Biological Limitations of *Protomyces gravidus* as a Mycoherbicide for Giant Ragweed, *Ambrosia trifida*

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ABSTRACT

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Stem gall disease of giant ragweed, *Ambrosia trifida*, was found widely distributed at endemic levels in lowland areas in eight northern Arkansas counties. Resting spores of the pathogen *Protomyces gravidus* germinated after 5 months of weathering in infected tissue in the field. Cultures of yeastlike ascospores were obtained on nutrient agar and increased in liquid shake culture to concentrations of 5.9×10^8 spores/ml in 5 days at 16–24 C. Inundative inoculation of giant ragweed seedlings produced galls on 100% of the plants when incubated in a dark dew chamber for 48 hr at 20 C. Plants were killed by the disease when systemically infected. Common ragweed, *A. artemisiifolia*, and cocklebur, *Xanthium strumarium*, were the only other hosts identified after inoculating 27 species in eight plant families. The major limitation to use of *P. gravidus* as a mycoherbicide is its low infectivity rate and lack of virulence in environmental conditions comparable to environments where it incites endemic disease.

Protomyces gravidus Davis, the causal organism of an endemic stem gall disease of giant ragweed, *Ambrosia trifida* L., is widely distributed throughout the geographic range of the weed in the United States. The pathogen was reported in 1884 by Peck (5) in New York as *P. macrosporus* Unger and described as a new species in 1907 by Davis (3) in Wisconsin from three species of *Bidens*. Reddy and Kramer (6) concluded that the species attacking *Ambrosia* and *Bidens* in North America was *P. gravidus*, based on resting spore morphology, size, and symptoms. It has not been previously reported from Arkansas. It is commonly collected in Ohio and Louisiana on *A. trifida* (S. C. Phatak and G. E. Holcomb, *unpublished*). Germination of the resting spores of this pathogen has not been reported.

We considered development of *P. gravidus* as a mycoherbicide for control of giant and common ragweed, *A. artemisiifolia* L., two weeds important for their competition in annual crops and medically important because of their copious production of allergenic pollen (1,11,14). Existing knowledge on the biology of other *Protomyces* spp. suggests that it could be suitable as a mycoherbicide (10,12,13), paralleling in many respects the biology of *Colletotrichum gloeosporioides* (Penz.) Sacc. f. sp. *aeschynomene*, the endemic pathogen developed as the mycoherbicide Collego (2,9). A mycoherbicide should be appropriately host-specific, have the capacity for large-scale spore production in submerged culture, and be virulent over a wide geographic (enviromental) range.

We found Protomyces stem gall widely distributed at endemic levels on giant ragweed in Arkansas in 1984 and began efforts to germinate the resting spore, culture the pathogen, and assess its potential as a mycoherbicide with seedling inoculations in the growth chamber and greenhouse.

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MATERIALS AND METHODS

Periodic field surveys for giant ragweed stem gall disease were conducted in eight counties in northern Arkansas from April through October in 1984, 1985, and 1986. Upland and lowland sites were examined. Disease incidence was estimated in 1985 and 1986 at selected sites by counting the number of stems with galls of *Protomyces* per 100 stems. Verification that each gall represented infection by *Protomyces* was done by microscopic examination of hand-cut sections of gall tissue.

Resting spores from fresh tissue were dormant. Galls collected in the field were overwintered in nylon mesh bags placed 10–20 cm above the ground on stakes at collection sites. Spores were obtained by blending the deteriorated gall tissue in water for 30 sec in a Waring Blendor. Debris was removed with a 75-μm soil sieve, and spores were collected on Whatman No. 4 filter paper by vacuum filtration. Spores were air-dried overnight at room temperature, then stored in screw-cap vials at 4 C. Germinability of resting spores was evaluated by placing air-dried spores in hanging drops of distilled water in van tiegham cells and incubating for 7 days at 20–24 C.

Pure cultures were obtained by germinating resting spores on 2% acidified water agar (two drops of 25% lactic acid per 15 ml of medium) at 20 C then transferring masses of exuded ascospores to nutrient agar (3 g of malt extract, 3 g of yeast extract, 5 g of peptone, 10 g of glucose, 18 g of agar, and 1 L of distilled water).

Isolates were maintained on nutrient agar slants at 4 C. For long-term storage, isolates were lyophilized and stored at 4 C. Subcultures of Arkansas isolate S1-9, the one used in the present studies, were entered in the American Type Culture Collection, Rockville, MD, as ATCC 64066 and the culture collection of the USDA Northern Regional Research Laboratory, Peoria, IL, as NRRL Y-17093.

Yeastlike cells derived from budding ascospores of the pathogen were increased in liquid shake culture on nutrient broth from 12 to 28 C. Cultures contained 50 ml of medium in 250-ml Erlenmeyer flasks and were incubated on a rotary shaker at 150 rpm. Each flask was inoculated with 0.1 ml of nutrient broth containing 5×10^5 log-phase cells grown at each temperature to be tested. Four flasks were harvested from each temperature after 50–120 hr of growth. They were held in an ice bath, and cell concentrations were determined by measurement of absorbance at 550 nm in a Spectronic 70 spectrophotometer and compared to a standard curve established by hemacytometer counts. Spores for greenhouse and growth chamber inoculations were produced in shake culture for 70–80 hr at 20 C and collected by centrifugation at 2,520 *g*. The pellet was resuspended in distilled water and spore concentration was adjusted to 1×10^7 cells per millileter by hemacytometer counts and dilution.

Seedling plants, two- to six-leaf stage, growing in 1:3 peat moss:soil mixture were inoculated by spraying spore suspensions with an aerosol sprayer until runoff. Check plants were sprayed with distilled water. Sprayed seedlings were incubated in a dew chamber for 48 hr in

the dark from 12 to 28 C in 4-degree increments then placed on a greenhouse bench. For dew period studies, sprayed plants were incubated for 16, 24, 36, or 48 hr at 20 C. After inoculation, host range test plants were incubated in the dew chamber for 48 hr at 20 C then placed on a greenhouse bench. Giant ragweed seedlings were included as inoculated checks in the host range tests.

Plants were observed for symptoms for a minimum of 8 wk after inoculation. Presence of galls containing resting spores of the fungus was the criterion for infection.

RESULTS

Stem gall disease of giant ragweed was found predominantly in lowland sites in eight northern Arkansas counties in 1984, 1985, and 1986. Incidence averaged 1.5 and 2.2% (range 1–6%) in infested sites during 1985 and 1986, respectively. Gall development was observed as early as April, and maximum gall size was noted from July to October (Fig. 1A). Most galls were situated near the base of the stems, less than 45 cm above the soil. Other types of galls, primarily caused by the stalk borer *Papaipema nebris* Guen. or the bacterium *Agrobacterium tumefaciens* (E. F. Smith & Town.) Conn., were common but could be distinguished from *P. gravidus* galls in the field by the distinctly different gall shape, color, size, and surface appearance. At one site, galls containing resting spores were found in another composite plant, *Bidens aristosa* (Michx.) Britt.

Cross sections of the galls revealed numerous spherical, thickwalled resting spores that averaged 38.5 μm in diameter (range 29–50 μm) (Fig. 1C). The resting spores were intercalary on intercellular, hyaline, septate hyphae. Resting spore size and shape were the same on *Bidens* and *Ambrosia*. The pathogen was identified as *P. gravidus* on both hosts, based on spore size and host specialization. It is distinguished from *P. andinus* and *P. grandisporus*, which also occur on these composite hosts, and *P. macrosporus*, which occurs on umbelliferae, because it has a smaller average spore size: *P. gravidus* = 37 μm, *P. andinus* = 61 μm, *P. grandisporus* = 69 μm and *P. macrosporus* = 51 μm.

Dormancy of the resting spores was broken after 5 mo of weathering in the field during autumn and winter. Germination in water at 20–24 C averaged 25–30% after 4 days. The thick outer wall of the resting spore ruptured and released the cytoplasmic contents (Fig. 1D). The cytoplasm, which consisted of differentiated ascospores, was in a vesicle that separated entirely from the enclosing thick spore wall (Fig. 1E). The vesicle membrane then ruptured, releasing masses of ascospores that dispersed in water. Conjugation of ascospores in water or nutrient solution was not observed.

In culture, the ascospores grew in a yeastlike manner by unipolar budding (Fig. 1B). They measured 3.2 (2–4) × 8.4 (6–10) μm. Colonies on nutrient agar were dull, flat, and salmon pink with smooth margins. Log-phase growth in shake culture occurred from 50 to 100 hr at temperatures from 16 to 24 C. Cell concentration reached 5.9×10^8 ($.22 \times 10^8$) cells per millileter after 120 hr at these temperatures. At 12 and 28 C, cell concentrations reached 1.3 and 0.5×10^8/ml, respectively, after 120 hr.

Infection of giant ragweed seedlings was achieved at 16–24 C when plants were incubated for 48 hr in the dark in a dew period immediately after inoculation. Percent of infection was 7.3 (3 of 40), 100 (60 of 60), and 60 (24 of 40) at 16, 20, and 24 C, respectively. No infection occurred at 12 C (0 of 20) and 28 C (0 of 40) in this test. At an optimum of 20 C, infection ranged from 5 to 100% after dew periods of 16–48 hr. Infection averaged 10% in dew periods up to 36 hr and was 100% when incubated in a dew period in continuous darkness for 48 hr.

Initial symptoms appeared on inoculated giant ragweed seedlings in the greenhouse after 10–14 days of incubation at temperatures ranging between 18 and 28 C. Small swellings on the stem usually occurred in the tissue that was just below the meristem node at the time of inoculation. All developing galls were clearly visible 3–4 wk following inoculation, and continued to elongate and increase in diameter and coalesce as the plant grew. The epidermis of the stem was disrupted, and galls up to 4 cm in diameter were produced 12–20 wk after inoculation. Plants were usually stunted but not killed, and eventually flowered in an apparently normal manner.

A few seedlings became systemically infected and produced grossly distorted plants that died before gall formation was evident. These plants contained resting spores and mycelium of the

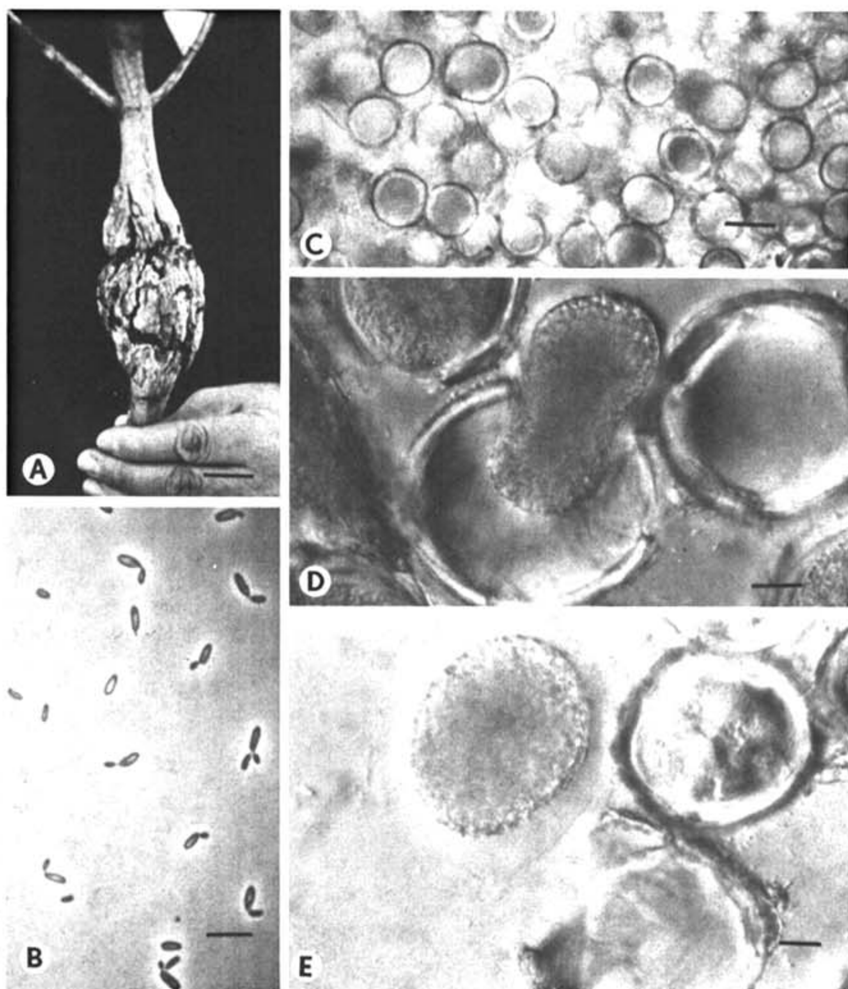

Fig. 1. (A) Symptoms of giant ragweed stem gall. Bar = 2.5 cm. **(B)** Budding ascospores, observed in distilled water with phase contrast microscopy. Bar = 10 μm. **(C)** Resting spores of *Protomyces gravidus* in gall tissue, observed in distilled water with bright field microscopy. Bar = 40 μm. **(D)** Emergence of vesicle from resting spore, observed with differential interference contrast microscopy. Bar = 10 μm. **(E)** Separation of vesicle from resting spore, observed with differential interference contrast microscopy. Bar = 10 μm.

fungus in stem, leaf, and bud tissue.

Specificity of *Protomyces* species for certain members of the compositae was confirmed in greenhouse inoculation tests. Giant ragweed and common ragweed were infected, but not equally, with this isolate of the fungus taken from giant ragweed. Common ragweed was infected to a lesser extent, four of eight plants versus 20 of 20 plants for giant ragweed.

Another host, not previously reported, was common cocklebur, *Xanthium strumarium* L. (15 of 20 plants). Symptoms on cocklebur and common ragweed were less pronounced than on giant ragweed, but both hosts produced resting spores in the tissue. In cocklebur, spores were very sparse and somewhat misshapen. *Bidens bipinnata* L., a member of a reported host genus, was not infected (0 of 40 plants) in this test nor were the following members of the compositae: *Helianthus annuus* L., *Lactuca sativa* L., *Taraxacum officinale* Weber, and *Zinnia elegans* Jacq. The following 20 plants in seven plant families also were nonhosts in these tests: Amaranthaceae, *Amaranthus retroflexus* L.; Apiaceae, *Anethum graveolens* L., *Cicuta maculata* L., *Coriandrum sativum* L., *Daucus carota* L., *Daucus carota* L. 'Red Core Danvers', *Sanicula canadensis* L., *Torilis arvensis* (Huds.) Link; Convolvulaceae, *Ipomoea hederacea* (L.) Jacq.; Fabaceae, *Aeschynomene virginica* (L.) B. S. P., *Cassia obtusifolia* L., *Cicer arietinum* L., *Glycine max* (L.) Merr., *Pisum sativum* L., *Vicia faba* L.; Gramineae, *Avena sativa* L., *Triticum aestivum* L.; Malvaceae, *Abutilon theophrasti* Medik.; Solanaceae, *Datura stramonium* L., and *Lycopersicon esculentum* Mill.

DISCUSSION

Germination of the resting spore of *P. gravidus* and host specialization in the compositae demonstrated in the host-range tests affirms the taxonomic position of this pathogen proposed by Reddy and Kramer (6). Lack of infection on *Bidens bipinnata* is not considered inimical to their classification because only one isolate of the fungus was tested and immune species and cultivars are known to occur in other host genera-pathogen combinations (7).

The germination process of this *Protomyces* is basically the same as that reported for other species of *Protomyces*, with the exception that the vesicular membrane containing spores extrudes completely from the resting spore wall before dehiscing rather than remaining attached as a *Taphrina*-like ascus (10,12). Complete emergence of the *P. gravidus* vesicle is more reminiscent of structures produced during germination of lower fungi (*Phycomycetes*), a group once considered to be the proper taxonomic position for these fungi (4). Ultrastructure studies would help clarify the phylogenic position of this fungus.

Prospects are limited for development of *P. gravidus* as a mycoherbicide for control of giant and common ragweed and cocklebur, despite the parallels between this pathogen and other pathogens successfully developed and commercialized as mycoherbicides (2,8,9). It is sufficiently specific, attacking only weed hosts, and inoculum can be produced abundantly in submerged culture and probably can be dried with the same technology currently used for commercial yeast or *Colletotrichum* species. Lack of secondary spread and resting spore dormancy would reduce the pathogen quickly to background levels and thus minimize environmental concerns and the economic deterrent imposed by a pathogen that gives residual weed control in successive years. In some other respects it is seriously inadequate.

The strain of *P. gravidus* we tested has several biological limitations. It did not kill the host seedlings, except in rare instances, or greatly affect their growth even when applied as inundative inoculum and incubated in environments suitable for infection and pathogenesis. Furthermore, infection and pathogenesis were not linear with inoculum concentration but were more dependent upon strict environmental regimes that would not likely exist in nature. The extent to which these barriers can be overcome is uncertain. It may be possible to predispose the plant with plant growth regulators. Also, it may be possible to select strains to improve the infection and pathogenesis process. If *P. gravidus* proves to be heterothallic as *P. inundatus* Dangeard has proved to be (13), genetic manipulation to overcome its biotic deficiencies would be simplified. The seriousness of the weed problem to which these and other *Protomyces* species could be directed encourages further study into this relatively poorly understood group of pathogens.

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