Production and Storage of Inoculum of *Cercospora kikuchii* for Field Studies

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ABSTRACT

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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 $CaCl_2$ 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 3.8×10^6 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.

Purple seed stain and Cercospora leaf blight are widespread and destructive diseases of soybeans [*Glycine max* (L.) Merr.] (8,13,14). Methodology is needed to enable researchers to produce enough inoculum for use in disease resistance screening. It is difficult to obtain abundant and consistent sporulation of *Cercospora kikuchii* (Matsumoto & Tomoyasu) Gardner, the causal organism, in agar culture. Early researchers claimed that sporulation occurred only on living host tissue (6,7). Sporulation in agar culture first was achieved on carrot-leaf-decoction agar (4). More recently, El-Gholl et al (3) reported that V-8 juice agar was the most suitable medium for sporulation of *C. kikuchii*, as well as for sporulation of several other *Cercospora* species. Roy and Abney (8) also induced sporulation on V-8 juice agar and potato-dextrose agar (PDA); Vathakos and Walters (10) were unable to repeat this work with their isolates of *C. kikuchii*, but reported profuse sporulation on dead-soybean-plant and senescent-soybean-plant agars. Yeh and Sinclair (15) induced sporulation on V-8 juice agar, dead-soybean-plant agar, and carrot-leaf-decoction agar and suggested that these media be used for producing inoculum for field studies.

Cultures of *C. kikuchii* grown in petri dishes are useful for producing inoculum for greenhouse, controlled environment, and small field plot studies, but this method is impractical for large-scale inoculum production for field evaluations of disease resistance and fungicides.

Technology has been developed for mass-producing spores of several different weed pathogens in submerged liquid culture (2), but because all fungi do not sporulate in submerged culture, alternative sporulation techniques also have been developed (12). A method for formulating granules of several fungal pathogens using homogenized mixtures of sodium alginate, fungal mycelium, and kaolin clay has been reported (11).

Because of the economic importance of *C. kikuchii* and the need to produce and store large amounts of inoculum of this pathogen for use in the field, we undertook experiments to determine if the sporulation technology that has been developed for mycoherbicides could be applied to *C. kikuchii*.

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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 mm^2 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 peristalic pump was used to drip the homogenates into a 0.25 M solution of $CaCl_2$ 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 $CaCl_2$. The granules were collected using sieves, rinsed with distilled water, spread one layer deep into plastic trays ($41 \times 27 \times 5.5$ 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

TABLE 1. Sporulation of four isolates of *Cercospora kikuchii* (CK-1 to CK-4) on rewetted sodium alginate-kaolin clay granules[v]

Light treatment	Conidia ($\times 10^5$) per gram of granules[w]			
	CK-1	CK-2	CK-3	CK-4
Continuous darkness	12 f[x]	2 g	39 c	26 d
Diurnal light[y]	26 d	11 f	41 c	25 d
Sunlamps[z]	40 c	20 e	44 b	49 a

[v] Air-dried granules were rewetted and immediately subjected to the various light treatments.
[w] Figures represent the mean of 18 counts; counts were made 7 days after the granules were rewetted.
[x] Values followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test. Statistical analyses were performed on logarithms of numbers of conidia.
[y] Alternating 12-hr fluorescent light/dark regimes.
[z] 10 min/12 hr exposure to sunlamps.

TABLE 2. Sporulation of four isolates of *Cercospora kikuchii* (CK-1 to CK-4) on V-8 juice agar

Light treatment	Conidia ($\times 10^5$) per gram of granules[w]			
	CK-1	CK-2	CK-3	CK-4
Continuous darkness	<1 d[x]	1 d	2 d	2 d
Diurnal light[y]	17 d	236 c	314 b	214 c
Sunlamps[z]	21 d	335 ab	374 a	253 c

[w] Figures represent the mean of eight replications; counts were made 7 days after inoculation with a 1 ml of an aqueous suspension of conidia and mycelial fragments.
[x] Values followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$). Statistical analyses were performed on logarithms of numbers of conidia.
[y] Alternating 12 hr fluorescent light/dark regimes.
[z] 10 min/12 hr exposure to sunlamps.

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 the number 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 1.5×10^5 conidia and mycelial fragments onto the agar surfaces (3). Cultures were subjected to the same temperatures and lighting conditions that the granules received. After 7 days, 10 ml of 0.05% Tween-80 solution was added to 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).

Infectivity of conidia from each isolate was determined by adjusting numbers of spores to 7.5×10^5 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 3.8×10^6 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 1.1×10^5 conidia per petri dish, while those incubated under alternating diurnal light or under sunlamps yielded approximately 1.95×10^7 and 2.45×10^7 conidia per plate, respectively (averages of all isolates). The counts that we obtained from V-8 agar agree fairly closely with the spore counts on V-8 agar reported 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.

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Cytology and Histology

Histopathology of *Cercospora sojina* in Soybean Seeds

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ABSTRACT

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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.

Many soybean (*Glycine max* (L.) Merr.) pathogens are seedborne (8,11). *Cercospora sojina* Hara (syn. *C. daizu* Miura), causal agent of frogeye leaf spot of soybean, is seedborne and reduces seed quality because of seed discoloration (7,10,14). The disease is found worldwide and causes yield reductions in the U.S. of 12–15% (6). Soybean seeds infected with *C. sojina* develop conspicuous light to dark gray to brown areas that vary from minute specks to large blotches covering the entire seed coat. Some lesions show alternating bands of light and dark brown. Occasionally, brown and gray lesions diffuse into each other. Usually the seed coat cracks or flakes. The symptoms are distinct from those produced on soybean seeds by *C. kikuchii*, *Colletotrichum truncatum*, the *Diaporthe-Phomopsis* complex, and *Fusarium* (11). No studies have been reported on the host-parasite relationship between *C. sojina* and soybean seed tissues. We present results from scanning electron microscopy and histopathological studies on the penetration and distribution of seven isolates of *C. sojina* in soybean seeds harvested from plants inoculated separately in the field with different isolates of the fungus.

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MATERIALS AND METHODS

The soybean seeds came from samples from studies by Yorinori (13–15) and were preserved for 3 yr in test tubes under ambient laboratory conditions. Seeds from a variety of cultivars were examined under a dissecting microscope and sorted into those with and without symptoms caused by *C. sojina*. The seed lots came from field-grown plants that each had been spray-inoculated 34, 40, 47, and 52 days after emergence with a conidial suspension (13) of one of eight isolates of *C. sojina* labeled F2, MS14, TN1, TN2, TN4, LA1, LA2, and LA5 (which are ATCC 44531, 44083, 44084, 44085, 44087, 44088, 44089, and 44090, respectively) at Urbana.

To verify the presence of *C. sojina*, randomly selected seeds with and without symptoms of infection by *C. sojina* were surface