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Full Length Research Paper

Effect of *Cryphonectria parasitica* toxin on lipid peroxidation and ultrastructure in two Chinese chestnut cultivars

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In order to clarify the responses of different chestnut cultivars to Cp-toxin stress, the effect of Cp-toxin from *Cryphonectria parasitica* (Murr.) Barr on *Castanea mollissima* Blume, especially on its cell structure, was examined. Chestnut shoots of both resistant (Beiyu No. 2) and susceptible (Hongguang) cultivars were treated with Cp-toxin for 24, 36, 48 and 72 h, respectively, and then electrolyte leakage (EL) and malondialdehyde (MDA) contents were measured in the leaf tissues. Also, ultrastructural changes of organelles were observed under transmission electron microscope. The results show that the EL and the MDA content increased notably when leaves were treated with Cp-toxin for 72 h, and the content of MDA and EL was 4.36 and 2.19 times that of the control samples, respectively. The damages on membrane systems of the cells were lighter and they occurred later than expected in the resistant cultivar than in the susceptible cultivar. At the treatment time of 72 h, Cp-toxin caused marked damage effects on membrane systems of chloroplasts and mitochondria. Finally, after 72 h treatment with Cp-toxin, the membrane systems were damaged, after which EL and the MDA contents increased and were significantly higher than those of the control ($P < 0.01$), while the plasma membrane and cell wall broke into pieces. The change of cell ultrastructure in different cultivars of chestnut in response to inoculation with *C. parasitica* that positively correlated with the resistance to the chestnut blight can be used as a criterion to evaluate disease resistance.

Key words: *Castanea mollissima*, *Cryphonectria parasitica*, Cp-toxin, cultivars, ultrastructure.

INTRODUCTION

Chestnut blight, or chestnut bark disease, is caused by an introduced fungus, *Cryphonectria parasitica* (Murrill) Barr, and is a widespread disease present in most chestnut producing areas. Researches have been carried out on different aspects of this disease because of its economic importance. Following the increase of cultivation area of Chinese chestnut in recent years, chestnut blight took place frequently and the disease incidence was up to 60%. Fungal culture filtrates (CFs) are widely used to study the early defense mechanisms in plant-pathogen interactions (Trillas and Araus, 1993; Davis et al., 1998; Zemanek et al., 2002). CFs contain toxic metabolites and phytotoxins, and they induce plant defense response (Jiang et al., 2005; Singh et al., 2006). CFs of *C. parasitica* can cause chestnut blight syndrome, alteration of mitochondria and plasmalemma proliferations (Plagnani et al., 1999). Rbeson (1982) did a research on the separation and purification of the toxin of *C. parasitica.* However, few studies have been done on the activity of the toxin and its effect on the ultrastructure of leaves of chestnut.

Malondialdehyde (MDA) is a major cytotoxic product of lipid peroxidation and has been used widely as an indicator of free radical production (Hong et al., 2000; Hua et al., 1997; Kavikishor et al., 1995); although electrolyte leakage (EL) reflects the damage of the

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Figure 1. The symptoms of chestnut leaves treated with Cp-toxin. T = Treatment and K = control.

plasmalemma.The main objective of this study was to examine the ultrastructure and physiological changes of chestnut leaf cells induced by Cp-toxin. For this purpose, MDA content and EL were measured, and the ultrastructure differences of leaf tissues between resistant and susceptible cultivars treated with Cp-toxin at different concentration were observed.

MATERIALS AND METHODS

Plants

The chestnut shoots of resistant (Beiyu No.2) and susceptible (Hongguang) cultivars were used as experiment material. All plants were grown in pots in a glasshouse under controlled conditions.

Isolation and purification of Cp-toxin from culture filtrates

A highly aggressive strain of *C. parasitica* originally isolated from diseased chestnut tissue was kindly provided by Prof. Tianhui Zhu, from Sichuan Agricultural University, and was cultured in PDA liquid medium (XU et al. 2006) on a shaker with 126 rpm at 25°C for 18 days. The culture medium was filtered and the filtrate was centrifuged at 10,000 *g* for 30 min to remove spores. The supernatant was filtered through 0.45 mm Millipore filter and was used as a crude toxin extract (Zhang et al., 1989; Dubery and Smit, 1994). Crude toxins were isolated from liquid culture filtrates of isolates by the use of methanol. The column was eluted with the eluant which had been eluted at a flow rate of 2 ml/min, and the fractions were collected for assay. According to the principle of same volume used to collect and reference to the order of the eluant, the eluant was evaporated to dryness. The procedure was repeated three times to increase toxin recovery. The eluant was evaporated until it was dried under reduced pressure at 40°C with a rotary evaporator. Cp-toxin was obtained and it was checked for activity by immersing the shoots of Beiyu 2 and Hongguang into about 1.0 ml filtrate (Han, 2009).

Treatment with Cp-toxin

The current shoots were cut and inserted into the solution at the same concentration (100 µg /ml). They were kept at 25°C in the moist chamber for 24, 36, 48 and 72 h, respectively. The ones treated with water were used as control. However, all experiments were repeated four times.

Determination of electrolyte leakage

Electrolyte leakage was calculated by the standard method of Huang et al. (2005). The leaves of two varieties were immersed in 10 ml of distilled water in test tube overnight at room temperature. The initial conductivity was determined using a conductivity meter (model kent EIL 5007). The test tubes were then placed in boiling water for 15 min and cooled to room temperature. After heating, the conductivity of the solution was measured again (as 100% leakage). The electrolyte leakage was calculated by the equation:

$$EL\ \% = initial\ conductivity / Total\ conductivity \times 100$$

Measurement of MDA contents

Lipid peroxidation was determined by estimating the malondialdehyde (MDA) content according to the method of Buege and Aust (1978). MDA is a product of lipid peroxidation, and its quantity was determined by thiobarbituric acid reaction. 1 g leaf was homogenized in 5 ml of 0.6% (v/v) TBA solution in 10% (v/v) trichloroacetic acid. The homogenate was centrifuged at 12,000 g for 15 min and the supernatant was heated in a boiling water bath for 15 min and then cooled quickly in an ice bath. The resulting mixture was centrifuged at 12,000 *g* for 15 min, and the absorbance of the supernatant was measured at 532 nm. Measurements were corrected for unspecific turbidity by subtracting the absorbance at 600 nm. MDA concentrations were calculated by means of an extinction coefficient of 155 (mmol/L)/cm (Zhang and Bramlage, 1992).

Ultrastructure studies

Leaf tissue samples were fixed in 2.5% glutaraldehyde, and postfixed in 1% OsO_4. After stepwise dehydration in acetone, the leaf tissue was embedded in Epon-812 resin. Ultrathin sections were cut with an ultra-microtome using a diamond knife. The ultrathin sections were collected on copper 200-mesh grids, and examined with H-600A-2 transmission electron microscope.

Statistical analysis

Electrolyte leakage and MDA contents were analyzed using commercially available software (SPSS 15.0, Chicago, IL, USA). Values followed by the same letter under the condition of the same variety were not significantly different at 0.05 level according to Duncan's multiple range test. All data are presented as mean ± S.E.

RESULTS

Biological characterization of Cp-toxin

The typical leaf wilt caused by Cp-toxin on susceptible

Table 1. Effect of Cp-П toxin on electric conductivity of chestnut leaves at different times (μ sec/cm).

Variety	Treatment time (h)				
	0	24	36	48	72
C. mollissima BL. Beiyu 2	31.17±1.567C^{c}	33.45±1.741^{Cc}	33.63±2.360^{Cc}	38.56±0.926^{Bb}	51.35±1.093^{Aa}
C. mollissima BL. Hongguang	32.29±1.301^{Ee}	52.09±1.302^{Dd}	57.52±1.404^{Cc}	61.28±1.231^{Bb}	91.16±1.509^{Aa}

Data are means ± S.D. (n=3). Values followed by the same letter under the condition of the same variety are not significantly different at 0.05 level according to Duncan's multiple range test.

Table 2. Effect of Cp-toxin on the MDA content of chestnut leaves at different times (μmol/g).

Variety	Treatment Time (h)				
	0	24	36	48	72
C. mollissima BL. Beiyu No.2	185.08±2.667^{Ee}	203.33±2.082^{Dd}	228.00±2.646^{Cc}	268.67±1.528^{Bb}	286.00±3.606^{Aa}
C. mollissima BL. Hongguang	211.27±2.845^{Ee}	287.30±2.910^{Dd}	355.57±0.510^{Cc}	384.00±4.359^{Bb}	495.67±4.509^{Aa}

Data are means ± S.D. (n=3). Values followed by the same letter under the condition of the same variety are not significantly different at 0.05 level according to Duncan's multiple range test.

cultivar is shown in Figure 1. Cp-toxin at a concentration of 50 to 100 μg/ml induced leaf wilt on susceptible cultivars, but not on resistant cultivars. Visible leaf wilt of resistant chestnut appeared at 48 h after immersion of shoots into Cp-toxin solution (100 μg/ml). Most of the chestnut leaves had symptoms of wilting after treating with Cp-toxin for 96 h. No wilt appeared after immersion of shoot into water without added toxin or when the toxin was sprayed on leaves.

Effect of Cp-toxin stress on electrolyte leakage of chestnut leaves

It is important to note that electrolyte leakage in var. Hongguang increased dramatically while that in var. Beiyu 2 showed minor variations, which indicated that Hongguang plants were more susceptible to Cp-toxin stress than Beiyu 2 (Table 1).

Effect of Cp-toxin on the MDA content of chestnut leaves

MDA is one of the end products which are produced as a result of lipid peroxidation damage by free radicals. In the two varieties, the MDA content significantly increased at about 1.55 and 2.35 times, respectively in response to 100 μg/ml toxin as compared to the control (Table 2). MDA content in Hongguang plants increased more rapidly when compared with that in Beiyu 2 plants at the same toxin levels, whereas that of leaves started to increase by the time treatment was applied. The MDA content in Hongguang reached peak levels after treatment with Cp-toxin for three days, while it was 211.27 μmol/g in the control plants.

Ultrastructure of compatible and incompatible response of chestnut leaves cells

An ultrastructure observation of the effects of Cp-toxin on leaf cells from the two chestnut cultivars was conducted. The response of leaves treated with distilled water was normal in both varieties. One of the first observable effects of Cp-toxin at the ultrastructure of Hongguang leaf cells was damage to the plasma membrane and cell wall. After treatment with Cp-toxin for 24 h, the susceptible Hongguang leaf cells showed an invaginated plasmalemma, and periplasmic space appeared between the cell wall and the plasma membrane (Figure 2B). The chloroplast lamellae began to swell and its ectad membrane inflated slightly. The double membrane of mitochondria became single after treatment with Cp-toxin for 36 h (Figure 2C and D). The structure of chloroplasts was completely damaged, while the mitochondria (cristae) disappeared and vacuolated after treatment with toxin for 48 h. The nuclei of these cells were vacuolated and the plasma membrane disintegrated into parts in most cells under treatment with Cp-toxin for 48 h (Figures 2E to G). Large amounts of Cp-toxin were intensively deposited in the cells by the 72 h Cp-toxin treatment, and the cell wall was broken into pieces by the same 72 h Cp-toxin treatment (Figure 2H).

When shoots of the resistant cultivar, Beiyu 2, were treated with Cp-toxin, most of the leaf cells still had dense cytoplasm with normal appearing organelles examined at earlier times (0 to 24 h) (Figure 3C). Interestingly, a noticeable electron-dense precipitate was observed by 36 h treatment with Cp-toxin within the cell wall of about

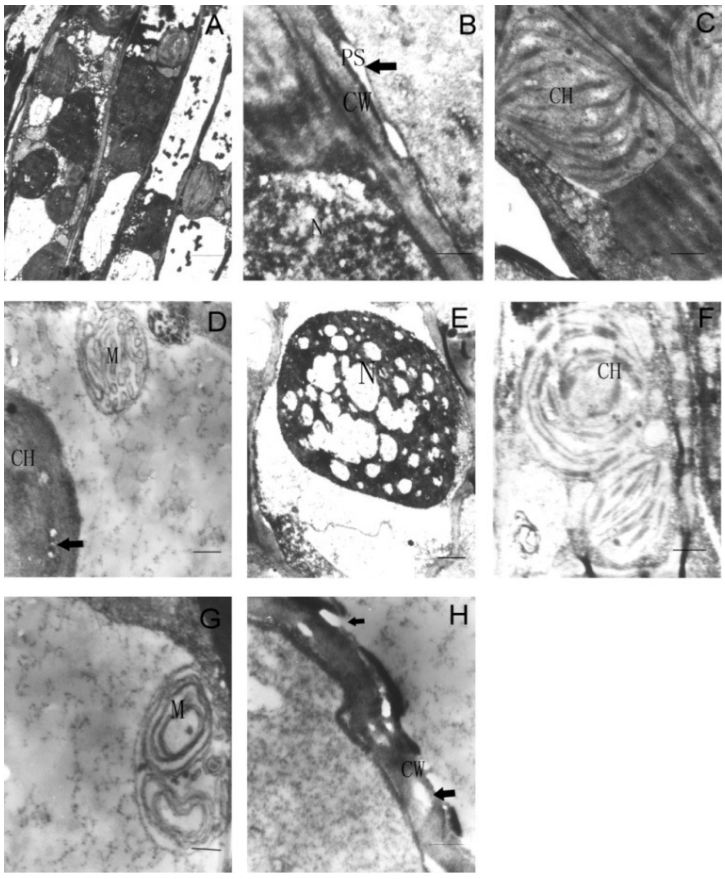

Figure 2. Transmission electron micrographs of chestnut leaves of the susceptible variety (Hongguang) treated by Cp-toxin. (A) The cells of the control, with no treatment of the susceptible variety, showed all the normal organelles. (B) The susceptible Hongguang cells at 24 h treatment with Cp-toxin. The arrow shows the invaginated plasmalemma. (C and D) The mitochondria's double membrane becoming single and the ridges are clear. There is disorder in the lamellae of chloroplasts after treatment with toxin for 36 h. (E) Nuclei was vacuolated after treatment with Cp-toxin for 36 h. (F) The structure of chloroplasts is completely damaged after treatment with Cp-toxin for 48 h. (G) Mitochondrion ridges disappeared and vacuolated after treatment with Cp-toxin for 48 h. (H) Cell wall broken into pieces after treatment with Cp-toxin for 72 h. Bars = 100 nm (A, H), 200 nm (B, C), 500 nm (D-G). CH = chloroplast, CW = cell wall, ER = endoplasmic reticulum, ES = extracellular space, M = mitochondria, N = nuclei, PS = periplasmic space, St = starch deposit, V = vacuoles.

20% of the cells. The electron-dense precipitate increased near the cell membrane (Figure 3D, E and H). There was a slight invagination of the plasma membrane,which appeared 36 to 48 h later after being treated with Cp-toxin (Figure 3F and G). The cytoplasm did not agglutinate; hence, the normal mitochondria, chloroplast and endoplasmic reticulum could be observed at 72 h treatment with Cp-toxin (Figure 3G and H). Starch was accumulated normally in chloroplasts after Cp-toxin treatment and the starch inclusions continued to enlarge in all stages of treated leaves cells (Figure 3H).

DISCUSSION

Toxin production in plant may be induced only by host factors, as was reported recently for AB-toxins produced by *Alternaria brassicicola* that caused black leaf spot of Brassica spp. (Otani et al., 1998). Stemphylium invades host tissues intercellularly (Shrestha et al., 1988), and SV-toxins were surveyed in the IFs of inoculated leaves.

Lower MDA induction in Beiyu 2 indicated a better protection from oxidative damage as compared to Hongguang. The high level of protection in Beiyu 2

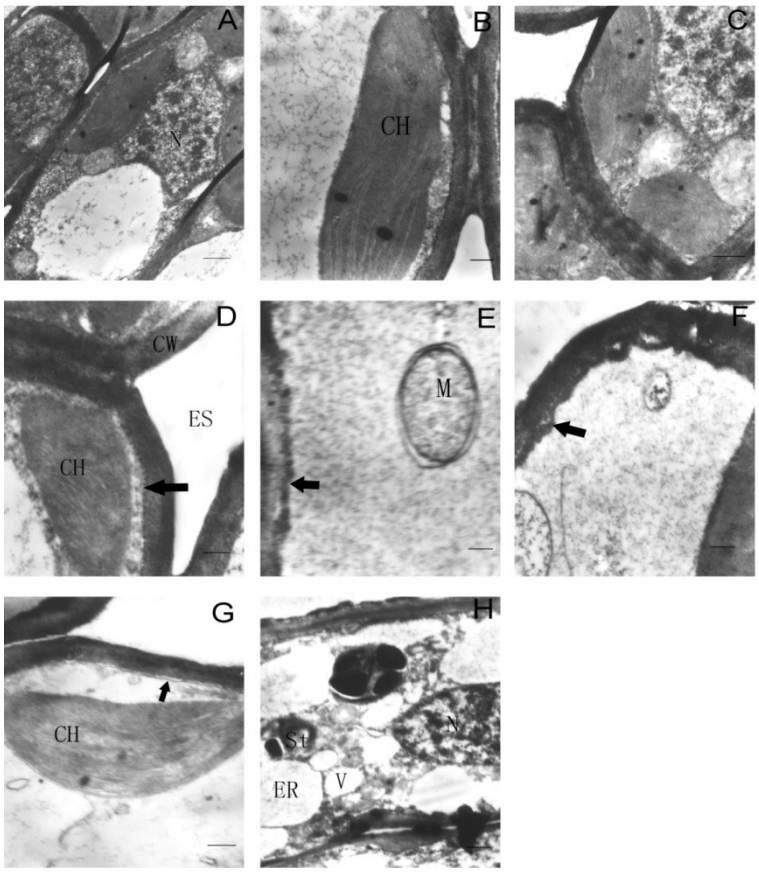

Figure 3. Transmission electron micrographs of leaf cells of the resistant variety (Beiyu 2) treated with Cp-toxin. (A and B) The cells of the control were treated with water. (C) Beiyu 2 leaf cells showed dense cytoplasm with normal appearing organelles at the 24 h treatment with Cp-toxin. (D and E) The electron-dense precipitate (arrow) was observed at the 36 h treatment with Cp-toxin within the cells, and the precipitate was present along the cell wall. (F and G) The invagination of plasma membrane (arrow) was slight and could be observed at the 48 h treatment with Cp-toxin. (H) Starch accumulated normally in chloroplasts after 72 h of Cp-toxin treatment. Bars=200 nm (A, H), 300 nm (D, G), 500 nm (B).

seemed to be a result of the more efficient antioxidative system, while significant increases in MDA levels in leaves of Hongguang appeared to be derived from low level of protection. The increased electrolyte leakage was positively correlated with accumulation of MDA, indicatingthat the plasmalemma injury by Cp-toxin stress resulted from oxidative damage. In Hongguang, increase in the electrolyte leakage in leaves is higher than that in Beiyu 2. Based on the present results, it appears that plants of Beiyu 2 variety have a better tolerance to Cp-toxin as compared to Hongguang variety. This result agrees with earlier findings on evaluation of the resistance of Chinese chestnut cultivars to *C. parasitica* reported by Qin (2002). Increased electrolyte leakage of Cp-toxin treated leaves indicates that the disruption of cell membrane integrity may play a role in the death of Cp-toxin treated leaves. When compared to the control, the cells of the leaves of different resistant varieties treated with Cp-toxin showed appreciable ultrastructural changes. The chloroplast lamellar swelling was observed earliest after 24 h when leaves were treated with Cp-toxin. After 48 h, the chloroplast lamellae were disordered and the membranes were ruptured. In addition, rupture of the membrane and swelling of the mitochondria lamellar was simultaneous or preceded by their occurrence when they were treated with Cp-toxin. When the treatment time was increased, the degree of destruction of the leaf ultrastructure became more difficult. With the exception of the harm done to the plasmalemma and the chloroplast lamellae, the nuclear membrane was harmed to some degree. It suggests that Cp-toxin affects not only the chloroplasts, but also other membrane systems of the cells. Electron microscope observations showed that the plasmalemma and nucleolemma were ruptured. The results presented in this study indicate that the plasma membrane might be the earliest and main target site of

Cp-toxin action. The damage and the development time of the resistant cultivar were lighter than the susceptible cultivar, respectively. The plasmolysis was harder in cells of the susceptible cultivar than in the resistant cultivar at the earlier stages of treatment. The process reaction of its plasma membrane occurred late than expected for more than 24 h, while the reaction of plasmolysis to toxin delayed for 36 h. These changes may be one of the reasons why the resistant cultivar had disease resistance. However, it explained that the resistance of the chestnut and cellular level was coincidental. Therefore, we could screen the material of anti-chestnut blight and identify its resistance by observing the changes of the cell ultrastructure.

Furthermore, we observed the electron-dense precipitate within the cell wall when the leaves of the resistant cultivars were treated with Cp-toxin for 36 h. It indicate that such compounds had been incorporated into the leaves' cells (infiltrated into the cells), which might also contribute to the restriction of the Cp-toxin damage. Pijut et al. (1990) characterized an electron-dense precipitate that was similar to phenolic deposits, whereas Uehara et al. (1997) found that the electron density of the mitochondrial matrix was in rice leaf cells after 1 h of toxin(s) treatment. Daayf et al. (1997) showed that the electron-dense compounds, produced in cotton roots infected with *Verticillium dahliae*, are terpenoids. Claudla et al. (2000) suggested that after a long period of time, the electron-dense material was associated with the moribund cellular features. The response seen here shows that the electron-dense precipitate could be associated with a defensive mechanism against the disruption caused by Cp-toxin, and as a permeability barrier or structural barrier, it could inhibit Cp-toxin entry into the cells, or restrict the release of hydrolytic enzymes which might degrade the cytoplasm of leaves cells. This electron-dense deposition could possibly account for the ability of resistant leaves to grow normally. However, the deposition of whether phenolic-like or its origin are still needed to assess the target site for Cp-toxin action remains definite by toxin mark.

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