

Etiology

### *Colletotrichum destructivum*, the Anamorph of *Glomerella glycines*

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#### ABSTRACT

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Morphology of acervuli, perithecia, conidia, and ascospores, development and morphology of appressoria, and discharge of ascospores of *Glomerella glycines* were studied on soybean (*Glycine max*) and in culture. *Colletotrichum destructivum* was shown to be the anamorph of *G. glycines*. Appressorial development and morphology are described for the first time. Sodium chloride-yeast extract agar was used for culturing acervuli of *C. destructivum*, potato-dextrose agar for culturing the

perithecia of *G. glycines*, and NaCl-yeast extract agar plus sucrose for culturing the fruiting structures of both forms. *Colletotrichum truncatum* did not produce the teleomorph, *G. glycines*, on any of the media tested. *C. destructivum* and *G. glycines* produced no symptoms in soybean tissues, but isolates from soybean pods and stems were pathogenic to soybean unifoliolate leaves in reinoculation studies.

*Additional key words:* *Colletotrichum lindemuthianum*, *Glomerella cingulata*.

Anthrachnose of soybean [*Glycine max* (L.) Merr.] is an economically important disease in the humid tropics and subtropics (12,13). Several *Colletotrichum* spp. have been isolated from soybeans (4,8,12,13,16), but the disease is caused mainly by *Colletotrichum truncatum* (Schw.) Andrus & W. D. Moore.

*Glomerella glycines* Lehman and Wolf also causes anthracnose, but is usually limited to aging soybean plants at harvest or to soybean stubble (5,7,8,12,16). *Colletotrichum glycines* Hori (= *C. truncatum*) was reported to be the anamorph of *G. glycines* (7). However, Lin and Wu (8) and Tiffany and Gilman (16) later presented evidence that a species similar to *Colletotrichum destructivum* O'Gara was the anamorph. Neither report gave detailed descriptions or illustrations of the two stages nor did they describe appressorial development. The description of appressoria

is one of the key characteristics used to separate species of *Colletotrichum* (14,17).

*C. destructivum* was first described on red clover; its teleomorph was unknown (10). Tiffany and Gilman (16) later presented evidence that *G. glycines* was its anamorph, but they had difficulty obtaining both conidia and ascospores in culture.

Using selective media, and histopathological and pathological data from inoculation of soybean seedlings, our objective was to provide a more detailed description of the morphology and pathogenicity of *G. glycines*.

#### MATERIALS AND METHODS

Species of *Colletotrichum* and *Glomerella* were isolated from soybean stems and pods in the R6 growth stage (3). Stem and pod tissue was washed for 6–8 hr in a pipette washer, surface sterilized in 0.5% NaOCl for 4 min, and rinsed twice in sterile deionized water. To induce sporulation (1), sterilized tissue was soaked in an aqueous solution (28.1% a.i.) of commercial paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride). Tissue was placed on moist

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cellulose pads and incubated at approximately 100% relative humidity under continuous light ( $800 \mu\text{Ein}/\text{m}^2/\text{sec}$ ) for 5–7 days at 25 C.

Conidial masses from 35 acervuli were picked from pod and stem tissue with a sterile loop and diluted ( $10^{-3}$ ) with sterile deionized water. A 0.1-ml sample of each suspension was spread separately on water agar by using a sterile bent glass rod. After 8–10 hr at 25 C, germinating conidia were located by using a binocular dissecting microscope and transferred to acidified (pH 4.5) potato-dextrose agar (PDA). Similarly, single ascospore colonies were obtained by transferring perithecial ooze to PDA.

We collected 35 single-spore isolates from soybean pod and stem tissue: One of *Glomerella cingulata* (Stonem.) Spauld. & Shrenk, nine of *G. glycines* (*C. destructivum*), and 25 of *C. truncatum* and other *Colletotrichum* spp. One culture each of *G. glycines* and *C. destructivum* was sent for verification to J. E. Mordue, Commonwealth Mycological Institute, Kew, Surrey, England. Three selected isolates of *G. glycines* (*C. destructivum*) from soybean were compared with *G. glycines* (*C. destructivum*) (ATCC-11871). In addition, we subcultured *Colletotrichum trifolii* Bain & Essary (ATCC-32358) and *G. cingulata* (ATCC-13743) on PDA and measured the conidia of each. We compared the various *Colletotrichum* spp. in culture to verify our identification of *C. destructivum*.

**Development of differential media.** Abundant acervuli and conidia, but no perithecia, were formed by the three isolates of *G. glycines* from soybean and the ATCC-11871 isolate on sodium chloride-yeast extract agar (SYA) which consisted of 5 g of NaCl, 3 g of yeast extract, and 20 g of agar per liter of deionized water. Both acervuli and perithecia were formed when 10 g of sucrose was added to SYA (SYAS). Fertile perithecia, but no acervuli, formed on acidified PDA, and a few acervuli formed on cornmeal agar with dextrose (17 g/L) (CMA). Culture color was determined using the Ridgway key (11). We studied the relationship of *G. glycines* and *C. destructivum* by culturing the nine single-spore isolates of *G. glycines* (*C. destructivum*) and the isolate of ATCC-11871 on the differential media.

**Ascospore discharge.** The mechanism of ascospore discharge by isolates of *G. glycines* on PDA and SYAS was determined in the laboratory. Five cultures per isolate were grown on each medium. Culture plate covers from each isolate of *G. glycines* on PDA and SYAS were replaced with culture plate bottoms containing sterile PDA or SYA and were secured around the seam with cellophane tape. Cultures were incubated under continuous light ( $400 \mu\text{Ein}/\text{m}^2/\text{sec}$ ) for 48 hr at 26 C, after which culture plate bottoms were separated from colonies of *G. glycines* and examined under a binocular bright-field microscope for germinating ascospores.

To determine whether *G. glycines* was homothallic or heterothallic, single-ascospore lines were grown in dual culture on PDA or SYAS in several combinations and incubated either under continuous fluorescent light ( $400 \mu\text{Ein}/\text{m}^2/\text{sec}$ ) or 12 hr of alternating dark and light for 15–20 days at 26 C.

Taschjian's slide culture technique (15) was used to study appressorial development of the isolates of *G. glycines*. Mycelial plugs, or conidial or ascospore suspensions, were used to inoculate PDA, SYA, or SYAS agar blocks in triplicate. Cultures were incubated under 12 hr of alternating light for 2, 3, or more days at 26 C.

**Seedling inoculations.** Seedlings of the susceptible soybean cultivars Amsoy, Corsoy 79, and Williams were inoculated while in the V1 growth stage (3). A suspension of conidia or ascospores ( $4\text{--}5 \times 10^6$  cells per milliliter) of the three isolates of *G. glycines* and the ATCC-11871 isolate was sprayed with an atomizer onto seedlings until runoff. Three seedlings in each of five pots were inoculated and an equal number sprayed with sterile deionized water served as controls. Seedlings were incubated in a mist chamber under alternating dark and cool fluorescent light ( $800 \mu\text{Ein}/\text{m}^2/\text{sec}$ ) at  $25 \pm 2$  C. After 3 days, seedlings were placed on a greenhouse bench, and development of acervuli and perithecia was monitored. After 5 days, stem sections were excised from seedlings, surface sterilized, treated with paraquat, and incubated as described previously. The experiment was done three times.

**Histopathological studies.** Histopathological studies of acervuli and perithecia in situ were conducted by using inoculated leaves from the above test. Leaf tissue containing acervuli and/or perithecia was excised; fixed in formaldehyde:acetic acid:50% ethyl alcohol (5:5:90, v/v) for 48 hr; dehydrated in tertiary butyl alcohol; and embedded in paraffin (Paraplast; Sherwood Medical Industries, St. Louis, MO) containing 0.4 to 1% beeswax and filtered through cotton). Paraffin sections 11–20  $\mu\text{m}$  thick were stained with safranin and light green and mounted in Canada balsam (6).

## RESULTS

**Cultural characteristics.** Colony diameter of the nine soybean isolates and the ATCC-11871 isolate of *C. destructivum* reached 50 to 60 mm after 5 days on PDA at 26 C (Fig. 1C). Depending upon isolate and culture age, color of mycelium varied from strawberry pink to carrot red. Perithecia were sparse after 10–15 days, and acervuli rarely formed on PDA. When present, acervuli usually developed near the middle (oldest portion) of the colonies.

Colonies of *C. destructivum* on CMA were colorless or off-white and produced few acervuli within 24 hr on SYA. They were off-white with abundant acervuli, and produced cinnamon-colored conidial masses (Fig. 1C). All isolates produced abundant acervuli and conidia when cultured on SYA and CMA under continuous fluorescent light at 26 C. Colonies on SYAS were similar to those on SYA, except they grew more rapidly and had fewer acervuli and more perithecia.

All isolates produced perithecia on PDA and SYAS and acervuli on CMA, SYA, and SYAS. Curved-spore *C. truncatum* grown on PDA, SYA, and SYAS always produced abundant acervuli and curved spores.

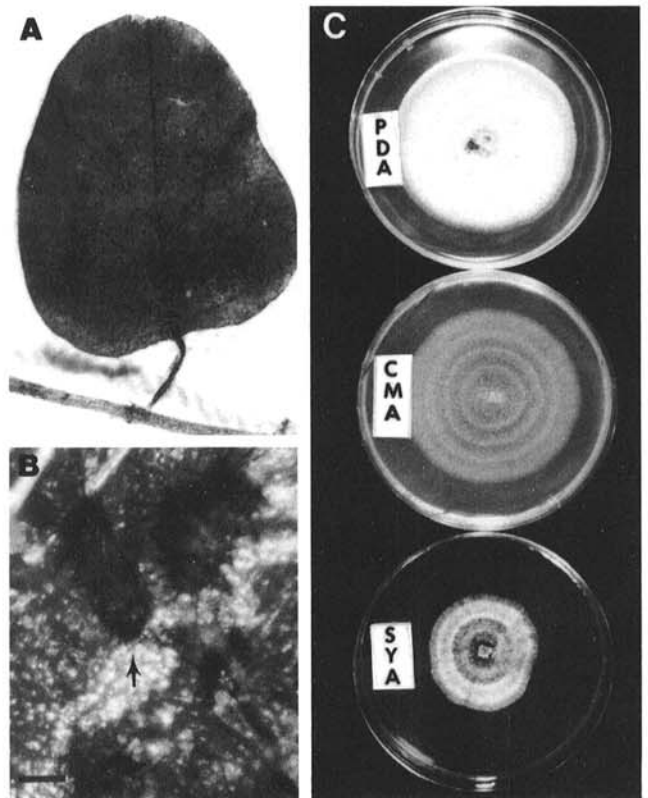


Fig. 1. A, Perithecia and acervuli of *Glomerella glycines* on a soybean leaf. B, Acervuli on soybean leaf surface (arrow) (bar =  $100 \mu\text{m}$ ). C, Comparative growth and sporulation of the fungus on three media in 9-cm-diameter cultural plates; PDA = potato-dextrose agar, CMA = cornmeal agar, and SYA = sodium chloride-yeast extract after 5 days under alternating 12-hr fluorescent light at 26 C.

*Colletotrichum destructivum*.

Conidiomata acervular, setose, rounded to obovate, superficial to subcuticular, compact, composed of hyaline *textura angularis*, 20 to 150  $\mu\text{m}$  across and 25 to 35  $\mu\text{m}$  thick (Figs. 2A and 3A). Conidiophores smooth, hyaline, and branched at the base. *Conidiogenous cells* smooth, cylindrical, hyaline, and phialidic, with collarettes sometimes present. *Conidia* smooth, hyaline, fusiform to constricted, mostly cylindrical, 12.7 to 18.2  $\times$  3.6 to 5.0 (mostly 14.5 to 17.3  $\times$  4.1 to 4.8)  $\mu\text{m}$ , guttulate, with rounded proximal ends and subconical distal ends bearing flattened attachments or scars, aseptate, forming septa in the middle at germination, each cell usually germinating with one germ tube to form an appressorium (Figs. 2A and B[e], 3B and C). *Appressoria* thick-walled, medium brown, mostly globose, some clavate, smooth, single celled, 6.5 to 10.2  $\times$  5.8 to 8.2 (mostly 8.0 to 9.0  $\times$  6.4 to 7.5)  $\mu\text{m}$  with a single germination pore near the middle. Appressoria never forming from hyphae. *Setae* thick-walled, sparse, long, straight to slightly curved, 57 to 155  $\mu\text{m}$  long and 4 to 6  $\mu\text{m}$  near the base (mostly 80 to 140  $\times$  4.5 to 5.5)  $\mu\text{m}$ , one- to four-septate (mostly two septate), tapered to apices (Figs. 2A and 3A).

*Glomerella glycines*.

*Perithecia* typically in glomerate masses of three to nine or more, mainly superficial, sometimes subepidermal, hyaline when young, becoming darker with age. Mature perithecia mostly ampulliform, some lageniform to subglobose with distinct necks projecting upward and variously

positioned in the glomerate mass. Perithecial wall soft with five to six cells, 5 to 7  $\mu\text{m}$  thick. Walls shared with adjoining perithecia but with separate and distinct necks (Figs. 2B[a] and 3D). Mature perithecia 110 to 250  $\times$  73 to 230  $\mu\text{m}$  (mostly 145 to 195  $\times$  83 to 140  $\mu\text{m}$ ). Perithecial neck short, 35 to 60  $\times$  32 to 42  $\mu\text{m}$  with few periphyses. *Asci* paraphysate, hyaline, clavate, and unitunicate with small apical pore, short stem, knobby base, 160 to 228  $\times$  18 to 30 (mostly 170 to 200  $\times$  18 to 23)  $\mu\text{m}$ , containing biseriolate ascospores (Figs. 2B and 3E). *Ascospores* smooth, hyaline, aseptate, 19 to 35  $\times$  3.0 to 4.7 (mostly 24.5 to 29.1  $\times$  3.3 to 4.0)  $\mu\text{m}$ , straight to sigmoid, mostly allantoid with rounded ends, forcibly discharge, often collecting on ostioles, germinating to form central septum, each cell forming a germ tube which produces an appressorium (Figs. 2B [c and d], 3E and F).

Single ascospore line of all isolates tested were homothallic.

**Characteristics of *Colletotrichum trifolii* and *Glomerella cingulata*.** Conidia of *C. trifolii* were 9.4 to 13.7  $\times$  4.1 to 6.4  $\mu\text{m}$  (mostly 10.2 to 12.4  $\times$  4.7 to 5.6  $\mu\text{m}$ ); those of *G. cingulata* 10.5 to 26.4  $\times$  3.6 to 6.8  $\mu\text{m}$  (mostly 12.3 to 17.7  $\times$  4.0 to 5.6  $\mu\text{m}$ ).

**Seedling infection.** Though only a few necrotic lesions developed on leaves of inoculated seedlings, acervuli of *C. destructivum* and

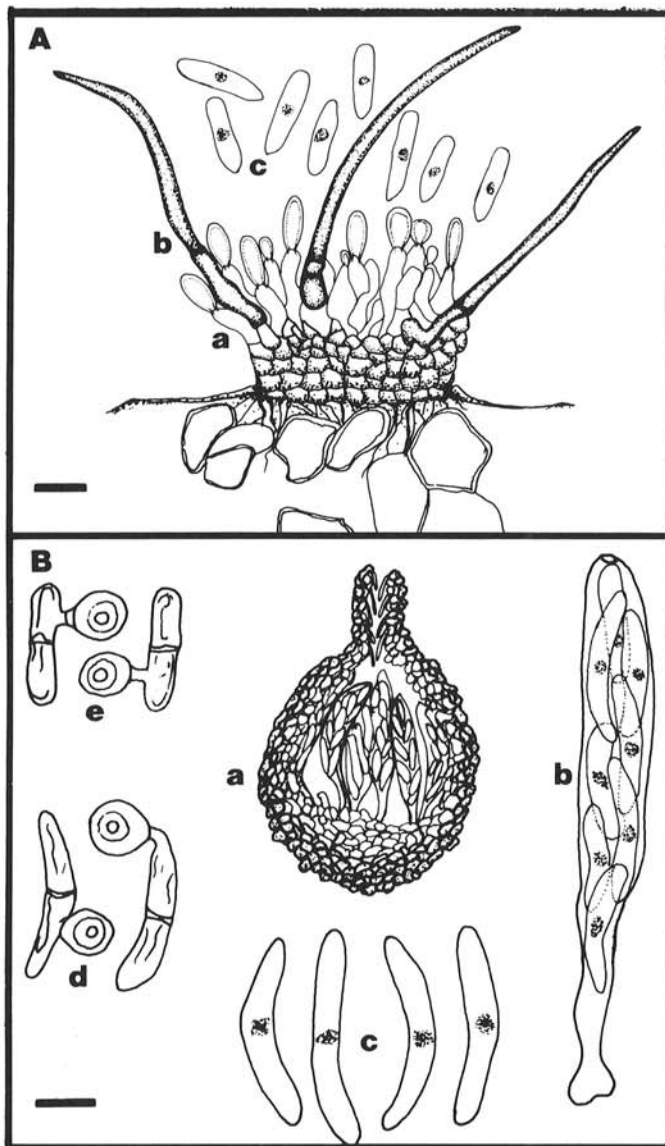


Fig. 2. Camera lucida drawings of *Glomerella glycines*: A, Vertical section of an acervulus with conidiogenous cells (a), setae (b), and conidia (c) (bar = 10  $\mu\text{m}$ ). B, (clockwise) Vertical section of a perithecium (a), an ascus (b), ascospores (c), appressoria from ascospores (d), and appressoria from conidia (e) (bar = 3  $\mu\text{m}$  for [a] and 10  $\mu\text{m}$  for [b] to [e]).

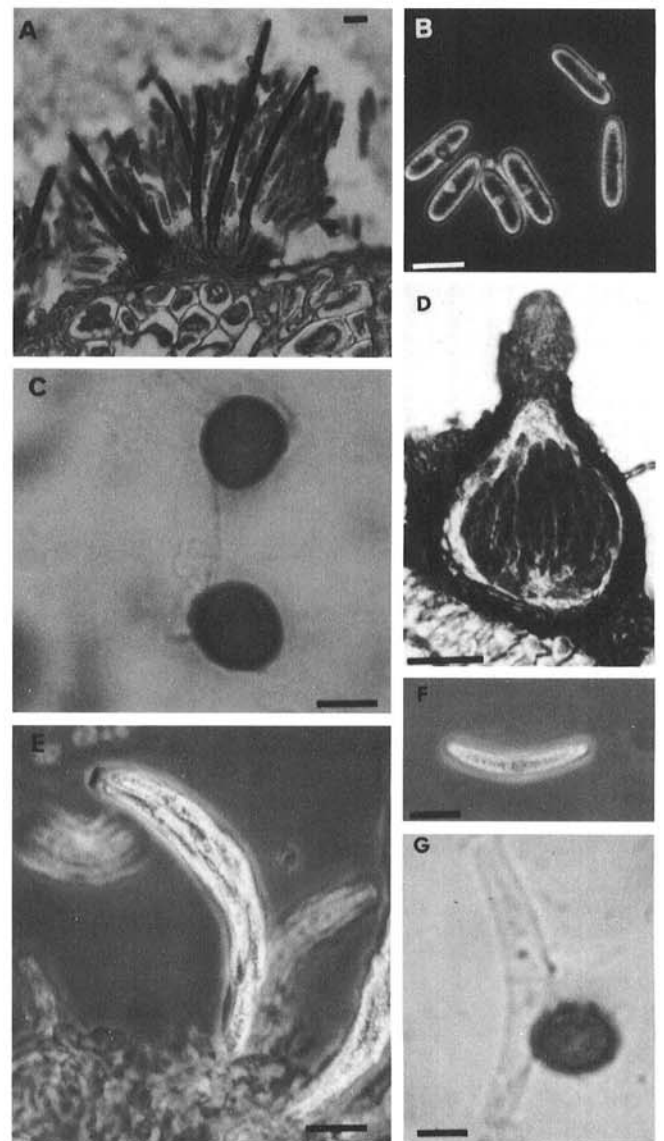


Fig. 3. Photomicrographs of *Glomerella glycines*: A, An 11- $\mu\text{m}$ -thick vertical section of an acervulus from a soybean petiole (bar = 10  $\mu\text{m}$ ). B, Conidia under phase contrast microscopy (bar = 10  $\mu\text{m}$ ). C, Conidial appressoria (bar = 5  $\mu\text{m}$ ). D, A 10- $\mu\text{m}$ -thick vertical section of a perithecium from a soybean petiole (bar = 3  $\mu\text{m}$ ). E, An ascus (bar = 5  $\mu\text{m}$ ). F, An ascospore (bar = 5  $\mu\text{m}$ ). G, An appressorium from an ascospore (bar = 2  $\mu\text{m}$ ).

perithecia of *G. glycines* developed on all seedling parts (Figs. 1A and B) within 3 days after surface sterilization, paraquat treatment, and incubation in a moist chamber.

## DISCUSSION

Using SYA to induce maximum sporulation of *C. destructivum* and SYAS for production of acervuli and perithecia, the connection between *G. glycines* and *C. destructivum* was established for the first time. Thus, the suggestion of Tiffany and Gilman (16) was confirmed. The conidia of the isolates of *C. destructivum* on PDA, including ATCC-11871, were longer and narrower than those of *C. trifolii* (ATCC-32358) but fell within the range of conidial size of *G. cingulata* (ATCC-13743). Measurements for acervuli, conidia, and setae of *C. destructivum* agreed with the original description of O'Gara (10).

Early workers (2,8,16) isolated and grew *G. glycines* only on PDA, on which the fungus sporulates poorly or not at all. Lin and Wu (8) mentioned sparse perithecia production on PDA and production of acervuli in a sucrose-free medium. Sectoring and differences in amounts of sporulation did not occur among the isolates tested although this has been reported (2,16). Chilton (2) noted that cultures which had lost the ability to produce conidia on PDA produced conidia on autoclaved red clover stems. We induced the production of acervuli and/or perithecia by using PDA, SYA, or SYAS.

The anamorph of *G. glycines*, originally described as *C. glycines* Hori (7), was later found to be *C. destructivum* (16). We described appressorial development for the first time. The appressorial size and conidial length of *C. destructivum* is similar to those of *C. lindemuthianum* (Sacc. & Magn.) Br. & Cav.; however, *C. destructivum* and *C. lindemuthianum* are differentiated by cultural characteristics, conidial width, and their respective teleomorphs. Mordue (9) stated that *C. lindemuthianum* grows 60 mm in 10 days at 22–24 C on PDA and is initially grey, rapidly becoming dark. Its teleomorph is *G. cingulata*. Lehman and Wolf (7) and von Arx (17) noted that the paraphysate perithecia with cylindrical short ascospores of *G. cingulata* separate it from *G. glycines*, which has aparaphysate perithecia with allantoid, long ascospores (7,8,16).

Our measurements of perithecia, asci, and ascospores agree with the original description of *G. glycines* by Lehman and Wolf (7). However, Hemmi (5) indicated the original description was made by Hori. In fact, that work of Hemmi (5) dealt only with the *Colletotrichum* state. Therefore, the teleomorph should be *Glomerella glycines* Lehman & Wolf, instead of *G. glycines* (Hori) Lehman & Wolf as often cited (13).

*Colletotrichum destructivum* was accepted by von Arx (17), who recognized *G. glycines* as a synonym of *G. cingulata* (18). The ascospore shape and size of the isolates of *G. glycines* that were studied, including the ATCC-11871 isolate, were longer than those of *G. cingulata* (ATCC-13743) or as has been previously reported (7,16,18). Aparaphysate perithecia and allantoid ascospores are used as differential characters other than size of ascospores. Sutton (14) did not consider *C. destructivum* as a separate species of

*Colletotrichum*. He probably thought of it as a synonym of an anamorph of *G. cingulata*.

Acervuli and perithecia of *G. glycines* frequently form on leaves, petioles, pods, and stems of field-grown soybean plants in Illinois. The fungus rarely causes necrotic lesions on young stems; they are more prevalent on senescent plant parts and on soybean stubble. Only a few necrotic lesions developed on seedling leaves after inoculation with high conidial and/or ascospores suspensions in a mist chamber. In addition, *G. glycines* produces no symptoms in soybeans and sporulates in nature when plants are under stress or reaching maturity (8,12,16). However, the fungus can be induced to form its fruiting structures by treating surface-sterilized tissues with paraquat (1). The role of *G. glycines* in soybean anthracnose is not clear. The fungus can cause disease in alfalfa (4) and was reported to cause disease in soybean plants more than 2-mo old (8).

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