

## Chlamydospore formation, production, and nuclear status in *Fusarium solani* f. sp. *glycines* soybean sudden death syndrome-causing isolates

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**Abstract:** Six isolates of *Fusarium solani* f. sp. *glycines* that cause soybean sudden death syndrome were examined for chlamydospore formation, production, and nuclear status. Chlamydospores formed from macroconidia either terminally, laterally by outward protrusion, or intercalarily. They also formed from germinated macroconidia and hyphae. Occasionally, a single macroconidium produced more than one chlamydospore. The percentage of chlamydospores produced differed significantly ( $P < 0.0001$ ) by incubation temperature and varied among fungal isolates. More chlamydospores formed at 30 C followed by 25 C, 20 C and 4 C. Nuclear stained chlamydospores usually were uninucleate but occasionally multinucleate. Nuclei migrated from macroconidia into chlamydospores through germ tubes.

**Key Words:** macroconidia, nuclear staining, soil-borne fungus, temperature

### INTRODUCTION

Sudden death syndrome (SDS) of soybean [*Glycine max* (L.) Merr.] is an economically important disease (Hartman et al., 1995; Wrather et al., 1995). The causal organism, *Fusarium solani* (Mart.) Sacc. f. sp. *glycines* (Roy, 1997), is a soilborne fungus, first identified in 1989 (Roy et al., 1989; Rupe, 1989). Research on its pathogenicity to soybean has been reported (Gray and Achenbach, 1996; Hartman et al., 1995; Melgar and Roy, 1994). To differentiate *F. solani* f. sp. *glycines* SDS-causing isolates from *F. solani* non-SDS-causing isolates, cultural and morphological characteristics (Roy et al., 1989; Roy, 1997) and random amplified polymorphic DNA (RAPD) (Achen-

bach et al., 1996) have been used. *Fusarium solani* f. sp. *glycines* SDS-causing isolates were reported to produce more macroconidia than *F. solani* non-SDS-causing isolates, which predominately produced microconidia (Roy et al., 1989; Roy, 1997).

Chlamydospores are survival structures for many soil fungi (Chakraborty et al., 1992; Couteaudier and Alabouvette, 1990; Nash et al., 1961) and are usually produced under certain environmental conditions (Park, 1954). Chlamydospores can be induced by certain chemicals or substances. Onion bulb extracts (Sood, 1996), root extracts of crop plants (Mondal et al., 1996), soil substances (Ford et al., 1970a, b), inorganic salt solutions (Hsu and Lockwood, 1973), and certain soil bacteria (Ford et al., 1970c; Vankat, 1952) were reported to induce chlamydospores in some *Fusarium* species. Osmotic potential was shown to influence chlamydospore formation (Nelson et al., 1990) while ammonia and high ion concentrations inhibited chlamydospore production in germinated macroconidia (Löffler and Schippers, 1985). Although chlamydospore formation in some strains of *F. solani* has been investigated (El-Ani, 1988), information about chlamydospore formation of *F. solani* f. sp. *glycines* SDS-causing isolates other than their occurrence is lacking. In this study, the formation and production of chlamydospores and the nuclear status of chlamydospores and macroconidia in *F. solani* f. sp. *glycines* SDS-causing isolates were investigated.

### MATERIALS AND METHODS

**Fungal isolates and cultural medium.**—The geographic origin and sources of *F. solani* f. sp. *glycines* SDS-causing isolates are listed in TABLE I. All isolates were maintained at 19 C on slants of Bilay's medium (Booth, 1971) prior to use. The pathogenicity of four of the six isolates (TABLE I) was previously reported (Achenbach et al., 1996; Gray and Achenbach, 1996). The pathogenicity of the other two isolates was tested and confirmed to cause SDS (L. E. Gray, unpublished). The phytotoxicity of their culture filtrates was evaluated by vital staining of soybean suspension cultured cells exposed to cultural filtrates and by a soybean stem-cutting assay (Li et al., 1997).

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TABLE I. Soybean sudden death syndrome-causing isolates of *Fusarium solani* used in this study

Isolate designation	Geographic origin	Source
Mont-1	Illinois	P. Stevens
B6-8v	Illinois	L. Gray
St90-1	Illinois	P. Stevens
K-1	Kansas	J. Rupe
UL-20	Illinois	L. Gray
17-1	Arkansas	J. Rupe

*Chlamyospore formation and production.*—Macroconidia from each isolate were harvested by adding 10 ml of sterile distilled water (pH 5.8) to a culture plate of a 14-da-old culture grown on Bilay's medium. The macroconidial suspension was diluted to  $5 \times 10^5$  macroconidia/mL, and 2.5 ml of this suspension was added into each well of a six-well plate (Corning Co., Corning, New York). Triplicate plates (one set for nuclear staining) were incubated at 4, 20, 25, and 30 C in the dark.

Macroconidial germination and chlamyospore production were directly observed in six-well plates using a Nikon TMS inverted microscope (Fryer, Inc., Huntley, Illinois). Macroconidia were considered germinated if the germ tube length exceeded the diameter of the macroconidial cells. Macroconidial germination and chlamyospore formation were determined by counting five randomly selected fields at  $\times 400$  for each of two replicates weekly from 1–4 wk. A minimum of 300 macroconidia of each isolate was examined for each wk at each temperature. The percentage macroconidial germination and chlamyospore production was calculated as follows: [(number of germinated macroconidia/total observed) \* 100] and [(number of chlamyospore/total macroconidia) \* 100]. Photographs were taken with Kodak Tri-X Pan black and white films.

*Nuclear staining and epifluorescence microscopy.*—Two methods were used. First, samples of 1.5 mL from each of the isolates were taken after the macroconidia suspension was incubated for 4 wk in a six-well plate as described above. Samples from each temperature and each isolate were added into a microcentrifuge tube, centrifuged at 5000 rpm for 10 min, and then fixed with freshly prepared methanol/acetic acid (3:1, v/v) for 3 h at room temperature or overnight at 4 C. The fixed cells were washed twice in 70% ethanol and either stored at 4 C in 70% ethanol until stained or washed in 50% ethanol and distilled water prior to staining with 0.15  $\mu\text{g}/\text{ml}$  of 4',6-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, Mis-

souri) (Li et al., 1993). The second method used one drop of a macroconidial suspension ( $2 \times 10^4$  macroconidia/mL in a 0.05% water agar solution) placed on a precleaned Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, Pennsylvania), and were incubated in a moist chamber at 25 C. Slides were removed weekly for up to 3 wk, and stained with DAPI as described above. Three slides from each of three isolates, B6-8v, Mont-1, and UL-20, that were incubated for 3 wk at 25 C were used to count the nuclei in each of 1004 chlamyospores. Slides were mounted in an anti-fade medium, Vector-shield (Vector, Burlingame, California) and examined with an Olympus BX 60 epifluorescence microscope at  $\times 400$  (Leco Co., Chicago, Illinois) equipped with a high-pressure mercury bulb. An Olympus U-MNU filter set was used that consisted of an exciter filter (360–370 nm), a dichroic mirror (400 nm) and emission filter (420 LP). Images were recorded with a cooled charged couple device video camera (DEI-750, Optronics Engineering, Goleta, California). Final image adjustment for color printing was done with Adobe Photoshop V.4.0 (Adobe Systems, Inc., Mountain View, California).

*Statistical analysis.*—The percentages of macroconidial germination and chlamyospore production were transformed using Arcsine and analyzed by analysis of variance (ANOVA) using version 3 of JMP statistical software (SAS Institute, Cary, North Carolina). Means were separated using least significant differences.

## RESULTS

*Formation of chlamyospores.*—Macroconidia of *F. solani* f. sp. *glycines* isolates were mostly sickle-shaped with four cells (FIG. 1), but 3-, 5-, and 6-celled macroconidia were occasionally found. After 3–5 d in distilled water, macroconidia began to form chlamyospores that were thick-walled, brownish and either globose or oval/ellipsoidal. While most of the chlamyospores were single-celled, bicellular chlamyospores also were observed (FIG. 2). Chlamyospores formed from either germinated macroconidia (FIG. 3) or hyphae (FIG. 4); and also formed terminally at either the apical or basal end of a macroconidium, or at both ends simultaneously (FIGS. 5, 6), and intercalarily within a central cell of a macroconidium (FIG. 7). Chlamyospores formed outside the macroconidia were either sessile or formed from a germ tube originating from a macroconidium. Germinating chlamyospores were observed after 3 wk incubation (FIG. 8).



FIGS. 1-8. Chlamydospore formation of *Fusarium solani* f. sp. *glycines* soybean sudden death syndrome-causing isolates. 1. Macroconidia. 2. Bicelled chlamydospore. 3. Chlamydospore formed from a germ tube. 4. Chlamydospore formed from hypha. 5. Intercalary and terminal chlamydospores formed endogenously within the macroconidia. 6. Terminal, exogenously formed chlamydospore. 7. Intercalary chlamydospore formed within a central cell of a macroconidium. 8. A germinated chlamydospore. Scale bars: 1-3, and 5 = 16  $\mu\text{m}$ , 4 = 15  $\mu\text{m}$ , 6 = 6  $\mu\text{m}$ , 7 = 11  $\mu\text{m}$ , 8 = 13  $\mu\text{m}$ .

*Effects of temperature on chlamydospore production.*—Macroconidia did not germinate at 4 C over a 4 wk period, but they did form chlamydospores. Macroconidial germination increased at 20, 25, and 30 C

over a 4-wk period (FIG. 9A). Macroconidial germination varied with isolates (FIG. 9B). After 4 wk, isolates B6-8v and UL-20 had significantly ( $P < 0.001$ ) higher rates of germination than the other isolates.

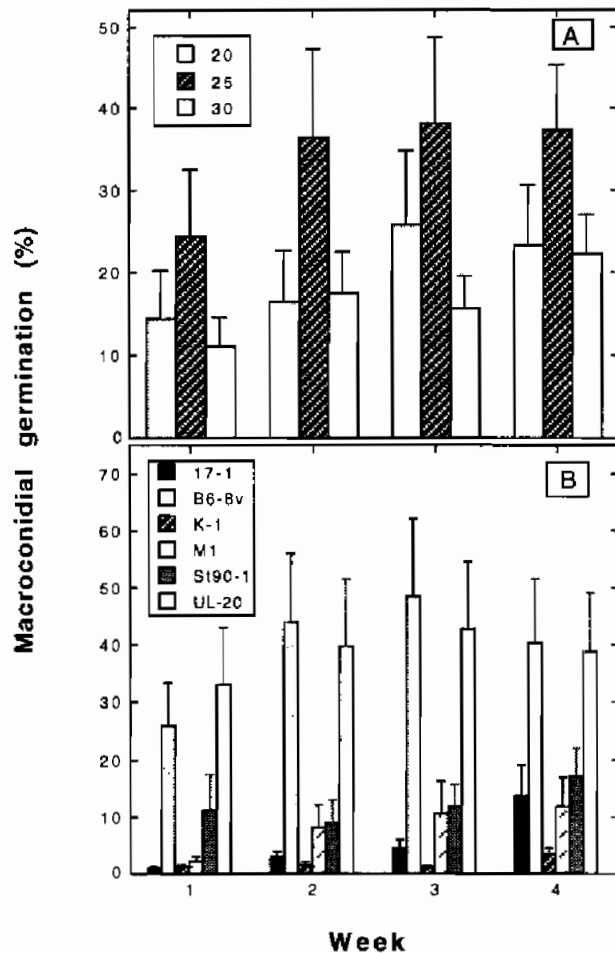


FIG. 9. Mean macroconidial germination. A. Six *Fusarium solani* f. sp. *glycines* sudden death syndrome-causing isolates over a 4 wk period at 20, 25, and 30 C. B. Macroconidial germination over a 4 wk period at 25 C for six *F. solani* isolates.

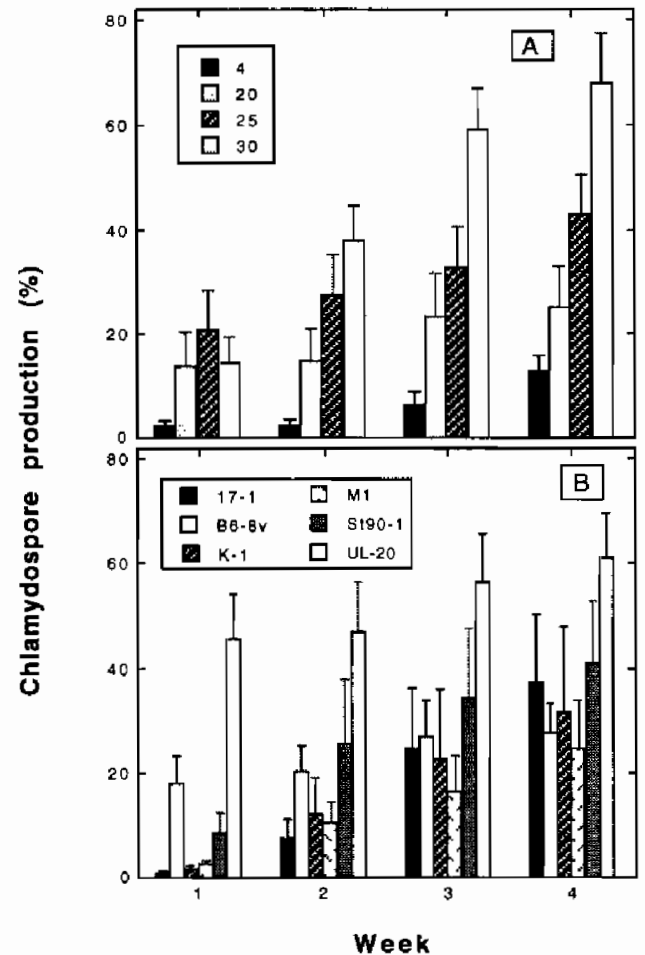
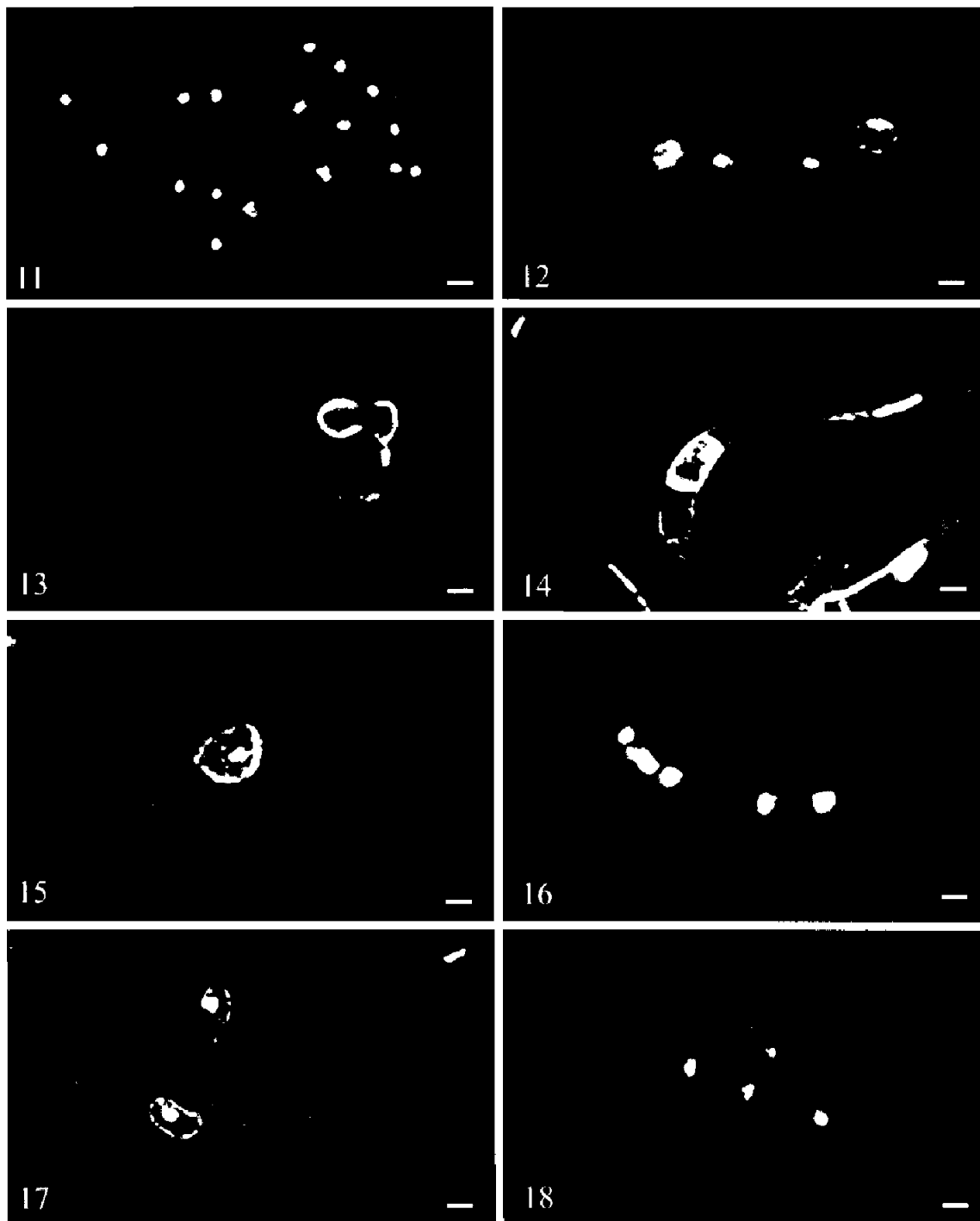


FIG. 10. Mean chlamydospore production. A. Six *Fusarium solani* f. sp. *glycines* sudden death syndrome-causing isolates over a 4 wk period at 4, 20, 25, and 30 C. B. Chlamydospore production over a 4 wk period at 25 C for six *Fusarium solani* f. sp. *glycines* isolates.

Chlamydospores were produced by all isolates at all temperatures tested (FIG. 10A), but the percentages of chlamydospores formed differed significantly ( $P < 0.0001$ ) with incubation temperature as there were more chlamydospores formed at 30 C. Production of chlamydospores increased over time for all the isolates (FIG. 10B).

*Nuclear status of macroconidia and chlamydospores.*—Of the two methods, primarily the second was used for micrographs and counts as the retention of macroconidia and chlamydospores was better. Cells of macroconidia were uninucleate (FIG. 11). When a macroconidium germinated, a nucleus from a macroconidial cell migrated into the germ tube that enlarged at the tip and formed a chlamydospore (FIG. 12). Repetition of this process in the chlamydospore may have resulted in the formation of bicelled chla-

mydospores (FIG. 13). Chlamydospores usually had a typical DAPI-stained nucleus no matter whether they formed intercalarily (FIGS. 14, 15), terminally (FIGS. 16, 17) or laterally (FIGS. 18, 19), or if they formed from hypha (FIG. 20). Macroconidia began to degrade after incubation in distilled water for 3 wk. Chlamydospores remained viable when their mother macroconidial cells became anucleate (FIGS. 13–15, 17). Most DAPI-stained chlamydospores had a single nucleus (90%), although two (7%), three (2%), or four (1%) nuclei (FIGS. 21–23) were found based on counts of 1004 chlamydospores from three isolates. Interestingly, in some cases, both macroconidium and chlamydospore were anucleate except for the germ tube, which contained a nucleus (FIG. 24). Germinated chlamydospores with a DAPI-stained nucleus also were observed (FIGS. 25–26).

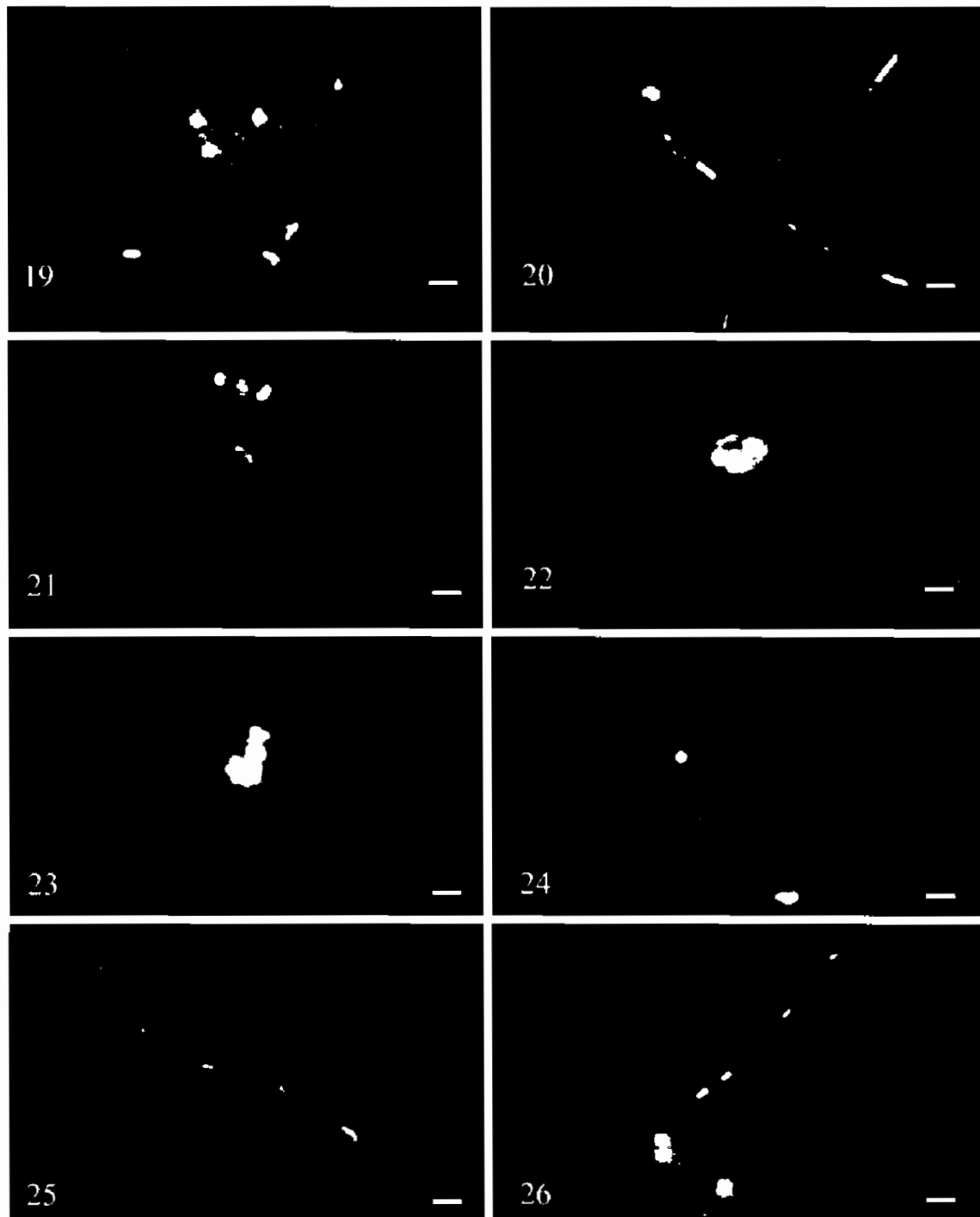


FIGS. 11–18. Nuclear status of *Fusarium solani* f. sp. *glycines* soybean sudden death syndrome-causing isolates. 11. Macroconidia. 12. Nuclear migration from macroconidium through germ tube to chlamyospore. 13. Anucleate bicelled chlamyospore. 14, 15. Uninucleate, intercalarily formed chlamyospore. 16, 17. Uninucleate, terminally formed chlamyospore. 18. Uninucleate, laterally formed chlamyospore. Scale bars: 11–15, 17, and 18 = 6  $\mu$ m, 16 = 4  $\mu$ m.

#### DISCUSSION

The main morphological features of chlamyospores in SDS-causing isolates of *F. solani* f. sp. *glycines* are the same as those described in other chlamyospore-producing fungi (Ainsworth, 1995). They are thick-

walled and intercalary or terminal asexual spores. However, unlike *F. culmarum*, *F. dimerum*, *F. merismoides* (Domsch et al., 1980) and *F. solani* f. sp. *phaseoli* (Ford et al., 1970b) that form chlamyospores in chains, *F. solani* f. sp. *glycines* SDS-causing isolates



FIGS. 19–26. Nuclear status of *Fusarium solani* f. sp. *glycines* soybean sudden death syndrome-causing isolates. 19. Uninucleate, laterally and intercalarily formed chlamydospores in a macroconidium. 20. Uninucleate chlamydospore formed from hypha. 21. Binucleate chlamydospore. 22. Trinucleate chlamydospore. 23. Tetranucleate chlamydospore. 24. Anucleate macroconidium and chlamydospore connected with a germ tube containing a nucleus. 25. Germinated uninucleate chlamydospore. 26. Germinated binucleate chlamydospore. Scale bars: 19–21, and 25 = 6  $\mu\text{m}$ , 22, 23 = 3  $\mu\text{m}$ , 24 = 5  $\mu\text{m}$ , 26 = 4  $\mu\text{m}$ .

usually produce single-celled and occasionally bicellular chlamydo-spores.

Chlamydo-spore formation of a *F. solani* isolate from a cancer patient was either intercalary endogenous, terminal endogenous, terminal exogenous, or laterally formed (El-Ani, 1988). In our study, chlamydo-spores formed as described by El-Ani (1988), but also formed from germinated macroconidia and hyphae. French and Nielson (1966) compared chlamydo-spore production of *F. oxysporum* f. sp. *batatas* in media containing various plant tissues and found that chlamydo-spores generally formed within a central cell of each macroconidium and only a few chlamydo-spores formed terminally. Our results showed that temperature did affect the frequency of chlamydo-spore production. Also, temperature played a role in their formation. At 4 and 30 C, all or most chlamydo-spores formed within macroconidia either terminally or intercalary while at 20 and 25 C, which favored macroconidial germination, most chlamydo-spores formed from germ tubes or hyphae. Another study (Sood, 1996) showed that 30 and 35 C favored chlamydo-spore production, but there was no mention in that study about chlamydo-spore formation at different temperatures. Moreover, temperature is an important factor for chlamydo-spore production. The isolates used in our study varied in chlamydo-spore production and some of these isolates also were reported to vary in virulence (Gray and Achenbach, 1996).

Most macroconidia of *F. solani* f. sp. *glycines* SDS-causing isolates had four nuclei. In some cases, we observed a chlamydo-spore with one nucleus originating from a four-celled macroconidium with four nuclei (FIG. 16). It is conceivable that in these cases nuclear division in the macroconidium occurred before or at the same time as chlamydo-spore formation; however, it is not known whether nuclear division is a prerequisite for chlamydo-spore formation as other uninucleate chlamydo-spores originated from four-celled macroconidia that had only three nuclei (FIG. 18). Nuclear migration from a macroconidium into a chlamydo-spore through a germ tube also was observed (FIG. 12). As the chlamydo-spore was forming from a macroconidium germ tube, two nuclei appeared to migrate leaving the mother macroconidium and the chlamydo-spore uninucleate. After 3 wk incubation in distilled water, most macroconidia were anucleate while most chlamydo-spores had one DAPI-stained nucleus. Most macroconidia had four cells and only one or two of these cells formed chlamydo-spores. Factors determining the ability of macroconidial cells to form chlamydo-spores are still unclear. The number of nuclei in fungal cells may have some biological and taxonomical importance. El-Ani

(1990) found that the nuclear condition in hyphae of *Fusarium* spp. often differs from one strain to another within the same species. Since our study seems to be the first report of the nuclear status of chlamydo-spores in *F. solani* f. sp. *glycines* SDS-causing isolates, it is not known if the nuclear status has any biological or taxonomical significance. More isolates of *F. solani* f. sp. *glycines*, *F. solani*, and other *Fusarium* spp. need to be examined and compared to determine if there is any consistent differences in nuclear status.

Investigating the basic biology of chlamydo-spore formation, production, and nuclear status may help us to better understand the survival mechanism, pathogenicity, and germination variability of *F. solani*. In addition, the relationship of chlamydo-spores to plant infection has been studied for *F. solani* f. sp. *phaseoli* to kidney beans (Mondal et al., 1995, 1996) and *F. oxysporum* f. sp. *ciceri* to chickpea (Arora et al., 1996). Infection of chlamydo-spores of *F. solani* f. sp. *glycines* SDS-causing isolates has not been determined. Further research needs to be done to determine their role in the infection process and if factors like host resistance and cropping patterns impact production, germination, and infectivity of chlamydo-spores.

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