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Acremonium isolates from *Stipa robusta*

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Abstract: Endophytic *Acremonium* isolates were obtained from 43 of 50 plants of *Stipa robusta* grown from seed collected from 10 locations in New Mexico and Colorado. Hyphae in the lower 2–3 cm of seedlings grown aseptically from infected seed were intercellular, about 2 μm in diameter, mostly straight. Coarse sinuous or convoluted hyphae were produced in culture to some extent by many isolates and conidia were seen in all cultures. Observations of fungal structures were easily made on water agar. When first cultured, mean conidial lengths of individual isolates varied from 5.9 to 8.7 μm. Considerable variation in conidial shapes occurred and average spore lengths of most isolates did not conform to described species, but were intermediate between *A. starrii* and *A. coenophialum*. The most common type of spore was too short for *A. coenophialum* and too long for *A. starrii*, and no isolate even faintly resembled *A. chisosum*. Spores of most isolates were dead in cultures 60 days old at 22 C. Serological analysis demonstrated the relatedness of the isolates to endophyte isolates from a broad range of other grasses.

Key Words: *Acremonium, Claviceps purpurea*, endophyte, sinuous hyphae, sporulation, *Stipa robusta*

INTRODUCTION

Stipa robusta (Vasey) Scribner was named sleepy grass by ranchers in north central Mexico and New Mexico because of its narcotic effect on horses (Bailey, 1903; Pammel, 1911). Lysergic acid and isolysergic acid amide were found in significant quantities in sleepy grass harvested near Cloudcroft, New Mexico (Petroski et al., 1992). White (1987) obtained a very slow-growing, nonsporulating isolate characteristic of fungi in *Acremonium* Link section *Albo-lanosa* as described by (Morgan-Jones and W. Gams, 1982) from grass near Cloudcroft, and Petroski et al. (1992), isolated a fungus resembling *A. chisosum* White and Morgan-Jones from sleepy grass from the same locality.

A. chisosum is prevalent in *Stipa eminens* Cav. in Texas and Mexico, yet this grass has no reputation for toxicity in horses (White and Morgan-Jones, 1987a) in the U.S.A. Kingsbury (1964) found no evidence of toxic *S. robusta*, except near Cloudcroft, New Mexico, and Marsh and Clawson (1929) fed sleepy grass from four widely separated localities to horses and only that from near Cloudcroft was toxic.

Strong circumstantial evidence that sleepy grass owes its reputation to unidentified *Acremonium* endophyte(s), the failure of an isolate from *S. robusta* to sporulate (White, 1987), and the isolation of a fungus resembling *A. chisosum* from *Stipa robusta* from the Cloudcraft area (Petroski et al., 1992), led us to test 10 accessions of *S. robusta* for endophytes and determine their relationship to other *Acremonium* spp., primarily by studying conidiogenesis.

MATERIALS AND METHODS

Collection sites and plant culture.—Forty seeds each from 10 locations in New Mexico (site 1, Monero; 2, Brazos; 3, Cebolla; 4, Los Pinos, all in Rio Arriba County, and 8, southeast of Raton, Colfax County) and Colorado (site 5, Antonito, Conejos County; 6, Monte Vista, Rio Grande County; 7, south of Cuchara Pass, Las Animas County; 9, Saguache, Saguache County; and 10, south of Meeker, Rio Blanco County) were incubated without surface disinfection or seed treatment on moist filter paper in deep Pyrex dishes at 23 C under fluorescent lights with a 12 h photoperiod starting 25 April 1994. Seedlings were transplanted in a commercial potting mix, 30 per site, in flats in the greenhouse by 26 May. All accessions of *S. robusta* developed rapidly without vernalization and true stems were 20–30 cm tall and a few panicles were exerted by 11–12 July. Isolates were la-

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TABLE I. Daily increase in colony diameter of *Acremonium* isolates from *Stipa robusta* grown on potato dextrose agar (PDA) for 32 days and on water agar (WA) for 24 days at 23 C

	Growth rate, mm/day							
	0.01–0.09	0.1	0.2	0.3	0.4	0.5	0.6	0.7
	Number of isolates							
PDA	0	1	1	7	5	14	9	2
WA	4[a]	0	3	10	12	3	0	0

[a] Isolates from site 5.

beled with the site location as the first numeral, which was followed by the individual plant within the sample as the second numeral. All plants in each flat were allowed to mature and seed was harvested in August 1994, and stored at −20 C until November 1995, when it was used to confirm seed transmission.

Endophyte isolation and growth rate.—A single stem of each of five plants per accession was cut 1 cm above the soil line and brought to the laboratory where an additional 1 cm of basal stem and senescent leaf sheaths were removed. Ten segments 1-cm-long were cut from each stem. The stem pieces were disinfected for at least 30 s in a 10% solution of commercial bleach (5.25% sodium hypochlorite) containing one drop of Tween-20 surfactant per 100 mL of disinfectant solution. The stem segments were placed without rinsing on the surface of 25 mL potato dextrose agar (PDA) containing 100 ppm of streptomycin sulfate and penicillin G (= PDA + SP) in 85-mm plastic Petri dishes. Each Petri dish, containing 10 stem segments, was sealed with Parafilm and incubated at 23 C on a laboratory bench.

If miscellaneous fungi or bacteria were seen within a few days, all "clean" host pieces within that Petri dish were transferred to a fresh PDA + SP dish. The host pieces were examined for endophyte emergence using a dissecting microscope starting on day 6 and the dates of emergence recorded. Several segments with fungus were transferred to 2% water agar (WA) as soon as endophytes had emerged from several or all of the stem segments of a sample to facilitate identification of the isolates. Mycelia were examined periodically at 100x, starting when mycelia were very young, and a few isolates were studied with the scanning electron microscope (SEM). Isolates with sufficient growth on 11 August 1994 were transferred to dishes containing either PDA or WA and growth rates were compared on these media at 23 C on day 32. Three dishes of each medium were inoculated with each isolate.

TABLE II. Mean length (L) and width (W) in μm of conidia of *Acremonium* isolates from *Stipa robusta* when first grown on potato dextrose agar at 23 C

Isolate	Number of spores	L	W
4-3	19	8.7	2.7
6-2	38	8.4	3.1
6-3	25	8.0	2.1
10-1	22	7.8	2.5
10-2	47	7.4	2.5
3-2	31	7.4	2.3
5-2	18	7.2	2.6
5-4	22	7.0	2.0
8-2	26	6.8	2.2
7-5	21	6.7	2.9
9-2	16	6.6	2.1
8-5	59	6.6	3.4
4-2	25	6.5	2.1
1-2	29	6.5	2.0
1-5	46	6.5	2.5
2-1	31	6.3	2.0
4-4	37	6.3	2.2
5-5	25	6.3	2.2
9-3	54	6.2	2.1
5-1	26	6.2	2.0
3-4	25	6.2	2.0
4-1	37	6.2	2.2
7-2	51	5.9	2.0

To test seed transmission, the greenhouse-grown seeds, harvested in August 1994, stored at −20 C until November 1995, were rubbed to remove awns, disinfected for 2 or more minutes in the commercial bleach + Tween-20 solution, and plated without drying or rinsing onto PDA + SP, 10 seeds per Petri dish, and incubated on the laboratory bench. Dishes were sealed with Parafilm. When seedlings in the Petri dishes reached 4–10 cm in length, the basal 2–3 cm of the shoot were removed for microscopic examination. The shoot samples, including coleoptile plus

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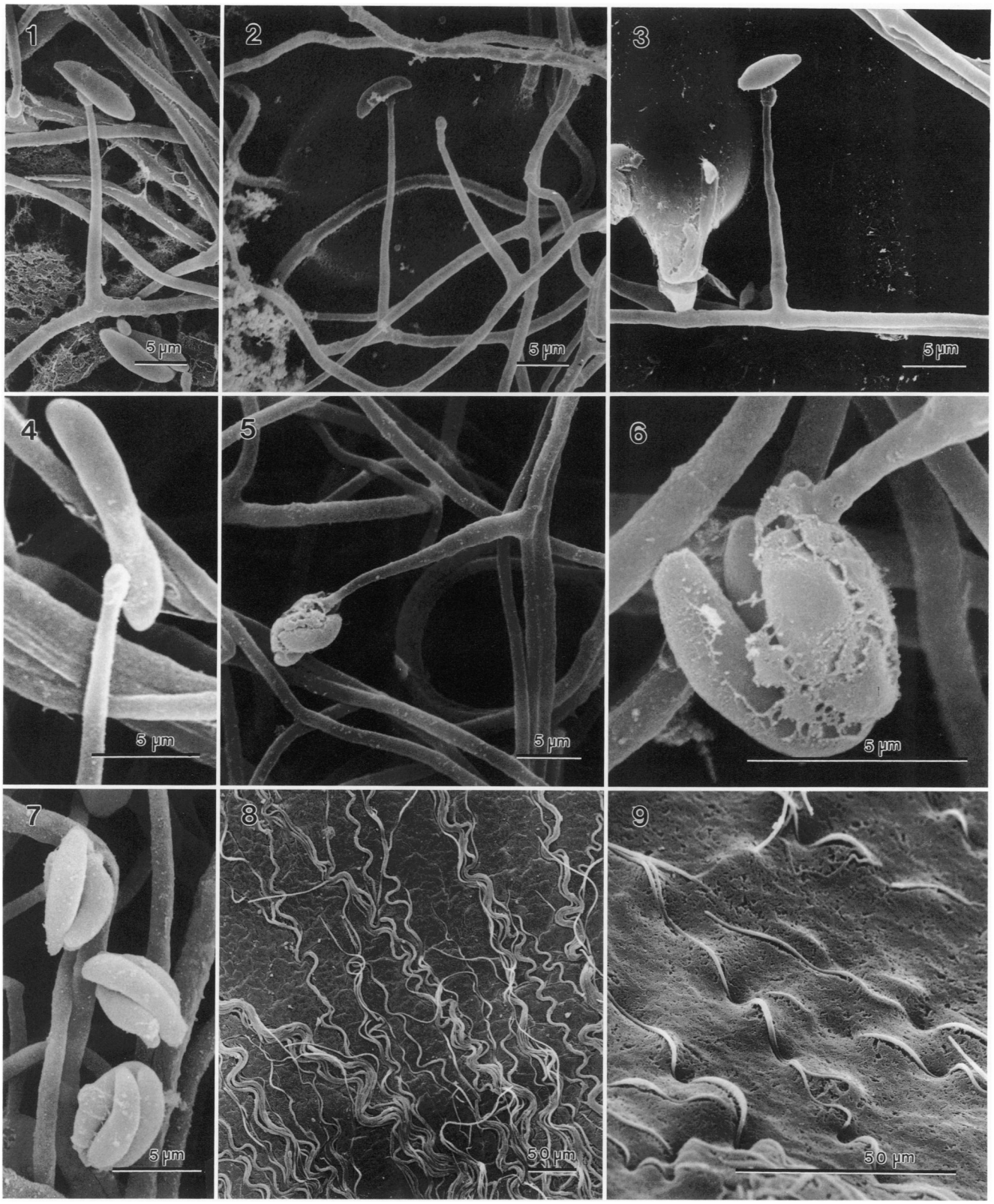
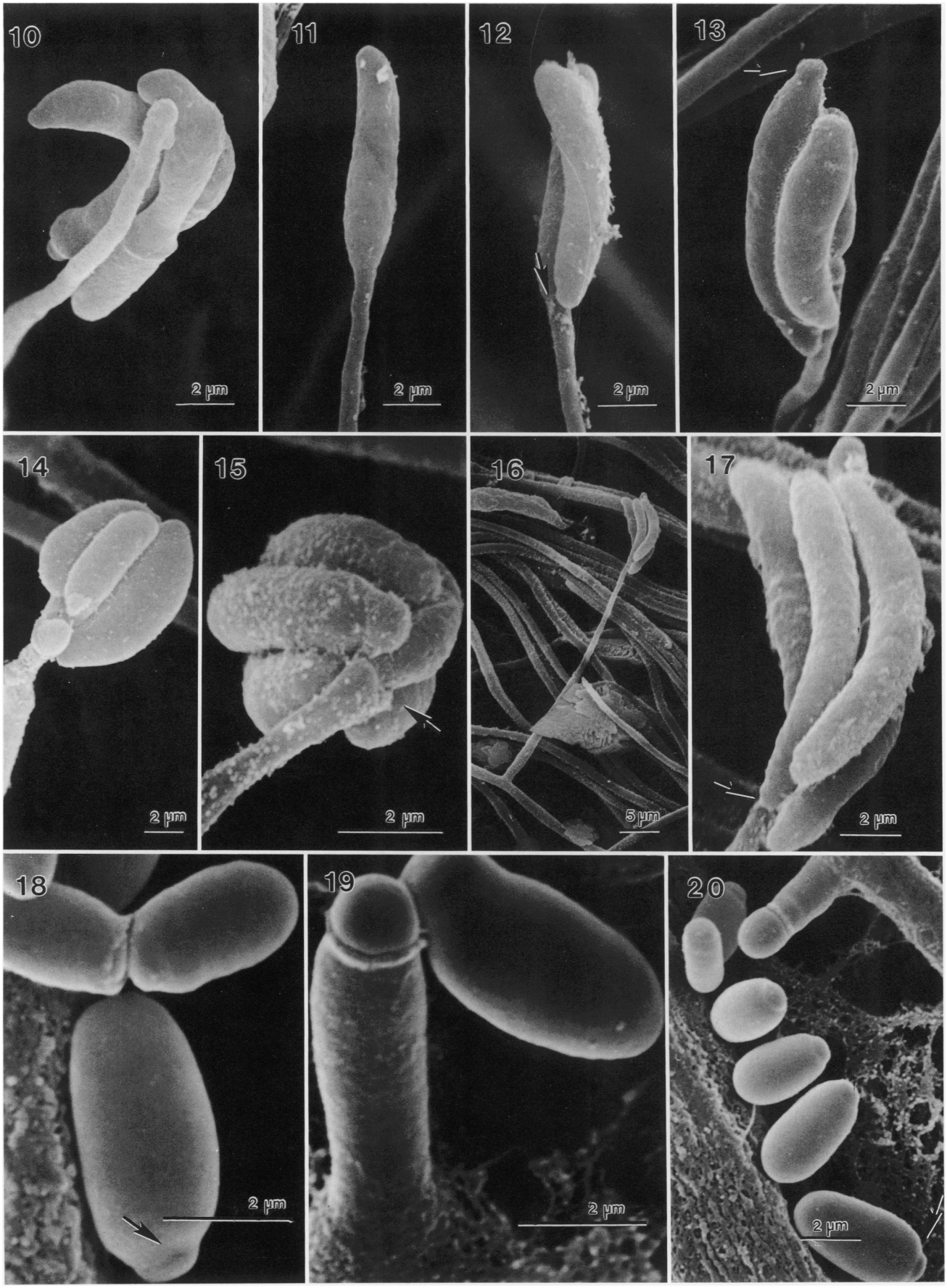
FIG. 1–9. *Acremonium* isolates. 1. Conidium and conidiogenous cell of *Acremonium coenophialum* from *Festuca arundinacea* selection GI 307, a control for isolates from *S. robusta* in western blot serology trial no. 2. 2. *Acremonium starrii* from *Hordeum bogdanii,* PI 269406, isolate 269406-A. A control for *S. robusta* isolates in western blot serology trial no. 1 (on water agar (WA) at 10 C). 3–9 *Acremonium* isolates from *S. robusta.* 3. Conidium and conidiogenous cell of isolate 4–1 growing on host piece from which the fungus was isolated. Trichome on left. 4. Large atypical conidium of isolate 8–5 with little differ-



entiation between apical and basal ends. 5. Conidiogenous cell of isolate 8–5 with a cluster of spores and residue of dried mucilage. 6. Cluster of conidia, isolate 8–5, with remnants of mucilage dried on the exterior of the cluster. 7. Spore clusters in isolate 10–2 not obscured by mucilage. Spore morphology is partially visible. 8. Mostly convoluted hyphae of isolate 8–5, with some strand formation. Hyphae formed on WA close to the host isolation piece. 9. Sinuous hyphae of isolate 10–2 on WA near the host piece from which the fungus emerged. Rhythmic oscillation is called "sinuous" in this paper.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enclosed structures, were teased apart to obtain thin tissue samples and examined with a light microscope in a water droplet plus a drop of lacto-phenol plus aniline blue solution for the presence of intercellular hyphae typical of these endophytes.

Scanning electron microscopy.—Samples for SEM were fixed in 2% glutaraldehyde/1.25% paraformaldehyde (buffered in 0.1 M Pipes buffer). The samples were dehydrated in an ethanol series, critical point dried and sputter coated with gold. Samples were examined and photographed using a Hitachi S-570 scanning electron microscope.

Endophyte identity and relatedness.—The relatedness of the *S. robusta* endophyte to other commonly encountered endophytes was tested by electroimmunoblot (western blot) analysis. Test cultures growing on PDA were ground in electrophoresis sample buffer, 1:10 w:v (150 mM Tris, 2% SDS, 10% glycerol, 2% mercaptoethanol, pH 7.5). Samples were diluted 1:2 with electrophoresis sample buffer containing a small amount of bromphenol blue and incubated in boiling water for 3 min prior to electrophoresis. Extracted proteins were separated by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) in a 10% gel in a MiniProtean II electrophesis cell (BioRad Laboratories, Hercules, CA). Proteins were transferred to 0.45 μm porosity nitrocellulose paper (MSI, Westbro, MA) in 25 mM Tris, 192 mM glycine, and 20% methanol with a BioRad Mini Transblot cell. Unbound sites on the nitrocellulose were blocked with phosphate buffered saline (PBS) (0.05M phosphate, 0.15 M NaCl, pH 7.4) containing 2% nonfat dry milk and 0.2% Tween-20 before incubation overnight at 4 C with a 1:2000 dilution of an anti-endophyte antiserum. The antiserum had been prepared against a potato-dextrose broth shake culture supernatant fraction of the endophyte (*Acremonium starrii* White & Morgan-Jones, isolate 269406-A (mean spore length 6.2 μm measured by SEM) isolated from *Hordeum bogdanii* Welensky, PI 269406. Bound antibody was detected using anti-rabbit IgG:alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, MO) in PBS with nitroblue tetrazolium and bromo-chloro-indoyl phosphate as a substrate. The relatedness of isolate 10–2 from *S. robusta* to endophyte isolates from *Hordeum bogdanii, Lolium perenne* L. cv. Lofts, *Festuca arundinacea* Schreb. cv. Tribute, *Achnatherum inebrians* (Hance) Keng and *Epichloë typhina* (Pers.) Tul. from *Dactylis glomerata* L. was tested in one blot. In a second blot, the variability among isolates from *Stipa robusta* was determined. *Didymella rabiei* (Kovachevski) v. Arx, an unrelated Ascomycete, served as a negative control in the second blot.

Spore dimensions were measured in lacto-phenol with cotton blue using a light microscope with an ocular micrometer. *Acremonium* isolates of this study have been deposited in the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852.

RESULTS

Endophytes began to emerge from the stem pieces in 10 d, mostly from the cut ends, and in 10–16 d fungi had emerged from all internodes and nodes of stem segments of 30 of 43 stems. At d 23 endophytes began emerging from most of the remainder of the stems, and continued to emerge sporadically until d 50 when observation ceased. The last isolates to emerge (from site 5) formed fungal cushions with a few short hyphae protruding from the cushions. The cushions were eventually over-grown by aerial hyphae and these isolates then formed "normal" mycelial colonies. We use the word "isolate" to represent the fungus from one stem.

Very sinuous, convoluted hyphae (FIGS. 8 & 9) radiated out from the majority of infected host pieces after transfer of the host pieces from PDA + SP to WA as soon as fungal egress was detected. This type of hyphae was especially characteristic of the isolates that emerged most rapidly from host tissues. The regular, rhythmic oscillations in direction of growth of sinuous hyphae was common in several isolates that sporulated poorly and may be a useful secondary trait in taxonomy. Hyphal strands (FIG. 8), both aerial and

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FIGS. 10–20. *Acremonium* isolates from *S. robusta*; and *Claviceps purpurea* isolate from sclerotium from *Secale cereale* L. 10–17. *Acremonium.* 10. Atypical conidia of isolate 4–4 on WA; strongly curved conidium on left. 11. Conidiogenous cell with conidium of isolate 4–4 on potato dextrose agar. 12. Tip of conidiogenous cell just below arrow. Basal end of the first conidium is downward. Isolate 4–4, PDA. 13. First and second conidium have slipped downward on conidiogenous cell of isolate 4–4 on PDA. Basal end (arrow) of one conidium. 14. Abnormal conidiogenous cell tip with conidia of isolate 4–3 that vary greatly in mass. 15. Abnormal flared conidiogenous cell tip. Remnants of dried mucus. Isolate 4–3, WA. 16 and 17. Conidiogenous cell supporting spores. Isolate 4–4, WA. 17. Conidia above and below cell tip. No evidence of apical branching of the conidiogenous cell. 18–20. *Claviceps purpurea* on WA at 23 C. Phialides, variation in spore sizes and shape, basal end of spores constricted when within the tip of the phialide (arrows in 18 and 20).

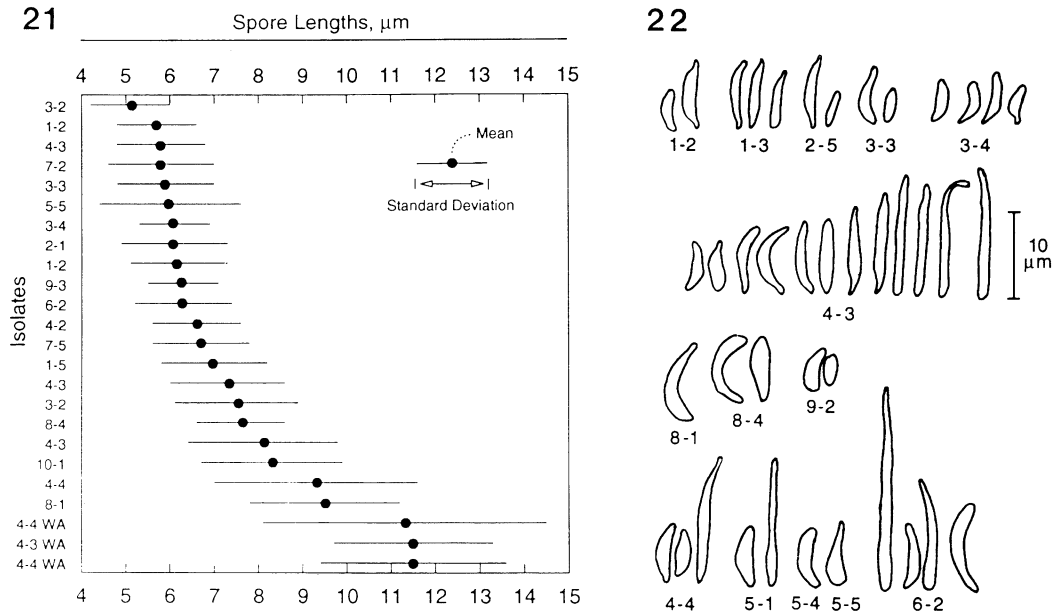

FIG. 21. Mean and standard deviations of spore lengths, all samples of 25 spores, on potato dextrose agar (PDA) except for the last three on water agar (WA). Note variations among samples of spores of isolate 4–3 and 4–4 taken from cultures of different ages and on different media. 22. Shapes and sizes of conidia observed in *Acremonium* isolates from *S. robusta* on PDA at 23 C. Small spores predominated in most isolates. Numbers below the spore groups refer to isolates. There were no sharp distinctions between "small" and "large" spores, either in size or shape, with much intergrading.

on the agar surface, were common. Hyphal coils were observed occasionally on WA.

Most young colonies on PDA were cottony, white to gray-tan, many with elevated central areas, and some sulcate. The underside of all colonies was tannish-gray centrally with whitish margins. As the colonies aged, tan replaced gray. When aerial hyphae collapsed the mycelium changed from white to tan and sulci in the culture became more apparent. Although hyphal growth on WA was sparse, sporulation was observed either on the agar or on aerial hyphae on the inoculum block.

The daily increase in colony diam varied from 0.1 to 0.7 mm on PDA and from trace to 0.5 mm on WA (TABLE I). A few isolates that emerged slowly from host tissue grew fairly rapidly in culture. The average daily increase in colony diam of fast-emerging isolates was 0.5 mm and of slow-emerging isolates 0.4 mm, except for isolates of site 5. Colonies from site 5 isolates increased in diam 0.2–0.3 mm/d after converting from the cushion growth form to normal hyphal growth.

All isolates produced conidia by the conclusion of these experiments. Dimensions of conidia produced by isolates on PDA when first cultured are given in TABLE II. Conidia were borne terminally (FIGS. 1–6, 10–17), usually two or more per conidiogenous cell. Mucilage in the conidial clusters varied from abundant to not apparent (FIGS. 5–7). Conidia perpendicular to the conidiophore (FIG. 3) were most frequently seen in WA cultures about 7–10 d old, early in sporulation, before spore clusters formed. Conidia were often quite variable in size (FIG. 21) and shape (FIG. 22), even from one culture, and they were mostly asymmetric along the long axis. Rarely (seen only in culture 4–1), the apex of a few conidiophores and attached spores were pendant. Spores borne in this manner often became strongly curved, suggestive of *A. uncinatum* Gams et al. (Gams et al., 1990).

Even though the relationship of endophytic *Acremonium* spp. to *Claviceps purpurea* (Fr.) Tul. is remote, study of the latter is useful. Most studies of the *Sphacelia* state of *C. purpurea* are based upon the dense hymenial layer, as formed in nature, often with much exudate (honey dew) and conidia, and details are difficult to discern. In contrast, on WA *C. purpurea* produces a sparse mycelium of coarse distributive hyphae with easily observed sporulation, both upon and within the medium (FIGS. 18–20). Short conidiogenous cells (FIGS. 19, 20) arise mostly at right angles (FIG. 20, and in Brefeld and Tavel, 1891, Plate V, FIG. 64) from distributive hyphae, as in *Acremonium* spp. In *Acremonium* spp. the hyphae are more delicate, and conidiogenous cells arise upright mainly from aerial hyphae, but the basic architecture of the two groups as revealed on WA is similar. Another similarity is the production of sinuous hyphae

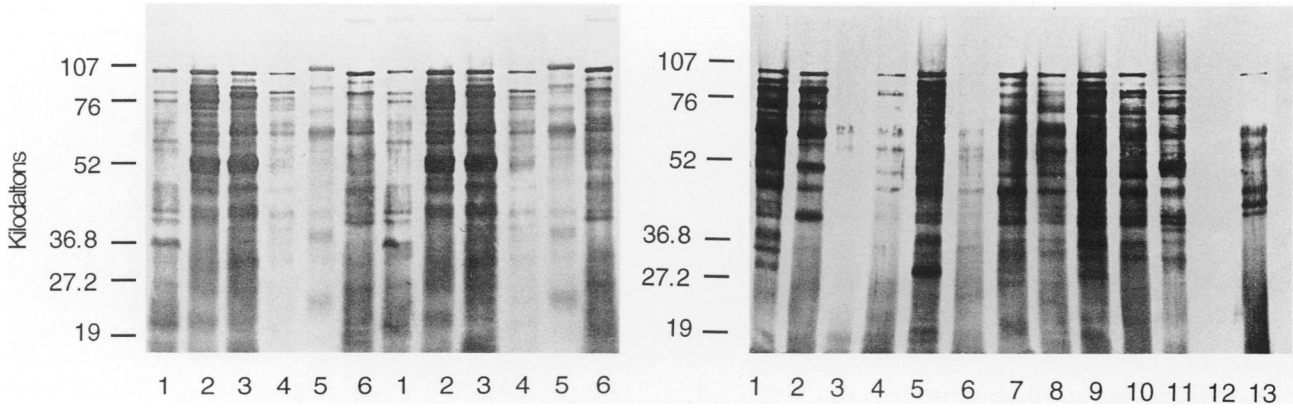

FIG. 23. Serology test, western blot 1, left. Lanes, left-to-right. 1) *Acremonium starrii* isolate 269406-A; 2) *Acremonium* isolate from Lofts perennial ryegrass. 3) Isolate from *Festuca arundinacea* cv. Tribute; 4) *Epichloë typhina* isolate Dg-5 from *Dactylis glomerata* (from J.F. White, Jr.) 5) Isolate 533 from *Achnatherum inebrians*; 6) isolate 10–2 from *S. robusta*. Western blot 2, right. All lanes identified by numbers only are from *Stipa robusta*. 1) 9–3b. 2) 9–1. 3) 8–2. 4) 7–5. 5) 6–5. 6) 5–5. 7) 4–1. 8) 3–2. 9) 2–1. 10) 1–2. 11) *Acremonium coenophialum* isolate GI 307. 12) *Didymella rabiei*. 13) 10–1.

like those in FIGS. 8 and 9, by many isolates of *C. purpurea* when grown on WA.

We sampled cultures 12–147 d old and concluded that viable single-spore colonies were produced most reliably from colonies 20–40 d old. During our spore measurement trials, many spores from older cultures did not stain or stained poorly with cotton blue, evidence that much of the cytoplasm had disintegrated.

The most common hyphae seen in all seedlings grown aseptically from infected seed were intercellular, about 2μm in diam, straight, unbranched, staining densely with aniline blue. Moderately convoluted hyphae were observed in all samples. Straight hyphae were seldom much thicker than 2 μm, but convoluted hyphal segments were in places 3.5–4.4 μm diam. Endophytes emerged from some roots well within the medium, often near the bottom of the Petri dish. Sometimes the fungus emerged from the entire expanded lamina of the most advanced leaf, clear to its tip, 6–10 cm from the seed.

All endophyte isolates, regardless of host, reacted with the antiserum in western blot tests (FIG. 23, left) and isolates from *Stipa robusta* varied in reaction (FIG. 23, right).

DISCUSSION

Isolates from Antonito, Colorado (Site 5) emerged very slowly, mostly forming cushions of fungal tissue at cut ends of the stem piece, but in time (60 d) all but a few cushions were obscured by mycelial growth that formed typical velutinous to cottony colonies on PDA. In contrast, *Acremonium* isolates from *Achnatherum inebrians* formed callus-like growths that did not revert to normal hyphal growth and no spores were observed on these isolates (Bruehl et al., 1994). The most common hyphae within the basal 1–3 cm of seedlings were straight, having grown within long, straight uninterrupted intercellular spaces. Convoluted hyphae were observed only in tissues lacking long, straight intercellular spaces. Convoluted hyphae were in places up to 4.4 μm in diam, but 2 μm was their usual thickness. In contrast, White and Morgan-Jones (1987a) found only thick-walled, straight hyphae 3–9 μm in diameter in hosts infected by *A. chisosum* and in herbarium specimens of *S. robusta*.

The mean spore lengths of individual isolates when first cultured (TABLE II) varied from 5.9 to 8.7 μm, with essentially a continuum between these extremes. Some isolates had spores long enough to be considered *A. coenophialum*, whose spores range from 6.5–13 μm (White and Morgan-Jones, 1987b) or 9–13 μm (White et al., 1993), whereas smaller conidia resembled *A. starrii*, whose spores range from 4–7 μm (White and Morgan-Jones, 1987b). Clay and Leuchtmann (1989) were unable to name several isolates and we do not attempt to name these isolates.

Acremonium chisosum from *Stipa eminens* produces solitary conidia that are cylindrical, symmetric along the longitudinal axis of the spore. *Stipa robusta* therefore harbors at least two endophyte species, *A. chisosum* and our unknown isolates.

We found several strongly curved spores (FIG. 10) in some isolates, especially in isolate 4–3, but our isolates produced mostly gently curved spores. The tendency of isolate 4–3 to produce some very curved spores suggested some affinity with *A. uncinatum* and spores of the latter species range in length from 5–14 μm, a broad size range. We also noted anastomosis of spores within spore heads (Gams et al., 1990).

While some isolates from *S. robusta* produce spores that are highly variable in shape and size, unifying characters among isolates include a strong tendency to produce several spores per conidiogenous cell and similar small spores in all isolates. The total range in length of spores of our isolates, from 2.5 to 16 μm, is greater than that for other grass endophytes. *Acremonium* isolates directly after isolation from the host are notable for their variability (Christensen et al., 1991), but to our knowledge no one has encountered variability in spore morphology comparable to that reported in this paper.

In addition to morphological diversity, endophyte isolates from *S. robusta* exhibit serological diversity in western blot analyses. The magnitude of serological differences among *S. robusta* endophytes seems similar to those observed among endophytes from diverse hosts.

Evidence is strong that *Acremonium* endophytes of grasses arose from *Epichloë* species (Leuchtmann and Clay, 1990; Schardl, 1993; Schardl and Siegel, 1993), but most puzzling is the variation in size and shape of spores of *Acremonium* species contrasted with the relative constancy of conidia of *Epichloë* species. White (1994) reported that the conidia of *E. amaryllans, E. baconii,* and *E. clarkii* (White, 1993) were similar in shape to those of *E. typhina* (White, 1992), lunate to navicular, with little variation in size. Spores of *E. typhina* averaged 3.1 ± 0.1 μm in length, those of *E. amaryllans* 4.5 ± 0.7 μm, the greatest difference in size within the four species. Possibly some unknown *Epichloë* or even an *Atkinsonella* Diehl species contributed to the variation among isolates from *S. robusta.* Morgan-Jones et al.(1990) noted some similarities of *A. uncinatum* and the *Ephelis* state of *Atkinsonella,* and of the microconidia of *Atkinsonella texensis* (Diehl) Leuchtmann & Clay and the conidia of *Acremonium chisosum* and *A. chilense* Morgan-Jones, White and Piontelli. *Atkinsonella texensis* occurs in the same geographic area as *S. robusta* and it attacks *Stipa leucotricha* Trin. & Rupr.

The general infection of *S. robusta* by endophytes suggests that most of these endophytes may not produce animal toxins. The results of White (1987) (eight of eight accessions infected) and of us (10 of 10) are evidence of a high incidence of endophytes in this grass. The feeding trials of Marsh and Clawson (1929) should be repeated and chemical analyses should be made of *S. robusta* from more localities.

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