Oxalic Acid Production by Sclerotinia sclerotiorum in Infected Bean and in Culture

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ABSTRACT

Sclerotinia sclerotiorum produced oxalic acid in a liquid-salts-yeast extract medium (initial pH 5.8) supplemented with either 110 mM D-glucose, 110 mM D-glucose and 0.2 M potassium phosphate, or 73.7 mM D-glucose and 56.0 mM sodium succinate. Oxalic acid concn in these media were 0, 17.8, and 70.8 mM, respectively, after 7 days' growth at 26 C. Growth was equivalent on these media.

On the glucose-succinate medium, fungal growth rate was correlated positively with rate of oxalate accumulation. Oxalic acid accumulation by 17 isolates varied from 1.4 to 78.3 mM in the glucose-succinate medium.

In bean hypocotyls infected by *S. sclerotiorum* (isolate Ss-3), oxalate was detected at 1.1, 31.4, and 48.3 mg/g dry wt of tissue 0, 2, and 4 days after inoculation, respectively. The pH value of tissue extracts was 6.1 on day 0, decreased to 4.1 on day 2, and increased to 5.8 on day 6. During the first 2 days after inoculation, 2-4 cm of the bean hypocotyl were colonized, and polygalacturonase activity of tissue extracts increased significantly.

This study suggests the possibility that oxalic acid plays an important role in pathogenesis of *S. sclerotiorum* infections. Phytopathology 60:1395 1398.

The involvement of oxalic acid in pathogenesis was first considered by de Bary in 1886 (5) for diseases caused by *Sclerotinia sclerotiorum* (Lib.) d By. From microscopic observations, he concluded that suscept cells were killed and middle lamellae were degraded, and that these symptoms preceded the invading hyphae. Oxalate was detected in infected tissues and in various liquid culture media. Plants treated with solutions of oxalic acid showed symptoms different from those of infected plants. Conversely, sap from infected plants, when applied to healthy plants, caused symptoms very similar to those associated with the disease. After this sap was boiled, it caused no adverse effects. De Bary (5) suggested from these studies that a ferment (enzyme) was probably responsible for the effect on the cell wall, although the nature of the "poison" that killed cells was not known. Other workers (4, 17, 18) have confirmed de Bary's histological observations concerning the action of various fungal products and/or products of pathogenesis which act in advance of the invading hyphae of *S. sclerotiorum*.

Overell (16) studied toxin production by *S. sclerotiorum* in liquid media and found that the highest concn of toxic substances occurred after 32 days, when filtrates contained 10 mM oxalic acid. Oxalate could not be detected in tissue lesions. Overell (16) concluded that oxalate was not involved in pathogenesis, as it only accumulated in aging cultures. Held (10) found that only a pathogenic isolate of *S. sclerotiorum* produced a toxic compound in a liquid culture medium. Toxicity was tested by the ability of heated culture filtrates to wilt clover leaves.

The involvement of cell-wall degrading enzymes in diseases incited by *S. sclerotiorum* has received considerable attention (6, 8, 9, 12). Hancock (8) found high endopolygalacturonase activity associated with *S. sclerotiorum*-infected sunflower and tomato stems and a decrease in the amount of pectic acid in these tissues. There was also a decrease in pH from 6.2 to about 4.5 associated with a 3-fold increase in the total acidity of *Sclerotinia*-infected stems. A synergistic action between oxalic acid and endopolygalacturonase in bean hypocotyls infected by *Sclerotium rolfsii* was suggested by Bateman & Beer (3) to account for the rapid collapse and death of infected tissues. Because a similar relationship might apply to *S. sclerotiorum* infections, these studies were undertaken to determine the production of oxalic acid by this pathogen in infected tissues during the early stages of pathogenesis and in liquid culture media.

MATERIALS AND METHODS.—*Sclerotinia sclerotiorum*, sensu Purdy (19), isolates were obtained by courtesy of various researchers (Table 1). All isolates were weakly virulent or avirulent on *Phaseolus vulgaris* L. 'Bountiful' except for isolates Ss-3 and Ss-9. Pathogenicity tests were not performed with other hosts. Some difficulty in maintaining highly virulent isolates was experienced. Stock cultures were maintained on potato-dextrose agar slants at 24 C and transferred every 1-2 months.

For in vitro studies of oxalic acid accumulation, the medium contained per liter of distilled water 180 mg $MgSO_4$, 149 mg KCl, 1.00 g NH_4NO_3, 680 mg KH_2PO_4, 11.3 mg $ZnSO_4 \cdot 7H_2O$, 13.9 mg $MnSO_4 \cdot H_2O$, 4.0 mg $FeCl_3$, 4.0 mg $CuSO_4$, and 0.5 g yeast extract (Difco Laboratories, Inc., Detroit, Mich.). The carbon source was either 110 mM D-glucose or 73.7 mM D-glucose and 56 mM sodium succinate, and the pH was adjusted to 3.0 or 5.8 by the addition of HCl or KOH. The culture media (25 ml/125 ml Erlenmeyer flask) were autoclaved for 13 min at 121 C. Plugs (1-cm diam) were cut from 5- to 8-day-old cultures of *S.*

TABLE 1. Growth of *Sclerotinia sclerotiorum* isolates and accumulation of oxalic acid in culture filtrates[a]

Isolate no.	Source of isolate	Final pH	Growth (mg dry wt/flask)[b]	Oxalic acid (mM)
Ss-1	N. Y., J. J. Natti, bean	7.4	145 ± 6	1.4 ± 0.4
0	Wis., L. Sequeira, lettuce	5.4	77 ± 8	5.9 ± 1.3
Ss-2	N. Y., J. J. Natti, bean	5.6	95 ± 5	6.6 ± 1.1
St-L1-81	Ky., L. Hensen, alfalfa	5.7	60 ± 5	6.9 ± 0.9
St-L38-27	Ky., L. Hensen, red clover	5.6	50 ± 4	6.9 ± 0.5
St-L1-69	Ky., L. Hensen, alfalfa	5.5	56 ± 34	13.8 ± 3.2
St-L38-70	Ky., L. Hensen, red clover	4.8	86 ± 9	16.5 ± 6.5
Ss-4	N. Y., J. J. Natti, bean	5.0	179 ± 4	21.8 ± 9.8
C-3	Wis., C. Newton, lettuce	5.1	95 ± 6	29.0 ± 1.9
Ss-1	Calif, J. G. Hancock, bean	4.6	118 ± 9	33.2 ± 2.3
Ss-9	N. C., R. D. Lumsden, bean	4.3	145 ± 22	43.4 ± 11.9
W	Fla., J. C. Walker, host unknown	4.8	168 ± 14	44.2 ± 9.4
F-58	Fla., L. R. Barker, celery	4.0	185 ± 9	60.0 ± 2.3
Ss-2	Calif., J. G. Hancock, host unknown	4.2	218 ± 15	65.2 ± 9.7
1	Wis., C. Newton, lettuce	3.9	106 ± 5	70.4 ± 1.1
Ss-3	Md., R. W. Goth, bean	3.5	195 ± 10	70.8 ± 4.1
SsM17	Pa., J. M. Skelly, crown vetch	3.7	163 ± 5	78.3 ± 8.1

[a] Each flask contained 25 ml of 73.7 mM D-glucose-56.0 mM sodium succinate culture medium, pH 6.0. The results represent the average of three replicates and their standard deviation.

[b] Dry wt of mycelial mats was determined after 6 or 7 days' growth at 26 C. Values were corrected for the wt of agar plug.

sclerotiorum grown on 0.1% yeast extract, basal salts, 110 mM D-glucose, and 2% agar, and one plug was transferred to each culture flask and incubated at 26 C.

Infected hypocotyls of *Phaseolus vulgaris* L. 'Top Crop' by *S. sclerotiorum* were obtained as described by Lumsden (12). Plants were rated by the following index: 0 = no symptoms; 1 = 1-2 cm of water-soaked hypocotyl; 2 = 2-4 cm of water-soaked hypocotyl; 3 = hypocotyl water-soaked to the level of cotyledon scars, and mycelium evident; 4 = same as 3 except hypocotyl covered with mycelium; and 5 = hypocotyl collapsed and dry. Polygalacturonase activity of infected bean tissues was estimated by the viscometric method (3). Activities were expressed in relative enzyme units calculated from 1,000/t, where t equals the time in min for 50% reduction in efflux time.

Oxalic acid content of culture filtrates and infected tissues was estimated by the $KMnO_4$ titration procedure employed by Bateman & Beer (3). D-glucose equivalents in the culture filtrates were estimated by the method of Nelson (15). For dry-wt determinations, fungal mats were collected on filter paper, washed, and dried to constant wt at 75-80 C.

RESULTS.—*Oxalic acid accumulation in culture.*—The accumulation of oxalic acid by *S. sclerotiorum* (isolate Ss-3) was influenced by the chemical composition of the culture medium (Table 2). When D-glucose was the carbon source and the concn of potassium phosphate was 5, 50, 100, and 200 mM, the oxalic acid concn in the culture filtrate after 7 days' growth was 0, 1.7, 9.9, and 17.8 mM, respectively. Dry wt ranged from 236 mg/flask on the 5 mM phosphate medium to 281 mg/flask on the 50 mM phosphate medium. Oxalic acid accumulation was 0 and 70.8 mM in a D-glucose-sodium succinate medium at initial pH values of 3.0 and 5.8, and the growth was 108 and 195 mg dry wt/flask, respectively. The pH decreased from 5.8 to 3.7 or lower. When yeast extract was omitted from the media containing D-glucose with various potassium phosphate concn, there was a considerable decrease (39%) in growth; but the oxalic acid concn was not appreciably altered. On the glucose-sodium succinate medium (pH 5.8) minus yeast extract, growth and oxalic acid concn were 62 mg dry wt/flask and 8.5 mM, respectively.

Time course of oxalic acid accumulation in culture.—Growth, change in pH, content of oxalic acid, and the decrease in D-glucose were determined over a 13-day incubation period for *S. sclerotiorum* (isolate Ss-3) grown on the glucose-sodium succinate culture medium (Fig. 1). The rate of oxalate accumulation in the culture filtrate was highest from day 2 to day

TABLE 2. Oxalic acid accumulation and growth of *Sclerotinia sclerotiorum* (isolate Ss-3) on various culture media[a]

Medium	Final pH	Growth (mg dry wt/flask)[b]	Oxalic acid (mM)
73.7 mM D-glucose + 56 mM sodium succinate	3.5	195 ± 10	70.8 ± 4.1
73.7 mM D-glucose + 56 mM sodium succinate[c]	3.0	108 ± 12	0
110 mM D-glucose + 5 mM potassium phosphate	2.2	236 ± 8	0
110 mM D-glucose + 50 mM potassium phosphate	3.1	281 ± 19	1.7 ± 0.7
110 mM D-glucose + 100 mM potassium phosphate	3.3	240 ± 14	9.9 ± 0.7
110 mM D-glucose + 200 mM potassium phosphate	3.7	250 ± 38	17.8 ± 1.8

[a] Each flask contained 25 ml basal medium with mineral salts at pH 5.8, 0.05% yeast extract, plus glucose and succinate or phosphate as indicated. The data represent the mean value from three replicate flasks and their standard deviation.

[b] Dry wt of mycelial mats was determined after 7 days' growth at 26 C, and values were corrected for wt of agar plug.

[c] Initial pH was 3.0.

5.5. This time period also corresponded to the rapid growth phase of the pathogen. By day 6, D-glucose was exhausted, oxalic acid accumulation ceased, and the growth rate began to decrease. From day 6 to day 13, there was essentially no change in oxalic acid concn, and the pH increased from 3.8 to 4.2.

Oxalic acid accumulation by isolates of S. sclerotiorum.—Glucose-sodium succinate medium (initial pH 6.0) was employed to evaluate the production of oxalic acid by 17 isolates of *S. sclerotiorum* (Table 1). After 6 or 7 days' incubation, growth of the isolates ranged from 50 to 218 mg dry wt/flask and oxalic acid concn ranged from 1.4 to 78.3 mM. The final pH of the culture medium was between 5.7 and 3.5 for all except one isolate. Because of differences in host range and growth rate of the isolates, it did not seem feasible to attempt to correlate virulence with oxalic acid production.

Oxalic acid content and polygalacturonase activity in diseased tissues.—Two days after inoculation of bean hypocotyls with *S. sclerotiorum* (isolate Ss-3), the plants had an average disease severity rating of 2 (Fig. 2-A). At this time, the oxalic acid accumulation had increased from 1.1 to 31.4 mg/g dry wt in diseased tissue, and the pH of tissue extracts had decreased from 6.1 to 4.1. The pH values of tissue extracts increased from 4.1 on day 3 to 5.7 on day 6. The oxalic acid concn reached a max of 48.3 mg/g dry wt on day 4 and then decreased to 39.7 mg/g dry wt by day 7.

Disease development and oxalic acid accumulation were delayed in plants inoculated with isolate Ss-9 of *S. sclerotiorum* as compared with beans inoculated with isolate Ss-3. The disease severity index was 1.5 on day 2, and only 3.5 mg/g dry wt of oxalic acid was detected (Fig. 2-B). By day 4 and day 7, oxalic acid concn reached 21.5 and 49.5 mg/g dry wt, and the disease severity index was 3.5 and 4.5, respectively. The pH values of diseased tissues on days 0, 2, and 6 were 6.1, 4.2, and 5.1, respectively.

In a separate experiment, polygalacturonase activity 1 day after inoculation of bean hypocotyls with either isolate Ss-3 or Ss-9 was 736 and 589 units/g dry wt, respectively. Negligible activity (less than 5 units/g dry wt) was associated with healthy tissues.

DISCUSSION.—In contrast to other reports (6, 16, 21), *S. sclerotiorum* grown in liquid culture accumulated large amounts of oxalic acid during the initial 6 days of the growth period. A probable explanation for this difference may be that the glucose-sodium succinate medium employed in this study had a high-buffering capacity, and the succinate was readily metabolized to oxalic acid. When the glucose medium lacking succinate was supplemented with increasing concn of potassium phosphate, there was an increase in oxalic acid accumulation. Similarly, Vega et al. (21) stated that as the buffering capacity of the standard salts medium was increased there was an increase in oxalate accumulation by *S. sclerotiorum*. *Sclerotium rolfsii* also accumulated high levels of oxalate only in media with a high-buffering capacity, and it was found that the pH of the culture medium had to be above 3.5 in order for *S. rolfsii* to accumulate large amounts

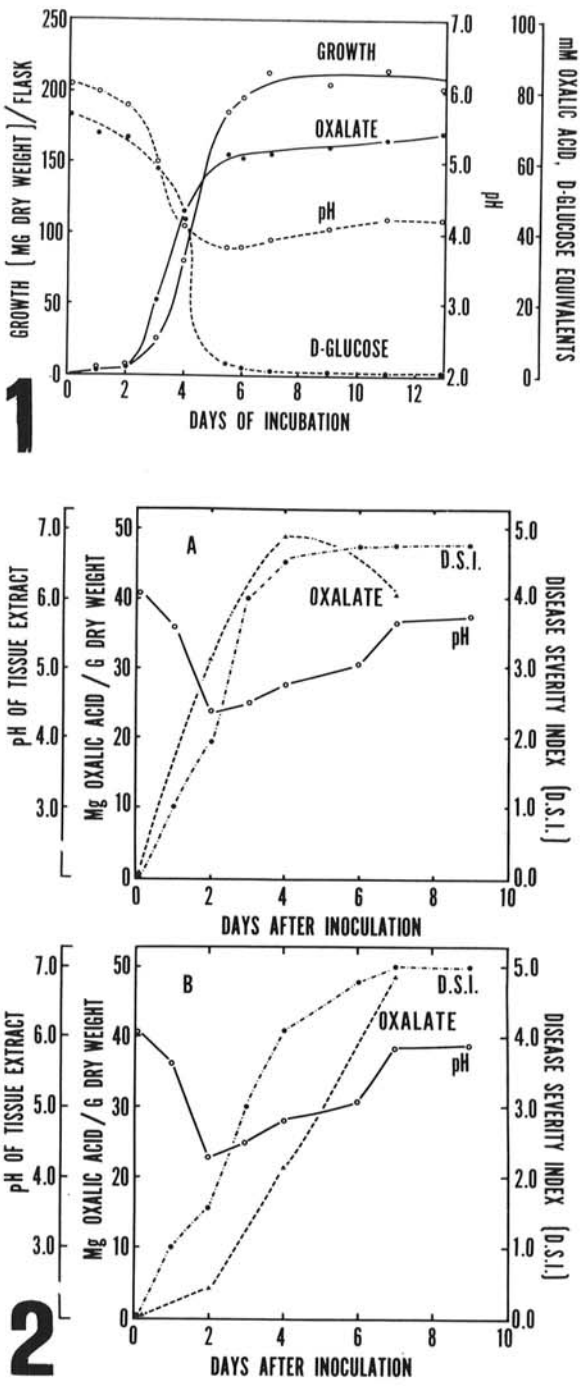

Fig. 1-2. **1)** Growth of *Sclerotinia sclerotiorum* (isolate Ss-3) on a 73.7 mM D-glucose-56.0 mM sodium succinate culture medium, accumulation of oxalic acid, pH of the culture filtrate, and utilization of D-glucose equivalents during a 13-day incubation period. **2)** Disease severity index (DSI), pH of hypocotyl extracts, and oxalic acid content in bean hypocotyl tissues infected with **A)** isolate Ss-3 of *S. sclerotiorum*; **B)** isolate Ss-9 of *S. sclerotiorum*. Disease severity index: 0 = healthy; 2 = 2-4 cm of hypocotyl water-soaked; 5 = entire hypocotyl collapsed and dry.

of oxalic acid (14). Likewise, *S. sclerotiorum* did not accumulate oxalic acid in the glucose-sodium succinate medium at initial pH 3.0. These data suggest that the pH of the growth medium is a primary factor in regulating oxalic acid accumulation in the medium.

Oxalic acid accumulation was not correlated with total growth of *S. sclerotiorum*. For example, growth on the nonbuffered glucose and the buffered glucose-sodium succinate media were equivalent, but oxalic acid accumulation was approximately 140 times greater in the latter than in the former (Table 2). Also, oxalic acid production occurred during the rapid growth phase of the fungus, at which time there was a decrease in the pH of the culture medium (Fig. 1).

In general, isolates of *S. sclerotiorum* which grew well on the glucose-sodium succinate medium also accumulated the largest amounts of oxalic acid (Table 1). But one isolate (Ss-1 from J. J. Natti) which grew well (145 mg dry wt/flask) accumulated only 1.4 mM oxalic acid.

The involvement of cell wall-degrading enzymes in pathogenesis by *S. sclerotiorum* was originally suggested by de Bary (5); others (4, 8, 9, 12) have reported a change in the composition of the cell walls of infected tissues. Bateman (2) suggested that endopolygalacturonase is one of the major enzymes responsible for maceration of plant tissues. Calcium ions greatly impeded the maceration of plant tissues by extracts from tissues infected with either *Rhizoctonia solani* (1) or *S. sclerotiorum* (8). Bateman & Beer (3) found that endopolygalacturonase would not hydrolyze a calcium-pectate complex except in the presence of oxalate. They suggested that oxalic acid enhances the activity of polygalacturonase by lowering the pH of the suscept tissues to a value closer to the optimum for the enzyme and by rendering the calcium-pectate complexes of the suscept cell walls more susceptible to hydrolysis by polygalacturonase.

Similar roles for the oxalic acid produced in *Sclerotinia*-infected tissues are suggested by the fact that oxalic acid accumulated early in pathogenesis; this was accompanied by a decrease in pH of the infected tissues to 4.5. This lowered pH would enhance the activities of hemicellulases (9), polygalacturonase (6, 8), cellulase (12), and phosphatidase (13), all of which have been implicated in diseases incited by *S. sclerotiorum*. There was also a positive correlation between disease severity index and oxalic acid accumulation in infected bean hypocotyls (Fig. 2). In the case of tissues infected by isolate Ss-9, disease development and rate of oxalic acid accumulation were slower than was the case for plants infected by isolate Ss-3.

The results from this study support previous suggestions (3, 5, 7, 11, 20) regarding a significant role for oxalic acid in disease development by pathogens which secrete large amounts of oxalic acid during the initial phase of pathogenesis.

Isolates of *S. sclerotiorum* from the same host are currently being investigated to determine if virulence is correlated with the production of oxalic acid and several extracellular enzymes which have been implicated in pathogenesis.

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