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Development of *Heterodera glycines* as Affected by *Fusarium solani*, the Causal Agent of Sudden Death Syndrome of Soybean

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Abstract: The effects of the blue form of *Fusarium solani*, the causal agent of sudden death syndrome (SDS), on *Heterodera glycines* were examined in the greenhouse. Roots of soybean cv. Coker 156 were inoculated with either *H. glycines* alone or *F. solani* + *H. glycines* in combination. Population levels of *H. glycines* were reduced 47% in the presence of *F. solani*. Life-stage development of *H. glycines* increased 3% in 30 days in the presence of *F. solani*. *Fusarium solani* colonized epidermal and cortical cells adjacent to developing juveniles of *H. glycines* and the nematode-induced syncytia within the soybean root tissue. At 40 days after inoculation, *F. solani* was isolated from 37% of the cysts in soil recovered from the *F. solani* + *H. glycines* combination treatment. *Fusarium solani* significantly affected *H. glycines* population density, life-stage development, and succeeding populations.

Key words: Fusarium solani, Heterodera glycines, histology, interaction, nematode, soybean, soybean cyst nematode, sudden death syndrome.

Sudden death syndrome (SDS) of soybean (*Glycine max* (L.) Merr.) is caused by a blue-pigmented, macroconidium-producing form of *Fusarium solani* (19,22). Rupe (22) suggested that SDS may involve a complex of pathogens. The soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe, is often associated with SDS and may be involved in a disease complex with the causal organism (6,8,19,22), although SDS can develop in the absence of SCN. The incidence of SDS may be positively correlated with numbers of *H. glycines* (7,21) and with soybean cultivars susceptible to *H. glycines* (6). Similarly, fields in Mississippi exhibiting symptoms of SDS also contained *H. glycines* (25). However, soybean yields were not correlated with *H. glycines* densities or SDS disease intensity.

The combination of *F. solani* and *H. glycines* in greenhouse tests resulted in the development of SDS foliar symptoms 3–5 days earlier, and symptoms were more severe than those produced by *F. solani* alone (10). *Fusarium solani* also colonizes the cyst stage of *H. glycines*, suggesting parasitism of both the nematode and the plant by the fungus (11).

The behavior, maturation, and pathogenicity of *H. glycines* can be altered by activities of associated soil-borne organisms. *Heterodera glycines* populations increased in the presence of *F. oxysporum* on the soybean cv. Lee (20) but were reduced when combined with *Phytophthora* Drechs. *sojae* Hild. (1). A mutually antagonistic relationship was observed between *H. glycines* and *Diaphorthe phaseolorum* (Cke. and Ell.) Sacc. var. *caulivora* (Athow and Caldwell) (23). However, the maturation rate of the *H. glycines* was substantially increased in the presence of *Calonectria crotalariae* (Loos) Bell and Sobers (13).

The objective of this research was to determine the influence of the blue form of *Fusarium solani* on development of *Heterodera glycines* race 3.

MATERIALS AND METHODS

Seed of soybean cv. Coker 156 were surface sterilized for 10 seconds in 100% ethyl alcohol, washed 5 minutes in 1.0% sodium hypochlorite, and placed on sheets of 26-cm × 39-cm sterile germination paper (Anchor Paper, St. Paul, MN) for 36 hours. Germinated seed with radicals 1–2 cm long were selected for inoculation.

Fusarium solani was isolated from SDS-symptomatic soybean roots (11) and grown

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on PDA/SA plates for 5–7 days for inoculum production. Agar disks 5 mm in diameter, containing *F. solani,* were aseptically transferred into sterile cornmeal–sand cultures (250 cm^3 dry sand, 14 cm^3 cornmeal, and 100 ml distilled water) and maintained in the dark for 21 days at 24 C. Cultures were shaken periodically to assure uniform fungal growth, and inoculum was combined, mixed, and weighed. The resulting inoculum was incorporated into a steam-sterilized mixture (1:1 [v/v]) of sand and Freestone fine sandy loam soil (72.4% sand, 15.6% clay, and 12.0% silt; 0.6% OM; 14.9 CEC; pH 6.1) to obtain an inoculum level of 0.05% (w/w). A volume of 150 cm^3 infested or sterile soil was placed into the appropriate conetainers (Stuewe & Sons Corvallis, OR).

A race 3 population of *H. glycines* was increased on soybean cv. Coker 156 in the greenhouse. After 60 days, roots were gently washed free of soil and light brown to tan cysts were dislodged from the roots with a stream of water. Cysts were suspended in water, then immediately poured through nested 850-μm-pore-d and 250-μm-pore-d sieves. Cysts were washed from the 250-μm-pore-d sieve into 20-ml glass test tubes with 10 ml of tap water and crushed with a modified Seinhorst cyst crusher for 1 minute (26). This suspension was passed through nested 75-μm-pore-d and 28-μm-pore-d sieves to remove broken cysts and debris. Nematode eggs and second-stage juveniles were standardized to 2,000 eggs and juveniles per ml.

Nematode suspensions were placed at a specific site on the soybean root using the agar cone method (4). A 1-ml suspension of eggs and second-stage *H. glycines* juveniles was pipetted near the root radical and covered with sterile soil to prevent dehydration. After 24 hours, each plant was removed from the filter cone and the inoculation zone marked with paraffin coated paper strips before transplanting into conetainers.

Eight soybean root systems were harvested at 3-day intervals for 30 days. Samples were stained to determine nematode infection and development (2,9). The root system was excised, weighed, cleared in 1.0% sodium hypochlorite for 4 minutes, stained with acid fuchsin, and destained in glycerine (2). The numbers of *H. glycines* per gram of root weight were calculated. Life stages of *H. glycines* were determined for 20 nematodes observed per root system. Second-stage juvenile (J2), swollen J2 (SJ2), third-stage juvenile (J3), fourth-stage juvenile (J4), male (M), and female (F) life stages were identified based on tail shape, gonad development, and rectum position (9,18). The mean stage of the *H. glycines* population (D) was calculated for individual harvest dates based on a 10-day developmental cycle where D = (% J2) + (% SJ2 × 2.33) + (% J3 × 3.67) + (% J4 × 5.83) + (% M × 6.33) + (% F × 6.67) (27). The mean percentage of nematodes in each life stage was weighted against the J2 to determine relative stage development after infection (9,27).

Histological study: To verify the presence of *F. solani,* alternate sequential sections of soybean roots were surface sterilized and placed on PDA/SA to allow fungal growth. The remaining sections were placed in the appropriate fixative for light and scanning electron microscopy. Light microscopy samples were fixed in formalin–acetic acid–alcohol for 24 hours (5). Root sections were stained for 1 minute in acid fuchsin to determine the presence and location of the developing SCN, excised, dehydrated in a T-butyl alcohol series, and embedded in paraplast. Serial longitudinal and cross sections, 10 μm thick, were cut with a rotary microtome and mounted on glass microscope slides with Haupt's adhesive. Sections were cleared of paraffin with xylene, stained with Johansen's quadruple stain, sealed with permount, and examined microscopically.

The tests were conducted in temperature-controlled growth chambers with minimum and maximum temperatures of 20 and 30 C, respectively. Fluorescent and incandescent light (ca. 3,000 lux) provided a 14:10 hour light:dark photoperiod each day. The test was repeated three times as a

randomized complete block with four replications. Data were subjected to analysis of variance using the SAS general linear models procedures (24).

Results

Second-stage juveniles were observed within root tissues by 3 days after inoculation regardless of the presence or absence of *F. solani* (Table 1). Juveniles were most frequently oriented parallel to the longitudinal axis of the root. Small necrotic lesions at the point of nematode entry were visible along the tap and lateral roots. No differences were noted in the location of J2 penetration in the presence of *F. solani*. By 6 days after inoculation, SJ2 were observed and presumed to be actively feeding in the presence or absence of *F. solani*. Third-stage juveniles, characterized by a prominent cuticular ridge, were observed 9 days after inoculation. At this stage, J3 protruded from the cortical and epidermal layers of the roots. Fourth-stage juveniles were observed 12 days after inoculation in both treatments. Males at 12 days were more elongated in contrast to the oval-shaped J4 females. At 12 days, males were observed in the *H. glycines* treatment, whereas females were found only in the *H. glycines* + *F. solani* treatment. At 12 days after inoculation, fewer J2 ($P = 0.05$) and more J3 ($P = 0.05$) were observed in the presence of *F. solani*. At day 15, females and males were observed in both treatments. The female to male ratio averaged 2.3:1 for *H. glycines* alone and 4.3:1 for the *F. solani* + *H. glycines* combination. Fewer SJ2 were observed in the *H. glycines* + *F. solani* treatment. Eggs were observed within the females egg mass by 18 days after inoculation in both treatments.

Total nematode numbers per gram of root were lower in the *F. solani* + *H. glycines* treatment compared to *H. glycines* alone at each sample date (Fig. 1). Lower population levels of *H. glycines* were observed at 6, 9, 12, 15, and 30 days after inoculation in the presence of *F. solani*. The number of *H. glycines* was 47% less in the presence of *F. solani* compared to *H.*

Table 1. Percentage of *Heterodera glycines* in each developmental life stage on soybean cv. Coker 156 infested with *H. glycines* alone (−) and in the presence of *Fusarium solani* (+) from 3 to 27 days after inoculation (DAI).

		Heterodera glycines %					
DAI	Treatment	J2	SJ2	J3	J4	M	F
3	+	94 a	6 a	0 a	0 a	0 a	0 a
	−	91 a	9 a	0 a	0 a	0 a	0 a
6	+	72 a	27 a	1 a	0 a	0 a	0 a
	−	63 a	36 a	1 a	0 a	0 a	0 a
9	+	28 a	59 a	13 a	0 a	0 a	0 a
	−	42 a	49 a	9 a	0 a	0 a	0 a
12	+	10 b	40 a	40 a	9 a	0 a	1 a
	−	16 a	37 b	24 b	21 a	2 a	0 a
15	+	8 a	24 b	21 a	15 a	12 a	20 a
	−	8 a	38 a	22 a	11 a	7 a	14 a
18	+	3 a	14 a	15 a	15 a	22 a	31 a
	−	2 a	12 a	14 a	23 a	25 a	24 a
21	+	3 a	5 a	6 a	14 a	16 a	56 a
	−	2 a	9 a	10 a	22 a	19 a	38 a
24	+	2 a	9 a	8 a	11 a	15 a	55 a
	−	4 a	8 a	8 a	12 a	14 a	54 a
27	+	4 a	4 a	3 a	8 a	12 a	69 a
	−	1 a	5 a	10 a	13 a	20 a	51 a
30	+	4 a	4 a	7 a	11 a	11 a	63 a
	−	5 a	3 a	2 a	10 a	10 a	69 a

Values are means of three tests of four replications.
Within time intervals, means in columns followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple-range test.

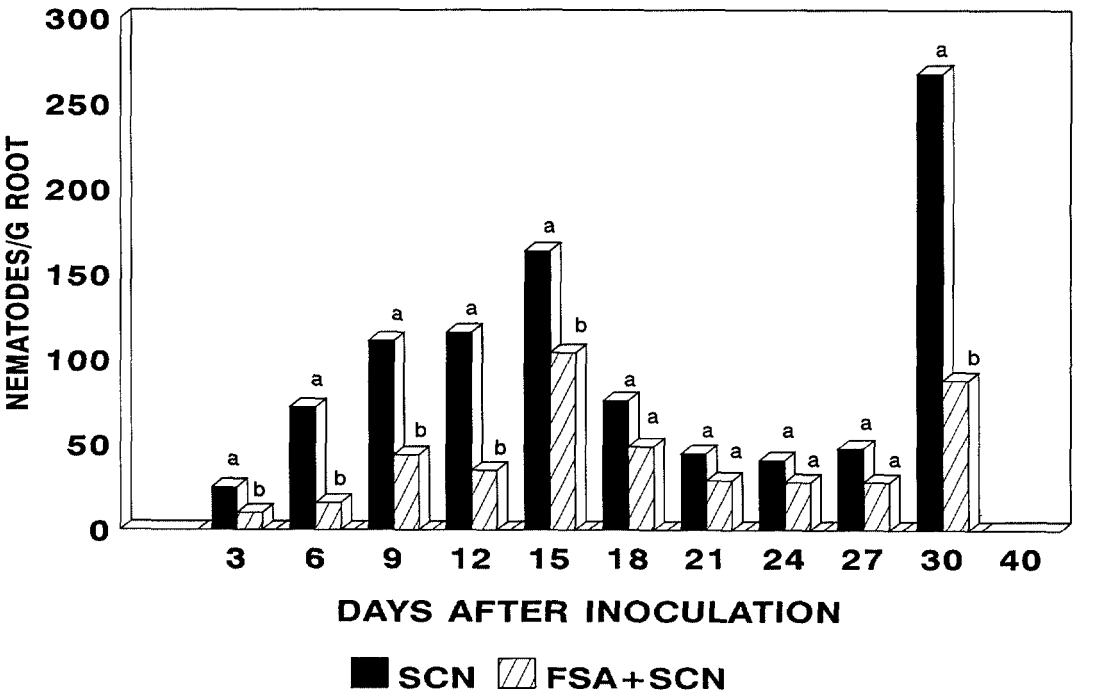

FIG. 1. Numbers of *Heterodera glycines* (SCN) per gram of Coker 156 soybean root in the presence or absence of *Fusarium solani* (FSA).

glycines alone when averaged across all sample dates.

At 40 days after inoculation, 37% of the cysts recovered from the *F. solani* + *H. glycines* combination were colonized by *F. solani* when plated on PDA/SA. Individual eggs from colonized cysts were also colonized by *F. solani*. Fungal mycelium and characteristic *F. solani* chlamydospores were observed in both the cysts and eggs. *Fusarium solani* was not reisolated or observed within *H. glycines* cysts or eggs from the *H. glycines* alone treatment.

Mean stage of the *H. glycines* population was altered by the presence of *F. solani*. The rate of development of *H. glycines* fit a linear model in the presence or absence of *F. solani* (Fig. 2). The slopes of the lines or regression coefficients were 20.9410 and 21.5127 for *H. glycines* alone and *H. glycines* + *F. solani* combined, respectively. The presence of *F. solani* increased the rate of *H. glycines* development by 3%.

Histological study: Heterodera glycines penetrated soybean cv. Coker 156 equally in the presence or absence of *F. solani*. The presence of *F. solani* did not appear to alter the location on the root or the numbers of J2 that penetrated. Juveniles (J2) entered the root randomly, disrupting cortical cells as they ingressed toward the vascular system. Second-stage juveniles in both treatments aligned themselves parallel to the vascular system during parasitism, as previously described (4). *Fusarium solani* mycelia were found more abundantly in areas of J2 penetration and ingress (Fig. 3A) compared with locations where no J2 penetration was observed. *Fusarium solani* mycelia in close proximity to *H. glycines* were found throughout the broken cortical cells ruptured by the J2. Intercellular and intracellular mycelia were found in the root cortex and closer to the vascular cylinder (Fig. 3B). *Fusarium solani* mycelia also colonized the cells located adjacent to the developing juveniles and were observed in the intercellular spaces around the nematode-induced syncytia 9 days after inoculation. At 12 days, the syncytial protoplast appeared granular and degenerated (Fig. 4A), with an absence of nuclei and protoplasm. Fungal mycelia could be observed within the degenerated syncytia,

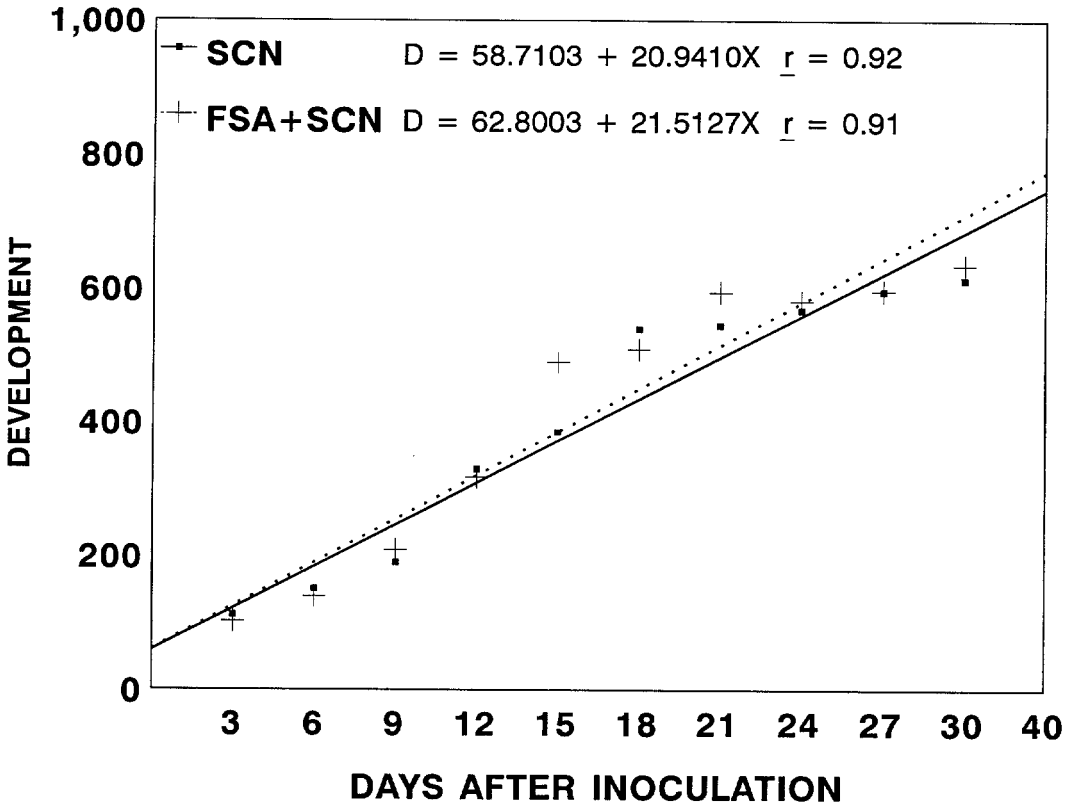

FIG. 2. *Heterodera glycines* (SCN) development on Coker 156 soybean in the presence or absence of *Fusarium solani* (FSA). Development is represented by D = (%J2) + (%SJ2 × 2.33) + (%J3 × 3.67) + (%J4 × 5.83) + (%M × 6.33) + (%F × 6.67).

and in many cases the cells were devoid of all contents. In later stages of fungal invasion, the syncytial cell walls were broken. *Fusarium solani* mycelium not associated with *H. glycines* remained in the intercellular spaces of the epidermal and outer cortical root cells. The syncytia associated with *H. glycines* without *F. solani* consisted of large cells containing a dense cytoplasm with multiple nuclei (Fig. 4B).

Fungal parasitism was not limited to just the soybean root cells. Developing juve-

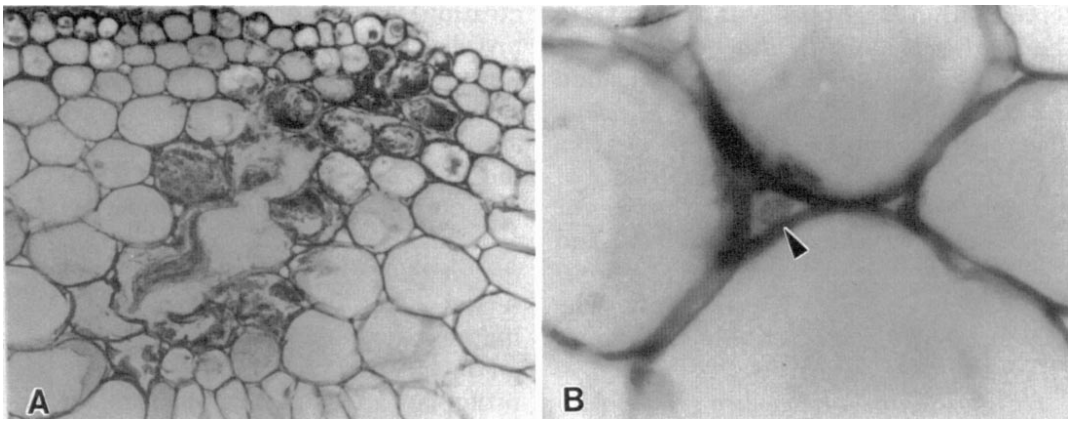

FIG. 3. *Fusarium solani* mycelia in Coker 156 soybean root. A) Mycelia associated with areas of *Heterodera glycines* penetration and migration in the root cortex (×200). B) Intercellular colonization of the cortex (×400).

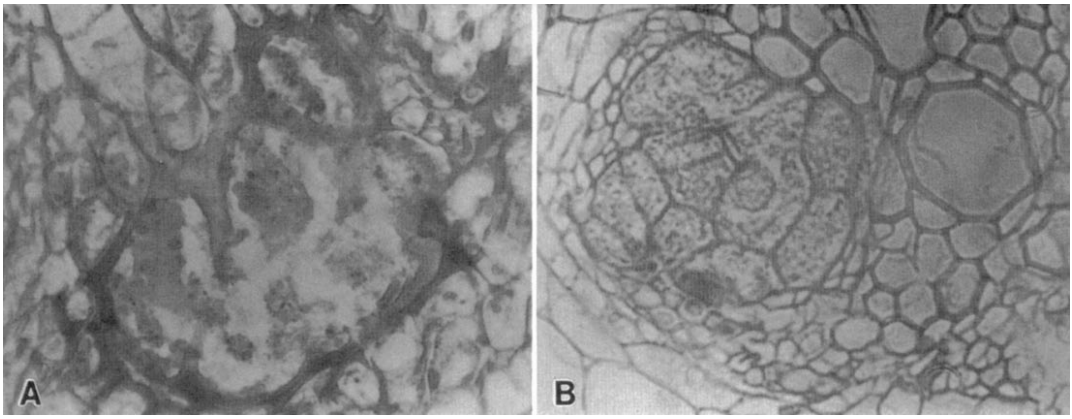

FIG. 4. A) Syncytium degeneration characterized by cell wall deterioration and the absence of nuclei in the presence of *Fusarium solani* (×400). B) Syncytium in the absence of *Fusarium solani* (×400).

niles appeared abnormal at 6 days after inoculation. At 9 days, fungal mycelium ramified throughout the developing juveniles (Fig. 5A,B), and colonized juveniles were devoid of body contents, with only the cuticle remaining. In addition, fungal mycelium and chlamydospores were observed in mature females 40 days after inoculation.

DISCUSSION

The blue form of *F. solani* significantly reduced *H. glycines* race 3 populations while affecting the maturation rate. *Heterodera glycines* maturation rate was increased 3%, whereas total population development was reduced 47%. The maturation rate of *H. glycines* J2, SJ2, and J3 life stages at 12 and 15 days after inoculation was increased when *F. solani* was present. Also, increased numbers of J4, males, and females occurred at 15 days when *F. solani* was present. Although fewer *H. glycines* males occurred in association with *F. solani,* the female-to-male ratios for both treatments were within the normal expected range (9). The maturation rate of each life stage increased in the presence of *F. solani,* and life stage developmental times closely coincided with previous studies (9). An increased rate of *H. glycines* maturation has also been reported when this species is associated with certain plant-parasitic fungi (13,14). The combination *H. glycines* and *C. crotalariae* increased maturation of the first generation; however, the effect on maturation was not as evident on later generations (13).

Numbers of *H. glycines* were lower in the

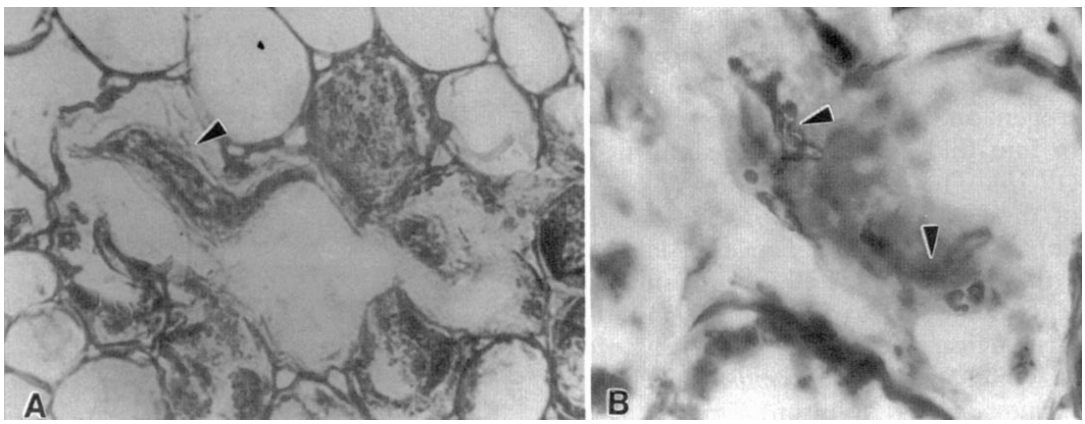

FIG. 5. A) Mycelia in cortical cells adjacent to a *Heterodera glycines* juvenile and within the juvenile body cavity (×400). B) Hyphae extending from the J2 (×1,000).

presence of *F. solani* at all sample dates. The suppression of nematode numbers in the presence of *F. solani* may be related to the effect of *F. solani* on soybean root growth and to parasitism of the nematode by the fungus. The suppression of nematode numbers corresponds directly with reductions of soybean root length and fresh weights. The initial decrease in numbers of *H. glycines* per gram of root in the presence of *F. solani* is a major factor in the reduced final population of *H. glycines.* Subsequent population densities would be affected—including at-harvest populations, as observed in previous field tests (11). Other investigators working with fungal nematode complexes have hypothesized that reductions in nematode populations may be partially attributed to a reduction in the food base (16,20) or changes in host physiology and morphology (14,16). Therefore, the reduction in *H. glycines* numbers may be attributed to soybean root necrosis. *Fusarium solani* can increase root necrosis ratings (11), and the two pathogens in combination additively increased root necrosis in these experiments. This results in a reduction in the amount of total nutrients available to the nematode, possibly suppressing development and reproduction.

Soybean root tissue modified by *H. glycines* was susceptible to fungal invasion. *Fusarium solani* colonized the intercellular and intracellular spaces of the cortex and syncytia adjacent to the developing juveniles. The syncytia produced by *H. glycines* are nutrient sinks (3). The concentration of proteins, nucleic acids, phosphorus, nitrogen, and amino acids is increased two- to four-fold compared to healthy tissues (12,15). The increase in nutrient content of these cells may also provide an additional food source for *F. solani.* Many syncytia were devoid of all protoplasmic contents at late stages of *F. solani* colonization. Histological studies of *M. incognita* (Kofoid & White) and *Fusarium oxysporum* Schlecht. *F. nicotianae* (J. Johnson) Synd. & Hans. (12) and *Phytophthora parasitica* var. *nicotianae* (Breda de Hann) Tucker indicated that nematode-colonized tissues and associated giant cells are susceptible to fungal invasion (17). A loss of the protoplasmic contents of these cells was associated with fungal infection (12,17). In our test, parenchyma cells replaced the empty syncytia at 30 days. This is characteristic of inactive syncytia (17).

No reference was found to an interaction in which a soybean fungal pathogen is also pathogenic to *H. glycines* and capable of reducing nematode numbers. When cysts recovered from the *H. glycines* + *F. solani* combination were surfaced sterilized and placed on culture medium, *F. solani* grew out of the cysts. *Fusarium solani* colonized 37% of *H. glycines* cysts in this test, which is consistent with other tests conducted with *F. solani* in the greenhouse and the field (10,11). The ability of *F. solani* to reduce numbers of *H. glycines* by direct parasitism is also supported by histological observations. Fungal mycelium was found in juveniles, adults, and cysts. Many *H. glycines* juveniles did not reach the adult stage because of parasitism by *F. solani.* Reductions in *H. glycines* populations would be directly related to this active parasitism by *F. solani.*

These studies demonstrate that the blue form of *F. solani,* which causes SDS, also reduces population levels of *H. glycines.* However, the population reduction would not be great enough to prevent damage to a subsequent soybean cultivar resistant to SDS but susceptible to *H. glycines.*

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