Microhumidity Chamber for Quantitative Inoculation of Attached Corn Leaves with Fungal Pathogens

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ABSTRACT

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A technique is described by which fungal spores can be deposited in a defined area on the surface of a corn leaf. This is accomplished by placing inoculum in cylindrical wells cut in a clear acrylic plastic sheet that rests on the upper leaf surface. Spores of the corn pathogens *Colletotrichum graminicola* and *Helminthosporium maydis* were uniformly distributed over the inoculation surface, and symptoms characteristic of each disease developed as expected. Moreover, infection occurred and symptoms developed without the addition of a wetting agent to the spore suspension. Applications of the technique are discussed.

Additional key words: *Zea mays*.

A common method for inoculating foliage with fungi is by spraying spore suspensions onto leaf surfaces followed by incubation of plants in humidity chambers. Spore concentration can be easily quantified in inoculum suspensions, but uniform and repeatable deposition of propagules over the leaf surface is difficult to ensure with inexpensive spray apparatus such as hand-held atomizers. An alternative method is to place droplets of defined inoculum concentration on specific areas of the leaf surface. Such methods are particularly useful on relatively small plants (3).

Settling towers that rely on random dispersal of spores have found wide application in quantitative inoculation of leaves with spores of the rust fungi (6). However, the technique is not convenient for use with aqueous spore suspensions, is best suited to inoculation of relatively small plants, and is not useful for the inoculation of a specific site.

The quantitative inoculation method of Schein (4) was a significant advance in the inoculation of leaves with aqueous spore suspensions because it allowed for accurate and repeatable deposition of any desired density of fungal spores on defined areas of foliar surface, and it allowed for different dosage reactions to be measured on the same leaf. However, the apparatus is not easily adaptable for use in the field and is expensive.

In studies in which the role of chemical substances on the plant-pathogen interaction is to be assessed, it is desirable to place droplets of defined volume on the leaf surface. Droplets are easily disturbed and after the droplet dries down the inoculation site, it may be difficult to locate. Van Tieghem cells (6) stuck to leaf surfaces with lanolin or other cementing agents can serve as effective inoculation chambers. The researcher must be certain, however, that the cementing agent does not alter normal spore germination, appressorium formation, or penetration.

The described technique was developed for the quantitative inoculation of corn leaves, using inexpensive materials suitable for use with plants of various sizes under a variety of environmental conditions.

MATERIALS AND METHODS

Microhumidity chamber. A diagram of the microhumidity chamber (MHC) is shown in Fig. 1. The upper and lower plates of the chamber were made of clear acrylic plastic. The lower plate was cut from 5-mm-thick sheeting. A groove (3 mm deep by 5 mm wide) cut in the center of the plate served as a seat for the leaf midrib; this anchored the leaf blade in place and prevented tissue from being crushed after the chamber was closed. A soft foam (polyethylene type) backing (3 mm thick) was placed between the lower plate and the leaf blade as a cushion. The upper plate was cut from 3-mm-thick sheeting. Holes bored in the plate served as inoculation wells. Although the diameter of the holes may be varied, we found that 4–5 mm was a convenient size. Spacing of the inoculation wells may be varied according to experimental needs. In Fig. 1 they are spaced 15 mm apart.

Inoculation procedure. For inoculation, the plant was positioned beside a frame or other adjustable support such as a ring stand on which the MHC was rested. A leaf was clamped in place between the layers of the MHC with no. 20 binder clips (IDL & Sales Corp., Carlstadt, NJ 07072) that had been spread open so that they just held the MHC together and did not crush the leaf tissue. The enclosed leaf was then placed in a horizontal position.

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Spore suspensions of desired concentration were pipetted (up to 20 μl) into inoculum wells, and the wells were sealed with Scotch Magic transparent tape (3M, St. Paul, MN 55144) to prevent evaporation. After the desired incubation period, the tape was removed to allow the droplet to evaporate. The apparatus was then dismantled, and symptom progress was followed.

Spore distribution. Uniform spore distribution is an important criterion for any inoculation procedure. Most methods of surface inoculation of corn leaves also include a wetting agent to promote uniform spore distribution. Thus, we wanted to determine whether spores in inoculum droplets would distribute themselves uniformly over the exposed leaf surface without the aid of a wetting agent.

To test these parameters we selected spores of the corn pathogens *Colletotrichum graminicola* (Ces.) Wils. and *Helminthosporium maydis* Nisik. and Miy. Spores were collected from cultures grown either on oatmeal agar (*C. graminicola*) or potato-dextrose agar (*H. maydis*) for 14 days at 24 C in the light (60 $\mu E \cdot m^{-2} \cdot s^{-1}$ across the range 400–700 nm). Suspensions of *H. maydis* spores were prepared with and without Tween 20 (20 μl/100 ml) at concentrations of 10^3, 7.5×10^3, 1.5×10^4, and 3.0×10^4 spores per milliliter. Suspensions of *C. graminicola* were similarly prepared, except that concentrations were 10^5, 2.5×10^5, 5×10^5, and 10^6 spores per milliliter. The third leaf of 3-wk-old seedlings of the corn hybrid $B73_{Ht} \times Va26_{Ht}$ was inoculated with 20-μl aliquots as described above.

Inoculation sites were photographed, and spore distribution was measured by dividing the photographs of the inoculated leaf surfaces into uniform grids. For *H. maydis*, spores were counted in 100 grid areas of 0.04 mm^2 each for each of four inoculation sites. Distribution of *C. graminicola* spores was similarly estimated, except that appressoria were counted in 60 areas of 0.01 mm^2 each for each of four inoculation sites.

RESULTS AND DISCUSSION

A drop of inoculum placed in a chamber well dispersed evenly across the exposed leaf surface. Figure 2a and b show that spores in the inoculum droplet distributed themselves evenly over the tissue. The black dots in Fig. 2a are pigmented appressoria of *C. graminicola* (5), the spores of which are hyaline and not readily visible on the leaf surface. Figure 2b shows spores of *H. maydis* on the leaf surface. Uniform spore distribution occurred at both low and high spore concentrations. High spore concentrations were used so that spores and appressoria would be more easily observed in the photographs represented by Fig. 2a and b. Such high spore concentrations are neither necessary for use of the MHC nor desirable for the investigation of either disease interaction. It was important that the MHC be positioned horizontally to ensure uniform spore distribution. At $P = 0.05$, distributions of *H. maydis* spores (inoculum level of 3×10^4 spores per milliliter) were 1.71 ± 0.30, 1.57 ± 0.27, 1.49 ± 0.28, and 1.50 ± 0.24 spores per 0.04 mm^2. The range of spores deposited in each area of 0.04 mm^2 for the above distributions was 0–7, 0–5, 0–7, and 0–5, respectively. At $P = 0.05$, distributions of appressoria of *C. graminicola* (inoculum level of 5×10^5 spores per milliliter) were 3.47 ± 0.51, 3.72 ± 0.48, 4.13 ± 0.51, and 4.13 ± 0.52 appressoria per 0.01 mm^2. The range of appressoria in each area for the above distributions was 0–9, 0–7, 0–8, and 0–9, respectively. The difference between the number of *C. graminicola* appressoria expected (based on spores applied) from the number observed resulted because not all spore germlings formed appressoria.

Figure 3 shows lesion development caused by *H. maydis* 8 days after inoculation using the MHC.

Problems encountered with the MHC were minor. The most

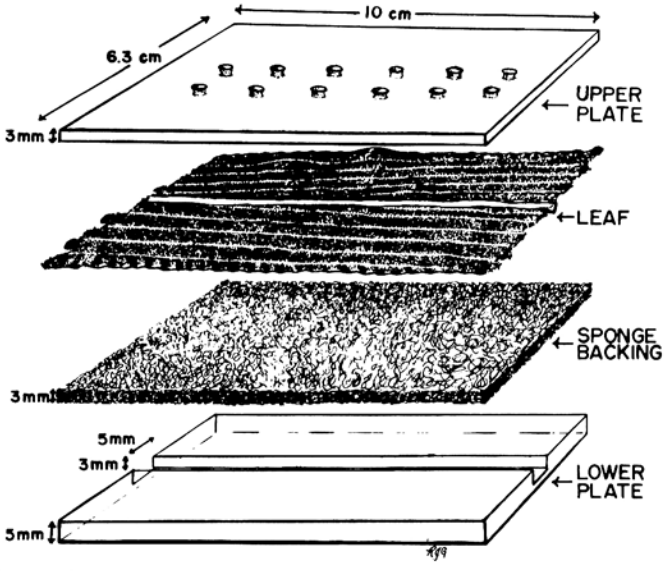
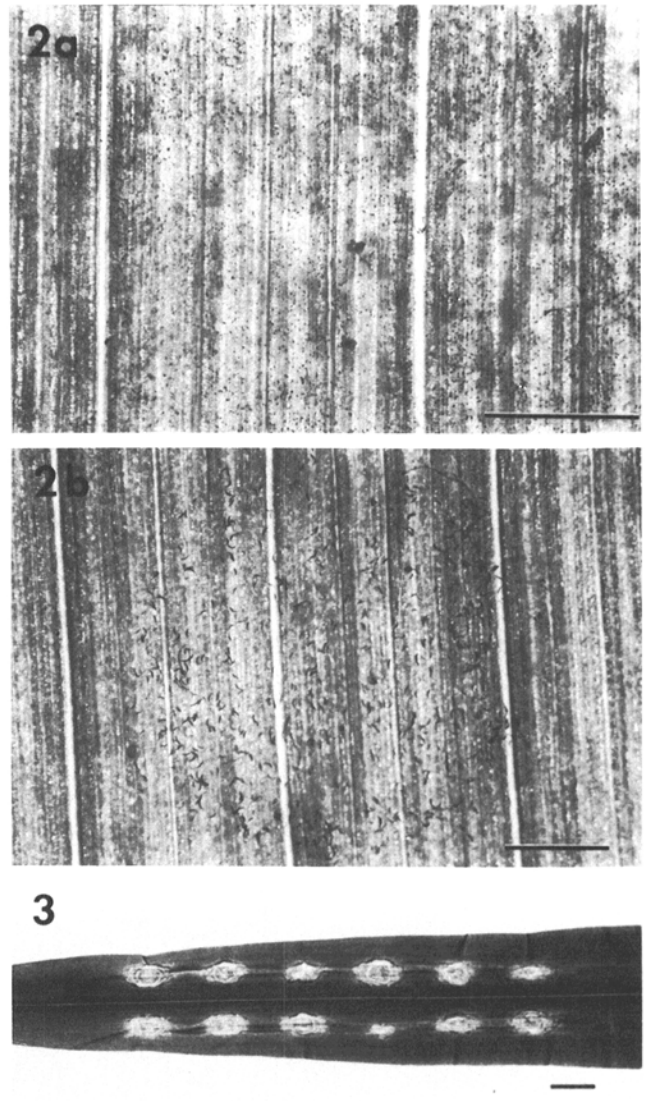


Fig. 1. Diagram of microhumidity chamber.



Figs. 2 and 3. 2, Distribution of spores at the inoculation site of a corn leaf using the microhumidity chamber. Magnification bars represent 1 mm. **a,** Black dots on the leaf surface are the pigmented appressoria of *Colletotrichum graminicola*. Spores applied in 20 μl of 10^6 spores per milliliter suspension without a wetting agent. Photographed at 18 hr after inoculation. **b,** Spores of *Helminthosporium maydis* applied in 20 μl from a 3×10^4 spores per milliliter suspension without a wetting agent. Photographed 2 hr after application. **3,** Symptoms caused by *Helminthosporium maydis* 8 days after inoculation using the microhumidity chamber. Arrows indicate inoculation sites where spores were applied. Bar represents 1 cm.

frequent problem was nonwettability of the cuticle surface, a result of the presence of epicuticular wax (1,7). If necessary, this can be easily remedied on corn leaves by rinsing the leaves and allowing them to dry before inoculation, or by filling the wells with water, suctioning off the water, and repeating the process several times.

Because wetting agents alter the hydrophobicity of the leaf surface, they might also alter the normal course of tropic responses that result in the successful penetration of the host by a spore germling (7). Also, surfactants stimulate enzyme synthesis by several fungi (2), and this should be a concern to plant pathologists since the enzymes stimulated include some of those that degrade cell wall components. In the present study, use of the wetting agent did allow for a more rapid wetting of the inoculation site; however, for the fungi and conditions reported, we have not observed differences in spore distribution or patterns of disease development when the MHC was used either with or without the wetting agent.

Problems with fluids leaking out of the wells by capillary action were encountered only occasionally. (When this occurs, one can easily remove the MHC and start again, or use other chambers on the plate.) When the MHC was clamped together securely, liquid from inoculation droplets did not flow beyond the intended inoculation surface.

Occasionally the inoculum droplet adheres to the wall of the inoculation well. This is particularly a problem where the diameter of the well is less than 5 mm. Maintaining the inoculum droplet in place was aided by spraying the walls of the wells of the MHC upper plate with MS-122 release agent (Miller-Stephenson Chemical Co., Inc., Danbury, CT 06810). This spray had no apparent influence on spore germination or the symptoms of disease development expected of the two pathogens tested.

Although the MHC is not the answer to all problems of inoculation of leaves with fungi, it is a simple and inexpensive tool, and it should have wide application in phytopathological research. Inoculation of mature plants such as corn is awkward if whole plants must be incubated in humidity chambers, and inoculation of detached plant parts may give results quite different from intact plants. The MHC also can be used for field inoculations, an awkward application for most other quantitative inoculation techniques.

Though the MHC is basically an adaptation of the Van Tieghem cell, it allows for more precise placement of treatment cells, and does not require the use of cementing agents. It also has advantages over the Schein (4) inoculator in that it can be used with small volumes of materials that are expensive or hard to acquire and that would be wasted in large spray volumes. Such materials could include enzymes, fungicides, host-specific toxins, or growth regulators that may influence the host-parasite interaction. A significant advantage is that the MHC allows screening the same plant for reaction to varied dosage of one or more pathogens on the same or different leaves.

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