Survey and Detection of Endophytic Fungi in *Lolium* Germ Plasm by Direct Staining and Aphid Assays

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ABSTRACT

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Clavicipitaceous anamorphic endophytes were detected in 28 of 85 accessions from five of eight species in a collection of *Lolium* germ plasm. Comparative descriptions of endophytic mycelium in seeds of *L. multiflorum, L. perenne, L. persicum, L. rigidum*, and *L. temulentum* revealed morphological characteristics distinguishing endophytes in each host species. Most endophyte-infected accessions originated from Eurasia, particularly from Middle Eastern countries. Endophyte incidence and infection percentages varied widely with host species and geographic origin. Feeding tests with *Diuraphis noxia* were useful in detecting endophyte-infected accessions of perennial ryegrass.

Additional keywords: *Acremonium*, bioassay, biological control, Clavicipitaceae, Russian wheat aphid

Clavicipitaceous fungi cause important diseases of grass inflorescences such as ergot of rye and wheat (39). Consumption of ergotized grain in Europe has resulted in outbreaks of convulsive and gangrenous ergotism in humans for centuries (3). Endophytic fungi in the tribe Balansiae of the Clavicipitaceae induce choke diseases of forage and turfgrasses and are systemic in their hosts (9). However, the importance of the lesser-known but presumably related anamorphic forms, placed in the *Albo-lanosa* section of the genus *Acremonium* (24), has been recognized only within the last decade. Their significance and abundance were overlooked because they are either mutualistic endosymbionts or weak parasites that do not cause disease symptoms, produce teleomorphic states, or form spores in or on their hosts (38).

Attention was focused on anamorphic endophytes when summer toxicosis of cattle in the eastern United States and ryegrass staggers of sheep in New Zealand were associated with endophyte-infected tall fescue (*Festuca arundinacea* Schreb.) and perennial ryegrass (*Lolium perenne* L.) (11,27). These maladies result in annual livestock losses that exceed $100 million for both countries (37). The toxigenic agents causing these disorders are ergot alkaloids and lolitrem neurotoxins (11,13,14,23,28,43). Additional interest was generated when it was found that some of these alkaloids may be responsible for beneficial effects in endophyte-infected grasses (17,28,32). After initial studies of resistance in endophyte-infected perennial ryegrass to the Argentine stem weevil (*Listronotus bonariensis* Kuschel) (4,30), reports of endophyte-associated resistance to other insects followed (1,12,15,17,19). Endophytes also may benefit hosts by increasing their resistance to environmental stresses and plant diseases, improving plant growth and vigor, and providing a competitive advantage over endophyte-free grasses and invading weed species (38). Moreover, Pedersen et al (26) reported that endophyte-infected annual ryegrass (*L. multiflorum* Lam.) used in rotation with soybeans reduced phytopathogenic nematode populations in soils and hence reduced disease severity in subsequent soybean crops.

The potential benefits and applications of *Acremonium* endophytes to the turfgrass industry has prompted considerable research in the United States and abroad. At present, little is known about the number of species, host ranges, and distributions of endophytic fungi in natural populations or in commercial cultivars and germ plasm collections of grasses. Endophytes have been found in seeds of major pasture grasses from many countries (21,22,33,35,36,41), yet surveys of endophyte populations have just begun. Since grasses infected with *Acremonium* endophytes are symptomless, a variety of techniques ranging from staining of seeds, leaf sheaths, and culm piths to more sophisticated serological methods (ELISA) (18,25,40) and chemical analyses for specific alkaloids (6,23,44) were developed to detect and identify them. Latch et al (20) first proposed that an aphid, *Rhopalosiphum padi* L., might be used to detect endophytes in tall fescue. Recently, Clement et al (8) showed that endophyte-infected perennial ryegrass and tall fescue were resistant to the Russian wheat aphid, *Diuraphis noxia* (Mordvilko).

The U.S. germ plasm collection of 667 plant inventory (PI) accessions of *Lolium* is maintained at the Western Regional Plant Introduction Station (WRPIS) in Pullman, Washington. Because fungal endophytes of grasses have broader significance after the discovery of their toxic effects on mammals and insect herbivores (2,7), knowledge of endophyte incidence in major germ plasm collections is needed. The objectives of this study were to determine the incidence of clavicipitaceous endophytes in a portion of the collection and to evaluate *D. noxia* as an assay tool to detect endophytes in perennial ryegrass.

MATERIALS AND METHODS

Seed source. Seed samples of 85 accessions were selected from eight *Lolium* species in the WRPIS seed storage facility. The samples had been stored at 4–5 C and 30–35% relative humidity for a varying duration of up to 37 yr. Most accessions had been increased several times since original seeds were received into the collection. Seeds from the most recent increase were selected because original seeds usually were unavailable.

Endophyte assays. Seeds from all accessions of ryegrass species represented by fewer than 20 accessions, including *L. canariense* Steud., *L. persicum* Boiss. & Hohen., *L. remotum* Schrank, and *L. subulatum* Vis., were examined for endophytic fungi. At least 10% of available accessions of species with more than 20 accessions, namely *L. multiflorum, L. perenne, L. rigidum* Gaudin, and *L. temulentum* L., also were examined.

Seed samples, 0.5–1.3 g depending on seed size, were soaked overnight in 5% sodium hydroxide at 22 C, rinsed with tap water, and stained for several days at 20–22 C in an aqueous solution of 0.07% aniline blue containing 12% lactic

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acid and 14% glycerol. Seeds were then rinsed, squashed, and mounted on slides in 1:1 (v/v) glycerol-distilled water and examined microscopically at ×100–400 magnification. Seed infection rates were based on examinations of 100 seeds in endophyte-free accessions and 150 seeds in endophyte-infected accessions. Measurements of hyphal diameters were based on examinations of hyphae in at least 25 seeds each in several accessions of each species.

Endophyte growth and morphology were assessed in seedlings of infected *L. perenne* accessions growing in two different soil and light environments. Seedlings were grown in the laboratory under low light (40 $\mu E \cdot m^{-2} \cdot s^{-1}$) in 75 × 90 mm glass petri dishes containing sterilized vermiculite or in a greenhouse under natural light in 15-cm pots with sterilized soil consisting of 55% peat moss, 35% pumice, and 10% sand. Leaf sheaths from 50–100 seedlings (6–10 wk old) were examined microscopically at ×400 to determine endophyte viability and hyphal diameters under each growing condition. Endophyte viability was determined in all other *Lolium* species by examining culm pith tissues at the nodes of 50–100 flowering adult plants.

Table 1. Occurrence of clavicipitaceous anamorphic endophytes in seeds of the WRPIS *Lolium* germ plasm collection[a]

Species	Accessions at WRPIS	Accessions examined	Accessions with endophyte	Accessions without endophyte[b]					
				NA	SA	EU	AF	AS	AU
L. canariense	1	1	0	0	0	1	0	0	0
L. multiflorum	89	9	3	0	1	2	0	1	2
L. perenne	394	40	6	2	0	18	3	7	4
L. persicum	12	12	9	0	0	0	0	3	0
L. remotum	3	3	0	0	0	2	0	1	0
L. rigidum	105	14	8	0	0	0	1	3	2
L. subulatum	2	2	0	0	1	0	0	0	1
L. temulentum	37	4	2	0	0	0	1	1	0
Total	643	85	28	2	2	23	5	16	9

[a] Based on examinations of 150 seeds in endophyte-infected accessions and 100 seeds in endophyte-free accessions of the Western Regional Plant Introduction Station (WRPIS) in Pullman, Washington.

[b] Continents of origin: North America (NA), South America (SA), Europe (EU), Africa (AF), Asia (AS), and Australia–New Zealand (AU). Information on specific endophyte-free accessions is available from the Germplasm Resources Information Network (GRIN) data base, USDA-ARS.

Table 2. Endophyte-infected accessions in the WRPIS *Lolium* germ plasm collection[a]

Species	Accession (PI)	Country of origin	Year[b]		Mean percent endophyte[c]
			Received	Increased	
L. multiflorum	376875	New Zealand	1972	1974	2
(annual ryegrass)	376876	New Zealand	1972	1974	2
	410154	South Africa	1976	1978	10
L. perenne	205278	Turkey	1953	1968	56 (56)
(perennial ryegrass)	231606	Portugal	1956	1968	47 (33)
	231618	Greece	1956	1968	3
	277846	Yugoslavia	1962	1980	2
	376878	New Zealand	1972	1975	1
	462339	New Zealand	1981	1984	99 (99)
L. persicum	222807	Iran	1955	1986	96 (70)
(Persian ryegrass)	229764	Iran	1956	1986	74
	230110	Iran	1956	1986	76
	239727	Iran	1957	1986	86
	239728	Iran	1957	1986	80
	239729	Iran	1957	1986	57
	269386	Afghanistan	1962	1986	68
	314446	Soviet Union	1966	1982	86
	317450	Afghanistan	1966	1988	82
L. rigidum	239730	Egypt	1957	1988	4
(Wimmera ryegrass)	239734	Israel	1957	1982	2
	239763	Morocco	1957	1988	2
	239782	Portugal	1957	1988	46
	239792	Greece	1957	1988	8
	250805	Egypt	1958	1988	44 (31)
	287857	Spain	1963	1988	48
	298416	Turkey	1964	1988	18
L. temulentum	206691	Turkey	1953	1988	48
(darnel)	249725	Greece	1958	1976	64 (37)

[a] The following accessions from the Western Regional Plant Introduction Station (WRPIS) have cultivar names: PI 376875 = G. Manawa, PI 376876 = G. Paroa, PI 410154 = Mid Mar, PI 376878 = G. Ruanui, PI 462339 = Ellett, PI 317450 = Alaff.

[b] Year accession was received into WRPIS *Lolium* germ plasm collection and year of increase from which seeds were sampled for examination.

[c] Infection rates were based on examinations of 150 seeds. Values in parentheses indicate viable endophyte as determined from examinations of 50–100 leaf sheaths of 9-wk-old seedlings or culm piths from nodes of flowering adult plants.

Aphid tests. A colony of *D. noxia* was established from aphids collected in August 1988 in a nursery of *Hordeum* spp. at Pullman and maintained on barley (*Hordeum vulgare* L. 'Steptoe') in an environmental chamber at 22 C, light intensity of 150 $\mu E \cdot m^{-2} \cdot s^{-1}$, and photoperiod of 14:10 hr (light:dark). Experiments were performed in a growth chamber under identical conditions. Test plants of *L. perenne* were grown individually from seed in a greenhouse in separate 15-cm pots containing the previously described soil mixture and maintained at 15–32 C under natural light and with weekly 20-20-20 (N-P-K) fertilizer applications. Before each test, plants were numbered and basal leaf sheaths were examined microscopically at ×400 for the presence of fungal endophyte when the plants were at least 8 wk old. Indexed test plants were placed randomly in the chamber next to each other so that aphids could move freely among plants. Data were recorded for each experiment by a separate observer without prior knowledge of the endophyte-infection status of test plants.

The first test evaluated *D. noxia* for its ability to detect endophytes in unclipped 9-wk-old seedlings. Ten endophyte-infected *L. perenne* seedlings of PI 462339 and 10 endophyte-free seedlings of PI 462340 from New Zealand (Mangere District) were infested with aphids by placing four barley leaves carrying numerous aphids on each plant. Aphids moved to the test plants as the barley leaves became desiccated. Live aphids, remaining on each plant 6 days after infestation, were counted at ×25 magnification.

The second test examined the ability of *D. noxia* to detect endophytes in regrowth foliage after plants were clipped to 2–4 cm above the soil surface at age 10 and 15 wk. The same experimental design with equal numbers of infected and uninfected plants and 6-day post-infestation aphid counts was used. However, the only variation from the first test was that plants from a single perennial ryegrass line PI 205278 from Turkey were tested. Aphid-infested barley leaves were placed on 20-wk-old test plants with 5-wk-old regrowth foliage that had regenerated after clipping.

Data from both tests were log $(x + 1)$ transformed and analyzed by one-way analysis of variance using general linear models. Treatment effects on aphid

counts were tested at the $P = 0.01$ level of significance.

RESULTS

Endophyte assays. Endophytic mycelia were observed in seeds of five of eight *Lolium* species from 34 countries in six continents (Table 1). The survey of 85 accessions represented 14% of the *Lolium* accessions in the WRPIS collection. Three species (*L. canariense, L. remotum*, and *L. subulatum*) were endophyte-free, but only six total accessions of these species were available. Approximately one-third of the *Lolium* accessions tested were infected with endophytes. The majority (84%) of surveyed accessions originated from Europe (35%), Asia (34%), and Australia (15%) (Table 1).

Incidence of endophyte infection, based on the percentages of examined accessions that were infected, varied with host species. Incidence of endophyte was highest in *L. persicum* (75%), followed by *L. rigidum* (57%), *L. temulentum* (50%), *L. multiflorum*, (33%), and *L. perenne* (15%).

Seed infection percentages and identifying characteristics of endophyte-infected accessions are summarized in Table 2. Endophyte levels among infected accessions varied widely with host species and geographic origin. Accessions of *L. persicum* had the highest range of infection rates (57–96%). The widest range of infection percentages (1–99%) was in *L. perenne*. The average infection rate for all accessions of a species was highest in *L. persicum* (78%) and *L. temulentum* (56%) and lower in *L. perenne* (35%), *L. rigidum* (22%), and *L. multiflorum* (5%). Most endophyte-infected accessions of *Lolium* originated from Asia (46%), particularly from the Middle East, and Europe (25%). Consequently, the highest infection rates in most species occurred in accessions from the Middle East. Very few infected accessions were found in germ plasm from Africa and New Zealand. No infected accessions were identified from North or South America, although only four accessions were examined.

Endophyte infection rates in seeds did not necessarily indicate the levels of viable endophyte that were observed in seedlings. For example, levels of infection in seedlings of *L. perenne* accession PI 231606 and in flowering adult plants of other *Lolium* accessions appeared lower than levels of infection in seeds (Table 2). None of the ryegrass species had signs or symptoms of choke disease during flowering.

Endophytic hyphae observed in leaf sheaths of *L. perenne* accessions were typical of *Acremonium lolii* Latch, Christensen & Samuels (Fig. 1A). They were less convoluted than hyphae of *A. coenophialum* Morgan-Jones & Gams that we observed in tall fescue, and they conformed closely to the shape of adjacent plant cells. Although most growth was parallel to the longitudinal axis of the leaf sheath, intercellular hyphae occasionally branched at the end walls of mesophyll cells to produce lateral growth. The rates hyphae entered and accumulated in leaf sheaths and hyphal diameters depended on the conditions under which plants were grown. Slow-growing seedlings of PI 205278, PI 231606, and PI 462339 grown under low light in nutrient-poor vermiculite and limited container volume had hyphae that had small diameters (< 1.0 μm) and entered leaf sheaths after 8–10 wk. Hyphae were significantly ($P < 0.001$) larger (2–3 μm) and entered leaf sheaths faster (6–8 wk) in seedlings of these accessions growing vigorously under brighter light and in a larger volume of nutrient-rich soil. Hyphae were not observed colonizing the entire leaf sheath in any of the annual ryegrasses, but hyphae were found in their culm piths.

Morphological differences were found that distinguished endophytic fungi in seeds of each ryegrass species. Endophytic hyphae were thin and stained poorly in seeds of *L. multiflorum* (< 1.0–1.5 μm) and *L. rigidum* (1.0–2.5 μm) (Fig. 2A,C). Hyphae stained well and were thicker in *L. perenne* (2–3 μm) (Fig. 1B) and thickest in *L. temulentum* (1.0–3.5 μm, rarely 5 μm) (Fig. 2D) and *L. persicum* (2–4 μm) (Fig. 2B). Endophytic mycelium was strongly convoluted in *L. persicum* (Fig. 2B) and moderately convoluted to straight in *L. multiflorum, L. rigidum*, and *L. temulentum*, as in *L. perenne*. Hyphal branching was observed commonly in *L. multiflorum, L. perenne*, and *L. temulentum*, occasionally in *L. rigidum*, and rarely in *L. persicum*. Hyphal constrictions at septa were observed in endophytic hyphae in all *Lolium* species, but they occurred less frequently with hyphae in *L. persicum*. Cell length was most variable in hyphae of the *L. temulentum* endophyte. Hyphae in seeds of *L. perenne* accessions (Fig. 1B) also branched more and were less convoluted than hyphae of *A. coenophialum* in tall fescue seeds.

Aphid tests. Significantly fewer Russian wheat aphids were found on perennial ryegrass plants containing endophytic mycelium than on plants lacking endophyte in tests 1 and 2 (Table 3). Endophyte-free plants in both tests supported aphid reproduction, but no nymphs were found on endophyte-infected plants.

DISCUSSION

Germ plasm is tested at the WRPIS to identify accessions infected with plant pathogens and those with resistance or tolerance to plant pests. The WRPIS also maintains PI germ plasm without selection and regardless of agronomic characteristics or phenotypic traits to sustain genetic diversity and maintain all potentially valuable traits for the future. Any selection to eliminate accessions infected with phytopathogens or lacking certain desirable traits such as pest resistance could result in loss of other as yet undiscovered traits that may be useful in future breeding programs. Nonselective maintenance may tend to dilute any one agronomically desirable trait in a germ plasm collection as more accessions are added.

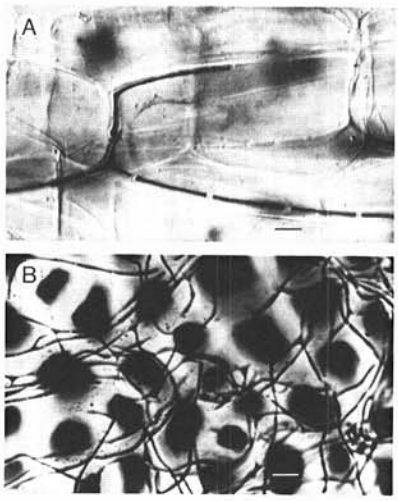

Fig. 1. Endophytic hyphae in **(A)** leaf sheath and **(B)** aleurone seed tissue of *Lolium perenne* (PI 205278). Scale bar = 5 μm.

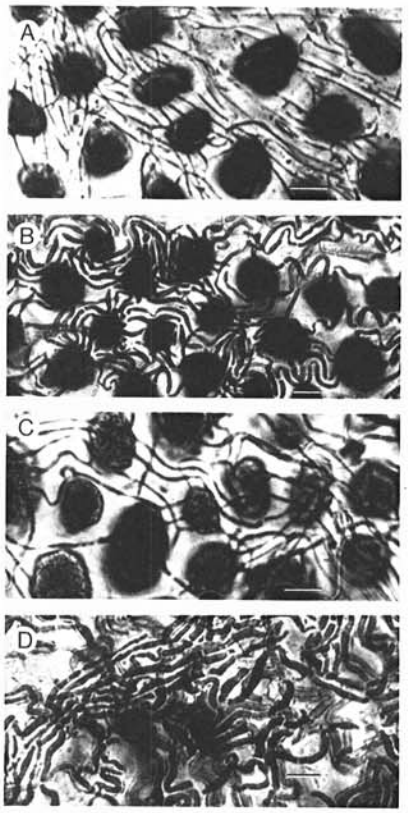

Fig. 2. Comparative morphology of endophytic hyphae in aleurone seed tissues of **(A)** *Lolium multiflorum* (PI 410154), **(B)** *L. persicum* (PI 222807), **(C)** *L. rigidum* (PI 250805), and **(D)** *L. temulentum* (PI 249725). Scale bar = 5 μm.

This hypothesis could explain the relatively low incidence and infection rates of clavicipitaceous endophytes in the WRPIS *Lolium* collection. The WRPIS collection is not subject to selection practices, but commercial collections and cultivars are often maintained under rigorous selection programs to enhance pest resistance. The *L. perenne* New Zealand cultivar Ellett (PI 462339) was the only endophyte-infected accession examined with a long history of commercial selection. The cultivar was selected for its vigor and resistance to *L. bonariensis* and grazing pressure in a 60-yr-old Auckland pasture of the Mangere District. It became a PI accession in 1981 and provided the highest infection percentage (99%) in this survey. Many endophyte-free cultivars have succumbed to *L. bonariensis* attack during field selection in New Zealand (5).

The 28 endophyte-infected *Lolium* accessions identified here were from 13 countries, mostly in Eurasia. Since endophytes are not endemic to South Africa or New Zealand, endophyte-infected germ plasm from these areas probably originated from the United Kingdom (22). Other surveys of *Festuca* and *Lolium* species have indicated a high incidence of endophytes in germ plasm from Europe (21,33), but to our knowledge, few surveys have included germ plasm from Asia. Because many wild and cultivated grasses have originated in the Middle East (42), this region is a probable area favoring evolution of endophyte-grass associations.

The morphology of endophytic fungi in seeds of *L. multiflorum, L. persicum*, and *L. rigidum* were compared here for the first time. The endophyte in each host appeared morphologically distinct on the basis of hyphal diameters, stainability, convolution, and branching. The endophyte of *L. persicum* was distinguished by its relatively wide, highly convoluted mycelium, similar to that of *A. coenophialum* (16). The endophytes in *L. multiflorum, L. rigidum*, and *L. temulentum* had moderately convoluted to straight mycelium, resembling the growth form of *A. lolii*, but the *L. rigidum* endophyte had infrequently branching hyphae. The fine, poorly staining mycelium of endophytes in *L. multiflorum* and *L. rigidum* required high magnification (×400) for detection. Endophytes were recognized only recently in these hosts (21). An endophyte of darnel (*L. temulentum*) has been known for many years (34), but it has not been identified (7). The endophyte of *L. perenne* cultivar Ellett was previously identified as *A. lolii* (22,29).

Endophytic mycelium was observed in culm piths at the nodes of infected accessions in all ryegrass species examined. However, mycelium was observed throughout leaf sheaths only in perennial ryegrass. Mycelium occasionally accumulated at the extreme base of leaf sheaths in *L. persicum*, but it did not grow to the sheath apex. Our observations support those of Latch et al (21), who found that endophytic mycelium was confined to nodal regions and portions of culm piths in annual ryegrasses.

Feeding tests indicated that the Russian wheat aphid is sensitive to an anamorphic endophyte in perennial ryegrass seedlings. Furthermore, we demonstrated the potential for using the aphid to assay for clavicipitaceous endophytes in accessions of *L. perenne* and to detect endophytes in individual plants of an accession. Determinations of infection in individual plants are based on criteria that may vary according to host species and accession, aphid species, foliage source (age), and number of postinfestation days before aphids are counted.

Continued use of the term "endophyte" without qualifying statements has led to considerable confusion because of its potential application to any organism found within plants. For example, both saprophytic fungi and bacteria commonly found as residents in plant tissues are considered endophytes by some (10,31). To avoid this confusion, we propose the term "clavicipitaceous anamorphic endophytes" (CAE) for the mutualistic fungi of the *Albo-lanosa* section of *Acremonium* and related anamorphic genera (e.g., *Phialophora, Gliocladium*) and the term "clavicipitaceous teleomorphic endophytes" (CTE) for the choke fungi of the Balansiae. These terms will distinguish the systemic, ergot alkaloid-producing fungi from nonclavicipitaceous fungal saprophytes and resident bacteria.

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Table 3. Counts of live Russian wheat aphids on endophyte-free and endophyte-infected perennial ryegrass plants[a]

Test no.	Accession (PI)	Endophyte presence	Number of aphids/plant[b] Mean ± SEM	Range	*F* value	*PR* > *F*
1	462339	+	0.8 ± 0.3	0–3	71.2	0.0001
	462340	−	31.4 ± 9.1	5–96		
2	205278	+	2.0 ± 0.6	0–7	21.8	0.0002
	205278	−	40.1 ± 11.8	9–138		

[a] Aphids were counted 6 days after infestation.
[b] Means were determined from aphid numbers on 10 replicate plants.

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