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Title: Distribution of Acremonium coenophialum in Developing Seedlings and Inflorescences of Festuca arundinacea

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Abstract approved: _____
Ronald E. Welty

Acremonium coenophialum is an endophytic fungus which infects the reproductive and vegetative tissue of tall fescue. Interest in this fungus was sparked by research which linked its presence in tall fescue with reduced weight gains and alkaloid-like poisoning in cattle. Incomplete information was available on the endophyte's life or disease cycle within the host grass. This current investigation traces the progression of A. coenophialum during plant development. Inflorescences of mature plants, in addition to seedlings, were histologically examined for the presence of the endophyte. The fungus grows from shoot apices into immature inflorescences and, eventually, into mature seed. From infected seeds, A. coenophialum grows into seedlings and occupies the shoot meristems of the plant. In contrast to previous information, the fungus invades the shoot primordia before seed germination, is capable of growing in roots, and is found inter/intracellularly.

Distribution of *Acremonium coenophialum* in Developing Seedlings and Inflorescences of *Festuca arundinacea*

by

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Typed by Dianne Simpson for Kathy L. Cook

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INTRODUCTION

Since its introduction into the United States from Europe, tall fescue (*Festuca arundinacea* Schreb.) has developed into one of the most important forage and turf grasses grown in the United States. Its commercial success can be attributed to its ease of establishment into a wide variety of soils and climates, and to its tolerance of poor grazing management. Although tall fescue has these favorable qualities, it has often been criticized as a forage grass because of associated livestock health problems and poor animal performance. Animals grazing tall fescue have shown symptoms of "fescue toxicoses" (Garner, 1983).

Fescue toxicoses in cows manifests itself in three ways: fescue foot (Cornell and Garner, 1980), summer syndrome or poor animal performance (Jacobson et al., 1970), and fat necrosis (Stuedemann et al., 1973). Bacon et al. (1977) associated symptoms of fescue toxicities with the presence of an endophytic fungus in the plant. Because no sexual stage was observed, the fungus was initially identified as *Sphacelia typhina* Sacc., the anamorph of *Epichloe typhina* (Fr.) Tulasne, the choke disease of grasses (Bacon et al., 1977; Saccardo, 1881; Sampson, 1933). However, the name was recently changed to *Acremonium coenophialum* (Morgan-Jones and Gams, 1982).

Grass infected by A. coenophialum is symptomless. The fungus is entirely endophytic in habit and only with microscopic examination can plant infection be determined. It has been conservatively estimated that 75% of all U.S. tall fescue acreage is endophyte infected (Bacon et al., 1986). Several European ecotypes have also been verified as infected (Siegel et al., 1984).

A tall fescue endophyte was first described in 1941 (Neill, 1941) and it has been hypothesized that this endophyte was A. coenophialum (Morgan-Jones and Gams, 1982; Latch et al., 1984). To date, fifteen species of fescue in addition to tall fescue have been observed to contain Acremonium endophytes (Halisky et al., 1985; White and Cole, 1985a). Acremonium-type endophytes have also been identified in Lolium (White and Cole, 1985a).

In 1983, Missouri, which produces 70% of the tall fescue seed in the U.S., had 100% of its seed lots infected (Rycyk and Sharpe, 1984). Of the examined seed, 91% of the lots had 50% or more infection by A. coenophialum. In Kentucky, 97% of the fields surveyed in 1981 were endophyte-infected (Siegel et al., 1983).

Many animal toxicity studies have been conducted since Bacon's initial observations. In tall fescue, poor animal performance and endophyte infection are positively linked (Hoveland et al., 1980; Schmidt et al., 1982). Weight gains for steers grazing endophyte-free tall fescue are twice as high versus gains for steers grazing endophyte-infected tall fescue (Hoveland et al., 1983). An inverse linear relationship has also been correlated between average daily gains for cattle and the level of endophyte infection (Williams

et al., 1984). Endophyte-infected tall fescue grasses are also suspected of causing reduced conception in grazing cattle, sheep and horses (Siegel et al., 1985).

Before it was established that tall fescue was infected with A. coenophialum, studies had linked the symptoms of fescue toxicoses to alkaloids present in the grass. Williams et al. (1975) demonstrated that fescue foot syndrome in cattle could be induced by an extract derived from a known toxic tall fescue (KY-31 variety) pasture. Summer syndrome was said to be caused by the accumulation of the alkaloid perloline (Bush et al., 1973). Poor average daily gain common in steers grazing KY-31, was attributed to this toxin. It is hypothesized that this type of toxin is responsible for decreased energy and nutrient availability to the animal. Studies correlating animal toxicities with perloline led to the development of a new tall fescue variety, G1-307, which contains low levels of perloline (Hemken, 1983).

Subsequent research by Bush et al. (1982) demonstrates a positive relationship between the presence of tall fescue endophyte and accumulation of loline alkaloids in the grass. Cattle maintained on a diet of G1-307, which was discovered to be highly infected and high in loline alkaloids, show severe symptoms indicative of summer syndrome (Hemken et al., 1981). Loline alkaloids have also been isolated from seed of endophyte-infected panicles of tall fescue, but were not extracted from seed of endophyte-free panicles (Jones et al., 1985). Definitive studies

linking loline production by A. coenophialum with fescue toxicity in cattle have not occurred.

Another group of alkaloids from tall fescue, unrelated to perloline and loline, were reported recently. Ergot alkaloids are produced in vitro by A. coenophialum isolated from tall fescue (Porter et al., 1979). This is the first report of a fungus, outside the genera Balansia and Claviceps, having the capability of producing alkaloids that are N-peptide substituted amides of lysergic acid. The 3 major ergot alkaloids produced by the tall fescue fungal isolate are ergovaline, ergovalinine, and chanoclavine I (Porter et al., 1981). These same alkaloids have recently been isolated from leaf sheaths and blades of endophyte-infected tall fescue (Lyons et al., 1986). Alkaloids are not found in non-infected samples. Ergovaline is the major alkaloid produced by the tall fescue endophyte, but, its effects on cattle have not been determined. However, the other endophyte-produced alkaloids are characterized as having a wide range of activities, many of which are symptomatic of fescue toxicity syndrome (Bacon et al., 1986).

In contrast, endophyte infection can be beneficial for grasses. In greenhouse and laboratory experiments the oat bird cherry aphid, Rhopalosiphum padi, and the greenbug, Schizaphis graminum, preferred feeding on endophyte-free tall fescue leaf sheaths by almost a 7 to 1 margin over Acremonium-infected leaf sheaths (Johnson et al., 1985). In Missouri, the abundance of some

types of leafhoppers and corn flea beetles also decreases with increasing endophyte levels in infected tall fescue (Kirfman et al., 1986). Furthermore, broth cultures of A. coenophialum isolated from tall fescue and the closely related Acremonium loliae isolated from perennial ryegrass have also been shown to be feeding deterents to Argentine stem weevil (Prestidge et al., 1985).

The importance of endophyte infection in field-grown tall fescue as a mechanism of insect resistance is not known. The ryegrass endophyte, A. loliae, is associated with insect resistance in ryegrass turf varieties. Resistance to sod webworms (Crambus spp.), bluegrass billbug (Sphenophorus parvulus), Argentine stem weevil, and fall armyworm is positively associated with A. loliae infection (Funk et al., 1983; Ahmad et al., 1986; Barker et al., 1984; Hardy et al., 1985; Clay et al., 1985).

The ryegrass endophyte shares other similarities with the endophyte of tall fescue, A. coenophialum. A. loliae infected ryegrass has been positively associated with ryegrass staggers, a tremorgenic disorder of grazing sheep (Fletcher and Harvey, 1981). Affected animals appear normal until disturbed whereupon they run, fall, and have uncontrollable muscle spasms (Siegel et al., 1985). Compounds called lolitrems have been isolated from infected grasses and are known to produce these same tremors in mice and sheep (Gallagher et al., 1981).

Endophyte infection of grasses is very important economically. It is estimated that hundreds of millions of dollars are lost each

year due to reduced animal productivity caused by fescue toxicoses and ryegrass staggers (Carlson, 1983).

Although conidia are produced by the endophyte *in vitro*, these spores have not been observed in association with infected plants *in vivo*. Therefore, the only known method of transmitting *A. coenophialum* and *A. loliae* in the field is by sowing infected seed. Non-infected perennial ryegrass plants that were grown with *A. loliae* infected plants remained free of the endophyte four years after being sown (Latch and Christensen, 1982). In laboratory experiments, successful inoculations of endophyte-free plants with *Acremonium* spp. are limited. Latch and Christensen (1985) infected tillers of tall fescue and perennial ryegrass with their respective endophytes by inoculating the meristems of 1-week-old seedlings. Johnson et al. (1986) were able to regenerate infected tall fescue plants by inoculating grass peduncle calli with *A. coenophialum*. However, in both experiments, seeds from inoculated plants were not examined for infection by their respective endophytes.

Since dissemination of the fungus occurs through sowing endophyte-infected seed, the most economical method of avoiding fungal toxicosis in tall fescue pastures is by sowing non-infected seed. Recognizing this and because of the associative data pointing to a correlation between the presence of the endophyte and fescue toxicity, some states have established examination programs to verify the amount of endophyte infection in seed.

Numerous detection methods have verified *A. coenophialum* in the aleurone layer of the seed, pith tissue of the stem, and leaf

sheaths (Clark et al., 1983; Johnson et al., 1982; Siegel et al., 1984; Latch et al., 1984; Welty et al., 1986a, 1986b). Hinton and Bacon (1985) observed mature tall fescue plants and determined that the hyphae occupies the leaf sheaths of tall fescue and are found between sclerenchyma cells of the reproductive culm and side branches. Hyphae were not observed in tissues of the root or floret glumes; the only below ground organs in which they observed the fungus were the lateral buds and rhizomes. They concluded that "sometime" before anthesis, hyphae were located in ovaries of the developing florets. Cole and White (1985) also noted that at an undetermined time before anthesis, the fungus was present in the ovule. When the seed matured, hyphae were located above the aleurone layer and beneath the pericarp of the seed. Neither paper reported the fungus hyphae penetrating the shoot primordium of the embryo at this time.

Growth into the vegetative tissues of tall fescue by A. coenophialum was studied by Lyons and Bacon (1985) and Bacon (1983). Lyons indicated that growth of the fungus from the seed into seedlings is not immediate, i.e., "infection" of the seedling does not occur until the sheath of the first leaf is apparent. Bacon hypothesizes that large intercellular spaces which result from the differentiation of sclerenchyma tissue must develop before the fungus can grow from the seed into the seedling.

The purpose of this study is to characterize further the life cycle of A. coenophialum. The appearance and distribution of the fungus within vegetative and reproductive tissues during tall

fescue development is documented. In an effort to describe accurately progression of the fungus during various growth stages of the grass, the research is divided into two parts: the first study traces the movement of hyphae into the developing inflorescence and its location in mature panicles; the second study examines transmission of the endophyte from mature seed into seedlings. This research defines the relationship of A. coenophialum with tall fescue and contributes to our general knowledge of host-parasite interactions.

MATERIALS AND METHODS

INFLORESCENCES

Tall fescue seed, variety G1-307, received from Dr. M. R. Siegel of the University of Kentucky, was sown at the Hyslop Field Laboratory, OSU, near Corvallis, OR. After the plants had been established for approximately two years, immature inflorescences were sampled weekly for 12 weeks or until the seed of the panicle was mature. For two consecutive years, reproductive tillers were collected and removed from their encompassing vegetative leaves.

Samples were fixed under vacuum at 4 C in 3% EM grade glutaraldehyde in 0.2 M sodium cacodylate-HCl buffer at pH 7.2. The inflorescences were dehydrated in a graded ethanol series (50, 70, 95%) for ca. four hours (8 hours in 95% ethanol). Samples were infiltrated 8-12 hours in 95% ethanol and LKB Historesin infiltration solution, 1:1 (v/v). Final infiltration was completed in full strength infiltration solution under a slight vacuum (15-20 mbar) at 4 C for approximately 12-16 hours before embedding in Historesin plastic. Embedded tissue was put into a 37 C oven for 2-3 hours or until the plastic polymerized.

Sections were cut (2-5 μm) with a steel blade on an AO-Reichert 820 rotary microtome, floated on a 10% ethanol solution at room temperature, and collected onto clean glass slides. Sections on slides were dried on a 60 C slide warmer for ca. 20 minutes.

Tissue was stained with freshly prepared Gill-3 Hematoxylin and Lee's Methylene Blue-Basic Fuchsin (Gallagher et al., 1985), as follows:

1. Stained in Gill-3 Hematoxylin for 8 minutes.
2. Rinsed in running tap water.
3. Decolorized by two quick dips in 0.5% acid alcohol (0.5 ml concentrated HCl in 95.5 ml of 95% ethanol).
4. Rinsed in tap water.
5. Dipped in saturated lithium carbonate for 30 seconds.
6. Rinsed in tap water.
7. Stained in Lee's Methylene Blue-Basic Fuchsin for 3 minutes.
8. Rinsed in tap water.
9. Air dried and coverslip applied.

Sections were observed with phase contrast and brightfield light microscopy. Photomicrographs were made with a Zeiss research microscope equipped with objectives of 2.5-100X (oil immersion), optivar settings at 1.25, 1.6, and 2.0, and an Olympus OM-2 35 mm camera.

To confirm that A. coenophialum was the fungus being observed in the sectioned tissue, immature inflorescences were dipped into 1% NaOCl for 10 seconds and transferred aseptically onto sterile Difco potato dextrose agar (PDA) plates. Plates were incubated at 15 C, 16 hours dark, and 25 C, 8 hours light for 4-6 weeks until

endophytic hyphae grew out onto the agar. Identification was made based on cultural characteristics and conidia morphology.

SEEDS/SEEDLINGS

G1-307 seed harvested in 1985 and 1986 from plants at Hyslop Field Laboratory were sterilized to remove any surface contaminants by modifying a tissue culture method developed by Lowe and Conger (1979). Seeds were stirred vigorously in 50% H_2SO_4 for 25-30 minutes, rinsed in sterile distilled water, 95% ethanol, and again in sterile distilled water; 5.25% NaOCl was added and the seeds were stirred vigorously again for 25-30 minutes. Seeds were then rinsed in 2-3 changes of sterile water, placed under a sterile transfer hood, and allowed to dry. Seeds were transferred aseptically to PDA test-tube slants and incubated at 15 C, 16 hours dark, 25 C and 8 hours light.

At 0, 3, 4, 7, 15, and 20 days after transfer to PDA slants, 5-7 seeds/seedlings were sampled. For seedlings, the leaf blade and sheath were cut aseptically ca. 5-10 mm above the point of seed attachment; approximately 3-5 mm of the primary and adventitious roots were left attached to these seedlings.

Seeds and seedlings were fixed under vacuum in 3.0% glutaraldehyde in 0.2 M sodium cacodylate-HCl buffer at pH 7.2. Dehydration in a graded ethanol series was followed by infiltration and embedment in Historesin (LKB) methacrylate plastic.

At the designated sampling times, four additional seeds/seedlings were placed aseptically on PDA plates to isolate

the endophytic fungus. Plates were incubated at 25 C, 8 hours light and 15 C, 16 hours dark. Approximately 4-6 weeks later, the plates were examined with a dissecting microscope for the presence of A. coenophialum.

Using a modified seed squash technique (Welty et al., 1986b), the percentage of seeds with endophyte was determined. To ascertain the percentage of viable endophyte in a seed, a seedling grow-out procedure was used (Welty et al., 1986a). One-hundred total seeds were examined for each year, i.e., fifty seeds were used for each technique.

RESULTS

STAINING REACTIONS

With brightfield microscopy, tall fescue cellulose cell walls stained with Gill-3 Hematoxylin and Lee's Methylene Blue-Basic Fuchsin appear colorless or tinted light blue. Chloroplasts, fats, and lipids are pink and the cytoplasm stains pinkish-blue. Nuclei stain dark blue and, in addition, any lignified elements appear blue.

Intercellular fungal hyphae can be distinguished from host cell walls by their purple-blue color. Fungal nuclei stain blue.

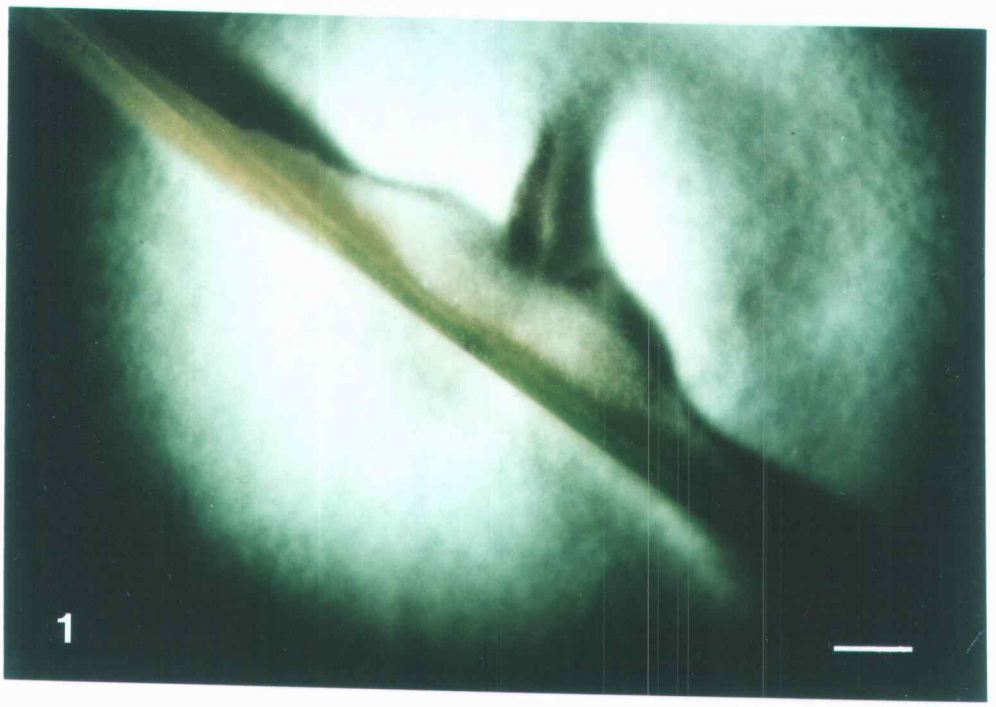
PERCENT ENDOPHYTE IN SEED

By microscopic examination, 99 and 96% of the seed sampled from 1985 and 1986, respectively, contain A. coenophialum. When the meristems and leaf sheaths of germinated seeds from 1985 and 1986 are examined by the grow-out procedure, 95 and 92% of the seedlings, respectively, contain viable endophyte.

ISOLATION OF ENDOPHYTE

After 4-6 weeks incubation, typical slow growing endophytic hyphae appear on the PDA and tissue surfaces. The fungal colonies are white and felty or cottony (Fig. 1). Conidiophores and conidia form abundantly on the medium. Conidia (7-12 X 2-3 μm) are ellipsoid, unicellular and hyaline. Cultural characteristics and conidia morphology confirm the identification of the isolated

Figure 1. Typical white felty colony of A. coenophialum isolated from leaf sheath and blade of tall fescue on PDA, 4-6 weeks after incubation. Bar = 96 μm.


1

fungus as Acremonium coenophialum (Morgan-Jones and Gams, 1982; Latch et al., 1984; White and Cole, 1985b). The endophytic hyphae can be isolated from inflorescences (Fig. 2), seeds, leaf, sheaths, and leaf blades.

INFLORESCENCE

Inflorescence Differentiation. During vegetative growth, Festuca spp. shoot apices typically bear 5-10 leaf primordia (Sharman, 1947). Primordia arise alternately as slight protuberances on the "shoulders" of the apical meristem. As these leaves mature new primordia are initiated and the growing point becomes encased within the cowl-like developing leaves.

When reproductive growth is triggered, the shoot apex elongates while continuing to produce leaf primordia. Almost simultaneously, secondary lateral bud (spikelet) primordia begin to develop in the axils of leaves. These leaf and bud primordia appear as distichously arranged double ridges on the growing point (Barnard, 1964). Growth of the subtending leaf primordia is suppressed as the spikelet buds develop, but leaf primordia at the base of the apex continue to develop.

In tall fescue, and other grasses with complex branched panicles, secondary buds on the shoot apex give rise to other lateral buds or primordia. These lateral buds eventually become the branches and spikelets of the inflorescences.

By the first sampling date, the shoot apex had elongated and numerous lateral buds had arisen and organized spikelet primordia

Figure 2. Endophytic hyphae surrounds immature inflorescence 4-6 weeks after incubation on PDA. Bar = 96 μm.


2

at their apices. The first ridges to form on these spikelet primordia are initials of the sterile glumes, which arise from periclinal divisions in the hypodermis of the spikelet primordia. These leaf-like organs envelop the growing point and its developing structures. Hyphae are sometimes found between basal cells of the lemma and palea and occasionally invade, intercellularly, the sterile glumes. However, developing anthers and ovaries are not infected at this stage of development.

It is not until the styles begin to develop that hyphae invade the cells of the ovary. In addition, the fungus remains concentrated at the base of the ovary.

As the florets proceed to differentiate, i.e., the stamens and pistils mature, unbranched hyphae are easily distinguished among the intercellular spaces of the sclerenchyma cells which connect the base of the floret to the rachilla (Fig. 3). Hyphae also grow between lignified cells of the vascular tissue at the base of the ovary. Hyphae can be the base of the ovary, in the funicular-chalazal region of the ovule, and in the nucellar tissue (Fig. 4). The ovary is well-developed at this stage and cells of the feathery stigma have differentiated. Mycelium is absent from the styles and integuments, but, the fungus is consistently found between cells of the nucellus adjacent to the embryo sac and in the funiculus. Hyphae are also observed in the lemma and palea and, rarely, in glumes of the spikelets. Occasionally, hyphae are found in the filaments of the anthers, but, are never observed in the lodicules or pollen grains.

Figure 3. Hypha (arrow) is clearly seen between cells of the rachilla (R) and the base of the ovary (O) in the developing inflorescence. Bar = 20 μm.

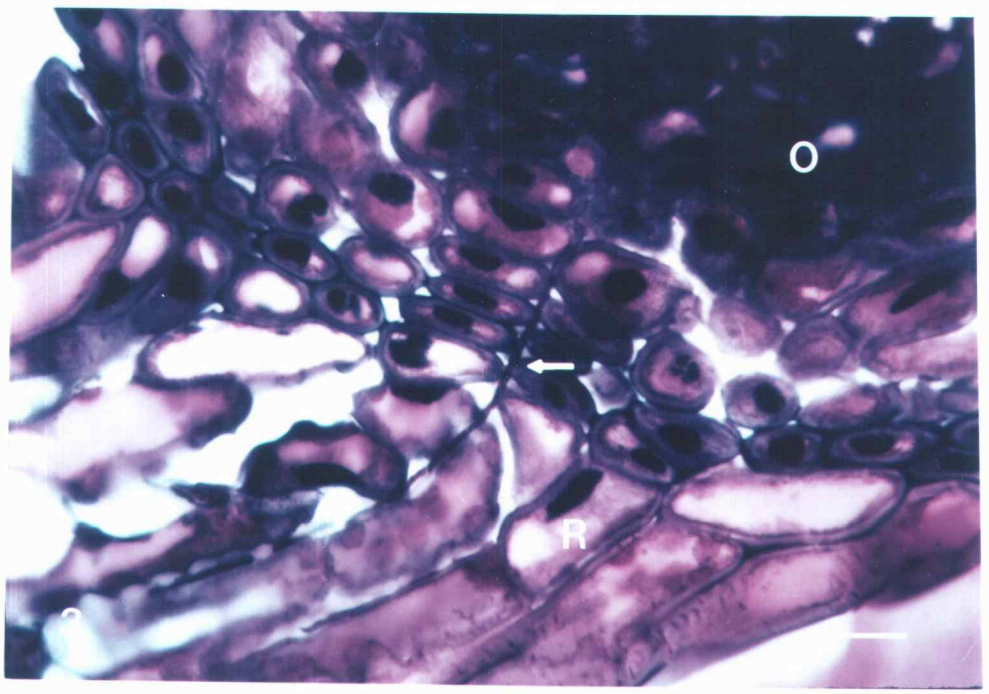
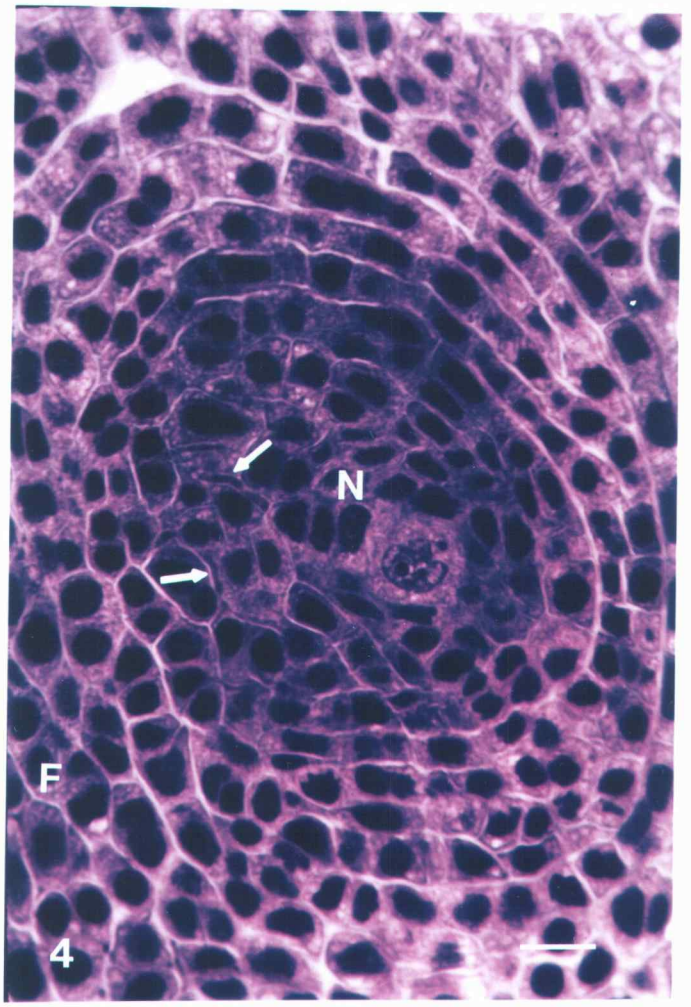

O
R

Figure 4. Endophytic hyphae (arrows) grow throughout the funiculus (F) of the ovary and enters the nucellus (N) of the ovule at the funicular-chalazal region early in megagametophyte development. Bar = 20 μm.


N
F
4

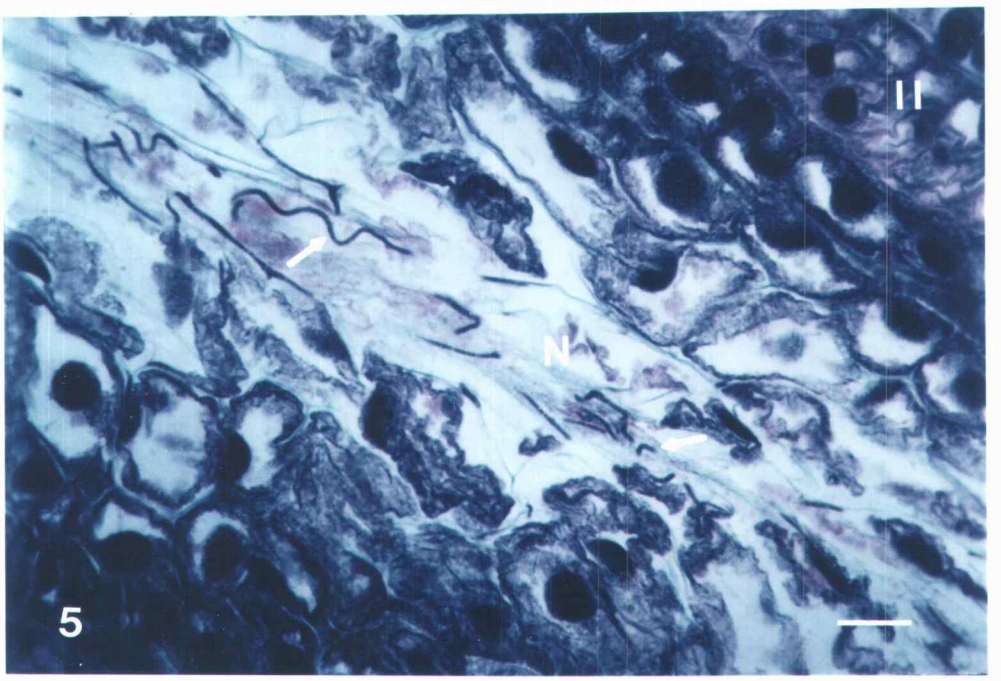
Seed Development. After fertilization the endosperm tissue and embryo enlarge and expand. The degrading nucellar tissue and the accompanying hyphae are "pushed" towards the edge of the seed (Figs. 5, 6 and 7). Hyphae in the nucellus are occasionally branched, fragmented, and guttulate, however, typical convoluted hyphae are also present. Intercellular hyphae can sometimes be seen among the cells of the ovary wall, which are eventually crushed and become part of the pericarp. Hyphae are still found in the rachilla and, occasionally, in the lemma and palea. However, the fungus cannot be found in the embryo at this stage of development.

Eventually, outer cells of the endosperm differentiate into the aleurone layer. The fungus is now located above the aleurone cells in the crushed nucellus (Fig. 8) and beneath the seed coat adjacent to the developing embryo.

At seed maturity, intercellular hyphae are observed adjacent to the base of the embryo, i.e., between the seed coat and embryo. Hyphae are also present between the epithelial cells of the scutellum and cells of the endosperm. Occasionally, hyphae are found intercellularly in the epithelial cells of the scutellum. In addition, the fungus is also present in cells of the embryo shoot apex (Fig. 9), coleoptile, and first foliage leaves. This is the first developmental stage in which hyphae are observed in the embryo of the seed.

Figure 5. Nucellar tissue (N) begins to degrade. During this time hyphae (arrows) are evident. II = inner integument. Bar = 26 um.

Figure 6. Development of endosperm (E) pushes degraded nucellus (N) and hyphae (arrows) to outer edge of seed. The inner integument (II) eventually becomes part of the pericarp. Bar = 12 μm.


II
N
5

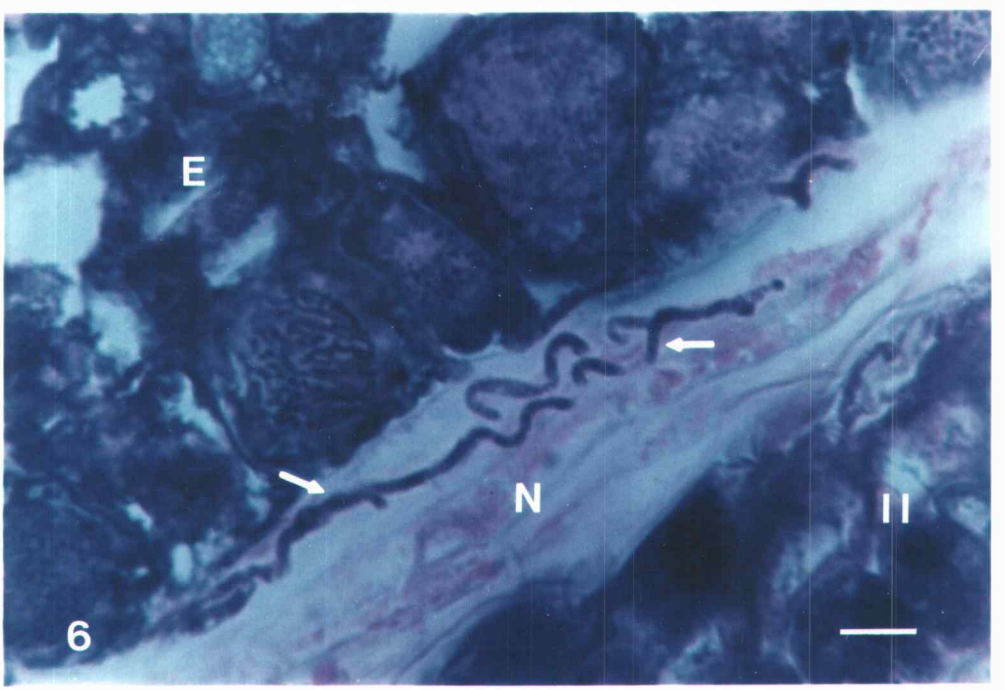
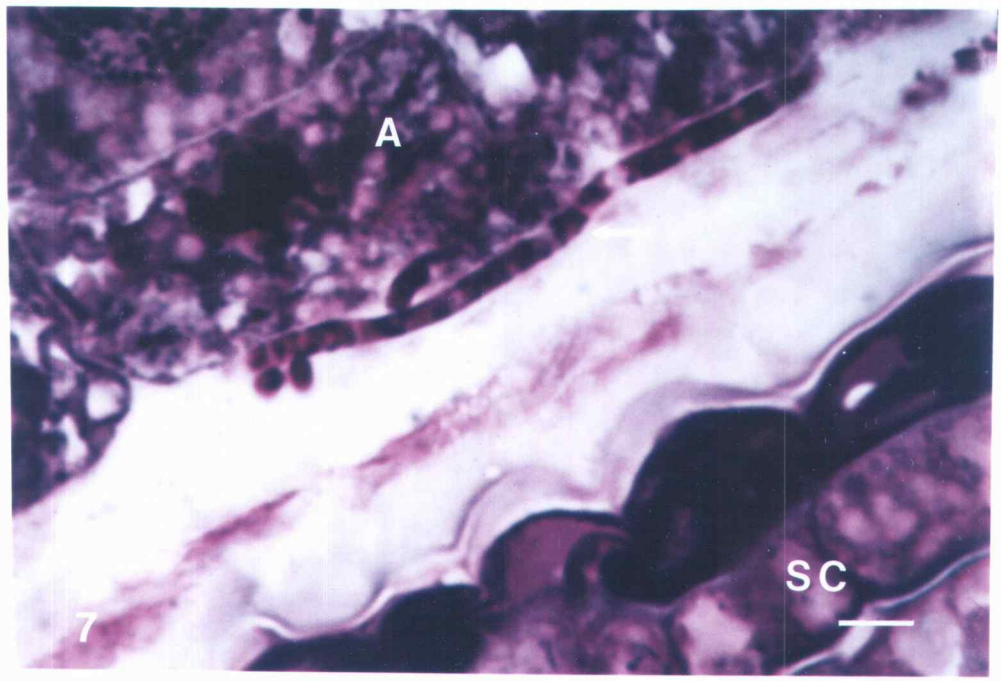

E
N
II
6

Figure 7. Outer endosperm cells begin to differentiate and become aleurone cells (A). Hyphae (arrow) are located above these cells in the nucellus and beneath the cutinized inner integument of the seed coat (SC). Bar = 7 μm.

Figure 8. Hyphae (arrows) are located above aleurone cells (A) of the mature seed. Bar = 5 μm.


A
SC
7

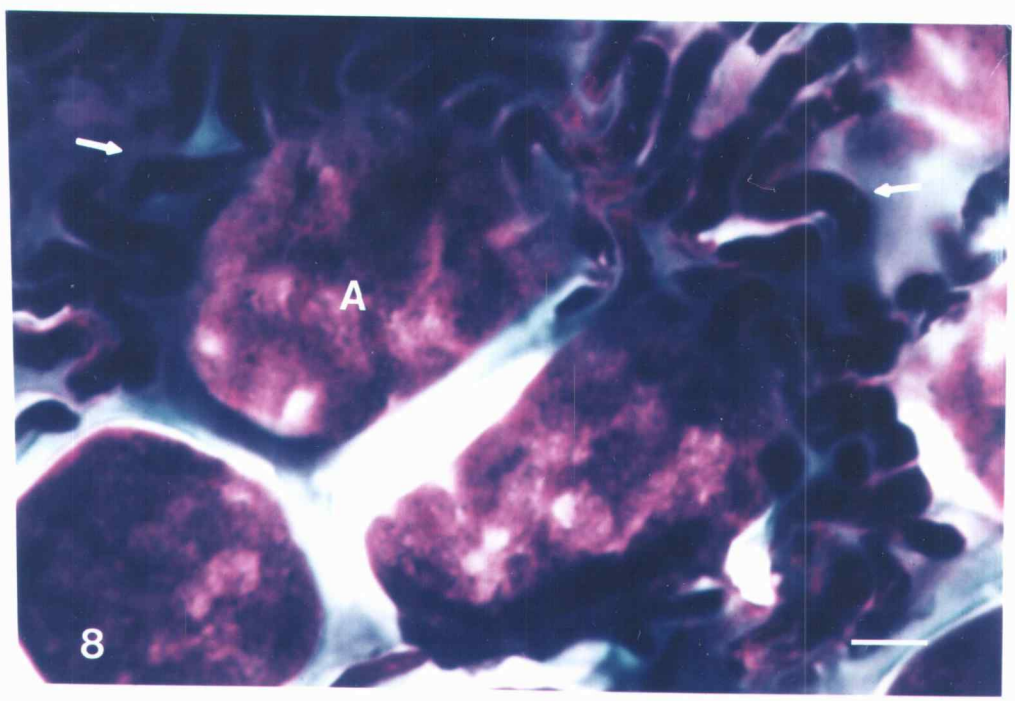
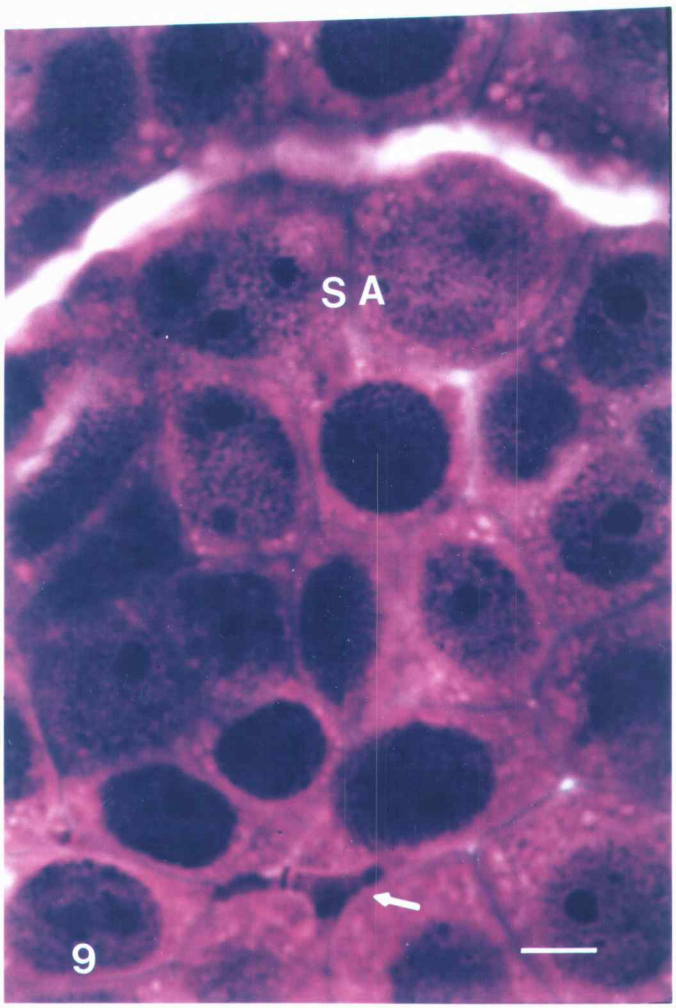

A
8

Figure 9. Endophyte (arrow) is located in shoot apex (SA) prior to germination. Bar = 7 μm.


SA
9

SEEDLINGS

Germination. During elongation of the grass embryo, convoluted hyphae are found concentrated in the crushed nucellar tissue above the aleurone layer. The endophyte is abundant above aleurone cells adjacent to the embryo and the closely positioned epithelial cells of the scutellum. Hyphae are also apparent between the seed coat and embryo. From here, the fungus appears to grow intercellularly into the scutellum and meristematic cells of the shoot apex augmenting hyphae that previously invaded the apex prior to germination (Fig. 10). Hyphae are seen between basal cells of the foliage leaves and coleoptile, but, do not advance very far into these tissues. The fungus in these regions is sometimes branched.

Intercellular hyphae are also observed in root primordia (Figs. 11 and 12). These hyphae are morphologically similar to hyphae found in shoot apices, and the root cells do not appear disrupted.

As tall fescue seeds germinate, the primary root pushes through the side of the coleorhiza. This is closely followed by the emergence of the coleoptile and the first foliage leaf. At this time, as the endosperm degrades, the fungus is observed above the aleurone cells. The hyphae appears fragmented compared to longer pieces at the base of the seed where the shoot is attached. Hyphae are located between cells of the scutellum and adjacent to cells of the vascular trace leading into the seedling. The fungus is easily observed in the internodal region between the shoot and

Figure 10. Pocket of hyphae (arrows) between the seed coat (SC) and embryo invades the scutellum (S) and grows into the previously infected shoot apex of the elongating embryo. Bar = 25 μm.

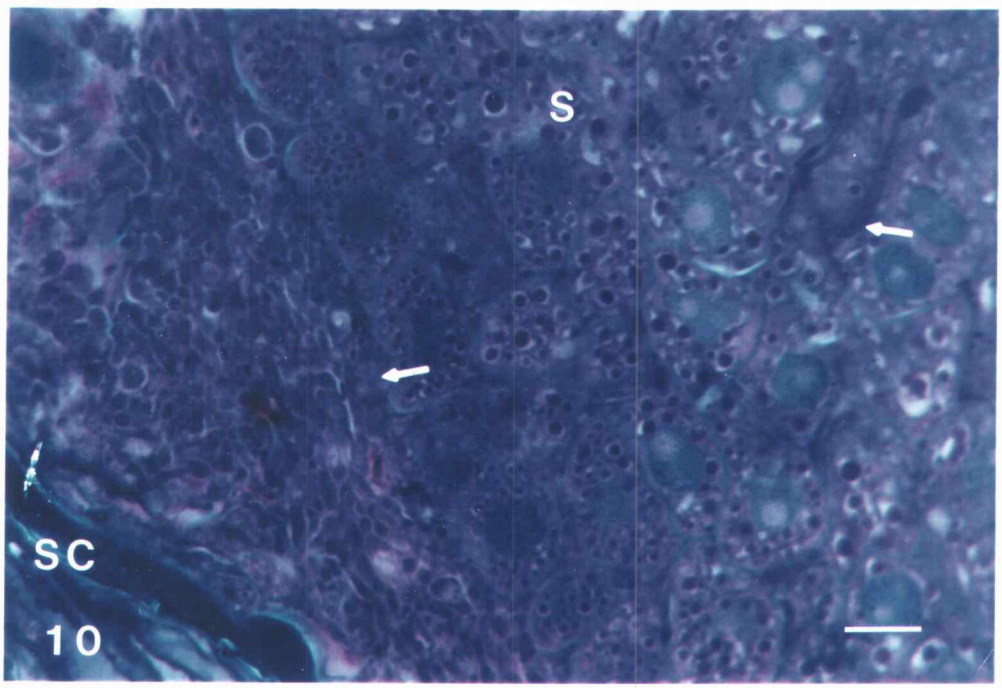
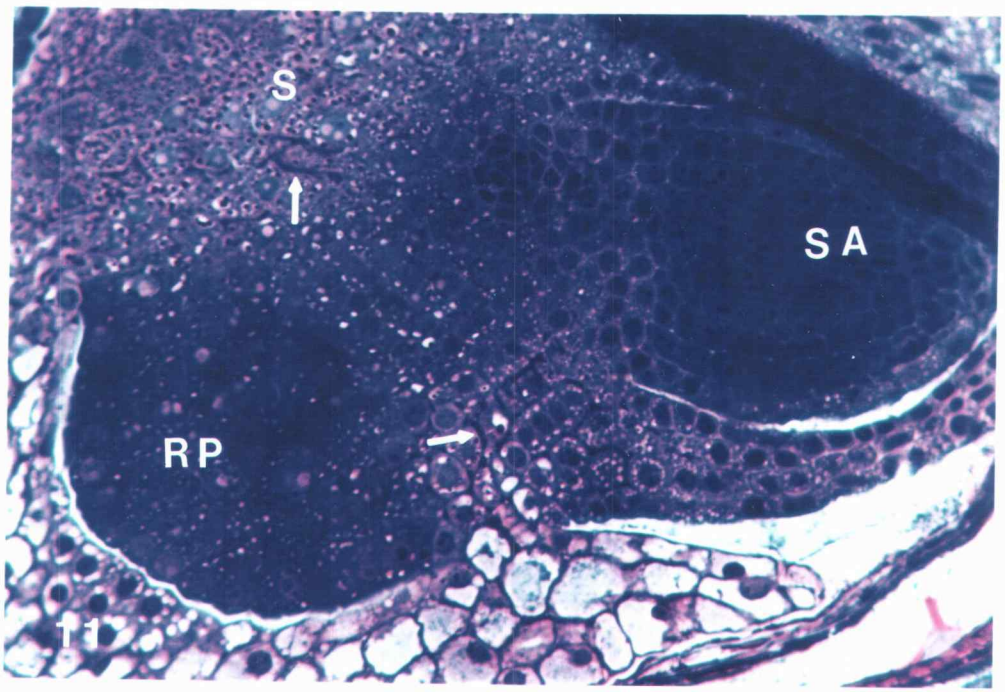
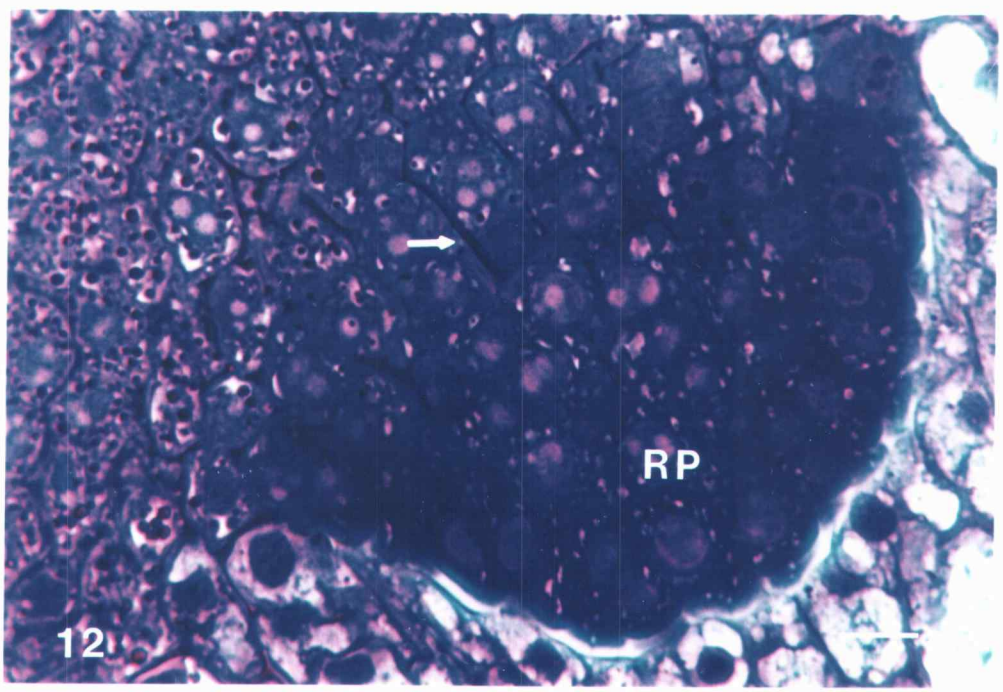

S
SC
10

Figure 11. A. coenophialum (arrows) is distributed throughout the embryo of the imbibed seed. Shoot apex = SA. Root Primordium = RP. Scutellum = S. Bar = 52 μm.

Figure 12. Root primordium (RP) of seed is infected with intercellular hyphae (arrow). Bar = 32 μm.


S
SA
RP
11


RP
12

root. The hyphae vary in shape among meristematic cells of the shoot apex, and parenchyma cells of the sheath. Branched globular hyphae, in addition to long septate straight hyphae, are found intercellularly. The greatest concentration of A. coenophialum in the young seedling is found in the sheath and at the root-shoot interface.

Hyphae are occasionally present in the coleorhiza and between cortical cells of the root. The fungus does not appear to infect the total length of the root but is located near the root-shoot interface.

Seedling Development. As the seedling grows and differentiates, the endosperm of the remaining seed disintegrates. At this stage hyphae are not easily discernible beneath the seed coat. However, the fungus proliferates throughout the scutellum and is distributed among the cells of the root-shoot internode, shoot apex, and leaf sheath as described previously.

Approximately 20 days after being placed in the germinator, the seedlings are well established and only remnants of the seed remain attached. Again, hyphae are consistently observed between cells of the root-shoot interface and the shoot apex. As before, the hyphae are sometimes branched and their width varies from 1-3 μm. Straight strands of hyphae are also located between parenchyma cells of the leaf sheath. New leaves initiated at the base of the growing point are also infected with hyphae.

In the area of the root-shoot internode, corresponding to the mesocotyl region of other grass species, hyphae are observed in the

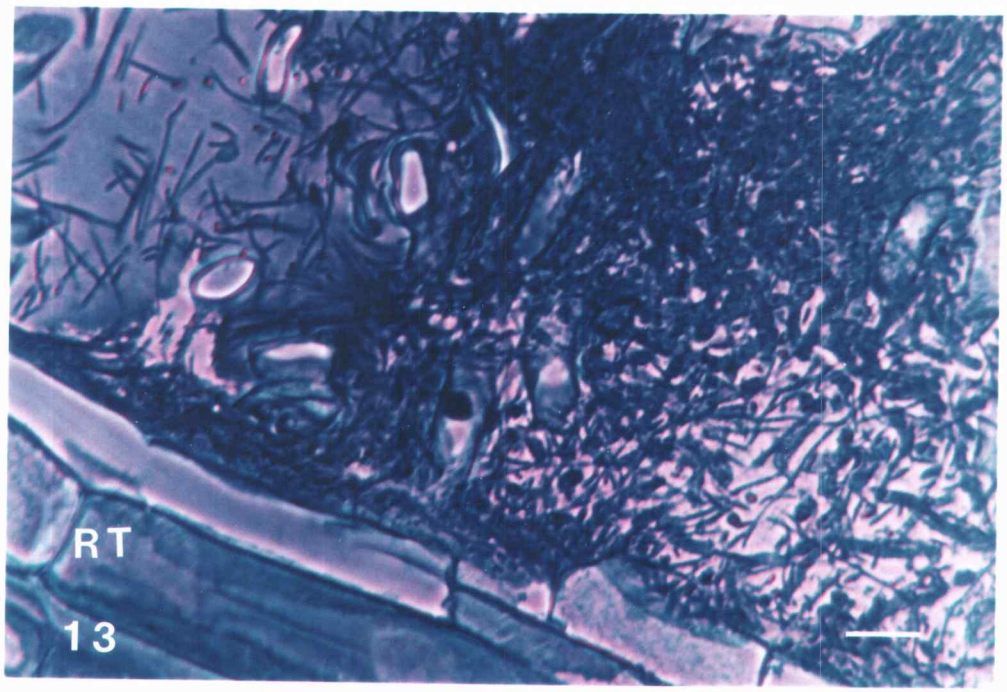
sheath surrounding adventitious root primordia. After these roots emerge, hyphae are evident between cortical cells of the root and parenchyma cells of this internodal region. The fungus is also located adjacent to vascular cells of these lateral roots as they converge into this meristematic internodal area. The endophyte is more often observed between cells of adventitious roots rather than the primary root.

Occasionally, a dense mantle of A. coenophialum can be observed surrounding the seedling at the root-shoot-seed interface or surrounding primary and adventitious roots. Hyphae surrounding the seedling appears to have originated from the infected seed. However, the source of the epiphytic fungus surrounding roots is not known. Conidia and conidiophores are produced by this epiphytic fungus. In addition, hyphae are intracellular and intercellular in root cells and hairs which are covered with epiphytic hyphae (Figs. 13 and 14).

Figure 15 summarizes this study's findings and illustrates A. coenophialum's infection cycle in tall fescue.

Figure 13. Epiphytic hyphae surrounds root (RT) of tall fescue. Bar = 26 μm.

Figure 14. Hyphae (arrows) is inter- and intracellular in root cells (RT). Bar = 26 μm.


RT
13

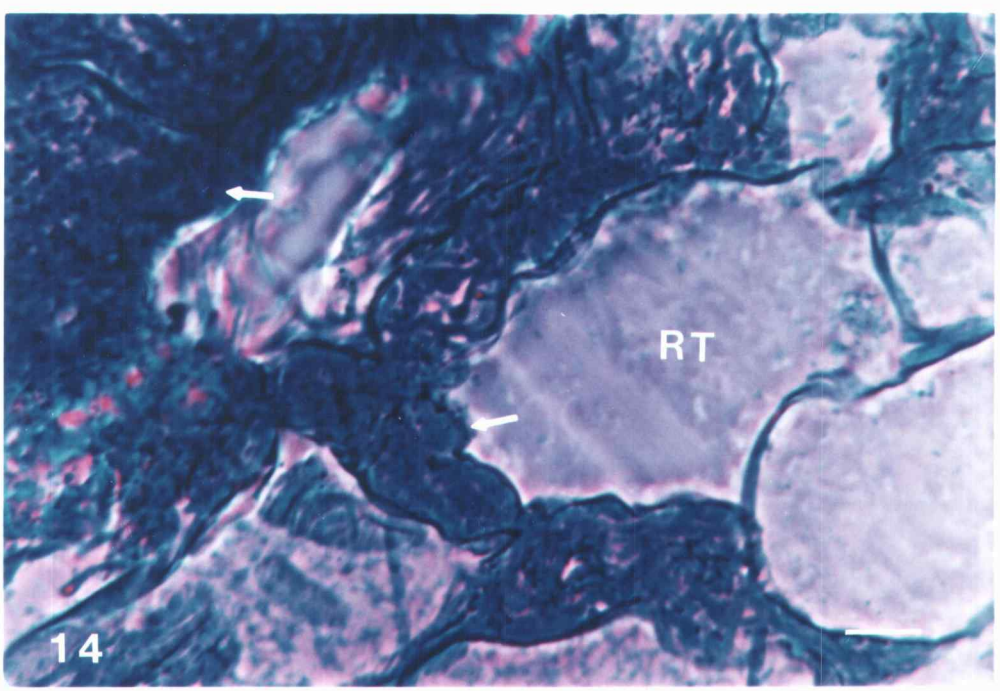
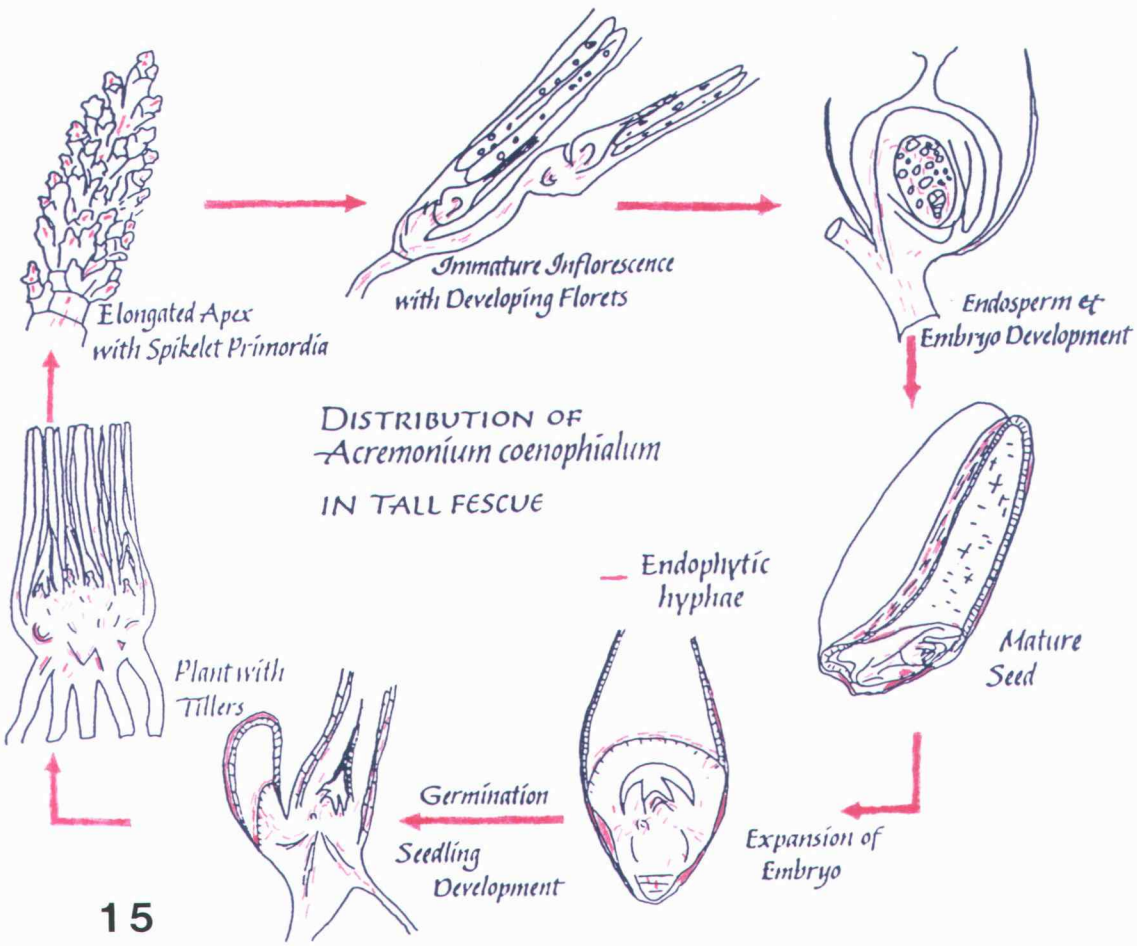

RT
14

Figure 15. A summary of the distribution of A . coenophialum in tall fescue. Red lines designate endophytic hyphae.


Elongated Apex
with Spikelet Primordia
Immature Inflorescence
with Developing Florets
Endosperm &
Embryo Development
DISTRIBUTION OF
Acremonium coenophialum
IN TALL FESCUE
Endophytic
hyphae
Mature
Seed
Plant with
Tillers
Germination
Seedling
Development
Expansion of
Embryo

15

DISCUSSION

Endophytic hyphae densely occupy meristematic areas surrounding the shoot apex of mature plants and seedlings of tall fescue. Other researchers have also confirmed the presence of A. coenophialum in these tissues (Bacon, 1983; Hinton and Bacon, 1985; Seigel et al., 1984). Association with these regions would be advantageous in accessing developing leaves and axillary buds. In addition, infection of adventitious roots and rhizomes arising from the base of the growing point ensures perennation of the fungus.

It is not uncommon to observe fungal hyphae in association with shoot apices of developing plants. In a species of Ephelis, which survives as both an epiphyte and endophyte in different phases of its host's life cycle, hyphae are observed surrounding the shoot apex and adjoining leaves of the grass, Danthonia spicata (Philipson and Christey, 1985). Mycelium of Epichloe typhina is also found intercellularly among young tiller buds in the crown region of Festuca rubra (Sampson, 1933). Similarly, Balansia epichloe and Myriogenospora atramentosa overwinter as mycelium in dormant buds in the crowns of various grasses (Rykard et al., 1985).

When tall fescue's reproductive phase of growth commences in early spring, A. coenophialum is in a position to invade tissue of the developing panicle. The fungus is present within the growing point as it differentiates into a reproductive structure. The endophyte survives as systemic intercellular hyphae in the immature

inflorescence and, as it expands, hyphae grow into the branches of the panicle and spikelet primordia.

As the inflorescence lengthens, hyphae grow from the main stem into the pedicels of spikelets. As noted previously by Hinton and Bacon (1985), a greater concentration of hyphae is found in the side branches of the inflorescence than in the main stem. This research indicates that the greatest amount of mycelium in these side branches is found leading from the rachillas into the bases of the developing florets.

It can be concluded, after examining the reproductive structures of the fescue grass flower, that pollen grains or lodicules are never infected with the endophyte. The filament of the anther is rarely invaded by hyphae. This could be of potential importance to plant breeders desiring to produce uninfected forage grass varieties from plants that are originally infected. Anther or pollen tissue culture could become a viable method of producing "clean" grass varieties.

A. coenophialum enters the ovary after the styles begin to differentiate. Similarly, ergot and smut fungi inhabit the reproductive structures of their respective monocot hosts during seed development and it is hypothesized that they replace their host's flower parts to take advantage of the natural flow of assimilates (Hancock and Huisman, 1981). Congruently, the "timed" appearance of A. coenphialum could possibly coincide with the active transport of metabolites into the developing reproductive structures.

Access to the nucellus in the ovule is gained via the funicular-chalazal region. A similar method of infection has been proposed for the endophyte of Festuca versuta (White and Cole, 1986a). A. coenophialum, and the endophyte of Festuca versuta grow throughout the nucellar tissue adjacent to the embryo sac. As the endosperm expands after fertilization, hyphae in the nucellus are pushed to the outer edge of the caryopsis. Analogous observations have been made for the perennial ryegrass endophyte, A. loliae (Lloyd, 1959). Consequently, endophytic hyphae are consistently observed above the aleurone layer of infected seeds as a result of the nucellus being pushed to the seed edge by the developing endosperm.

Invasion of the embryo by the fungus occurs before the seed is mature. Embryo shoot apices are consistently infected with hyphae, thus enhancing successful transmission of the fungus into subsequent generations of grasses. Despite earlier assertions that fungal invasion of the shoot occurs after germination (Bacon, 1983; Cole and White, 1985; Hinton and Bacon, 1985; Lyons and Bacon, 1985; Siegel et al., 1985), this research found that the endophyte is present in the shoot primordia before germination. Development of sclerenchyma cells and their intercellular spaces is not a prerequisite for invasion of seedling shoot apices by A. coenophialum.

Hyphae are never found within the endosperm but are seen between epithelial cells of the scutellum and the endosperm. With development of the seedling, hyphae are observed leading from the

scutellum into the shoot. The endophyte is also visible adjacent to cells of the vascular trace in the scutellum. As the seed disintegrates, fungal hyphae grow from the scutellum into the actively metabolizing seedling.

The fungus is located in areas of the plant which are nutritionally advantageous. Evidence of this relationship can be substantiated by the predominance of the fungus in meristematic regions, in the funiculus of the developing ovule, between the epithelium of the scutellum and the endosperm. Kirby (1961) hypothesized that closely packed meristematic cells are necessary to maintain a sufficient supply of nutrients for the intercellular hyphae of Epichloe typhina. Conjecture would lead to a similar hypothesis for A. coenophialum. Meristematic regions, roots, and reproductive tissues are nutrient sinks (Kosuge, 1978) which could maintain a constant supply of nutrients for the endophytes intercellular growth. In these areas of high metabolic activity more leachates could be available for use by the fungus.

Many species of grasses have been recently identified as infected with endophytic hyphae. Based on morphology and distribution within the plants, endophytes inhabiting species of Bromus, Poa, Festuca, Stipa, and Lolium appear to be closely related to the endophyte of tall fescue (White and Cole, 1985a, 1986a, 1986b; White et al., 1987; White and Morgan-Jones, 1987; Latch et al., 1984; Halisky et al., 1985). Microscopic examination of these grasses reveals convoluted intercellular hyphae in stem

parenchyma tissue, tissue of the leaf sheath, and above the aleurone layer of the seed.

In Festuca versuta, systemic hyphae are located in the leaf sheaths, glumes, rachillas, and appear to enter the embryo through cells of the scutellum (White and Cole, 1986a). Because of differences in growth characteristics in culture media and the absence of conidia production in vitro, this endophyte is considered distinct or different than A. coenophilaum or A. loliae. Information concerning the endophytes of Bromus spp., Poa spp. and Festuca spp. is largely observational and additional research is necessary to define their possible associations with A. coenophialum.

A. coenophialum shares many similarities with systemic parasitic fungi of the tribe Balansiae in the family Clavicipitaceae. Many members of this group are ovarian parasites (Bacon et al., 1983; Clay, 1984; Diehl, 1950; Kirby, 1961; Rykard et al., 1985; Sampson, 1933). Unlike A. coenophialum, infection by these fungi commonly result in sterility of their grass hosts. Infection by Balansiae fungi is symptomless until mycelium emerges from the host and superficial stromata with conidia and perithecia are produced.

Like the endophyte of tall fescue, these fungi typically inhabit meristematic apices of their grass hosts. A recent addition to the Balansiae, Myriogenospora atramentosa associates with the meristematic growing point of Bahia grass (Luttrell and

Bacon, 1977; Rykard et al., 1985). However, the mycelium of this fungus is entirely superficial and does not penetrate its host.

Analogous to A. coenophialum and tall fescue, inoculations of uninfected host plants with Myriogenospora spp., and other culturable biotrophs of the Balansiae, have rarely yielded infected plants. Out of many attempts, Diehl (1950) was only successful once in artificially infecting a host grass with Balansia sp. After several different approaches, Western and Cavett (1959) infected Dactylis glomerata with E. typhina by inoculating freshly cut stubble. Muhle and Frauenstein (1970) attempted to repeat their findings but were unsuccessful.

The many similarities between the endophyte of tall fescue and members of the Balansiae indicate that A. coenophialum may be a clavicipitaceous anamorph. Additional research is needed to define the relationship of A. coenophialum with other biotrophs of the Balansiae and to investigate the nutritional interactions of these endophytic fungi with their respective hosts.

Three important additions have been made to the previously established information concerning the tall fescue endophyte: first, as shown, the fungus occupies shoot primordia of seeds prior to germination; secondly, the endophyte can be found in root tissue; finally, hyphal growth has been observed to be both intercellular and intracellular.

Examination of tall fescue roots reveals intercellular and intracellular hyphae of A. coenophialum. Hyphae are seen in the root-shoot internode leading into cortical cells of the root. This

intercellular fungus does not advance into the root for a great distance, but, is observed adjacent to vascular tissue connecting the root and internode. In addition, intercellular and intracellular hyphae are located in cortical cells of roots surrounded by epiphytic hyphae of A. coenophialum.

With brightfield or phase microscopy, it cannot be determined if intracellular hyphae enters cells via mechanical pressure or through enzymatic degradation of host cell walls. Kulkarni and Nielsen (1986) stated that A. coenophialum cannot grow in vitro on pectin, cellulose, galacturonic acid, or polygalacturonic acid. This could indicate an inability of the fungus to produce the complement of enzymes necessary to degrade cell walls. However, degradation enzymes can be induced by host products as demonstrated by research with pathogenic soft-rot fungi and bacteria (Collmer et al., 1982; Kolattukudy and Koller, 1983). Host plants also possess many cell wall degrading enzymes which, although normally involved in germination, pollination, abscission, etc., may be induced by a pathogen and utilized for cell wall degradation (Cooper, 1983). Therefore, it cannot be concluded from in vitro experiments that cell wall degrading enzymes are not participatory in this fungus-host interaction.

Additional research is required to determine if a "triggering" mechanism exists which permits the fungus to infect intracellularly. Previous data suggests that the endophyte is entirely intercellular in growth (Hinton and Bacon, 1985; Siegel

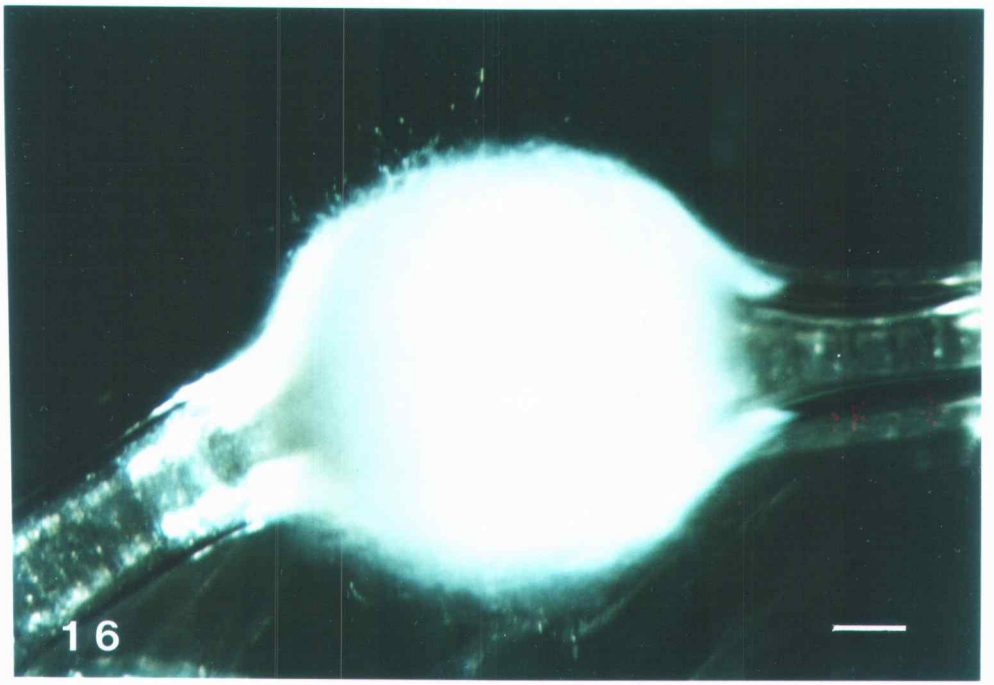
et al., 1984). The fungus-plant relationship is termed "mutualistically symbiotic" or commensal by some researchers (Siegel et al., 1984; Siegel et al., 1985). One of the criteria used to define mutualistic symbiosis is a "lack of destruction of host cells or tissue" (Siegel et al., 1987). However, based on observations of intracellular hyphae and the limited destruction of root cortical cells, A. coenophialum may be described more accurately as a parasitic symbiont in certain growing conditions. Re-examination of the endophyte-host association is warranted.

Observations of epiphytic hyphae in the root zone have been substantiated by other research conducted in this laboratory. Sterile seedlings used for a non-destructive viable endophyte grow-out test (unpubl. data) are observed to have A. coenophialum growing around their roots approximately 30 days after sowing on PDA media (Fig. 16). A "ball" of hyphae commonly surrounds lateral roots approximately 5-20 mm distant from the point of seed attachment. It is also noted during microscopic examination of 3-week-old seedlings for determination of viable endophyte content (Welty et al., 1986a) that epiphytic hyphae grow around root primordia of both tall fescue and perennial ryegrass (unpubl. data) (Fig. 17). Microscopic examinations of shoot meristem inoculated plants (unpubl. data) reveal epiphytic hyphae surrounding roots. In these samples intracellular hyphae are also observed in cells of roots and root hairs (Fig. 18).

Although A. coenophialum has been observed in vitro on adventitious roots and in root meristems during this investigation,

Figure 16. Adventitious root of tall fescue is covered with a white "ball" of A. coenophialum hyphae. Bar = 96 μm.

Figure 17. Squash preparation of 3-week-old tall fescue seedling shows typical convoluted endophytic hyphae above a root primordium (out of focus) (RP). Bar = 17 μm.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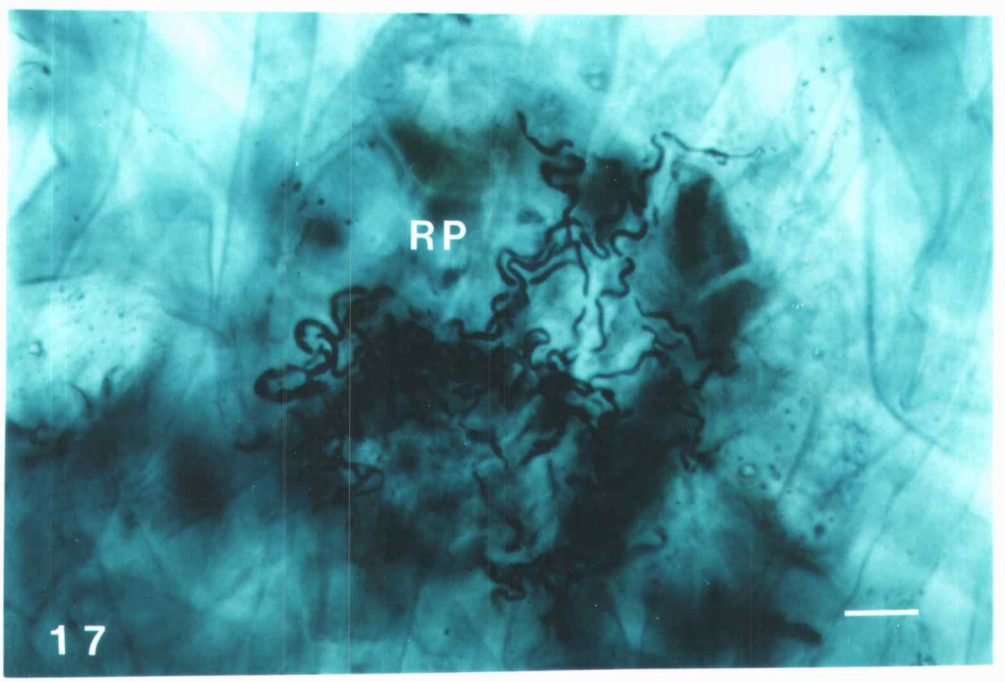
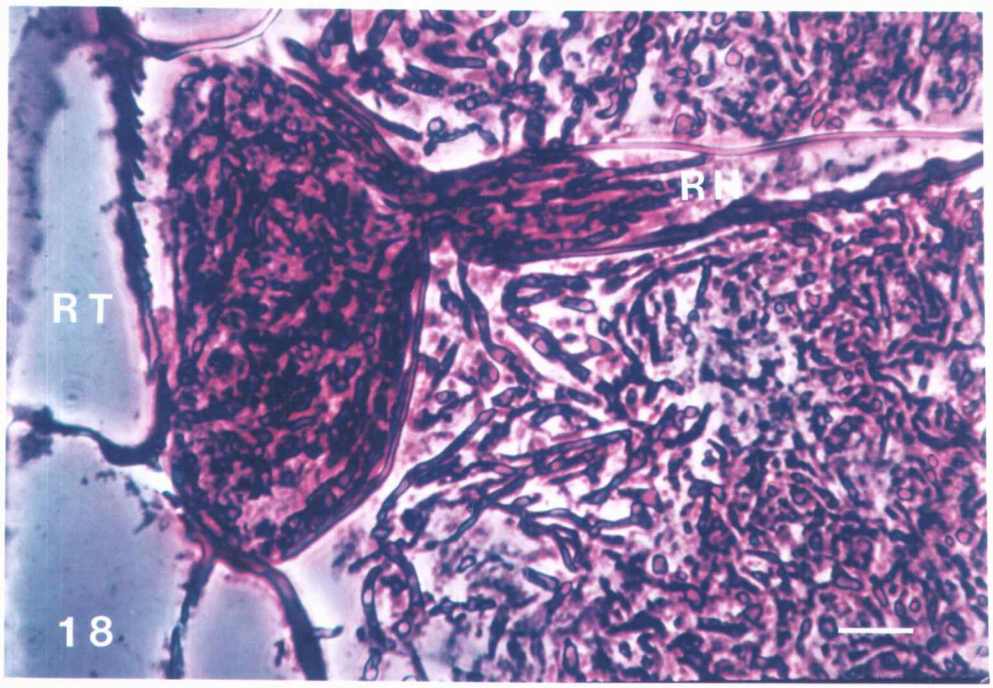

RP
17

Figure 18. Root hair (RH) of shoot meristem inoculated sterile tall fescue seedling is infected with intracellular hyphae of A. coenophialum. Epiphytic hyphae are also visible in the surrounding medium. RT = root. Bar = 26 μm.


RT
RT
18

hyphal root growth on an artificial medium may not accurately represent fungal growth characteristics in the natural environment. Therefore, it is important that the distribution of A. coenophialum in roots of field-grown tall fescue is determined. If proven capable of successfully colonizing the rhizosphere, the endophyte-root association could be potentially responsible for observed growth differences between infected and non-infected grass plants. A. coenophialum infected grass is more tolerant of environmental stresses such as high temperatures and drought (Read et al., 1984; Funk et al., 1985). Consequently, in adverse growing conditions endophyte-infected plants are more productive and persistent than non-infected plants. The mechanism for this improved growth is unknown. However, many mycorrhizal fungi improve host growth and survival ability by increasing the water absorption and inorganic nutrient uptake capacity of host feeder roots (Gerdemann, 1968; Harley and Smith, 1983).

In addition, cultures of A. coenophialum are shown to inhibit the growth of some saprophytic and soil fungi in vitro (White and Cole, 1985C; Siegel et al., 1987). If antagonistic products are produced by the endophyte and these occur in the rhizosphere, the endophyte could gain a competitive advantage over soil-inhabiting microorganisms. Tall fescue could also benefit from this type of root association by having potential pathogens deterred by endophyte produced antagonistic products. However, speculations can only be answered by additional research which verifies or

negates endophytic presence in roots of tall fescue and evaluates the importance of this in the endophyte-host association.

It is hypothesized that mycorrhizal relationships may be essential adaptations for many grasses growing in semi-arid regions. Similarly, the vast host range of Acremonium-type endophytes in grasses could indicate that endophyte-infected plants have a selective advantage over non-infected grasses. Acremonium-infection of grass plants may represent an important ecological adaptation.

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