A Conductivity Assay for Measuring Virulence of Sclerotinia sclerotiorum

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ABSTRACT

Water-soaking of host tissues is generally associated with infection by *Sclerotinia sclerotiorum*. The early occurrence of this symptom in advance of the mycelium of certain isolates of the fungus suggested a possible relation of electrolyte loss to virulence. Liquid exuded from damaged tissues accumulated around mycelial inoculum placed on carrot root discs and this infection drop contained electrolytes. Conductivity increased rapidly within the first 10 min after infected discs were placed in distilled water, but the rate of increase declined thereafter. The initial change in conductance was apparently due to electrolytes in the infection drop; subsequent increases in conductance were due to leakage from affected tissues. Electrolyte loss increased as the incubation of infected carrot discs increased from 3 to 24 hr at 20 C; losses were greater from tissues incubated at 20 C than at 16 or 24 C. Five isolates of *S. sclerotiorum* were rated for virulence in terms of degree of colonization and symptom expression following inoculation of lettuce and bean seedlings, or of excised carrot, turnip, lettuce, and celery tissues. Virulence of most isolates was correlated with the rate and total amount of electrolyte loss from infected carrot discs incubated for 12 hr at 20 C. Total conductance after 10-min immersion in the bathing solution was 40 μmhos for the least virulent isolate vs. 95 μmhos for the most virulent; other isolates had conductance values between these extremes. The procedure appears to provide a reliable estimate of the virulence of different isolates of the fungus.

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An increase in membrane permeability is considered one of the earliest events in pathogenesis for many different plant pathogens (15). The extensive investigations of Thatcher (12, 13, 14) were the first to call attention to the broad spectrum of host-pathogen complexes in which permeability alterations were evident. His data indicated that early membrane permeability changes might have a role in the nutrition of the pathogen and in resistance of the host.

With regard to soft-rotting fungi, Thatcher (12, 13) demonstrated that increased permeability of host cells preceded the colonizing hyphae of Botrytis and Sclerotinia. More recently, Hancock (5) reported a significant increase in permeability in excised sunflower hypocotyl tissues infected by *Sclerotinia sclerotiorum* d By. This change in permeability of affected cells could result in increased leakage of materials which could be utilized by the fungus for growth. The ability of these pathogens to disrupt normal membrane function, therefore, may be closely related to virulence.

Sclerotinia sclerotiorum produces phosphatidase in culture (11) and in infected tissue (8). It also produces endopolygalacturonase (2, 3), hemicellulases (4), and cellulase (7) which cause cell wall modifications (1, 6). More importantly, oxalic acid is produced during early stages of pathogenesis (9) and Hancock (5) reported that the concentration of this acid in infected tissues was sufficient to kill host cells. These observations suggest that ability to induce membrane permeability changes could be related to virulence of *S. sclerotiorum* and that virulence might be correlated with the production by this pathogen of one or more cell membrane- and cell wall-modifying enzymes or toxic metabolites. The objectives of this investigation, therefore, were to develop and evaluate a method which could be used to determine the relation between virulence of *S. sclerotiorum* isolates and water-soaking (membrane alteration) of infected suscept tissues.

MATERIALS AND METHODS.—*Source and maintenance of cultures.*—*Sclerotinia sclerotiorum* isolates were obtained from infected lettuce tissues collected in Wisconsin and Florida. Stock cultures were maintained on potato-dextrose agar (PDA) slants at 4 C. The cultural characteristics and the influence of temperature on linear growth rate were determined for five isolates on PDA plates. One-cm plugs, removed from the advancing mycelial front of each isolate, were transferred to each plate. After initiation of growth (1 day) at 22±2 C, plates with uniform growth were selected for incubation at 4, 8, 16, 20, 24, 28, and 32 C. There were three plates for isolates C-3 and F-48, but only one plate for each of the remaining isolates, at each temperature. Preliminary experiments had shown that the variation in linear growth between replicates was within 10% (e.g., ranges 18-20 mm at 26 hr and 55-57 mm at 76 hr for five replicates of F-48 at 22±2 C). The mycelial fronts were marked at the start of incubation and at 12- to 24-hr intervals thereafter until the mycelium reached the edge of the plate. The linear growth rate for each interval was then calculated.

Inoculation procedures.—The virulence of isolates C-3, 1s, 1, F-48, and F-49 to *Lactuca sativa* L. 'Great Lakes' and *Phaseolus vulgaris* L. 'Bountiful' was determined in the greenhouse by stem-inoculating 8- to 12-cm tall plants. The inoculum (a 1.0-cm plug from the advancing margin of a PDA culture or an oat kernel infested with the isolate) was placed next to the stem. The inoculated plants were incubated in a mist chamber or under a plastic tent at 16-24 C for 7-14 days.

Bean pod, carrot taproot, celery petiole, and lettuce leaf tissues were inoculated with PDA plugs prepared as above. Both cut and uncut surfaces were inoculated. The tissues were incubated in moist chambers at 22 C. Virulence of the five isolates was visually ranked after 3-10 days. Ranking was based on the extent of colonization and tissue damage.

Electrolyte loss measurements.—Electrolyte loss from infected tissues was determined by the increase in conductance (electrolytes) of the bathing medium. Conductivity was measured with a Model RC 16-2B conductivity bridge (Industrial Instruments, Inc., Cedar Grove, N. J.) equipped with a dip-type conductivity electrode of medium range (5-5,000 μmhos, cell constant = 1.0). The conductivity of a standard solution of NaCl or KCl was linear from 5×10^{-5} to 1×10^{-2} M (8-1,200 μmhos) with this method.

The relationship between virulence and electrolyte loss was assayed as follows: Carrot root discs (5-mm thick; 10-mm diam) were randomized and placed on moist filter paper in a 15-cm diam petri plate, and a 6-mm inoculum plug (from the advancing mycelial front of a PDA culture) was placed with the mycelial side down on top of the disc. Three to five replicates, each consisting of two discs per isolate, and a control disc (incubated with a sterile PDA plug) were included in each plate. Immediately following inoculation, each plate was covered with a plastic film and incubated at 20±1 C for the designated time. After incubation, two discs were placed in 10 ml of glass-distilled water (conductance 3-5 μmhos). After thorough stirring, the conductance of the bathing solution was measured at 10-min intervals for 40-60 min. The effects of isolate, incubation time, and incubation temperature on electrolyte loss were determined.

RESULTS.—*Cultural characteristics of isolates of S. sclerotiorum.*—Only minor cultural differences in mycelial characteristics (dense to sparse, appressed to aerial) and sclerotial size, number, and distribution were noted among the five isolates of *S. sclerotiorum*. The average linear growth rates at 22 C (in mm/hr) of the isolates were as follows: C-3 (1.30), 1s (1.24), F-48 (1.23), 1 (0.95), and F-49 (0.50). It is evident that there were marked differences in growth rate at 22 C; similar relative growth differences also occurred between isolates at the other temperatures tested.

The minimum, optimum, and maximum temperatures for growth of *S. sclerotiorum* isolates in culture were 4, 20-24, and 32-36 C, respectively. Growth, although very slow at 4 and 32 C, quickly resumed when plates previously incubated at these temperatures were placed at 22 C. No growth occurred at 36 C and there was no resumption of growth at 22 C of cultures previously held at 36 C for 24 hr.

Determination of virulence.—Environment and inoculation procedures greatly influenced disease development. High humidity and soil moisture near field capacity particularly favored the incidence of disease. Variations in soil and air temperatures in the range 16-24 C did not appreciably alter disease initiation or development, however. Infested oat-kernel inoculum resulted in a higher percentage of infected plants than did PDA inoculum. The incidence of infection was highest when the inoculum was placed in contact with or within 2 mm of the stem of the suscept.

The results of several inoculation experiments with lettuce and bean plants showed that isolate C-3 was the most virulent, followed by isolate 1s (Table 1). Isolate C-3 rapidly girdled the stems and killed the plants. F-49, which caused small necrotic lesions, but did not kill the plants, was the least virulent. Isolates 1 and F-48 colonized the stems and caused water-soaked lesions of varying size, but did not kill the plants. On the basis of cumulative data from several inoculation experiments, the isolates were ranked for virulence as follows: C-3 > 1s > 1 > F-48 > F-49.

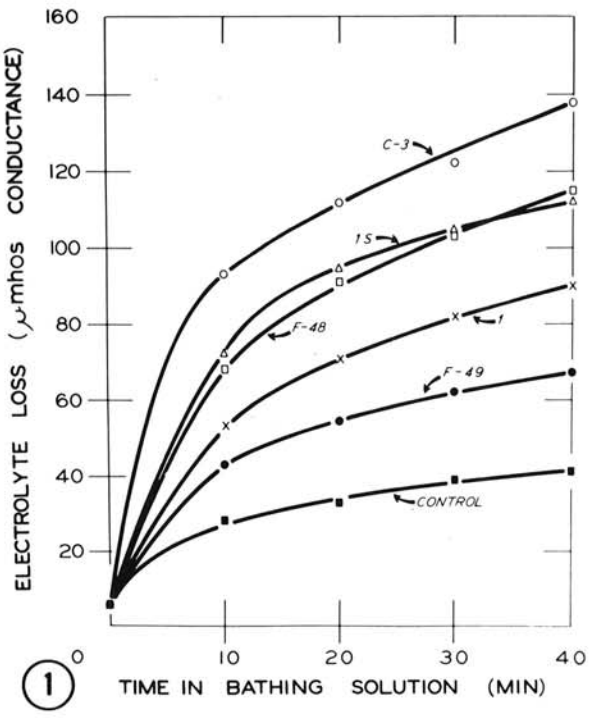
Inoculation experiments with excised tissues gave similar results. Carrot, turnip, lettuce, and celery tissues were all colonized and rotted within 3-10 days by isolate C-3. F-49 usually initiated rot, but then ceased development and did not cause extensive rot in most cases. Based on rate of colonization and degree of tissue destruction of excised plant parts, the relative virulence of the isolates was also judged to be C-3 > 1s > 1 > F-48 > F-49.

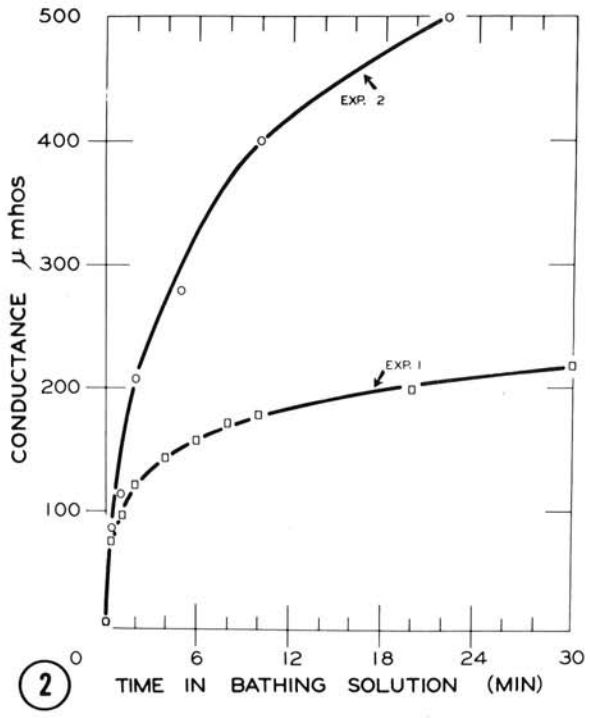
Relationship of virulence to electrolyte loss.—Water-soaking of the host tissue was always associated with *S. sclerotiorum* infection and the early occurrence of this symptom in advance of mycelium of virulent isolates suggested that it might be related to virulence. Liquid from damaged tissues

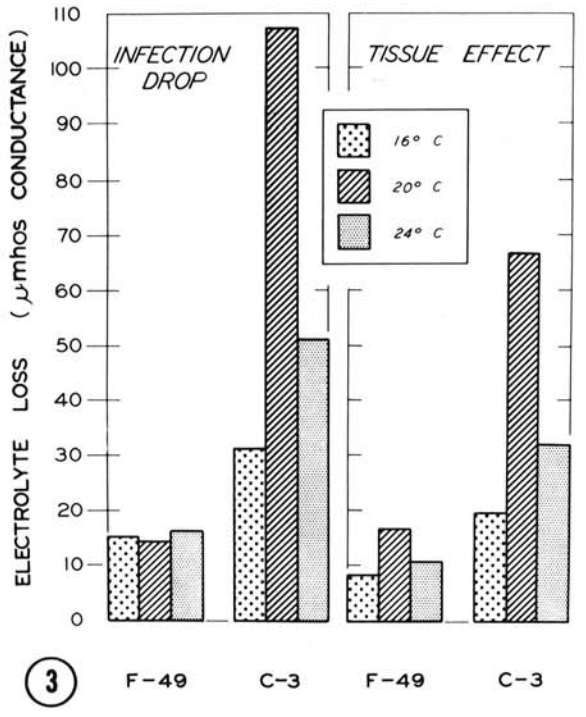
TABLE 1. Virulence of five isolates of *Sclerotinia sclerotiorum* to lettuce

Isolate designation	Plants surviving after 4 days	Symptoms on survivors	Virulence ranking[b]
C-3	0/8[a]	All stems girdled	5
1s	4/8	Large stem lesions	4
1	8/8	Small stem lesions	3
F-48	8/8	Small stem lesions	2
F-49	8/8	Small, arrested stem lesions	1

[a] (Number of plants surviving)/(number of plants inoculated).
[b] Relative ranking based on plant death and lesion size. 1 = least virulent; 5 = most virulent.







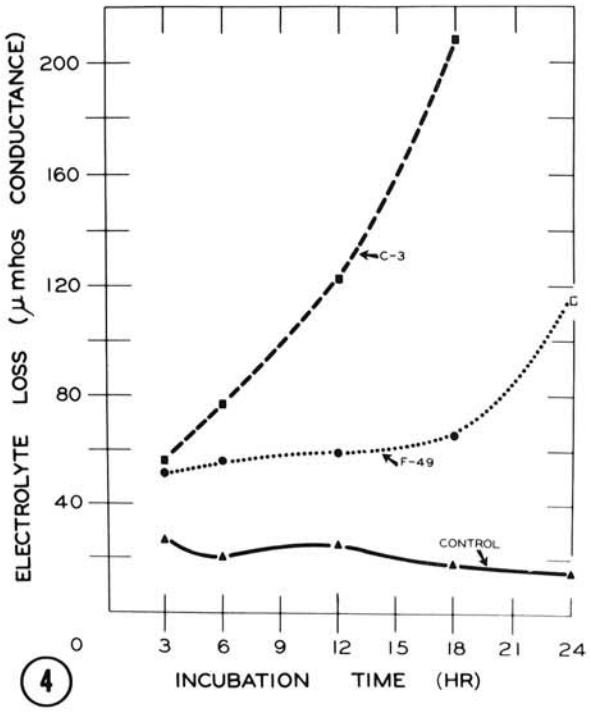


Fig. 1-4. 1) Electrolyte leakage from carrot discs induced by five isolates of *Sclerotinia sclerotiorum* after 12 hr incubation at 20 C. Each point represents the average of three replicates. Initial conductance is that of bathing solution prior to addition of discs. Control = carrot discs with sterile PDA. **2)** Change in conductance of the solution bathing carrot discs which had been incubated 12 hr at 20 C with *Sclerotinia sclerotiorum* isolate C-3. For Experiment 2, carrots had been stored 5 months longer than for Experiment 1. **3)** Effect of incubation temperature on electrolyte content of the infection drop and on the rate of tissue leakage from carrot discs incubated for 12 hr with *Sclerotinia sclerotiorum* isolates C-3 and F-49. Values represent the average of three replicates corrected for electrolyte leakage from control discs. **4)** Influence of incubation time on electrolyte loss from carrot discs inoculated with *Sclerotinia sclerotiorum* isolates C-3 and F-49. Readings were taken after 30 min in bathing solution. Each point represents the average of three replicates.

accumulated around the PDA culture plugs within 12-40 hr after placing them on carrot root cross sections. The amount of accumulated liquid appeared correlated with the extent of tissue damage, hence with virulence of each isolate. The observation that the infection drop contained electrolytes prompted the use of a conductivity assay to obtain data that would express on a quantitative basis the relationship of electrolyte loss to virulence.

Since noninoculated carrot discs caused a smaller increase in conductivity of the bathing solution than did turnip root, potato tuber, cucumber fruit, or celery root discs, the former were used as the test tissue. The effect of inoculation with the five isolates on electrolyte loss was positively correlated with their virulence ranking (Fig. 1, Table 1) except for isolates 1 and F-48 which reversed positions. Although the relative ranking of the isolates in terms of electrolyte loss was constant from experiment to experiment, differences in total conductance between isolates and in rates of increase in conductance fluctuated depending on age and condition of test tissues.

To determine whether the rapid initial increase in conductance was primarily due to electrolytes present in the infection drop, conductance measurements were made at 1- to 2-min intervals during the first 10 min for C-3-infected tissue (Fig. 2). In two experiments, 50 and 40% of the total conductance change measured occurred within the first 2 min. The conductance of the bathing medium was increased by a similar amount if the infection drop liquid was added alone. The first increment of change in conductance, therefore, was referred to as the infection drop effect; all subsequent leaching was referred to as the tissue leakage effect.

The effect of incubation temperature on electrolyte loss from tissues was analyzed with respect to infection drop and tissue leakage components for isolates C-3 and F-49, the most virulent and the least virulent isolate, respectively (Fig. 3). It is apparent that 20 C is close to the optimum temperature for maximum electrolyte loss with isolate C-3 and that the bulk of the electrolytes were present in the infection drop. The differences observed for isolate F-49 at 16, 20, and 24 C were not considered significant.

Electrolyte loss increased as the incubation period increased (Fig. 4). Isolates C-3 and F-49, which caused the highest and lowest electrolyte losses, respectively, had been previously rated (Table 1) as the highest and lowest in virulence.

DISCUSSION.–The major aim of these studies was to determine the possible relationship between virulence in *S. sclerotiorum* and the release of electrolytes from infected tissues, which presumably results from alteration of host cell permeability. This relationship was suggested by initial experiments in which the amount of water-soaking in infected carrot discs was positively correlated with the virulence ranking of several isolates of the pathogen. The release of electrolytes from carrot discs inoculated with *S. sclerotiorum* provided a simple method to quantitate the degree of virulence of each isolate.

The initial release of electrolytes from infected carrot tissues into the bathing solution probably represents host and pathogen materials, such as oxalic acid (9), which accumulated in the infection drop during incubation. The release of electrolytes to the bathing solution during the first 10 min accounted for 60-75% of the total release obtained in 40 min. It was in this initial time interval that major differences in the induction of electrolyte release among the isolates were apparent. In general, the more virulent isolates induced greater electrolyte loss from tissues, or released more electrolytes themselves, than the less virulent ones. Between 10-40 min, however, conductance of the bathing solution increased at similar rates for all isolates. Due to the small size of the carrot discs in relation to the inoculum plug, this possibly represents a constant leakage rate from equivalent cross-sectional areas of infected and noninfected tissues.

Permeability changes induced in host cells by *S. sclerotiorum* and measured in terms of electrolyte leakage appear to result from the action of several cell wall- and cell membrane-modifying enzymes and oxalic acid in concert with other, as yet unknown, factors (3, 5, 8, 10). Our evidence indicates that a combination of enzymes produced by the fungus is more effective than are individual enzymes in causing electrolyte leakage (10), but it is clear that additional products of the pathogen must be involved. Elucidation of the cause of electrolyte leakage, an important phenomenon in the initial stages of pathogenesis, would be essential in establishing the nature of the factors involved in virulence of the pathogen.

Although virulence of a pathogen such as *S. sclerotiorum* can be determined visually in terms of degree of colonization of plant tissues or symptom expression, it is difficult to measure these parameters quantitatively. Electrolyte loss, as determined by the techniques described here, provides a simple, convenient parameter which can be used to predict the virulence of a given isolate. Additional tests with several isolates of *S. sclerotiorum*, other than those included in this report, indicate that the procedure is highly reliable. However, there are differences in the permeability changes induced in excised tissues as opposed to attached plant parts and results should be interpreted with caution (5).

In our experience, electrolyte loss is the only parameter that is positively correlated with degree of colonization of host tissues. Growth rate in culture media, for instance, is not a reliable measure of virulence. Mycelial growth of isolates C-3 and F-48 on PDA was similar, but they differed greatly in virulence. There is an obvious difference, therefore, between growth in culture and ability to colonize host tissue. This difference may reside in the production of metabolites that affect cell membrane integrity and result in release of electrolytes.

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