifolia* with crude elicitor preparation from *Cfp* induced the synthesis of these two compounds (ALAMI *et al.* 1998). On the other hand, the phytoalexins umbelliferone and glyceollin production was induced in *P. acerifolia* leaves and in soybean by treatment with CP (PAZZAGLI *et al*. 1999; SCALA *et al.* 2004). More recently, ALAMI *et al.* (1998; 1999) reported the synthesis of umbelliferone, scopoletin and also xanthoarnol which were elicited by another protein isolated from germinating *C. fimbriata* spores in *P. acerifolia* cell suspension cultures. In the plant-pathogen interaction the production of biologically active secondary metabolites, and among these the phytoalexins, able to trigger defence reactions, is a general phenomenon (JACKSON and TAYLOR 1996; AGRIOS 1997). This induction process is caused by the rapid perception in the host of an external stimulus which may consist in a physical or chemical factor, such as a toxin (SCALA *et al.* 2004). Phytoalexins formed only after the primary metabolic precursors were diverted into novel secondary metabolic pathways (HAMMOND-KOSACK and JONES 2000). In this context, the more abundant electrondense material detected after 24 h of CP treatment in the plane cell cytoplasm and walls may be due to the accumulation of defence-related phenolic substances induced by CP (EL MODAFAR *et al*.1996).

Regarding the decrease of starch after 24 h, either in the controls or in the treated leaves, it is generally accepted that this reserve material is reorganized during the night because the enzymes responsible for starch degradation are also present by day, when they could be active during chloroplast starch synthesis (BENNICI *et al.* 2005).

The second type of plane leaf cell/tissue response to CP consisted in several negative changes involving all cell components of the palisade and spongy tissue. The ultrastructural analysis showed strong chloroplast alterations in the form and at level of thylakoids. The most serious effect due to CP occurred in cytoplasm and nucleus. These two cell components are often indistinct and opaque or with the same electrondensity. The nucleus, in particular, did not showed the normal chromatin structure. In addition the organelles were not well discernible. These modifications or damages were evident in the spongy tissue where the organelles showed strong structural disorganization or disruption. It is probable that CP accumulates in these cells, or they resulted more sensible to the toxic action of this substance than the palisade cells. In this case all these negative intracellular processes might contribute to the final cell collapsis as their distorted and flattened forms seems to demonstrate. This cell behaviour may be indicative also of a cell death process. The host cell death is reported as an hypersensitive response of many plant species against the attack of microbial pathogens and may be considered a programmed cell death in order to deprive the pathogens of their nutrition base (GREENBERG *et al.* 1997; PONTIER *et al.* 1998; BULOW *et al.* 2004). Characteristics of the hypersensitive response include an oxidative burst, leading to generation of hydrogen peroxide, accumulation of wall-bound phenolic compounds, synthesis of hydrolytic enzymes and anti-microbial substances (SOYLU 2006). These defence responses may be elicited by CP also in a susceptible species to the canker stain disease such as *P. acerifolia* (SCALA *et al.* 2004). It is very difficult in our case to distinguish between a response of this type and, more simply, a direct toxic effect of CP on the leaf cells able to induce irreversible cellular death or necrosis (FIETTA 2006). However, cytoplasmatic structural injures that involve also membranes, induce ion effluxes which might be a perequisite for the activation of defence mechanisms (HAMMOND-KOSACK and JONES 2000).

In conclusion, all these observed well-defined plane leaf cell manifestations following CP treatment taken as a whole phenomenon, indicate a localized strong response activated by this protein which can be one of the principal *Cfp* elicitor involved in canker stain pathogenesis. This statement is strengthened by the effect of CP like the damage caused by many other phytotoxins (DALY 1981; SCHEFFER 1983).

Undoubtedly, further studies are necessary to better understand the interaction between CP and host plane and, especially, the machanism of its toxicity at citoplasmic level.

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