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**An Enhanced Endophyte for Grass Systems**

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Abstract

The invention relates to a new endophyte strain that produces epoxy-janthitrems with improved stability and compatibility with respect to higher endophyte infection frequency over known commercial epoxy-janthitrem producing strains. The endophyte strain is grown in association with a variety of grasses. These include but are not limited to perennial, Italian, annual and hybrid ryegrasses and festulolium hybrids. The endophyte strain has a broader intra specific interspecific and inter generic compatibility and stability over known strains. The invention also provides a method of increasing the resistance of a host grass to biotic and/or abiotic stress/es by inoculating the grass with an endophyte strain according to the invention and a host grass which, confers improved resistance to biotic and abiotic stresses. Such stresses may include insect and pest damages and heat and water stress.

## Title

An Enhanced Novel Endophyte for Grass Systems Improvement

## 1. Field of the Invention

The invention relates to a novel, epoxy-janthitrem producing *Epichloë* fungal endophyte with improved properties. More specifically the invention relates to an endophyte with: i) improved fungicide resistance, ii) broad intra-specific, inter-specific and inter-generic compatibility and stability iii) which, in combination with a variety of grasses acts as a bio-protection agent against biotic stresses such as insect pests, and abiotic stresses, while minimizing livestock toxicity due to its alkaloid profile. More specifically the endophyte strain is defined as E142.

## 2. Background of the Invention

New Zealand's livestock farming system commonly relies on a pasture mixture of perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*). Some key factors such as dry matter production, nutritive value and persistence, affect the economic value of the pasture to the farmer. Environmental pressures such as heat, moisture and insect predation are major elements affecting pasture persistence and production. The presence of an asexual fungal endophyte from the genus *Epichloë* is an important factor for pasture persistence in New Zealand (Popay and Hume 2011) and it is valued as a key component to improving persistence reliability and the economic value of pasture-based systems.

Fungal endophytes of the genus *Epichloë*, infect and form mutualistic associations with many temperate climate *Pooideae* grasses. The mutualistic associations result in the production of alkaloids by the endophyte which in turn are considered to play a role in conferring pest, disease, and drought resistance (Rowan and Gaynor 1986, Bush et al. 1997, Malinowski et al. 1997) to the host grass. Some *Epichloë* endophytes transmit horizontally via sporulation outside of the host. However, all of the *Epichloë* endophytes that are considered beneficial to modern agriculture are exclusively vertically transmitted via the endophyte colonizing the seed of the grass host (Bush et al. 1997).

Many of the common or 'wild type' endophyte infections of improved grass cultivars used for pastoral agriculture production can cause significant animal health implications, such as ryegrass staggers and fescue toxicosis (Bacon et al. 1977, Fletcher and Harvey 1981). These may be complex toxic reactions by grazing livestock to particular alkaloids produced by the endophyte under different environmental conditions. Significant economic losses within modern agricultural systems can occur due to these animal toxicosis (Woodfield and Easton 2004). However, the presence of endophyte may be essential for the persistence of a chosen grass in agricultural and horticultural systems.

Novel endophytes act as bio-protection agents which can reduce the use of the pesticides. The worldwide trend toward environmentally friendly modes of production, is enhanced by reductions in pesticide use and the banning of others is facilitated by these technologies. Organic and conventional farming systems can embrace these improvements and provide a higher quality end product without resorting to contentious technologies.

Improved grass breeding lines can be artificially infected with selected endophytes (Latch and Christensen 1985). Endophytes can be discovered, screened for desirable characteristics relating to a particular end use, isolated to axenic culture outside of the host, and inserted into a new host that has been improved via plant breeding. Endophytes selected and used in such a way are often referred to as 'novel' endophytes.

The rate at which novel endophytes can be successfully inoculated and stabilised in grass breeding lines varies, both intra-specifically and inter-specifically (Easton et al. 2009, Kaur et al. 2016). Novel endophytes which have high artificial inoculation rates and vertically transmit at high percentages once

in the host, can be brought to the commercial market more efficiently than endophytes that lack these characteristics.
Novel endophytes in association with different grass hosts, vary in their susceptibility to fungicides used during seed production (Chynoweth et al. 2012, US Patent 8,465,963). Novel endophyte infected breeding lines which have enhanced resistance to fungicide application, can be increased more efficiently and the endophyte infection percentage maintained more easily during production than for breeding lines hosting susceptible novel endophytes.

This technology has been exploited over the past 25 years to include the following but not limited to: AR1 (US Patent 6,072,107), MaxP (NZ patents 541,606 and 567,558), AR37 (NZ Patent 543,849), Avanex (NZ Patent 579,801), NEA2 (NZ PVR Grant 2,714), and U2 (NZ PVR Grant 2,716) endophytes. These endophytes have been successfully inserted into a range of grass hosts and have delivered considerable economic benefits to New Zealand and internationally.

## 3. Object of the Invention

It is an objective of the present invention to provide an endophyte that goes some way to overcome or mitigate the disadvantages of known endophytes, or to at least provide an alternative.

## 4. Summary of the Invention

The invention provides an endophyte strain producing epoxy-janthitrems with improved stability and compatibility with respect to higher endophyte infection frequency over known commercial epoxy-janthitrem producing strains when grown in association with a variety of grasses such as, but not limited to, perennial, Italian, annual and hybrid ryegrasses, and festulolium hybrids.

The invention also provides an endophyte strain producing epoxy-janthitrems, with a broader intra-specific, inter-specific and inter-generic compatibility and stability over known commercial epoxy-janthitrem producing strains when grown in combination with a variety of grasses such as, but not limited to, perennial, Italian, annual and hybrid ryegrasses, festulolium hybrids and fescue species.

Preferably the endophyte strain provides improved inoculation ability when inoculated into a variety of grasses such as, but not limited to, perennial, Italian, annual and hybrid ryegrasses, and festulolium hybrids.

The endophyte strain preferably comprises improved vegetative stability over known commercial epoxy-janthitrem producing strains when grown in combination with a variety of grasses such as, but not limited to, perennial, Italian, annual and hybrid ryegrasses, and festulolium hybrids.

The endophyte strain preferably shows improved vertical transmission rates over known commercial epoxy-janthitrem producing strains when grown in combination with a variety of grasses such as, but not limited to, perennial, Italian, annual and hybrid ryegrasses, and festulolium hybrids.

The endophyte strain producing epoxy-janthitrems preferably comprises improved fungicide resistance with respect to higher endophyte infection frequency over known commercial epoxy-janthitrem producing endophyte strains.

The endophyte strain producing epoxy-janthitrems may provide improved fungicide resistance over known commercial epoxy-janthitrem producing endophyte strains and preferably the fungicide is from the triazole group.

The endophyte strain producing epoxy-janthitrems may provide improved fungicide resistance over known commercial epoxy-janthitrem producing endophyte strains and preferably the fungicide is from the strobilurin group.

Preferably the fungicide is from the strobilurin group in combination with fungicide from the triazole group.

There is provided an endophyte strain which does not produce detectable levels of loline, ergovaline, peramine and lolitrem B alkaloids, but which does produce varied levels of epoxy-janthitrem compounds in the host grass.

The invention also provides a method of increasing the resistance of a host grass to biotic and /or abiotic stress/es by inoculating the grass with an endophyte strain which produces varied levels of epoxy-janthitrem compounds in the host grass due to the presence of an endophyte.

The invention also provides a host grass produced according to the method above in which its association with the endophyte confers improved resistance to biotic and abiotic stresses such as but not limited to insect and pest damages and heat and water stress.

The endophyte preferably comprises a B10 allele size of approximately 162.1bp-162.7bp, a B11 allele size of approximately 126.8bp to 127.1bp.

The endophyte preferably comprises a NCESTA1CC05, NCESTA1QA09 allele size of approximately 143.6bp and 173.6bp respectively.

The endophyte preferably comprises an ANS054 and ANS056 allele size of approximately 275.4bp and 240.0bp respectively.

The endophyte preferably comprises an ANS033 allele sizes of approximately 134.4bp and 143.9bp.

The endophyte preferably comprises an EF-22 allele sizes of approximately of 130.0bp and 139.4bp.

The endophyte preferably comprises the presence of the genes identified as EfM3.04570, EfM3.044580, EfM3.050800, EfM3.062040.

The endophyte preferably comprises the absence of the genes identified as EfM3.005360, EfM3.062860.

The endophyte is preferably termed E142 (*Epichloë festucae var. lolii*).

The endophyte E142 is found in the *Lolium perenne* accession coded Lp12059 which is accession A22356 in the Margot Forde germplasm center database in Palmerston North, New Zealand.

The endophyte E142, was deposited on 31/08/2017 at The National Measurements Institute of Australia, accession number VQ17/00179.

The endophyte according to the described invention is preferably in the form of an endophyte culture.

It is acknowledged that the terms "comprise", "comprises" and "comprising" may, under varying jurisdictions, be attributed with either an exclusive or an inclusive meaning. For the purpose of this specification, and unless otherwise noted, these terms are intended to have an inclusive meaning i.e. they will be taken to mean an inclusion of not only the listed components which the use directly references, but also to other non-specified components or elements.

The invention is described below with reference to examples. The examples are only preferred embodiments of one or more ways that the invention can be carried out and should not be read as limiting the scope of the claimed invention.

## 5. Brief Description of the Drawings

Figures 1-4 are transmission electron micrographs of Lolium perenne pseudo-stem cross-section infected with E142. Hyphae (marked with asterisks) growing within the intercellular spaces between plant cells (PC).

## 6. Detailed Description of the Invention

### 6.1 Origin

E142 was found in the *Lolium perenne* accession coded Lp12059. Lp12059 refers to the accession A22356 in the Margot Forde germplasm center database which was requested by Cropmark Seeds. A22356 results from a seed increase of the accession A17882 which is the original germplasm collected in Greece in 2012. A17882 has been collected by an expedition organized by the Margot Forde Germplasm Centre and the Greek gene bank.

The GPS coordinates linked to the collection site of A17882 are presented in the Table 1.

**Table 1**: Geographical location data of where the original Lp12059 germplasm was collected

| | |
|---|---|
| Latitude | 38.008920000000003 |
| Longitude | 22.152180000000001 |
| Altitude | 1225 m |
| Map link | https://www.google.com/maps/@38.0082065,22.1515717,495m/data=!3m1!1e3 |

E142 originates from Greece, a comparison between the country of origin of the most common commercialised endophytes which have NZ PVR grants is shown in Table 2.

**Table 2:** Geographical origin (country where discovered) of some novel endophytes

| **Endophyte** | **Geographical origin** |
|---|---|
| E142 | Greece |
| AR1 | Italy |
| AR37 | Portugal |
| NEA2 | Spain |
| NEA3 | Romania |
| NEA10 | Spain |
| NEA11 | France |
| U2 | Norway |
| AR542 (Max P) | Morocco |

### 6.2 Discovery

Seeds of Lp12059 were initially screened for endophyte presence using SSR makers. These DNA markers showed that Lp12059 was hosting two endophyte strains both with a distinct DNA profile. Seedlings of Lp12059 were established into root-trainers and blotted to discard non-infected plants. All the remaining plants infected with endophyte were individually tested using the same SSR primers. Plants hosting E142 were isolated from the other plants and used for the endophyte isolation and alkaloid profiling.

E142 was isolated from Lp12059 via a technique modified from Christensen et al 1991. Pseudo stem segments (3-4mm) were sterilized by rinsing them in 80% ethanol for 10 seconds, 20% bleach for five minutes, and distilled water for one minute. The segments were then submerged in potato dextrose agar (PDA) and placed in an incubator at 20°C. Mycelia that emerged from the segments within six weeks were then transferred to a new PDA plate.

### 6.3 Morphology

Colonies of E142 grown on potato dextrose agar at 20°C display a slow rate of growth. Colonies are moderately convoluted in shape with the colony margin not visibly submerged in the media. Colonies are tan in color on top with a pale brown underside. The aerial mycelia of colonies have a waxy appearance and formation of conidia/sporulation has not been observed. The morphology of E142 is distinctly different to AR37 and all other *Epichloë festucae var. lolii* endophytes currently granted a plant variety right (PVR) in New Zealand. E142 is currently under examination to be granted PVR in New Zealand.

Despite these differences in axenic culture morphology, the hyphal morphology of E142 *in planta*, as determined by transmission electron microscopy, was indistinguishable from AR37. Methodology for the transmission electron microscopy is described in Green et al 2016. As shown in photos one to four, one to two hyphae were observed per intercellular space for samples taken from the pseudo-stem of *Lolium perenne* infected with either E142 or AR37. No hyphae were observed within the vascular bundles, which is a defining feature of mutualistic symbiotic associations.

### 6.4 Genetic Identification of E142: DNA polymorphism using microsatellite analysis

E142 can be distinguished from other *Epichloë* endophytes by the characterisation of alleles at several genetic loci. The distinguishing characteristics are the number and size of distinct microsatellite sequences amplified by the polymerase chain reaction (PCR) technique using selected amplification primers.

Nine sets of PCR primer pairs, one of each pair labelled with a fluorescent dye at the 5'- end for detection purposes, have been selected to define the microsatellite loci of E142. The fluorescent dye labels were 6-FAMTM, NEDTM, VIC® (all Life Technologies). The primer names, sequences, and 5'-end modifications are listed in Table 3.

DNA was extracted from endophyte cultures grown on PDA using the Macherey-Nagel NucleoSpin® Plant II DNA extraction kit.

Five different PCR programs were used to characterise the microsatellite sequences, the program relating to each primer pair is listed in Table 3. The programs were as follows:

1. 94°C for 5 min, 31 cycles of (94°C for 1 min, 65°C for 2 min, 72°C for 1 min), 72°C for 10 min, samples were then held at 4°C until analysis.

2. 95°C for 10 min, 10 cycles of (94°C for 30s, 65°C (-1°C per cycle) for 30s, 72°C for 1 min), 20 cycles of (94°C for 30s, 55°C for 30s, 72°C for 1 min), samples were then held at 4°C until analysis.

3. 95°C for 10 min, 10 cycles of (94°C for 30s, 55°C (-1°C per cycle) for 30s, 72°C for 1 min), 20 cycles of (94°C for 30s, 45°C for 30s, 72°C for 1 min), samples were then held at 4°C until analysis.

4. 94°C for 4 min, 30 cycles of (94°C for 30s, 55°C for 30s,72°C for 30s), 8 cycles of (94°C for 30s, 53°C for 30s, 72°C for 30s), 72°C for 30 min, samples were then held at 4°C until analysis.

5. 95°C for 2 mins, 30 cycles of (95°C for 30s, 57°C for 30s, 73°C for 30s), 73°C for 5 min, samples were then held at 4°C until analysis.

**Table 3:** Primer names, sequences, modifications, and PCR programs

| Primer Name | Reference | Primer Sequence 5'-3' | 5'-end modification | PCR program |
|---|---|---|---|---|
| B10.1-Forward(F) | Moon et al. 1999 | CGCTCAGGGCTACATACACCATGG | NED | 1 |
| B10.2-Reverse(R) | Moon et al. 1999 | CTCATCGAGTAACGCAGGCGACG | | 1 |
| B11.1-F | Moon et al. 1999 | CATGGATGGACAAGAGATTGCACG | 6-FAM | 1 |
| B11.4-R | Moon et al. 1999 | TTCACTGCTACAATTCTGTCCAGC | | 1 |
| NCESTA1CC05-F | van Zijll de Jong et al. 2003 | CGCATACACGTTATGAAGCAGAGG | NED | 2 |
| NCESTA1CC05-R | van Zijll de Jong et al. 2003 | TTGGGACTTTCCAGAGTTGAGCAG | | 2 |
| NCESTA1NG03-F | van Zijll de Jong et al. 2003 | CGGGCGCACTTGCTTCTCGG | VIC | 2 |
| NCESTA1NG03-R | van Zijll de Jong et al. 2003 | GCCCCGCAGCCTTGTCGTTG | | 2 |
| NCESTA1QA09-F | van Zijll de Jong et al. 2003 | TGGATATTTTGAAGAAGTTCCAGG | 6-FAM | 3 |
| NCESTA1QA09-R | van Zijll de Jong et al. 2003 | CTAACGATGTATGCGTTTGTTTGG | | 3 |
| ANS033-F | Kirkby et al. 2011 | GCGTTGAGGAGGCTAGATAGAA | 6-FAM | 4 |
| ANS033-R | Kirkby et al. 2011 | TTCCAAGCTGAACAAAAGTCAA | | 4 |
| ANS054-F | Kirkby et al. 2011 | CTTGTGATGAGAATCGTCAAGC | 6-FAM | 4 |
| ANS054-R | Kirkby et al. 2011 | CCCTACCGAAACTACATCGTTC | | 4 |
| ANS056-F | Kirkby et al. 2011 | AGACCATCATGTGAGCGACTT | 6-FAM | 4 |
| ANS056-R | Kirkby et al. 2011 | GTGATGCCCTCTATGTGTATGC | | 4 |
| EF22-F | Von Cräutlein et al. 2014 | GCAATCCCAAAACATGACGC | NED | 5 |
| EF22-R | Von Cräutlein et al. 2014 | GCAAAACATGTGAAACGGCC | | 5 |

PCR products were analysed using an ABI 3130xl genetic analyser (Applied Biosystems®) and the product length measured using Peak ScannerTM software. Allele sizes for E142, AR1, AR37, and wild type *E. festucae var. lolii* (WT) are listed in Table 4.

**Table 4:** Summary of microsatellite data distinguishing E142 endophyte from other *E. festucae var. lolii endophytes.*

| Endophyte Allele size/Microsatellite locus | E142 | AR37 | AR1 | WT |
|---|---|---|---|---|
| B10 | 162.4 | 162.3 | 177.0 | 176.8 |
| B11 | 126.9 | 130.9 | 148.0 | 176.1 |
| NCESTA1CC05 | 143.6 | 140.1 | 218.5 | 165.8 |
| NCESTA1NG03 | 218.1 | 218.1 | 227.0 | 226.9 |
| NCESTA1QA09 | 173.6 | 188.2 | 190.1 | 148.7 |
| ANS033 | 134.4, 143.9 | 137.8, 147.3 | 147.5, 157.2 | 147.3, 157.2 |
| ANS054 | 275.4 | 272.5 | 260.9 | 260.8 |
| ANS056 | 240.0 | 239.0 | 227.1, 238.8 | 226.8, 238.8 |
| EF22 | 130.0, 139.4 | 154.3, 164.0 | 131.0, 139.4 | 141.8, 150.9 |

Note: Caution should be applied in comparing SSR results across-methods. Whilst there is a high level of consistency within a method, a small level of variation for given base pair values will exist between methods due to factors such as the reagents and equipment used.

### 6.5 Genetic analysis of E142: Whole genome sequencing

Whole genome sequencing was carried out on E142 and AR37 strains. Genome assemblies were performed for these two strains alongside the reference genome Fl1.

A comparative genomic analysis of the two genomes confirmed all the SSR distinctions presented above and enabled further comparison of other regions with key genes of interest such as secondary metabolite genes and other genes across the entire genomes.

#### 6.5.1 Material and methods

a. Preparation of genomic DNA

Cultures of AR37 and E142 were sub-cultured onto PDA medium then further sub-cultured by grinding blocks of mycelium and placing on cellophane discs on PDA media. Mycelium was harvested after 10 days' growth, washed and freeze dried. Genomic DNA was isolated using a modified protocol of Byrd et al. (1990) and shown to be of high quality and high molecular weight.

Both genomic DNA samples met the quality specifications required by New Zealand Genomics Limited (NZGL) for sequencing as outlined in their Bioanalyzer report.

b. Assembly software testing

Test genome assemblies were produced using ABySS v1.3.6 (Simpson et al., 2009), IDBA-UD v1.1.3 (Peng et al., 2012), MaSuRCA v3.2.1 (Zimin et al., 2013) and SPAdes v3.10.0 (Bankevich et al., 2012). The quality of these assemblies was compared using N50 (a measure of genome contiguity), the total number of bases in contigs >1 kb, and the proportion of gene models from a closely related species (the reference strain *E. festucae,* E2368) that could be aligned to each assembly. SPAdes performed well across all categories, so this assembler was used for further analyses.

c. Data

NZGL provided 250 bp paired end reads as FastQ files for AR37 (14,099,403 read pairs) and E142 (17,882,552 read pairs). Initially assuming a genome size of 35 Mb, this equates to 201-fold coverage for AR37 and 255-fold coverage for E142.

d. Data cleaning and assembly

Sequencing adapters were removed using the 'fastq-mcf' function in the ea-utils suite (v1.1.2−621; https://expressionanalysis.github.io/ea-utils/). Reads were dynamically trimmed to obtain the longest sub-sequence for which each base had a probability of error $P \leqslant 0.05$ using the SolexaQA++ package v3.1.4 (Cox et al., 2010). Paired end reads <150 nucleotides were discarded. Following trimming, AR37 had an estimated 121-fold coverage and E142 had 135-fold coverage, again initially assuming a genome size of 35 Mb. These paired end reads were assembled with SPAdes v3.10.0 (Bankevich et al., 2012) using a k-mer sweep from 37−127 in 10-unit steps, with subsequent correction of mismatches and short indels (i.e., using the –careful flag). The final scaffold files for these AR37 and E142 assemblies are defined as the v1.0 reference genomes.

e. Sequence analysis

Homologues of *E. festucae* Fl1 genes were identified in the genome assemblies of E142 and AR37 using MegaBlast within Geneious (v7.1.9).

### 6.5.2 Analysis of ltm and eas cluster genes and perA regions in Fl1, E142 and AR37

Ltm and per genes but not eas genes were identified in the genomes of E142 and AR37 using *E. festucae* Fl1 sequences deposited in GenBank as search baits (Table 5).

**Table 5:** Presence and absence of the ltm and eas cluster genes and perA in E142 and AR37

| Gene Group | Gene | GenBank Accession No | Fl1 | E142 | AR37 |
|---|---|---|---|---|---|
| ltm cluster | *ltmG* | JN613320.1 | + | + | + |
| | *ltmS* | JN613320.1 | + | + | + |
| | *ltmM* | JN613320.1 | + | + | + |
| | *ltmK* | JN613320.1 | + | + | + |
| | *ltmP* | JN613319.1 | + | + | + |
| | *ltmQ* | JN613319.1 | + | + | + |
| | *ltmF* | JN613319.1 | + | + | + |
| | *ltmC* | JN613319.1 | + | + | + |
| | *ltmB* | JN613319.1 | + | + | + |
| | *ltmJ* | JN613318.1 | + | - | - |
| | *ltmE* | JN613318.1 | + | - | - |
| eas cluster | *lpsB* | JN177500.1 | + | - | - |
| | *easE* | JN177500.1 | + | - | - |
| | *easF* | JN177500.1 | + | - | - |
| | *easG* | JN177500.1 | + | - | - |
| | *easA* | JN177500.1 | + | - | - |
| | *easH* | JN177500.1 | + | - | - |
| | *dmaW* | JN177501.1 | + | - | - |
| | *cloA* | JN177501.1 | + | - | - |
| | *easC* | JN177501.1 | + | - | - |
| | *easD* | JN177501.1 | + | - | - |
| | *lpsA* | JN177502.1 | + | - | - |
| perA domain | *perA* | AB205145.1 | + | + | + |

Note: + = gene presence, - = gene absence

a. Results

Nine of the 11 ltm genes are present in both E142 and AR37. The sequences of all nine genes are identical between the two strains.

All 11 eas genes are absent in both E142 and AR37.

Both E142 and AR37 contain the perA-2 allele, which lacks the terminal reductase domain present in perA that is required for product release. However, both sequences contain an additional nonsense mutation resulting in premature translational termination of the gene. Therefore, both genes are non-functional. There is one non-synonymous SNP difference [MPC1] between E142 and AR37.

### 6.5.3 Comparison of reference genes tub2 and EF-2

The house keeping genes ®[MPC2]-tubulin (tub2) and elongation factor 2 (EF-2) were also analysed (Table 6). The tub2 genes are identical between E142 and AR37 but there is a missense mutation in the EF-2 gene of E142 that is not found in AR37 [BS3] [MPC4].

**Table 6**: Presence of the reference genes tub2 and EF-2 genes in Fl1, E142 and AR37.

| Gene | Identifier/Gene Model | Fl1 | E142 | AR37 |
|---|---|---|---|---|
| *Tub2* | EfM3.014010 | + | + | + |
| *EF-2* | EfM3.021210 | + | + | + |

### 6.5.4 Gene differences between E142 and AR37

a. Method

To identify genes that may be present in one strain but absent in the other, reads from both strains were independently mapped to the EfM3 v5 gene models using Bowtie2 v2.3.2 (Langmead & Salzberg, 2012). The number of reads that mapped to each gene was calculated with Mapcount v1 (http://mpcox.github.io/mapcount/). Because only parts of genes may be deleted, the focus was not placed on genes with zero reads in one strain, but some reads in the other. Instead, a ratio of read counts between E142 and AR37 was calculated, and attention was focused on genes with high differential read count ratios. A small subset of primers (Table 7) of this enriched list of genes with putative differential presence/absence between the two strains were designed for validation by PCR testing (Table 8).

**Table 7:** Gene names, primer names, primer sequences and product size in Fl1 (base pairs). Section A of this table shows those present in AR37 and absent in E142, and Section B shows those present in E142 and absent in AR37.

| Putative Gene Identification | Identifier/Gene Model | Primer Name | Primer Sequence (5'->3) | Fl1 Size (BP) |
|---|---|---|---|---|
| **Section A** | | | | |
| Cytochrome P450 mono-oxygenase | EfM3.005360 | CP450M-F | CAGCTTCGACGACATCATGACG | 602 |
| | | CP450M-R | AGTCATGATTGCGTGCGGTTG | |
| LysM containing protein | EfM3.062860 | LysMConP-F | AACGAGACGTGCTTCGACATC | 681 |
| | | LysMConP-R | TGTGGCTGTCAGTAGGAGAACG | |
| **Section B** | | | | |
| Hypothetical | EfM3.044570 | Hypo570-F | TCTGGACGGCATTTCGATTCG | 653 |
| | | Hypo570-R | TGCAATCAAGACAGCCACGAG | |
| Hypothetical | EfM3.044580 | Hypo580-F | AATTCGACCACGGCGTGTTCC | 483 |
| | | Hypo580-R | TGTCACTCGGGTCATTCTCGTC | |
| GTP cyclohydrolase | EfM3.050800 | GTPCyc-F | ACAATGCGCTCTTCTACGAGG | 444 |
| | | GTPCyc-R | TTGCGCGTCTTGTACTTGCTC | |
| TIM complex component tim54 | EfM3.062040 | Tim54CC-F | GCTTCAGGCTGATCTTCGCTTG | 741 |
| | | Tim54CC-R | ATCAGTTCATCACGCGCGATC | |

DNA was extracted from endophyte cultures grown on PDA using the Macherey-Nagel NucleoSpin® Plant II DNA extraction kit in an automated system. The DNA was then checked by B10/B11 analysis (as

per section 6.4) to ensure strain identity. All samples used were quantified to ensure the quantity of DNA used for each PCR reaction was of a similar enough concentration to avoid false negative results. These custom primers were manufactured by Life Technologies ThermoFisher. The PCR mix per sample was: sterile $H_2O$ (16.5µl), 10x Ex Taq Buffer Mg2+ plus (2.5µl), dNTP Mix-2.5mM each (2µl), 20µM Primer-Forward (1.25µl), 20µM Primer-Reverse (1.25µl) and Ex Taq Hot Start polymerase -5U/µl (0.5µl). The Ex Taq Buffer, dNTPs and Taq were Takara RR006A. The DNA template volume varied slightly dependent on primer set.

PCR was carried out using the following Thermocycler (Eppendorf Mastercycler) program: 94°C for 1 min, 30 cycles of (94°C for 15s, 56°C for 30s and 72°C for 45s), 72°C for 10 min, samples were then held at 4°C until analysis.

PCR products (5µl including loading dye run in duplicate on the gel), were processed for analysis via gel electrophoresis using 1.5% agarose gels containing Sybr Safe DNA Gel Stain (Thermofisher), run for ~75 minutes at 90V in 1xTBE buffer. The gels were imaged using a DNR MiniBIS Pro bioimaging system with Gelquant software. A 100-base pair ladder (Thermofisher) and a no-template negative control were run on each gel. For analysis, presence of a product band of the expected size was taken as confirmation of gene presence in the sample and absence of a product band of the expected size was taken as confirmation that the sample lacked the gene of interest. Any non-specific priming was considered inconsequential as it was not near the size of the gene of interest. Table 8 displays the results.

b. Results

The comparison between the two genomes enabled the identification of approximately 40 candidate genes showing either presence or absence differences between E142 and AR37. A subset of six genes from that group were selected for validation using PCR (Table 8).

**Table 8.** Presence and absence of a six gene subset between E142 and AR37.

| Putative Gene Identification | Identifier/ Gene Model | Primer name | AR37 | E142 | No-Template Control |
|---|---|---|---|---|---|
| Cytochrome P450 mono-oxygenase | EfM3.005360 | CP450M | + | - | - |
| LysM containing protein | EfM3.062860 | LysMConP | + | - | - |
| Hypothetical | EfM3.044570 | Hypo570 | - | + | - |
| Hypothetical | EfM3.044580 | Hypo580 | - | + | - |
| GTP cyclohydrolase | EfM3.050800 | GTPCyc | - | + | - |
| TIM complex component tim54 | EfM3.062040 | Tim54CC | - | + | - |

Note: + = gene presence, - = gene absence

### 6.6 E142 Alkaloid profile

#### 6.6.1 Alkaloid profile; description and measurement

E142 does not produce detectable levels of loline, ergovaline, peramine and lolitrem B alkaloids, but does produce varied levels of epoxy-janthitrem compounds.

Loline analysis was performed via the method described in Barker et al 2015, LOD=25 ppm. Ergovaline analysis via a modified method described by Spiering et al 2002, LOD=0.1ppm. Lolitrem B analysis via a modified method described by Gallagher et al 1985, LOD=0.05ppm. Peramine analysis via a modified method described by Vázquez-de-Aldana et al 2010, LOD=0.5ppm. None of these alkaloids have been detected at or above the assay limits of detection (LOD) in any of the analysed grass and seed samples infected with E142.

Epoxy-janthitrem analysis was performed by AgResearch New Zealand or via a modified method described by Hennessy et al. 2016, LOD=<0.1ppm. E142 produces varied concentrations of the five forms of epoxy-janthitrems namely: Epoxy-janthitriol and epoxy-janthitrem I, II, III and IV.

Table 9 compares the E142 alkaloid profile with the most common commercialised endophytes which have NZ PVR grants.

**Table 9**: Alkaloid production by some novel endophytes

| Taxon | Endophyte Strain | Epoxy-janthitrems | Lolitrem B | Ergovaline | Peramine | Lolines |
|---|---|---|---|---|---|---|
| *Epichloë festucae var. lolii* | E142 | + | - | - | - | - |
| | AR1 | - | - | - | + | - |
| | AR37 | + | - | - | - | - |
| | NEA2 | - | + | + | + | - |
| | NEA3 | - | - | + | + | - |
| | NEA10 | - | - | + | - | - |
| *LpTG - 2* | NEA11 | - | - | + | + | - |
| *Epichloë uncinata* | U2 | - | - | - | - | + |
| *Epichloë coenophiala* | AR542 (Max P) | - | - | - | + | + |

Note: + = Alkaloid produced *in planta*, - = Alkaloid not produced *in planta.*

#### 6.6.2 Epoxy-janthitrem chemical structure

Epoxy-janthitrems are part of a group of indole-diterpenoid alkaloids with structural similarity to lolitrems (McKenzie, 2014). Both epoxy-janthitrems and lolitrem B are lipophilic compounds, which are not easily translocated around the plant, hence affecting the way these alkaloids are distributed throughout the plant (Ruppert, 2016, Munday-Finch & Garthwaite, 1999).

Epoxy-janthitrems are a group of five compounds: epoxy-janthitriol (epoxy-janthitrem triol), epoxy-janthitrem I, epoxy-janthitrem II, epoxy-janthitrem III, and epoxy-janthitrem IV, (Ruppert, 2016) which are highly unstable and will readily degrade in light, heat and certain solvents (Hennessy, 2015). The four main epoxy-janthitrem compounds known to be produced in *Epichloë* are shown in Figure 1.

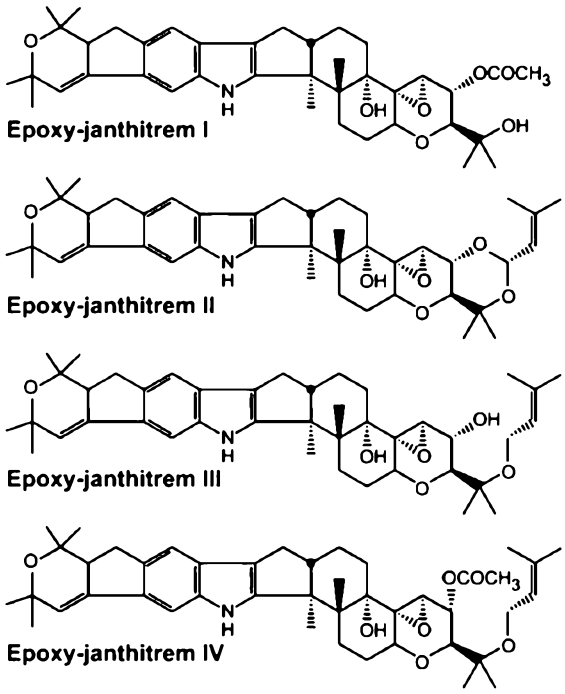


**Figure 1:** The chemical structures of epoxy-janthitrem forms I – IV. Epoxy-janthitrem triol is not shown in this figure. (Hennessy, 2015).

### 6.6.3 E142: Epoxy-janthitrem seasonal variation and plant distribution

Alkaloid concentrations are known to be seasonally affected and have been shown to be lower during the winter when temperatures are cooler (Ball et al., 1995). Two other important factors which are known to affect alkaloid concentrations are plant genotype and temperature where higher temperatures induce greater concentrations of epoxy-janthitrems (Hennessy, 2015). On this basis, a trial was conducted to evaluate E142's variation of epoxy-janthitrem levels across seasons within three different genetic backgrounds.

#### 6.6.3.1 Materials and methods

Two diploid perennial ryegrass cultivars Excess and One50, and one Italian ryegrass cultivar Asset, were used in this study. These cultivars were infected with *Epichloë festucae var. lolii* strain E142. The Excess, Asset and One 50 material infected with E142 were originally inoculated following the inoculation method detailed in section 10. Sixteen genotypes were selected from each line based on 100% endophyte infection and transplanted on the 1st November 2016 to a field plot at Templeton HQ, NZ. Every single genotype was DNA tested to confirm endophyte strain identity. The experiment was set up as a randomised block design with four replicates where each plot is represented by a row of four individual genotypes of the same cultivar, resulting in a total of 16 plants per entry.

Plants were spaced at 25cm intervals with 50cm between plots and one metre between rows. Plants were sampled every six weeks across one year giving a total of nine harvests.

Six tillers per genotype were sampled with each of those separated into three groups: i) leaf blade, ii) pseudo-stem and iii) immature seed head when present. The four genotypes were subsequently bulked together for each plot.

Plants were kept into a cooled chilly bin directly after harvest, separated into their sample type group and placed into a freezer until being freeze dried. Epoxy-janthitrem levels were determined by HPLC analysis.

**6.6.3.2 Results**

a. Seasonal variation

E142 epoxy-janthitrems' seasonal variation followed a similar pattern that of lolitrem B reported by Ball et al. 1995. Epoxy-janthitrem concentrations were found to be the lowest during winter and spring and were higher in the summer and autumn months. Peak epoxy-janthitrem production was in March (Chart 1). Despite the differences in the genetic background of the three host cultivars, no real variation was noted in the annual distribution of epoxy-janthitrem levels. A partial explanation for the increase in production seen in summer and autumn relates to the increase in temperature and when plants are under some degree of moisture stress.

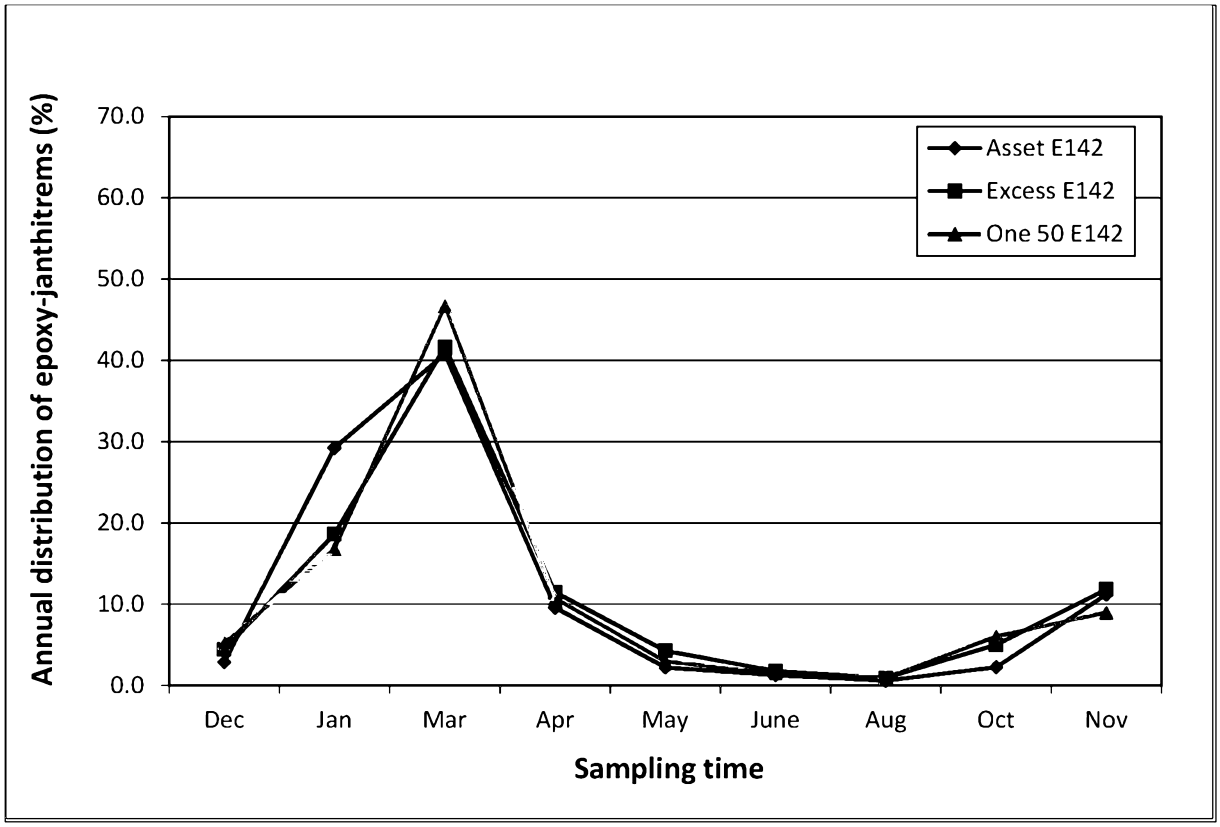


**Chart 1:** Seasonal distribution in percent of epoxy-janthitrems in the leaf blade and pseudo-stem of three ryegrasses cultivars (December 2016 to November 2017).

b. Plant distribution

E142 epoxy-janthitrem concentrations were found to be considerably higher in the pseudo-stem than in the leaf blade for all nine measurements. From November to April, epoxy-janthitrems were also measured in the immature seed heads which generally had higher concentrations than the pseudo-stem and leaf blade; the exception was the November and March measurements (Chart 2).

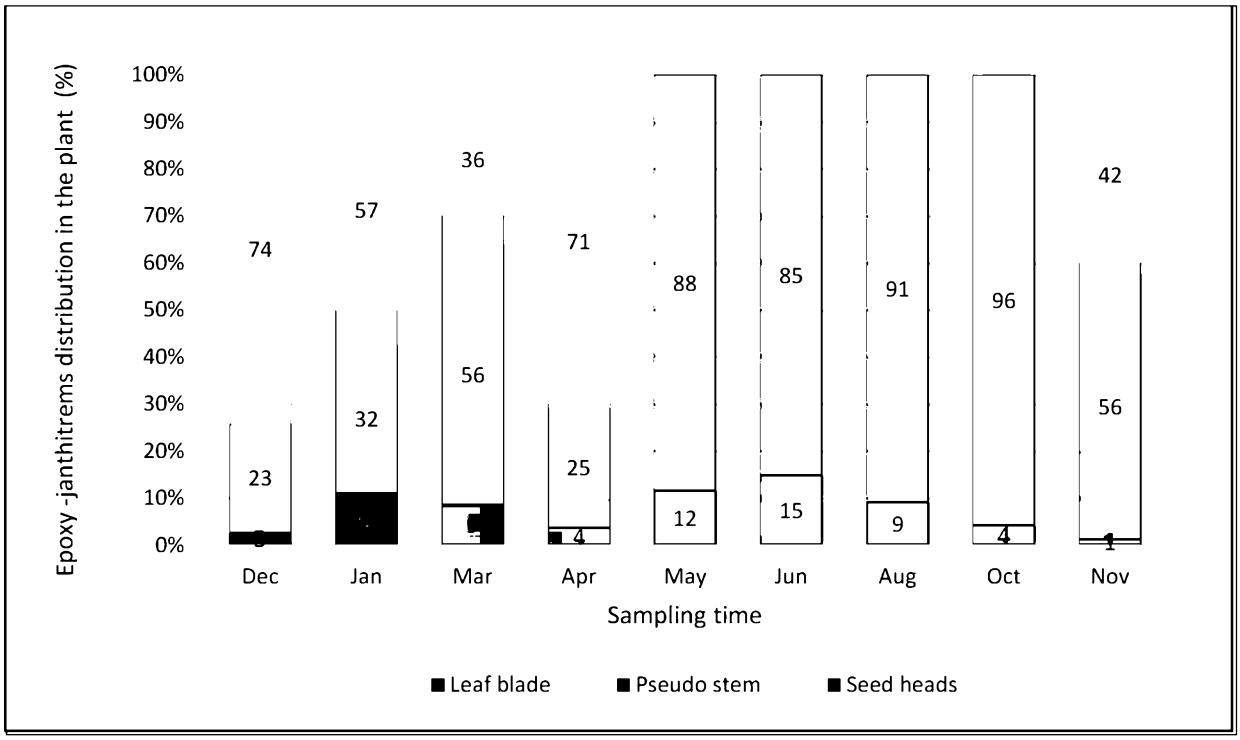


**Chart 2:** Annual distribution of epoxy-janthitrems throughout the plant: leaf blade, pseudo-stem and immature seed head at nine sampling dates. The data presented is an average concentration of three ryegrass cultivars (Excess, One50 and Asset) infected with E142.

From a survival point of view, having higher alkaloid concentrations in the seed head and pseudo-stem than in the leaves, is advantageous for both the host ryegrass and the endophyte. Survival of the ryegrass is strongly dependent on the initiation of new tillers which occur at the lower part of the plant where the meristem is located, while propagation and subsistence of the endophyte depends on its host survival and transmission into the seed.

During the trial, it was also apparent that variation in the total level of epoxy-janthitrems produced by E142 was present not only between cultivar genotypes, but also within a cultivar. This supports and reinforces the influence of the host and endophyte interaction as being one of the major factors influencing epoxy-janthitrem expression. This offers an opportunity to breed an endophyte/cultivar association with an enhanced or reduced capacity of epoxy-janthitrem expression.

## 7. Insect pest protection and pasture persistence

In New Zealand, it is well documented that one of the main factors positively affecting growth and persistence of perennial ryegrass is the association with fungal endophyte *Epichloë festucae var. spp.* in the plant. Pasture established with non-endophyte infected cultivars fail to persist in the insect prone areas such as in the upper North Island. This symbiosis has been shown to give protection against numerous pasture pests and increased pasture persistence from their secondary metabolites (McKenzie, 2014). Each endophyte offers a different degree of protection according to their alkaloid profile with AR37 showing increased pasture production and persistence over that of AR1 and nil. This is due to the higher pasture pest resistant properties from the epoxy-janthitrem alkaloids (McKenzie, 2014); epoxy-janthitrems are produced by both E142 and AR37 endophyte strains.

Several studies to assess AR37 infected ryegrass have been undertaken. The epoxy-janthitrems are thought to be involved in the bioactivity of the endophyte (Hennessy, 2015). An experiment utilizing a semi-synthetic diet was conducted by Hennessy et al., 2016 on porina larvae consuming a high *in planta*

concentration of epoxy-janthitrems (30.6 µg/g in leaves and 83.9 µg/g in pseudo-stems). Results showed that a strong antifeedant effect was present reducing both porina growth and their survival rate. Finch et al., 2010 and Hennessy 2015 also conducted bioassays which showed that epoxy-janthitrem I behaved as a feeding deterrent against larval porina and increased the larvae's mortality rate. Evidence of toxicity was observed during that work.

AR37 (an epoxy-janthitrem producer), reduces Argentine Stem Weevil larval (*Listronotus bonariensis*) damage (Popay and Wyatt 1995, Popay and Thom 2009) and deters black beetle adult (*Heteronychus arator*) feeding (Ball et al 1994) leading to reduced black beetle population increases in the field (Popay and Thom 2009). AR37 also reduces damage by pasture mealybug (*Balanococcus poae*) (Pennell et al 2005), root aphid (*Aploneura lentisci*) and porina (*Wiseana cervinata*) (Popay and Gerard 2007, Jensen and Popay 2004). AR37 infected *Lolium perenne* pasture has led to improved agronomic persistence where certain insect pest pressure has been evident (Popay and Hume 2011).

## 8. Alkaloids and animal health

Epoxy-janthitrems have a low tremorgenic potential (Fletcher and Sutherland, 2009) and are much less toxic than lolitrem B, however, in some cases ryegrass staggers have been identified in animal grazing AR37, although cases are infrequent and in short duration (McKenzie, 2014; Fletcher, 2005).

Severity and duration of ryegrass staggers in sheep grazing *Lolium perenne* infected with AR37 was shown to be significantly less than in sheep grazing *L. perenne* infected with wild type *E. festucae var. lolii* (Finch et al., 2012; Fletcher et al., 2017).

A trial on cattle showed that the average milk yields (kg/cow/day) in cows grazing of *Lolium perenne* infected with AR37 were similar in spring but were reduced in autumn when compared to *Lolium perenne* infected with AR1 endophyte. The reduction in average milk yield was less for cows grazing *Lolium perenne* infected with AR37 than that for *Lolium perenne* infected with wild type *E. festucae var. lolii*. There were no observed effects on cow health over the period the trial was run 2005-2006 (Thom et al. 2007).

## 9. Protein expression via PAGE

### 9.1 Method

Using the P-Per Plant Protein Extraction Kit (Thermofisher Scientific), protein was extracted from solid endophyte cultures of the same age and run on NuPAGE 4-12% Bis-Tris gels (Life Technologies/Thermofisher) using LDS sample buffer (Life Technologies/Thermofisher) and a 1x MOPS (Life Technologies/Thermofisher) running buffer system. The gel was run at 200 constant volts.

Post electrophoresis, the gel was fixed, then stained using Simply Blue Safestain (Life Technologies/Thermofisher) and de-stained in water. The gel was then photographed using a MiniBIS Pro Gel Imager (DNR Bio-Imaging Systems). The image was visually analysed for between sample differences; namely in band presence/absence and major band intensity differences. This analysis was performed for E142 versus AR37.

### 9.2 Results

Four notable differences were found between the protein profiles for endophyte strains E142 and AR37. The differences were present in the 50kDa to approximately 110kDa region of the gel. There were presence/absence differences of a protein band and large intensity variability of major protein bands.

## 10. Inoculation methods

Inoculation of E142 into various grass hosts was performed by a modified micro-slit technique (Latch & Christensen 1985). Nil endophyte seed was sterilized in ethanol and bleach before being rinsed in distilled water and sown on water agar (3% w/v) containing sucrose (3% w/v). Seedlings were then grown in the dark for 6-10 days. Using a syringe needle (0.3mm x 13mm) a slit was made in the basal meristem of the seedling and a piece of endophyte, which had been growing in potato dextrose broth for a minimum of 7 days, inserted into the slit. Seedlings were then placed back in the dark for 7 days. Following this the seedlings were adapted to light, planted and transferred to a controlled growth room (20°C, 24 hours light) to grow for approximately 4 weeks.

Endophyte presence was confirmed via histological staining with aniline blue or via a tissue print immunoblot test modified from Gwinn et al (1991).

Whilst the invention has been described with reference to specific embodiments, it will be appreciated that modifications and improvements to those embodiments are envisaged as being within the scope of this invention.

## 11. E142: improved fungicide resistance and stability

E142 endophyte has a superior fungicide resistance when grown in combination with, but not limited to, perennial ryegrass.

An experiment was conducted to assess the impact of various fungicide treatments on the vertical transmission of E142 and AR37. AR37 was used as a bench mark due to its alkaloid profile being similar to E142 and therefore to assess whether E142 was an improvement over the AR37 invention for this characteristic.

### 11.1 Materials and Methods

Perennial ryegrass plants from the diploid cultivar One50 (line reference: KR1304.NU) and tetraploid cultivar Base (line reference: K1405.1) infected with *Epichloë festucae var. lolii* strains E142 and AR37 were used in this experiment. The plant inflorescence emergence time of Base and One50 are only two days apart and were selected on that basis to ensure uniform growth stage at spraying time. Plants infected with AR37 were sourced from the two lines described above. E142 plants were originally inoculated (following the inoculation method described in section 10) into the same two lines once the AR37 strain was removed by heat treatment. Endophyte presence was confirmed for each of the genotypes using the tissue immunoblot technique by testing five tillers per plant. Only plants with full infection were selected in order to remove potentially unstable genotypes. All genotypes were subsequently sampled to confirm endophyte strain identity using the B10 and B11 SSR primers as described in section 6.4.

The experimental design was a single replicate of a split-split-plot design, with four fungicide treatments applied to the main-plots and two cultivars applied to the sub-plots. Each sub-plot consisted of eight plots of the same cultivar with four plots of E142 and four plots of AR37. Each plot was composed of four genotypes with a total of 16 genotypes per cultivar/endophyte combination, was assigned for every fungicide treatment.

The sixteen genotypes were selected from each line and divided by hand into ramets composed of four individual plants and transplanted to PB3/4 bags on the 30th October 2016. Plants were kept in the shade house in Darfield for one month and hand transplanted to a field plot at Templeton, NZ. A month prior to inflorescence emergence, all plants were trimmed in order to even out inflorescence emergence date and flowering time before spraying.

Plants were spaced at 50 cm intervals with 75 cm between rows and one meter between treatments.

The treatments included the following combinations of fungicides namely: Opus® (i.e., 125g/L epoxiconazole, BASF Crop Protection Ltd.); Proline® (i.e., 250 g/L prothioconazole, Bayer Crop Science Ltd.); and Proline® (i.e., 250 g/L prothioconazole, Bayer Crop Science Ltd.) plus Amistar® (i.e., 250 g/L azoxystrobin, Syngenta Crop Protection Ltd.) (Table 10).

Three applications were applied for each treatment. The first at mid to late inflorescence emergence (GS 55-50), which was on the 20th December 2016, the second at the early to mid-flowering (GS 65), which was on the 31st December 2016, and the third on the 11th January 2017 at the end of flowering, early grain fill (GS75-80).

**Table 10:** Treatments consisting of various combinations of fungicides applied to perennial ryegrass cultivar Base and One50 seed crops.

| Treatment # | Fungicide | Rate | Reference |
|---|---|---|---|
| 1 | Untreated | 0 | NT |
| 2 | Opus® | 1L/ha | Opus 1L |
| 3 | Proline® | 0.6L/ha | Proline |
| 4 | Proline® + Amistar® | 0.6L/ha + 0.5L/ha | P+A |

Each individual plant was hand bagged once pollination was finished and seed heads started browning off. Seed was hand harvested from individual plants from mid to late February 2017, dried (30°C/24h), hand threshed and stored in brown paper envelopes kept at 5°C with 30% relative humidity (RH). One month later, 40 seeds from each plant and for all treatments were hand sown into trays by QuikStart Seedlings 2007 Limited and placed in one of their glasshouses and watered as required. Eight weeks after sowing, one tiller per plant was tested to determine the viable endophyte transmission rate using the tissue immunoblot technique. Statistical analysis was performed using GenStat (VSN International 2013, GenStat for Windows 18th Edition, VSN International, Hemel Hempstead, UK). Analysis of Variance (ANOVA) and Fisher's unprotected test of least significant difference (LSD; $P<0.05$) were performed to compare treatment effects of the fungicides on the endophytes viable transmission rates. The data was analyzed as eight separate ANOVA analyses, one for each sub-plot, with one fungicide treatment, one cultivar and two endophytes. The design of each sub plot was treated as a randomized block design with four plots assigned to each of the two endophyte treatments.

### 11.2 Results

It is worth noting that the E142 material used in this experiment for both cultivars was the original inoculated plants (F0 generation). Being the F0 generation no breeding selection or re-selection cycles to improve the overall endophyte stability was carried out. Therefore, it gives an underlying advantage to the AR37 material which has been bred to accommodate the endophyte stability in both cultivars.

a. <u>E142 and AR37 response to fungicide in cultivar Base</u>

As shown in Table 11, there was no significant difference in the viable endophyte transmission between Base E142 and Base AR37 for the fungicide free treatment (NT). E142 however displayed a 4.6% greater transmission rate over AR37. For all the other treatments the viable endophyte transmission rate of Base E142 was significantly higher than Base AR37 with considerable differences present between the two strains; Opus 1L (+12.5%), Proline (+13.6%) and P+A (+25.6%) treatments.

The effect of the fungicide treatments for each endophyte was also assessed by comparing the means of the three fungicide treatments, Opus 1L, Proline and P+A, to their respective fungicide free treatment (NT).
For Base E142, a minor decrease in the viable endophyte transmission rate was present with a range varying between -0.8% to -6.5% when compared to its respective fungicide free treatment.
For Base AR37, however, the fungicide treatments appear the have a strong impact on the endophyte viable transmission rate, with a substantial decrease of -8.6% (Opus 1L), -15.4% (Proline) and -25.9% (P + A).

**Table 11:** The effect of four fungicide treatments on E142 and AR37 endophytes' viable transmission rates in the cultivars Base.

| Endophyte viable transmission rate (%), Cultivar Base | | | | |
|---|---|---|---|---|
| | NT | Opus 1L | Proline | P + A |
| E142 | 99.2 | 98.4 | 92.7 | 94.2 |
| AR37 | 94.5 | 85.9 | 79.1 | 68.6 |
| LSD (5%) | 5.9 | 10.4 | 8.5 | 13.5 |
| Significance | NS | p<0.05 | p<0.05 | p<0.01 |

b. E142 and AR37 response to fungicide in cultivar One 50

As shown in Table 12, there was no significant difference in the viable endophyte transmission between One50 E142 and One50 AR37 for the fungicide free treatment (NT). E142 however, displayed a slightly superior transmission rate of 1.6% over AR37.No significant effect was present between One50 E142 and One50 AR37 when treated with Opus 1L although E142 showed a 2.7% greater transmission over AR37. For the remaining two treatments, the viable endophyte transmission rate of One50 E142 was significantly higher than One50 AR37 with large differences present between the two strains; E142 displayed a superior transmission rate over AR37 when treated with Proline (+27.5%) and P+A (+29.4%).

The means of the 3 fungicide treatments, Opus 1L, Proline and P+A, were compared to their respective fungicide free treatment (NT) to assess the effect of the fungicides on each endophyte.
For One50 E142, different degrees of decrease in the viable endophyte transmission rate was measured, with a minor to moderate loss of -0.4% and -7.5% when treated with Opus 1L and Proline respectively. A -17.6% decrease was observed when treated with the P + A fungicide combo.
For One50 AR37, the decrease was small when treated with Opus 1L (-1.6%) but a very large reduction was present when treated with Proline (-33.5%) and P+A (-45.5%).

**Table 12:** The effect of four fungicide treatments on E142 and AR37 endophytes' viable transmission rates in the cultivar One50.

| | Endophyte viable transmission rate (%), Cultivar One50 | | | |
|---|---|---|---|---|
| | NT | Opus 1L | Proline | P + A |
| E142 | 99.0 | 98.6 | 91.5 | 81.4 |
| AR37 | 97.5 | 95.9 | 64.0 | 52.0 |
| LSD (5%) | 4.7 | 6.2 | 24.1 | 18.5 |
| Significance | NS | NS | p<0.05 | p<0.05 |

Comparing the mean of the three fungicide treatments across both endophytes and cultivars, the results suggest that fungicides vary in terms of the resulting reduction in endophytes' viable transmission rate, with P+A > Proline > Opus 1L.

### 11.3 Conclusion

The various fungicide treatments show little effect on E142 viable transmission rate in association with either Base or One50 cultivars, except for One50 E142 treated with Proline + Amistar which was considerably lower than its respective free fungicide treatment, but still significantly greater than One50 AR37.

Overall the results exhibit a good tolerance of E142 to various fungicide treatments as well as a significantly superior resistance of E142 over AR37 for all fungicide treatments in both cultivars. The results are supporting a strong improvement of E142 over AR37 for this characteristic. Such a trait is a real attribute to commercial seed when marketed in combination with endophyte:

i) it allows a wider range of fungicides to be used during seed production to improve the control of fungal diseases, which offers the ability to increase both seed yield and seed quality while decreasing production cost,

ii) a lower production cost enables seed marketers to supply a product at a competitive price to the end user,

iii) it increases product reliability and security for the buyers to purchase seed with good levels of viable endophyte.

It will be appreciated by those in the relevant field that modifications to the above examples can be made that will still fall within the scope of the present invention.

## 12. E142: Superior intra/inter-specific and inter-generic compatibility

E142 has a broad intraspecific, interspecific and inter-generic host compatibility when inoculated into material of various genetic backgrounds. The overall endophyte-host compatibility of E142 was experimentally assessed across three successive levels:

Level 1: inoculation ability into the various host material,
Level 2: stability at six months post inoculation,
Level 3: vertical transmission frequency into the seed of the inoculated host.

Each experiment was conducted using the endophyte strains E142 and AR37 inoculated into cultivars of *Lolium perenne, Lolium multiflorum, Festulolium loliaceum, Festuca pratensis* and *Festuca arundinacea*. AR37 was used as a bench-mark due to its similar alkaloid profile to E142 and to assess whether E142 was an improvement over the AR37 invention for this characteristic. The dual aims of this work were to assess E142's performance at all levels and to compare it against AR37 as a standard.

The species were divided into three groups:
Group 1: contained *Lolium multiflorum*,
Group 2: contained *Lolium perenne* and *Festulolium loliaceum*,
Group 3: contained *Festuca pratensis* and *Festuca arundinacea*. It should be noted that the plants in group 3 were only assessed for compatibility with E142 and therefore there was no comparison with AR37 for this material.

### 12.1 Level 1: Inoculation ability

#### 12.1.1 Materials and Methods

All seeds were heat treated (50°C and 45% RH for 30 days), prior to the experiments in order to eliminate any potential pre-existing viable endophyte and to avoid any other fungal contamination. A sample of these seeds were grown and checked via tiller tissue immunoblot to ensure the heat treatment was successful. All cultivars used in these experiments are not the natural host cultivar nor have they been bred for stability of either endophyte thus eliminating any bias towards one endophyte strain.

Inoculation was carried out as per the method detailed in section 10 of this document. For group one there were four treatments; namely two cultivars with E142 and AR37 endophytes. For group two there were six treatments; namely three cultivars with E142 and AR37 endophytes. For the experiment of group's one and two, seedlings from each cultivar were inoculated with *Epichloë festucae var. lolii* strains E142 and AR37; this process was replicated three times over consecutive weeks. An average of 254 seedlings (with a range of 196 to 318) were inoculated for each cultivar.

One replicate of one cultivar was inoculated with both endophytes per day of inoculation. The endophyte inoculation order for any given treatment was randomised across the inoculation days to reduce any bias from operator fatigue. The experiment for group 3 focused on E142's compatibility with the *Festuca* genera and only one replicate was carried out per cultivar.

Inoculated seedlings were planted into 10 cells (5x2) plastic trays containing commercial potting mix (Intelligro). For each replicate the same cultivar/endophyte combination was planted in a tray to minimize potential for mix up. While being maintained in their replicates as a block, the trays were randomly placed into an enclosed growth chamber with a controlled environment of 20°C constant temperature and 24hr light for approximately five weeks, then transferred into a glasshouse in Darfield (Central Canterbury). Eight to twelve weeks after planting, the inoculation ability of each strain was assessed by testing two tillers per plant via the tissue immunoblot technique to determine the endophyte infection frequency (E+). It should be noted that not all seedlings survive inoculation and planting, so the number of plants for testing is reduced compared to the number inoculated. An average of 141 seedlings (with a range of 112 to 196) were tested for each cultivar. The inoculation success of E142 vs AR37 was determined to show the compatibility level of each endophyte within hosts of various genetic backgrounds across species and genera. Statistical analysis was performed using GenStat (VSN International 2013. GenStat for Windows 18th Edition. VSN International, Hemel Hempstead, UK). Analysis of variance (ANOVA) with three replicates (dates) and two factors, namely cultivar and

endophyte, and the least significant difference test (LSD; P<0.05) were performed to compare the main treatment effects of the groups of cultivars on the endophyte infection frequency.

#### 12.1.1.1 Group 1- *Lolium multiflorum*

Two *Lolium multiflorum* cultivars were used in this experiment; namely Tabu and Vibe.

Tabu was bred in the 1990s from a mass selection made from an 18-month old field trial of the variety Flanker which was itself bred from a collection of single plants from an old pasture.

Vibe was bred in 2012 and originated from a five-cycle recurrent breeding program aiming at selecting for improved persistency under dryland conditions and set stock sheep grazing. Breeding started in 1999 from cultivars such as Concord, Tabu and Te Rahu ecotypes.

#### 12.1.1.2 Group 2- *Lolium perenne and Festulolium loliaceum*

Two *Lolium perenne* and one *Festulolium* cultivars were used for this experiment. The *L. perenne* cultivars used were Bronsyn and LpD1402N, and the *Festulolium* was Matrix (*Festulolium loliaceum*). Bronsyn is an older cultivar bred in the 1990s from the Mangere ecotypes collected in the 1960s in Northern New Zealand. The Mangere ecotypes have improved winter activity and suggest introgression from *L. multiflorum*.

LpD1402N is an improved breeding line bred by Cropmark post 2010. It originated by outcrossing the modern *L. perenne* cultivar Eiffel, which is a late heading European variety, with other very late heading European cultivars such as Mezzo and Samsara. The final cross was made in 2014 by crossing that material with some New Zealand bred germplasm. Overall it remains closer to its European background being a late heading line with moderate to strong winter dormancy.

The genetic origin of Matrix is a combination of Mangere ecotypes, Hawkes Bay ecotypes, North West Spain germplasm and European meadow fescue germplasm. It was bred in the 2000s from the *L. perenne* cultivars Aries HD, Grasslands Impact and a meadow fescue ecotype originating from Switzerland. It is a classified as a *Festulolium* with around 8% introgression of *Festuca pratensis*.

#### 12.1.1.3 Group 3: *Festuca* species

The inter-generic compatibility of E142 was assessed using Easton, a tall fescue cultivar (*Festuca arundinacea*) and Fp1202N, a meadow fescue cultivar (*Festuca pratensis*) respectively.

Easton has been developed from Advance tall fescue for increased production combined with superior persistence.

The meadow fescue Fp1202N is a breeding line bred from European germplasm and developed by Cropmark Seeds for increased production, improved winter activity and disease resistance.

### 12.1.2 Results

#### 12.1.2.1 Level 1- Group 1: *Lolium multiflorum*

a. Endophyte and endophyte by cultivar effect

The endophyte infection frequency of E142 for the combined *L. multiflorum* cultivars was 42% higher than for AR37. This is a highly significant difference between the two endophytes with a p<0.001 as shown in Table 13. No cultivar x endophyte interaction was present in that experiment (Table 13).

In the cultivar Vibe, the infection frequency of E142 (57.7%) was nearly three times higher than the infection frequency of AR37 (21.6%).

A similar pattern was present with Tabu where the infection frequency of E142 was nearly four times higher than the infection frequency of AR37, 63.2% vs 16.1% respectively (Table 13).

**Table 13**: Endophyte infection frequency means of each endophyte strain for the two *L. multiflorum* cultivars.

| Endophyte | Vibe E+ (%) | Tabu E+ (%) | All Cultivars E+ % |
|---|---|---|---|
| E142 | 57.7 | 63.2 | 60.4 |
| AR37 | 21.6 | 16.1 | 18.8 |
| LSD 5% | 23.6 | 23.6 | 16.7 |
| Significance | p<0.01 | p<0.01 | p<0.001 |
| F probability (all cultivars) | | | |
| Cultivar x Endophyte | | | 0.452 |

b. Cultivar effect

There was no significant difference between cultivars reinforcing the significance of the endophyte strain effect rather than the cultivar (Table 14).

**Table 14**: Effect of cultivars on the endophyte infection frequency.

| Cultivar | E+ % |
|---|---|
| Vibe | 39.7 |
| Tabu | 39.6 |
| LSD 5% | 16.7 |
| Significance | (NS) |

**12.1.2.2 Level 1 - Group 2: *Lolium perenne and Festulolium loliaceum***

a. Endophyte and endophyte by cultivar effect

The endophyte infection frequency of E142 for the combined *L. perenne* and *Festulolium* cultivars was 37% higher than for AR37. This is a highly significant difference (P<0.001) between the two endophytes, with no cultivar by endophyte interaction present (Table 15).

In the cultivar Bronsyn, the infection frequency of E142 (49.1%) was over six times higher than the infection frequency of AR37 (7.9%).

The infection frequency of E142 (54.2% and 56.9%) was nearly three times higher than the infection frequency of AR37 (20.1% and 20.2%) in the LpD1402N and Matrix backgrounds respectively (Table 15).

**Table 15:** Endophyte infection frequency means of each endophyte strain for the two *L. perenne* and the *Festulolium* cultivars.

| Endophyte | Bronsyn E+ (%) | LpD1402N E+ (%) | Matrix E+ (%) | All Cultivars E+ (%) |
|---|---|---|---|---|
| E142 | 49.1 | 54.2 | 56.9 | 53.4 |
| AR37 | 7.9 | 20.1 | 20.2 | 16.1 |
| LSD 5% | 25.3 | 25.3 | 25.3 | 14.6 |
| Significance | p<0.01 | p<0.05 | p<0.01 | p<0.001 |
| F probability (all cultivars) | | | | |
| Cultivar x Endophyte | | | | 0.906 |

b. Cultivar effect

The endophyte infection frequency rate is not significantly different between the three cultivars as shown in Table 16. This reinforces the significance of the endophyte effect over the cultivar.

**Table 16**: Effect of cultivar on the endophyte infection frequency.

| Cultivar | E+ % |
|---|---|
| Bronsyn | 28.5 |
| LpD1402N | 37.1 |
| Matrix | 38.6 |
| LSD 5% | 17.9 |
| Significance | (NS) |

**12.1.2.3 Level 1: inter-specific effect**

A comparison of the mean endophyte infection frequency was carried out to look at whether the ryegrass species had any impact on the inoculation success rate (Table 17). The results show that E142 is compatible with a broad range of genetic backgrounds, with a high infection frequency ranging from 49.1-63.2% across all cultivars tested and showing no real differences across the ryegrass species. AR37 was also inoculated into all cultivars however, it has a greatly reduced inoculation frequency rate when compared to E142, ranging from 7.9-21.6%.

**Table 17**: Interspecies comparison of endophytes infection frequency means of the different *Lolium perenne*, *Lolium multiflorum* and *Festulolium loliaceum* cultivars.

| Cultivar Species | Cultivar | E142+(%) | AR37+(%) |
|---|---|---|---|
| *L. perenne* | Bronsyn | 49.1 | 7.9 |
| | LpD1402N | 54.2 | 20.1 |
| *Festulolium* | Matrix | 56.9 | 20.2 |
| *L. multiflorum* | Vibe | 57.7 | 21.6 |
| | Tabu | 63.2 | 16.1 |

#### 12.1.2.4 Level 1: Group 3-Fescue species

As shown in Table 18, an average of 140 seedling were inoculated per cultivars. Note that not all inoculated seedling survived the inoculation and transplanting phase therefore an average of 35 seedling per cultivar were tested to calculate the percentage of successful inoculation.

The attempt to inoculate E142 into another genera than its original host was successful. Fp1202N (F. *pratensis*) and Easton (F. *arundinacea*) were both successfully inoculated at an infection frequency of 20.9% and 37.0% respectively (Table 18).

**Table 18:** Percentage of successful inoculations of E142 into Fp1202N meadow fescue and Easton tall fescue.

| Cultivar Species | Cultivar | Number of seedlings inoculated | E142 infection frequency (%) |
|---|---|---|---|
| *Festuca pratensis* | Fp1202N | 153 | 20.9 |
| *Festuca arundinacea* | Easton | 127 | 37.0 |

The inoculation success rate was relatively high despite the genetic barrier which is usually incurred when inoculating a symbiotic endophyte into another genera and species other than its original host. Often times poor inoculation ability and host dwarfism is found whilst attempting this. There was no sign of dwarfism in any of the successfully inoculated host plants. These results validate the broad compatibility of E142 across a wide range of genetic backgrounds and offer the opportunity to use its favorable alkaloid profile in new host backgrounds.

### 12.2 Level 2: Endophyte vegetative stability at six months post inoculation

Endophyte vegetative stability refers to the stability of the inoculated endophyte within its vegetative growing host. Various degrees of endophyte vegetative instability occur a few months post inoculation. This is partially due to the plant's defensive mechanisms being more active as the plant is ageing. The degree of that instability or endophyte rejection varies depending on the compatibility of each endophyte strain and the endophyte by host interaction. This level of vegetative stability was tested at six months post inoculation at which stage various degrees of synthetic symbioses were recorded:

i) Complete instability; no endophyte infection remaining in the plant.
ii) Partial stability; only a partial endophyte infection remains in the inoculated plants with a mix between infected and non-infected tillers.
iii) Complete stability; the endophyte remains fully in the inoculated plant with all tillers being infected.

#### 12.2.1 Materials and Methods

Plants successfully inoculated in level one of this work were transplanted individually into PB3 bags, containing commercial potting mix (Intelligro). Plants were placed into a tunnel-house in Templeton (Central Canterbury) with plants from the four treatments of 'group 1' and plants from the six treatments of 'group 2' kept apart. Within each group, plants from the various treatments were kept in their respective blocks and randomly placed within each replicate.

Approximately six months post inoculation, five tillers per plant were assessed via the tissue immunoblot technique to determine the remaining level of endophyte infection. As per the inoculation work this experiment remained in the groupings as detailed previously.

Complete instability was determined as 0% endophyte infection i.e. 0/5 tillers showing endophyte presence. The endophyte was deemed as being fully stable in its host if the blot results were 100% (5/5 tillers showing endophyte presence).

The proportion of completely stable and unstable plants was analysed as a generalized linear model (GLM) with a binomial distribution and a logit link as is standard for this type of data (McCullagh and Nelder, 1989). The dispersion in the analysis of deviance was estimated to allow for any aggregation in the counts. The GLM model was fitted using Genstat 19 (VSN International, 2017). Note analysis of variance is not appropriate for this data because of the varying weights due to the number of plants observed being variable between plots (as a result of the difference in inoculation success rate between the two strains) and the unequal variation of proportions over the range of 0-100%.

**12.2.2 Results**

**12.2.2.1 Level 2: Group 1- *Lolium multiflorum***

a. Complete Stability

The proportion of fully stable plants of E142 endophyte was significantly higher than AR37 ($p<0.01$) in both cultivars combined (Table 19). The share of fully stable plants inoculated with E142 averaged 78.7% for all treatments which was 37% higher than AR37 averaging 41.8% (Table 19).

The percentage of plants with complete stability of Vibe E142 (68.7%) was over two times higher than those of Vibe AR37 (29.9%).

The Tabu and E142 association displayed a similar trend with 34% more stable plants than Tabu AR37 (Table 19).

**Table 19**: Mean proportion of plants showing complete vegetative stability of E142 and AR37 in association with two *L. multiflorum* cultivars at six months post inoculation.

| Endophyte | All Cultivars E+(%) | Vibe E+ (%) | Tabu E+ (%) |
|---|---|---|---|
| E142 | 78.7 | 68.7 | 88.7 |
| AR37 | 41.8 | 29.9 | 53.7 |

| F probability (all cultivars) | |
|---|---|
| Endophyte | 0.004 |
| Cultivar x Endophyte | 0.747 |

b. Complete Instability

The proportion of fully unstable plants (0% endophyte infection remaining) in the combined L. multiflorum cultivars was nearly nine times higher with AR37 than with E142. This is a significant difference between the two endophytes strains with a $p<0.05$ (Table 20).

The percent of fully unstable plants of Vibe AR37 averaged 15.8% which was 12% higher than in Vibe E142 (4.3%).

In Tabu, complete instability of AR37 was 21.5% while no E142 plants were found with complete instability.

These results come as a complement to the complete stability analyses presented in section 'a' to reinforce E142's superior vegetative stability post inoculation over AR37.

**Table 20**: Mean proportion of plants showing complete vegetative instability of E142 and AR37 in association with two *L. multiflorum* cultivars at 6 months post inoculation.

| Endophyte | All Cultivars E+(%) | Vibe E+ (%) | Tabu E+ (%) |
|---|---|---|---|
| E142 | 2.1 | 4.3 | 0 |
| AR37 | 18.7 | 15.8 | 21.5 |

| F probability (all cultivars) | |
|---|---|
| Endophyte | 0.011 |
| Cultivar x Endophyte | 0.106 |

**12.2.2.2 Level 2 - Group 2: *Lolium perenne and Festulolium loliaceum***

a. Complete Stability

The proportion of fully stable plants of the E142 endophyte was significantly higher than for AR37 ($p<0.01$) in all cultivars (Table 21). The share of fully stable plants inoculated with E142 averaged 72.3% for all treatments which was 38% higher than AR37 averaging 34.5% (Table 21).

E142 showed a higher percent of complete stability in all three cultivars used in this study when compared to AR37. Plants with complete stability represented 77.3% in Bronsyn E142 which was over 1.5 times higher than Bronsyn AR37 (41.3%). LpD1402N E142 had 47% more fully stable plants than LpD1402N AR37. A similar trend was present with Matrix E142 having 39% more stable plants than Matrix AR37 (Table 21).

**Table 21:** Mean proportion of plants showing complete vegetative stability of E142 and AR37 in association with the two *L. perenne* and one *Festulolium* cultivars.

| Endophyte | All Cultivars E+(%) | Bronsyn E+(%) | LpD1402N E+(%) | Matrix E+(%) |
|---|---|---|---|---|
| E142 | 72.3 | 77.3 | 66.2 | 73.2 |
| AR37 | 34.5 | 49.9 | 19.3 | 34.3 |

| F probability | |
|---|---|
| Endophyte | 0.009 |
| Cultivar x Endophyte | 0.850 |

b. Complete Instability

The proportion of fully unstable plants (0% endophyte infection remaining) for the combined *L. perenne* and *Festulolium* cultivars when inoculated with E142 was approximately two and a half times lower than with AR37 (Table 22). For all treatments the percentage of unstable plants averaged 10% with E142 and 26.2% with AR37. While there is a significant difference ($p<0.05$) between the two strains the F probability shows a cultivar by endophyte interaction due to an inconstant degree of instability of the

endophytes across cultivars. The inconstancy was mostly present across the cultivars infected with AR37 where significant differences were present at all levels between Bronsyn, LpD1402N and Matrix. This suggest a stronger importance played by the host interaction in the stability of AR37. No significant difference was present between all three cultivars for E142.

Both E142 and AR37 had less than 10% of fully unstable plants in Bronsyn. With LpD1402N a much larger difference was present with a greater proportion of plants showing complete instability for AR37 (59.8%) when compared to E142 (8.5%). In the cultivar Matrix, the percentage of fully unstable plants was relatively similar between the two endophytes with Matrix E142 having 6% less than Matrix AR37 (Table 22).

**Table 22:** Mean proportion of plants showing complete vegetative instability of E142 and AR37 in association with the two *L. perenne* and one *Festulolium* cultivars.

| Endophyte | All Cultivars E+(%) | Bronsyn E+(%) | LpD1402N E+(%) | Matrix E+(%) |
|---|---|---|---|---|
| E142 | 10.0 | 8.9 | 8.5 | 12.5 |
| AR37 | 26.2 | 0 | 59.8 | 18.9 |

| F probability | |
|---|---|
| Endophyte | 0.012 |
| Cultivar x Endophyte | 0.026 |

**12.2.2.3 Level 2: Across species effect**

A comparison of the means of plants showing complete stability was carried out to look at whether the species had any impact on the endophyte vegetative stability at six months post inoculation (Table 23). The results show that E142 is stable across a broad range of genetic backgrounds, with a high level of fully stable plants across all cultivars tested regardless of the species (ranging from 66.2%-88.7%).

The proportion of plants with complete stability of E142 within each species ranged from 66.2-77.3% for *L. perenne* with 11% difference between cultivars Bronsyn and LpD1402N. A larger range was observed for *L. multiflorum* with values of 68.7-88.7% (20% difference between cultivars Vibe and Tabu).

The lower proportion of stable plants seen with AR37 (ranging from 19.3-53.7%) indicates frequent rejection of the endophyte by the host plant which results in a higher loss of the synthetic symbiosis post inoculation. A degree of endophyte loss in E142 is also present but at much lower frequency.

It appears that the success in achieving a stable synthetic symbiosis with AR37 could also be more dependent on the host specificity. This was illustrated in the *Lolium perenne* work by a large difference (30%) when comparing the proportion of fully stable plants in Bronsyn AR37 (49.9%) with LpD1402N AR37 (19.3%). The number of fully stable plants was also affected by the host cultivar with E142, but again the variation was much lower than the one reported for AR37.

**Table 23**: Interspecies comparison of 100% endophyte infection (complete stability) frequency means of the different *Lolium perenne*, *Lolium multiflorum* and *Festulolium* cultivars.

| Cultivar Species | Cultivar | E142+(%) | AR37+(%) |
|---|---|---|---|
| *L. perenne* | Bronsyn | 77.3 | 49.9 |
| | LpD1402N | 66.2 | 19.3 |
| *Festulolium* | Matrix | 73.2 | 34.3 |
| *L. multiflorum* | Vibe | 68.7 | 29.9 |
| | Tabu | 88.7 | 53.7 |

#### 12.2.2.4 Level 2: Group 3: *Festuca* species

E142 vegetative stability for this group of material was tested at six- and twelve-months post inoculation. As shown in Table 24, all Fp1202N and Easton plants successfully inoculated with E142 were found to be fully stable with all tillers tested found to be infected. No sign of instability was recorded during the post inoculation checks.

**Table 24:** E142 vegetative stability at six and twelve months, post inoculation in cultivars Fp1202N and Easton. Shown as a percentage of plants being fully stable.

| **Cultivars** | **Complete stability at 6 months (%)** | **Complete stability at 12 months (%)** |
|---|---|---|
| **Fp1202N** | 100 | 100 |
| **Easton** | 100 | 100 |

### 12.3 Level 3: Vertical Transmission

#### 12.3.1 Materials and Methods

All inoculated plants which retained some endophyte at the six months post inoculation check (vegetative stability) were hand transplanted into an open pollination block at Templeton New Zealand (Central Canterbury) in Spring 2017. Plants from group one, two and three were separated and planted into open pollination isolation thus avoiding any cross pollination between the two *Lolium* species. Within each group plants from the various treatments were kept in their respective blocks and randomly placed within each replicate.

Each individual plant was bagged by hand once pollination was finished and the seed head had started browning off. The seed was hand harvested from individual plants from mid to late February 2017, dried in a drying bin (30°C/24h), hand threshed and packaged into brown paper envelopes. They were stored at 5°C with 30% relative humidity (RH).

Approximately one month after harvest and threshing, 40 seeds per plant (from all treatments), were hand sown into trays by QuikStart Seedlings 2007 Limited. These were then placed in one of the QuikStart glasshouses and watered as required. Eight weeks after sowing, one tiller per seedling was tested to determine the viable endophyte transmission rate using the tissue immunoblot technique. The viable endophyte infection frequency of E142 vs AR37 was determined to show the vertical transmission rate of each endophyte within the hosts of various genetic backgrounds across species and genera.

The proportion of responding plants was analysed as a generalized linear model with a binomial distribution and a logit link as is standard for this type of data (McCullagh and Nelder, 1989). The dispersion in the analysis of deviance was estimated to allow for any aggregation in the counts. The GLM model was fitted using Genstat 19 (VSN International, 2017). Note analysis of variance was not deemed appropriate for this data because of the varying weights due to the number of plants observed varying between treatment. Difference in numbers originated from the disparity in the inoculation success rate and vegetative stability between the 2 endophyte strains.

The vertical transmission was assessed using two different parameters:

a) the comparison of all E142 vs AR37 plants which had retained endophyte infection at the six-month post inoculation check and,
b) the comparison of only those E142 vs AR37 plants which had shown 100% endophyte infection (complete vegetative stability) at the six-month post inoculation check.

### 12.3.2 Results

#### 12.3.2.1 Level 3: Group 1- *Lolium multiflorum*

a. Parameter 'a' analysis

The endophyte vertical transmission rate of E142 for all *L. multiflorum* cultivars was significantly higher ($p<0.05$) than AR37, 86.8% vs 48.2% respectively (Table 25). Within both individual cultivars E142 displayed a greater vertical transmission rate over AR37. In Vibe, E142 had a vertical transmission rate of 78.5% which was over two times higher than for Vibe infected with AR37 (38.3%). A similar result was observed in Tabu where the E142 vertical transmission rate was 40.3% greater than AR37, 95.2% vs 58.2% respectively.

**Table 25**: Vertical transmission rate of E142 and AR37 for the two *L. multiflorum* cultivars (all infected plants).

| Endophyte | All cultivars E+ (%) | Tabu E+ (%) | Vibe E+ (%) |
|---|---|---|---|
| E142 | 86.8 | 95.2 | 78.5 |
| AR37 | 48.2 | 58.2 | 38.3 |

| F probability (all cultivars) | |
|---|---|
| Endophyte | 0.011 |
| Cultivar x Endophyte | 0.510 |

b. Parameter 'b' analysis

The overall vertical transmission rate for the parameter "b" group (completely stable plant) was superior over the parameter "a" were all plant were included.

The endophyte vertical transmission of E142 endophyte was significantly higher than AR37 ($p<0.05$) in both cultivars combined 96.5% vs 69.1% respectively (Table 26). Within Tabu, E142 displayed as vertical transmission of 98.6% which was 32% higher than Tabu AR37 (66.8%). A similar trend was observed with the cultivar Vibe where E142 had also a considerably higher transmission over AR37, 94.3 vs 71.3 respectively (Table 26).

**Table 26**: Vertical transmission rate of E142 and AR37 for the two *L. multiflorum* cultivars (only 100% infected plants).

| Endophyte | All cultivars E+ (%) | Tabu E+ (%) | Vibe E+ (%) |
|---|---|---|---|
| E142 | 96.5 | 98.6 | 94.3 |
| AR37 | 69.1 | 66.8 | 71.3 |

| F probability (all cultivars) | |
|---|---|
| Endophyte | 0.012 |
| Cultivar x Endophyte | 0.339 |

**12.3.2.2 Level 3 - Group 2: *Lolium perenne and Festulolium loliaceum***

a. Parameter 'a' analysis

The endophyte vertical transmission rate of E142 for the combined *L. perenne* and *Festulolium* cultivars was 24% higher than for AR37; this is a highly significant difference as shown in Table 27 ($p<0.05$).

Within each individual cultivar this experiment shows that E142 has a higher vertical transmission rate over AR37 for Bronsyn, LpD1402N and Matrix (Table 27). In Bronsyn, E142 had transmission rate of 88.5% which was 34% higher than of AR37 (54.9%). In association with LpD1402N, the E142 vertical transmission rate was 18% higher than AR37, 89.6% vs 71.7% respectively (Table 27). A similar pattern was observed with Matrix where the E142 vertical transmission rate was 22% higher when compared with AR37, 76.5 vs 55% respectively.

**Table 27:** Vertical transmission rate of each endophyte strain for the two *L. perenne* and the *Festulolium* cultivars (all infected plants).

| Endophyte | All cultivars E+ (%) | Bronsyn E+(%) | LpD1402N E+(%) | Matrix E+(%) |
|---|---|---|---|---|
| E142 | 84.6 | 88.5 | 89.6 | 76.5 |
| AR37 | 60.5 | 54.9 | 71.7 | 55.0 |

| F probability (all cultivars) | |
|---|---|
| Endophyte | 0.05 |
| Cultivar x Endophyte | 0.86 |

b. Parameter 'b' analysis

As seen with the Lolium multiflorum group the overall vertical transmission rate for the parameter 'b' group (completely stable plant) was superior over the parameter 'a' were all plant were included. This indicates that early instability recorded at six months post inoculation correlates with uncomplete vertical transmission.

The endophyte vertical transmission of E142 endophyte was significantly higher than AR37 ($p<0.05$) in all cultivars combined 99% vs 92.6% respectively (Table 28). Within Bronsyn, the vertical transmission

of E142 was similar to AR37 with only 1% difference between the 2 strains. In association with LpD1402N and Matrix E142 had a higher transmission rate than AR37 of 9% and 11% respectively (Table 28).

**Table 28**: Vertical transmission rate of each endophyte strain for the two *L. perenne* and the *Festulolium* cultivars (only 100% infected plants).

| Endophyte | All cultivars E+ (%) | Bronsyn E+(%) | LpD1402N E+(%) | Matrix E+(%) |
|---|---|---|---|---|
| E142 | 99.0 | 99.0 | 98.1 | 100 |
| AR37 | 92.6 | 100 | 88.9 | 88.9 |

| F probability (all cultivars) | |
|---|---|
| Endophyte | 0.034 |
| Cultivar x Endophyte | 0.405 |

**12.3.2.4 Level 3 - Group 3: Fescue species**

All progeny of the successfully inoculated Fp1202N plants tested positive for endophyte infection resulting in 100% vertical transmission rate. Three plants did not produce viable seed so could not be tested. With Easton the vertical transmission rate averaged 94.6% with a range of 84-100%. One plant that did not produce viable seed so could not be tested (Table 29).

**Table 29**: The vertical transmission rate of the E142 endophyte in association with the two *Festuca* cultivars.

| Cultivars | Vertical transmission rate (%) | Standard error |
|---|---|---|
| **Fp1202N** | 100 | 0 |
| **Easton** | 94.6 | 1.82 |

These results reinforce the intergeneric compatibility of E142 when inoculated into a different genetic background than its original host.

Herbage samples were taken from the innoculated fescue plants (in March 2018 post-harvest) and tested via HPLC analysis for the concentration of epoxy-janthitrems. This was done to ensure that the functionality of E142 was maintained even when the host-endophyte relationship was greatly modified from its original association. Levels of epoxy-janthitrems found in the Fp1202N and Easton material were comparative to the levels observed for E142 in the *Lolium perenne* species. This supports the conclusion that a regular symbiotic association of E142 can be maintained at an intergeneric level.

## 12.4 Conclusion

E142 has displayed a broad intraspecific, interspecific and inter-generic host compatibility when inoculated into material of various genetic backgrounds. It has shown a strong inoculation ability into the species *Lolium perenne, Lolium multiflorum, Festulolium loliaceum, Festuca pratensis* and *Festuca arundinacea*. Following on from this E142 has shown high vegetative stability in these same species at the time point six months post inoculation and has displayed outstanding vertical transmission rates. E142 has been shown to be superior to AR37 at each of these three assessment levels in all of comparitive species.

E142 displays a very high vertical transmission rate at the F0 generation. This is greatly beneficial as E142 can potentially be inoculated into a cultivar with excellent agronomic traits and that cultivar will

not require further lengthy breeding for endophyte stability. This could reduce the time by years between inoculation into a host to a host-endophyte association being ready for sale to the market. This also reduces the risk of losing desirable agronomic traits due to substantial further breeding for improving endophyte stability.

Additionally, E142 has been shown to maintain its functionality in terms of epoxy-janthitrem production when in association with genera outside its natural symbiosis.

The results showed that the endophyte strain E142 has a broader compatibility over AR37 for all *Lolium* and *Festulolium* species used in these experiments. E142 was also successfully inoculated into two other genera than its natural host; *Festuca arundinacea* and *Festuca pratensis*. Broader compatibility is a real commercial attribute because:

i) it increases the range of host species and genera that can be provided to the end user with a valuable endophyte,

ii) it offers a broader genetic base for plant breeders to form their base population to breed superior and improved cultivars,

iii) it increases product reliability and security for the buyers to purchase seed with a good level of viable endophyte.

It will be appreciated by those in the relevant field that modifications to the above examples can be made that will still fall within the scope of the present invention.

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Claims:

1. An endophyte strain producing epoxy-janthitrems with improved stability and compatibility with respect to higher endophyte infection frequency over known commercial epoxy-janthitrem producing strains when grown in association with a variety of grasses such as, but not limited to, perennial, Italian, annual and hybrid ryegrasses, and festulolium hybrids.

2. An endophyte strain producing epoxy-janthitrems, with a broader intra-specific, inter-specific and inter-generic compatibility and stability over known commercial epoxy-janthitrem producing strains when grown in combination with a variety of grasses such as, but not limited to, perennial, Italian, annual and hybrid ryegrasses, festulolium hybrids and fescue species.

3. An endophyte strain according to claim 1 or claim 2 with improved inoculation ability when inoculated into a variety of grasses such as, but not limited to, perennial, Italian, annual and hybrid ryegrasses, and festulolium hybrids.

4. An endophyte strain according to any one of claims 1-3, with improved vegetative stability over known commercial epoxy-janthitrem producing strains when grown in combination with a variety of grasses such as, but not limited to, perennial, Italian, annual and hybrid ryegrasses, and festulolium hybrids.

5. An endophyte strain according to any one of claims 1-4, with improved vertical transmission rates over known commercial epoxy-janthitrem producing strains when grown in combination with a variety of grasses such as, but not limited to, perennial, Italian, annual and hybrid ryegrasses, and festulolium hybrids.

6. An endophyte strain producing epoxy-janthitrems as claimed in any one of claims 1-5, with improved fungicide resistance with respect to higher endophyte infection frequency over known commercial epoxy-janthitrem producing endophyte strains.

7. An endophyte strain producing epoxy-janthitrems with improved fungicide resistance over known commercial epoxy-janthitrem producing endophyte strains as claimed in claim 6 where the fungicide is from the triazole group.

8. An endophyte strain producing epoxy-janthitrems with improved fungicide resistance over known commercial epoxy-janthitrem producing endophyte strains as claimed in claim 6 where preferably the fungicide is from the strobilurin group.

9. An endophyte strain producing epoxy-janthitrems according to claim 6 where the fungicide is from the strobilurin group in combination with fungicide from the triazole group.

10. An endophyte strain according to claim 1 or claim 2 which does not produce detectable levels of loline, ergovaline, peramine and lolitrem B alkaloids, but which does produce varied levels of epoxy-janthitrem compounds in the host grass.

11. A method of increasing the resistance of a host grass to biotic and /or abiotic stress/es by inoculating the grass with an endophyte strain which produces varied levels of epoxy-janthitrem compounds in the host grass due to the presence of an endophyte according to any one of the preceding claims.

12. A host grass produced according to the method of claim 11 in which its association with the endophyte confers improved resistance to biotic and abiotic stresses such as but not limited to insect and pest damages and heat and water stress.

13. An endophyte according to any one of claims 1-10 which comprises a B10 allele size of approximately 162.1bp-162.7bp, a B11 allele size of approximately 126.8bp to 127.1bp.

14. An endophyte according to any one of claims 1-10 or 13 which comprises a NCESTA1CC05, NCESTA1QA09 allele size of approximately 143.6bp and 173.6bp respectively.

15. An endophyte according to any one of claims 1-10, or 13-14 which comprises an ANS054 and ANS056 allele size of approximately 275.4bp and 240.0bp respectively.

16. An endophyte according to any one of claims 1-10, or 13-15 which comprises an ANS033 allele sizes of approximately 134.4bp and 143.9bp.

17. An endophyte according to any one of claims 1-10, or 13-16 which comprises an EF-22 allele sizes of approximately of 130.0bp and 139.4bp.

18. An endophyte according to any one of claims 1-10, or 13-17 which comprises the presence of the genes identified as EfM3.04570, EfM3.044580, EfM3.050800, EfM3.062040.

19. An endophyte according to any one of claims 1-10, or 13-18 which comprises the absence of the genes identified as EfM3.005360, EfM3.062860.

20. The endophyte E142 (*Epichloë festucae var. lolii*).

21. The endophyte E142 found in the *Lolium perenne* accession coded Lp12059 which is accession A22356 in the Margot Forde germplasm center database in Palmerston North, New Zealand.

22. The endophyte E142, deposited on 31/08/2017 at The National Measurements Institute of Australia, accession number VQ17/00179.

23. An endophyte according to any one of claims 1-10 or 13-22 which is in the form of an endophyte culture.

Figure 1.

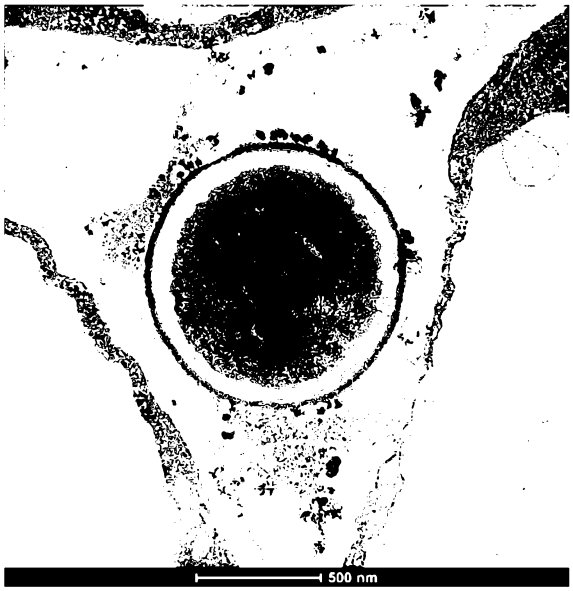


Figure 2.

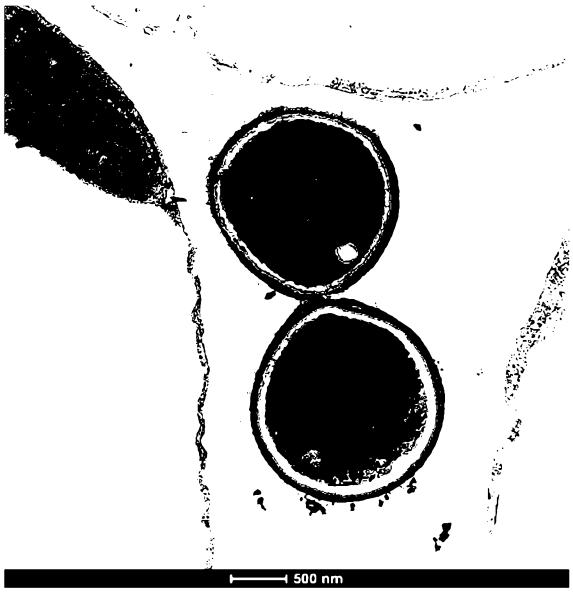

Figure 3.

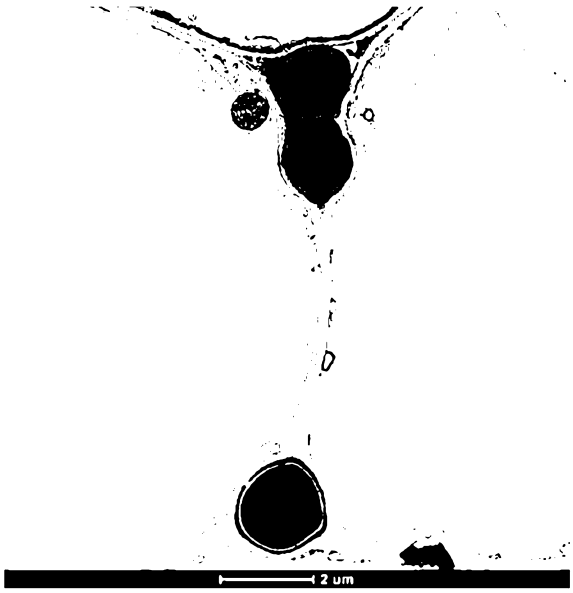


Figure 4.