Resistance to African black beetle (*Heteronychus arator*) in perennial ryegrass (*Lolium perenne* L.) infected with AR1 endophyte

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Abstract

The insect pest, African black beetle (*Heteronychus arator* (Fabricus, 1775)) is of considerable economic cost to the New Zealand agricultural industry, in regions where the insect is established and regular outbreaks are now occurring. Selection for *Epichloë festucae* var. *lolii* (Latch, M.J. Chr. & Samuels) C. W. Bacon & Schardl, stat. nov. et comb. nov. endophyte-perennial ryegrass (*Lolium perenne* L.) associations which do not cause toxicity to livestock and with strong resistance to African black beetle (*H. arator*), would be of significant value to farmers in regions where this pest is a problem.

Epichloë festucae var. *lolii* strain AR1 endophyte does not produce ergovaline, the alkaloid known to deter African black beetle (*H. arator*), yet pastures infected with this endophyte have moderate resistance to adult African black beetle (*H. arator*). Research into AR1 is of importance, as to date there have been no reports of toxicity in livestock brought about by consumption of from AR1-infected pastures and in the absence of African black beetle (*H. arator*) this 'novel' endophyte can provide highly productive pastures.

AR1 endophyte produces secondary metabolites that are simple indole diterpenes, including paxilline and paxilline-like compounds. The numerous paxilline-like compounds produced by AR1 are detected by a paxilline ELISA (enzyme linked immunosorbant assay) and quantified collectively as paxilline immunoreactive equivalents. Earlier work suggested that these paxilline-like compounds could be the bioactive, or linked-marker associated with the bioactive compound(s), that provide some resistance to adult African black beetle (*H. arator*) feeding.

The overall aim of this research was to determine if increased concentration of paxilline immunoreactivity was associated with a reduction in feeding damage using a series of adult African black beetle (*H. arator*) feeding trials on closely related AR1-infected perennial ryegrasses (*L. perenne*).

A negative relationship was established between feeding damage from adult African black beetle (*H. arator*) and plant pseudostem paxilline immunoreactivity in AR1-infected perennial ryegrass (*L. perenne*) post exposure to beetles. However, it was not a simple relationship being complicated by: i) paxilline immunoreactivity

not reflecting endophyte concentrations; ii) the influence of cultivar on the expression levels of paxilline immunoreactivity; iii) the effect of African black beetle (*H. arator*) presence on expression levels of paxilline immunoreactivity; and iv) the paxilline ELISA quantifying the complete complex of paxilline-like compounds and not simply those that are bioactive.

The highest concentrations of paxilline immunoreactivity were found in plant undamaged pseudostem and were not strongly correlated with the lower concentrations found in herbage. Therefore undamaged pseudostem is recommended as the section sampled if the entire plant pseudostem was not available. Low levels of feeding from adult African black beetle (*H. arator*) accentuated plant tiller production. However, the negative effects of high feeding pressure still affected plants four weeks post-exposure to beetles, with reduced paxilline immunoreactivity production and plant tiller numbers. Adult African black beetle (*H. arator*) were able to compensate the deterrent effects of AR1 once an endophyte-free food source was available.

Results from this research contribute towards further understanding the bioactivity of AR1 endophyte against adult African black beetle (*H. arator*) and will underpin further research into the chemical basis for this resistance. The paxilline ELISA could potentially be used for screening *E. festucae* endophyte ryegrass associations that produce unknown compounds which deter African black beetle (*H. arator*) and which do not cause toxicity to livestock.

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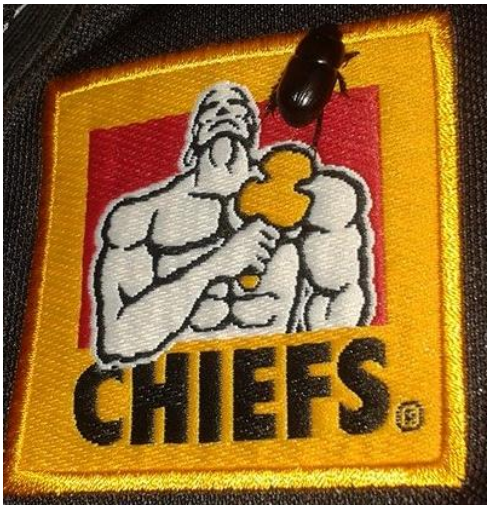

Dedication

This thesis and PhD is dedicated to:

my late father, Gavin John Ross, who always believed in me

to my mother Carole Shirley Ross, who gave her all to support me in this journey

to my partner Reimana, we have struggled and battled through these tough times together, it has only made us stronger and closer

to my beautiful son, Kahurangi for being so wise beyond your years, and for being so helpful, caring, thoughtful and understanding

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Abbreviations

Abbreviation	Definition
1º	primary
2º	secondary
A0	assessment 0
A1	assessment 1
A2	assessment 2
A3	assessment 3
A4	assessment 4
A5	assessment 5
A6	assessment 6
A7	assessment 7
A0–A1	cumulative data from assessment 0 to assessment 1
A0–A2	cumulative data from assessment 0 to assessment 2
A0–A3	cumulative data from assessment 0 to assessment 3
ANOVA	analysis of variance
AR1	An *Epichloë festucae* var. *lolii* novel endophyte strain
-AR1	plant-line infected with AR1 endophyte
AR37	An *Epichloë festucae* var. *lolii* novel endophyte strain (has also been referred to as Lp14)
-AR37	plant-line infected with AR37 endophyte
BL-1	breeding line 1
BSA	bovine serum albumin
BT	back transformed
C	cultivar
CA	California
cELISA	competitive ELISA
CO	Colorado

Abbreviation	Definition
Cum	cumulative
CV	coefficient of variation
df	degrees of freedom
DP	damaged pseudostem
dwt	dry weight
E-	endophyte-free
E+	endophyte-infected
ELISA	enzyme-linked immunosorbent assay
F	F-statistic
F1–F23	half-sibling families 1 to 23
GLMM	generalised linear mixed model
H_2SO_4	sulphuric acid
Herb	herbage
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
-IRE	immunoreactive equivalents
ln	natural logarithm
LpTG-1	*Lolium* taxonomic group 1 (*Epichloë festucae* var. *lolii*)
LpTG-2	*Lolium* taxonomic group 2 (Heteroploid endophyte containing up to two gene sequence variants similar to the single gene sequence variance found in *Epichloë festucae* and *Epichloë typhina*)
LSD or LSD-25	lysergic acid diethylamide (psychedelic drug)
LSD(5%)	Fisher's least significant difference
M1	model 1
M2	model 2
MN	Minnesota
NC-1	non-commerical cultivar 1

Abbreviation	Definition
NC-2	non-commerical cultivar 2
NJ	New Jersey
NZ	New Zealand
P or P-value	probability value
paxilline-IRE	paxilline immunoreactive equivalents
PBS	phosphate buffered saline
PBST	phosphate buffered saline containing 0.05% Tween 20 (v/v)
rdf	residual degrees of freedom
REML	restricted maximum likelihood
SED	standard error of the difference
SEM	standard error of the mean
sqrt	square root
TMB	3,3',5,5'-tetramethylbenzidine
UdP	undamaged pseudostem
USA	United States of America
w BB	with African black beetle treatment
w/o BB	without African black beetle treatment
WT	wild-type

Taxonomy

Binomial name	Synonyms	Common or other name/s
Microorganisms		
Echinodothis tuberiformis (Berk. & Ravenel) G.F. Atk.	*Dussiella tuberiformis* (Berk. & Ravenel) Pat. *Hypocrea tuberiformis* Berk. & Ravenel *Hypocrella tuberiformis* (Berk. & Ravenel) G.F. Atk.	
Epichloë coenophiala (Morgan-Jones & W. Gams) C.W. Bacon & Schardl, comb. nov.	*Neotyphodium coenophialum* (Morgan-Jones & W. Gams) Glenn, C. W. Bacon & Hanlin *Acremonium coenophialum* Morgan-Jones & W. Gams	
Epichloë festucae var. *lolii* (Latch, M.J. Chr. & Samuels) C. W. Bacon & Schardl, stat. nov. et comb. nov.	*Neotyphodium lolii* (Latch, M.J. Chr. & Samuels) Glenn, C.W. Bacon & Hanlin *Acremonium lolii* (as *Acremonium loliae*) Latch, M.J. Chr. & Samuels	LpTG-1 *(Lolium* taxonomic group 1)
Epichloë occultans (C.D. Moon, B. Scott & M.J. Chr.) Schardl, comb. nov.	*Neotyphodium occultans* C.D. Moon, B. Scott & M.J. Chr.	

Binomial name	Synonyms	Common or other name/s
Microorganisms continued		
Epichloë tembladerae (Cabral & J.F. White) Iannone & Schardl	*Neotyphodium tembladerae* Cabral & J.F. White	
Epichloë typhina (Pers.) Tul. & C. Tul.		
Epichloë uncinata (W. Gams, Petrini & D. Schmidt) Leuchtm. & Schardl, comb. nov.	*Neotyphodium uncinatum* (W. Gams, Petrini & D. Schmidt) Glenn, C.W. Bacon & Hanlin	
Neotyphodium chilense (Morgan-Jones, J.F. White & Piont.) Glenn, C. W. Bacon & Hanlin	*Acremonium chilense* Morgan-Jones, J.F. White & Piont.	
Neotyphodium starrii (J.F. White & Morgan-Jones) Glen, C.W. Bacon & Hanlin, nomen dubium	*Acremonium starrii* J.F. White & Morgan-Jones	
Pencillium paxilli Bainier		
Pythium graminicola Subraman.		Crown and root rot disease
Typhula ishikariensis Imai		Winter or speckled snow mould

Binomial name	Synonyms	Common or other name/s
Plants		
Bromus setifolius J. Presl	*Bromus macranthos* E. Desv. (= *Bromus setifolius* var. *brevifolius*)	
Festuca arizonica (Vasey) Hackel ex Beal		Arizona fescue, mountain bunchgrass or pinegrass
Festuca rubra L.		Red fescue
Pennistum clandestinum Hochst. Ex Chiov.		Kikuyu
Lolium arundinaceum (Schreb.) Darbysh.	*Festuca arundinacea* Schreb.	Tall fescue
Lolium giganteum (L.) Darbysh.	*Festuca gigantea* (L.) Vill.	Giant fescue
Lolium multiforum Lam.		Italian or annual ryegrass
Lolium perenne L.		Perennial ryegrass
Lolium pratense (Huds.) Darbysh.	*Festuca pratensis* Huds.	Meadow fescue
Lolium temulentum L.	*Lolium cuneatum* Nevski ex Malzev	Darnel ryegrass
Paspalum dilatatum Poir.		Paspalum
Poa annua L.		Poa, annual meadow grass or annual bluegrass

Binomial name	Synonyms	Common or other name/s
Insects		
Aploneura lentisci (Passerini, 1865)		Root aphid
Balanococcus poae (Maskell, 1879)		Pasture mealy bug
Costelytra zealandica (White, 1846) (Given, 1952)		Grass grub
Graphania mutans (Walker, 1857)		Grey-brown cutworm
Heteronychus arator (Fabricius, 1775)	*Scarabaeus arator* (Fabricius, 1775) *Heteronychus arator* (Burmiester, 1847 (*nec*. Fabricus)) *Heteronychus sanctae-helenae* (Blanchard, 1853)	African black beetle
Listronotus bonariensis (Kuschel, 1955)		Argentine stem weevil
Oscinella frit (Linnaeus, 1758)		Frit fly
Oulema melanopus (Linnaeus, 1758)		Cereal leaf beetles
Spodoptera frugiperda (Smith, 1797)		Fall armyworm
Wiseana cervinata (Walker, 1865)		Porina

Chapter 1

Introduction

1.1 Perennial ryegrass, fungal endophytes and African black beetle

Perennial ryegrass (*Lolium perenne* L.) is the most predominant and intensely grazed pasture in New Zealand (Belgrave *et al.*, 1990; Easton & Tapper, 2005). It is easily established, very productive and highly digestible. In New Zealand perennial ryegrass (*L. perenne*) contains the clavicipitaceous endophytic fungus, *Epichloë festucae* var. *lolii* (Latch, M.J. Chr. & Samuels) C. W. Bacon & Schardl, stat. nov. et comb. nov., formerly *Neotyphodium lolii* (Latch, M.J. Chr. & Samuels) Glenn, C.W. Bacon & Hanlin and *Acremonium lolii* Latch, M.J. Chr. & Samuels (Leuchtmann *et al.*, 2014) (as *Acremonium loliae*), which causes toxicosis in grazing livestock (Fletcher *et al.*, 1999; Easton & Tapper, 2005) but is essential for plant persistence in pastures through tolerance and resistance to invertebrate pests (Prestidge & Ball, 1993; Easton & Tapper, 2005). The last four decades of endophyte research have primarily focused on endophytes present in forage and turf grasses due to their agronomic significance and importance. This includes: detrimental effects on grazing livestock, effects on host plant responses to biotic and abiotic stresses and effects on the biodiversity and trophic interactions in wild populations (Roberts *et al.*, 2005). The endophyte *Epichloë coenophiala* (Morgan-Jones & W. Gams) C.W. Bacon & Schardl, comb. nov. (formerly *Neotyphodium coenophialum* (Morgan-Jones & W. Gams) Glenn, C. W. Bacon & Hanlin and *Acremonium coenophialum* Morgan-Jones & W. Gams) (Leuchtmann *et al.*, 2014) of tall fescue (*Lolium arundinaceum* (Schreb.) Darbysh., formerly *Festuca arundinacea* Shreb.) and *E. festucae* var. *lolii* of perennial ryegrass (*L. perenne*) are the two most extensively researched endophytes due to their agronomic importance particularly in the United States of America (USA) and New Zealand (NZ), respectively.

The anamorphs of *Epichloë* species previously described as genus *Neotyphodium* (Leuchtmann *et al.*, 2014), infect cool-season grasses (C3 grasses) in the subfamily

Pooideae. The feature that distinguishes the anamorphs of *Epichloë* endophytes from the other clavicipitaceous fungi is their inability to reproduce sexually, and instead they 'reproduce' (propagate) by vertical transmission via the host seed. They are obligate biotrophic endosymbionts, solely reliant on their host grass for survival and reproduction. The symbiont-host grass relationship is generally accepted as being a defensive mutalistic association with the fungus providing its host with protection against biotic and abiotic stress through the production of secondary metabolites, known as alkaloids. The wild-type *E. festucae* var. *lolii* of perennial ryegrass (*L. perenne*) that was introduced into New Zealand produces the following secondary metabolites: ergovaline, lolitrems and peramine. Wild-type *E. festucae* var. *lolii* causes mammalian toxicity, which is attributed mainly to two classes of alkaloids, the ergot alkaloids (ergovaline) and the indole diterpenes (lolitrems). Ergovaline is responsible for heat stress in animals and the lolitrems for ryegrass staggers. Peramine is not known to be toxic to grazing animals, but is a potent feeding deterrent to Argentine stem weevil (*Listronotus bonariensis* (Kuschel, 1995)). The ergot alkaloids and indole diterpenes are known to demonstrate anti-insect activity and toxicity (Ball *et al.*, 1997a; Bush *et al.*, 1997; Byrne *et al.*, 2002). Endophyte strain, plant genotype, tissue type, season, plant age and abiotic and biotic stresses influence alkaloid profile and concentrations.

In New Zealand, selected 'novel' *E. festucae* var. *lolii* strains are used in commercial cultivars and do not produce, or produce less of, the alkaloids known to be toxic to mammalian animals, whilst still maintaining anti-insect activity against Argentine stem weevil (*L. bonariensis*), which is a major pasture pest. The novel endophyte AR1 was a major step forward in overcoming mammalian toxicity. It produces peramine, a potent Argentine stem weevil (*L. bonariensis*) deterrent but not lolitrems or ergovaline, the major causative agents of mammalian toxicity. AR1-infected ryegrass has been extensively researched and is considered robust, with well-defined codes of practice for pasture establishment and management. It is considered a major success story, being widely adopted by the farming community and is now the benchmark for the New Zealand pastoral industry.

African black beetle (*Heteronychus arator* (Fabricius, 1775)) is a major pest of regions in the upper and mid-west and mid-east of the North Island. Although regarded as a sporadic pasture pest, in some regions it is a significant and regular

problem and compromises the survival and productivity of AR1-infected cultivars. AR1 lacks any of the known alkaloids that deter adult African black beetle (*H. arator*), but shows moderate resistance to this insect. Testing of a range of AR1-infected ryegrass cultivars indicated a plant genotype influence on resistance to adult African black beetle (*H. arator*). Selection of AR1 cultivars with optimal resistance to African black beetle (*H. arator*) would be of significant value to both plant breeders and farmers in regions where this pest is a problem.

1.2 Perennial ryegrass – one of the 'true grasses'

1.2.1 Classification of perennial ryegrass

The two most commonly used classification systems for flowering plants are the morphologically based Cronquist system (Cronquist, 1988), and the more recent phylogenetically based Angiosperm Phylogeny Group classification system (APG II) (APG II 2003 *et al.*, 2003). APG II is a modern cladistic classification system; its predecessor was the APG system. Table 1-1 shows the classification of perennial ryegrass (*L. perenne*) using the APG II & Cronquist systems.

Monocotyledons or monocots are one of two major groups of flowering plants (angiosperms) that are traditionally recognised. The second group is dicotyledons or dicots. Monocot seedlings typically have one cotyledon (embryonic leaf in their seed), in comparison to the dicots which have two cotyledons. Monocots comprise the majority of biomass produced in agriculture (Campbell, 1996). There are approximately 59,000 species within this group (Mabberley, 1993). The most economically important family group among all plants is Poaceae (Constable, 1985; Raven & Johnson, 1995; Bremer, 2002). The Poaceae family includes all the true grains (rice, wheat, maize etc.), the bamboos, sugar cane and the pasture grasses (Constable, 1985). Plants from this family are called 'true grasses' because the term 'grass' is also applied (although incorrectly) to many grass-like plants that are not members of the Poaceae family, including rushes (Juncaceae) and sedges (Cyperaceae). Plant communities dominated by the 'true grasses' are called grasslands and it is estimated that grasslands comprise 20% of the vegetation cover of the earth (Constable, 1985). Poaceae grasses also occur in many other habitats

Table 1-1: Classification systems for perennial ryegrass.

AGP II		Cronquist	
Kingdom:	Plantae	Kingdom:	Plantae
(unranked):	Angiosperms	Phylum/Division:	Tracheophyta (vascular plants)
(unranked):	Monocots	Subphylum:	Magnoliophytina (flowering plants, angiosperms)
(unranked):	Commelinids	Class:	Lilopsida (Monocotyledons)
Order:	Poales	Order:	Cyperales (sedges and grasses)
Family:	Poaceae	Family	Poaceae (Gramineae)
Subfamily:	Pooideae	Subfamily:	Pooideae
Tribe:	Poeae	Tribe:	Poeae
Genus:	*Lolium*	Genus:	*Lolium*
Species:	*Lolium perenne*	Species:	*Lolium perenne*
Binomial Name:	*Lolium perenne* L.	Binomial Name:	*Lolium perenne* L.

including wetlands, forests and tundra. They have evolved to become highly specialised for wind pollination. They produce much smaller flowers, which are gathered in highly visible plumes (inflorescences) (Kuldau & Bacon, 2008). Compared to dicots, grasses lack the biosynthetic capacity for the production of secondary metabolites which are useful in the long-term survival strategy of each species (Kuldau & Bacon, 2008). Some grasses overcome this problem by cohabitating with microorganisms which produce these secondary metabolites (Kuldau & Bacon, 2008). Most of the grasses divide into two physiological groups, using the C3 and C4 photosynthetic pathways for carbon fixing. The C3 grasses are referred to as 'cool-season grasses' and the C4 grasses as 'warm season grasses'. All grasses can be annual or perennial (lasts for more than two growing seasons).

1.2.2 The genus *Lolium*

Ryegrass (*Lolium*) is a genus of tufted grasses native to Europe, Asia and northern Africa but widely cultivated and naturalised globally. Ryegrasses are naturally diploid and are described as being closely related to the fescues (*Festuca*) and some

of the fescues (*Festuca*) have now been reclassified as *Lolium* species (*L. arundinaceum* (tall fescue) = *F. arundinacea*, *Lolium giganteum* (L.) Darbysh. (giant fescue) = *Festuca gigantea* (L.) Vill, *Lolium pratense* (Huds.) Darbysh. (meadow fescue) = *Festuca pratensis* Huds.). Perennial ryegrass (*L. perenne*) is a low-growing, tufted, hairless, cool-season grass found often infected with fungal endophytes in their native environments. Asexual *Epichloë* endophytes in particular are associated with several species of cool-season grasses, particularly those belonging to the *Lolium* genera. The most common asexual *Epichloë*-infected species of *Lolium* are tall fescue (*L. arundinaceum*) and perennial ryegrass (*L. perenne*) (Roberts *et al.*, 2005). Cultivated varieties of these two grasses are widely grown in temperate climates around the world as forage grasses for cattle, sheep and horses, turf and conservation uses (Roberts *et al.*, 2005). Cultivated varieties of perennial ryegrass (*L. perenne*) are the predominant pasture grass in New Zealand. It is considered to be the most productive pasture and can be intensively grazed.

1.3 Endophytic fungi

1.3.1 General description and traditional classification

An endophytic fungus is a fungus that lives inside another plant and it may or may not be a parasite of its host plant. Fungal endophytes are endosymbionts, i.e., they live within a plant for at least part of their lifecycle without causing apparent disease. An endosymbiont is any organism that lives within the body or cells of another organism forming an endosymbiosis. In an endosymbiotic relationship, one organism lives inside the body of another and both function as a single organism. Symbionts that are vertically transmitted (in the case of plants, via the seed) should evolve a more benign relationship with their host than those that are not vertically transmitted, as any net benefit they have on host fitness would likely promote their own transmission (Ewald, 1987). In particular, the association of cool-season grasses (family Poaceae, subfamily Pooideae) and the *Epichloë* endophytes, formerly known as the *Epichloë-Neotyphodium* group (Leuchtmann *et al.*, 2014) of fungi appear to demonstrate this and occupy a unique place in the realm of host-symbiotic relationships.

Historically, two types of endophytic fungi have been recognised; the clavicipitaceous and the nonclavicipitaceous endophytes, based on phylogeny and

life history traits (Rodriguez *et al.*, 2009). In their review Rodriguez *et al.* (2009) classified the clavicipitaceous endophytes as Class 1 endophytes and the nonclavicipitaceous as Classes 2, 3 & 4.

Unlike mycorrhizal fungi that colonise plant roots and grow into the rhizosphere, endophytes reside entirely within the plant tissues and may grow within roots, stems and/or leaves emerging to sporulate at plant or host tissue senescence (Sherwood & Carroll, 1974; Carroll, 1988; Stone *et al.*, 2004). The clavicipitaceous endophytic fungi (classification shown in Table 1-2) represent a small number of phylogentically related species that are fastidious in culture and limited to some cool- and warm-season grasses (Bischoff & White Jr., 2005).

Table 1-2: Classification of Clavicipitaceae fungi.

Classification Level	Name
Kingdom	Fungi
Division/Phylum	Ascomycota
Subdivision	Pezizomycotina
Class	Sordariomycetidae
Subclass	Hypocreomycetidae
Order	Hypocreales
Family	Clavicipitaceae

The Hypocreales lineage is well known for plant pathogens, saprotrophs and endophytes of which many produce bioactive compounds (Rodriguez *et al.*, 2009). Diehl (1950) divided the Clavicipitaceae family into three subfamilies; Clavicipitoideae, Oomycetoideae and Cordycipitoideae. Within the Clavicipitoideae subfamily, Diehl (1950) further divided it into three tribes (Clavicipiteae, Balansiae and Ustilaginoideae). Since then the Clavicipitaceae family has been reassessed and divided into four divisions (tribes) (Kuldau *et al.*, 1997). Using this common classification for Clavicipitaceae the four tribes are:

1. Ustilaginoidieae (genus *Ustilaginoidea*): A tropical tribe that form hardened stromata on stems.

2. Clavicipeae (genus *Claviceps*): A tribe that infect florets of grasses and replace the host ovules with mycelia that develop into sclerotia (Tydzynski *et al.*, 1995; Kuldau *et al.*, 1997) and parasitises a wide range of grasses.
3. Balansieae: This is the most diverse tribe, consisting of several genera, including the *Epichloë* (which now includes the previously described *Neotyphodium* genus (Leuchtmann *et al.*, 2014), which included most asexual *Epichloë* descendants), *Echinodothis, Balansia, Atkinsonella and Myriogenospora* genera.
4. Cordycipieae (genus *Cordyceps*): This tribe are pathogens of insects and fungi.

The first three tribes are biotrophs of grasses (Poaceae) or sedges (Cyperaceae) (Kuldau *et al.*, 1997).

1.3.2 Recent cladistic classifications using molecular techniques and phylogenetics

Using molecular techniques and phylogentic analyses, the theory of monophyly (a clade, consisting of a single ancestor and all its descendants) of the family Clavicipitaceae has been both supported and rejected by recent authors. Sung *et al.* (2007) state that the discrepancies in the numerous phylogenetic analyses of Clavicipitaceae species carried out to resolve the theory of a monophyletic family, have arisen because these studies had limited taxon sampling that did not cover the morphologicial and ecological diversity of the family. Kuldau *et al.* (1997) support the theory of monophyly, whereas Sung *et al.* (2007), Spatafora *et al.* (2007) and Tanaka and Tanaka (2008) reject it. Leuchtmann *et al.* (2014) propose that all previously described *Neotyphodium* species (with two exceptions) be synonymised under the monophyletic genus *Epichloë*.

1.3.2.1 Work of Kuldau et al. (1997)

The phylogenetic results of Kuldau *et al.* (1997) from the analyses of 28S rDNA sequences supported the theory of monophyly. Kuldau *et al,* (1997) distinguished four clades among the plant-associated Clavicipitaceae: *1)* Monophyletic genus *Epichloë* and related asexual species*; 2)* Monophyletic genus *Claviceps*; *3) Echinodothis tuberiformis* (Berk. & Ravenel) G.F. Atk., the only representative; *4)*

Ephelis anamorphs (the teleomorphic genus *Balansia* probably being paraphyletic with other genera possessing *Ephelis* anamorphs).

1.3.2.2 Work of Sung et al. (2007) and Spatafora et al. (2007)

Sung *et al*. (2007) conducted a series of phylogenetic analyses (multi-gene) with seventy representatives from Clavicipitaceae to test the monophyly of the family. Their results provided evidence for rejecting monophyly in favour of a paraphyly, with Clavicipitaceae comprised of three clades (Clavicipitaceae clades A, B and C). Clavicipitaceae clade A consisted of four subclades (A1–A4). Species of the subfamily Clavicipitoideae (*Claviceps, Balansia, Epichloë* and *Neotyphodium*) formed subclade A1; clade B consisted of three major subclades (B1–B3); clade C consisted of two major subclades (C1–C2). Common to all three clades were species from the genus *Cordyceps*.

Spatafora *et al.* (2007) proposed from results of their multi-gene phylogenetic analyses and ancestral character state reconstruction that their data did not support the monophyly of Clavicipitaceae. In this study Spatafora *et al.* (2007) divided Clavicipitaceae into three distinct clades (Clade A, B and C). Clade A contained three subclades, the paraphyletic endophytic fungi (including *Epichloë and Neotyphodium*) and two additional subclades which included two major groups of arthropod pathogens. Clades B and C also contained numerous taxa that are pathogens of animals including arthropods and nematodes. All three clades contained pathogens of fungi. Spatafora *et al.* (2007) found species of the genus *Cordyceps* in all three clades as did Sung *et al.* (2007).

The results of their study support the theory that the endophytic fungi (grass symbionts) in the Clavicipitaceae family are a group that were derived from an animal pathogen through a dynamic process of interkingdom host jumping. Although the endophytic fungi were traced back to a plant-associated common ancestor, the data support the theory that the common ancestor of clade A was associated with an animal. Therefore the common ancestor of the Clavicipitaceae endophytic fungi (grass symbionts) likely originated via an interkingdom host jump from animals to plants. With the host shift from animal to plants the biochemical arsenal of endophytes was likely to already be in place for defence against animal herbivores (Clay, 2009).

Jumps to new hosts among organisms closely associated in a common habitat have been referred to as the "host habitat hypothesis" (Nikoh & Fukatsu, 2000). This hypothesis was put forward to explain shifts to distantly related hosts of Hypocreales (Nikoh & Fukatsu, 2000; Spatafora *et al.*, 2007).

1.3.2.3 Work of Tanaka and Tanaka (2008)

More recently, Tanaka and Tanaka (2008) have suggested a new classification of the Clavicipitaceae from their phylogenetic results from the analyses of Exon 3 nucleotide sequences of the ALDH1-1 gene. Their results also reject the monophyly of Clavicipitaceae. They have divided Clavicipitaceae into two groups, the paraphyletic group (Cordyceps-Nomuraea-Ustilaginoidea) and a monophyletic group (Clavicipitoideae). The monophyletic group was further divided into three clades (*Aciculosporium-Claviceps, Epichloë-Neotyphodium* and *Parepichloë-Heteroepichloë-Ephelis*).

1.3.2.4 Work of Leuchtmann et al. (2014)

In 2011, at the 18th International Botanical Congress in Melbourne, Australia, nomenclatural rule changes were adopted, with the principle stating a single name should be used for each fungal species (McNeill *et al.*, 2012). The genus *Neotyphodium*, which consists of the anamorphs of *Epichloë* species and includes most asexual *Epichloë* descendants, is proposed to be realigned and classified in to the *Epichloë* genus (Leuchtmann *et al.*, 2014) in line with nomenclature rule changes at the 18th International Botanical Congress (2011). Leuchtmann *et al.* (2014) have re-examined the classification of several described *Epichloë* and *Neotyphodium* species and varieties, proposed new combinations and names, resulting in the realignment of *Neotyphodium* species within genus *Epichloë*. They accept 43 unique taxa in *Epichloë* including distinct species, subspecies and varieties, and excluding two taxa *Neotyphodium starrii* (J.F. White & Morgan-Jones) Glen, C.W. Bacon & Hanlin (formerly *Acremonium starrii* J.F. White & Morgan-Jones) and *Neotypodium chilense* (Morgan-Jones, J.F. White & Piont.) Glenn, C. W. Bacon & Hanlin (formerly *Acremonium chilense* Morgan-Jones, J.F. White & Piont.). *Epichloë festucae* var. *lolii*, formerly *N. lolii* and *A. lolii* (as *A. loliae*) has been included in this realignment and renaming (Leuchtmann *et al.*, 2014).

1.3.3 Summary of the classification of Clavicipitaceae

Classification of fungi within the Clavicipitaceae family is under constant review, refinement and reassessment as new phylogenetic classification tools evolve and new studies are completed, especially in the area of molecular genetics. In all of these studies it is apparent that the *Epichloë–Neotyphodium* group of fungal endophytes are unique and form a distinct grouping within the various proposed classifications. The *Epichloë–Neotyphodium* group of fungal endophytes have been realigned and renamed under the single monophyletic genus *Epichloë* (Leuchtmann *et al.*, 2014), in the family Clavicipitaceae. This was done in line with rule changes in the International Code of Nomenclature for algae, fungi and plants adopted at the 18th International Botanical Congress (2011).

1.4 Endophytic Clavicipitaceae – The genus *Epichloë*

1.4.1 Lifecycles and types of endophytic Clavicipitaceae

White Jr. (1988) first proposed three types of clavicipitaceous endophytic fungi; symptomatic and pathogenic, sexual life cycle (Type I), asymptomatic, asexual life cycle (Type III) and mixed, sexual and asexual life cycles (Type II). The *Epichloë* genus of fungal endophytes in the clavicipitaceous family (Clavicipitaceae, Ascomycota), are symbionts of temperate grasses (subfamily Pooidaea). The *Epichloë* genus is composed of haploid species that all undergo an external contagious stage. When the grass produces flowers, the fungus grows over the developing inflorescence to form stroma. The inflorescence primordium remains at an arrested stage of development within the fungal mycelium, and development of the seed head is prevented (choke disease). The spermatia of the fungus are vectored between compatible mating types of the fungus by 'pollinating' symbiotic flies (genus *Botanophila* Lioy), which lay eggs on the fungal stroma and the fly larvae feed on the post-fertilised mycelium of the fungus (Bultman & White 1988). The fungus depends on the fly stage to complete its sexual mating cycle and the fly larvae depends on the fungus for nourishment.

Some *Epichloë* species, defined as Type II endophytes by Clay and Shardl (2002), exhibit stromata only in a proportion of the tillers, allowing partial seed production and thus vertical transmission within seeds; while others (Type I endophytes)

produce stromata on all (or most) of the tillers (any tillers that do not exhibit stromata do not contain the fungus) (White Jr., 1988; Rodriguez *et al.*, 2009). At least nine different sexual species are recognised in the *Epichloë* genus. This includes *E. festucae* a natural endophyte of *Festuca* species (Leuchtmann *et al.*, 1994; Scott *et al.*, 2005). This endophyte is also capable of forming compatible associations with perennial ryegrass (*L. perenne*) (Christensen *et al.*, 1997; Scott *et al.*, 2005). The ability of *E. festucae* to infect *L. perenne* aligns with the subsequent reclassification of some host plant *Festuca* species as *Lolium* species.

The *Epichloë* genus has evolved a diversity of asexual forms in cool-season grasses in the subfamily Pooideae (Schardl, 1996). Glenn *et al.* (1996) reclassified the asexual genus *Acremonium* to the genus *Neotyphodium. Neotyphodium* species, using the Clay and Shardl (2002) classification, are referred to as type III endophytes reproducing only by vertical transmission via the host seed. Leuchtmann *et al.* (2014) realigned the nomenclature of *Neotyphodium* species with the genus *Epichloë.* These asexual *Epichloë* species are obligate biotrophic endosymbionts. The occurrence of regular vertical transmission via the host seed is unique to the *Epichloë* genus.

1.4.2 History of the Clavicipitaceae endophytes

Clavicipitaceous endophytes were first studied in Europe in plants in the late 19th century (Guerin, 1898; Hanausek, 1898; Vogl, 1898). Vogl (1898) first reported the presence of fungal mycelium in plants of darnel ryegrass, *Lolium temulentum* L., that showed no external signs of infection. *L. temulentum* was considered to be toxic and with the discovery of the fungus it was theorised that the presence of the fungus was possibly the cause of the toxicosis. Freeman (1902, 1903, 1904) conducted detailed studies of the endophyte of darnel and noted the presence of the fungal mycelium in the seeds of *L. perenne*. The darnel endophyte has only recently been identified, originally as *Neotyphodium occultans* C.D. Moon, B. Scott & M.J. Chr. (Moon *et al.*, 2000) now renamed *Epichloë occultans* (C.D. Moon, B. Scott & M.J. Chr.) Schardl, comb. nov. (Leuchtmann *et al.*, 2014). Neill (1940) reported that hyphae are intercellular and infected host plants show no external symptoms of infection. The fungus can only be transferred/spread by the transmission of the endophyte via the seed of an infected ryegrass plant (vertical transmission)

(Sampson, 1935; Neill, 1941). Bacon *et al.* (1977) linked the endophyte *E. coenophiala* (Leuchtmann *et al.*, 2014), to the widespread occurrence of 'summer syndrome' toxicosis in cattle grazing tall fescue (*L. arundinaceum*) pastures, confirming the early hypothesis of toxicosis linked with animals ingesting endophyte-infected grasses.

Sampson (1933) noted that the endophytes of *L. perenne* and *L. temulentum* were very similar to the asexual stage of *Epichloë typhina* (Pers.) Tul. & C. Tul., infecting many species of Poaceae (Gramineae). Sampson (1933) also reported that for a large part of the year, *E. typhina* existed in an anamorph state as a symptomless intercellular mycelium within the host plant, which he termed 'latent infection' and drew a comparison with the 'non-choke inducing' endophytes of perennial ryegrass (*L. perenne*). White Jr. (1988) also noted this and suggested the asexual *Epichloë* endophytes probably evolved from the 'choke inducing' endophytes that had an anamorphic stage.

The origin of the asexual *Epichloë* species indicates that they may be due to both clonal and interspecific hybridizations with the sexual *Epichloë* species (Moon *et al.*, 2002; Schardl *et al.*, 2004). Hybrid origins are inferred from phylogenetic analyses where there is the tendency for multiple loci of isozymes that are a single copy in the sexual *Epichloë* species. All but *E. festucae* var. *lolii* are apparent interspecific hybrids (Scott *et al.*, 2005). New endophyte species have been based, in part, on their hybrid origins and relationships to the sexual *Epichloë* species. *Epichloë festucae* var. *lolii* is a haploid asexual derivative of *E. festucae* (Christensen *et al.*, 1993; Schardl *et al.*, 1994) and is the predominant endophyte of *L. perenne*.

1.4.3 Distribution of the Clavicipitaceous endophytes in the plant host

The *Epichloë* genus are biotrophic fungi that systemically colonise the intercellular spaces of leaf primordia, leaf sheaths and leaf blades of vegetative tillers and the inflorescence tissues of reproductive tillers (Scott *et al.*, 2005). In the sexual stage, the fungus prevents the growth of the flowering structure of the host plant (choke disease). Endophytic mycelia are systemic throughout the above-ground portions of the grass plant because they proliferate in the shoot meristem and are situated in the intercellular places of the newly forming aerial plant parts. The fungi do not

colonise root meristems, and roots typically lack these fungi (Rodriguez *et al.*, 2009).

The asexual *Epichloë* species have lost the capacity for development of the sexual stage. In these species no obvious symptoms of infection are observable at any stage of plant development. These fungi are endophytes of leaves, culms and rhizomes, and they frequently colonise inflorescence primordia. As inflorescences develop the mycelium grows into ovules, and within seeds it colonises the scutellum and embryo axis (Philipson & Christey, 1986) before germination (Rodriguez *et al.*, 2009). Asexual *Epichloë* endophytes are transmitted vertically through seeds and by clonal reproduction of host plant through vegetative propagation of new plants (Clay, 1986; Clay & Kover, 1996; Clay & Schardl, 2002). Many retain an epiphyllous mycelium where conidia form, suggesting the potential for horizontal transmission (Rodriguez *et al.*, 2009) and most produce conidia in culture. Tadych *et al.* (2007) demonstrated that epiphyllous conidia are released from conidiophores only in water, suggesting they may spread among neighbouring plants via rain or dew. The likely, but unconfirmed, sites of infection are tillers, where the fungi colonise meristems epiphyllously in plant crowns. Colonisation of new tillers may then occur, possibly resulting in the eventual infection of some ovules and seeds. The original mature plant tissues of neighbouring plants would not bear endophytic mycelium as clavicipitaceous endophytes grow rapidly in nutrient-rich meristematic tissue but show limited capacity to grow through mature plant tissue (Western & Cavett, 1959; White Jr. *et al.*, 1991). If horizontal transmission to a neighbouring plant that already contained an endophyte occurred, there is the potential for interspecific hybridization that is a feature of the phylogenetic analysis of asexual *Epichloë* endophytes (Tadych *et al.*, 2009).

Christensen *et al.* (2008) have published evidence that the *Epichloë* fungal endophytes grow by two methods in the host plant. Most significantly they have described how the fungal endophytes grow by intercalary hyphal extension in elongating grass leaves. Secondly they have shown that the fungal endophytes grow by hyphal division and expansion at the apex, forming heavily branched mycelium amongst dividing plant cells in the meristem. In intercalary extension, the hyphae are attached to enlarging host cells, and cumulative growth along the length of the filaments enables the fungus to extend at the same rate as the host. When plant cells

divide and then enlarge to form the leaf, hyphal compartments are subjected to stretch, but this stress is relieved by intercalary extension accompanied by cellular division along the length of the filament. Christensen *et al.* (2008) suggest that intercalary extension may be activated by this stretching because endophyte expansion ceases once the hyphae emerge beyond the leaf expansion zone (Tan *et al.*, 2001). Also, hyphae in mature tissue are no longer growing but still remain metabolically active (Tan *et al.*, 2001). Fungal growth parallels development of the adjacent leaf tissue which also undergoes intercalary extension. Paralleled intercalary extension allows for synchronised growth of the endophyte and host. It explains the two different fungal morphologies found in the host – a heavily branched mycelial net in the plant meristematic zones where plant cells are rapidly dividing but not expanding; and the intercellular hyphae in the leaf expansion zone, oriented in the direction of plant growth and following the longitudinal axis of the leaf. The proposed model also explains the inability of endophytes to infect mature tissues that are no longer growing in plants. It also accounts for the fixed number of hyphae present in mature leaves and that the number of hyphae in old and new sheaths is similar. It is unknown if this mechanism of growth is unique to the *Epichloë* endophytes (Christensen *et al.*, 2008).

1.4.4 Costs and benefits of the endophyte-plant host symbiotic relationship

1.4.4.1 Defensive Mutualism

Evolutionarily, symbioses exist because of enhanced fitness in at least one of the participant populations. If the fitness of the host population is reduced, the association may be characterised as ‘parasitism’ or ‘pathogenicity’; if fitness is not affected, as commensalism; if fitness is enhanced, as mutualism (Douglas, 1994). Where the endophyte-plant host symbiotic relationship lies on this parasitic-mutualistic continuum is variable and dependent on the endophtye type and association (type I, II or III) and the environmental factors and stressors at any one place and time. The fungal endophyte-plant host symbiotic relationship is generally described as mutalistic (Clay, 1988b, 1988a). Clay (1988a) coined the term ‘defensive mutualism’ for the endophyte-plant host symbiotic relationship. The primary benefit to the host is the defence against insect and mammalian herbivory

via the production of secondary metabolites. The defensive mutualism hypothesis now includes any modification or enhancement in host physiology that enables the hosts to better tolerate stress of any origin and survive and reproduce as a result of it (White Jr. & Torres, 2009). The host plant provides the fungus with nourishment, protection from environmental extremes and predation. The host plant receives in return, protection from certain biotic and abiotic stresses. Effects of asexual *Epichloë* species on grazing animals (Ball, 1997; Fletcher *et al.*, 1999) and on over forty species of insects (Breen, 1994; Clement *et al.*, 1994; Popay & Rowan, 1994) provide plenty of support for the defensive mutualism hypothesis.

Wild and native grasses are frequently infected with *Epichloë* group endophytes. In natural populations the proportion of plants infected ranges from low to 100%. This suggests that there is no consistent increase in plant host fitness that can be attributed to the presence of the *Epichloë* endophytes. Where high infection levels in native populations are observed, this suggests a selective advantage over uninfected plants (Wei *et al.*, 2006). There are exceptions to the defensive mutualism theory and in some cases a high endophyte infection level in a host plant population has not been found to increase the fitness of the population. For example, no resistance to herbivory or plant pathogens account for the high frequency of asexual *Epichloë* endophyte infection (80%) in a native grass population of Arizona fescue (*Festuca arizonica* Vasey) and infection was found to generally decrease the host fitness (Faeth & Sullivan, 2003). These authors speculate that the infection is parasitic rather than mutualistic. In another study looking at abiotic factors (different watering and nutrient regimes), the authors concluded that the costs outweighed the benefits to the plant hosting an endophyte in a resource-limiting environment (Ahlholm *et al.*, 2002). In some studies the endophyte infection levels of a population showed spatial patterns, directly in response to grazing pressure of herbivores (Koh & Hik, 2007). Other studies, that tracked endophyte infection levels over time, found that infection levels in populations increased over time (Neill, 1940; Lloyd, 1959; Clay *et al.*, 2005). Levels of endophyte-infection in host populations can be a direct response to environmental factors (Constable, 1985; Lewis, 2000; White Jr. *et al.*, 2001). A stable, decreasing or low endophyte-infection level in a host population does not necessarily reject the defensive mutualism hypothesis as it may just reflect where the endophyte-host association is

situated on the parasitic-mutualistic scale (parasitic, commensalistic or mutualistic) and which way the association is currently heading (Popay, 2009). Especially noted in the literature is herbivory pressure and how it can affect endophyte infection levels in host populations (White Jr. *et al.*, 2001; Li *et al.*, 2007; Palmer *et al.*, 2008).

In cultivated populations the selective advantages are often very obvious and the selective disadvantages unwanted. This was the case with pastures and turf grasses whereby endophyte-infected plants were (and still are) used as they gave insect resistance even though they had adverse effects on livestock. Initially, before the role of the endophytes was known, the endophyte-infected state was unwittingly selected for in both the USA and New Zealand because it gave better yield and persistence. Saikkonen *et al.* (1998, 2006) disagree with the defensive mutualism theory, suggesting that the data supporting this come from agronomic, cultivated forage and turf grasses that have been artificially selected. They point out that defensive mutualism is not often seen in native grass populations. Agronomic (cultivated) grasses and the endophytes used in these associations were originally isolated from natural populations (Clay, 1990). Where the endophyte-host plant symbiosis lies on the parasitic-mutualistic continuum is depicted by environmental factors and pressures (stressors) at any given time and place. The association is not fixed; instead it is able to slide along this scale depending on circumstances. This dynamic process needs to be applied when considering the costs and benefits shaping the endophyte-host symbiosis association (Bronstein, 2001).

Therefore, grass populations maintaining heterogeneity of endophyte-infected and endophyte-free individuals will evolutionarily have considerable advantages to the species (not an individual) (Popay, 2009). Frequency-dependent selection (Ayala & Campbell, 1974) can be seen in the population dynamics of many insect herbivores affected by endophtye-infection of host plants. Insect populations are characterised by short periods of high density resulting in severe damage to their host plants followed by long periods of low density (Varley *et al.*, 1973; Berryman, 1987; Abbott & Dwyer, 2007). These outbreaks may be localised or widespread. During periods of low insect density the proportion of endophyte-infected plants in the population may reduce. This reduction would be radically reversed during an insect outbreak. In a native grass population, the outbreak may be partly triggered by the availability of the insect's food source, the endophyte-free host plants. The

outbreak would initially significantly reduce the total plant population as the endophyte-free plants are decimated, resulting in an increase in the proportion of endophyte-infected host plants. This would then be followed by high numbers of endophyte-infected host plants in the recovering population as a result of the original surviving endophyte-infected plants reproducing. This high level of endophyte-infection in the host plant population would cause the insect population to decline as their food resource (endophyte-free host plants) is now reduced. Insect numbers would return back to low levels as long as there were no alternative food sources available. Once the insect density had returned to low enough levels, the selective advantage of a plant hosting an endophyte would have decreased, possibly even to the point of being a cost to the plant. The endophyte-free plants may now have a small selective advantage and this would in turn lead to a decline in endophyte-infected host plants in the population (Popay, 2009).

For the endophyte-host plant symbiosis itself there are also likely to be advantages to both the endophyte and the host plant in maintaining variability in the expression of the endophyte-host plant interaction within populations. This allows for greater adaptations to changes in the environment. A natural grass population made up of genetically variable endophyte-host plant associations is more likely to be resilient in responding to abiotic or biotic selection pressures. In agronomic cultivated grass populations less genetic variability in the endophyte-plant host association is deemed more advantageous in terms of plant benefit (vegetative growth and plant persistence) due to the more constant selective pressures (particularly insect herbivory in agricultural environments) (Popay, 2009).

1.4.4.2 Benefits

There are numerous clavicipitaceous endophyte benefits to hosts reported in the literature. These include: resistance to mammalian and insect herbivory (Clay, 1990; Clay & Schardl, 2002), drought tolerance (Arachevaleta *et al.*, 1989; West, 1994), resistance to nematodes (West *et al.*, 1988; Kimmons *et al.*, 1990; Prestidge & Ball, 1993), resistance to fungal pathogens (Gwinn & Gavin, 1992), growth enhancements (Malinowski & Belesky, 2000) and greater field persistence (West *et al.*, 1988; Hill *et al.*, 1990). Other benefits include reduced seed and seedling predation (Clay, 1996; Clay & Holah, 1999). The defence against insect pests spans

several taxonomic orders and includes leaf- and root-chewing and plant-sucking herbivores. There are some insects, however, that have been found not to be affected by endophyte infection. The common *E. festucae* var. *lolii* strain in perennial ryegrass (*L. perenne*) has no effect on frit fly (*Oscinella frit* (Linnaeus, 1758)), leather jackets (*Tipula* species) (Lewis & Clements, 1986; Lewis & Vaughan, 1997) and cereal leaf beetles (*Oulema melanopus* (Linnaeus, 1758)) (Clement *et al.*, 2007). The fitness of other insects has been found to be enhanced by the endophyte (Lopez *et al.*, 1995; Saikkonen *et al.*, 1999; Tibbets & Faeth, 1999; Bultman & Bell, 2003; Popay *et al.*, 2004). The reasons for improved individual insect fitness are unknown but are likely to be related to altered plant chemistry due to the presence of the endophyte (Rasmussen *et al.*, 2008). In some cases the endophyte's alkaloids have no effect, in other cases the negative effects caused by the insects are offset by increased host plant quality (Popay, 2009).

Host benefits to the clavicipitaceous endophyte include, provision of nutrients and propagation through the seed (Scott *et al.*, 2005), and an enclosed protective environment.

1.4.4.3 Costs

Some infected plants may grow less well compared with endophyte-free plants under some conditions; this can be dependent on endophyte strain, host strain and environmental conditions. There is some evidence that there is a cost to the plant host supporting clavicipitaceous endophytes. There have been dramatic examples of this reported in the literature. These include increased disease susceptibility of plants carrying endophytes compared to endophyte-free equivalents. For example, it was observed plots of an endophyte-containing cultivar of tall fescue grass (*L. arundinaceum*) showed an increased incidence or severity of crown and root rot disease caused by *Pythium graminicola* Subraman. when compared with endophyte-free plots at Rutgers University turfgrass breeding station (Rodriguez *et al.*, 2009). Other cultivars, with and without endophyte, were examined and none showed the same effect. Further investigation showed an unusually high number of intercellular hyphal strands. It was postulated that the cultivar had enhanced susceptibility due to the abnormally high endophyte content which increased the nutrient demands and stress on the grass host (Rodriguez *et al.*, 2009). In other

examples, although endophyte-containing grasses were more susceptible to certain diseases, they also recovered better once the environmental conditions changed. Wäli *et al.* (2006) observed this in natural populations of red fescue (*Festuca rubra* L.) damaged by winter snow mould (*Typhula ishikariensis* Imai) and subsequent recovery of the plants in spring. In another example, the incidence of endophyte in a host plant population can be dependent on need, such as, when herbivore pressure is high. This was observed by White Jr. *et al.* (2001), when damage by leaf-cutting ants in a desert environment to populations of *Bromus setifolius* J. Presl was extensive, endophyte *Epichloë tembladerae* (Cabral & J.F. White) Iannone & Schardl, formerly *Neotyphodium tembladerae* Cabral & J.F. White (Leuchtmann *et al.*, 2014), incidence was high (80–100% of sampled plants). In contrast, where leaf-cutting ant populations were low or rare, there was a low incidence of endophyte infection (0–20% of sampled plants). These examples fit with the hypothesis that hosting an endophyte is costly and the benefit/s may be dependent on specific circumstances and/or environmental conditions. Thus under certain circumstances and environmental conditions, the hosting of an endophyte may result in reduced plant fitness and disadvantage the plant. This may be significant in both natural and artificial plant populations and associated ecosystems. Costs to the endophyte include reliance on the host plant for nutrition, transmission and continued survival. For the asexual endophytic fungi, this reliance is absolute.

1.4.5 Effects on pasture grass invertebrates

Some of the host fitness enhancements resulting from infection of an endophyte are protection from biotic stresses such as herbivory and disease, which is mediated by the production of secondary metabolites (Popay & Bonos, 2005). As discussed earlier, hosting an endophyte involves both costs and benefits, and if a host plant contains an endophyte this is no guarantee of resistance to both biotic (herbivory) and abiotic (drought) stresses. In fact insect resistance may be enhanced (desirable), reduced or not affected (undesirable) by the presence of endophyte. The effects are strain specific and substantially influenced by the plant host genotype.

Prestidge *et al.* (1982) found a correlation between decreased Argentine stem weevil (*L. bonariensis*) adults and *E. festucae* var. *lolii* infection levels. By 1994, many species of insects had been found to be adversely affected by endophytes

(Breen, 1994; Clement *et al.*, 1994; Popay & Rowan, 1994). Since 1994, few insects have been added to the list as the emphasis has shifted to exploring the biotic responses in the diversity of endophyte-plant host associations that exist. The effects of endophyte infection have mainly been shown in economically important grasses (tall fescue (*L. arundinaceum*) and perennial ryegrass (*L. perenne*)) or by bioassaying the alkaloids produced by endophyte infection (Popay & Bonos, 2005). The alkaloid profile is important directly in the deterrence or toxicity to invertebrates but also indirectly by altering insect behaviour, e.g., deterring adults so oviposition does not occur or is greatly reduced, as for example with Argentine stem weevil (*L. bonariensis*) adults; in increasing emigration of the insect to more 'preferred' feeding material; deterring feeding, thus reducing larval or pupal survival. Insect herbivores that feed near the base of the plant in the region of the meristem inflict more severe damage than leaf grazers or sucking insects and they appear more sensitive to the presence of endophytes (Popay & Bonos, 2005). Although the endophytes do not extend into the roots, root-feeding invertebrates can be affected, though to a lesser extent than those feeding on above-ground plant parts (Popay & Bonos, 2005). Sensitivity to endophytes in the plant-sucking and leaf- and root-grazing invertebrates is much more diverse in the range of responses compared with those insects that feed near the base of the plant.

1.4.6 Alkaloid production and expression

Alkaloids produced by *Epichloë*-host grass associations are considered to be important in providing protection to pastures against damage by insects and response to grazing animal toxicosis. Four classes of alkaloids are common but not universally produced by this group of fungi.

1.4.6.1 Indole diterpenes

The indole diterpenes are a structurally diverse group of metabolites, principally found in the filamentous fungi of the genera *Penicillium*, *Aspergillus*, *Claviceps* and *Epichloë* (Steyn & Vleggaar, 1985; Scott *et al.*, 2005). The group is characterised by the presence of a cyclic diterpene skeleton derived from four isoprene units, and an indole moiety derived from tryptophan or a tryptophan precursor (Byrne *et al.*, 2002; Scott *et al.*, 2005). Many exhibit potent mammalian tremorgenic activity; others give anti-insect activity (Parker & Scott, 2004; Scott *et*

al., 2005). Most notable examples of mammalian toxicity are the lolitrems from *E. festucae* and most *E. festucae* var. *lolii*, and are known to be responsible for ryegrass staggers in livestock (Siegel *et al.*, 1990; Christensen *et al.*, 1993; Bush *et al.*, 1997). This group of alkaloids also includes the terpendoles and the epoxy-janthitrems (isolated from the novel endophyte AR37). Fungi, such as *Pencillium paxilli* Bainier, produce simple indole diterpene alkaloids (paxilline, paxitriol and paspaline). Indole diterpenes are confirmed as fungal metabolites in asexual *Epichloë* (formerly *Neotyphodium*) species (Penn *et al.*, 1993; Reinholz & Paul, 2001). Paxilline, first identified by Weedon and Mantle (1987), is a tremogenic indole diterpene (Miles *et al.*, 1992), previously thought to be the key intermediate in the biosynthetic pathway for lolitriol and lolitrem B production (Miles *et al.*, 1993) in *Neotyphodium* species. The paxilline enzyme-linked immunosorbent assay (ELISA) and high pressure liquid chromatography (HPLC) were used to screen asexual *Epichloë* isolates for their potential to produce indole diterpenoid tremogenic compounds (Penn *et al.*, 1993). Non-tremogenic paspaline is considered a key intermediate for the production of indole diterpenes, including the lolitrems and paxilline, by multiple pathways (Munday-Finch *et al.*, 1996; Saikia *et al.*, 2012). The preferential synthesis of these metabolites *in planta* suggests that genes for lolitrem biosynthesis are symbiotically regulated (Scott *et al.*, 2005).

1.4.6.2 Ergot alkaloids

The most notable example of the ergot alkaloids is ergovaline. Ergovaline is well known for pest protection and mammalian toxicity, especially in tall fescue pastures. The clavines and some derivatives may also be present and add to bioactivity. They are known as potent toxins with neurotrophic activities. Ergovaline causes vasoconstrictive effects and heat stress in animals. Ergot alkaloid toxicity (ergotism) has been known for many centuries (Groger, 1972; Clay & Schardl, 2002) and several ergot alkaloids now have pharmaceutical applications (and illegal recreational applications), such as lysergic acid diethylamide (also known as LSD, LSD-25 or acid).

1.4.6.3 Peramine

Peramine is a pyrrolopyrazine alkaloid that has no known effects on grazing animals or other mammals (Keogh *et al.*, 1996). This compound is a metabolite

unique to the *Epichloë* genus of fungi (Scott *et al.*, 2005). It is found throughout the host plant tissues above ground. Although it has no known effects on grazing animals it is a known powerful feeding deterrent for Argentine stem weevil (*L. bonariensis*) adults and to a lesser extent also to larvae (Rowan *et al.*, 1990). Although not found to be a deterrent to grey-brown cutworm (*Graphania mutans* (Walker, 1857)), it impaired larval development and increased pupal mortality (Dymock *et al.*, 1988). It is commonly inferred in the literature to be a potent insect deterrent, but other than its effects on Argentine stem weevil (*L. bonariensis*) and cutworm (*G. mutans)* there is little published evidence for effects on other insects. Ball *et al.* (1997a) showed that peramine does not deter adult African black beetle (*H. arator*) from feeding.

1.4.6.4 Lolines

Lolines are pyrrolizidine alkaloids that have potent insecticidal activity, especially for sucking insects of pasture plants (Johnson *et al.*, 1985; Tapper *et al.*, 2004). Although pyrrolizidine alkaloids are found in approximately 3% of flowering plants (Smith & Culvenor, 1981) the lolines (saturated amino pyrrolizidine alkaloids) are not hepatotoxic and are not significant animal and human toxins like the 1, 2–unsaturated pyrrolizidine alkaloids (Bush *et al.*, 1993). They were first isolated from darnel ryegrass (*L. temulentum*) and later several derivatives were identified in tall fescue (*L. arundinaceum*) (Hofmeister, 1892; Guerin, 1898; Yates *et al.*, 1989; Bush *et al.*, 1993; Schardl *et al.*, 2007). Concentrations have been reported that exceed the biomass of the endophyte in infected plants (Tapper *et al.*, 2004). They are known as potent toxins with neurotrophic activities and have a broad spectrum of activity against insects. Lolines are not produced by *E. festucae* var. *lolii* in association with perennial ryegrass (*L. perenne*) (Clay & Schardl, 2002). They are produced by other asexual *Epichloë* species, *E. coenophiala, Epichloë uncinata* (W. Gams, Petrini & D. Schmidt) Leuchtm. & Schardl, comb. nov. (formerly *Neotyphodium uncinatum* (W. Gams, Petrini & D. Schmidt) Glenn, C.W. Bacon & Hanlin) and *E. occultans,* and are a characteristic of associations of tall fescue (*L. arundinaceum*), meadow fescue (*L. pratense*) and Italian (or annual) ryegrass (*Lolium multiforum* Lam.*)* respectively. Unlike the other three major alkaloid classes, they have adverse effects on root-feeding pasture pests such as the native grass grub larvae (*Costelytra zealandica* (White, 1846) (Given, 1952)) (Popay &

Lane, 2000). There is no conclusive evidence for endophytes in perennial ryegrass (*L. perenne*) affecting white grubs (Scarabaeidae), which graze on grass roots (Prestidge & Ball, 1993).

1.4.6.5 Alkaloid expression

Siegel *et al.* (1989) found that peramine was present in the majority of clavicipitaceous endophyte-infected host grasses. A smaller number of these endophyte-infected host grasses were found to have ergot alkaloids, loline alkaloids and lolitrems (from the indole diterpenes group). The lolitrems are mainly produced by *E. festucae* var. *lolii* endophytes infecting ryegrass (*L. perenne*) and usually *E. coenophiala,* but certain isozyme phenotypes infecting tall fescue (*L. arundinaceum*) produce this alkaloid (Christensen *et al.,* 1993). Loline alkaloids are produced by *E. coenophiala* in tall fescue and *E. uncinata* in meadow fescue but not by the endophyte in perennial ryegrass (*L. perenne*) (Schardl *et al.*, 2007). Ergot alkaloids are widespread in the plant-parasitic Clavicipitaceae. Detailed reviews of endophyte alkaloids have been published (Porter, 1994; Bush *et al.*, 1997; Siegel & Bush, 1997) as reviewed by Clay and Shardl (2002). All four classes of alkaloids have been produced by pure fungal cultures (Porter, 1994; Blankenship *et al.*, 2001) and are absent in uninfected grasses, clearly demonstrating their fungal origin (Clay & Schardl, 2002).

The specific alkaloid profiles are a function of the fungal species and/or strain, while concentrations vary with the symbiotic endophyte-host plant combination and are modulated by the host plant. Even the same endophyte genotypes inoculated into different plant genotypes from the same species can have major effects on alkaloid production *in planta*. Plant genotype, tissue type, season, plant age and other abiotic or biotic stresses influence alkaloid profile and concentrations (Clay & Schardl, 2002; Rodriguez *et al.*, 2009). The host plant genotype has a major influence on the quantities of alkaloids that are produced. A four- to ten-fold difference in concentrations of different alkaloids has been recorded between individual ryegrass plants taken from the field and then grown under the same conditions as each other (Latch, 1994; Ball *et al.*, 1995a; Ball *et al.*, 1995b). Easton *et al*. (2002) found that concentrations of peramine and ergovaline consistently varied across two perennial ryegrass (*L. perenne*) families. Seasonal changes in

alkaloid levels in plants correlated with seasonal changes in endophyte concentration (di Menna *et al.*, 1992; Ball *et al.*, 1995a; Ball *et al.*, 1995b; Justus *et al.*, 1997) and may alter the resistance to herbivory at different times of the year (Popay & Mainland, 1991). It has also been demonstrated in ryegrass plants that endophyte metabolic activity varies between different plant genotypes, primarily due to variation in the number of metabolically active hyphae (Schmid *et al.*, 2000). Environmental stresses often alter the alkaloid content in plants: usually concentration is increased. Water deficit is known to elevate ergovaline levels in ryegrass (Barker *et al.*, 1993; Lane *et al.*, 1997).

While the alkaloids are always present in the plant and are regarded as a constitutive defence system, they are also inducible. That is, alkaloid levels are raised in response to a specific stimulus. The most obvious inducible responses are triggered by damage to the plant by herbivores. Re-growth following defoliation often contains higher levels of alkaloids than equivalent older plants, probably as a result of mycelium being concentrated with meristematic tissue and up-regulation of alkaloid genes (Sullivan *et al.*, 2007). This results in the key plant tissues involved in re-growth of the plant being protected. Grazing (particularly by livestock) or artificial clipping, although generally increasing alkaloid levels, may have no adverse effects on the grass and may actually stimulate growth (Popay, 2009). Insect damage to the crown and pseudostem, however, increases alkaloids levels significantly. This 'true' induced response may be significant, with much higher levels of alkaloids produced in the crown and pseudostem in response to damage to these more vulnerable regions of the plant. Less herbivory on or near the crown and on the pseudostems of grasses not only protects the plant but also the endophyte (Popay, 2009) and their long term survival.

1.4.7 Distribution of the four major alkaloids in grasses

The study of Spiering *et al.* (2005) study suggests that the levels and distribution of the endophyte is not a major determinant of the distribution of the fungal alkaloids in perennial ryegrass (*L. perenne*) (noting that lolines are not produced by perennial ryegrass (*L. perenne*) as stated earlier). The distribution of the four main alkaloid classes has been found to be different within the grass.

1. The indole diterpenes alkaloid class contains the lolitrems, terpendoles and the epoxy-janthitrems). Lolitrem B accumulates over time in older tissue and is present only at low levels in young tissue. Compared to other plant components the highest concentration of lolitrem B is found in the seed (Ball *et al.*, 1995b).
2. The ergot alkaloids class contains ergovaline, clavines and the clavine derivatives. Ergovaline is concentrated in the stem and basal leaf sheath of intermediate age (Koulman *et al.*, 2007), showing a very heterogenous vertical distribution (Spiering *et al.*, 2005).
3. Peramine is found fairly evenly distributed in plant tissues and does not accumulate in older tissues (Ball *et al.*, 1997b; Spiering *et al.*, 2002; Spiering *et al.*, 2005). It appears to be continuously produced by the endophyte, but does not progressively accumulate. Compared with other plant components, the highest concentration of peramine is found in the seed (Ball *et al.*, 1995a).
4. Lolines are distributed throughout the plant including the roots. They are found in greatest amounts in the seed, followed in decreasing amounts in the rachis, stem, leaf sheath and leaf blade (Bush *et al.*, 1993). During the growing season there is little change in the accumulation of the loline alkaloids in the leaf blade (Bush *et al.*, 1993). In leaf sheaths, the accumulation of loline alkaloids is positively associated with endophyte mycelium concentration (Bush *et al.*, 1993).

Ball *et al.* (1995b) found that in perennial ryegrass (*L. perenne*) the production of the alkaloids peramine and lolitrem B were lowest in the winter months. From this work it appears an increase in endophyte content was correlated with the reproductive development of perennial ryegrass (*L. perenne*) and content was higher in leaf sheafs compared with leaf blades. The two alkaloid concentrations were largely determined by endophyte concentration (mycelium mass). This is contrary to the later study by Spiering *et al.* (2005) which found alkaloid and endophyte concentrations were not always directly related.

Distribution of the fungus (Musgrave, 1984) and the alkaloids ensures that protection is strongest at the base of plants where herbivory is most likely to threaten the survival of both plant and fungus (Popay, 2009). In addition,

concentrated alkaloids in the seed are likely to reduce predation of both seed and seedlings (Popay, 2009). It has been shown that reduced rodent herbivory of seed is a major factor in giving endophyte-infected tall fescue a selective advantage in the field (Clay, 1996; Clay & Holah, 1999).

1.5 Asexual *Epichloë* (formerly *Neotyphodium*) in New Zealand pastures

1.5.1 Overview

Epichloë festucae var. *lolii*, also known as LpTG-1 (*Lolium* taxonomic group 1), is the predominant endophyte of perennial ryegrass (*L. perenne)* and is a haploid sexual derivative of *E. festucae* (Christensen *et al.*, 1993; Schardl *et al.*, 1994). The second endophyte hosted by *L. perenne* is the heteroploid LpTG-2 (*Lolium* taxonomic group 2) (Christensen *et al.*, 1993), which contains up to two gene sequence variants that are similar to the single gene sequence variants found in *E. festucae* and *E. typhina* (Schardl *et al.*, 1994). Perennial ryegrass (*L. perenne),* is the predominant pasture grass in New Zealand. In New Zealand *E. festucae* var. *lolii* is commonly found in many commercial cultivars and native ryegrass. Selected *E. festucae* var. *lolii* strains are used in the commercial cultivars whereas the native ryegrass contains 'wild-type' *E. festucae* var. *lolii.* In New Zealand all wild-type *E. festucae* var. *lolii* endophytes produce ergovaline, lolitrems and peramine (Tapper & Latch, 1999). It has long been observed that older ryegrass pastures contain a disproportionately high frequency of wild-type *E. festucae* var. *lolii* infection (Neill, 1940; Lloyd, 1959). This natural shift in endophyte frequency is very indicative that plants containing the endophyte were in some way at a selective advantage to endophyte-free plants. The shift of natural endophyte-infection frequency in the pasture grasses over time, in conjunction with trial results comparing endophyte-infected and endophyte-free pastures that resulted in complete pasture devastation by Argentine stem weevil (*L. bonariensis*) adults in the endophyte-free pastures, confirmed that the development of endophyte-free pastures was not a viable option for turf and forage pastures in New Zealand.

1.5.2 History of endophyte and New Zealand pastures

In the 1980s important discoveries were made in regards to *E. festucae* var. *lolii* in perennial ryegrass (*L. perenne*). These discoveries had significant effects on New Zealand's pastoral industry.

The first discovery was the cause of ryegrass staggers, made by Fletcher and Harvey (1981). They established the link between the presence of *E. festucae* var. *lolii* in perennial ryegrass (*L. perenne*) and ryegrass staggers. The most obvious solution was to remove the endophyte-infected pastures and replace them with endophyte-free pastures so that the adverse effects on the health of grazing livestock were removed. Research began into the elimination of *E. festucae* var. *lolii* from infected seeds and plants (Harvey *et al.*, 1982; Latch & Christensen, 1982).

The second discovery was the link between the presence of *E. festucae* var. *lolii* and increased resistance to Argentine stem weevil (*L. bonariensis*) attack (Mortimer *et al.*, 1982; Prestidge *et al.*, 1982). Argentine stem weevil (*L. bonariensis*) in the 1980s was ranked as one of New Zealand's most important pasture pests as it infests all improved ryegrass pastures nationwide and it is still an important pasture pest today. In the 1980s Argentine stem weevil (*L. bonariensis*) effects were estimated to cost New Zealand 80 to 2500 million dollars annually due to lost production and pasture renovation (Prestidge *et al.*, 1991).

These two initial discoveries put the farmers in a dilemma in New Zealand: Whether to sow high endophyte pastures and significantly improve Argentine stem weevil (*L. bonariensis*) resistance but increase ryegrass staggers risk to stock; or whether to plant low endophyte pastures with significant increased susceptibility to Argentine stem weevil (*L. bonariensis*) attack but very low risk of ryegrass staggers to stock. A survey of farmers in 1985 indicated that farmers preferred to sow high endophyte seed lines as they considered the advantages of pasture persistence (resistance/tolerance of Argentine stem weevil (*L. bonariensis*) attack) and production to be of greater value than the risk of ryegrass staggers to stock that they could manage when and if it occurred (Prestidge *et al.*, 1985a).

The most obvious symptom of *E. festucae* var. *lolii* toxicosis to livestock, particularly sheep, is ryegrass staggers, which in severe cases can be fatal (Easton

& Tapper, 2005). *Epichloë festucae* var. *lolii* toxicosis also affects the livestock's thermoregulation, faecal moisture regulation, and growth rate (Easton & Tapper, 2005). Technically, treatment for ryegrass staggers is basic; remove livestock from toxic pastures onto non-toxic pastures. In some circumstances, however, this can be difficult because if endophyte-free pastures are not available then transfer is not a viable option. With *E. festucae* var. *lolii* considered to be an effective biocontrol agent for Argentine stem weevil (*L. bonariensis*), if the detrimental effects of the endophyte on grazing livestock could be significantly reduced or removed, the potential alternative was to find a novel endophyte that did not have detrimental effects on grazing livestock but retained insect resistance.

A third discovery was made by Gallagher *et al.* (1981, 1982b, 1982a, 1984). They found a group of substituted indole diterpenes, named lolitrems, were the likely cause of the ryegrass staggers. Initially lolitrem B was implicated as the major indole diterpenoid, but since then many other compounds have been discovered and characterised by laboratories around the world, and the biosynthetic pathways as well (Tapper *et al.*, 2004).

The fourth major discovery was made by Rowan and Gaynor (1986) that Argentine stem weevil (*L. bonariensis*) resistance was caused by an unrelated alkaloid to that of the lolitrems. It is now known as peramine, a pyrrolopyrazine alkaloid (Rowan *et al.*, 1986). Peramine is a unique metabolite to the *Epichloë* group of fungi (Scott *et al.*, 2005). Rowan (1993) and Schardl *et al.* (1991) showed peramine was produced in cultures of *E. festucae* var. *lolii* and *E. typhina* respectively, confirming it is a fungal metabolite. It is now known to be a potent feeding deterrent against Argentine stem weevil (*L. bonariensis*) (Prestidge *et al.*, 1985b; Rowan & Gaynor, 1986; Rowan *et al.*, 1990). Although often implicated, peramine has not been definitively shown to deter any other insects, suggesting peramine functions primarily as a marker for the presence of endophyte, which is detected by the Argentine stem weevil (*L. bonariensis*) adults. The detection of peramine by adult Argentine stem weevils (*L. bonariensis*) means they tend not to lays eggs on endophyte-infected perennial ryegrass (*L. perenne*) pasture. Fewer eggs, results in fewer larvae on infected pasture. Any larvae that are produced are exposed to the toxic effects of the other endophyte alkaloids.

1.6 Evolution of the 'novel' endophytes

1.6.1 Overview

Some endophytes have been deliberately sought to resolve the problem of mammalian toxicity associated with the wild-type endophytes in tall fescue and perennial ryegrass (*L. perenne*). These 'novel' endophytes are now widely used in agriculture in USA, Australia and New Zealand. The key to exploiting endophytes for biotic stress protection in turf and forage grasses without negative effects on grazing animals lies in the diversity of endophytes that exist in the *Lolium* plant genera in natural habitats. Endophytes from paddocks with no or minor incidence of livestock toxicosis were genetically indistinguishable from endophytes from paddocks with major incidence. The genetic difference or diversity was found amongst the grass host (de Jong *et al.*, 2005), suggesting that quantitative variability in endophyte–related effects may result from variation in host genotype. Other studies support host-mediated genetic control of endophyte phenotypic traits: Christensen *et al.*, (1998) showed that artificial inoculations of different tall fescue cultivars with different *E. coenophiala* isolates revealed that certain isolates and certain cultivars had consistently higher levels of ergovaline. Host genetic control of endophyte toxin levels has been detected in perennial ryegrass (*L. perenne*) by Easton *et al.* (2002) in a partial diallele crossing scheme between different endophyte-containing genotypes. In this study as much as 65% of the genetically controlled variation in toxin concentration was a function of mycelial mass. Results from QTL (quantitative trait locus) of a perennial ryegrass (*L. perenne*) mapping family also support the role of host genetic factors in the control of endophytic traits (de Jong *et al.*, 2005).

1.6.2 AR1 History and other 'novel' endophytes

The primary focus of endophyte research in New Zealand has been the association of perennial ryegrass (*L. perenne*) with *E. festucae* var. *lolii* which causes toxicosis in grazing livestock (Fletcher *et al.*, 1999), but it is essential to sward persistence (Prestidge & Ball, 1993). *Epichloë festucae* var. *lolii* can readily be manipulated, i.e., it can be isolated from a host plant, cultured in artificial media and reinoculated into another grass plant of the same species (Latch & Christensen, 1985; Scott *et al.*, 2005). Seed from plants of *Lolium* species were collected mainly in Europe and

screened for the presence of known classes of compounds in order to identify strains that lack mammalian toxins (the lolitrems, particularly lolitrem B and the ergot alkaloids). New strains were sought that initially did not produce the mammalian lolitrem toxins but produced ergovaline and peramine. The majority of collections screened in New Zealand had chemical profiles similar to the wild-type endophytes, suggesting that these strains of endophytes predominate in the naturalised and wild populations of tall fescue (*L. arundinaceum*) and perennial ryegrass (*L. perenne*).

There were strains of endophytes that differed to the wild-type, producing different known alkaloid combinations. Comparative insect studies of the endophytes in-planta identified potentially useful strains (Popay & Bonos, 2005). Subsequently, perennial ryegrass (*L. perenne*) cultivars infected with 'novel' endophytes were obtained (Grasslands Pacific Endosafe, Grasslands Greenstone Endosafe, AR1, AR37, NEA2, AR542, etc,). Endophytes deficient in the production of lolitrem B but still producing ergovaline and peramine gave strong resistance to Argentine stem weevil (*L. bonariensis*) in perennial ryegrass (*L. perenne*) (Popay *et al.*, 1995; Popay & Wyatt, 1995).

Ergovaline was subsequently found to also cause negative and unwanted effects on livestock (Fletcher & Easton, 1997; Fletcher *et al.*, 1999). Initially, the Grasslands Pacific Endosafe and Grasslands Greenstone Endosafe were thought to be the same endophyte (Tapper & Latch, 1999) and were introduced into ryegrass cultivars because they did not produce lolitrem B were and deemed to be safe for grazing livestock. Grasslands Pacific Endosafe was taken off the market because it was discovered it caused significant detrimental effects on the livestock and subsequently was found to produce high levels of ergovaline (Latch & Christensen, 1985; Davies *et al.*, 1993; Easton *et al.*, 1993). Grasslands Greenstone Endosafe was found to produce lower levels of ergovaline (Latch *et al.*, 1985; Tapper & Latch, 1999).

The novel endophyte, AR1 was a major step forward in overcoming the ruminant toxicity associated with wild-type endophytes and the earlier generation of 'novel' endophytes. AR1 produces no lolitrems or ergovaline (Tapper & Latch, 1999) but it does produce peramine, a known potent deterrent for Argentine stem weevil (*L. bonariensis*) adults. AR1 can only produce simple indole diterpenes (including

paxilline and paxilline-like compounds such as the terpendoles) as it does not have the full complement of genes for the production of more complex indole diterpenes such as lolitrem B (Young *et al.*, 2009). Although AR1 genetically can produce paxilline, a mild tremogen (Miles *et al.*, 1992) the amounts produced are considered minimal (Young *et al.*, 2009) instead production of the other simple indole diterpenes including the paxilline-like compounds being more predominant (Young *et al.*, 2009). Also there have never been any reports of mammalian neurotoxicosis in livestock from AR1-infected pastures (Bluett *et al.*, 2005b; Bluett *et al.*, 2005a). AR1 also showed strong resistance to pasture mealy bug (*Balanococcus poae* (Maskell, 1879)) (Popay *et al.*, 2000), reduced resistance to African black beetle (*H. arator*) (Popay & Baltus, 2001) little or no effect on porina (*Wiseana cervinata* (Walker, 1865)) (Jensen & Popay, 2004) and increased susceptibility to root aphid (*Aploneura lentisci* (Passerini, 1865)) (Popay *et al.*, 2004).

Ryegrass (*L. perenne*) infected with the endophyte AR37 was found to be more productive and persistent, and gave a better range of insect protection than ryegrass infected with AR1 endophyte. AR37 had no effect on adult Argentine stem weevil (*L. bonariensis*), but potent activity against its larvae (Popay & Wyatt, 1995). It reduced African black beetle (*H. arator*) adult feeding, as Lp14 in Ball *et al.* (1994) reduced pasture mealy bug (*B. poae*) populations (Pennell *et al.*, 2004), reduced survival of porina (*W. cervinata)* (Jensen & Popay, 2004), and almost completely eliminated root aphid infestations (*A. lentisci*) (Popay *et al.*, 2004). At the time it produced none of the known common alkaloids. It was however, subsequently found, on occasion, to cause ryegrass staggers in livestock (Fletcher, 1999; Fletcher & Sutherland, 2009), but to date not cattle (Thom *et al.*, 2013b). AR37 was found to produce novel indole diterpenes, the epoxy-janthitrems (the same alkaloid class as the lolitrems) (Tapper & Lane, 2004). The risk of ryegrass staggers meant that some of the focus returned to AR1.

All companies marketing propriety ryegrass cultivars were invited to have their material inoculated with AR1. AR1 was first available for sale in 2002, and up to 2005, seven different companies have had more than 20 cultivars inoculated. AR1 was extensively researched in the late 1990s (Hume, 1999; Popay *et al.*, 1999; Fletcher & Easton, 2000; Popay *et al.*, 2000), with significant research effort going into defining the best practice for its effective use. In 2003, the second year AR1

was available, 40% of all proprietary perennial ryegrass (*L. perenne*) seed sold in New Zealand was infected with AR1. On the farm, AR1 has performed to expectations, with more than 80% of proprietary perennial ryegrass (*L. perenne*) seed sold now containing this endophyte. Almost all early work determining health and productivity of livestock grazing AR1 used sheep as the research animal (Fletcher & Easton, 2000). Live weight gains of lambs grazing AR1 are 10–15% higher than for lambs grazing wild-type endophyte (Fletcher, 1999; Fletcher *et al.*, 1999). Dairy farms using AR1-infected perennial ryegrass (*L. perenne*) have undergone intense monitoring (Keogh & Blackwell, 2001) and long term on-farm trials are ongoing (Easton & Tapper, 2005). Trial results show ~ 20% improved milk production through summer and autumn for cows grazing AR1-infected pasture compared with cows grazing ryegrass naturally infected with ergovaline- and lolitrem- producing endophyte. Milk production of cows on AR1 has been increased by 9% over the whole lactation period (Bluett *et al.*, 2005b; Bluett *et al.*, 2005a).

Although AR1-infected ryegrass is robust and research into best practice for establishing and managing AR1 pastures was widely distributed (Hume, 1999), AR1 did not provide adequate protection against severe outbreaks of African black beetle (*H. arator*) (Popay & Baltus, 2001). AR1-infected ryegrass has been planted on farms in potential African black beetle (*H. arator*) problem areas and has provided adequate protection against minor African black beetle (*H. arator*) pressure. However, an outbreak in the Waikato and Bay of Plenty in 2007/8 persisted over 3–4 seasons (Bell *et al.*, 2011) and AR1-infected pastures were devastated from the prolonged high pressure beetle attack. AR1 is now sold with a warning to farmers that in the northern New Zealand, the host pasture may lack persistence in the face of severe African black beetle (*H. arator*) attack.

Research trials are ongoing and at several sites: plots have been monitored for percent infection with AR1 and percent contamination with naturally occurring endophyte. From ongoing trials and monitoring it has been shown that where contamination was high initially at pasture establishment, this contamination has increased with time. Where AR1 pasture has been successfully established, it has persisted and remained uncontaminated (Bluett *et al.*, 2001). Seed is sold with the

expectation that at least 70% are infected with live endophyte. Wholesale seed companies have had to provide retailers with protocols for dealing with seed retained at the end of the season, and farmers have been discouraged from taking possession of seed before they are ready to plant. Demand for seed has been such that carryover volumes have not been an issue. AgResearch, a New Zealand government-owned agricultural research institute has been involved with quality control, assaying certified seed lots containing AR1 endophyte to determine if there is any contamination with seed infected with toxin-producing wild-type endophyte. Few seed lines to date have failed the quality control check indicating high quality seed production by the local seed industry.

1.7 African black beetle biology and lifecycle in New Zealand

1.7.1 African black beetle distribution

African black beetle (*H. arator*) (Coleoptera: Scarabaeidae: Dynastinae) is a native of South Africa. The nomenclature of *Heteronychus arator* Fabricius, 1775 (*H. arator* F.) has a complex history that Landin (1964) discusses in detail and it is now accepted to be based on Fabricius's original description in 1775 from material held in the Banks collection at the British Museum (Kuijten, 1983; Watt, 1984). The complex history surrounding the nomenclature of *H. arator* F. has resulted in the following synonyms being used to describe the beetle in the literature; *Scarabaeus arator* Fabricius, 1775 (*S. arator* F.), the original nomenclature used, *Heteronychus. arator* Burmiester, 1847 (*nec.* Fabricus) and *Heteronychus sanctae-helenae* Blanchard, 1853 (*H. sanctae-heleane* Blanch.). The nomenclatural history even included the reclassification of from the genus *Heteronychus* to *Hybosorus* but this was material held in the Kiel, Germany and not from the British Museum. It was later determined that the specimens held at the two museums, although labelled the same, were not the same species. Landin (1964) emphasises that Fabricius came to Kiel in late 1775 and his study was published earlier that year, so the material held in the Kiel collection could not have existed at the time of the original publication. Therefore the original description in 1775 had to be based on material from the British Museum and the specimen in the Kiel museum is not *H. arator* F., and its subsequent placement in the *Hybosorus* genus is now accepted (Kuijten, 1983). African black beetle (*H. arator*) was first recorded from Waiheke Island in New

Zealand in March 1937 (Spiller & Turbott, 1944) and is believed to have arrived from Australia. Beetles were first reported in the Auckland region in the late 1930s. In the review by Watt (1984), it was deemed unnecessary to use the subspecies *australis* as described by Encrödi (1961) (which is based on New Zealand material) until the geographical variation of the beetle in its natural range in Africa is determined owing to the variability in the characters used to distinguish the subspecies. By the late 1970s the African black beetle (*H. arator*) range extended from the top of the North Island and southwards to Raglan on the west coast and to Gisborne on the east coast (Esson, 1973). It is now established throughout the upper regions of the North Island of New Zealand (Northland, Waikato, Bay of Plenty, Hawkes Bay and coastal Taranaki) and is found mainly in peat and ash soils that are light and free-draining. The distribution of African black beetle (*H. arator*) is likely determined by temperature, with the beetle typically only found in areas with a mean annual surface air temperature of 12.8°C or higher. The map in Figure 1-1 illustrates the current expected African black beetle (*H. arator*) distribution range, which has extended beyond that predicted by Watson (1979). In the lower North Island there are pockets of land on both coastlines as far south as Wellington that are also potential African black beetle (*H. arator*) areas owing to a mean air temperature of $\geq$ 12.8°C.

1.7.2 African black beetle lifecycle

The African black beetle (*H. arator*) has an annual life-cycle, that is, one generation per year, as shown in Figure 1-2. Adult African black beetle (*H. arator*) are active in spring from late September. Over-wintering adults resume feeding as temperatures increase and begin to mate. Emergence times vary year to year depending on climatic conditions. In warmer areas of the North Island, the African black beetle (*H. arator*) spring development may be quicker than that shown in Figure 1-2 as first-instar larvae have been observed in sandy soil in Dargaville in early October (Esson, 1973).

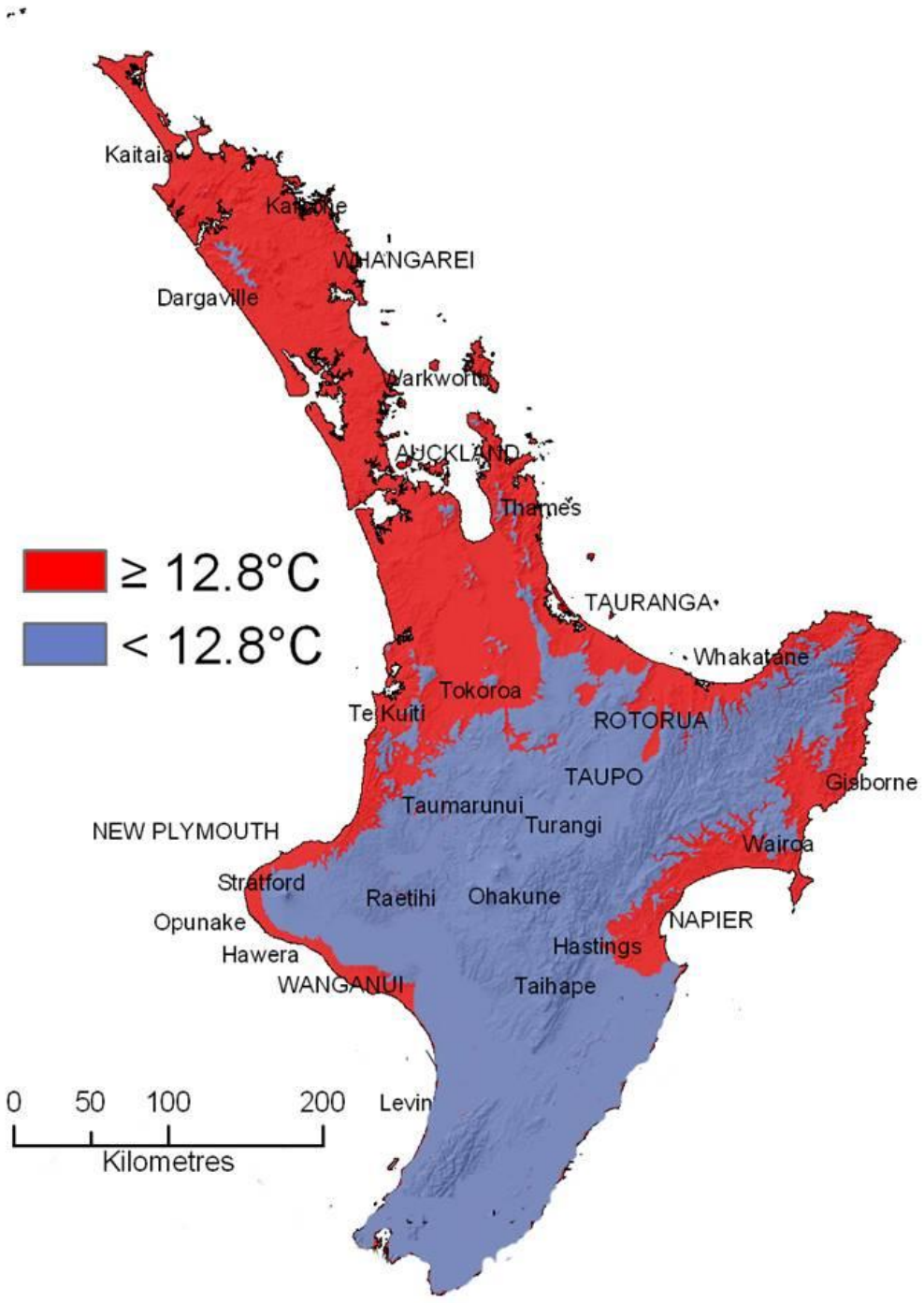

Figure 1-1: Expected distribution of African black beetle in New Zealand.
The distribution is based on the 12.8°C mean annual air temperature isotherm (NIWA 1971–2000). Note small pockets of coast as far south as Wellington where the 12.8°C isotherm is met or exceeded. Image adapted from Popay, A. J., AgResearch, New Zealand.

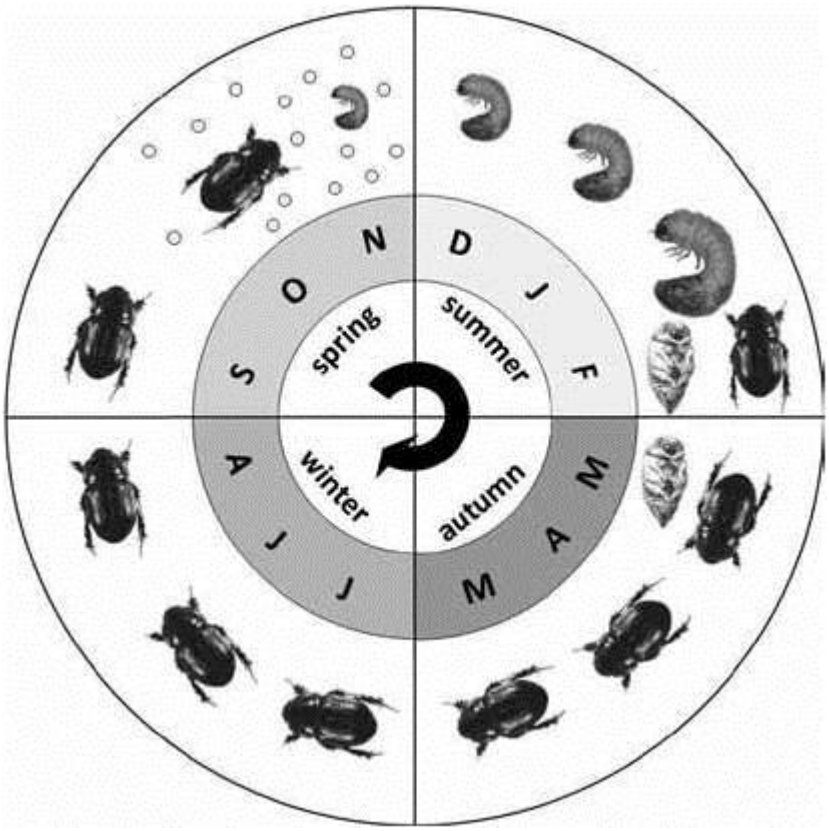

Figure 1-2: African black beetle lifecycle in New Zealand.
Letters – Months of the year. Figure from Bell et al. (2011).

Copulation takes place in the soil and has been observed to last as long as 12 h, and is thought to last considerably longer (Harrington, 1953). The female adult lays eggs from October through to November and the adult African black beetles (*H. arator*) then die off. The eggs are laid singly and are found about 1.25 cm below the soil, but have been found at greater depths (Todd, 1959). The incubation period is thought to be about six weeks early in the season, but probably decreases as the soil temperatures increase (Todd, 1959). The eggs are ovoid and opaque in colour (creamy-white) and just before hatching they become larger and almost spherical (Todd, 1959). Larvae are present from late spring through to late summer (November to February) and pass through three stages, with fully grown third-instars about 2.5 cm long (Figure 1-3 D, E & F). The larvae lie in the soil in a curled up position. The head capsule is light brown and the body is creamy-white or greyish-white, except for the hind segments which appear black (or brown) where the contents of the gut show through the gut wall as seen in Figure 1-3 D, E and F.

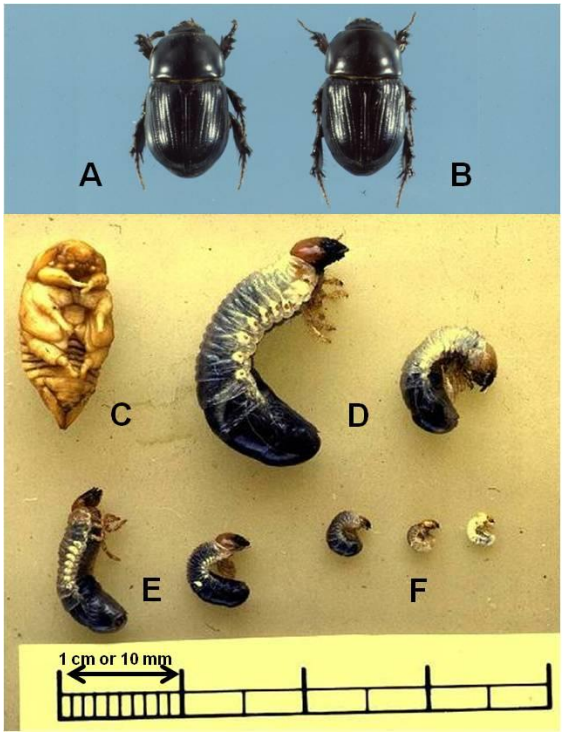

Figure 1-3: African black beetle larvae and pupa.
A) Adult male, B) Adult female, C) Pupa, D) 3rd instar larvae, E) 2nd instar larvae, F) 1st instar larvae. Images adapted from Popay, A. J., AgResearch, New Zealand.

In comparison, fully grown third-instar larvae are uniformly creamy-white because the gut content has been excreted before pupation (Todd, 1959). Larvae begin to pupate in February and emerge as adults in late summer/early autumn from late February/March to April. The pupa is initially pale to yellow (Figure 1-3 C) and just before the adult emerges, it changes to reddish-brown with the adult form visible through the pupal skin (Todd, 1959). Adult beetles are active in early autumn from March to April when pupae are transforming into adults. The newly-emerged adult beetle is chestnut in colour, but soon changes to a glossy black. The female is about 1.4 cm long and is generally larger than the male (Todd, 1959) as shown in Figure 1-3 A & B. This weight and size difference is known as sexual size dimorphism (Shine, 1989; Fairbairn, 1997) and for many animals including African black beetle (*H. arator*), it is associated with female fecundity and egg production (Darwin, 1874). Adults are dormant over winter, from May to August, depending on temperatures. Throughout winter the adults periodically come to the surface and feed and active adults have been observed on warm winter days on the ground surface (Ross, pers. obs.).

The root-feeding larvae cause the most damage to plants, and during outbreaks they can severely damage pastures. The third-instar larvae, present from January to February/March, feed on the grass roots close to the soil surface at a time when plants are already under considerable moisture and temperature stresses. Drought stressed grasses stop producing new roots, making them prone to pulling because of larval root feeding (Watson *et al.*, 2000; DairyNZ, 2010; Eden *et al.*, 2011). If African black beetle (*H. arator*) numbers are high, many of the plants will not survive and in severe cases the pasture composition changes as the bare ground is taken over by clover and weeds (Todd, 1964).

Adult African black beetles (*H. arator*) are normally active at dusk when they emerge from underground to feed and/or mate. The adult beetles feed on the pseudostem near the base of the plant, often destroying the growing point and killing the tillers. Adult beetles can cause significant damage to seedlings in newly sown pastures. The adult beetles can also fly, and in outbreak years large massed flights can be observed in autumn and spring. Flights occur when the soil surface temperatures exceed 17 °C at dusk with calm wind conditions (Watson, 1979; Bell *et al.*, 2011). Density-dependant spring migration flights may occur before oviposition (King *et al.*, 1981e). Adult African black beetle (*H. arator*) flights during autumn and spring are most likely very important in the dispersal of the beetle and in the infestation of new pastures and areas especially during outbreak years.

1.7.3 African black beetle hosts

Grasses are the preferred hosts (food sources) for all life stages of African black beetle (*H. arator*) (Bell *et al.*, 2011). This includes paspalum (*Paspalum dilatatum* Poir.), ryegrasses (*Lolium* spp.), poa (*Poa annua* L.; annual meadow grass or annual bluegrass), kikuyu (*Pennistum clandestinum* Hochst. Ex Chiov.) (Bell *et al.*, 2011) and other C_4 grasses in intensive dairy grazed pastures (Todd, 1959; King, 1976; King *et al.*, 1981a, 1981f, 1981d, 1981c; Blank & Olson, 1988; Tozer *et al.*, 2008). Unsurprisingly, grasses are also the preferred oviposition sites (King *et al.*, 1981b; Bell *et al.*, 2011). So the main breeding grounds for African black beetle (*H. arator*) in New Zealand are grassland pastures, particularly in coastal regions where the soil is light and sandy or inland on peaty soils. Legumes are generally unfavourable

hosts, although larvae will consume white clover roots when given no other choice (King *et al.*, 1981a). In the laboratory, carrots in an artificial diet form (King *et al.*, 1981c; Bell *et al.*, 2011; Ross, K, M., pers. obs.), are suitable food for both larvae and adults. Raw carrot and kumara are also suitable food sources for maintaining adult African black beetle (*H. arator*) in the laboratory (Ross, K, M., pers. obs.)

1.7.4 On-farm African black beetle monitoring

Watson *et al.* (1980b) showed that it was possible to predict paddocks that were at risk of developing damaging summer populations of African black beetle (*H. arator*) larvae by monitoring African black beetle (*H. arator*) adult numbers in the paddock in the preceding spring by taking spade square samples. King *et al*. (1980) found that using pitfall traps for monitoring was unreliable. If the number of adults was >10/m^2 the pasture was determined to be at risk of suffering severe African black beetle (*H. arator*) damage from larval feeding in the following summer. A damaging larval population over summer was defined to be 40−60 larvae/m^2 and was dependant on a range of factors, such as, soil moisture, soil temperature, and availability of appropriate host plants (King, 1979; King *et al.*, 1982; Bell *et al.*, 2011).

1.7.5 African black beetle population dynamics

The population of adult beetles varies widely from year to year due to climatic variation and the availability of a feed source. In areas where African black beetle (*H. arator*) occurs, outbreaks have occurred sporadically. African black beetle (*H. arator*) outbreaks are associated with warmer and drier than normal conditions during spring to autumn, typically droughts. These outbreaks have coincided with La Niña weather patterns where warmer than normal conditions occur (Eden *et al.*, 2011). Warm temperatures allow beetles to begin feeding in early spring, increasing their survival. As a consequence the oviposition rate increases and occurs earlier than usual, both of which increase the likelihood the larvae will reach adulthood (East *et al.*, 1981). Dry conditions in the summer increase the survival of young larvae and speeds up larval development; ensuring adults emerge in early autumn (East *et al.*, 1981; Bell *et al.*, 2011; Eden *et al.*, 2011). The early emergence of the adults and/or extended periods of warm temperatures in autumn, together with the availability of an appropriate food source, allow the adult beetles more time to gain

fat reserves for when they are mainly dormant, therefore increasing their probability of over-wintering survival.

Availability of suitable food sources (plant hosts) affects the African black beetle (*H. arator*) population by influencing the ability of the beetle to complete its lifecycle, including the number of eggs laid and survival at all stages. Grass plants that are endophyte-free (E-) or hosting an ineffective endophyte for African black beetle (*H. arator*) control are suitable food sources.

Population modelling studies in the 1980s (King *et al.*, 1981d) did not take into account the role of food resources for overwintering adults or the effect of endophyte in ryegrass on feeding activity. Wild-type endophyte-infected ryegrasses would have been common in the pastures resulting in reduced over-wintering adult survival and a reduction in the number of eggs laid in spring by the surviving beetles (Popay & Baltus, 2001). Pastures have significantly changed in composition since then so future modelling needs to take into account food resources (Bell *et al.*, 2011), pasture composition and endophyte type and status to enable better prediction of population outbreaks.

A particularly severe outbreak of African black beetle (*H. arator*) began in 2007/8 in the Waikato and Bay of Plenty regions and population densities are still being monitored. In February 2010 population densities of up to 80 larvae/m^2 were still being found (Bell N., AgResearch, New Zealand; unpublished work, 2013). Damage has been severe and exacerbated by drought, with dramatic consequences for pasture persistence. Bell *et al.* (2011) among many other queries, questions if the widespread planting of AR1 in African black beetle (*H. arator*) prone areas has contributed to the current ongoing outbreak.

1.7.6 Control of African black beetle

1.7.6.1 Crop rotation

Bell *et al.* (2011) recommends the sowing of an unsuitable host plant or crop in spring, such as, brassicas, legumes or chicory as a control measure as this breaks the African black beetle (*H. arator*) lifecycle by disrupting larval feeding over summer, therefore reducing larval survival and development through to the adult beetle. It is also suggested that crop rotation should lower the population density

once pastures are resown in autumn and that a cropping phase allows for the control of weedy host grasses such as paspalum (*P. dilatatum*) and poa (*P. annua*) (Bell *et al.*, 2011).

1.7.6.2 Insecticide

There are no currently marketed insecticides recommended for African black beetle (*H. arator*) control in established pastures (Bell *et al.*, 2011; Eden *et al.*, 2011). Bell *et al.* (2011) suggested, from previous trials (Watson & Webber, 1975, 1976; Watson *et al.*, 1978; Watson *et al.*, 1980a; Watson & Wrenn, 1980; King *et al.*, 1982; Blank & Olson, 1988) that the best beetle control achieved appears to be when insecticides are used against the early summer populations. Modelling work also suggested that targeting the early larval stages in December will have a greater effect than controls applied in early spring, before the eggs are laid (East *et al.*, 1981). Eden *et al.* (2011) evaluated the control of African black beetle (*H. arator*) when insecticides are applied in spring and autumn targeting three life stages; newly emerged adults in autumn, mating and egg-laying adults in spring and newly hatched larvae in summer. Although significant adult mortality occurred in both autumn and spring, the remaining live beetles were unaffected and no treatment reduced the subsequent larval population in the following summer. The following factors were said to have likely contributed to the failure to control or reduce African black beetle (*H. arator*) population numbers. Timing of the insecticide application; if applied too early or late the targeted life stage can be missed. Soil factors; high organic matter inactivates the insecticide, and granular insecticides require rainfall or high soil moisture to penetrate the soil. Lastly, the possibility of little or no insecticide contact with targeted life stage and the high mobility of adult beetles.

Pasture renewal is a stage where insecticides are readily available for use in the form of seed coats (Anonymous, 2009; Bell *et al.*, 2011). This may reduce adult African black beetle (*H. arator*) numbers in autumn-sown pastures, therefore reducing the number of over-wintering beetles which re-emerge in spring for mating and egg-laying. This in turn reduces the following spring larval population. The use of insecticidal seed coating is likely to be crucial to pasture establishment

in outbreak years and for reducing beetle populations building up after pasture renewal in the intervening years (Bell *et al.*, 2011).

1.7.6.3 Biocontrol

Pathogens of African black beetle (*H. arator*) have been identified; the protozoa *Adelina Beauveria* fungus, a rickettsia, a RNA virus and entomopathogenic nematodes (Archibald *et al.*, 1975; Longworth & Archibald, 1975; King *et al.*, 1985; Ford *et al.*) and a selection of these are undergoing further investigation for possible use as biological control agents (Bell *et al.*, 2011).

1.7.6.4 Endophyte selection

Sowing a pasture grass that is infected with a suitable endophyte, such as AR37, Endo 5, NEA2 in ryegrasses and MaxP^{TM} in tall fescue (*L. arundinaceum*), to deter adult African black beetle (*H. arator*) is currently the only control measure available to reduce populations of this pest. AR1 infected-ryegrasses are not recommended for use in African black beetle (*H. arator*) prone areas (Popay & Baltus, 2001; DairyNZ, 2010; Bell *et al.*, 2011). Ergovaline is the only alkaloid known to deter adult African black beetle (*H. arator*) feeding and is produced by wild-type endophyte (Ball *et al.*, 1997a). The AR1 strain does not produce ergovaline, but does have a weakly deterrent effect, reducing adult feeding relative to endophyte-free ryegrass (Popay & Baltus, 2001). In the field AR1-infected ryegrass is considerably more vulnerable compared to wild-type-infected ryegrass (Popay & Baltus, 2001; Hume *et al.*, 2007; Popay & Thom, 2009). NEA2 and Endo 5 are two endophytes available in tetraploid ryegrasses that produce low levels of ergovaline, but enough to reduce African black beetle (*H. arator*) populations. AR37 endophyte does not produce ergovaline but still has a strong effect on adult African black beetle (*H. arator*) (Ball *et al.*, 1994) and in the field reduces African black beetle (*H. arator*) populations to the same extent as wild-type endophyte (Hume *et al.*, 2007; Thom *et al.*, 2008; Popay & Thom, 2009; Thom *et al.*, 2013a). However during an outbreak the adult deterrence provided by even the best selected endophytes may not be sufficient to prevent damaging larval populations from building up or new infestations from mass adult beetle migration in late autumn or early spring (Bell *et al.*, 2011; Thom *et al.*, 2013a). Although loline alkaloids are not produced by *E. festucae* var. *lolii*, loline concentration (in offered food) has

been reported to be negatively related to feeding and live weight gain of African black beetle (*H. arator*) larvae and is associated with reduced feeding by adults in *Festulolium* grasses infected with *E. uncinata* (Barker *et al.*, 2014).

1.7.6.5 Combinations of control measures

Bell *et al.* (2011) suggests control measures can and should be combined. The use of an incorrect endophyte-infected grass and weedy pastures provide food sources for African black beetle (*H. arator*) and can assist in the build-up of African black beetle (*H. arator*) populations. Therefore the sowing of the correct endophyte-infected grasses to deter African black beetle (*H. arator*) is crucial, especially during an outbreak. In addition, combining crop rotation by using a non-host break crop and then insecticidal seed coating with pasture renewal should result in reduced adult beetle numbers and aid in the disruption of the beetle lifecycle. In turn this should result in the general reduction of adults available for egg-laying, and hence reduced larval populations in the following spring.

1.8 Immunoassays for endophyte detection

1.8.1 Immunology and antibodies

Antibodies, a large family of glycoproteins known as immunoglobulins (Ig), can be produced by animals in response to exposure to foreign molecules (antigens) in the body to provide adaptive or specific immunity. Antibodies bind specifically to antigens and once bound, the circulating antibody-antigen complexes are removed through phagocytosis by macrophages (Harlow & Lane, 1988). Antibodies are produced by B-lymphocytes and have two main parts, one region is constant and the second region is variable. Antibodies can be visualised as forming a 'Y' shape and each 'Y' contains four polypeptides; two identical copies known as the heavy chain and two identical copies known as the light chain (Harlow & Lane, 1988). There are five classes of antibodies; IgM, IgG, IgA, IgE and IgD. The class of an antibody is determined by the heavy-chain-constant region (Fc) (Crowther, 1995).

The basic structure of an antibody is shown in Figure 1-4. The constant region binds to Fc receptor cells on phagocytes and activates the complement system. This is a series of some 30 proteins that enhance the bacterial killing effect of antibodies by facilitating phagocytosis or by puncturing of the bacterial membrane. The second

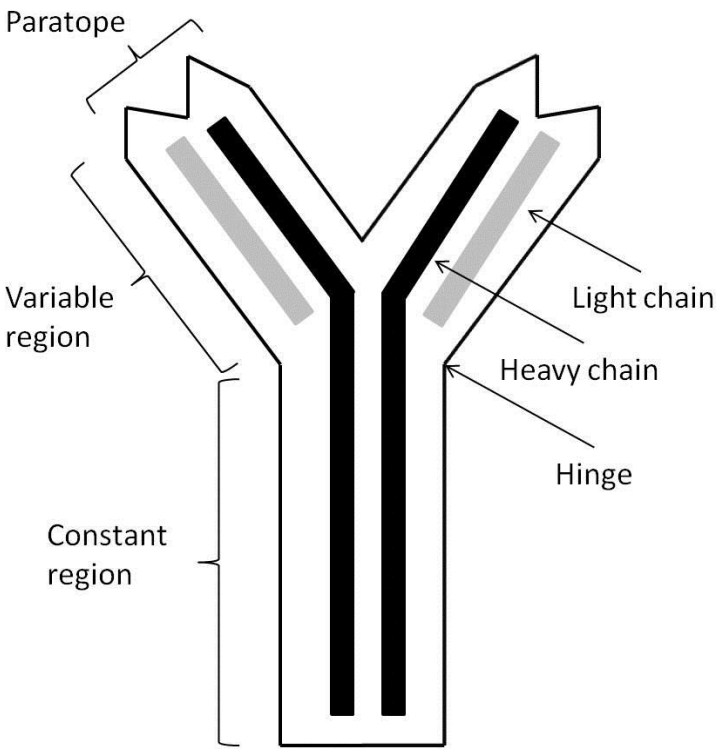

Figure 1-4: Antibody Structure.
Paratope = antigen binding site, Variable region = Fab domain (fragment having the antigen binding site), Constant region = Fc domain (fragment that crystallizes). Adapted from figures 2.2, 2.3 & 2.4 Harlow and Lane (1988).

region of the antibody is extremely variable between antibodies and it is this end that binds the various antigens. The antibody binds to a portion of the antigen known as an epitope (antibody binding site or antigenic determinant). Although this response at the variable region is to a specific antigen, as well as binding to the antigen, antibodies can also cross-react with similar antigens having one or more similar epitopes. The portion of the antibody that binds the antigen epitope is known as the paratope (antigen binding site). Antibodies from different classes can contain the exact same paratope and variable region, whereas antibodies within a class cannot. The segment between the variable and constant regions is called the hinge. The hinge allows lateral and rotational movement of the two identical variable regions and in practice this allows the variable regions to interact with a large number of different antigen conformations (Harlow & Lane, 1988).

An antigen that elicits an immune response is referred to as an immunogen. All immunogens are antigens, but not all antigens are immunogens. While

microorganisms and macromolecules (foreign proteins, nucleic acids, carbohydrates, polysaccharides etc.,) are usually effective immunogens, low molecular weight molecules (below 5000 Da) usually do not elicit an immune response and are termed haptens (Crowther, 1995). If haptens are conjugated to a large molecule (carrier), such as a protein, they can stimulate an immune response. An antibody will be specific to either an epitope on the hapten, or the bridging group linking hapten to carrier, or on the carrier itself. Polyclonal antibodies are mixtures of serum immunoglobulins and collectively are likely to bind to multiple epitopes on the antigen. Monoclonal antibodies by definition contain only a single antibody clone and bind specifically with one epitope.

If an animal has never been exposed to an immunogen it is termed a naïve animal. When an antigen is introduced into a naïve animal the initial antibody response is called the primary response with the majority of antibodies produced being from the IgM class. Mounting an antibody response requires a number of very complex processes to occur, involving the following: antigen presenting cells (APCs), helper T cells, B cells, phagocytosis, antigen binding, processing and presenting by APCs and B cells, helper T cell proliferation, and B cell proliferation and differentiation. B cells are differentiated in to plasma cells and memory cells. B plasma cells secrete large amounts of antibody and are short-lived (3–4 days). B memory cells are long lived, do not secrete antibodies but retain the cell-surface antigen as their specific antigen receptor ready to respond to subsequent exposure to the immunogen. For a primary response the level of antibody detected in the serum peaks around 7–10 days after immunization (Harlow & Lane, 1988). If the no further immunogen is introduced, the response declines as the B plasma cells die but the memory B cells (and helper T cells) remain. When an animal is subsequently exposed to the immunogen a second time (weeks, months or years) the antibody response is much faster, more potent and more persistent (Harlow & Lane, 1988). This secondary response again is very complex and similar to that of the primary response, however, the predominant class of antibodies produced is now IgG. IgG antibodies are bivalent, that is, they contain two identical paratopes, one on each arm of the antibody, as shown in Figure 1-4. Antibodies produced in the secondary response tend to bind the immunogen with a higher affinity than those produced in the

primary response owing to higher numbers of helper T cells and B memory cells in the immune system of the 'primed' animal (Harlow & Lane, 1988).

1.8.2 Immunological techniques

Antigens and antibodies are used and manipulated in various immunological techniques in which the antigen-antibody interaction is exploited. These technologies can be used to detect, quantify or locate an analyte of interest. High affinity and reversible binding of the antibody to antigen and the use of sensitive detection labels enables immunological techniques to achieve high sensitivity and specificity. The design of the immunogen (antigen or antigen-conjugate), use of an appropriate adjuvant (nonspecific stimulator of the immune response), the immunising route and protocol are crucial to the successful production of high affinity antibodies. The choice of animal for immunising is determined by; how much serum is needed, what species the immunogen is isolated from, how much immunogen is available and required, and whether polyclonal or monoclonal antibodies needed. The labelling systems used include enzymes, fluorescent or chemiluminescent compounds, radioactive nucleotides or combinations of these. The two most common enzyme-labels are horseradish peroxidase (HRP) and alkaline phosphatase (AP).

There are two basic immunotechnology formats for detecting the antigen (analyte) of interest by antibodies (antibody capture methods); the direct and indirect format and are shown in Figure 1-5. In the direct format the analyte is detected directly by a labelled specific antibody. Whereas in the indirect format the analyte is detected by an unlabelled specific antibody (1°) and then an enzyme-labelled anti-species antibody (2°) detects the specific antibody, therefore the analyte is being indirectly detected. An advantage of the indirect format is that the enzyme-labelled anti-species antibodies are commercially available.

1.8.2.1 The endophyte ELISAs

The ELISA technique was first developed by Engvall and Perlman (Engvall & Perlmann, 1971, 1972). The most common ELISA design that has been applied for the detection of endophyte secondary metabolites, as with other small molecules, is the competitive ELISA (cELISA) (Candlish, 1991, Stanker & Beier, 1996) (Stanker

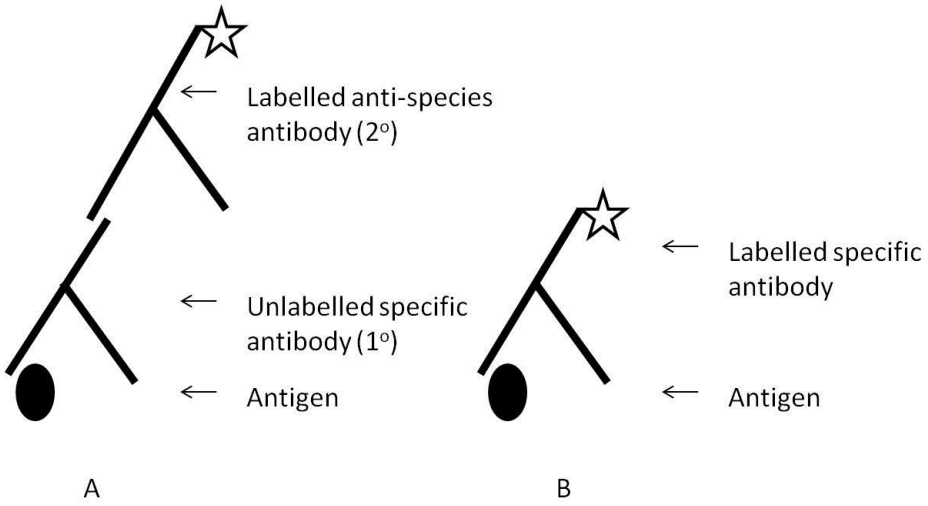

Figure 1-5: Antibody Capture Immunotechnology Formats.
A) Indirect format; the labelled anti-species antibody (2°) binds to the specific antibody (1°), which is bound to the antigen (analyte). B) Direct format; the labelled specific antibody binds to the antigen.

& Beier, 1996). Competition ELISAs are assays in which measurement involves the quantification of a substance by its ability to interfere (compete) with an established pre-titrated system and can be used to measure either antibody or antigen (Crowther, 1995). Microtitre plates can be coated with either antibody or antigen depending on what system is being used. When conjugated antigen is bound to the solid phase (microtitre plate) the cELISA involves competition of free analyte (antigen) in standard or sample against antigen coated to the plate for binding with the specific antibody. The resulting colour intensity is inversely proportional to the concentration of the analyte in the sample. There are two formats that can be used for this type of cELISA, indirect and direct as described in Section 1.8.2 and shown previously in Figure 1-5.

The basic indirect cELISA steps are shown in Figure 1-6. The microtitre plate is coated with antigen or antigen-carrier protein conjugate. Antigen binds to the polystyrene microtitre plate wells by non-specific binding. Excess coating antigen is removed from the plate by washing. Any remaining non-specific binding sites on the plate are blocked using blocking buffer, usually a protein solution. Excess blocking buffer is removed by washing. In the competition step, standards or

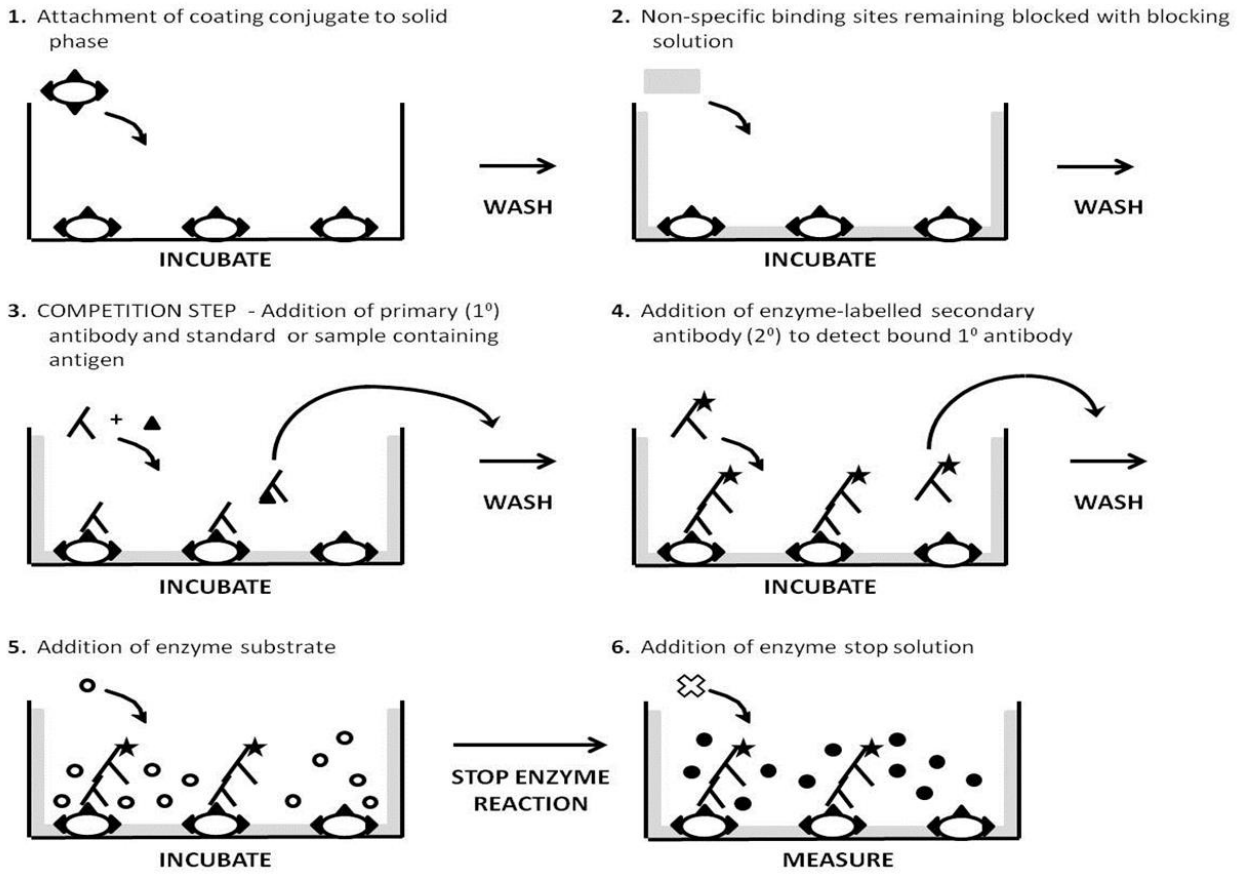

Figure 1-6: The indirect competitive ELISA (cELISA).

samples containing free antigen (analyte) are added to the plate followed by the unlabelled specific primary antibody. The specific primary antibody can bind either to the plate-bound coating antigen or to the free antigen in the standards or samples. The specific primary antibody is used at a dilution that is limiting, such that the maximum absorbance in the assay, in the absence of free antigen, is approximately 1.0 absorbance (A_{max}). The plate is washed to remove free antigen-specific antibody complexes, leaving only plate-bound antigen-specific antibody complexes. In the antibody detection step, the enzyme-labelled anti-species secondary antibodies are added to bind plate-bound specific primary antibodies. The plates are washed removing any excess enzyme-labelled anti-species secondary antibodies, leaving only plate bound antibodies. Substrate is added and enzyme present converts the substrate to a coloured product, the intensity of which is inversely proportional to the concentration of the free antigen in the standard or sample. The enzyme reaction is stopped by the addition of a stop solution and the absorbances of the wells are measured using a microplate reader. A standard curve is generated using reference standards and concentrations of analyte in samples are determined.

When the analyte is present in the sample at a high concentration, few specific antibodies remain available to bind the plate-bound antigen; therefore the binding of the enzyme-labelled anti-species antibodies to the plate-bound specific antibodies is reduced. With enzyme-label reduced, colour development is reduced.

1.8.2.2 The endophyte immunoblot

The endophyte immunoblot (tissue print-immunoblot) was first described by Gwinn *et al*. (1991b, 1991a). Endophyte antigens immobilised onto nitrocellulose were detected using polyclonal antibodies specific for *E. coenophiala* (formerly *N. coenophialum*). The endophyte immunoblot (Simpson *et al.*, 2012) uses an indirect format (Figure 1-5 A) and polyclonal antibodies that were raised in rabbits against the endophyte, *E. festucae* var. *lolii* (formerly *N. lolii*) and goat anti-rabbit IgG antibodies conjugated with alkaline phosphatase (goat anti-rabbit IgG-AP). The endophyte immunoblot method is a qualitative method detecting the presence of endophyte-specific surface antigens immobilised on nitrocellulose membranes, confirming either the presence or absence of the endophyte in a grass plant. Specific

antibodies are used at saturation and antibody concentration is not limited as in an ELISA assay.

1.9 Overview of thesis

1.9.1 AR1 and African black beetle

African black beetle (*H. arator*) is a major pest of grasses throughout the northern part of the North Island, including Waikato and Bay of Plenty, and its range now extends into Hawkes Bay. African black beetle (*H. arator*) is generally regarded as a sporadic pest and occasional widespread outbreaks of this insect are devastating for farmers. Adult beetles feed at the base of tillers and can destroy new pasture, but the root-feeding larvae do the most damage. The adult beetle is deterred by certain fungal endophytes producing specific secondary metabolites in ryegrass. This reduces the number of eggs the beetle lays and results in fewer root-feeding larvae in the pasture. Adult African black beetle (*H. arator*) were found to be deterred from feeding by the presence of asexual *Epichloë* (*Neotyphodium*) endophytes in perennial ryegrass (*L. perenne*) (Ball & Prestidge, 1992). The asexual *Epichloë* fungal secondary metabolites, peramine, lolitrem B and paxilline did not deter adult African black beetle (*H. arator*) (Ball *et al.*, 1997a), however, the fungal secondary metabolite ergovaline, and the structurally similar ergopeptine ergot alkaloids ergotamine, α-ergosine and α-ergocryptine did. (Ball & Prestidge, 1993; Ball *et al.*, 1997a). Studies with ryegrass plants infected with asexual *Epichloë* endophytes (producing different alkaloid spectra), confirmed that ergovaline (known to deter African black beetle (*H. arator*)), peramine and lolitrem B were not required for African black beetle (*H. arator*) resistance (Ball *et al.*, 1994).

The fungal endophyte, AR1, was developed to provide its host perennial ryegrass (*L. perenne*) with resistance to Argentine stem weevil (*L. bonariensis*) without any adverse effects on farm animal production as it produces peramine but does not produce the mammalian toxins, ergovaline and lolitrem B (Tapper & Latch, 1999). This endophyte lacks any of the alkaloids known to deter adult African black beetles (*H. arator*) but, despite this, shows moderate resistance to this insect (Popay & Baltus, 2001). The biochemical basis for this resistance is not understood.

Fungal endophytes interact strongly with their host plant genotype and this interaction affects resistance to insects. In the AR1-African black beetle (*H. arator*) relationship, some cultivars infected with this endophyte are more resistant than others. In a preliminary study (Lincoln trial, Figure 1-7), feeding damage by adult African black beetles (*H. arator*) in a range of different cultivars showed some association with the response elicited by infected plant material in a paxilline ELISA (Popay A. J., Fletcher L. R., Briggs L. R., AgResearch, New Zealand; unpublished work, 2006). Although AR1 genetically is capable of producing paxilline, a mild tremogen it does so only at very low levels (Young *et al.*, 2009). AR1 can only produce simple indole diterpenes (paxilline and paxilline-like compounds such as the terpendoles).

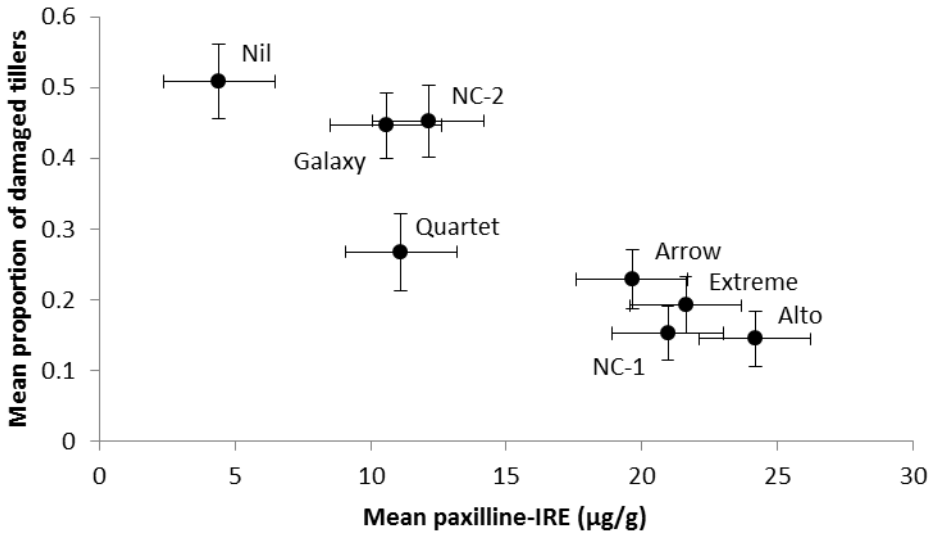

Figure 1-7: Mean feeding damage by adult African black beetle compared with mean levels of paxilline immunoreactive equivalents (paxilline-IRE) in AR1-infected commercial cultivars and a breeding line (PG189) with an endophyte-free control (Nil). *(Mean ± SEM). (Popay A. J., Fletcher L. R., Briggs L. R., AgResearch, New Zealand; unpublished work, 2006).*

The immunoassay (paxilline ELISA) recognises compounds that the AR1 and host plant associations are producing, these compounds have a paxilline-like structure, with epitopes that are the same or similar to that of paxilline. The active component

(or compound produced in association with the bioactive) in the infected plant material deterring adult African black beetle(*H. arator*), appears to be one that cross-reacts with the antibody used in that paxilline immunoassay.

AR1-infected ryegrass has been highly successful in reducing the effects of Argentine stem weevil (*L. bonariensis*) without having adverse effects on grazing livestock. In fact it is still the only commercially available endophyte that poses no risk to animal health or to the occurrence of secondary metabolite residues in animal products. It has been widely adopted by New Zealand farmers, increasing live weight gains of lambs and milk production of cows.

Although yields of AR1-infected ryegrass are generally similar to the wild-type, persistence, and on occasions its productivity, is lower. In African black beetle (*H. arator*) prone regions, the prolonged outbreak of beetles over many seasons (2007/2008–2011/2012) has resulted in AR1 cultivars now being sold with a warning about lack of persistence when under severe African black beetle (*H. arator*) attack (Bell *et al.*, 2011). In areas where African black beetle (*H. arator*) is a problem, there is no doubt that this pest compromises both persistence and productivity of ryegrass with this endophyte. Selection of AR1 cultivars with optimal resistance to African black beetle (*H. arator*) would provide a significant advance to plant breeders.

1.9.2 Intention of thesis

The results of the Lincoln trial (Figure 1-7) suggest increasing levels of paxilline immunoreactive equivalents (paxilline-IRE) in the host plant is associated with a reduction in adult African black beetle (*H. arator*) feeding damage (Popay A. J., Fletcher L. R., Briggs L. R., AgResearch, New Zealand; unpublished work, 2006). It was the discovery of the potential association that motivated the research for this thesis. The inferred relationship may be misleading as the negative correlation found was between different ryegrass cultivars and species. The differences observed between the cultivars may have been predominantly a cultivar effect irrespective of endophyte infection, rather than a host plant's influence on the levels of endophyte alkaloid production.

ELISA was specifically chosen, as the priority was detection (not quantification) of paxilline-like compounds, both known and unknown. ELISA is a low cost, fast, robust, reliable, repeatable and high throughput system. Furthermore, it was from this methodology that the initial trend was observed in the Lincoln Trial. The paxilline ELISA quantitatively measures levels of paxilline immunoreactive equivalents which is determined by the variable cross-reactivity's of known and unknown paxillline-like compounds. It cannot quantify the paxilline-like compounds collectively or individually. Analytical methods could be used to quantify known paxilline-like compounds, but not unknkown paxilline-like compounds. Further, these analytical methods are high cost with low throughput compared to ELISA, and resources were not available.

This research will investigate the variation in resistance to African black beetle (*H. arator*) within a breeding line (and among 23 half-sibling ryegrass families) infected with AR1 and aims to identify the basis for that resistance. The heritability of the resistance and plant and environmental factors that impact on expression of resistance will also be studied. The study used a series of feeding trials with adult African black beetles (*H. arator*) to investigate what variation there was in paxilline ELISA immunoreactivity determined on extracts taken from AR1-infected half-sibling perennial ryegrass (*L. perenne*) families from within a breeding-line (GA97), and whether any differences were related to feeding by adult African black beetle (*H. arator*). The plant-based feeding trials used either, plants grown from seed or cloned plants. Plants grown from seed within a family are a representative plant from that family. These plants are closely related but not genetically identical and the family variation for characteristics can be determined. In comparison when a single plant is separated (cloned) into many smaller plants (ramets), each plant clone is genetically identical and from the same original family representative. Cloning of plants allows multiple testing on an individual plant within a family, and for the variation of that individual plant (inter-clonal variation) for characteristics to be determined. This knowledge will ultimately aid the plant breeders to effectively screen and select for optimal AR1 and host plant associations.

1.9.3 Hypothesis and aims

1.9.3.1 Hypothesis

Deterrence of African black beetle (*H. arator*) feeding is associated with greater production of specific active compounds in closely related AR1-infected ryegrasses.

1.9.3.2 Aims

- Determine the variation among half-sibling perennial families infected with the strain AR1 that deter African black beetle (*H. arator*) feeding (Chapters 4 and 5).
- Determine the variation among half-sibling perennial families infected with the strain AR1 in response to ELISAs, which detect particular classes of compounds that are associated with the deterrence of African black beetle (*H. arator*) feeding (Chapters 3, 4 and 5).
- Determine the biochemical basis for the bioactivity providing the host plant resistance to African black beetle (*H. arator*) (Chapters 4 and 8).
- Ascertain if the production of these active compounds is genetically based (Chapters 3, 4 and 5).
- Determine the various effects of field parameters (season, plant age, etc.) on the production of the compounds identified in the plants (Chapters 5, and 7).
- Determine the relationships between endophyte concentration, production of active compounds and resistance to African black beetle (*H. arator*) (Chapters 6 and 8).
- Determine the distribution and concentrations of the active compound/s is in various parts of the plants (Chapters 4 and 5).

1.9.4 Thesis structure

This thesis consists of a series of five adult African black beetle (*H. arator*) feeding trials conducted at AgResearch, Hamilton, New Zealand (2008–2012) to investigate if feeding damage by adult beetles is related to paxilline immunoreactivty in ryegrass. Chapter 2 explains the general materials and methods used to conduct this research. Chapter 3 describes the preliminary work required before research could commence, including the initial selection of plant genotypes from 23 closely related families by paxilline ELISA. Chapters 4 (Trial 1) and 5 (Trial 2) present the initial

feeding trials using cloned plants from the half-sibling families for the determination of plant variation in feeding damage and paxilline immunoreactivity, and for further plant and family selection. In addition, the research of Chapter 5 allows a preliminary assessment of the influence of both beetle presence and season on plant responses. Chapter 6 (Trial 3) explores how AR1 endophyte affects adult African black beetle (*H. arator*) in an artificial diet trial, using AR1-infected seed from a commercially available ryegrass cultivar. Chapters 7 and 8 are presented as papers to be submitted for publication. Chapter 7 presents a further in planta feeding trial (Trial 4), from a selected number of families using plants grown from seed, to investigate the use of paxilline immunoreactivity to select for plant resistance to African black beetle (*H. arator*). The final feeding trial, described in Chapter 8 (Trial 5), uses plants grown from seed to explore how long term exposure to African black beetle (*H. arator*) from late autumn–late spring affects plant responses from AR1-infected ryegrass families and cultivars, including resistance to African black beetle (*H. arator*), paxilline immunoreactivity and dry matter production. In addition; Chapter 8 examines what influence these plant responses have on adult African black beetle (*H. arator*) survival and fecundity.

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Chapter 2

Materials and Methods

2.1 Plant and insect techniques

2.1.1 Seed germination and plant identification

Seeds of the 23 half-sibling perennial ryegrass (*Lolium perenne* L.) families from the GA97 breeding-line were sourced from Forage Improvement, AgResearch, Grasslands, Palmerston North, New Zealand. Seeds of the commercially available cultivars containing either AR1 or AR37 endophyte and the endophyte-free equivalent were obtained from the AgResearch Margot-Forde Germoplasm Centre, Palmerston North, New Zealand. Unused seed was stored in airtight packages in the dark at 4°C. Seeds were germinated by spreading on damp filter paper in petri-dishes sealed with parafilm and left to germinate in the dark at 20°C for 7 to 10 days. Germinated seedlings were carefully planted out into trays containing a general purpose potting mix (Daltons GB Potting Mix) for establishment.

For perennial ryegrass (*L. perenne*) plant identification, morphology matched the description in the literature (See Champion *et al.*, (2012), p128-130) and seed was obtained from reputable sources.

2.1.2 Plant cloning

Plants were cloned by splitting the original plant into ramets of four to six tillers and re-planted. This always included at least one spare plant clone being re-planted back into the original tray or pot. In the first plant/insect trials (Trial 1), cloned plants were transferred into the trial trays containing potting mix. From Trial 2, the cloned plants were first planted out into mortar sand to encourage root development for 10–14 days and then transferred into trays containing general purpose potting mix for establishment.

2.1.3 Planting out

Cloned plants or seedlings were planted out into polystyrene trays, metal rings or pots containing soil : sand (2 : 1) mix, sand or Daltons GB potting mix. Seedlings or cloned plants were left to establish either in the glasshouse (late autumn to early

spring) or the screenhouse (late spring to early autumn) depending on the ambient air temperature outside.

2.1.4 Plant maintenance

Yates Thrive All Purpose Soluble Fertiliser (NPK analysis; 27 : 5.5 : 9, with trace elements) and urea (plant grade) were sourced from local retail stockists.

Spare perennial ryegrass (*L. perenne*) plants and post-trial plants were maintained in the screenhouse at ambient temperature with automated watering. Plants were trimmed every 4–6 weeks to 3 cm or 4 cm above the crown. The plants were fertilised post-trimming using Thrive fertiliser at the recommended label rate (1.8 g/L) with the addition of urea (2.5 g/l) dissolved in tapwater).

2.1.5 Adult African black beetle collection and identification

Pitfall traps as described by King *et al.* (1980) with minor modifications, for adult African black beetle (*Heteronychus arator* (Fabricius 1775)) collection were made from round plastic containers (425 ml, Uni-pack, New Zealand). Small holes, made in the bottom of the containers using a soldering iron, were large enough to allow water drainage but not to allow an adult African black beetle (*H. arator*) to escape. Cored holes were made in the pasture approximately 1.5 m apart using a 100 mm corer and the pitfall trap was placed in the hollow, with 150–200 traps placed out at one time. The beetles emerge in the evening and fall into traps when searching for food. Figure 2-1 shows the setting of a pitfall trap.

Figure 2-1: Setting a pitfall trap.
A) Corer (100 mm). B) Cored hole and soil core. C) Plastic pitfall trap with drainage holes placed in cored hole, with a captured adult African black beetle (H. arator).

Adult African black beetle (*H. arator*) were collected from the traps daily, first thing in the morning after other insects were released. This was before the beetles died from being exposed to the sun and high temperatures or by being eaten by predators. If pitfall traps were not checked daily or first thing in the morning, a small layer of soil was placed in the traps with a food source on top (slices of carrot or kumara) to ensure survival of adult African black beetle (*H. arator*). Pitfall traps were placed in paddocks with AR1 sown in, or in paddocks where in previous seasons the paddock had high numbers of African black beetle (*H. arator*) recorded or damage to pastures. They were placed alongside the fence-line to reduce damage from stock. Adult African black beetles (*H. arator*) were also collected by hand just after dusk at places where there was sufficient grass surrounds and strongly lit-up large concreted areas, as beetles are attracted to these well-lit areas during flight activity. Upon landing the beetles walk on the ground towards the dark, and are easily collected because they cannot burrow into soil. Beetles were transferred into containers partly filled with damp soil, and perforated lids were placed on the container. When beetles were not present in the Waikato they were sourced from Kerikeri in the Upper North Island.

Collection of adult African black beetle (*H. arator*) was in early autumn from March to April when pupae were transforming into adults, or in spring from late September when over-wintering dormant adults emerge to mate and feed, as discussed in Section 1.7.2 and shown in Figure 1-2.

For adult African black beetle (*H. arator*) identification, adults were collected when abundant, matching the lifecycle (Bell *et al.*, 2011; Todd, 1959) (also see Section 1.7.2) and beetle morphology met the description and key indicators (Klimaszewki & Watt, 1997; Watt 1984) reported in the literature.

2.1.6 Adult African black beetle maintenance

Captured male and female African black beetles (*H. arator)* were placed into separate containers (2–4 litres) with perforated lids. The containers had damp soil in the bottom. Each container held 20 to 50 beetles. Males were distinguished from females as the last segment on the front legs (fore tarsus) of males is much thicker than that on the female leg which elongate and narrow, as shown in Figure 2-2. Soil in the containers was kept damp but not too moist. Slices of carrot or kumara were

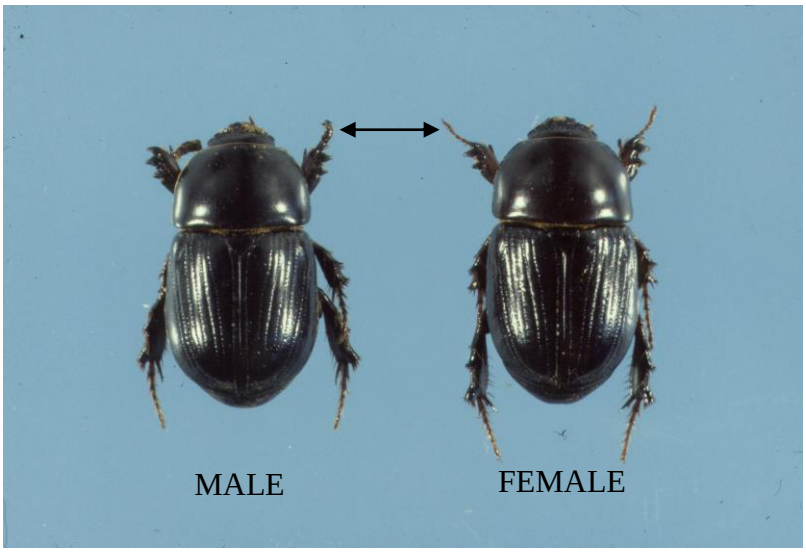

Figure 2-2: Adult African black beetle gender.
Showing the difference in the last segment of the front legs (fore tarsus) for gender determination (Image from Popay, A. J., AgResearch, New Zealand).

put in the containers to feed the beetles and the slices were changed as necessary (approximately weekly for carrot and fortnightly for kumara).

2.1.7 Adult African black beetle feeding trials

Three types of adult African black beetle (*H. arator)* feeding trials were performed:

- Feeding trial with choice of plants.
- Feeding trial with no choice of plants.
- Feeding trial with artificial diet (with no choice).

In the experimental unit of the feeding trial with choice of plants the beetles had the choice of more than one type of plant to feed from. In comparison, with a trial with no choice of plants the experimental unit consists of a single 'plant type'. Plant type may refer to plant genera, plant species, plant cultivar, plant breeding line and plant family or any combination. Generally plants were trimmed prior to trial assessments. The impact of trimming was deemed minimal because of the following reasons: all plants were treated the same (including plants in the control group), perennial ryegrass is adapted to defoliation, trimming mimics (to a certain extent) defoliation by mammalian herbivores, trimming was not lower than 3 cm from the crown whereas adult African black beetle feed at the base of the crown

and in severe cases kill the tiller, defoliation does not kill the tiller. Therefore any response found was over and above any impact of trimming.

The experimental unit of a feeding trial with artificial diet with no choice consisted of one beetle and one artificial diet plug of a specific formulation. The actual number of diet formulations, beetles, plants or diets, and the number of replications was specific for each trial.

2.2 Endophyte detection in plant material

Presence or absence of endophyte (E+ or E-) in a plant was determined primarily using the endophyte tissue print-immunoblot (immuno-detection) technique and reagents as described by Gwinn *et al.* (1991) and Simpson *et al.* (2012). Endophyte status was also determined using the compound microscope method as described by Simpson *et al.* (2012) with minor modifications. This method is also similar to that described by Latch and Christensen (1985). When it was not possible to get a clear positive or negative result the immunoblot was repeated and a plant sample was also checked under the microscope. The compound microscope method was used to keep the tiller intact and attached to the plant when there were insufficient tillers for immunoblotting.

2.2.1 Endophyte immunoblot

Seedlings or cloned plants were established (3–4 tillers) before examination for endophyte status. Sheets (10.25 cm × 10.25 cm) of nitrocellulose membrane (0.45 µm pore size) (Sigma) that had gridlines (printed or hand-drawn 1 cm^2 grid squares) were used. A single tiller sample from a plant was taken by cutting it at the base. Any necrotic (dead) material was removed and a second cut was made by a scalpel at the base of the tiller sample to obtain a clean transverse cross-section. The cut end was blotted directly onto the nitrocellulose membrane paper within a grid square, leaving a circular outline (print) from the plant sap. Five tillers were blotted per grid square in the positions shown in Figure 2-3. This was done without touching fingers on the nitrocellulose membrane surface.

The immunoblot sheet was blotted using the multiple grid pattern shown in Figure 2-4. Prints (blots) of samples of individual plant tillers were recorded using this grid reference system. Each sheet was labelled with a sheet number and the collection

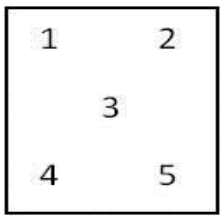

Figure 2-3: Immunoblot designated positions within a 1 cm square in the grid.

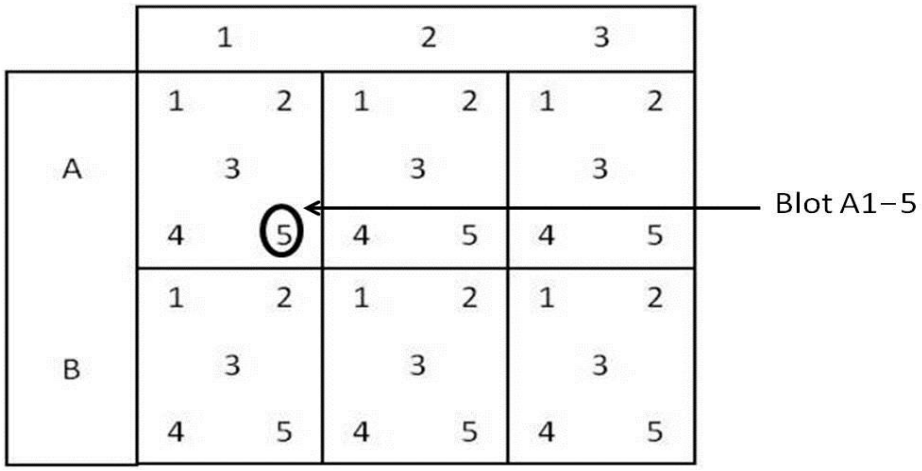

Figure 2-4: Immunoblot position of grid-squares and blots on a sheet.
Circled blot (A1-5) is recorded by grid-square position (A1) then by blot position (5) next to the record of the corresponding plant that the tiller used for the immnoblot came from.

date and stored, with the blue cover paper back on it, in a sealed plastic bag at 4°C. The sheet was sent to AgResearch, Grasslands, Palmerston. North, New Zealand for development of the immunoblot. A red colour on the developed blots indicated that endophyte was present (E+) while a light pink colour indicated that endophyte was absent (E-). An example of a developed blot is shown in Figure 2-5.

2.2.2 Examination of endophytes with a microscope

Plants were checked under the microscope for the presence (E+) or absence (E-) of endophytes. A single tiller sample was taken by cutting the tiller at the base of the plant. When there were insufficient tillers for a tiller sample to be taken a leaf blade was removed down to the base of the plant. Any necrotic material was removed from the pseudostem to leave clean and live sheath material. Using a scalpel blade a single layer of epidermal plant cells was removed from the leaf sheath and

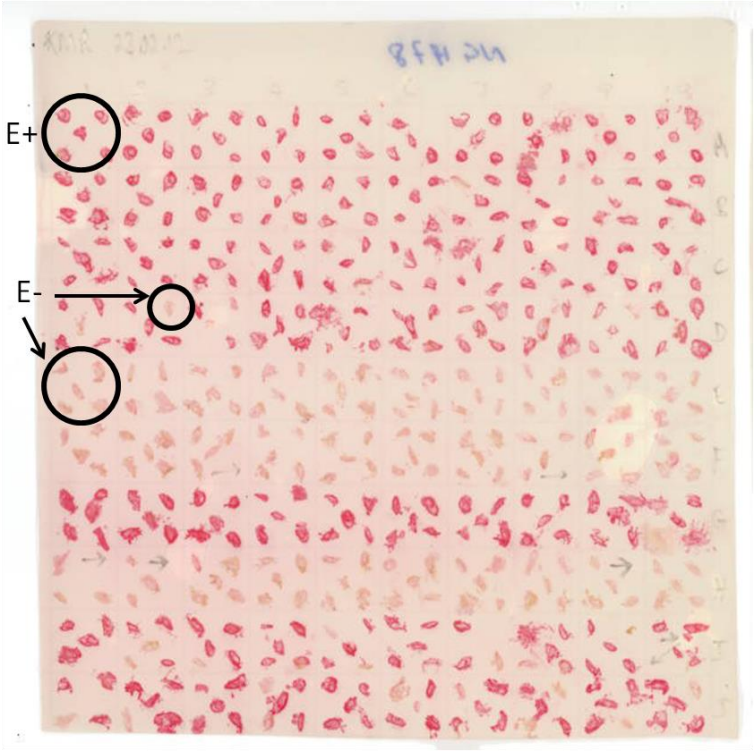

Figure 2-5: A developed immunoblot.
Showing positive, endophyte present (E+; Grid A1) and negative, endophyte absent (E-; GridE1 and blot D2-2) blots.

mounted on a microscope glass slide and then stained with a drop of aniline blue stain (glycerol 50%, lactic acid 25%, deionised water 24.95%, Sigma aniline blue 0.05%). The mounted sample was covered with a coverslip and left at room temperature for at least ten minutes for the stain to be absorbed into the plant tissue. The sample was then examined at 100× and 400× magnifications. Fungal hyphae present stain blue and show up as strands attached to the sides of the plant cell as shown in Figure 2-6.

2.3 Sample preparation for ELISA and chemical analysis

2.3.1 Freeze-drying

Plant samples (herbage and pseudostem) were frozen at –20°C. Once samples were completely frozen the plastic bags were opened and placed on the freeze-drying trays ensuring adequate airflow for drying. The trays were loaded into the large-scale freeze-drier (Cuddons, 15 litre condenser capacity, Plant and Food, Ruakura Research Centre, Hamilton, New Zealand) and were dried for 72 h.

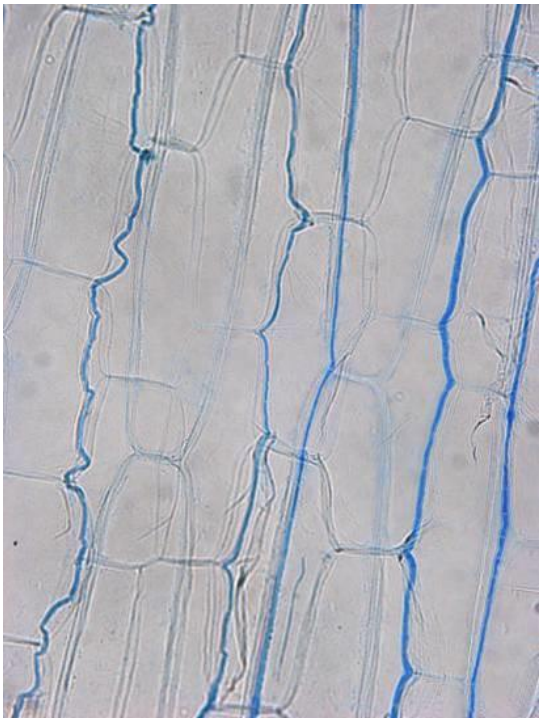

Figure 2-6: Fungal endophyte stained by aniline blue.
(Image from Christensen, M. J., AgResearch, New Zealand, 2008).

Samples were removed from the freeze-drier and air was expelled from the plastic bags prior to sealing. Freeze-dried samples were stored at –20°C until they were milled.

2.3.2 Milling

All samples were equilibrated to ambient room temperature (21°C) in their sealed containers before milling commenced. Seed samples (stored at 4°C) or freeze-dried plant samples (Section 2.3.1) were ground either by the IKA-A10 blade mill (IKA®-WERKE, Staufen, Germany) for small samples (≤2 g) or the Udy Cyclone mill (Udy Corporation, CO, USA) for the medium and larger samples (>2 g). Milled samples were then stored in sealed containers at –20°C until required for analysis.

2.4 Analytical techniques

2.4.1 Materials and reagents

All inorganic chemicals and organic solvents were of reagent grade or better and, unless stated otherwise, were supplied from a local chemical supplier. Maxisorp polystyrene 96-well microtiter plates, plate lids and plate sealers, manufactured by Nunc A/S, Roskilde, Denmark and bovine serum albumin (BSA), Fraction V, fatty acid poor, manufactured by Gibco, New Zealand, were supplied by Invitrogen Corporation, New Zealand. Ovalbumin (OVA), 98% was obtained from Neova, Novatech, Canada. Tween 20® was obtained from Merck Schuchardt, Hohenbrunn, Germany. Methanol, HPLC grade, and sulphuric acid, concentrated, were obtained from BDH, Poole, England.

The following reagents were prepared for use in the ELISA assays of paxilline, peramine, lolitriol/lolitrem and endophyte mycelium mass:

- Coating buffer: sodium carbonate/bicarbonate buffer, 0.05 mol/L, pH 9.6.
- Phosphate buffered saline (PBS): sodium chloride (0.15 mol/L), sodium dihydrogen orthophosphate (0.017 mol/L), disodium hydrogen orthophosphate (0.0084 mol/L), pH 7.4.
- Wash buffer (PBST): 0.05% Tween 20 (v/v) in PBS.
- Blocking/antibody buffer: 1% bovine serum albumin (w/v) in PBST (1% BSA/PBST) or 1% ovalbumin (w/v) in PBST (1% OVA/PBST).
- Standard/Sample buffer: 10% methanol (v/v) in PBST (10% methanol/PBST) or PBST.
- Substrate buffer: BioFX TMB One Component HRP microwell substrate from SurModics, MN, USA; or K-Blue aqueous TMB substrate from Neogen, KY, USA.
- Stop solution: 0.3 mol/l sulphuric acid (0.3 mol/l H_2SO_4).
- Plate coating antigens: OVA-paxilline conjugate (Garthwaite *et al.*, 1993) or BSA-peramine conjugate (Garthwaite *et al.*, 1994) or BSA-lolitriol conjugate (Briggs *et al.*, 2007) or *E. festucae* var. *lolii* mycelium coating antigen (Faville *et al.*, 2007).
- Specific primary antibodies (in-house): anti-paxilline M03/01 mouse monoclonal antibody (Garthwaite *et al.*, 1993); or anti-peramine 165-2

sheep polyclonal antibody (Garthwaite *et al.*, 1994); or anti-lolitriol 134 sheep polyclonal antibody (Briggs *et al.*, 2007); or anti-*E. festucae* var. *lolii* SAPU F2 rabbit polyclonal antibody (Faville *et al.*, 2007).

- Anti-species secondary antibodies (commercially available): sheep anti-mouse conjugate with HRP from Chemicon, CA, USA; or rabbit anti-sheep conjugated with HRP or goat anti-rabbit conjugated with HRP from DAKO, Denmark.

2.4.2 ELISA analysis

Extraction and ELISA methods followed were those previously developed in-house at AgResearch, Ruakura.

For all ELISA analyses:

- All freeze-dried milled grass samples were weighed using a four-place balance (Mettler AE 260 Delta Range).
- A bench-top centrifuge (Eppendorf Centrifuge 5418) was used for the centrifugation of samples contained in microfuge tubes.
- For the coating and substrate ELISA steps, plates were sealed with a plate sealer, covered with a lid and then wrapped in tinfoil.
- For all remaining ELISA steps the plates were sealed with a plate sealer and covered with a lid and kept in the dark.
- Plates were washed four times after each step with PBST on an automated plate washer (Dynex, MRW AM60).
- At the substrate step the plates were placed on an IKA-SCHÜTTLER MTS 4 (which enhances the enzymatic colour development and reduces the incubation time required). Note, the colour development is from clear to blue and once the enzyme reaction is stopped by the addition of sulphuric acid the colour changes from blue to yellow.
- The absorbance of the wells on the ELISA plates was determined at 450 nm using a VERSA$_{max}$ tunable microplate reader.
- Data analysis was performed using software developed in-house (4-parameter curve fit) at AgResearch. The results generated take into account the dilutions used on the ELISA plates.

2.4.3 Paxilline analysis

2.4.3.1 Sample extraction

2.4.3.1.1 Grass (herbage and pseudostem) extraction

Grass samples prepared as in Section 2.3 were equilibrated to 21°C in their sealed containers, then 50 mg of sample was weighed into a microfuge tube (2 ml). Weighed samples were stored at –20°C until required. Samples were extracted on the same day the paxilline ELISA was performed, the immunoreactivity determined by ELISA is not stable on storage. Extracting solvent (1 ml, 90% methanol in water) was added to the microfuge tubes and samples were rotated end-over-end (ensuring all sample was wetted) on a rotation mixer (Labnet mini labroller, NJ, USA), for 20 min. Samples were then centrifuged at 8 609 ×*g* for 5 min. Supernatant was collected and analysed by ELISA. In case of the need for re-analysis and because of limited availability of plant sample, extracts were stored in screw-top glass vials (1.5 ml) at 4°C, or for longer term at –20°C, with the knowledge that the samples would have undergone varying degrees of degradation.

2.4.3.1.2 Seed extraction

Milled seed samples were equilibrated to room temperature (21°C) in their sealed containers then 200 mg samples were weighed into microfuge tubes (2 ml). Weighed samples were stored at –20°C until required. Samples were extracted on the same day the paxilline ELISA was performed because of the instability of the immunoreactivity in the seed samples. Extracting solvent (1 ml, 100% methanol) was added to the microfuge tubes and samples were extracted by end-over-end rotation for 20 min, ensuring all sample was wetted. The tubes were then centrifuged at 8 609 ×*g* for 5 min. From each the supernatant was recovered for ELISA, and transferred to a screw-top glass vial (1.5 ml) for storage at 4°C. Long term storage at –20°C was necessary as there was limited seed sample available for the majority of trial samples if repeated extraction and analysis was required. This was done knowing that sample extracts would have undergone varying degrees of degradation on storage.

2.4.3.1.3 Artificial diet extraction

Until required the weighed artificial diet samples were stored at –20°C and prior to extraction were equilibrated to room temperature (21°C) in their sealed microfuge tubes (2 ml). Samples were extracted on the same day the paxilline ELISA was performed because of the known instability of the immunoreactivity in seed samples (2.4.3.1.2). Extracting solvent (1 ml, 100% methanol) was added to the microfuge tubes and samples were extracted by end-over-end rotation for 20 min, ensuring all sample was wetted. Tubes were then centrifuged at 8 609 ×*g* for 5 min. From each tube the supernatant (1 ml) was recovered. Extracts were concentrated (10×) with the 1 ml methanol removed under nitrogen blowdown and then the extract resuspended in 100 µl methanol for ELISA analysis. Long term storage of artificial diet extract at –20°C was necessary (there was no more sample available) in case repeated ELISA analysis was required. This was done knowing that sample extracts could have undergone varying degrees of degradation on storage.

2.4.3.2 Paxilline ELISA

The paxilline ELISA is outlined as shown in Table 2-1. The coating antigen (OVA-paxilline) and specific primary antibody (monoclonal mouse anti-paxilline M03/01 antibody) used in this cELISA are those described in Section 2.4.1. The assay described by Garthwaite *et al.* (1993) has since been re-formatted using 1% BSA/PBST as the blocking agent and conjugate buffer, Chemicon sheep anti-mouse conjugated with HRP for the anti-species secondary antibody and BioFX TMB substrate buffer. The assay working range and sensitivity differs from that described by Garthwaite *et al.* (1993) because of the optimisation.

Microtiter plates were coated with coating conjugate, OVA-paxilline at 3.0 µg/ml in bicarbonate coating buffer (100 µl/well). Coated plates were sealed, covered and incubated overnight at 4°C, then washed as described in Section 2.4.2. Plates were blocked with blocking/antibody buffer, (1% BSA/PBST, 200 µl/well), sealed (Section 2.4.2) and incubated for 1 h at 21°C. Paxilline standard curve concentrations (0.1–1 000 ng/ml) and sample extract dilutions (minimum extract dilutions of 1 in 9 for herbage and 1 in 10 for seed) were prepared in standard/sample buffer (PBST and 10% methanol/PBST) to give a final methanol

Table 2-1: Outline of the paxilline indirect competitive ELISA.

STEP	REAGENT	TEMP	TIME	VOLUME (µl/Well)
Coat	OVA-paxilline conjugate 3 µg/ml in coating buffer	4°C	18 h	100
Wash 4X with PBST				
Block	1% BSA/PBST	21°C	1 h	200
Wash 4X with PBST				
Competition Standards/samples + Specific (1°) antibody	Diluted sample extract or paxilline standard (0.1–1000 ng/ml) in 10% methanol/PBST Anti-paxilline M03/01 diluted 1/23 ×10^6 in 1% BSA/PBST	21°C	1 h	50 50
Wash 4X with PBST				
Antibody detection HRP labelled anti-species (2°) antibody	Chemicon sheep anti-mouse-HRP diluted in 1/2 500 in 1%BSA/PBST	21°C	2 h	100
Wash 4X with PBST				
Substrate	BioFX TMB substrate	21°C	0.5 h	100
Stop	Stop solution (0.3 mol/l H_2SO_4)	21°C		100
Read plate		21°C		

concentration of 10% (v/v). Specific primary mouse monoclonal antibody, anti-paxilline was diluted 1 in 23×10^6 in 1% BSA/PBST. Plates were washed prior to the addition of standards and samples (50 µl/well), followed by the specific antibody (50 µl/well) giving a total volume of 100 µl/well. Sealed plates were incubated for 1 h at 21°C. All sample extracts were analysed using a minimum of two dilutions. All standard concentrations and sample dilutions were analysed in duplicate wells. The same positive control sample of herbage was extracted and analysed on every plate. Pates were washed prior to the addition of the anti-species secondary antibody, (sheep anti-mouse conjugated with HRP, Chemicon), which was diluted 1 in 2 500 in 1% BSA/PBST (100 µl/well). Plates were sealed and incubated for 2 h at 21°C. Plates were washed prior to the addition of the substrate solution, BioFX TMB substrate (100 µl/well). Plates were then sealed, covered, placed on a plate shaker and incubated for 0.5 h at 21°C to develop the colour (Section 2.4.2). The enzyme reaction was stopped by the addition of stop solution, 0.3 mol/l H_2SO_4 (100 µl/well). The plates were read and data analysed as described in Section 2.4.2. The results generated take into account the dilutions used on the ELISA plate.

Results were expressed as µg of paxilline-immunoreactive equivalents (paxilline-IRE) per g of milled grass, as paxilline was the reference compound used to generate the standard curve in the assay. ELISA analysis of samples was repeated within 24 h if required. The assay working range was 1–40 ng/ml with a detection limit for the undiluted grass extract of 0.18 µg/g dwt. The analysis was repeated either because a result obtained was out of the working range of the assay or there was >10 % CV on replicate determination.

2.4.4 Peramine analysis

2.4.4.1 Grass (herbage and pseudostem) extraction

Grass samples (50 mg) as prepared in Section 2.3 were extracted for 1 h in 90% methanol in water (1 ml). Owing to the limited availability of plant sample, extracts were stored for up to seven days at –20°C as the immunoreactivity measured in the peramine ELISA was found to be stable for at least one week.

2.4.4.2 Peramine ELISA

Reagents used for the peramie ELISA (BSA-peramine coating conjugate, specific primary polyclonal sheep anti-peramine 165-2 antibody) were those described by Garthwaite *et al*. (1994). The reformatted assay (now using 1% BSA/PBST as the blocking agent and conjugate buffer, DAKO rabbit anti-sheep conjugated with HRP for the anti-species secondary antibody and BioFX TMB substrate buffer) followed a similar protocol as that for the paxilline ELISA (Section 2.4.3.2) and is outlined in Table 2-2. The differences from the paxilline ELISA were the coating conjugate, standard, specific primary and anti-species secondary antibodies used and the plate coating incubation temperature.

Microtiter plates were coated with coating conjugate, BSA-peramine at 0.2 µg/ml in bicarbonate coating buffer (100 µl/well). Plates were sealed, covered and incubated overnight at ambient room temperature (21°C). Plates were washed then blocked with blocking/antibody buffer (1% OVA/PBST, 200 µl/well), sealed and incubated for 1 h at 21°C. Peramine standard curve concentrations (0.1–10 000 ng/ml) and sample extract dilutions (1/9 minimum dilution) were prepared in standard/sample buffer (10% methanol/PBST and PBST) to give a final methanol concentration of 10% (v/v). The specific primary sheep polyclonal, anti-peramine 165-2 antibody was diluted 1 in 3.5 $\times 10^5$ in 1% OVA/PBST. Plates were washed prior to addition of the standards and samples (50 µl/well), followed by the specific primary antibody, 50 µl/well) giving a total volume of 100 µl/well. Sealed plates were incubated for 1 h at 21°C. All sample extracts were analysed using a minimum of two dilutions. All standard concentrations and sample dilutions were analysed in duplicate wells. The same positive control sample of herbage was analysed on every plate, and this was the same positive control grass sample that was used for the paxilline analyses. Plates were washed prior to addition of the anti-species secondary antibody (rabbit anti-sheep conjugated with HRP, DAKO), which was diluted 1 in 4 500 in 1% OVA/PBST (100 µl/well). Plates were then sealed, incubated for 2 h at 21°C. Plates were washed prior to addition of the substrate solution, BioFX TMB substrate (100 µl/well). Plates were then sealed, covered, placed on a plate shaker and incubated for 0.5 h at 21°C to develop the colour (Section 2.4.2). The enzyme reaction was stopped by addition of the stop solution,

Table 2-2: Outline of the peramine indirect competitive ELISA.

STEP	REAGENT	TEMP	TIME	VOLUME (µl/well)
Coat	BSA-peramine conjugate 0.2 µg/ml in coating buffer	21°C	18 h	100
Wash 4X with PBST				
Block	1% OVA/PBST	21°C	1 h	200
Wash 4X with PBST				
Competition Standards/samples + Specific (1°) antibody	Diluted sample extract or peramine standard (0.1–10 000 ng/ml) in 10% *Methanol/PBST* Anti-peramine 165-2 diluted 1/350 ×10^3 in 1% BSA/PBST	21°C	1 h	50 50
Wash 4X with PBST				
Antibody detection HRP labelled anti-species (2°) antibody	DAKO rabbit anti-sheep-HRP diluted 1/4 500 in 1% BSA/PBST	21°C	2 h	100
Wash 4X with PBST				
Substrate	BioFX TMB substrate	21°C	0.5 h	100
Stop	Stop solution 0.3 mol/l H_2SO_4	21°C		100
Read plate		21°C		

0.3 mol/l H_2SO_4 (100 µl/well). Plates were read and data analysed as described in Section 2.4.2.

Results were expressed as µg of peramine-immunoreactive equivalents (peramine-IRE) per g of milled grass, as peramine was the reference compound used to generate the standard curve in the assay. Although it would be anticipated that there should be no other peramine-like compounds that would be detected by the assay, the assay has not been quantitatively validated against a reference method therefore results are expressed as immunoreactive equivalents of the reference standard used. The assay working range was 2.5–350 ng/ml with a detection limit for the undiluted grass extract of 0.45 µg/g dwt. The ELISA analysis of sample extracts was repeated either because a result obtained was out of the working range of the assay or there was >10 % CV on replicate determination.

2.4.5 Lolitrem analysis

2.4.5.1 Extraction of single tiller samples

A qualitative test for *Epichloë festucae* var. *lolii* (Latch, M.J. Chr. & Samuels) C. W. Bacon & Schardl, stat. nov. et comb. nov. wild-type endophyte contamination was carried out using methodology developed and validated at AgResearch. Nunc 96-well untreated plates (Nunc catalogue number 267245) and covers (Nunc catalogue number 276002) were used for the single tiller extraction. One basal tiller section (2.5 mm long) was placed in a well, with every second row left empty (rows; A, C, E, and G contained tiller samples and rows B, D, F, and H remained empty). Details of sample and their location on the extraction plate were recorded. Once the plate was full, it was sealed using a plate sealer and lid, ensuring all wells were sealed. Plates were stored at −20°C until ready for tiller extraction and ELISA.

To each well containing a tiller, 90% methanol in water (125 µl) was added. The plate was sealed (with a plate sealer and lid) and incubated on the plate shaker at 21°C for 1 h. Extracts (100 µl) were removed and placed in the corresponding well in the adjacent row and 0.1 mol/l HCl (50 µl) was added. The plate was sealed and samples were hydrolysed for 15 min at 37°C. The hydrolysates (50 µl) were removed and added to PBST (250 µl) in titretubes. The resulting hydrolysed extracts were at a final dilution of 1 in 9, in 10% methanol/PBST. Residual acid in the diluted extracts had been previously shown to not affect results. Extracts were

not stored: if the analyses required repeating, fresh tiller samples were collected and extracted.

2.4.5.2 Lolitrem ELISA

The lolitrem ELISA followed a similar protocol as that for the paxilline and peramine ELISAs (Sections 2.4.3.2 & 2.4.4.2) and is outlined in Table 2-3. Coating antigen (BSA-lolitriol) and antibodies (specific anti-lolitriol sheep 165-2 polyclonal antibody and anti-species antibody, DAKO rabbit anti-sheep conjugated with HRP) used in this cELISA are those described by Briggs *et al.* (2007) with modifications required for analysis of fresh tiller samples as in Section 2.4.5.1.

Microtiter plates were coated with the coating conjugate, BSA-lolitriol at 1.0 µg/ml in bicarbonate coating buffer (100 µl/well). Plates were sealed, covered and incubated overnight at ambient room temperature (21°C). The plates were washed then blocked with blocking/antibody buffer (1% BSA/PBST, 200 µl/well), sealed and incubated for 1 h at 21°C. Plates were washed prior to addition of the standards, samples and specific antibody. Lolitriol standard curve concentrations (0.01–50 ng/ml) and the hydrolysed sample extracts (diluted at least 1 in 9 to remove matrix interferences) were prepared in standard/sample buffer, 10% methanol/PBST or PBST to give a final methanol concentration of 10%. Specific primary sheep polyclonal antibody, anti-lolitriol 134 was diluted 1 in 125 $\times 10^3$ in 1% BSA/PBST. Standards and samples (50 µl/well) followed by the specific antibody (50 µl/well) were added to the plate giving a total volume of 100 µl/well. Plates were sealed and incubated for 1 h at 21°C. All sample extracts were analysed initially at a 1 in 9 dilution and extracts of two tillers were analysed per individual plant. All standard concentrations and sample dilutions were analysed in duplicate wells. The same positive control sample (extracts of two tillers from a *L. perenne* plant infected with wildtype *E. festucae var. lolii*) was analysed on every plate. Plates were washed prior to addition of the species-specific secondary antibody, rabbit anti-sheep-HRP (DAKO), which was diluted 1 in 4 500 in 1% BSA/PBST (100 µl/well). Plates were then sealed and incubated for 2 h at 21°C. The plates were washed then substrate solution (BioFX TMB) was added to the plate (100 µl/well). Plates were then sealed, covered, placed on the plate shaker and incubated for 0.5 h at 21°C to develop the colour. The enzyme reaction was stopped by addition of the stop solution, 0.3 M

Table 2-3: Outline of the lolitrem indirect ELISA.

STEP	REAGENT	TEMP	TIME	VOLUME (µl/Well)
Coat	BSA-lolitriol conjugate 1.0 µg/ml in coating buffer	4°C	18 h	100
Wash 4X with PBST				
Block	1% BSA/PBST	21°C	1 h	200
Wash 4X with PBST				
Competition Standards/samples + Specific (1°) antibody	Diluted hydrolysed sample extract or lolitriol standard (0.01–50 ng/ml) in 10% methanol/PBST Anti-lolitriol 134 diluted 1/125 $\times 10^3$ in 1% BSA/PBST	21°C	1 h	50 50
Wash 4X with PBST				
Antibody detection HRP labelled anti-species (2°) antibody	DAKO rabbit anti-sheep-HRP diluted 1/4 500 in 1% BSA/PBST	21°C	2 h	100
Wash 4X with PBST				
Substrate	BioFX TMB substrate	21°C	0.5 h	100
Stop	Stop solution 0.3 mol/l H_2SO_4	21°C		100
Read plate		21°C		

H_2SO_4 (100 µl/well). Plates were read and data analysed as described in Section 2.4.2.

Lolitriol was the reference compound used to generate the standard curve in the assay. Although results were expressed as ng of lolitriol-immunoreactive equivalents (lolitriol-IREs) per ml of tiller extract, when applied to the single tiller test (Section 2.4.5.1) these were only qualitative giving a yes or no result for wild-type contamination.

2.4.6 Analysis of endophyte mycelium mass

2.4.6.1 Endophyte mycelium mass sample extraction

Milled grass samples as prepared in Section 2.3 were weighed (20 mg) into a Kimax tube (12 ml). PBST (10 ml) was added to the Kimax tube and samples were mixed thoroughly, ensuring all sample was wetted. These were then incubated for 3 h at 30°C. A representative portion was then transferred to a microfuge tube (2 ml) and centrifuged at 2 150 ×*g* for 3 min. Supernatant (1 ml) from each was transferred undiluted to screw-top glass vials (1.5 ml). Extracts were stored at 4°C until analysis by ELISA. Samples are stable for one week at 4°C or at –20°C for long term storage. Owing to the long extraction time (>3 h) the extracts are prepared a day ahead of the ELISA being performed.

2.4.6.2 ELISA of endophyte mycelium mass

The ELISA for endophyte mycelium mass followed a similar protocol as that for the previous ELISAs (paxilline, peramine and lolitrem; Sections 2.4.3.2, 2.4.4.2 & 2.4.5.2), except it had an extra pre-incubation step before the standards, samples and specific antibody were added to the plate. The protocol, coating antigen (*E. festucae* var. *lolii* mycelium and antibodies (anti-*E. festucae* var. *lolii* SAPU F2 specific primary rabbit polyclonal antibody, DAKO goat anti-rabbit conjugated with HRP anti-species secondary antibody), used in this cELISA are those as described by Faville *et al.* (2007) but modified with a pre-incubation step, and are outlined in Table 2-4.

Microtiter plates were coated with the *E. festucae* var. *lolii* mycelium coater at 20 µg/ml in bicarbonate coating buffer (100 µl/well). Plates were then sealed, covered and incubated overnight at ambient room temperature (21°C). Plates were washed,

Table 2-4: Outline of the indirect competitive ELISA of endophyte mycelium mass.

STEP	REAGENT	TEMP	TIME	VOLUME (µl/Well)
Coat	*E. festucae* var. *lolii* coater 20 µg/ml in coating buffer	21°C	o/n	100
Wash 4X with PBST				
Block	1% BSA/PBST	21°C	1 h	200
Wash 4X with PBST				
Pre-incubation Standards/samples + Specific (1°) antibody	Sample extract or *E. festucae* var. *lolii* endophyte standard (0.05–200 µg/ml) in PBST Anti-*E. festucae* var. *lolii* SAPU (F2) diluted 1/10 000 in 1% BSA/PBST	21°C	2 h	150 150
Competition	Add aliquot of pre-incubation solution to ELISA plate	21°C	1 h	100
Wash 4X with PBST				
Antibody detection HRP labelled species-specific (2°) antibody	DAKO goat anti-rabbit-HRP diluted 1/10 000 in 1% BSA/PBST	21°C	2 h	100
Wash 4X with PBST				
Substrate	Neogen K-Blue Aqueous TMB substrate	21°C	0.5 h	100
Stop	Stop solution 0.3 mol/l H_2SO_4	21°C		100
Read plate		21°C		

then blocked with blocking/antibody buffer (1% BSA/PBST, 200 µl/well), sealed and incubated for 1 h at 21°C. Prior to performing the blocking step the 2 h pre-incubation step of standards and samples with the specific antibody was started. The *E. festucae* var. *lolii* endophyte standard curve concentrations (0.05–200 ng/ml) and sample extract dilutions (undiluted and 1 in 2 dilution) were prepared in PBST. Specific primary rabbit polyclonal antibody, anti-*E. festucae* var. *lolii* SAPU F2 was diluted 1 in 10 000 in 1% BSA/PBST. Standards and samples (150 µl) and the specific antibody (150 µl) were combined in a micro-titretube and incubated for 2 h at 21°C. Plates were washed and the pre-incubated standard- and sample-antibody solutions (100 µl/well) were added to the plates. Sealed plates were incubated for 1 h at 21°C. All sample extracts were analysed using a minimum of two dilutions. All standard concentrations and sample dilutions were analysed in duplicate wells. The same positive control sample of herbage was analysed on every plate. Plates were washed prior to the addition of the anti-species secondary antibody, goat anti-rabbit-HRP (DAKO), which was diluted 1 in 10 000 in 1% BSA/PBST (100 µl/well). Plates were then sealed and incubated for 2 h at 21°C. Plates were washed, then substrate solution (Neogen K-Blue Aqueous TMB substrate) was added to the plate (100 µl/well). Plates were then sealed, covered, placed on the plate shaker and incubated for 0.5 h at 21°C. The enzyme reaction was stopped by addition of the stop solution, 0.3 M H_2SO_4 (100 µl/well). Plates were read and data analysed as described in Section 2.4.2. Results were expressed as µg of *E. festucae* var. *lolii*-immunoreactive equivalents (*E. festucae* var. *lolii*-IREs) per g of milled grass, as *E. festucae* var. *lolii* mycelium was the reference material used to generate the standard curve in the assay. The assay working range is 0.614–10.62 µg/ml with a detection limit for the undiluted grass extract of 6.14 mg/g dwt. The ELISA analysis of samples was repeated either because a result obtained was out of the working range of the assay (at greater dilutions) or there was >10 % CV on replicate determination.

2.5 Statistical analyses

2.5.1 Statistical Models

A brief introduction to the main statistical methods used in this thesis is given below. In each experimental chapter, more detailed descriptions of the statistical analyses performed are provided.

Analysis of variance (ANOVA) is a widely used statistical method for comparing several means (See Moore and McCabe (1989), Chapters 12 and 13, p743–825). Underlying ANOVA is the assumption that the residuals are normally distributed with mean zero and constant variance. ANOVA deals mainly with balanced and orthogonal designs. When the data are unbalanced or non-orthogonal, restricted maximum likelihood (REML) can be used. REML fits a linear mixed model which consists of two components: 1) a fixed model and 2) a random model. The fixed model is formed from fixed terms which typically represent the experimental treatments. Structural components of the experimental design are usually accounted for by the random model. For more details, see Welham *et al.* (2014) (Chapter 16, p427–450).

Both ANOVA and REML are based on linear models, and where the response variable is assumed to be normally distributed. Data transformations can sometimes be used to normalise the data, removing skewness and stabilising the variance, allowing the continued use of ANOVA or REML to reach valid statistical conclusions. When required, concentrations (such as, paxilline immunoreactivity) can usually be normalised using a log transformation, and counts ≥ 0 (such as, plant tiller number) using a square root transformation. When standard transformations fail to normalise the data and/or stablize the variance, a rank transformation is sometimes useful. Here the responses are ordered from smallest to largest, and the rank is the position within this ordering.

In certain cases, a generalised linear mixed model (GLMM), an extension of REML, can be used to analysis non-normally distributed data (Genstat manual). GLMM requires a transformation, known as the link function, in order to fit a linear model. For example, the number of damaged tillers on a plant, assumed to be binomially distributed, can be modelled using a GLMM with a logit link function.

2.5.2 Statistical Significance

The probability value (*P*) is used to quantify the evidence against the null hypothesis. Although throughout this thesis a significance level of 5% was used, when interpreting the evidence against a null hypothesis, the following definitions were used to describe *P* to avoid the sharp cut-off between "significant" and "not significant" (See Moore and McCabe (1989), Chapter 6, p476–477):

- $P \leq 0.001$= strong evidence (statistically significant).
- $0.001 < P \leq 0.05$ = evidence (statistically significant).
- $0.05 < P \leq 0.1$ = weak evidence (statistically not significant).
- $P > 0.1$ = no evidence (statistically not significant).

It is important to recognise that statistical significance doesn't imply biological significance (Moore and McCabe (1989), Chapter 6, p476–477). For example, a statistically significant difference in the mean paxilline immunoreactivity between plants may not be biological significant if the difference between the means is not large enough to have a meaningful biological impact.

Post-hoc tests were conducted Fisher's least significant differences, at the 5% significance level (LSD(5%)) (Welham *et al.* (2014), Chapter 4, p86–87).

2.6 References

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Chapter 3

Preliminary work and ELISA pre-screening

The purpose of this preliminary work was to screen 230 individual AR1-infected perennial ryegrass (Lolium perenne L.) plants (23 half-sibling families) grown from seed to find the highest and lowest paxilline-like metabolite producers and thus select a smaller number for the study of resistance to adult African black beetle (Heteronychus arator (Fabricius, 1775)) feeding. Included in this preliminary work was the screening of plants for their endophyte status (presence or absence) and for any wild-type endophyte contamination. The two grinding mills used to mill samples throughout this study were compared to ensure either mill could be used depending on sample size.

3.1 Introduction

The level of protection endophyte secondary metabolites (alkaloids) give ryegrass from being eaten by insects is dependent on what and how much of these compounds are produced. It is known that different endophyte strains produce different secondary metabolites and that the amounts produced are determined by the plant and endophyte genetic combination. The effect of host genotype means that genetically different ryegrass plants infected with the same endophyte will vary in levels of secondary metabolites and therefore also the degree of resistance to insects (Clay & Schardl, 2002; Rodriguez *et al.*, 2009). This allows plant breeders to select for plant and endophyte associations with high expressions of alkaloids that can provide resistance to insects such as Argentine stem weevil (*Listronotus bonariensis* (Kuschel, 1955)) and African black beetle (*H. arator*). Two fungal secondary metabolites that provide resistance to insects are peramine, against Argentine stem weevil (*L. bonariensis*) (Prestidge *et al.*, 1985; Rowan & Gaynor, 1986; Rowan *et al.*, 1990) and ergovaline, against African black beetle (*H. arator*) (Ball *et al.*, 1997). However ergovaline causes adverse effects to livestock, whereas peramine does not (Fletcher & Easton, 1997; Fletcher *et al.*, 1999).

The novel *Epichloë festucae* var. *lolii* (Latch, M.J. Chr. & Samuels) C. W. Bacon & Schardl, stat. nov. et comb. nov. AR1 endophyte was developed to provide

perennial ryegrass (*L. perenne*) with resistance to insects without causing ill-health to livestock. AR1 produces peramine and simple indole diterpenes, including paxilline and paxilline-like compounds such as the terpendoles (Young *et al.*, 2009), but does not produce the mammalian toxins, ergovaline and lolitrem B (Tapper & Latch, 1999). Although AR1 genetically can produce paxilline a mild tremogen, the amounts produced are minimal with production of the other simple indole diterpenes being more predominant (Young *et al.*, 2009). Also there have never been any reports of livestock ill-health from AR1-infected pastures (Bluett *et al.*, 2005b; Bluett *et al.*, 2005a). Paxilline and peramine do not deter adult African black beetle (*H. arator*) from feeding (Ball *et al.*, 1997), yet AR1 still shows low to moderate resistance to this insect (Popay & Baltus, 2001). Some of the known and unknown paxilline-like compounds produced by AR1-infected grasses are detected by the paxilline ELISA (enzyme linked immunosorbent assay) (Briggs L. R., AgResearch, Hamilton, New Zealand; personal communication, 2006).

The results of the Lincoln trial (Figure 1-7) for selected commercial cultivars suggested increasing levels of paxilline immunoreactive equivalents (paxilline-IRE) in the host plant were associated with a reduction in adult African black beetle (*H. arator*) feeding damage for a selection of commercial cultivars (Popay A. J., Fletcher L. R., Briggs L. R., AgResearch, New Zealand; unpublished work, 2006). The testing of genetically related material (within a cultivar or breeding line) was undertaken to investigate further, the resistance of AR1-infected plants to feeding from adult African black beetle (*H. arator*) and to determine if plant resistance to African black beetle (*H. arator*) is related to the levels of paxilline-IRE (paxilline immunoreactivity) produced by the hosted endophyte, AR1. A breeding-line is made up of half-sibling families, that is, of the two parent plants one parent plant is common for all half-sibling families and the other different. Half-sibling families from within the breeding line were studied as plants are genetically closely related, yet should contain sufficient genetic differences for both levels of resistance to feeding from adult African black beetle (*H. arator*) and for levels of secondary metabolite production.

The GA97 breeding-line studied consists of 23 half-sibling perennial ryegrass (*L. perenne*) families. Although plants from within a family that are grown from seed

are very similar, they are not genetically identical. Selections of individual plants from within each half-sibling family were chosen for feeding trials with adult African black beetle (*H. arator*). Prior to this plant selection for feeding trials with adult African black beetle (*H. arator*), preliminary work was necessary before a pilot trial was undertaken:

- Depending on sample size, two grinding mills were used to mill plant samples. The two mills were compared to ensure that results obtained by ELISA or chemical analyses were both comparable and reproducible whichever mill was used. A grass sample was milled by each grinding mill, and extracts of the milled grass samples were analysed by the paxilline ELISA.
- All plants from the half-sibling families (23) were grown from seed and screened for endophyte status (presence or absence) by immunoblot and microscopy, and for endophyte wild-type contamination by the lolitriol/lolitrem ELISA.
- Concentrations of paxilline-IRE in plants from the half-sibling families were determined prior to exposure to adult African black beetle (*H. arator*) to determine variation in levels between plants, within and between families.

The purpose of the initial paxilline ELISA pre-screen was to establish baseline levels of paxilline immunoreactivity in plants and to aid in the selection of plants. Furthermore, the number of plants for used in trial work had to be reduced because logistically it was not possible for a single person to experiment on 230 plants. The paxilline ELISA was also used in the comparison of the two grinding mills.

Because AR1 endophyte is not capable of producing lolitrems but wild-type *E. festucae* var. *lolii* does (Tapper & Latch, 1999; Young *et al.*, 2009), the lolitriol/lolitrem ELISA was performed on all plants from each half-sibling family using the qualitative single tiller test (Section 2.4.5.1) to determine if any of the families were contaminated with wild-type endophyte.

3.1.1 Paxilline ELISA

The paxilline ELISA uses an anti-paxilline antibody, a paxilline coating conjugate (protein-hapten) and paxilline as the reference standard as described in Section

2.4.2. The paxilline-like compounds (paxilline-IRE) produced by AR1 cross-react with the monoclonal antibody used in the paxilline ELISA (Briggs L. R., AgResearch, New Zealand; personal communication 2009) because they possess the same or very similar epitopes to which the antibody can bind (see Section 1.8.1). These paxilline-like compounds are thought to be the bioactive compounds (or marker compounds) that provide some resistance to adult African black beetle (*H. arator*) and are measured by ELISA as paxilline-IRE (see Figure 1-7).

3.1.2 Lolitrem ELISA

The lolitrem ELISA detects lolitrem-like structures and uses an anti-lolitriol antibody, a lolitriol coating conjugate (protein-hapten) and lolitriol as the reference standard as described in Section 2.4.5.2. Positive results for this ELISA indicated that a particular family was contaminated with wild-type endophyte. When a sample is extracted for the ELISA the final hydrolysate contains a range of hydrolysed lolitrem derivatives. The ELISA measures the immunoreactivity of the lolitriol-like analogues, which have been hydrolysed to lolitrem derivatives. The results are expressed as lolitriol immunoreactive equivalents (lolitriol-IRE) and are an indirect measure of the lolitrem analogues present in a host plant and hence wild-type endophyte. When testing weighed milled grass samples the results are quantitative. However, when applied to the single tiller test for contamination by wild-type endophyte the results are qualitative, giving a yes or no answer.

3.2 Methods

3.2.1 Planting out

Seed was sourced from Forage Improvement, AgResearch, Grasslands, Palmerston North, New Zealand. Perennial ryegrass (*L. perenne*) seeds infected with the novel endophyte AR1, from 23 half-sibling families (accession) from the breeding-line GA97 were germinated and planted out as described in Sections 2.1.1 and 2.1.3 into polystyrene trays containing Daltons GB commercial potting mix. Ten germinated seeds were planted out for each half-sibling family (230 seedlings total), with each seedling plant considered a family representative. In total five trays were planted. Each tray contained five families and 50 seedlings per tray (except the final tray which contained three families and 30 seedlings) as shown in Figure 3-1. Seedlings

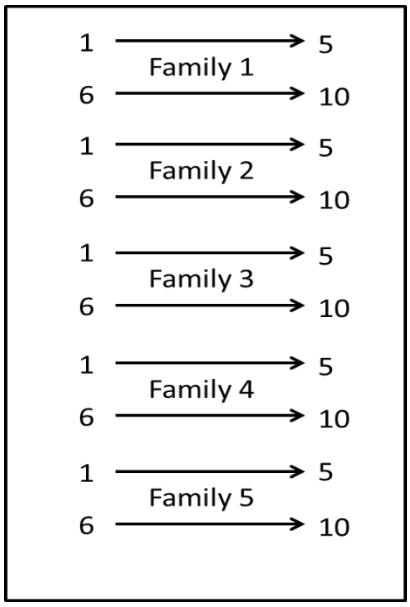

Figure 3-1: Planting positions of half-sibling family seedlings planted out per tray.
1–10 = seedling plant from each family.

were left to establish and were maintained as described in Section 2.1.4. The half-sibling families investigated are listed in Table 3-1.

Table 3-1: *Lolium perenne* half-sibling families from the GA97 breeding line containing AR1 endophyte.

Family Number	Accession (half-sibling family)	Family Number	Accession (half-sibling family)
1	A12061	*13*	A12075
2	A12063	*14*	A12077
3	A12064	*15*	A12078
4	A12065	*16*	A12080
5	A12066	*17*	A12081
6	A12068	*18*	A12082
7	A12069	*19*	A12083
8	A12070	*20*	A12084
9	A12071	*21*	A12085
10	A12072	*22*	A12086
11	A12073	*23*	A12087
12	A12074		

3.2.2 Endophyte detection

Endophyte status was determined by immunoblot and when required, by microscopy (Section 2.2). One tiller was checked at each sampling as AR1-infected perennial ryegrass (*L. perenne*) plants are known to have a tiller endophyte-infection rate of almost 100% (Card, S. D., AgResearch, New Zealand; personal communication, 2009).

3.2.3 Wild-type endophyte contamination – analysis by Lolitrem ELISA

Wild-type contamination of family lines was determined by the lolitriol/lolitrem ELISA as explained previously in Section 3.1.2. Two single tillers were taken from each individual plant from the twenty-three half-sibling families for lolitriol/lolitrem analysis as described in Section 2.4.5.

3.2.4 Comparison of grinding mills using the paxilline ELISA

The two grinding mills, blade mill (IKA-10) and cyclone mill (Udy) were first compared using the paxilline ELISA (Section 3.1.1) to ensure the same immunoassay result was obtained using either mill. An AR1-infected grass herbage test sample (E+) was prepared as described in Section 2.3.1. The grass sample was cut using scissors into smaller sections (25 mm long) and mixed thoroughly. The AR1-infected herbage sample was then split into six sub-samples. Three sub-samples were milled using the blade mill and three sub-samples using the cyclone mill (Section 2.3.2). Three lots from each milled sub-sample were weighed, extracted then analysed using the paxilline ELISA (Section 2.4.3) and the mean concentration of paxilline-IRE per gram of milled grass (µg/g) were compared within and between grinding mills.

3.2.5 Paxilline ELISA pre-screen

Levels of paxilline immunoreactivity were determined by the paxilline ELISA (Section 2.4.3 and 3.1.1) for each plant from the 23 half-sibling families as part of the criteria for the selection of plants for the initial pilot trial (including the wild-type contaminated family lines). As shown in Figure 3-2, all plants were initially cut to a height of 8 cm from the crown and the herbage removed was discarded.

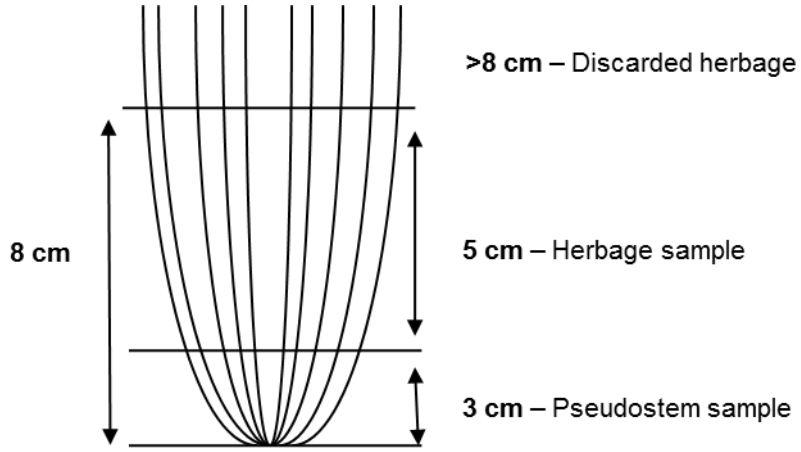

Figure 3-2: Trimming and herbage sampling of plants.

Plants were trimmed further to 3 cm from the crown, and the 5 cm herbage harvested kept for the initial paxilline analyses. Herbage samples were prepared as described in Section 2.3 using the blade mill (samples <2 g). Levels of paxilline immunoreactivity were determined on the extracts from milled herbage samples as described in Section 2.4.3.

3.2.6 Plant selection for pilot trial

Selection of 50 plants out of 250 plants was based on the following criteria with a minimum of two representatives from each family:

- Endophyte status: ensuring the plants were infected with the AR1 endophyte.
- Individual plant paxilline-IRE result: a range of low (<1–4 µg/g), medium (>4–8 µg/g), high (>8 µg/g), from the paxilline ELISA were chosen.
- The family from which the plant originated.
- Plant health; plants were scored using a scale of 1–5 (5 = very strong, 1 = very weak). The plant health score was a visual assessment that took into consideration plant tiller number (≥10), tiller strength and general growth of plant. Plants with a health score of three or more were chosen.

3.3 Results

3.3.1 Endophyte detection

Of the eight plants that did not survive, three were from family A12072, and one from each of the following five families; A12077, A12065, A12073, A12068 and A12083. For all families there were at least nine surviving plants, except for A12072 of which there were seven. When plants were older than 6 weeks and had established, each plant was checked to determine its endophyte status (222 plants had survived). From the primary immunoblot test six of 222 plants returned a negative or inconclusive result. These were re-tested by immunoblot and by microscopy. All of these plants returned a positive result for infection with endophyte.

3.3.2 Wild-type endophyte contamination – analysis by Lolitrem ELISA

Three half-sibling families (A12074, A12085 and 12086) were positive for lolitrems and confirmed to be contaminated with wild-type endophyte. The remaining 20 half-sibling families were negative for contamination with wild-type endophyte.

3.3.3 Comparison of grinding mills using the paxilline ELISA

Two grinders were used in this study owing to sample size. The blade mill (IKA–10) was used for samples of weight up to 2 g and the cyclone mill (Udy) for samples of more than 2 g.

There were no significant differences (%CV <10) between the two mills for the paxilline ELISA result as sub-samples produced by both mills had very similar immunoreactivity levels, as shown in Table 3-2. This result provided confidence that either mill could be used for milling plant samples for analysis by immunoassay and analytical chemistry. From the triplicate extractions from each sub-sample it was also confirmed that the paxilline analysis provides comparable and reproducible results within normal ELISA working parameters (%CV <10).

Table 3-2: Comparison of milled grass samples between and within blade and cyclone grinding mills using the grass extracts of these analysed by the paxilline ELISA.

Mill	Sub-sample	[a]Mean paxilline-IRE (µg/g) from each sub-sample	%CV between sub-samples
Blade	1	2.62 (1.72)	
Blade	2	2.42 (1.95)	
Blade	3	2.54 (2.30)	
Mean of all sub-samples with blade		***2.53***	***3.71***
Cyclone	1	2.52 (2.00)	
Cyclone	2	2.46 (3.47)	
Cyclone	3	2.64 (2.96)	
Mean of all sub-samples with cyclone		***2.54***	***3.79***
Mean of all sub-samples with both mills		**2.53**	**3.64**

[a]Paxilline-IRE (paxilline immunoreactive equivalents) values shown are the mean of triplicate extractions of each milled sub-sample lot. Values shown in brackets are the %CV of the paxilline-IRE triplicate extractions for each sub-sample.

3.3.4 Paxilline ELISA pre-screen

The herbage paxilline-IRE levels were square-root transformed prior to analysis to normalise the data. The data were analysed using residual maximum likelihood (REML) in GenStat version 15 (Section 2.5). Date of ELISA analysis was included as a random effect. The square-root transformed values observed for each individual plant, illustrated a large variation between individual plants within the same family across all families (Figure 3-3). The predicted mean square-root paxilline-IRE value for each family is also shown. There was weak evidence that the mean square-root paxilline-IRE differed between families ($F_{(22,177)} = 1.53$; $P = 0.070$). The analysis was repeated with the omission for the family lines contaminated with wild-type endophyte and similar results were obtained

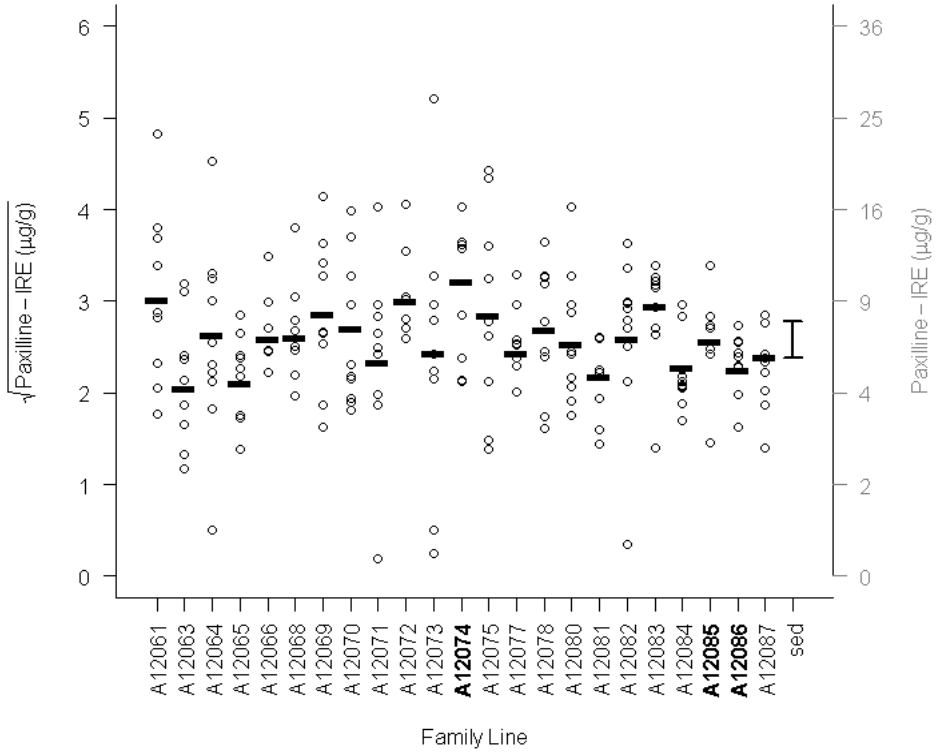

Figure 3-3: Dotplot of levels of paxilline immunoreactive equivalents (paxilline-IRE) across families.

The data graphed has been square-root transformed (with the back-transformed scale on the right-hand vertical axis). The circles represent the observed values of individual plants in a family. The bars represent the predicted square-root mean for the families. Family lines contaminated with wild-type endophyte are highlighted in bold font.

($F_{(19,149)}$ = 1.54; P = 0.081). Note, the suggestion of a family effect, albeit weak, was supported when the data were examined on the basic rank scale (Section 2.5), with P <0.5 (contaminated lines included ($F_{(22,\ 173)}$ = 2.00; P = 0.007) and contaminated lines excluded ($F_{(19,149)}$ = 1.94; P =0.015).

3.3.5 Plant selection for pilot trial

The 50 plants selected for the pilot trial are shown in Table 3-3 and range from very low to very high results in the paxilline ELISA; 12 graded low, 18 graded medium and 20 graded high. The three family lines contaminated with wild-type endophyte were also included for comparison as a benchmark for African black beetle (*H. arator*) resistance.

Table 3-3: Plants selected for pilot trial.

*****The identification number of the family and individual plant (grown from seed) in that family is shown for each plant selected. The concentration of paxilline-IRE (Pax-IRE) for each is shown, and the grading of this as very high (VH), high (H), medium (M), low (L) and very low (VL) is shown. The plant health score (PHS) of each plant selected is shown, graded on a scale of 1–5, with 1 = weak unhealthy plant and 5 = strong healthy plant. The number of tillers (Tiller #) a plant had is shown. Family lines and individual seedling plants shown to be contaminated with wild-type endophyte are highlighted in bold font.

Family	Plant*	Pax–IRE (µg/g)	Pax–IRE Grade	PHS	Tiller #
A12061	61/6	11.47	VH	3	19
	61/9	14.43	VH	4	20
A12063	63/4	5.84	M	4	15
	63/9	3.50	L	3	25
A12064	64/1	3.33	L	3	28
	64/6	5.34	M	3	27
	64/7	10.56	H	5	44
	64/10	20.46	VH	3	28
A12065	65/1	5.78	M	3	23
	65/6	3.07	L	3	20
	65/7	1.93	L	3	22
A12066	66/7	7.37	M	3	13
	66/8	6.09	M	3	13
A12068	68/6	14.48	VH	4	27
	68/8	6.27	M	3	18
A12069	69/6	10.74	H	3	26
	69/9	17.23	VH	4	28
A12070	70/5	4.65	M	3	33
	70/6	8.81	H	4	49
	70/8	3.28	L	3	23
A12071	71/3	6.24	M	3	18
	71/5	16.28	VH	3	14
A12072	72/2	9.16	H	4	32
	72/9	16.47	VH	5	41
A12073	73/1	0.26	VL	3	28
	73/2	8.75	H	4	19
A12074	**74/1**	**13.06**	**VH**	**5**	**49**
	74/10	**4.56**	**M**	**4**	**38**
A12075	75/6	18.87	VH	4	35

Table 3–3 continued on next page

Table 3–3 continued

Family	Plant*	Pax–IRE (µg/g)	Pax–IRE Grade	PHS	Tiller #
A12077	77/5	5.26	M	4	29
	77/8	5.64	M	4	32
A12078	78/1	2.59	L	5	30
	78/2	10.21	H	4	19
	78/7	3.04	L	4	19
A12080	80/2	4.67	M	3	23
	80/7	3.10	L	3	18
	80/10	10.72	H	5	59
A12081	81/1	6.71	M	4	43
	81/10	5.10	M	4	15
A12082	82/1	8.54	H	4	19
	82/4	8.87	H	3	22
A12083	83/1	6.98	M	3	17
	83/2	9.92	H	4	26
A12084	84/1	3.53	L	4	40
	84/5	4.33	M	4	43
A12085	**85/6**	**11.46**	**VH**	**4**	**23**
A12086	**86/2**	**2.65**	**L**	**3**	**19**
	86/4	**5.95**	**M**	**5**	**32**
A12087	87/5	3.50	L	4	36
	87/10	4.93	M	5	54

3.4 Discussion

Previous unpublished work, the Lincoln trial (Popay A. J., Fletcher L. R., Briggs L. R., AgResearch, New Zealand; unpublished work, 2006), suggested that AR1-infected plants with high levels of paxilline ELISA immunoreactivity were associated with low feeding damage from adult African black beetle (*H. arator*). The Lincoln study had used plants from different cultivars and not plants from within a cultivar, therefore the perceived relationship between paxilline immunoreactivity and feeding damage may have been a cultivar effect irrespective of endophyte infection and not because of the host plant's influence on the production of the paxilline-like compounds detected as paxilline immunoreactivity (by the paxilline ELISA).

The ten plants grown from seed (family representatives) and planted out for each half-sibling family are closely related but not genetically identical and gave a reasonable representation of the varied characteristics of each family. For each half-sibling family there was a maximum of ten family representatives that underwent the preliminary testing for: endophyte status, wild-type endophyte contamination, tiller number, plant health score and level of paxilline immunoreactivity in herbage. Family differences for the level of paxilline immunoreactivity were indicated, although the evidence was weak and not statistically significant, the result provided incentive to continue the investigation into paxilline immunoreactivity differences between families and how this relates to feeding damage from adult African black beetle (*H. arator*).

Plants (and families) from the 23 half-sibling families (GA97 breeding line) were screened and plants identified as high, medium or low producers of paxilline immunoreactivity. Fifty individual AR1-infected plants were selected for the pilot trial based on the level of paxilline immunoreacitivity in herbage, plant tiller number (≥10) and plant health score (≥3), across all families and across the range of levels of paxilline immunoreactivity. Representatives from the three family lines contaminated with wild-type endophyte were also included in the selection as benchmark plants and families for levels of resistance to feeding by adult African black beetle (*H. arator*).

In addition to determining the levels of paxilline immunoreactivity in AR1-infected plants (and families) the variability in the levels of paxilline immunoreactivity across plants within the same family was found. Families with larger variability provided greater scope for improving the average family level of paxilline immunoreactivity by breeding compared with families with small variability. Families with moderate to large plant variation (grown from seed) enables the average value of a trait for the family to be potentially increased (or decreased) through breeding trials. Therefore families with sufficient variation in plant levels of paxilline immunoreactivity, and plants from within these families with moderate to high levels of paxilline immunoreactivity, high tiller numbers and high plant health scores could be identified as potential candidates for breeding to increase the level of paxilline immunoreactivity. Family variation for paxilline

immunoreactivity and other characteristics (plant size and robustness) gave valuable information used in the selection process and for identifying potential families and plants (from within those families) for breeding trials.

Ideal ryegrass plants wanted for agricultural purposes are plants that are robust (high plant health score), have a high herbage production (large plant size and high tiller number), and high levels of the beneficial fungal secondary metabolites that are not harmful to livestock (e.g. peramine for resistance to Argentine stem weevil (*L. bonariensis*) and nil or negligible levels of the secondary metabolites that are harmful to livestock (e.g. ergovaline, although provides for resistance to African black beetle (*H. arator*), is toxic to livestock). Because AR1-infected ryegrass does not produce the known secondary metabolites that cause ill health to mammals (livestock), but does produce some of the beneficial secondary metabolites for insect pest resistance it is still the safest endophyte-plant association available. If there is a relationship between paxilline immunoreactivity and feeding damage from adult African black beetle (*H. arator*), increasing the levels of paxilline immunoreactivity in AR1-infected cultivars through breeding programs could be viable option for improving AR1-infected plant resistance to adult African black beetle (*H. arator*).

3.5 Summary

Plants from the 23 half-sibling families (GA97 breeding line) were screened and plants were identified as high, medium or low producers of paxilline immunoreactivity with family differences indicated. Fifty plants across the range of paxilline immunoreactivity (low, medium and high levels) representing all 23 half-sibling families were selected for further study (Table 3-3) to investigate if plant resistance to feeding by adult African black beetle (*H. arator*) is related to the concentration of paxilline immunoreactivity.

It was established that all plants were infected with endophyte, however, three families were identified as being contaminated with wild-type endophyte (A12074, A12085 and A12086).

It was confirmed that either grinding mill, blade or cyclone (IKA-10 for samples ≤2 g and Udy for samples >2 g respectively) could be used to mill plant samples for analysis by ELISA and analytical chemistry.

3.6 References

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Chapter 4

Trial 1 – Pilot Study

The purpose of the feeding pilot trial with adult African black beetle (Heteronychus arator (Fabricius, 1775)) was to screen selected AR1-infected perennial ryegrass (Lolium perenne L.) plants from 23 genetically related half-sibling families (within a breeding-line) for:

- *Expression levels of paxilline-like and peramine fungal metabolites throughout.*
- *Resistance to adult African black beetle (H. arator) feeding by assessing both the proportion of tillers damaged on a plant and the scale of feeding damage to each tiller.*
- *Plant tiller production and any evidence of a relationship between plant size and feeding damage from adult African black beetle (H. arator).*
- *Any evidence of an association between feeding damage by adult African black beetle (H. arator) and endophyte metabolite production of paxilline-like compounds and peramine-like compounds.*

Cloned plants were used to determine plant variation within a genotype. With this information, a reduced number of AR1-infected plants would be selected for further study of plant resistance to adult African black beetle (H. arator) feeding.

4.1 Introduction

AR1-infected ryegrass cultivar results from the initial Lincoln trial (Popay A.J, Fletcher L.R., Briggs L. R., AgResearch, New Zealand; unpublished work, 2006) as mentioned in Section 3.1, suggested the possibility that a relationship between damage from adult African black beetle (*H. arator*) feeding and levels of paxilline immunoreactivity exists (also see Figure 1-7). AR1 produces peramine, a potent feeding deterrent to Argentine stem weevil (*Listronotus bonariensis* (Kuschel, 1955)) adults (Rowan *et al.*, 1990a). Although often stated to be a potent insect deterrent, there is little evidence from the literature for effects on other insects, other than it affects cutworm (*Graphania mutans* (Walker, 1857)) larval development (Dymock *et al.*, 1988). Ball *et al.* (1997) showed that peramine does not deter adult

African black beetle (*H. arator*) from feeding. Levels of peramine produced by AR1-infected grasses are never-the-less important for the deterrence of Argentine stem weevil (*L. bonariensis*) adults, a major pasture pest in New Zealand.

This pilot trial was a large scale feeding trial (with choice of plants) with adult African black beetles (*H. arator*) (Section 2.1.7) that used plants selected (Table 3-3) from half-sibling family lines within a breeding line which exhibited a range of paxilline ELISA immunoreactivity results from low to high (0–40 µg/g).The purpose of this pilot trial was to determine if there were plant and family differences for adult African black beetle (*H. arator*) feeding damage, and if there were differences, then to determine if these related to immunoreactivity measured by the paxilline ELISA. The results from the paxilline ELISA pre-screen (Section 3.2.5) suggested that there was a family effect related to the levels of paxilline immunoreactive equivalents (paxilline-IRE) produced in the plants. Plant clones were used rather than individual plants grown from seed for this trial to reduce the host genetic variation. This was in response to the large variation found within the family lines from the pre-screen with the paxilline ELISA (Figure 3-3).

Levels of immunoreactivity for paxilline-like metabolites were measured pre- and post-exposure to adult African black beetle (*H. arator*) feeding and peramine measured only for the latter. It was anticipated that peramine would be the only compound detected in the plants by the peramine ELISA (Section 2.4.4.2) which uses an anti-peramine antibody, a peramine coating conjugate (protein-hapten) and peramine as the reference standard as described in Section 2.4.4.2.

4.2 Materials and methods

This research (Section 2.1.7) was undertaken from October 2008 to November 2011 at AgResearch, Ruakura Research Centre, Hamilton, New Zealand. Fifty individual plants from 23 half-sibling families within the AR1-infected, diploid perennial ryegrass (*L. perenne*) breeding line, GA97 were selected. Individual plants were chosen on the basis that they exhibited a range of paxilline ELISA immunoreactivity between 0 and 40 µg/g (Table 3-3). Families and plants infected with wild-type endophyte were included for comparison of plant resistance and

levels of immunoreactivity. The pilot trial was also used to further reduce the number of selected plants for continued experimental work.

4.2.1 Beetle collection

In spring, active adult African black beetle (*H. arator*) were collected (Section 2.1.5) from the field in the Waikato region and maintained (Section 2.1.6) in the laboratory until required.

4.2.2 Plants

Original plants (Section 3.2.1) grown from seed were used as the parent plants for cloning. Selected plants were cloned as described in Section 2.1.2. Three clones of each plant were planted into replicate polystyrene trays (internal 49.5 cm × 30 cm) containing potting mix (one cloned plant per tray) and the fourth clone was replanted back into the same position in the original plant tray.

4.2.3 Trial design and methodology

The trial consisted of three replicate trays (three experimental units) each containing 50 cloned plants (1–4 plants per family) planted in ten rows of five plants per row, with the position of the individual plants randomised (rows 5 cm apart, plants 5 cm apart). Planting was based on a randomised planting design for each tray. Figure 4-1 illustrates the experimental unit planting positions. The three replicates were laid out next to each other in a 10 row by 15 column grid.

Cages were constructed for each experimental unit using green knitted windbreak; (Ultrapro windbreak.LT 915 mm wide, 55% wind porosity, Cosio Plastics, New Zealand), acetate sheets and velcro strips (Figure 4-2). Cages were needed to prevent adult African black beetles (*H. arator*) from escaping. They were resistant to water damage and easily removed and replaced for African black beetle (*H. arator*) plant damage assessments through the duration of the trial.

Plant clones were planted in mid-September 2008 and were allowed to recover and establish for 5 weeks prior to the start of the trial in mid-October. Plant establishment and the trial were conducted in the glasshouse (temperature range 15–25°C). Plants were watered by hand as required (2–3 times a week).

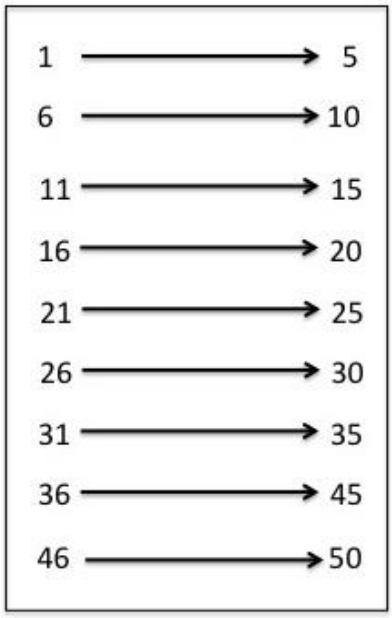

Figure 4-1: Experimental unit (tray) planting positions.

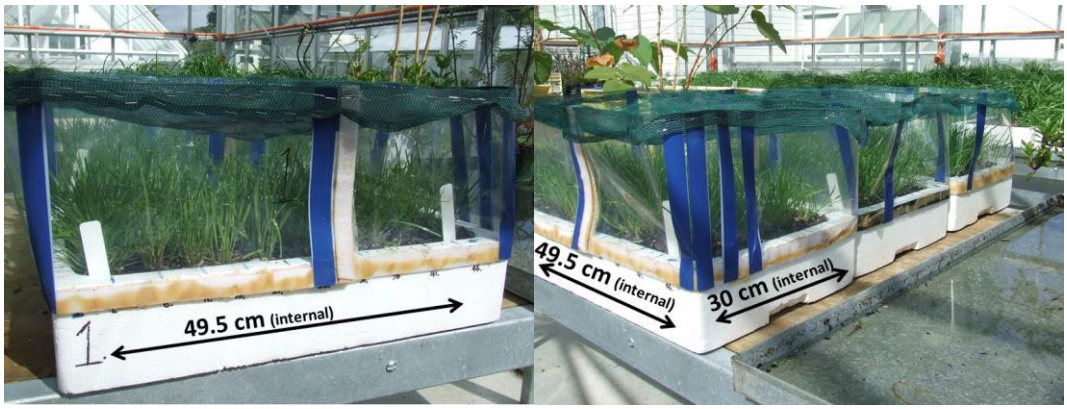

Figure 4-2: Trays (experimental units) with cages attached.

Prior to introducing the African black beetle (*H. arator*), the plants were trimmed to 3 cm, fertilised (as described in Section 2.1.4), tillers counted per plant at the start of the trial (Assessment 0; A0). The first 20 adult African black beetles (*H. arator*) were then added to each tray. Depending on availability, 16–18 adult African black beetles (*H. arator*) were added once a week for the next 3 weeks to each tray to give a total of 70 adult African black beetles per tray (Table 4-1).

Table 4-1: Addition of adult African black beetles to each tray in Trial 1 (pilot trial).

				Cumulative total		
Time	Female	Male	Total	Female	Male	All beetles
Week 0	3	17	20	3	17	20
Week 1	5	11	16	8	28	36
Week 2	7	9	16	15	37	52
Week 3	7	11	18	22	48	70

In the trial schedule (Table 4-2) feeding damage assessments from adult African black beetle (*H. arator*) were performed mid-trial after 2 weeks at Assessment 1 (A1) and after 4 weeks at the end of the trial at Assessment 2 (A2). Prior to assessments A1 and A2 plants were trimmed to 8 cm then herbage samples (5 cm) collected (Section 3.2.5; Figure 3-2). At the end of each assessment plants were fertilised.

Total tiller number, and number of damaged and undamaged tillers were recorded at each assessment. Damaged tillers were scored on a scale of 1–3; 1 = minor damage (surface feeding only), 2 = moderate damage (damage had partially penetrated the tiller) and 3 = severe damage (tiller shredded completely and unlikely to survive).

Herbage (5 cm) and damaged pseudostem samples (3 cm) were collected mid-trial (A1), and herbage, damaged and undamaged pseudostem samples at the end of the trial (A2) as shown in Figure 3-2. All grass samples (herbage and pseudostem) were then stored at –20°C. Undamaged pseudostem samples were collected from plants that had ≥10 undamaged tillers remaining at the end of the trial, with either 30% or a minimum of five undamaged pseudostems collected. This ensured a plant had a minimum of five undamaged tillers remaining for regrowth.

After the trial, the cages were removed from the trays and the experimental units (trays) were moved outside for adult African black beetles (*H. arator*) to disperse. The trays were then transferred back to the screenhouse and the plants were maintained alongside the trays containing the original 230 plants (as described in Section 2.1.4).

Table 4-2: Schedule for feeding-choice Trial 1 (pilot trial) with adult African black beetle.

Time	Procedure
Week –5	Plants cloned and planted into trays (experimental units)
Week 0	Plants trimmed and fertilised
(A0)	Tillers counted
	African black beetles added
Week 1	African black beetles added
Week 2	Plants trimmed and herbage collected
(A1)	African black beetle damage assessed, tillers counted and damaged pseudostem collected
	Plants fertilised
	African black beetles added
Week 3	African black beetles added
Week 4	Plants trimmed and herbage collected
(A2)	Feeding damage from African black beetles assessed, tillers counted and damaged pseudostem collected
	Undamaged pseudostem collected
	Plants fertilised

A0, A1, A2 = Assessments 0 (start of trial), 1 (mid-trial), 2 (end of trial).

Grass samples consisting separately of herbage, damaged and undamaged pseudostems, were prepared for analyses as described in Section 2.3. Grass samples were analysed using both the paxilline and peramine ELISAs (Sections 2.4.3 and 2.4.4, respectively). The peramine and paxilline ELISAs were only performed on a subset of samples (14 of the 23 half-sibling families) taken at A2.

4.2.4 Statistical Analyses

The data from each assessment were analysed separately and cumulatively. Repeated measures analyses were not used because different experiment protocols were imposed prior to each assessment, such as the addition of beetles (See Table 4-2). The following variables were analysed: total number of tillers, number of tillers damaged from feeding adult African black beetles (*H. arator*), damage score of tiller and levels of paxilline and peramine immunoreactivity. Family lines contaminated with wild-type endophyte were not removed from the data prior to analyses because family was a factor accounted for in the statistical analyses. The data were analysed using either generalised linear mixed model (GLMM) or REML in GenStat version 15 (Section 2.5) with random effects to account for spatial and replicate effects. Two parametrisations of the fixed model were considered:

- M1: The basic model, tests for differences between plants (*Plant*).
- M2: Tests for plant differences within families and between families (*Family + Family.Plant*).

Note: The term '*Plant'* allowed for differences between plants, '*Family'* allowed for differences between families and *'Family.Plant'* allowed for differences between plants from within a family.

Post hoc tests were conducted using Fisher's least significant difference at the 5% significance level (LSD(5%)).

Tiller number data were analysed using REML and were square root transformed prior to analysis to stabilise the variance. The tiller number data were also analysed cumulatively (total tiller number at A2 plus number of damaged tillers from A1 which had been removed), so total tiller production could be examined.

Both the feeding damage and damage score data were assumed to have overdispersed binomial distributions, and therefore were analysed using GLMM, with a logit link function. Overdispersion was considered appropriate, as damage to tillers on the same plant was unlikely to be independent. The damage data were also analysed cumulatively (number of damaged tillers at A2 plus the number of damaged tillers from A1 which had been removed), enabling damage over the duration of the trial to be examined.

ELISA data were analysed using REML and were natural log transformed prior to analysis to stabilise the variance. Statistical analyses were only performed on the herbage and damaged pseudostem samples, as there were insufficient data from the undamaged pseudostem samples. Damage from adult black beetle feeding was correlated with both paxilline and peramine immunoreactivity.

4.3 Results

For all statistical analyses above, analogous results were obtained when initial tiller number, a proxy for plant size, was included as a covariate.

Families contaminated with wild-type endophyte are indicated with the superscript 'WT'in the text, and highlighted in bold in the tables and figures.

4.3.1 Total tiller number

For initial tiller number there was strong evidence (P <0.001) that the mean number of tillers differed between all plants and between plants from within a family for all assessments, and between plants for total tillers produced in the trial (Appendix I, Table A). However, a positive relationship was found between initial tiller number and plant tiller number at subsequent assessments (A1 $F_{(49,31)} = 0.42$; $P < 0.001$ and A2 $F_{(49,46)} = 0.73$; $P < 0.001$).

It can be seen that both prior to (Figure 4-3 A) and after African black beetle (*H. arator*) attack (Figure 4-4 A) for median plant tiller number, there was variation between families and some families were more variable than others. Within an individual plant genotype initially grown from seed, the variation between the genetically identical cloned plants (inter-clonal variation) for plant tiller number was in some cases quite large and in other cases very small (Figure 4-3 B and Figure 4-4 B). From the unadjusted cumulative tiller number data (Figure 4-4 A), families were grouped into high, medium and low median tiller numbers per plant (Table 4-3). The cumulative square root transformed means for tiller number of the clones from plants grown from seed ranged from 1.52–7.50 (Appendix I, Table A). Plants from the family lines contaminated with wild-type endophyte (highlighted in bold font) were not significantly different from the AR1-infected lines and had medium–high median tiller number.

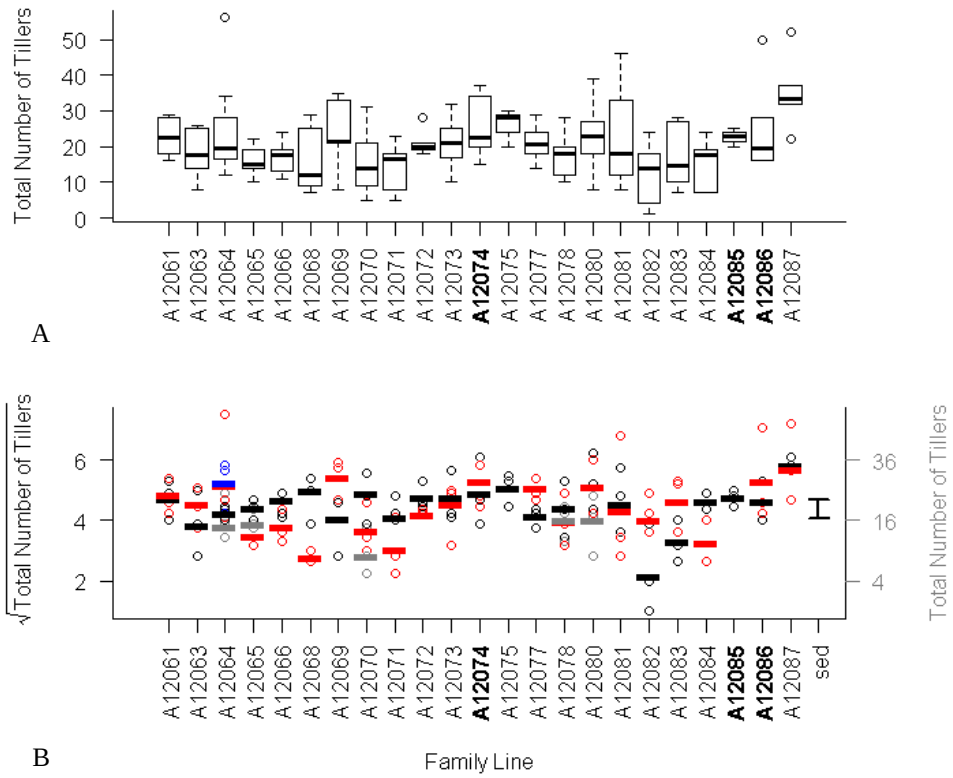

Figure 4-3: Total tiller number at the start of the trial (A0).
A) Boxplot; displaying family median, upper and lower quartiles and showing variation within and between families.
B) Dotplot; displaying individual plants within a family (circles) and the corresponding mean (bars), showing the variation within a family and between plants cloned from a single individual plant grown from seed. The data graphed has been square root transformed (with the back-transformed scale on the right vertical axis). The dotplot is coloured by individual plants grown from seed within a family, with each individual circle of the same colouring representing a cloned plant with a matching coloured bar denoting the mean of the clones for the individual plant. sed = standard error of the difference (fixed effects model, M1).
Family lines contaminated with wild-type endophyte are highlighted in bold font.

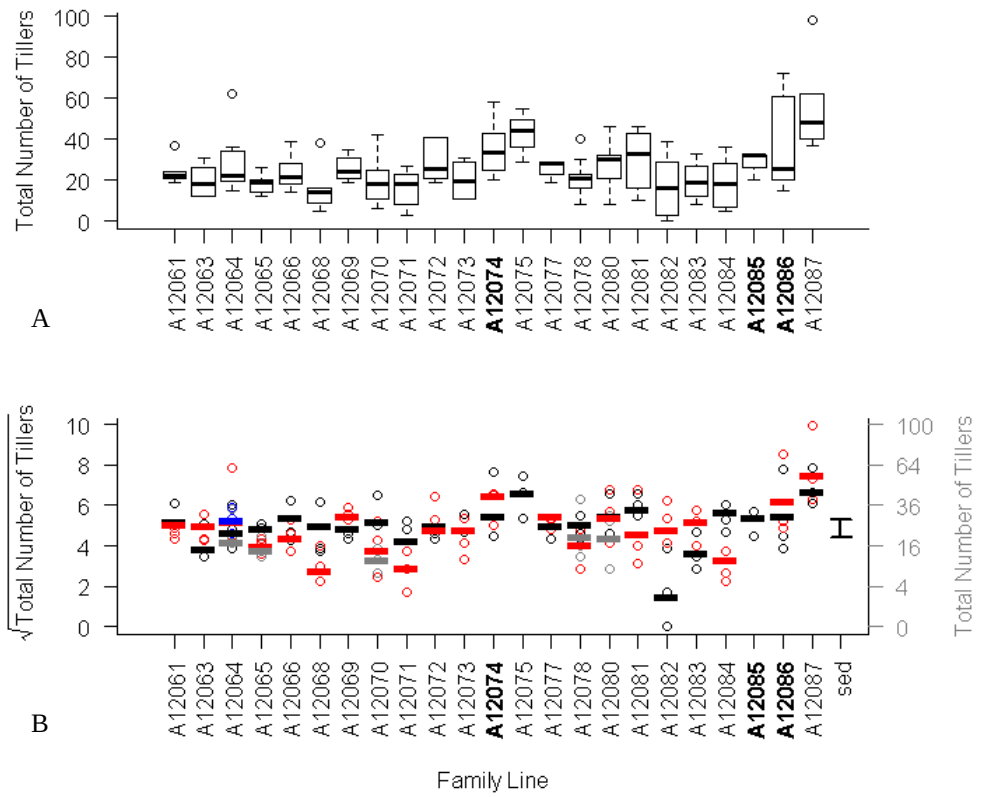

Figure 4-4: Cumulative tiller number at the end of the trial (A2).
A) Boxplot; displaying family median, upper and lower quartiles and showing variation within and between families.
B) Dotplot; displaying individual plants within a family (circles) and the corresponding mean (bars), showing the variation within a family and between plants cloned from a single individual plant grown from seed. The data graphed has been square root transformed (with the back-transformed scale on the right vertical axis). The dotplot is coloured by individual plants grown from seed within a family, with each individual circle of the same colouring representing a cloned plant with a matching coloured bar denoting the mean of the clones for the individual plant. sed = standard error of the difference (fixed effects model, M1).
Family lines contaminated with wild-type endophyte are highlighted in bold font.

Table 4-3: Median cumulative tiller number per plant for the half-sibling families.

Median plant tiller number	Half-sibling families
High (>30 tillers/plant)	**A12074**, A12075*, A12080, A12081, **A12085*****, A12087
Medium (20–30 tillers/plant)	A12061, A12064, A12066, A12069, A12072, A12077, A12078, **A12086**
Low (<20 tillers/plant)	A12063, A12065, A12068, A12070, A12071, A12073, A12082, A12083, A12084

*A single individual family representative (plant) grown from seed. Family lines contaminated with wild-type endophyte are highlighted in bold font.

4.3.2 Feeding damage

There was statistical evidence that the proportion of tillers damaged differed between individual plants and also between plants within families for assessments (Table 4-4). There was no evidence that the proportion of tillers damaged was related to the initial tiller number (A1 $F_{(1,76)} = 0.46$; $P = 0.501$ and A2 $F_{(1,84)} = 0.97$; $P = 0.328$). The data were analysed from the three perspectives; start to mid-trial (A1; Figure 4-5) and mid-trial to end of trial (A2) and total feeding damage (cumulative data A0–A2; Figure 4-6) to identify what level of feeding damage each of the plants sustained (low to high feeding damage).

There was considerable variation throughout the trial in the proportion of damaged tillers for all plants and also from plants within familes (Figure 4-5 A and Figure 4-6 A) and some families were more variable than others; A12064 had two of three plants (family representatives grown from seed) in the top ten and the remaining plant was in the bottom ten. Similarly within an individual plant genotype, the inter-clonal variation for proportion of tillers damaged was in some cases quite large and in other cases very small (Figure 4-5 B and Figure 4-6 B).

Table 4-4: Mean plant proportion of damaged tillers (logit transformed), of the clones from individual plants grown from seed (from the 23 half-sibling families), from feeding by adult African black beetles.

The data was analysed unadjusted for initial tiller number (plant size) at start of trial. The unadjusted data were also analysed cumulatively (cumulative damaged tiller number = the number of damaged tillers at Assessment 2 plus the number of damaged tillers from Assessment 1 which had been removed).

		Mean plant logit(proportion of damaged tillers) (M1)		
Family	**Plant**	**A1**	**A2**	**Cum A0–A2**
A12061	61/6	0.21 (0.55)	0.89 (0.71)	1.95 (0.88)
	61/9	-2.45 (0.08)	0.16 (0.54)	0.28 (0.57)
A12063	63/4	-1.95 (0.12)	-0.23 (0.44)	0.00 (0.50)
	63/9	-1.07 (0.26)	1.10 (0.75)	1.60 (0.83)
A12064	64/1	-1.04 (0.26)	0.19 (0.55)	0.74 (0.68)
	64/6	-0.24 (0.44)	2.18 (0.90)	-0.50 (0.38)
	64/7	-2.40 (0.08)	-0.34 (0.42)	2.57 (0.93)
	64/10	-2.92 (0.05)	-0.71 (0.33)	-0.04 (0.49)
A12065	65/1	-1.16 (0.24)	1.55 (0.82)	1.72 (0.85)
	65/6	-1.09 (0.25)	2.34 (0.91)	2.64 (0.93)
	65/7	-1.96 (0.12)	0.64 (0.66)	0.85 (0.7)
A12066	66/7	0.14 (0.54)	1.23 (0.77)	1.92 (0.87)
	66/8	-1.12 (0.25)	-0.02 (0.49)	0.57 (0.64)
A12068	68/6	-0.95 (0.28)	-0.65 (0.34)	0.06 (0.51)
	68/8	-3.43 (0.03)	-1.20 (0.23)	-1.06 (0.26)
A12069	69/6	-0.55 (0.37)	0.61 (0.65)	1.51 (0.82)
	69/9	-0.98 (0.27)	1.38 (0.80)	1.80 (0.86)
A12070	70/5	-1.86 (0.13)	-0.33 (0.42)	0.00 (0.50)
	70/6	-1.23 (0.23)	-0.29 (0.43)	0.24 (0.56)
	70/8	-0.27 (0.43)	-0.60 (0.35)	0.57 (0.64)
A12071	71/3	-0.66 (0.34)	0.49 (0.62)	1.31 (0.79)
	71/5	0.22 (0.56)	1.40 (0.80)	2.85 (0.95)
A12072	72/2	-0.97 (0.27)	1.16 (0.76)	1.55 (0.82)
	72/9	-3.04 (0.05)	-0.24 (0.44)	-0.14 (0.47)
A12073	73/1	-0.42 (0.40)	1.01 (0.73)	1.50 (0.82)
	73/2	-1.48 (0.19)	0.95 (0.72)	1.22 (0.77)
A12074	**74/1**	-1.75 (0.15)	-0.49 (0.38)	-0.03 (0.49)
	74/10	-1.09 (0.25)	0.60 (0.64)	0.88 (0.71)
A12075	75/6	-1.76 (0.15)	1.53 (0.82)	1.72 (0.85)
A12077	77/5	-1.79 (0.14)	-0.27 (0.43)	0.09 (0.52)
	77/8	-1.58 (0.17)	-0.09 (0.48)	0.30 (0.57)

Table 4–4 continued on next page

Table 4–4 continued

		Mean plant logit(proportion of damaged tillers) (M1)		
Family	**Plant**	**A1**	**A2**	**Cum A0–A2**
A12078	78/1	-1.89 (0.13)	0.06 (0.51)	0.46 (0.61)
	78/2	-1.76 (0.15)	-1.02 (0.26)	-0.52 (0.37)
	78/7	-1.92 (0.13)	-1.17 (0.24)	-0.78 (0.31)
A12080	80/2	-2.09 (0.11)	0.39 (0.60)	0.66 (0.66)
	80/7	-1.39 (0.20)	0.69 (0.67)	0.54 (0.63)
	80/10	-2.57 (0.07)	0.57 (0.64)	0.94 (0.72)
A12081	81/1	-0.76 (0.32)	0.62 (0.65)	1.18 (0.76)
	81/10	-0.66 (0.34)	-0.01 (0.50)	0.85 (0.70)
A12082	82/1	-1.86 (0.14)	1.39 (0.80)	1.70 (0.85)
	82/4	-0.11 (0.47)	0.52 (0.63)	1.55 (0.82)
A12083	83/1	-0.82 (0.30)	0.27 (0.57)	0.97 (0.73)
	83/2	-3.59 (0.03)	-0.27 (0.43)	-0.21 (0.45)
A12084	84/1	0.17 (0.54)	0.79 (0.69)	1.34 (0.79)
	84/5	-1.54 (0.18)	0.11 (0.53)	0.69 (0.67)
A12085	**85/6**	-1.41 (0.20)	0.50 (0.62)	0.82 (0.69)
A12086	**86/2**	-1.67 (0.16)	0.37 (0.59)	0.55 (0.63)
	86/4	-1.49 (0.18)	-1.05 (0.26)	-0.32 (0.42)
A12087	87/5	-1.43 (0.19)	-0.28 (0.43)	1.48 (0.81)
	87/10	-0.71 (0.33)	0.89 (0.71)	0.29 (0.57)
Fisher's LSD(5%)		4.411	3.285	3.185
Plant Effect (Individual Plants^{P})				
F-statistic$_{df}$		$1.83_{49,75}$	$2.27_{49,70}$	$2.49_{49,63}$
P-value		***0.009***	***<0.001***	***<0.001***
Fisher's LSD(5%)		4.411	3.285	3.185
Plant Effect (Within Family^{FP})				
Wald-statistic$_{df}$		51.26_{27}	58.92_{27}	58.22_{27}
P-value		***0.003***	***0.004***	***<0.001***

A0, A1, A2 = Assessments 0 (start of trial and treatment phase), 1 (mid-trial), 2 (end of trial). Cum = cumulative. $_{df}$ = Degrees of freedom. LSD(5%) = Least significance difference at the 5% significance level. M1 = fixed effect model 1; ^{FP} = *Family.Plant.* ^{P} = *Plant*.
Back transformed values are in brackets. Family lines and plants contaminated with wild-type endophyte are highlighted in bold font. Statistically significant results are highlighted in bold and italic font. Weak evidence of effects (0.05< *P* ≤1.0) are highlighted in italic font.

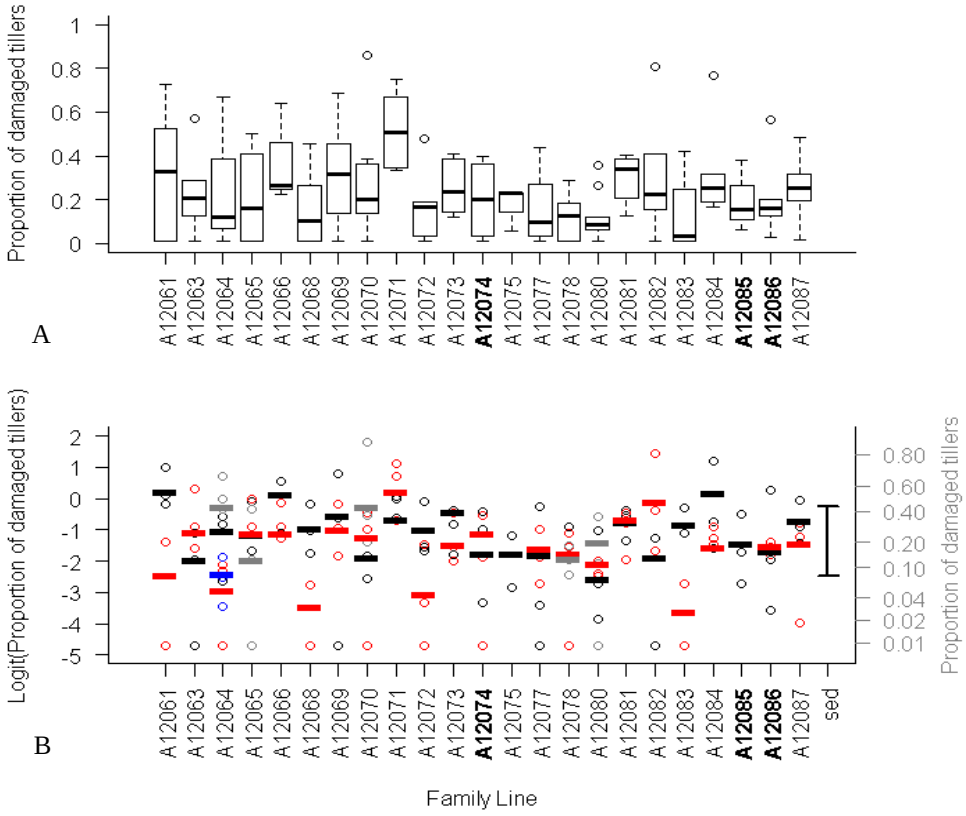

Figure 4-5: Proportion of tillers damaged at mid-trial (A1).
A) Boxplot; displaying family median, upper and lower quartiles and showing variation within and between families.
B) Dotplot; displaying individual plants within a family (circles) and the corresponding mean (bars), showing the variation within a family and between plants cloned from a single individual plant grown from seed. The data graphed has been logit transformed (with the back-transformed scale on the right vertical axis). The dotplot is coloured by individual plants grown from seed within a family, with each individual circle of the same colouring representing a cloned plant with a matching coloured bar denoting the mean of the clones for the individual plant. sed = standard error of the difference (fixed effects model, M1).
Family lines contaminated with wild-type endophyte are highlighted in bold font.

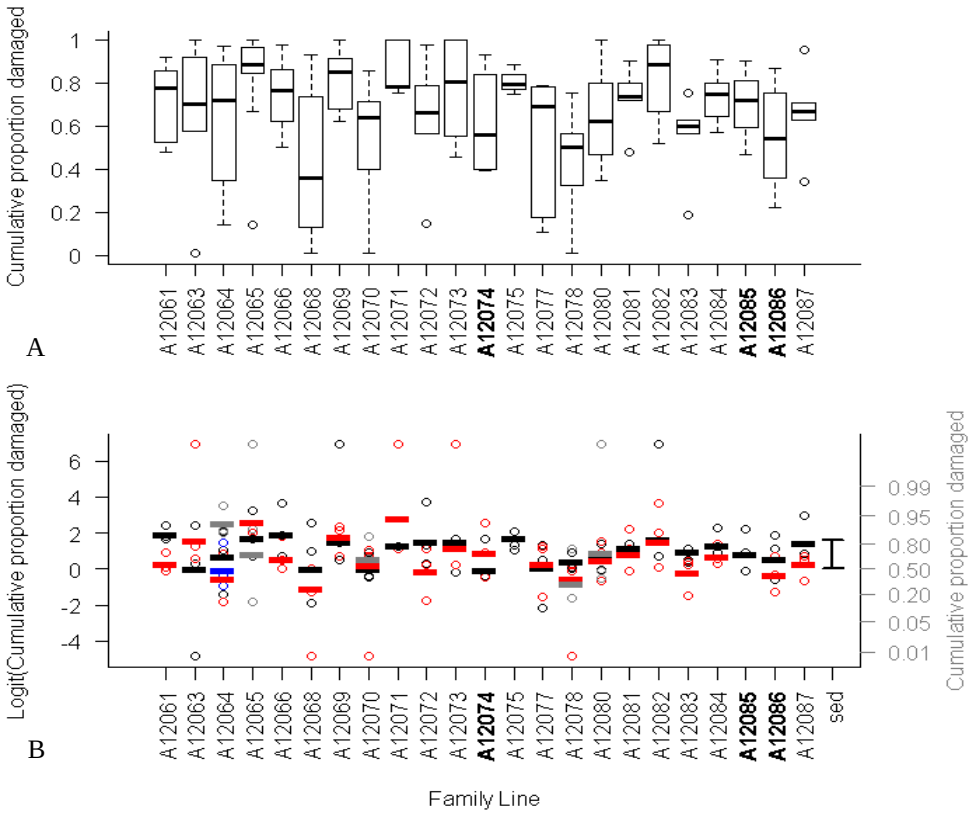

Figure 4-6: Cumulative proportion of tillers damaged at end of trial (A2).
A) Boxplot; displaying family median, upper and lower quartiles and showing variation within and between families.
B) Dotplot; displaying individual plants within a family (circles) and the corresponding mean (bars), showing the variation within a family and between plants cloned from a single individual plant grown from seed. The data graphed has been logit transformed (with the back-transformed scale on the right vertical axis). The dotplot is coloured by individual plants grown from seed within a family, with each individual circle of the same colouring representing a cloned plant with a matching coloured bar denoting the mean of the clones for the individual plant. sed = standard error of the difference (fixed effects model, M1).
Family lines contaminated with wild-type endophyte are highlighted in bold font.

Plants were under high pressure from feeding by adult African black beetle (*H. arator*) (>1 beetle per plant), resulting in the median cumulative proportion of damaged tillers per plant for the majority of families (21 of 23) being >0.5, including wild-type–contaminated family lines (Table 4-5). Only families A12068 and A12078 had medians ≤0.5 (0.36 and 0.50 respectively) as illustrated in Figure 4-6 A.

Table 4-5: Median cumulative proportion of damaged tillers per plant for the half-sibling families.

Median proportion of damaged tillers	Half-sibling families
Very High (>0.75 proportion of damaged tillers)	A12061, A12065, A12066, A12069, A12071, A12073, A12075*, A12082
High (>0.5–≤0.75 proportion of damaged tillers)	A12063, A12064, A12070, A12072, **A12074**, A12077, A12080, A12081,A12083, A12084, **A12085****, **A12086**, A12087
Medium (≤0.5 proportion of damaged tillers)	A12068, A12078

Family lines contaminated with wild-type endophyte are highlighted in bold font. *a single individual family representative (plant) grown from seed.

Families that had all or the majority of family representatives in the top 25 least damaged plants were A12064, A12068, A12070, A12077, A12078, A12080 and $A12086^{WT}$ (seven of the top ten plants were from the the families underlined). Plants from the family lines contaminated with wild-type endophyte were not significantly different from many of the plants from the uncontaminated AR1-infected family lines (Table 4-4).

4.3.3 Peramine and paxilline ELISA results

The peramine and paxilline ELISAs were only performed on a subset of samples (14 of the 23 half-sibling families) taken at the end of the trial (A2). From the results for tiller number and feeding damage, only plants that were considered likely to be proceeding on with, in subsequent trials were analysed. The subgoup of ELISA data

refers to the set of ELISA results from plants with peramine or paxilline ELISA results for all three plant sections; herbage, damaged and undamaged pseudostems. This enabled the calculation of an overall plant pseudostem level of peramine and paxilline immunoreactivity, by summing the damaged and undamaged pseudostem levels weighted by the proportion of tillers damaged (for the subgroup of ELISA data).

For both peramine and paxilline immunoreactivity, there was strong evidence (P <0.001) of differences between plants (Table 4-6), within families and indicated between families. For both fungal secondary metabolites, variation was larger within some families than others although this depended on the section of plant analysed (herbage, damaged or undamaged pseudostem). In general, the inter-clonal variation between cloned plants within a single genotype was small, but there were significant differences between the plant means of different family representatives and these differences could account for the majority of the variation within the family (Table 4-6).

Examples of the peramine and paxilline immunoreactivity in herbage, damaged pseudostem and undamaged pseudostem showing plant and family variation are illustrated for paxilline immunoreactivity (see Figure 4-7 to Figure 4-9 respectively). Levels of peramine and paxilline immunoreactivity were generally higher in the undamaged pseudostem than the damaged pseudostem, with the lowest levels in the herbage (Figure 4-10).

For family A12073, one of the family representatives (73/1) had no detectable levels of peramine immunoreactivity, minimal levels of paxilline immunoreactivity in herbage and damaged pseudostem (and insufficient sample available to measure immunoreactivity levels in the undamaged pseudostem) therefore family A12073 was omitted from the immunoreactivity data. The subgroup of ELISA data (Appendix I, Table B) that had recorded levels of peramine and paxilline immunoreactivity for all three plant sections (herbage, damaged and undamaged pseudostem) was analysed.

Table 4-6: Mean plant concentrations (natural log transformed) of the clones from individual plants grown from seed (from the 23 half-sibling families) for peramine-IRE and paxilline-IRE in herbage and damaged pseudostem plant sections at A2.

		Mean natural log transformed concentrations of ELISA immunoreactivity (M1)			
		Peramine-IRE (µg/g)		Paxilline-IRE (µg/g)	
Family	Plant	Herb	DP	Herb	DP
A12061	61/6	2.78 (15.1)	3.02 (19.49)	1.74 (5.69)	2.13 (8.41)
A12061	61/9	3.23 (24.23)	3.28 (25.66)	2.29 (9.87)	2.56 (12.94)
A12063	63/4	3.13 (21.92)	3.42 (29.48)	2.08 (7.98)	2.49 (12.03)
A12063	63/9	2.59 (12.28)	2.55 (11.82)	1.33 (3.79)	1.77 (5.85)
A12064	64/1	3.05 (20.09)	3.15 (22.29)	2.28 (9.77)	2.48 (11.88)
A12064	64/6	2.62 (12.74)	2.84 (16.15)	1.81 (6.09)	1.86 (6.42)
A12064	64/7	2.98 (18.67)	3.19 (23.24)	2.25 (9.53)	2.52 (12.37)
A12064	64/10	3.96 (51.61)	3.22 (24.08)	3.09 (21.89)	2.83 (16.98)
A12069	69/6	3.17 (22.86)	3.37 (28.02)	1.92 (6.83)	2.37 (10.69)
A12069	69/9	3.21 (23.78)	3.09 (20.98)	1.77 (5.84)	2.22 (9.17)
A12072	72/2	2.83 (15.86)	3.00 (19.03)	1.55 (4.73)	2.48 (11.88)
A12072	72/9	3.15 (22.34)	3.27 (25.23)	2.51 (12.30)	2.60 (13.41)
A12073	73/1	-0.02 (-0.02)	0.03 (0.03)	-1.32 (0.27)	-1.36 (0.26)
A12073	73/2	3.10 (21.13)	3.43 (29.97)	2.00 (7.37)	2.37 (10.73)
A12074	**74/1**	3.06 (20.22)	3.21 (23.78)	2.45 (11.54)	2.73 (15.30)
A12074	**74/10**	3.11 (21.33)	2.94 (17.86)	2.03 (7.58)	1.88 (6.58)
A12077	77/5	2.84 (16.03)	2.67 (13.37)	1.12 (3.07)	1.54 (4.65)
A12077	77/8	2.97 (18.41)	3.12 (21.62)	1.55 (4.69)	1.97 (7.16)
A12078	78/1	3.18 (23.14)	3.22 (23.9)	1.5 (4.49)	1.71 (5.5)
A12078	78/2	3.10 (21.26)	3.28 (25.68)	1.88 (6.53)	2.32 (10.18)
A12078	78/7	2.65 (13.15)	3.05 (20.07)	1.17 (3.23)	2.00 (7.37)
A12080	80/2	3.26 (25.05)	3.55 (33.95)	1.78 (5.91)	2.50 (12.15)
A12080	80/7	3.02 (19.53)	3.06 (20.31)	1.35 (3.86)	1.85 (6.34)
A12080	80/10	3.74 (41.01)	3.42 (29.69)	2.45 (11.54)	2.31 (10.11)
A12081	81/1	2.90 (17.14)	2.85 (16.29)	1.64 (5.14)	2.12 (8.35)
A12081	81/10	2.76 (14.72)	2.54 (11.67)	0.84 (2.31)	1.19 (3.28)
A12083	83/1	3.40 (29.02)	3.39 (28.76)	1.48 (4.37)	2.37 (10.73)
A12083	83/2	2.95 (18.14)	3.05 (20.07)	1.87 (6.48)	1.84 (6.30)

Table 4–6 continued on next page

Table 4–6 continued

		Mean natural log transformed concentrations of ELISA immunoreactivity (M1)			
		Peramine-IRE (µg/g)		Paxilline-IRE (µg/g)	
Family	**Plant**	**Herb**	**DP**	**Herb**	**DP**
A12086	**86/2**	2.88 (16.73)	2.61 (12.63)	1.98 (7.21)	2.18 (8.82)
A12086	**86/4**	3.09 (20.93)	3.19 (23.22)	2.7 (14.88)	2.79 (16.28)
A12087	87/5	3.22 (23.93)	3.16 (22.57)	2.1 (8.15)	2.38 (10.75)
A12087	87/10	3.14 (22.01)	3.39 (28.78)	2.17 (8.73)	2.68 (14.61)
Fisher's LSD(5%)		0.394	0.451	0.538	0.518
Plant Effect (Individual Plants^{P})					
F-value$_{df}$		$21.08_{31,52}$	$15.82_{31,37}$	$16.19_{31,43}$	$19.46_{31,41}$
P-value		***<0.001***	***<0.001***	***<0.001***	***<0.001***
Plant Effect (Within family^{FP})					
Wald-statistic$_{df}$		368.36_{18}	342.51_{18}	257.19_{18}	370.88_{18}
P-value		***<0.001***	***<0.001***	***<0.001***	***<0.001***

A2 = Assessment 2 (end of trial). $_{df}$ = Degrees of freedom. DP = Damaged pseudostem. Herb = Herbage. LSD(5%) = least significance difference at the 5% significance level. M1 = fixed effects model 1; ^{FP} = *Family.Plant.* ^{P} = *Plant*. Peramine- and Paxilline-IRE = Peramine and paxilline immunoreactive equivalents.

Back transformed values are in brackets. Family lines and plants contaminated with wild-type endophyte are highlighted in bold font. Statistically significant results are highlighted in bold and italic font. Weak evidence of effects ($0.05 < P \leq 1.0$) are highlighted in italic font.

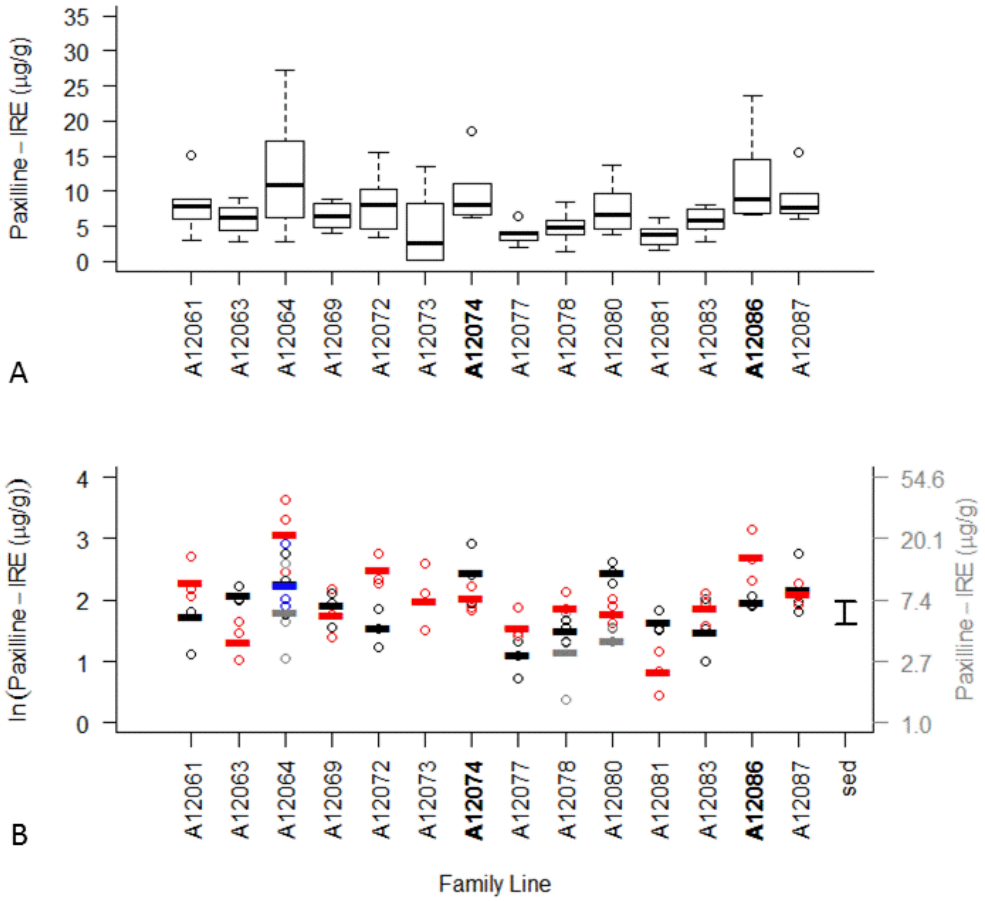

Figure 4-7: Levels of paxilline immunoreactive equivalents (paxilline-IRE) in herbage.
A) Boxplot; displaying family median, upper and lower quartiles and showing variation within and between families.
B) Dotplot; displaying individual cloned plants within a family and the corresponding mean, showing the variation within a family and between plants cloned from a single individual plant grown from seed (family representative). The data has been square root transformed (with the back-transformed scale on the right vertical axis). The dotplot is coloured to distinguish individual family representatives within a family, with each individual circle of the same colouring representing a cloned plant with a matching coloured bar denoting the mean of the clones for the individual family representative plant. . sed = standard error of the difference (fixed effects model, M1).
Family lines contaminated with wild-type endophyte are highlighted in bold font.

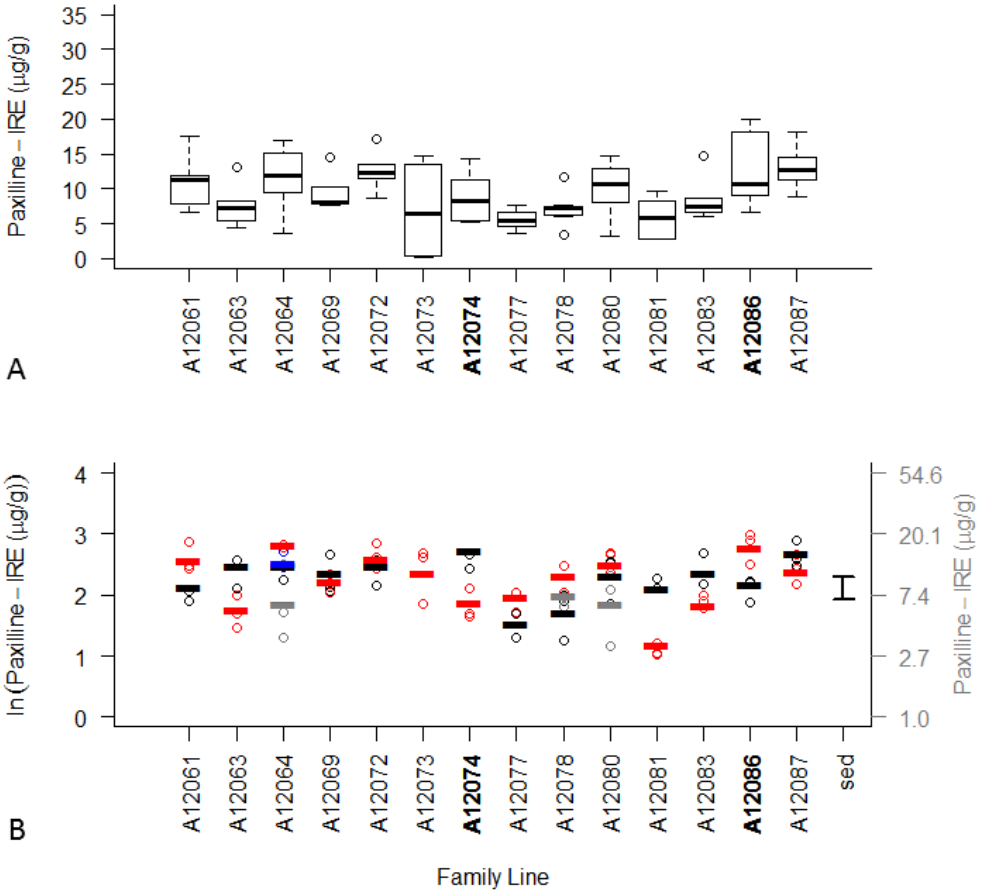

Figure 4-8: Levels of paxilline immunoreactive equivalents (paxilline-IRE) in damaged pseudostem.

A) Boxplot; displaying family median, upper and lower quartiles and showing variation within and between families.

B) Dotplot; displaying individual cloned plants within a family and the corresponding mean, showing the variation within a family and between plants cloned from a single individual plant grown from seed (family representative). The data has been square root transformed (with the back-transformed scale on the right vertical axis). The dotplot is coloured to distinguish individual family representatives within a family, with each individual circle of the same colouring representing a cloned plant with a matching coloured bar denoting the mean of the clones for the individual family representative plant. sed = standard error of the difference (fixed effects model, M1).

Family lines contaminated with wild-type endophyte are highlighted in bold font.

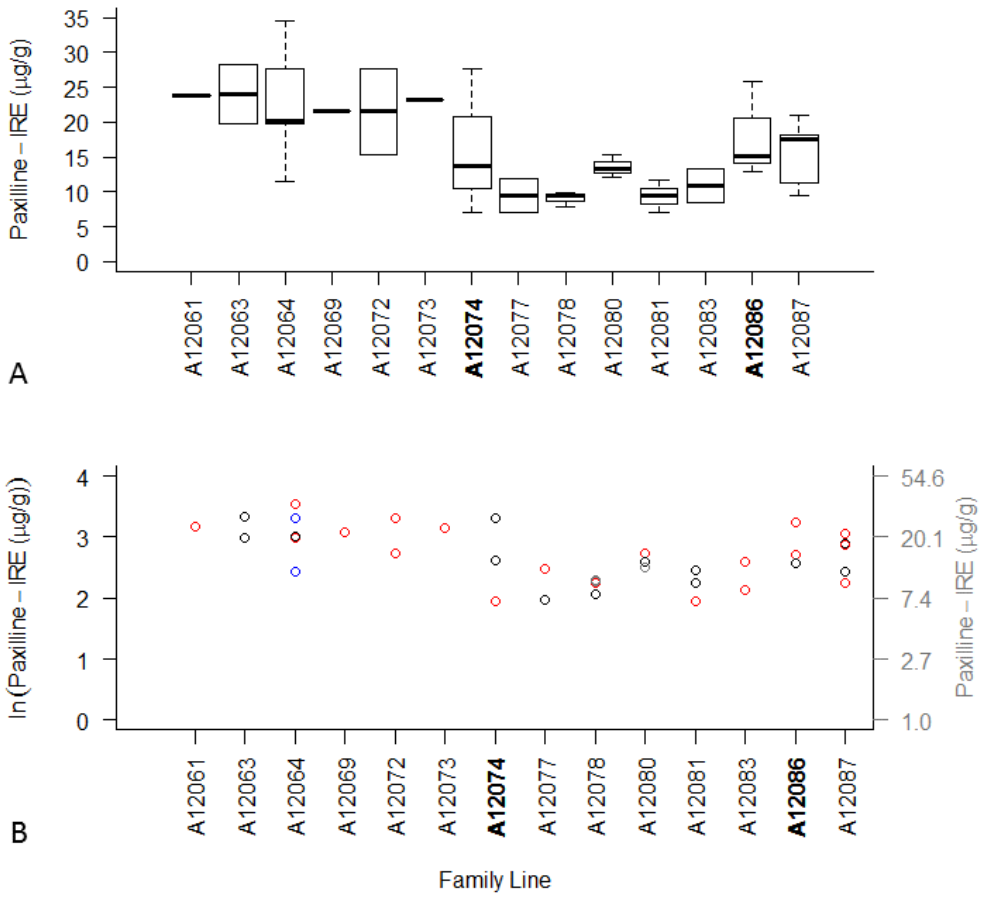

Figure 4-9: Levels of paxilline immunoreactive equivalents (paxilline-IRE) in undamaged pseudostem.

A) Boxplot; displaying family median, upper and lower quartiles and showing variation within and between families.

B) Dotplot; displaying individual cloned plants within a family showing the variation within a family and between plants cloned from a single individual plant grown from seed (family representative). The data has been square root transformed (with the back-transformed scale on the right vertical axis).The dotplot is coloured to distinguish individual family representatives within a family, with each individual circle of the same colouring representing a cloned plant from a single family representative.

Family lines contaminated with wild-type endophyte are highlighted in bold font.

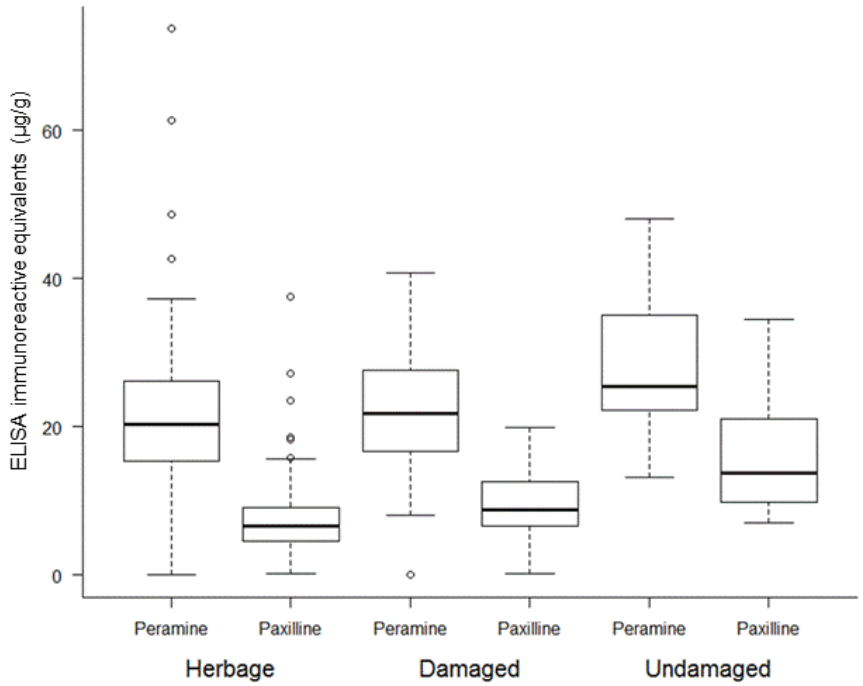

Figure 4-10: Comparison of the concentration of peramine and paxilline immunoreactivity in the three different plant sections; herbage, damaged and undamaged pseudostem from all ELISA data.

From scatterplots, positive correlations were found between the various plant sections for each secondary metabolite group, ranging from weak to strong, however, some relationships appear non-linear, (Appendix I, Figure A and Figure B). Strong correlations (Pearson's correlation coefficient, CC >0.85) were observed for both peramine and paxilline immunoreactivity between the overall pseudostem with both damaged and undamaged pseudostem subsections, with the strongest correlations (CC >0.95) found with the undamaged pseudostem plant subsection (Appendix I, Figure B c-f). When comparing levels of peramine and paxilline immunoreactivity within a plant section, however, only weak positive correlations were found for the undamaged and overall plant sections (Appendix I, Figure B a-b). For peramine immunoreactivity, only relatively weak correlations (CC ≤0.65) were found between herbage with both pseudostem plant subsections and the overall plant pseudostem, and for paxilline immunoreactivity, moderate correlations (0.65< CC ≤0.85) (Appendix I, Figure A). Yet when comparing the two secondary metabolite groups within a plant section moderate correlations were

found for both herbage and damaged pseudostem (Appendix I, Figure C a and b) and at best weak correlations in the undamaged and overall pseudsotem sections Appendix I, Figure C c and d). In addition, in a scatterplot comparing levels of paxilline immunoreactivity in herbage between the initial paxilline screen pre-trial (Section 3.3.4) and at end of this trial only a relatively weak positive correlation was apparent (CC = 0.63) (Appendix I, Figure D).

4.3.4 Relationships between feeding damage and the ELISA results for peramine and paxilline immunoreactivity

Damage from adult African black beetle (*H. arator*) feeding was correlated with both paxilline and peramine immunoreactivity. The levels of peramine and paxilline immunoreactivity in the different plant sections were compared with the proportion of damaged tillers for the subset of samples (from 14 of the 23 half-sibling families) that were analysed by ELISA and the subgoup of ELISA data (the set ELISA results from plants with peramine or paxilline ELISA results for all three plant sections; herbage, damaged and undamaged pseudostems). An estimation of the overall plant pseudostem level of peramine and paxilline immunoreactivity was calculated weighted by the proportion of tillers damaged.

The relationship between feeding damage from adult African black beetle (*H. arator*) with peramine immunoreactivity, and with paxilline immunoreactivity are presented using scatterplots in Figure 4-11 (for the three plant sections) and in Figure 4-12 (overall plant pseudostem). No relationships were found between feeding damage with both peramine and paxilline immunoreactivity for any of the three plant sections, including the calculated overall pseudostem.

However, in the plot comparing the overall plant pseudostem with feeding damage for paxilline immunoreactivity (Figure 4-12 B) a cluster of data points (circled) was evident.

The group of plants making up this cluster had high levels of paxilline immunoreactivity in the overall pseudostem (≥25 μg/g) and low proportions of damaged tillers (≤0.2 or 20%). This cluster of data points can also be seen in the plot comparing levels of paxilline immunoreactivity in undamaged pseudostem and feeding damage from adult African black beetle (*H. arator*) (Figure 4-11 F).

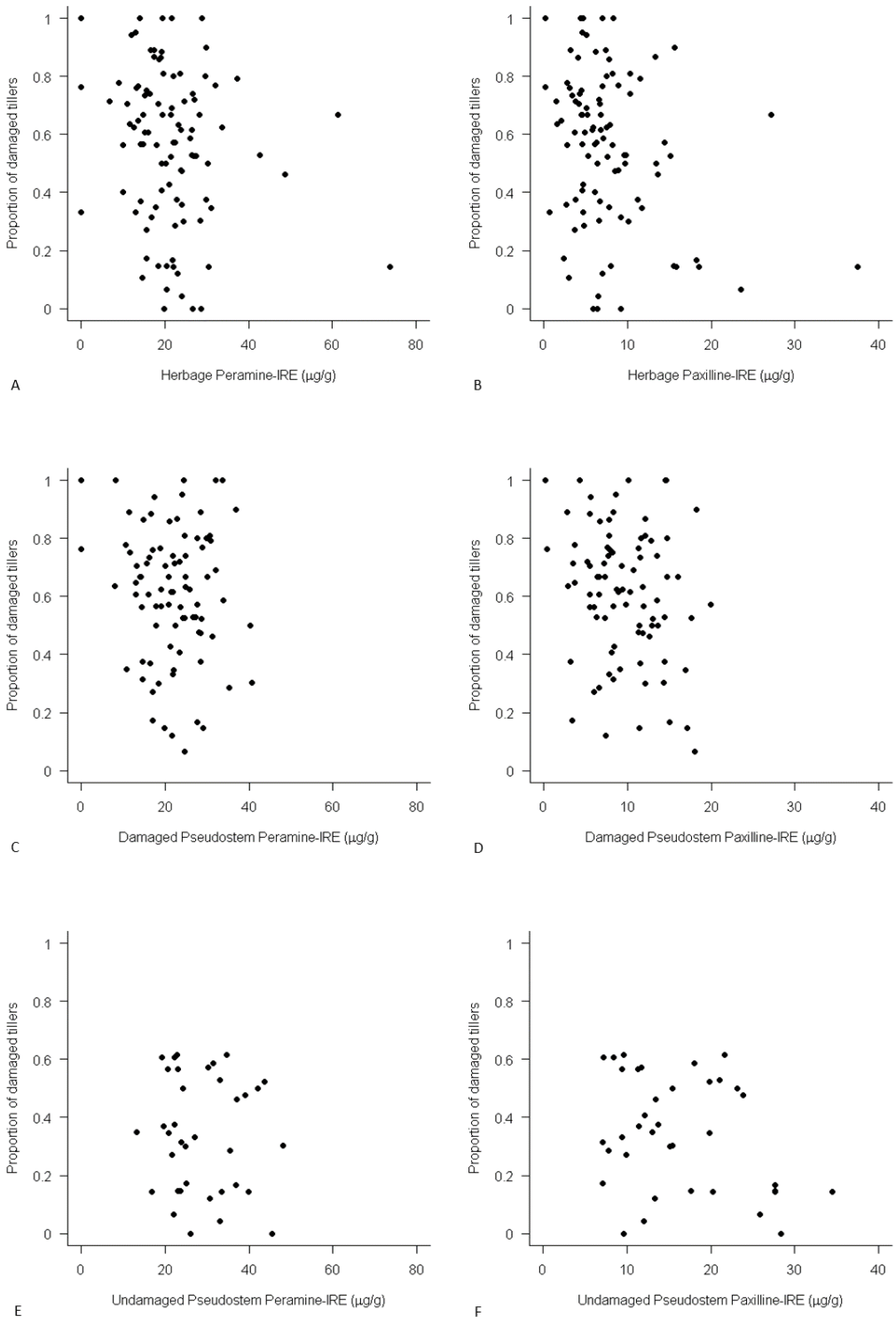

Figure 4-11: Scatterplots (raw data) between adult African black beetle feeding damage with peramine (A, C and E) and paxilline (B, D and F) ELISA immunoreactivity in the three plant sections; herbage, damaged and undamaged pseudostem.

Plots A–F; A and B) herbage, C and D) damaged pseudostem, E and F) undamaged pseudostem. Peramine- and paxilline-IRE = peramine and paxilline immunoreactive equivalents.

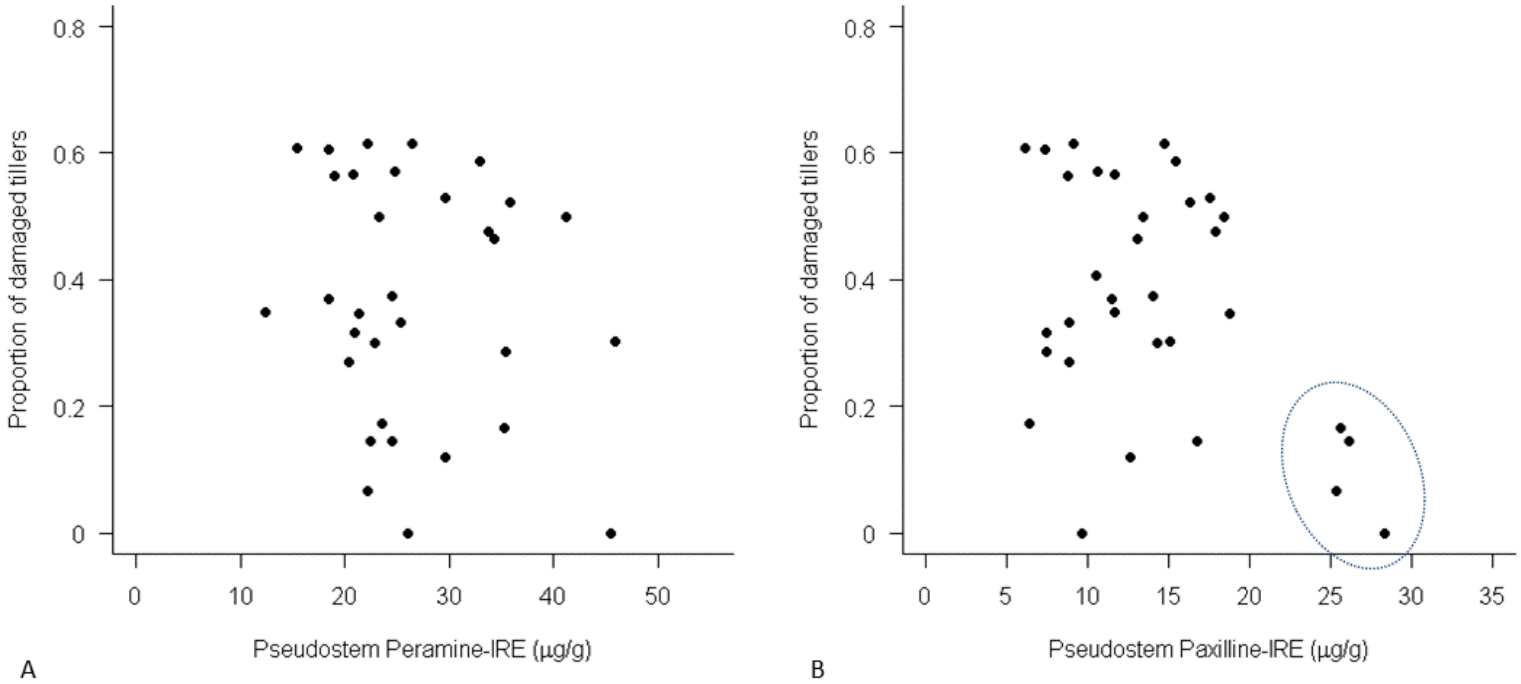

Figure 4-12: Scatterplots (raw data) between adult African black beetle feeding damage with peramine and paxilline ELISA immunoreactivity in the overall pseudostem (Subgroup data).

Plots A and B; A) Peramine data, B) Paxilline data. Subgroup data = subgroup of peramine or paxilline ELISA data (Appendix I Table B) in which individual cloned plants had measured levels of peramine or paxilline immunoreactivity for all three plant sections; herbage, damaged and undamaged pseudostem. Overall pseudostem levels of peramine or paxilline immunoreactivity were calculated by summing the damaged and undamaged pseudostem immunoreactivity levels weighted by the proportion of tillers damaged. Peramine- and paxilline-IRE = peramine and paxilline immunoreactive equivalents.

4.3.5 Damage score

The analyses of plant tillers for the severity of damage from feeding by adult African black beetle (*H. arator*) (1 = minor damage, 2 = moderate damage and 3 = severe damage causing tiller death, with undamaged tillers = 0) were examined from two perspectives; the proportion of all tillers with a damage score of three and the proportion of damaged tillers with a score of three. This was because of the low number of tillers with damage scores of one or two at each assessment (A1 and A2; Figure 4-13). Owing to the sparsity of the data (the proportion of tillers damaged at A1 was low relative to A2; Figure 4-13) only the analysis of the proportion of tillers with a damage score of three produced sensible results and was used. Plant means for the proportion tillers damaged with a damage score of three at A2 are tabled in Appendix I, Table C.

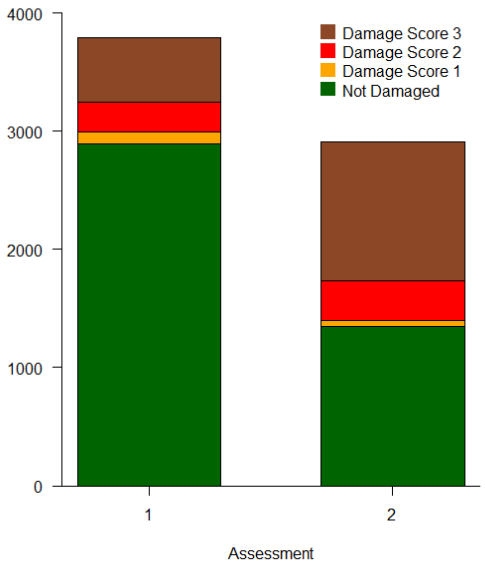

Figure 4-13: Total number of plant tillers at each assessment (A1 and A2) partitioned by tiller damage scores.
1 = Assessment 1 (A1). 2 = Assessment 2 (A2). Not Damaged = undamaged tiller; damage score = 0.

The proportion of tillers with a damage score of three differed significantly between plants ($F_{(19,73)}$ = 1.85; P = 0.008) at A2, with weak evidence (Wald $statistic_{df}$ = 38.63_{27}; P = 0.068) of differences within families. No evidence was found of an initial tiller number (plant size) effect on the proportion of tillers with a damage score of three. The results mirrored those found for the proportion of all tillers damaged (feeding damage) because a tiller was either undamaged or if fed on by a beetle it was generally severely damaged causing death of the tiller. Seven of the ten plants with lowest proportion of damaged tillers also had the lowest proportion of tillers with a damage score of three. Eight of the ten plants with the greatest damage also had the highest proportion of tillers with a damage score of three.

4.3.6 Comparison of current trial results with Lincoln trial results

The Lincoln trial data were re-analysed using only the undamaged pseudostem samples for direct comparison with the current trial. Undamaged pseudostem samples were used as earlier analyses suggest that these best reflect overall pseudostem immunoreactivity levels (Section 4.3.3). The current trial had much higher levels of adult African black beetle (*H. arator*) damage than the Lincoln trial. A scatterplot of the mean level of paxilline-IRE against the mean proportion damage (Figure 4-14) provides little evidence of a relationship for the Lincoln trial. Conversely, the current trial suggests a positive relationship. However, when the raw data is examined (Figure 4-15) within certain cultivars and families there were suggestions of a negative association with feeding damage and paxilline-IRE levels in the pseudostem (for example, ‘Arrow’ and A12064), in others a positive association (for example ‘Alto’ and ‘PG150’) and in others no association (for example, ‘Extreme’ and A12087). For the Lincoln Trial, large variation was found within the cultivars (Figure 4-15 A), consistent with the variation found within the half-sibling famililes in the current trial (Figure 4-15 B).

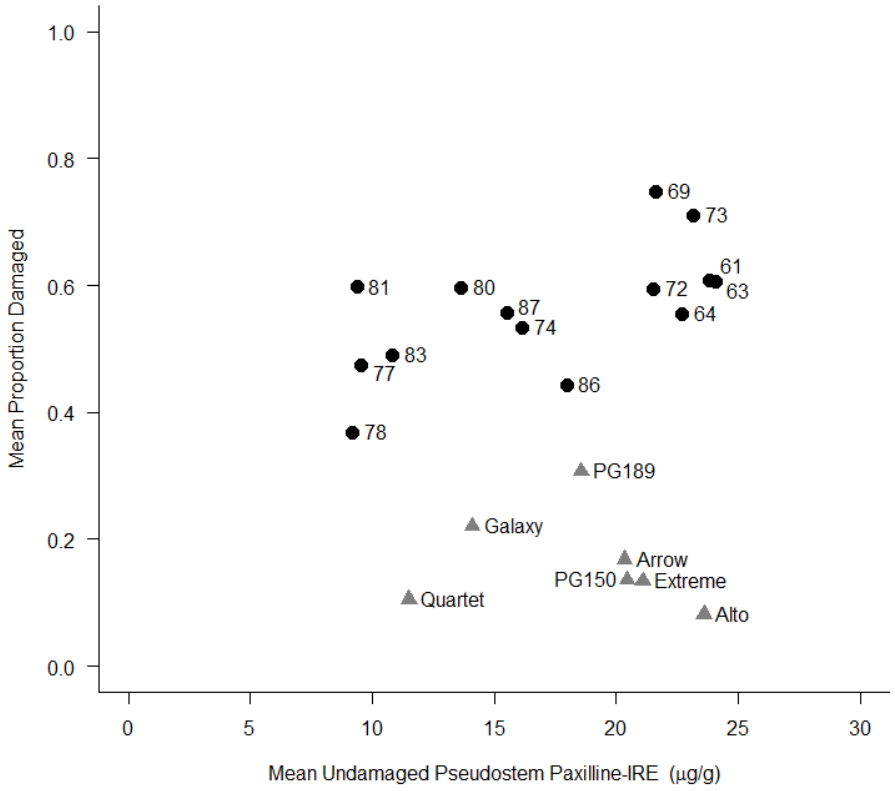

Figure 4-14: Scatterplot of the mean values for the level of paxilline-IRE in half-sibling families of the GA97 breeding line in the present study and commercially available cultivars in the Lincoln trial.

The identity of the family or cultivar is shown with its data point. 64–87 = Families A12061–A12087. Paxilline-IRE = Paxilline immunoreactive equivalents.

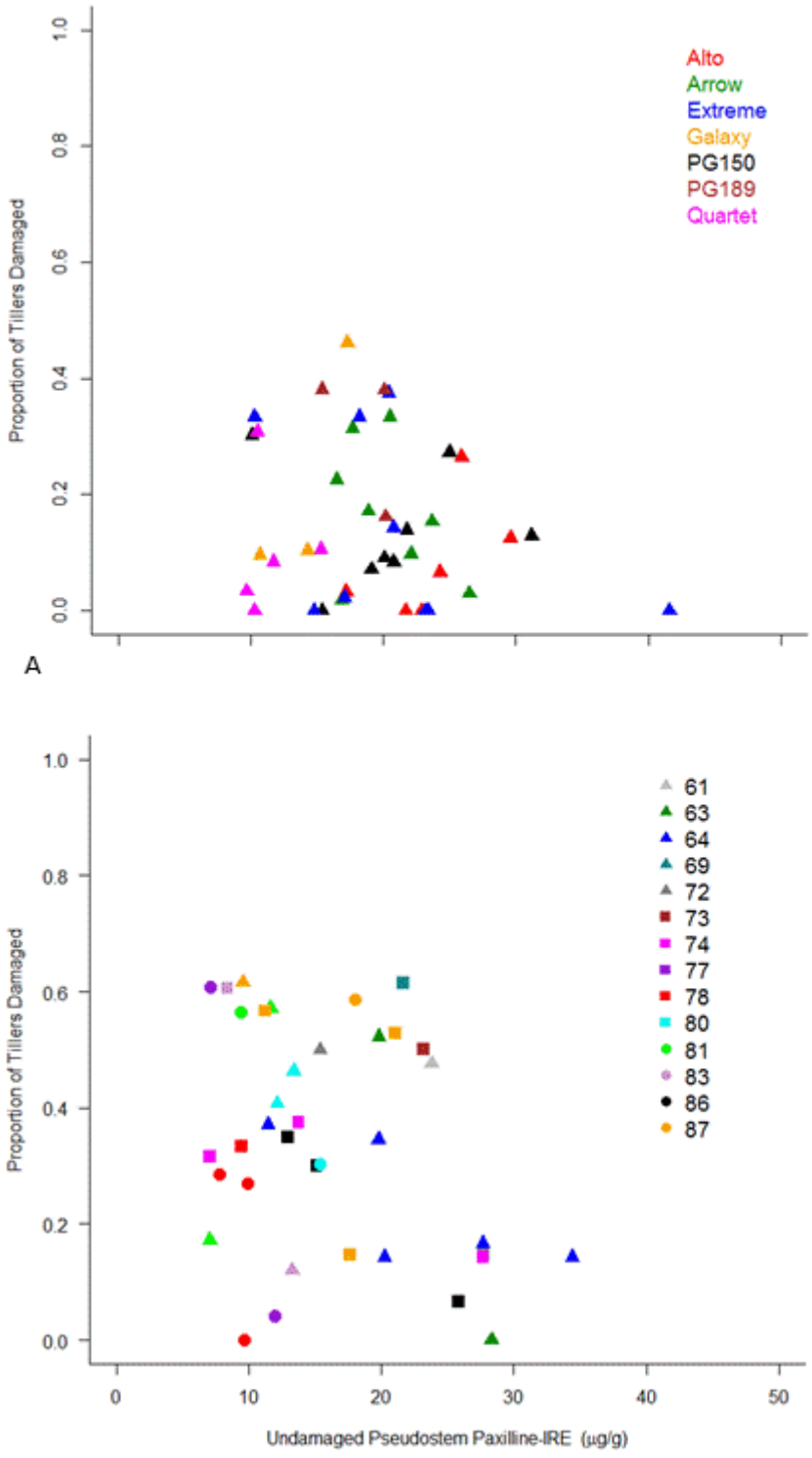

Figure 4-15: Individual plants and proportion of tillers damaged.
A) Lincoln trial. B) PhD pilot trial. The identity of the family or cultivar is shown by the colour of the data point. 64–87 = Families A12061–A12087. Paxilline-IRE = Paxilline immunoreactive equivalents.

4.4 Discussion

The purpose of the pilot trial with African black beetle (*H. arator*) was to screen AR1-infected ryegrass plants from 23 half-sibling families (within the GA97 breeding line) to determine resistance of plants to feeding from adult African black beetle (*H. arator*), levels of the paxilline-like and peramine-like fungal metabolites, tiller production and any associations between fungal metabolites and feeding damage; and whether there were associated plant and family differences. Using this information, a number of AR1-infected plants were selected for further study of plant resistance to feeding by adult African black beetle (*H. arator*). Inclusion of the plants and familes contaminated with wild-type endophyte not only allowed the identification of AR1-infected plants and families with at least the same level of resistance to African black beetle (*H. arator*) as the wild-type infected plants, but also those AR1-infected plants with at least the same level of secondary metabolite production (peramine and paxilline immunoreactivity) and tiller production. All of which are useful grass-breeding traits. The trial was run in mid-late spring when adult African black beetle (*H. arator*) were in their reproductive phase and levels of alkaloid production in grass were increasing. Seasonal trends have been reported for the level of fungal endophytes (Fletcher, 1983; Mortimer *et al.*, 1984; di Menna & Waller, 1986; Fletcher, 1986; di Menna *et al.*, 1992; Ball *et al.*, 1995a) and many of the metabolites they produce, with general alkaloid production slightly lagging endophyte concentration, increasing during spring, through summer and early autumn, then falling in late autumn and winter (di Menna *et al.*, 1992; Woodburn *et al.*, 1993; Ball *et al.*, 1995a; Easton *et al.*, 1996). Different metabolites may have production peaks at slightly different times, with lolitrem B peaking in summer and early autumn (Prestidge & Gallagher, 1988; Ball *et al.*, 1991; di Menna *et al.*, 1992; Ball *et al.*, 1995a) and with peramine being high from mid-spring through to mid-autumn (Ball *et al.*, 1995a).

Differences identified among plants found in the present work were very promising and in line with the intention to identify plants and families with high resistance to feeding by adult African black beetle (*H. arator*), average to high tiller production and high production of paxilline-like compounds. Differences between plants for the levels of the endophyte and endophyte metabolites has been reported previously

(Musgrave, 1984; Jones *et al.*, 1985; Belesky *et al.*, 1989; Rowan *et al.*, 1990b; Ball *et al.*, 1991; Hill *et al.*, 1991; Breen, 1992; Azevedo *et al.*, 1993; Davies *et al.*, 1993; Keogh & Tapper Brian, 1993; Agee & Hill, 1994; Ball *et al.*, 1995a). It has been found that the genetic characteristics of both endophyte and host plant are important in the levels of alkaloid production (Fannin *et al.*, 1990; Christensen *et al.*, 1991; Hill *et al.*, 1991; Christensen *et al.*, 1993; Agee & Hill, 1994), with individual plants infected with the same endophyte found to contain different levels of endophyte and endophyte metabolites (Breen, 1992; Ball *et al.*, 1995a). Other work has shown that within a host plant species (e.g. *L. perenne*) the level of insect resistance is variable both between individual plants (Easton *et al.*, 2000) and between cultivars infected with the same endophyte (Popay *et al.*, 2003). The number of tillers a plant produced did not appear to be influenced by adult African black beetle (*H. arator*) attack, *i.e.*, plants that had high tiller numbers at the start of the trial, prior to exposure to feeding by adult African black beetle (*H. arator*), had high tiller numbers at the end of the trial, post exposure. The number of tillers a plant had did not influence the proportion of tillers damaged from feeding by adult African black beetle (*H. arator*). The results imply that feeding by beetles is not influenced by plant size and feeding by adult African black beetle (*H. arator*) does not influence tiller production for specific plants and families. Instead plant resistance to attack by African black beetle (*H. arator*), plant size and tiller production, are traits strongly influenced by host plant genetics. Identification of plants with high tiller production is a useful grass breeding trait and was used in the selection of plants for further investigation as high tiller number in general reflects, a larger plant size resulting in higher drymatter producton.

Epichloë festucae var. *lolii* and several of the fungal metabolites it produces are known to be concentrated at the base of the plant (Musgrave, 1984; Gallagher *et al.*, 1987). In agreement with published data, in the present study, the highest levels of peramine and paxilline immunoreactivity were found in the pseudostem sections (undamaged pseudostem, then damaged pseudostem) and the lowest in the herbage. Fungal secondary metabolites differ in solubility properties and therefore translocation within the plant will differ. In agreement with the literature, the more polar (water soluble) alkaloids, such as peramine and lolines will be more mobile within the plant (Tapper *et al.*, 1989; Fannin *et al.*, 1990; Davies *et al.*, 1993; Ball

et al., 1995a) and the lipophilic alkaloids, such as lolitrems, paxilline and ergovaline are less polar and will be less mobile within the plant (Gallagher *et al.*, 1987; di Menna *et al.*, 1992; Davies *et al.*, 1993; Keogh & Tapper Brian, 1993).

In this current study, concentrations of peramine immunoreactivity between the herbage and both pseudostem plant sections (damaged and undamaged) were only weakly correlated. Yet concentrations of peramine immunoreactivity between the damaged and undamaged pseudostem sections were moderately correlated. Ball *et al.* (1995a) suggests that peramine is translocated from the basal part of the plant (8 cm from the crown) to the upper parts of the plant (>8 cm). Therefore in the present study, the equivalent 'basal part of the plant' was separated into the pseudostem (3 cm from the crown) and herbage (from 3 to 8 cm from the crown) and the equivalent 'upper part of the plant' was not measured and was discarded. This may in part explain why, in the current work, higher levels of peramine immunoreactivity were found in pseudostem than in the herbage. The pseudostem section includes the base of the plant where endophyte and fungal alkaloid concentrations are known to be high. Whereas in the herbage section, peramine is possibly being transported to the upper parts of the plant (>8cm).

With less polar alkaloids not as broadly distributed within the plant, comparisons between the different plant sections for each secondary metabolite group may reflect this difference in solubility properties, with stronger relationships found for the paxilline group than for the peramine group. Herbage sections were weakly correlated with the damaged, undamaged and overall pseudostem (weighted by the proportion of damaged tillers) sections for the peramine group of secondary metabolites and moderately correlated for the paxilline group. Whereas the damaged and undamaged pseudostem sections were moderately or strongly correlated with each other for peramine and paxilline immunoreactivity respectively, and both pseudotstem sections were strongly correlated with the overall pseudostem for each metabolite group.

This difference in alkaloid solubility properties may also be reflected in the results from comparisons between the two metabolite groups within each plant section. Weak correlations were found in the undamaged and overall pseudostem plant sections and moderate correlations in herbage and damaged pseudostem. This is

unlike correlations found between metabolites reported in the literature (Ball *et al.*, 1995b; Ball *et al.*, 1995a) and may suggest that there is more than just an environmental influence on the undamaged pseudostem samples.

Davies *et al.* (1993) suggested that the distribution of paxilline-like compounds may relate to hydrophilic components and that the patterns of distribution of individual paxilline-like compounds between plant sections may differ depending on both seasonal fluctuations and on other environmental effects, such as, pest-resistance.

Given that adult (*H. arator*) feed at the base of the plant and the distribution of the fungus and the alkaloids at the base of the plant (Musgrave, 1984; Ball *et al.*, 1995a) ensures plant and fungus have the strongest protection where herbivory will threaten their survival (Popay, 2009); the levels of specific secondary metabolites found in the pseudostem section are presumed to be more relevlant for resistance to feeding from African black beetle (*H. arator*) than herbage in this work. Though, if the level of specific secondary metabolites in the herbage were relative to the level found in the pseudostem section, the herbage section could be used for sampling. Herbage is the most practical plant section for sampling throughout the duration of a trial because no tillers are required to be scarified for sampling, as is required when sampling the pseudostem section. However, it was found pseudostem sampling is required. With the undamaged pseudostem section having the strongest correlation with the overall pseudostem for both peramine and paxilline immunoreactvity it was considered the best plant section for sampling. With higher levels of immunoreactivity found in the undamaged than damaged pseudostem (especially paxilline immunoreactivity) it is important to be measuring the same sample types (plant sections) for plant comparisons as mixed pseudostem samples could skew the results if the entire plant pseudostem is not available for testing.

Comparison of paxilline immunoreactivity concentrations in herbage from the initial pre-screen (Chapter 3), prior to adult African black beetle (*H. arator*) attack and at the end of this trial, after exposure to beetles were not strongly correlated (Appendix I, Figure D). This result may indicate that paxilline immunoreactivity could be influenced by adult black beetle feeding. If the levels of paxilline immunoreactivity were constitutively produced or from a generalised response due

to seasonal and/or insect attack, a strong correlation would be expected as the plants were genetically identical. It is known that the production levels of secondary metabolites including paxilline immunoreactivity, are strongly influenced by the host plant (Fannin *et al.*, 1990; Christensen *et al.*, 1991; Hill *et al.*, 1991; Christensen *et al.*, 1993; Agee & Hill, 1994) and it was anticipated that the relative production level would be consistent within the specific endophyte and plant association. Although production levels of the metabolites are influenced by seasonal variation (di Menna *et al.*, 1992; Woodburn *et al.*, 1993; Ball *et al.*, 1995a; Easton *et al.*, 1996) and Davies *et al.* (1993) suggested distribution of individual paxilline-like metabolites in plant sections may differ if exposed to insect attack, it was anticipated that the relative levels of paxilline immunoreactivity between plants would remain the same over time as reported by Ball *et al*. (1995). Peramine was not measured prior to plant exposure to actively feeding adult African black beetle (*H. arator*).

Although it was anticipated that there would be no other peramine-like compounds detected by the peramine ELISA, the ELISA has not been validated against a reference method therefore results were expressed as immunoreactive equivalents of the reference standard used (peramine). In this current study there was no evidence found that peramine immunoreactivity influences adult African black beetle (*H. arator*) feeding. This was in agreement with data published by Ball *et al.* (1997). The antibody in the paxilline ELISA is generic and each paxilline-like compound (known and unknown) will cross-react with the antibody to varying degrees. The paxilline ELISA uses paxilline as the reference standard and quantitatively measures equivalents of paxilline immunoreactivity, but it is not a quantitative measure of the mix of paxilline-like compounds. The ELISA detects both known and unknown paxilline-like compounds by measuring equivalents of paxilline immunoreactivity determined by the different cross-reactivities of the mix of paxilline-like compounds and the level of each compound. Although no correlations were found between paxilline immunoreactivity and feeding damage by adult African black beetle (*H. arator*), a cluster of data points was observed with an apparent association between high levels of paxilline-IRE (≥25 μg/g) in the overall pseudostem (Figure 4-12 B) and undamaged pseudostem sections and corresponding low feeding damage (Figure 4-12 B and Figure 4-11 F respectively).

Owing to the small sample size the cluster results from this present work can only be treated as indicative. In the paxilline-like mix of compounds, those that are not associated with feeding may be produced at lower levels or stay the same, while those that are associated with feeding may be produced at higher levels. However, because the cross-reactivity of the different compounds will be different, the overall level of paxilline-IRE may stay the same, lower or increase depending on the production profile of alkaloids. Therefore, initially until the paxilline-like compounds associated with feeding are produced at high enough levels, they may be masked by those not associated with feeding and the bioactivity is only detectable by ELISA once the profile of paxilline-like compounds changes sufficiently to allow detection. Therefore, it is possible that a subset of paxilline-like compounds is being masked by others recognised by the ELISA that do not affect adult African black beetle (*H. arator*) feeding, for example, the simple indole diterpene, paxilline itself (Ball *et al.*, 1997), which is produced in low amounts in AR1-infected plants (Young *et al.*, 2009) but has high cross-reactivity (100%).

It has been shown that grasses with endophytes deter or are toxic to many insect herbivores (Popay & Rowan, 1994) and this has not been considered as induced resistance because the endophytes produce compounds that are directly active rather than inducing changes in the host plant (Karban & Baldwin, 1997). There is evidence, however, in the literature that suggests some beneficial effects of endophytes against insect herbivory are induced and that the endophyte mediates the induced response by its host grass (Bultman & Ganey, 1995; Bultman *et al.*, 2004; Sullivan *et al.*, 2007). Bultman & Ganey (1995) first showed that endophyte-infected ryegrass was detrimental to fall armyworm (*Spodoptera frugiperda* (Smith 1797)) larvae and pupae compared with endophyte-free ryegrass. They also showed that endophyte-infected ryegrass that had been damaged (clipped) produced larger deleterious effects on fall armyworm pupal mass and development times compared with endophyte-infected plants that were not artificially clipped. The interaction between prior damage and endophyte infection suggests that induced plant responses accentuate the effects of the endophytes (Karban & Baldwin, 1997). Bultman *et al.* (2004) reported inducible loline production and Sullivan *et al.* (2007) showed that this increase in loline concentration involved the upregulation of genes encoding for loline production following damage in endophyte-infected tall fescue

(*L. arundinaceum*). Bultman *et al.* (2004) and Sullivan *et al.* (2007) also showed that damaged plants containing the fungus were more resistant to insects and had negative effects on these insects. In comparison, endophyte-free plants had increased susceptibility to insects following damage. Foliar N% is postively correlated with photosynthesis and plant growth rate (Reich *et al.*, 1997; Wright *et al.*, 2004) and high foliar N% in new leaves is attractive to herbivores (Sullivan *et al.*, 2007). The Bultman *et al.* (2004) and Sullivan *et al.* (2007) studies suggest that the endophyte does not enhance the plants strategy for dealing with herbivores, instead it changes the plant's strategy competely; from a strategy of compensation (high foliar N%, increased foliar growth) found in endophyte-free plants, to resistance (low foliar N%, increased fungal alkaloid production) by diverting nitrogen to the endophyte for defence. Low foliar N% in new leaves has the added advantage of being less palatable to herbivores.

The response of increased levels of paxilline (and peramine) immunoreactivity appears to be concentrated in the undamaged pseudostem plant section. After attack from adult African black beetle (*H. arator*) the AR1-infected plant potentially responds by increasing fungal metabolite production in general plus increasing the production levels of specific metabolites over and above that of the general increase in fungal metabolite production (the accentuated response). All plant tillers would be affected, but damaged tillers would have fewer resources available for increased metabolite production because of the physical interruption in the vascular tissues for the movement of resources up and down the plant tiller and for both metabolite production and photosynthesis in the leaf blades. This would further limit the resources available in the damaged tiller for fungal metabolite production. The higher levels of peramine and paxilline immunoreactivity found in the undamaged pseudsostem than in the damaged pseudostem futher support this theory of limited resources for metabolite production in damaged tillers.

The weak correlation found between pre and post exposure to beetles for levels of paxilline immunoreactivity in herbage and the potential cluster of plants found with low damage and high paxilline immunoreactivity in undamaged and overall pseudostem, indicates that exposure to adult African black beetle (*H. arator*) attack could influence levels of paxilline immunoreactivity in plants. Potentially, a subset

of paxilline-like secondary metabolites was produced in large enough quantities (≥25 µg/g) in some plants to be detected by the paxilline ELISA, forming the cluster. The other plants that have low adult African black beetle (*H. arator*) feeding damage (≤20%), but not high levels of paxilline immunoreactivity were possibly producing this subset of paxilline-like compounds at levels high enough to reduce feeding but this was not identified by ELISA due to a low cross-reactivity of these compounds and the paxilline-like alkaloid profile of the plants. Or the reduction of feeding may have been associated with other unrelated compounds.

The original plot of mean feeding damage versus mean levels of paxilline immunoreactivity from the Lincoln trial (Figure 1-7; also see Section 1.9.2 and Chatper 7) suggests increasing levels of paxilline immunoreactive equivalents (paxilline-IRE) in the host plant is associated with a reduction in adult African black beetle (*H. arator*) feeding damage (Popay A. J., Fletcher L. R., Briggs L. R., AgResearch, New Zealand; unpublished work, 2006). The Lincoln trial results, however, were not directly comparable with the results from the current work because the samples were not consistent, with varying ratios of damaged and undamaged tillers in the samples. To statistically compare results of these two trials, only undamaged pseudostem samples were compared. With the mixed pseudostem samples removed, the proposed negative association for the Lincoln trial was not as strong (Figure 4-14). Inclusion of the samples containing damaged pseudostem appears to have accentuated the initial proposed association (Figure 1-7). In the present trial, there was very high feeding pressure on the plants (≥1 adult/plant) and the half-sibling families sustained much higher adult African black beetle (*H. arator*) damage than the Lincoln trial. The families appeared to be grouped in bunches and instead of an overall proposed negative association with paxilline-IRE levels and feeding damage by adult African black beetle (*H. arator*), there appeared to be a positive association (Figure 4-14).

When comparing the means between paxilline immunoreactivity and adult African black beetle (*H. arator*) damage of the families (Trial 1) and cultivars (Lincoln Trial), the relationship within these families and cultivars was not being examined. Although the Lincoln trial had more individual family representatives, further investigation found large variation within the cultivars, consistent with the results

of the present study (Figure 4-15). Within certain cultivars and families there was the suggestion of negative, positive and no associations with feeding damage and paxilline-IRE levels in the pseudostem.

The beetles had the choice of 50 genetically different plant types for feeding. Although the use of plant clones reduced the number of plants representing each family, it allowed for multiple testing on individual plants within a family and the plant inter-clonal variation for various traits to be examined. Small plant inter-clonal variation ensures the trait is consistent and can be maintained. The limitations of low clonal replication (3) and analysis of a subset of samples by ELISA (Section 4.3.3) and low family representation were unavoidable concessions that had to be made to ensure the pilot trial work could be completed with the available resources, of which labour was a major limitation, yet still encompass representatives from all 23 half-sibling families. These aspects were addressed in subsequent trial work (Chapters 5, 7 and 8).

Although this trial was only a preliminary study, results from this work suggest that levels of paxilline immunoreactivity may be influenced by adult African black beetle (*H. arator*) feeding. The results indicate the possibility that AR1-infected ryegrass produces a specific subset of paxilline-like secondary metabolites that are associated with reduced adult African black beetle feeding damage. Other metabolites produced by the endophyte and detected by the paxilline ELISA and known to have no effect on African black beetle (*H. arator*), such as paxilline, likely mask the production of this subset of compounds. This could account for a lack of correlation between feeding damage and paxilline immunoreactivity. It is important to consider that this proposed subset of paxilline-like compounds may not be bioactive, instead associated with reduced feeding damage. They could, however, act as marker compounds for plant resistance to feeding by adult African black beetle (*H. arator*). The families contaminated with wild-type endophyte did not stand out from the other families.

The results from the Lincoln trial and this current trial provide incentive to examine more extensively, the relationship between feeding damage and associated pseudostem levels of paxilline immunoreactivity within and between half-sibling family lines. Using the results from the unadjusted cumulative data for the

proportion of damaged tillers, paxilline immunoreactivity and tiller number, plants and families were identified and selected for further investigation into the AR1-African black beetle (*H. arator*) relationship.

4.5 Summary

Fifty AR1-infected ryegrass plants were selected from 23 half-sibling families (GA97 breeding line) and screened for resistance to feeding by adult African black beetle (*H. arator*) and for their levels of paxilline and peramine immunoreactivity. The results from this trial showed differences found between plants for feeding damage and levels of paxilline and peramine immunoreactivity. The level of peramine immunoreactivity appears to be a generalised response to both seasonal conditions and herbivory damage and does not influence adult African black beetle (*H. arator*) feeding.

The results indicate feeding adult African black beetle (*H. arator*) could influence levels of paxilline immunoreactivity in plants and potentially a subset of paxilline-like compounds could be associated with reduced feeding damage. Other paxilline-like compounds could be masking the detection by ELISA of these 'bioactive' paxilline-like compounds until immunoreactivity levels reach a threshold of ≥25 µg/g in the base of the tillers.

Higher levels of both peramine and paxilline immunoreactivity were found at the base of the plant which is consistent with reports from the literature, providing the strongest level of protection at the most vulnerable part of the plant against herbivory. The highest concentrations were found in the undamaged pseudostems, next in the damaged pseudostem and the lowest in the herbage. The undamaged pseudostem plant section is considered the best option for sampling in subsequent feeding trials with adult African black beetle (*H. arator*).

4.6 References

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Chapter 5

Trial 2 – Family and plant selection

The purpose of the second feeding trial with adult African black beetle (Heteronychus arator (Fabricius, 1775)) was to further study, plant resistance to adult African black beetle (H. arator) using a reduced number of AR1-infected plant genotypes from the genetically related half-sibling families. The suggested association between feeding damage and concentration of paxilline immunoreactivity was further investigated using cloned plants by:

- *Examining the expression levels of paxilline-like fungal metabolites for plants exposed and not exposed to adult African black beetle (H. arator).*
- *Assessing plant resistance in plants exposed to adult African black beetle (H. arator) feeding by measuring both the proportion of tillers damaged on a plant and the scale of feeding damage to each tiller.*
- *Comparing feeding damage by adult African black beetle (H. arator) with endophyte production of paxilline-like compounds for any evidence of a relationship or association between the two.*
- *Comparing plant tiller production between plants exposed and not exposed to adult African black beetle (H. arator).*
- *Looking at the consistency of results between Trials 1 and 2 for plant resistance against adult African black beetle (H. arator), paxilline immunoreactivity concentrations and plant tiller production for plants and families.*

With this information plants and families would be identified and classified into different groupings for levels of plant resistance to adult African black beetle (H. arator), paxilline immunoreactivity and tiller production. From these groupings a reduced number of AR1-infected plants would be identified for further study.

5.1 Introduction

The results from the preliminary trial (Chapter 4) using AR1-infected half-sibling perennial ryegrass (*Lolium perenne* L.) families from within a breeding-line (GA97) indicated that differences can be found between plants, within families, results were indicative for differences between families for adult African black beetle (*H. arator*) feeding damage, paxilline (and peramine) immunoreactivity and tiller number. Results suggested that black beetle influence paxilline immunoreactivity levels in plant psuedostem. Although no correlations were found between adult African black beetle (*H. arator*) feeding damage and paxilline (or peramine) immunoreactivity, of particular interest was a small cluster of plants which showed high concentrations of paxilline immunoreactivity in the undamaged and overall pseudostem plant sections and low adult (*H. arator*) feeding damage in the plant. The indicative result suggested the possibility that a subset of paxilline-like compounds paxilline-like compounds were associated with reduced plant feeding damage and detection of these compounds by ELISA was being masked by other paxilline-like compounds (that were not associated with bioactivity, such as, paxilline). Results from Trial 1 (Chapter 42) are consistent with the theory proposed from the Lincoln trial results, that paxilline immunoreactivity is negatively associated with feeding damage from adult African black beetle (*H. arator*).

Using the information gained from the preliminary trial a second large scale feeding-choice trial was planned and conducted using the AR1-infected half-sibling perennial ryegrass (*L. perenne*) families from within the breeding-line GA97, exposed and not exposed to feeding from adult African black beetle (*H. arator*). The trial had two phases, a treatment phase (plants exposed and not exposed to beetles) and a post-treatment phase (no beetle exposure) to examine African black beetle (*H. arator*) and seasonal effects on plants. A selection of plants (20) from half-sibling families (10 of 23) was chosen for further investigation into the AR1-African black beetle (*H. arator*) relationship. In addition, valuable information was gained on how the presence of beetles affects levels of paxilline immunoreactivity and tiller production in plants during and after exposure.

The number of cloned replicate plants used in this second large scale feeding-choice trial was increased from that used in Trial 1. The trial was also used to further reduce the number of original plants (grown from seed) for continued experimental work.

5.2 Materials and methods

This research (Trial 2) was undertaken from November 2009 to January 2010 at AgResearch, Ruakura Research Centre, Hamilton, New Zealand.

5.2.1 Beetle collection

In spring active adult African black beetle (*H. arator*) were collected (Section 2.1.5) from the field in the Waikato region and maintained (Section 2.1.6) in the laboratory until required.

5.2.2 Plants

Plants (20) were selected for Trial 2 (Table 5-1) from 10 out of 23 half-sibling families. The plants chosen ranged from low to high graded plants for tiller number, feeding damage and paxilline immunoreactivty. One family (A12086) contaminated with wild-type endophyte was included for comparison. Two representatives (plants orginally grown from seed) from each family were chosen.

The plants were selected on the following criteria:

- Endophyte status ensuring plants were infected with endophyte (presumed to be AR1 from the specified seed except for the half-sibling family that tested positive for wild-type endophyte contamination).
- Adult African black beetle (*H. arator*) feeding damage results in regard to individual plants and family ensuring representatives from the cluster in Trial 1 were chosen.
- Individual plant paxilline ELISA results; a range of low (0–10 µg/g), medium (11–20 µg/g), high responses (>20 µg/g) from the paxilline ELISA were chosen.
- Plant health. The plants were scored using a scale of 1–5 (5 = very strong, 1 = very weak). The plant health score was a visual assessment that took

Table 5-1: Plants selected for Trial 2.

Family	Plant	Tiller number and feeding damage rank	Paxilline ELISA rank		
			Mean Herb	Mean DP	Mean UdP
A12061	61/6	L	L	L	n/a
	61/9*	H	L	M	H
A12063	63/4*/†/††	L	L	M	H
	63/9	L	L	L	n/a
A12064	64/7*/†/††	H	M	M	M
	64/10*/†/††	H	H	M	H
A12069	69/6	L	L	M	n/a
	69/9*	L	L	L	H
A12073	73/1	M	L	L	n/a
	73/2*	H	L	M	H
A12077	77/5*/†	M	L	L	L
	77/8*/†	M	L	L	M
A12078	78/1	H	L	L	L
	78/7*/†	M	L	L	L
A12083	83/1	L	L	M	n/a
	83/2*/†	H	L	L	M
A12086	**86/2***	H	L	L	M
	86/4*/†/††	H	M	M	H
A12087	87/5*/†	H	L	M	M
	87/10	L	L	M	M

Herb = herbage. DP = damaged pseudostem. UdP = undamaged pseudostem. L= low, M = medium, H = high. n/a = not applicable.
From Trial 1: Tiller number ranked from low to high number of tillers and feeding damaged ranked from high damage to low damage; L = low tiller number and high feeding damage, H = high tiller number and low feeding damage. Paxilline ELISA rank; L ≤10 µg/g, 10< M ≤20 µg/g, H >20 µg/g paxilline immunoreactive equivalents. *elevated paxilline ELISA response in undamaged pseudostem. †<20% feeding damage. ††undamaged pseudostem paxilline ELISA response of >25µg/g and <20% feeding damage (cluster).
Families and plants contaminated with wild-type endophyte are highlighted in bold font.

into consideration plant tiller number (≥10), tiller size and general growth of plant. Plants with a health score of ≥3 were chosen.

- Family means and variation were taken into consideration for feeding damage, paxilline ELISA immunoreactivity responses and tiller number.

5.2.3 Trial design and methodology

Selected plants (20) were cloned as described in Section 2.1.2 into seven plants and six were placed in polystyrene (internal 49.5 cm × 30 cm) trays in September 2009 and the final clone was replanted back into the original plant pot. The cloned plants were initially planted into sand for 10 days to encourage root development and were then re-planted into polystyrene trays containing Daltons GB potting mix for establishment. Once plants had recovered and grown sufficiently they were screened by immunoblot (Section 2.2) to ensure they were still infected with endophyte and presumed to be AR1 (or wild-type if previously identified as being one of the half-sibling families contaminated with wild-type endophyte).

Two months later (November 2009) six plant clones from each selected individual plant were planted into polystyrene trays containing Daltons GB potting mix for the trial (one cloned plant per tray and each tray was considered an experimental unit). The trial consisted of six replicates (trays), and 20 cloned plants per tray. Plant clones were planted using a randomised planting plan for each tray with rows 10 cm apart and plants 5 cm apart.

The cages to enclose the African black beetle (*H. arator*) were the same design as those used for Trial 1 (Section 4.2.3, Figure 4-2). Plant establishment and the trial were conducted in the screenhouse with automated watering.

When the replanted cloned plants had sufficiently recovered and grown, the trial commenced in late spring–early summer (November–December 2009). The trial consisted of two treatments; with adult African black beetle (*H. arator*) (four replicates) and without adult African black beetle (*H. arator*) (two replicates). Both treatments were caged once the adult beetles were added, to ensure plants were exposed to the same environmental conditions. Depending on availability, adult African black beetle (*H. arator*) were added once a week for the next 3 weeks. A minimum of at least one adult beetle per plant was added as shown in Table 5-2 with a total of 41 beetles per tray. The ratio of female to male adult African black beetle (*H. arator*) was approximately 1 : 1.

Table 5-2: Addition of adult African black beetle to Trial 2.

				Total on Tray		
Time	**Female**	**Male**	**Total**	**Female**	**Male**	**All**
Week 0	9	11	20	9	11	20
Week 1	4	5	9	13	16	29
Week 2	6	6	12	19	22	41

From the trial schedule (Table 5-3) assessments were performed at the start of trial (A0), mid-treatment phase (A1), end of treatment phase (A2) and the end of the post-treatment phase (A3; end of trial). The plants were not trimmed and no herbage sample was taken at the start of the trial (A0), but the numbers of tillers per plant were counted prior to addition of beetles.

African black beetle (*H. arator*) feeding damage assessments were performed at A1 and A2, with no feeding damage at the start (A0) and end of the trial (A3). Prior to assessments A1−A3 plants were trimmed to 8 cm and herbage samples (5 cm) collected (Section 3.2.5, Figure 3-2). At the end of each assessment plants were fertilised (Section 2.1.4).

Total tiller number (A0–A3), damaged tillers and undamaged tillers were recorded at each assessment (A1–A3). Damaged tillers were scored on a scale of 1–3 as described for Trial 1 (Section 4.2.3). Herbage (both treatments) and damaged pseudostem samples were collected at A1 and undamaged pseudostems collected at the end of treatment phase (A2). Plant trimming, herbage and pseudostem sampling were the same as that for Trial 1, (Section 4.2.3). Data on tiller damage was only collected from trays with beetles.

After the treatment phase of the trial, cages were removed and the trays were moved outside for adult African black beetle (*H. arator*) to disperse. The trays were then transferred to the screenhouse and plants maintained as described in Section 2.1.4. The post-treatment assessment (A3) was performed in the screenhouse.

Table 5-3: Adult African black beetle feeding Trial 2 schedule.

Time	Description
Week –1	Cloned plants transplanted into trays (experimental units)
Week 0	*Start of Trial (treatment phase):*
(A0)	Tillers counted
	African black beetle added
Week 1	African black beetle added
Week 2	*Mid-treatment phase:*
(A1)	Plants trimmed and fertilised
	African black beetle damage assessed
	Tillers counted
	Herbage and damaged pseudostem collected
	African black beetle added
Week 4	*End of treatment phase:*
(A2)	Plants trimmed and fertilised
	Herbage collected
	African black beetle damage assessed
	Tillers counted
	Herbage, damaged and undamaged pseudostem collected
	Cages removed to allow adult African black beetle to disperse
Week 8	*End of post-treatment phase:*
(A3)	Plants trimmed and fertilised
	Tillers counted
	Herbage collected

A0, A1, A2, A3 = Assessments 0 (start of trial and treatment phase), 1 (mid-treatment phase), 2 (end of treatment phase), 3 (End of post-treatment phase and trial).

The grass samples (herbage and, damaged and undamaged pseudostem) were prepared for analysis as described in Section 2.1.3. Grass samples were analysed by paxilline ELISA (Section 2.4.3).

5.2.4 Statistical analyses

All statistical analyses were conducted in GenStat version 15. The following variables were analysed: adult African black beetle (*H. arator*) feeding damage (number of damaged and undamaged tillers), levels of paxilline-IRE, damage score and tiller number. Repeated measures analyses were not used because different experiment protocols were imposed prior to each assessment, such as addition or removal of beetles, removal of cages and movement of experimental units during trial. The data were analysed using either REML or GLMM, as appropriate with random terms accounting for temporal replicate effects. Two parametrisations of the fixed model were considered:

- M1:*Plant+Treatment+Plant.Treatment+Location*

The term '*Plant'* allowed differences between plants. For tiller number and paxilline immunoreactivity data the term '*Treatment'* allowed for an effect of adult African black beetle (*H. arator*) presence, and '*Plant.Treatment'* allowed the treatment effect to differ between plants. *Location* allowed for an overall effect of inner and outer location in the tray. The full model M1 was initially fitted; *Plant + Treatment + Plant.Treatment + Location*. If '*Plant.Treatment'* was not statistically significant it was dropped and the additive model was used; *Plant + Treatment + Location*.

- M2: Family + Treatment + Family.Treatment + Family.Plant + *Family.Plant.Treatment + Location*

The term '*Family'* allowed differences between families. '*Family.Plant'* allowed differences between plants within a family. For tiller number and paxilline immunoreactivity the term ''*Family.Treatment'* allowed for family specific treatment effects. The term '*Family.Plant.Treatment'* allowed specific plant treatment effects within a family. The effects of the terms 'Location' and 'Treatment' are as described above for M1. The full model M2 was used initially; *Family + Family.Plant + Family.Treatment + Family.Plant.Treatment + Location*. If '*Family.Plant.Treatment'* was not significant it was dropped from the model.

Post hoc tests were conducted using Fisher's least significant difference at the 5% significance level (LSD(5%)). The wild-type endophyte contaminated family line

was not removed from the data because family (or plant) was a factor accounted for in the statistical analyses. Adjusting for the covariate initial tiller number, a proxy for plant size, produced analogous results.

Total tiller number was analysed using a linear mixed model fitted by REML. To stabilise the variance the total tiller number was square root transformed. The number of damaged tillers, assumed to be binomially distributed, was analysed using a GLMM with a logit link function. For feeding damage and tiller number, the data from each assessment were analysed separately and cumulatively.

Damage score data were analysed using GLMM analogous to the adult African black beetle (*H. arator*) feeding damage data. Two variables were examined: a) proportion of **total tillers** with a damage score of three and b) proportion of **damaged tillers** with a damage score of three. Owing to convergence issues with GLMM, the proportion of damaged tillers with a score of three could only be analysed by logistic regression. Only data mid-treatment (A1) were analysed because at the end of the treatment phase (A2), all but five of the damaged tillers were given a score of three.

Level of paxilline immunoreactivity in herbage and pseudostem grass samples were analysed using one-way analysis of variance (ANOVA). Data from the three assessments were independently analysed. All paxilline immunoreactivity data were log transformed prior to analysis. Analyses of herbage samples by the paxilline ELISA was performed on samples from both the end of treatment phase (A2) and the end of the post-treatment phase (A3), whereas damaged and undamaged pseudostem samples were only measured at the end of the treatment phase (A2). No statistical analyses were performed on the undamaged pseudostem paxilline immunoreactivity data as it was extremely sparse. Damage from adult African black beetle (*H. arator*) feeding was correlated with paxilline immunoreactivity.

5.3 Results

5.3.1 Feeding damage

There was no evidence of a relationship between the proportion of tillers damaged and initial tiller number, so the covariate adjustment for initial tiller number had very little impact on the estimated proportions. For all analyses, there was significant evidence (P <0.05) that the mean proportion of damaged tillers differed between plants at the end of the treatment phase (A2; Table 5–4) but not at the mid-treatment phase (A1). With no evidence of plant differences prior to the end of the treatment phase, the data from the cumulative proportion of damaged tillers (Figure 5-1) was used to identify which plants and families sustained high and low feeding damage, and for comparison with the paxilline ELISA results. Large variation in feeding damage was found within families (Figure 5-1 A), and for some families (family 78) between the two family representatives (means of cloned plants; Figure 5-1 B) with the interclonal variation (between cloned plants) larger for some plants (86/4) than others (78/1); also see Table 5–4 for plant clone means.

5.3.2 Paxilline ELISA result

Paxilline immunoreactivity was measured at the end of the treatment phase (A2) and at the end of the post-treatment phase (A3) for herbage, and at the end of the treatment phase (A2) for pseudsotem. For mean herbage paxilline immunoreactivity there was significant evidence (P <0.05) of differences between plants at end of the treatment phase (A2) and end of the post-treatment phase (A3) (Table 5-5). For plants within families the evidence of plant differences was found only at the end of the treatment phase. There was weak evidence ($0.05< P \leq 0.1$) of an African black beetle (*H. arator*) feeding effect with paxilline immunoreactivity levels higher in the plants that had not been exposed to beetles (Table 5-6). Evidence of this effect was stronger at the end of the treatment phase (A2) and close to being statistically significant ($F_{(1,4)} = 7.34$; $P = 0.053$). For mean damaged pseudostem paxilline immunoreactivity at the end of the treatment phase (A2) there were significant differences (P <0.05) found between plants and between plants within families at end of the treatment phase (Table 5–7).

Table 5-4: Mean plant proportion of damaged tillers (logit transformed) from feeding by adult African black beetle.

The data was analysed unadjusted for initial tiller number (plant size) at start of trial. The unadjusted data were also analysed cumulatively (cumulative damaged tiller number = the number of damaged tillers at Assessment 2 plus the number of damaged tillers from Assessment 1 which had been removed).

		Mean plant logit(proportion of damaged tillers)		
Family	**Plant**	**A1**	**A2**	**Cum A0–A2**
A12061	61/6	-1.69 (0.16)	1.28 (0.78)	1.50 (0.82)
	61/9	-1.06 (0.26)	-0.30 (0.43)	0.08 (0.52)
A12063	63/4	-0.72 (0.33)	-0.52 (0.37)	0.45 (0.61)
	63/9	-0.71 (0.33)	0.36 (0.59)	0.92 (0.72)
A12064	64/7	-1.69 (0.16)	0.27 (0.57)	0.56 (0.64)
	64/10	-1.09 (0.25)	0.52 (0.63)	0.85 (0.70)
A12069	69/6	-0.81 (0.31)	0.74 (0.68)	1.25 (0.78)
	69/9	-0.55 (0.37)	0.27 (0.57)	0.95 (0.72)
A12073	73/1	-0.32 (0.42)	2.02 (0.88)	2.78 (0.94)
	73/2	-1.26 (0.22)	-0.21 (0.45)	0.14 (0.54)
A12077	77/5	-1.07 (0.25)	0.22 (0.56)	0.62 (0.65)
	77/8	-0.93 (0.28)	1.15 (0.76)	1.56 (0.83)
A12078	78/1	-0.28 (0.43)	0.98 (0.73)	1.61 (0.83)
	78/7	-2.80 (0.06)	-1.08 (0.25)	-0.93 (0.28)
A12083	83/1	-1.01 (0.27)	-0.09 (0.48)	0.48 (0.62)
	83/2	-0.94 (0.28)	0.62 (0.65)	1.14 (0.76)
A12086	**86/2**	-0.98 (0.27)	0.25 (0.56)	0.69 (0.67)
	86/4	-1.59 (0.17)	0.64 (0.66)	0.89 (0.71)
A12087	87/5	-1.28 (0.22)	0.31 (0.58)	0.65 (0.66)
	87/10	-0.30 (0.43)	1.36 (0.80)	2.04 (0.89)
Fisher's LSD(5%)		2.012	2.348	2.368
Plant Effect (Individual Plants^{P})				
F-statistic$_{df}$		$1.17_{19,51}$	$2.22_{19,56}$	$2.76_{19,56}$
P-value		0.315	***0.011***	***0.002***
*Plant Effect (*Within Family^{FP}*)*				
Wald-statistic$_{df}$		15.79_{10}	33.98_{10}	43.05_{10}
P-value		0.106	***<0.001***	***<0.001***

A0, A1 and A2 = Assessments 0 (start of trial and treatment phase), 1 (mid-treatment phase), 2 (end of treatment phase). Cum = Cumulative. $_{df}$ = Degrees of freedom. LSD(5%) = least significance difference at the 5% significance level. M1 = fixed effects model 1; ^{FP} = *Family.Plant*, ^{P} = *Plant*. Back transformed values are in brackets. Family lines and plants contaminated with wild-type endophyte are highlighted in bold font. Statistically significant results are highlighted in bold and italic font. Weak evidence of effects (0.05< *P* ≤1.0) are highlighted in italic font.

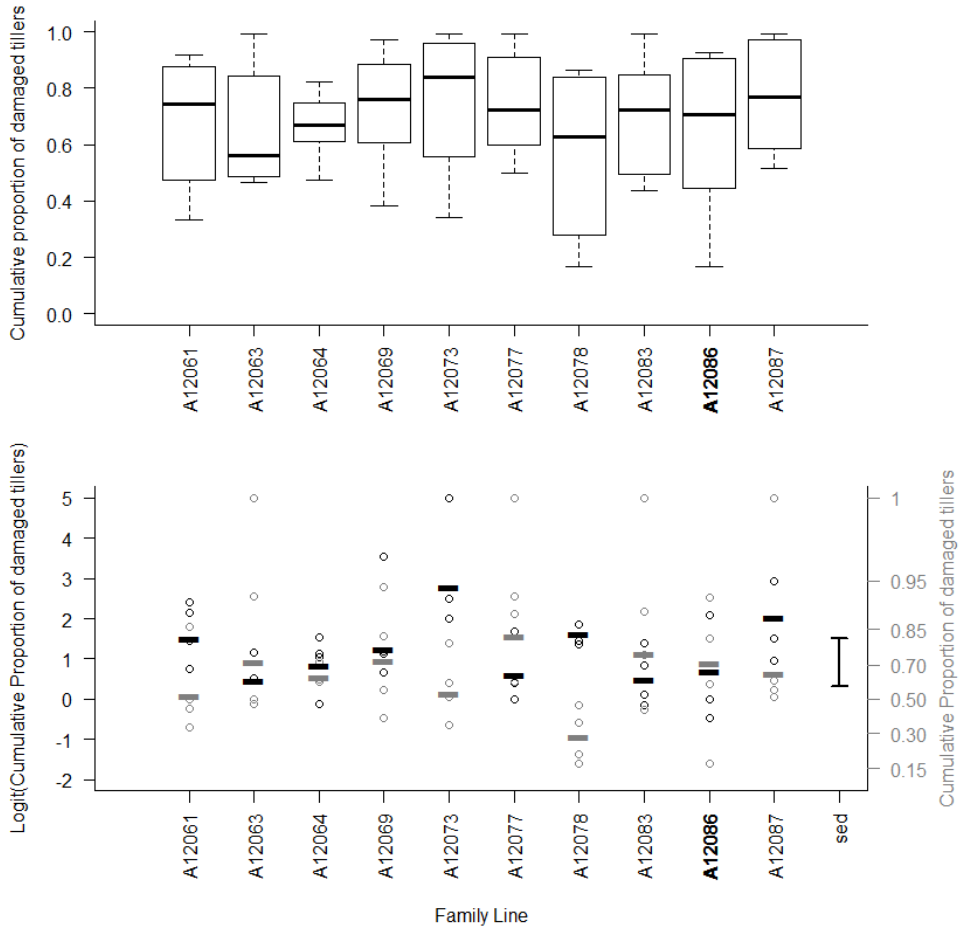

Figure 5-1: Cumulative proportion of damaged tillers at the end of the treatment phase (A2).

A) Boxplot; displaying family median, upper and lower quartiles and showing variation within and between families.

B) Dotplot; displaying individual plants within a family and the corresponding mean, showing the variation within a family and between cloned plants from a single individual seedling plant. The dotplot is coloured by individual plants within a family, with each individual circle of the same colouring representing a cloned plant with a matching coloured bar denoting the mean for the individual seedling plant. sed = standard error of the difference (fixed effects model, M1).

Wild-type contaminated family lines are highlighted in bold font.

Table 5-5: Mean plant paxilline-IRE concentrations (natural log transformed) in herbage of the clones from individual plants grown from seed (from 10 of the 23 half-sibling familes).

		Mean paxilline-IRE (µg/g) in Herbage (natural log (ln), M1)					
		End of trial (A2)			Post-trial (A3)		
			Plant by treatment			Plant by treatment	
Family	Plant	By plant	w BB	w/o BB	By plant	W BB	w/o BB
A12061	61/6	0.79 (2.19)	0.61 (1.85)	1.02 (2.78)	1.36 (3.88)	0.97 (2.63)	1.81 (6.10)
	61/9	0.77 (2.16)	0.58 (1.78)	1.12 (3.07)	1.20 (3.32)	0.82 (2.28)	1.63 (5.12)
A12063	63/4	0.33 (1.40)	0.19 (1.21)	0.56 (1.75)	1.23 (3.41)	1.15 (3.14)	1.30 (3.66)
	63/9	0.00 (1.00)	-0.14 (0.87)	0.16 (1.17)	0.71 (2.04)	0.61 (1.83)	0.73 (2.08)
A12064	64/7	1.31 (3.71)	1.22 (3.38)	1.50 (4.47)	1.59 (4.89)	1.39 (4.00)	1.85 (6.39)
	64/10	1.65 (5.19)	1.48 (4.39)	1.82 (6.15)	1.71 (5.54)	1.32 (3.74)	2.19 (8.91)
A12069	69/6	0.94 (2.55)	0.84 (2.32)	0.99 (2.69)	1.39 (4.00)	1.12 (3.06)	1.63 (5.08)
	69/9	0.92 (2.52)	0.86 (2.35)	0.95 (2.59)	1.15 (3.17)	0.91 (2.47)	1.42 (4.15)
A12073	73/1						
	73/2	0.67 (1.95)	0.55 (1.74)	0.77 (2.16)	0.75 (2.12)	0.47 (1.60)	
A12077	77/5	0.01 (1.01)	-0.16 (0.85)	0.04 (1.04)	1.21 (3.35)	1.04 (2.83)	1.24 (3.45)
	77/8	0.38 (1.46)	0.43 (1.53)	0.32 (1.38)	0.47 (1.60)		0.73 (2.08)
A12078	78/1	0.38 (1.46)	0.11 (1.12)	0.67 (1.95)	0.69 (1.99)	0.41 (1.51)	0.93 (2.54)
	78/7	-0.03 (0.97)	0.03 (1.03)	-0.22 (0.80)	0.52 (1.69)	0.43 (1.53)	0.52 (1.69)
A12083	83/1	1.08 (2.93)	0.88 (2.41)	1.24 (3.46)	1.06 (2.90)	0.67 (1.95)	1.39 (4.02)
	83/2	1.04 (2.82)	0.88 (2.40)	1.26 (3.53)	1.46 (4.32)	1.19 (3.27)	1.67 (5.32)

Table 5–5 continued on next page

Table 5–5 continued

		Mean paxilline-IRE (µg/g) in herbage (natural log (ln), M1)					
		End of trial (A2)			Post-trial (A3)		
			Plant by treatment			Plant by treatment	
Family	**Plant**	**By plant**	**w BB**	**w/o BB**	**By plant**	**w BB**	**w/o BB**
A12086	**86/2**	0.98 (2.65)	0.80 (2.21)	1.29 (3.64)	0.89 (2.44)	0.28 (1.32)	1.53 (4.60)
	86/4	1.48 (4.38)	1.40 (4.06)	1.60 (4.95)			
A12087	87/5	1.65 (5.19)	1.55 (4.71)	1.72 (5.57)	1.76 (5.82)	1.68 (5.38)	1.68 (5.36)
	87/10	0.78 (2.17)	0.60 (1.82)	1.01 (2.73)	1.32 (3.76)	1.15 (3.16)	1.60 (4.96)
†Fisher's LSD(5%)		†0.379			†0.880		
‡Fisher's LSD(5%) within treatment			‡0.790			‡1.401	
‡Fisher's LSD(5%) within plant			‡0.716			‡1.416	
Plant Effect (†Individual Plants^{P}) or (‡Individual Plants^{PTrt})							
F-statistic$_{df}$		†$17.73_{18,73}$	‡$0.59_{18,53}$		†$3.62_{17,45}$	‡$0.58_{15,27}$	
P-value		***†<0.001***	‡0.891		†<0.001	‡0.862	
Plant Effect (Within Family^{FP})							
F-statistic$_{df}$		$5.15_{9,65}$			$1.05_{8,36}$		
P-value		***<0.001***			0.420		

A2 and A3 = Assessments 2 (end of treatment phase) and 3 (end of post-treatment phase and trial). Cum = cumulative. $_{df}$ = Degrees of freedom. LSD(5%) = Least significance difference at the 5% significance level. M1 = fixed effects model 1; ^{FP} = *Family.Plant*, ^{P} = *Plant*, ^{PTrt} = *Plant.Treatment*. Paxilline-IRE = Paxilline immunoreactive equivalents. w BB = with African black beetle (*H. arator*) treatement. w/o = without African black beetle (*H. arator*) treatment. Back transformed values are in brackets. Family lines and plants contaminated with wild-type endophyte are highlighted in bold font. Statistically significant results ($P \leq 0.05$) are highlighted in bold and italic font. Weak evidence of effects ($0.05 < P \leq 1.0$) are highlighted in italic font.

Table 5-6: Concentrations of paxilline immunoreactivity in herbage between the two treatments; with and without adult African black beetle.

	Mean paxilline-IRE (µg/g)in herbage (natural log (ln), M1)	
Treatment	**A2**	**A3**
w BB	0.66 (1.94)	0.87 (2.39)
w/o BB	0.93 (2.53)	1.41 (4.08)
Treatment Effect (Individual Plants^{Trt})		
F-statistic$_{df}$	$7.34_{1,4}$	$5.99_{1,4}$
P-value	*0.053*	*0.075*
Fisher's LSD(5%)	2.749	0.605

A2 and A3 = Assessments 2 (end of treatment phase) and 3 (end of post-treatment phase and trial). Cum = cumulative. *df* = degrees of freedom. LSD(5%) = least significance difference at the 5% significance level. M1 = fixed effects model 1; *Trt* = *Treatment*. Paxilline-IRE = Paxilline immunoreactive equivalents. w BB = with African black beetle (*H.* arator) treatment. w/o = without African black beetle (*H.* arator) treatment.
Back transformed values are in brackets. Statistically significant results ($P \leq 0.05$) are highlighted in bold and italic font. Weak evidence of effects ($0.05 < P \leq 1.0$) are highlighted in italic font.

There was no evidence ($P > 0.05$) of a relationship between the proportions of damaged tillers and the level of paxilline immunoreactivity in herbage or damaged pseudostem.

In general, the mean concentrations of paxilline immunoreactivity was higher in the undamaged pseudostem than in the damaged pseudostem (Figure 5-2) and the herbage plant sections; also see Table 5-5 and Table 5-7. Overall, paxilline immunoreactivity concentrations in pseudostem from plants not exposed to beetle feeding were higher than those found for exposed plants in both the weighted pseudostem and undamaged pseudostem (Figure 5-2) which is consistent with the results found for herbage (Table 5-6). Significant differences were found between control plants (without exposure to beetles) for paxilline immunoreactivity concentrations in pseudostem ($F_{(12,11)} = 8.57$; $P < 0.001$).

Table 5-7: Mean plant paxilline immunoreactivity in the adult African black beetle treatment group for damaged and undamaged pseudostem at end of the treatment phase (A2).

		Mean paxilline-IRE (µg/g) (natural log (ln), M1)	
Family	**Plant**	**Damaged**	**Undamaged**
A12061	61/6	1.60 (4.95)	1.67 (5.31)
	61/9	1.75 (5.74)	1.83 (6.45)
A12063	63/4	1.35 (3.86)	1.56 (4.88)
	63/9	1.21 (3.35)	
A12064	64/7	1.21 (3.35)	
	64/10	2.19 (8.97)	2.79 (16.86)
A12069	69/6	1.79 (6.00)	
	69/9	1.64 (5.16)	
A12073	73/1		
	73/2	1.95 (7.01)	2.39 (10.90)
A12077	77/5	1.08 (2.93)	1.77 (5.89)
	77/8	1.00 (2.72)	2.30 (10.00)
A12078	78/1	1.48 (4.38)	
	78/7	1.00 (2.73)	1.52 (4.72)
A12083	83/1	1.68 (5.35)	
	83/2	2.31 (10.11)	
A12086	**86/2**	1.26 (3.51)	1.74 (5.69)
	86/4	1.94 (6.94)	2.39 (10.92)
A12087	87/5	2.04 (7.71)	2.62 (13.74)
	87/10	1.90 (6.68)	
Fisher's LSD(5%)		0.564	
Plant Effect (Individual Plants^{P})			
F-statistic$_{df}$		$5.53_{18,29}$	
P-value		***<0.001***	
Plant Effect (Within Family^{FP})			
Wald-statistic$_{df}$		26.71_{9}	
P-value		***0.002***	

A0, A1, A2 and A3 = Assessments 0 (start of trial and treatment phase), 1 (mid-treatment phase), 2 (end of treatment phase), 3 (end of post-treatment phase and trial). *df* = Degrees of freedom. LSD = Least significance difference at the 5% significance level. M1 = Fixed effect model 1; ^{FP} = *Family.Plant,* ^{P} = *Plant*. Paxilline-IRE = Paxilline immunoreactive equivalents.

Back transformed values are in brackets. Family lines and plants contaminated with wild-type endophyte are highlighted in bold font. Statistically significant results are highlighted in bold and italic font. Weak evidence of effects ($0.05< P \leq 1.0$) are highlighted in italic font.

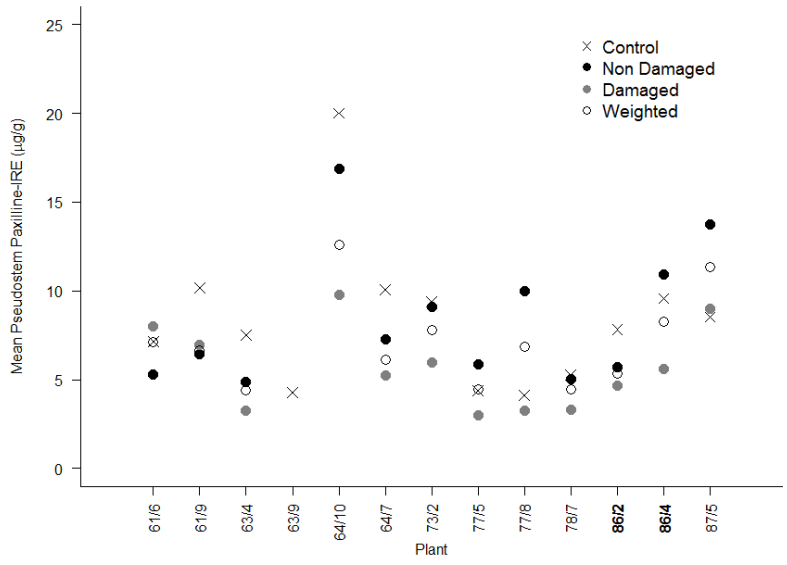

Figure 5-2: Mean levels of paxilline immunoreactvity from plants exposed (damaged, undamaged and weighted overall pseudostem) and not exposed (control means) to adult African black beetle feeding.
Weighted overall pseudostem levels of paxilline immunoreactivity were calculated by summing the damaged and undamaged pseudostem immunoreactivity levels weighted by the proportion of tillers damaged. Paxilline-IRE = Paxilline immunoreactive equivalents. Plants contaminated with wild-type endophyte are highlighted in bold font.

5.3.3 Damage score

The analyses of plant tillers for the severity of damage from feeding by adult African black beetle (*H. arator*) (1 = minor damage, 2 = moderate damage and 3 = severe damage causing tiller death, with undamaged tillers = 0) was examined from two perspectives; the proportion of all tillers with a damage score of three and the proportion of damaged tillers with a score of three. Statistical results for the proportion of tillers with a damage score of three are summarised in Table 5-8. When data were unadjusted for initial tiller number, the proportion of tillers with a damage score of three differed between plants (Table 5-8) and within families at end of trial ($P \leq 0.5$). These results mirrored the results of the total proportion of damaged tillers at A2 because the majority of tillers were severely damaged at this stage. For the same reason, only mid-treatment (A1) data were analysed when examining the proportion of damaged tillers with a score of three and there was no evidence of differences between plants or within families at this time.

Table 5-8: Mean proportion of all tillers and damaged tillers with a damage score of 3 for plant clones.

		Mean proportion of tillers and damaged tillers with a damage score of three (logit Scale, M1)		
		All tillers		Damaged tillers
Family	**Plant**	**A1**	**A2**	**A1**
A12061	61/6	-1.94 (0.13)	1.28 (0.78)	0.79 (0.69)
	61/9	-1.45 (0.19)	-0.30 (0.43)	0.73 (0.68)
A12063	63/4	-0.90 (0.29)	-0.52 (0.37)	0.89 (0.71)
	63/9	-0.84 (0.30)	0.36 (0.59)	0.92 (0.71)
A12064	64/7	-1.66 (0.16)	0.28 (0.57)	0.97 (0.73)
	64/10	-1.51 (0.18)	0.53 (0.63)	0.73 (0.67)
A12069	69/6	-0.93 (0.28)	0.74 (0.68)	0.92 (0.72)
	69/9	-0.86 (0.30)	0.26 (0.56)	0.80 (0.69)
A12073	73/1	-0.39 (0.40)	2.03 (0.88)	0.96 (0.72)
	73/2	-1.65 (0.16)	-0.21 (0.45)	0.72 (0.67)
A12077	77/5	-1.26 (0.22)	0.22 (0.55)	0.87 (0.70)
	77/8	-1.05 (0.26)	1.16 (0.76)	0.92 (0.71)
A12078	78/1	-0.29 (0.43)	0.99 (0.73)	0.96 (0.72)
	78/7	-3.16 (0.04)	-1.07 (0.26)	0.69 (0.67)
A12083	83/1	-1.30 (0.21)	-0.10 (0.48)	0.75 (0.68)
	83/2	-1.42 (0.19)	0.64 (0.65)	0.69 (0.66)
A12086	**86/2**	-1.15 (0.24)	0.18 (0.55)	0.84 (0.70)
	86/4	-1.63 (0.16)	0.35 (0.59)	1.00 (0.73)
A12087	87/5	-1.73 (0.15)	0.33 (0.58)	0.68 (0.66)
	87/10	-0.44 (0.39)	1.36 (0.80)	0.92 (0.72)
Fisher's LSD(5%)		2.173	2.362	0.636
Plant Effect (Individual Plant^{P})				
F-statistic$_{df}$ or †*Wald*-statistic$_{df}$		$1.46_{19,50}$	$2.17_{19,56}$	$^{\dagger}18.96_{19}$
P-value		0.144	***0.013***	0.478
Plant Effect (Within Family^{FP})				
F-statistic$_{df}$ or †*Wald*-statistic$_{df}$		$^{\dagger}17.23_{10}$	$^{\dagger}33.44_{10}$	$^{\dagger}11.23_{10}$
P-value		*0.070*	***<0.001***	0.363

A1 and A2 = Assessments 1 (mid-trial), 2 (end of trial). $_{df}$ = Degrees of freedom. LSD(5%) = Least significance difference at the 5% significance level. M1 = Fixed effect model 1; ^{FP} = *Family.Plant,* ^{P} = *Plant*.

Back transformed values are in brackets. Family lines and plants contaminated with wild-type endophyte are highlighted in bold font. Statistically significant results are highlighted in bold and italic font. Weak evidence of effects (0.05< *P* ≤1.0) are highlighted in italic font.

5.3.4 Tiller number

Strong evidence (P <0.001) was found for mean plant tiller number differences between plants at all assessment dates (Table 5-9). As shown in Table 5-10, there was weak evidence ($F_{(1,4)}$ = 5.80; P = 0.074) of an adult African black beetle (*H. arator*) feeding effect with mean plant tiller number greater at the mid-treatment phase for plants exposed to adult African black beetle (*H. arator*) feeding. At the end of the treatment phase (A2) there was no evidence of mean plant tiller differences (P >0.1). However, four weeks later at the end of the post-treatment phase and trial (A3) the beetle feeding effect had reversed with plants not exposed to beetles having the greater mean plant tiller number ($F_{(1,4)}$ = 4.86; P = 0.092). For the total number of tillers produced throughout the entire trial (cumulative tiller number) plants exposed to adult African black beetle (*H. arator*) on average produced more tillers than plants not exposed (M1 A0–A2 $F_{(1,4)}$ = 6.78; P = 0.060 and M1 A0–A3 $F_{(1,4)}$ = 10.02; P = 0.034).

Plant differences were evident within a family for plant tiller number unadjusted for initial tiller number at assessments and cumulatively ($P \leq 0.5$; Table 5-9). Positive relationships were found between initial plant tiller number and plant tiller numbers at the other assessments (A1, A2, A3); A1 ($F_{(1,87)}$ = 110.02; P < 0.001), A2 ($F_{(1,83)}$ = 26.52; P < 0.001) and A3 ($F_{(1,97)}$ = 3.91; P = 0.051).

The top performing families with high tiller numbers were 64, 78, and 77 (Table 5-9). Results for tiller number were similar with those from Trial 1 with the top performing plants and families in this current trial (Trial 2) coming from the medium and high grouped families for median plant tiller number, determined in Trial 1 (Table 4-5).

5.4 Discussion

As for Trial 1, this second feeding choice trial was performed in late spring–early summer when adult African black beetle (*H. arator*) were emerging to feed and reproduce (Todd, 1959; Bell *et al.*, 2011) and when it was anticipated that there would be high alkaloid levels in the endophyte-infected ryegrass plants. Samples were not analysed by peramine ELISA owing to the results obtained in Trial 1. The

Table 5-9: Mean plant tiller number (square root transformed) for plant clones.

		Mean square root transformed tiller number (M1)					
Family	**Plant**	**A0**	**A1**	**A2**	**A3**	**Cum A0–A2**	**Cum A0–A3**
A12061	61/6	4.30 (18.46)	4.40 (19.39)	4.37 (19.13)	2.97 (8.79)	4.53 (20.48)	4.33 (18.71)
	61/9	3.58 (3.58)	3.78 (14.27)	4.09 (16.74	5.25 (27.52)	4.27 (18.20)	5.31 (28.20)
A12063	63/4	3.94 (15.50)	4.02 (16.19)	3.68 (13.56)	3.63 (13.17)	4.15 (17.26)	4.22 (17.83)
	63/9	4.83 (23.34)	4.76 (22.61)	4.34 (18.82)	2.93 (8.58)	4.87 (23.67)	4.24 (17.99)
A12064	64/7	6.40 (40.93)	6.97 (48.58)	6.88 (47.38)	5.44 (29.60)	7.19 (51.67)	7.16 (51.22)
	64/10	6.41 (41.13)	6.92 (47.86)	7.20 (51.90)	7.38 (54.41)	7.70 (59.35)	8.80 (77.35)
A12069	69/6	5.71 (32.64)	5.64 (31.83)	5.63 (31.65)	5.34 (28.52)	6.21 (38.60)	7.10 (50.38)
	69/9	3.64 (13.27)	3.83 (14.68)	3.60 (12.93)	3.92 (15.40)	4.11 (16.91)	4.82 (23.20)
A12073	73/1	3.82 (14.57)	3.89 (15.14)	3.32 (11.00)	1.28 (1.64)	3.87 (14.98)	3.16 (9.99)
	73/2	4.37 (19.10)	4.42 (19.54)	4.67 (21.78)	2.96 (8.76)	4.86 (23.64)	3.55 (12.61)
A12077	77/5	4.45 (19.80)	4.79 (22.92)	4.75 (22.55)	3.18 (10.11)	5.09 (25.86)	4.53 (20.52)
	77/8	4.15 (17.26)	4.72 (22.23)	4.56 (20.82)	1.54 (2.36)	5.02 (25.22)	4.17 (17.42)
A12078	78/1	4.82 (23.22)	5.31 (28.21)	5.15 (26.52)	6.02 (36.24)	5.88 (34.62)	7.28 (52.98)
	78/7	4.22 (17.84)	4.43 (19.61)	5.17 (26.71)	5.15 (26.53)	5.14 (26.41)	5.17 (26.71)
A12083	83/1	4.16 (17.31)	4.34 (18.79)	4.26 (18.18)	2.28 (5.18)	4.58 (20.97)	3.93 (15.46)
	83/2	4.01 (16.09)	3.87 (14.98)	3.37 (11.36)	2.79 (7.78)	3.83 (14.65)	4.04 (16.31)
A12086	**86/2**	4.41 (19.46)	4.47 (19.98)	4.48 (20.03)	3.25 (10.57)	4.81 (23.11)	4.42 (19.55)
	86/4	3.89 (15.12)	3.67 (13.50)	3.75 (14.09)	0.94 (0.88)	3.81 (14.49)	2.47 (6.11)

Table 5–9 continued on next page

Table 5–9 continued

		Mean square root transformed tiller number (M1)					
Family	**Plant**	**A0**	**A1**	**A2**	**A3**	**Cum A0–A2**	**Cum A0–A3**
A12087	87/5	5.22 (27.25)	5.30 (28.09)	4.95 (24.52)	2.01 (4.05)	5.30 (28.07)	3.97 (15.78)
	87/10	3.81 (15.53)	3.80 (14.43)	3.41 (11.66)	2.13 (4.54)	3.96 (15.65)	3.84 (14.71)
Fisher's LSD(5%)		0.884	0.771	1.095	2.510	0.962	1.906
Plant Effect (Individual Plants^{P})							
F-statistic$_{df}$		$4.47_{19,88}$	$12.67_{19,89}$	$7.82_{19,98}$	$3.99_{19,87}$	$10.44_{19,88}$	$5.71_{19,88}$
P-value		***<0.001***	***<0.001***	***<0.001***	***<0.001***	***<0.001***	***<0.001***
Plant Effect (Within family^{FP})							
F-statistic$_{df}$		$4.02_{10,85}$	$5.81_{10,82}$	$3.50_{10,89}$	$1.61_{10,81}$	$4.56_{10,82}$	$2.04_{10,82}$
P-value		***<0.001***	***<0.001***	***<0.001***	0.118	***<0.001***	***0.039***

A0, A1, A2 and A3 = Assessments 0 (start of treatment phase and trial), 1 (mid-treatment phase), 2 (end of treatment phase) and 3 (end of post-treatment phase and end of trial). Cum = Cumulative. $_{df}$ = Degrees of freedom. LSD(5%) = Least significance difference at the 5% significance level. M1 = Fixed effect model 1; ^{FP} = *Family.Plant,* ^{P} = *Plant*.

Back transformed values are in brackets. Family lines and plants contaminated with wild-type endophyte are highlighted in bold font. Statistically significant results are highlighted in bold and italic font. Weak evidence of effects (0.05< *P* ≤1.0) are highlighted in italic font.

Table 5-10: Mean plant tiller number (square root transformed) by treatment group, with and without African black beetle for plant clones.

	Mean square root transformed tiller number (M1)					
Treatment group	**A0**	**A1**	**A2**	**A3**	**Cum A0–A2**	**Cum A0–A3**
w BB	4.82 (23.23)	5.02 (25.15)	4.48 (20.11)	2.76 (7.61)	5.23 (27.35)	5.48 (30.06)
w/o BB	4.20 (17.60)	4.32 (16.65)	4.68 (21.90)	4.28 (18.30)	4.69 (21.97)	4.17 (17.36)
Fisher's LSD(5%)	0.821	0.804	0.357	1.913	0.579	1.154
Treatment Effect (Individual Plant^{Trt})						
F-statistic$_{df}$	$4.47_{1,4}$	$5.80_{1,4}$	$1.18_{1,98}$	$4.86_{1,4}$	$6.78_{1,4}$	$10.02_{1,4}$
P-value	0.102	*0.074*	0.280	*0.092*	*0.060*	***0.034***
Plant by Treatment Effect (Individual Plant^{PTrt})						
F-statistic$_{df}$	$0.59_{19,75}$	$1.07_{19,67}$	$1.10_{19,79}$	$1.36_{19,66}$	$1.06_{19,66}$	$1.42_{19,61}$
P-value	0.899	0.402	0.371	0.177	0.406	0.154

A0, A1, A2 and A3 = Assessments 0 (start of treatment phase and trial), 1 (mid-treatment phase), 2 (end of treatment phase) and 3 (end of post-treatment phase and end of trial). Cum = Cumulative. $_{df}$ = Degrees of freedom. LSD(5%) = Least significance difference at the 5% significance level. M1 = Fixed effect model 1, ^{PTrt} = *Plant.Treatment*, ^{Trt} = *Treatment*. w BB = with African black beetle (*Heteronychus arator*) treatment. w/o = without African black beetle (*H. arator*) treatment. Back transformed values are in brackets. Statistically significant results are highlighted in bold and italic font. Weak evidence of effects (0.05< *P* ≤1.0) are highlighted in italic font.

paxilline ELISA (Sections 2.4.3 and 3.1.1) was used in this section of work as a subset of unknown paxilline-like compounds were suggested to be associated with reduced adult African black beetle (*H. arator*) feeding, although currently, cannot be detected separately from the other paxilline-like compounds (Trial 1, Chapter 4).

Although the paxilline ELISA detects paxilline-like compounds, it cannot measure the concentration of each paxilline-like compound, instead quantitatively measuring levels of immunoreactivity as paxilline immunoreactive equivalents. Levels of paxilline immunoreactivity were higher in the herbage of plants that were not exposed to adult African black beetle (*H. arator*) feeding, although this was not statistically significant. In general, the undamaged pseudostem from plants not exposed to adult African black beetle (*H. arator*) had higher concentrations of paxilline-IRE than those exposed to adult African black beetle (*H. arator*) (Figure 3). This observation was still unexpected because if the proposed subset of paxilline-like secondary metabolites were produced in association with feeding damage as suggested in Chapter 4, the concentration of immunoreactivity in the plants exposed to beetles was predicted to be higher than plants not exposed. The observed result may simply be because unexposed plants, having optimal conditions for growth and not being under any stress, did not have limiting resources for secondary metabolite production including paxilline immunoreactivity. In addition, in the undamaged plants, paxilline-like compounds not associated with feeding damage but with high cross-reactivities in the ELISA, may have been produced at higher levels than in damaged plants. Or in damaged plants, production of paxilline-like compounds was simply down regulated, resulting in lower levels of paxilline immunoreactivity.

In herbage the negative effects of feeding adult beetles were still apparent one month post-exposure, with plants previously exposed to African black beetle (*H. arator*) still with lower levels of paxilline immunoreactivity than those not exposed. In addition, a seasonal effect could be identified in herbage with higher levels of paxilline immunoreactivity at the post-treatment phase (mid-summer) compared with the end of the treatment phase (early summer) in both the presence and absence of beetles.

Greater tiller production was found for plants exposed to beetles intially, but then under high beetle pressure, tiller production did not keep ahead of the sustained feeding damage to tillers. Then following exposure to beetles, a long recovery time was needed for plant tiller numbers (undamaged) to return to the same level as those of the unexposed plants. These results for tiller number suggest that plants may respond to low levels of herbivory by producing more tillers than plants not exposed and this effect was common to all plants, however, high levels of herbivory are detrimental to the plants. Maschinski and Whitham (1989) reported evidence that a plant's response to herbivory is plastic and varies according to the prevalent biotic and abiotic conditions it experiences. Herbivory can be detrimental, of no consequence, or even beneficial, depending on the conditions governing a plant's ability to replace tissue consumed by herbivores and that the negative and positive responses reported in the literature are not in conflict but are extremes of the same continuum (Maschinski & Whitham, 1989; Whitham *et al.*, 1991).

The negative effect of African black beetle (*H. arator*) feeding on tiller number was still apparent four weeks post trial, so plants took a long time to recover. This suggests tiller production is costly to the plant. In addition, the plant may be putting resources into protection via the endophyte, for increased alkaloid production (Bultman *et al.*, 2004; Sullivan *et al.*, 2007) for the protection of both old and new tillers. If resources are diverted for protection and recovery against herbivory, it is possible fewer resources are available for other aspects of plant growth, such as root formation, which in turn may further hinder the recovery of tiller numbers. Plant interclonal variation for portion of damaged tillers was more variable for some plants and families and consistent with Trial 1 results suggesting some plants would respond better than others in a breeding program.

For plants exposed to adult African black beetle (*H. arator*), results from this trial were consistent with results from Trial 1, although the cluster of plants with low feeding damage and high paxilline immunoreactivity was not observed in Trial 2. The relative performance of the plant genotypes for feeding damage, paxilline immunoreactivity and tiller production is consistent between the two trials, however, the actual concentration of paxilline-IRE in Trial 2 plants was lower than that in Trial 1 plants. Consequently for Trial 2, lower alkaloid production may explain the

lack of a negative association observed between feeding damage and paxilline immunoreactivity that was in seen in both the Lincoln Trial and Trial 1.

Significant plant and within family plant differences were found for feeding damage, paxilline immunoreactivity and tiller number. Plants that produced a lot of tillers at the start of the trials still produced a lot of tillers during and at the end of the trials and the initial tiller number (plant size) was not related to the proportion of tillers damaged. The distribution of paxilline immunoreactivity in the plant sections was consistent, with the highest concentrations of paxilline-IRE found in the undamaged pseudostem and the lowest concentrations in the herbage. The severity of the feeding damage to damaged tillers (damage score; 1–3) could not be analysed as the majority of tillers were severely damaged (damage score three).

High beetle pressure may explain the lack of differences found between plants in the severity of damage the tillers sustained. Another possibility is that the beetles were not detecting the presence and levels of either the endophyte or certain alkaloids produced through feeding but instead by other mechanisms, possibly detecting volatiles released by endophyte-infected plants. Qawasmeh *et al.* (2015) describe how adult African black beetle (*H. arator*) are less attracted to wild-type and AR1-infected plants than endophyte-free plants. Although they mention that adults do not discriminate between host plants solely on these volatiles but also on visual and leaf-chemical cues (Qawasmeh *et al.*, 2015).

The beetles used in these spring trials were mature beetles that had first emerged the previous autumn. Beetle age may be important when assessing the deterrent effects of endophyte-plant associations, with a reduction in sensitivity to the adverse effects of the bioactives with age. Plants infected with wild-type endophyte also had high damage, which is unusual and supports the suggestion of reduced beetle sensitivity to the bioactive secondary metabolites with age. Also, when feeding by beetles is deterred by endophyte, the beetle moves onto another food source, however, in the trial the beetles were confined for 4 weeks. With the beetles in the phase of regular feeding, it is likely deterrent effects of the endopyte were not as influential as food choices were limited. This may in part explain some of the

variation found between plants within a family and also the large plant interclonal variation seen for some plant genotypes.

Differences between families were undetermined because of plant differences found within families and low family representation, but the data from both trials indicated the possibility of family differences. Differences found between plants and between plants within families for levels of feeding damage and paxilline immunoreactivity (in both the presence and absence of beetles) demonstrate the importance of the host plant genotype influence as previously reported in the literature (Easton *et al.*, 2000; Popay & Baltus, 2001; Easton *et al.*, 2002; Popay *et al.*, 2003), and provide potential for selecting plants with more resistance to adult African black beetle (*H. arator*) feeding on the basis of the AR1-host plant association. In general plant genotypes (and families) performed consistently in both trials and the following three families with low–moderate feeding damage and medium–high levels of paxilline immunoreactivity and tiller production were selected for further investigation; A12064, A12078 and A12087.

The reduction of the number individual plant genotypes used, from 50 in Trial 1 to 20 in Trial 2, allowed the number of clonal experimental units to be increased from three to six, two of which weren't exposed to African black beetle (*H. arator*). The low number of representatives from within each family, however, has reduced the ability to investigate family differences. The limitations of low numbers of individuals from within a family and low clonal replication (for both trials and for each treatment in Trial 2) were unavoidable concessions made to ensure the trials could be completed with the labour available. These limitations are addressed in subsequent trial work (Chapters 7 and 8).

5.5 Summary

Higher levels of paxilline immunoreactivity were found in plants not exposed to adult African black beetle (*H. arator*). This may be a simple reflection of the unexposed plants being under minimal stress and resources for secondary metabolite production were not as limited, or a simple down regulation of secondary metabolite production. A seasonal effect was seen with higher levels of paxilline immunoreactivity found in herbage mid-summer than early summer. Low levels of

feeding from adult African black beetle (*H. arator*) appears to accentuate plant tiller production, however, high levels of feeding are detrimental to plants and plant tiller number is reduced. The negative effects of high feeding pressure from adult African black beetle (*H. arator*) were still apparent one month post exposure to beetles, with lower levels of paxilline immunoreactivity and lower plant tiller numbers.

Distribution of paxilline immunoreactivity in the plant was consistent between Trials 1 and 2, with the highest levels found in undamaged pseudostem, then damaged pseudostem and the lowest in herbage. Relative plant performance for the measured variables (feeding damage, paxilline immunoreactivity, damage score and tiller production) were consistent between Trials 1 and 2, however, paxilline-IRE concentrations were lower in Trial 2. This may explain why a negative association between feeding damage and paxilline immunoreactivity was not apparent in Trial 2. Initial tiller number (plant size) was related to plant tiller number throughout both trials. Plants that had high tiller numbers at the start of a trial had relatively high plant tiller numbers at the end of a trial. Initial plant size, however, did not affect the proportion of damaged tillers, the severity of the tiller damage or the level of paxilline immunoreactivity.

5.6 References

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Chapter 6

Trial 3 – An artificial diet feeding trial with adult African black beetle using AR1-infected and endophyte-free perennial ryegrass seed.

6.1 Introduction

Despite the absence of significant correlations between adult African black beetle (*Heteronychus arator* (Fabricius, 1775)) feeding damage and paxilline ELISA (enzyme-linked immunosorbant assay) immunoreactivity, results from the first two feeding trials using plants (Chapters 4 and 5) from AR1-infected half-sibling perennial ryegrass (*Lolium perenne* L.) families from within a breeding line (GA97), did show some support for the hypothesis that high paxilline immunoreactivity is associated with low feeding damage from adult African black beetle (*H. arator*). Of particular interest was the cluster of plants, with low adult African black beetle (*H. arator*) feeding damage and high paxilline immunoreactivity in the plant pseudostem (>25 µg/g). This cluster was not observed in the second feeding-choice trial (Chapter 5), although levels of paxilline immunoreactive equivalents (paxilline-IRE) in pseudostem were lower than Trial 1 and did not exceed 25 µg/g grass.

Although adult African black beetle (*H. arator*) feed on the pseudostem near the crown of the plant, seed incorporated into the artificial diet rather than plant material was chosen for Trials 3a and 3b, because endophyte-infected (E+) seed acts as a reservoir for alkaloids produced by the endophyte (Miles *et al.*, 1993; Miles *et al.*, 1994). The endophytic fungus is contained within the seed as a means of transmission (Neill, 1940; Clay, 1986; Clay & Kover, 1996; Clay & Schardl, 2002) and often contains higher levels of alkaloids than the plants themselves do (Gallagher *et al.*, 1982). The standard method used by chemists to isolate and characterise endophyte-produced compounds is by bulk extraction of seed (Miles *et al.*, 1993; Miles *et al.*, 1994), as this provides a much cleaner matrix compared with plant material (Finch S.C., AgResearch, New Zealand; personal

communication, 2010). Seed also provides consistency and reproducibility of sample that would be difficult to obtain when using freshly grown plant pseudostem (freeze-dried or fresh) for individual diet trials owing to plant-age, growing conditions and seasonal trends influencing both alkaloid expression and the palatability of the diet for the insect. Incorporation of ryegrass seed into artificial diets for consumption by adult African black beetle (*H. arator*) was already an established AgResearch method (Popay A.J., AgResearch, New Zealand; personal communication, 2010) and the seed from infected plants may produce the same or stronger effects seen with vegetative plant material (Gallagher *et al.*, 1982).

Feeding trials using AR1-infected perennial ryegrass (*L. perenne*) seed incorporated into artificial diet were undertaken to determine if presence of AR1 endophyte is detrimental to adult African black beetle (*H. arator*) and to provide evidence supporting the hypothesis that paxilline immunoreactivity is associated with low or reduced feeding damage from adult African black beetle (*H. arator*). Because there was insufficient seed from the GA97 breeding line used in plant trials available, seed from a commercially available ryegrass cultivar 'Extreme' that was infected with AR1 endophyte or was endophyte-free (E-) was used. This cultivar is known to contain high levels of paxilline-IRE when analysed by the paxilline ELISA, and to provide moderately high resistance to feeding by adult African black beetle (*H. arator*) (Popay A.J., AgResearch, New Zealand; personal communication, 2006) as shown in the initial Lincoln trial discussed in Chapter 1, Section 1.9.2 and Chapter 7.

The feeding trials (3a and 3b) using artificial diets were performed in spring (September–November 2010) when over-wintering adult African black beetles (*H. arator*) had come out of dormancy and were emerging to feed and mate. The objective of the first trial (3a) was to establish that the presence of the AR1 endophyte in seed of 'Extreme' reduces adult African black beetle (*H. arator*) feeding. A second objective of Trial 3a was to determine if the presence of AR1 endophyte modified the behaviour of adult African black beetles (*H. arator*). In the second trial, 3b, seed of 'Extreme' and extracts from seed were tested for their effects on African black beetle (*H. arator*) feeding. Indole diterpenoid profiles were determined for seed and pseudostem of cultivar 'Extreme'.

6.2 Methods

6.2.1 Adult African black beetle

6.2.1.1 Beetle collection

In spring active adult African black beetles (*H. arator*) were collected (Section 2.1.5) from the field in Waikato region and maintained (Section 2.1.6) in the laboratory until required.

6.2.1.2 Beetle weight and replicate allocation

The day before a trial started the beetles, field-collected in the Waikato region (Section 6.2.1.1), were sorted by gender, weighed and assigned to replicates based on similarity of weight. Beetles were first separated according to gender, and then for each gender the beetle weights were recorded. Beetles within each gender, of similar weight were allocated to a replicate with individual beetles randomly assigned to each treatment within the replicate group. For example, in replicate one (A1, B1, C1 and D1, where A, B, C and D were four different treatment groups) all beetles were of the same gender and had weights preferably within a 10–20 mg range depending on beetle availability. Each treatment group had 20 replicates consisting of 10 female and 10 male beetles.

6.2.1.3 Beetle replicate set-up

Single beetles were placed into their allocated individual containers (three quarters filled with moist soil). A 9 cm diameter petri-dish lid was placed on the container (experimental unit) to ensure the adult African black beetle (*H. arator*) did not escape. Spare experimental units for each replicate containing a single beetle were also set up to replace any experimental units removed owing to inactive beetles. After the beetles had buried themselves, the soil in the containers was tamped down. The next day the containers were checked to ensure the beetles were active. Containers that did not appear to have had the soil disturbed were deemed to contain inactive beetles. These containers were removed and replaced with containers from the ‘spares’ that were deemed to contain an ‘active’ beetle (that is, the soil was disturbed and indicated that the beetles had been up on top of the soil overnight, probably foraging for food) within the same weight group.

6.2.2 Ryegrass seed.

Whole seed was stored in the dark at 4°C and milled seed in the dark at –20°C until required for use. The two "Extreme" cultivar ryegrass (*L. perenne*) seed lines (E+, accession 15150 and E- accession 15149) were extracted using the standard paxilline ELISA extraction procedure (Section 2.4.3.1.2). Extracts were analysed by the paxilline ELISA (Section 2.4.3.2) prior to the start of each feeding trial to confirm levels of paxilline-IRE in each seed line and the endophyte status (E+ or E-) of the seed.

6.2.2.1 Bulk extraction of ryegrass seed

Seed was milled using the Udy cyclone mill (Section 2.3.2). HPLC grade methanol (2× 12.5 mL) was added to milled seed (2× 2.5 g) at a ratio of 1 : 5 (w/v) in glass Kimax tubes (2× 12 mL). The solutions were rotated end over end for 20 minutes then centrifuged at 1294 rcf for 5 mins (Sorvall RT7 centrifuge, RTH-750 rotor with buckets at 2500 rpm). The supernatant from each tube was collected and combined into a 50 mL glass round bottom flask and the methanol removed via rotary evaporation. The extract was stored at –20°C until required and re-suspended in ethanol (250 µl) for use. The extracted milled seed from each Kimax tube was combined, left overnight in a fume-hood to remove residue methanol and then stored in the dark at –20°C until required for use.

Extracts and seed (both E+ and E-) from the bulk extraction procedure were used for diet preparation (see below in Table 6-1). In addition, the bulk extracted seed, both E+ and E-, were re-extracted using the standard paxilline ELISA extraction procedure (Section 2.4.3.1.2) and analysed by paxilline ELISA.

6.2.3 Diet preparation

6.2.3.1 Carrot agar

The following diet as described by Ball *et al.* (1997) was used. Fresh diced carrot (150 g), distilled water (75 mL), penicillin-G (0.032 g) and streptomycin sulphate (0.032 g) were thoroughly liquidised in a 400 W commercial Waring Blender (Model HGB2WTG4). During blending, 1.5 mL of absolute ethanol (BDH) was added to the carrot mix. In a 250 mL glass beaker, agar (7.5 g) was dissolved in

distilled water (94 mL) by heating over a Bunsen burner (or in a microwave oven). Once the agar mix had cooled to 60°C 4-hydroxybenzoic acid (0.112 g) and sorbic acid (0.094 g) were added as mould inhibitors. The liquidised carrot and the agar solution were then added together and mixed thoroughly.

6.2.3.2 Artificial diet treatments

Eight diet treatments using carrot agar as the base were prepared as described in Table 6-1. For the milled seed diets the appropriate seed (2g) was added to carrot agar (20g); also see Section 6.2.2. For the bulk extract of seed diets the appropriate extract (100 µl of dried methanol extract re-suspended in ethanol) was added to carrot agar (22g); also see Sections 6.2.2 and 6.2.2.1. The amount of extract added (100 µl) was equivalent to 2 g seed. The agar control diet was carrot agar (22 gm). The eight diet treatments used in artificial diet trials are described in Table 6-1.

6.2.3.3 Diet and feeding tube set-up

Diets were made on the first day of a trial, Day 0 (Sections 6.2.3.1 and 6.2.3.2). After diets had cooled and firmly set, discs were cut using a number 7 cork-borer (11 mm diameter). The diet discs were weighed prior to placement into feeding tubes. Feeding tubes were constructed using a cut 5 mL Eppendorf pipette tip attached to the centre of a small plastic lid as shown in Figure 6-1 A. Feeding tubes were then placed into the appropriately labelled container on the soil surface (Figure 6-1 B) and the beetles could access the diet from either end of the feeding tube (Figure 6-1 C). The petri-dishes containing the remaining diets were sealed using parafilm and stored at 4°C until used for replacing diet during the trial. Diet discs were replaced weekly (or sooner if required because of rapid consumption).

Table 6-1: Description of treatments used in artificial diet trials.

Treatment code	Diet treatment
A	Milled E+ seed in carrot agar
B	Milled E- seed in carrot agar (endophyte control)
C	Bulk extracted milled E+ seed in carrot agar
D	Bulk extracted milled E- seed in carrot agar (endophtye control)
E	Bulk extract of E+ seed in carrot agar
F	Bulk extract of E- seed in carrot agar (endophyte control)
G	Carrot agar diet (agar control)
H	Carrot agar diet with additional 100 µl ethanol (extract solvent control)
I	Raw carrot (maintenance food)

Milled E+ seed = AR1-infected Lolium perenne ryegrass seed cv. "Extreme", Accession 15150. Milled E- seed = endophyte-free Lolium perenne ryegrass seed cv. "Extreme", Accession 15149.

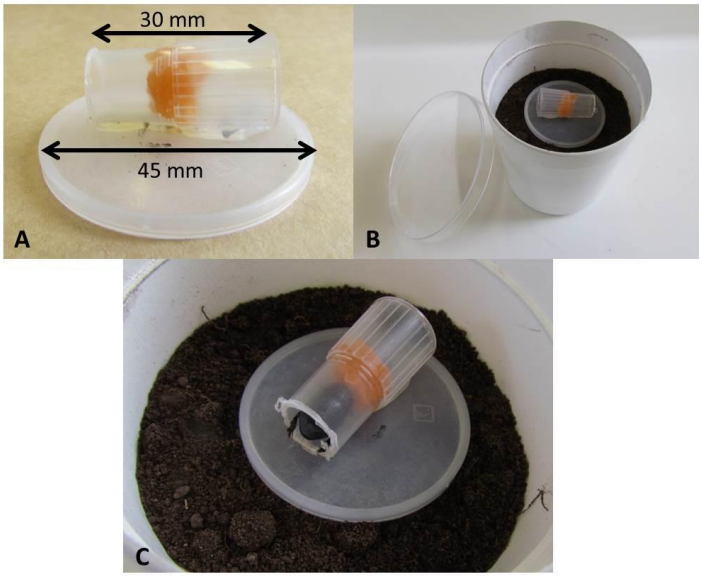

Figure 6-1: The artificial diet experimental unit set up.
A) The feeding tube (made from a cut 5 mL pipette tip attached to the center of a small plastic lid) containing a carrot agar diet disc. B) The feeding tube placed on the soil surface in the container, with the 9 cm petri-dish lid removed and placed next to the container. C) An adult African black beetle feeding on the carrot agar diet disc inside the feeding tube which is on the soil surface.

6.2.4 Trial Assessments

6.2.4.1 Daily assessment

- Soil disturbance; was the soil disturbed, yes or no?
- Adult African black beetle (*H. arator*) position; was the beetle buried or on the surface? If on the surface, was the adult African black beetle (*H. arator*): Active or inactive, in the feeding tube or on the soil, feeding or not feeding?
- Diet position; was the diet disc in place or had it been moved? (Any moved diet disc or pieces were returned to the feeding tube).

Diet score; the diet disc was scored visually for amount of diet consumed, on a scale of 0–10; 0 = no feeding, 1 = 10% and 10 = 100% of diet disc consumed. After each daily assessment the soil was tamped down again so that beetle activity could be recorded again the following day.

6.2.4.2 Final assessment in diet phase

- Daily assessment was performed as above.
- Remaining diet was weighed and stored at –20°C.
- Beetles were recovered and weighed.
- Beetles were returned to containers and feeding tubes were removed for post-trial recovery.
- Fresh carrot was placed into containers (¼ sliced ring).

6.2.4.3 Post-diet phase

- Daily assessments were performed as described in Section 6.2.4.1 with the following alteration; instead of a feeding score it was recorded if the beetle had fed or not fed, by checking the carrot for signs of feeding.
- Carrot was replaced daily (¼ sliced ring).
- On final day of post-treatment phase in addition to the daily assessments, the adult African black beetles (*H. arator*) were recovered and weighed.

6.2.5 Feeding trials using artificial diets

6.2.5.1 Feeding Trial 3a using artificial diets

Trial 3a with milled ryegrass seed incorporated in an artificial diet was performed in early spring (September 2010). The trial consisted of four treatments (A, B, G and I, Table 6-1) and was set-up as described in Sections 6.2.1, 6.2.2 and 6.2.3. The raw carrot (treatment I, Table 6-1) was used to ensure the adult African black beetles (*H. arator*) were active and feeding after their over-wintering dormancy cycle. Trial 3a had two phases, diet phase and post-diet phase. The diet phase of this current trial lasted 15 days with daily assessments (Section 6.2.4.1) and a final assessment in the diet phase (Section 6.2.4.2). At the end of the diet phase all beetles were put back on maintenance food (raw carrot) for a post-diet phase which lasted 5 days. Beetles were monitored and assessed as described in Section 6.2.4.3.

6.2.5.2 Feeding Trial 3b using artificial diets

Trial 3b with milled ryegrass seed and extracts of milled seed incorporated in an artificial diet was performed mid-late spring (October-November 2010). The trial consisted of eight treatments (A–H, Table 6-1), a single diet-phase and was set-up as described in Sections 6.2.1, 6.2.2 and 6.2.3. In addition to the standard experimental units containing African black beetle (*H. arator*) (Section 6.2.1.2), further experimental units were set up containing artificial diet discs but no adult African black beetle (*H. arator*) for each treatment. The experimental units without African black beetle (*H. arator*) were set up for testing the levels of paxilline-immunoreactivity in the diet discs each time they were replaced to determine the stability of the paxilline immunoreactivity. When the diet discs were replaced, a single old and new disc from each treatment were collected and stored at −20°C. These were later extracted (Section 2.4.3.1.3) and analysed by the paxilline ELISA (Section 2.4.3.2).

6.2.6 Statistical analyses

For Trial 3a the following variables were analysed:

Non behavioural;

- Weight at start of diet phase (start of trial).
- Weight change from start to end of diet phase of trial.
- Weight at end of post-diet phase (end of trial).
- Weight change from end of diet phase to end of post-diet phase.
- Diet consumed.
- Cumulative diet score.

Behavioural;

- Days soil disturbed (by beetle overnight; overall trial).
- Days soil disturbed (by beetle overnight; diet phase).
- Days soil disturbed (by beetle overnight; post-diet phase).
- Days buried (beetle buried during assessment; overall trial).
- Days buried (beetle buried during assessment; diet phase).
- Days buried (beetle buried during assessment; post-diet phase).
- Days diet undisturbed (diet remained in place inside the feeding tube and not removed by the beetle).

Raw carrot (Treatment I), was excluded from the diet consumed, diet score and diet disturbance measurements during the diet phase. The data for each variable were analysed using a two-way blocked analysis of variance (ANOVA) initially using the full model, *Gender+Treatment+Gender.Treatment* whereby:

- *Gender.Treatment:* The F test assesses whether the means for each treatment by gender group are the same.
- *Gender:* (If *Gender.Treatment* is dropped) The F test assesses whether the means for female and male beetles are the same.
- *Treatment:* (If *Gender.Treatment* is dropped) The F test treatment assesses whether the means for all treatment groups (diets) are the same.

The block was the African black beetle (*H. arator*) replicate. Blocking controls for the differences in the initial weights between beetles, and by accounting for this source of variation the treatment effects are more easily detected. Insignificant terms at the 5% level were sequentially dropped. The model assumptions of normal data with constant variance were checked by examining residual plots.

6.2.7 Analysis by LCMS/MS for indole diterpenes

Milled seed and plant (pseudostem) samples from perennial ryegrass (*L. perenne*) cultivars, "Extreme" AR1-infected and "Extreme" endophyte-free were sent to AgResearch, Palmerston North. As described by Rasmussen *et al.* (2012), samples were extracted in 80% acetonitrile with 1% acetic acid and extracts analysed by LC-MS/MS for known indole diterpenes, including paxilline-like compounds, with the numbers reported being standardised relative peak areas.

6.3 Results

From analysis by the paxilline ELISA, it was confirmed 'Extreme E+' seed (AR1-infected) was positive for the paxilline-like endophyte secondary metabolites (18.11 µg/g), and that 'Extreme E-' seed did not contain detectable levels of these metabolites. Analysis by LCMS/MS of the seed from the 'Extreme E-' cultivar detected the presence of lolitrems indicating a low level of contamination by the wild-type endophyte. Lolitrems are not detected by the paxilline ELISA, and pseudostem of plants grown from the same batch of seed tested negative for paxilline-immunoreactivity by the paxilline ELISA, negative for endophyte infection by immunoblot and negative for lolitrems by LCMS/MS. It was likely that some of the endophyte-free seed contained wild-type endophyte that was no longer viable but still contained alkaloids.

6.3.1 Feeding Trial 3a results

Initially all diet treatments (A, B, G and I; Table 6-1) were analysed together, but owing to gender by treatment interactions (P <0.05; Table 6-2 and Table 6-4) in Treatment G (carrot agar only), the two seed diet treatments, A and B, were also analysed separately (mean values for measured variables are shown in Table 6-3 and Table 6-5). From the concentration of paxilline immunoreactivity in the

Table 6-2: Summary table of statistical analyses for weights of adult African black beetle, diet consumed and cumulative diet score, in a feeding trial with endophyte-infected (E+) and endophtye-free (E-) ryegrass seed incorporated in the diet, carrot agar diet and raw carrot.

	All diet treatments (A, B, G and I)			Seed diet treatments (A and B)		
Variable	**Effect**	**F-statistic**	**P-value**	**Effect**	**F-statistic**	**P-value**
Beetle weight at start of diet phase (start of trial)	Gender by treatment	$F_{(3,54)} = 0.07$	0.975	Gender by treatment	$F_{(1,18)} = 0.23$	0.637
	Gender	***$F_{(1,18)} = 7.43$***	***0.014***	***Gender***	***$F_{(1,18)} = 6.92$***	***0.017***
	Treatment	$F_{(3,57)} = 0.45$	0.715	Treatment	$F_{(1,19)} = 0.78$	0.389
Weight change (start to end of diet phase)	Gender by treatment	$F_{(3,54)} = 0.93$	0.432	Gender by treatment	$F_{(1,18)} = 0.13$	0.724
	Gender	$F_{(1,18)} = 0.81$	0.380	Gender	$F_{(1,18)} = 0.52$	0.480
	Treatment	***$F_{(3,57)} = 3.70$***	***0.017***	***Treatment***	***$F_{(1,19)} = 10.75$***	***0.004***
Beetle weight at end of post-diet phase (end of trial)	Gender by treatment	$F_{(3,54)} = 1.75$	0.168	***Gender by treatment****	***$F_{(1,18)} = 4.59$***	***0.046***
	Gender	***$F_{(1,18)} = 17.95$***	***<0.001***			
	Treatment	$F_{(3,57)} = 0.57$	0.637			
Weight change (end of diet phase to end of post-diet phase)	Gender by treatment	$F_{(3,54)} = 2.51$	*0.068*	***Gender by treatment****	***$F_{(1,18)} = 10.51$***	***0.005***
	Gender	$F_{(1,18)} = 0.13$	0.727			
	Treatment	***$F_{(3,57)} = 5.86$***	***0.001***			
Diet consumed	***Gender by treatment***	***$F_{(2,36)} = 4.96$***	***0.013***	Gender by Treatment	$F_{(1,18)} = 0.04$	0.845
				Gender	$F_{(1,18)} = 0.24$	0.632
				Treatment*	***$F_{(1,19)} = 7.70$***	***0.012***
Cumulative diet score	***Gender by treatment***	***$F_{(2,36)} = 3.50$***	***0.041***	Gender by treatment	$F_{(1,18)} = 0.38$	0.547
				Gender	$F_{(1,18)} = 0.11$	0.744
				Treatment*	***$F_{(1,19)} = 5.65$***	***0.028***

Values that are statistically significantly different ($P \leq 0.05$) are highlighted in bold and italics. Values that show weak evidence ($0.05 < P \leq 0.10$) but are not statistically significant are highlighted in italics. Diet treatments; A = Carrot agar diet with AR1-infected seed. B = Carrot agar diet with endophyte-free seed. G = Carrot agar diet. I = Raw carrot. * = change in effect between all diet treatments analysed and diet treatments A and B only.

Table 6-3: Mean values for weights of adult African black beetle, diet consumed and cumulative diet score in a feeding trial with endophyte-infected (E+) and endophtye-free (E-) ryegrass seed incorporated in the diet, carrot agar diet and raw carrot.

		Beetle weight (mg)					
Gender	**Diet Treatment**	**At start of diet phase**	**Weight change (start to end of diet phase)**	**Weight at end of post-diet phase**	**Weight change (end of diet phase to end of post-diet phase)**	**Diet consumed (mg)**	**Cumulative diet score**
Female	A	189.9	***-12.0***	211.0	***33.1***	*232*	*4.2*
Female	B	190.6	2.9	190.8	-2.7	377	7.3
Female	G	190.3	6.5	198.1	1.3	***822***	***19.1***
Female	I	189.7	11.3	208.6	7.6		
Male	A	167.3	-8.0	174.6	15.3	*255*	*2.4*
Male	B	167.5	3.9	181.2	9.8	422	7.8
Male	G	167.5	-1.6	170.5	4.6	364	6.5
Male	I	167.1	0.4	170.6	3.1		
All diet treatments (A, B, G, I)	LSD	17.61	14.92	21.52	16.69	292.8	8.24
	LSD (within gender)	1.78	14.81	19.75	16.22	259.1	7.60
Seed diet treatments (A and B)	LSD	18.27	11.03	22.30	17.90	184.40	5.36
	LSD (within gender)	1.55	12.43	18.58	13.90	171.50	5.39

Table 6-3 continued on next page

Table 6-3 continued

Gender	Diet Treatment	Beetle weight (mg)				Diet consumed (mg)	Cumulative diet score
		At start of diet phase	Weight change (start to end of diet phase)	Weight at end of post-diet phase	Weight change (end of diet phase to end of post-diet phase)		
All diet treatments (A, B, G, I)							
Female	All (A, B, G and I)	***190.1***		***202.1***			
Male	All (A, B, G and I)	***167.4***		***174.2***			
Combined	A		***-10.0***		***24.2***		
Combined	B		3.4		3.5		
Combined	G		2.4		2.9		
Combined	I		5.8		5.3		
Fisher's LSD		17.56	10.44	13.84	11.90		
Significant effects ($P \leq 0.05$)		Gender	Treatment	Gender	Treatment	Gender by Treatment	Gender by Treatment
Seed diet treatments (A and B)							
Female	A–B	***190.3***					
Male	A–B	***167.4***					
Combined	A		***-10.0***			***244***	***3.3***
Combined	B		***3.4***			***400***	***7.5***
Fisher's LSD		18.25	8.55			117.7	3.73
Significant effects ($P \leq 0.05$)		Gender	Treatment	Gender by Treatment*	Gender by Treatment*	Treatment*	Treatment*

Diet treatments; A = Carrot agar diet with AR1-infected seed. B = Carrot agar diet with endophyte-free seed. G = Carrot agar diet. I = Raw carrot. LSD = Fisher's least significant difference at the 5% significance level. Values that are significantly different are highlighted in bold and italics. Values that are of interest but not significantly different between are highlighted in italics. * = change in effect between all diet treatments analysed and diet treatments A and B only.

Table 6-4: Summary table of statistical analyses for adult African black beetle activity (days soil disturbed overnight, days adults buried during assessment) and the diet position (days the diet was undisturbed and remained in the feeding tube) in a feeding trial with endophyte-infected (E+) and endophtye-free (E-) ryegrass seed incorporated in the diet, carrot agar diet and raw carrot.

	All diet treatments (A, B, G and I)			Seed diet treatments (A and B)		
Variable	**Effect**	**F-statistic**	***P*-value**	**Effect**	**F-statistic**	***P*-value**
Days soil disturbed (overall trial)	Gender by treatment	$F_{(3,54)} = 0.86$	0.465	Gender by treatment	$F_{(1,18)} = 1.64$	0.217
	Gender	***$F_{(1,18)} = 8.90$***	***0.008***	***Gender***	***$F_{(1,18)} = 5.29$***	***0.034***
	Treatment	$F_{(3,57)} = 0.30$	0.828	Treatment	$F_{(1,19)} = 0.54$	0.470
Days soil disturbed (diet phase)	Gender by treatment	$F_{(3,54)} = 0.83$	0.481	Gender by treatment	$F_{(1,18)} = 1.32$	0.266
	Gender	***$F_{(1,18)} = 11.52$***	***0.003***	***Gender***	***$F_{(1,18)} = 7.67$***	***0.013***
	Treatment	$F_{(3,57)} = 0.49$	0.688	Treatment	$F_{(1,19)} = 0.61$	0.446
Days soil disturbed (post-diet phase)	Gender by treatment	$F_{(3,54)} = 0.61$	0.611	Gender by treatment	$F_{(1,18)} = 1.50$	0.237
	Gender	$F_{(1,18)} = 0.92$	0.350	Gender	$F_{(1,18)} = 0.27$	0.609
	Treatment	$F_{(3,57)} = 0.87$	0.462	Treatment	$F_{(1,19)} = 0.23$	0.635
Days buried (overall trial)	Gender by treatment	$F_{(3,54)} = 1.13$	0.347	Gender by treatment	$F_{(1,18)} = 1.39$	0.254
	Gender	$F_{(1,18)} = 0.95$	0.342	Gender	$F_{(1,18)} = 0.68$	0.422
	Treatment	$F_{(3,57)} = 1.06$	0.375	Treatment	$F_{(1,19)} = 0.82$	0.376
Days buried (diet phase)	Gender by treatment	$F_{(3,54)} = 0.64$	0.591	Gender by treatment	$F_{(1,18)} = 0.67$	0.424
	Gender	$F_{(1,18)} = 0.76$	0.396	Gender	$F_{(1,18)} = 0.95$	0.342
	Treatment	$F_{(3,57)} = 1.27$	0.293	Treatment	$F_{(1,19)} = 0.68$	0.419
Days buried (post-diet phase)	Gender by treatment	$F_{(3,54)} = 0.96$	0.418	Gender by treatment	$F_{(1,18)} = 1.40$	0.253
	Gender	$F_{(1,18)} = 0.46$	0.507	Gender	$F_{(1,18)} = 0.17$	0.688
	Treatment	***$F_{(3,57)} = 2.85$***	***0.045***	*Treatment**	*$F_{(1,19)} = 3.80$*	*0.066*

Table 6-4 continued on next page

Table 6-4 continued

	All diet treatments (A, B, G and I)			Seed diet treatments (A and B)		
Variable	**Effect**	**F-statistic**	***P*-value**	**Effect**	**F-statistic**	***P*-value**
Days diet undisturbed (diet phase)	***Gender by treatment***	***$F_{(2,36)}$ = 4.07***	***0.026***	Gender by treatment	$F_{(1,18)}$ = 0.51	0.484
				Gender	$F_{(1,18)}$ = 0.55	0.469
				Treatment*	***$F_{(1,19)}$ = 6.29***	***0.021***

Values that are statistically significantly different (P ≤0.05) are highlighted in bold and italics. Values that show weak evidence (0.05< P ≤0.10) but are not statistically significant are highlighted in italics. Diet treatments; A = Carrot agar diet with AR1-infected seed. B = Carrot agar diet with endophyte-free seed. G = Carrot agar diet. I = Raw carrot. * = change in effect between all diet treatments analysed and diet treatments A and B only.

Table 6-5: Mean values for adult African black beetle activity (days soil disturbed overnight, days adults buried during assessment) and the diet position (days the diet was undisturbed and remained in the feeding tube) in a feeding trial with endophyte-infected (E+) and endophtye-free (E-) ryegrass seed incorporated in the diet, carrot agar diet and raw carrot.

		Adult African black beetle activity						Diet position
		Days soil disturbed			Days buried			
Gender	**Treatment**	**Trial overall**	**Diet phase**	**Post-diet phase**	**Trial overall**	**Diet phase**	**Post-diet phase**	**Days diet undisturbed**
Female	A	17.1	12.4	4.7	19.5	15.0	4.5	12.8
Female	B	14.8	10.8	4.0	19.4	14.7	4.7	11.2
Female	G	16.5	12.2	4.3	19.6	14.7	4.9	***8.5***
Female	I	16.6	12.7	3.9	19.6	14.8	4.8	
Male	A	18.1	13.7	4.4	18.8	14.7	4.1	12.5
Male	B	18.7	14.0	4.7	19.6	14.7	4.9	9.6
Male	G	18.5	13.8	4.7	19.0	14.4	4.6	12.5
Male	I	17.9	13.8	4.1	19.8	14.9	4.9	
All diet treatments (A, B, G, I)	LSD	2.78	2.09	1.06	0.92	0.53	0.60	3.39
	LSD (within gender)	2.81	2.10	1.08	0.93	0.52	0.60	2.95
Seed diet treatments (A and B)	LSD	3.16	2.35	1.14	0.99	0.49	0.72	3.21
	LSD (within gender)	3.37	2.46	1.22	1.14	0.53	0.75	2.70
		All diet treatments (A, B, G, I)						
Female	All (A, B, G and I)	***16.3***	***12.0***					
Male	All (A, B, G and I	***18.3***	***13.8***					

Table 6-5 continued on next page

Table 6-5 continued

Gender	Treatment	Adult African black beetle activity: Days soil disturbed: Trial overall	Days soil disturbed: Diet phase	Days soil disturbed: Post-diet phase	Days buried: Trial overall	Days buried: Diet phase	Days buried: Post-diet phase	Diet position: Days diet undisturbed
All diet treatments (A, B, G, I) continued								
Both	All (A, B, G and I			4.4	19.4	14.7		
Both	A						***4.3***	
Both	B						4.8	
Both	C						4.8	
Both	D						4.9	
	LSD	1.44	1.11	na	na	na	0.42	
Significant Effects ($P \leq 0.05$)		Gender	Gender	Nil	Nil	Nil	Treatment	Gender by Treatment
Seed diet treatments (A and B)								
Female	A–B	***16.0***	***11.6***					
Male	A–B	***18.4***	***13.9***					
Combined	A–B			4.4	19.4	14.7	4.7	
Combined	A							***12.7***
Combined	B							***10.4***
	LSD	2.24	1.11					1.88
Significant Effects ($P \leq 0.05$)		Gender	Gender	Nil	Nil	Nil	Nil*	Treatment*

Diet treatments; A = Carrot agar diet with AR1-infected seed. B = Carrot agar diet with endophyte-free seed. G = Carrot agar diet. I = Raw carrot. LSD = Fisher's least significant difference at the 5% significance level. Values that are significantly different are highlighted in bold and italics. Values that are of interest but not significantly different between are highlighted in italics. * = change in effect between all diet treatments analysed and diet treatments A and B only.

'Extreme E+' *L. perenne* seed measured by the paxilline ELISA, Diet A was estimated to contain paxilline-IRE at a concentration of 1.65 µg/g of diet (wet weight).

6.3.1.1 Adult African black beetle weight, diet consumed and diet score

Table 6-2 summarises the statistical analyses and Table 6-3 the mean values, for beetle weights, diet consumed and total diet score. For the mean beetle weights at the start of the trial (Table 6-3) there was no evidence (P >0.05; Table 6-2) of a gender by treatment interaction or a treatment effect. However there was evidence of sexual size dimorphism, i.e., a gender effect (P ≤0.05; Table 6-2) with female beetles on average, heavier than male beetles by 22.7 mg (Table 6-3).

Diet phase. The weight change of beetles from the start of the trial to the end of the diet phase was significantly less (P ≤0.05; Table 6-2) on AR1-Extreme *L. perenne* seed (Diet A), the only diet with a mean negative weight change, than for the other diet treatments. There were no significant differences for weight change of beetles from the other diets; Diet B (endophyte-free seed; endophyte control), Diet C (carrot agar; agar control) and Diet D (raw carrot; African black beetle (*H. arator*) feeding control). There was no evidence (P >0.05; Table 6-2) of gender or gender by treatment effects.

For the diet consumed and diet score there was evidence of gender by treatment interactions ($F_{(2,36)}$ = 4.96; P = 0.013 and $F_{(2,36)}$ = 3.50; P = 0.041 respectively). Significantly more diet was consumed by female beetles on the carrot agar diet (Diet G) than by males on the same diet. Female beetles on carrot agar diet also consumed significantly more diet than beetles (male and female) on the two diets containing seed (Diets A: E+ seed and B: E- seed), also see Table 6-3. When only the two seed diets (A and B) were compared, beetles consumed significantly more of the diet containing endophyte-free seed than of diet with AR1-infected seed (Weight change of diet: F(1,19) = 7.70; P = 0.012; Diet score: F(1,19) = 5.65; P = 0.028) (Table 6-3).

Post-diet phase. Average weight change during the post-diet phase was significantly higher ($F_{(3,57)}$ = 5.86; P <0.001) for beetles on Diet A (E+ seed) than the other diets (Table 6-3). There was evidence of a weak gender by treatment

interaction, ($F_{(3,54)}$ = 2.51; *P* = 0.068) with the diet A (E+ seed) female beetles having the highest weight change (Table 6-3). The gender by treatment interaction was confirmed for comparisons of Treatments A and B ($F_{(1,18)}$ = 10.51; *P* = 0.005), with female beetles on the AR1-infected seed diet gaining the most weight (Table 6-3).

Overall trial. At the end of the post-diet phase (end of trial) female beetles weighed more than male beetles by 27.9 mg ($F_{(1,18)}$ = 17.95; *P* = <0.001), as they did at the start of the trial, and there was no evidence (P >0.05, Table 6-2) of gender by treatment interactions or treatment effects. Beetles from Diet A (E+ seed) recovered the weight that was lost during the diet phase and gained sufficient weight during the post-diet phase that resulted in beetle weights (gender specific) that were no different to the other diet treatments. When comparing only the seed diets, however, female beetles in Treatment A weighed more than female beetles in Treatment B and males on both diets A and B at the end of the trial ($F_{(1,18)}$ = 4.59; *P* = 0.046) (Table 6-3).

6.3.1.2 Adult African black beetle activity and diet position

Adult African black beetle (*H. arator*) activity (number of days the soil was disturbed overnight and the number of days adults were buried during assessment) and diet position (number of days the diet was undisturbed and remained in the feeding tube) were monitored to determine if the presence of the diet treatments influenced adult beetle behaviour. Diet position related only to the diet-phase of the trial. The statistical results are summarised in Table 6-4 and the mean values in Table 6-5, for African black beetle (*H. arator*) activity and diet position.

During the diet phase, no treatment effects were found (*P* >0.05; Table 6-4) but overall (*P* ≤0.05; Table 6-4) male beetles on average emerged from the soil overnight significantly more often (by ~2 more days; Table 6-5) than females. No significant effects (P >0.05, Table 6-4), were found in the post-diet phase although interestingly female beetles on the AR1-infected seed diet (Diet A) emerged from the soil overnight more frequently than the males on this diet, whereas for all other diet treatments (endophyte-free, carrot agar and raw carrot) the males had the higher average, albeit not significantly so (Table 6-5).

The average number of days a beetle was buried in soil (at assessment) was not affected by gender or treatment during the diet phase and overall trial (P >0.05; Table 6-4). During the post-diet phase, however, beetles that had been on the E+ seed diet (Diet A) were on average buried on significantly fewer occasions than those on the other diets ($F_{(3,57)}$ = 2.85; P = 0.045) (Table 6-5). This result is consistent with the increased diet consumption and large positive weight change found for beetles on E+ seed diet during the post-diet phase of the trial. However, once the statistical analyses were redone omitting the carrot agar diet and raw carrot treatments, there was only weak evidence of a treatment effect ($F_{(1,19)}$ = 3.80; P = 0.066) between the two seed diet treatments. There was evidence of a gender by treatment effect ($F_{(2,36)}$ = 4.07; P = 0.026) for the average number of times the diet was disturbed with female beetles from the carrot agar diet (Diet G, highlighted in bold and italics in Table 6-5) disturbing the diet more often than beetles from diets containing seed, Diets A (E+) and B (E-), and with male beetles from the carrot agar diet. When only the two seed diets were re-analysed there was evidence of a treatment effect ($F_{(1,19)}$ = 6.29; P = 0.021) for the number of days the diet was undisturbed and in place inside the feeding tube (diet position) with the diets containing AR1-infected seed left undisturbed more often than diets with endophyte-free seed (Table 6-5).

6.3.2 Feeding Trial 3b results

The results are not reported for this trial because the beetles, whilst still active, had ceased regular feeding and therefore the trial results were unusable.

Important information, however, was gained in regard to the extraction of paxilline-like compounds from the E+ seed. The E- seed used in Trial 3b was confirmed (as it was for Trial 3a), to have no detectable levels of paxilline immunoreactivity by paxilline ELISA. For the E+ seed it was found that the bulk extraction procedure used did not adequately extract the paxilline immunoreactivity, with the level of the paxilline immunoreactivity remaining in the bulk extracted seed found to be higher than that in the bulk extract when re-extracted and analysed by paxilline ELISA (19.01 µg/g, 10.01 µg/g and 14.82 µg/g of seed for whole seed, bulk extract and bulk extracted seed, respectively). The actual measured levels of paxilline-IRE in Diets A (containing E+ seed), C (containing E+ seed extract) and E (containing

extracted E+ seed) were 1.14, 0.58 and 0.69 µg/g diet (wet weight) respectively. The level of paxilline immunoreactivity in the diets containing E+ seed (not extracted and extracted) remained constant, indicating the immunoreactive compounds were stable for up to 5 days. However, in the diets containing extract, the paxilline immunoreactivity became non-detectable within 5 days.

6.3.3 Instrumental chemical analyses (3c) – Indole-diterpernoid chemical profiles determined by LCMS/MS

Non-polar extracts (80% acetonitrile with 1% acetic acid) of seed and plant pseudostem were analysed by LCMS/MS, with the results reported as standardised relative peak areas (Table 6-6). The values do not allow comparison between analytes/compounds, but do allow comparison of a single analyte/compound between samples. Suitable standards were not available to enable quantitative analyses. Compounds terpendole E, terpendole K and terpendole N have been tentatively identified by Wade Mace, AgResearch, NZ, by comparison of fragmentation and retention times with known compounds.

The majority of the compounds detected were more concentrated in seed than in the pseudostem, ranging from 3.8–48 times more in the seed (terpendole C 48×, paspaline 7.8×, and 13-desoxypaxilline 3.8×). However, terpendole K and terpendole N were more concentrated in the pseudostem (3.2× and 2.2× respectively) compared with that of seed.

The distribution of expression of detected compounds within seed and pseudostem, for the ‘Extreme’ cultivar infected with AR1 were not the same (Table 6-6). In AR1-infected seed the early pathway indole diterpenes (highlighted by terpendole C, paspaline and 13-desoxypaxilline) were more highly expressed relative to the pseudostem (Table 6-6 and Figure 6-2). Compared with seed, the pseudostem showed relatively higher proportions of terpendole K and terpendole N, both late pathway indole diterpenes. The pseudsostem samples that were analysed by LCMS/MS were taken from plants that had not been exposed to adult African black beetle (*H. arator*) feeding.

Table 6-6: LCMS/MS indole diterpene alkaloid screen and % cross-reactivity of detected indole diterpenoids in the paxilline ELISA.

	Standardised peak area				
Detected indole diterpenoid	**Extreme E- pseudostem**	**Extreme E+ pseudostem**	**Extreme E- seed**	**Extreme E+ seed**	**%CR on paxilline ELISA**
Paspaline	ND	0.36	ND	2.80	NT
†Terpendole-E	ND	0.00	ND	0.19	NT
Paspaline-B	ND	ND	ND	ND	NT
13-Desoxypaxilline	ND	0.22	ND	0.82	NT
Paxilline	ND	ND	ND	ND	100
Terpendole-C	ND	0.09	ND	4.42	35
†Terpendole-K	ND	0.09	ND	0.03	NT
†Terpendole-N	ND	0.14	ND	0.06	NT
Lolitriol	ND	ND	0.05	ND	<0.6
Lolitrem-B	ND	ND	0.13	ND	<0.3

†Compounds tentatively identified by Mace, W. J., AgResearch, New Zealand, by comparison of fragmentation and retention times with known compounds. ND = non-detectable. NT = not tested % CR = percent cross-reactivity. %CR data supplied by Briggs, L. R., AgResearch, New Zealand 2008.

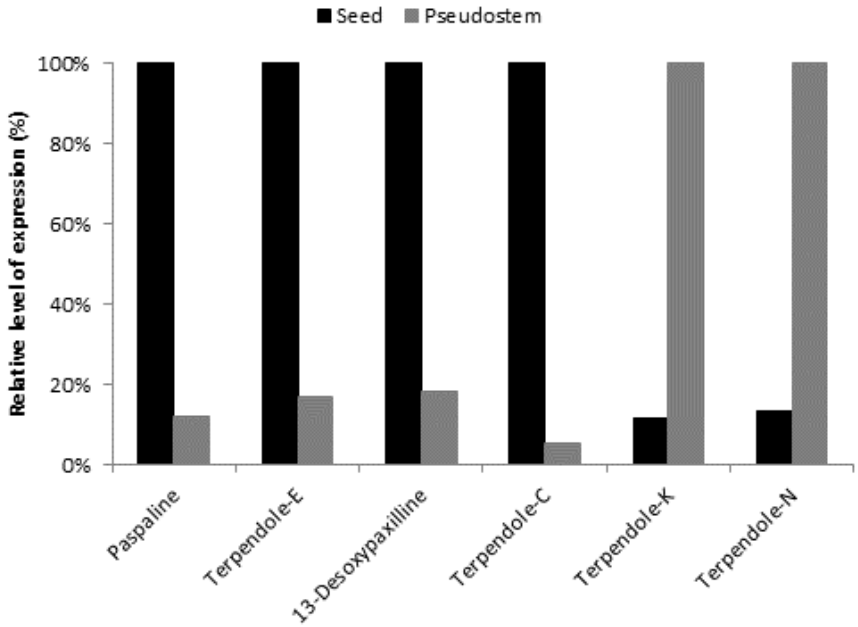

Figure 6-2: Relative expression levels of indole diterpenoids from standardised relative peak areas detected by LCMS/MS in AR1-infected pseudostem and seed.

The endophyte-free seed (also used for Trials 3a and 3b) was found to have low levels of wild-type endophyte contamination by LCMS/MS as it had a minor peak for lolitrem B and lolitriol. However, plants grown from the same batch of seed (pseudostem samples) did not test positive for lolitrems (wild-type endophyte contamination) or other paxilline-like indole diterpenoids by LCMS/MS. Endophyte-free seed and pseudostem did not test positive by paxilline ELISA for any detectable levels of paxilline-like immunoreactivity.

6.4 Discussion

Bulk extraction of seed is the preferred method for isolation and characterisation of secondary metabolites produced by endophytes. Artificial diet containing seed of "Extreme" cultivar infected with AR1 endophyte (Trial 3a) was found to adversely affect adult African black beetle (*H. arator*) feeding resulting in significant weight loss. The weight loss, reduced feeding and the finding that beetles were able to recover from this weight loss once they had access to a food supply that did not contain AR1 endophyte, suggests that the seed was a strong feeding deterrent but was not toxic.

The female beetles had the highest beetle weights at the end of the trial (consistent with the sexual size dimorphism observed in adult African black beetle (*H. arator*)). Female beetles on average gained more weight than male beetles (6.3% and 4.3% weight gain respectively), which may reflect the greater need for energy resources for egg production. Therefore in this current trial (Trial 3a), the large weight loss for the female beetles found at this time of the African black beetle (*H. arator*) lifecycle (prior to egg production), may adversely affect egg production. If female beetles gain access to a suitable food source, however, they could compensate for the weight loss quite quickly.

Adult African black beetle (*H. arator*) in pastures do not feed on seed, instead they feed on vegetative plant material (Bell *et al.*, 2011) and evidence of strong deterrence in plants has not been reported (Popay & Baltus, 2001; Thom *et al.*, 2013), with levels of secondary metabolites often lower in plants compared with seed (Gallagher *et al.*, 1982). Grasses are the preferred food source for all life stages of African black beetle (*H. arator*) (Bell *et al.*, 2011) and preferred oviposition sites (King *et al.*, 1981; Bell *et al.*, 2011). From the literature (Popay & Baltus, 2001;

Bell *et al.*, 2011) female beetles lay fewer eggs in environments that contain high levels of certain alkaloids. Popay and Baltus (2001), however, reported that in planta there were no significant differences in egg counts on AR1-infected and endophyte-free perennial ryegrass (*L. perenne*) plants, and significantly fewer eggs were found under wild-type infected plants in a pot trial. Thom *et al.* (2013) reported that African black beetle (*H. arator*) populations build up more quickly under AR1-infected pastures than pastures with grasses infected with AR37 or wild-type endophyte. From the results of Trial 3a and the evidence reported in the literature (Gallagher *et al.*, 1982; Popay & Baltus, 2001; Thom *et al.*, 2013), the concentration of secondary metabolites (and level of bioactivity) providing deterrence from AR1 endophyte in planta is considered to be a lot lower than in seed.

Although the indole diterpenoid profiles of the AR1-infected seed and pseudostem material from the ryegrass cultivar 'Extreme' were similar for the known detectable compounds, the relative expression levels of compounds in the seed was different to that found in the pseudostem. The relative expression levels of indole diterpenoids (3c) from standardised relative peak areas showed the majority of alkaloids were higher in seed than in pseudostem, consistent with the literature (Gallagher *et al.*, 1982). Relative expression levels of terpendoles K and N, however, indicated higher expression levels in pseusostem than seed indicating that vegetative plants are metabolising and are producing fungal secondary metabolites at different ratios and expression levels than found in the seed. There may also be other intermediate metabolites that may or may not be accumulated in the seed.

Compounds deterrent to adult African black beetle (*H. arator*) feeding in seed may not be present in the plant at high enough quantities to be detrimental in the plant. For example, terpendole C had high expression in seed but low expression in pseudostem. Terpendole C detected by LCMS/MS had moderate cross-reactivity in the paxilline ELISA, whereas, the cross-reactivity for the other detected indole diterpenes (excluding paxilline) has not been tested. Another group of compounds in the metabolising plant may be causing the adverse effects (albeit reduced) and may not being detected by ELISA or LCMS/MS. Young *et al.* (2009) speculated the less tremorgenic indole-diterpenes could be beneficial to their host by acting

against insects, as has been demonstrated for the potent insecticide, nodulisiporic acid A (Ondeyka *et al.*, 1997), a biogenically related yet structurally distinct compound (Byrne *et al.*, 2002). Nodulisporic acid has good insecticidal activity against a range of insects.

In the current trial using seed in artificial diet, the dry weight concentration of paxilline IRE at which feeding deterrent effects occurred was 18 µg/g (equivalent to a wet weight concentration of 1.65 µg/g), less than the 25 µg/g threshold estimated in plants suggest in Trial 1 Chapter 4. This may be explained by the concentration and proportion of the proposed specific paxilline-like secondary metabolites associated with reduced African black beetle (*H. arator*) feeding being higher in seed than pseudostem. Alternately, the deterrence observed in this current trial may have been associated with other unrelated compounds.

The indole diterpenoid chemical profile (of known compounds) was determined by LCMS/MS (Section 6.2.7) for pseudostem plant material collected from plants that were not previously exposed to feeding by adult African black beetle (*H. arator*). The proposed specific paxilline-like secondary metabolites associated with reduced feeding damage from adult African black beetle (*H. arator*) *in planta*, therefore, may not have been produced or detected in this material. A comparison of the indole diterpene profile (including paxilline-like alkaloids) profile of plants exposed to feeding adult African black beetle (*H. arator*) with those that are not exposed prior to analysis by LCMS/MS may indicate if, and which indole diterpenoids are associated with bioactivity *in planta*. Feeding by adult African black beetle (*H. arator*) is complex, influenced by a balance of both positive feeding stimulants and anti-feedants.

The adverse effect of AR1 in seed on adult African black beetle (*H. arator*) feeding is likely to be due to one or a group of compounds concentrated in the seed. These compounds may or may not be detected by LCMS/MS or by paxilline ELISA. The two detection systems, LCMS/MS and ELISA, use different extract solvent systems (non-polar and methanolic respectively), each optimised carefully to overcome matrix effects for the method of analysis. The different solvents extract a different range of compounds and in different proportions, although the extracts will contain compounds in common. The active compound or group of compounds in seed may

not be found in the plant (pseudostem section) or be produced in large enough quantities to be effective until the plant has been damaged. A further possibility is that other, unidentified active compounds could be produced that are not present in extracts from either solvent system or if present in the extract, not detected by either detection system (paxilline ELISA or LCMS/MS).

Given the strong effect of AR1-infected seed on African black beetle (*H. arator*), the use of seed for the isolation and characterisation of bioactive compounds produced by AR1 endophyte that provide in planta resistance against adult African black beetle (*H. arator*) appears to be the best option available. However, the use of pseudostem plant material for feeding trials and the methods for the bulk extraction of alkaloids from seed, do need further investigating.

Female beetles have generally been observed to be larger than the male beetles (Todd, 1959) and this is confirmed by their higher weight in these experiments. This gender difference in size is believed to be caused by natural selection for large females due to a fecundity advantage, a common occurrence observed in many insects, fish, birds and certain mammals (Vollrath & Parker, 1992; Hayssen & Kunz, 1996; Arnqvist *et al.*, 2003; Bornholdt *et al.*, 2008). The difference observed between the genders in weight gain from the start to the end of the trial (6.31% and 4.06% for females and males respectively) are consistent with the gender disparity in energy requirements for reproduction and sexual size dimorphism (Darwin, 1874; Shine, 1989; Fairbairn, 1997).

Diet consumed and diet score were consistent with average change in beetle weights except for the females from the carrot agar treatment. Females on carrot agar consumed very large amounts of this diet compared to those on endophyte-free seed diets but this was not reflected in higher weight gains. This may be due to the seed-based diets (and raw carrot) having a greater nutritional value or calorie count and lower moisture content than the carrot-agar diet for the same amount of diet eaten. Males on the other hand did not consume significantly more carrot agar than males on seed diets, suggesting the nutritional value was sufficient at this time for male beetles to maintain and gain weight. This may be due to larger energy requirements of the larger females at this time in the African black beetle (*H. arator*) lifecycle for reproduction, as sperm production is less costly than egg production. It would

be interesting to determine if this gender difference in consumption would be as large in autumn, when the new generation of adult African black beetle (*H. arator*) are feeding at high intensity to build up their fat energy stores before the winter dormancy period but when sexual reproduction is not occurring.

Despite the general differences in feeding, male beetles were on average more active than the female beetles during the treatment phase and overall. This may have been due to behaviourally related influences other than feeding, with the male beetles searching for a mate. This gender difference was masked in the post-treatment phase of the trial because the beetles that had been on the AR1-infected seed diet that lost weight significantly increased their feeding and, thus emerged more often from the soil and were buried significantly less often during the daytime than the beetles that had been on the other treatments. In spring and autumn adult African black beetle (*H. arator*) are normally active at dusk and remain buried during the daytime. The increased activity of the beetles exposed to AR1 likely results from a compensatory requirement for food. Therefore the presence of endophytes that deter beetle feeding, including AR1, may modify the behaviour of beetles after exposure, and in the field this may increase their vulnerability to diurnal predators, such as birds.

Results from Trial 3b (not reported) were not useable because adult African black beetle (*H. arator*) had ceased regular feeding. Regular feeding occurs in spring (post-winter) prior to egg production and again in autumn prior to the African black beetle (*H. arator*) overwintering dormancy period (Todd, 1959, 1964). It is surmised that in late spring, once the beetle fat reserves have built up post-winter and for sexual reproduction, feeding becomes irregular but beetles were observed remaining active searching for potential mates. In late autumn/early winter once the beetle has built up sufficient fat reserves, they enter a dormancy phase (Todd, 1959, 1964) and feeding becomes irregular, then appears to largely cease although beetles were observed to periodically emerge on warm days to feed during this period. To date, regular feeding by adult African black beetle (*H. arator*) in the laboratory cannot be maintained and the dormancy period cannot be averted.

From Trial 3b, it was found that high levels of immunoreactivity remained in the bulk extracted seed when re-extracted and analysed by the paxilline ELISA. The

concentration found was lower than that in seed without bulk extraction and higher than that contained in the extract. This result indicated that further work needs to be done to improve or modify the bulk extraction procedure of paxilline-immunoreactive compounds from seed, including multiple extraction steps. In addition, the level of paxilline immunoreactivity in the diet containing extract (E+ seed) was unstable and lost within 5 days, and may be due to removal of the alkaloids from protective components in the seed, such as antioxidants. For future diet work, the diets containing extracts would either need to be made up on a more regular basis (possibly daily) and work would need to be done to improve the stability of the extracted immunoreactive compounds in African black beetle (*H. arator*) artificial diets. In addition, feeding trials using artificial diets made with undamaged pseudostem material (Chapter 4) would be useful.

Although the methanolic bulk extraction of AR1-infected ryegrass seed was found not to be optimal, this does not affect the immunoreactivity results from Trials 1 and 2. Trial 1 and 2 results were based on a small scale (50mg) methanolic extraction optimised for ELISA. The small scale extraction, although may not extract 100% of paxilline immunoreactive equivalents, is reproducible. Therefore results can be compared between Trials 1 and 2. The large scale bulk extraction (Trial 3b), however, had not been optimised for ELISA. In addition, different substrates were extracted, with vegetative plant material used in Trials 1 and 2, and seed in Trial 3.

The indole diterpenoid profiling and work with components incorporated in artificial diet requires a significant amount of developmental work to determine how to best incorporate pseudostem plant material (freeze-dried or fresh) into artificial diets for adult African black beetle (*H. arator*), and would involve large chemistry- and biochemistry-based inputs, which are outside the bounds of this PhD study. The decision was then made to not continue with artificial diet experiments but to seek further evidence that there was an endophyte-host heritable factor in AR1-infected plants that may be used to enhance the effect of AR1 on African black beetle (*H. arator*).

6.5 Summary

Artificial diet containing seed of cultivar 'Extreme AR1' showed evidence of adverse effects that resulted in beetles unable to maintain current weight or gain weight. Once beetles had an endophyte-free food source the beetles recovered and the beetle weights returned to normal. These results (Trial 3a) suggest a strong deterrent effect from AR1 endophtye.

The presence of AR1 endophyte did not modify the behaviour of the adult African black beetle (*H. arator*) when considering emergence activity at dusk and the time spent on the surface during diurnal hours. However, it did influence feeding behaviour by reducing the level of feeding. Adult African black beetle (*H. arator*) behaviour was affected post-exposure to AR1 endophyte, with both emergence activity at dusk and diurnal surface time increasing, potentially increasing beetle vulnerability to predators (these behaviour changes were consistent with large weight gain).

The methanolic bulk extraction of AR1-infected ryegrass seed did not extract all the immunoreactivity detected by the paxilline ELISA, with higher levels of immunoreactivity left in the bulk extracted seed than in the extract. This immunoreactivity was stable in the artificial diets containing seed, but was unstable and not detectable within 5 days from the artificial diets containing extracts. Although the bulk extraction of AR1-infected seed was not optimal, this does not affect the immunoreactivity results from Trials 1 and 2. In Trials 1 and 2 a small scale extraction of vegetative plant material, optimised for ELISA was used. Whereas in Trial 3b, a large scale bulk extraction of seed, which had not been optimised was used.

The non-polar indole diterpenoid profiles (determined by LCMS/MS) of 'Extreme AR1' although similar are different for seed and pseudostem. The majority of the compounds detected in both seed and pseudostem were more concentrated in seed and had higher relative expression levels in seed than in plant pseudostem (not exposed to adult African black beetle (*H. arator*)), but not for terpendoles K and N. These compounds had much higher relative expression levels in the pseudostem, than in the seed. An indole diterpenoid LCMS/MS profile from plants (pseudostem) that had been exposed to feeding from adult African black beetle (*H. arator*) may

be different (by relative expression levels) from that of an unexposed plant and from seed.

The decision was made to focus on trials with AR1-infected plants with the aim of identifying AR1-infected ryegrass combinations with high resistance to feeding by adult African black beetle (*H. arator*) and to cease any further trial work using artificial diets. Further work is required for optimising the composition of diet, optimising extraction of seed and plant material, and to overcome stability issues.

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Chapter 7

Selection for resistance to *Heteronychus arator* (Coleoptera: Scarabaeidae) in perennial ryegrass infected with *Epichloë* strain, AR1, on the basis of paxilline immunoreactivity

This chapter is written as a journal paper. It includes trial work that was not performed by this author but has been discussed earlier in this thesis (Lincoln Trial), previous work already presented in this thesis (Trials 1 and 2) and work new to this thesis (Trial 4):

- *Trial A = Lincoln Trial; Popay A. J., Fletcher L. R., Briggs L. R., AgResearch, New Zealand; unpublished work, 2006 (first presented on p51, also see Figure 1-7 and Section 4.3.6).*
- *Trial B = Trial 1 (first presented in Chapter 4).*
- *Trial C = Trial 2 (first presented in Chapter 5).*
- *Trial D = Trial 4 (first presented in Chapter 7).*

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7.1 Abstract

A series of adult African black beetle (*Heteronychus arator* (Fabricius, 1775)) choice feeding trials (A–D) using AR1-infected perennial ryegrass (*Lolium perenne* L.) cultivars (plus endophyte-free) and half-sibling families selected from within a breeding line, found differences between lines for levels of feeding damage, paxilline immunoreactivity and tiller production. The presence of endophyte was only an advantage when adult beetle feeding levels were high. At low feeding levels there was no advantage. A negative trend between mean feeding damage and mean paxilline immunoreactivity was observed in Trial A. Although significant differences in feeding damage and paxilline immunoreactivity were found in subsequent trials, there was no significant relationship evident. The correlation results in Trial B, however, suggest there is a subset of paxilline-like compounds that could be associated with reduced feeding damage from adult beetles. Other paxilline-like compounds are likely masking the detection (by paxilline ELISA) of this association until the paxilline-IRE concentrations reach a threshold of ≥25 µg/g in the base of the tillers. Plant resistance to attack by adult African black beetle (*H. arator*), plant size and tiller production, were traits strongly influenced by host plant genetics although a general increase in tiller production was accentuated by herbivory and was not cultivar or family specific. The highest concentrations of paxilline immunoreactivity were found in the undamaged pseudostems, next in the damaged pseudostem and the lowest in the herbage, with levels higher in plants not exposed to feeding from adult beetles. There was strong evidence of endophyte and family or cultivar effects for both feeding damage and plant tiller number with benefits for hosting the AR1 endophyte.

7.2 Introduction

African black beetle (*H. arator*) is a major pest of grasses throughout the northern part of New Zealand's North Island, including Waikato, Bay of Plenty and Hawkes Bay. It was generally regarded as a sporadic pest but an outbreak in the Waikato and Bay of Plenty in 2007–8 persisted for three seasons (Bell *et al.*, 2011). Widespread outbreaks of this insect are economically devastating for farmers. Adult beetles feed at the base of tillers and can destroy new grass seedlings but it is the root-feeding larvae that do the most damage to the persistence and productivity of

grass-based pastures (Popay & Baltus, 2001; DairyNZ, 2010). The adult beetle is deterred by certain fungal endophytes in ryegrass and tall fescue resulting in fewer eggs and therefore fewer root-feeding larvae in the pasture (Popay & Baltus, 2001; Thom *et al.*, 2013).

Perennial ryegrass (*L. perenne*) is the most predominant and intensely grazed pasture in New Zealand (Belgrave *et al.*, 1990; Easton & Tapper, 2005). It is easily established, very productive and highly digestible. In New Zealand perennial ryegrass (*L. perenne*) contains the clavicipitaceous endophytic fungus, *Epichloë festucae* var. *lolii*[1], formerly *Neotyphodium lolii*[2] (Leuchtmann *et al.*, 2014). This endophyte causes toxicosis in grazing livestock (Fletcher *et al.*, 1999; Easton & Tapper, 2005) but is essential for plant persistence in pastures through resistance to invertebrate pests (Prestidge & Ball, 1993; Easton & Tapper, 2005). *Epichloë coenophiala*[3] of tall fescue (*Lolium arundinaceum*[4]) and *E. festucae* var. *lolii* of perennial ryegrass (*L. perenne*) are the two most extensively researched endophytes due to their agronomic importance particularly in the United States of America and New Zealand, respectively.

The anamorphs of *Epichloë* species previously described in the genus *Neotyphodium* (Leuchtmann *et al.*, 2014), infect cool-season grasses (C3 grasses) in the subfamily Pooideae. The feature that distinguishes these *Epichloë* anamorph endophytes from the other clavicipitaceous fungi is they lack the capacity to reproduce sexually, and instead they are propagated by vertical transmission via the host seed. They are obligate biotrophic endosymbionts, solely reliant on their host grass for survival and reproduction (Ewald, 1987). The asexual fungus provides its host with protection against biotic and abiotic stress through the production of alkaloids (Clay, 1988a, 1988b, 2009). The wild-type *E. festucae* var. *lolii* of

[1] *Epichloë festucae* var. *lolii* (Latch, M.J. Chr. & Samuels) C. W. Bacon & Schardl, stat. nov. et comb. nov..

[2] *Neotyphodium lolii* (Latch, M.J. Chr. & Samuels) Glenn, C.W. Bacon & Hanlin.

[3] *Epichloë coenophiala* (Morgan-Jones & W. Gams) C.W. Bacon & Schardl, comb. nov. (formerly *Neotyphodium coenophialum* (Morgan-Jones & W. Gams) Glenn, C. W. Bacon & Hanlin and *Acremonium coenophialum* Morgan-Jones & W. Gams).

[4] *Lolium arundinaceum* (Schreb.) Darbysh. formerly *Festuca arundinacea* Schreb.

perennial ryegrass (*L. perenne*) that was introduced into New Zealand produces the following secondary metabolites: ergovaline, lolitrems and peramine (Tapper & Latch, 1999). Wild-type *E. festucae* var. *lolii* causes both mammalian toxicity (Fletcher & Harvey, 1981) and resistance to Argentine stem weevil (*Listronotus bonariensis* (Kuschel, 1995)) (Mortimer *et al.*, 1982; Prestidge *et al.*, 1982). Mammalian toxicity was attributed mainly to two classes of alkaloids, the ergot alkaloids (ergovaline) and the indole diterpenes (lolitrems). Ergovaline is responsible for heat stress (Fletcher & Easton, 1997; Fletcher *et al.*, 1999) in animals and the lolitrems for ryegrass staggers (Gallagher *et al.*, 1981, 1982b, 1982a, 1984; Tapper *et al.*, 2004). Peramine is not known to be toxic to grazing animals, but is a potent feeding deterrent to Argentine stem weevil (*L. bonariensis*) (Prestidge *et al.*, 1985; Rowan & Gaynor, 1986; Rowan *et al.*, 1990a). The ergot alkaloids and indole diterpenes are known to demonstrate anti-insect activity and toxicity. Endophyte strain, plant genotype, tissue type, season, plant age and abiotic and biotic stresses influence alkaloid profile and concentrations (Clay & Schardl, 2002; Easton *et al.*, 2002; Popay *et al.*, 2003; Rodriguez *et al.*, 2009).

There are diverse strains of *E. festucae* var. *lolii* that occur in natural grasslands worldwide. In New Zealand, many of these strains are being collected and screened, for potential use in the forage industry. Selected 'novel' *E. festucae* var. *lolii* strains are used in commercial cultivars that do not produce, or produce less of, the alkaloids known to be toxic to mammalian animals, whilst still maintaining anti-insect activity.

The *E. festucae* var *lolii* strain, AR1 was developed to provide its host perennial ryegrass (*L. perenne*) with resistance to Argentine stem weevil (*L. bonariensis*) through the production of peramine without any adverse effects on animal production. AR1 produces simple indole diterpenes (including paxilline and paxilline-like compounds such as the terpendoles) but it lacks the genes for the production of more complex indole diterpenes such as lolitrem B (Young *et al.*, 2009), which is highly detrimental to livestock (Tapper *et al.*, 2004). Although AR1 genetically can produce paxilline, a mild tremogen (Miles *et al.*, 1992) the amounts produced are considered minimal (Young *et al.*, 2009). Instead production of the other simple indole diterpenes including the paxilline-like compounds is more

predominant (Young *et al.*, 2009). To date there have never been any reports of mammalian toxicity in livestock from AR1-infected pastures (Bluett *et al.*, 2005b; Bluett *et al.*, 2005a). AR1 endophyte does not produce ergovaline, the only alkaloid known to deter adult beetles (Ball *et al.*, 1997) but, despite this, plants infected with AR1 show some resistance to this insect (Popay & Baltus, 2001; Thom *et al.*, 2013). Lolines have also been indicated to deter African black beetle (*H. arator*) (Barker *et al.*, 2014), but *E. festucae* var. *lolii* in perennial ryegrass (*L. perenne*) does not produce lolines (Clay & Schardl, 2002). AR1-infected ryegrass is not recommended for use in black-beetle-prone areas, because other endophytes are available to farmers that have stronger resistance.

The chemical basis for the mild deterrent effects of AR1-infected ryegrass on African black beetle (*H. arator*) is not understood. In this study we sought to determine if African black beetle (*H. arator*) resistance was related to the differences in paxilline immunoreactivity and whether this could be used to select breeding lines with improved resistance to African black beetle (*H. arator*). Selection of AR1 cultivars highly resistant to African black beetle (*H. arator*) would provide a significant advantage to farmers given the lack of animal toxicity associated with this endophtye.

7.3 Materials and Methods

Feeding trials involving adult African black beetle (*H. arator*) were undertaken between 2006 and 2011 at Ruakura Research Centre, Hamilton, New Zealand.

Plants. *Seed germination and seedling plants*. Seed of cultivars containing AR1 endophyte or endophyte-free equivalents were obtained from the AgResearch Margot-Forde Germoplasm Centre, Palmerston North, New Zealand. Seed of the half-sibling families from the BL-1 breeding-line were sourced from Forage Improvement, AgResearch, Grasslands, Palmerston North, New Zealand. Germination was achieved by spreading seeds on damp filter paper in petri-dishes sealed with parafilm and left in the dark at 20°C for 7 to 10 days. Germinated seedlings were planted out into plant pots (100 mm depth × 125 mm top diameter) or polystyrene trays (internal 495 mm L × 300 mm W) containing a general purpose potting mix (Daltons GB Potting Mix) for establishment (6 weeks minimum).

Cloned plants. Plants were cloned by splitting the original plant into ramets of four to six tillers and re-planted. This always included at least one spare clone being re-planted back into the original tray or pot. The cloned plants were first planted out into mortar sand to encourage root development for 10–14 days and then transferred into polystyrene trays containing general purpose potting mix for establishment (2 weeks minimum).

Plant maintenance. Seedling and cloned plants were housed in either a glasshouse (late autumn–early spring) or screenhouse (late spring–early autumn) depending on the ambient air temperature outside for both establishment and plant-based trials. Plants were fertilised post-trimming, using Thrive All Purpose Soluble Fertiliser (NPK analysis; 27 : 5.5 : 9, with trace elements) from Yates (Auckland, New Zealand; sourced from local stockists) at the recommended label rate (1.8 g/L) with the addition of urea (2.5 g/L) dissolved in tapwater).

Endophyte status. After plant establishment the endophyte status (endophyte-infected, E+ or endophyte-free, E-) of all seedling and cloned plants (>6 weeks old) were checked using the endophyte tissue immunoblot technique (Gwinn *et al.*, 1991; Simpson *et al.*, 2012). A single tiller sample was tested from each plant. Selected plants with the correct endophyte status and a minimum of five tillers were replanted into containers as required for each specific trial.

African black beetle. Adult African black beetle (*H. arator*) were collected from the field when they were active and feeding regularly either in autumn when teneral adults had recently emerged or spring when having overwintered and entering their reproductive phase. Beetle collection was done by pitfall trapping with traps checked daily in the early morning. In the laboratory, African black beetle (*H. arator*) were separated by gender and maintained at ambient temperature (~20–25°C) in containers with perforated lids and partly filled with moist soil. A diet of sliced carrot was fed.

Grass sample preparation. Plant samples (herbage and pseudostem) were frozen at –20ºC then freeze-dried. After storage at –20°C freeze-dried samples were equilibrated to ambient room temperature (21°C) and then ground either by the IKA-A10 blade mill (IKA®-WERKE, Staufen, Germany) for small samples (≤2 g)

or by the Udy Cyclone mill (Udy Corporation, CO, USA) for larger samples (≥2 g). Milled samples were stored in sealed containers at –20°C until required for analysis by ELISA.

Extraction of grass samples for paxilline and peramine analyses. All grass samples (herbage and pseudostem) were equilibrated to 21°C and then accurately weighed (50 mg ± 0.5 mg) using a four place balance (Mettler AE 260 Delta Range) in an Eppendorf microfuge tube (2 ml) and stored at –20°C until required. Samples that required analyses by both the paxilline and peramine ELISAs had the same extract analysed concurrently. Samples were extracted on the same day the paxilline ELISA was performed, as previous studies have shown that immunoreactivity determined by the paxilline ELISA decreases on storage. After addition of extracting solvent (1 ml, 90% HPLC grade methanol in water), samples were rotated end-over-end using a Labnet mini labroller (NJ, USA) for 20 min. Samples were centrifuged at 8 609 ×g for 5 min (Eppendorf Centrifuge 5418), the supernatant collected and analysed by ELISA.

ELISA analyses. ELISA data analysis was performed using software developed in-house (4-parameter curve fit) at AgResearch. The ELISA analysis of samples was repeated within 24 h if a result obtained was out of the working range of the assay or if there was >10 % CV on replicate determination.

Paxilline ELISA. Plant (herbage and pseudostem) and seed extracts were analysed using an indirect competitive paxilline ELISA developed in-house at AgResearch (Garthwaite *et al.,* 1993) with the following modifications. Phosphate buffered saline (PBS) containing 0.05% Tween 20 (v/v) was the wash buffer (PBST). The blocking agent and antibody buffer was 1% bovine serum albumin (w/v) in PBST (1% BSA/PBST). Sheep anti-mouse conjugated to HRP (Chemicon, CA, USA) was the secondary antibody. The HRP substrate was BioFX TMB One Component HRP microwell substrate buffer (SurModics, MN, USA). The results generated were expressed as µg of paxilline-immunoreactive equivalents (paxilline-IRE) per g of milled grass, as paxilline was the reference compound used to generate the standard curve in the assay. Assay working range was 1–40 ng/ml with a detection limit for the undiluted grass extract of 0.18 µg/g dwt.

Peramine ELISA. Plant (herbage and pseudostem) extracts were analysed using an indirect competitive peramine ELISA developed in-house at AgResearch (Garthwaite *et al.*, 1994) with the same modifications as for the paxilline ELISA except the secondary antibody was rabbit anti-sheep conjugated to HRP (DAKO, Denmark). Results generated take into account the dilutions used on the ELISA plate and were expressed as µg of peramine-immunoreactive equivalents (peramine-IRE) per g of milled grass, as peramine was the reference compound used to generate the standard curve in the assay. Although it was anticipated that there should be no other peramine-like compounds that would be detected by the assay, results were expressed as peramine-IRE because the assay has not been quantitatively validated against a reference method. The assay working range was 2.5–350 ng/ml with a detection limit for the undiluted grass extract of 0.45 µg/g dwt.

Lolitrem ELISA. Single tiller extracts were analysed using an indirect competitive lolitrem ELISA developed in-house at AgResearch (Briggs *et al.*, 2007), with the following sample extraction modification for the qualitative analysis of fresh single tiller samples (developed and validated in-house at AgResearch). One basal tiller section (2.5 mm long) per well was placed in a Nunc 96-well untreated plate, with every second row left empty. Plates were sealed using Nunc sealing tape and lids and stored at −20°C until ready for tiller extraction and ELISA analysis. To each well containing a tiller, 90% methanol in water (125 µl) was added. The plate was sealed and incubated on a plate shaker (IKA-SCHÜTTLER MTS 4) at 21°C for 1 h. Extracts (100 µl) were removed and placed in the corresponding well in the adjacent row and 0.1 mol/l HCl (50 µl) was added. The plate was sealed and the samples were hydrolysed for 15 min at 37°C. The hydrolysates (50 µl) were removed and added to PBST (250 µl) in titretubes. The resulting hydrolysed extracts were at a final dilution of 1 in 9, in 10% methanol/PBST. Residual acid in the diluted extracts had been previously shown to not affect results. Although results were expressed as ng of lolitriol-immunoreactive equivalents (lolitriol-IREs) per ml of tiller extract, these were only qualitative giving a yes or no result for wild-type contamination.

Trial Design. *Trial A.* In a pilot trial to determine if African black beetle (*H. arator*) feeding differed on seven ryegrass cultivars known to vary in paxilline immunoreactivity, four diploid lines ('Alto', 'Arrow', 'Extreme' and a non-commercial cultivar (NC-1) and three tetraploid lines; 'Galaxy', 'Quartet' and a second non-commercial cultivar (NC-2), each infected with AR1 (E+) were used. The endophyte-free (E-) cultivar 'Extreme' was also used as the nil control. Seed was sown in 0.15 ha blocks in autumn 2005 at Lincoln, Canterbury, New Zealand. The fields had been grazed regularly by sheep. Twenty plants from each block were randomly selected and transferred to Ruakura Research Centre in autumn 2006. Plants were potted into commercial potting mix and maintained in a screenhouse. After each plant was tested for endophyte presence by immunoblot, one plant of each treatment with the appropriate endophyte status was transplanted into a polystyrene tray to provide a choice feeding test for the African black beetle (*H. arator*). Plants were arranged in 2 × 4 grid pattern within each planter box (500 × 300 mm) with 100 mm spacing between each plant. The position of each treatment within the row was fully randomised. Fifteen replicate trays were prepared and maintained in a glasshouse.

Eight weeks later, eight adult African black beetle (*H. arator*), collected from the field, were released onto each of 10 replicates, chosen as having the healthiest plants. Each tray was then covered with fine nylon mesh material supported by wire frames. African black beetle (*H. arator*) damage assessments were carried out on each plant 4 weeks later. The number of new and old undamaged tillers was recorded, with new tillers being those that had emerged since the beetles were introduced to the plants. Damage was categorised as minor where there was surface feeding only, moderate where damage had partially penetrated the tiller and severe where the tiller was shredded completely and unlikely to survive.

At the same time as African black beetle (*H. arator*) feeding was assessed, the basal 20 mm of leaf sheath was sampled from 10 tillers on each plant for analysis of paxilline immunoreactivity by ELISA. Where possible, only old undamaged tillers were sampled but to ensure that 10 tillers were sampled per plant new undamaged tillers and/or old damaged tillers were also taken to provide sufficient material for

the ELISA. Two tillers from each plant were also tested for endophyte by immunoblot.

Trial B. In this choice feeding experiment 23 half-sibling families of *L. perenne* infected with AR1 from a single breeding line BL-1 (diploid) were tested to determine if there were differences in adult African black beetle (*H. arator*) feeding damage and if this was related to paxilline immunoreactivity. Plants had been grown from seed in polystyrene trays and paxilline immunoreactivity tested for by ELISA in herbage (50 mm section; 30–80 mm from the crown). Plants (and families) had also been screened for wild-type *E. festucae* var. *lolii* endophyte contamination using the lolitrem ELISA. From this initial screening, 50 individual AR1-infected plants were then selected for further testing based on the level of paxilline immunoreacitivity in herbage, plant tiller number (≥10) and plant vigour score (≥3; graded on a scale of 1–5, with 1 = weak unhealthy plant and 5 = strong healthy plant), across all families and across the range of concentrations of paxilline immunoreactivity. Representatives from the three family lines contaminated with wild-type endophyte were also included in the selection as benchmark plants and families with high levels of resistance to feeding by adult African black beetle (*H. arator*). Selected plants (50) were cloned to provide four genetically identical plants with one clonal plant used in each of three experimental replicates and the remaining clone replanted back into the original tray in early spring (September 2008). Cloned plants were tested for endophyte presence using immunoblot. For each replicate, cloned plants were planted in a randomised design into polystyrene trays (internal 495 mm × 300 mm) in ten rows of five plants per row, (rows 50 mm apart, plants 50 mm apart). The three replicates were laid out next to each other in a 10 row by 15 column grid. Plant establishment and the trial (in mid-spring, October 2008) were conducted in the glasshouse (temperature range 15–25°C) and plants were watered by hand as required.

Cages were constructed for each replicate using green knitted windbreak (Ultrapro windbreak.LT 915 mm wide, 55% wind porosity, Cosio Plastics, New Zealand), acetate sheets and velcro strips. Prior to addition of adult African black beetle (*H. arator*) at the start of the trial, plants were trimmed to 3 cm from the crown, fertilised and tillers per plant counted (Assessment A0). The first 20 adult African

black beetles (*H. arator*) were then added to each replicate. Depending on availability, 16–18 adult African black beetles (*H. arator*) (per replicate) were added once a week for the next 3 weeks to all replicates to give a total of 70 adult beetles per replicate (48 male, 22 female).

Assessments of feeding damage by adult African black beetles (*H. arator*) were performed after 2 weeks at Assessment 1 (A1) and at the end at Assessment 2 (A2) after 4 weeks. Prior to each assessment plants were initially cut to a height of 80 mm from the crown and the herbage removed was discarded. Plants were trimmed further to 30 mm from the crown, and the 50 mm herbage harvested for analysis by ELISA (paxilline and peramine). At the end of each assessment plants were fertilised. Total tiller number, and number of damaged and undamaged tillers were recorded at each assessment. Damaged tillers were scored on a scale of 1–3; 1 = minor damage (penetrated the outer layers of the tiller only, cuticle and epidermal layer with minimal damage), 2 = moderate damage (penetrated the inner layers of the tiller reaching the vascular bundle but tiller likely to survive) and 3 = severe damage (tiller severed or nearly severed and tiller unlikely to survive). Damaged pseudostem samples (3 cm) were collected at A1 and damaged and undamaged pseudostem samples (separately) at A2 for measurement of paxilline immunoreactivity. Undamaged pseudostem samples were collected from plants that had ≥10 undamaged tillers remaining at the end of the trial, with either 30% or a minimum of five undamaged pseudostems collected.

Trial C. In this choice feeding experiment 10 of the 23 half-sibling families from breeding line BL-1 underwent further testing to determine if feeding damage was related to paxilline immunoreactivity. The original plants grown from seed were the same as those used in Trial B, with 20 individual plants selected (two representatives per family) on the basis of feeding damage and tiller number results from Trial B; high ranked plants (9, high tiller number and low feeding damage), medium ranked plants (4) and low ranked plants (7). A family line contaminated with wild-type endophyte was also included in the selection as benchmark plants and families with high levels of resistance to feeding by adult African black beetle (*H. arator*). Selected plants underwent a two-step cloning process to provide sufficient clonal plants for Trial C. In the first step, plants were cloned to provide

three genetically identical plants. Two clones were replanted into two plant pots (100 mm depth × 125 mm top diameter) and the third clone replanted back into the original tray. Once clonal plants (in the pots) had established the endophyte status of every plant was confirmed to be positive by the endophyte tissue immunblot and was presumed to be AR1 (or wild-type) infected. In the second step, plants from a single pot were cloned into seven clonal plants, six of which were used in Trial C (one per replicate) and the final clone was replanted back into the plant pot. For each replicate, cloned plants (20) were planted in a randomised design into polystyrene trays (internal 495 mm × 300 mm) in four rows of five plants per row, (rows 100 mm apart, plants 50 mm apart). Plant establishment and the trial (in early to mid-summer, December 2009–January 2010) were conducted in the screenhouse (temperature range 15–25°C) and plants were watered by an automated system for 2 hours, 3 times a week.

The trial lasted 8 weeks and was divided into two phases, each lasting 4 weeks; a treatment phase (with and without African black beetle (*H. arator*)) and a post-treatment phase (without beetles). There were four replicates for the treatment with beetles and two replicates for the treatment without beetles. In the treatment phase of the trial both treatments were caged (same design as those used in Trial B). The first 20 beetles were then added to each replicate. Depending on availability, 9–12 adult African black beetles (*H. arator*) were added (per replicate) once a week for 2 weeks (during the treatment phase of the trial). A minimum of at least two beetles per plant was added with a total of 41 beetles per replicate (19 female : 22 male).

Assessments in the treatment phase of the trial were performed as for Trial B, at start of trial and treatment phase (A0), after 2 weeks in the mid-treatment phase (A1) and at the end of treatment phase (A2) after 4 weeks. At the end of the treatment phase, cages were removed and all replicates were left outside (24 h) for adult African black beetle (*H. arator*) to disperse. Replicates (uncaged) were returned to the screenhouse for the post-treatment phase. At the end of the trial and post-treatment phase (A3), plants were trimmed, herbage samples collected (as for Trial B) and tillers counted. Grass samples (herbage, damaged and undamaged pseudostem) were analysed by the paxilline ELISA.

Trial D. In this choice feeding experiment ten lines of perennial ryegrass (*L. perenne*) (plant-line) were selected; three cultivars (AR1-infected and the equivalent endophyte-free), 'Extreme' (diploid), 'Quartet' (tetraploid) and 'Galaxy' (tetraploid), and four diploid AR1-infected half-sibling families (E+) from the breeding line BL-1 (F3, F14, F15 and F23; from a total of 23 families). The seven AR1-infected lines were chosen on the basis of four combinations of two parameters; feeding damage from adult African black beetle (*H. arator*) and paxilline immunoreactivity. Plants were grown initially from seed, after seedling establishment the endophtyte status of each plant was checked using the endophyte tissue immunoblot before individual plants selected for the trial from each plant line were replanted into black planter bags (PB1½; 1.5 Pints, 0.9 L, 90 × 90 × 150 mm, Caranz, New Zealand).

Trial (25 replicates, 250 plants in total) set-up and subsequent assessments were staggered over a 5 day period (day 1–day 5), with five replicates randomly assigned to each of the five sampling periods. Selected plants (transplanted into planter bags) were then placed into metal rings (diameter 330 mm) with a 20 mm base of mortar sand. The location of the ten plant-lines per replicate ring was randomised. Mortar sand was then used to fill the surrounds of the planter bags and made flush (30–40 mm below the top of the metal ring). Plants were left to establish in the glasshouse.

Twelve adult African black beetle (*H. arator*) were added per replicate at a ratio of 1 male :1 female beetle (a high stocking rate of 1.2 beetles per plant). Replicates were covered with green knitted windbreak; (Ultrapro windbreak.LT 915 mm wide, 55% wind porosity, Cosio Plastics, New Zealand) supported by wire frames to enclose the adult African black beetle (*H. arator*) and prevent escape while allowing space for plant growth.

Four trial assessments (A0–A3) were performed over a period of 4 weeks; A0 = day 0, A1 = day14, A2 = day 21, A3 = day 28. Prior to each assessment plants were trimmed to 40 mm from the crown. At the initial assessment (A0), before addition of the adult African black beetle (*H. arator*), a sub-sample of tillers 40 mm from the crown (pseudostem sample) was collected. At each subsequent assessment (A1 to A3) the total number of tillers, undamaged, damaged (feeding by adult African black beetle (*H. arator*)) and dead tillers were recorded. At assessments A1 and A2

the trimmed African black beetle (*H. arator*) damaged tillers were removed and collected (pseudostem sample); the dead tillers were removed and discarded. At assessment A3 all trimmed plant tillers (undamaged, damaged and dead) were removed and collected (pseudostem sample). To ensure enough sample for analysis by ELISA, collected tillers were pooled according to plant-line and day of collection at each assessment, i.e., five replicates were pooled for each day. This resulted in five pooled pseudostem tiller samples per plant-line per assessment (a reduction from a total of 250 individual samples to 50 pooled samples per assessment). All collected tillers were frozen at –20°C at the time of collection and subsequently freeze-dried for analysis by the paxilline ELISA.

Statistical Analyses. *Trial A.* Total tiller number and paxilline immunoreactivity were analysed using one-way analysis of variance (ANOVA) blocked by replicate. To stabilise the variance total tiller number was square root transformed. The number of damaged tillers was analysed using an overdispersed logistic regression model, with rep incorporated as a fixed effect.

Trial B. Total tiller number, peramine immunoreactivity, and paxilline immunoreactivity were analysed using restricted maximum likelihood (REML), with random effects of replicate, row, and column to account for spatial variability. To stabilise the variance total tiller number was square root transformed and immunoreactivity data natural log transformed. The number of damaged tillers was analysed using generalised linear mixed model (GLMM) with an overdispersed Binomial distribution and a logit link. Replicate, row, and column random effects were used to model spatial variability. To assess whether there were differences between plants from a family line, the fixed effect of Plant was reparameterised in terms of Plant within Family. Data from each assessment was analysed both independently and cumulatively.

Trial C. Data from Trial C was analysed analogously to Trial B, but with random effects of replicate, row within replicate, and column within replicate. Plant, Beetle and their interaction were included as fixed effects. A fixed term was also included to account for potential differences between plants within the interior, versus those on the edges, of the tray.

Trial D. Data on total tiller number at each assessment was analysed using repeated measures REML. Random terms, allowing for temporal replicate effects, were included. To stabilise the variance the total tiller number was square root transformed. The number of damaged tillers, assumed to be binomially distributed, was analysed using a repeated measures GLMM with a logit link function. Cumulative tiller number, and cumulative damage, was similarly analysed using REML and GLMM, respectively, with replicate a random effect. Level of paxilline immunoreactivity in pseudostem grass samples was analysed using one-way (ANOVA).

Post hoc tests were conducted using Fisher's least significant difference at the 5% significance level (LSD(5%)). Repeated measures analyses were not used for Trials A–C because there were either no repeated measures (Trial A) or different experiment protocols were imposed prior to each assessment, such as addition (Trials B and C) or removal of beetles, removal of cages and movement of experimental units during trial. All statistical analyses were conducted in GenStat version 16.

7.4 Results

Half-sibling family lines and plants contaminated with wild-type endophyte are highlighted with the superscript WT in the text, and in bold font in tables and figures. For Trials B and C, when comparing the scale of tiller damage (1–3) in the damaged tillers, the data were too sparse to be analysed because the majority of African black beetle (*H. arator*) damaged tillers scored level 3, severely damaged, causing death of the tiller.

Trial A. *Tiller number.* Cultivars listed in rank order from highest to lowest for mean plant tiller number: 'Galaxy,' 'Arrow', 'Extreme', NC-2, 'Alto', NC-1, 'Extreme Nil' and Quartet. Differences ($F_{(7,63)}$ = 2.43; P = 0.029) were found between cultivars for mean plant tiller number Table 7-1 with the tetraploid cultivar 'Quartet' significantly different from 'Arrow', 'Extreme' (diploids), 'Galaxy' and NC-2 (tetraploids) but not 'Alto', NC-1 or 'Extreme Nil' (diploids).

Table 7-1: Trial A; mean plant tiller numbers, mean proportions of feeding damage and mean concentrations of paxilline immunoreactivity, in plant pseudostem for AR1-infected cultivars.

		Plant tiller number*		Proportion of damaged tillers from African black beetle feeding**		Paxilline immunoreactivity (Paxilline-IRE µg/g)	
Ploidy	**Cultivar**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
Diploid	Alto	5.41 (29.3)	0.294	-1.97 (0.12)	0.334	24.19	2.057
	Arrow	6.04 (36.5)	0.294	-1.38 (0.20)	0.255	19.65	2.057
	Extreme	5.58 (31.1)	0.294	-1.61 (0.17)	0.278	21.63	2.057
	NC-1	5.29 (28.0)	0.294	-1.9 (0.13)	0.315	20.97	2.057
	Extreme Nil	5.20 (27.0)	0.294	-0.01 (0.50)	0.245	4.4	2.057
Tetraploid	Galaxy	6.01 (36.1)	0.294	-0.29 (0.43)	0.213	10.58	2.057
	Quartet	4.62 (21.3)	0.294	-1.16 (0.24)	0.302	11.11	2.057
	NC-2	5.59 (31.2)	0.294	-0.26 (0.43)	0.231	12.14	2.057
Fisher's LSD(5%)		0.832		0.906		5.815	
Culitvar Effect							
F-statistic$_{df}$		$2.43_{7,63}$		$8.68_{7,63}$		$11.41_{7,62}$	
P-value		0.029		<0.001		<0.001	

SEM = standard errors of means. LSD(5%) = least significant differences of means (5% level). Max = maximum. *Square-root transformed. **Logit transformed. Back transformed values are in brackets.

Feeding damage. Differences were found between cultivars ($F_{(7,63)}$ = 8.68; *P* <0.001) for mean proportion of damaged tillers from adult African black beetle (*H. arator*) feeding Table 7-1. Listed in rank order from lowest to highest mean proportion of damaged tillers from adult African black beetle (*H. arator*) feeding: 'Alto', NC-1, 'Extreme', 'Arrow', 'Quartet', 'Galaxy', NC-2 and 'Extreme Nil', no significant differences were found between the top five ranked cultivars, and between the bottom five ranked cultivars. However, significant differences were found between the following cultivars; 'Alto' and NC-1 had significantly less damage than 'Galaxy', NC-2 and 'Extreme Nil' and 'Extreme' had significantly less damage than 'Extreme Nil'. A replicate effect was found ($F_{(9,63)}$ = 4.37; *P* <0.001) with some beetles in certain replicates not actively feeding.

Paxilline ELISA. Differences were found between cultivars ($F_{(7,62)}$ = 11.41; *P* <0.001) for mean concentration of paxilline-IRE Table 7-1. Listed in rank order from highest to lowest concentrations of paxilline immunoreactivity ('Alto', 'Extreme', NC-1, 'Arrow', NC-2, 'Quartet', 'Galaxy' and 'Extreme Nil'), all AR1-infected cultivars had significantly higher concentrations of paxilline immunoreactivity than 'Extreme Nil'. No significant differences were found between the top four ranked cultivars or between the bottom three ranked AR1-infected cultivars. Samples were not consistently collected across the replicates and had different compositions, from all old undamaged tillers through to all damaged tillers. This resulted in the proportion of damaged tillers being under-represented in the tiller samples analysed for paxilline immunoreactivity.

Relationship between feeding damage and paxilline immunoreactivity. When cultivar means for feeding damage and paxilline immunoreactivity were plotted it appeared there was an association between feeding damage and paxilline immunoreactivity. However a regression analysis was not significant ($F_{(7,57)}$ = 1.05; *P* = 0.409). Within a cultivar the relationship or trend between feeding damage and paxilline immunoreactivity appeared different depending on the cultivar.

Trial B. *Tiller number.* Significant differences (P <0.05) were found for plant tiller number at all three assessments (A0, A1 and A2) for total tiller number (A0–A2; Table 7-2) between plants[5] irrespective of family, and between plants from the same family line (within family[6]). Initial tiller number (plant size) was related to plant tiller number at subsequent assessments; A1 ($F_{(1,95)}$ = 198.88; P <0.001) and A2 ($F_{(49,69)}$ = 1.46; P <0.001), and this relationship was consistant across all plants. Therefore plants and families that were high tiller producers at the start of the trial were high producers at the end of the trial. Using total tiller production (cumulative tiller number results; Table 7-2) families were grouped by median tiller numbers per plant into high (>30), medium (20–30) and low (<20) categories.

Feeding damage. The proportion of tillers damaged differed ($P \leq 0.05$) between plants[7] and within family[8] at A1, A2 and for the total proportion of tillers damaged in trial (A0–A2; cumulatively; Table 7-2) between plants and within families. A large range was found for the cumulative proportion of feeding damage in plants, with some families being more variable than others (Figure 7-1). If the family variation was large, this was from either clonal plant mean differences within the family or because of large clonal plant variation between replicates. The median cumulative proportion of damaged tillers per plant for the majority of families (21 of 23) was >0.5, including family lines contaminated with wild-type endophyte (Table 7-2; also see Figure 7-1).

[5] Plant effect (individual plants): A0 ($F_{(49,77)}$ = 3.52; P <0.001), A1 ($F_{(49,78)}$ = 3.67; P <0.001), A2 ($F_{(49,75)}$ = 3.12; P <0.001), A0–A2 ($F_{(49,80)}$ = 3.88; P <0.001).

[6] Plant effect (within family): A0 ($Wald_{27}$ = 82.83; P <0.001), A1 ($Wald_{27}$ = 66.60; P <0.001), A2 ($Wald_{27}$ = 51.58; P = 0.003), A0–A2 ($Wald_{27}$ = 69.82; P <0.001).

[7] Plant effect (individual plants): A1 ($F_{(49,75)}$ = 1.83; P =0.009), A2 ($F_{(49,70)}$ = 2.27; P =<0.001), A0–A2 ($F_{(49,80)}$ = 3.88; P <0.001).

[8] Plant effect (within family): A1 ($Wald_{(27)}$ = 51.26; P = 0.003), A2 ($Wald_{(27)}$ = 58.92; P = 0.004), A0–A2 ($Wald_{(27)}$ = 69.82; P <0.001).

Table 7-2: Trial B; mean cumulative plant tiller number, mean proportion damaged tillers and mean plant ELISA immunoreactivity of the clones from individual plants grown from seed (from the 23 half-sibling families). The data were analysed unadjusted for initial tiller number.

		Plant tiller number (Sqrt)*		Proportion damaged tillers (Logit)**		ELISA immunoreactivity (µg/g) at A2 (ln)***							
		Cum		Cum		Peramine				Paxilline			
Family	Plant	A0–A2	SEM	A0–A2	SEM	Herb	SEM	DP	SEM	Herb	SEM	DP	SEM
F1	F1-6	5.21 (27.14)	0.576	1.95 (0.88)	0.754	2.78 (15.1)	0.143	3.02 (19.49)	0.165	1.74 (5.69)	0.231	2.13 (8.41)	0.201
	F1-9	5.06 (25.60)	0.601	0.28 (0.57)	0.669	3.23 (24.23)	0.143	3.28 (25.66)	0.136	2.29 (9.87)	0.232	2.56 (12.94)	0.171
F2	F2-4	3.90 (15.21)	0.579	0.00 (0.5)	0.704	3.13 (21.92)	0.143	3.42 (29.48)	0.165	2.08 (7.98)	0.232	2.49 (12.03)	0.201
	F2-9	5.02 (25.2)	0.582	1.6 (0.83)	0.724	2.59 (12.28)	0.142	2.55 (11.82)	0.136	1.33 (3.79)	0.23	1.77 (5.85)	0.169
F3	F3-1	4.69 (22.0)	0.579	0.74 (0.68)	0.669	3.05 (20.09)	0.142	3.15 (22.29)	0.165	2.28 (9.77)	0.231	2.48 (11.88)	0.199
	F3-6	5.21 (27.14)	0.577	-0.50 (0.38)	0.950	2.62 (12.74)	0.142	2.84 (16.15)	0.136	1.81 (6.09)	0.231	1.86 (6.42)	0.169
	F3-7	4.25 (18.06)	0.580	2.57 (0.93)	0.627	2.98 (18.67)	0.143	3.19 (23.24)	0.136	2.25 (9.53)	0.231	2.52 (12.37)	0.17
	F3-10	5.32 (28.3)	0.579	-0.04 (0.49)	0.605	3.96 (51.61)	0.143	3.22 (24.08)	0.165	3.09 (21.89)	0.231	2.83 (16.98)	0.201
F4	F4-1	4.87 (23.72)	0.584	1.72 (0.85)	0.749								

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Table 7–2 continued

Family	Plant	Plant tiller number (Sqrt)* Cum A0–A2	SEM	Proportion damaged tillers (Logit)** Cum A0–A2	SEM	ELISA immunoreactivity (µg/g) at A2 (ln)*** Peramine Herb	SEM	Peramine DP	SEM	Paxilline Herb	SEM	Paxilline DP	SEM
F4	F4-6	4.01 (16.08)	0.577	2.64 (0.93)	1.039								
	F4-7	3.82 (14.59)	0.592	0.85 (0.70)	0.758								
F5	F5-7	5.45 (29.7)	0.593	1.92 (0.87)	0.774								
	F5-8	4.41 (19.45)	0.599	0.57 (0.64)	0.678								
F6	F6-6	4.97 (24.7)	0.578	0.06 (0.51)	0.666								
	F6-8	2.81 (7.9)	0.580	-1.06 (0.26)	0.844								
F7	F7-6	4.91 (24.11)	0.581	1.51 (0.82)	0.754	3.17 (22.86)	0.143	3.37 (28.02)	0.136	1.92 (6.83)	0.231	2.37 (10.69)	0.169
	F7-9	5.5 (30.25)	0.590	1.8 (0.86)	0.690	3.21 (23.78)	0.142	3.09 (20.98)	0.136	1.77 (5.84)	0.231	2.22 (9.17)	0.169
F8	F8-5	5.22 (27.25)	0.578	0.00 (0.50)	0.628								
	F8-6	3.85 (14.82)	0.580	0.24 (0.56)	0.710								

Table 7–2 continued on next page

Table 7–2 continued

		Plant tiller number (Sqrt)*		Proportion damaged tillers (Logit)**		ELISA immunoreactivity (µg/g) at A2 (ln)***							
						Peramine				Paxilline			
Family	Plant	Cum A0–A2	SEM	Cum A0–A2	SEM	Herb	SEM	DP	SEM	Herb	SEM	DP	SEM
F8	F8-8	3.28 (10.76)	0.579	0.57 (0.64)	0.824								
F9	F9-3	4.29 (18.4)	0.579	1.31 (0.79)	0.702								
	F9-5	2.85 (8.12)	0.582	2.85 (0.95)	1.381								
F10	F10-2	5.04 (25.4)	0.584	1.55 (0.82)	0.717	2.83 (15.86)	0.143	3 (19.03)	0.136	1.55 (4.73)	0.232	2.48 (11.88)	0.17
	F10-9	4.81 (23.14)	0.589	-0.14 (0.47)	0.621	3.15 (22.34)	0.143	3.27 (25.23)	0.136	2.51 (12.3)	0.232	2.6 (13.41)	0.171
F11	F11-1	4.83 (23.33)	0.591	1.5 (0.82)	0.764	-0.02 (-0.02)	0.143	0.03 (0.03)	0.165	-1.32 (0.27)	0.232	-1.36 (0.26)	0.199
	F11-2	4.81 (23.14)	0.591	1.22 (0.77)	0.716	3.1 (21.13)	0.143	3.43 (29.97)	0.136	2 (7.37)	0.231	2.37 (10.73)	0.171
F12	**F12-1**	5.48 (30.03)	0.579	-0.03 (0.49)	0.602	3.06 (20.22)	0.142	3.21 (23.78)	0.165	2.45 (11.54)	0.231	2.73 (15.3)	0.198
	F12-10	6.48 (41.99)	0.596	0.88 (0.71)	0.613	3.11 (21.33)	0.142	2.94 (17.86)	0.136	2.03 (7.58)	0.231	1.88 (6.58)	0.17
F13	F13-6	6.62 (43.82)	0.577	1.72 (0.85)	0.630								

Table 7–2 continued on next page

Table 7–2 continued

		Plant tiller number (Sqrt)*		Proportion damaged tillers (Logit)**		ELISA immunoreactivity (µg/g) at A2 (ln)***							
						Peramine				Paxilline			
Family	Plant	Cum A0–A2	SEM	Cum A0–A2	SEM	Herb	SEM	DP	SEM	Herb	SEM	DP	SEM
F14	F14-5	5 (25)	0.583	0.09 (0.52)	0.642	2.84 (16.03)	0.143	2.67 (13.37)	0.165	1.12 (3.07)	0.231	1.54 (4.65)	0.2
	F14-8	5.54 (30.69)	0.579	0.3 (0.57)	0.637	2.97 (18.41)	0.142	3.12 (21.62)	0.165	1.55 (4.69)	0.23	1.97 (7.16)	0.198
F15	F15-1	5.07 (25.7)	0.579	0.46 (0.61)	0.653	3.18 (23.14)	0.143	3.22 (23.9)	0.136	1.5 (4.49)	0.231	1.71 (5.5)	0.17
	F15-2	4.12 (16.97)	0.581	-0.52 (0.37)	0.689	3.1 (21.26)	0.143	3.28 (25.68)	0.165	1.88 (6.53)	0.232	2.32 (10.18)	0.2
	F15-7	4.51 (20.34)	0.579	-0.78 (0.31)	0.660	2.65 (13.15)	0.143	3.05 (20.07)	0.165	1.17 (3.23)	0.231	2 (7.37)	0.199
F16	F16-2	5.41 (29.27)	0.597	0.66 (0.66)	0.616	3.26 (25.05)	0.143	3.55 (33.95)	0.136	1.78 (5.91)	0.231	2.5 (12.15)	0.17
	F16-7	4.39 (19.27)	0.580	0.54 (0.63)	0.691	3.02 (19.53)	0.142	3.06 (20.31)	0.136	1.35 (3.86)	0.231	1.85 (6.34)	0.17
	F16-10	5.52 (30.47)	0.581	0.94 (0.72)	0.620	3.74 (41.01)	0.142	3.42 (29.69)	0.136	2.45 (11.54)	0.231	2.31 (10.11)	0.17
F17	F17-1	5.78 (33.41)	0.580	1.18 (0.76)	0.627	2.9 (17.14)	0.143	2.85 (16.29)	0.136	1.64 (5.14)	0.231	2.12 (8.35)	0.17
	F17-10	4.58 (20.98)	0.584	0.85 (0.7)	0.657	2.76 (14.72)	0.143	2.54 (11.67)	0.136	0.84 (2.31)	0.232	1.19 (3.28)	0.17

Table 7–2 continued on next page

Table 7–2 continued

		Plant tiller number (Sqrt)*		Proportion damaged tillers (Logit)**		ELISA immunoreactivity (µg/g) at A2 (ln)***							
						Peramine				Paxilline			
Family	Plant	Cum A0–A2	SEM	Cum A0–A2	SEM	Herb	SEM	DP	SEM	Herb	SEM	DP	SEM
F18	F18-1	1.52 (2.31)	0.581	1.7 (0.85)	1.047								
	F18-4	4.84 (23.43)	0.580	1.55 (0.82)	0.688								
F19	F19-1	3.7 (13.69)	0.579	0.97 (0.73)	0.764	3.4 (29.02)	0.143	3.39 (28.76)	0.165	1.48 (4.37)	0.231	2.37 (10.73)	0.2
	F19-2	5.19 (26.94)	0.594	-0.21 (0.45)	0.639	2.95 (18.14)	0.143	3.05 (20.07)	0.136	1.87 (6.48)	0.232	1.84 (6.3)	0.17
F20	F20-1	5.7 (32.49)	0.589	1.34 (0.79)	0.680								
	F20-5	5.04 (25.4)	0.593	0.69 (0.67)	0.887								
F21	**F21-6**	5.39 (29.05)	0.600	0.82 (0.69)	0.645								
F22	**F22-2**	5.5 (30.25)	0.582	0.55 (0.63)	0.616	2.88 (16.73)	0.143	2.61 (12.63)	0.136	1.98 (7.21)	0.231	2.18 (8.82)	0.17
	F22-4	6.18 (38.19)	0.580	-0.32 (0.42)	0.593	3.09 (20.93)	0.143	3.19 (23.22)	0.136	2.7 (14.88)	0.231	2.79 (16.28)	0.17
F23	F23-5	6.74 (45.43)	0.581	1.48 (0.81)	0.553	3.22 (23.93)	0.143	3.16 (22.57)	0.136	2.1 (8.15)	0.231	2.38 (10.75)	0.17

Table 7–2 continued on next page

Table 7–2 continued

		Plant tiller number (Sqrt)*		Proportion damaged tillers (Logit)**		ELISA immunoreactivity (µg/g) at A2 (ln)***							
						Peramine				Paxilline			
Family	**Plant**	**Cum A0–A2**	**SEM**	**Cum A0–A2**	**SEM**	**Herb**	**SEM**	**DP**	**SEM**	**Herb**	**SEM**	**DP**	**SEM**
F23	F23-5	6.74 (45.43)	0.581	1.48 (0.81)	0.553	3.22 (23.93)	0.143	3.16 (22.57)	0.136	2.1 (8.15)	0.231	2.38 (10.75)	0.17
	F23-10	7.5 (56.25)	0.579	0.29 (0.57)	0.617	3.14 (22.01)	0.143	3.39 (28.78)	0.136	2.17 (8.73)	0.231	2.68 (14.61)	0.17
Fisher's LSD(5%)		1.640		3.185		0.394		0.451		0.538		0.518	
Plant Effect (Individual Plants)													
F-statistic$_{df}$		$3.88_{49,80}$		$2.49_{49,63}$		$21.08_{31,52}$		$15.82_{31,37}$		$16.19_{31,43}$		$19.46_{31,41}$	
P-value		***<0.001***		***<0.001***		***<0.001***		***<0.001***		***<0.001***		***<0.001***	
Plant Effect (Within family)													
Wald-statistic$_{df}$		69.82_{27}		58.22_{27}		368.36_{18}		342.51_{18}		257.19_{18}		370.88_{18}	
P-value		***<0.001***		***<0.001***		***<0.001***		***<0.001***		***<0.001***		***<0.001***	

A0 and A2 = Assessments 0 (start of trial) and 2 (end of trial). Cum = Cumulative. DP = Damaged pseudostem. $_{df}$ = Degrees of freedom. Herb = Herbage LSD(5%) = least significance difference at the 5% significance level. M1 = fixed effect models 1. (M1). *Square-root transformed. **Logit transformed. ***Natural log transformed. Back transformed values are in brackets. Family lines and plants contaminated with wild-type endophyte are highlighted in bold font. Statistically significant results are highlighted in bold and italic font. Weak evidence of effects (0.05< P ≤1.0) are highlighted in italic font.

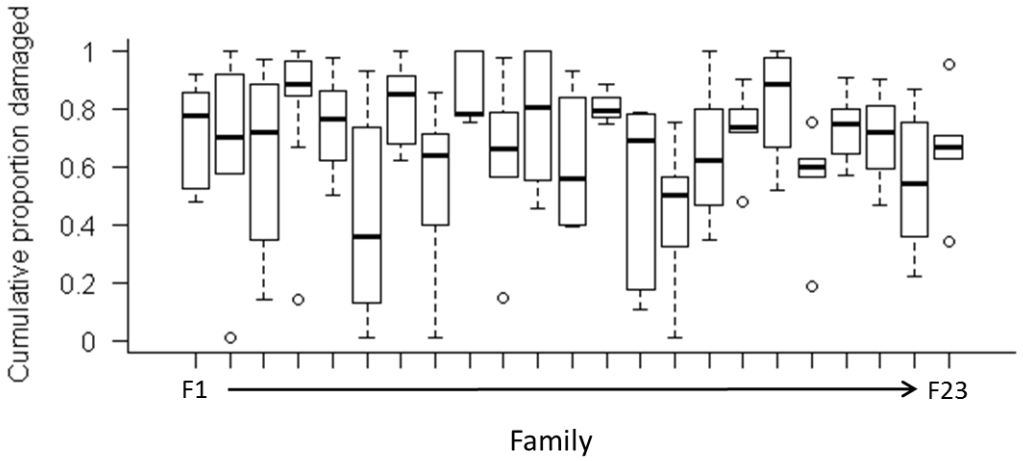

Figure 7-1: Trial B; Cumulative proportion of tillers damaged at end of trial (A2).
Boxplot; displaying family median, upper and lower quartiles and showing variation within and between families. Family lines contaminated with wild-type endophyte are highlighted in bold font.

Families were grouped by the median proportion of damaged tillers into low (≤0.5), medium (>0.5–≤0.75) and high (>0.75) categories, with and only families F6 and F15 in the low category. Families F3, F6, F8, F14, F15, F16 and $F22^{WT}$ had all or the majority of family representatives in the top 25 plants (least damaged) and families underlined accounted for seven of the top ten plants for least amount of damage. Plants from the family lines contaminated with wild-type endophyte were not significantly different from many of the AR1-infected plants (Table 7-2). The proportion of damaged tillers was not related to the initial tiller number of a plant (plant size).

Peramine and paxilline ELISAs. The peramine and paxilline ELISAs were only performed on a subset of samples from 14 of the 23 half-sibling families taken at A2 (Table 7-2). Samples selected for ELISA analyses were on the basis of the tiller number and feeding damage results, with samples from plants likely to be continued with in subsequent trial work analysed. For both peramine (per) and paxilline (pax) immunoreactivity, there were significant differences (P <0.001) between plants and

within family for both the mean herbage [9] and mean damaged pseudostem [10] immunoreactivity (Table 7-2); between plants and within families. Undamaged data were too sparse to be analysed. For both fungal secondary metabolites, variation was larger within some families than others (as shown for concentrations of paxilline immunoreactivity in damaged pseudostem; Figure 7-2), although family variability depended on the plant section analysed (herbage, damaged or undamaged pseudostem). The plant inter-clonal variation in general was small, but for some families there were significant differences between the plant means of the different family representatives and these differences could account for the majority of the variation within the family (Table 7-2). Generally higher levels of peramine and paxilline immunoreactivity were found in the undamaged pseudostem, the next highest being the damaged pseudostem, with the lowest levels found in the herbage (Table 7-3).

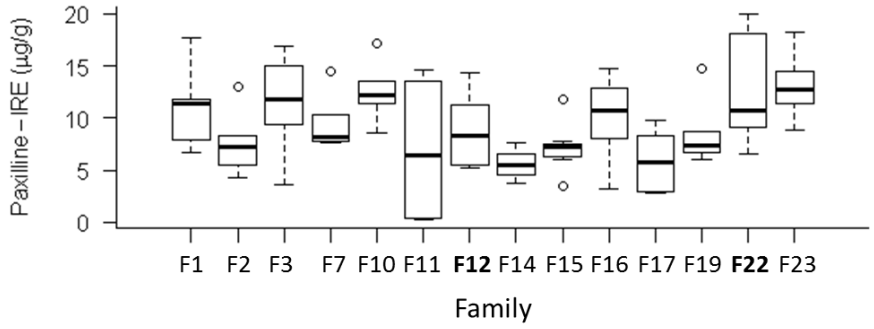

Figure 7-2: Trial B; concentrations of paxilline immunoreactive equivalents (paxilline-IRE) in damaged pseudostem.
Boxplot; displaying family median, upper and lower quartiles and showing variation within and between families. Family lines contaminated with wild-type endophyte are highlighted in bold font.

[9] Herbage: 1) Plant effect (individual plants); per ($F_{(31,52)}$ = 21.08: P <0.001), pax ($F_{(31,43)}$ = 16.19; P <0.001). 2) Plant effect (within family); per ($Wald_{(18)}$ = 368.36; P <0.001) pax ($Wald_{(18)}$ = 257.19; P <0.001).
[10] Damaged pseudostem: 1) Plant effect (individual plants); per ($F_{(31,37)}$ = 15.82; P <0.001), pax ($F_{(31,41)}$ = 19.46; P < 0.001). 2) Plant effect (within family); per ($Wald_{(18)}$ = 342.51; P <0.001), pax ($Wald_{(18)}$ = 370.88; P < 0.001).

Table 7-3: Trial B; distribution of peramine and paxilline immunoreactivity in the three plant sectons, herbage, damaged pseudostem and undamaged pseudostem.

	Peramine-IRE (µg/g)			Paxilline-IRE (µg/g)		
Raw data	**Herbage**	**Damaged pseudostem**	**Undamaged pseudostem**	**Herbage**	**Damaged pseudostem**	**Undamaged pseudostem**
			All ELISA data			
range	1.45–37.49	8.06–40.75	13.13–48.09	1.45–37.49	2.78–19.91	7.05–34.43
median	21.02	21.84	25.48	6.73	9.09	13.76
mean	9.87	21.86	28.77	7.93	9.83	16.21
			Subgroup ELISA data			
range	12.94–48.66	10.85–40.75	13.13–48.09	2.34–23.51	3.37–18.07	7.05–27.68
median	21.55	21.79	24.48	7.15	11.37	13.38
mean	22.02	23.52	28.03	8.53	10.92	15.09

Peramine- and paxilline-IRE = Peramine and paxilline immunoreactive equivalents Subgroup data = subgroup of peramine or paxilline ELISA data in which individual cloned plants had measured levels of peramine or paxilline immunoreactivity for all three plant sections; herbage, damaged and undamaged pseudostem.

Relationships between feeding damage and the ELISA results for peramine and paxilline immunoreactivity. The concentrations of peramine and paxilline immunoreactivity in the different plant sections were compared to the proportion of damaged tillers. No correlations were found between feeding damage and peramine immunoreactivity. Although no correlations were found with paxilline immunoreactivity when comparing the levels in the overall plant pseudostem and in undamaged pseudostem with feeding damage, an interesting cluster of data points (circled) was found (Figure 7-3). The group of four plants making up this cluster had high concentrations of paxilline immunoreactivity in the pseudostem (≥25 μg/g) and low proportions of damaged tillers (≤0.2 or 20%).

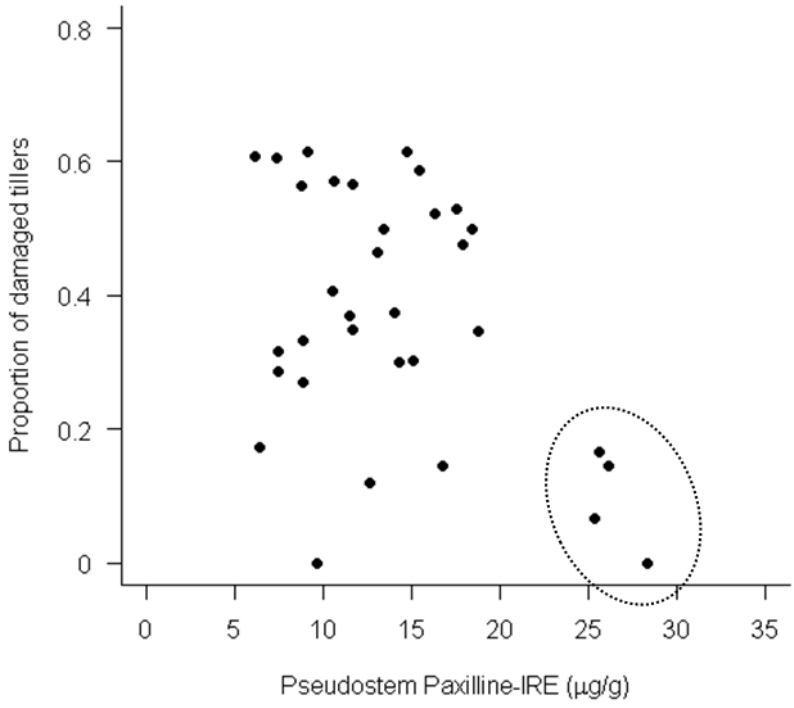

Figure 7-3: Trial B; scatterplot (raw data) between adult African black beetle feeding damage and paxilline ELISA immunoreactivity in overall pseudostem.
Pseudostem = overall pseudostem, in which the concentrations of paxilline immunoreactivity were calculated by summing the two paxilline immunoreactivity levels in damaged and undamaged pseudsotem weighted by the proportion of tillers damaged.

Trial C. *Tiller number.* As for Trial B, positive relationships were found between initial plant tiller number and plant tiller numbers at subsequent assessments; A1 ($F_{(1,87)}$ = 100.02; P <0.001), A2 ($F_{(1,83)}$ = 26.52; P <0.001) and A3 ($F_{(1,97)}$ = 3.91; P = 0.051). This accounts for the significant differences ($P \leq 0.05$) found between plants[11] and within families[12] at all assessments including cumulative tiller number and for cumulative tiller production being constant across assessments and for cumulative tiller production being constant across assessments (Also see Table 7-4; cumulative tiller number A0–A2).

There was evidence of an adult African black beetle (*H. arator*) feeding effect and this effect changed in the different phases of the trial (Table 7-5). Although not significant, mean plant tiller number was higher at the mid-treatment phase (A1) for plants exposed to adult beetle feeding ($F_{(1,4)}$ = 5.80; P = 0.074). By the end of the treatment phase (A2) there was no evidence of mean plant tiller number differences (P >0.1). However, by the end of the post-treatment phase and trial (A3) the effect had reversed by which plants not exposed to beetles had the greater mean plant tiller number ($F_{(1,4)}$ = 4.86; P = 0.092). When the cumulative proportion of damaged tillers was assessed, at the end of the treatment phase (A0–A2) there was weak evidence of a beetle feeding effect ($F_{(1,4)}$ = 6.78; P = 0.060) with greater tiller production from plants exposed to beetles, and by the end of the post-treatment phase this effect was significant ($F_{(1,4)}$ = 10.02; P = 0.034).

For plants exposed to beetles, results for tiller number were consistent with Trial B results (including the post-treatment assessment, A3) with the top performing family representatives in Trial C for plant tiller number coming from the medium and high grouped families from Trial B for median plant tiller number (Table 7-2).

[11] Plant effect (individual plants): A0 ($F_{(19,88)}$ = 7.31; P <0.001), A1 ($F_{(19,89)}$ = 12.67; P <0.001), A2 ($F_{(19,98)}$ = 7.82; P <0.001), A3 ($F_{(19,87)}$ = 3.99; P <0.001), A0–A2 ($F_{(19,88)}$ = 10.44; P <0.001) and A0–A3 ($F_{(19,88)}$ = 5.71; P <0.001).

[12] Plant effect (within family): A0 ($F_{(10,85)}$ = 4.02; P <0.001), A1 ($F_{(10,82)}$ = 5.81; P <0.001), A2 ($F_{(10,89)}$ = 3.50; P <0.001), A0–A2 ($F_{(10,82)}$ = 4.56; P <0.001) and A0–A3 ($F_{(10,82)}$ = 2.04; P = 0.039).

Table 7-4: Trial C; mean plant tiller number (square root scale*), mean proportion of damaged tillers (logit scale) and mean concentration of paxilline immunoreactivity (natural log (ln) scale***) unadjusted for initial tiller number.**

		Tiller number* (Cum A0–A2)				Proportion of damaged tillers** (Cum A0–A2)		Paxilline-IRE (µg/g)*** (A2)								
								w BB					w/o BB			
Family	Plant	w BB	SEM	w/o BB	SEM	Mean	SEM	Herb	SEM	DP	SEM	UdP	Herb	SEM	UdP	SEM
F1	F1-6	4.68 (21.92)	0.426	4.58 (21.01)	0.586	1.50 (0.82)	0.552	0.61 (1.85)	0.171	1.60 (4.95)	0.188	1.67 (5.31)	1.02 (2.78)	0.227	1.97 (7.16)	0.147
	F1-9	4.52 (20.41)	0.417	4.24 (18.01)	0.593	0.08 (0.52)	0.460	0.58 (1.78)	0.161	1.75 (5.74)	0.203	1.83 (6.23)	1.12 (3.07)	0.230	2.29 (9.84)	0.161
F2	F2-4	4.45 (19.8)	0.414	3.85 (14.83)	0.593	0.45 (0.61)	0.475	0.19 (1.21)	0.161	1.35 (3.86)	0.179	1.56 (4.76)	0.56 (1.75)	0.231	1.95 (6.99)	0.161
	F2-9	5.11 (26.11)	0.417	4.55 (20.69)	0.586	0.92 (0.72)	0.453	-0.14 (0.87)	0.159	1.21 (3.35)	0.178		0.16 (1.17)	0.229	1.43 (4.17)	0.218
F3	F3-7	7.55 (56.96)	0.417	6.82 (46.54)	0.594	0.56 (0.64)	0.337	1.22 (3.38)	0.162	1.52 (4.59)	0.177		1.50 (4.47)	0.237	2.28 (9.8)	0.161
	F3-10	8.08 (65.33)	0.425	7.29 (53.2)	0.586	0.85 (0.7)	0.345	1.48 (4.39)	0.163	2.19 (8.97)	0.187	2.79 (16.28)	1.82 (6.15)	0.228	3.00 (20.01)	0.147
F7	F7-6	6.41 (41.09)	0.417	6.23 (38.78)	0.586	1.25 (0.78)	0.408	0.84 (2.32)	0.161	1.79 (6.00)	0.180		0.99 (2.69)	0.228		
	F7-9	4.26 (18.11)	0.417	4.07 (16.55)	0.594	0.95 (0.72)	0.528	0.86 (2.35)	0.162	1.64 (5.16)	0.181		0.95 (2.59)	0.235		
F11	F11-1	3.87 (14.98)	0.417	4.27 (18.21)	0.594	2.78 (0.94)	0.978									

Table 7–4 continued on next page

Table 7–4 continued

Family	Plant	Tiller number* (Cum A0–A2)				Proportion of damaged tillers** (Cum A0–A2)		Paxilline-IRE (µg/g)*** (A2)								
								w BB					w/o BB			
		w BB	SEM	w/o BB	SEM	Mean	SEM	Herb	SEM	DP	SEM	UdP	Herb	SEM	UdP	SEM
	F11-2	5.45 (29.68)	0.417	3.98 (15.83)	0.586	0.14 (0.54)	0.403	0.55 (1.74)	0.160	1.95 (7.01)	0.179	2.39 (10.91)	0.77 (2.16)	0.230	2.24 (9.35)	0.147
F14	F14-5	5.51 (30.34)	0.414	4.47 (19.95)	0.586	0.62 (0.65)	0.408	-0.16 (0.85)	0.160	1.08 (2.93)	0.176	1.77 (5.87)	0.04 (1.04)	0.230	1.47 (4.33)	0.147
F14	F14-8	5.52 (30.44)	0.417	4.36 (19)	0.594	1.56 (0.83)	0.485	0.43 (1.53)	0.187	1.00 (2.72)	0.177	2.30 (9.97)	0.32 (1.38)	0.236	1.38 (3.98)	0.161
F15	F15-1	6.16 (37.98)	0.417	5.67 (32.14)	0.586	1.61 (0.83)	0.453	0.11 (1.12)	0.160	1.48 (4.38)	0.178		0.67 (1.95)	0.229		
	F15-7	5.83 (33.95)	0.417	4.07 (16.56)	0.594	-0.93 (0.28)	0.406	0.03 (1.03)	0.163	1.00 (2.73)	0.204	1.52 (4.57)	-0.22 (0.80)	0.233	1.66 (5.28)	0.161
F19	F19-1	4.75 (22.55)	0.417	4.46 (19.91)	0.586	0.48 (0.62)	0.461	0.88 (2.41)	0.160	1.68 (5.35)	0.178		1.24 (3.46)	0.231		
	F19-2	4.69 (21.96)	0.417	2.53 (6.39)	0.593	1.14 (0.76)	0.489	0.88 (2.4)	0.160	2.31 (10.11)	0.199		1.26 (3.53)	0.320		
F22	**F22-2**	4.63 (21.41)	0.417	5.65 (31.9)	0.593	0.69 (0.67)	0.469	0.80 (2.21)	0.160	1.26 (3.51)	0.179	1.74 (5.70)	1.29 (3.64)	0.230	2.02 (7.51)	0.161
	F22-4	3.98 (15.84)	0.417	3.8 (14.42)	0.586	0.89 (0.71)	0.542	1.40 (4.06)	0.162	1.94 (6.94)	0.206	2.39 (10.91)	1.6 (4.95)	0.234	2.25 (9.49)	0.147
F23	F23-5	5.58 (31.13)	0.426	5.11 (26.08)	0.593	0.65 (0.66)	0.421	1.55 (4.71)	0.169	2.04 (7.71)	0.183	2.62 (13.74)	1.72 (5.57)	0.236	2.11 (8.27)	0.161

Table 7–4 continued on next page

Table 7–4 continued

Family	Plant	Tiller number* (Cum A0–A2) w BB	SEM	w/o BB	SEM	Proportion of damaged tillers** (Cum A0–A2) Mean	SEM	Paxilline-IRE (µg/g)*** (A2) w BB Herb	SEM	DP	SEM	UdP	w/o BB Herb	SEM	UdP	SEM
	F23-10	3.97 (15.75)	0.414	4.13 (17.02)	0.586	2.04 (0.89)	0.730	0.60 (1.82)	0.160	1.90 (6.68)	0.179		1.01 (2.73)	0.228		
Fisher's LSD(5%)			†0.962			2.368		†0.379		0.564			†0.379		0.632	
Fisher's LSD(5%) within trt			1.672					0.790					0.790			
Fisher's LSD(5%) within plant			1.461					0.716					0.716			
Plant Effect (Individual plants)																
F-statistic$_{df}$			$10.44_{19,88}$			$2.76_{19,56}$		$17.73_{18,73}$		$5.53_{18,29}$			$17.73_{18,73}$		$8.57_{12,11}$	
P-value			***<0.001***			***0.002***		***<0.001***		***<0.001***			***<0.001***		***<0.001***	
Plant Effect (Within family)																
F-statistic$_{df}$ or †*Wald*-statistic$_{df}$			$4.56_{10,82}$			$^{\dagger}43.05_{10}$		$5.15_{9,65}$		$^{\dagger}26.71_{9}$			$5.15_{9,65}$			
P-value			***<0.001***			***<0.001***		***<0.001***		***0.002***			***<0.001***			

A0, A2 and A3 = Assessments 1 (start of trial and treatment phase), 2 (end of treatment phase) and 3 (end of post-treatment phase and trial). Cum = cumulative. Paxilline-IRE = Paxilline immunoreactive equivalents. $_{df}$ = degrees of freedom. LSD(5%) = least significance difference at the 5% significance level. †For herbage paxilline immunoreactivity or tiller number, weak evidence of treatment differences (but not significant), plant means for each treatment are tabled separately. Back transformed values are in brackets. Family lines and plants contaminated with wild-type endophyte are highlighted in bold font. Statistically significant results are highlighted in bold and italic font. Weak evidence of effects ($0.05 < P \leq 1.0$) are highlighted in italic font. trt = treatment. w BB = with African black beetle (*H. arator)* treatment. w/o BB = without African black beetle (*H. arator)* treatment. Herb = Herbage. DP = Damaged pseudostem. UdP = Undamaged pseudostem. SEM = standard error of the mean.

Table 7-5: Trial C; African black beetle feeding (treatment) effects.
Mean plant tiller number and herbage paxilline immunoreactivity in the treatment phase (A0, A1 and A2), at the end of the post-treatment (A3) phase and cumulatively; at the end of the treatment (A0–A2) and post-treatment phases (A0–A3) of the trial. The data was analysed unadjusted initial tiller number.

	A0	A1	A2	A3	Cum A0–A2	Cum A0–A3
			†Plant tiller number (M1)			
Mean (w BB)	4.82 (23.23)	5.02 (25.15)	4.48 (20.11)	2.76 (7.61)	5.23 (27.35)	5.48 (30.06)
Mean (w/o BB)	4.2 (17.60)	4.32 (18.65)	4.68 (21.90)	4.28 (18.30)	4.69 (21.97)	4.17 (17.36)
Fisher's LSD(5%)	0.821	0.804	0.357	1.913	0.579	1.154
Treatment Effect						
F-value$_{df}$	$4.47_{1,4}$	$5.80_{1,4}$	$1.18_{1,98}$	$4.86_{1,4}$	$6.78_{1,4}$	$10.02_{1,4}$
P-value	0.102	*0.074*	0.280	*0.092*	*0.060*	***0.034***
			‡Herbage Paxilline-IRE (µg/g) (M1)			
Mean (w BB)			0.66 (1.94)	0.87 (2.39)		
Mean (w/o BB)			0.93 (2.53)	1.41 (4.08)		
Fisher's LSD(5%)			0.275	0.606		
Treatment Effect						
F-value$_{df}$			$7.34_{1,4}$	$5.99_{1,4}$		
P-value			*0.053*	*0.075*		

A0 and A2 = Assessments 0 (start of trial) and 2 (end of treatment phase). Cum = Cumulative. DP = Damaged pseudostem. $_{df}$ = degrees of freedom. LSD(5%) = least significance difference at the 5% significance level. M1 = fixed effect models 1. w BB = with adult beetles treatment. w/o BB = without adult beetles treatment. †Means of square root transformed data. ‡Means of natural log transformed data. Back transformed values are in brackets. Family lines and plants contaminated with wild-type endophyte are highlighted in bold font. Statistically significant results are highlighted in bold and italic font. Weak evidence of effects ($0.05 < P \leq 1.0$) are highlighted in italic font.

Feeding damage. For plants exposed to adult African black beetle (*H. arator*), results at the end of the treatment phase (A2) and for cumulative feeding damage during the treatment phase (A0−A2) for feeding damage were consistent with Trial B results with differences found between plants (Table 7-4), and between plants from within the same family; A2 ($Wald_{(10)} = 33.98$; $P < 0.001$) and A0−A2 ($Wald_{(10)} = 43.05$; $P < 0.001$). Eight of the ten top performing family representatives (low feeding damage) came from the low and medium grouped families from Trial B (Table 7-2) for median cumulative proportion of damaged tillers. The proportion of damaged tillers was not related to the initial tiller number of a plant (plant size).

Paxilline ELISA. In the treatment phase (A2) of the trial, differences were found ($P \leq 0.05$) between plants [13] and within family [14] for concentrations of paxilline immunoreactivity for plants exposed and not exposed (controls) to adult African black beetle (*H. arator*) in herbage (Herb), damaged pseudsotem (DP) and undamaged pseudostem (UdP). Mean herbage was used when there were no beetle effects found. In general the paxilline immunoreactivity levels found in plants not exposed to adult African black beetle (*H. arator*) feeding were higher than those in exposed plants, for both herbage and pseudostem plant sections (Table 7-4). Weak evidence ($0.05 < P \leq 0.10$) of a African black beetle (*H. arator*) treatment effect for herbage paxilline immunoreactivity levels (Table 7-5) was found at the end of both the treatment and post-treatment phases of the trial. At the end of the treatment phase evidence of the effect was stronger and close to being significant. An overall pseudostem paxilline immunoreactivity mean was calculated, weighted by the proportion of damaged tillers, for exposed plants because too few undamaged tillers were available for analysis of paxilline immunoreactivity (Figure 7-4).

[13] Plant effects (individual plants); mean $Herb_{(pax)}$ ($F_{(18,72.6)} = 17.73$; $P < 0.001$), plants exposed to black beetle $DP_{(pax)}$ ($F_{(18,29.4)} = 5.53$; $P < 0.001$) and plants not exposed to African black beetle (*H. arator)* (controls) $UdP_{(pax)}$ ($F_{(12,11)} = 8.57$; $P < 0.001$).

[14] Plant effects (within family); plants exposed to frican black beetle (*H. arator)* $Herb_{(pax)}$ ($F_{(9,65.4)} = 5.15$; $P < 0.001$) and plants exposed to black beetle $DP_{(pax)}$ ($Wald_{(9)} = 26.71$; $P = 0.002$).

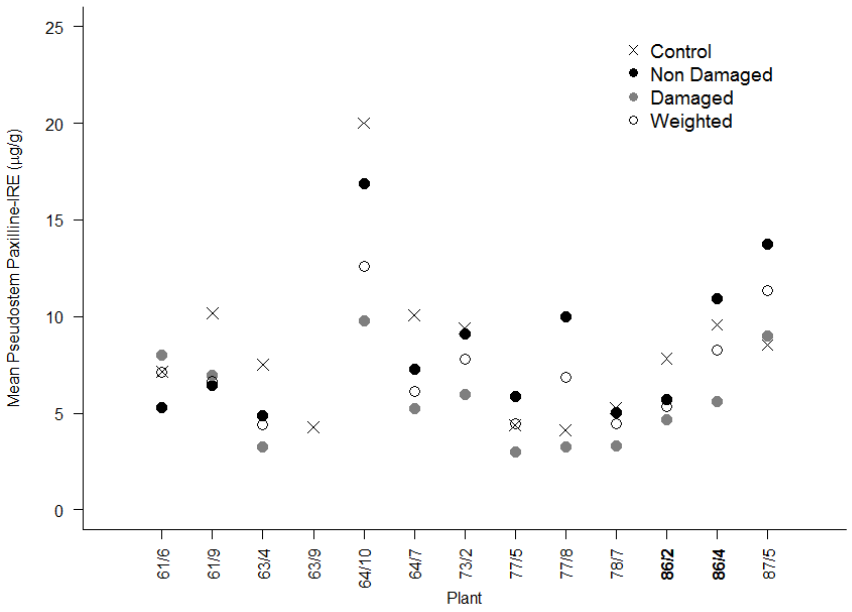

Figure 7-4: Trial C; mean concentrations of paxilline immunoreactvity from plants exposed (damaged, undamaged and weighted overall pseudostem) and not exposed (control means) to adult African black beetle feeding.
Weighted overall pseudostem concentrations of paxilline immunoreactivity were calculated by summing the damaged and undamaged pseudostem immunoreactivity levels weighted by the proportion of tillers damaged. Paxilline-IRE = Paxilline immunoreactive equivalents. Plants contaminated with wild-type endophyte are highlighted in bold font.

In general plants not exposed to African black beetle (*H. arator*) (controls) had higher concentrations of paxilline immunoreactivity in the pseudostem than from plants exposed to beetles (weighted pseudostem). When comparing the paxilline concentrations in the controls with the undamaged pseudostem in plants exposed to African black beetle (*H. arator*), approximately four of 12 plants had higher levels recorded in the undamaged pseudostem from exposed plants. When paxilline immunoreactivity levels in undamaged pseudostem for plants exposed and not exposed to beetles (Table 7-4), were compared in a scatterplot (not shown) no correlations were observed. Consistent with Trial B results, plants exposed to adult beetle feeding, in general had higher concentrations of paxilline immunoreactivity in the undamaged pseudostem than in the damaged pseudostem, with lowest levels found in the herbage (Table 7-4). Seasonal increases in concentrations of paxilline

immunoreactivity were found in the herbage plant sections when comparing the end of the treatment phase (A2) with the end of the post-treatment phase (A3) for both treatments (Table 7-5; also see Table 7-4 for plant means).

Relationship between feeding damage and paxilline immunoreactivity. For the plants in the treatment group exposed to beetles, no relationship was found between levels of immunoreactivity and adult African black beetle (*H. arator*) feeding for herbage or damaged pseudostem plant sections, consistent with Trial B results. Many plants had insufficient undamaged pseudostem for comparison. However, 10 of 12 plants that had measured concentrations of paxilline immunoreactivity in undamaged pseudostem, were also the top performing plants for feeding damage (least to most damage) and seven of eight plants that had insufficient undamaged pseudostem for analyses were the bottom performing plants for feeding damage.

Trial D. *Feeding damage.* The feeding damage results are summarised in Table 7-6. There was strong evidence of an interaction between plant-line and assessment time ($F_{(18,440)}$ = 4.62; P <0.001). In general, plant-lines with endophyte infection sustained the least damage and overall damage was higher at A1 than at other assessment times (Figure 7-5). This result was mirrored when plants were re-parameterised in terms of endophyte status, i.e., endophtye-infected (E+) or endophyte-free (E-) with an assessment time by endophyte interaction found ($F_{(2,445)}$ = 29.34; P <0.001). For the endophyte-free plants the level of damage was significantly higher at A1 than at subsequent assessments (LSD(5%)) when the beetles were feeding at their highest level, whereas, for the endophyte-infected plants, the level of damage was consistent throughout the trial (Figure 7-5).

When plants were classified in terms of family or cultivar ('F3', 'F14', 'F15', 'F23', 'Extreme', 'Galaxy' and 'Quartet') there was strong evidence of a family-cultivar effect ($F_{(6,168)}$ = 6.85; P <0.001). This was for the cultivar 'Galaxy' with significantly higher levels of feeding damage from adult African black beetle (*H. arator*) than for the other half-sibling families and cultivars (Figure 7-6 A). There was no evidence (P >0.05) that the family-cultivar effect depended on endophyte status or varied over time.

Table 7-6: Trial D; mean plant tiller number (square root transformed*), mean proportion of feeding damage (logit transformed) and mean concentrations of paxilline immunoreactivity, in plant pseudostem for cultivars and half-sibling families.**

Ploidy	Family or Cultivar	Cum plant tiller number* (A0–A3)		Cum proportion of damaged tillers from African black beetle feeding** (A0–A3)		Paxilline immunoreactivity (A3) (Paxilline-IRE µg/g)	
		Mean	SEM	Mean	SEM	Mean	SEM
Diploid	Extreme E+	4.81 (23.1)	0.232	0.76 (0.68)	0.214	27.86	3.260
	Extreme E-	4.88 (23.8)	0.232	1.95 (0.88)	0.263	0.00	3.260
	F3	5.83 (34.0)	0.232	0.57 (0.64)	0.194	27.96	3.260
	F14	5.31 (28.2)	0.232	0.88 (0.71)	0.208	34.73	3.260
	F15	5.82 (33.9)	0.232	0.77 (0.68)	0.197	39.65	3.260
	F23	6.69 (44.7)	0.232	0.86 (0.70)	0.187	38.66	3.260
Tetraploid	Galaxy E+	2.83 (8.0)	0.232	1.94 (0.87)	0.387	21.39	3.260
	Galaxy E-	4.01 (14.1)	0.232	3.27 (0.96)	0.453	0.00	3.260
	Quartet E+	3.58 (12.8)	0.232	1.28 (0.78)	0.279	26.02	3.260
	Quartet E-	4.03 (16.2)	0.232	1.72 (0.85)	0.280	0.00	3.260
	Fisher's LSD(5%)	0.655		0.646		9.220	
	Plant-line Effect						
	F-statistic$_{df}$	$24.62_{9,240}$		$9.01_{9,215}$		$4.53_{6,28}$	
	P-value	<0.001		<0.001		0.003	

SEM = standard errors of means. LSD(5%) = least significant differences of means (5% level). * Square-root transformed. **Logit transformed. Back transformed values are in brackets.

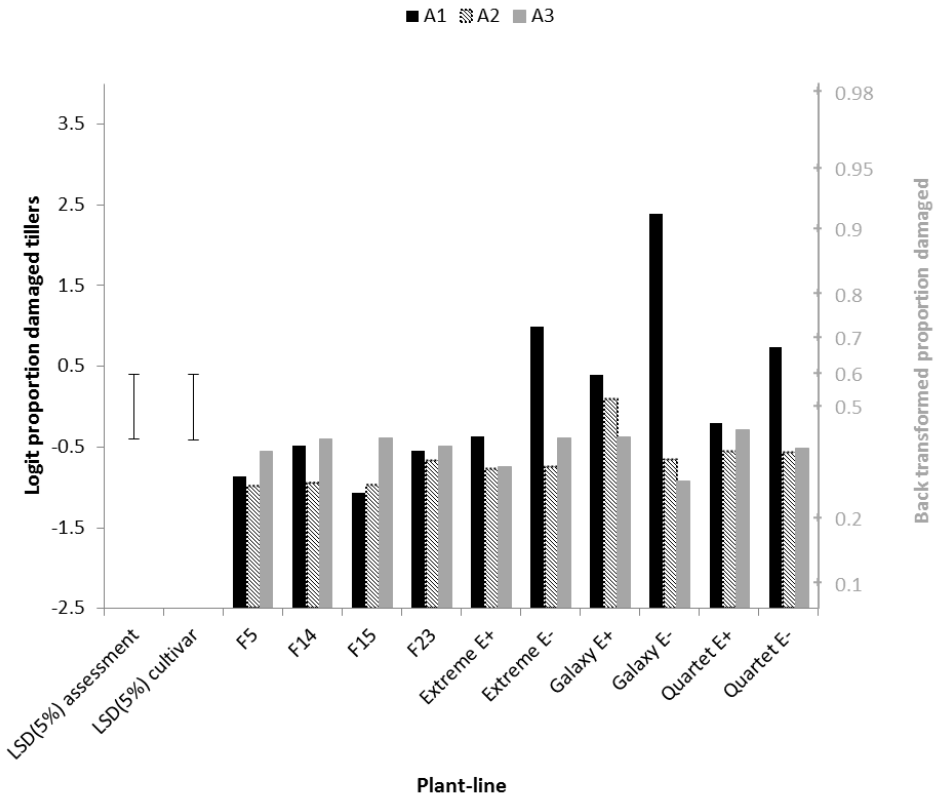

Figure 7-5: Trial D; comparison of feeding damage by adult African black beetle for individual plant-lines at each assessment.

Mean data unadjusted for the covariate, initial tiller number (initial plant size) on the logit scale for proportion of damaged tillers, with the back transformed scale on the right-hand vertical axis of the graph. LSD(5%) = Fisher's least significant difference (LSD) post hoc test at the 5% significance level. A1, A2 and A3 = assessments 1, 2 and 3 respectively.

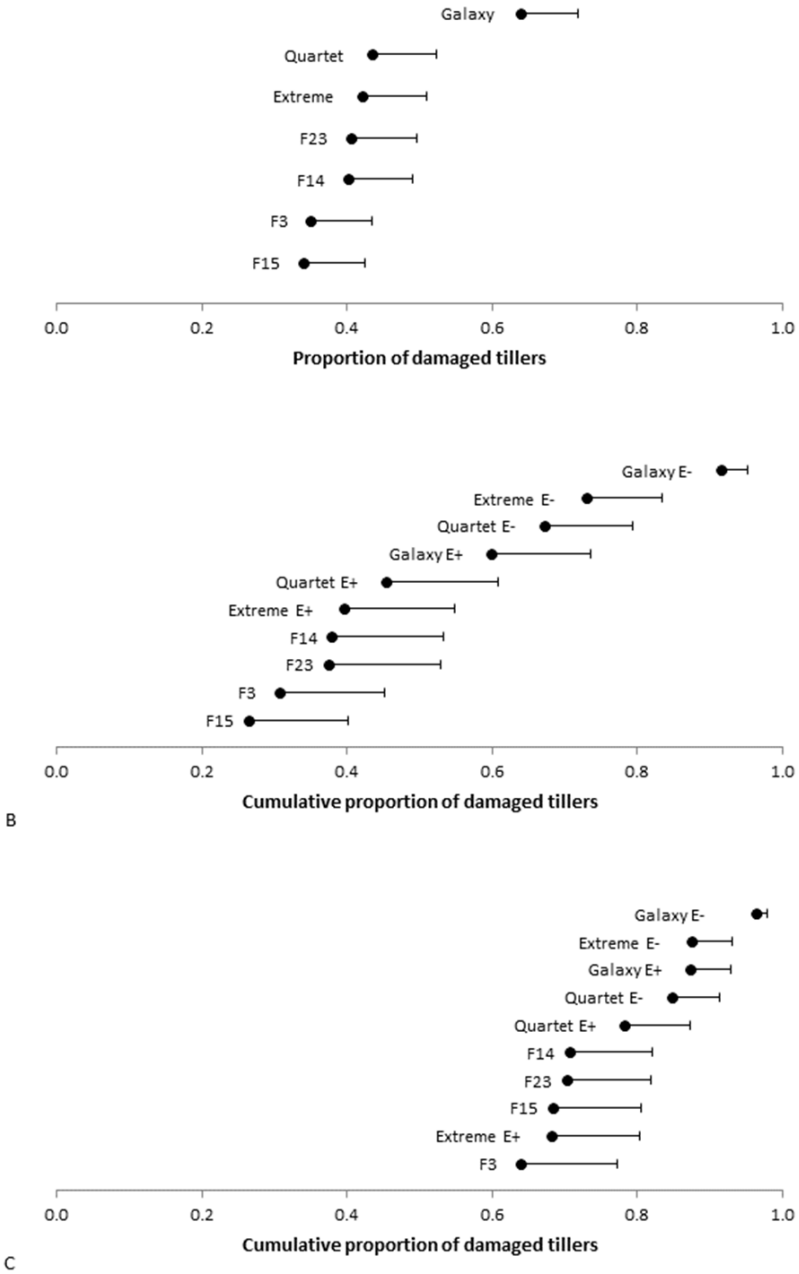

Figure 7-6: Trial D; feeding damage by adult African black beetle.

Mean + LSD(5%). A) Family-cultivar effects; plants separated by cultivar or half-sibling family for the proportion of damaged tillers in rank order from lowest to highest, from the pooled data from all three damage assessments (A1–A3) as there was no assessment by plant family-cultivar effect ($F_{(12,439)}$ = 1.19; P = 0.077). B–C = Cumulative feeding damage from adult African black beetle for plant-lines in rank order from lowest to highest. B) Cumulative proportion of damaged tillers from the start to mid-trial, days 0–14 (A0–A1). C) Cumulative proportion of damaged tillers from start to end of trial, days 0–28 (A0–A3). A0, A1 and A3 = assessments 0 (day 0), 1 (day 14) and 3(day 28) respectively. LSD(5%) = Fisher's least significant difference (LSD) post hoc test at the 5% significance level.

The cumulative proportion of damaged tillers was analysed for plant-line with each assessment interval analysed separately, and strong evidence of plant-line effects were found at each assessment level; A0–A1 ($F_{(9,216)}$ = 16.97; P <0.001), A0–A2 ($F_{(9,215)}$ = 13.54; P <0.001) and A0–A3 ($F_{(9,215)}$ = 9.01; P <0.001). Total damage, that is, the cumulative proportion of damaged tillers, by day 14 (A0–A1) and by day 28 (A0–A3) are shown in Figure 7-6 B and C respectively, with plant-lines that are significantly different from each other easily distinguished.

By day 14 (A0–A1; Figure 7-6 B) there were no differences between the half-sibling families for feeding damage and for all cultivars ('Extreme', 'Quartet' and 'Galaxy'), the endophyte-infected plant-lines were significantly different from their equivalent endophyte-free plant-lines with 'Galaxy E-' significantly more damaged than any other plant-line. Although plant-line 'F15' was not significantly different from the other half-sibling families for feeding damage it was significantly different from all cultivar lines (E+ and E- lines), except for plant-line 'Extreme E+'.

By the end of the trial (A0–A3; Figure 7-6 C) the order from least to most damaged had changed minimally. The five least damaged plant-lines were the same as those at day 14 (A0–A1). These plant-lines remained significantly different from all endophyte-free lines and not significantly different from each other. For the cultivar 'Quartet', plant-line 'Quartet E+' was now not significantly different from 'Quartet E-'. Plant-line 'Galaxy E-' remained significantly different from all other plant-lines, sustaining the highest level of feeding damage from adult African black beetle (*H. arator*). 'Galaxy' was the most susceptible family or cultivar to feeding from adult African black beetle (*H. arator*) (Figure 7-6).

Paxilline immunoreactivity in plant pseudostem. The data from each assessment for the level of paxilline immunoreactivity in pseudostem samples were analysed separately (Table 7-6). Pseudostem samples taken at the start of the trial, day 0 (A0), prior to addition of adult African black beetles (*H. arator*) were all from undamaged tillers and were a subset of the pseudostems from the whole plant. Samples at assessments day 14 (A1) and day 28 (A2) consisted of only damaged tillers. Samples at day 28 (A3) consisted of all the tillers from the entire plant (undamaged, dead and damaged). Statistically significant differences for mean concentrations of paxilline immunoreactivity between plant-lines were found at all assessments; A0

($F_{(6,28)}$ = 3.87; *P* = 0.006), A1 ($F_{(6,28)}$ = 2.56; *P* = 0.0.42), A2 ($F_{(6,28)}$ = 5.67; *P* <0.001), and A3 ($F_{(6,28)}$ = 4.53; *P* = 0.003).

The order of AR1-infected plant-lines from highest to lowest for concentrations of paxilline immunoreactivity at the start and end of the trial (before and after addition of adult African black beetle (*H. arator*)) (Figure 7-7 A and B respectively) differed considerably after addition of feeding adult African black beetle (*H. arator*). When plotted in rank order, plant-lines that were significantly different (LSD(5%)) from each other for concentrations of paxilline immunoreactivity were clearly distinguishable at each assessment Figure 7-7. The top five ranked AR1-infected plant-lines with the highest concentrations of paxilline immunoreactivity at the end of the trial, A3 (Figure 7-7 B; 'F15', 'F23', 'F14', 'F3', 'Extreme E+') were also the top five ranked plant-lines for least feeding damage (cumulative damage) from adult African black beetle (*H. arator*) (Figure 7-6 C). The three half-sibling families 'F14', 'F15' and 'F23' had the largest increases in mean paxilline immunoreactivity from the start to the end of the trial (Figure 7-7). There was no trend observed between concentrations of paxilline immunoreactivity in pseudostem measured at the start (A0) and end (A3) of the trial.

*Feeding damage and paxilline immunoreactivity***.** A regression analysis was not able to be performed as there were not enough ELISA data points (from pooling samples). When comparing the family and cultivar means for levels of feeding damage with concentrations of paxilline immunoreactivity there was a suggestion of an association (negative trend) with differences found between cultivars and half-sibling families (Figure 7-8). That negative trend was still apparent when the endophyte-free lines were omitted from the analysis and the AR1-infected plant-lines were plotted (Figure 7-8; squares and circles only). However, for the AR1-infected half-sibling families (within a breeding line), no trend was evident (Figure 7-8; patterned circles only), consistent with Trial B results. Within a cultivar or half-sibling family line when simple scatterplots were plotted for individual plant-lines there were no consistent trends observed (data not shown).

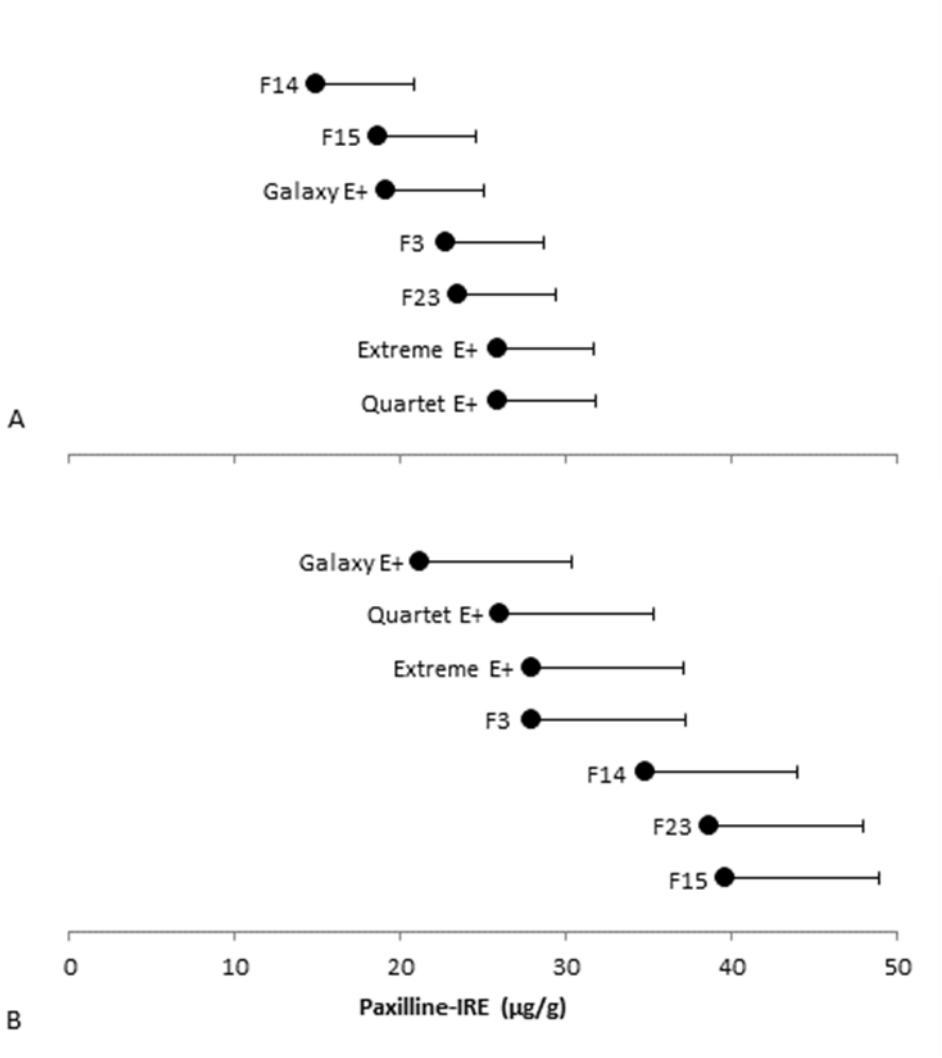

Figure 7-7: Trial D; concentrations of paxilline immunoreactivity in pseudostem plant sections of the seven AR1-infected plant-lines measured as paxilline-IRE (paxilline immunoreactive equivalents) in rank order from highest to lowest.
Mean + LSD(5%). A = Levels of pseudostem paxilline immunoreactivity at the start of the trial, pre-addition of feeding adult African black beetle (H. arator) at assessment 0 (A0). B = Levels of pseudostem paxilline immunoreactivity at the end of the trial, post addition of feeding adult African black beetle (H. arator) at assessment 3 (A3). LSD(5%) = Fisher's least significant difference (LSD) post hoc test at the 5% significance level.

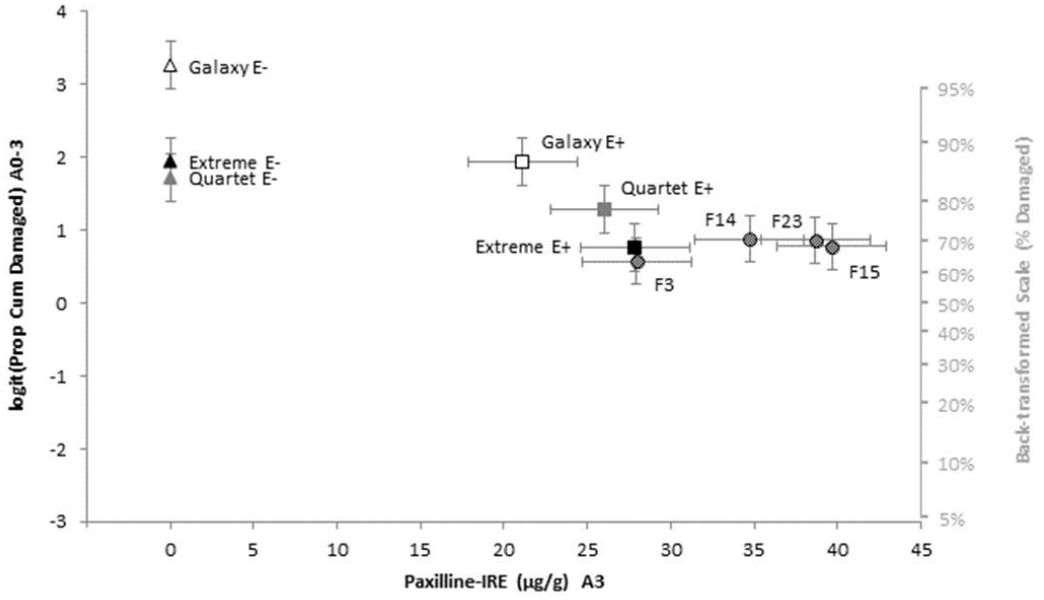

Figure 7-8: Trial D; scatterplot of predicted means of pseudostem paxilline immunoreactivity measured as paxilline-IRE (paxilline immunoreactive equivalents) at A3 versus the logit proportion of cumulative damaged tillers from the start of the trial to the end of the trial (A0–A3) for all ten plant-lines, with the back transformed scale on the right-hand vertical axis of the graph.
Mean ± SEM. Triangle = endophyte-free (E-) cultivars. Square = AR1-infected (E+) cultivars. Circle with pattern fill = AR1-infected (E+) half-sibling families. Black fill = cultivar "Extreme'. White fill = cultivar "Galaxy". Grey fill = cultivar "Quartet'. A0 and A3 = assessments 0 (day 0) and 3 (day 28) respectively.

Tiller number. There was strong evidence of an assessment by plant-line interaction ($F_{(27,476)} = 6.23$; $P < 0.001$) (Table 7-6). However, with the half-sibling families had consistently the highest mean number of tillers at each assessment and 'Galaxy E+' the lowest. For all plant-lines the highest mean number of tillers was at A1 and lowest at A3. The overall family-cultivar effect did not depend on endophyte status at any time. A positive relationship with initial tiller number was found at all subsequent assessments (A1, A2 and A3) and at A2 and A3 the slope of relationship depended on the cultivar: A1 ($F_{(1,225)} = 3840.23$; $P < 0.001$), A2 ($F_{(9,217)} = 2.23$; $P = 0.021$) and A3 ($F_{(9,214)} = 3.17$; $P = 0.001$).

When plants were parameterised in terms of family or cultivar, the highest mean tiller numbers were from the BL-1 breeding line families, followed by the cultivars,

'Extreme', then 'Quartet', and the lowest 'Galaxy' (LSD(5%)). The top five family-cultivars for tiller number were also the same as those with the least feeding damage (Figure 7-6 C), that is, all four half-sibling families from BL-1 breeding line and the cultivar 'Extreme'. When plants were parameterised in terms of endophyte status (E+ or E-) for tiller number, there was strong evidence of an assessment by endophyte interaction ($F_{(3,233)} = 32.17$; $P < 0.001$) with endophyte-free plants having more tillers in the first half of the trial, and the least in the second half. There was no evidence that the overall family-cultivar effect depended on the endophyte status at any time.

For total tiller production (cumulative tiller number) there was strong evidence of differences for mean cumulative tiller number between plant-lines at all assessments; A0–A1 ($F_{(9,240)} = 22.84$; $P < 0.001$), A0–A2 ($F_{(9,240)} = 24.62$; $P < 0.001$) and A0–A3 ($F_{(9,240)} = 25.97$; $P < 0.001$). The rank order of the cumulative number of tillers for each plant-line when plotted remained relatively constant from start of the trial to finish of the trial and there were no indications that feeding by adult African black beetle (*H. arator*) altered tiller production for any of the cultivars or half-sibling families from breeding line BL-1. These results were consistent with Trials B and C.

7.5 Discussion

The severity of African black beetle (*H. arator*) feeding damage depended on both the choice of cultivar and plant endophyte status (E+ or E-). AR1-infected ryegrasses had more resistance to African black beetle (*H. arator*) when compared to endophyte-free ryegrasses, consistent with results reported by Popay and Baltus (2001). However, once feeding levels of African black beetle (*H. arator*) declined, the benefit of hosting an endophyte diminished.

AR1-infected plant-lines had increased concentrations of paxilline and peramine immunoreactivity post African black beetle (*H. arator*) feeeding, and this is consistent with a general plant-endophtye response to insect attack protecting the most vulnerable section of the plant above ground (Popay, 2009). Distribution of the fungus and the alkaloids at the base of the plant (Musgrave, 1984; Gallagher *et al.*, 1987) ensures the plant and fungus have the strongest protection from herbivory

(Popay, 2009). In addition, plants generally had higher concentrations of paxilline and peramine immunoreactivity in the undamaged pseudostem sections than in the damaged pseudostem, with lowest levels found in the herbage. This difference in levels of immunoreactivity between damaged and undamaged pseudostem is likely due to reduced resources for alkaloid production in the damaged tillers.

The chemical basis of African black beetle (*H. arator*) resistance in AR1-ryegrass associations is not well understood, however, it is known that fungal endophytes interact strongly with their host plant genotype and that this interaction affects resistance to insects, as does the ryegrass species (Fannin *et al.*, 1990; Christensen *et al.*, 1991; Hill *et al.*, 1991; Christensen *et al.*, 1993; Agee & Hill, 1994; Popay *et al.*, 2003). In the initial trial, Trial A, the African black beetle (*H. arator*) deterrent properties of a range of diploid and tetraploid ryegrass cultivars infected with AR1 endophyte were explored. Overall the tetraploid cultivars incurred greater beetle feeding damage than the diploid cultivars. This is consistent with the finding of Popay *et al.* (2003) that, in general, tetraploids and hybrids sustain more damage by African black beetle (*H. arator*) than diploids or perennials. The three diploid cultivars, 'Alto', 'Extreme' and 'Arrow' in rank order for highest resistance to beetle feeding, also had high paxilline immunoreactivity and high plant tiller numbers.

At the ryegrass cultivar level, a negative relationship between mean paxilline immunoreactivity (in pseudostem) and mean feeding damage was discernible. However, at an individual plant level no relationship was found between paxilline immunoreactivity and feeding damage. Despite inconsistencies in sample collection for analysis by the paxilline ELISA, subsequent trials revealed AR1-interactions with the host plant genotype strongly influenced alkaloid expression even within cultivars.

To better understand AR1 bioactivity, the cultivar effect was removed and host plant genotype variability reduced, by studying closely related plant-genotypes from within a breeding line. In Trials B and C, an AR1-infected diploid, perennial ryegrass (*L. perenne*) breeding line (BL-1), consisting of 23 half-sibling families with clonal replication, was chosen for study of resistance to adult African black beetle (*H. arator*) and concentrations of paxilline immunoreactivity. Despite

indications of differences between half-sibling families for levels of feeding damage, paxilline immunoreactivity and tiller number, there was no evidence of a relationship between any of these variables.

Trial D established which tested AR1-infected cultivars and half-sibling families (from BL-1) had strong resistance to feeding from adult African black beetle (*H. arator*) using a feeding trial with a choice of plants grown from seeds (i.e. not clones). As with Trial A, no relationship was found between paxilline immunoreactivity and feeding damage at either the cultivar or family level. Nor was a relationship found within cultivars or within families. Sample limitations could account for this lack of relationships. Firstly, a lack of families from within the breeding line was represented (four of 23) and secondly, a reduced number of samples were measured for paxilline immunoreactivity (the 25 individual replicate samples were pooled by assessment day to make five pooled samples per plant-line to ensure there was adequate sample for analysis by paxilline ELISA). However, a negative trend was observed between mean paxilline immunoreactivity and mean feeding damage between cultivars and families that was consistent with Trial A. Yet no associations were found between feeding damage and peramine immunoreactivity, consistent with Ball *et al.* (1997) who concluded that peramine does not deter adult African black beetle (*H. arator*) from feeding.

Trial B provided indications of a potential subset of the paxilline-like compounds detected by the paxilline ELISA, associated with reduced feeding damage with a cluster of plants with high paxilline immunoreactivity (≥25 µg/g) and low feeding damage (≤20%). In addition, no relationship was found between paxilline and peramine immunoreactivity suggesting more than just an environmental influence on paxilline-like alkaloid production.

Results from Trials C and D also support the suggestion that a subset of paxilline-like compounds are associated with reduced feeding damage from adult beetles. In Trial D, concentrations of paxilline immunoreactivity differed between plant cultivars and families throughout the trial. The level of paxilline immunoreactivity when compared at start with end of the trial, increased for all AR1-infected plant-lines, with families F14, F15 and F23 having the greatest increase. The change in level of paxilline immunoreactivity was dependent on the host plant and the final

concentrations of paxilline immunoreactivity were not related to initial levels. In Trial C there was no correlation between plants exposed and not exposed to beetles for concentrations of paxilline immunoreactivity, consistent with the suggestion that adult African black beetle feeding influences levels of paxilline immunoreactivity.

There is also evidence that African black beetle (*H. arator*) feeding affects tiller production, with greater production by plants exposed to feeding beetles. Plants exposed to adult African black beetle (*H. arator*) initially had more tillers than plants not exposed, suggesting that plants may respond to low levels of herbivory and produce more tillers than plants not exposed. However, with high feeding pressure, exposed plants could not sustain tiller production at high enough levels to compensate for tiller damage from feeding African black beetle (*H. arator*). The negative effect from high levels of African black beetle (*H. arator*) feeding on tiller number was still apparent up to 4 weeks post-exposure. The long plant recovery period, suggests that tiller production is costly to the plant. Herbivory can be detrimental, of no consequence, or even beneficial, depending on the conditions governing a plant's ability to replace tissue consumed by herbivores (Maschinski & Whitham, 1989; Whitham *et al.*, 1991).

Time of year the adult African black beetle (*H. arator*) feeding trials were run (four trials spring and one trial in autumn) was based on two considerations. First consideration was the African black beetle (*H. arator*) lifecycle when adult beetles were actively feeding. This was either as overwintering beetles emerging from their dormancy period to actively feed and reproduce in spring (Todd, 1959; Todd, 1964; Bell *et al.*, 2011) or as new beetles (from pupae) in autumn. Secondly, trials were performed in the glasshouse or screenhouse (depending on the time of the year), to provide climatic conditions known for high alkaloid production. Seasonal trends have been reported for the level of fungal endophytes (Fletcher, 1983; Mortimer *et al.*, 1984; di Menna & Waller, 1986; Fletcher, 1986; di Menna *et al.*, 1992; Ball *et al.*, 1995) and many of the metabolites they produce, with general alkaloid production slightly lagging behind endophyte concentration, increasing during spring, through summer and early autumn, then falling in late autumn and winter (di Menna *et al.*, 1992; Woodburn *et al.*, 1993; Ball *et al.*, 1995; Easton *et al.*, 1996).

Different metabolites may have production peaks at slightly different times, with lolitrem B peaking in summer and early autumn (Prestidge & Gallagher, 1988; Ball *et al.*, 1991; di Menna *et al.*, 1992; Ball *et al.*, 1995) and with peramine being high from mid-spring through to mid-autumn (Ball *et al.*, 1995). Seasonal trends were found in Trial C, conducted in late spring–early summer with increasing concentrations of paxilline immunoreactivity found in herbage for the plants not exposed to adult African black beetle (*H. arator*).

Given that adult African black beetle (*H. arator*) feed at the base of the plant, the levels of the specific fungal secondary metabolites in the pseudostem plant section for resistance to feeding from African black beetle (*H. arator*), were considered more relevant than levels in the herbage plant section. For Trials A and D concentrations of paxilline immunoreactivity were measured by paxilline ELISA in the pseudostem sections of the plants (3 or 4 cm from the crown). In practical terms, herbage collection is preferable to pseudsotem collection as it doesn't require destructive sampling. Therefore, it was interesting to investigate whether concentrations of paxilline immunoreactivity in herbage reflect that in pseudostem. This was not found.

Differences between plants for the levels of endophyte and endophyte metabolites have been reported previously (Jones *et al.*, 1985; Belesky *et al.*, 1989; Rowan *et al.*, 1990b; Ball *et al.*, 1991; Hill *et al.*, 1991; Breen, 1992; Azevedo *et al.*, 1993; Davies *et al.*, 1993; Agee & Hill, 1994; Ball *et al.*, 1995). It has been found that the genetic characteristics of both endophyte and host plant are important in the levels of alkaloid production (Fannin *et al.*, 1990; Christensen *et al.*, 1991; Hill *et al.*, 1991; Christensen *et al.*, 1993; Agee & Hill, 1994). Research has shown that within a host plant species (e.g. *L. perenne*) the level of insect resistance is variable both between individual plants (Easton *et al.*, 2000), and between cultivars infected with the same endophyte (Trial A).

Results from Trials A–D provide further encouragement to look more extensively at the half-sibling families and the proposed negative association of paxilline immunoreactivity with resistance to adult black feeding, both between families within a breeding line and within cultivars or families. The sample limitations incurred in these studies of family representation and number of samples analysed

by the paxilline ELISA will need to be addressed to establish if there are any relationships within a breeding line and within a cultivar or family.

Further study is required to establish if the paxilline immunoreactivity response is associated with reduced feeding damage and if exposure to feeding from African black beetle (*H. arator*) accentuates the response. This further work is required before it can be suggested that the paxilline ELISA can be used as a screening tool by plant breeders for plant resistance to feeding from adult African black beetle (*H. arator*) in *Epichloë* endophyte-ryegrass associations. However, for any further work with the paxilline ELISA in developing a screening tool, it would be recommended that either entire plant or undamaged pseudostem samples would need to be collected for analysis by ELISA, post-exposure to feeding from adult African black beetle (*H. arator*).

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Chapter 8

Correlation of paxilline immunoreactivity with African black beetle resistance in AR1-perennial ryegrass associations

This chapter is written as a journal paper and presents Trial 5 of this thesis.

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8.1 Abstract

African black beetle (*Heteronychus arator* (Fabricius, 1775)) is a major pasture pest in northern New Zealand. The adult beetle is deterred by ergotpeptine alkaloids produced by fungal endophytes in grasses, but these compounds are also toxic to grazing mammals. A novel fungal endophyte strain, AR1 provides weak resistance to African black beetle (*H. arator*) without any adverse effects on animal production. Although AR37 provides stronger resistance to African black beetle (*H. arator*), adverse effects on animals have been reported. Both novel endophytes do not produce any of the known compounds that deter African black beetle (*H. arator*). A mid-autumn to late spring non-choice feeding trial was performed with endophyte-free, AR1- and AR37-infected perennial ryegrass (*Lolium perenne* L.) exposed and not exposed to feeding from adult African black beetle (*H. arator*). The trial provided evidence of a negative relationship between feeding damage and paxilline immunoreactivity and this relationship was influenced by the cultivar or half-sibling family. However, endophyte mycelium mass was not strongly correlated with paxilline immunoreactivity and no evidence of a relationship between endophyte mycelium mass and feeding damage was found. Plant-line differences for herbage and pseudostem dry matter, live African black beetle (*H. arator*) and number of offspring were found. Positive relationships were established for paxilline immunoreactivity with both herbage and pseudostem dry matter, which were steeper in the presence of beetles. In general for the AR1-infected ryegrasses, the average amount of paxilline immunoreactivity, endophyte mycelium mass, herbage and pseudostem dry matter, was higher in the absence of African black beetle (*H. arator*). Further studies are needed to determine if the negative association between feeding damage and paxilline immunoreactivity found in plants exposed to actively feeding adult beetles is either a general or induced plant response.

8.2 Introduction

Perennial ryegrass (*L. perenne*) is the predominant component of intensely grazed pasture in New Zealand (Belgrave *et al.*, 1990; Easton & Tapper, 2005). It is easily established, very productive and highly digestible. In New Zealand perennial ryegrass (*L. perenne*) contains the clavicipitaceous endophytic fungus, *Epichloë*

festucae var. *lolii* (Latch, M.J. Chr. & Samuels) C. W. Bacon & Schardl, stat. nov. et comb. nov., which causes toxicosis in grazing livestock (Fletcher *et al.*, 1999; Easton & Tapper, 2005) but is essential for plant persistence in pastures through tolerance and resistance to invertebrate pests (Prestidge & Ball, 1993; Easton & Tapper, 2005). The anamorphs of *Epichloë* species are distinguished from the other clavicipitaceous fungi because they have no external stage and infected plants look no different from uninfected (endophyte-free) plants. Lacking the capacity to reproduce sexually and progagating by vertical transmission via the host seed, these anamorphs infecting cool-season grasses (C3 grasses) in the sub-family Pooideae, are solely reliant on their host grass for survival and reproduction.

The last four decades of endophyte research have primarily focused on endophytes present in forage and turf grasses due to their agronomic significance and importance. This includes; detrimental effects on grazing livestock, effects on host plant responses to biotic and abiotic stresses, effects on the biodiversity and trophic interactions in wild populations (Roberts *et al.*, 2005). The endophytes *Epichloë coenophiala* (Morgan-Jones & W. Gams) C.W. Bacon & Schardl, comb. nov., of tall fescue (*Lolium arundinaceum* (Schreb.) Darbysh.) and *E. festucae* var. *lolii* of perennial ryegrass (*L. perenne*) are the two most extensively researched endophytes due to their agronomic importance particularly in the United States of America (USA) and New Zealand (NZ), respectively.

The wild-type *E. festucae* var. *lolii* of perennial ryegrass (*L. perenne*) that was introduced into New Zealand produces the following secondary metabolites: ergovaline, lolitrems and peramine (Tapper & Latch, 1999). This endophyte causes mammalian toxicity, which is attributed mainly to two classes of alkaloids, the ergot alkaloids (ergovaline) and the indole diterpenes (lolitrems). Ergovaline is responsible for heat stress in animals (Fletcher & Easton, 1997; Fletcher *et al.*, 1999) and the lolitrems for ryegrass staggers (1981; Gallagher *et al.*, 1982b; 1982a; 1984; Tapper *et al.*, 2004). Peramine is not known to be toxic to grazing animals, but is a potent feeding deterrent to major ryegrass pest, Argentine stem weevil (*Listronotus bonariensis* (Kuschel, 1955)) (Prestidge *et al.*, 1985; Rowan & Gaynor, 1986; Rowan *et al.*, 1990). The ergot alkaloids and indole diterpenes also demonstrate anti-insect activity and toxicity. Endophyte strain, plant genotype, tissue type,

season, plant age and abiotic and biotic stresses influence alkaloid profile and concentrations (Clay & Schardl, 2002; Easton *et al.*, 2002; Popay *et al.*, 2003; Rodriguez *et al.*, 2009).

In New Zealand, selected 'novel' *E. festucae* var. *lolii* strains have been inoculated into local commercial cultivars that do not produce, or produce less of, the alkaloids known to be toxic to mammalian animals, whilst still maintaining activity against Argentine stem weevil (*L. bonariensis*). The novel endophyte strain AR1 produces peramine, a potent Argentine stem weevil (*L. bonariensis*) deterrent but not lolitrems or ergovaline, the major causative agents of mammalian toxicity. AR1 can only produce simple indole diterpenes (including paxilline and paxilline-like compounds such as the terpendoles) as it does not have the full complement of genes for the production of more complex indole diterpenes such as lolitrem B (Young *et al.*, 2009). Although AR1 genetically can produce paxilline, a mild tremogen (Miles *et al.*, 1992), the amounts produced are considered minimal (Young *et al.*, 2009), with production of the other simple indole diterpenes including the paxilline-like compounds being more predominant (Young *et al.*, 2009). In New Zealand the endophyte strain AR1 has been available in proprietary diploid perennial ryegrasses (*L. perenne*) since 2000 (Thom *et al.*, 2013). To date there have never been any reports of mammalian neurotoxicosis in livestock from AR1-infected pastures (Bluett *et al.*, 2005b; Bluett *et al.*, 2005a). AR1-infected ryegrass has been extensively researched and is considered robust, with well-defined codes of practice for pasture establishment and management. However, in areas where African black beetle (*H. arator*) is a problem, there is no doubt that this pest compromises both persistence and productivity of AR1-infected pastures (Thom *et al.*, 2013). Although other commercial endophytes provide their host with resistance to African black beetle (*H. arator*) some, such as AR37 are associated with livestock toxicity (Fletcher, 1999; Fletcher & Sutherland, 2009). Selection for AR1-infected ryegrass with strong resistance to African black beetle (*H. arator*) would be of significant value to farmers in regions where this pest is a problem.

African black beetle (*H. arator*) is a major pest of regions in the upper and mid-west and mid-east of the North Island, New Zealand. Sporadic region-wide outbreaks of this pest cause significant damage to ryegrass pastures. An outbreak

in the Waikato and Bay of Plenty in 2007/8 persisted over 3–4 seasons (Bell *et al.*, 2011). Adult beetles feed at the base of tillers and can destroy new pasture but the root-feeding larvae do the most damage. The adult beetle is deterred by certain fungal endophytes in ryegrass and tall fescue. This reduces the number of eggs the beetle lays and results in fewer root-feeding larvae in the pasture (Popay & Baltus, 2001; Thom *et al.*, 2013). Both AR1 and AR37 lack any of the known alkaloids that deter adult African black beetle (*H. arator*), but show moderate (AR1) to strong (AR37) resistance to this insect (Popay & Baltus, 2001; Hume *et al.*, 2007). Although AR1 did not reduce egg numbers in a pot trial (Popay & Baltus, 2001), build-up of populations in AR1 pastures was delayed in a 4-year large scale paddock experiment compared with endophyte-free paddocks (Thom *et al.*, 2013). The chemical basis for this resistance is not understood. However, testing a range of AR1-infected ryegrass cultivars (diploids and tetraploids) and half-sibling families from within a diploid breeding line indicated a cultivar genotype influence on resistance to adult African black beetle (*H. arator*) with paxilline immunoreactivity measured by ELISA associated with resistance (Ross *et al*, unpublished 2015, Chapter 7). It may be possible to use the paxilline ELISA as a tool for screening AR1 endophyte ryegrass associations that produce unknown compounds that deter African black beetle (*H. arator*), which do not cause toxicity to livestock.

This study presents results from an African black beetle (*H. arator*) feeding trial using AR1-infected plants (and an equivalent AR37-infected cultivar as the benchmark comparison) from mid-autumn to late spring 2012. The first objective of this study was to examine more extensively the associations between paxilline immunoreactivity and feeding damage from adult African black beetle (*H. arator*) between cultivars and half sibling families (from within a breeding line) and within a cultivar or family. The second objective was to determine if paxilline immunoreactivity and endophyte mass differed between cultivars, and if so, was paxilline immunoreactivity correlated with endophtye mass and feeding damage. The third objective was to determine if there were relationships between dry matter production and paxilline immunoreactivity. The fourth objective was to determine if adult survival and offspring abundance were related to paxilline immunoreactivity, endophyte mass or dry matter.

8.3 Materials and methods

This research was undertaken from May 2012 to November 2012 at AgResearch, Ruakura Research Centre, Hamilton, New Zealand. Eight lines of seed (plant type) were selected; two cultivars infected with endophtye (E+; 'Extreme AR1', 'Extreme AR37' and 'Quartet AR1') and the equivalent endophyte-free (E-; 'Extreme Nil' and 'Quartet Nil'), and three AR1-infected half-sibling families from the breeding line BL-1 (F3-AR1, F15-AR1 and F23-AR1). The AR1-infected lines were chosen based on results of plant resistance to adult African black beetle (*H. arator*) and levels of paxilline immunoreactivity reported in previous AgResearch in-house studies.

Plants. Individual plants were grown from seed. For the cultivars, seed containing endophyte (AR1 and AR37) or the endophyte-free equivalent were obtained from the AgResearch Margot-Forde Germoplasm Centre, Palmerston North, New Zealand. Seed of the half-sibling families from the BL-1 breeding-line were sourced from Forage Improvement, AgResearch, Grasslands, Palmerston North, New Zealand. Seeds were germinated by spreading on damp filter paper in petri-dishes sealed with parafilm and left in the dark at 20°C for 7 to 10 days. Germinated seedlings were planted out into polystyrene trays (internal 495 mm L by 300 mm W) containing a general purpose potting mix (Daltons GB Potting Mix) and left to establish (minimum 6 weeks) in the shadehouse over mid to late summer (January–February 2012). After 8 weeks a single tiller sample from each plant was cut at the tiller base and checked for endophyte infection using the endophyte tissue immunoblot technique and reagents as described by (Gwinn *et al.*, 1991) and Simpson *et al.* (2012). In early autumn (March 2012) plants with the correct endophyte status and a minimum of five tillers were trimmed to 4 cm from the crown and replanted into 10 L buckets containing soil : sand (2 : 1 v/v) mix (3/4 full) in a ring formation. Plants were left to re-establish for a minimum of 4 weeks before the trial commenced in late autumn (May 2012).

The feeding trial consisted of a single plant-line per replicate bucket (8 plants), two treatments (with and without adult African black beetle (*H. arator*)) and seven replicates per treatment. The trial was a fully randomised split plot design, blocked by replicate. The trial set-up and subsequent assessments were staggered over a 7

day period (days 1–7), a single replicate per day was assessed. All buckets were covered with green knitted windbreak (Ultrapro windbreak.LT 915 mm wide, 55% wind porosity, Cosio Plastics, NZ). The windbreak prevented adult African black beetle (*H. arator*) escaping whilst allowing space for plant growth.

Eight trial assessments were conducted every 4 weeks over a period of 28 weeks from mid-late autumn to late spring. At each assessment plants were trimmed to 4 cm from the crown, and the trimmed herbage (per bucket) stored at –20ºC. For the initial assessment (A0) all tillers were undamaged as the assessment was performed prior to the addition of the adult African black beetle (*H. arator*). At each subsequent assessment (A1–A7) feeding damage was measured for each plant in each replicate i.e., the total number of tillers, undamaged, damaged (from feeding by adult African black beetle (*H. arator*)) and dead tillers were recorded per bucket. At the end of A7, all the tillers from the trimmed plants (pseudostem sample; consisting of undamaged, damaged and dead tillers per bucket) were removed at ground level and frozen at –20ºC. Adult African black beetle (*H. arator*) were recovered, recorded if dead or alive and number of offspring (eggs and larvae) counted and recorded per bucket. The frozen herbage and pseudostem samples taken during and at the end of the experiment were freeze-dried and weighed. Pseudostem grass samples were analysed by the paxilline and endophyte mycelium mass ELISAs.

ELISA analysis of grass pseudostem samples. Freeze-dried pseudostem samples (stored at –20°C) were equilibrated to ambient room temperature (21°C) prior to milling. Samples were then ground by the Udy Cyclone mill (Udy Corporation, CO, USA). Milled pseudostem samples were stored in sealed containers at –20°C until required. The samples were equilibrated to 21°C in their sealed containers before being weighed out for sample extraction and analysis by ELISA.

Extraction of pseudostem samples for paxilline ELISA analysis; 50 mg of sample was weighed out to the nearest 0.1 mg (Mettler AE 260 Delta Range) into an Eppendorf microfuge tube (2 ml) and stored at –20°C until required. Samples were extracted on the same day the paxilline ELISA was performed, as the paxilline immunoreactivity determined by the paxilline ELISA decreases on storage. After addition of extracting solvent (1 ml, 90% HPLC grade methanol in water), samples

were rotated end-over-end on a rotation mixer (Labnet mini labroller, NJ, USA) for 20 min. Samples were then centrifuged at 8 609 ×g for 5 min (Eppendorf Centrifuge 5418), the supernatant collected and analysed by ELISA.

Extraction of pseudostem samples for analysis by endophyte mycelium mass ELISA; 20 mg of sample was weighed out to the nearest 0.1 mg (Mettler AE 260 Delta Range) into a Kimax tube (12 ml). PBST (10 ml) was added and samples were mixed thoroughly ensuring all sample was wetted. Kimax tubes were incubated for 3 h at 30°C. A representative sample was then transferred to a microfuge tube (2 ml) and centrifuged at 2 150 ×g for 3 min. Supernatant (1 ml) from each was transferred undiluted to screw-top glass vials (1.5 ml). Extracts were stored at 4°C until analysis by ELISA. Samples are stable for one week at 4°C or at –20°C for long term storage. Owing to the long extraction time (>3 h) the extracts are prepared a day ahead of the ELISA being performed.

ELISA data analysis was performed using software developed in-house (4-parameter curve fit) at AgResearch.

Paxilline ELISA. Plant (pseudostem) extracts were analysed using an indirect competitive paxilline ELISA developed in-house at AgResearch (Garthwaite *et al.*, 1993) with the following modifications. Phosphate buffered saline (PBS) containing 0.05% Tween 20 (v/v) was the wash buffer (PBST). The blocking agent and antibody buffer was 1% bovine serum albumin (w/v) in PBST (1% BSA/PBST). Sheep anti-mouse conjugated to HRP (Chemicon, CA, USA) was the secondary antibody. The HRP substrate was BioFX TMB One Component HRP microwell substrate buffer (SurModics, MN, USA) and the stop solution was sulphuric acid (0.3 M H_2SO_4). Results were expressed as µg of paxilline-immunoreactive equivalents (paxilline-IRE) per g of milled grass, as paxilline was the reference compound used to generate the standard curve in the assay. The assay working range was 1–40 ng/ml with a detection limit for the undiluted grass extract of 0.18 µg/g dwt.

Endophyte mycelium mass ELISA. Plant (pseudostem) extracts were analysed using an indirect competitive paxilline ELISA developed in-house at AgResearch. The coating antigen (*E. festucae var. lolii* mycelium) and antibodies (anti-*E. festucae*

var. lolii SAPU F2 specific primary rabbit polyclonal antibody, DAKO goat anti-rabbit conjugated with HRP anti-species secondary antibody) used in this cELISA are those described by Faville *et al.* (2007), but modified with a 2 h pre-incubation step before the standards, samples and specific antibody were added to the plate. The assay working range is 0.614–10.62 µg/ml with a detection limit for the undiluted grass extract of 6.14 mg/g dwt. Results were expressed as mg of *E. festucae* var. *lolii*-immunoreactive equivalents (*E. festucae* var. *lolii* -IREs) per g of milled grass, as *E. festucae* var. *lolii* mycelium was the reference material used to generate the standard curve in the assay.

ELISA data analysis was performed using software developed in-house (4-parameter curve fit) at AgResearch.

African black beetle. Adult African black beetle (*H. arator*) were collected from the field by pitfall trapping in autumn soon after teneral adults had emerged to feed. African black beetle (*H. arator*) were separated by gender and maintained in the laboratory at ambient temperature (20–25°C) in containers with perforated lids, damp soil and sliced carrot. Six adult African black beetle (*H. arator*) were added per replicate at the commencement of the trial after initial tiller count (A0) at a ratio of 1:1 male to female beetles.

Statistical Analysis. All statistical analyses were conducted in GenStat version 15. The arcsine-square root transformed proportion of damaged tillers was analysed using one way analysis of variance (ANOVA), blocked by replicate. The proportion of damaged tillers was transformed to stabilise the variance. Data from the seven assessments were analysed independently. Levels of paxilline immunoreactivity and endophyte mass in pseudostem grass samples, levels of dry matter for herbage and pseudostem, and the number of live tillers were analysed using split plot ANOVA. At each of the seven assessments, dry matter herbage and number of live tillers was analysed independently. The number of African black beetles (*H. arator*) found alive and the number of offspring were analysed using one-way ANOVA, blocked by replicate. To normalise the data, the number of offspring was natural log transformed prior to analysis. Post hoc tests were conducted using Fisher's least significant difference at the 5% significance level (LSD(5%)). The relationship

between African black beetle (*H. arator*) feeding damage with paxilline immunoreactivity and with endophyte mycelium mass was analysed using REML with replicate included as a random effect. To linearise the relationship, the African black beetle (*H. arator*) feeding damage data were converted to a proportion and arcsine-square root transformed. Only the replicate buckets containing African black beetle (*H. arator*) were used in the analysis.

Between assessments A6 and A7, extra plants were added to some of the buckets to ensure sufficient food for the African black beetle (*H. arator*). The addition of plants has been taken into account in the analyses of feeding damage (resulting in some plants lines having values >1 for proportion of damaged tillers), endophyte data (excluded) and dry matter (excluded).

The repeated analyses were not presented for simplicity due to the presence of treatment by time interactions and evidence of heterogeneity in variance between time-points.

8.4 Results

Feeding damage. In late autumn-early winter and from early spring (A1, A5–A7) there were differences ($P \leq 0.05$; Table 8-1) in feeding with 'Extreme AR37' and half-sibling family F15-AR1 having significantly lower feeding damage than both endophyte-free lines ('Extreme Nil' and 'Quartet Nil') and family F3-AR1 significantly less damage than 'Extreme Nil'.

In early spring only the three half-sibling families had significantly lower feeding damage than the two cultivar endophyte-free lines ('Extreme Nil' and 'Quartet Nil'). By mid-spring all AR1-infected lines (except 'Extreme AR1') had significantly lower damage than the endophyte-free cultivar lines ('Extreme Nil' and 'Quartet Nil'). There was no evidence of significant differences between the AR1-infected cultivars and families for feeding damage with the exception of family F23-AR1 which had significantly less feeding damage than 'Extreme AR1'.

Table 8-1: Mean proportion of tillers damaged by African black beetle (on arcsine-square root scale) for three half sibling families infected with AR1 and two commercial cultivars infected with AR1 and/or AR37 or without endophyte.

	Proportion of damaged tillers (arcsine-square root transformed) per bucket						
Family or cultivar	**A1**	**A2**	**A3**	**A4**	**A5**	**A6**	**A7***
F3-AR1	0.077	0.095	0.059	0.086	0.197	0.360	0.802
F15-AR1	0.055	0.055	0.027	0.068	0.184	0.463	1.010
F23-AR1	0.106	0.114	0.048	0.086	0.173	0.332	0.810
'Extreme AR1'	0.138	0.155	0.083	0.230	0.362	0.555	1.014
'Extreme AR37'	0.042	0.075	0.037	0.117	0.266	0.383	0.688
'Extreme Nil'	0.228	0.229	0.088	0.312	0.549	0.754	1.294
'Quartet AR1'	0.128	0.112	0.077	0.175	0.241	0.376	0.874
'Quartet Nil'	0.192	0.187	0.181	0.311	0.436	0.683	1.203
Mean cultivar	0.121	0.128	0.075	0.173	0.301	0.488	0.962
LSD(5%)	0.116	0.1233	0.1286	0.1929	0.1887	0.1919	0.1993
Cultivar effect							
F-statistic	$F_{(7,42)} = 2.58$	$F_{(7,42)} = 1.84$	$F_{(7,42)} = 1.84$	$F_{(7,42)} = 2.22$	$F_{(7,42)} = 4.22$	$F_{(7,42)} = 5.62$	$F_{(7,41)} = 7.33$
P-value	***0.027***	0.105	0.355	*0.052*	***0.001***	***<0.001***	***<0.001***

*A1–A7 = Assessments 1 (week 4, late autumn–early winter), 2 (week 8, early winter), 3 (week 12, mid-winter), 4 (week 16 late-winter), 5 (week 20, early spring), 6 (week 24 mid-spring), 7 (week 28, late spring). *Proportion of damaged tillers adjusted for added plants between A6 and A7. Statistically significant evidence (P ≤0.05) is highlighted in bold and italics. Weak evidence (0.05 < P ≤0.01) although not statistically significant is highlighted in italics. LSD(5%) = Fisher's least significant difference (LSD) post hoc test at the 5% significance level.*

By late spring all endophyte-infected lines (AR1 and AR37) were significantly less damaged than the two endophyte-free lines ('Extreme Nil' and 'Quartet Nil'). 'Extreme AR37' had significantly less feeding damage than 'Quartet AR1', F15-AR1 and 'Extreme AR1'. Of the three half-sibling families, F3-AR1 and F23-AR1 were the best performing with significantly less feeding damage than F15-AR1 and 'Extreme AR1'. In the cultivar 'Extreme', endophyte AR37 out-performed AR1 for resistance to feeding from adult African black beetle (*H. arator*), although damage was still very high (>50% of tillers damaged) in AR37-infected plant-line.

Dry matter. Herbage refers to plant material >4 cm from the crown and pseudostem to plant material ≤4 cm from the crown. At all assessments, mean herbage dry matter per bucket differed (P <0.001; Table 8-2) between cultivars. In the late autumn–early winter (A1 and A2) and in early–late spring (A5–A7) on average plants exposed to adult African black beetle (*H. arator*) had lower mean levels of dry herbage matter than plants not exposed (P ≤0.05; Table 8-2). This difference between treatments in herbage dry matter was not seen in the mid–late winter time (A3 and A4).

By late-spring (A7), there was a cultivar by treatment effect $F_{(4,48)} = 2.99$; $P = 0.11$; Figure 8-1), by which the endophyte-free lines ('Extreme Nil' and 'Quartet Nil') had the largest, and family F23-A and 'Extreme AR37' had the smallest differences between with and without African black beetle (*H. arator*) treatments, with all differences significant. For plants not exposed to adult African black beetle (*H. arator*), herbage dry matter was similar for all plant-lines. By comparison, in the presence of beetles, herbage dry matter production significantly differed between certain plant-lines. Families F23-AR1, F3-AR1 and 'Extreme AR37' (no significant differences) had the highest levels of herbage dry matter production (4.21, 3.30 and 3.30 g/bucket respectively), and were significantly different from the remaining plant-lines. Endophyte-free plant-lines had the lowest levels of herbage dry matter production ('Extreme Nil' = 0.37 and 'Quartet Nil' = 0.70 g/bucket), with 'Extreme AR1' the lowest of the endophyte-infected plant-lines (1.58 g/bucket) which was not significantly different from the the endophyte-free plant-lines. Even under African black beetle (*H. arator*) attack, family F23-AR1 had high herbage dry matter production (Figure 8-1), with mean production similar to unexposed plants.

Table 8-2: Mean herbage dry matter production from half-sibling families and cultivars, with and without adult African black beetle, at seven assessments over 28 weeks.

	Mean herbage dry matter per bucket (g)							
Plant type	**A0**	**A1**	**A2**	**A3**	**A4**	**A5**	**A6**	**A7***
Without beetle								
F3-AR1	4.40	4.11	3.70	2.73	3.32	4.28	5.82	6.18
F15-AR1	3.36	3.52	2.90	2.27	3.33	4.32	6.25	5.17
F23-AR1	3.28	2.98	2.62	1.96	2.53	3.78	5.58	6.01
'Extreme AR1'	3.49	2.97	1.96	1.29	1.93	2.91	5.26	5.45
'Extreme AR37'	3.30	3.56	3.05	1.95	2.16	2.81	4.26	4.80
'Extreme Nil'	2.82	3.32	2.80	1.80	2.10	3.13	4.76	5.04
'Quartet AR1'	3.75	3.43	2.82	2.08	2.82	3.85	5.48	5.13
'Quartet Nil'	3.58	3.32	2.59	1.67	2.17	3.06	4.85	5.03
With beetle								
F3-AR1	4.25	3.80	3.38	2.64	3.24	3.56	4.00	3.30
F15-AR1	3.69	3.45	2.79	2.28	3.03	3.74	3.97	1.83
F23-AR1	3.62	3.22	2.54	1.90	2.69	3.71	4.58	4.21
'Extreme AR1'	3.37	3.10	2.41	1.59	1.79	2.13	2.58	1.58
'Extreme AR37'	3.05	3.46	2.90	1.86	2.10	2.28	2.71	3.30
'Extreme Nil'	3.05	2.71	2.30	1.64	1.88	1.90	1.42	0.37
'Quartet AR1'	3.70	3.27	2.57	1.81	2.30	2.71	3.44	2.47
'Quartet Nil'	3.35	2.67	1.93	1.64	2.06	2.10	1.81	0.70
LSD(5%) between cultivars	0.805	0.541	0.548	0.371	0.588	0.779	1.182	1.405
LSD(5%) within cultivars	0.535	0.470	0.566	0.417	0.672	0.809	1.147	1.437
Cultivar effect								
F-statistic, $F_{(7,42)}$	2.60	4.87	10.15	22.53	18.43	12.70	7.10	5.73
P-value	***0.025***	***<0.001***	***<0.001***	***<0.001***	***<0.001***	***<0.001***	***<0.001***	***<0.001***
Treatment effect								
F-statistic, $F_{(1,48)}$	0.02	5.21	4.28	0.43	1.86	27.74	121.01	153.58
P-value	0.894	***0.027***	***0.044***	0.517	0.179	***<0.001***	***<0.001***	***<0.001***
Cultivar by Treatment effect								
F-statistic, $F_{(7,48)}$	0.89	1.87	1.39	0.63	0.36	0.87	1.87	2.55
P-value	0.525	*0.095*	0.233	0.729	0.922	0.539	*0.095*	***0.026***

A0–A7 = Assessments 0 (week 0, mid-late autumn), 1 (week 4, late autumn–early winter), 2 (week 8, early winter), 3 (week 12, mid-winter), 4 (week 16 late-winter), 5 (week 20, early spring), 6 (week 24 mid-spring), 7 (week 28, late spring). Statistically significant evidence (P ≤0.05) is highlighted in bold and italics. Weak evidence (0.05 < P ≤0.01) although not statistically significant is highlighted in italics. LSD(5%) = Fisher's least significant difference (LSD) post hoc test at the 5% significance level.

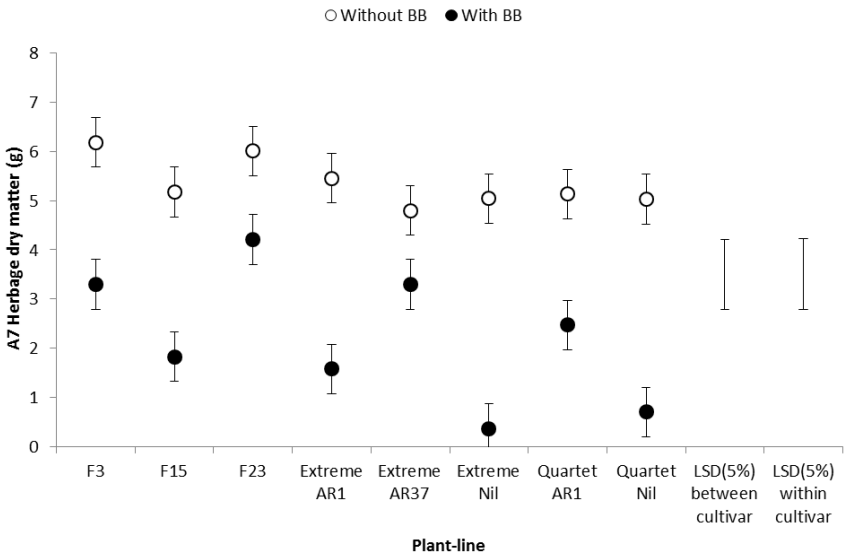

Figure 8-1: Herbage dry matter in late spring (A7) for half-sibling families and cultivars with and without adult African black beetle.
Mean ± SEM. A7 = Assessment 7 (week 28, late spring). BB = Adult African black beetle (H. arator). LSD(5%) = Fisher's least significant difference (LSD) post hoc test at the 5% significance level.

For pseudostem dry matter taken at the final assessment (A7) in late spring (Table 8-3), a cultivar by treatment interaction was found ($F_{(4,48)} = 2.99$; $P = 0.011$; Figure 8-2). In the absence of African black beetle (*H. arator*), family F3-AR1 had significantly higher production than the other plant-lines.

In the presence of African black beetle (*H. arator*), F3-AR1 had significantly higher dry matter production (2.22 g/bucket) than the other plant-lines except family F23-AR1 (2.08 g/bucket). The two endophyte-free cultivars ('Extreme Nil' and 'Quartet Nil') were not significantly different (1.007 and 1.176 g/bucket respectively), and both of these treatments had significantly lower pseudostem dry matter than all or some of the AR1-infected cultivars and families. The four plant-lines, F3-AR1, F15-AR1, 'Extreme Nil' and 'Quartet Nil' had significantly higher pseudostem dry matter production in the absence of adult African black beetle (*H. arator*) than in their presence. For the remaining families, although pseudostem dry matter in general was higher in the absence rather than in the presence of adult African black beetle (*H. arator*), the difference was not significant.

Table 8-3: Mean total pseudostem dry matter production from half-sibling families and cultivars, with and without adult African black beetle in late spring (A7).

	Mean total pseudostem dry matter per bucket (g) at A7	
Family or cultivar	**Without beetle**	**With beetle**
F3-AR1	2.79	2.22
F15-AR1	1.96	1.50
F23-AR1	2.05	2.08
'Extreme AR1'	1.86	1.59
'Extreme AR37'	1.73	1.81
'Extreme Nil'	2.05	1.01
'Quartet AR1'	1.82	1.60
'Quartet Nil'	1.91	1.18
LSD(5%) between cultivars	0.4234	
LSD(5%) within cultivars	0.4451	
Cultivar by treatment effect		
F-statistic	$F_{(4,48)} = 2.99$	
P-value	***0.011***	

A7 = Assessment 7 (week 28, late spring). Statistically significant evidence (P ≤0.05) is highlighted in bold and italics. LSD(5%) = Fisher's least significant difference (LSD) post hoc test at the 5% significance level.

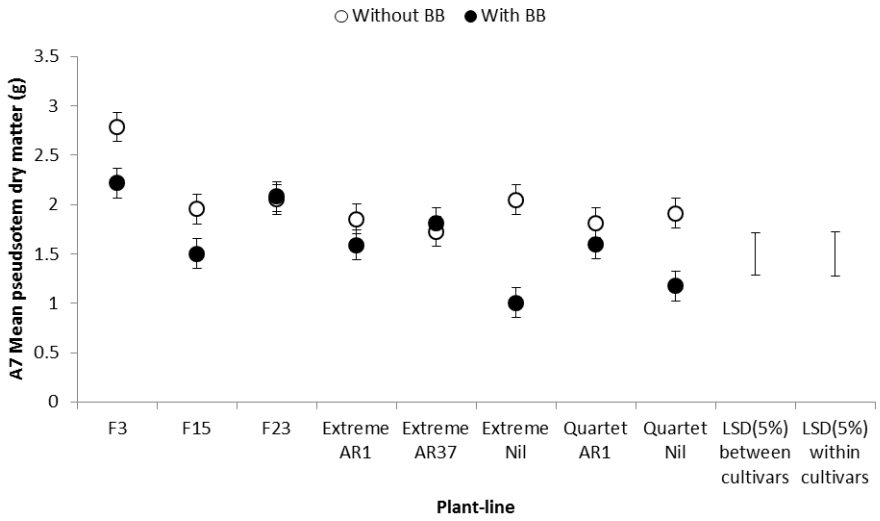

Figure 8-2: Pseudostem dry matter in late spring (A7) for half-sibling families and cultivars with and without adult African black beetle.
Mean ± SEM. A7 = Assessment 7 (week 28, late spring). BB = Adult African black beetle (H. arator). LSD(5%) = Fisher's least significant difference (LSD) post hoc test at the 5% significance level.

Live Tillers. There was strong evidence at all assessments (A0–A7) that the mean total number of live tillers per bucket differed between cultivars (P <0.001). From mid-spring (A6 and A7) there was a cultivar by beetle effect ($F_{(7,48)}$ = 3.00; P = 0.011 and $F_{(7,48)}$ = 7.48; P <0.001 respectively) and in late spring (A7) families F3-AR1, F23-AR and cultivars 'Extreme AR37' and 'Quartet AR1' had more live tillers in the presence of adult African black beetle (*H. arator*) than without, whereas, family F15 and cultivars 'Extreme AR1', 'Extreme Nil' and 'Quartet Nil' had less tillers in the presence of beetles. The number of live tillers a family or cultivar had in the presence or absence of African black beetle (*H. arator*) in late spring were significantly different for family F23-AR1, cultivars 'Extreme AR1', 'Extreme AR37', 'Extreme Nil' and 'Quartet Nil' (Table 8-4).

Endophyte data. Mean paxilline-IRE was influenced by cultivar and treatment, and these effects were interactive ($F_{(5,36)}$ = 2.54; P = 0.046), with the average concentration of paxilline-IRE significantly higher in plant lines that had not been exposed to feeding from adult African black beetle (*H. arator*) (Table 8-5). The exceptions to this were family F23-AR1 and 'Extreme AR37'. When exposed to adult African black beetle (*H. arator*), half-sibling family F23-AR1 had a significantly higher concentration of paxilline-IRE than the other endophyte-infected lines. When not exposed to adult African black beetle (*H. arator*), 'Extreme AR37' had significantly lower paxilline-IRE than the other endophyte-infected lines. 'Quartet AR1' had significantly lower paxilline-IRE than F23-AR1 in unexposed plants and the half-sibling families (F3-AR1, F15-AR1 and F23-AR1) were not significantly different from each other.

Endophyte mycelium mass was affected by both cultivar ($F_{(5,30)}$ = 28.48; P <0.001) and treatment ($F_{(1,36)}$ = 6.39; P = 0.016), but there was no evidence of an interaction among these factors (Table 8-5). Family F23-AR1 had on average significantly higher endophyte mycelium mass than all other endophyte-infected lines (range 10.15–21.24 mg/g, median = 13.61, mean = 14.37). There was no evidence of significant differences between families F3-AR1 and F15-AR1, or between 'Quartet AR1', 'Extreme AR1' or 'Extreme AR37'. However the three endophyte-infected cultivars ('Quartet AR1', 'Extreme AR1' and 'Extreme AR37') had

Table 8-4: Mean number of live tillers from half-sibling families and cultivars, with and without adult African black beetle, at seven assessments over 28 weeks.

	Mean live tiller number per bucket							
Plant type	**A0**	**A1**	**A2**	**A3**	**A4**	**A5**	**A6**	**A7***
Without beetle means								
F3-AR1	224.7	252.4	274.9	277.7	257.4	261.9	307.3	282.1
F15-AR1	168.1	210.7	227.7	244.3	229.9	224.4	210.7	201.0
F23-AR1	169.6	184.6	205.4	206.3	198.1	220.0	243.9	250.1
'Extreme AR1'	157.7	168.7	153.7	129.4	124.6	148.6	184.1	179.6
'Extreme AR37'	185.3	200.6	204.6	180.3	128.0	137.0	161.0	173.6
'Extreme Nil'	173.9	185.0	195.9	172.9	134.3	148.0	173.9	179.1
'Quartet AR1'	120.1	137.7	145.7	141.9	136.1	151.3	154.1	151.4
'Quartet Nil'	116.3	128.7	133.4	119.7	112.9	137.6	150.0	146.1
With beetle means								
F3-AR1	218.6	253.3	279.0	264.9	259.3	260.9	298.0	324.1
F15-AR1	180.0	211.7	245.4	234.3	247.0	230.3	258.7	193.6
F23-AR1	174.3	201.6	225.7	215.0	226.1	264.4	302.9	324.6
'Extreme AR1'	154.9	192.1	187.3	158.6	142.6	151.3	141.9	116.0
'Extreme AR37'	180.3	213.3	223.3	192.1	161.7	168.1	188.6	250.9
'Extreme Nil'	169.3	197.0	189.6	151.0	151.4	144.4	95.6	28.6
'Quartet AR1'	122.7	135.7	147.9	135.6	135.4	152.3	168.4	180.6
'Quartet Nil'	115.7	130.9	128.9	112.3	109.4	119.3	116.7	47.0
LSD(5%) between cultivars	22.28	29.85	28.47	31.76	35.10	35.40	55.90	62.98
LSD(5%) within cultivars	20.61	27.42	28.82	32.42	32.34	33.78	55.17	61.82
Cultivar effect								
F-statistic, $F_{(7,42)}$	31.74	24.20	44.97	45.83	33.78	30.73	20.15	22.21
P-value	***<0.001***	***<0.001***	***<0.001***	***<0.001***	***<0.001***	***<0.001***	***<0.001***	***<0.001***
Treatment effect (without and with beetle)								
F-statistic, $F_{(1,48)}$	0.00	3.03	4.47	0.04	6.03	1.72	0.03	1.27
P-value	1.000	*0.088*	***0.040***	0.849	***0.018***	0.196	0.855	0.266
Cultivar by Treatment effect								
F-statistic, $F_{(7,48)}$	0.36	0.45	0.95	1.04	0.71	1.44	3.00	7.48
P-value	0.923	0.863	0.478	0.416	0.662	0.211	***0.011***	***<0.001***

A0–A7 = Assessments 0 (week 0, mid-late autumn), 1 (week 4, late autumn–early winter), 2 (week 8, early winter), 3 (week 12, mid-winter), 4 (week 16 late-winter), 5 (week 20, early spring), 6 (week 24 mid-spring), 7 (week 28, late spring). Statistically significant evidence (P ≤0.05) is highlighted in bold and italics. Weak evidence (0.05 < P ≤0.01) although not statistically significant is highlighted in italics. LSD(5%) = Fisher's least significant difference (LSD) post hoc test at the 5% significance level.

Table 8-5: Mean concentrations of paxilline-IRE and endophyte mycelium mass (µg/g) in late spring (A7) with and without African black beetle; with summary of statistical analyses.

	Paxilline-IRE concentration (µg/g)		Endophyte mycelium mass (mg/g)	
Family or cultivar	**With beetles**	**Without beetles**	**With beetles**	**Without beetles**
F3-AR1	6.64	12.52	15.53	16.08
F15-AR1	5.56	11.41	14.21	16.42
F23-AR1	11.33	13.42	20.58	21.90
'Extreme AR1'	5.99	12.11	10.59	12.97
'Extreme AR37'	5.45	6.62	10.60	13.19
'Quartet AR1'	4.64	9.77	9.49	10.80
LSD(5%) between cultivars	2.892		3.179	
LSD(5%) within cultivar	2.761		3.394	
Cultivar Effect *F*-statistic; *P*-value	$F_{(5,30)}$ = 8.12; ***P <0.001***		$F_{(5,30)}$ = 28.48; ***P <0.001***	
Treatment Effect *F*-statistic; *P*-value	$F_{(1,36)}$ = 61.95; ***P <0.001***		$F_{(1,36)}$ = 6.39; ***P = 0.016***	
Cultivar by Treatment *F*-statistic; *P*-value	$F_{(5,36)}$ = 2.54; ***P = 0.046***		$F_{(5,36)}$ = 0.22; *P* = 0.950	

Paxiline-IRE = paxilline immunoreactive equivalents. A7 = Assessment 7 (week 28, late spring). LSD(5%) = Fisher's least significant difference (LSD) post hoc test at the 5% significance level. Statistically significant evidence and effects are highlighted in bold and italics.

significantly lower endophyte mycelium mass than the three half-sibling families (F23-AR1, F15-AR1 and F3-AR1). There was a significantly higher overall mean endophyte mycelium mass in ryegrass associations not exposed to adult African black beetle (*H. arator*) (mean difference = 1.73 µg/g; Table 8-5). There was no evidence of a relationship between paxilline immunoreactivity and endophyte mycelium mass (P >0.05).

Relationships between feeding damage and endophyte data. In late spring (A7), there was strong evidence ($F_{(1,42)}$ = 29.93; P <0.001) of a negative relationship between feeding damage and paxilline immunoreactivity (Figure 8-3), and that this relationship differed between cultivars and half-sibling families ($F_{(5,42)}$ = 2.45; P = 0.049) (Table 8-6 and Figure 8-3 B–G). These relationships still held when endophyte-free cultivars were excluded from the analysis. No evidence was found of a relationship between feeding damage and endophyte mycelium mass (P >0.05).

Table 8-6: Regression analyses between African black beetle feeding damage and paxilline immunoreactivity in late spring (A7), omitting endophyte-free cultivars.

Family or cultivar	Intercept	SE (Intercept)	Slope	SE (Slope)
F3-AR1	0.906	0.119	-0.03021	0.01606
F15-AR1	1.221	0.129	-0.04528	0.02117
F23-AR1	0.746	0.244	-0.00292	0.02103
'Extreme AR1'	1.552	0.131	-0.09207	0.02003
'Extreme AR37'	0.927	0.175	-0.05663	0.03064
'Quartet AR1'	1.202	0.128	-0.07949	0.02518
Feeding damage-paxilline relationship			$F_{(1,23)}$ = 29.67: ***P* <0.001**	
Cultivar interaction (different slopes for each cultivar)			$F_{(5,29)}$ = 2.48; ***P* = 0.055**	
†Conditional R^2			0.68	

A7 = Assessment 7 (week 28, late spring). SE = Standard error. Statistically significant evidence and effects are highlighted in bold and italics. †Nakagawa and Schielzeth (2013).

Relationships between dry matter and endophyte data. For herbage and pseudostem dry matter there was evidence of a positive relationship with paxilline immunoreactivity, and the slope of this relationship was affected by the presence of beetles (treatment by paxilline immunoreactivity interaction; $F_{(1,84)}$ = 4.49; P = 0.037 and $F_{(1,83)}$ = 4.96; P = 0.029 respectively), but there was no evidence that it differed between plant-lines The outcome ($F_{(1,68)}$ = 4.14; P = 0.046 and $F_{(1,63)}$ = 3.00; P = 0.088 for herbage and pesudostem respectively), was similar when the analysis was repeated with the endophyte-free plant-lines omitted (intercepts and slopes with the endophyte-free plant-lines omitted are reported in Table 8-7.

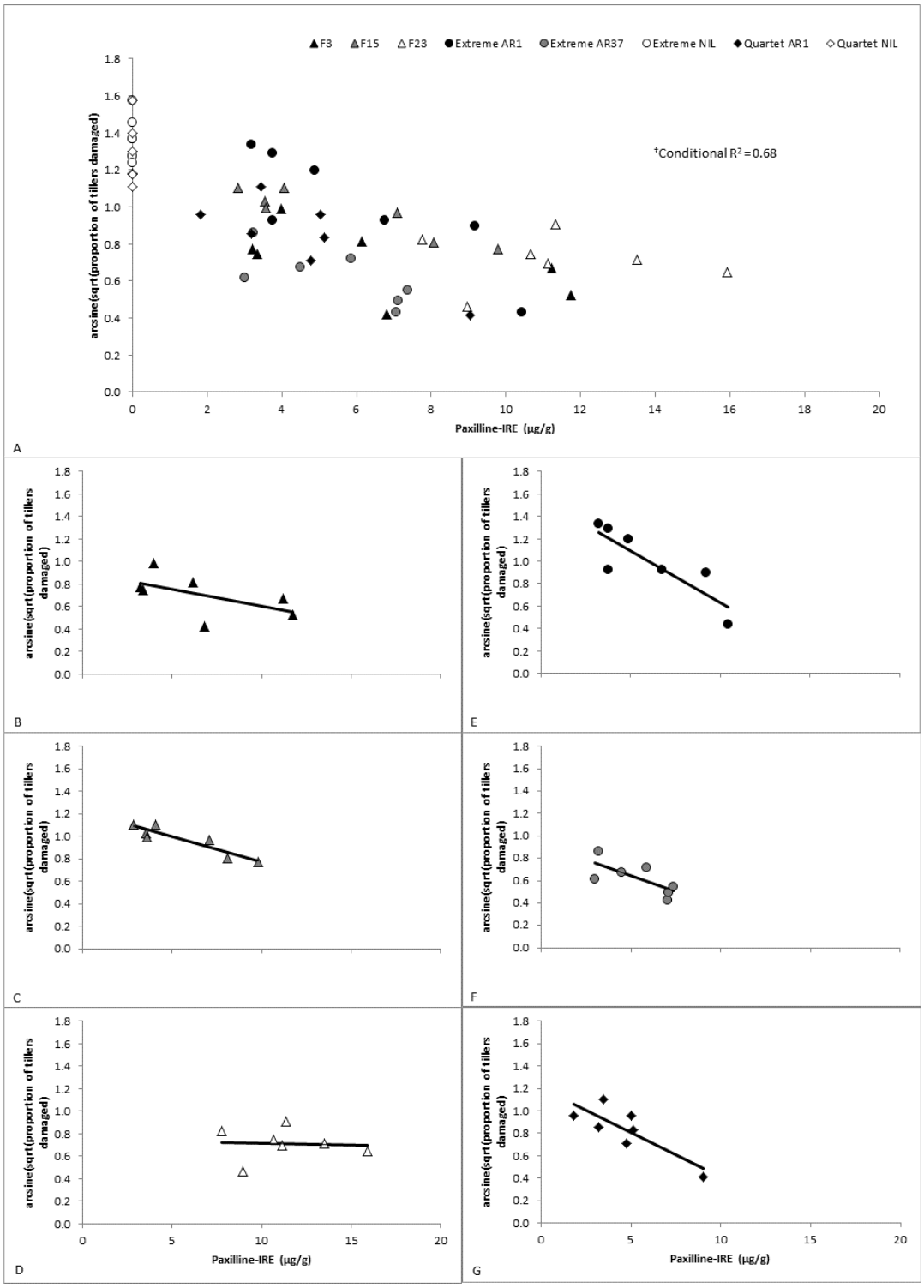

Figure 8-3: Regression analyses of feeding damage and paxilline immunoreactivity including the endophyte-free cultivars.

A) Scatterplot of all data from half-sibling families and cultivars. †Nakagawa and Schielzeth (2013). B) Slope of half-sibling family F3-AR1. C) Slope of half-sibling family F15-AR1. D) Slope of half-sibling family F23-AR1. E) Slope of cultivar 'Extreme AR1'. F) Slope of cultivar 'Extreme AR37'. G) Slope of cultivar 'Quartet AR1'. Data points for half-sibling families and cultivars are coded by symbol and colour.

Table 8-7: Herbage and pseudostem dry matter regression analyses with paxilline immunoreactivity in late spring (A7) with and without African black beetle; with endophyte-free cultivars omitted.

	Herbage				Pseudostem			
Family or cultivar	**Intercept**	**SE (Intercept)**	**Slope**	**SE (Slope)**	**Intercept**	**SE (Intercept)**	**Slope**	**SE (Slope)**
				Without beetles				
F3-AR1	5.475	0.501	0.190	0.062	2.691	0.184	0.026	0.023
F15-AR1	4.679	0.474	0.190	0.062	1.889	0.173	0.026	0.023
F23-AR1	5.127	0.529	0.190	0.062	1.934	0.194	0.026	0.023
'Extreme AR1'	4.822	0.490	0.190	0.062	1.770	0.179	0.026	0.023
'Extreme AR37'	5.211	0.465	0.190	0.062	1.785	0.170	0.026	0.023
'Quartet AR1'	4.948	0.450	0.190	0.062	1.790	0.163	0.026	0.023
				With beetles				
F3-AR1	4.112	0.470	0.376	0.069	2.402	0.171	0.085	0.026
F15-AR1	3.041	0.498	0.376	0.069	1.776	0.182	0.085	0.026
F23-AR1	3.256	0.479	0.376	0.069	1.864	0.175	0.085	0.026
'Extreme AR1'	2.633	0.486	0.376	0.069	1.830	0.177	0.085	0.026
'Extreme AR37'	4.553	0.502	0.376	0.069	2.098	0.184	0.085	0.026
'Quartet AR1'	4.031	0.503	0.376	0.069	1.953	0.195	0.085	0.026
Plant dry matter-paxilline immunoreactivity relationship			$F_{(1,69)} = 33.86$; ***P* <0.001**		$F_{(1,69)} = 8.87$; ***P* = 0.004**			
Treatment by beetle interaction (different slope without and with African black beetle)			$F_{(1,68)} = 4.14$; ***P* <0.046**		$F_{(1,68)} = 4.14$; *P = 0.088*			
†Conditional R^2			0.74		0.50			

A7 = Assessment 7 (week 28, late spring). SE = Standard error. Statistically significant evidence and effects are highlighted in bold and italics. Weak evidence (0.05 < P ≤0.01) although not statistically significant is highlighted in italics. †Nakagawa and Schielzeth (2013).

Recovered live adults and offspring data. The number of live beetles found at the end of the trial differed between cultivars ($F_{(7,42)}$ = 4.06; P = 0.002), with significantly fewer beetles recovered from the three half-sibling AR1-infected families and 'Extreme AR37' than from the endophyte-free plant-lines (Table 8-8). The number of beetles recovered from the endophyte-infected plant-lines was similar. The number of eggs and larvae recovered also differed between cultivars ($F_{(7,42)}$ = 2.27; P = 0.047), with 'Extreme AR1' and the three half-sibling families (F23-AR1, F15-AR1 and F3-AR1) having significantly fewer offspring recovered than the endophyte-free plant types. Because plants were added between assessments A6 and A7, regression analyses were not performed for recovered live adult African black beetle (*H. arator*) and number of offspring with herbage dry matter, pseudostem dry matter, endophyte mass and paxilline immunoreactivity.

Table 8-8: Mean number of adult African black beetle recovered alive and mean number of offspring recovered per bucket.

Family or cultivar	Mean number of beetles recovered	Mean number of offspring recovered (log)
F3-AR1	2.57	1.21
F15-AR1	2.29	1.18
F23-AR1	1.86	1.14
'Extreme AR1'	2.86	0.93
'Extreme AR37'	1.86	1.25
'Extreme Nil'	3.86	2.13
'Quartet AR1'	3.14	1.52
'Quartet Nil'	4.29	2.04
SEM	0.62	0.41
LSD(5%)	1.26	0.83
F-statistic; *P*-value	$F_{(7,42)}$ = 4.06: ***P* = 0.002**	$F_{(7,42)}$ = 2.27: ***P* = 0.047**
Effect	Cultivar	Cultivar

SEM = standard error of the mean. LSD(5%) = Fisher's Least Significant Difference (LSD) at the 5% significance level. Statistically significant evidence and effects are highlighted in bold and italics.

8.5 Discussion

Endophyte-infected ryegrass plants, suitable for agricultural purposes, are plants that are robust, have high dry matter production and have high levels of the beneficial fungal secondary metabolites to provide plant resistance to biotic factors without being harmful to livestock. The novel *E. festucae* var. *lolii* strain AR1 was developed to provide New Zealand farmers with an endophyte that had no adverse effects on livestock but provided resistance to Argentine stem weevil (*L. bonariensis*). The endophyte was not expected to affect African black beetle (*H. arator*) given the absence of ergovaline production. However, moderate effects on adults were reported by Popay & Jensen (2001), presenting an opportunity to identify the cause of the deterrent effects and if possible select for stronger resistance. Previously it had been shown that the effects of AR1 on African black beetle (*H. arator*) damage appeared to be associated with paxilline immunoreactivity where in general, high levels of paxilline immunoreactivity (detected by ELISA) reflected low levels of feeding damage (Ross *et al*, unpublished 2015, Chapter 7).

In this current trial a negative relationship between feeding damage and paxilline immunoreactivity was established (Table 8-6 and Figure 8-3), with different slopes dependent on the host plant-endophtye association (plant-line; cultivar or half-sibling family and endophyte strain, AR1 or AR37). The different slopes for plant-lines may explain why relationships were not able to be established in the earlier work by Ross *et al.* (unpublished 2015, Chapter 7).

The paxilline ELISA recognises paxilline and an array of other simple paxilline-like indole diterpenes, quantified by paxilline-IRE. These compounds are likely intermediates or precursors of the more complex alkaloids. The paxilline-like compounds associated with reduced feeding damage from adult African black beetle (*H. arator*) may be acting as bioactives or as marker compounds, and may differ between endophyte strains.

Ross *et al.* (unpublished 2015, Chapter 7) provided evidence that plant resistance to feeding damage from adult African black beetle (*H. arator*) is dependent on both plant-line and endophyte status (endophyte-infected or endophtye-free), with

endophyte-infection reducing tiller damage. By late spring all AR1-infected plant-lines were out performing the endophyte-free lines, with 'Extreme AR37', F3-AR1 and F23-AR1 the most resistant plants. Although feeding damage on endophyte-infected ryegrass was high, it must be noted that the trial did not reflect a normal field situation where beetles have free movement and multiple food sources to choose from. However, the results likely reflect how each plant-line would perform under high pressure from adult beetles feeding in an outbreak situation.

Levels of paxilline immunoreactivity differed between plant-lines and in the presence of beetles. Levels were higher in plants not exposed to adult African black beetle (*H. arator*). In addition, for damaged plants, the damaged tillers had lower levels of paxilline immunoreactivity than the undamaged tillers. Both the reduction in paxilline immunoreactivity between unexposed and exposed plants, and between undamaged and damaged tillers within a damaged plant, can be explained by the disruption of the plant tissue and meristem where hypae and alkaloids are concentrated. The negative relationship between feeding damage and paxilline immunoreactivity established in this trial was found following exposure to African black beetle (*H. arator*) and is consistent with the suggestion a subset of paxilline-like alkaloids are associated with feeding damage (Ross *et al.*, unpublished 2015, Chapter 7). Both *E. festucae* var. *lolii* strains AR1 and AR37 in association with perennial ryegrass (*L. perenne*) do not produce the known African black beetle (*H. arator*) deterrent, ergovaline (Ball *et al.*, 1997). Although they differ in alkaloid profiles (Thom *et al.*, 2013), the negative relationship found between feeding damage and paxilline immunoreactivity for both strains, suggests they may share the same subset of bioactive alkaloids.

The performance of individual plant-lines used in this current trial for studying resistance to adult African black beetle (*H. arator*) and paxilline immunoreactivity production was consistent with that found by Ross *et al.* (unpublished 2015, Chapter 7). This consistency of performance in multiple feeding trials allows identification of plant-lines suitable for breeding programs to improve plant resistance to adult beetles. Through the various trials, effects of host plant, cultivar or family, endophyte and beetle feeding on the AR1-ryegrass-insect relationship were examined. The combination of host plant and endophyte was found to affect

plant resistance to adult beetles. This is consistent with previous findings that the genetic characteristics of both endophyte and host plant are important in levels of alkaloid production (Fannin *et al.*, 1990; Christensen *et al.*, 1991; Hill *et al.*, 1991; Christensen *et al.*, 1993; Agee & Hill, 1994). In addition, differences between plant-lines were found for endophyte concentration and this was consistent with reported differences in concentrations of endophyte and their endophyte metabolites between plant-lines (Breen, 1992; Ball *et al.*, 1995b).

No evidence of a relationship between paxilline immunoreactivity and endophyte mycelium mass was found. In general alkaloid production lags endophyte concentration (di Menna *et al.*, 1992; Ball *et al.*, 1995b), and different metabolites have production peaks at slightly different times (Prestidge & Gallagher, 1988; Ball *et al.*, 1991; di Menna *et al.*, 1992; Ball *et al.*, 1995b). Evidence of relationships between endophyte concentration and alkaloid production have been reported for some of the known alkaloids (Ball *et al.*, 1995b). For some alkaloids the relationship is found throughout the year, for others only at certain times of the year (Ball *et al.*, 1995a). The latter may explain that although the rank order for levels of paxilline immunoreactivity and endophyte mycelium mass was very similar, no relationship was found.

Consistent with the lack of a relationship between paxilline immunoreactivity and endophyte concentration, there was also no evidence of a relationship between feeding damage and endophyte mass, suggesting that the plant-endophyte association is responding to beetle attack. Therefore, the relationship between feeding damage and paxilline immunoreactivity is likely a response to insect attack (Karban & Baldwin, 1997), and likely includes the production of specific paxilline-like alkaloids as suggested by Ross *et al.* (unpublished 2015, Chapter 7).

Although the paxilline ELISA detects paxilline-like compounds, it cannot measure actual amounts of each paxilline-like compound, instead quantitatively measuring levels of immunoreactivity as paxilline immunoreactive equivalents. Paxilline immunoreactivity levels were significantly higher in plants not exposed than exposed to adult African black beetle (*H. arator*). This observation was still unexpected because if the proposed subset of paxilline-like secondary metabolites were produced in association with feeding damage, the concentration of paxilline

immunoreactivity in plants exposed to beetles was predicted to be higher than plants not exposed. Resource availability has an effect on alkaloid production, with high resource costs associated with hosting an endophyte and producing alkaloids (Rodriguez *et al.*, 2009). The observed result may simply be because unexposed plants, having optimal conditions for growth and minimal stress, did not have limiting resources for secondary metabolite production including paxilline immunoreactivity. Whereas, for exposed plants the supply of resources was reduced, such as a lower photosynthetic capability due to tiller damage. Mean endophyte mass was also significantly higher in plants not exposed than those exposed to adult African black beetle (*H. arator*), which could also be attributed to resource limitations. In addition, in the undamaged plants, paxilline-like compounds not associated with feeding damage but with high cross-reactivities in the ELISA, may have been produced at higher levels than in damaged plants. Or for damaged plants, all paxilline-like alkaloid production was reduced, therefore lowering the level of paxilline immunoreactivity. Alternatively, paxilline-like alkaloid production was simply down-regulated.

There is evidence in the literature that shows some beneficial effects of endophytes against insect herbivory are induced and that the endophyte mediates the induced response by its host grass (Bultman & Ganey, 1995; Bultman *et al.*, 2004; Sullivan *et al.*, 2007). The interaction between prior damage and endophyte infection suggests that induced plant responses accentuate the effects of the endophytes (Karban & Baldwin, 1997). Bultman *et al.* (2004) reported inducible loline production and Sullivan *et al.* (2007) showed that this increase in loline concentration involved the upregulation of genes encoding for loline production following damage in endophyte-infected tall fescue (*L. arundinaceum*). Bultman *et al.* (2004) and Sullivan *et al.* (2007) also showed that damaged plants containing the fungus were more resistant to insects and had negative effects on these insects. In comparison, endophyte-free plants had increased susceptibility to insects following damage. Patchett *et al.* (2008) has shown an increase (26%) in total loline alkaloids in the roots of meadow fescue exposed to grass grub larvae and a decrease (10%) in the concentration in the crown and no difference in the concentration in above ground herbage. Patchett *et al.* (2008) suggest this may be an induced effect with alkaloid transport to the region of the plant under attack and propose it is a

redistribution of lolines in response to an insect attack instead of an overall increase in alkaloid production in the plant.

Differences were found between plant-lines for herbage dry matter production. When beetles were actively feeding (in late autumn–early winter and in early–late spring) the dry matter production significantly reduced for all plant-lines. In comparison, there were no significant differences in herbage dry matter production between plant-lines when beetles were not present. Of the plant-lines studied, family F23-AR1 performed the best with regards to dry matter production in the presence of actively feeding adult African black beetles (*H. arator*) (Figure 8-1). Under African black beetle (*H. arator*) pressure, 'Extreme AR37' had higher yields for dry matter production than 'Extreme AR1' and 'Extreme Nil'. However, 'Extreme AR37' herbage dry matter yields were not significantly higher than the two AR1-infected families, F23-AR1 and F15-AR1. Such findings highlight the importance and influence of the host plant (cultivar or family) genotype in endophyte-ryegrass associations.

For pseudostem dry matter production in late spring there was evidence of plant-line and African black beetle (*H. arator*) influences, with F3-AR1, F15-AR1 and the endophyte-free plant-lines having reduced production in the presence of adult beetles (Figure 8-2). Plant-lines F3-AR1, F23-AR1 and 'Extreme AR37' (although not significantly different from each other) had the highest levels of pseudostem dry matter production in the presence of beetles than the other plant-lines.

Live tiller numbers in early spring reflected dry matter production in late spring. The ability of endophyte-host plant associations to increase tiller production while under African black beetle (*H. arator*) pressure is an important factor in compensating for feeding damage in both herbage and pseudostem dry matter yields.

A positive relationship was found between paxilline immunoreactivity with both herbage and pseudostem dry matter. The slope of this relationship was steeper in the presence of beetles. Although we cannot conclude cause and effect, it does suggest that changes in the level of paxilline immunoreactivity will have a greater influence on plant dry matter production in the presence of actively feeding African black beetles (*H. arator*) than in the absence.

The number of live adult African black beetle (*H. arator*) recovered, a proxy for survival, differed between plant-lines, with 'Extreme AR37' and the three half-sibling families having significantly fewer beetles recovered than the other plant-lines. In agreement with the literature (Popay & Baltus, 2001), there was no evidence of difference in recovery between the AR1-infected plant-lines of 'Extreme' and 'Quartet' and their respective endophyte-free lines. The results provide further evidence that hosting an endophyte is beneficial to the plant and that the detrimental effects of the endophyte-plant association to adult beetles are influenced by both the endophyte strain and the host-plant.

The number of African black beetle (*H. arator*) offspring differed between plant-lines, with endophyte-free lines recording the highest numbers consistent with the literature (Popay & Baltus, 2001; Hume *et al.*, 2007). However, in this current trial there was no evidence that the number of offspring differed between plant-lines infected with endophyte, although differences have been reported in field trials (Thom *et al.*, 2013). In addition fewer offspring were found for both endophyte-infected 'Extreme' lines compared with 'Extreme Nil'. This is a reflection of the greater beetle feeding on endophyte-free plant-lines than on endophyte-infected plant-lines, which resulted in a higher fecundity level. These results provide evidence that hosting an endophyte is beneficial to the plant by reducing beetle oviposition, and in turn reducing larval herbivory.

Beetles actively feed in autumn, as newly emerged beetles (from pupae) and build-up fat reserves for the overwintering dormancy period when feeding is intermittent. In spring they emerge from their dormant period to feed and reproduce (Todd, 1959; Todd, 1964; Bell *et al.*, 2011). This study was conducted from mid-autumn to late spring 2012 (New Zealand), incorporating the two periods in the African black beetle (*H. arator*) lifecycle when adult beetles are actively feeding. Over this period alkaloid levels are still high when compared with levels in the winter (di Menna *et al.*, 1992; Woodburn *et al.*, 1993; Ball *et al.*, 1995b; Easton *et al.*, 1996).

In summary, levels of paxilline immunoreactivity, endophyte mass and feeding damage from adult African black beetle (*H. arator*) differed between plant-lines. After examining more extensively the associations between paxilline immunoreactivity and feeding damage from adult beetles plant-line specific

negative relationships were determined. These relationships included both AR1- and AR37-infected ryegrass associations. Both endophytes do not produce ergovaline, known to deter African black beetle (*H. arator*). There was no evidence of relationships between feeding damage and endophyte mass, or between paxilline immunoreactivity and endophyte mass. However, a positive relationship was found between paxilline immunoreactivity and dry matter production and it appears that in the presence of adult African black beetle (*H. arator*), changes in the level of paxilline immunoreactivity are associated with a greater effect on dry matter production. Hosting an endophyte in general was beneficial, with reduced feeding damage, higher dry matter production, fewer live adult beetles and offspring. The AR1-infected half-sibling families and 'Extreme-AR37' consistently outperformed the AR1-infected cultivar lines and endophyte-free lines.

Further work is warranted to investigate the negative relationship between feeding damage from adult African black beetle (*H. arator*) and paxilline immunoreactivity from *E. festucae* endophyte-ryegrass associations that produce unknown compounds that deter adult beetles and do not cause toxicity to livestock. This relationship supports the potential use of the paxilline ELISA as a screening tool for asexual *E. festucae* endophyte ryegrass associations that do not produce the known compounds that deter adult beetles. Further investigation of the plant response to adult African black beetle (*H. arator*) feeding would be useful knowledge for understanding and identifying the bioactive compounds, or marker compounds, associated with increased plant resistance to adult beetles. This knowledge would underpin improved screening of plant-lines in a breeding program for the selection of endophyte-ryegrass associations with strong resistance to African black beetle (*H. arator*).

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Chapter 9

Thesis Summary

9.1 Thesis overview

The insect pest, African black beetle (*Heteronychus arator* (Fabricius, 1775)) is of considerable economic cost to the New Zealand agricultural industry, in regions where the insect is established and regular outbreaks are now occurring. Selection for *E. festucae* endophyte ryegrass associations that do not cause toxicity to livestock and with strong resistance to African black beetle (*H. arator*), would be of significant value to farmers in regions where this pest is a problem. The scientific research contained within this thesis is an important step in the development of non-toxic African black beetle (*H. arator*) resistant ryegrasses.

Prior to commencing my thesis research, there was a lack of understanding regarding the bioactivity of AR1 endophyte in how it provides pasture with moderate resistance to African black beetle (*H. arator*). The commercially important AR1 endophyte does not produce the known alkaloid, ergovaline (Tapper & Latch, 1999), which deters African black beetle (*H. arator*) (Ball *et al.*, 1997). Earlier work suggested that paxilline-like compounds could be the bioactive compound that is providing the resistance, or be a marker linked with the bioactive compound (Popay A. J., Fletcher L. R., Briggs L. R., AgResearch, New Zealand; unpublished work, 2006; also see Chapter 7). However, this earlier observation was drawn from mean responses across several different cultivars, some of which had different ploidy levels. As a consequence, it is unclear whether this observation is simply an artefact of cultivar differences. My thesis research advances our understanding of AR1 bioactivity, providing scientific knowledge of the AR1-African black beetle (*H. arator*) relationship using closely related plant-genotypes.

Research into the resistance properties of AR1 is of importance to the pastoral sector as to date there have been no reports of toxicity in livestock from AR1-infected pastures (Bluett *et al.*, 2005b; Bluett *et al.*, 2005a). Moreover, AR1 does not produce any of the alkaloids known to cause mammalian toxicity, such as, lolitrem, which induces ryegrass staggers or ergovaline, which disrupts

thermoregulation (Tapper & Latch, 1999). My research has contributed to a greater understanding of AR1 bioactivity through examining: 1) how different AR1-ryegrass associations affect plant resistance to adult African black beetle (*H. arator*); 2) distribution of paxilline-like compounds in ryegrass; 3) relationships between feeding damage and paxilline immunoreactivity; 4) African black beetle (*H. arator*) exposure and plant responses, including paxilline immunoreactivity and dry matter production; and 5) the influence of different AR1-ryegrass associations on adult African black beetle (*H. arator*) behaviour, survival and fecundity.

The overarching aim of my research was to determine if increased concentrations of paxilline immunoreactivity were associated with a reduction in feeding damage from adult African black beetle (*H. arator*) in closely related AR1-infected perennial ryegrasses (*Lolium perenne* L.). To address this, a series of laboratory trials were performed on ryegrass plants using half-sibling families from a breeding line (GA97). These trials substantiated the unpublished work (Popay A. J., Fletcher L. R., Briggs L. R., AgResearch, New Zealand; unpublished work, 2006; also see Chapter 7), which suggested a relationship between increased levels of paxilline immunoreactivity and reduction in feeding damage at the cultivar level. Furthermore, my trials extended previous work to investigate the relationship between feeding damage and paxilline immunoreactivity within half-sibling families.

I found a negative relationship between feeding damage from African adult African black beetle (*H. arator*) and paxilline immunoreactivity (detected by ELISA) in perennial ryegrass (*L. perenne*) infected with the *Epichloë festucae* var. *lolii* (Latch, M.J. Chr. & Samuels) C. W. Bacon & Schardl, stat. nov. et comb. nov. strain AR1 post exposure to beetles. My research has further revealed that the relationship between feeding damage and paxilline immunoreactivity is complicated by: 1) endophyte concentrations not being simply reflected by paxilline immunoreactivity; 2) the influence of cultivar on the expression levels of paxilline immunoreactivity; 3) the effect of African black beetle (*H. arator*) on expression levels of paxilline immunoreactivity; and 4) the paxilline ELISA quantifying the array of paxilline-like compounds collectively.

9.2 Variation and distribution of paxilline in ryegrass plants

Key findings and implications

A negative relationship has been determined between feeding damage from African adult African black beetle (*H. arator*) and paxilline immunoreactivity (detected by ELISA) in perennial ryegrass (*L. perenne*) infected with the *Epichloë festucae* var. *lolii* strain AR1 post exposure to beetles.

The paxilline immunoreactivity levels in planta were found to be highest in the basal part of the plant (undamaged pseudostem > damaged pseudostem) and lowest in the herbage section, consistent with the literature (Musgrave, 1984; Gallagher *et al.*, 1987), providing the strongest level of protection at the most vulnerable part of the plant against insect herbivory (Popay, 2009). For studies on adult African black beetle (*H. arator*) resistance, the pseudostem plant section was the most appropriate for sampling. I recommend undamaged pseudostem if the entire plant pseudostem is not available. This conclusion has implications for trial design and plant sampling in studies for ensuring the most appropriate sample is collected. However, pseudostem sampling is more invasive than herbage sampling because tillers are removed from the plant. This will have to be considered when designing and running trials.

The variation in feeding damage, and in paxilline immunoreactivity, is large both within and among families. This suggests that the identification of half-sibling families and plants could be used in breeding programs to increase the production levels of paxilline immunoreactivity and thereby increase resistance to African black beetle (*H. arator*).

Future work

The high variability observed between plants derived from the same seed lot, and even between cloned plants, provides scope for increasing resistance to African black beetle (*H. arator*) through increasing concentrations of paxilline immunoreactivity. Data on feeding damage and pseudostem paxilline immunoreactivity need to be collected from a vast number of plants within a

cultivar or half-sibling family, from multiple trials, in order to fully explore the variability and heritable aspects of these two traits.

To determine if the negative relationship between feeding damage and paxilline immunoreactivity can also be found pre-exposure to adult African black beetle (*H. arator*), cloned plants, i.e. a single genotype, would need to be used in trials that compare paxilline immunoreactivity levels in plants exposed and not exposed to beetles. This research would provide further information on how the paxilline ELISA could be adapted for use as a rapid screening tool that ranks African black beetle (*H. arator*) resistance in the AR1-infected ryegrasses, eliminating the need to determine resistance on every plant by direct testing with African black beetle (*H. arator*). Insect feeding trials are costly. However, the number needed would be vastly reduced in a breeding program that used ELISA-based techniques for the initial screening of plants for resistance.

9.3 Paxilline-like compounds and bioactivity

Key findings and implications

My research has suggested the presence of a subset of paxilline-like compounds that are associated with plant resistance through reducing or deterring feeding, and levels of paxilline immunoreactivity are influenced by adult African black beetle (*H. arator*) feeding. Furthermore, other paxilline-like compounds, which do not afford protection against African black beetle (*H. arator*), may mask the detection of the bioactive subset by ELISA. The presence of masking compounds will complicate any relationship between feeding damage and paxilline immunoreactivity.

Higher levels of paxilline immunoreactivity in herbage were found in mid-summer than in early summer, which was consistent with the published literature showing that alkaloid levels increase over the summer months (di Menna *et al.*, 1992; Ball *et al.*, 1995). Surprisingly, higher levels of paxilline immunoreactivity were found in plants not exposed to adult African black beetle (*H. arator*). However, the ELISA measures levels of immunoreactivity not levels of paxilline-like compunds. It may simply be a reflection of the unexposed plants being under minimal stress and therefore resources for secondary metabolite production not being as limited, lower

production of masking paxilline-like compounds or a down regulation of all paxilline-like alkaloid production. Regardless, our understanding of the feeding damage and paxilline immunoreactivity relationship, warrants further investigation. Moreover, it has important implications in the design of future trials.

Future work

To determine if plant response is elicited by African black beetle (*H. arator*) feeding in plants, feeding studies using artificial diets comprising of pseudostem that had either been exposed or not exposed to beetle feeding are required. In addition to the artificial diet trials, feeding studies using cloned plants that have been previously exposed and not exposed to beetle would help determine if the plant response was accentuated by African black beetle (*H. arator*) feeding, and provide information on the effect this has on paxilline immunoreactivity in plants. The use of cloned plants is importance in ensuring that any genetic differences are controlled for.

Future research should also seek to understand why higher levels of paxilline immunoreactivity were found in plants not exposed to adult African black beetle (*H. arator*) compared to those exposed. Plant studies incorporating different feeding pressures from adult African black beetle (*H. arator*) could be used to determine if this finding is related to resource availability for secondary metabolite production. Complementary screening studies of both seed and plant by both paxilline ELISA and instrumental chemical analyses, such as, LCMS/MS or metabolomic analyses are needed to provide a fundamental knowledge on the paxilline-like compounds. This should include comparisons of plants exposed and not exposed to African black beetle (*H. arator*). Such information is vital for the identification of the bioactive compounds, or linked-markers to the bioactive compounds providing resistance to feeding from adult African black beetle (*H. arator*).

9.4 African black beetle–plant interactions

Key findings and implications

Exposure to adult African black beetle (*H. arator*) was found to be detrimental to plants only when plants were rapidly growing and beetles were actively feeding (i.e., in the spring and autumn months). Low levels of feeding from adult African black beetle (*H. arator*) accentuated plant tiller production, whereas high levels of feeding were detrimental to plants with reduced plant tiller numbers. A plant's response to herbivory is plastic and varies according to the biotic and abiotic conditions (Maschinski & Whitham, 1989). Therefore, herbivory can be detrimental, of no consequence, or beneficial, depending on the conditions and negative and positive effects are not in conflict but are extremes of the same continuum (Maschinski & Whitham, 1989; Whitham *et al.*, 1991). The negative effects of high feeding pressure on plants were long lasting, still affecting plants at least one month post exposure to beetles, with lower levels of both tiller number and paxilline immunoreactivity.

Live plant tiller numbers in early spring reflected dry matter production in late spring. This was consistent with the relationship between feeding damage and pseudostem paxilline immunoreactivity found in late spring being dependent on the cultivar or half-sibling family. Therefore, live tiller number counts in early spring could potentially be used to identify endophyte-ryegrass associations with high dry matter production despite being under African black beetle (*H. arator*) attack.

Future Work

Plant studies incorporating different feeding pressures from adult African black beetle (*H. arator*), as well as data collected post exposure, are necessary for determining both the level of feeding that is no longer beneficial to plants, and also how long plant recovery after detrimental feeding from African black beetle (*H. arator*) takes. This information will be essential for estimating the impact of feeding by African black beetle (*H. arator*). Specifically, are the beetle population levels likely to be beneficial or detrimental to pasture production and pasture persistence? Information gained from such studies would also allow for improved estimation of pasture damage from feeding by adult African black beetle (*H. arator*). This would

aid in the management of pastures that have sustained significant beetle attack, and be of obvious economic benefit.

Long-term trials (≥ 1 year) using cloned plants to investigate how varying levels of African black beetle (*H. arator*) pressure affect endophyte-infected and endophyte-free grass associations measuring feeding damage, alkaloid levels, herbage production, beetle survival and fecundity, would provide important long term information about AR1–plant-insect interactions both over the lifecycle of the beetle and throughout the different seasons. By providing insights into the long term effects of African black beetle (*H. arator*) on AR1-infected cultivars or families, such research could help develop simple diagnostic tools for the monitoring of African black beetle (*H. arator*) populations in pastures, and estimating pasture damage.

9.5 Wider implications and the future

Results from this research have contributed to a deeper understanding of the bioactivity of AR1-endophyte against adult African black beetle (*H. arator*). With AR1 endophyte still the only commercially available endophyte known to have never caused toxicity in livestock, effort and research towards breeding AR1-infected pasture grasses with high resistance to adult African black beetle (*H. arator*) is warranted. As the weather patterns in our regions change, the environment may become more favourable to African black beetle (*H. arator*) survival and reproduction, increasing base-line populations and expanding the pastoral farming areas under threat from African black beetle (*H. arator*).

For some endophyte-infected pastures it is known that environmental conditions such as high temperatures correspond with toxicity (Armstrong, 1994). Moreover, certain alkaloid concentrations are known to increase with temperature (Easton *et al.*, 1996). With global summer conditions becoming hotter and drier, endophyte-infected pastures may exhibit more frequent incidents of toxicity to livestock. Therefore, they may become a less preferred grass option in African black beetle (*H. arator*) prone areas, leaving farmers looking for other non-toxic alternatives.

In the development of AR1-infected cultivars with high resistance to African black beetle (*H. arator*), researchers have to work with very large numbers of plants to

overcome high variability observed between plants derived from the same seed line, and even between cloned plants. For significant and meaningful results to be obtained data need to be collected from very large numbers of plants. The approach taken in this thesis to address this problem was to undertake research to characterise the plant property of interest, African black beetle (*H. arator*) resistance, and to find strategies and technologies that could speed up the selection of 'premium' plants for further development.

In plant breeding, selection trials are very expensive and time consuming. The approach adopted in this thesis, of researching and identifying the bioactive or linked-markers (e.g. chemical or molecular) associated with the characteristic of interest, could be applied to facilitate the development of cost-effective rapid tests for use in plant breeding programs targeting other beneficial characteristics.

9.6 References

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Appendix I: Chapter 4 Additional Information

Tables A–C and Figures A–D

Table A: Mean plant tiller number (square root transformed) of the clones from individual plants grown from seed (from the 23 half-sibling families). The data was analysed unadjusted for initial tiller number (plant size) at start of trial. The data were analysed cumulatively (cumulative total tiller number = total tiller number at Assessment 2 plus the number of damaged tillers from Assessment 1 which had been removed).

		Mean plant tiller number (square root scale, M1)			
Family	**Plant**	**A0**	**A1**	**A2**	**Cum A0–A2**
A12061	61/6	4.72 (22.23)	4.78 (22.87)	3.69 (13.63)	5.21 (27.14)
	61/9	4.86 (23.65)	5.27 (27.79)	4.62 (21.30)	5.06 (25.60)
A12063	63/4	3.85 (14.85)	3.80 (14.40)	3.72 (13.80)	3.90 (15.21)
	63/9	4.53 (20.56)	5.11 (26.11)	3.91 (15.31)	5.02 (25.2)
A12064	64/1	4.25 (18.02)	4.81 (23.16)	4.04 (16.28)	4.69 (22.0)
	64/6	3.82 (14.55)	3.77 (14.24)	3.40 (11.56)	5.21 (27.14)
	64/7	5.24 (27.45)	5.72 (32.76)	5.05 (25.47)	4.25 (18.06)
	64/10	5.18 (26.78)	5.42 (29.40)	5.34 (28.56)	5.32 (28.3)
A12065	65/1	4.44 (19.69)	5.05 (25.49)	4.15 (17.25)	4.87 (23.72)
	65/6	3.49 (12.20)	4.03 (16.27)	3.46 (11.98)	4.01 (16.08)
	65/7	3.91 (15.26)	3.96 (15.71)	3.63 (13.16)	3.82 (14.59)
A12066	66/7	4.67 (21.79)	5.50 (30.29)	3.57 (12.72)	5.45 (29.7)
	66/8	3.81 (15.54)	4.25 (18.02)	3.99 (15.94)	4.41 (19.45)
A12068	68/6	5.01 (25.11)	5.03 (25.28)	4.07 (16.55)	4.97 (24.7)
	68/8	2.80 (7.84)	2.82 (7.97)	2.92 (8.50)	2.81 (7.9)
A12069	69/6	4.08 (16.67)	4.68 (21.93)	3.71 (13.77)	4.91 (24.11)
	69/9	5.45 (29.69)	6.22 (38.69)	4.42 (19.49)	5.5 (30.25)
A12070	70/5	4.89 (23.88)	5.54 (30.64)	4.84 (23.44)	5.22 (27.25)
	70/6	3.69 (13.59)	4.06 (16.51)	3.32 (11.05)	3.85 (14.82)
	70/8	2.82 (7.96)	3.32 (10.99)	2.37 (5.63)	3.28 (10.76)
A12071	71/3	4.10 (16.83)	4.32 (18.64)	3.51 (12.29)	4.29 (18.4)
	71/5	3.03 (9.20)	3.12 (9.73)	1.34 (1.79)	2.85 (8.12)
A12072	72/2	4.77 (22.78)	5.07 (25.72)	4.16 (17.34)	5.04 (25.4)
	72/9	4.21 (17.74)	4.31 (18.56)	5.13 (26.32)	4.81 (23.14)
A12073	73/1	4.75 (22.57)	4.95 (24.48)	3.70 (13.71)	4.83 (23.33)
	73/2	4.55 (20.67)	4.85 (23.54)	4.02 (16.14)	4.81 (23.14)
A12074	**74/1**	4.91 (24.06)	5.36 (28.77)	5.05 (25.49)	5.48 (30.03)
	74/10	5.31 (28.17)	6.52 (42.51)	5.57 (30.99)	6.48 (41.99)

Table A continued on next page

Table A continued

Family	Plant	Mean plant square root transformed tiller number (M1) A0	A1	A2	Cum A0–A2
A12075	75/6	5.07 (25.65)	6.10 (37.19)	6.11 (37.34)	6.62 (43.82)
A12077	77/5	4.14 (17.17)	5.22 (27.25)	4.65 (21.66)	5.00 (25.00)
	77/8	5.10 (25.97)	5.48 (29.99)	4.83 (23.31)	5.54 (30.69)
A12078	78/1	4.40 (19.36)	5.15 (26.52)	4.57 (20.92)	5.07 (25.70)
	78/2	3.97 (15.79)	4.25 (18.02)	3.83 (14.70)	4.12 (16.97)
	78/7	4.00 (15.97)	4.55 (20.73)	4.19 (17.54)	4.51 (20.34)
A12080	80/2	5.14 (26.45)	5.25 (27.57)	5.09 (25.89)	5.41 (29.27)
	80/7	4.00 (15.97)	4.29 (18.36)	3.88 (15.05)	4.39 (19.27)
	80/10	5.14 (26.42)	5.38 (28.91)	5.31 (28.19)	5.52 (30.47)
A12081	81/1	4.54 (20.64)	5.77 (33.30)	4.90 (24.01)	5.78 (33.41)
	81/10	4.35 (18.92)	4.30 (18.45)	3.87 (14.97)	4.58 (20.98)
A12082	82/1	2.15 (4.63)	1.79 (3.20)	1.49 (2.21)	1.52 (2.31)
	82/4	4.03 (16.22)	4.77 (22.76)	3.40 (11.54)	4.84 (23.43)
A12083	83/1	3.33 (11.09)	3.70 (13.67)	3.21 (10.28)	3.70 (13.69)
	83/2	4.62 (21.35)	4.39 (19.29)	5.15 (26.54)	5.19 (26.94)
A12084	84/1	4.62 (21.34)	4.73 (22.37)	4.36 (19.03)	5.70 (32.49)
	84/5	3.28 (10.75)	3.60 (12.94)	2.68 (7.20)	5.04 (25.40)
A12085	**85/6**	4.79 (22.91)	5.48 (30.02)	4.79 (22.93)	5.39 (29.05)
A12086	**86/2**	4.66 (21.68)	5.20 (27.05)	4.84 (23.41)	5.5 (30.25)
	86/4	5.28 (27.92)	6.16 (37.95)	5.53 (30.59)	6.18 (38.19)
A12087	87/5	5.70 (32.43)	7.67 (58.75)	6.85 (46.98)	6.74 (45.43)
	87/10	5.82 (33.91)	7.00 (48.94)	5.38 (28.91)	7.50 (56.25)
Fisher's LSD(5%)		1.213	1.654	1.735	1.640
Plant Effect (Individual Plants^{P})					
F-statistic$_{df}$		$3.52_{49,77}$	$3.67_{49,78}$	$3.12_{49,75}$	$3.88_{49,80}$
P-value		***<0.001***	***<0.001***	***<0.001***	***<0.001***
Plant Effect (Within Family^{FP})					
Wald-statistic$_{df}$		82.83_{27}	66.60_{27}	51.58_{27}	69.82_{27}
P-value		***<0.001***	***<0.001***	***0.003***	***<0.001***

A0, A1 and A2 = Assessments 0 (start of trial and treatment phase), 1 (mid-trial), 2 (end of trial. Cum = Cumulative. $_{df}$ = Degrees of freedom. LSD(5%) = Least significance difference at the 5% significance level. M1 = fixed effect model 1; ^{FP} = *Family.Plant*, ^{P} = *Plant*. Back transformed mean values are in brackets. Family lines and plants contaminated with wild-type endophyte are highlighted in bold font. Statistically significant results are highlighted in bold and italic font. Weak evidence of effects (0.05< *P* ≤1.0) are highlighted in italic font.

Table B: Levels of peramine and paxilline ELISA immunoreactivity (raw data) in plant sections for plant clones that had three sections measured; herbage, damaged and undamaged pseudostem (subgroup ELISA data).

		Peramine-IRE (µg/g)			Paxilline-IRE (µg/g)		
Family	Plant	Herbage	Damaged pseudostem	Undamaged pseudostem	Herbage	Damaged pseudostem	Undamaged pseudostem
A12061	61/9	23.82	28.00	39.04	8.91	11.34	23.81
A12063	63/4	21.35	28.62	43.58	7.59	13.04	19.81
A12064	64/7	14.14	16.47	19.67	18.27	15.04	27.66
A12064	64/7	21.75	27.53	36.89	11.70	16.88	19.80
A12064	64/10	30.99	22.02	20.88	6.73	11.53	11.45
A12069	69/9	23.78	21.42	34.65	5.79	10.34	21.63
A12072	72/9	18.45	29.03	23.66	9.73	11.43	15.38
A12072	72/9	19.14	22.32	24.18	15.56	17.14	27.68
A12073	73/2	30.20	40.24	42.14	13.45	13.58	23.17
A12074	**74/1**	29.89	28.37	22.17	11.19	14.38	13.76
A12074	**74/10**	16.86	14.62	23.79	9.25	8.26	7.07
A12077	77/5	15.95	12.94	19.21	3.74	5.46	7.15
A12078	78/1	22.35	35.20	35.39	4.78	6.64	7.81
A12078	78/2	12.94	21.84	27.01	4.69	7.75	9.43
A12078	78/7	15.55	17.01	21.59	3.65	6.04	9.89
A12080	80/2	28.42	40.75	48.09	6.63	14.32	15.41
A12080	80/7				4.59	8.07	12.14

Table B continued on next page

Table B continued

		Peramine-IRE (µg/g)			Paxilline-IRE (µg/g)		
Family	**Plant**	**Herbage**	**Damaged pseudostem**	**Undamaged pseudostem**	**Herbage**	**Damaged pseudostem**	**Undamaged pseudostem**
A12080	80/10	48.66	31.17	36.97	13.66	12.62	13.38
A12081	81/1	14.17	17.80	20.53	4.60	8.27	9.38
A12081	81/1	22.00	20.79	30.20	6.32	9.78	11.67
A12081	81/10	15.60	17.02	24.94	2.34	3.37	7.05
A12083	83/2	15.19	15.93	22.29	4.90	6.64	8.38
A12083	83/2	23.03	21.53	30.66	6.98	7.38	13.28
A12086	**86/2**	17.86	10.85	13.13	7.84	9.09	12.98
A12086	**86/4**	20.32	24.58	22.03	10.06	12.14	15.14
A12086	**86/4**	24.35	18.41	24.77	23.51	18.07	25.82
A12087	87/5	26.34	21.74	22.92	6.83	8.87	9.56
A12087	87/5	20.32	19.80	22.94	8.02	11.37	17.62
A12087	87/5	26.41	26.71	32.95	9.76	14.44	21.05
A12087	87/10	14.78	19.09	23.01	6.12	11.88	11.27
A12087	87/10	25.94	33.81	31.54	7.15	13.50	18.06
range		12.94–48.66	10.85–40.75	13.13–48.09	2.34–23.51	3.37–18.07	7.05–27.68
median		21.55	21.79	24.48	7.15	11.37	13.38
mean		22.02	23.52	28.03	8.53	10.92	15.09

Peramine- and paxilline-IRE = Peramine and paxilline immunoreactive equivalents Subgroup data = subgroup of peramine or paxilline ELISA data in which individual cloned plants had measured levels of peramine or paxilline immunoreactivity for all three plant sections; herbage, damaged and undamaged pseudostem. Family lines and plants contaminated with wild-type endophyte are highlighted in bold font.

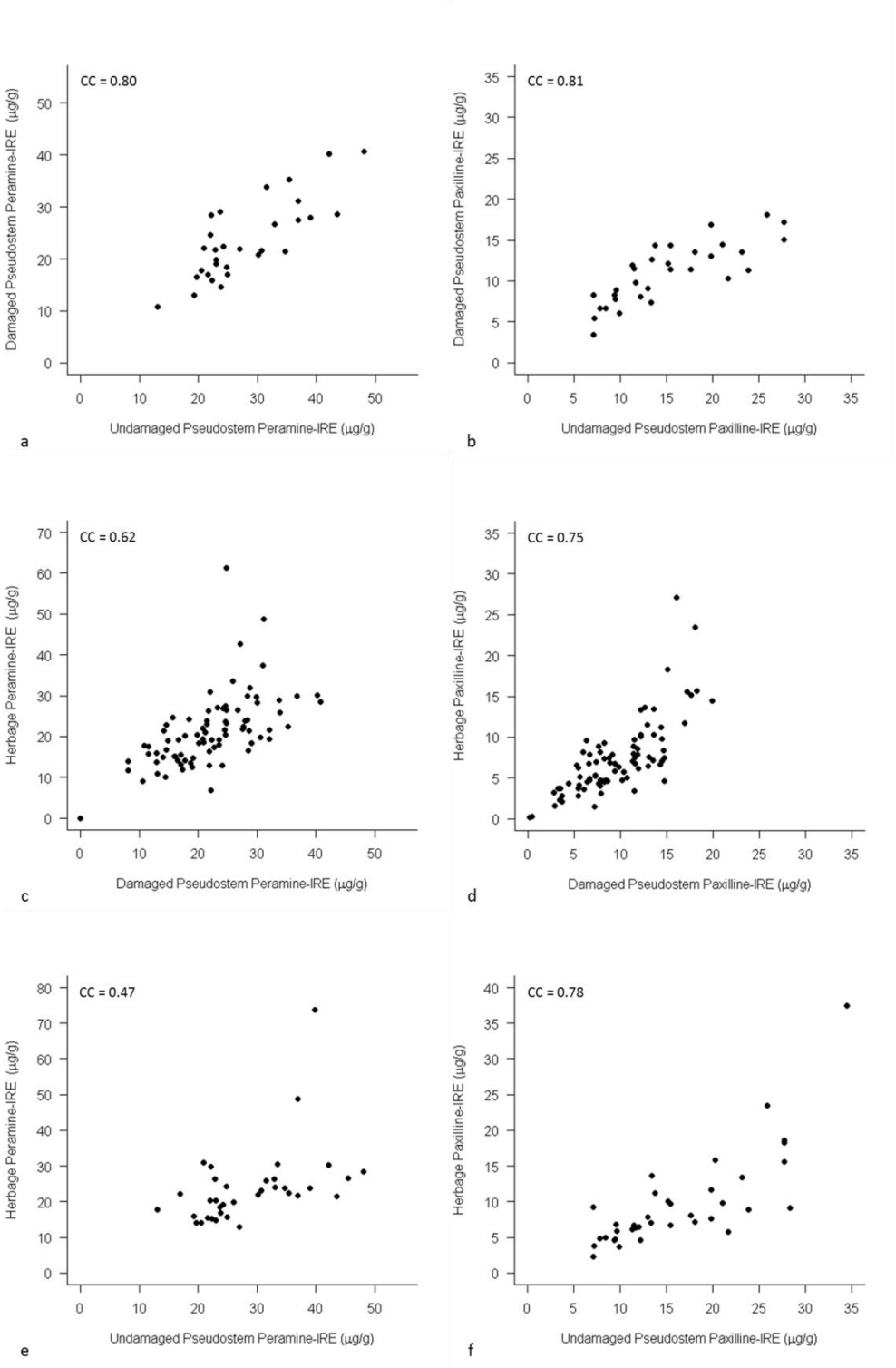

Figure A: Scatterplots (raw data) comparing the levels of peramine (a, c and e) or paxilline (b, d and f) immunoreactivity between the three plant sections; herbage, damaged and undamaged pseudostem. *a and b) Damaged pseudostem vs. undamaged pseudostem. c and d) Herbage vs. damaged pseudostem. e and f) Herbage vs. undamaged pseudostem. Peramine- or paxilline-IRE = peramine or paxilline immunoreactive equivalents. CC = Pearson's correlation coefficient.*

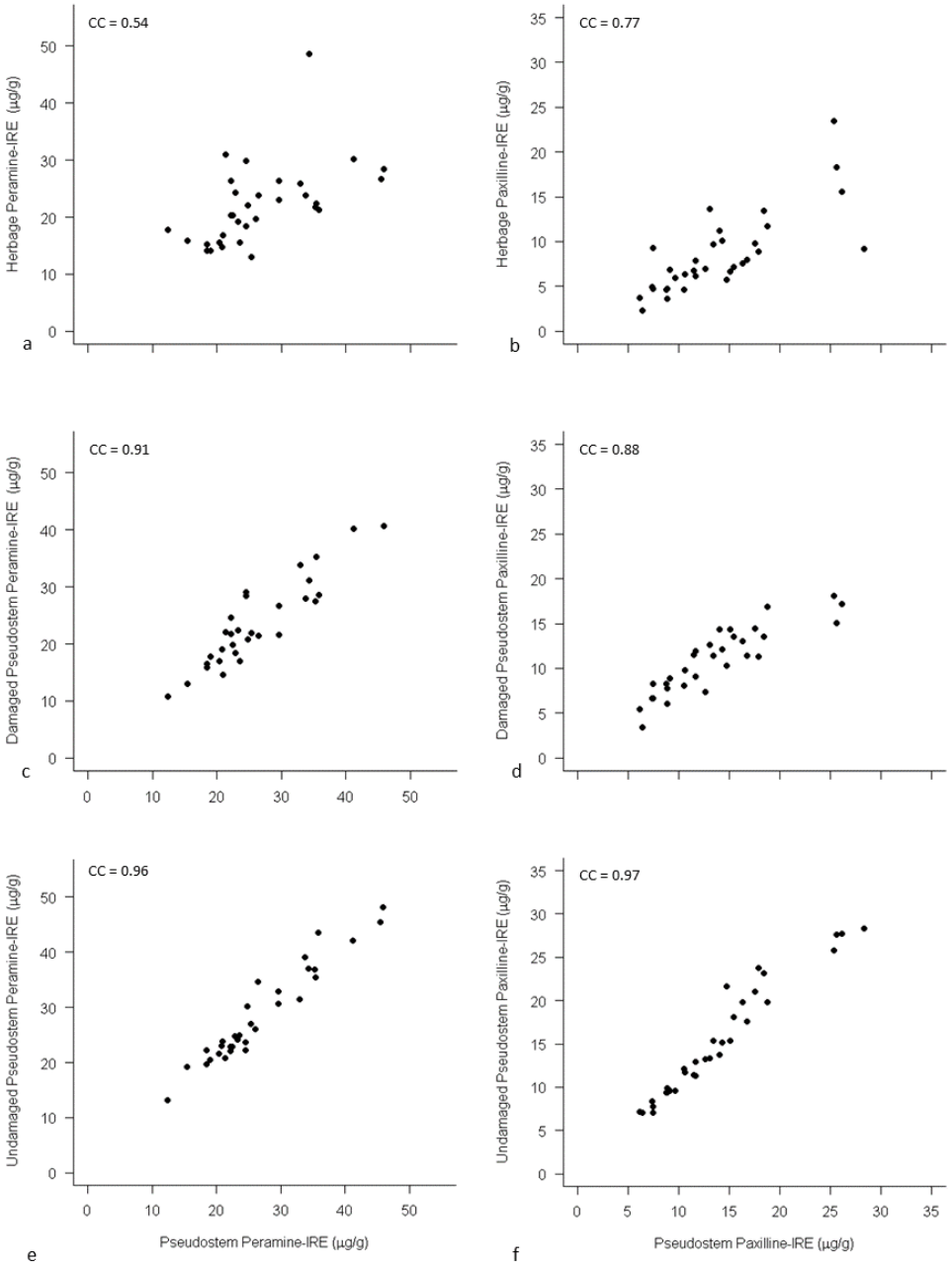

Figure B: Scatterplots (subgroup raw data) comparing overall pseudstem levels of peramine (a, c and e) and paxilline (b, d and f) immunoreactivity with levels in the three plant sections; herbage, damaged and undamaged pseudostem. *Plots a–f; a and b) herbage, c and d) damaged pseudostem, e and f) undamaged pseudostem. Subgroup data = subgroup of peramine or paxilline ELISA data (Appendix I Table B) in which individual cloned plants had measured levels of peramine or paxilline immunoreactivity for all three plant sections; herbage, damaged and undamaged pseudostem. Overall pseudostem levels of peramine or paxilline immunoreactivity were calculated by summing the damaged and undamaged pseudostem immunoreactivity levels weighted by the proportion of tillers damaged. Peramine- and paxilline-IRE = peramine and paxilline immunoreactive equivalents. CC = Pearson's correlation coefficents.*

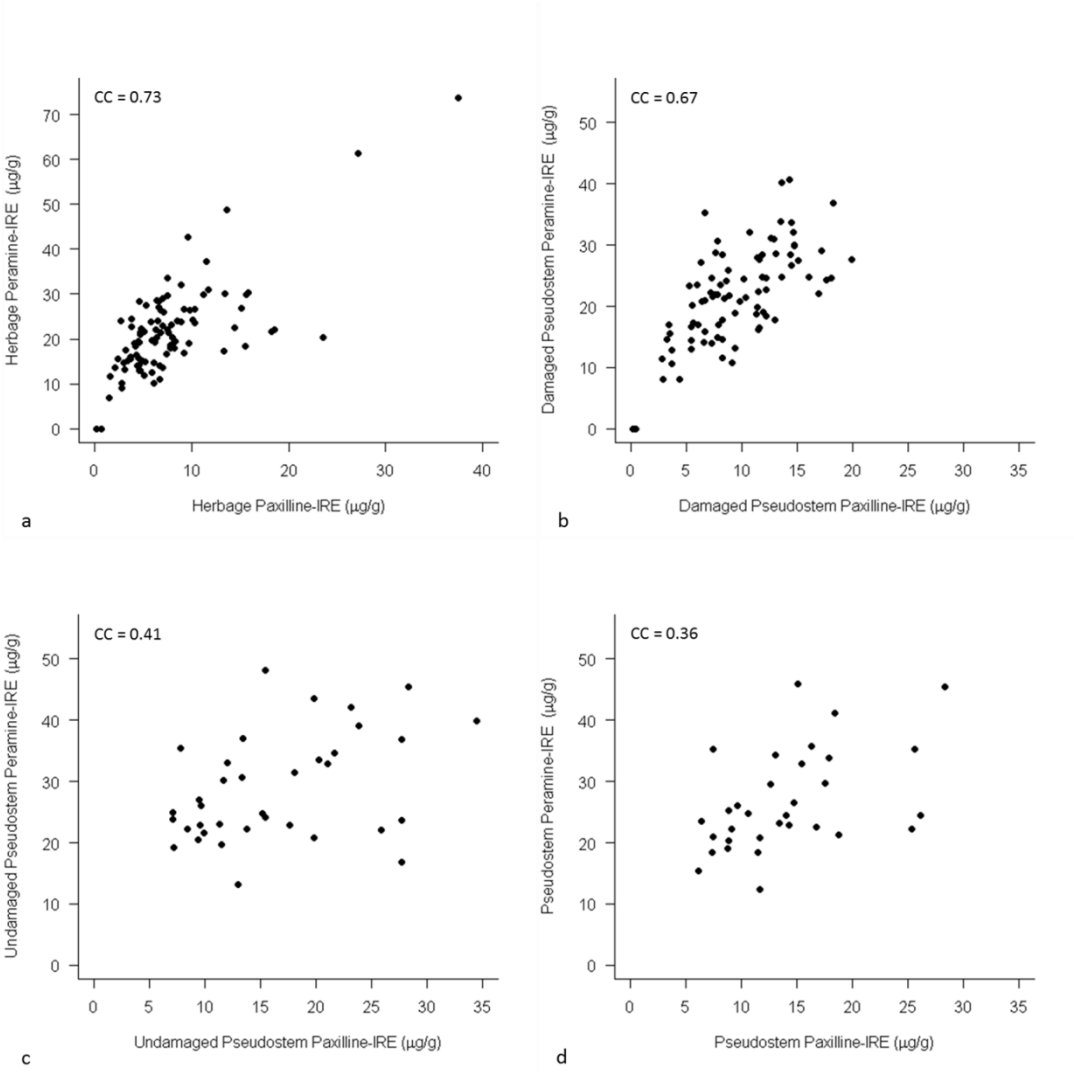

Figure C: Correlation (raw data) of paxilline immunoreactivity vs peramine immunoreactivity in the different plant sections. *Plots a–d; a) Herbage, b) Damaged pseudostem, c) Undamaged pseudostem, d) Overall pseudostem (subgroup data). Subgroup data = subgroup of peramine or paxilline ELISA data (Appendix I Table B) in which individual cloned plants had measured levels of peramine or paxilline immunoreactivity for all three plant sections; herbage, damaged and undamaged pseudostem. Overall pseudostem levels of paxilline immunoreactivity were calculated by summing the damaged and undamaged pseudostem immunoreactivity levels weighted by the proportion of tillers damaged. Peramine- and paxilline-IRE = peramine and paxilline immunoreactive equivalents. CC = Pearson's correlation coefficient.*

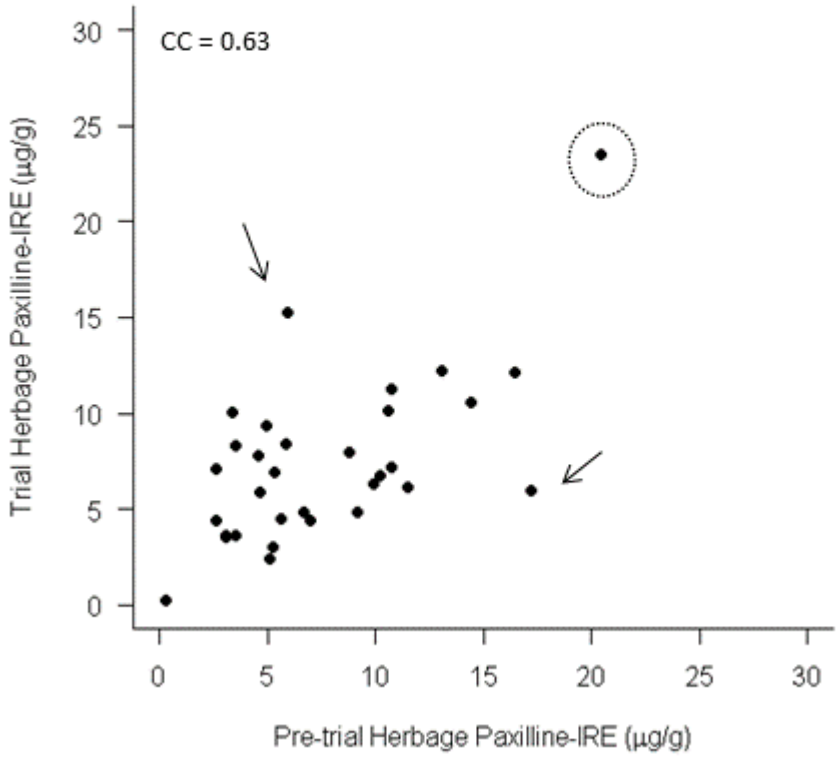

Figure D: Scatterplot (raw data) of paxilline immunoreactivity levels in pre-trial herbage versus end of trial herbage. *Paxilline-IRE = Paxilline immunoreactive equivalents. CC = Pearson's correlation coefficient. One outlier data point is circled and removal of this circled outlier would greatly influence the observable association and correlation coefficient and in this case removal resulted in no observable relationship between the two variables (changing the correlation coefficient from 0.63 to 0.46). The two data points identified by arrows spread the width of the data and may be the outliers, if removed with the circled outlier the correlation is strenghtened (CC = 0.76), or if these data points are removed instead of the circled data point, the correlation remains similar (CC = 0.64).*

Table C: Mean plant proportion of all tillers with a damage score of three (logit transformed) of the clones from individual plants grown from seed (from the 23 half-sibling families) at Assessment 2 from feeding by adult African black beetle. The data was analysed unadjusted for initial tiller number (plant size) at start of trial.

Family	Plant	Mean proportion of tillers with a damage score of three at A2 (logit scale, M1)
A12061	61/6	0.25 (0.56)
A12061	61/9	-0.23 (0.44)
A12063	63/4	-1.06 (0.26)
A12063	63/9	0.81 (0.69)
A12064	64/1	-1.28 (0.22)
A12064	64/6	0.29 (0.57)
A12064	64/7	-1.22 (0.23)
A12064	64/10	-1.07 (0.26)
A12065	65/1	0.88 (0.71)
A12065	65/6	1.85 (0.86)
A12065	65/7	0.18 (0.55)
A12066	66/7	1.21 (0.77)
A12066	66/8	-0.63 (0.35)
A12068	68/6	-1.58 (0.17)
A12068	68/8	-1.20 (0.23)
A12069	69/6	-0.74 (0.32)
A12069	69/9	0.85 (0.70)
A12070	70/5	-0.80 (0.31)
A12070	70/6	-0.87 (0.30)
A12070	70/8	-0.77 (0.32)
A12071	71/3	-0.02 (0.49)
A12071	71/5	0.33 (0.58)
A12072	72/2	-0.57 (0.36)
A12072	72/9	-0.87 (0.30)
A12073	73/1	-0.13 (0.47)
A12073	73/2	-0.41 (0.40)
A12074	**74/1**	-1.05 (0.26)
A12074	**74/10**	-0.13 (0.47)
A12075	75/6	1.03 (0.74)
A12077	77/5	-1.07 (0.26)
A12077	77/8	-0.70 (0.33)
A12078	78/1	-0.89 (0.29)
A12078	78/2	-1.98 (0.12)
A12078	78/7	-1.65 (0.16)

Table C continued on next page

Table C continued

Family	Plant	Mean proportion of tillers with a damage score of three at A2 (logit scale, M1)
A12080	80/2	-0.34 (0.42)
A12080	80/7	0.27 (0.57)
A12080	80/10	-0.44 (0.39)
A12081	81/1	0.51 (0.63)
A12081	81/10	-0.47 (0.39)
A12082	82/1	1.14 (0.76)
A12082	82/4	-0.51 (0.38)
A12083	83/1	-0.81 (0.31)
A12083	83/2	-0.69 (0.33)
A12084	84/1	0.74 (0.68)
A12084	84/5	-0.76 (0.32)
A12085	**85/6**	0.16 (0.54)
A12086	**86/2**	-0.04 (0.49)
A12086	**86/4**	-1.56 (0.17)
A12087	87/5	-0.98 (0.27)
A12087	87/10	-0.01 (0.50)
Fisher's LSD(5%)		3.422
Plant Effect (Individual plants)		
F-statistic$_{df}$		$1.85_{49,73}$
P-value		***0.008***
Plant Effect (Within family)		
Wald-statistic$_{df}$		38.63_{27}
P-value		*0.068*

A2 = Assessment 2 (end of treatment phase). $_{df}$ = Degrees of freedom. ^{FP} = *Family.Plant*. LSD(5%) = least significance difference at the 5% significance level. M1 = fixed effects model 1. ^{P} = *Plant*. Back transformed values are in brackets. Family lines and plants contaminated with wild-type endophyte are highlighted in bold font. Statistically significant results are highlighted in bold and italic font. Weak evidence of effects ($0.05< P \leq 1.0$) are highlighted in italic font.