A Vacuum Injection Method for Quantitative Leaf Inoculation of *Poa pratensis* with *Helminthosporium sorokinianum*

Clinton F. Hodges

Associate Professor of Horticulture and Agronomy, Departments of Horticulture and Agronomy, Iowa State University, Ames 50010.

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ABSTRACT

A vacuum injection inoculation method was devised for inoculating *Poa pratensis* L. with *Helminthosporium sorokinianum* for leaf lesion development. Individual tillers were inoculated by inserting a hypodermic needle into the sheath fold; then, under vacuum, a suspension of conidia was siphoned into the sheath fold. Evaluation of lesion development was made on the youngest visible leaf of inoculated tillers 4 to 5 days after inoculation. On the basis of numbers of conidia introduced per lesion developed, inoculation was most efficient at low concentrations of conidia. As conidia concentrations were increased, mean number of lesions and disease severity increased; however, direct lesion-to-conidia efficiency decreased. The method provides a quantitatively repeatable means of leaf inoculation and eliminates potential saprophytic development of *H. sorokinianum* on senescing leaves.

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Helminthosporium leaf spot caused by *H. sorokinianum* Sacc. ex Sorok. (*H. sativum* P. K. & B.) is a common and widespread disease of *Poa pratensis* L. and *Agrostis palustris* Hud. throughout the north central states. Much of the research concerned with leaf infection and subsequent lesion development has been conducted by inoculating leaves with conidia and mycelium fragments in water with an atomizer or sprayer (1, 2, 3, 4, 7, 9). Studies involving the reproducibility of spray inoculations indicate that reliable results can be obtained under precise conditions (6, 8). Reproducibility, however, depends on the skill of the operator, movement of the spray nozzle, constant spray pressure, and uniformity and size of plants (6). If these factors vary, it cannot be assumed that all leaves of a plant receive the same number of spores. The intravaginal and extravaginal branching of *P. pratensis* results in plants that are not very uniform in size, shape, and density; the absence of uniformity makes such plants poor specimens for even foliage inoculations (6).

Inoculation studies with *H. sorokinianum* on *P. pratensis* and *A. palustris* disregard leaf age in evaluating lesion development (1, 3, 7, 9). Total lesion counts per tiller, per plant, or turf area, disregard the facultative nature of *H. sorokinianum* and fail to distinguish between the saprophytic and parasitic capabilities of the organism. Such evaluations also fail to recognize the progressive senescence of each older leaf on a tiller. There is evidence that, with each older leaf of perennial grasses, there is a progressive decline in photosynthesis and a loss of assimilates (5, 10). Therefore, lesions produced on the oldest leaves probably are not truly representative of the parasitic capabilities of *H. sorokinianum*. The research herein was initiated to develop a quantitatively reproducible method for inoculating *P. pratensis* leaves with *H. sorokinianum* that would result in lesions representative of true parasitism and eliminate potential saprophytic infestation of senescing leaves.

MATERIALS AND METHODS.–*Preparation of plant materials for inoculations.–Helminthosporium sorokinianum* was isolated from leaf lesions on *P. pratensis* on 4% Bacto agar in distilled water and transferred to V-8 juice agar [20% (v/v) V-8 juice in

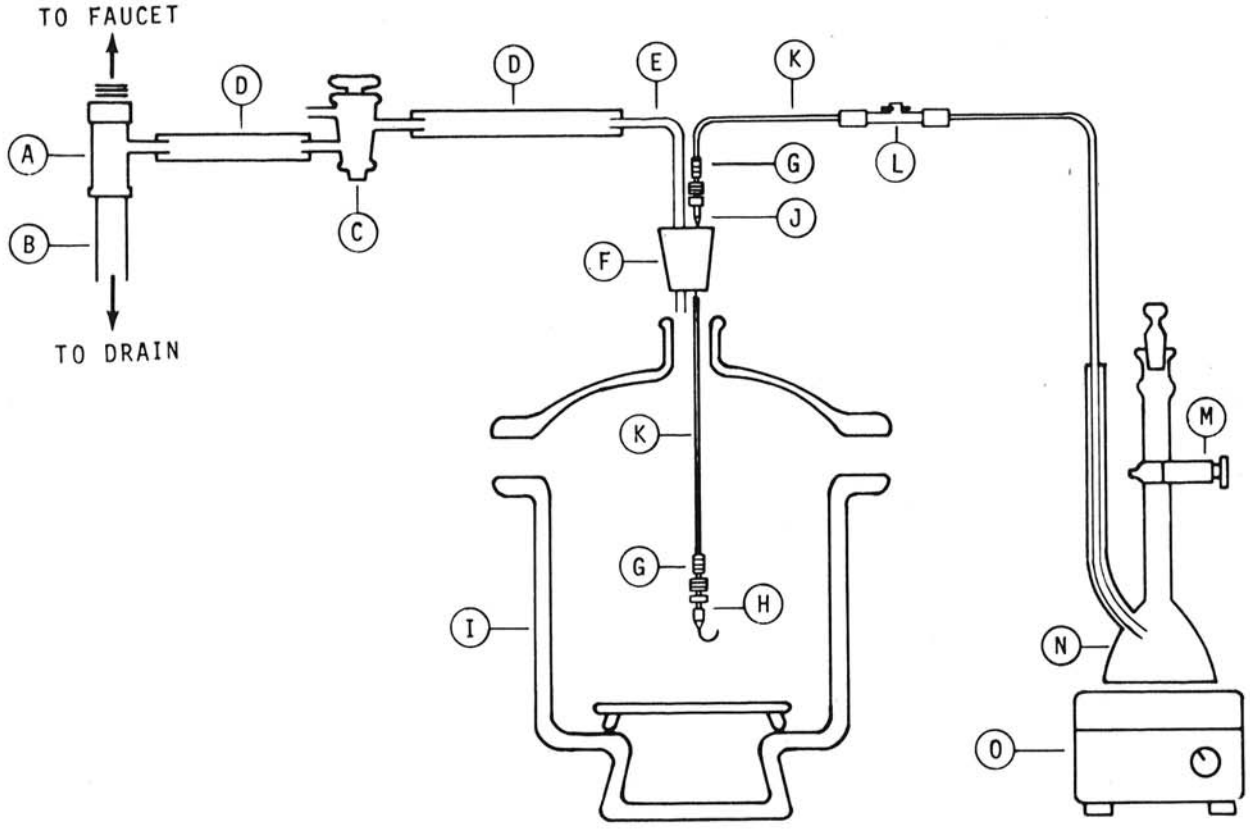

Fig. 1. Vacuum injection inoculation apparatus and component parts. **A)** Brass aspirator filter pump. **B)** Tygon tubing [9.5 mm (3/8 in.) ID]. **C)** Three-way glass stopcock (8 mm OD). **D)** Rubber vacuum tubing (1/4 in. ID). **E)** Glass tubing (8 mm OD). **F)** Rubber stopper (No. 6). **G)** Plastic tubing to male Luer-Lok adapter (Clay Adams, B7551). **H)** Hypodermic needle (No. 25, 5/8 in.). **I)** Scheibler desiccator (250 mm) with tubulated cover for No. 6 rubber stopper. **J)** Hypodermic needle (No. 18, 2 in.). **K)** Polyethylene tubing (.045 ID - .062 OD). **L)** Chromaflex straight valve (Kontes K423500-0000). **M)** Buret meniscus reader (Fisher 3-872). **N)** Cassia volumetric flask, 110 ml in 1/10 ml (Kimble 28066) with custom fit 8 mm (OD) capillary tubing sidearm. **O)** Magnetic stirrer.

4% Bacto agar in distilled water]. Cultures were grown on V-8 juice agar in 30-ml disposable plastic culture flasks at 22 C under 2,150 lx (200 ft-c) of continuous light. All inoculations were made with conidia from 20-day-old cultures for maximum virulence (4). Conidia were collected by placing 15 ml of sterile distilled water in each flask and shaking the flask to suspend conidia. Mycelial fragments were separated from conidia by passing the suspension through a 43- to 47-μ micro-sieve. Inoculum concentrations of 10, 100, and 1,000 conidia per 0.2 ml of sterile distilled water were prepared with an automatic particle counter (High Accuracy Products Corp.).

Poa pratensis 'Newport' was used for all inoculations. Plants were vegetatively propagated in a steamed 2:1 (v/v) loam-peat soil mixture in 7.6-cm (3-inch) diam plastic pots and grown in the greenhouse 60 days at 18 to 28 C with a 16-hr daylength supplemented by incandescent lights. Plants selected for inoculation were reduced to two tillers, and all but the three youngest leaves of each tiller were removed. Only tillers with their youngest visible leaf about one-half expanded from the leaf sheath fold were used for inoculations. Each tiller selected for inoculation also was 5- to 6-cm long from its base to the blade-sheath junction of the third leaf (Fig. 2-B). Three separate lots of 20 tillers each were inoculated with each concentration of conidia. Control plants were inoculated with sterile distilled water.

Vacuum injection inoculation apparatus and procedure.—Components of the vacuum injection inoculation apparatus are illustrated in Fig. 1. Individual tillers of *P. pratensis* were inoculated by inserting a hypodermic needle (Fig. 1-H; Fig. 2-C) into the leaf sheaths about 1.5 cm above the crown. The entire plant and pot was then placed in the desiccator and the three-way stopcock (Fig. 1-I, C) was positioned to evacuate the desiccator. After evacuation, the Chromaflex valve (Fig. 1-L) was opened, and 0.2 ml of conidial suspension was siphoned into the sheath fold. When the needle has been placed properly, a droplet of conidia suspension may appear at the inner side of the second leaf at the junction of the blade and sheath (Fig. 2-A). During inoculation, conidia should be kept in suspension by use of a magnetic stirrer (Fig. 1-O), and the polyethylene tubing (Fig. 1-K) should be minimal in length to prevent settling of conidia. After the proper volume of conidia suspension was siphoned into the sheath fold, as seen by movement of the meniscus in the flask with a meniscus reader (Fig. 1-M), the Chromaflex valve was closed, the three-way stopcock opened to release the vacuum, and the plant was removed from the desiccator. The procedure was repeated for each tiller inoculated. Inoculated plants were placed in growth chambers at 22 C (± 2 C) with a 16-hr daylength ca. 19,380 lx (1,800 ft-c). After 4 to 5 days, the youngest leaf of each inoculated tiller was pulled from the tiller and examined under a dissecting microscope for developing lesions. The mean number of lesions on the youngest visible leaf of each group of inoculated tillers were counted, and the efficiency of each inoculation was expressed as a percentage of the number of conidia used for inoculation. Infected tissue around needle holes was counted as one lesion. Disease severity was determined on the basis of number of countable lesions and leaf blighting. Leaves were considered blighted when any portion of the blade was killed from margin to margin.

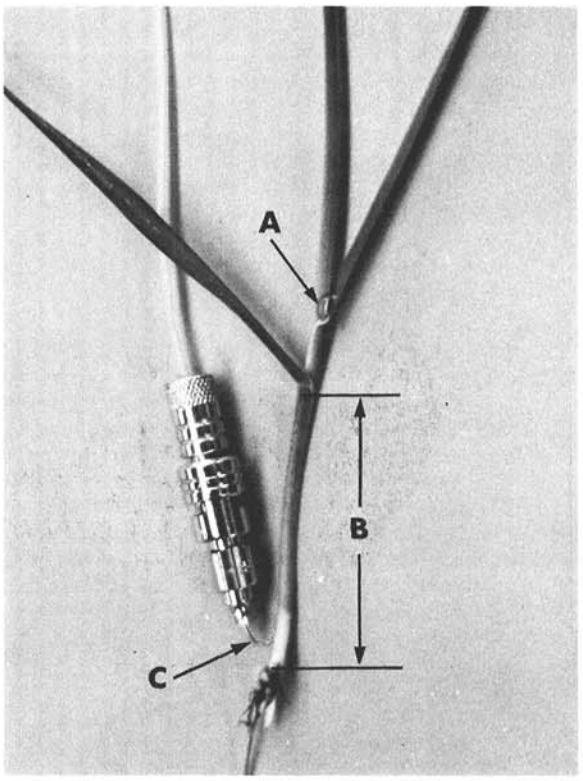

Fig. 2. Placement of hypodermic needle into tiller for vacuum injection inoculation. **A)** Droplet of conidia suspension that may appear at the junction of the blade and sheath of the second leaf when needle (C) has been inserted properly. **B)** Tillers selected for inoculation should be 5-6 cm long from their base to the blade - sheath junction of the third leaf. **C)** Hypodermic needle should be inserted into the sheath fold of the tiller about 1.5 cm above the crown.

RESULTS.—Disease severity increased on the youngest visible leaf as inoculum concentration was increased from 10 to 1,000 conidia per tiller. Mean number of lesions increased as conidia concentration increased; the greatest number of lesions occurred on the youngest leaves of tillers receiving 1,000 conidia (Table 1). Tillers from all experiments inoculated with 100 and 1,000 conidia produced 4 and 10 blighted leaves, respectively, within 4 to 5 days after inoculation (Fig. 3-D, E). Seven days after inoculation of tillers with 100 and 1,000 conidia, individual lesions on many of the youngest leaves were not discernible because of extensive blighting.

Although disease severity and the mean number of

TABLE 1. Mean number of lesions on the youngest visible leaf of *Poa pratensis* and lesion-to-conidia efficiency of vacuum injection inoculations with three concentrations of conidia

Expt. no.	Number of conidia injected into each tiller[a]					
	10		100		1,000	
	Mean[b]	Efficiency[c] (%)	Mean	Efficiency (%)	Mean	Efficiency (%)
1	4.3	43.0	7.3	7.3	15.9	1.6
2	3.9	39.0	7.7	7.7	13.7	1.4
3	4.1	41.0	9.2	9.2	19.4	1.9

[a] Each concentration of conidia was injected into tillers in 0.2 ml of sterile distilled water.

[b] Each mean was determined from 20 tillers from each experiment and each concentration of conidia.

[c] Efficiency was determined on the basis of the mean number of countable lesions expressed as a percentage of the number of conidia applied to each tiller.

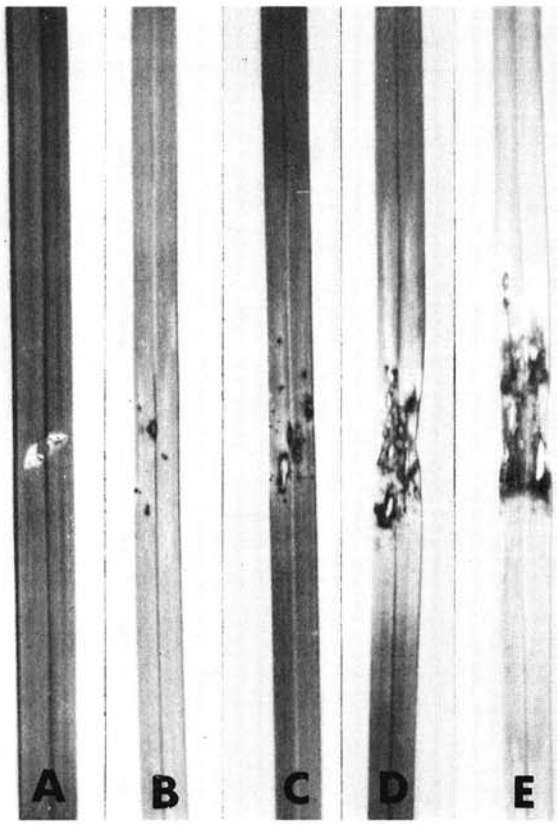

Fig. 3. Lesion development on *Poa pratensis* leaf blades following vacuum injection inoculation with three concentrations of *Helminthosporium sorokinianum* conidia. **A)** Control showing needle puncture. **B)** Ten conidia. **C)** One hundred conidia. **D)** One thousand conidia; 4 days after inoculation with some individual lesions still discernible. **E)** One thousand conidia; 7 days after inoculation leaf is blighted with few individual lesions visible.

lesions produced on the youngest leaves of inoculated tillers increased with greater concentrations of conidia, the lesion-to-conidia efficiency of the inoculations decreased. The mean number of lesions increased as conidia inoculum was increased from 10 to 1,000; however, the mean number of lesions as a percentage of the number of conidia applied to each inoculated tiller decreased (Table 1). The most efficient lesion-to-conidia inoculations occurred on tillers inoculated with 10 conidia (Table 1; Fig. 3-B).

DISCUSSION.—Vacuum injection inoculation is quantitatively repeatable, and it eliminates potential saprophytic development of *H. sorokinianum* on aging leaves. Maximum efficiency, on the basis of lesions to conidia applied, ranged from 39 to 43% on the youngest leaves of the three lots of tillers inoculated with 10 conidia (Table 1). Although this percentage range accounts for less than half the conidia applied, it shows that the inoculation procedure is quantitatively repeatable on a lesion-to-conidia basis at low concentrations of conidia. Of the conidia not accounted for on an individual lesion basis, it is probable that some failed to germinate or did not infect after germination; others may have infected leaf sheaths instead of the expanding youngest, visible leaf; and several conidia may have infected the tissue injured by the puncture of the hypodermic needle.

Vacuum injection inoculation eliminates potential saprophytic development of *H. sorokinianum* on aging leaves. Evaluation of lesion development irrespective of leaf age probably is not representative of the true parasitic and subsequent pathogenic potential of *H. sorokinianum*. Research with other perennial grasses indicates that each older leaf has a lower rate of photosynthesis than its successor (5) and that actively growing leaves retain their assimilates and import assimilates from older leaves (10). In that only the three youngest leaves were retained on inoculated tillers, and that evaluation of infection and lesion development was done on the youngest visible leaf, potential saprophytic development of lesions on aging leaves was eliminated, and all lesion development on the youngest leaf was representative of the parasitic ability of the pathogen. Exclusive use of the youngest leaf for determining the degree of infection also has the advantage of minimizing the time between inoculation and evaluation of infection.

The vacuum injection inoculating system has several potential uses for studying leaf-infecting pathogens of grasses. The quantitative repeatability and rapid evaluation of infection suggest that this inoculation system might be useful for rapid screening of relatively small numbers of plants for resistance to *H. sorokinianum* and other leaf-infecting organisms. The system may also provide a more accurate means of evaluating how various cultural practices applied to grasses may influence leaf-infecting organisms. It is probable that vacuum injection could be useful in studying interactions of organisms; i.e., various organisms might be intermixed and injected to determine whether a given pathogen is

enhanced or inhibited. Such inoculations also could be of value in studying potential "disease complexes" incited by two or more pathogens. There is also the potential for examining the succession of organisms often associated with grass diseases; i.e., the progression of organisms ranging from parasites to possible parasites to saprophytes.

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