

Infection and Mycelial Colonization of Gramineous Hosts by *Sclerophthora macrospora*

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## ABSTRACT

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Seedlings of *Lolium perenne* were more susceptible to infection than were more mature plants following inoculation with zoospores of *Sclerophthora macrospora*. Repeated exposure of seedlings to high concentrations of zoospores resulted in higher infection and damping-off percentages than a single exposure to zoospores. The ratio of infected plants to inoculum levels tended to decrease as the amount of inoculum increased. The mesocotyl

region of seedlings and the shoot apical meristem, axillary buds, and the intercalary meristem of mature plants were likely portals of entry. Following penetration, mycelium rapidly colonized developing leaf primordia. Mycelial growth was most extensive in older portions of the youngest leaves. Numbers of substomatal sporangiophoric pads were proportional to the percent leaf area colonized by secondary mycelium.

*Additional key words:* yellow tuft disease, turfgrasses, downy mildew, *Secale cereale*.

*Sclerophthora macrospora* (Sacc.) Thirum., Shaw and Naras., an obligate parasite and member of the Peronosporales (13), incites yellow tuft disease of turfgrasses (8). Gramineous hosts of *S. macrospora*, *S. rayssiae* var. *zeae* Payak and Renfro, and several species of *Sclerospora* are most susceptible to infection during the seedling stage (5,6,10,11,14,15). In turf, yellow tuft symptoms are observed most frequently in the spring following fall seedlings (7).

In spite of the rapidly expanding knowledge of *S. macrospora* and the diseases that it causes, more information is needed on the infection process in turfgrasses. This study was conducted to determine the relationship of plant age at inoculation to susceptibility, sites of penetration, and the development of systemic mycelium in the host.

## MATERIALS AND METHODS

Unless stated otherwise, all plants used in this study were perennial ryegrass, (*Lolium perenne* L. 'Yorktown').

**Relationship of seedling age to susceptibility.** Seeds of cultivar Yorktown were germinated on moist filter paper. Plants of three developmental stages were inoculated: radicles emerging from the coleorhiza and about 1 mm long; seedlings with coleoptiles 1-4 mm long not ruptured by the first leaf; and seedlings with first leaves 1-4 mm in length emerging from the coleoptile. Ten seedlings at each developmental stage were placed in each of three replicate 60 × 15 mm plastic petri dishes containing zoospore suspensions ( $23 \times 10^3$  spores per milliliter) produced by submerging detached leaves of infected Kentucky bluegrass (*Poa pratensis* L.), or rye (*Secale cereale* L.) in distilled water for 15 hr at 15 C. Inoculum and seedlings were incubated together on three successive occasions for 6, 15, and 6 hr, respectively, at 15 C. On each occasion thirty seedlings in distilled water served as controls.

After the final submersion, 10 seedlings per treatment per replicate were transplanted into each of three 10-cm-diameter pots containing a sterilized potting mix, and then placed on a sunny laboratory bench and observed for disease development. After 23 days, the plants were examined for the presence of *S. macrospora* mycelium by harvesting the three oldest leaves per plant, clearing them of chlorophyll in warm 5% potassium hydroxide, staining them with zinc chloriodide (16), and examining them at ×100 magnification.

The percentage of seedlings that became systemically infected,

and the type of hyphae present in the leaves were recorded.

**Pathogen ingress. Seedlings.** The lemma and palea were removed from perennial ryegrass seedlings when their coleoptiles were 1-4 mm long. The seedlings were exposed to a zoospore suspension for 24 hr at 15 C and maintained 2 days on moist filter paper; then the first leaf, coleoptile, endosperm, and roots were removed. The remaining tissues were stained with phloxine, mounted in water on glass slides, and observed by light microscopy at ×150 and ×600.

**Four-month-old plants.** Four-month-old greenhouse-grown perennial ryegrass plants were washed free of soil, and senescent leaves and sheaths were removed. To determine potential penetration sites on these plants, the following inoculation techniques were used: roots, crowns, and axillary buds were submerged in zoospore suspensions; leaves distal to the collar region of inverted plants were submerged in zoospore suspensions; or entire plants were submerged in the inoculum suspensions. Four plants in each of three 250-ml beakers were used per treatment. The inverted crowns and roots of plants in the second treatment were wrapped in moist filter paper to prevent desiccation and all zoospore suspensions were produced as described previously. The beakers containing plants and zoospore suspensions were incubated on four successive occasions for 6, 16, 6, and 16 hr, respectively, at 15 C. The successive replenishment of inoculum provided active, living zoospores. The mean number of zoospores was  $22.5 \times 10^3$  spores per milliliter on each occasion, and each beaker contained 50 ml of the suspension.

Following the final exposure to zoospores, individual plants were transferred to 4-cm-diameter pots containing a sterilized potting mix. After a 20-day growth period all leaves, sheaths, crowns, and axillary buds were removed and examined for *S. macrospora* mycelium as previously described.

**Influence of zoospore concentration and duration of exposure to inoculum on infection.** Perennial ryegrass seeds were germinated on moist filter paper. Seedlings with unruptured coleoptiles 1-4 mm long were selected because of the marked susceptibility to infection at that developmental stage (Table 1).

Ten seedlings per treatment for each of three replicates were placed in plastic petri dishes (60 × 15 mm) containing 4.4, 8.1, 14.4, or  $27.5 \times 10^3$  zoospores per milliliter of suspension, and were incubated at 15 C for 6, 12, or 24 hr. Control seedlings were incubated in distilled water at 15 C for the same period.

After the exposure periods, seedlings were transferred to moist filter paper and, upon appearance of the first leaf, planted in 10-cm-diameter plastic pots containing sterilized potting mix. Twenty-two days later, the youngest leaf of each plant was

removed, cleared, stained, and examined for mycelium of *S. macrospora*.

**Development of mycelium in systemically infected plants.** Eight infected rye seedlings in the first-leaf stage were planted in 4-cm-diameter pots containing sterilized potting mix; four were grown at 15 C and four at 26 C, each group under 14-hr days and cool white fluorescent light (about  $75 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$ ) in separate growth chambers.

Twenty-one days following transplanting, leaves were excised from plants at the collar region. A 2-cm segment of lamina was excised from the tips, mid-sections, and bases of the second, third, and fourth oldest leaves and examined for *S. macrospora* mycelium after being cleared and stained as previously described.

The mean percent area of the leaf section colonized by mycelium and the mean number of substomatal sporangiophoric pads per  $0.5\text{-mm}^2$  leaf section surface were determined from three random fields of observation. The substomatal sporangiophoric pads were counted and the percent area colonized by mycelium was visually estimated.

## RESULTS

**Relationship of seedling age to susceptibility.** Of the three developmental stages tested, the coleoptile stage (coleoptile 1–4 mm long and not ruptured by the first leaf) was most susceptible to *S. macrospora*; 100% of the plants became systemically infected (Table 1). Seedlings inoculated at the radicle-emergence stage also were very susceptible (83.3% became systemically infected). Seedlings slightly more advanced in development (first leaf extending 1–4 mm beyond the ruptured coleoptile apex) developed only 56.7% infection. Although only a few hours separated the developmental stages, their infection percentages differed

TABLE 1. Percentage of *Lolium perenne* seedlings at three developmental stages that became systemically infected after 6-, 15-, and 6-hr successive exposures to zoospores of *Sclerophthora macrospora*<sup>a</sup>

Developmental stage	Infection (%)	Damped-off (%)
Radicles 1.0 mm long	83.3 a <sup>x,y</sup>	16.7 a <sup>x,y</sup>
Coleoptiles 1.0–4.0 mm long	100.0 b	6.7 b
First leaf 1.0–4.0 mm long	56.7 c	3.3 b
Control <sup>f</sup>	0.0 d	0.0 b

<sup>a</sup> Plants were inoculated by submergence in zoospore suspensions ( $23 \times 10^3$  spores per milliliter) at 15 C and examined for systemic infection 23 days later.

<sup>x</sup> Data represent means of 10 plants in each of three replications.

<sup>y</sup> Means followed by the same letter in the same column are not significantly different ( $P = 0.05$ ) according to Duncan's multiple range test.

<sup>f</sup> Thirty seedlings similarly submerged in distilled water served as controls.

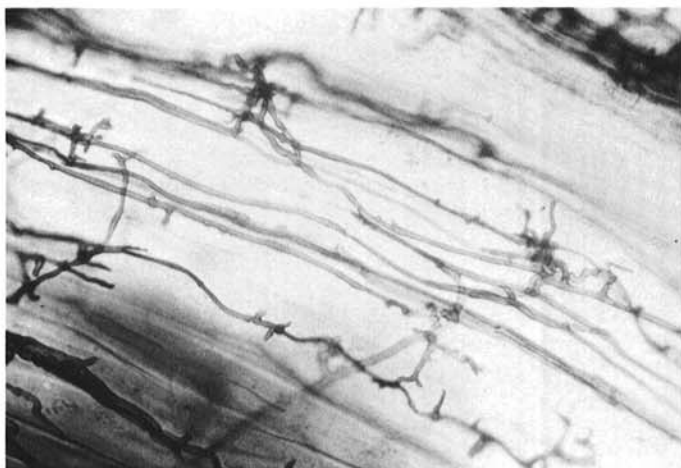


Fig. 1. Narrow primary extension hyphae of *Sclerophthora macrospora* in a leaf sheath of *Lolium perenne*,  $\times 150$ .

significantly.

Results indicated that seedlings infected at a less advanced developmental stage were more prone to subsequent damping-off (Table 1). By 23 days after inoculation, 16.7% of seedlings inoculated at the coleoptile stage, 6.7% at the radicle-emergence stage, and 3.3% of seedlings at the first-leaf stage had died.

*S. macrospora* produces two types of hyphae: narrow, extension hyphae (primary type) and robust, polymorphic hyphae (secondary type) (Figs. 1 and 2). Mycelial development in the second and third leaves of infected plants 23 days following inoculation was exclusively of the secondary type. The first leaf typically contained only primary hyphae that developed from the crown and extended upward toward the apex of the lamina. The acropetal development of only primary hyphae into the first leaf suggests that the coleoptile and the laminae of the first leaf were not sites of penetration.

The exclusive presence of secondary mycelium in the second and third leaves suggests that their developing primordia were extensively colonized by mycelium long before these leaves emerged through the sheath of the first leaf. Because the first leaf was in a more advanced state of maturation at the time of inoculation, there would have been no opportunity for the fungus to colonize the primordium. Consequently, at 23 days after inoculation the fungus had only begun to colonize the first leaf from the crown.

In a few infected plants observed at 23 days following inoculation, primary hyphae had grown from the crown region along vascular bundles and into the seminal roots. Primary hyphae comprised the only mycelium in infected plants which had damped-off.

**Pathogen ingress.** *Seedlings.* Germ tubes of zoospores appeared to penetrate the anticlinal walls of epidermal cells throughout the entire mesocotyl region. Owing to the lack of a specific stain for *S. macrospora* germ tubes, precise histological documentation of this process was not obtained.

*Four-month-old plants.* Four-month-old plants were more likely to become infected when immersed in a highly concentrated zoospore suspension (Table 2).

Of the two plants that had their crowns, axillary buds, and roots submerged in inoculum, one contained mycelium in the crown region, axillary buds, and the second leaf produced after inoculation; the other had mycelium only in two axillary buds. No mycelium was observed in the roots of either plant.

Infected plants subjected to total submersion during inoculation also possessed mycelium in the second leaf developing after inoculation. Primary hyphae were observed in the collar region or intercalary meristem of one plant, and in the sheath of a second plant. In the former plant, mycelium could not be traced any appreciable distance above or below the collar region, and in the latter, primary hyphae were detected growing upward from the

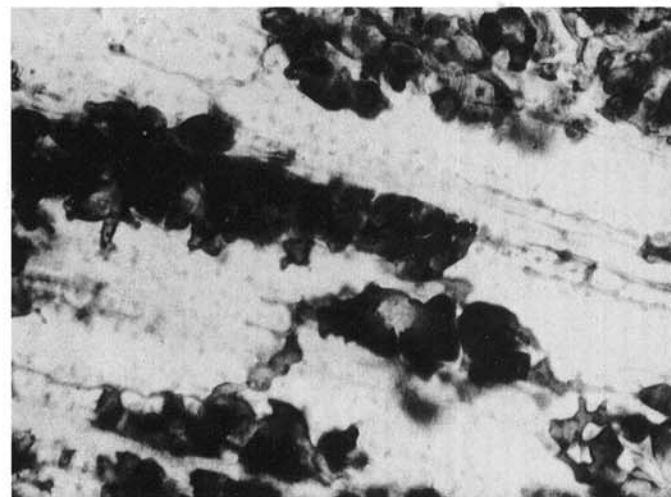


Fig. 2. Robust secondary polymorphic hyphae of *Sclerophthora macrospora* in leaf mesophyll of *Poa pratensis*,  $\times 60$ .

crown. In both instances, only the youngest of the three original leaves present during inoculation contained mycelium.

None of the plants in which only leaves were exposed to zoospores were infected. Aggregations of germinated zoospores were observed on the surface of intact leaves and also at the tips of cut leaves. No direct germ tube penetration of epidermal cells or indirect entry via stomates or wounds could be discerned.

**Influence of zoospore concentration and duration of exposure to inoculum on infection.** A single inundation with zoospores when seedlings were most susceptible resulted in high infection percentages (Fig. 3). Percentage infection of seedlings tended to increase with increased zoospore concentrations up to  $8.1 \times 10^3$  zoospores per milliliter and with increases in duration of exposure up to 6 hr. There was a nonsignificant trend of increasing infection percentages with inoculum levels greater than  $8.1 \times 10^3$  zoospores per milliliter and exposure periods of 12 and 24 hr.

**Development of mycelium in systemically infected plants.** Temperature during the initial 21 days after inoculation of plants of the same age had little effect on percent leaf area colonized by mycelium or on the number of substomatal sporangiophoric pads produced (Table 3). Combined data from the temperature parameters used showed that the older portions (ie, tips and midsections) of the youngest leaves had more mycelium and substomatal sporangiophoric pads than did the basal portions of these leaves (Table 4). There was a direct relationship between numbers of substomatal sporangiophoric pads and percent area colonized by secondary mycelium (Table 4).

## DISCUSSION

In this study, seedlings of *L. perenne* were considerably more susceptible to infection by zoospores than were more mature plants. The preferred penetration sites on 4-mo-old plants were the shoot apical meristem, axillary buds, and possibly the intercalary meristem in the collar region of the lamina. Tasugi (cf, Semeniuk and Mankin, [12]) report that rice axillary buds were penetrated by *S. macrospora*, and that *Sclerophthora rayssiae* var. *zeae* zoospores apparently penetrate corn leaves in the whorl in the

region of intercalary meristems (9). Observations reported here indicate that anticlinal walls of epidermal cells in the mesocotyl region of seedlings also were penetrated. Akai and Fukutomi (2) describe a similar mode of penetration by *S. macrospora* in ventral scale and epiblast of developing rice seedlings. Zoospores of *S. macrospora* are chemotactically attracted to the germinating seed (4), especially the mesocotyl, suggesting that this region is the primary site of penetration.

The position of mycelium within infected plants suggests zoospore germ tubes are capable of penetrating only juvenile, meristematic tissues. The absence of secondary mycelium in old leaves and roots of plants exposed to zoospores when 4 mo of age, and the absence of secondary mycelium in the first leaf, coleoptile, and roots of seedlings similarly exposed to zoospores, indicate that zoospore germ tubes are not capable of penetrating these tissues.

Other graminicolous plant pathogenic fungi, including *Physoderma maydis* Miyabe and *Tilletia* and *Ustilago* species, also

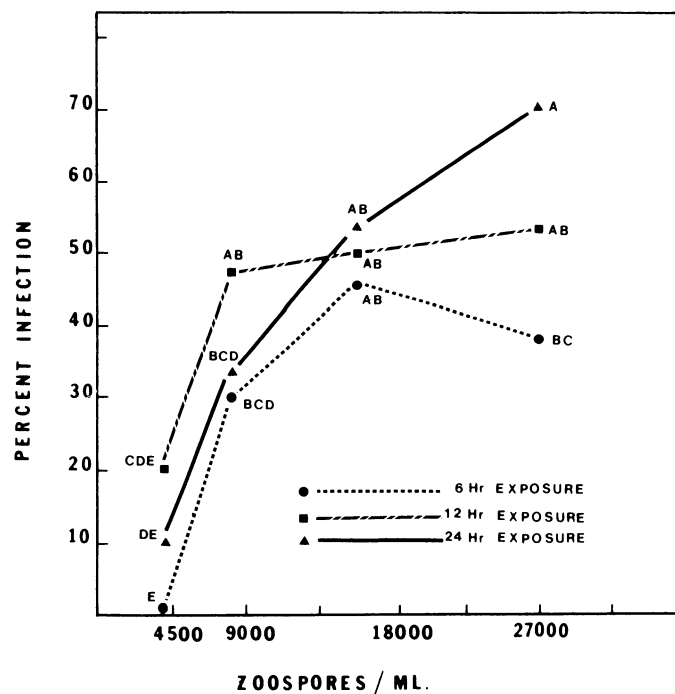


Fig. 3. Percent *Lolium perenne* seedlings infected at four specific inoculum levels and three durations of exposure to zoospores of *Sclerophthora macrospora*. Each point represents the mean percent infection of ten plants in each of three replications. Points marked by a common letter are not significantly different ( $P=0.05$ ) according to Duncan's multiple range test.

TABLE 2. Percentage of *Lolium perenne* plants, 4 mo old at time of inoculation, systemically infected after 20 days. Plants were exposed to *Sclerophthora macrospora* zoospores in three regions: crowns, axillary buds, and roots; leaves above the collar (ie, above the intercalary meristem); and total submergence of plants<sup>a</sup>

Sites of exposure	Infection <sup>y</sup> (%)
Crowns, axillary buds, roots	16.7
Leaves above collar region	0
Total submergence	33.2
Control <sup>z</sup>	0

<sup>a</sup>Plants were exposed to zoospore suspensions ( $22.5 \times 10^3$  spores per milliliter) on four successive occasions at 15 C for 6, 16, 6, and 16 hr, respectively.

<sup>y</sup>Data represent means of four replicate plants in each of three replications.

<sup>z</sup>Twelve plants similarly treated in distilled water served as controls.

TABLE 3. Percentage leaf area colonized by *Sclerophthora macrospora*, and mean number of substomatal sporangiophoric pads produced per 0.5 mm<sup>2</sup> in the second, third, and fourth leaves of infected *Secale cereale* plants incubated at 15 or 26 C 21 days following inoculation

Leaf number	Incubation temp (C)	Area colonized (mean %)	Substomatal pads (mean no. per 0.5 mm <sup>2</sup> )
Fourth leaf	15 C	20.5 <sup>y</sup>	6.8 a <sup>z</sup>
	26 C	17.5	3.2 b
Third leaf	15 C	15.5 <sup>y</sup>	4.9 <sup>y</sup>
	26 C	11.1	3.0
Second leaf	15 C	9.1 <sup>y</sup>	2.2 <sup>y</sup>
	26 C	12.1	1.8

<sup>y</sup>No significant differences within leaf number treatment.

<sup>z</sup>Means followed by the same letter within leaf number treatment are not significantly different ( $P=0.05$ ) according to Duncan's multiple range test.

TABLE 4. The mean percent leaf area colonized by *Sclerophthora macrospora* mycelium, and mean number of substomatal pads per 0.5 mm<sup>2</sup> in the tip, midsection, and base of the second, third, and fourth leaves of eight *Secale cereale* plants<sup>x</sup>

Leaf number/leaf section	Area colonized (mean %)	Substomatal pads (mean no. per 0.5 mm <sup>2</sup> )
Fourth leaf, tip	25.3 a <sup>y,z</sup>	7.5 a <sup>y,z</sup>
Fourth leaf, mid-section	18.1 ab	5.2 ab
Third leaf, mid-section	18.1 ab	4.8 ab
Third leaf, tip	14.5 ab	5.3 ab
Second leaf, base	13.0 ab	3.4 ab
Fourth leaf, base	12.8 ab	2.2 b
Second leaf, mid-section	10.0 ab	0.4 b
Second leaf, tip	8.8 b	2.4 b
Third leaf, base	7.3 b	1.4 b

<sup>x</sup>Plants incubated at 15 or 26 C for 21 days following inoculation. Data from both temperature parameters were combined for statistical analysis.

<sup>y</sup>Means followed by the same letter in the same column are not significantly different ( $P=0.05$ ) according to Duncan's multiple range test.

<sup>z</sup>Correlation coefficient,  $r^2 = .91$  (calculated by using mean percentages).

primarily penetrate meristematic tissues (1,3).

It also was observed that infected plants that had been inoculated at 4 mo of age by submergence in zoospore suspensions were characterized by extensive mycelial development in the crowns, shoot apical meristem, and the second leaf developed after inoculation. Apparently, zoospores swim down the successive layers of leaf sheaths and make contact with the penetrable shoot apical meristem and axillary buds. Extravaginally developing axillary buds, and possibly intercalary meristems, also would provide entry sites.

Field observations (authors', unpublished) provided additional evidence that ingress leading to infection was via juvenile meristematic tissues. In the field, extravaginally developing stolons of *Agrostis canina* L. show conspicuous yellow tuft symptoms after heavy rains during cool weather. Our inspections revealed that mycelium was usually confined to terminally produced tillers, while mother plants producing many of the infected stolons were healthy. In early fall, new leaves emanating from the center of many healthy looking crabgrass (*Digitaria sanguinalis* (L.) Scop.) plants showed yellow tuft symptoms. Sporangial production was massive on these young, yellowed leaves. Histological examination revealed that they were heavily colonized by mycelium, but the older, green, healthy appearing leaves were free of *S. macrospora*.

Akai and Fukutomi (2) show that *S. macrospora* hyphae do not colonize the first leaf of infected rice plants and believe that the pathogen has difficulty invading differentiated tissues. We determined *S. macrospora* has difficulty in colonizing the first leaf of *L. perenne* seedlings. Only primary hyphae were observed in the basal portions of the first leaf 23 days following inoculation, whereas secondary hyphae were present in all other leaves. Only two leaves present at the time of inoculation of a total of six 4-mo-old plants that had become infected contained mycelium. In both instances, these were the youngest leaves at the time of inoculation; only primary hyphae were present. The second leaf, and all leaves produced subsequently on infected mature plants which had been inoculated at 4 mo of age contained secondary mycelium. These observations indicated that differentiated tissues are resistant to systemic invasion by *S. macrospora*.

Once plants became infected, mycelia rapidly colonized leaf primordia. The leaf primordia initially were invaded by primary hyphae. Secondary mycelium is produced by intermittent expansion of regions along primary hyphae. Eventually, expanding regions of secondary hyphae grew close together connected by small sections of primary hyphae. From secondary hyphae, substomatal sporangiophoric pads were produced. The number of substomatal sporangiophoric pads was observed to increase proportionally with increase in area of mycelial colonization within leaves.

It is possible that mature plants were less likely to become infected because of structural barriers. Most meristematic tissues of older plants are protected by several layers of leaf sheaths. Because penetration of mature leaves and sheaths is unlikely, zoospores would be required to swim down between sheaths to gain access to penetration sites. Seedlings, during the period between radicle emergence and coleoptile elongation were most susceptible to the establishment of infection. Meristematic tissues of the mesocotyl region provide readily accessible sites for penetration by germ tubes of *S. macrospora* zoospores.

Repeated inundation of seedlings in zoospore suspensions resulted in 3–17% damping-off within 23 days of inoculation. Safeeulla (11) reports that seedlings of finger millet, *Eleusine coracana* (L.) Gaertn., also may die when infected by *S. macrospora*. None of our infected 4-mo-old perennial ryegrass plants damped-off. Multiple infection, enhanced by attraction of zoospores to potential penetration sites, and subsequent rapid mycelial colonization, weakened young plants and increased the probability of damping-off.

Repeated immersion of seedlings in suspensions containing high populations of zoospores resulted in 57–100% infection, the amount depended upon the stage of seedling development at inoculation. These high infection percentages are not unrealistic for field situations. In commercial sod-producing areas of Rhode

Island, we have observed that infection percentages of Kentucky bluegrass plants reach 100% in low-lying areas.

When seedlings received a single exposure to zoospores, infection ranged from 10–70%, the percentage depended on inoculum level and length of exposure of seedlings to inoculum. Percent infection achieved tended to increase with increasing zoospore concentrations and exposure times. The ratio of the number of infected plants to inoculum level decreased as the amount of inoculum increased. This is the most common relationship between amount of inoculum and amount of plant disease (17). Results of this and other investigations (8,12) show that increasing *S. macrospora* zoospore inoculum levels to a critical level increases the probability that seedlings will be infected. In this study, increases beyond  $8.1 \times 10^3$  zoospores per milliliter and an exposure period of 6 hr produced no significant increase in infection percentages.

The importance of the influence of time in the infection process by *S. macrospora* was documented in these studies. Seedlings were more likely than mature plants to become infected, indicating that plant age was an integral factor in susceptibility. An exposure time of 6 hr was adequate for obtaining high infection percentages with seedlings. The time of year when seed is sown would also be an important factor in infection. During cool moist weather the ability of the fungus within infected plants to produce zoospores is greatly enhanced. This is corroborated by conspicuous symptom expression and the presence of prodigious numbers of sporangia found on the leaves of infected cool season turfgrasses during spring and fall. Conversely, symptom expression and production of asexual sporangia generally decline in infected cool-season turfgrasses with the advent of hot, dry weather. Spring and particularly fall, are the normal periods when grass seed crops are sown and new axillary buds and tillers are produced. This coincides with the time of year when temperature and moisture for sporangial production and dissemination of zoospores is optimum, and when plants are most vulnerable to infection, and explains why swards often exhibit pronounced yellow tuft symptoms in the spring following fall seeding.

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