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NOTES

Formation of Appressoria in Vitro by the Entomopathogenic Fungus *Zoophthora radicans* (Zygomycetes: Entomophthorales)

Conidial germlings of the entompathogenic fungus *Zoophthora radicans* (Bref.) Batko (Zygomycetes: Entomophthorales) develop a range of appressorial types to assist in the penetration of the cuticle of *Empoasca fabae* (Homoptera: Cicadellidae) (S. P. Wraight, T. M. Butt, S. Galaini-Wraight, L. L. Allee, and D. W. Roberts, *J. Invertebr, Pathol.* in press). There is, however, no previous study in the formation of appressoria in vitro by this fungus. We describe here an in vitro system whereby conidia and capilliconidia of *Z. radicans* are encouraged to produce appressoria on water agar.

The isolates of *Z. radicans* (ARSEF, 1588, 1590, 1938, and 2196) used in this study are in the U.S. Department of Agriculture, Agricultural Research Service Collection of Entompathogenic Fungi (ARSEF), Ithaca, New York. The principal isolate used was 1590, which was previously passed once through fifth instar nymphs of *E. fabae* while 1588, 1938, and 2196 were subcultured twice on SDAY (1% soytone, 2% dextrose, 1.5% agar, 1% yeast extract) after retrieval from liquid nitrogen storage. All isolates were maintained on SDAY in 35 × 10-mm Petri dishes at 23°C with a 14:10 light/dark cycle.

Conidia of *Z. radicans* were discharged from 10 (2-day-old) SDAY sporulating cultures and inoculated onto a 0.5 to 1.0-mm-thick layer of 1-day-old water agar or SDAY. In order to ensure a relatively uniform distribution of conidia, each 35-mm-diam water agar plate was serially exposed for 2 min to each of the 10 sporulating cultures. Conidia were then overlaid with 0.2 ml of SDY (2% dextrose, 1% soytone, 1% yeast extract) or sterile distilled water and covered with a 22 × 22-mm glass coverslip. Conidial development was also tested on water agar and SDAY without adding any liquid or coverslip. After 48 hr of incubation at 23°C in the dark, 100 conidia in each replicate were scored for the formation of appressoria, germ tubes, passively dispersed capilliconidia, or forcibly discharged secondary conidia.

Conidial germination was evaluated at 7, 11, 14, and 20 hr postinoculation on water agar and SDY (+coverslip); appressorial formation by germinating capilliconidia was also evaluated. Capilliconidia produced by incubating conidia on water agar for 2 hr were treated in the same way as primary conidia except that SDY was diluted 50-fold, and the period of incubation after flooding was 30–32 hr. Diluted SDY was used because capilliconidia germinated better at this concentration than in full-strength SDY or 1% (w/v) yeast extract (unpublished).

Appressoria formed in vitro were com-

TABLE 1

DIFFERENTIATION (PERCENTAGE; MEAN ± STANDARD DEVIATION)[a] OF *Zoophthora radicans* AT 23°C ON DIFFERENT COMBINATIONS OF WATER AGAR, SOLID AND LIQUID MEDIA, AND COVERSLIP 20 hr POSTINOCULATION

Basal/ overlaid medium	GT	APP	NG
SDAY/SDY	82.7 ± 5.2	0.0	17.3 ± 5.2
SDAY/W	77.8 ± 5.17	0.7 ± 0.7	21.4 ± 4.4
WA/SDY	58.8 ± 11.5	33.5 ± 12.5	7.7 ± 3.0
WA/W	84.1 ± 3.6	0.0	15.9 ± 3.7

[a] From 10 replicates of 100 conidia each; SDAY, 1% soytone, 2% dextrose, 1.5% agar, and 1% yeast extract; SDY, 1% soytone, 2% dextrose, and 1% yeast extract; WA, 2% water agar; W, water; GT, germ tube; APP, appressorium; NG, nongerminated.

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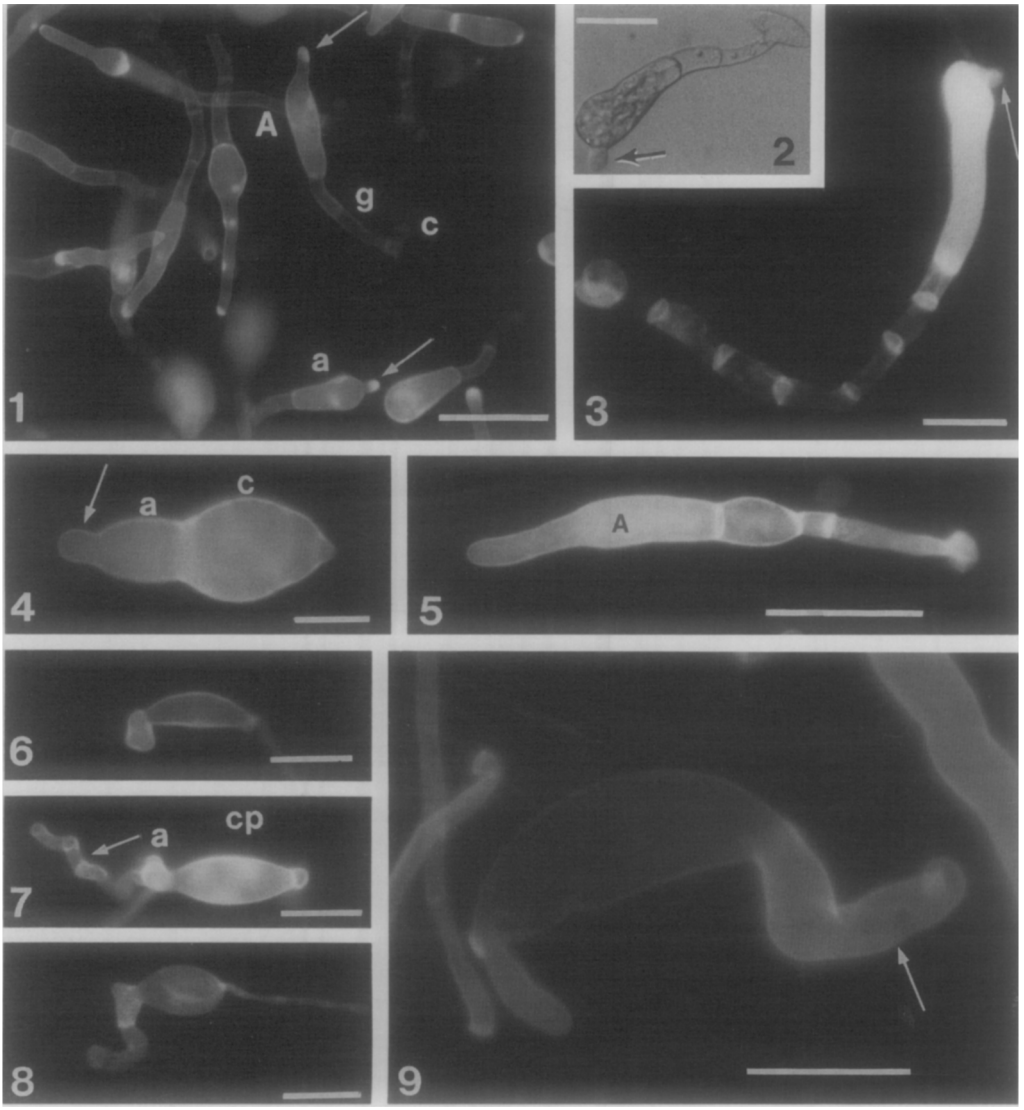

FIGS. 1–9. Appressorial formation by *Zoophthora radicans* in vitro (Figs. 1, 2, and 4–8) and in vivo (Figs. 3 and 9) (c, conidium; cp, capilliconidium; g, germ tube; a, appressorium; A, appressorium-like structure; arrow, penetration hypha).

FIG. 1. Appressoria and appressorium-like structure of *Zoophthora radicans*, 20 hr postinoculation (PI), on water agar covered by SDY and coverslip; bar = 60 μm.

FIG. 2. Appressorium and penetration hypha, 21 hr PI, on water agar covered by SDY and coverslip; bar = 30 μm.

FIG. 3. Appressorium and penetration hypha, 13 hr PI, breaching the cuticle of *Empoasca fabae;* bar = 30 μm.

FIG. 4. Appressorium and short penetration hypha formed directly from primary conidium, 20 hr PI, on water agar covered by SDY and coverslip; bar = 30 μm.

FIG. 5. Appressorium-like structure, 48 hr PI, on SDAY covered by SDY and coverslip (bar = 60 μm).

FIGS. 6–8. Appressoria and penetration hyphae formed from capilliconidia, 30–32 hr PI, on water agar covered by diluted (1/50) SDY and coverslip (bar = 30 μm).

FIG. 6. Short appressorium formed directly from capilliconidium.

FIG. 7. Appressorium and penetration hypha formed from fusiform capilliconidium.

FIG. 8. Appressorium and penetration hypha formed from an oval capilliconidium.

FIG. 9. Germ tube and penetration hyphae formed on *E. fabae* from capilliconidium, 13 hr PI; bar = 12 μm.

pared to those formed on fifth instar nymphs of *E. fabae* following methods described earlier (T. M. Butt, S. P. Wraight, S. Galaini-Wraight, R. A. Humber, D. W. Roberts, and R. S. Soper, *J. Invertebr. Pathol.* **52**, 49–56, 1988). ARSEF 1590 from both in vitro and in vivo systems was stained with 0.01% aq (w/v) Tinopal (Sigma, St. Louis) and examined with an Olympus BH-2 microscope with epifluorescence attachments; the excitation, the beam splitter, and barrier filters used were 390–500, 455, and 455 nm, respectively. Preparations were examined using bright-field and/or epifluorescence optics and photographed with Kodak Tri-X Pan or TMAX 400 film.

Most treatments induced germ tube formation by more than 84% of primary conidia; however, only water agar overlaid with 0.2 ml of SDY and a coverslip favored appressorial formation (Table 1; Figs 1 and 2) and appressorium-like structures (ALS) (Fig. 1). Appressoria produced in vitro resembled those formed on *E. fabae* (Fig. 3). Appressoria of *Z. radicans* were usually formed at the end of a septate germ tube of variable length and often terminated in a bulb from which a narrow hypha (penetration peg) emerged (Figs. 1–3). Vacuoles developed in mature appressoria as the cytoplasm moved forward into the appressorial tip and penetration hypha. At 48 hr postinoculation (PI) in vitro, the pegs or penetration hyphae were 14.0–291.2 μm long and 5.2–14.0 μm wide (av 85 ± 62.5 × 7.8 ± 2.2 μm; n = 100, 10 appressoria randomly selected from each of 10 plates). These structures resembled those formed in vivo on *E. fabae* (T. M. Butt et al., 1988) although the latter were usually thinner and shorter (S. P. Wraight et al., 1989). Appressoria were occasionally formed apically on short germ tubes or directly from conidia (Fig. 4). This mode of differentiation was also seen on *E. fabae* by S. P. Wraight et al. (1989). All treatments encouraged formation of ALS with only slightly narrower penetration hyphae (Fig. 5). The ALS of *Entomophaga aulicae* did not form penetration hyphae in vitro (F. Murrin and R. A. Nolan, Canad. J. Bot. **67**, 754–762, 1989). While few "penetration" hyphae actually entered the agar (Fig. 2), the germ tubes, appressoria, and ALS remained attached to the agar surface even after removing the coverslip. Hyphae produced on water agar overlaid by SDY plus coverslip sometimes produced uninucleate ovoid conidia at the end of extremely long multinucleate filaments.

In the time-course experiment, more than 81% of primary conidia germinated 11–14 hr PI and most appressoria were formed within 20 hr on water agar plus SDY and coverslip (Fig. 10). In vivo, it takes 6 hr for appressorial formation and penetration (S. P. Wraight *et al.*, 1989). The differing rates of appressorial formation in the former experiment (33.5%) and here (58.1%) may be a result of variability in the physiology of the fungus.

Conidia developing on water agar with no added liquid and coverslip strongly tended to form capilliconidia whereas conidia developing on SDAY showed a much greater tendency to produce forcibly discharged secondary conidia (Table 2). H. J. W. van Roermund, D. F. Perry, and D. Tyrrell (*Trans. Brit. Mycol. Soc.* **82**, 31–38, 1984) obtained similar results with *Z. radicans* on SDAY. These observations suggest that the absence of nutrients in the substrate favors

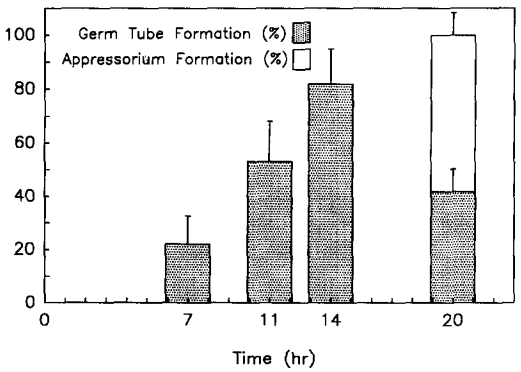

FIG. 10. Time course of *Zoophthora radicans* differentiation at 23°C on 2% water agar overlaid by SDY and a coverslip.

TABLE 2
DIFFERENTIATION (PERCENTAGE; MEAN ± STANDARD DEVIATION)[a] OF *Zoophthora radicans* ON SOLID SUBSTRATE AT 23°C IN THE DARK 20 hr POSTINOCULATION WITH NO LIQUID OVERLAY OR COVERSLIP

Substrate	GT	SC	SC + EPC	CC	NG
Water agar	0.0	1.3 ± 1.5	16.5 ± 13.3	81.9 ± 15.5	1.6 ± 1.6
SDAY	0.7 ± 0.3	23.1 ± 7.0	76.7 ± 12.7	7.4 ± 4.4	15.2 ± 2.3

[a] From 10 replicates of 100 conidia each; GT, germ tube; SC, secondary conidium (remaining attached to the conidiophore); EPC, empty primary conidium; CC, capilliconidium; NG, nongerminated primary or secondary conidium.

the formation of secondary capilliconidia. Cues other than the concentration of nutrients or physical conditions differing from those of these experiments must be necessary to induce formation of germ tubes and appressoria.

Capilliconidia (96 ± 1.1%) readily germinated on water agar covered with dilute SDY (1/50) and a coverslip. Within 48 hr, most had produced appressoria with (26 ± 4.4%) and without (61 ± 3.5%) penetration hyphae (Figs. 6–8), while 9 ± 2.2% produced only germ tubes. Capilliconidia were predominantly lunate to fusiform (Figs. 6 and 7), but ovoid spores were also produced (Fig. 8); only 4 ± 0.9% of the spores failed to germinate. The brightly fluorescing, bulbous appressoria produced by capilliconidia developed close to the spore (Figs. 6–8) and resembled those produced on *E. fabae* (Fig. 9). Similar structures were produced in vivo by capilliconidia of other entomophthoralean fungi (T. M. Butt, *J. Invertebr. Pathol.* **50**, 72–74, 1987; T. R. Glare and G. A. Chilvers, *Trans. Brit. Mycol. Soc.* **85**, 463–470, 1985). Appressoria of *Z. radicans* occasionally produced a narrow, septate, and filamentous penetration hypha (Fig. 7).

That primary conidia and capilliconidia produced appressoria only on water agar covered by a liquid medium and coverslip implies a requirement for specific nutritional and physical conditions. The role of the coverslip has not yet been determined but it may modify the gas composition around conidia so as to encourage appressorial formation. As was noted above, *Z. radicans* capilliconidia require a far lower concentration of nutrients to form appressoria than do primary conidia. Similarly, R. J. St. Leger, T. M. Butt, M. S. Goettel, R. C. Staples, and D. W. Roberts (*Exp. Mycol.*, in press) found that low nutrient levels encouraged appressorial formation by the hyphomycete *Metarhizium anisopliae* while high nutrient levels favored lush hyphal growth.

Appressorial formation by primary conidia of *Z. radicans* takes longer in vitro than in vivo; we are currently studying the factors that accelerate this differentiation. The method described here opens up the possibility for detailed studies on factors inducing and preventing the formation of these infection structures as well as the mechanisms involved.

KEY WORDS: *Zoophthora radicans; Empoasca fabae;* appressoria; conidia; capilliconidia; differentiation.

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