Sporulation and Variation in Size of Conidia and Conidiophores Among Five Isolates of *Cercospora kikuchii*

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ABSTRACT

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The sporulation of five soybean (*Glycine max*) seed isolates of *Cercospora kikuchii*, causal fungus of purple seed stain of soybean—Ck-1 (ATCC 36864), Ck-A, and Ck-B from Illinois, Ck-T from Taiwan, and PS-161 from Indiana—was compared on carrot leaf-decoction agar (CLDA), dead soybean plant tissue agar (DSPT), potato-dextrose agar (PDA), and V-8 juice agar (V-8A) at either alternating 12 hr of light and dark or continuous dark at 25 C. Lighting regimen, type of medium, and source of inoculum (mycelia or mycelia plus conidia) influenced spore production. All isolates produced conidia at 12 hr of alternating light and dark; production was sparse under continuous dark. More spores were produced by all isolates on CLDA, DSPT, and V-8A than on PDA. Conidia production was not significantly different among isolates on all media tested. Conidia and conidiophores of Ck-B were smaller than those of the other four isolates. The sizes of conidia and conidiophores varied within each isolate. It is suggested that inoculum of *C. kikuchii* for field inoculations be grown on V-8A, DSPT, or CLDA under alternating 12 hr of light and dark at 25 C and that the plates be inoculated by spreading mycelia plus conidia over the agar surface.

The literature is confusing concerning the correct medium to use for the study of sporulation of *Cercospora kikuchii* (T. Matsumoto & Tomoyasu) Gardner, causal fungus of purple seed stain of soybean (*Glycine max*) (9). Crane and Crittenden (1) obtained only mycelial growth of *C. kikuchii* on potato-dextrose (PDA), Czapek, and Bacto agars. The isolate of *C. kikuchii* studied by Matsumoto and Tomoyasu (6) sporulated on raisin decoction agar but not on 13 other decotion agars or on three defined liquid media. Matsumoto (5) and Murakishi (7) claimed that the fungus would sporulate only on living host tissues. Kilpatrick and Johnson (3) and Lyda et al (4) induced sporulation of the fungus by using carrot leaf-decotion agar, but Vathakos and Walters (10,11) failed to verify their results. Roy and Abney (8) reported that their isolates of *C. kikuchii* sporulated on PDA and V-8 juice agar (V-8A), but Vathakos and Walters (10) could not obtain sporulation on these two media. However, Vathakos and Walters (10) obtained abundant conidia production on dead soybean plant tissue agar (10) and senescent soybean plant tissue (11) under alternating 8 hr of light and 16 hr of dark; conidia production under continuous dark was sparse. Lyda et al (4) obtained conidia production on V-8A in both complete light and dark. These differences may be due in part to the isolates studied, the media used, and environmental conditions.

To develop a standard method for preparing *C. kikuchii* inoculum for field inoculation of soybean plants, we compared the influence of alternating light and dark, four types of media, and two sources of inoculum on the sporulation of five isolates of *C. kikuchii*.

MATERIALS AND METHODS

The five isolates of *C. kikuchii* were obtained from soybean seeds showing symptoms of purple seed stain (9). Ck-1 (ATCC 36864) was obtained from Amsoy soybean seeds grown at the University of Illinois Plant Pathology Research Center, Urbana, and Ck-T was obtained from Shih-Shih seeds grown in Taiwan. Seeds of these two cultivars were surface-disinfected with 0.5% sodium hypochlorite (10% Clorox) for 5 min and rinsed for 1 min with sterile distilled water. The seeds were plated on PDA. Amsoy seeds from the same source also were plated on moist filter paper in 9-cm culture plates. Abundant conidia were produced on the seed surfaces after 24 hr at room temperature (23–27 C). Two single-conidium isolates were obtained from infected seeds, as was Ck-1, and labeled Ck-A and Ck-B. PS-161 was obtained from T. S. Abney, Purdue University, Lafayette, Indiana. Ck-1, Ck-T, and Ck-A are pinkish-gray and produce the characteristic purple pigment on potato-dextrose agar, whereas PS-161 and Ck-B are white to gray and do not produce the purple pigment.

The growth and sporulation of the five isolates were compared on four media: potato-dextrose agar (PDA) prepared by autoclaving 1 L of distilled water containing 39 g of Difco PDA powder; carrot leaf-decoction agar (CLDA) prepared by the recipe of Kilpatrick and Johnson (3); V-8 juice agar (V-8A) prepared by autoclaving 800 ml of distilled water containing 200 ml of V-8 juice, 3 g of $CaCO_3$, and 20 g of Difco agar; and dead soybean plant tissue agar (DSPT) prepared by autoclaving an autoclaved (15 min at 121 C) extract of 200 g of chopped mature soybean (Amsoy 71) chaff composed of dry stems and pods from field-grown plants and 20 g of Difco agar adjusted to 1 L in distilled water. All agars except CLDA were autoclaved at 121.6 C for 15 min and poured into 9-cm culture plates at 20 ml per plate.

Inocula of the five isolates were prepared by adding 2 ml of sterile distilled water to a 9-cm culture plate of the test fungus grown on PDA. The plate then was swept with a soft, camel's hair brush to loosen mycelia and conidia. By controlling the environmental conditions, cultures could either be induced to produce conidia or prevented from producing conidia. Thus, two types of inoculum were tested: mycelia alone and mycelia plus conidia.

For sporulation tests, agar plates were prepared for each isolate and brushed with one of the inoculum types to give a uniform distribution, then incubated either under continuous darkness or with 12 hr of alternating dark and light at 25 C. The light source was four 20-W cool white fluorescent tubes mounted 30 cm above the plates. After 3 or 4 days, measurements of conidia and conidiophores were made under a microscope with a micrometer. After the same length of time, a plate of each isolate was washed twice with 20 ml of sterile distilled water, combined, then diluted to 100 ml with 60 ml of sterile distilled water. Five separate spore counts were made from each plate, and the number of conidia produced per square centimeter was recorded, using a hemacytometer on an Olympus BHA compound microscope. The study was done three times, and the data were combined.

RESULTS AND DISCUSSION

Several environmental factors influenced the sporulation of *C. kikuchii* in vitro, including light period, type of

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inoculum, media used, and isolate (Table 1). Among these, light was the most important. All isolates sporulated on all media tested under alternating 12 hr of light and dark, but sporulation was sparse under continuous dark. On the average, 2.085×10^5 and 0.133×10^5 condia per square centimeter (FLSD at 5% = 0.372) were recorded on cultures under alternating light and dark and continuous dark, respectively. Lyda et al (4) found no significant difference in sporulation of *C. kikuchii* between continuous light and 12 hr of alternating light and dark at 28 C. This agreed in part with the results of Vathakos and Walters (10). Veiga and Kimati (12) reported that sporulation of *C. sojina* also was increased under light. More conidia were produced ($2.490 \times 10^5/cm^2$) when mycelia plus conidia was used as the source of inoculum than when mycelia alone was used ($1.715 \times 10^5/cm^2$) (FLSD at 5% = 0.719) under 12 hr of light. These results are similar to those of Ekpo and Esuruoso (2), who reported that the sporulation of *C. cruenta* Sacc. and *C. canescens* Ell. & Martin were more profuse when a conidial suspension was used for inoculation than when fragmented mycelium was used. The media used also affected sporulation. Over all isolates, the conidia produced per square centimeter for the four agars were: V-8A = 2.54×10^5, CLDA = 2.46×10^5, DSPT = 2.37×10^5, and PDA = 1.04×10^5. Significantly fewer conidia were produced on PDA than on the other media, but there was no difference in the number of conidia produced on V-8A, CLDA, and DSPT (FLSD at 5% = 0.95). Conidia production was not significantly different among isolates over all media tested. Thus, differences among reports on the sporulation of *C. kikuchii* can be influenced by the factors tested as well as by pH and relative humidity (6,7) and by interactions among any of the factors.

The average conidial size did not differ among isolates Ck-T, Ck-1, Ck-A, and PS-161, but the conidia of Ck-B were smaller than those of any of the others (Table 2). However, the range within each isolate was wide. There was a wide range in the lengths of conidiophores among the isolates. Conidiophores of isolates Ck-T, Ck-1, and PS-161 tended to be longer than those of Ck-A and Ck-B. The lack of variation in Ck-A and Ck-B was expected, since each was cultured from a single conidium.

We suggest that inoculum of *C. kikuchii* for field inoculation be grown on V-8A, DSPT, or CLDA under alternating 12 hr of light and dark at 25 C and that the plates be inoculated by spreading mycelia plus conidia over the agar surface. Yeh and Sinclair (13) reported that isolate Ck-1 of *C. kikuchii* grown on CLDA under alternating 12 hr of light and darkness produced conidiogenous cells within 36 hr.

Table 1. Conidia production by five isolates of *Cercospora kikuchii* grown on four media at 12 hr of alternating light and dark for 4 days at 25 C

C. kikuchii isolate[a]	Media[b]	Number of conidia $\times 10^5/cm^2$[c]	
		Mycelia alone	Mycelia + conidia
Ck-1	CLDA	2.1	2.4
	DSPT	2.3	2.3
	PDA	1.2	1.8
	V-8A	2.3	2.7
Ck-A	CLDA	0.7	1.9
	DSPT	1.7	2.7
	PDA	0.1	0.2
	V-8A	1.2	2.5
Ck-B	CLDA	1.4	2.7
	DSPT	2.3	2.7
	PDA	1.0	0.8
	V-8A	2.8	4.3
Ck-T	CLDA	1.4	2.7
	DSPT	1.8	2.7
	PDA	0.8	1.1
	V-8A	1.2	2.7
PS-161	CLDA	2.4	6.9
	DSPT	1.9	3.3
	PDA	2.2	1.2
	V-8A	3.5	2.2

[a]Ck-1, Ck-A, and Ck-B from Illinois-grown soybean seeds, Ck-T from Taiwan-grown soybean seeds, and PS-161 from Indiana-grown soybean seeds.
[b]CLDA = carrot leaf-decoction agar, DSPT = dead soybean plant tissue agar, PDA = potato-dextrose agar, and V-8A = V-8 juice agar.
[c]Inoculum came from identical cultures of each isolate except that cultures for mycelia alone were grown on PDA under continuous dark and cultures for conidia production were grown under alternating 12 hr of light and dark. Means of three replicates.

Table 2. Range in size of conidia and conidiophores of five isolates of *Cercospora kikuchii* grown on carrot leaf-decoction agar at 12 hr of alternating light and dark after 4 days at 25 C

C. kikuchii isolate[a]	Conidia[b] (μm)	Conidiophores[b] (μm)
Ck-1	2.8–3.6 × 95–305	3.6–5.5 × 120–382
Ck-A	3.0–4.0 × 112.5–275	4.2–5.5 × 112.5–180
Ck-B	2.25–3.0 × 50–112	2.5–3.25 × 50–125
Ck-T	4.0–5.0 × 62.5–300	3.75–5.5 × 162.5–375
PS-161	3.0–4.2 × 75–325	4.0–5.5 × 125–360

[a]Ck-1, Ck-A, and Ck-B from Illinois-grown soybean seeds, Ck-T from Taiwan-grown soybean seeds, and PS-161 from Indiana-grown soybean seeds.
[b]Based on 50 measurements.

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