Sporulation of the Fungus *Hirsutella rhossiliensis* from the Nematode *Criconemella xenoplax*

B. A. JAFFEE, Visiting Assistant Professor, and E. I. ZEHR, Professor, Department of Plant Pathology and Physiology, Clemson University, Clemson, SC 29631

ABSTRACT

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Hirsutella rhossiliensis emerged from colonized *C. xenoplax* when incubated for 1 day at 25 C in moist chambers or soil. Emergent hyphae branched infrequently and developed phialides and spores after 2–3 days. With the host nematode as the only substrate, about 700 spores per nematode were produced in moist chambers. Sporulation ceased after 21 days. Optimum, maximum, and minimum temperatures for sporulation from colonized nematodes on agarose were about 25, 30, and 10 C, respectively. Spore production was generally high between pH 5.5 and 7.0, the range common in South Carolina peach orchard soils. Sporulation on agarose was not stimulated by KCl or NaCl. Decreasing the osmotic potential below −5 bars with these salts suppressed spore production until few or no spores were observed at −40 bars.

The ectoparasitic nematode *Criconemella xenoplax* (Raski) Luc & Raski is an important pest of peach in South Carolina because of its role in the peach tree short-life syndrome (5,8). We reported previously (2,3) that the fungus *Hirsutella rhossiliensis* Minter & Brady parasitizes juvenile and adult *C. xenoplax*. This fungus produces nonmotile, adhesive spores that stick to the nematode's cuticle. *H. rhossiliensis* penetrates the cuticle, kills the nematode, and continues to grow within the nematode's body (2,6). Once the nematode is filled with hyphae (colonized), the fungus emerges and sporulates. Because sporulation of *H. rhossiliensis* may influence its ability to suppress nematode population densities, we studied the manner of sporulation and the effects of temperature, pH, and osmotic potential on sporulation from colonized *C. xenoplax*.

MATERIALS AND METHODS

Colonized *C. xenoplax*. Healthy adult females obtained from greenhouse pot cultures were surface-disinfested in 0.5% NaOCl for 15 sec, rinsed in sterile distilled water, inoculated with about 20 spores of *H. rhossiliensis* (ATCC 46487) per nematode (2), and incubated at 25 C in sterile distilled water adjusted to −6 bars with KCl (3). After 3 days of incubation, the nematodes were filled with hyphae (colonized).

Extent and manner of sporulation. Colonized *C. xenoplax* were rinsed in sterile distilled water for 1 hr and incubated at 25 C in moist chambers, petri plates containing agarose, or on glass slides covered with soil (buried slides). Moist chambers consisted of 9-cm plastic petri plates containing a moist Whatman No. 2 filter paper with a 6-cm-diameter circle removed from the center. Colonized *C. xenoplax* were placed in the center of the plate (one nematode per plate). One colonized *C. xenoplax* per plate was also placed on 0.5% SeaPlaque agarose (FMC Corp., Rockland, ME 04841) in 5.5-cm plastic petri plates. After incubation, moist chambers and agarose plates were examined at ×140 with a dissecting microscope to determine the number of spores produced per nematode. Buried slides were prepared by attaching a square (0.25 mm^2) of double-stick transparent tape to a glass microscope slide. A colonized *C. xenoplax* was placed on the tape and the slide was placed in a 10-cm glass petri plate and covered with 1 cm of peach orchard soil at 8% moisture (−0.5 bars). After incubation, slides were removed carefully from the soil, washed gently, stained with 0.01% cotton blue in lactophenol, and examined at ×125 or 500 with a compound microscope.

Effects of temperature, pH, and osmotic potential on sporulation. Colonized nematodes were placed on 0.5% agarose (unamended, amended with buffers, or amended with KCl or NaCl for temperature, pH, or osmotic potential studies, respectively) in 5.5-cm petri plates (one nematode per plate). Sporulation was measured after 6 days of incubation at 25 C unless otherwise indicated. The pH of 0.5% agarose was adjusted to 4.0, 5.0, and 6.0 with 0.025 M potassium citrate buffer; to 5.2, 6.0, and 7.0 with 0.05 M potassium 2[N-morpholino]ethanesulfonic acid (MES) buffer (Sigma Chemical Co., St. Louis, MO 63178); and to 6.0, 7.0, and 8.0 with 0.05 M potassium phosphate buffer. At pH 4.0, the buffer and agarose were mixed after autoclaving to permit hardening of the agarose; for other pH values, agarose powder was dissolved in the appropriate buffer before autoclaving. The pH of the solidified agarose was measured with a surface electrode before and after incubation. In a separate experiment, the K^+ content of all buffers was adjusted to 100 mM with KCl to determine if the response to pH was influenced by K^+ concentration. The osmotic potential of unbuffered agarose was adjusted with KCl or NaCl (3). The osmotic potential of the solidified agarose was confirmed by direct measurement with a dew-point hygrometer (HR-33T Dew Point Microvoltmeter and C-52 sample chamber, Wescor, Inc., Logan, UT 84321) calibrated with KCl solutions.

Except for the pH tests, which were performed twice, all experiments were performed at least four times with four to 10 nematodes per treatment. Length of incubation was measured from day zero, when colonized nematodes were transferred to moist chambers, buried slides, or agarose.

RESULTS AND DISCUSSION

Extent and manner of sporulation in moist chambers, soil, and agarose. Within 1 day, hyphae emerged from the heads and tails of the colonized nematodes in moist chambers. Phialides and spores developed on emerging hyphae within 3 days; vegetative hyphae without phialides were not observed. The hyphae radiated from the nematode and branched infrequently. When first placed in moist chambers, colonized nematodes were filled with hyphae, but as sporulation slowed (about day 14), the hyphae within the nematodes contracted (see Fig. 2 of ref. 2). By day 21, sporulation ceased; the fungus appeared to have assimilated all of the nematode except the cuticle and had produced about 700 spores per nematode (Fig. 1). At day 21, the mean ±SD number of hyphae, hyphal branches, and hyphal length per colonized nematode

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Present address of first author: Pennsylvania State University, Fruit Research Laboratory, Biglerville, PA 17307.

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was 24 ± 7, 5 ± 3, and 3.6 ± 0.8 mm, respectively.

Although fewer spores per nematode were observed on buried slides (Fig. 1), the manner of sporulation in soil was similar to that in moist chambers. By day 6, some hyphae were damaged and lost during separation from the soil; therefore, sporulation in soil after that time was not assessed.

Agarose was used with the intention that it would not support fungal growth by itself but would facilitate both observation of sporulation from colonized nematodes and accurate adjustment of pH and osmotic potential. The manner of sporulation from colonized nematodes on agarose was initially similar to that in moist chambers or soil, but some hyphae grew into the agarose and did not produce phialides (Fig. 2). Spore counts at days 3 and 6 were similar on agarose and in moist chambers (Fig. 1). After day 6, however, branching of hyphae on agarose made spore counting difficult. Furthermore, colonies did not stop growing as they did in moist chambers. The ability of agarose alone to support growth of *H. rhossiliensis* was demonstrated by placing spores on 0.5% agarose made with 3X distilled water. The spores germinated and slowly developed into sporulating colonies. Because growth and sporulation on agarose alone were slow and sparse, sporulation from colonized nematodes through day 6 probably resulted from nutrients supplied by the nematode rather than by the agarose.

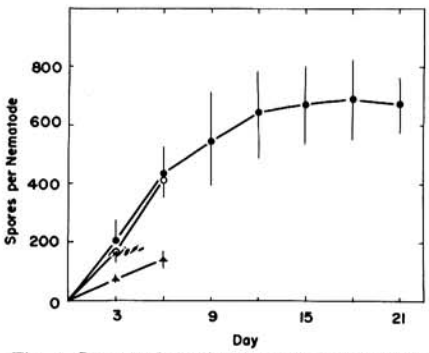

Fig. 1. Sporulation of *Hirsutella rhossiliensis* from *Criconemella xenoplax* at 25 C in moist chambers (dark circles), soil (triangles), or agarose (open circles). Each value is the mean of four replicates, six to 10 nematodes per replicate. Vertical bars indicate standard deviation.

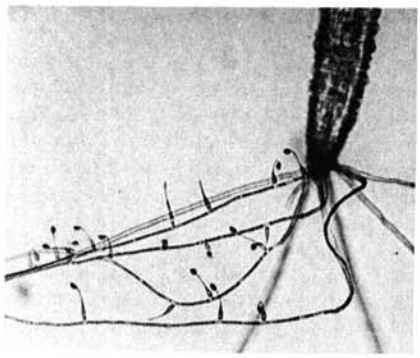

Fig. 2. Sporulation of *Hirsutella rhossiliensis* from a colonized *Criconemella xenoplax* after incubation for 3 days on agarose at 25 C (×200). Initial sporulation on agarose was similar to that in moisture chambers or soil.

Effects of temperature, pH, and osmotic potential on sporulation. The optimum, maximum, and minimum temperatures for sporulation from nematodes on unbuffered agarose were about 25, 30, and 10 C, respectively (Fig. 3A). Based on soil temperature data for South Carolina (4), maximum sporulation would be expected in spring and fall, moderate sporulation in summer, and minimum sporulation in winter (Fig. 3B).

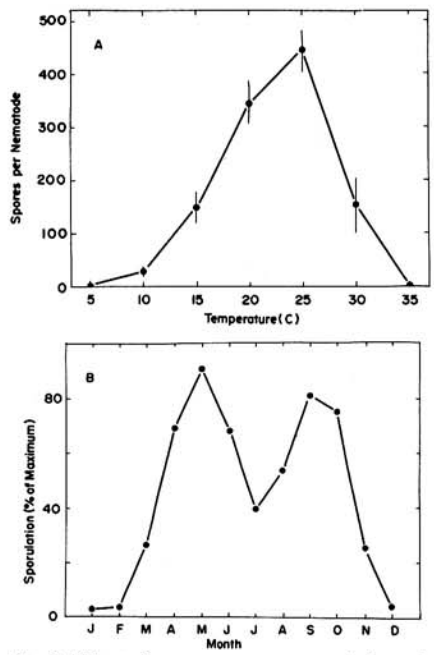

Fig. 3. Effect of temperature on sporulation of *Hirsutella rhossiliensis* from *Criconemella xenoplax*. **(A)** Sporulation from colonized nematodes incubated for 6 days on 0.5% agarose in petri plates. Each value is the mean of four replicates, four nematodes per replicate. Vertical bars indicate standard deviation. **(B)** Expected sporulation (percent of maximum) based on soil temperature data (10-cm depth) for South Carolina and sporulation data from (A).

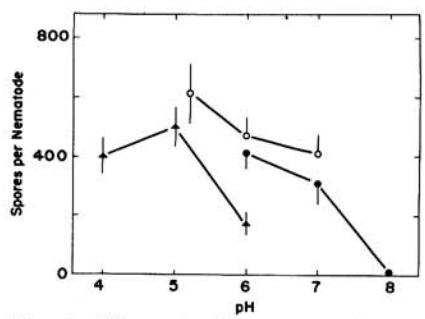

Fig. 4. Effect of pH on sporulation of *Hirsutella rhossiliensis* from *Criconemella xenoplax*. Colonized *C. xenoplax* were incubated at 25 C for 6 days on 0.5% agarose made with 0.025 M citrate buffer (triangles), 0.05 M MES buffer (open circles), or 0.05 M phosphate buffer (dark circles). Each value is the mean of eight replicates, one nematode per replicate. Vertical bars indicate standard deviation.

Sporulation from nematodes on buffered agarose was generally high at pH 5.5–7.0 (Fig. 4), the range common in South Carolina peach orchard soils. Although the data suggest a pH optimum between 4 and 6, the response to pH varied with the buffer. For example, at pH 6.0, sporulation was high with MES and phosphate buffers but low with citrate buffer. The differential response to pH was not influenced by K^+ concentration because when the K^+ content of all buffers was adjusted to 100 mM with KCl, results were similar to those obtained with unequal K^+ concentration (*unpublished*). The pH of unbuffered agarose varied from 5.5 to 6.5 among experiments but did not vary within experiments. The pH of the agarose did not change after 6 days of incubation.

Certain salts, including KCl, stimulate infection of *C. xenoplax* by *H. rhossiliensis* (3). Although stimulation of growth and sporulation by salts or lowered osmotic potential has been reported for other fungi (1,7), sporulation of *H. rhossiliensis* from colonized *C. xenoplax* on unbuffered agarose was not stimulated by KCl or NaCl (Fig. 5). Decreasing the osmotic potential to −10, −20, or −40 bars resulted in decreased sporulation. Only a few spores were produced on KCl-amended agarose at −40 bars and none at −80 bars, and no sporulation occurred on NaCl-amended agarose at −40 or −80 bars. As reported for *Fusarium roseum* by Sung and Cook (7), sporulation was generally greater in agarose osmotically adjusted with KCl rather than with NaCl.

South Carolina peach orchards infested with damaging levels of *C. xenoplax* are usually sandy and well leached. The osmotic potentials of saturation extracts from five such soils ranged from −0.1 to −0.9 bars (3). Thus, the osmotic potentials in these soils are

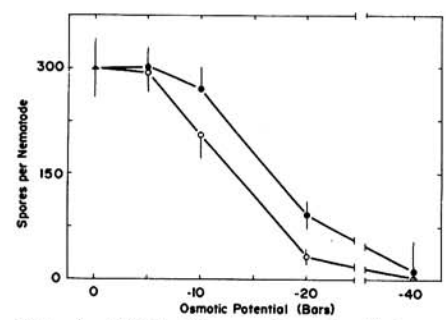

Fig. 5. Effect of osmotic potential on sporulation of *Hirsutella rhossiliensis* from *Criconemella xenoplax*. Colonized *C. xenoplax* were incubated at 25 C for 6 days on 0.5% agarose adjusted to 0 (triangle), −5, −10, −20, or −40 bars with KCl (dark circles) or NaCl (open circles). Each value is the mean of four replicates, four nematodes per replicate. Vertical bars indicate standard deviation.

probably too high to limit sporulation of *H. rhossiliensis*.

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