Production and Storage of Inoculum of Cercospora kikuchii for Field Studies

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ABSTRACT


A procedure is described for granulating mycelial inoculum of Cercospora kikuchii by dropwise addition of homogenized mixtures of sodium alginate, kaolin clay, and mycelium into a 0.25 M CaCl₂ solution. After 6 mo under refrigeration, the granular preparations produced profuse viable, infective conidia when air-dried granules were rehydrated and exposed to irradiation from sunlamps (10 min/12 hr) at 25 C. An average of 3.8 × 10⁷ conidia were produced per gram of air-dried granules of four isolates of C. kikuchii. This technique should prove useful for storage and production of large quantities of inoculum when needed for field evaluation of disease resistance and fungicide evaluation.

Additional key words: Cercospora leaf blight, Glycine max, mycoherbicides, purple seed stain.

MATERIALS AND METHODS

Four isolates of C. kikuchii were used: CK-1 (ATCC 36864), CK-2 (ATCC 42151), CK-3 (isolated from a purple-stained soybean in our laboratory), and CK-4 (obtained from H. J. Walters, University of Arkansas, Fayetteville). All isolates were stored in screw-cap test tubes that contained twice-sterilized sandy loam soil. Mycelium of each isolate was transferred to potato-dextrose agar (PDA) (Difco Laboratories, Detroit, MI) that was contained in 100-mm-diameter plastic petri dishes. Five 1,000-ml flasks containing 500 ml of a liquid growth medium described previously (12) were inoculated with a 5-mm³ agar plug taken from the outer edge of an actively growing colony, and were grown for 72–120 hr with constant agitation at 125 rpm at 25 C. The mycelia and growth media were diluted 1:1 (v/v) with 2% (w/v) sodium alginate (Kelgin MV; Kelco, Chicago, IL) and 20% (w/v) kaolin clay (Thiele Kaolin Co., Wrens, GA) in distilled water. This resulted in final concentrations of 1 and 10% for sodium alginate and kaolin clay, respectively. Streptomycin sulfate (125 mg/L) and chloramphenicol (75 mg/L) were added to suppress bacterial growth. The homogenized mixtures of all isolates had a pH of 6.5.

Granules were prepared from the homogenate by modification of previously described techniques (11). A peristaltic pump was used to drip the homogenates into a 0.25 M solution of CaCl₂ contained in a 12-L plastic reservoir. Silicone tubing from the pump head was connected to a plastic funnel (8.5 cm in diameter) that was fitted into the bottom half of a 100-mm-diameter polystyrene petri dish. Silicone tubing (2.0 mm outside diameter) was split lengthwise and fitted around the circumference of each funnel to serve as a gasket between the funnel and the petri dish bottom. Approximately 50 holes, each 3 mm in diameter, were made in the petri dish bottom by using a hot dissecting needle. Granules 2–3 mm in diameter were formed as the alginate-clay-mycelial homogenates were dripped into 0.25 M CaCl₂. The granules were collected using sieves, rinsed with distilled water, spread one layer deep into plastic trays (41 × 27 × 5.5 cm) lined with aluminum foil, and air-dried in a greenhouse for 48 hr at 28–32 C.

Nine samples (1 g each) of each air-dried preparation were rewetted on moistened 9-cm-diameter filter paper in the bottom half of petri dishes. Replicates of three samples immediately were subjected to one of the following lighting conditions that were established in separate incubators: continual darkness at 25 C, alternating 12 hr light/12 hr dark regimes provided by 40 W cool-white fluorescent bulbs at 25 C, or 10 min/12 hr exposures to 275 W
sunlamps (General Electric Company, Cleveland, OH) at 23–26°C. After 7 days, the granules were transferred to 100-ml beakers containing 50 ml of 0.05% Tween-80 [(nonoxyl-20)polyoxyethylene sorbitan monooleate] in distilled water, and stirred for 10 min on a magnetic stirrer to rinse the conidia from the granules. The experiment was arranged in a split plot factorial design with isolates of *Cercospora kikuchii* as main plots and lighting conditions as subplots. The test was repeated three times. Significant differences were determined at P = 0.05 according to Duncan’s multiple range test.

The number of conidia produced on granules was compared to that of conidia produced in petri dishes. Plastic petri dishes containing clear V-8 juice agar were inoculated by pipetting 1 ml of an aqueous suspension containing approximately 1.5 × 10⁵ conidia per ml into each plate and the surfaces of the cultures were rubbed with an index finger to detach the conidia. Conidial concentrations were determined with a hemacytometer. Logarithmic transformation of data of conidial production on petri dishes was necessary according to Bartlett’s test for homogeneity of variances (9).

Injectivity of conidia from each isolate was determined by adjusting numbers of spores to 7.5 × 10⁵ conidia per milliliter in 0.05% Tween-80 in distilled water and spraying soybeans (cultivar Forrest) in the unifoliate leaf stage until run-off (14). Inoculated plants were placed in a dew chamber at 25°C for 48 hr, then moved to subirrigated fiberglass trays in a greenhouse (28–32°C with approximately 12-hr photoperiods), and observed 14 days for disease development. Remaining granules were stored in paper bags at 4°C and tested for sporulation and conidial infectivity periodically during a 6-mo period.

**RESULTS AND DISCUSSION**

Approximately 0.3 L/min of the mycelial, clay, sodium alginate homogenate was processed with the described apparatus. By using multiple pump heads and funnels, this rate could be increased with little effort. Approximately 110 g of air-dried granules were produced per liter of homogenate. Conidiophores developed on the granules within 48 hr after rehydration and conidia were observed within 72 hr.

Variations in conidial production occurred both among isolates and lighting conditions, which corroborates the findings of others (5,8,10,15). Isolates CK-3 and CK-4 produced the most conidia, and CK-2 the least (Table 1). All isolates yielded maximum numbers of conidia when exposed to the sunlamps, producing an average of 3.8 × 10⁶ conidia per gram of air-dried granules. The numbers of conidia produced on granules by isolates CK-1 and CK-2 subjected to diurnal light were intermediate to the numbers produced on granules in continuous darkness or exposed to the sunlamps. The numbers of conidia produced by isolates CK-3 and CK-4 on granules in continuous darkness were not significantly different from the numbers of conidia produced on granules of these isolates exposed to diurnal light, but sporulation on granules of isolates CK-1 and CK-2 in continuous darkness was significantly less than on granules of these isolates exposed to either diurnal light or the sunlamps. Lyda et al (5) found no differences in sporulation of some cultures of *C. kikuchii* that were incubated either in continuous darkness or continuous light; Chen et al (1) reported that sporulation of one isolate of *C. kikuchii* actually increased in continuous darkness as compared to sporulation in continuous light.

Sporulation on V-8 juice agar also varied greatly among isolates and light treatments (Table 2). Cultures incubated in continuous darkness yielded approximately 1.1 × 10⁶ conidia per petri dish, while those incubated under alternating diurnal light or under sunlamps yielded approximately 1.95 × 10⁶ and 2.45 × 10⁶ conidia per plate, respectively (averages of all isolates). The counts that we obtained from V-8 agar plate were similar to those obtained by Yeh and Sinclair (15) and are approximately seven times greater than the counts reported by Chen et al (1). However, our counts were over 20 times greater than those reported by El-Gholl et al (3). Therefore, mycelium from 1 L of growth medium, which would yield approximately 220 g of air-dried granules, could potentially produce the same number of conidia that would otherwise require approximately 20 to 80 petri dishes of V-8 agar to produce, based on our spore counts and that of others.

Conidia of all isolates, produced either on granules or on V-8 agar were uniformly infective on soybeans. Typical lesions (9,13,14) developed on leaves, petioles, and stems within 10 days after inoculation; severely infected plants became defoliated.

The sodium alginate-kaolin clay method of producing inoculum of *C. kikuchii* has advantages over petri dish culture. All isolates of the fungus that were tested sporulated profusely on the granules. In addition, the granules can be produced rapidly and stored in bulk for several months under nonsterile conditions. The materials used in this technique are inexpensive and readily available. This technique, with appropriate modifications, may also be useful for producing inoculum of other pathogens which sporulate only sparingly with existing methods.

**LITERATURE CITED**


ABSTRACT


Soybean seeds were collected from plants either uninoculated or inoculated separately with one of eight isolates of Cercospora sojina. Seeds infected by C. sojina were discolored gray to dark brown. Histopathological and scanning electron microscope studies showed the presence of hyphae of C. sojina within the seed coat tissues of seeds from plants inoculated with all but one isolate. The fungus penetrated seeds both indirectly through pores and cracks in the seed coat and directly through hilar tracheids. In seeds inoculated with four of the isolates and in infected seeds from naturally inoculated plants, hyphal mats in parenchymatous seed coat tissues as well as hyphal aggregates, which varied in size and number, were associated with fungal hyphae. Hyphal aggregates were abundant in the hilar region, moderately common in the seed coat layers, found occasionally on the seed surface and in the space between the seed coat and embryo, and rarely observed in the hypocotyl-radicle axis. Fungal infection was not found in the cotyledons. Hyphae without hyphal aggregates were found in seeds from plants inoculated with three of the isolates.

Additional key words: Cercospora kikuchii, Glycine max, Phomopsis spp.