Ascochyta chrysanthemi Toxin: Production and Properties

D. L. Schadler and D. F. Bateman

National Science Foundation Graduate Trainee, and Professor and Chairman, respectively, Department of Plant Pathology, Cornell University, Ithaca, New York 14850.

Based on portions of a M.S. thesis by the senior author.

Accepted for publication 17 December 1973.

ABSTRACT

Filtrates from 14-day-old cultures of *Ascochyta chrysanthemi* contained a toxin capable of inducing a complex of foliar symptoms in chrysanthemum similar to those that occur on plants that are stem-inoculated with the pathogen. *A. chrysanthemi* toxin sufficient to produce typical foliar symptoms on chrysanthemum accumulated within 6 days in liquid culture; dilution endpoints of filtrates of 14-day-old cultures for symptom induction on chrysanthemum were between 1:100 and 1:1,000. The toxin, in culture filtrates or partially purified preparations, was stable in storage at 4 C for 3 yr. *A. chrysanthemi* toxin was destroyed by boiling and by charring, but not by autoclaving at 121 C unless autoclaved in strong acid or strong base; it was not extracted from culture filtrates by organic solvents but was absorbed by activated charcoal and was retained by DEAE cellulose at pH 8.0. This toxin was not precipitated in 66% methanol; it was dialyzable but was retarded in Sephadex gels with exclusion limits above 1,000 molecular weight.

Phytopathology 64:779-784.

Additional key words: chrysanthemum ray blight, *Mycosphaerella ligulicola*.

Florists' chrysanthemum [*Chrysanthemum morifolium* (Ramat.) Hemsl.] naturally infected or stem-inoculated with *Ascochyta chrysanthemi* Stevens *(Mycosphaerella ligulicola* Baker, Dimock and Davis) displays a foliar symptom complex consisting of leaf spotting, wilting, and necrosis of tissue distant from the infected area. Culture filtrates of *A. chrysanthemi* when injected into stems of healthy chrysanthemum plants induced a symptom complex essentially identical to that caused by the fungus (7). The pattern of symptom development induced by the pathogen or its culture filtrate indicates that a translocatable toxin may be involved in this disease.

Antibiotics and/or phytotoxins have been isolated from several *Ascochyta* form-species. Oku and Nakanishi (8, 9) have crystalized the antibiotic ascochitine from mycelium and culture filtrates of *Ascochyta fabae*; Bertini (3) has crystalized this antibiotic from culture filtrates of *Ascochyta pisi*. Susuki, et al. (13) isolated ascotoxin from dried mycelium of *Ascochyta imperfecta*; this compound is identical with decumbin, which has been purified from *Penicillium decumbens* by Singleton, et al. (12). Tamura, et al. (14) have isolated ascochlorin from mycelium and culture filtrates of *Ascochyta viciae* and Sasaki, et al. (11) have isolated ascofuranone from these same sources. All of the above compounds are products of secondary metabolism, and are soluble in various organic solvents. Although the organisms producing them are all plant pathogens, only ascochitine (8, 9) has been implicated as having a role in disease development.

The objectives of this study were to investigate the toxic nature of culture filtrates of *A. chrysanthemi* and to attempt purification and characterization of the toxic factor(s) that induce a characteristic foliar symptom complex in chrysanthemum.

MATERIALS AND METHODS.—*A. chrysanthemi* isolates LI-1, Sakae, and N_a were used throughout this study. Isolates LI-1 and Sakae do not sporulate readily in culture (6), and where conidia were needed, isolate N_a, which produces abundant pycnidia in culture, was used. All isolates were maintained in pure culture on potato-dextrose agar (PDA) (Difco Laboratories). PDA supplemented with KNO_3 (10 mg/ml) was used for production of conidia (pycnidiospores). Conidia were harvested by flooding the culture with 10 to 15 ml of water, and pouring or pipetting off the spore suspension.

A modification of Joham's basal salts medium as reported by Watkins (15) (Watkins' medium) was used for production of culture filtrates. The medium was adjusted to pH 6 with 1 N HCl and sterilized by autoclaving for 20 min at 121 C; the pH of the medium after autoclaving was 4.0 - 4.5.

Isolate LI-1 was used for production of culture filtrates. Six 5-mm diam plugs of mycelium taken from the edge of a young colony growing on PDA were introduced into 0.5 liter of Watkins' medium in 3-liter Florence flasks. The cultures were incubated for 14 days at 20 C in the dark. Periodically, early in the incubation period, the cultures were gently swirled to distribute the growing mycelium throughout the medium. Abundant mycelium was produced during the culture period.

The culture liquid was filtered twice through two layers of cheesecloth, once through a single disk of Whatman #2 filter paper, and once through two disks of Whatman #2 filter paper. These filtrations removed the mycelial fragments and most of the polysaccharide gum from the filtrate. Filtrates were then passed through a Gelman Metricel filter (Gelman Instrument Co., 0.20-μ pore size) and stored at 4 C until used.

Young chrysanthemum (*C. morifolium* 'Iceberg') plants were grown in Cornell mix (10) in 12.7-cm (5-inch) diam pots in a greenhouse at ca. 24 C to a height of 30 to 45 cm before they were used in experiments. Plants, after use in one experiment, were cut back to the third or fourth node and an axillary bud was permitted to grow into a shoot that was used in a second experiment. Such "second growth" shoots always appeared healthy and yielded results in experiments identical to "first growth" shoots. Pathogenicity of the fungal isolates used was checked periodically.

For routine determination of toxin activity, a "petiole method" of bioassay was developed. This involved immersing a leaf on an intact plant in water, and cutting away the leaf blade with a scalpel, leaving only a tongue of

tissue (leaf-tongue) containing the midvein. Best results were obtained with a leaf-tongue 1.0 and 1.5 cm wide and 7.0 to 8.0 cm long (Fig. 1-B). Two ml of test solution was placed in a 12-ml plastic vial (Kerr Glass Manufacturing Corp., Packaging Products Division) with a wedge-shaped section cut from its cap (Fig. 1-A). The leaf-tongue was held in place by snapping the cap onto the vial in such a way that a cotton ball held in the cut in the cap was snug against the leaf-tongue (Fig. 1-C). The vials were removed after 2 days at which time a first evaluation of symptom development was made on leaves above and below the point of test solution introduction. Two days later, a final evaluation of symptom development was made. Vials were put in place between 8:00 and 10:00 a.m.; symptoms developed most rapidly under conditions favoring rapid transpiration (i.e., bright sunlight, high ambient temp, and circulating air). In some cases, plants would take up the full 2-ml volume of test solution; tests showed that little or no test solution was lost by evaporation to the atmosphere. The pH of nonbuffered toxic test solution, in the range from pH 3 to pH 9, had no significant effect on the assay. Only *A. chrysanthemi* filtrates or preparations of *A. chrysanthemi* toxin induced characteristic foliar symptoms in the chrysanthemum bioassay, and "escapes" (toxin solutions not producing symptoms) were extremely rare.

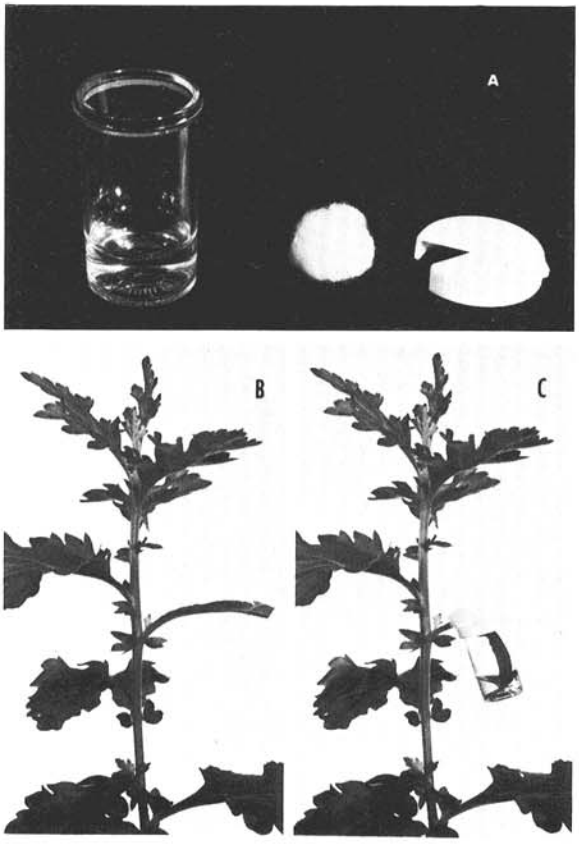

Fig. 1. The petiole method for the bioassay of *Ascochyta chrysanthemi* toxin. **A)** Twelve-ml plastic vial containing test solution. Cotton ball fits into the wedge-shaped section of the cap. **B)** Chrysanthemum plant prior to toxin treatment showing leaf tongue. **C)** Vial in place on the chrysanthemum plant with tip of the leaf tongue immersed in the test solution.

Leaves on treated plants were evaluated by the following rating system: 0 = no apparent symptoms; 1 = spots and/or veinal necrosis on 25% or less of the leaf surface; 2 = spots and/or veinal necrosis on 25-75% of the leaf surface, but no marginal or interveinal necrosis; 3 = spots and/or veinal necrosis on 75% or more of the leaf surface, with limited marginal and interveinal necrosis; 4 = spots and/or veinal necrosis on the entire leaf surface, with extensive marginal and interveinal necrosis, and sometimes wilting of the leaf. Symptom rating for a given plant was based on the leaf showing the most severe symptoms on that plant. Plants with one leaf with a rating of 3 or 4 usually had many other leaves with this rating, but plants with leaves with a rating of 1 or 2 often had only a few leaves showing symptoms.

For tests of toxin activity on flower petals, droplets of the test solution were applied to tips of petals on intact cut chrysanthemum flowers. Wounding of petal tissue with a needle through the droplet gave more consistent results and more rapid symptom development than when the petal was not wounded. Flowers were sometimes covered with clear polyethylene bags to maintain high humidity, but this was not essential for symptom development; symptoms developed on flowers held at either 9 or 21 C.

All experiments were repeated at least once, some as many as seven times, with one or more replicates of each treatment in each experiment.

RESULTS.—*Foliar symptoms.*—The symptoms that developed on chrysanthemum plants when 14-day-old culture filtrates of *A. chrysanthemi* were introduced by the petiole method were strikingly similar to the symptoms which appeared on plants following stem inoculation with the pathogen (Fig. 2). The most characteristic symptom of both inoculated and culture filtrate-treated plants was the development of circular to elliptical leaf spots ranging in size from 0.1 to 1 mm in diam on leaves above and below the point of inoculation or culture filtrate introduction. Generally, each spot had a small necrotic veinlet at its center. Spots were randomly distributed over the leaves, but often they were found only on portions of the leaves collateral with the inoculation point or introduction petiole. When the lower surface of the leaf was viewed by reflected light, small raised areas associated with each spot were observed. Once developed, the spots were permanent. Extensive veinlet necrosis developed on some leaves irrespective of spot development. Extensive veinlet necrosis developed simultaneously with wilting of the leaf. An assymetric necrosis of leaf margins sometimes developed, especially if large amounts of fungal mycelium were used for stem inoculation, or if high-titer toxin solutions were introduced through a petiole. New terminal growth developing after initial symptom development in toxin-treated plants was free of symptoms, but leaves in the bud stage at the time of symptom development on foliage were often subsequently malformed.

Eosin (eosin yellowish; Allied Chemical Co.) (0.05% in 0.05 M Tris-HCl buffer, pH 8.0) when introduced into plants by the petiole method, showed a distribution pattern in the veinlets of leaves similar to that of symptom

development induced by *A. chrysanthemi* toxin.

Treatment of chrysanthemum plants with *A. chrysanthemi* toxin did not result in the development of extensive leaf and stem decay as often observed in diseased plants in the field (2); only the leaf-spotting complex as described above was observed.

Necrotic tissue and a small callus generally developed on stem-inoculated plants near the point of inoculation. Neither necrotic tissue nor callus developed around petioles through which toxin or culture filtrates had been introduced. *A. chrysanthemi* was isolated from stem-inoculated plants only from the site of inoculation and from tissues immediately surrounding it. The pathogen was not isolated from any other part of the plant, even though severe symptoms may have developed some distance from the point of inoculation.

Lyophilized culture filtrates containing toxin induced discoloration, red streaks, and necrosis of treated flower petals similar to those occurring on naturally infected plants (1). Neither unseeded culture medium nor culture filtrate of *A. pisi* Libert. caused development of any discoloration or necrosis of chrysanthemum flower tissue. Fractions from DEAE-cellulose anion exchange chromatography of culture filtrates, which showed leaf spotting activity, were pooled, lyophilized and dissolved in a thick sucrose solution. Within 36 h after application of this solution to petals, extensive red streaking had developed. Petals treated with sucrose solution alone did not develop symptoms. Treatment of flower petals with *A. chrysanthemi* toxin did not result in the extensive destruction of the flower as observed in field infections by *A. chrysanthemi* (1).

Conidia of *A. chrysanthemi* isolate N_a suspended in water and atomized onto flowers kept at room temp and high relative humidities produced typical symptoms, consisting of necrosis and red streaks on petals within 24 h. Microscopic examination of fresh mounts of petals stained with methylene blue revealed that by the time extensive symptoms had developed, the spores of *A. chrysanthemi* had germinated and produced appressoria, but had not penetrated the host.

Production and accumulation of A. chrysanthemi toxin in culture filtrates.—Watkins' medium supported adequate growth of *A. chrysanthemi* and accumulation of toxin at levels deemed adequate for experimentation. Yeast extract proved essential for fungal growth. The pH of filtrates of 14-day-old cultures was 3.0 - 4.0. All three *A. chrysanthemi* isolates tested produced toxin and were virulent when tested by stem inoculation; isolate LI-1 was used for routine production of culture filtrates. Most culture filtrates had dilution endpoints for foliar symptom induction between 1:100 and 1:1,000, although dilution endpoints between 1:1,000 and 1:10,000 were sometimes observed.

The time-course of toxin accumulation was determined in 100-ml aliquots of Watkins' medium dispensed into 500-ml Erlenmeyer flasks. Each flask was seeded with four 5-mm plugs of mycelium taken from the edge of an expanding colony of *A. chrysanthemi* growing on PDA. Cultures were incubated without agitation at 20 C in the dark. Culture filtrates were harvested daily and subsequently assayed for toxin by the petiole method. Sufficient toxin to produce typical leaf symptoms had accumulated by day 6. Toxin levels reached an apparent plateau by day 10 or 11. Mycelial dry weight of the organism increased throughout the course of the experiment.

Watkins' medium was not toxic to chrysanthemum. Various treatments of the medium (concn, aging, lyophilization, etc.) did not render it toxic to chrysanthemum as determined with the petiole method of assay. Unseeded culture filtrate was routinely used as a control treatment.

Properties of A. chrysanthemi toxin.—Sterile culture filtrates of *A. chrysanthemi* stored at 4 C for 3 yr showed no loss in leaf-spotting ability when tested on

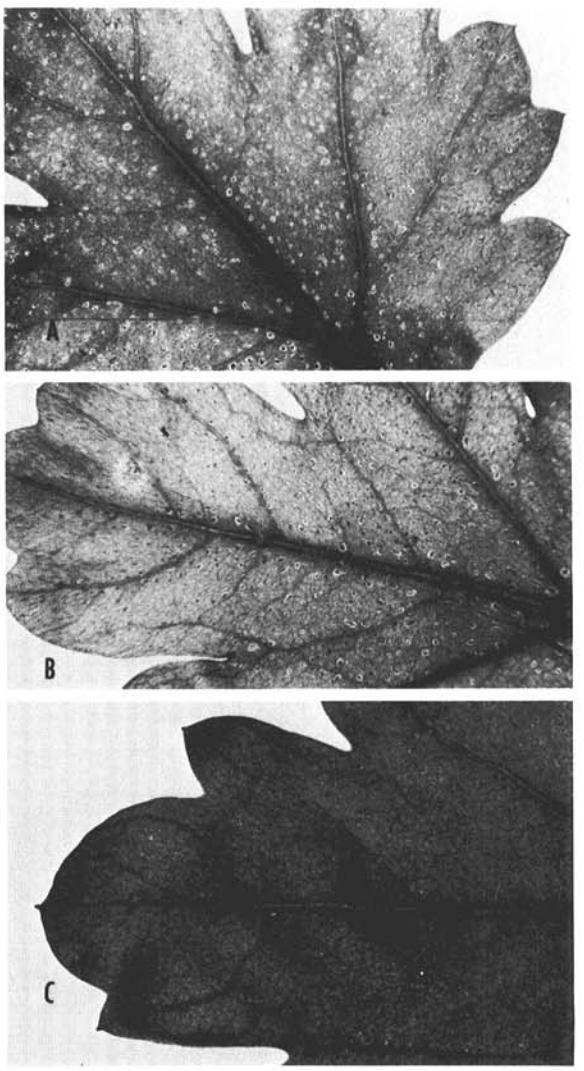

Fig. 2. Foliar symptoms induced by *Ascochyta chrysanthemi.* **A)** Leaf from a stem-inoculated plant; **B)** Leaf from a toxin-treated (petiole method) plant; **C)** Healthy leaf. Note the small necrotic veinlets at the centers of most spots and the necrosis of some veinlets near the leaf margin in A. All photographs were taken with backlighting.

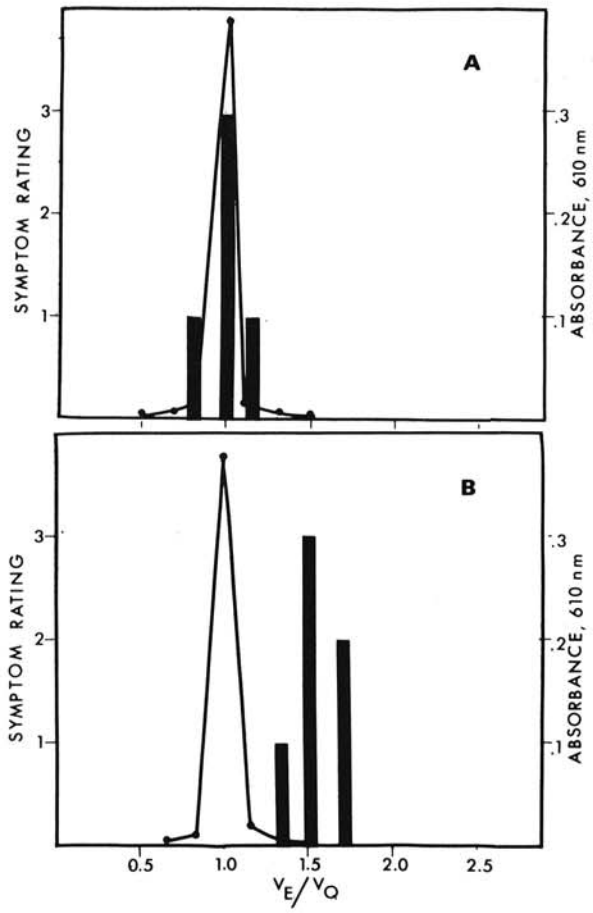

Fig. 3. Elution patterns of toxin in culture filtrates of *Ascochyta chrysanthemi* from columns of Sephadex gels in the presence of 0.1 M sodium phosphate buffer (pH 7.0) and 0.1 M NaCl. **A)** Sephadex G-10; **B)** Sephadex G-75. The petiole method bioassay was used to detect the presence of toxin. On Sephadex G-25 and Sephadex G-50, the V_E/V_O ratio for the toxin was 1.20 and 1.35, respectively. Solid line indicates absorbance at 610 nm of the void volume marker; bars indicate symptom ratings.

TABLE 1. Stability of *Ascochyta chrysanthemi* toxin to acid and base[a]

Initial pH	Time of autoclaving, min			
	0	20	40	60
1	+/+[b]	+/−	−/−	−/−
3	+/+	+/+	+/+	+/+
7	+/+	+/+	+/+	+/+
10	+/+	−/−	−/−	−/−
13	+/+	−/−	−/−	−/−

[a]Filtrate from 14-day-old cultures of *A. chrysanthemi* was adjusted to pH 1, 3, 7, 10, or 13 with 1 N HCl or 1 N NaOH, autoclaved, and bioassayed. Two replicates of this experiment gave results identical to those recorded here. Unseeded medium did not contain toxin after any of the above treatments.

[b](+/+) indicates that toxin was detectable in both the full-strength sample and in 1:2 dilution; (+/−) indicates that toxin was detectable in the full-strength sample but absent in a 1:2 dilution; (−/−) indicates that toxin was not detectable in either the full-strength sample or a 1:2 dilution.

chrysanthemum. Culture filtrates adjusted to pH values between 1 and 13 and stored at 4 C showed no loss in toxin activity when stored for 18 mo. The toxin was stable to lyophilization in culture filtrates and in active fractions from anion exchange and molecular sieve gel chromatography.

Experiments were carried out to determine the stability of *A. chrysanthemi* toxin to acid and base. The pH of culture filtrates was adjusted to pH 1, 3, 7, 10, or 13 with 1 N HCl or 1 N NaOH; aliquots at each pH were autoclaved at 121 C, in sealed tubes for 0, 20, 40, or 60 min in continuous runs. After cooling, the tubes were opened and the pH of the sample adjusted to pH 5.0 and bioassayed on chrysanthemum by the petiole method. Undiluted samples and samples diluted 1:2 with distilled water were tested (Table 1). The toxin appeared to be completely stable to autoclaving at pH 7. Toxin activity was lost on autoclaving for 40 min at pH 1, or on autoclaving at pH 10 or 13 for any of the time periods tested.

A. chrysanthemi toxin was dialyzable and could be recovered from the water into which it had dialyzed; it also passed through an Amicon UM-2 ultra-filter (Amicon Corp.; general retentivity greater than 1,000 mol wt). The toxin was absorbed from culture filtrates by activated charcoal (Norit A; Arthur H. Thomas Co.). Supernatants of culture filtrates treated with 5 mg/ml of activated charcoal retained a weak toxin activity; supernatants of culture filtrates treated with 10 mg/ml were not toxic to chrysanthemum. Toxin was desorbed from activated charcoal by solutions of acetone or ethanol. Toxin in culture filtrates was progressively inactivated by boiling; however, it was not inactivated in culture filtrates autoclaved (121 C) for 20 min. Toxin in lyophilized culture filtrates was destroyed by ashing (850 C for 8 h) in a muffle furnace.

To determine the solubility of the toxin in organic solvents, culture filtrates were brought to room temp, and adjusted to the desired pH with 1 N HCl or 1 N NaOH. Fifty ml of the culture filtrate was placed in a 250-ml separatory funnel, and shaken with 15 ml of water-immiscible solvent. The organic solvent phase was drawn off, dehydrated over anhydrous Na_2SO_4, and evaporated in an air stream. The residue remaining after evaporation was taken up in 0.2 ml of acetone, and diluted to 2.0 - 2.5 ml with distilled water. This solution was allowed to stand at 4 C for 18 h and then was assayed for toxin activity by the petiole method. Extracted culture filtrates (aqueous phase) were concd in vacuo for 15-30 min to remove any residual solvent. Filtrates were then adjusted to pH 5 with 1 N HCl or 1 N NaOH, and assayed for toxin by the petiole method. Nine organic solvents (*n*-hexane, benzene, toluene, diethyl ether, chloroform, ethyl acetate, 1,2-dichloroethane, 2,3-dimethyl-4-heptanone, and *n*-butanol) with dielectric constants ranging from 1.9 to 17.8 were used in attempts to extract the toxin from culture filtrates at pH's of 1, 3, 7, 10, and 13. The toxin was not extracted by any of the solvents at any pH; the toxin always remained in the aqueous phase. Attempts were made to extract the toxin from lyophilized culture filtrates with water-miscible organic solvents. The solubility of *A. chrysanthemi* toxin in the three solvents tested (ethanol, methanol, and acetone) was negligible.

The toxin did not precipitate when absolute methanol was added to tenfold concd culture filtrate to give a 66% methanol solution.

Diethylaminoethyl (DEAE) cellulose (Whatman DE 11) was prepared according to the manufacturer's directions (5). Culture filtrates diluted fivefold with 0.05 M Tris [Tris(hydroxymethyl)aminomethane, Sigma Chemical Co.]-HCl buffer at pH 8.0 were applied to columns (1.76 × 26 cm) of DEAE cellulose. Eluant was 0.05 M Tris-HCl buffer at pH 8.0 with concns of NaCl increasing in a stepwise manner (0.0, 0.1, 0.2, and 0.5 M NaCl). The toxin was bound by DEAE cellulose, and was eluted with 0.1 M NaCl in 0.05 Tris-HCl buffer at pH 8.0.

Sephadex gel chromatography of *A. chrysanthemi* toxin was carried out using Sephadex G-10, G-25, G-50, or G-75 (Pharmacia Fine Chemicals) in eluant containing 0.1 M sodium phosphate buffer (pH 7.0) - 0.1 M NaCl solution. Gel filtration was done at 20 C using 1.76×23.0 - 26.0 cm columns with an eluant flow rate of 15 ml/h. Samples applied to the columns were lyophilized culture filtrate dissolved in 0.1 M sodium phosphate buffer (pH 7.0) - 0.1 M NaCl to 0.2 of the original volume of the culture filtrate. A small amount of Blue Dextran 2000 (Pharmacia Fine Chemicals) was added to each sample to mark the void volume of the column. The toxin was retarded by Sephadex G-25, Sephadex G-50, and Sephadex G-75 (Fig. 3).

Ability of other form-species of the form-genus Ascochyta to produce disease symptoms on chrysanthemum.—*Ascochyta imperfecta* Peck, *A. fabae* Speg., and *A. pisi* were tested for ability to produce symptoms on chrysanthemum. Cultures of *A. imperfecta* and *A. pisi* were obtained from local sources; *A. fabae* was obtained from Dr. Hachiro Oku, Okayama University, Tsushima Okayama, Japan. All organisms grew well on Watkins' medium. Filtrates prepared according to the procedures given above for *A. chrysanthemi* were tested for toxicity to chrysanthemum by the petiole method. In no case was leaf spotting or any other abnormality induced on chrysanthemum by filtrates of *Ascochyta* form-species other than *A. chrysanthemi*. Ascochitine was crystalized from *A. pisi* filtrate (3), and ethanol solutions of chloroform extracts of *A. fabae* filtrate gave the ultraviolet and visible spectra reported (8) for ascochitine. Thus, the antibiotic factor produced by *A. pisi* and *A. fabae* was present in their culture filtrates. Chrysanthemum plants were stem inoculated with mycelium of *A. imperfecta, A. pisi,* and *A. fabae*. In no case did leaf spotting or any other symptom characteristic of chrysanthemum Ascochyta blight develop.

DISCUSSION.—The experiments reported here confirm the earlier observation that *A. chrysanthemi* produces a stable toxin (7). The nature and distribution of symptoms (Fig. 2) are identical to those that develop in *A. chrysanthemi*-infected plants. This similarity suggests that the toxin plays a role in symptom expression and may be a factor in pathogenesis by the organism. It has not, however, been demonstrated that the toxin is essential for pathogenesis. On the basis of the information reported, *A. chrysanthemi* toxin should be classified as a phytotoxin (16).

Crossan (4) reported that isolates of *Ascochyta* from seven different hosts, conforming to the descriptions for seven form-species, produced identical symptoms on each host, irrespective of the original source of the inoculum. In reciprocal pathogenicity studies, he found that *A. chrysanthemi* was pathogenic only to chrysanthemum. The studies reported here indicate that *A. pisi, A. fabae,* and *A. imperfecta* lack the ability to induce symptoms on chrysanthemum. Hence, some degree of host specificity is indicated in pathogenesis caused by *A. chrysanthemi* on chrysanthemum.

The toxin produced by *A. chrysanthemi* was apparently not produced by the three other form-species of *Ascochyta* tested. *A. pisi, A. fabae* and *A. imperfecta* have been shown to produce phytotoxins or antibiotics (3, 8, 13) and the filtrates of *A. pisi* and *A. fabae* cultures tested in the studies reported here were shown to contain their respective toxins. The toxins produced by these form-species are soluble in various organic solvents; *A. chrysanthemi* toxin is not. The other toxins are low molecular weight compounds. The Sephadex gel chromatography data (Fig. 3) and the behavior of *A. chrysanthemi* toxin in dialysis and ultrafiltration suggest that it also is a relatively small molecule.

In all of the anion exchange and Sephadex gel chromatographic studies with *A. chrysanthemi* toxin (Fig. 3), the toxic principle was observed to behave as a single compound. It seems reasonable to conclude that the toxin is either a single chemical entity, or several very similar compounds.

The failure to separate foliar symptom-inducing activity from petal symptom-inducing activity suggests that the same toxin may be involved in both symptom complexes.

A. chrysanthemi toxin apparently is a relatively stable, nonvolatile, low molecular wt, organic compound that behaves as a functional anion at pH 8. The rapid accumulation of toxin in culture filtrates suggests that it is not a staling product of *A. chrysanthemi*. The toxin does accumulate in liquid cultures of *A. chrysanthemi*, but not to exceedingly high levels. *A. chrysanthemi* toxin is highly mobile within chrysanthemum plants. The similarity between the patterns of nonmetabolizable eosin dye movement and accumulation, and of the distribution of symptoms induced by the toxin on leaves suggest that the same routes are utilized for transport of these two compounds in intact plants.

Efforts in our laboratory are currently being directed to isolation and determination of the chemical composition of *A. chrysanthemi* toxin.

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