Use of Oil and Liquid Nitrogen for Quantitative Work with Helminthosporium maydis Race T

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ABSTRACT

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Standardized inoculations with *Helminthosporium maydis* race T were possible over time when conidia were produced at one time, collected dry, stored in liquid nitrogen, dispensed by weight, suspended in oil, and sprayed onto plants with an aliquot inoculator.

Additional key words: southern corn leaf blight, *Zea mays*.

Many types of studies on plant diseases call for precise quantitative inoculations. For such inoculations, it is necessary to have high quality inoculum, a uniform means of applying inoculum to plants, and precisely controlled environmental conditions. This allows different treatments of the same experiment to be conducted at different times (12).

Some quantitative studies on cereal rust fungi have involved producing a large quantity of uredospores at one time (12), storing them dry in liquid nitrogen (5, 6, 7, 12), and inoculating plants with a quantitative inoculator using a known weight of spores suspended in oil (12, 14). Most work on *Helminthosporium* has involved producing spores as needed on agar (4, 10, 17), filter paper (2, 8, 17), or leaves (10, 17); determining the concentration by counting the number of spores/unit area or volume (2, 4, 10); suspending the spores in water (2, 4, 10, 17) or mixing them with talc (3, 11, 16); and spraying (4, 10, 17) or dusting (3, 11, 16) them on plants by hand. The talc method is good and does not require sophisticated equipment, but it requires more spores than the oil method and increases the chances of airborne contamination from one pot or culture to the next; oil can confine spores better. Also, it may be desirable to have a more permanent type of inoculum storage, such as a liquid nitrogen cryostat. For example, rust fungi lose some germinability and vigor in a few months at 4 C. Although *Helminthosporium* spores deteriorate more slowly, they, like other fungal spores, will eventually lose some germinability or vigor. Because oil is a more efficient carrier than talc, it serves very well with the small vials of spores stored in liquid nitrogen.

The purpose of my study was to determine whether, in addition to the methods already accepted for *Helminthosporium* research, some of the techniques that work well for the cereal rust fungi could be used for quantitative studies with *Helminthosporium maydis* Nisikado & Miyake race T.

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MATERIALS AND METHODS

Host.—Five seeds of the susceptible corn inbred W64A*cms*T were planted in a sand:field-loam:peat (1:2:1,v/v) mixture in a circle in 10-cm clay pots. The seedlings, grown on a greenhouse bench, were inoculated about 2 wk after planting when the plants were in the three- to four-leaf stage.

Inoculum.—*Helminthosporium maydis* race T was supplied by C. A. Martinson, Iowa State University at Ames. It was isolated from a single lesion on an ear of corn found near Mitchellville, Iowa, in 1969. An infected kernel from this stored ear was placed on moist filter paper in a petri dish for sporulation; then I transferred the resulting spores en masse to corn-leaf agar (CLA) which was made by autoclaving a 125-ml volume of seedling corn leaves in 875 ml of distilled water for 15 min, straining the broth through cheesecloth, and adjusting the volume to 1 liter. The broth was amended with 30 g of sucrose and 20 g of agar and autoclaved for 15 min. Subcultures were produced by mass transfer at 1-wk intervals. After spores had formed, the petri dish lid was removed so that the agar and spores would dry quickly. Spores were harvested with a cyclone separator (15).

Plants were inoculated with spores from the first CLA culture. A few days later, leaf pieces with lesions were removed and placed on moist filter paper in a petri dish at room temperature. After spores had formed and dried, they were harvested with a cyclone separator. Most spores were collected from the filter paper surrounding the leaf tissue.

Conidia also were produced on filter paper according to Lukens' method (8), but with modified Fries medium (13) that was further modified to contain 20 μg/ml $FeSO_4 \cdot 7H_2O$. Mycelium from the liquid medium was macerated in a blender, washed, resuspended in 0.1 M phosphate buffer at pH 6.4, and spread on dry filter paper in petri dishes. After spores had formed and dried, they were harvested with a cyclone separator.

Some spores collected from CLA, corn leaves, or filter paper were sealed in 5-mm-wide glass vials and stored in a liquid nitrogen cryostat at −196 C (7). The remaining spores were stored dry at room temperature.

Inoculation and incubation.—The plants in all experiments were inoculated quantitatively with spores suspended in Soltrol 170, a nonphytotoxic oil (manufactured by the Phillips Petroleum Co., Bartlesville, OK 74003), by means of an aliquot inoculator (J. A. Browning, M. D. Simons, and G. D. Booth, *unpublished*). The method of inoculation was similar to that used by Politowski and Browning (12) to inoculate oats with *Puccinia coronata avenae*. The plants revolved at 20 rpm 60 cm from the atomizer that was set to spray for 9 sec. When the oil had evaporated, the plants were placed dry in the dew chamber.

A dew-deposition environment chamber (1) that maintains temperature within ± 0.5 C was used with a constant dew-period temperature of 23 C for 16 hr. After incubation, the plants were placed in a 21 ± 3 C greenhouse until lesions developed.

RESULTS AND DISCUSSION

Testing of spores from all methods of production and storage conditions.—Spores stored in liquid nitrogen or at room temperature for 1 yr were tested for germinability on water agar and used to inoculate plants. On removal from liquid nitrogen, most vials of spores were given a 45 C water-bath heat-shock treatment for 5 min to break dormancy, as is required for rust spores (6); other nonheat-shocked vials served as checks. The inoculum concentration was 1 mg of spores per 4 ml of oil.

All spores stored in liquid nitrogen and used with or without heat shock, and those stored at room temperature from dried filter paper and dried leaves, germinated about 100% on water agar. Spores produced on CLA and stored dry at room temperature had only 50% germination after 16 hr at 23 C. Spores from all methods of production and storage conditions produced lesions typical of southern corn leaf blight on plants in 4-5 days. Lesions were crowded; this was caused by the high concentration of spores in the inoculum suspension.

Heat-shock treatment was not necessary to stimulate germination of *H. maydis* spores that had been held in liquid nitrogen storage. Evidently, *H. maydis* spores differed from those of the cereal rust fungi in that cryogenic storage did not induce a heat-reversible dormancy. Because oil is superior to water as a carrier, water was not used in these experiments; the spores do not wet and suspend well in water and they may germinate in water if not used quickly.

It seems advantageous to increase spores for mass storage on agar or filter paper rather than leaves; the fungus, however, should have been isolated recently from diseased material because prolonged culturing of an isolate in vitro may result in loss of virulence (9). I harvested considerably fewer spores from leaves, and had difficulty not harvesting pieces of leaf tissue along with spores.

Production of spores on filter paper resulted in some pieces of filter paper with very few spores whereas other pieces were covered densely. This does not seem to be a serious problem because the fungus obtained from 1 liter of modified Fries medium can be used to seed many pieces of filter paper.

Repeated subculturing on agar resulted in some cultures that produced few spores. This might not have occurred if each subculture had been started from a single spore. However, a large quantity of inoculum can be produced for long-term storage by making many one-generation subcultures directly from the original culture.

Repeatability.—Four pots of corn (four plants per pot) were inoculated using 1 mg of spores in 8 ml of oil. The spores, which were collected from filter paper and freshly removed from liquid nitrogen storage, were used without heat shock treatment. The next day four pots that had been planted 1 day later than the first group were inoculated similarly with spores freshly removed from storage. Lesions were counted 4 days later on the second and third leaves.

The average number of lesions per second leaf was 18 (with a range of 14-23) on both the first and second groups inoculated. The number per third leaf averaged 18 for the first group and 19 for the second group (with a range of 16-22). This indicates good repeatability.

Relationship between spore concentration and resultant lesions.—Effective serial dilutions of 1 mg of spores per 8, 16, 32, 64, 128, and 256 ml of oil were made by diluting the previous concentration by half with oil. The spores, collected from filter paper and stored in liquid nitrogen, were used without heat shock. Three pots of corn (four plants per pot) were inoculated with each concentration. Lesions were counted 4 days later on the second and third leaves.

The average number of lesions per second leaf, starting with the highest concentration, was 19, 13, 9, 6, 3, and 3; that per third leaf was 23, 12, 9, 5, 3, and 3. Even though the numbers were similar for the second and third leaves, the lesions were more widely separated on the third leaf. There was a direct relationship between concentration of spores applied and the resulting number of lesions for the five higher concentrations. The two middle concentrations (1 mg of spores/32 or 64 ml of oil) probably would be best for most work because the resultant lesions were not too crowded.

Comparison with other techniques.—My procedure can be summarized as follows: a quantity of spores was produced at one time on CLA or filter paper, stored in liquid nitrogen, used without heat shock, dispensed by weight, suspended in oil, and sprayed onto plants with a quantitative inoculator. This procedure is believed to have major advantages over earlier methods with dry inoculum or water. First, spores produced at one time and stored in liquid nitrogen are expected to retain high quality (5) so that different treatments of the same experiment can be conducted at different times. Second, oil is superior to water as a carrier because spores wet and suspend better in oil. Third, oil is a more efficient carrier than talc and it can confine spores better than talc. Additionally, for repeatable inoculations, it is much easier to dispense spores by weight than to determine the spore concentration by some other method (e.g., counting the number of spores/unit area or volume). Of course, the spore-talc mixture also can be dispensed by weight.

For small grain seedlings, the most efficient quantitative means of inoculation with rust spores is the

use of an aliquot inoculator with spores suspended in oil (J. A. Browning, M. D. Simons, and G. D. Booth, *unpublished*). The desired concentration of spores and oil can be used each time by simply adjusting quantities. This may be an efficient method for other fungal spores, too.

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