The Synergistic Role of Oxalic Acid and Endopolygalacturonase in Bean Leaves Infected by *Cristulariella pyramidalis*

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ABSTRACT

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Amounts of oxalic acid were elevated in lesions of bean leaves that were infected for 3 days with *Cristulariella pyramidalis*. Amounts in the green tissue adjacent to these lesions were considerably higher than those in healthy leaves. Endopolygalacturonase was not detectable in extracts of healthy bean leaves but was present in leaves infected with *C. pyramidalis* and in pectin-containing medium supporting growth of this fungus. This enzyme was inhibited by $CaCl_2$ and the inhibition was reversed by oxalic acid. Oxalic acid depolarized cell potentials at a concentration as low as 10^{-4} M but only if the solution was approximately pH 4 or lower. This depolarization was not rapidly reversed when the bathing solution was changed to one free of oxalic acid. The data presented are consistent with synergistic roles for oxalic acid and endopolygalacturonase during invasion of bean leaves by *C. pyramidalis*.

The leaf-spotting pathogen, *Cristulariella pyramidalis* Waterman and Marshall, and other fungi (4,7,11) produce oxalic acid in axenic culture. Bateman and Beer (2) showed that *Sclerotium rolfsii* synthesized oxalic acid and pectic enzymes both in culture and in infected bean hypocotyls. They reported that oxalic acid and especially oxalic acid plus pectic enzymes were toxic to the bean tissue. They concluded that oxalic acid caused injury directly because of the low pH and indirectly because it activated pectic enzymes. The activation was due to (i) an oxalic acid-mediated decrease in pH to that optimal for enzyme activity and (ii) chelation of cell wall Ca^{+2}, which enable the enzyme to more rapidly degrade the carbohydrate of the middle lamella. A similar function of oxalic acid was suggested by Maxwell and Lumsden (12) in plants infected with *Sclerotinia sclerotiorum*.

The purpose of our research was to study the pectic enzymes produced by *C. pyramidalis* and to determine the role of these enzymes and oxalic acid in bean leaves infected with this fungus.

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MATERIALS AND METHODS

Fungal and plant tissues and tissue extracts. *C. pyramidalis* was grown in 250-ml Erlenmeyer flasks containing 40 ml of the medium previously described (8) and in similar medium at pH 4.5 or 6.0 in which 1% pectin (General Biochemicals, Inc., Chagrin Falls, OH 44022) was the carbon source. Liquid portions of the cultures were filtered through Whatman No. 1 filter paper and then through a 0.22-μ Millipore filter at 3-day intervals. The filtrates were dialyzed against 20 volumes of 0.01 M sodium citrate buffer, pH 5.0, at 5 C for 18 hr with two changes of buffer. The dialyzed solutions were used for enzyme assays immediately or were stored at 5 C under toluene for a maximum of 3 days. The enzyme activity remained constant during this time, and the solutions did not become turbid, even when stored for 1 mo.

Garden bean (*Phaseolus vulgaris* L.) plants were grown in the greenhouse for 14 days and then inoculated with *C. pyramidalis* according to Trolinger's method (14). Agar plugs approximately 0.25 cm^2 from the leading edge of the mycelium of 8-day-old cultures were placed across the midveins of the bean leaves. The leaves were punctured through each corner and the center of the agar plug inocula. The plants then were transferred to a 100% humidity chamber that was maintained at 23 C. After 3 days the plants were placed on a laboratory bench for 1 hr and the necrotic lesions including approximately 5 mm of surrounding green tissue were excised, as were analogous sections from leaves of plants that were treated with an uninoculated agar plug and incubated in an identical manner. The sections from approximately 75 control or diseased leaves were combined and homogenized with 2 ml of 0.25 M NaCl for each gram of excised tissue in a Virtis homogenizer at medium speed for 2 min. The homogenate was centrifuged at 16,000 *g* for 20 min at 4 C and the supernatant was used for the enzyme studies.

Oxalic acid. Oxalic acid was detected in healthy and *C. pyramidalis*-infected leaves of bean plants that were grown as described above. Tissue samples were taken from necrotic areas including 3-mm borders of green tissue, 10-mm strips of green tissue adjacent to the first sample, and areas from leaves of healthy bean plants. The extraction and assay procedures were a modification of the methods of Sasson et al (13). Each sample, containing 2–3 g of tissue, was homogenized with 3 ml of 70% ethanol per 0.1 g of tissue using a Virtis homogenizer. Each homogenate was centrifuged at 16,000 *g* for 20 min and the supernatant was concentrated to dryness in a flash evaporator at 45 C. The residue was mixed with 2 ml of 14% BF_3 in methanol, incubated overnight at room temperature, and then mixed with 4 ml of 33% $(NH_4)_2SO_4$ and 2 ml of CCl_4. The mixture was centrifuged at 16,000 *g* for 20 min and the CCl_4 layer was subjected to gas chromatography using authentic oxalic acid treated in an identical way as the standard. The gas chromatograph was a Varian Model 2100 equipped with a flame ionization detector and a 5 ft × 1/8 in. glass column packed with 12% HI-EFF-IBP, 80-100 mesh, Gas-chrom P (Applied Science Laboratories, State College, PA 16801). Nitrogen was used as carrier gas at 30 cm^3/min, and the hydrogen and air flow rates were 30 and 350 cm^3/min, respectively. The column, injector, and detector temperatures were 115, 195, and 220 C, respectively.

Polygalacturonase. The cup-plate method (3) was used to semiquantitatively assay polygalacturonase activity in extracts of healthy bean leaves, leaves that were infected with *C. pyramidalis* for 3 days, and filtrates of liquid media that had supported fungal growth. The filtrates of pectin-containing medium that had supported fungal growth for 9 days and extracts of leaves that had been infected for 3 days were also assayed quantitatively by viscosimetric (2) and reducing sugar (5) methods.

The cup-plate method was performed as described by Dingle et al (3) using 1.5% agar, 1% sodium polypectate (Sunkist Growers, Inc., Ontario, CA 91764), and 0.1 M sodium acetate buffer, pH 4.5. The diameters of the clear zones in millimeters were taken as units of relative polygalacturonase activity.

Polygalacturonase activity was measured viscosimetrically as described by Bateman and Beer (2) using 5.5 ml of 1% sodium polypectate in 0.05 M sodium citrate buffer, pH 4.5, and 0.5 ml of enzyme preparation. The reciprocal of the time required for 50% reduction in viscosity was multiplied by 1,000 to obtain the units of enzyme. These mixtures were subjected to ascending paper chromatography with 77% ethanol and 90% formic acid (85:15, v/v) (9). Authentic galaturonic acid was chromatographed as a reference. The developed papers were air dried, sprayed with aniline-diphenylamine reagent (Sigma Chemical Co., St. Louis, MO 63178), and heated at 85 C for 10 min.

In the reducing sugar assay for polygalacturonase activity, tubes containing 1.7 ml of 0.25% polygalacturonic acid (Sigma Chemical Co., St. Louis, MO 63178) in 0.05 M sodium citrate (pH 3–6.5) or 0.05 M sodium HEPES (pH 6.5–8.5), 0.1 ml of enzyme, and 0.2 ml of water were incubated at 30 C for 10 min. The reaction was stopped by placing the tubes in a boiling water bath and the reducing sugar then was measured by the Nelson colorimetric method as described by Hodge and Hofrieter (5). One unit of activity is defined as the amount of enzyme required to release one microequivalent of reducing sugar in the 10-min assay.

Effect of Ca^{+2} and oxalate on polygalacturonase. In some assays for reducing sugars, 0.1 ml of water was replaced with 0.1 ml of $CaCl_2$ to give final calcium concentrations from 10^{-5} M to 10^{-2} M. Another set of experiments was conducted in which the calcium concentration was 10^{-2} M and 0.1 ml of aqueous solutions of sodium oxalate was used in place of 0.1 ml of water so that the final oxalate concentrations were from 10^{-5} M to 10^{-2} M.

The effects of calcium and of calcium and oxalate on polygalacturonase activity also were studied by the cup-plate method (3). The plates were made as described except that ammonium oxalate was omitted. The plates were used as such or were treated with 5 ml of 0.5 M $CaCl_2$ for 3 hr and then rinsed with water. In some experiments this treatment was followed by 5 ml of 0.6 M ammonium oxalate for 3 hr and a subsequent water rinse. Enzyme preparations were then added to each type of plate.

Effect of oxalate and pH on cell potential differences. The potential differences between the external solution and the vacuole of a cortical parenchyma cell of a potato (cv. Kennebec) petiole segment was measured by the methods described by Mancarella (10). Petiole segments approximately 1 cm long were incubated overnight in a dilute salt solution to allow sufficient time for recovery from the damage caused by cutting (10). The aged petioles were perfused with this same salt solution at a rate of 5 ml/min and a cell at the cut surface then was impaled with a microelectrode. When a steady potential was obtained, the flow was diverted to 2.5×10^{-3} M KCl. When the potential was steady again, the flow was diverted to aqueous KCl containing various concentrations of oxalic acid, which sometimes was adjusted to desired pH values with 1 N NaOH.

TABLE 1. Oxalic acid levels in healthy bean leaves and in the necrotic and surrounding green areas of bean leaves infected with *Cristulariella pyramidalis* as a function of time after inoculation

Sample	Time (days)	Oxalic acid (mg/g fresh wt)
Control leaf	···	0.02
Necrotic zone	1	0.23
Necrotic zone	2	0.49
Necrotic zone	3	1.31
Surrounding green tissue	3	0.13

RESULTS

The concentration of oxalic acid increased dramatically in the area of infected bean leaves developing necrosis (Table 1). Three days after the plants were inoculated, the level of oxalic acid in the green tissue surrounding the necrosis was six times as great as the level in healthy leaves. No fungal mycelium was observed in this area when the tissues were sectioned and stained with lactophenol-cotton blue (data not shown).

Polygalacturonase was not detected with the cup-plate method in culture filtrates from media not containing sodium polypectate or in extracts of healthy bean leaves. The medium containing pectin had no polygalacturonase activity on day 3 but was highly active on day 6 (Table 2). Because the activity was slightly greater on day 9 and had started to decrease by day 12, the medium that had supported fungal growth for 9 days was used in subsequent studies. The enzyme was also present in bean leaves 3 days after they had been inoculated with *C. pyramidalis*. Leaves infected for other time periods were not assayed. The polygalacturonase from infected bean leaves and from culture filtrates (Table 2) apparently is the same enzyme or combination of enzymes. This conclusion is based on the observations that fungus-free leaves had no polygalacturonase activity and that both preparations had a single pH optimum of 4.5 (Fig. 1).

The polygalacturonase from infected leaves and culture filtrate is largely an endoenzyme, since each preparation rapidly decreased

the viscosity of a solution of sodium polypectate. This is substantiated by the paper chromatographic analyses of these digests. Even after 30-min incubations with the enzymes, only a small amount of material migrated with an R_f corresponding to that of authentic galacturonic acid. Two equally intense spots were seen at lower R_f values, but most of the carbohydrate in the reaction mixture remained at the origin during chromatography.

Calcium chloride inhibited the polygalacturonase, but the inhibition was partially reversed in the presence of an equimolar concentration of oxalate (Fig. 2). When the enzyme was assayed by the cup-plate method, no polygalacturonase activity could be detected on plates that had been flooded with 0.5 M $CaCl_2$. The activity was completely recovered when the plates that were treated with $CaCl_2$ were flooded with 0.6 M ammonium oxalate before the enzyme assay. This was true for enzyme from culture filtrates and infected bean leaf extracts.

The lowest concentration of oxalic acid that causes necrosis on wounded bean leaves (7) and potato petioles (authors, *unpublished data*) is 0.01 M. Substantially lower concentrations depolarized potato petiole cell potentials (Table 3). Some depolarization occurred with a 10^{-4} M solution of oxalic acid, and the depolarization was 100% with a 10^{-3} M solution. The depolarization was substantially less when partially neutralized solutions of oxalic acid were used. No depolarization occurred with 10^{-3} M oxalic acid if the pH was 4.8 or 5.3.

TABLE 2. Polygalacturonase (PG) activity at pH 5.5 in culture filtrates that had supported growth of *Cristulariella pyramidalis* for various lengths of time and in extracts of lesions of bean leaves infected with the fungus for 3 days.

Assay Method	PG (units/ml)				
	Culture filtrate				Tissue extract
	3 day	6 day	9 day	12 day	
Cup-plate	0	22	24	21	32
Viscosity	...	...	667	...	1,142
Reducing sugar	...	...	7	...	14

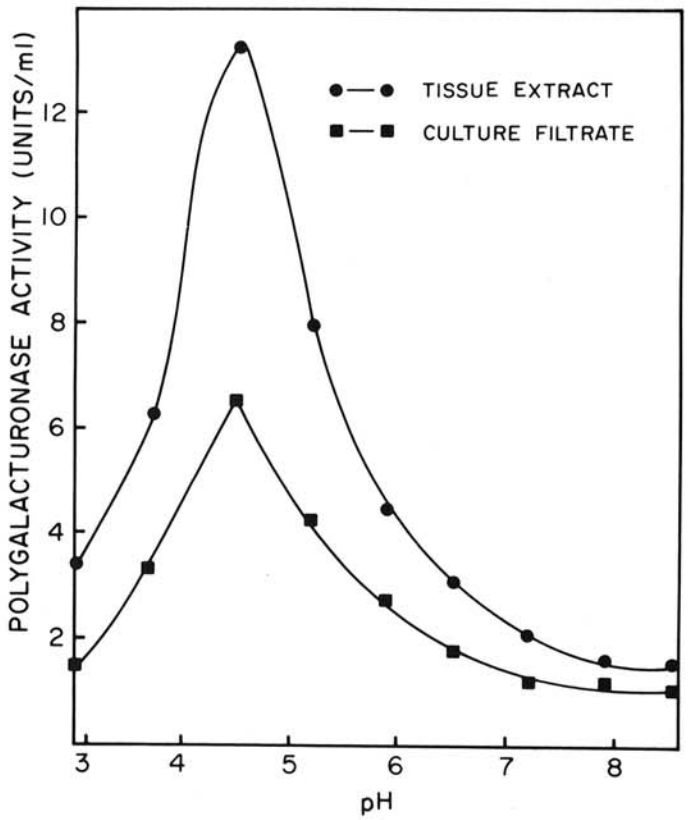

Fig. 1. Polygalacturonase activity (reducing sugar assay) as a function of pH in culture filtrates and bean leaf extracts that had supported *Cristulariella pyramidalis* for 9 days and 3 days, respectively. Each point is the average value of three assays.

DISCUSSION

The amount of oxalic acid isolated from infected bean leaves in this study was low compared with that reported by others (2,12). We found only 1.31 mg/g of necrotic lesion 3 days after inoculating the bean plants with *C. pyramidalis*. Maxwell and Lumsden (12) obtained up to 48.3 mg/g dry weight in bean leaves 4 days after inoculation with *S. sclerotiorum*. Bateman and Beer (2) reported that bean hypocotyls infected with *S. rolfsii* for 56 hr contained 32.74 mg of oxalic acid per gram dry weight. The oxalic acid levels found in this study seem low partially because we reported the results on a fresh weight basis. In addition, the method used extracted free oxalic acid but not most of the oxalate salts.

Ayers et al (1) reported that the polygalacturonase of *Rhizoctonia solani* was inhibited when the $CaCl_2$ concentration was higher than 5×10^{-4} M. Our study showed that the enzyme from *C. pyramidalis* was inhibited by 10^{-3} M $CaCl_2$ (Fig. 2). This could be due to a direct inhibition of the enzyme, but it is more likely because of calcium chelation of the polygalacturonic acid. This is supported by the observation that the substrate solution became quite viscous in the presence of 10^{-2} M $CaCl_2$.

Oxalate completely reversed the calcium inhibition of polygalacturonase from *S. rolfsii* (2). We observed only a slight inhibitory effect of calcium when the enzyme was assayed in the presence of an equal concentration of oxalate (Fig. 2) and a

TABLE 3. Depolarization of potato petiole cell potentials in solutions containing 2.5×10^{-3} M KCl and oxalic acid

Oxalate Concentration (M)	pH	Depolarization (%)
1×10^{-5}	4.85	0
1×10^{-4}	3.95	34
1×10^{-3}	3.10	100
1×10^{-3}	3.70[a]	76
1×10^{-3}	4.10[a]	10
1×10^{-3}	4.80[a]	0
1×10^{-3}	5.30[a]	0

[a]Adjusted to the indicated pH with 1 N NaOH.

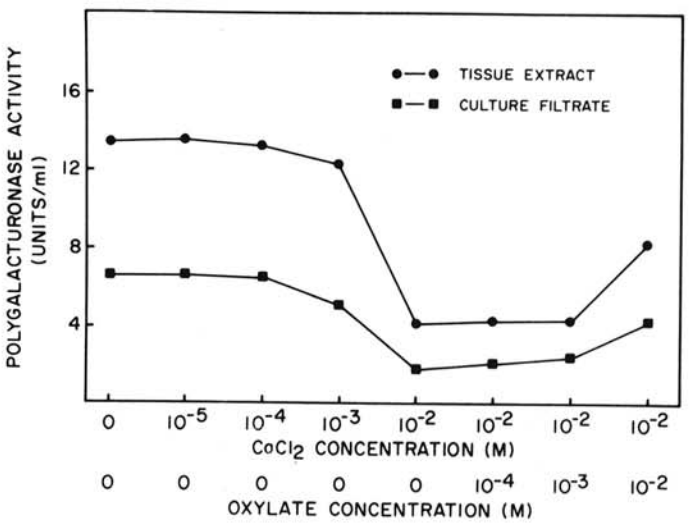

Fig. 2. Polygalacturonase activity (reducing sugar assay) in the presence of various amounts of calcium and of calcium plus sodium oxalate in culture filtrates and bean leaf extracts that had supported *Cristulariella pyramidalis* for 9 days and 3 days, respectively. Each point is the average value of three assays.

complete reversal of the calcium inhibition by oxalate with the cup-plate method. The difference in the results of these two experiments may be due to the fact that there was an excess of oxalate in the cup-plate method. Alternately, the precipitate of calcium oxalate that was seen in the assay in solution may have interfered with the enzyme activity.

The depolarization of the potato cell potential after treatment with sublethal concentrations of oxalic acid at approximately pH 4 or below was probably caused by the hydrogen ion concentration. Similar depolarizations were noted by Jones et al (6), but they reported that these pH effects were easily reversible. In this study the cell had little tendency to repolarize when the oxalic acid solution was replaced with aqueous KCl. Repolarization was still slow but more effective when oxalic acid was replaced with bathing solution containing calcium (data not shown). A plausible explanation is that at low pH the flow of hydrogen ions into the cell is passive and this increases the tendency of oxalate to move into the membrane. The oxalate might then chelate divalent cations in the membrane, thus disrupting the structural integrity of the membrane and increasing the permeability. Such bound oxalate would be difficult to remove, especially if the perfusion solution did not contain calcium.

C. pyramidalis synthesizes sufficient oxalic acid in axenic cultures to cause cell death when the medium is applied to wounded bean leaves. This toxic effect if probably mainly the result of low pH of the solutions rather than of the presence of oxalic acid per se (7).

The furthest advance of *C. pyramidalis* hyphae in infected bean leaves is at or slightly behind the leading edge of the necrotic lesions. Water droplets on this region of the leaves had a pH of 4–4.5, as measured with pH paper. In contrast, droplets on the necrotic and surrounding green regions had pH values of 2.5 and 5.5, respectively. The pH at the leading edge of the necrotic lesions is approximately the pH of optimal growth for this fungus (7). It is also the optimal pH of the endopolygalacturonase produced by *C. pyramidalis* (Fig. 1). In addition, oxalic acid solutions caused cell depolarization, which may be a measure of increased permeability, only if the solutions were at approximately this pH or lower (Table 3).

We hypothesize that the probable roles of oxalic acid during infection caused by *C. pyramidalis* are similar to those proposed for other plant-pathogen combinations (2,12). Specifically, we suggest that oxalic acid (i) increases the permeability of the host plasma membrane, thus providing nutrients for the parasite, (ii) decreases the pH to a value favorable for the growth of *C. pyramidalis* and the activity of the fungal endopolygalacturonase, and (iii) chelates the calcium of the cell wall calcium pectate, thus changing this polysaccharide to a form that can be hydrolyzed by the fungal enzyme. The necrosis associated with *C. pyramidalis* infection is probably caused by oxalic acid in concert with fungal endopolygalacturonase.

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