Pathological Anatomy of *Dactylis glomerata* Infected by *Stagonospora arenaria*

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ABSTRACT

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Stagonospora arenaria penetrated guard cells, subsidiary cells, and long cells of orchardgrass leaves via penetration pegs from appressoria and via hyphal wedges between cells. Most penetration attempts at guard and subsidiary cells were successful. A few penetrations occurred through stomatal openings. Penetration attempts at long cells were usually unsuccessful. Resistance to penetration was associated with papilla formation. Papillae were more frequently formed in long cells than in guard or subsidiary cells. Penetrated epidermal cells were colonized, and then the pathogen ramified intercellularly, but sparsely, in the mesophyll. Host responses began outside the margin of hyphal growth. As the typical purple leaf spot lesions developed, epidermal nuclei migrated toward the infection center, leucoplasts of mesophyll cells enlarged and became amber, and a dark amber gel formed between mesophyll cells. Some leaf tips, leaves, or plants formed large tan lesions. Tan lesions were characterized by development of diffuse light-brown pigment, collapse of mesophyll cells, and early disintegration of plastids, chloroplasts, and nuclei. In tan lesions, hyphae emerged from stomata, grew across the surface, and entered other stomata. Hyphae rarely emerged from purple lesions.

Additional key words: *Stagonospora maculata*, nuclear migration, fluorescence.

Stagonospora arenaria Sacc. causes purple leaf spot of orchardgrass (*Dactylis glomerata* L.). In the northeastern United States, the disease reduces forage yield (1,9) and quality (6). Purple leaf spot lesions are typically dark brown or purplish-black and 1–5 mm long by 0.5–1 mm wide (2,5). During the course of a program of breeding for resistance to this disease (11), it became apparent that in addition to the typical purple lesions, the fungus can incite large spreading tan lesions on leaf tips, entire leaves, or entire plants. The pathological anatomy of purple and tan lesions is of interest in understanding the ontogeny of lesions. Aside from a report that *S. arenaria* penetrates through stomata or guard cells (2), no information is available on the association of the fungus with host tissue or on host cell reactions.

The present study was undertaken to ascertain, by light microscopy, the characteristic penetration, colonization, and host responses associated with the two lesion types.

MATERIALS AND METHODS

S. arenaria was isolated from orchardgrass in Centre County, PA. Conidial inoculum was produced by culturing the isolate for 10 days on 20% V-8 juice agar at 22 C with 12 hr of fluorescent light daily. The inoculum, consisting of about 500,000 conidia per 100 ml, was suspended in distilled water containing one drop of Tween-20.

Eight susceptible plants were selected among third-cycle progeny in a recurrent-selection breeding program (11) at the U.S. Regional Pasture Research Laboratory. The plants were trimmed and transplanted into a commercial peat moss:vermiculite mixture (1:1, v/v) in 10.5-cm-diameter pots. Approximately 4 g of 15-15-15 (N-P-K) fertilizer was added each time the tops were trimmed. Leaves produced within 6 wk after trimming were sprayed with the inoculum suspension. The plants were placed in a dew chamber at 100% RH and 24 C for 2 days and then were transferred to a glasshouse at about 30 C day (14 hr) and 20 C night. Four of the plants developed purple lesions, and four developed tan lesions.

One expanded leaf was harvested from each plant 2, 3, 4, 6, and 10 days after inoculation. Several 2 × 5-mm pieces excised from each leaf at each harvest were cleared in lactophenol and examined with interference contrast and fluorescence microscopy. Other pieces were fixed in FAA, embedded in Paraplast, sectioned at 7 μm, and stained with safranin and fast green (3). For each sampling time, two leaf pieces from each plant were examined in transverse sections and two pieces were examined in longitudinal sections. Whole mounts were made of fresh samples in water, samples cleared 1–3 days in saturated chloral hydrate solution, and samples cleared 2–4 days in 1 N NaOH.

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Penetrations and papillae were quantified with respect to location in the abaxial epidermis of three 10-mm^2 leaf samples from each of six plants. The experiment was conducted in April 1980 and repeated in April 1981. Samples were harvested 48 hr after inoculation, cleared in lactophenol, and scanned with a fluorescence microscope equipped with a 355- to 425-μm exciter filter and a 460-μm barrier filter. All papillae were readily located by looking for green autofluorescence. Sites of penetration were detected by autofluorescence of the entire penetrated cell. In both trials, all autofluorescent epidermal cells were examined by interference contrast microscopy (×100 objective) to determine whether penetration had occurred. In less than 10% of autofluorescent guard cells, penetration was not completed, although pegs had formed; these are not considered further in this paper. Penetration was found in all other autofluorescent cells. In extensive random examinations, no penetrations were found in cells lacking autofluorescence. Thus, autofluorescence provided a reliable means for locating penetrations. Events were categorized according to whether they occurred at, or away from, the "stomatal region." The stomatal region was defined as the stomata, guard cells, and subsidiary cells. The area of the leaf occupied by stomatal regions was determined by counting all stomatal regions and measuring the average area of 10 regions per sample.

RESULTS

Penetration. Penetration occurred by several routes. Occasionally, hyphae grew through stomata into substomatal cavities (Fig. 1). Most frequently, however, the pathogen produced a small appressorium on a guard or subsidiary cell (Fig. 2) and penetrated the cell via a thin peg. Mycelium filled the cell and then exited through the end wall into the substomatal cavity (Figs. 2 and 3). Epidermal long cells were occasionally penetrated directly from appressoria in the same manner. Sometimes the fungus produced a multicellular wedge between lateral walls of epidermal cells and then grew directly into the mesophyll or penetrated and colonized an adjacent cell before passing into the mesophyll (Fig. 4). Only rarely were subcuticular hyphae formed before penetration.

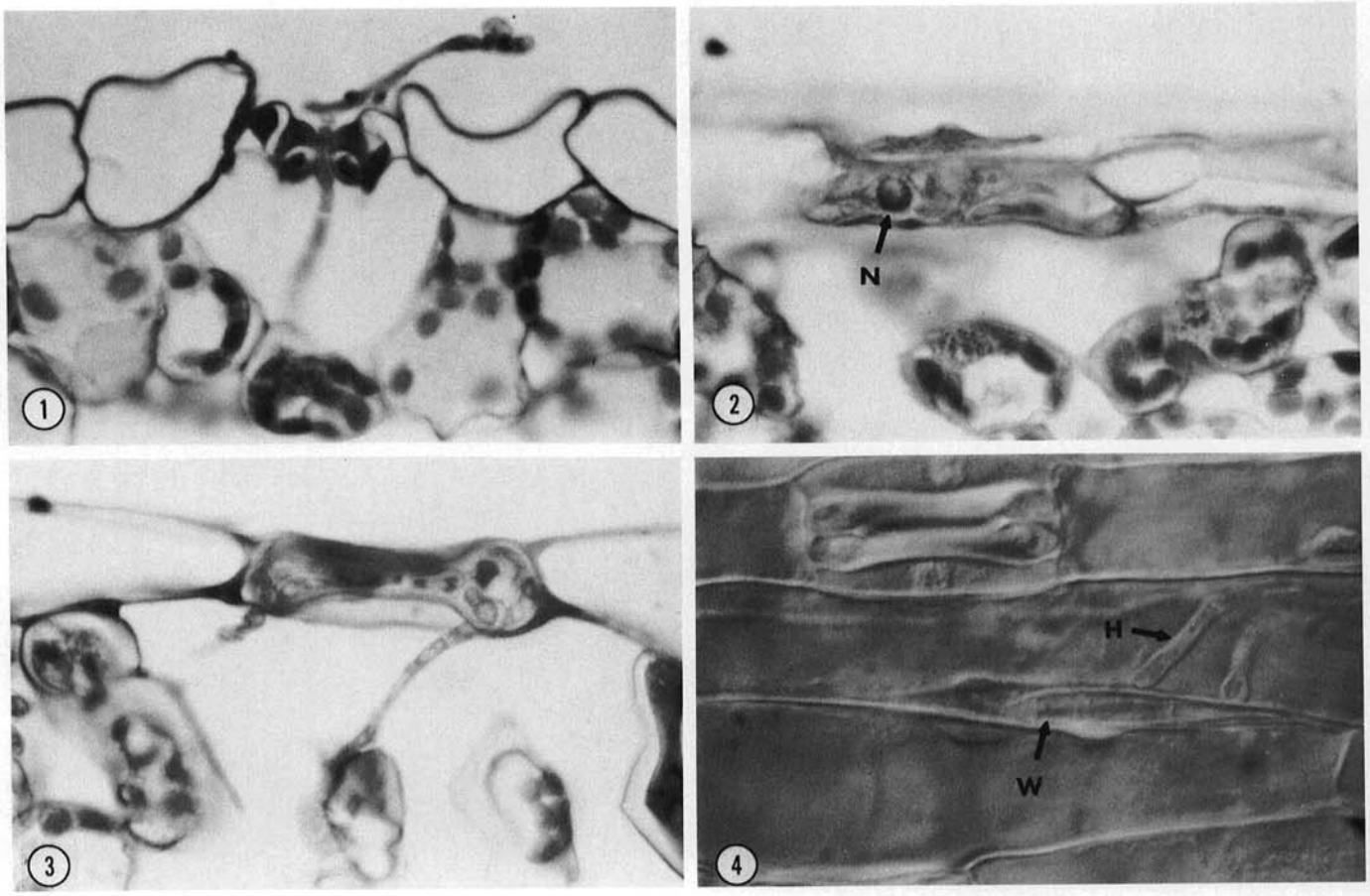
The frequency of papillae was far greater than that of penetrations in long cells of all six plants tested (Table 1). Conversely, penetrations greatly outnumbered papillae in guard and subsidiary cells. In each of two trials, plants 5 and 6 had fewer penetrations per square millimeter than plants 1, 2, and 3. Plant 1 developed tan lesions in both the April 1980 and April 1981 trials.

TABLE 1. Penetrations and papillae in orchardgrass leaves inoculated with *Stagonospora arenaria*

	Stomatal region[a]		Nonstomatal region	
Plant	Penetrations[b]	Papillae[b]	Penetrations	Papillae
1	43.2	0	0.04	19.0
2	38.2	0.4	0.06	17.8
3	29.2	3.2	0.20	27.3
4	24.3	0.6	0.12	17.2
5	15.8	0.8	0.05	13.7
6	7.4	1.6	0.04	11.6
Avg	26.4	1.1	0.08	17.8

[a] Stomatal region includes stomatal opening, guard cells, and subsidiary cells.

[b] Events per square millimeter. Each value is the average of two inoculation trials (April 1980 and April 1981). In each trial the abaxial surface of three replicate 10-mm^2 leaf pieces was examined for each plant.



Figs. 1–4. Infection of orchardgrass leaves by *Stagonospora arenaria*. **1,** Penetration by passage of a hypha through an open stomate (×1,100). **2,** A subsidiary cell with an appressorium on its outer surface, a penetration hyphae filling the subsidiary cell, and a hypha that has exited the cell at the lower right corner into the mesophyll 2 days after inoculation. Note the host nucleus (N) (×1,100). **3,** A guard cell filled with hyphae, and hyphae that have exited from the guard cell into the substomatal cavity (×1,100). **4,** A hyphal wedge (W) between lateral walls of adjacent long cells, and hyphae (H) within a long cell (×1,100).

Plant 2 developed only purple lesions in both trials. The other plants developed mixtures of purple and tan lesions in one or another trial.

Lesion development. Two days after inoculation, infections were visible in cleared tissues as faint-yellow areas involving one to two epidermal cells and four to five palisade cells. Mycelium extended four palisade cells away from the initial penetration. At 72 hr, lesions of fresh, unfixed leaves showed blue, water-soaked areas involving about four by four epidermal cells, surrounded by faint translucent yellowing. Tan lesions could be distinguished visually from purple lesions at 6 days. Purple lesions reached full pigmentation at 9–10 days and expanded slowly thereafter.

The purple lesions often spread laterally across veins which were capped by chlorenchymous cells. Lateral expansion usually stopped at large girder veins which contained sclerenchyma from surface to surface (Fig. 6), although chlorosis did extend to adjacent intercostal areas.

Colonization. During the first 10 days after inoculation, hyphae grew only in spaces between mesophyll cells. They were mostly uniform in width, occasionally branched, and sometimes anastomosed, but never were abundant or aggregated. Hyphae were more sparse in the palisade layer than in the substomatal cavities or spongy mesophyll, and more sparse in purple lesions than in tan lesions (Fig. 5). They ramified throughout the entire mesophyll between the upper and lower epidermises. In purple lesions, hyphae were present as frequently in dark-pigmented areas as in light-pigmented areas. Within 6 days, hyphae commonly had exited through the stomata of tan lesions, grown several millimeters over the surface, and sometimes entered other stomata near the lesion margin. Hyphae rarely emerged from purple lesions.

Host wall responses. Host wall, nuclear, and plastid alterations began outside the margin of hyphal advance and continued within the colonized area.

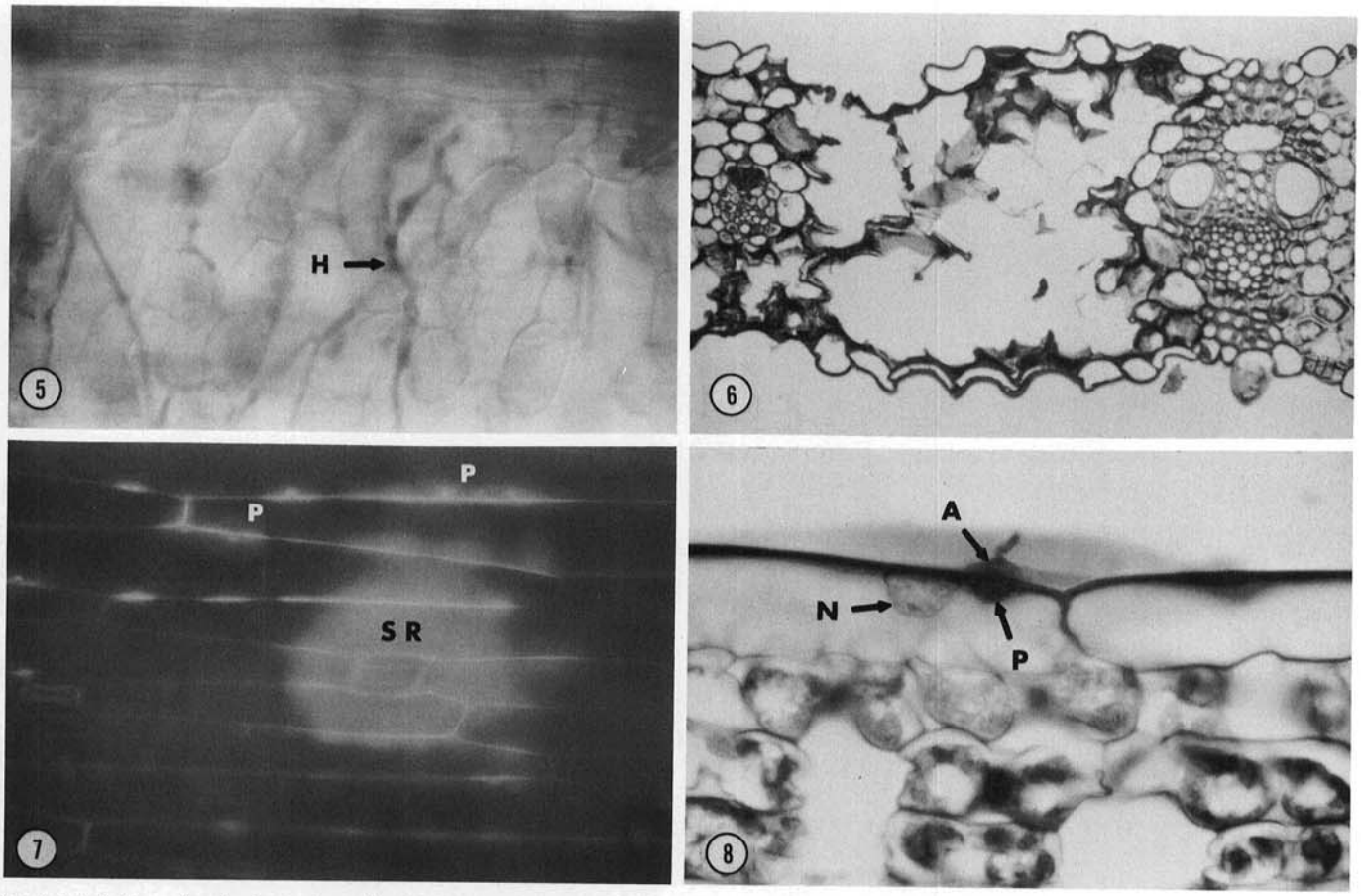
Papilla formation and autofluorescence were the most conspicuous wall responses (Figs. 7 and 8). Papillae were usually located in the lateral walls of long cells (Fig. 7). They were associated with appressoria, and apparently represented points of unsuccessful penetration. The papillae and a halo of the surrounding epidermal wall were autofluorescent. In addition, entire walls of penetrated cells, and of mesophyll cells bordered by colonizing hyphae, autofluoresced. Epidermal walls bordering hyphal wedges were swollen and discolored. Mesophyll walls of tan lesions were thin and distorted. They collapsed readily when the tissue was manipulated.

Nuclei. Nuclei of uninoculated long cells were located in the middle one-third of the cell (Fig. 12). In long cells that had a single prominent papilla, the host nucleus became stationed close to the papilla (Figs. 8 and 12). Nuclei of long cells near the edge of the young lesions were usually located in the half of the cell toward the focus of infection. Nuclei of palisade and subsidiary cells in and near lesions became conspicuously vacuolated.

Pigmentation. Chloroplasts lined the periphery of healthy palisade cells, but became scattered throughout infected cells. In purple lesions, chloroplasts diminished in volume and color from the edge to the dark part. In tan lesions, chloroplasts early became tiny, dark-amber spheres.

Healthy palisade cells contained a single, colorless plastid, slightly smaller than the chloroplasts. In water mounts of purple lesions, plastids enlarged greatly and became dark amber (Fig. 9). In tan lesions, the plastids were smaller and lighter in color (Fig. 10).

The color of purple lesions was primarily due to deep amber



Figs. 5–8. Infection of orchardgrass leaves by *Stagonospora arenaria*. **5,** A vertical view of the spongy mesophyll showing intercellular hyphae (H) in a tan lesion 8 days after inoculation (×440). **6,** A purple lesion 10 days after inoculation showing intercellular hyphae and collapse of epidermal and mesophyll cells. The lesion is bound at the right by a girder vein but extends across the small vein on the left (×275). **7,** A vertical view of the epidermis 2 days after inoculation as seen with fluorescence microscopy, showing autofluorescence of unpenetrated papillae (P), surrounding halos in the lateral walls, and the large area of autofluorescence around a stomatal region (SR) that has been successfully penetrated (×275). **8,** A leaf section 4 days after inoculation showing an appressorium (A) on the epidermal surface, a papilla (P) where penetration failed, and the host epidermal nucleus (N) nearby (×1,100).

pigmentation in mesophyll intercellular spaces (Fig. 9). The intercellular material retained its coherence and color when cells were separated by micromanipulation and soaked in water or organic solvents. Thus, the material appeared to be a firm, homogeneous gel. It had surrounded many of the palisade cells and some spongy cells beginning 6–7 days after inoculation. Cellular lumina remained lightly colored. By 10–16 days, dark amorphous beadlike deposits were present on the interior surfaces of the epidermal cell walls (Fig. 11).

Tan lesions at 5 days had faint-yellow or tan color that was not concentrated in any location. Later there was light-brown pigmentation of some mesophyll lumina and epidermal walls.

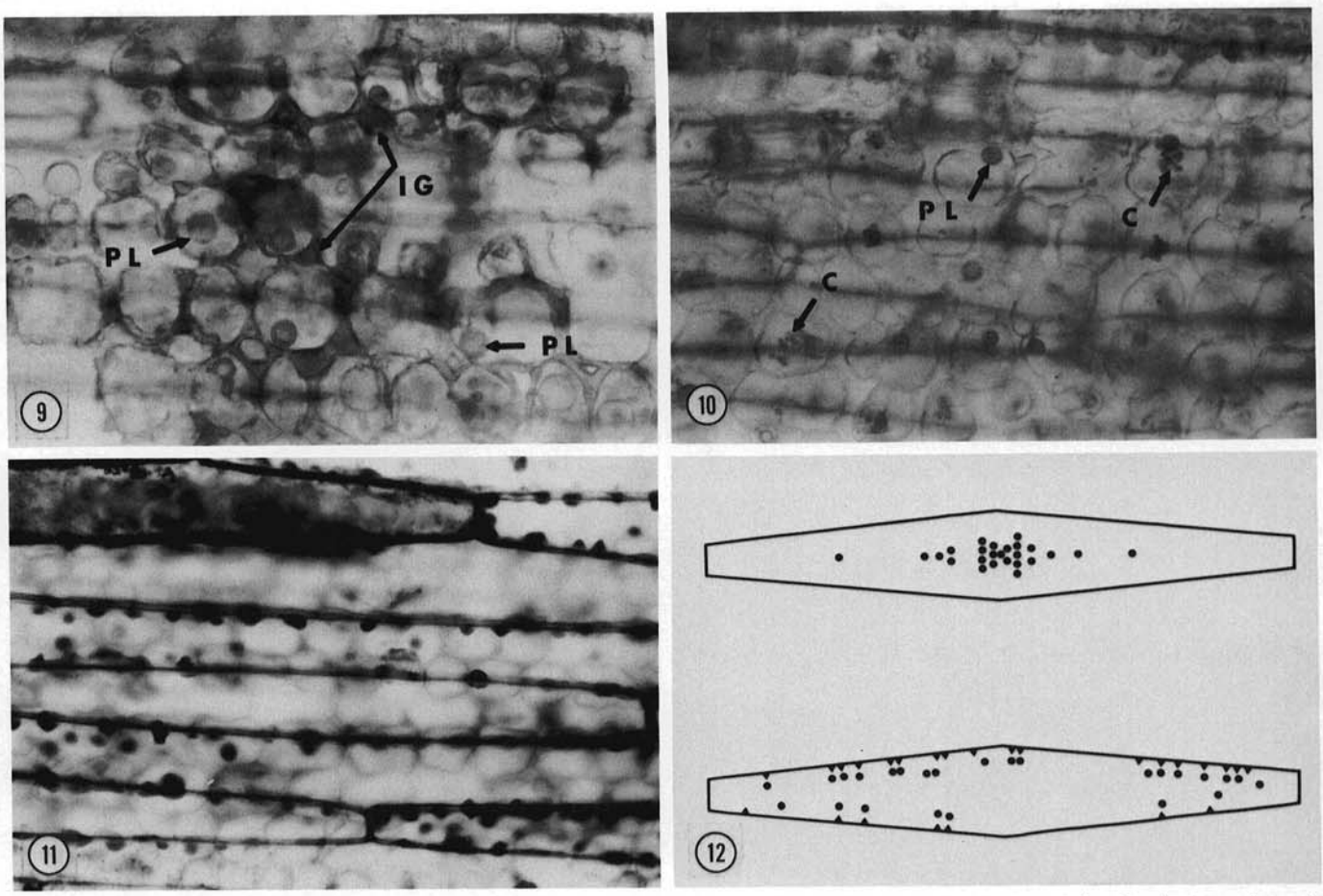
DISCUSSION

Graham (2) reported that appressoria of *S. maculata* (=*S. arenaria*) form at the point where epidermal cells adjoin, that infection hyphae can enter between a guard cell and an adjacent epidermal cell, and that penetration can occur through stomata. In addition to corroborating Graham's findings, results of the present study revealed that epidermal long cells can be penetrated by pegs from appressoria or by hyphal wedges in lateral walls, and that guard, subsidiary, and long cells can be colonized by fungal hyphae before the pathogen passes into the mesophyll.

The present study also established that colonization of leaf tissue, after penetration of the epidermis, proceeds via ramification of hyphae between mesophyll cells. At least until the time when typical purple lesions and the tan lesions reach full size and pigmentation, the mycelium remains intercellular within the mesophyll.

Although there is no assurance that graminicolous *Stagonospora* spp. are phylogenetically related, reports on penetration and colonization by two other *Stagonospora* spp. show interesting contrasts with *S. arenaria*. According to Mathre (7), *S. bromi* S. Smith & Rambs. penetrates cells directly without benefit of appressoria and grows not only between cells, but also inside mesophyll cells with little or no effect. Matsumoto (8) demonstrated that *S. sacchari* Lo & Ling, in addition to colonizing mesophyll intercellularly, enters bundle sheaths and xylem vessels. I have never observed *S. arenaria* inside mesophyll cells or vascular tissue.

Orchardgrass long cells strongly resisted penetration. The reaction was associated with papilla formation. Papilla formation has been implicated in epidermal resistance in many of the grasses (10), and the present study provides an additional example of this possible mechanism. At the light microscopic level of magnification, however, the mechanisms for papilla formation appeared to be largely absent in guard and subsidiary cells. Enhanced susceptibility of cells near stomata resembles the situation noted by Johnson et al (4) for certain barley genotypes inoculated with powdery mildew. With *S. arenaria*, successful penetrations near stomata outnumbered unsuccessful attempts approximately 24:1 (Table 1). Conversely, away from stomata, there were approximately 200 unsuccessful attempts for every successful penetration. Host nuclei were close to sites of papilla formation in long cells. In guard and subsidiary cells, nuclei were



Figs. 9–12. Infection of orchardgrass leaves by *Stagonospora arenaria*. **9,** Water mount of a purple lesion 8 days after inoculation showing large pigmented plastids (PL) and amber colored, intercellular gel (IG) in the palisades (×440). **10,** Water mount of a tan lesion 8 days after inoculation. Compare with Fig. 9 and note smaller plastids (PL), aggregations of chloroplasts (C), and absence of the amber, intercellular gel (×440). **11,** Water mount of a purple lesion 17 days after inoculation showing dark, beadlike deposits on the interior surfaces of epidermal walls (×275). **12,** Diagrammatic representation of nuclear positions in epidermal long cells. The upper diagram shows the relative lateral distribution of nuclei (•) in 25 uninoculated (control) long cells. The lower diagram shows the distribution of nuclei in 25 inoculated long cells; each cell had a single large papilla (▲) in the lateral wall, and the host nucleus (•) always was located adjacent to the papilla.

also close to penetration sites. Therefore, some feature of host cells other than nuclear proximity may be involved in their vulnerability. Such features might include small lateral walls and restricted cytoplasmic and nuclear volume.

The number of penetration attempts per square millimeter at stomatal regions of plants 1 and 2 (Table 1) was greater than that away from stomata. The reason is unknown. Possibly, detection of sites by autofluorescence is less effective in discerning unsuccessful attempts than in discovering successful attempts. Possibly, proportionately more attempts were made at stomatal regions due to a stimulation of appressorial and penetration peg differentiation at the stomatal region. Further studies are necessary to determine whether frequency of penetration is related to frequency of macroscopic lesions.

No clear differences were discerned between leaves forming tan lesions and leaves forming purple lesions with respect to site and frequency of penetration. Events determining lesion type apparently come into play after penetration. The variation in lesion type of certain plants from one inoculation trial to another indicates an environmental × genotype interaction for lesion type. The fate of a given encounter can be environmentally determined.

The results indicate that one approach for elucidating the regulation of lesion size and color would be to assess properties of the amber pigments of the plastids and intercellular gel as they relate to pathogen growth and to host wall integrity. Girder veins were a barrier to lateral expansion of purple lesions. Presumably, tightly packed, thick-walled cells of girders impede growth of the fungus. Additional studies are needed to clarify the etiological differences between purple and tan lesions. Since the tan lesion response has not been widely reported for purple leaf spot disease, a significant component of the damage caused by *S. arenaria* may have been overlooked.

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