Storage and Behavior in Soil of Septoria Species Isolated from Cereals

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ABSTRACT

Twenty-four isolates of five *Septoria* spp. (*S. avenae*, *S. avenae* f. sp. *triticea*, *S. nodorum*, *S. passerinii*, and *S. tritici*) were stored in soil-spore preparations at 4 C, in the dark, for more than 20 mo without loss of sporulating ability or pathogenicity. The storage of single-spore isolates in sterilized soil minimized changes compared to those which may occur on nutrient agar, and was more convenient and reliable than previously described methods of *Septoria* storage. Observations of fungal behavior during and following storage in sterilized soil were made using light microscopy and scanning electron microscopy. The behavior of these species during storage in sterilized soil mimicked their saprophytic behavior in dead leaf tissue. Also, their growth from portions of soil-spore preparations incubated on nutrient agar, following storage, was the same as that following isolation from their respective hosts.

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Additional key words: cirrhus matrix, host range studies.

Like many plant pathogenic fungi, *Septoria* spp. may lose the ability to sporulate and/or pathogenic characteristics when grown continuously in culture (1, 3, 12, 14, 20, 22, 23). A storage procedure for isolates used in host-parasite relationship studies should be simple and economical and maintain the sporulating ability and pathogenicity of the isolates (6, 17).

The possibility of storing any *Septoria* spp. in sterilized soil has largely been neglected except for one limited attempt (16). To date, *Septoria* spp. isolated from cereals have been stored on porcelain beads (15), in liquid nitrogen (1), under oil (A. A. Rosielle, *personal communication*) and by lyophilization (Shearer, *unpublished*). Reported here is a method of storage of *Septoria* species, isolated from cereals, in sterilized soil and an account of their behavior in this storage medium.

MATERIALS AND METHODS.— *Isolation from cereals.*—Pycnidium-containing tissues (ca. 2 cm^2) were soaked in ca. 2 ml of sterile deionized water for 15 to 30 min to cause extrusion of pycnidiospores. The resulting spore suspensions were streaked onto Difco potato-dextrose agar (PDA) in plastic petri plates. The plates were incubated at 18 C with 6,270±270 lx of continuous irradiation from cool-white fluorescent lamps; these conditions were standard throughout this study.

Single germinated spores of *S. avenae* Frank (perfect state *Leptosphaeria avenaria* G. F. Weber), *S. avenae* Frank f. sp. *triticea* T. Johnson (perfect state *L. avenaria* Weber f. sp. *triticea* T. Johnson), and *S. nodorum* (Berk.) Berk. (perfect state *L. nodorum* Müller) were transferred to specific nutrient agar for increase of inoculum after 12 hr. Single germinated spores of *S. tritici* Rob. ex Desm. and *S. passerinii* Sacc. have slow growth rates and were transferred to nutrient media after 36 to 48 h.

Increase of inoculum.—*Septoria avenae* and *S. avenae* f. sp. *triticea* were increased on potato-marmite agar (PDA+0.1% marmite) and *S. nodorum* on wheatmeal agar (2% wheatmeal+2% agar) (21). *Septoria passerinii* was increased on cornmeal agar (Difco) supplemented with 2% dextrose+2% peptone (1), while *S. tritici* was increased on Elliot-V-8 juice agar (11).

Cultural characteristics.— The various *Septoria* spp. isolated from cereals show two types of cultural characteristics when grown on nutrient agar. Mycelial colonies with spores produced in pycnidia (Fig. 1) are characteristic of *S. avenae*, *S. avenae* f. sp. *triticea*, and *S. nodorum* (3, 4, 12, 20, 21). Failure to subculture these species to fresh nutrient agar within 7 to 10 days resulted in their inability to form abundant pycnidia with spores. Pink yeast-like colonies (Fig. 2), composed mainly of spores, are characteristic of *S. passerinii* and *S. tritici* (1, 9, 11, 22, 23), although *S. tritici* may also form mycelial colonies with spores formed in pycnidia (4). Failure to subculture *S. passerinii* to fresh nutrient agar within 7 to 10 days often resulted in the formation of nonviable, leathery-brown colonies, while failure to frequently subculture *S. tritici* resulted in a change to slow growing, greyish-white, non-sporulating colonies (see also 22, 23).

Soil-spore preparations,— Five-g samples of a coarse-sandy clay loam at 1% moisture (10 to 15% clay, C/N ratio=25, and pH 7.1) were each placed in 59.0-ml (2-oz) bottles and autoclaved twice (20 min at a 12 h interval). Spores of the *Septoria* species were harvested from 5- to 10-day-old cultures and suspended in 2-ml aliquots of sterile deionized water. The aliquots were poured onto the surface of the soil preparations which were sealed, thoroughly shaken to ensure even distribution of the spores throughout the soil, and immediately stored in the dark at 4 C. This procedure is somewhat similar to the technique used for other fungi (17).

Inoculation of plants in the glasshouse and field with Septoria isolates stored in sterilized soil.— Soil from soil-spore preparations was suspended in 2 ml of sterile deionized water and dispensed onto the surface of nutrient agar, suitable for increase of inoculum of the respective *Septoria* species. The nutrient plates were incubated for 5 to 10 days, following which spore suspensions of approximately 5×10^5 spores/ml in 0.5% gelatin were sprayed onto plants as previously described (18, 21). Inoculated plants were further incubated in a glasshouse at 21 (range 19 to 31) C and irradiated by natural sunlight supplemented by light from 300-W incandescent lamps between 0500 h

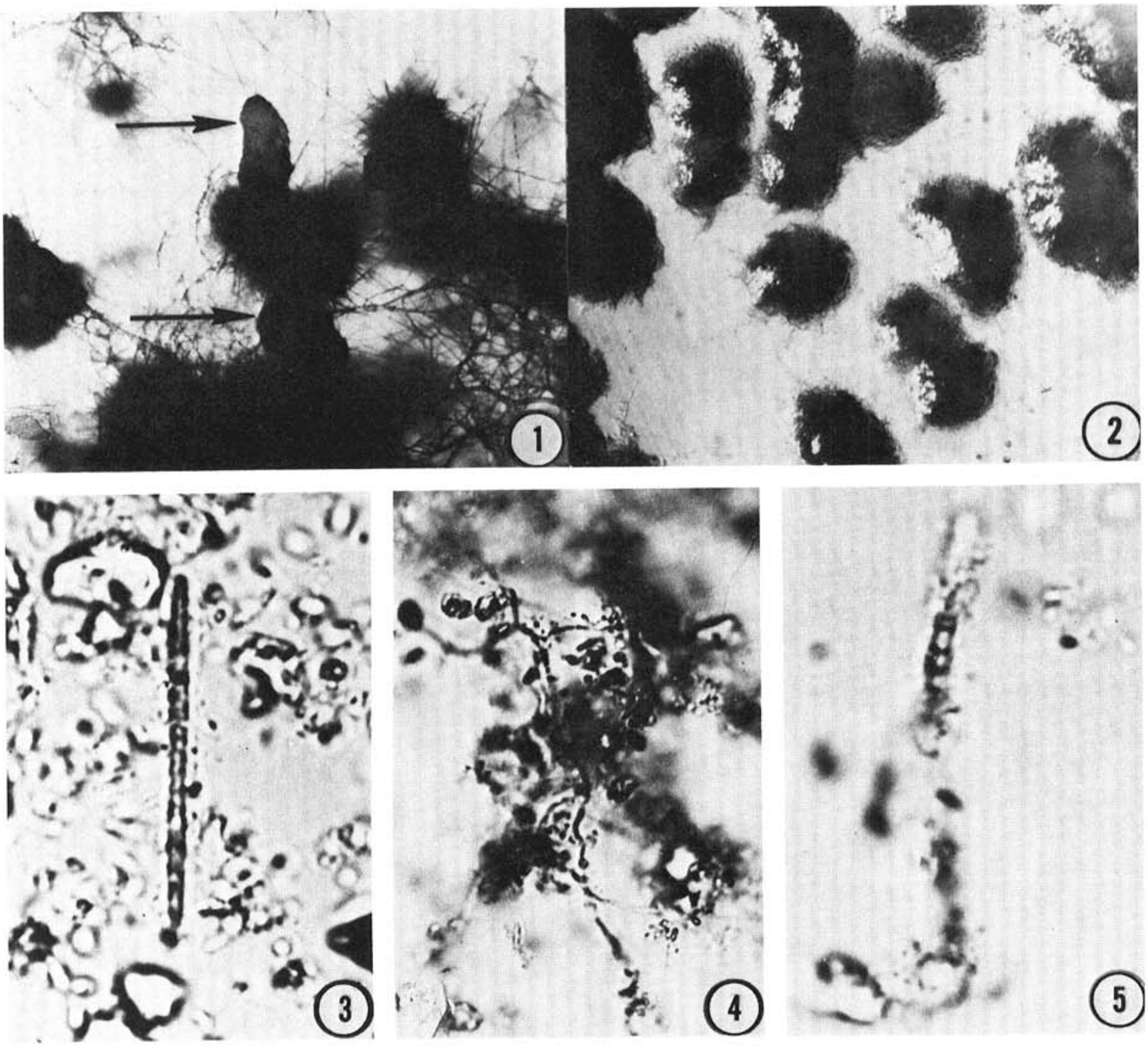


Fig. 1-5. 1) Pycnidia extruding pycnidiospores in pink cirrhi (indicated by an arrow) in a culture of *Septoria avenae* following isolation from oats and incubation on potato-marmite agar for 5 days (× 150). **2)** Glistening pink yeast-like colonies of *Septoria tritici* following isolation from wheat and incubation on Elliot-V-8 juice agar for 5 days. These masses are composed mainly of spores, as shown in Fig. 7 (× 150). **3)** A nongerminated spore of *Septoria tritici* in a 12-mo-old soil-spore preparation stored in the dark at 4 C (× 1,250). **4)** Mycelium of *Septoria avenae* in a 12-mo-old soil-spore preparation stored in the dark at 4 C. Note silt particles adhering to the surface of the mycelium (× 400). **5)** Silt particles adhering to a nongerminated spore of *Septoria tritici* from a 12-mo-old soil-spore preparation stored in the dark at 4 C (× 1,250).

and 2300 h. Light intensity at plant height varied between 1,530±130 and 23,600±2,270 lx. The methods used in field inoculation of plants were similar to those described by Hooker (13).

Preparation of specimens for light microscopy and scanning electron microscopy.— For light microscopy, portions of soil-spore preparations were suspended in sterile deionized water and stained with lactophenol cotton blue prior to examination. for scanning electron microscopy, small portions of soil-spore preparations were suspended in 2-ml aliquots of sterile deionized water and dispensed onto sterile filter paper disks (7-mm diam) to facilitate manipulation of soil particles. Pycnidiospores were examined after fixation in 6% glutaraldehyde (0.01 M phosphate buffer pH 7.0) for about 3 days at 24 C. All specimens were outgassed in a vacuum of 10^{-4} Torr for 3 h and then affixed to aluminum pegs with silver conductive paint. Specimens were then coated with carbon followed by an Au-Pd alloy, as described by Greenhalgh and Evans (10).

RESULTS.—*Longevity in soil.*— Isolates of *S. avenae*, *S. passerinii*, and *S. tritici* have been stored in sterilized soil for more than 24 mo and isolates of *S. avenae* f. sp. *triticea* and *S. nodorum* for more than 20 mo without total loss of viability. Preparations of *S. nodorum* and *S. passer-*

inii dried out during storage, but when reconstituted with deionized water and dispensed onto nutrient agar, the isolates were still viable. None of the 24 isolates stored in soil, to date, have totally lost viability during storage; by comparison, several isolates have totally lost viability during routine maintenance on nutrient agars.

Behavior during storage in soil.— The various *Septoria* species differed in their behavior during storage in soil. Twelve-month-old soil-spore preparations of *S. avenae*, *S. passerinii*, and *S. tritici* and 7-month-old preparations of *S. nodorum* were examined, using light microscopy, to determine if the spores had germinated or undergone morphological changes during storage. Only nongerminated spores were observed in soil preparations of *S. tritici* (Fig. 3), while germinated spores with germ tubes approximately half a spore length were observed in *S. passerinii* preparations. *Septoria avenae* and *S. nodorum* preparations contained mycelium originating from germinated spores (Fig. 4); nongerminated spores were not detected.

All species had silt particles adhering to the surfaces of mycelium (Fig. 4) or spores (Fig. 5) and no changes reminiscent of resting structures were observed. Neither spores nor mycelium could be detected by scanning electron microscopy (SEM) on the surface of soil particles during storage.

Growth from soil-spore preparations following storage.— Using SEM, observations were made on the growth of *S. tritici* and *S. avenae* from portions of 12-mo-old soil-spore preparations, incubated on PDA. Observations were made on these species because: (i) they represent the two extremes, that is no germination vs. germination during storage; and (ii) they are representative of the two growth habits on nutrient agar; that is, the yeast-like vs. mycelial-pycnidial.

The growth of *S. tritici* from a portion of a soil-spore preparation, incubated on PDA, changed from an occasional mycelial strand after 36 h (Fig. 6) to a pink mucilaginous growth, composed mainly of spores, which covered most of the soil surface after 3 days (Fig. 7). This budding, yeast-like growth habit was identical to that found on agar following isolation from wheat (Fig. 2).

Growth of *S. avenae* from portions of soil-spore preparation incubated on PDA for 36 h was mycelial in habit and was greater than that exhibited by *S. tritici* (compare Fig. 6 with 8). If soil-spore preparations of *S. avenae* were incubated on potato-marmite agar for about 9 days, the fungus formed mature pycnidia on the soil particles (Fig. 9); these pycnidia extruded spores in a cirrhus (Fig. 10). The mycelial-pycnidial growth habit, exhibited by *S. avenae*, after 12 mo of storage in soil, was identical to that following isolation from oats (Fig. 1).

Pathogenicity.— There were no detectable changes in pathogenicity when spores from the various *Septoria* spp. isolates, stored in soil, were used to inoculate their respective hosts in the glasshouse. Inoculum from an isolate of *S. tritici*, stored in soil for 12 mo, was used to inoculate *Triticum aestivum* L. 'Era' under field conditions and infection was obtained on the initial attempt, resulting in typical field symptoms.

DISCUSSION.— When maintained on agar, *Septoria* spp. undergo rapid changes in growth rate, sporulation and pathogenicity; even single spore isolation does not yield stable cultures (1, 3, 12, 14, 20, 22, 23). Storage of single-spore isolates of *Septoria* in sterilized soil minimizes the frequency of changes that may occur during maintenance on nutrient agar, and requires a minimum of time and equipment.

To our knowledge the limited attempt by Miller et al. (16) is the only reported study of a *Septoria* species stored in soil. They (16) reported a maximum of 6-mo survival of *S. glycines* Hemmi in soil, but did not elaborate on experimental conditions or procedure. The survival of fungi stored in soil may involve many factors, such as: soil type, fungal isolate, condition of culture prior to storage, water content of soil during storage, gaseous content of storage container, and the environmental conditions under which the preparations are kept. In our study we have used techniques and conditions thought to be conducive to survival of the *Septoria* spp. studied, such as moist, sandy clay loam, high concentration of inoculum, and tightly sealed vials immediately stored in darkness at 4 C.

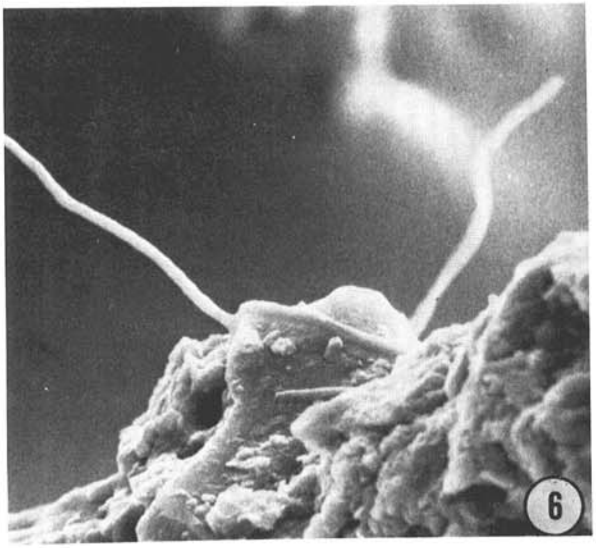
We have maintained all isolates for more than 20 mo but do not know the maximum longevity under our conditions. Atkinson (2) and Fennell (6) have reported up to a 5-yr survival record for some species of fungi in sterilized soil.

The behavior, in sterilized soil, of *Septoria* species isolated from cereals, parallels their saprophytic behavior in leaf tissues. Spores of *S. tritici* did not germinate in the soil during storage and this species may not grow and sporulate in dead leaf tissues (5). In contrast, *S. avenae* and *S. nodorum* did germinate in the storage soil and both are capable of growth and sporulation in dead leaf tissue (19, 24, Shearer, *unpublished*).

The behavior of *Septoria* spp. in sterilized soil may not be indicative of their behavior in nonsterile soil under field conditions. Hilu and Bever (11) observed that pycnidia of *S. tritici* disappeared from infected leaf tissue buried 76-mm (3-in) deep in soil for one mo. Von Wechmar (24) found no viable pycnidia of *S. nodorum* in infected wheat straw buried 152-mm (6-in) deep in soil for one mo and suggested decomposition of pycnidial contents by soil microorganisms. Viable pycnidia were found for up to 8 mo in infected straw left on the soil surface (24). The *Septoria* species infecting cereals are probably poor competitors in unsterilized soil.

The scanning electron microscope (SEM) was used to observe fungal growth and behavior in soil because of its depth of focus and resolving power. We observed no spores or mycelial fragments on soil particles immediately following removal from the soil-spore preparations. In studies on natural soil, using EM, Gray (8) rarely observed fungal spores on the soil surface. The failure to observe spores or small mycelial fragments with SEM may be due to the amount of relief on the soil particle surface and/or to silt particles adhering to spores and mycelium.

Pycnidiospores of *Septoria* spp. are extruded in a mucilaginous matrix which covers the surface of the spore (Fig. 11) and often renders observation with SEM difficult. This mucilaginous matrix has been reported to be a germination inhibitor at high concns and a germination stimulator at low concns (7). This substance, in addition to coating the spores, probably aids in the adherence of silt particles to the spores and may act as a buffer against physicochemical reactions at the soil-spore interface. Spores of species which have a yeast-like growth habit on nutrient agar are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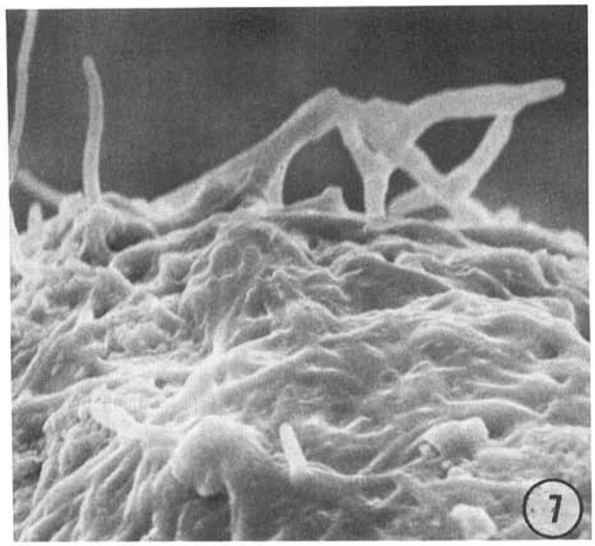
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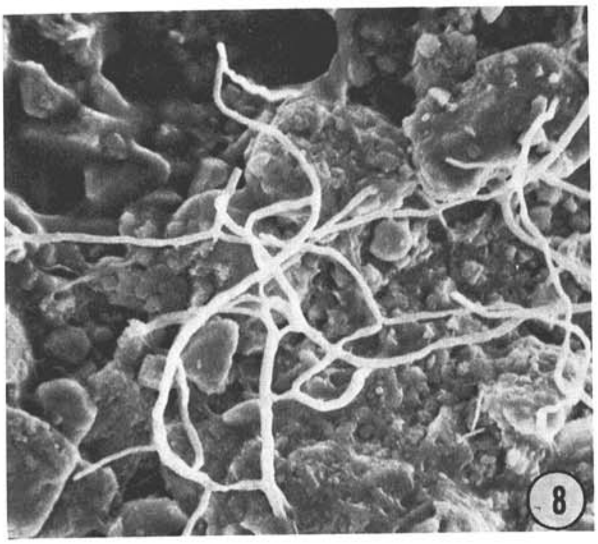
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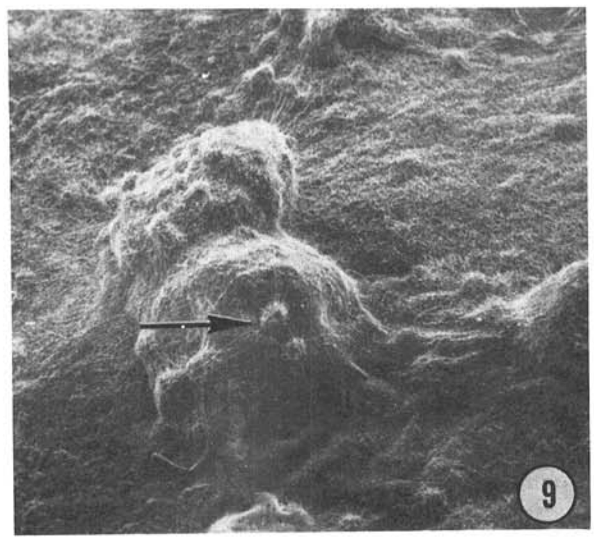
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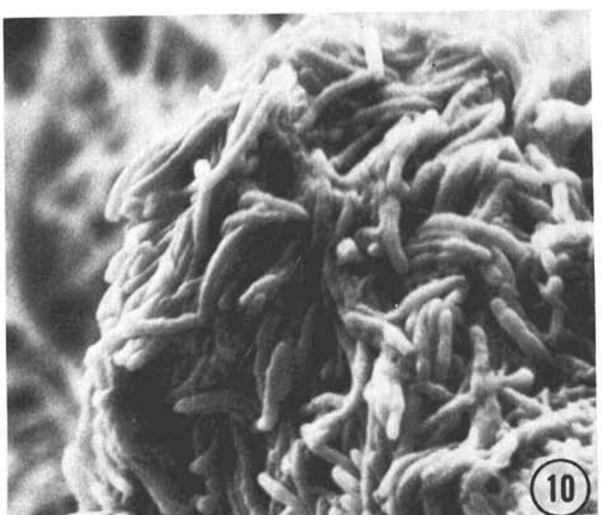
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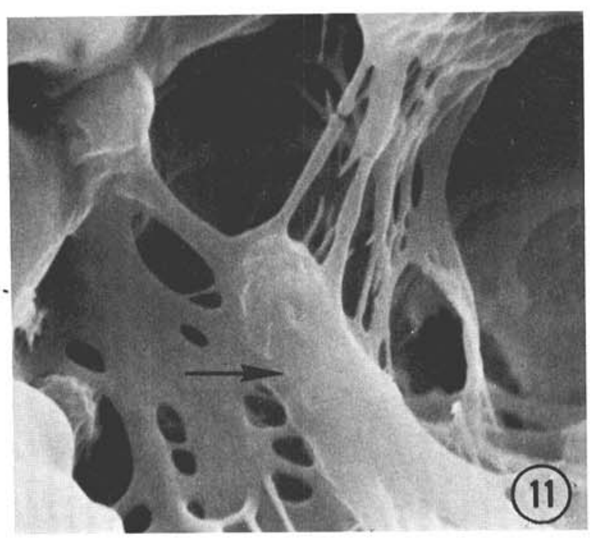
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Fig. 6-11. 6) Mycelium of *Septoria tritici* from a portion of a 12-month-old soil-spore preparation incubated on potato-dextrose agar for 36 h (× 1,200). **7)** Mucilaginous, budding growth of *Septoria tritici* on the surface of a portion of a 12-mo-old soil-spore preparation incubated on potato-dextrose agar for 3 days (×1,200). **8)** Mycelium of *Septoria avenae* growing from a portion of a 12-mo-old soil-spore preparation incubated on potato-dextrose agar for 36 h (× 650). **9)** Pycnidia of *Septoria avenae* on the surface of soil particles (arrow) from a portion of 12-mo-old soil-spore preparation incubated on potato-marmite agar for 9 days (× 22). **10)** Pycnidiospores of *Septoria avenae* extruded in a cirrhus from a pycnidium as illustrated in Fig. 9 (× 650). **11)** A pycnidiospore (arrow) of *Septoria avenae* situated within the ostiole of a pycnidium in leaf tissue of *Avena sativa* 'Rodney', and covered by the matrix of the cirrhus (×5500).

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also covered by a similar mucilaginous matrix (Fig. 2, 7).

The storage of *Septoria* spp. in soil has proven to be especially useful in host range studies. Often a species is isolated long before cross-inoculation experiments can be carried out. Since subculturing on nutrient agar may change the characteristics of an isolate, all isolates were routinely stored in soil immediately following isolation and purification. Changes in pathogenicity have not been detected when inoculum was obtained from these soil-spore preparations. This storage procedure, because of its economy and simplicity, is ideal for storing large numbers of isolates for long intervals of time.

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