The Role of Oxalic Acid in the Pathogenesis of *Endothia parasitica*

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ABSTRACT.—Oxalic acid is present in increased amounts along the advancing edge of *E. parasitica* cankers and appears to play a role in pathogenesis. It acts synergistically *in vitro* with polygalacturonase activity in degradation of calcium polypectate and displays a toxicity toward chestnut protoplasts.

Oxalate was isolated from media beneath cultures of *Endothia parasitica* (Murr.) P. J. and H. W. And. by Englander and Corden (1971). Oxalate is an inhibitor of succinate dehydrogenase as well as divalent cation-containing enzymes. It is a strong chelator of calcium and has been shown to act synergistically with the polygalacturonase of such fungi as *Sclerotium rolfsii* and *Rhizoctonia solani* (Bateman and Beer, 1965; Bateman, 1964).

This paper reports on the production of oxalate and polygalacturonase activity by *E. parasitica* and their activities toward Chinese *(Castanea mollissima* Bl.) and American *(C. dentata* [Marsh.] Borkh.) chestnuts.

MATERIALS AND METHODS

Four isolates of *E. parasitica* were used in this study. Isolate Al was obtained from an active canker in the University of Tennessee chestnut breeding orchard at Alcoa, Tennessee. The American hypovirulent, H', was obtained from R. A. Jaynes, Connecticut Agricultural Experiment Station, New Haven. The European hypovirulent, 2103-B, was supplied by J. Grente, INRA-Station de Pathologie Vegetale, Clermont-Ferrand, France. The European virulent, M-272, was obtained from S. Naef-Roth, Institut fur Spezielle Botanik, Zurich, Switzerland.

The bioassay was performed on inner bark from Chinese and American chestnuts. Longitudinal cuts were made along 8-12-year-old shoots and 1-cm wide strips of bark peeled off. Discs, 0.24 cm^2, of Whatman #4 filter paper were placed on the inner surface and the test solutions applied in a volume of 5 or 10 /21. The bark strips were incubated 24 hours in a humidity chamber. Browning of cells due to polyphenol oxidase activity was used to monitor cell disruption (Joslyn and Ponting, 1951). Responses were scored from 0 to 4, with 0 indicating no responses and 1 indicating light browning with brown cells distinguishable under a stereoscopic microscope. A score of 2 and 3 indicated light and dark general browning, and the score of 4 indicated heavy browning with the effect extending beyond the edge of the paper disc.

Oxalate was determined by the method of Pucher *et al.,* (1941) as applied by Bateman and Beer (1965). This method employs an ether extraction of an acidified sample, calcium precipitation of oxalate and titration with permanganate. It is specific for oxalate.

The pH of various zones in a canker was estimated using pHydrion short range pH papers. A slit was made in the bark and the paper inserted and compressed. The pH was estimated by comparing the color with the color chart provided.

Cultures were grown on the minimal media of Puhalla and Anagnostakis (1971), with sodium or calcium polypectate replacing dextrose as the sole carbon source. Polygalacturonase was partially purified from 50 m stationary cultures grown for 100 hours on 1 percent sodium polypectate (Sunkist Growers). The mycelium was removed by centrifugation and an equal volume of ice cold acetone added to the supernatant. The flocculant was harvested by centrifugation (20 min., 20,000 xg.) and redissolved in 0.12 *M* sodium acetate buffer, pH 5.3. Oxalate was determined in 25 ml stationary cultures grown on 0.5 percent calcium polypectate prepared by adding 2 ml of 50 percent CaCl to the 25 ml of freshly autoclaved culture media containing 0.5 percent sodium polypectate. Cultures were harvested by lyophilization.

Polygalacturonase activity was detected in cankers, utilizing an agar diffusion method (Dingle, Reid and Solomons, 1953). Sections of canker were placed on plates containing 40 ml of 1 percent sodium polypectate, 2 percent agar, 0.015 percent salicy-

lanilide and buffered to pH 5.3 with 0.2 *M* potassium phosphate buffer. Plates were incubated 48 hours under an atmosphere of propylene oxide before clarified zones were visualized with 5 *N* HCl .

The effect of oxalate on polygalacturonase degradation of calcium polypectate was determined using the cup-plate method described by Bateman and Beer (1965). Plates prepared as described above were either subjected to: 1) no further treatment, 2) incubated 12 hours with 8 ml of 0.5 *N* CaCl, or 3) treated with CaCl and incubated another 12 hours with 8 ml of 0.6 *M* ammonium oxalate. Cups were cut using a #5 cork borer and their bottoms sealed with melted agar. Polygalacturonase containing solutions were added to the cups in volumes up to 0.3 ml and incubated overnight before development with 5 *N* HO. The diameters of resulting clear zones were proportional to enzyme activity.

RESULTS AND DISCUSSION

E. parasitica acidifies the canker, particularly along the advancing edge of the mycelium (Table 1). A broad range of pH values was found in tissue in advance of nascent mycelium, described as the "gelatinous" zone by Rankin (1914), suggesting a "titration" of the tissues in advance of the mycelium. Estimates of pH from Chinese and American cankers were similar and subsequently averaged.

Oxalate is found in increased amounts along the advancing edge of the canker (Table 2) and in liquid cultures containing calcium polypectate (Fig. 1). Oxalate is produced by all isolates, including the hypovirulents. Quantities of oxalate accumulating in infected tissue or liquid cultures are small in comparison to those of such pathogens as *S. rolfsii* (Bateman and Beer, 1965) where 30 mg/g dry wt. and 16 mg/ml may be found in infected bean hypocotyls and culture fluid, respectively. The woody nature of chestnut inner bark may account for the small ratios seen in Table 2. However, the liquid cultures yield only 0.1 percent of the level reported for *S. rolfsii,* suggesting a stringent control over oxalate synthesis by the fungus.

The effect of pH and various buffers on chestnut inner bark was determined using the bioassy (Figs. 2 and 3). Each buffer elicits a browning response at or above the pH found along the advancing edge of the canker (Table 1). Further, oxalate buffer elicits a response at the pH of sound tissue, from 1/2 to 1 1/2 pH units above that necessary for a response from the same molar concentration of the other carboxylic acid buffers. Assuming strictly passive permeability, oxalate buffer will supply less of the permeable undissociated acid than will equimolar citrate or acetate buffers at the same pH. Utilizing the Henderson-Hasselbalch equation and the pH at which a minimally detectable (threshold) response was seen on American chestnut inner bark, it was calculated that approximately 0.9; 0.6 and 0.3 $_{1,2}$ moles of undissociated phosphoric, acetic and citric acids, respectively, were necessary to achieve a threshold response, while only approximately 2 X 10^{-5} μ moles of undissociated oxalic was necessary to achieve this threshold (Fig. 2). These cal-

Table 1
pH of canker zones from Chinese and American chestnuts.

Zone	Average pH	Standard Deviation[a]
Sound Inner Bark	5.5	0.5
"Gelatinous"	4.7	0.9
Advancing Edge of Mycelium	2.8	0.7
Old Necrotic	3.4	0.4

[a] Based on observations from 12 cankers each from American and Chinese trees.

Table 2
Oxalate obtained from canker zones of American chestnuts.

Zone	Dry Weight mg / g	Standard Deviation
Sound Outer Bark	3.78	0.08
Sound Inner Bark	6.10	0.78
Advancing Edge of Canker[a]	9.26	0.64
Old Necrotic	4.44	0.14

[a] Gelatinous zone and 0.5 cm of nascent mycelium.

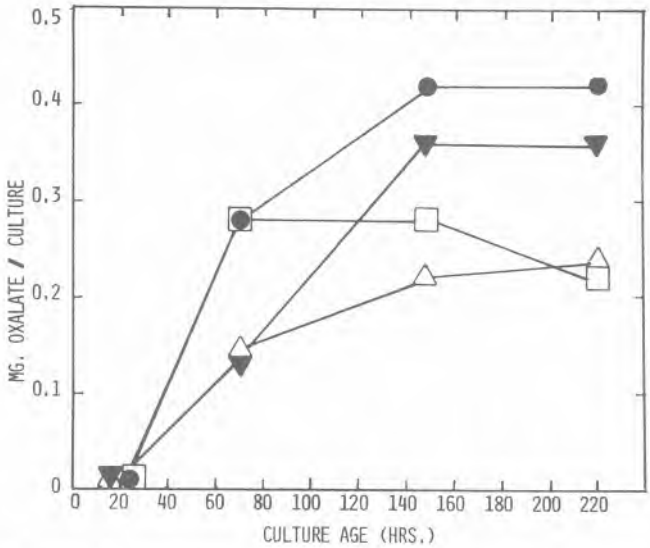

Figure 1. Isolates A1 (•), M-272 (▲), H' (□) and 2103-B (△) were grown on calcium polypectate and oxalate determined as described under Methods.

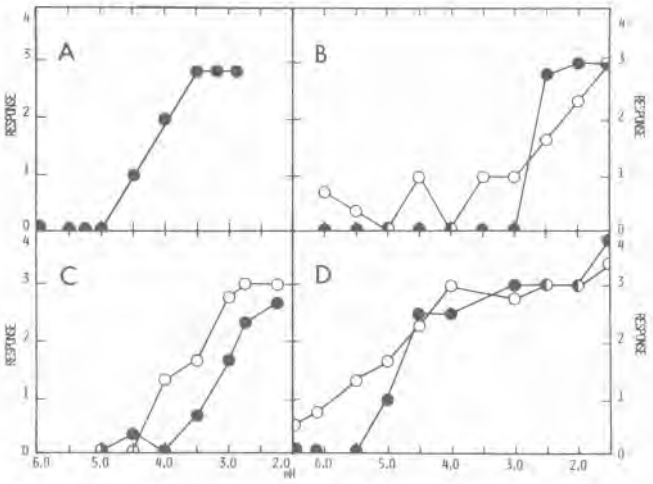

Figure 2. Bioassays of buffers on Chinese and American chestnut inner bark were performed as described under Methods. In this and the following figures, each point represents the average of a minimum of six replications. Each disc contained 1.0 μ mole of its respective buffer applied in 10 μl, except that 10 μ moles phosphate buffer were applied per disc. Plot A represents the bioassay of acetate; B, phosphate; C, citrate; and D oxalate buffers. Open symbols give the response of Chinese chestnut, and closed symbols the response of American chestnut inner barks.

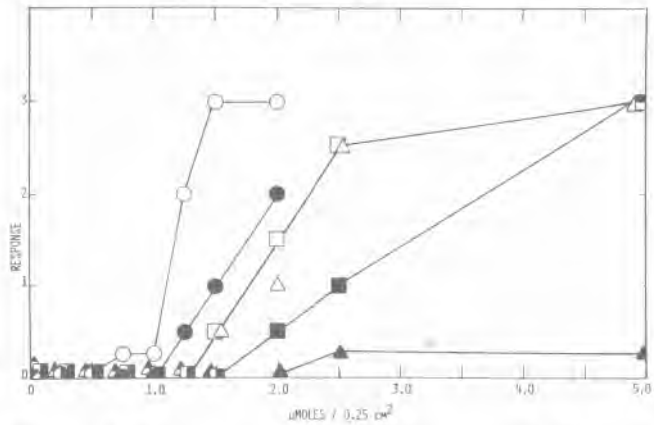

Figure 3. Bioassays of carboxylic acid buffers at pH 5.7 were performed as described under Methods. The open symbols represent the response of Chinese inner bark, the closed symbols, American inner bark. Acetate (△▲), citrate (□ ■) and oxalate (○ •) buffers were used.

culated levels agree with those determined by direct bioassay of unbuffered acids (Fig. 4) and calculation of the undissociated acid in the same manner. Approximately 0.7 and 0.2 μ moles of acetic and citric acid, respectively, were necessary to achieve a response in this experiment, while 8 X 10^{-4} μ moles oxalic acid was necessary to reach this threshold. Thus, oxalic acid appears to effect protoplasts at levels considerably below those effective for the other acids, suggesting an effect other than one due

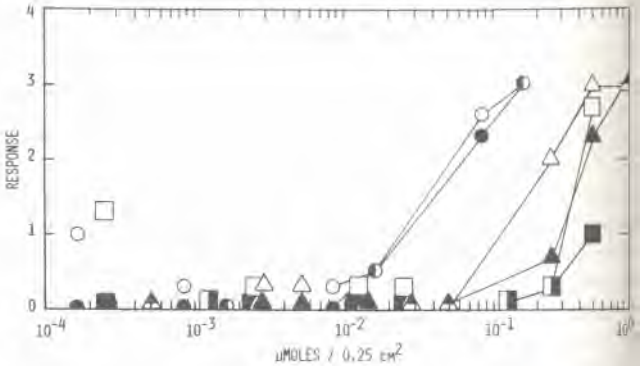

Figure 4. Bioassays of unbuffered carboxylic acids were performed as described under Methods. Open symbols represent the response of Chinese, and closed symbols American inner bark. Five μl of acetic (△▲), citric (□ ■) and oxalic (○ •) acid solutions were applied per disc. Measured pH of the acid solutions at their threshold concentrations were 2.5 for oxalic and citric and 3.1 for acetic acid.

strictly to acidification.

The strong affinity of oxalate for divalent cations, however, precludes an interpretation that this toxic effect is expressed solely within the protoplast. For example, oxalate might remove calcium from acidic phospholipids, disrupting the plasma membrane.

The Chinese chestnut inner bark bioassays display an increased browning response in comparison to the American (Fig. 3). This response may be important in the resistance of this species to *E. parasitica* as the polyphenolic products formed after cell disruption may inhibit enzymes produced by the fungus and exert a toxic effect against the pathogen (Williams, 1963).

Oxalate also acts synergistically with polygalacturonase activity produced by the fungus (Table 3). Polygalacturonase activity is detectable in the canker and appears maximally after 100 hours in liquid cultures. Polygalacturonase is ineffective, however, against calcium polypectate in the cup-plate assay. Oxalate restores the activity, by removing calcium from the polypectate and exposing this substrate to the enzyme.

Table 3

The effect of oxalate on enzymatic degradation of pectate.

Substrate	Radius Clear Zone[a]	Standard Deviation
1) Sodium polypectate	4.6	1.6
2) Calcium polypectate	0	—
3) Calcium polypectate and Oxalate	4.5	1.8

[a] The radius is reported in mm / 0.1 ml enzyme solution used in three separate experiments with six replications of each treatment.

Oxalate, therefore, appears to play a dual role in *E. parasitica* pathogenesis. It may act synergistically with polygalacturonase, advancing tissue maceration. It is also toxic toward protoplasts and aids in acidifying the canker.

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