Fungi belonging to the *Neotyphodium* and *Epichloë* genera are among the many types of microbes termed *endophytes* by virtue of their *in planta* life cycle.

The complex functions of these endophytes have received intensive study during the past three decades because of their roles in causing production losses in livestock and enhancing biotic and abiotic stress resistances in hosts.
Endophytes of cool-season grasses
sexual *Epichloë* species
asexual *Neotyphodium* derivatives
Ascomycete fungi (family Clavicipitaceae) that are ecologically obligate symbionts of grasses.

All establish asymptomatic associations with their host during the vegetative phase of growth, but the sexual species are capable of forming a stroma around the developing inflorescence (*choke disease*)
Tall fescue (*Festuca arundinacea*)  
- *Neotyphodium coenophialum*

Perennial ryegrass (*Lolium perenne*)  
- *Neotyphodium lolii*

Meadow fescue (*Festuca pratensis*)  
- *Neotyphodium uncinatum*

Italian ryegrass (*Lolium multiflorum*), Annual ryegrass (*Lolium rigidum*)  
- *Neotyphodium occultans*

Red fescue (*Festuca rubra*)  
- *Epichloë festucae*

Broad host range  
- *Epichloë typhina*
Tall fescue and perennial ryegrass are widely grown in temperate climates throughout the world as forage for cattle, sheep and horses, as well as for turf and conservation uses. Because of their economic relevance, the respective associations with *Neotyphodium coenophialum* and *Neotyphodium lolii* are the most studied worldwide.
Tall fescue

(*Festuca arundinacea*)
Tall fescue is the most important forage grass in the USA

14 million ha

8.5 million cattle and horse heads

‘Transition zone’ (east central and southeast) characterised by poor soil fertility and unfavourable climatic conditions, where only this grass ensures sufficient persistence
Native to much of Europe and North Africa, two ‘types’ or ‘races’ are morphophysiologically distinguishable

- the **Continental** (or European) type (widespread)

- the **Mediterranean** type (restricted to the Mediterranean basin)
Perennial ryegrass
(*Lolium perenne*)
Well adapted to temperate areas, perennial ryegrass is the primary grass used for pasture and silage in animal production.

In New Zealand, perennial ryegrass is the predominant component in the permanent pastures for wool and dairy production (10 M ha).

In Australia it covers more than 6 M ha in temperate, winter-spring rainfall zones.

In continental Europe, the UK and Ireland, perennial ryegrass forms the basis of most pastures for cattle and sheep grazing.
The asexual (or anamorphic) *Neotyphodium* endophytes systemically colonise the intercellular spaces of leaf primordia, leaf sheaths and leaf blades of vegetative tillers, the inflorescence tissues of reproductive tillers, and eventually the seeds.

Having suppressed the sexual reproduction, they can only propagate through the host’s ‘infected’ seeds (vertical or maternal transmission).
Grass-endophyte association

*Antagonism* (symptomatic, sexual forms)

*Mutualism* (asymptomatic, asexual forms)

Endophyte benefits to the host: greater persistence/fitness (through a mosaic of positive attributes, more or less effective according to the conditions: resistance/deterrence to mammalian and pest feeding; drought tolerance; resistance to nematodes and fungal pathogens; enhanced mineral nutrition; etc.)

Host benefits to the endophyte: provision of nutrients and a means of dissemination through the seed
A possible co-evolution between the two symbionts, driven by both their mutualistic association and the vertical transmission of the fungus, may have occurred, resulting in specific host-endophyte associations.
Genetic variation of endophytes

Taxonomic classification of endophyte isolates was firstly carried out based on distinctive morphological and cultural characters.

Taxonomic Groupings (TG) within the main species *Neotyphodium coenophialum* (FaTG-1) and *Neotyphodium lolii* (LpTG-1):
- FaTG-2
- FaTG-3
- LpTG-2
Neotyphodium coenophialum (FaTG-1): long conidia (> 10 _m), synthesis of loline alkaloids

FaTG-2: short conidia (< 8 _m), lack of loline alkaloids

FaTG-3: short conidia (< 8 _m), synthesis of loline alkaloids

Neotyphodium lolii (LpTG-1): synthesis of lolitrem B alkaloid

LpTG-2: lack of lolitrem B alkaloid
The variation of endophyte isolates assessed by isoenzyme polymorphism analysis corresponded closely to the taxonomic distinction based on morphophysiological characters.

Isoenzymes may reveal only a subset of the possible genetic variation, providing a relatively conservative measure of endophyte variation.
DNA-based markers have been developed

Non-specific markers (RAPDs, AFLPs): owing to nonspecificity, use limited to the analysis of endophytes in fungal cultures

Recently, a large set of SSR markers have been generated

The specificity of SSR markers permits in planta endophyte detection
- SSRs derived from genomic DNA libraries
- SSRs derived from clones of cDNA libraries (associated with ESTs)

EST-derived SSR markers more sensitive for *in planta* detection than genomic DNA-derived SSR markers. However, genomic DNA-derived SSR markers revealed more polymorphism than EST-derived SSR markers.
Intraspecific genetic diversity

Genetic diversity of *Neotyphodium lolii* assessed with 18 EST-derived SSR markers. The geographic pattern of diversity in *N. lolii* was not randomly distributed. Endophytes in accessions from western Europe (and, hence, the New World), eastern Europe, the Mediterranean basin, and North Africa clustered to form four distinct groups. The geographical pattern of endophyte diversity showed similarities with both ancient and recent routes of dispersion of perennial ryegrass.
A possible co-evolution between the two symbionts, driven by both their mutualistic association and the vertical transmission of the fungus, may have occurred, resulting in specific host-endophyte associations.
In tall fescue

*Neotyphodium coenophialum* associated with the Continental type

FaTG-2 variant associated with the Mediterranean type

FaTG-3 variant possibly associated with North-African Mediterranean germplasm
<table>
<thead>
<tr>
<th></th>
<th>Length of conidia (μm)</th>
<th>Total lolines (g/g DM)</th>
<th>Ergovaline (g/g DM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>N. coenophialum</em></td>
<td>11.6 a</td>
<td>2435 a</td>
<td>5.8 a</td>
</tr>
<tr>
<td>(Continental germplasm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FaTG-2</td>
<td>5.9 b</td>
<td>10 b</td>
<td>1.2 b</td>
</tr>
<tr>
<td>(Mediterranean germplasm)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Presence of endophytes in grasses known for decades

Received growing attention since in 1977 a link was established between tall fescue endophyte infection and animal toxicosis
Epichloë typhina from Toxic Tall Fescue Grasses

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Epichloë typhina, a clavicipitaceous systemic phytopathogen, was isolated from two varieties and three hybrids of tall fescue (Festuca arundinacea). The morphology of the fescue isolates was compared with E. typhina isolated from bent grass (Agrostis perennans). In all isolates, conidia were identical and were typical of E. typhina. In fescue grasses the endophyte failed to produce stromata, but on bent grass the fungus seasonally produced stromata, typical of the genus. Cattle grazing the fescue grasses showed signs of the fescue toxicity syndrome, the E. typhina was found in frequencies of 100%; in grasses from pastures in which cattle showed no signs of the syndrome, frequencies were 0 to 50%. Nutritional factors in vitro were more complex for the isolates from fescue than for the isolate from bent grass. These studies suggested that E. typhina includes biotypes that might be involved in the toxicity syndrome. The fescue biotypes grew poorly on media, and yields were inadequate for toxicity studies. However, the bent grass isolate grew well on three media, and extracts from two of these were toxic to chicken embryos. All isolates produced in vitro the nontoxic fungal sterol tetraene [3α,4α,6α,8α(11),22-tetraene]-one, which has been isolated from toxic fescue grasses.

The fescue toxicity syndrome of cattle (6) is a sporadic but serious problem in all sections of the United States where varieties of tall fescue grass (Festuca arundinacea Schreber) are grown as forage. Although the fescue toxicity syndrome has been experimentally induced in cattle by intramuscular or intraperitoneal injection of grass extracts (4, 17), the chemical identity of the toxic compound is unknown. On the basis of the clinical signs and the sporadic and seasonal nature of the syndrome, a vasococontracting mycotoxin has been implicated (18).

Indeed, the severe clinical signs of the syndrome, gangrene of the tail tip, hooves, and ears, are similar to those of the gangrenous-type alkaloid poisoning caused by the alkaloids from species of Claviceps. Species of Claviceps have been ruled out as the cause of the fescue toxicity syndrome (18). The involvement of fescue alkaloids in the syndrome has been examined. Per洛line, one of several alkaloids of tall fescue, has been implicated from in vitro studies as a possible cause of poor performance of cattle that graze fescue during the summer (5). The effect of per洛line on sheep was examined, but the effect of feeding this compound to cattle has not been determined. However, cattle grazing fescue hybrids selected for low per洛line content showed signs of the summer syndrome of toxic fescue (J. Bond, J. B. Powell, D. J. Undersander, L. I. Colbert, and R. R. Otten, Abstr. Annu. Meet. Am. Soc. Anim. Sci., Southern Division, Abstr. 61, 1977).

Many fungi have been studied as causes of the fescue toxicity syndrome (19). Recently, the isolation of Balansia epichloë from grasses of a toxic fescue pasture and the in vitro toxicity of this clavicipitaceous systemic phytopathogen were reported (2, 10). Moreover, studies on the identity of toxic alkaloids produced by B. epichloë (11) and the possible involvement of other species of Balansia in the poor performance of animals grazing infected grasses prompted a search for other endophytic fungi in toxic grasses. We now report the isolation and cultivation of another clavicipitaceous endophyte, Epichloë typhina (Fries) Tulase, from hybrids and varieties of toxic tall fescue and the demonstration of in vitro toxicity of one isolate from Agrostis perennans (Walter) Tuckerman, bent grass.

MATERIALS AND METHODS

Examination of samples. Toxic and nontoxic fescue pastures in the United States were randomly sampled (see Table 1 for sources) and grass tillers were kept moist and as fresh as possible until analyzed in the laboratory. Grasses were examined from pit scramappings removed from longitudinally slit culms of fertile tillers. For uniformity, the internode of the...
Impact of endophyte-infected grasses on animal health

Fescue toxicosis

Syndrome affecting:
- performance and production
- behaviour
- physiology (respiration, heart rate, body temperature, etc.)
- tissue concentrations (serum or plasma concentrations of enzymes, hormones, minerals, etc.)

In horses (mares): increased gestation length, agalactia, foal and mare mortality, thickened placentas, dysmature foals, reduced serum prolactine and progesterone
**Fescue foot**
Gangrenous condition of the extremities (hooves, tail, ears) as a result of vasoconstriction

**Fat necrosis**
Masses of hard fat primarily located in the abdominal cavity

Estimated economic losses over 600 M USD
Ryegrass staggers
Intense neurological symptoms (head tremors, nystagmus, poor coordination, stiff legs, muscle contraction, tetanic spasms), increased body temperature, low ewe fertility, poor production, etc.
In Europe

The widespread use of botanically diverse permanent pastures for livestock production seems to explain why the economic impact of endophytes in Europe is still uncertain. The use of artificial, monospecies pastures is quite limited in this continent.
Positive effects of endophyte-infection on grasses
Insect resistance/deterrence

Bioactivity of grass endophytes against a range of insects (over 40 species reported)

Foliar-chewing lepidopterans (fall armyworm and sod webworm: *Parapediasia* spp.)

Stem-boring larvae (bluegrass billbug: *Stenophorus parvulus*; Argentine stem weevil: *Listronotus bonariensis*)

Plant sap-sucking pests (hairy chinch bug: *Blissus leucopterus*; pasture mealybug: *Balanococcus poae*)

Leafhoppers

Aphids
Experimental evidence proved that endophyte effects on livestock or insects are mediated by a set of biologically-active, secondary metabolites.

Four classes of compounds:

Ergot alkaloids
Pyrrolizidine alkaloids
Lolitrems
Pyrrolopyrazine alkaloids
Ergot alkaloids (clavine alkaloids, ergoline alkaloids, ergopeptine alkaloids), all sharing a lysergic acid moiety

Present in endophyte-infected *Festuca* and *Lolium*

Potent toxins with neurotropic activities: chemical structure similar to the receptors noradrenaline, dopamine and serotonin, thus are capable of producing a toxic effect for the associated receptors

Major problem to livestock producers because of their antimammalian activities (e.g., ergotism by *Claviceps purpurea*)

Tryptophan and mevalonate are precursors to ergot alkaloids. It is estimated that at least 11 enzymatic steps are involved in ergot alkaloid synthesis
Fig. 12.2. Structure of the ergot alkaloids.
The ergopeptine alkaloid ergovaline has historically been identified as the putative toxin causing fescue toxicosis in livestock.

However, ruminant absorption is an active system favouring the ergoline alkaloids over ergopeptine alkaloid transport, especially lysergic acid.
Pyrrolizidine alkaloids

Lolines are saturated amino-pyrrolizidines with an ether linkage between C-2 and C-7
<table>
<thead>
<tr>
<th>Composto</th>
<th>(R_1)</th>
<th>(R_2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>lolina</td>
<td>-H</td>
<td>-(\text{CH}_3)</td>
</tr>
<tr>
<td>N-formillolina</td>
<td>-CHO</td>
<td>-(\text{CH}_3)</td>
</tr>
<tr>
<td>N-acetillolina</td>
<td>-(\text{CH}_3\text{CO})</td>
<td>-(\text{CH}_3)</td>
</tr>
<tr>
<td>N-metillolina</td>
<td>-(\text{CH}_3)</td>
<td>-(\text{CH}_3)</td>
</tr>
<tr>
<td>norlolina</td>
<td>-H</td>
<td>-H</td>
</tr>
<tr>
<td>N-acetilnorlolina</td>
<td>-(\text{CH}_3\text{CO})</td>
<td>-H</td>
</tr>
<tr>
<td>N-formilnorlolina</td>
<td>-CHO</td>
<td>-H</td>
</tr>
</tbody>
</table>
Present in several host-endophyte combinations
Absent in tall fescue infected by FaTG-2 variant

Highest concentration of all alkaloids

_N-formyloline, N-acetylloleine and N-acetylNorloine usually are the most abundant ones

L-proline and L-homoserine are precursors to loline alkaloids. It is estimated that at least 8 enzymatic steps are involved in loline alkaloid synthesis
Lolines (specifically $N$-formylololine and $N$-acetylololine) have proved to be toxic/deterrent against insects: e.g. aphids *Rhopalosiphum padi* and *Schizaphis graminum*, and species belonging to other orders.
Lolitrems are an important subgroup of indole-diterpene metabolites relatively abundant in leaf sheaths and seeds of perennial ryegrass infected by *Neotyphodium lolii*, although they can also be found in tall fescue infected by endophytes other than *Neotyphodium coenophialum*.

**Lolitrem B** is thought to be responsible for the livestock disorder known as ryegrass staggers.
Peramine
It is a unique pyrrolopyrazine alkaloid. Proline and arginine are possible precursors but, to date, there is no experimental information on the byosynthesis of this compound.

Peramine is a potent antifeeding deterrent against adult Argentine stem weevil (*Listronotus bonariensis*), the major pest of perennial ryegrass in New Zealand. In this country, perennial ryegrass can only be grown if high level of peramine is provided by endophyte infection.
Positive effects of endophyte-infection on grasses

Enhanced persistence in water-limited environments
Enhanced mineral nutrition
Nematicidal activity
Anti-fungal activity
Competitive advantage (rhizosphere allelopathy)
Drought tolerance

Possible adaptive features of endophyte-infected plants in water-limited environments:
- extensive root system
- reduced stomatal conductance
- accumulation of osmotically active metabolites
- enhanced concentration of phenolics (resistance to oxidation stress)
- Earlier/greater accumulation of dehydrins
### Tifton, GA (300 mm mean June-August rainfall)

<table>
<thead>
<tr>
<th></th>
<th>Mean stand persistence (%)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Year 1</td>
<td>Year 2</td>
<td>Year 3</td>
</tr>
<tr>
<td>Endophyte Infected</td>
<td></td>
<td>99 a</td>
<td>98 a</td>
<td>63.5 a</td>
</tr>
<tr>
<td>Endophyte Free</td>
<td></td>
<td>96.5 a</td>
<td>84.2 b</td>
<td>27 b</td>
</tr>
</tbody>
</table>

### Fayetteville, AR

<table>
<thead>
<tr>
<th></th>
<th>Tiller density (No./m²)</th>
<th></th>
<th>April-October water: 577 mm</th>
<th>April-October water: 952 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endophyte Infected</td>
<td>2396 a</td>
<td></td>
<td>3400 a</td>
<td></td>
</tr>
<tr>
<td>Endophyte Free</td>
<td>2117 b</td>
<td></td>
<td>2704 b</td>
<td></td>
</tr>
</tbody>
</table>
A lower limit is set by extremely dry conditions beyond which the endophyte exerts little effect in contributing to plant persistence and the survival is mainly due to intrinsic adaptation of the host to harsh conditions.
Enhanced mineral nutrition

Possible higher nitrogen use efficiency of grasses in response to endophyte infection when grown at low N availability (observations in perennial ryegrass, Arizona fescue, tall fescue; controversial results)
### Lodi

<table>
<thead>
<tr>
<th></th>
<th>4-yr cumulated DMY (t/ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(N0 treat.: 40 kg/ha N pre-sowing only)</td>
<td></td>
</tr>
<tr>
<td>Endophyte Infected</td>
<td>33.46 a (+7.4%)</td>
</tr>
<tr>
<td>Endophyte Free</td>
<td>31.15 b</td>
</tr>
</tbody>
</table>

No significant difference (+2.1%) in the treatment with 450 kg/ha N total across years.
Present limits
Although still incomplete, some information on the relationships between alkaloids and animal toxicosis, or alkaloids and pest resistance has been gathered. Other beneficial aspects of the endophyte-grass interaction have not yet been characterised. Very little is known yet regarding the factors important in host colonisation or nutrient exchange between plant and fungus. The mechanisms underlying defense responses of endophyte-infected grasses against plant pathogens and nematodes are not yet understood, although they appear to be dependent on endophyte strain, secondary metabolite production, and specific interactions between fungus and plant genotype. The physiological mechanisms leading to enhanced tolerance to abiotic stresses of endophyte-infected grasses are still largely unknown.
In many instances there is a need to maintain the endophyte infection as a mechanism of persistence for the cultivated cool-season grasses.

Both the plant genotype and the endophyte strain play an integral role in determining the outcome of the host-endophyte-(livestock)-(insect) interactions, primarily by modifying the expression of alkaloids produced by endophyte-infected plants.

Exploiting the inherent variation in alkaloid production to minimise the effects on grazing animals by selecting for plant-endophyte associations with low concentrations of toxic alkaloids.
Incorporating non-toxic-alkaloid–producing endophytes in grasses (endophyte-safe approach)

“Reinfecting” (or “inoculating”) – usually on seedlings – endophyte-free tall fescue and perennial ryegrass cultivars with “safe” endophyte strains (not producing toxic alkaloids)
Neotyphodium lolii in perennial ryegrass

[N. 187 (“Endosafe R”): low lolitrem B synthesis reinfected on Grasslands Pacific and Grasslands Nui cvs]
AR1 strain: no lolitrem B, no ergovaline, yes peramine reinfected on > 20 New Zealand cvs

Neotyphodium coenophialum in tall fescue

AR542 (Max Q™): no ergot alkaloids, yes lolines reinfected on Jesup, Georgia 5, Grasslands Flecha cvs
ArkPlus™: no ergot alkaloids, yes lolines reinfected on HiMag cv (HiMag5, HiMag9)
Most recent approach: molecular breeding

Characterisation of fungal genome and its functions would enable genetic transformation of the endophyte with the aim of eliminating the genes responsible for the production of toxic alkaloids while maintaining the desirable features.
High-throughput gene discovery by expressed sequence tag (EST) sequencing has become a powerful tool.

An endophyte EST resource comprising 9507 sequences from *Epichloë* and *Neotyphodium* endophytes has been established, and a total of 3942 unique genes have been identified.

SNPs are becoming increasingly important markers in genetic analysis. 1979 SNPs have been identified from the endophyte EST resource.
Transcriptome analysis

Measurement of gene expression

Hybridisation-based **microarrays** have become the tool of choice for transcriptomic analysis, primarily because of their ability to analyse simultaneously multiple samples.

The availability of fungal genomic and EST sequences has made it possible to construct DNA microarrays for the simultaneous analysis of the expression levels of large sets of genes.
Putative genes involved in alkaloid synthesis

*Neotyphodium coenophialum*

*dmaW, lpsA* for ergot alkaloids  
*lolC* for lolines

*Neotyphodium lolii*

*ltmM* for lolitreem B  
*perA* for peramine
Transcriptome analysis by microarray approach can be used to identify novel endophyte genes putatively involved in specific metabolic pathways and other cellular processes (through gene disruption or gene silencing).

Microarray analysis has proved capable of assessing gene expression changes in response to the introduction of chimeric transgenes in transformed endophyte.
In the future, major advances in the study of grass-endophyte interactions are likely to be made through co-genotyping of endophyte and host grass and the molecular dissection of the grass-endophyte association at the transcriptomic level.

The identification of host-specific and endophyte-specific genomic regions controlling the interaction phenotype will permit an efficient marker-assisted breeding of improved symbioses.