Influence of Nutrition, Temperature, Moisture, and Gas Composition on Parasitism of Rhizopus oryzae by Syncephalis californica

W. E. Hunter, J. M. Duniway, and E. E. Butler

Department of Plant Pathology, University of California, Davis, CA 95616. Present address of first author: Diamond Shamrock Chemical Co., 5639 Camellia Ave., Sacramento, CA 95819.

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ABSTRACT

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The influence of environment on parasitism of *Rhizopus oryzae* by *Syncephalis californica* was studied in vitro and in nonsterilized field soil. *Syncephalis californica* frequently was observed parasitizing *R. oryzae* in nature on decaying apricot fruit on soil in an orchard at Winters, California. Host and parasite populations in soil were measured at depths of 3 and 20 cm in the orchard from May through November 1973; propagules per gram of dry soil ranged from 23 to 1,847 for *Rhizopus* spp. and from 40 to 233 for *S. californica*. There were no consistent relationships between the populations of the hosts and parasite in the field. Parasitism occurred in vitro at temperatures of 15-36 C, at pH values of 4.0-7.5, and at four different carbon:nitrogen ratios. Parasitism was observed in vitro and in nonsterilized orchard soil at oxygen concentrations of 1-21% and at carbon dioxide concentrations of 0.03-5%. Soil moisture levels between saturation and field capacity, but not including saturation, were suitable for parasitism. Infection by *S. californica* suppressed sporulation of *R. oryzae* under most conditions that were suitable for growth of the host. The results indicate that *S. californica* is an aggressive mycoparasite capable of attacking *R. oryzae* under a wide set of soil environments.

Additional key words: biocontrol, soil fungi.

Many fungi have been reported to be mycoparasites. As early as 1890 Zopf (12) listed more than 50 species of fungi parasitic on other fungi and subsequent workers have greatly increased this number (2, 7). Nevertheless, little is known about the occurrence of mycoparasitism in natural soils. The only critical work that we are aware of is by Boosalis (1) who estimated the frequency of parasitism of *Rhizoctonia solani* by *Penicillium vermiculatum* in soil by observing hyphae of *P. vermiculatum* inside the hyphae of *R. solani*. In that study, the probabilities are high that the fragments with internal hyphae developed while *R. solani* was alive, but soil is biologically complex and the hyphae of *R. solani* could have become colonized by *P. vermiculatum* after being killed by unknown factors. Thus, in the best studies available there is some doubt that mycoparasitism is actually the phenomenon being observed in soil. Even if the foregoing were not a limitation, there is doubt about the frequency of mycoparasitism in soil. In a recent book on the ecology of soil fungi, Griffin (5) offers the opinion that mycoparasitism is relatively rare and probably not of much ecological importance in soil. It is possible that Griffin is correct, but many studies will be needed to make a meaningful evaluation.

In the present paper we present the results of studies on *Syncephalis californica* Hunter & Butler as a parasite of *Rhizopus oryzae* Fischer in natural field soil and in vitro and the influence of environmental and nutritional factors on mycoparasitism. The parasitic relationship between *S. californica* and *R. oryzae* lends itself to these kinds of studies. Infection of the host is followed by the development of large swellings in host hyphae (Fig. 1) and relatively large reductions in the number of host sporangia formed. These criteria were used to measure the occurrence and frequency of parasitism.

MATERIALS AND METHODS

Field observations.—Populations of host *Rhizopus* spp. and *S. californica* both were monitored from May through November 1973, in the soil of an apricot orchard at Winters, California. Benomyl malt agar made with 25 mg benomyl, 30 g Blue Ribbon malt extract, 10 g glucose, 20 g agar, and 1 liter of distilled water was used to assay soil for propagules of *Rhizopus* spp. Freshly collected soil from 3 and 20 cm below the soil surface was diluted 100-fold with 0.1% water agar. One ml of diluted soil suspension and 10-15 ml of benomyl malt agar at 45 C were added to each of five petri plates for each soil sample. Plates were swirled to distribute the soil and incubated for 24 hr at 36 C. Only *R. oryzae* and *R. arrhizus* Fisch., each a host for *S. californica* (6), produced visible colonies on the medium. The numbers of infectious propagules of *S. californica* were determined by mixing the soil with mycelial fragments of *R. oryzae* and incubating the mixture on malt agar supplemented with 50 mg of chlortetracycline; benomyl was omitted. *Rhizopus oryzae* was grown on potato-dextrose broth for 3 days at 24 C and mycelial mats were blended in 0.1%

water agar. The resulting suspension was used to dilute soil 100-fold and the final dilution of each soil sample was spread on 10 plates of solidified chlortetracycline malt agar. The number of swellings produced by *S. californica* on mycelium of *R. oryzae* was counted after 48 hr at 33 C. Populations of fungi are expressed as numbers of propagules per gram dry soil.

Soil temperature in the orchard was measured at depths of 3 and 20 cm with a recording thermometer (Model 2200, Marshalltown Manufacturing, Inc., Marshalltown, IA 50158). Soil pH was determined to be 7.3 by the method of Schofield and Taylor (9). To

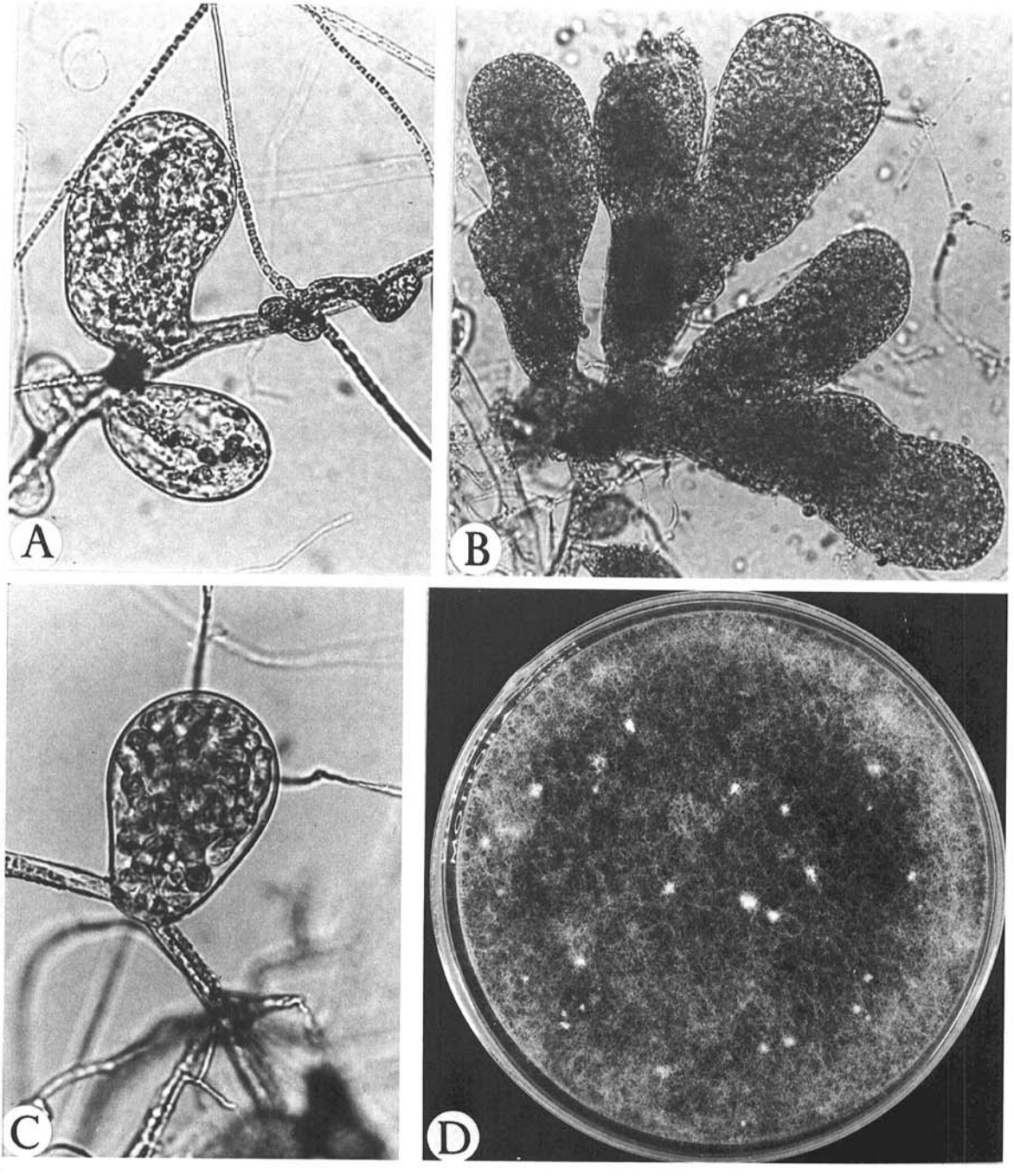

Fig. 1-(A to D). **A, C)** Swellings in hyphae of *Rhizopus oryzae* with internal hyphae of *Syncephalis californica* from culture (×300); **B)** Swellings in hyphae of *R. oryzae* from nonsterilized field soil (×300); **D)** Assay plate with *R. oryzae* infected by propagules of *S. californica* from soil in which the dense white patches are colonies of *S. californica*.

determine concentrations of O_2 and CO_2 in field soil, capillary tubes were buried so that the open ends were either 3 or 20 cm below and the closed ends were above the soil surface. The first gas samples were taken 1 wk later. Samples were collected with 1-ml syringes and samples were transported to the laboratory with rubber stoppers on the needles. One milliliter of gas was removed from each capillary tube and discarded; a second milliliter was collected for analysis. Gas samples were analyzed for O_2 and CO_2 with a Carle Model 8000 gas chromatograph (Carle Instruments, Inc., Fullerton, CA 92631). The orchard soil was a Yolo fine sandy loam containing 47.1% sand, 35.5% silt, and 17.4% clay.

In vitro experiments.—For all in vitro experiments, *R. oryzae* was grown on potato-dextrose broth at 24 C. Three-day-old hyphal mats were rinsed and blended in distilled water at the rate of one mat per liter (ca. 0.5 g dry weight/liter). Sporangiospores of *S. californica* were obtained by transferring infected mycelium of *R. oryzae* to the center of water agar plates (6). *Syncephalis californica*, but not *R. oryzae*, sporulated after 3-4 wk of incubation at 24 C. Sporangiospores were washed from the plates with water and the spore suspension was filtered through sterile cheesecloth to remove mycelial fragments. In vitro experiments on mycoparasitism were initiated by mixing 1 ml of blended mycelium of healthy *R. oryzae* with 3 to 4 × 10^4 sporangiospores of *S. californica* and spreading the mixture on basal agar (BA). The inoculum was spread evenly over the agar surface and petri plates of BA were incubated in the dark. Basal agar contained 10 g glucose, 2 g anhydrous L-asparagine, 0.5 g $MgSO_4 \cdot 7H_2O$, 1.0 g K_2HPO_4, 1.5 mg $Fe(NO_3)_2 \cdot 9H_2O$, 1 mg $ZnSO_4 \cdot 7H_2O$, 0.5 mg $MnSO_4 \cdot 4H_2O$, 15 g purified agar (General Biochemical Co., Chagrin Falls, OH 44022), and 1 liter of glass-distilled water.

The effects of pH, carbon:nitrogen (C:N) ratio, temperature, and gas composition on mycoparasitism were examined in vitro. The pH of BA was adjusted with 1 N NaOH or HCl after autoclaving. The levels of carbon and C:N ratio were adjusted by varying the amounts of glucose and L-asparagine. Gas composition was varied by mixing air, CO_2 and N_2 to give a final flow rate of 15 liters/hr. The desired gas mixture was passed through sealed plastic boxes (approximately 5 liters volume) containing petri plate cultures. Plates for the pH and C:N ratio experiments were incubated at 33 C; those for the gas composition experiments were incubated at 20 C.

Soil experiments.—Mycelial mats of *R. oryzae* from potato-dextrose broth were cut into 13-mm diameter disks. The disks were dipped in a suspension of *S. californica* sporangiospores before being buried in soil. The soil was not sterilized and was collected from the orchard where field observations were made. Glass funnels with porous plates and suspended columns of water (4) were used to control soil matric potential (ψ_m). (Matric potential is equivalent to soil moisture tension or suction; 1 bar = 0.99 atmosphere). The gas composition over soil was varied by passing the desired gas mixture through the tops of enclosed glass funnels containing soil

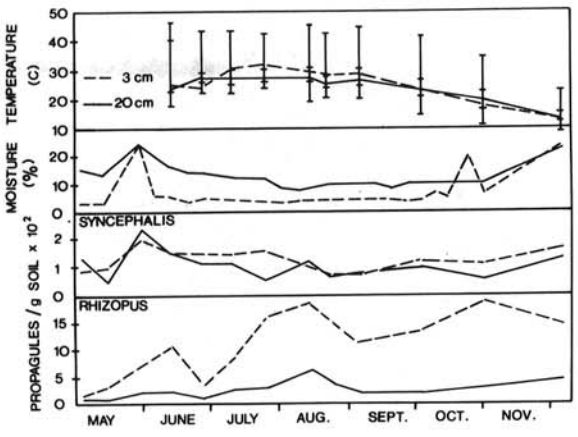

Fig. 2. Temperature, moisture, and the populations of *Syncephalis californica* and *Rhizopus* spp. observed in the soil of an apricot orchard at Winters, California. Data were obtained for soil at depths of 3 cm (broken lines) and 20 cm (solid lines). Temperatures plotted are weekly means and the vertical bars show the extreme temperatures observed. Upper- and lowermost bar limits indicate temperature extremes at 3 cm. Soil moisture is expressed as a percentage by weight of dry soil.

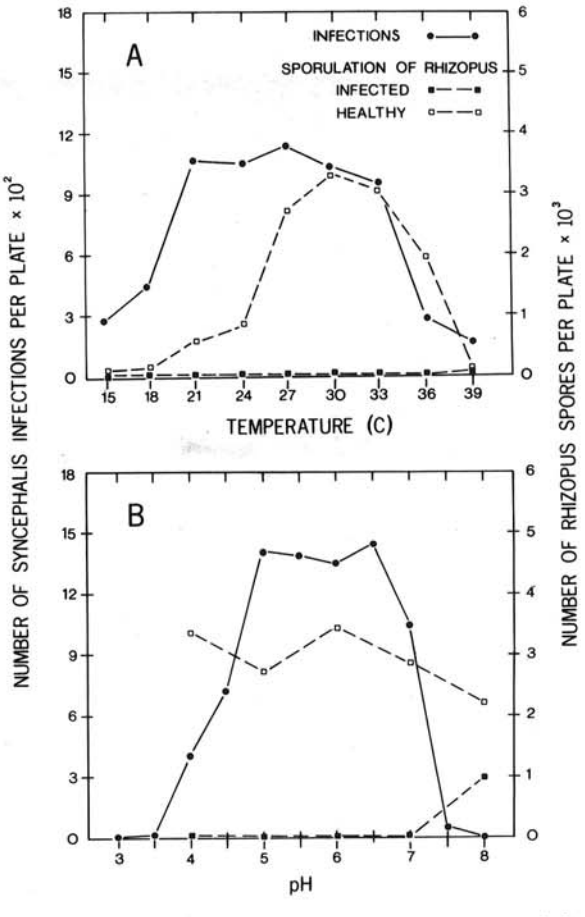

Fig. 3-(A, B). Infection of *Rhizopus oryzae* by *Syncephalis californica* and sporulation of healthy and infected *R. oryzae* in vitro at various temperatures **A)** and pH values **B)**. *Syncephalis californica* sporangiospores from culture were used as inoculum.

(approximately 0.5 liters of air space) at 15 liters/hr. Soil temperature was 22-27 C during the experiments.

In some experiments, sporangiospores of *S. californica* produced in soil were used as inoculum. Infected mycelial mats of *R. oryzae* were buried 3 mm below the surface of field soil and were maintained at $\psi_m = -0.3$ bar. An abundance of *S. californica* sporangiophores formed on the soil surface in 3 days. Sporangiospores were vacuumed from the soil surface with a Pasteur pipette containing a cotton plug to trap the spores.

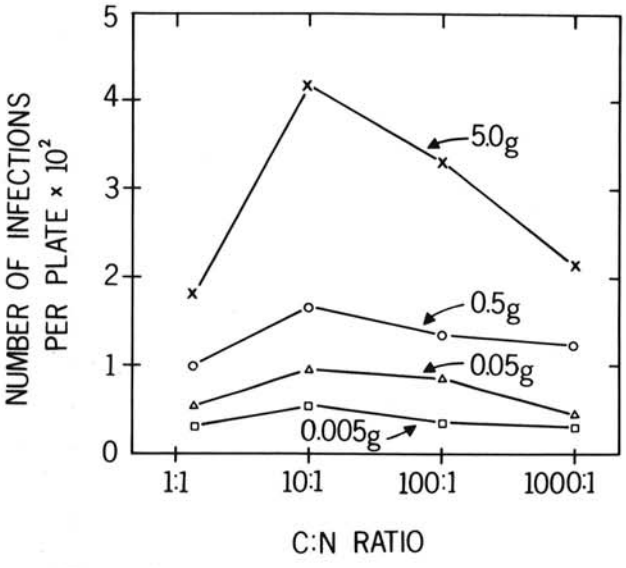

Fig. 4. Influence of the carbon to nitrogen ratio of medium with four different levels of carbon on infection of *Rhizopus oryzae* by *Syncephalis californica*. Total amounts of carbon were equivalent ot 5.0, 0.5, 0.05, and 0.005 g of elemental carbon per liter. Carbon:nitrogen ratios are expressed as moles of carbon per mole of nitrogen. A water agar control yielded 28.6 infections per plate.

Measurements of mycoparasitism.—For in vitro experiments, the numbers of primary infections were estimated by counting swellings [Fig. 1-(A to C)] in hyphae of *R. oryzae* when they first became visible to the unaided eye (Fig. 1-D). Secondary swellings, resulting from growth of *S. californica* within the host, had not yet developed at this time (6). The swellings in four randomly selected areas (1.44 cm^2) of each petri plate culture were counted with the aid of a dissecting microscope (×45). The swellings on mycelial disks from soil were counted 48 hr after inoculation.

Sporulation by healthy and infected colonies of *R. oryzae* was compared because reduction in sporulation is a major pathologic effect of *S. californica* on *R. oryzae* (6). One wk after inoculation, cultures of *R. oryzae* were scraped and rinsed with water to remove the spores which then were counted in a hemacytometer. Sporulation of *R. oryzae* in soil was measured by counting sporangia on the soil surface. Data were analyzed for significance by a Duncan's multiple range test at $P = 0.01$.

RESULTS

Field observations.—To determine initially if *S. californica* could be found parasitizing *Rhizopus* spp. in the field, apricot fruit on the orchard floor were examined

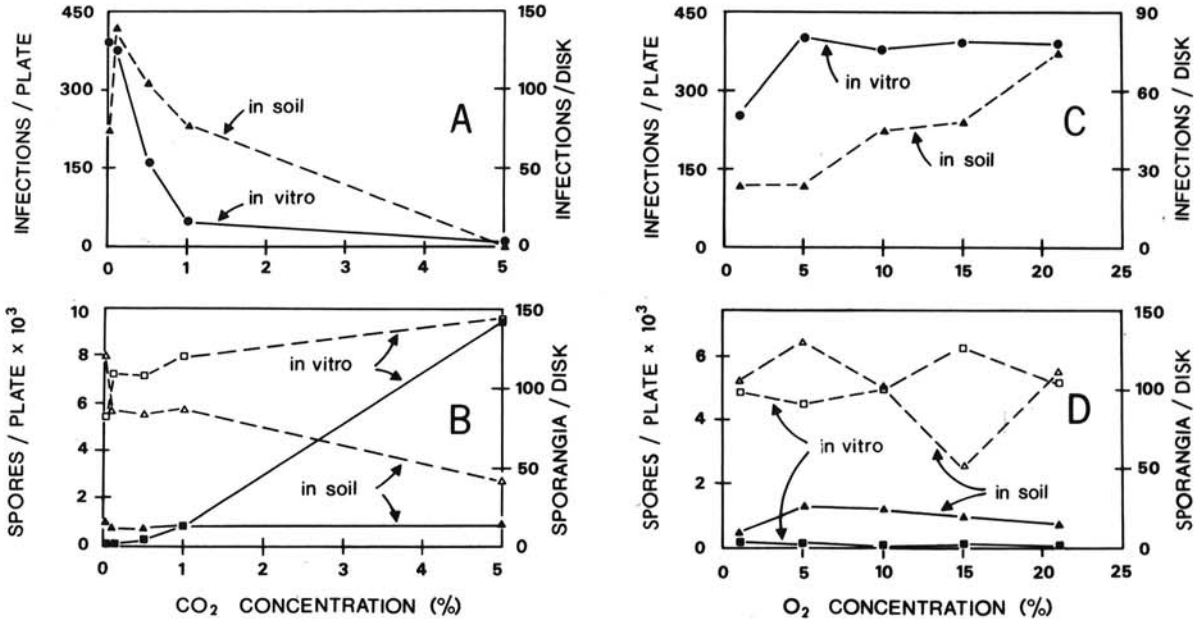

Fig. 5-(A to D). Infection of *Rhizopus oryzae* by *Syncephalis californica* and sporulation of healthy and infected *R. oryzae* in vitro and in nonsterilized soil at various CO_2 (A, B) and O_2 (C, D) concentrations; **A, C)** Number of infections by *S. californica*; **B, D)** Sporulation of healthy *R. oryzae* (open symbols) and infected *R. oryzae* (closed symbols). The oxygen concentration was 21% when carbon dioxide was varied (A, B) and the carbon dioxide concentration was 0.03% when oxygen was varied (C, D). Soil moisture was maintained at −0.3 bar matric potential.

with a ×15 hand lens for the presence of infected *Rhizopus* hyphae during the growing season of 1973. The greatest incidence of fruit with infected hyphae was 7% and that occurred about 1 mo before harvest when the fruit were beginning to ripen. The orchard floor was still damp from a previous irrigation at this time. After the fruit were taken to the laboratory and incubated for 3-4 days at 24 C in moist chambers, a maximum of 30% of the fruit had hyphal swellings which could be seen with a dissecting microscope. These observations indicated that parasitism of *Rhizopus* spp. by *S. californica* was fairly common in the field.

The results of other field observations are shown in Fig. 2. The population of *Rhizopus* spp. at a depth of 3 cm in the soil greatly increased from May to August and remained high through November. Only small fluctuations were observed in the population of *Rhizopus* spp. at a soil depth of 20 cm. The population of *S. californica* did not show any large changes with time at either soil depth and there was no demonstrable correlation between populations of hosts and the parasite. At depths of 3 and 20 cm, respectively, CO_2 concentrations in the soil atmosphere were 0.08-0.42% and 0.19-0.85% by volume. The respective O_2 concentrations were 20.2-21.4% and 17.8-21.4%.

Effects of temperature and pH.—*Syncephalis californica* was capable of infecting *R. oryzae* in vitro over the entire temperature range of 15-39 C (Fig. 3-A). Optimum temperatures for infection were 21-33 C. Numerous infections occurred over a pH range of 4.5-7.0; pH values of 5.0-6.5 were optimum for infection (Fig. 3-B). Even at extreme temperatures and pH values at which comparatively few infections occurred, sporulation of the infected host was greatly reduced [Fig. 3-(A, B)]. Replicate experiments with *S. californica* sporangiospores from culture and soil yielded results nearly identical to those in Fig. 3.

Effect of carbon level and C:N ratio.—As shown in Fig. 4, most infections occurred on modified BA at a C:N ratio of 10:1 at all carbon levels tested. The influence of C:N ratio, however, decreased as the level of carbon was decreased to 0.005 g carbon per liter when there was no longer a significant effect of C:N ratio. Infection greatly reduced sporulation at all levels of carbon and C:N ratios shown in Fig. 4.

Effect of gas composition.—Concentrations of CO_2 higher than 0.1% significantly reduced infection of *R. oryzae* by *S. californica*, both in vitro and in soil (Fig. 5-A). Almost no infection occurred in 5% CO_2 even though *R. oryzae* continued to grow and sporulate (Fig. 5-B). Although infection of *R. oryzae* by *S. californica* decreased as O_2 concentration decreased from 21 to 1% (Fig. 5-C), the decreases in infection were not statistically significant. *Syncephalis californica* significantly reduced sporulation by *R. oryzae* at all CO_2 and O_2 concentrations tested except 5% CO_2 in vitro and in soil (Fig. 5-B) and 15% O_2 in soil (Fig. 5-D).

Effect of soil moisture.—Mycelial mats of healthy *R. oryzae* did not grow or sporulate in saturated soil ($\psi_m = 0$) but did grow and sporulate abundantly in soil at ψ_m values of −0.02 to −0.38 bar (Fig. 6). The number of infections of *R. oryzae* by *S. californica* increased significantly to a maximum as ψ_m decreased from zero to −0.3 bar and decreased significantly as ψ_m decreased from −0.3 to −0.38 bar (Fig. 6). Sporulation of *R. oryzae* infected by *S. californica* at the ψ_m values used tended to be inversely related to the number of infections.

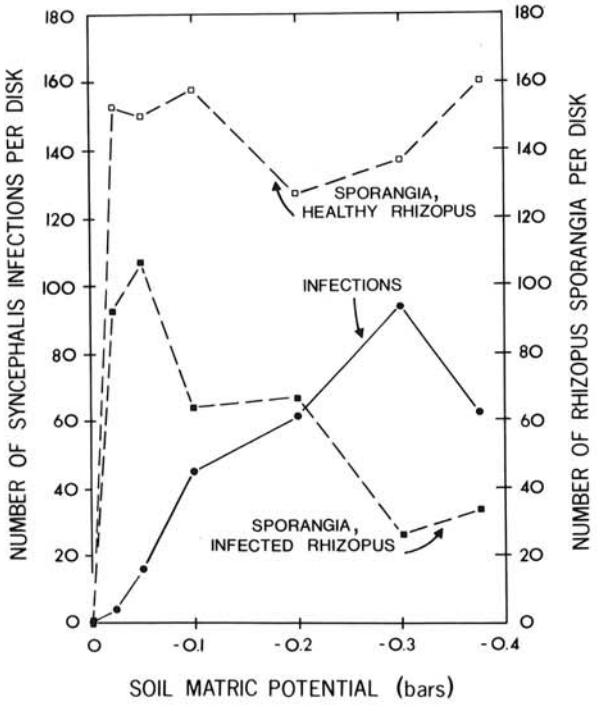

Fig. 6. Infection of *Rhizopus oryzae* by *Syncephalis californica* and sporulation of healthy and infected *R. oryzae* in nonsterilized soil at various matric potentials (ψ_m).

DISCUSSION

This is probably the first report of clear evidence for the occurrence of mycoparasitism in natural soil (1, 3, 10, 11). The development of characteristic swellings on the hyphae of *Rhizopus oryzae* (Fig. 1) is the key to parasitism; when swellings are present there is little doubt that infection by *S. californica* has occurred. In our experience, swellings occur only on living host hyphae and they are always induced by *S. californica*. They are not caused by other soil organisms nor do they appear in noninfected hyphae.

Aside from the induction of swellings, the predominate observable effect of infection by *S. californica* on the host is a suppression of sporulation (6). The in vitro experiments indicate that large reductions in sporulation can be effected by small numbers of infections. For example, sporulation by *R. oryzae* was reduced tenfold by 44 infections of *S. californica* (less than one per cm^2) per culture plate (1% CO_2 in Fig. 5) and sporulation was inhibited entirely by as few as 280 infections per plate (36 C in Fig. 3). More importantly, in nonsterilized soil, sporulation of the host was almost halved by one to four infections per mycelial disk (5% CO_2 in Fig. 5, −0.02 bar in Fig. 6), and a tenfold reduction in sporulation occurred with 24 infections per disk (1% O_2 in Fig. 5). Sporangiospores almost certainly have an important role

in the life cycle and ecology of *R. oryzae*. Zygospores of *R. oryzae* are rarely found in nature and its vegetative hyphae probably are ephemeral, persisting only while actively growing in a suitable, but temporary, ecological niche. Healthy *R. oryzae* usually forms an abundance of sporangiospores which are almost certainly more persistent than mycelium. Furthermore, sporangiospores are the predominate means by which *R. oryzae* is dispersed. Because of the probable importance of sporangiospores to *R. oryzae*, suppressions of sporulation of a magnitude caused by *S. californica* are very likely to influence the population dynamics of *R. oryzae* in nature. In this example of mycoparasitism it appears that the population dynamics of the host can be altered, and perhaps some measure of biological control can be achieved, without the parasite actually killing its host.

Syncephalis californica parasitized *R. oryzae* under almost all conditions tested that were suitable for growth of host mycelium. In vitro, parasitism by *S. californica* was influenced less by the level of carbon or the carbon to nitrogen ratio of the medium than is parasitism by some other mycoparasites (8). Although the numbers of infections by *S. californica* were reduced under some of the nutrient regimes tested (Fig. 4), the reductions probably reflect the influence of nutrition on growth of host mycelium rather than on the parasite because sporulation by infected *R. oryzae* was markedly reduced in all cases as compared to healthy controls (W. E. Hunter, *unpublished*). The temperatures and pH values (Fig. 3) as well as the O_2 and CO_2 concentrations (Fig. 5) conducive to mycoparasitism by *S. californica* can be expected to prevail in many soils for significant periods (e.g., Fig. 2). Likewise, the water regime between saturation and field capacity (i.e., -0.02 to -0.4 bar) was conducive to mycoparasitism (Fig. 6); no parasitism occurred at saturation ($\psi_m = 0$) because the host failed to grow (Fig. 6). One experiment using thermocouple psychrometers indicated that infection by *S. californica* can occur in soil at water potentials down to -25 bars, the lowest water potential at which host mycelium grew (W. E. Hunter, *unpublished*).

In view of the results presented here, *S. californica* is a versatile biotrophic mycoparasite capable of interfering with host growth and asexual reproduction under a wide range of natural conditions. *Syncephalis californica* was found in many different soils from several localities (6) indicating that it is a natural soil inhabitant in diverse environments. The *Syncephalis* sp. studied here is but one member of a cosmopolitan genus of which all 42 species are mycoparasites (6). During our investigations we isolated *S. nodosa* v. Tiegh. from several soils; it grows rapidly, produces large numbers of sporangiospores, and almost completely suppresses sporulation in *R. oryzae*. This suggests that mycoparasitism by *Syncephalis* spp. may play an active role in the biology of *Rhizopus* spp. and other species of the Mucorales.

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