Infection, Disease Development, and Axenic Culture of *Entyloma compositarum*, the Cause of Hamakua Pamakani Blight in Hawaii

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ABSTRACT

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A Hamakua pamakani pathogen introduced from Jamaica in 1974 on *Ageratina riparia* leaves was isolated and pure cultures were grown on yeast morphology agar. Inoculations of axenically grown *A. riparia* seedlings resulted in typical leaf spots and abundant pathogen sporulation after 13 days of incubation at 18 C. There was no significant difference in numbers of spots produced on leaves inoculated through abaxial or adaxial surfaces. Germ tube penetration occurred between epidermal cells by dissolution of the middle lamella. This fungus is tentatively placed in the genus *Entyloma*, based on the two distinct types of conidia produced: short, broadly falcate conidia (15–20 × 3–3.5 μm) and long, slender, arcuate conidia (30–40 × 1.5–2.5 μm), characteristic of *E. compositarum*.

The 1974 importation of the Jamaican fungus *Cercosporella* sp., cause of leaf blight on *Ageratina riparia* K. & R. (= *Eupatorium riparium* Regel) (6), marked the beginning of biological control of exotic weeds in Hawaii with plant pathogens introduced for this purpose. The striking control of *A. riparia*, accomplished in less than 10 yr by this pathogen on extensive Hamakua pamakani infestations on Oahu, Hawaii, and Maui (8), demonstrated the potential of pathogens other than rust fungi (1) to effectively control introduced weeds. During the course of host-range studies with the Jamaican fungus in 1975, it became apparent that the pamakani fungus did not belong in *Cercosporella*. The lack of growth on potato-dextrose agar (PDA), V-8 juice agar, and corn-meal agar prompted us to find a suitable medium to characterize the fungus.

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MATERIALS AND METHODS

Conidia for inoculation and taxonomic studies were obtained from naturally infected leaves in the field. Material was collected during early morning from plants growing at Tantalus Ridge, Oahu, at an elevation of 450 m. Inoculation and disease development studies were done on detached Hamakua pamakani leaves from plants grown in a disease-free Manoa greenhouse. Detached leaves of *A. riparia* produced roots on wet filter papers placed in petri dishes and stored in an illuminated incubator at 12–24 C. To isolate the pathogen, spores were mass transferred to 5% V-8 juice agar with sterile glass needles. This medium was adequate to observe spore germination and to detect the presence or absence of bacterial contaminants on spore surfaces.

Yeast morphology agar (YMA) (Difco) was used to grow isolates of the pathogen

from spores that were free from bacterial contaminants on V-8 juice agar. YMA, at 25% of Difco's recommended concentration and with the agar level restored to 1.8%, was used in most of these studies. Spore suspensions for inoculations were made by washing spores from young sporulating lesions with tap water into glass beakers. Suspensions were then adjusted to 2×10^5 spores/ml using a Howard mold counting chamber (5). Leaves were inoculated by wetting the surfaces with a camel's-hair brush immersed in the spore suspension. Epidermal strips and freehand cross sections of leaves were cleared and stained in 0.1% acid fuchsin in lactic acid (9) to study penetration and hyphal development within the leaves. Measurements were made of spores from freshly collected field specimens and from spores produced on YMA at 18 C.

Isolates were tested for pathogenicity on seedlings grown axenically on Hoagland and Arnon solution (4) with 2% agar in 7-cm-high large-mouth growth flasks. Field-collected seed was surface-sterilized for 30 min in 1% sodium hypochlorite solution. Four or five seeds were placed on the surface of 5% V-8 juice agar in petri plates and incubated for 4–5 days under 2,700-lx continuous fluorescent illumination. At the end of the incubation period, germinated seeds with no evidence of fungal or bacterial contamination were transferred to culture jars containing 40 ml of Hoagland and Arnon agar and grown for 2 mo under continuous illumination before inoculation. Seedlings were inoculated by applying conidial suspensions in sterile water to the leaf surfaces with a sterile pipet, then incubated for 18 days in a Percival growth chamber set for 12 hr at 18 C with light (5,400 lx) and 12 hr at 16 C without light.

RESULTS AND DISCUSSION

Penetration and infection. Under optimal conditions of humidity and temperature (18 C and 100% RH), conidial germination and penetration of the leaf epidermis occurred in 24 hr. Penetration was effected by an infection peg (1–1.5 μm diameter) dissolving the middle lamella between epidermal cells (Fig. 1A). The pathogen invaded the intercellular spaces of the palisade and/or spongy tissues of the leaf in 48–72 hr, with massive hyphal development occurring in the substomatal chamber (Fig. 1B). Enzymatic degradation of the host cuticle has been shown to occur with some pathogenic fungi (10), but this mechanism alone could not account for the unique germ tube penetration through the middle lamella of leaf epidermal cells to invade intercellular spaces of the mesophyll. In 4–5 days, all the mesophyll intercellular space in a developing leaf spot was filled with hyphae, and hyaline sporodochial cushions developed in 6–7 days in the substomatal chambers (Fig. 1D). Subsequent sporulation occurred 7 days after inoculation on short hyaline conidiophores protruding through stomata. Conidia were produced singly, without cross-walls, and were hyaline and highly vacuolated (Fig. 1C). Masses of hyaline conidia showed as white clumps on the abaxial surface of 12-day-old lesions (Fig. 1E). Necroses and cessation of sporulation occurred in the oldest section of lesions 19 days after inoculation (Fig. 1F).

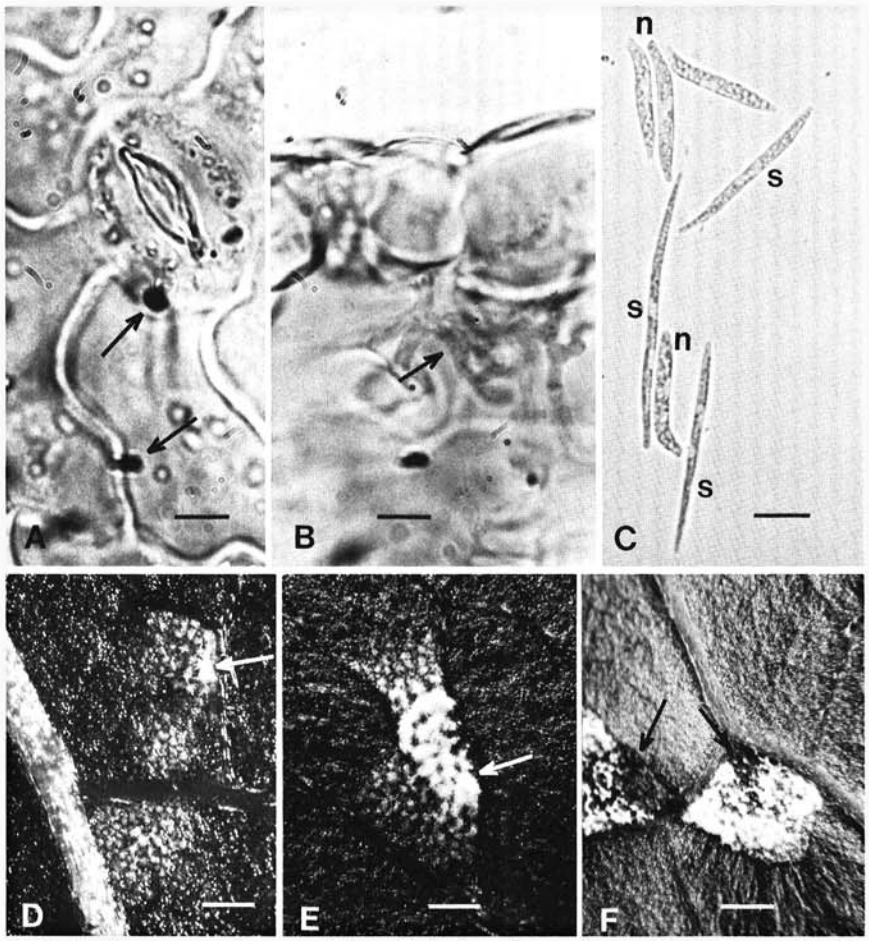

Fig. 1. *Entyloma compositarum* conidia and infection of *Ageratina riparia*. **(A)** Penetration sites indicated by circular dark spots (arrows) on the middle lamella between epidermal cells and stomatal guard cell. Scale bar = 8.5 μm. **(B)** Leaf cross section through a stoma showing hyphal development in the substomatal chamber. Scale bar = 8.5 μm. **(C)** Two types of single-cell conidia of the biocontrol fungus *E. compositarum*. n = Short, broad, falcate conidia and s = long, slender, arcuate conidia showing highly vacuolated protoplasm. Scale bar = 11 μm. **(D)** Seven-day-old lesion showing hyaline sporodochia and initial sporulation (arrow). Scale bar = 250 μm. **(E)** Twelve-day-old lesion showing white clumps representing masses of hyaline conidia (arrow) produced on the leaf abaxial surface. Scale bar = 250 μm. **(F)** Nineteen-day-old lesions, focused on older portion of the lesions showing dark necrotic areas where sporulation has ceased (arrows). Scale bar = 650 μm.

Inoculation of the adaxial surface of three sets of five *A. riparia* leaves incubated under 100% RH at 16, 20, and 24 C, when compared to similar inoculations of the abaxial surface of equal sets incubated under the same environmental conditions, showed that infection occurred through both leaf surfaces irrespective of stomatal openings. Penetration was always by dissolution of the middle lamella between epidermal cells (Fig. 1A). No penetration was observed through stomatal openings despite the presence of approximately 5 $\times 10^4$ stomata per square centimeter on *A. riparia* abaxial leaf surfaces. There was no statistically significant difference between the infection frequencies through the upper or the lower epidermis at 16 and 20 C after 12 days of incubation. The mean number of lesions was 122 on five leaves inoculated on the adaxial surface and incubated at 16 C and 92 on five leaves incubated at 20 C. The mean number of lesions was 122 on five leaves inoculated on the abaxial surface and isolated at 16 C and 103 on five leaves incubated at 20 C. No lesions developed at an incubation temperature of 24 C on leaves inoculated on either surface. The ability of the pathogen to penetrate readily through either leaf surface was perhaps responsible for the speed at which disease epidemics occurred when the pathogen was first field-released in Hawaii (8).

Pathogen isolation and growth in

culture. Leaves with small lesions were surface-disinfested by being dipped in 0.2% sodium hypochlorite for less than 5 sec, and allowed to dry. The lesions were cut out with the aid of a dissecting microscope and plated on water agar. After 2–4 days at 16–20 C, lesions without signs of microbial growth were placed on YMA plates at 16–20 C. Growth of the pathogen was visible 4 wk after initial plating.

Conidia washed from leaves onto thin water agar plates germinated in 20–24 hr at temperatures of 16–20 C. Growth ceased on this medium 72 hr after germination, however, and there was no growth after transfer to V-8 juice agar or PDA. Spores incubated at temperatures above 22 C failed to germinate.

Axenically grown seedlings on Hoagland and Arnon agar that were inoculated with pure cultures of the pathogen on YMA and incubated at 18 C at 100% RH developed lesions and sporulated in 13 days.

Morphology and taxonomy. Conidia produced on inoculated host leaves and incubated at optimum conditions were filiform, hyaline, and nonseptate, with granular vacuolated protoplasm (Fig. 1C), and 30–55 μm long and 2.3–3.2 μm in diameter. Conidiophores were hyaline and short, less than 15 μm long. Substomatal sporodochia were also hyaline. One-month-old colonies on YMA agar were powdery white, circular, and 2–3 mm in diameter. Conidia produced on this medium were morphologically similar to those on host tissue.

The biocontrol fungus was previously reported as *Cercosporella* sp. (6) and *Cercosporella ageratina* (nomen nudem) (7). However, further study indicated that it closely resembles the anamorph of the smut *Entyloma compositarum* Farlow. The anamorph of *E. compositarum* was described as *Cercosporella columbrina* Ellis & Everhart, according to Fischer (3) and Deighton (2). The original description seems to apply to both conidial forms, but the dimensions given, 30–40 × 1–1.25 μm, are more appropriate for the long, slender, needlelike conidia. We have searched for teliospores in the host material infected with the biocontrol fungus but have not found them. Nevertheless, the evidence for the match is convincing because the biocontrol fungus produces two distinct types of conidia like those found in *E. compositarum*: short, broad, falcate conidia (15–20 × 3–3.5 μm) and long, slender, arcuate conidia (30–40 × 1.5–2.5μm). The absence of teliospores in the biocontrol fungus is analogous to many tropical rust fungi that produce only uredinia and urediniospores. Illustrations are provided for both conidial types of the biocontrol fungus and conidial types and teliospores from material determined as *E. compositarum* (Fig. 2).

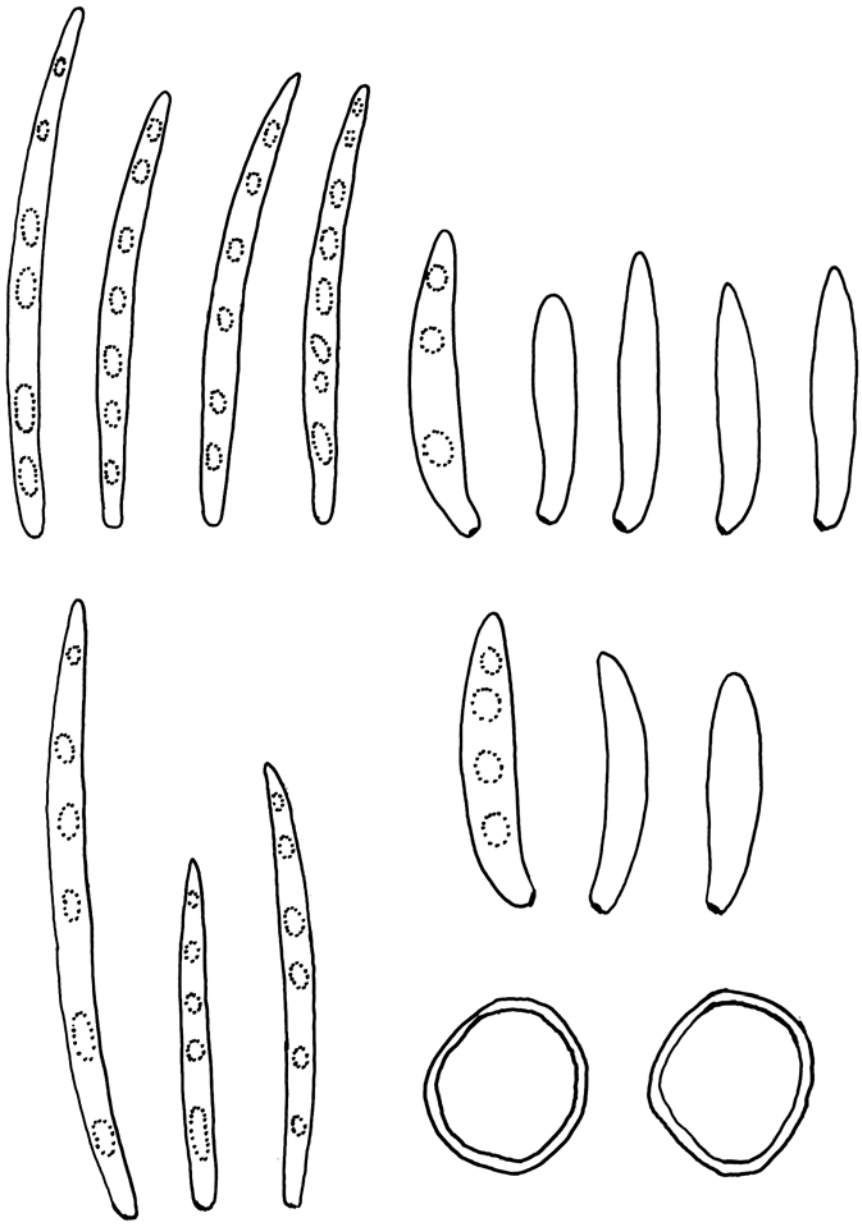

Fig. 2. (Upper row) Two types of conidia produced by the biocontrol fungus isolated from *Ageratina riparia*. (Lower row) Conidial types and teliospore of *Entyloma compositarum*, DAOM 157643. (×2,000)

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