Host Polyphenoloxidase Activity and Fungal Oxalate Production in the Host-Parasite Interaction *Castanea sativa-Cryphonectria parasitica**

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ABSTRACT. Polyphenoloxidase (PPO) activity of European chestnut *(Castanea sativa)* bark tissues inoculated with the hypovirulent strain TR57/a of *Cryphonectria* and its monoconidial virulent isolate M18, was analyzed qualitatively and compared with activity in healthy control bark. PPO activity of *C. sativa* was higher during spring and fall season than during summer. An inverse relationship was found between PPO activity and size of the necrosis produced by the fungus. A selective inhibition of one isoenzyme by the virulent strain was found in isoelectrofocusing (IEF) experiments. Oxalate accumulation was found in necrotic chestnut bark tissues inoculated with the hypovirulent strain and with the virulent isolate. No significant differences were found in oxalate accumulation between the hypovirulent strain and the virulent isolate. No inhibition of PPO from healthy chestnut bark was found at different concentrations of oxalic acid.

The virulent strains of the chestnut blight pathogen *Cryphonectria parasitica* (Murr.) Barr produce in culture, larger amounts of oxalic acid (OA) than the hypovirulent ones (6, 12, 20). Vannini and Fulbright (26) reported an accumulation of OA in tissues of apple infected with hypovirulent and virulent strains of *C. parasitica.* However, no data are available about production of OA in chestnut bark tissues infected by virulent and hypovirulent strains of the fungus.

OA is reported to play a role in the pathogenesis of several host-pathogen combinations (7, 11, 15, 22, 25) by its capacity to chelate divalent cations. Sato (23) showed an inhibitory effect by oxalic acid on polyphenoloxidase (EC14.1.18.1:PPO) activity in spinach leaves. One of the possible roles of PPO in diseased plants is to oxidize phenols, producing compounds, such as quinones, that are toxic to the invading microorganisms (3, 4, 9, 24).

The aim of this work was to investigate the relation between PPO activity and OA accumulation in European chestnut *(Castanea saliva* Mill.) bark tissues infected with a hypovirulent strain and an isogenic virulent isolate of *C. parasitica.*

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MATERIALS AND METHODS

Biological materials. *C. parasitica* used in this study included the dsRNA-containing Italian hypovirulent strain TR57/a and its monoconidial dsRNA-free isolate M18. The strain and the isolate were grown and maintained on PDA (Difco) at 25 C with a 14-h photoperiod. Four experiments were performed, in May, June, September and October.

Plant materials utilized for the experiments were one-year-old suckers collected from a stump of *C. sativa* in a natural stand in central Italy. The suckers were maintained with the cut end in a test tube containing deionized water and incubated in a growth chamber with a 12-h photoperiod at 25 C and 80% relative humidity.

dsRNA isolation. The fungus was grown in complete liquid medium for *C. parasitica* with the omission of glucose (19). Extraction of dsRNA was performed by the cellulose column method as described by Morris and Dodds (19) and modified by Fulbright et al. (10).

Inoculations. The suckers were inoculated with strain TR57/a and isolate M18 by removing a disc of bark with a 3 mm cork borer and by placing a plug of 3 mm of mycelium taken from the advancing edge of a 7-day-old culture grown on PDA. The inocula were taped with parafilm to avoid desiccation and the suckers incubated in the above condition for 5, 10, 20 and 30 days. Non-inoculated and wounded suckers were used as controls. After 5, 10, 20 and 30 days, the size of the necrosis was measured. Chestnut trees were also inoculated in experimental plots with TR57/a and M18 for the virulence assay. The severity of symptoms was assessed after 1 year.

PPO extraction and assay. Infected tissues were collected at 5, 10, 20 and 30 days from inoculations. Old necrotic tissues and the advancing edge of cankers were collected separately. The tissues from about 20 cankers were utilized for each sample. Healthy and wounded bark tissues were collected from the controls. One gram of tissue, ground to a fine powder in a mortar with liquid N_2, was extracted in 4 ml of 0.1 M Pi-citrate buffer pH 5.0, 0.2% L-cysteine, at 4 C for 1 h with gentle agitation. The suspension was centrifuged at 40,000 g for 20 min at 4 C. After centrifugation the supernatant was collected and dialyzed exhaustively against several changes of distilled water at 4 C. PPO activity was measured with a double-beam spectrophotometer (Lamda 15, Perkin Elmer, Oak Brook, Ill.). The reaction mixture was 1 ml of 0.1 M Pi-citrate buffer, pH 7.0, 10 mM L-Dopa and 200 uL of crude extract. The

reaction was followed for 10 min at 30 C and the increase of absorbance at 450 nm recorded. PPO activity was expressed as increase of absorbance at 450 nm/min/g fresh weight at 30 C. The reference cuvette contained the same reaction mixture but with boiled crude extract.

Isoelectrofocusing. IEF was performed using a 10% acrylamide gel in a vertical slab unit with ampholine pH 2.5-10 (Sigma, St. Louis, Mo.). Focusing was carried out at 4 C and at 250 V until the current dropped to 1.0 mA. pH measurements were made by cutting 1-cm sections from the gel, soaking them in distilled water, and measuring the pH. The gel was subjected to staining by soaking it in 0.1 M Pi-citrate buffer pH 7.0 with 10 mM L-Dopa as substrate.

Extraction and determination of soluble and insoluble oxalic acid from the infected tissues. Infected and healthy bark tissues were dried at 60 C for 72 h and ground to a powder with a pestle in a mortar.

Soluble oxalic acid extraction. The chestnut powder (about 5 g) was extracted twice at 20 C for 30 min with 10 ml of 0.05 M Tris-HCI buffer, pH 7.8, and centrifuged at 40,000 g for 20 min. The supernatant was collected, adjusted to pH 5.0 with 0.1 N HC1, and treated with ascorbate oxidase spatula (Boheringer Mannheim) to eliminate ascorbic acid (13). The supernatant was then used for soluble oxalic acid determination.

Insoluble oxalic acid extraction. The pellet from the above centrifugation was extracted twice at 60 C for 30 min with 10 ml of 3 N HC1. After centrifugation as above, the supernatant was collected and used for the insoluble oxalic acid determination.

Oxalic acid content of the extracts was determined by the oxalate oxidase enzymatic kit (Sigma) modified by changing the time of incubation of the enzymatic reaction to 10 min at 35 C. Absorbance of the indamine dye was measured at 590 nm with a double beam spectrophotometer (Perkin Elmer Lamda 15). The data were expressed as mg/g fresh weight.

Effect of oxalic acid on PPO activity. 0.1 ml of healthy bark crude extract was incubated for 5 min at 25 C in 1 ml of 0.1 M Pi-citrate buffer pH 7.0, at different concentrations of oxalic acid (Sigma), 0, 10, 20, 40, 60 and 80 mM; pH values of the reaction mixture were eventually adjusted with KOH. The reaction was started by adding 10mM L-Dopa and measured as above.

RESULTS

dsRNA isolation. The dsRNA pattern of strain TR57/a and isolate M18 were confirmed (Figure 1) (26).

Inoculations. The size of the necrosis produced in each experiment by strain TR57/a and isolate M18 after 30 days from inoculation is reported in Figure 2. The progression of the disease was faster during June and September than during May and October. Isolate M18 produced larger necrosis than strain TR57/a. From the experimental plot inoculations, strain M18 was judged virulent and strain TR57/a hypovirulent.

Figure 1. dsRNA pattern of Italian strain TR57/a (line 1) and isolate M18 (line 2). dsRNA extracts were layered in 5% polyacrylamide slab gel and electrophoresed at 40 mA for 12 h. Isolate M18 lacks dsRNA.

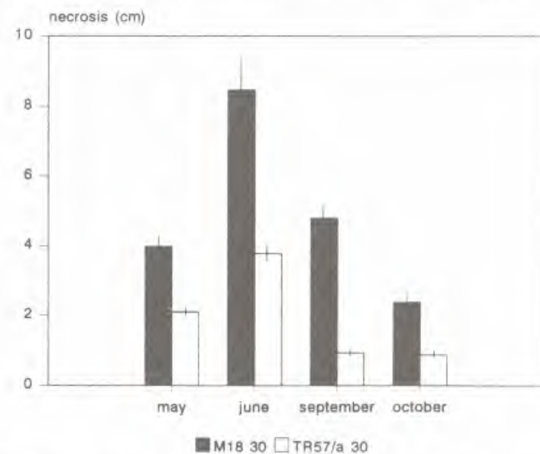

Figure 2. Length of the necrosis produced by strain TR57/a (dsRNA-containing) and isolate M18 (dsRNA-free), after 30 days from inoculation on cut suckers. Vertical bars indicate the standard error.

PPO activity. PPO activity in all the sampled tissues was higher in the months of May and October than in June and September (Figure 3 a, b). PPO activity at the advancing edge of the necrosis caused by M18 slightly declined at 30 days compared with the healthy control. PPO activity in the necrotic tissues was very low or absent. The plot of the size of the necrosis versus PPO activity of the control tissues are reported in Figure **4.**

Isoelectrofocusing. The isoenzyme patterns of PPO are reported in Figure 5. The healthy control tissues showed four isoenzymes in May and June and five isoenzymes in September and October; one isoenzyme focused at pH 4.5,

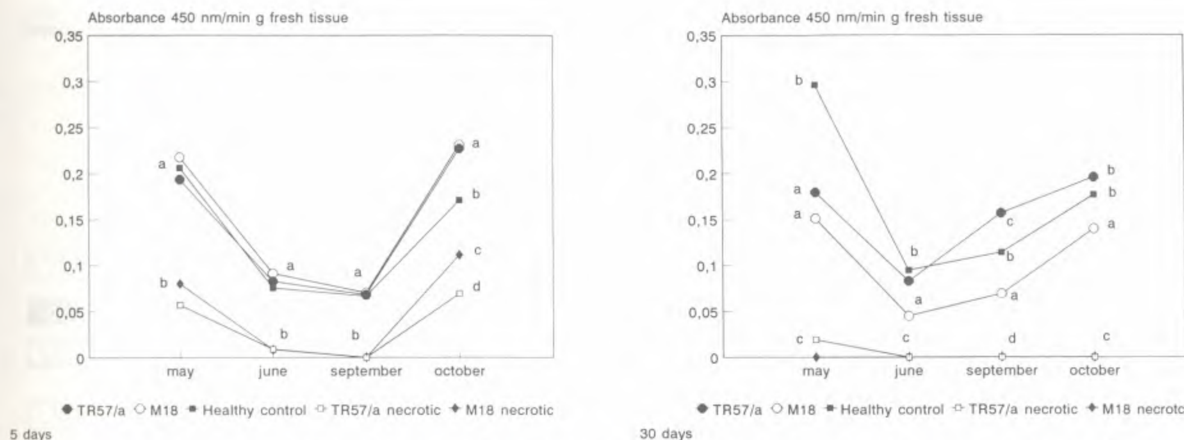

Figure 3. PPO activity at 5 and 30 days in the healthy control, in the advancing edge of the lesion of the tissues inoculated with strain TR57/a and isolate M18 and in the necrotic area of tissues inoculated with strain TR57/a and isolate M18. Activity is expressed in increase of absorbance at 450 nm/min/g of fresh tissues at 30 C. Points in each month followed by different letters differ significantly at P < 0.05 in Duncan's Multiple Range Test.

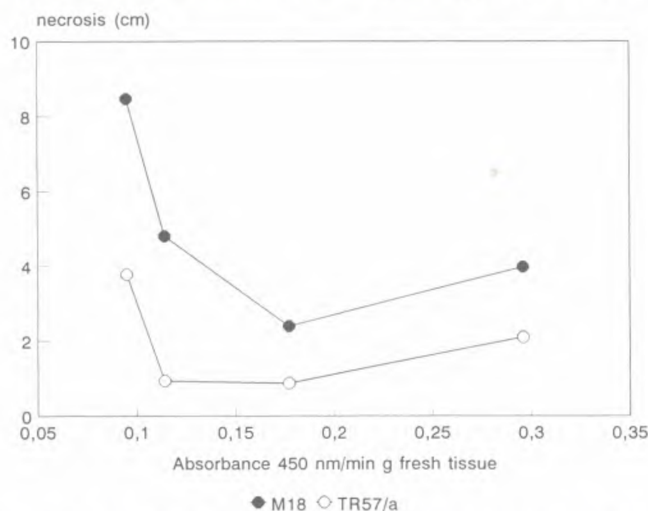

Figure 4. Plot of the length of the necrosis caused by strain TR57/a and isolate M18, after 30 days from inoculation, versus PPO activity of the healthy control. Vertical bars indicate the standard error.

while the remaining ones focused at alkaline pH values. The advancing edge of the necrotic tissues inoculated with strain TR57/a always showed the pattern of the control, while the isoenzyme at pI 8.5 and the one at pI 7.8 were lacking in the advancing edge of the necrotic tissues inoculated with the isolate M18 in the May and June experiments, and in the fall experiments.

In the necrotic tissues from infection with strain TR57/a and isolate M18, PPO isoenzymes were weaker and fewer than in the control healthy tissue. However, in the necrosis caused by the virulent isolate M18, had fewer PPO isoenzymes than necrosis caused by the hypovirulent strain TR57/a.

Extraction and determination of soluble and insoluble oxalic acid from the infected tissues. Oxalic acid accumulated in the necrotic tissues only (Figure 6c); no accumulation of oxalic acid was recorded in the advancing edge of the infections produced by either the hypovirulent strain or the virulent isolate, compared with the control. Most of the oxalic acid was accumulated as insoluble oxalate (Figure 6b); about one tenth of total oxalic acid was accumulated in soluble form. No differences were recorded in accumulation of oxalic acid between the hypovirulent strain and the virulent isolate.

Effect of oxalic acid on PPO activity. No inhibition of PPO activity was found at all the concentration of oxalic acid used.

DISCUSSION

Studies of herbaceous tissues of several plant species, in short-term experiments (4-7 days), showed an increase of PPO activity following infections by virus, bacteria, fungi or mechanical injury (1, 2, 4, 8, 16, 18, 21). Our results on chestnut bark tissues and in long-term experiments indicated that in the advancing edge of lesions caused by both the hypovirulent strain (TR57/a) and the virulent isolate (M18) of *C. parasitica,* the PPO activity of *C. sativa* is not increased compared with the healthy tissue. The kind of tissues examined and the period of time investigated from

Figure 5. Isoenzymatic patterns of PPO of the healthy control, of the advancing edge of the lesion of the tissues inoculated with strain TR57/a and isolate M18 and of the necrotic area of tissues inoculated with strain TR57/a and isolate M18. Samples were layered in 10% polyacrylamide vertical slab gel and isoelectrofocused at 250 V at 4 C until the current dropped to 1 mA.

the inoculation could account for the different behavior of *C. sativa*-*C. parasitica* interactions. However, PPO analysis 5 days after inoculation showed that the enzymatic activity of the inoculated tissues was significantly higher than the control in the October experiment. In long-term experiments (30 days), the virulent isolate dropped the PPO activity of the host tissues around the infection site. Kaul and Munjal (14) also reported a marked decrease in PPO activity of apple fruits inoculated with different pathogenic fungi after 10 and 15 days from inoculation. The IEF experiments gave a further evidence of the repression of PPO activity in the tissues infected with the virulent isolate of the fungus. The mechanism of selective inhibition of the alkaline isoenzymes at pI 8.5 in the May and June experiments and at pI 7.8 in the fall experiments takes relevant interest but needs further investigations. The hypovirulent strain did not interfere with the PPO activity of the host in the advancing edge of the lesion. On the contrary the virulent isolate was able to repress the PPO activity of the host. An inverse relationship was found between the seasonal PPO activity of the healthy tissues and the size of the necrosis caused by the virulent isolate of the fungus, suggesting a role of PPO in the mechanisms of defense against the blight. A similar relationship was found by Bashan et al. (4) in the interaction *tomato*-*Pseudomonas syringae* pv *tomato* van Hall and by Bashan (3) in the interaction cotton-A *Itemaria macrospora* Nees. between the index of the disease and the PPO activity. However, the qualitative and quantitative changes of PPO activity registered at different sampling seasons could be explained as a result of general metabolic changes occurring in the plant at different phenological stages; consequently the PPO could be not the only factor responsible for limiting the diffusion of the fungus in the host.

The repression of PPO activity during pathogenesis was also observed by Marciano et al. (17) in the interaction *Sclerotinia sclerotiorum* (Lib.)-sunflower. Repression of the enzyme was related with the production of oxalic acid by the fungus. In the interaction *C. parasitica*-*chestnut* an alternative mechanism of PPO inhibition other than the OA may operate. Although production of OA *in vitro* by virulent strains of *C. parasitica* is reported to be higher than the hypovirulent ones (6, 12, 20), data obtained from *in vivo* experiments showed no differences in accumulation of OA between the hypovirulent strain and the virulent isolate in the old necrotic tissues, and no accumulation of OA in the advancing edge of the lesions compared with the healthy control. The lack of differences in the production of OA between hypovirulent strain and virulent isolate *in vivo* may be explained by the composition of the substrate of growth of the fungus that can influence the production of OA; indeed Bennett and Hindal (6) reported that the production of oxalate by several hypovirulent and virulent strains of the fungus on selected media was not controlled by the cytoplasmic agent of hypovirulence.

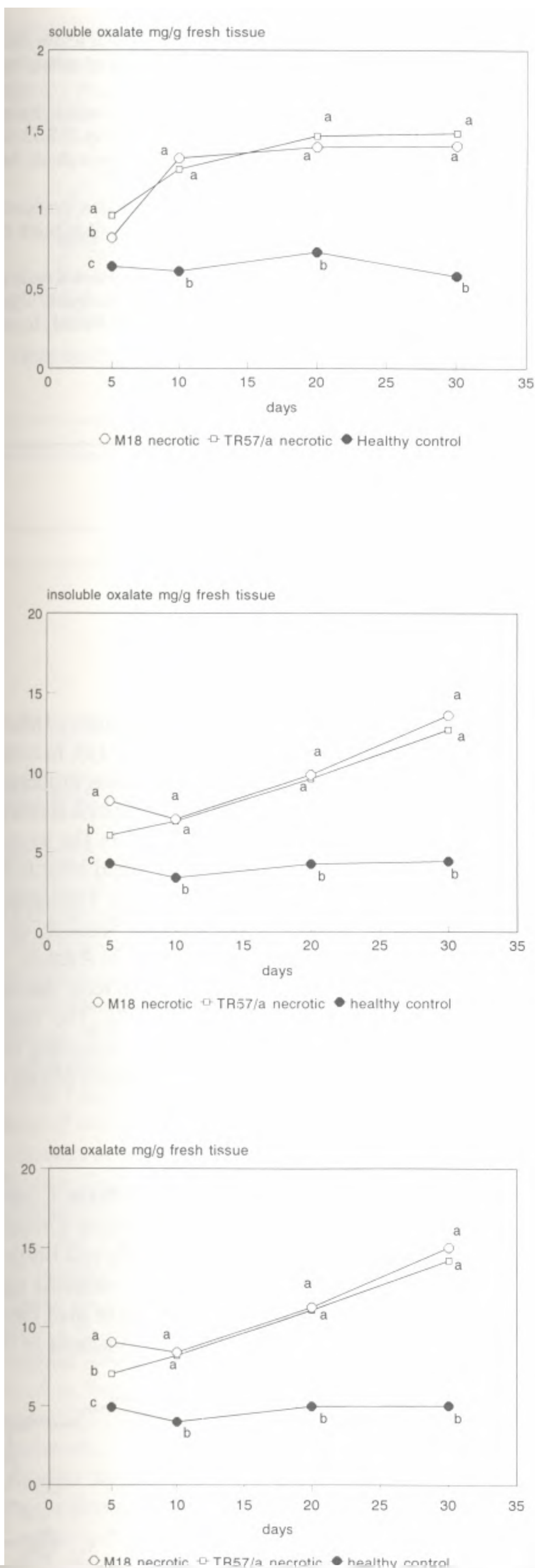

Figure 6. Soluble, insoluble and total oxalic acid produced by strain TR57/a and isolate M18 in the necrotic tissues of chestnut after 5, 10, 20 and 30 days from inoculation, compared with the healthy control. Data are expressed in mg/g fresh weight. Points 5, 10, 20, 30 days followed by different letters differ significantly at $P<0.05$ in Duncan's Multiple Range Test.

The above results together with the lack of inhibition of PPO in *vitro* with OA at different concentrations do not seem to support the hypothesis about a direct role of OA in the pathogenesis of *C. parasitica.* Moreover OA is also present in high amounts (about 5 mg/g fresh tissues), in soluble and insoluble form, in the healthy tissues of *C. sativa.* Further investigations are needed to explain the role of PPO of the host and the OA produced by *C. parasitica* during the first stage of pathogenesis, by using different hypovirulent and virulent isogenic strains.

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