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Endophytes in New Zealand grass seeds: occurrence and implications for conservation of grass species

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Abstract Grass seeds from 24 native species and 3 introduced, naturalised species were collected during the summer of 1999/2000 in New Zealand, mainly from the South Island. These species represented 12 grass genera. Of the native grasses, 19 are endemic. These seeds were examined for the presence of *Epichloë* or *Neotyphodium* endophytic fungi. No endophyte was detected in any of the 64 seed collections of native species. These included seven native *Poa* and two native *Festuca* species, and contrast with the common occurrence of endophytes in Northern Hemisphere and South American populations of these genera. This may be linked to the time of colonisation of these grasses by endophytes occurring after the isolation of New Zealand from continental populations, or low herbivore pressure (vertebrate and invertebrate) in New Zealand. No endophyte was found in *Elymus recticetus*, a naturalised grass species introduced from Australia. However, endophyte (*E. festucae*) was detected in 6 of the 20 naturalised *Festuca ovina* and *F. rubra* collections, of which 4 had 82–87% endophyte-infected seed. Endophyte is known to be widespread in roadside collections of the two agricultural species *Lolium perenne* (perennial ryegrass) and *Schedonorus phoenix* (syn. *F. arundinacea*, tall fescue). Published records show that to date the only native New Zealand species known to contain endophyte is *Echinopogon ovatus*. Germplasm conservation of native grasses by seed, and the regimes required to maintain viable endophyte in such collections, needs to ensure that seed is stored between 0°C and –15°C, or at 5°C in aluminium laminated packaging.

Keywords endophyte; *Epichloë*; *Neotyphodium*; *Elymus*; *Festuca*; *Poa*; native grasses; seed storage

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INTRODUCTION

Endophytic fungal associations occur in many grass genera and are common in temperate grasses from the Northern Hemisphere, but they are much less common in the Southern Hemisphere grasses (Moon et al. 2002). In the context of this paper, endophytic fungi represent the sexual fungi of *Epichloë* Tul. and the asexual grass symbionts now classified in the form genus *Neotyphodium* Glenn, Bacon, & Hanlin (= *Acremonium* Link sect. *Albo-lanosa* Morgan-Jones & Gams) (Schardl 1996). Some *Epichloë* species and all related *Neotyphodium* species are seed transmissible. These fungi are obligately symbiotic with host grasses, and the grasses are facultative hosts (Wilkinson & Schardl 1997). These associations are widely studied as the hosts often receive significant benefits from the symbiosis through greater resistance to biotic and abiotic stresses. Plant persistence is improved, especially through better insect pest tolerance and sometimes by reduced herbivore grazing, due to a number of alkaloids produced by the association. Strains of both *Epichloë* and *Neotyphodium* are known to synthesise ergot alkaloids (resulting in fescue toxicosis in livestock), indolediterpenes (including lolitrems that act as tremogens in livestock), peramine (that deters insects), saturated aminopyrrolizides (lolines) (that deter insects), or combinations of these alkaloids (Bush et al. 1997; Lane et al. 2000).

Endophytic associations involving *Epichloë* and *Neotyphodium* endophytes occur in many grass genera, including *Festuca*, *Lolium*, and *Poa* (Schardl & Phillips 1997; Leyronas & Raynal 2001). Of particular interest for New Zealand indigenous grasses is a recent survey in China that identified endophytic associations in 25 species of grasses including *Elymus* (100% infection), low levels of infection in three *Poa* species (2–6% infection), and a species of *Deschampsia* (2% infection) (Nan & Li 2001). Also, an endophyte, *Neotyphodium tembladerae*, has been identified in several South American *Festuca* and *Poa* species (Cabral et al. 1999). In New Zealand, endophytic associations are commonly found in several introduced pasture and turf species (Hume 1993; Fletcher et al. 2001), with *Neotyphodium lolii* in perennial ryegrass (*Lolium perenne*), *Neotyphodium coenophialum* in tall fescue (*Schedonorus phoenix* syn. *Festuca arundinacea*), and *N. uncinatum* in meadow fescue (*S. pratensis* syn. *F. pratensis*). As a result of their importance in agricultural situations, the endophyte associations in ryegrass and tall fescue have received considerable attention in New Zealand (see review by Easton et al. 2001). Similarly, roadside collections of perennial ryegrass and tall fescue typically have a high % infection with endophyte (G. C. M. Latch & A. V. Stewart unpubl. data).

In contrast, there is little information on the occurrence of endophyte associations in other introduced grasses that have naturalised in New Zealand and in grasses that are indigenous to New Zealand. To date, an *Epichloë* endophyte has been found in *Festuca glauca* (Hedley & Braithwaite 1978), and an endophyte that is phylogenetically related to *Epichloë* and *Neotyphodium* species, with the proposed name *N. aotearoae* (Moon et al. 2002), has been reported in *Echinopogon ovatus* (hedgehog grass) (Miles et al. 1998). The New Zealand-based research on the transmission of endophyte into seed of *Danthonia spicata* reported by Philipson & Christey (1985) used seed of Canadian origin. We could find no published studies on fungal endophytes in the tribe *Danthonieae* in New Zealand.

Previous seed storage studies on endophyte viability in perennial ryegrass (Rolston et al. 1986, 1993; Hare et al. 1990) and tall fescue (Welty et al. 1987) have shown that storage temperature, and seed moisture content which is in equilibrium with relative humidity, are critical. Endophyte viability is more susceptible than seed viability to the effects of higher temperatures and higher seed moisture contents. This has important implications for the conservation as seed of any New Zealand indigenous or naturalised grasses that harbour endophytes.

The purpose of this study was to examine a wide range of New Zealand grasses to determine the extent of endophytic associations and to assess the implications of endophytic associations for germplasm storage as grass seed.

MATERIALS AND METHODS

Collection details

In the summer of 1999/2000, seeds of grass species were collected in New Zealand mainly from sites in the South Island, with a few in the North Island. Each species was identified in the field or by collecting seedheads for later identification. A further 14 seed samples were obtained from private New Zealand or international collections. The exact ages of these samples were generally unknown, but were most probably between 6 months and 3 years. In most cases the number of plants harvested for seed at each site was also recorded. Of the 24 native grass species collected (Table 1), 19 are endemic, while the 3 introduced grass species collected (*Festuca ovina*, *F. rubra*, *Elymus recticetus*) (Table 2), are naturalised in New Zealand. Nomenclature follows Edgar & Connor (2000).

Detection of *Epichloë* or *Neotyphodium* endophytes

Twenty seeds from each collection were sown in trays of potting mix in a green house. At the 3–4 tiller stage, presence of *Epichloë* or *Neotyphodium* endophyte was determined for one tiller per plant using a modification of the immunoblot technique of Gwinn et al. (1991) where a goat anti-rabbit conjugate was substituted for the protein-A conjugate. In total, 692 plants were examined, with an average of approximately 9 seedlings (range 2–20) per collection for the native grasses and 12 seedlings (range 1–20) per collection for the 3 naturalised species. As the antisera may not have reacted to the endophytes in these grasses, one tiller per plant for up to three plants per collection was microscopically examined for the presence of endophyte. This was done by stripping the epidermis from the adaxial surface of

Table 1 Native grass collection details. Seed status: "fresh" was seed collected in January–March 2000; "unknown" was where the exact seed collection time was unknown but seed was most probably 6 months to 3 years old.

Species	Region	Collection site	Seed status	Number of plants sampled
Anemanthele lessoniana	Christchurch	garden origin	fresh	10
Austrofestuca littoralis	Canterbury	Banks Peninsula	fresh	2
Deschampsia chapmanii	Canterbury	Lake Lyndon	fresh	20
Dichelachne crinata	Canterbury	Fernhurst	fresh	20
	Canterbury	Lake Pukaki	fresh	3
	Canterbury	Port Hills	fresh	25
	Canterbury	Rangitata Gorge	fresh	20
	Otago	Lake Hawea	fresh	10
	Otago	Lindis Pass	fresh	8
	Otago	Ohau	fresh	5
	Westland	Coast Road	fresh	2
	Westland	Coast Road	fresh	2
Elymus apricus	Otago	Lindis Pass	fresh	1
	Otago	Lindis Pass	fresh	1
Elymus enysii	Canterbury	Mt Cheeseman	unknown	unknown
	Canterbury	Porters Pass	unknown	unknown
Elymus falcis	Canterbury	Castle Hill	fresh	5
	Canterbury	Tekapo	fresh	5
Elymus multiflorus	Marlborough	Deadmans Creek	fresh	1
	Canterbury	Governors Bay	fresh	25
	Canterbury	Long Bay	fresh	25
Elymus solandri	Canterbury	Cave Stream	fresh	10
	Canterbury	Glenrock	fresh	5
	Canterbury	Long Bay	fresh	10
	Canterbury	Rangitata Gorge	fresh	15
	Canterbury	Lake Pukaki	fresh	10
	Otago	Lindis River	fresh	10
	Otago	Lake Hawea	fresh	10
	Otago	Ohau	fresh	10
	Wellington	Point Gordon	fresh	1
Elymus tenuis	Canterbury	Lake Coleridege	unknown	unknown
Festuca actae	Canterbury	garden origin	unknown	unknown
	Canterbury	Godley Head, Banks Peninsula, high altitude	fresh	25
	Canterbury	Godley Head, Banks Peninsula, medium altitude	fresh	100
	Canterbury	Godley Head, Banks Peninsula, sea level	fresh	5
	Canterbury	Mt Cavendish	fresh	25
Festuca novae-zealandiae	Canterbury	Lincoln, garden origin	fresh	5
	Canterbury	Porters Pass	unknown	5
Hierochloe redolens	Canterbury	Long Bay	fresh	25
	Canterbury	Mt Cavendish	fresh	5
Koeleria sp.	Canterbury	Lake Tekapo	fresh	5
Lachnagrostis lyallii	Canterbury	Lake Tekapo	fresh	100
	Otago	Ohau	fresh	100
Poa anceps	Westland	Westland	fresh	1
	Taranaki	New Plymouth	fresh	3
Poa buchananii	Canterbury	Porters Pass	unknown	unknown
Poa cita	Canterbury	garden origin	unknown	unknown
	Canterbury	Long Bay	fresh	1
	Canterbury	Porters Pass	fresh	5

Continued over page

Table 1 *Continued*

Species	Region	Collection site	Seed status	Number of plants sampled
Poa colensoi	Westland	Arthur's Pass	fresh	5
	Canterbury	Cass	fresh	25
	Canterbury	Glenrock	fresh	10
	Canterbury	McKenzie Pass	fresh	20
	Canterbury	Tekapo	fresh	1000
	Canterbury	Porters Pass	unknown	unknown
	Otago	Lake Hawea	fresh	10
	Otago	Lindis Pass	fresh	20
	Otago	Lindis River	fresh	20
	Otago	Ohau	fresh	100
Poa imbecilla	Westland	Westland	fresh	3
Poa lindsayi	Canterbury	Porters Pass	unknown	unknown
Poa matthewsii	Otago	Ohau	fresh	5
Puccinellia sp.	Canterbury	Sumner, estuary	fresh	1
Stenostachys gracilis	Otago	Ohau	fresh	1

the leaf sheath and mounting the tissue in aniline blue (1:2:1 v/v lactic acid, glycerol, water, 0.15% aniline blue) on a microscope slide for examination at a magnification of 100×. If *Epichloë* or *Neotyphodium* endophytic mycelium was present it appeared as intercellular, sparsely branched hyphae. In all cases, results from the microscopic examination matched those of the immunoblot.

No seed germinated for 13 collections and, in these cases, an examination of three seeds per collection was undertaken for the presence of endophyte. This required the seeds to be soaked overnight in 5% sodium hydroxide and washed in water. The seed coat was removed and the seed was squashed on a microscope slide, stained with aniline blue, and examined under a microscope.

Seed storage trial

The seed storage trial was initiated in 1984 using perennial ryegrass (cv. Grasslands Nui) infected with wild-type endophyte (Rolston et al. 1986). Seeds were dried to four moisture contents (8.6, 10.0, 12.1, 13.8%), and packaged into three bag types (permeable cloth bags, heat sealed semi-permeable polyethylene of different thickness (9 to 70 μm), and water impermeable aluminium-polyethylene laminated bags). The seed samples were stored in four environments, ambient (10–20°C), 5°C refrigeration, 3°C/30% relative humidity, and –15°C. Seeds were removed at regular intervals over a period of 15 years and tested for viable endophyte presence in 50 germinated seedlings by either immunoblot or light microscope as outlined above.

RESULTS

Occurrence of endophyte

No endophytic associations were found in the 24 native grass species examined (Table 1). No endophytic associations were found for the 17 collections of naturalised *Elymus recticetus* (Table 2), while endophyte was found in the 2 naturalised species of *Festuca* (Table 2). For the one *F. ovina* sample, one seedling was endophyte-infected out of the three seedlings examined. Of the 19 *F. rubra* collections, 4 had 82–87% infection and 1 collection at Simons Pass had 25% infection (Table 2).

Seed storage trial

The long-term seed storage trial of perennial ryegrass seed infected with wild-type endophyte demonstrates the importance of storage temperature and packaging. Seed in heat-sealed vapour/moisture-proof aluminium-polyethylene laminated bags maintained the highest viable endophyte levels after 15 years of storage (Table 3). Seed stored at either –15 or 0°C maintained viable endophyte for 15 years, except in cotton bags at –15°C due to the very high humidity of the freezer. Viable endophyte declined with increasing seed moisture content (data not shown).

Table 2 Collection details for introduced *Festuca* species and introduced *Elymus recticetus*. Seed status: "fresh" was seed collected in January–March 2000; "unknown" was where the exact seed collection time was unknown but seed was most probably 6 months to 3 years old. * shows seed collections of *Festuca* that were infected with endophyte.

Species	Region	Collection site	Seed status	Number of plants sampled
Festuca ovina	Otago	Ohau	fresh	1*
Festuca rubra	Canterbury	Bealey River	fresh	25
	Canterbury	Bourkes Pass	fresh	25
	Canterbury	Cass	fresh	25
	Canterbury	Church Bay	fresh	5*
	Canterbury	Gebbes Pass	fresh	5
	Canterbury	Lake Pukaki	fresh	50*
	Canterbury	Lake Tekapo	fresh	100
	Canterbury	McKenzie Pass	fresh	25
	Canterbury	Mt Peel	fresh	1
	Canterbury	Simons Pass	fresh	10*
	Otago	Glendhu Bay	fresh	10
	Otago	Lindis Pass	fresh	10*
	Otago	Lindis Pass	fresh	10*
	Otago	Lindis River	fresh	10
	Otago	Ohau	fresh	100
	Otago	Ohau	fresh	20
	Otago	Ohau, flat paddock	fresh	25
	Otago	Tarras-Wanaka	fresh	25
	Westland	Deaths Corner	fresh	2
Elymus rectisetus	Canterbury	Allandale	fresh	25
	Canterbury	Church Bay	fresh	10
	Canterbury	Darfield	unknown	unknown
	Canterbury	Fernhurst	fresh	25
	Canterbury	Gebbes Pass	fresh	10
	Canterbury	Glenrock	fresh	5
	Canterbury	Governors Bay	fresh	5
	Canterbury	Halswell	fresh	10
	Canterbury	Kirwee	unknown	unknown
	Canterbury	Lake Lyndon	unknown	unknown
	Canterbury	Porters Pass	unknown	unknown
	Canterbury	Purau	fresh	10
	Canterbury	Sumner	fresh	25
	Canterbury	Taitapu	unknown	unknown
	Otago	Ohau	fresh	10
	Otago	Tarras	fresh	25
	Marlborough	Waipapa	fresh	100

Table 3 Percent viable endophyte in perennial ryegrass after 15 years storage at three temperatures using three packaging materials.

	Storage temperature		
Packaging material	5°C	0°C	–15°C
Cotton	2	78	8
Aluminium laminate	76	74	82
Plastic 70 µm	6	80	58

DISCUSSION

This first New Zealand survey was unable to detect endophytes in the native grasses examined, which suggests that if endophytes are present they occur at low frequencies. Additional collections are needed to determine whether endophytes occur in other native grass species or in the surveyed species collected from other regions within the country. In particular, *Festuca novae-zealandiae* was not well represented in the survey with one garden sample and one old seed sample, while species of *Rytidosperma* were not collected. The only New Zealand species currently known to contain an endophyte is *Echinopogon ovatus* (Miles et al. 1998).

These results are interesting from an evolutionary viewpoint when we consider that many species of the same genera in the Northern Hemisphere and South America contain endophyte. One might postulate that endophyte-mediated protection from herbivore pressure (vertebrate and invertebrate) was not a decisive evolutionary factor in New Zealand. Another scenario is that colonisation of grasses by this group of endophytic fungi occurred more recently than did the isolation of New Zealand biota from continental populations, as it appears that these endophytes evolved in the Northern Hemisphere and later spread into the Southern Hemisphere (see review by White et al. 2001; Moon et al. 2002). However, new evidence from Moon et al. (2002) indicates that there are some distinct endophytes that have diverged relatively early in the evolution of grass endophytes that are unique to the Southern Hemisphere, typified by *N. aotearoae* infecting *Echinopogon ovatus* in New Zealand and Australia. These authors discuss transoceanic endophyte geneflow.

Similarly, a survey in Australia of 13 grass genera, 20 grass species, showed no endophytes of the *Epichloë/Neotyphodium* complex (Aldous et al. 1999). This Australian study had the grass genera *Elymus* and *Poa* in common with our study, but none of the same species. Notably, *Echinopogon ovatus*, and other species of this genus, are the only native grass species in Australia that are endophyte-infected (Miles et al. 1998). *Echinopogon* species are endemic to New Zealand and Australia. Again, it may be postulated that the same evolutionary processes or timing of events may have resulted in Australia having few native grasses that are infected with *Epichloë/Neotyphodium* endophytes.

The survey results for the three introduced grasses are in keeping with other studies. *Elymus recticetus*, which had no endophyte present in any of the 17 collections, is naturalised from Australia, where Aldous et al. (1999) also reported no endophyte in *Elymus scaber*, or any of the other 51 native Australian grasses surveyed. Results for Northern Hemisphere surveys of *Festuca rubra* and *F. ovina* show a wide range of endophyte infection. In Spain, Zabalgogeazcoa et al. (1999) reported all 27 populations of *F. rubra* to be endophyte-infected. Saikkonen et al. (2000) reported that approximately 30% of the populations of *F. ovina* and *F. rubra* were endophyte-infected in Finland, which is similar to the 30% infection in our survey. As discussed by White et al. (1993) for *Festuca arizonica*, endophyte infection can vary markedly between populations.

Many of New Zealand's native grass species have a vastly reduced habitat today because of agricultural development and the introduction of grazing mammals. Many are now quite difficult to find even though they may not be threatened, and collecting seed at the appropriate time is even more difficult. There is a risk that some indigenous species such as *Deschampsia cespitosa* may become contaminated from introduced germplasm if the newly developed turf cultivars of this species are imported from Europe and North America. Seed collections can be an aid to germplasm conservation, complementing New Zealand's in situ conservation effort (Anon. 2000).

In the conservation of grasses by seed there is a need to be aware of the importance of storage conditions in maintaining grass germplasm with endophyte. The lesson learnt in New Zealand with pasture species is that endophyte viability is easily lost when seed operators are unaware of the special needs required to maintain endophyte. Apart from the aim of conserving the endophytes themselves, maintenance of viable endophyte in the seed is important as it relates to the effect endophytes may have on improving plant persistence primarily through alkaloids. This has been well documented in New Zealand regarding species used in agriculture (Easton et al. 2001), and is also likely to apply to native grasses. For example, Miles et al. (1998) found that endophyte-free plants of *E. ovatus* were more palatable to the pasture pest *Listronotus bonariensis* (Argentine stem weevil) than endophyte-infected plants. This related closely to two alkaloids produced by the association, *N*-formylloline (a known insect deterrent) and analogs of the indole-diterpeniod paxilline (known to be toxic to vertebrate herbivores).

To enhance endophyte viability (and seed viability) for long-term storage, endophytic seeds should be stored at 5°C (or colder), with seed moisture content < 8% (Hare et al. 1990). Seed should be packaged in aluminium, laminated bags when using conventional freezing (–15°C), while cloth bags are suitable in a controlled low temperature (0°C), low relative humidity (30%) environment.

The collection described in this paper represents an addition to a very small collection (now 29 of our 187 species) of native grass seed held in the Margot

Forde Germplasm Centre, Palmerston North, and will be a valuable source of material for studies on live plants of New Zealand native grasses and their endophytes. Further collection expeditions throughout remote areas of New Zealand will be required to extend this collection.

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