

A quantitative confined inoculation method for studies of pathogenicity of fungi on plants

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Abstract. A technique for inoculation with precise numbers of fungal spores on leaves and stems of plants was developed. The technique consisted of placing 1- μ l drops with a fixed number of spores on the surface of leaves and stems, and covering each inoculum drop with a 10- μ l drop of low-temperature gelling SeaPlaque agarose to fix the inoculum on the target site. With this technique single zoospores of *Phytophthora capsici* were able to cause local lesions on leaves and stems of peppers, and the size of the lesions directly correlated with the number of spores in the inoculum drops. Similar results were obtained when the technique was used to inoculate taro leaves with zoospores of *Phytophthora colocasiae* and black mustard leaves with *Alternaria brassicae*. This method has the advantages of being accurate and precise, and it is also easy to handle the inoculated plants. It may also be applicable to other pathogens.

Keywords: Black mustard; Confined inoculation; Conidia; Pepper; Taro; Zoospores.

Introduction

Establishment of disease by artificial inoculation is essential for studies of various aspects of plant pathology, including epidemiology, etiology, disease resistance, host-parasite interaction and disease control. The conventional methods of depositing prescribed amounts of fungal inoculum on plant surfaces via a settling tower or horizontal spraying have the disadvantage of causing considerable variation in spore distribution (Tuite, 1969). Although accuracy and precision were improved by applying a drop of inoculum with a modified hypodermic needle (Lapwood and Mckee, 1966) or capillary pipette (Toussoun et al., 1960), the number of spores in each drop was indeterminate. Moreover, it is difficult to maintain the inoculum drop in a fixed position. In this report, we describe a simple procedure to apply an inoculum drop containing a precise number of spores with a microliter pipette and stabilizing the drop on a target area with low-temperature gelling agarose.

Materials and Methods

Microorganisms

Alternaria brassicae (Berk.) Sacc. (XL-11) was derived from a conidium produced on a diseased leaf of black mustard [*Brassica nigra* (L.) Koch]. *Phytophthora capsici* Leonian (XL-12) and *Phytophthora colocasiae* Racib. (XL-10) were isolated from pepper (*Capsicum annuum* L.)

stem and taro [*Colocasia esculenta* (L.) Schott] leaf, respectively, with a selective medium (Ko et al., 1978).

Production of Spores

Conidiation of *A. brassicae* was induced by growing the fungus on V-8 agar (10% V-8 juice, 0.02% CaCO₃ and 2% agar) at 24°C for 3–4 days under cool white fluorescent light (2,000 lux) followed by 2 days incubation at 18°C (Aragaki, 1964). A conidial suspension was prepared by placing three pieces of culture blocks (ca. 5 × 5 × 3 mm) in 5 ml wheat grain extract in a test tube, and by agitating the test tube for 1 min with a Vortex mixer. Wheat grain extract was prepared by autoclaving whole wheat grains in distilled water at the ratio of 1 to 6 by volume. Only the top layer of clear extract was used. *Phytophthora capsici* and *P. colocasiae* were separately grown on V-8 agar at 24°C for 4 days under light to induce sporangial formation (Aragaki and Hine, 1963). Zoospore suspension was obtained by transferring two pieces of culture blocks (ca. 20 × 10 × 3 mm) from each species to 5 ml sterile distilled water in a plastic Petri plate (60 mm diam.) and placing the plates at 5°C for 30 min to induce zoospore release from sporangia.

Host Plants

Seedlings of black mustard and cv. California Wonder of pepper were established from seeds planted in Supersoil potting mix in rectangular plastic pots (6.5 × 6 × 6 cm), while those of taro were established from suckers planted in the same medium in round plastic pots (17 cm diam., 12.5 cm high). All the test plants were fertilized with Osmocote 14-14-14 and used after 1 to 2 months.

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Inoculation

The spore concentration in the suspensions was adjusted to approximately the desired number per μl as described by Ko et al. (1973) with a Pipeman microliter pipette (P-20D, West Coast Scientific, Inc., Oakland, CA) (Ko and Nishijima, 1985). Five 1- μl drops of the spore suspension were then placed on a glass slide and examined under the microscope. Those drops with the exact number of spores needed were individually transferred with the microliter pipette to leaves or stems about 3 to 5 cm about the pots and temporarily positioned horizontally during inoculation. The emptied spots on the glass slide were examined again with the microscope to assure that all the spores in each drop were transferred. One drop per leaf or stem was used.

SeaPlaque agarose (FMC Bioproducts, Rockland, Maine) which gels at about 30°C, was used to stabilize inoculum drops on target areas to prevent injuring to spores due to high temperature. Five ml of 0.8% agarose in a test tube was heated in a microwave oven at high power for 40 sec to melt the agarose. The test tube was then placed in 150-ml water bath at about 32°C in a 250-ml beaker to keep the agarose in the liquid state at this temperature. Each inoculum site on the leaf or stem was covered with 10 μl agarose. The agarose drop appeared as a clear dome on the plant surface. The agarose solidified and fixed the inoculum on the target site within 5 min. Inoculated plants were placed in plastic bag moist chambers (50 cm diam., 90 cm high), each with 100 ml water on the bottom, and kept on the laboratory floor at 24°C for observation at different time intervals. Three plants were used for each treatment and the experiments were repeated at least once with similar results.

Results and Discussion

Agarose drops on plant surfaces will dry up at different rates under different environmental conditions. An estimate of these rates were determined on inoculated plants placed on a screenhouse bench, laboratory floor, or in plastic bag moist chamber on laboratory floor. Agarose drops on both leaves and stems retained moisture for 3 h under the three conditions tested (Table 1). However,

Table 1. Maintenance time of agarose drops on leaves and stems under different conditions.

Condition	Maintenance time (h)				
	3	5	24	48	72
Leaves					
Screenhouse	+ ^a	-	-	-	-
Laboratory	+	-	-	-	-
Plastic bag	+	+	+	+	+
Stems					
Screenhouse	+	-	-	-	-
Laboratory	+	-	-	-	-
Plastic bag	+	+	+	+	+

+^a, original dome shape; -, dried and formed a thin film.

those exposed to open air in the screenhouse and laboratory became dry and formed a thin film on each target site after 5 h, while those in the plastic bags retained moisture during the entire test period of 72 h.

To determine if the agarose drop can adequately cover the inoculum site, 1- μl drops of yellow food color (Schilling, McCormick & Co., Hunt Valley, MD) were pipetted onto the surfaces of pepper leaves and stems. Each color drop was overlaid with 10 μl agarose. The food color was confined to the agarose drop in every test, indicating that the agarose drops are suitable for use in confining the inocula to the target sites.

To determine if the spores in the agarose drops can infect host plants, 1- μl inoculum drops containing 1, 3, or 5 zoospores of *P. capsici* were placed on pepper leaves and stems and covered with agarose drops. The inoculated plants were enclosed in plastic bags. No symptoms were observed on leaves after 1 day (Table 2). After 2 days, water-soaked lesions developed on all the inoculated leaves. Only those leaves inoculated with 5 zoospores dropped after 3 days. All the inoculated leaves dropped on the 4th day. Inoculated stems did not develop local lesions after 2 days (Table 2). Only those stems inoculated with 5 zoospores developed symptoms after 3 days. On the 4th day, those inoculation sites on stems with 1 or 3 zoospores each developed into water-soaked lesions, while those inoculated with 5 zoospores each developed seedling blight. The results show that with the method developed even a single zoospore of *P. capsici* can infect and cause local lesions on leaves and stems of pepper. Therefore, it can be a useful technique for quantitative studies of disease of plants.

Table 2. Symptom development on pepper leaves and stems after confined inoculation with zoospores of *Phytophthora capsici*.

Zoospore no./inoculum drop	Days after inoculation			
	1	2	3	4
Leaves				
1	- ^a	L	L	F
3	-	L	L	F
5	-	L	F	F
Stems				
1	-	-	-	L
3	-	-	-	L
5	-	-	L	D

^a-, no symptoms; L, water-soaked lesion; F, leaf drop; D, seedling blight.

Table 3. Lesion development on pepper leaves after confined inoculation with zoospores of *Phytophthora capsici*.

Zoospore no./inoculum drop	Lesion size (mm, diam.) after	
	4	5 (days)
1	0	0
5	4.3 ± 1.3	22.0 ± 2.9
25	19.0 ± 2.9	34.5 ± 4.9

Table 4. Lesion development on taro leaves after confined inoculation with zoospores of *Phytophthora colocasiae*.

Zoospore no./inoculum drop	Lesion size (mm, diam.) after	
	3	4 (days)
1	0	0
5	13.5 ± 4.9	18.0 ± 5.7
25	18.0 ± 0.7	24.5 ± 0.7

To determine the effects of humidity and inoculum density on disease development, inoculum drops containing 1, 5, or 25 zoospores of *P. capsici* were placed on pepper leaves and covered with agarose drops. Inoculated plants were enclosed in plastic bags for 1 day before exposure to open air in the laboratory. Leaves inoculated with single zoospores did not develop disease symptoms. However, those inoculated with 5, or 25 zoospores developed expanding lesions with sizes positively correlated with spore numbers (Table 3). Results suggest that the method developed can be used to study inoculum potential and disease development under controlled environments. With this method moisture can also be used to regulate spore penetration, but not disease development after penetration.

To determine if the method developed can be applied to other pathogens, inoculum drops containing 1, 5, or 25 zoospores of *P. colocasiae* were placed on taro leaves and covered with agarose drops. Inoculated plants were incubated in moist chambers for 2 days before exposure to open air. Under such conditions, single zoospores did not cause any symptoms. However, those inoculated with 5 or 25 zoospores developed expanding lesions with sizes positively correlated with spore numbers (Table 4). Leaves of black mustard were similarly inoculated with inoculum drops containing 1, 5, or 25 conidia of *A. brassicae*. In this case, single conidia were able to induce the development of local lesions. The lesion size also correlated positively with spore number (Table 5).

The procedure described in this report has the advantage of being able to inoculate plants with exact numbers of spores on target sites positioned at different angles. It

Table 5. Lesion development on black mustard leaves after confined inoculation with conidia of *Alternaria brassicae*.

Conidium no./inoculum drop	Lesion size (mm, diam.) after 4 days	
	Exp. I	Exp. II
1	1.0 ± 1.4	1.3 ± 1.5
5	5.0 ± 2.2	6.3 ± 1.5
25	11.8 ± 1.3	10.5 ± 2.4

is also easy to handle plants inoculated with this method. The technique may also be applicable to other types of pathogens such as bacteria or nematodes.

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