Etiology

Conidial Morphology, Axenic Growth, and Sporulation of *Stegophora ulmea*

G. H. McGranahan and E. B. Smalley

Graduate research assistant and professor, respectively, Department of Plant Pathology, University of Wisconsin, Madison 53706. Current address of the senior author is USDA-ARS, Department of Pomology, University of California, Davis 95616.
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ABSTRACT

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Techniques were developed for axenic culture of the fungus *Stegophora ulmea* [= *Gnomonia ulmea*], the cause of black spot of elm, previously reported to be an obligate parasite. The fungus was isolated by the surface-sterilized leaf-disk method. Radial growth was optimal on oatmeal agar. Growth was limited on malt, Czapek's, dilute potato-dextrose, and elm leaf extract agars. Maximum sporulation was obtained in a medium composed of the water-soluble fraction of ground oats. Production of macroconidia was minimal on potato-dextrose broth. Temperature had a significant effect on both growth and sporulation in liquid media. Optimum temperature for growth ranged between 16 and 24 C; growth was substantially reduced at 28 C. Macroconidia were produced between 16 and 24 C with an optimum at 20 C. Aeration moderately reduced growth and prevented sporulation. Growth was not influenced by absence of light; however, sporulation was five times greater in cultures grown in the dark. Temperature also had a significant effect on germination of ascospores (optimum at 8 C) but was not a significant variable in macroconidial germination. Microconidia were not produced in culture and did not germinate under the test conditions. Field studies confirmed the presence of two conidial forms occurring in lesions of *S. ulmea*. Macroconidia (= *Gloeosporium ulmicolum*) were common from the time of leaf emergence through June (in Wisconsin) at which time a transition occurred within the lesion to the microconidial form (= *Cylindrosporella ulmeum*). It is suggested that the spores of *C. ulmeum* may serve as spermatia.

Black spot of elm, which is caused by the ascomycete *Stegophora ulmea* (Schw.: Fries) Sydow & Sydow [= *Gnomonia ulmea* (Schw.) Thum.], is a serious foliar disease on many elm (*Ulmus*) species (9,15). Because several *Ulmus* species (some highly susceptible to black spot) are now being used in elm breeding programs (5,6,16), an increased understanding of this pathogen is essential.

Although several studies have been published on the biology, ecology, cytology, and chemical control of *S. ulmea* (10,11,14,17), the life cycle and methods for handling the organism in culture require further investigation. Pomerleau (14) observed germination of ascospores that were ejected onto glass slides in moist chambers. He rarely obtained germination of conidia. Morgan-Jones (11) reported limited germination of macroconidia after 48 hr in water or elm leaf extract, but no germination of microconidia. Growth in culture by this fungus has not been reported and both Pomerleau (14) and Morgan-Jones (12) considered *S. ulmea* to be an obligate parasite.

At present, the accepted anamorph of *S. ulmea* is *Cylindrosporella ulmeum* (Miles) von Arx (1), which is characterized by small spores (Fig. 1) referred to here as microconidia. Morgan-Jones (11) reported the existence of another form with larger spores (macroconidia) which resembles *Gloeosporium ulmicolum* Miles (Fig. 1). He observed that the

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spore form produced within a lesion changed during the growing season.

The aims of the present study were to develop techniques for germination, isolation, and growth of *S. ulmea* in pure culture and to determine quantitatively whether a change in spore form occurs during the growing season.

MATERIALS AND METHODS

Axenic culture and culture media. In attempted pure culture isolations, freshly sporulating lesions from naturally infected elm leaves were removed with 3- or 5-mm-diameter cork borers and surface disinfected in 1% NaClO (prepared freshly from commercial bleach [5.5% NaClO active ingredient]) for 2–5 min. Disinfected leaf disks were incubated on various agar media in 100 × 15 mm plastic petri plates (Falcon Labware, Becton-Dickinson Labware, 1950 Williams Dr., Oxnard, CA 93030) wrapped with Parafilm (American Can Co., Neenah, WI) to prevent dehydration.

Culture media included 2% water agar (Difco), potato-dextrose agar (PDA) (broth of 250 g of peeled potatoes, 20 g of dextrose, and 20 g of Bacto agar in 1 L of glass-distilled H_2O), similarly prepared PD broth (PDB) and dilute PDA (125 g of potatoes, 10 g of dextrose, and 20 g of Bacto agar), oatmeal agar (4), malt agar, Czapek's sucrose nitrate agar (18), elm leaf extract agar (25 g of dried American elm leaves steamed in 1 L of glass-distilled H_2O and filtered, 20 g of Bacto agar made up to 1 L with distilled H_2O), and "oat water-solubles" media (OWS). OWS was prepared as follows: 40 g of dry rolled oats (Quaker Oats Co., Chicago, IL) were ground in a Waring blender and steamed for 1 hr at 100 C in 1.0–1.5 L of distilled H_2O. The liquid extract was then strained through cheesecloth and centrifuged 20 min at 9 *g* and 0 C. The supernatant was decanted, made up to 1 L and 50 ml of the OWS liquid was dispensed into 125-ml flasks for sterilization. When used in studies on the effect of crude, water-soluble, nonstarchy polysaccharides of oats (7) on growth and sporulation, OWS concentrate contained extract from 60 g of rolled oats per 500 ml of medium. Dilutions of the concentrate (three flasks per concentrate) utilized in the studies included 1:0, 1:1, 1:2, 1:3, 1:4, and 1:5 prepared with distilled H_2O.

Inoculum for liquid media consisted of plugs of mycelium from the edges of 2- to 3-mo-old cultures of *S. ulmea* ground under aseptic conditions in an Omni-mixer (Sorvall; E. I. DuPont de Nemours, Newtown, CT) for at least 1 min and made up to a solution of ~100 propagules per milliliter. One milliliter of solution was used to inoculate 50 ml of medium in 125-ml flasks. Flasks were plugged with cotton after inoculation.

Colony diameters on solid media were measured after 2 mo. In liquid media, growth was measured after 5 wk of incubation in milligrams dry weight (3) on tared #4 or #41 Whatman filter paper disks. Uninoculated controls were used to adjust for differences in

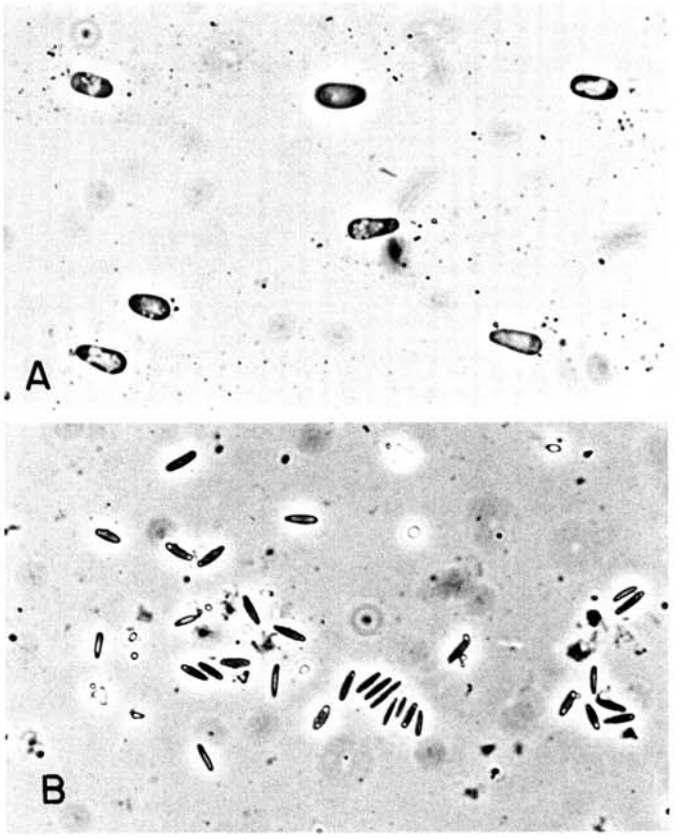

Fig. 1. A, Macroconidia of *Stegophora ulmea* (anamorph = *Gloeosporium ulmicolum*) and **B,** microconidia of *S. ulmea* (anamorph = *Cylindrosporella ulmeum*) (×1,650).

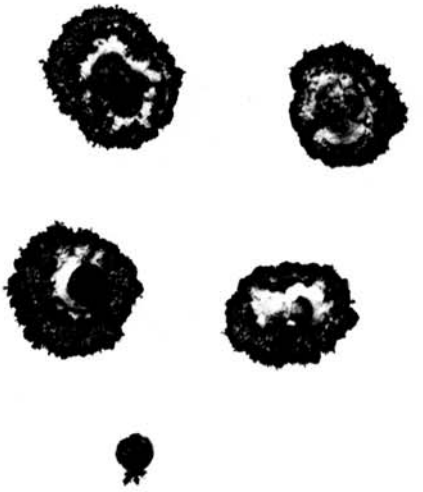

Fig. 2. Growth of *Stegophora ulmea* on PDA after 2 mo (isolation was from surface-sterilized leaf disks).

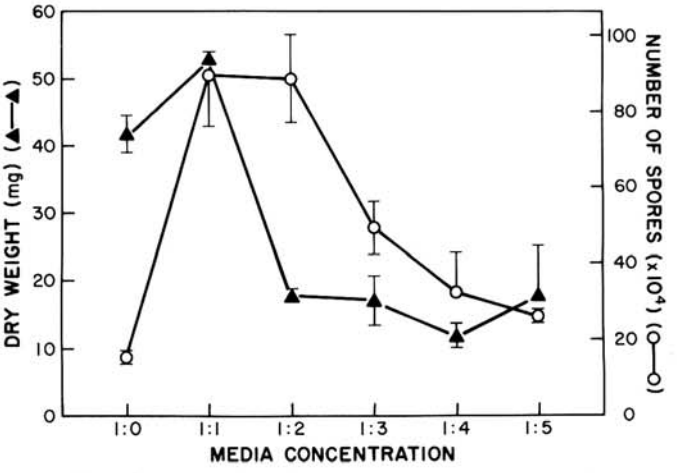

Fig. 3. Effect of concentration of the water-soluble (ws) fraction of oatmeal (OWS) in liquid media on growth (milligrams dry weight) and sporulation of *Stegophora ulmea*. A concentrate made up from the ws fraction of 60 g of rolled oats in 500 ml of H_2O was diluted such that 1:0 = ws of 120 g of oats per liter, 1:1 = ws of 60 g of oats per liter, 1:2 = ws of 40 g of oats per liter, 1:3 = ws of 30 g of oats per liter, 1:4 = ws of 25 g of oats per liter, and 1:5 = ws of 20 g of oats per liter. Each point represents the mean of data on four flasks, each containing 50 ml of medium, 5 wk after seeding with a suspension (~100 propagules per milliliter) of *S. ulmea*.

viscosity of the media. Sporulation was measured in numbers of conidia per milliliter. Four samples were counted per flask with a hemacytometer.

To determine optimum conditions for growth and sporulation in liquid media (OWS and PDB), the effect of aeration (rotary shaker, 150 rpm), light (aluminum foil wrapped versus unwrapped flasks), and temperatures (4, 8, 12, 16, 20, 24, 28, and 32 ± 2 C) were evaluated on four to seven seeded flasks and compared with an equivalent set of controls.

Germination of ascospores and conidia. Mature ascospores were obtained from infected, overwintered leaves collected near Madison, WI, in early April and stored frozen for 4–6 mo. For ascospore germination studies, sufficient numbers of perithecia were removed from the lesions and crushed in sterile distilled water to produce a spore concentration of $\sim 10^6$ spores per milliliter. A similar concentration of macroconidia was obtained by rinsing spores from lesions on leaves of plants inoculated with ascospores 3 wk previously in growth chambers. A microconidial suspension of 10^4 spores per milliliter was made up by rinsing spores from lesions present on leaves in the field in late July and August.

Loopfuls of spore suspension were deposited on punched 5-mm-diameter disks of water agar in standard petri plates. Seeded plates were placed in incubators at 4, 8, 12, 16, 20, 24, 28, and 32 C (± 2 C). Four disks per spore type per temperature were removed and evaluated for germination after 24 hr. Germination was measured by the percent of spores with germ tubes greater than half (4–6 μm) the length of the spore. At least 50 spores were counted per disk. The experiment was repeated twice.

Seasonal change in conidial morphology. Studies on seasonal changes in conidial morphology involved sampling of naturally infected elm leaves at weekly intervals between 4 June and 27 August 1981. At each sampling time, three randomly selected branches were removed from a planting of highly susceptible *Ulmus laevis* Pall. (W366—an introduction from Finland) growing at the University of Wisconsin Experimental Farms near Arlington, WI. Three leaves from each of five shoots per branch (45 leaves total) were examined microscopically each week for type of spore present within the lesion.

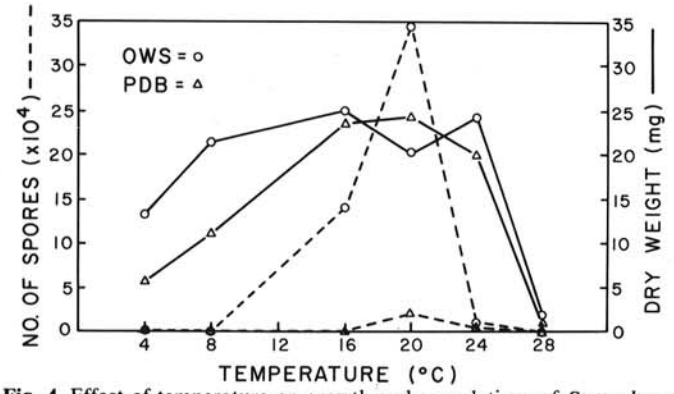

Fig. 4. Effect of temperature on growth and sporulations of *Stegophora ulmea* in oat water solubles medium (OWS) (water-soluble fraction of 40 g of rolled oats per liter of media) and potato-dextrose broth (PDB). Each point represents the mean for four flasks, each containing 50 ml of medium 5 wk after seeding with a suspension (~100 propagules per milliliter) of *S. ulmea*.

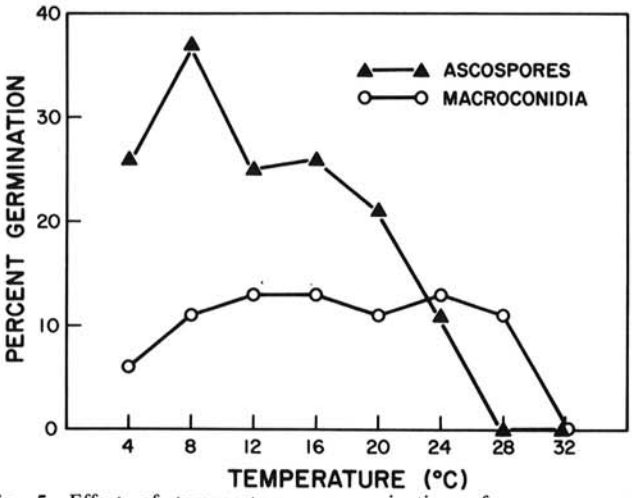

Fig. 5. Effect of temperature on germination of ascospores and macroconidia of *Stegophora ulmea*. Germination is expressed as percent of spores with germ tubes greater than half the length of the spore. Each point represents mean percent germination for ~400 spores.

RESULTS

Axenic culture. The surface-sterilized leaf-disk technique was a successful method for initial isolation of *S. ulmea*. Although the fungus proved to be a very slow-growing ascomycete in pure culture, it can no longer be considered an obligate parasite. The surface-sterilized leaf-disk technique was a successful method for initial isolation. Growth on solid media was usually not visible for at least 3 wk and sometimes not for 6 wk. After 2 mo on the most successful media, PDA and oatmeal agar, colony diameters averaged 19.7 ± 3.8 mm and 20.1 ± 5.4 mm, respectively. Growth was severely limited on malt agar, Czapek's sucrose nitrate agar, dilute PDA, and elm leaf extract agar.

Plugs of mycelium from the margins of 2-mo-old isolation cultures of *S. ulmea* grew readily when transferred to fresh culture media and were utilized as inoculum in the various studies on in vitro growth and sporulation.

Colonies on solid media were initially white, raised, and calluslike. As a culture aged, a dark margin of acervuli developed, and eventually the whole culture turned dark brown (Fig. 2). Only macroconidia were observed in culture and, after repeated transfers, sporulation was greatly reduced.

Optimal growth of *S. ulmea* on standard oatmeal agar in the isolations from infected elm leaves, and the failure to obtain growth on elm leaf extract media, suggested the use of the water-soluble fraction of ground oats for improved pure culture growth and sporulation. Standard oatmeal agar is also the recommended media for a related fungus, *Gnomonia leptostyla* (Fries) Ces. & de Not. (8). The concentration of the water-soluble fraction from ground oatmeal in the OWS medium substantially influenced the

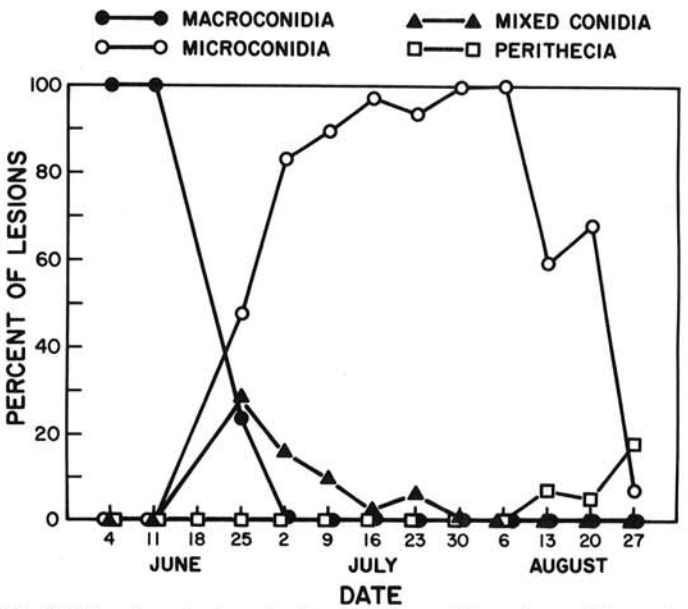

Fig. 6. Natural seasonal production of macroconidia, microconidia, and perithecia of *Stegophora ulmea*. Each point represents the percent of the lesions with each spore type on 45 leaves of *Ulmus laevis* sampled at weekly intervals at the University of Wisconsin's experimental farm at Arlington.

growth and sporulation of *S. ulmea* in culture (Fig. 3). Maximum dry weight accumulation was recorded for the 1:1 concentration. This is equivalent to using 60 g of rolled oats to produce 1 L of media. Optimum sporulation occurred at concentrations between 1:1 and 1:2. A concentration of 1:2 requires 40 g of rolled oats to make 1 L of media.

Aeration moderately reduced growth and prevented sporulation. Average dry weight per flask in shake cultures was 36.00 mg (SE = 1.13) compared with 46.86 mg (SE = 1.03) in still culture. Growth was not influenced by the absence of light; however, sporulation was five times greater in cultures grown in the dark; 3.14×10^4 (SE = 1.10) spores per milliliter were produced under light while 15.64×10^4 (SE = 3.42) spores per milliliter were produced in foil-wrapped flasks.

Temperature had a highly significant effect on growth and sporulation in both the OWS media and in PDB (Fig. 4). Optimum temperatures for growth ranged between 16–24 C, with growth substantially reduced at 28 C. Macroconidia were produced between 16–24 C; a peak was reached at 20 C. OWS media greatly improved spore production. At the optimum temperature, 20 C, 34.8×10^4 (SE = 8.16) spores per milliliter were produced in OWS compared with 2.44×10^4 (SE = 1.15) spores per milliliter produced in PDB.

Germination of ascospores and conidia. The influence of incubation temperature on germination was more pronounced for the ascospores (highly significant) than for the macroconidia (not significant) (Fig. 5). The optimum temperature for ascospore germination ($37 \pm 10.26\%$) was 8 C. No germination was observed at temperatures >24 C. Macroconidial germination was reduced at temperatures <8 C and >28 C. Microconidia did not germinate at any of the temperatures tested.

Seasonal change in conidial morphology. Spore type within a lesion changed over the season (Fig. 6), confirming the existence of two distinct spore forms. All of the lesions present on 4 June and 11 June contained macroconidia. By 25 June, 24% of the lesions contained macroconidia and 47% contained solely microconidia. On 9 July, lesions with macroconidia predominated.

By 13 August, perithecial necks were observed protruding through the abaxial surface of the leaf, the first indication of the presence of the perfect state. By this time, many leaves had fallen because of severe infection and many of the lesions on the remaining leaves lacked spores of any type. No further production of spores in the lesions occurred for the rest of the season.

DISCUSSION

This study shows clearly that *S. ulmea* is not an obligate parasite. The difficulties encountered by early workers who attempted to grow *S. ulmea* in culture may be attributed to the method of isolation, the long time required for growth on media, and the choice of media. The OWS medium made with 40 g of oats per liter and incubated in the dark at 20 C proved to be the superior medium of those tested. At 5 wk, the entire fungal contents of seeded OWS flasks may be used as inoculum. This medium may be useful for culturing other slow-growing fungi.

All attempts to obtain cultures from single germinated spores were unsuccessful, but clumps of spores removed from lesions that sporulated after surface disinfection did produce viable cultures. Although the concentration of spores needed to obtain a culture was not quantified, it appeared that the higher the concentration of spores, the greater the chances of obtaining growth. In any case, the most successful technique for obtaining cultures required the use of surface-disinfected leaf tissue.

The change in spore type produced during the season is probably responsible for the contradictions concerning the anamorph of *S. ulmea*. The original type specimen of *Cylindrosporella ulmeum* (= *Gloeosporium ulmeum*) (the anamorph with microconidia) was collected by Miles (10) on 19 August in Illinois and was found associated with the ascigerous state of *S. ulmea*. This association is consistent with our findings. The type specimen of *G. ulmicolum* (the anamorph with macroconidia) also was collected by Miles (10) in August and was not associated with *S. ulmea*. The presence of the type of spores that characterize *G. ulmicolum* is rare in August in Wisconsin. In the original description of this fungus, Miles (10) reported that it was found only on a single tree in a nursery in which most trees were severely infected with black spot. Late-season lesions containing the spores that typify *G. ulmicolum* usually are found only on trees that continue to produce new shoots throughout the summer or on vigorously growing seedling elms. Since the spore type changes before the perithecial necks are visible, it is not surprising that *G. ulmicolum* was not clearly associated with *S. ulmea*.

Pomerleau (14), who studied the ecology, epidemiology, and cytology of *S. ulmea*, probably was observing both spore forms since his measurements conform to the macroconidial type and he reported that among these were smaller, more pointed spores. The presence of more than one imperfect spore type is not unusual in the Diaporthales. Both *G. leptostyla* (2) and *Gnomonia platani* Kleb. (13) have a number of fruiting forms.

The lack of an increase in lesion frequency suggests either that the microconidia (which predominate after mid-July) are not pathogenic or that there is a lack of susceptible host tissue available at that time. Because our attempts to inoculate susceptible tissue with microconidia have been unsuccessful, it is possible that microconidia serve only as spermatia as Morgan-Jones (11) has suggested. However, when a suspension of microconidia was applied to sporulating lesions in the growth chambers, there was no evidence of the development of the perfect stage. Further work is required to establish the role of the microconidia.

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