ASPECTS OF THE ROOT ECOLOGY

OF *NEOTYPHODIUM* ENDOPHYTES

IN *LOLIUM PERENNE*

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ABSTRACT

Three different strains of the biotrophic endosymbiont, *Neotyphodium*, in perennial ryegrass were compared with endophyte-free ryegrass (Nil) for their effects on root herbivory, soil biota, root growth, root morphology and alkaloid and nutrient content in pot trials.

Ryegrass infected with the endophyte strain, AR37, which produces janthitrem alkaloids, was toxic to a root aphid, *Aploneura lentisci.* Relative to Nil plants, ryegrass with Wild-type endophyte (producing the alkaloids peramine, ergovaline and lolitrem B) showed occasional resistance to *A. lentisci* whereas ryegrass with AR1 (producing peramine only) was highly susceptible. A high variability in root aphid populations among individual AR1 plants was related to plant genotype or a plant genotype/endophyte interaction. Differential effects of endophyte on *A. lentisci* were maintained under nutrient stress. Neither AR37 nor AR22 (a strain similar to AR1) in ryegrass affected feeding or survival of root-feeding larvae of the scarab *Costelytra zealandica.*

Endophyte infection had no discernible adverse effects on populations of Collembola, oribatid mites or dorylaimid nematodes. Higher root aphid populations were associated with higher populations of Collembola and lower populations of nematodes. Rate of mycorrhizal colonisation of ryegrass infected with AR37 and Wild-type was slower than in AR1 and Nil but then proceeded rapidly to the extent that they became more heavily infected than AR1. After 2 years, infection of roots of AR1-infected plants was less than on AR37, Wild-type and Nil plants.

Alkaloid content of roots was very low relative to that of leaf sheaths. Two janthitrem fractions were consistently found in roots of AR37 plants and may be the cause of toxicity to root aphid. Ergovaline and lolitrem B were only found in roots under certain conditions and adverse effects of Wild-type infection on *A. lentisci* could not be attributed conclusively to the presence of either alkaloid.

Manipulation of herbivory by insecticide showed that root and foliar growth of AR1 and Nil plants were reduced by *A. lentisci* and also by an unidentified pseudococcid species infesting tillers. Root/shoot ratios were occasionally reduced in the presence of both species and in the presence of *A. lentisci* alone suggesting plant allocation to foliar growth in response to damage by these insects.

Not all effects of endophyte infection were mediated by herbivory. AR1-infected ryegrass had a higher specific root length than Nil plants with AR37 and Wild-type intermediate between these. Insecticide treatment increased specific root length in all plants but relative differences between endophyte treatments remained the same. AR37 differed from other endophyte treatments in having a lower investment in root growth and increasing root nitrogen concentrations during summer followed by a relatively larger investment in root growth in autumn and early winter. Protection from herbivory meant that actual root biomass of AR37 plants was not less than in other treatments.

Endophyte-infected plants had higher concentrations of potassium and phosphorus than Nil plants in roots but not in leaf blades. Nitrogen concentrations in roots were inversely related to root biomass resulting in AR1-infected plants having the highest concentrations of nitrogen due to the adverse effects of *A. lentisci* on root growth. The higher nutrient content of AR1-infected ryegrass compared with Nil may increase its susceptibility to *A. lentisci*.

It is concluded that *Neotyphodium* infection has multiple but highly strain-specific effects on root ecology of perennial ryegrass.

CHAPTER ONE

LITERATURE REVIEW

1.1 *NEOTYPHODIUM* ENDOPHYTES OF GRASSES

The term endophyte literally means within (= *endo*) plant (= *phyte*). De Bary (1879) originally defined the term as any fungus whose hyphae invaded tissues or cells of living autotrophic organisms. This broad definition encompassed every form of fungal infection from pathogens through to mycorrhizal symbionts. Through common usage the term endophyte has come to refer to those fungi which live asymptomatically within the tissues of living plants (Carroll 1988), although fungi are not the only organisms existing in such a relationship with plants. Considerable research has also been carried out in recent years on the role of bacteria living endophytically within plants (eg. Kobayashi & Palumbo 2000). Endophytic infections abound in nature and have been isolated from almost every plant thus far studied (Petrini 1991). For most of these endophytic organisms, the functional relationship between the endophyte (bacteria or fungi) and its host has not been defined. There is, however, one group of endophytic fungi which, because of their economic importance, have been extensively studied in the last 25 years and whose association with their hosts is more comprehensively understood than any other endophyte-host relationship. These are the endophytic fungi belonging to the genus *Neotyphodium* Glenn, Bacon & Hanlin (= *Acremonium* sect. *Albanosa* Morgan-Jones and Gams) which infect species of grasses (Graminae), principally perennial ryegrass (*Lolium perenne* L.) and tall fescue (*Festuca arundinacea* Schreb.). These fungi, belonging to the tribe Balansia (family Clavicipitacea, class Ascomycetes), are obligate biotrophic endosymbionts which, with some exceptions, have no structures that are external to their host. Henceforth, for the purposes of this thesis, the term endophyte will be used to refer solely to these clavicipitaceous fungi.

1.1.1 History

Vogl (1898; cited in Freeman 1903) was the first to report the presence of fungal mycelium in the remains of the nucellus, just outside the aleurone layer of the endosperm, in a high proportion of the seed of *Lolium temulentum* L. Since Roman times it had been known that darnel (= *L. temulentum*) contained a poisonous substance and it was this observation that prompted several published reports of the association of a fungus with *L. temulentum* at that time, including one by Guerin (1898, cited in Freeman 1903) who suggested that the presence of mycelia in the seed indicated a case of symbiosis rather than parasitism. Subsequently Freeman (1903) provided a detailed description of the lifecycle of the fungus, noting the presence of hyphae in the growing points and developing inflorescences. The lack of sporulation was also noted and this author commented that it was "conceivable that a symbiotic relationship advantageous to both fungus and host" had arisen which resulted in the loss of spore formation. This author also reported the presence of similar hyphae in five other *Lolium* spp. including *L. perenne*. Further taxonomic and biological studies (Sampson 1933; Neill 1941; Diehl 1950) followed that of Freeman but essentially the endophyte-grass association remained nothing more than a scientific curiosity until 1977 when Bacon et al. (1977) suggested that a fungus was the probable cause of fescue toxicity in cattle in the USA. This assertion was substantiated by Hoveland et al. (1980). Fletcher and Harvey (1981) then demonstrated that ryegrass staggers, a disorder of grazing animals in New Zealand, was associated with fungal endophyte infection of ryegrass. Shortly after this, resistance to Argentine stem weevil (*Listronotus bonariensis* (Kuschel)) a major pest of ryegrass was also found to be due to the presence of the endophyte (Prestidge et al. 1982). These discoveries provided the catalyst for a large amount of research and an extensive literature on many different aspects of the grass – endophyte relationship that shows no signs of declining 25 years later.

1.1.2 Taxonomy

The family Clavicipitaceae is largely comprised of fungi that are parasites of grasses, insects and other fungi (Jones and Clay 1987). The Balansia tribe, within which *Neotyphodium* spp. reside, are distinct from other members of the Clavicipitaceae because their infections are generally both perennial and systemic

in their hosts. Other genera within this tribe include *Atkinosella*, *Balansia*, *Myriogenospora, Epichoë* and possibly *Balansiopsis* (Diehl 1950; Siegel et al. 1987; Clay 1990). Infections of fungi belonging to these latter genera are all capable of a teleomorphic state resulting in the production of external stromata on leaves or inflorescences of host plants, that often result in host sterilisation. Species of *Balansia* and *Myriogenospora* infect a wide range of grasses and sedges that are common in the tropics and have a C_4-type photosynthetic pathway while the two known species of *Atkinosella* each infect a single known genus, *Danthonia* and *Stipa*. *Epichloë*, of which there are fewer than 10 known species, infect temperate grasses. The most common, *Epichloë typhina* (Fr.) Tul., occurs primarily in members of the subfamily Pooideae which includes several important forage and turf grass genera such as *Lolium*, *Festuca*, *Holcus*, *Hordeum*, *Agrostis* and *Dactylis*. A characteristic of *E. typhina* infections are the 'choke' symptoms on its host caused by the production of a weft of mycelium which arrests inflorescence development.

The feature that distinguishes the *Neotyphodium* endophytes from the other clavicipitaceous fungi is their inability to reproduce sexually. Species with this anamorphic state were originally assigned to the genus *Acremonium*, sect. *Alba-lanosa* on the basis of their growth characteristics and conidial production in pure culture (Morgan-Jones and Gams 1982). In 1996 they were reclassified as a new genus, *Neotyphodium* (Glenn, Bacon & Hanlin) (Glenn et al. 1996). Four species of *Neotyphodium* are now recognised endophytes in *Festuca* and *Lolium*; viz. *N. coenophialum* in tall fescue (*F. arundinacea*), *N. lolii* in perennial ryegrass (*L. perenne*), *N. occultans* in annual ryegrass (*L. multiflorum* L.) and *N. uncinatum* in meadow fescue (*F. pratensis*). Other species almost certainly exist. Christensen et al. (1993) identified six taxonomic groupings of these endophytic fungi based on isozyme analysis, of which three occurred in tall fescue and two in perennial ryegrass. Two other seed-borne fungi described by Latch et al. (1984) as *Gliocladium*-like and *Phialophora*-like endophytes also occur in species of *Lolium* and *Festuca*, sometimes concurrently with *Neotyphodium* infections. They are now known not to belong to these genera and are referred to as p-endophytes (An et al. 1993). Relatively little is known of the effects of these endophytes but some have potent anti-fungal activity in agar culture (Siegel and Latch 1991).

Like *Epichloë, Neotyphodium* species infect the temperate pooid grasses. Infections are common in the economically important species of *Festuca* and *Lolium* and also occur in *Poa, Bromus* and *Stipa*. Aside from similarities in host range, the *Neotyphodium* and *Epichloë* endophytes have other characteristics in common, including morphology, secondary product biochemistry and similarities in DNA sequences (Schardl and Tsai 1997). This commonality of features has led to a general agreement in the literature that there is a phylogenetic relationship between these two genera (eg. Wilkinson & Schardl 1997). The three types of infection of *F. rubra* by *E. typhina* observed by Sampson (1933) illustrate the evolutionary sequence that has culminated in the development of the *Neotyphodium* endophytes. Two types of infection result, respectively, in all, or some, of the inflorescences on individual plants being aborted by the presence of the fruiting body of the endophyte. A third type of infection was termed "latent" when all inflorescences on infected plants developed normally but the pith, ovules and seed of the host plant were found to contain abundant hyphae. This latter type of infection represents a transition between the teleomorphic state of the Balansiae and the anamorphic one of the *Neotyphodium* species.

1.1.3 Colonisation of the plant

Neotyphodium species are typical hyphal fungi that grow within plants. Hyphae are concentrated in the stem apex region, infecting the axillary buds from which new tillers develop. Hyphae colonise the intercellular spaces while maintaining close contact with cell walls, are seldom branched and often appear convoluted as they run parallel with the long axis of the leaf sheath and leaf lamina. Mycelia are usually confined to the leaf sheath area but can extend to the leaf lamina in some plant/endophyte associations (Christensen et al. 1997; Moy et al. 2000). Colonisation of the leaf by hyphae continues as long as the leaf is growing and ceases when leaf growth ceases although they remain metabolically active throughout the life of the leaf (Schmid et al. 2000; Christensen et al. 2002). Hyphae are also able to penetrate the vascular bundles but seldom do so (Christensen et al. 2001). Hyphae come to reside in the mature seed in reproductive tillers after first invading the inflorescence primordium and floral apices and then the ovaries and developing ovules, and in this way are maternally transmitted to the next generation (Philipson and Christey 1986). Although almost

always considered to only inhabit internal tissues, epiphyllous mycelia belonging to the genus *Neotyphodium* have been identified in *Poa ampla* (Moy et al. 2000). The endophyte does not occur in the roots (Hinton and Bacon 1985) but it can be transferred vegetatively to new plants via stolons or rhizomes (Hinton and Bacon 1985). Nutrients are absorbed from the host cell to which hyphae are attached.

1.1.4 Chemistry

The production of secondary metabolites by the fungus is fundamental to the endophyte-host interactions. At least 20 secondary metabolites are known to be produced by *Neotyphodium* species in ryegrass and tall fescue with another 17 produced by *Balansia* symbiota (Bacon & White 2000). Of this diverse array, only four classes of compounds have been the focus of much research (reviewed by Lane et al. 2000). Two, the ergopeptine alkaloids and a water soluble guanidinium alkaloid called peramine, are common to fungal endophytes in tall fescue and ryegrass. Tremorgenic indole diterpenoids (lolitrems) are produced only by certain endophytes in perennial ryegrass and the pyrrolizidine alkaloids (lolines) generally only by *N. coenophialum, N. uncinatum* and *N. occultans*.

Mammalian toxicity is attributed mainly to two specific compounds, an ergopeptide, ergovaline, and lolitrem B. Ergovaline is believed to be responsible for the symptoms of fescue toxicity and heat stress in animals grazing tall fescue (Stuedemann & Thompson 1993) and ryegrass (Fletcher & Easton 1997), while lolitrem B is thought to be the causal agent of ryegrass staggers (Gallagher et al. 1984). The wild-type endophyte that was introduced naturally into New Zealand with perennial ryegrass produces ergovaline, lolitrem B and peramine. Ergovaline is also produced by the equivalent wild-type endophyte in tall fescue in the United States along with three loline derivatives.

These alkaloids also affect a variety of insect herbivores. Peramine is a potent feeding deterrent to Argentine stem weevil (Rowan et al. 1990) and the primary alkaloid responsible for resistance in endophyte-infected ryegrass to this pest. Peramine has been implicated in observed adverse effects of ryegrass infected with *N. lolii* on the aphid *Schizaphis graminum* (Rondani) (Siegel et al. 1990) but other insects such as black beetle (*Heteronychus arator* (F)) show no

sensitivity to this alkaloid (Ball et al. 1997a). Peramine concentrations are highest in the leaf lamina in infected perennial ryegrass and only very low levels are found in the roots (Ball et al. 1997b). The ergopeptine alkaloids show deterrency and/or toxicity to a range of insects including Argentine stem weevil adults (Dymock et al. 1988), black beetle adults (Ball et al. 1997a), fall armyworm (*Spodoptera frugiperda* Smith) (Clay & Cheplick 1989), the large milkweed bug (*Oncopeltus fasciatus* Dallas) (Yates et al. 1989) and Japanese beetle larvae (*Popillia japonica* Newman) (Patterson et al. 1991). Lolitrem B reduces growth and development of Argentine stem weevil larvae (Prestidge & Gallagher 1985) but has no effect on adults (Dymock et al. 1989) or on black beetle (Ball et al. 1997a). Distribution of ergovaline (Lane et al. 1997a) and lolitrem B (Ball et al. 1997c) in the plant is similar with concentrations highest in the leaf sheaths and developing inflorescences. The loline alkaloids have a broad spectrum of activity against insects, including fall armyworm and European corn borer (*Ostrinia nubilalis* Hübner) (Riedell et al. 1991), porina caterpillars (*Wiseana* spp.) and grass grub (*Costelytra zealandica* (White)) larvae (Popay & Lane 2000), Japanese beetle larvae (Patterson et al. 1991), aphids (Seigel et al.1990; Wilkinson et al. 2000) and the large milkweed bug (Yates et al. 1989). Several other insects which do not feed directly on plants are killed by contact or oral activity of *N*-formyl loline (Dahlmann et al. 1997). The loline alkaloids are distributed throughout the plant including in the roots (Bush et al. 1993).

1.1.5 Mutualism

The nature of the symbiotic relationship between host and endophyte is one of mutualism, defined as "an interaction between individuals of two species that increases the fitness of both" (Clay 1988). The host plant provides the endophytic fungus with nourishment, protection from environmental extremes and predation and receives in return protection from certain biotic and abiotic stresses. The association is often described as defensive mutualism where defence against insect and mammalian herbivory through the production of secondary metabolites is the primary benefit to the host. Effects of *Neotyphodium* spp. on grazing animals (Fletcher & Easton 1997; Ball 1997) and over 40 species of insects (Popay & Rowan 1994; Breen 1994) provide ample support for the defensive mutualism hypothesis. The defence against insect pests spans several taxonomic

insect orders and includes leaf and root chewing and plant sucking herbivores. Increasingly, however, there is also evidence that the fungus can alter the morphology and physiology of its host in ways that increases its tolerance to drought and mineral imbalances (Belesky & Malinowski 2000). Some authors (eg. Saikkonen et al. 1998, 1999) have suggested that abiotic stress tolerance may be the primary benefit of the endophyte mutualism rather than protection against herbivory.

Mutualism between host plant and endophyte manifests itself as improved growth and survival of individual plants and a predominance of infected plants in grassland communities in natural and managed landscapes. Increased growth due to the presence of endophyte has been demonstrated many times in tall fescue and is often attributed to the ability of endophyte-infected plants to cope with drought (Bacon 1993; West 1994; Hill et al. 1996) although the mechanisms are not well understood (Belesky & Malinowski 2000). Nevertheless, herbivory has also been shown to be a major factor in giving endophyte-infected tall fescue a selective advantage in the field (Clay 1996; Clay & Holah 1999). In perennial ryegrass Latch et al. (1985) found a growth response to infection by *N. lolii* in the absence of any apparent insect herbivory or abiotic stresses. Several other studies, however, have been unable to demonstrate that growth of endophyte-infected ryegrass is greater than endophyte-free ryegrass when herbivory is not a factor, even under drought conditions (Hume et al. 1993; Barker et al. 1997; Eerens et al. 1998a; Cheplick et al. 2000). Neither defoliation nor nitrogen economy of ryegrass interact in any major way with endophyte infection to improve ryegrass host fitness (Lewis et al. 1996; Cheplick & Cho 2003). On the other hand, increasing yield differences of ryegrass with varying levels of infection by a range of endophyte strains were significantly correlated with decreasing incidence of insect damage in several field trials (Popay et al. 1999). In both fescue and ryegrass pastures a natural change in endophyte infection frequency from low to high over relatively short periods of time is a well known phenomenon and indicative of the competitive advantage of infected plants (eg. Prestidge et al. 1984, 1985; Popay et al. 1999; 2003a).

In natural populations of grasses infection frequency ranges from very low to 100% suggesting no consistent increase in host fitness can be attributed to endophyte in these situations, although few have been extensively studied. Nevertheless there are examples of high infection frequency in native habitats which are most likely maintained by infected plants having a competitive advantage over uninfected ones. In Switzerland the native woodland grass *Brachypodium sylvaticum* is always infected by the host-specific endophyte *E. sylvatica* which is mainly seed-borne and seldom reproduces sexually (Bucheli and Leuchtmann 1996; Leuchtmann and Schardl 1998). This endophyte adversely affects development and survival of the fall armyworm indicating it possesses strong anti-herbivore properties which may be the reason for its dominance in the environment (Brem and Leuchtmann 2001). Interestingly, in this study microherbivores showed a clear preference for feeding on tillers bearing fungal stromata over asymptomatic tillers which has implications for the evolutionary development of the asexual *Neotyphodium* endophytes. In another study, infection frequency of *Bromus setifolius* by an endophyte *N. tembladerae* in its native habitat was highly correlated with the presence of leaf-cutting ants (*Acromyrmex* sp.) (White et al. 2001).

Not all examples of high endophyte infection rates in natural grass populations can be ascribed to increased host fitness. Arizona fescue (*F. arizona*) with an infection frequency by *Neotyphodium* of over 80% in its native environment is a case in point. Despite this high rate of infection, no resistance to herbivory or plant pathogens which could account for the high frequency of infection have been identified. Moreover, in a field study in which plant genotype and environmental conditions were controlled, endophyte infection was found to generally decrease the performance of the host, contrary to expectations (Faeth and Sullivan 2003). These authors speculated that infection is parasitic rather than mutualistic and high infection rates may be maintained by horizontal transmission of the endophyte. In another study, species-specific interactions in growth response of endophyte-infected *F. rubra* and *F. pratensis* plants to different nutrient and watering regimes were recorded but the authors concluded for both species that the cost of endophyte infection outweighed the benefits in a resource-

limiting environment (Ahlholm et al. 2002). Thus there are exceptions to the generally held belief that all these endophytes are plant mutualists.

1.1.6 Factors affecting the symbiosis

Different endophyte strains are operationally characterised by their geographic origins and the type of alkaloids they produce. When isolated from their natural hosts and inoculated into a different host, these endophytes retain the ability to produce the same range of alkaloids (Davies et al. 1993) but concentrations *in planta* vary considerably as a result of various factors. Plant genotype, in particular, 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 (Latch 1994; Ball 1995a & b). Similarly Easton et al (2002) found that concentrations of peramine and ergovaline consistently varied across two perennial ryegrass families. In two of these studies (Ball et al. 1995 a & b; Easton et al. 2002) the concentration of alkaloids was correlated with the amount of mycelium in the plants as determined by ELISA. It has also been demonstrated in ryegrass plants that endophyte metabolic activity varies between different plant genotypes primarily due to the variation in number of metabolically active hyphae (Schmid et al. 2000). Corresponding host-plant influences on alkaloid expression have been recorded in fescue associations (eg. Agee & Hill 1994; Adcock et al. 1997; Hiatt & Hill 1997; Faeth et al. 2002). It may be possible to utilise the host genetic control of alkaloid expression to reduce the impact of endophytes on animal toxicosis (Prestidge & Ball 1993; Easton et al. 2002)

Environmental factors can also influence the expression of the symbiosis. Seasonal changes in alkaloid content of plants occur in concert with seasonal changes in concentration of endophyte (di Menna et al. 1992; Ball et al. 1995a, b) and may alter the strength of resistance to herbivory (Popay & Mainland 1991). In addition, environmental stresses often increase alkaloid content in plants. Water deficit, for instance, elevates ergovaline concentrations in ryegrass (Barker et al. 1993; Lane et al. 1997b) and tall fescue (Arechavaleta et al. 1992) although

peramine and lolitrem B levels are less consistently affected by such conditions (Barker et al. 1993; Lane et al. 2000).

While the alkaloids are always present in the plant and can therefore be regarded as a constitutive defence system, there is also some evidence that they are inducible. That is levels are raised in response to damage to the plant usually caused by herbivores. Pupal weight of fall armyworm reared on endophyte-infected tall fescue previously damaged by clipping was found to be lower than those reared on undamaged tissues (Bultman & Murphy 2000). Regrowth following defoliation often contains higher levels of alkaloids than equivalent older plant parts, probably as a result of a strong association of mycelium with meristematic tissue. In this context the increased expression could be regarded as an inducible effect which, in evolutionary terms, is designed to protect key plant tissues involved in growth and propagation of the plant.

1.1.7 Economic importance of endophytes

It has been estimated that in the southeastern United States alone, 10 million hectares of tall fescue are used primarily for cattle grazing (Shelby & Dalrymple 1987). Similarly, perennial ryegrass forms the basis of the majority of pastures used for agriculture throughout New Zealand. In both countries there is a high frequency of *Neotyphodium* infection in these grasses primarily because endophyte-free plants are at a competitive disadvantage to endophyte-infected plants and fail to persist, but also as a result of plant breeding which has coincidentally selected for infected plants. Clearly, while endophyte infection of tall fescue and perennial ryegrass has obvious benefits for pasture productivity and persistence, it also has significant disadvantages for grazing mammals, including sheep, cattle, deer and horses.

N. coenophialum infection of tall fescue is associated with several animal disorders including fescue foot, fat necrosis and fescue toxicosis, a condition of ill thrift and heat stress (Stuedemann and Hoveland 1988). Heat stress and lower liveweight gains are also caused by infection of ryegrass with the wild-type *N. lolii* endophyte in New Zealand but the most visible symptoms of the toxicity is the occurrence of the neuromuscular disorder, ryegrass staggers, which causes affected animals to tremor and fall (Fletcher & Harvey 1981). Two mammalian

toxins synthesised by the endophytes, ergovaline and lolitrem B are believed to be the cause of these disorders.

In the United States the mechanisms behind the abiotic stress tolerance thought to give *N. coenophialum*-infected fescue a significant advantage over endophyte-free are not understood. The failure of endophyte-free ryegrass to thrive and persist in New Zealand, however, is attributed mainly to the damage caused by Argentine stem weevil (Prestidge et al. 1991). *N. lolii* protects the ryegrass from this pest primarily by the production of peramine, a potent feeding deterrent to the adult weevil (Rowan et al. 1990). Peramine is not toxic to grazing mammals, unlike ergovaline and lolitrem B, the other two major products of the wild-type endophyte. These latter two alkaloids also have effects on Argentine stem weevil but are not essential for maintaining strong resistance (Popay et al. 1995, 1999).

In both New Zealand and the United States the key to exploiting the advantages of endophyte without the disadvantages of mammalian toxicity lay in the diversity of endophytes that exist in naturalised populations of tall fescue and ryegrass particularly in Europe. Endophytes in ryegrass were found that produced peramine but not ergovaline and lolitrem B. These endophytes were removed from their parent plants, cultured and then inoculated into ryegrass cultivars that were adapted to New Zealand conditions (Latch & Christensen 1985). One of these endophytes, AR1, after rigorous animal safety testing, is now commercially available in a range of perennial ryegrass cultivars and has been readily adopted by farmers. Similarly, endophytes in tall fescue that do not produce ergovaline but still produce loline alkaloids offered a similar solution to the problem of fescue toxicosis in the United States. An endophyte with the trade name "Max-Q" that originated in Europe and which does not produce the mammalian toxin ergovaline is now available in the USA.

Each of the main groups of alkaloids studied in the ryegrass and tall fescue endosymbiosis have effects only on certain insects. Any change in the alkaloid profile of endophytes in either ryegrass or tall fescue will therefore have consequences for the spectrum of herbivorous invertebrates that the endophyte protects against. In the case of AR1 the absence of ergovaline increases the

susceptibility of its host ryegrass to black beetle (*H. arator)* compared to the wild-type endophyte (Popay et al. 1999; Popay & Baltus 2001). On the other hand the diversity of endophytes that exist naturally and the variety of alkaloids that they produce also offers opportunities for extending protection to a wider range of invertebrate pests in ryegrass and tall fescue (Popay et al. 2000; Popay unpublished).

1.1.8 Effect of endophytes in roots

Because the continuing interest in endophytes has been largely driven by their ability to improve vegetative production in agricultural environments, research has focussed mainly on above-ground interactions between the endophyte and its host and there is relatively little information on effects in roots. What information there is illustrates a diversity of abiotic and biotic responses associated with *Neotyphodium* infection. Endophyte-infected grasses may have larger root systems than endophyte-free (Arechavaleta et al. 1989; Belesky et al. 1989; Hill et al. 1990; Malinowski et al. 1997) but whether this is a consequence of enhanced vegetative growth mediated by resistance to herbivorous pests, abiotic factors or a direct result of endophyte infection is not known. The alkaloids responsible for bioactivity against insects have all been found in varying quantities in the roots but the low levels of peramine (Ball et al. 1997b), lolitrem B (Ball et al. 1997c) and ergovaline (Azevedo et al. 1993; Lane et al. 1997a) recorded suggest these are unlikely to be of any biological significance. In tall fescue infected by *N. coenophialum*, however, as much as 10 - 15% of the total loline content in a plant can be found in the roots (Bush et al. 1993).

Root-feeding invertebrates show some sensitivity to endophye-infection in tall fescue. Populations of various species of plant endo-parasitic nematodes have been found to be lower in endophyte-infected tall fescue than in endophyte-free (Pederson et al. 1988; West et al. 1988, 1990; Kimmons et al. 1990; Elmi et al. 2000) whereas ecto-parasitic nematodes appear not to be affected (Bernard et al. 1997). Root-feeding grubs, members of the Scarabaeidae, may also be affected by endophyte infection of tall fescue. Murphy et al. (1993) found fewer *Popillia japonica* in endophyte-infected than in endophyte-free tall fescue field plots and in rearing tests growth and survival of this grub and another, *Cyclocephala lurida*

Bland, were reduced by endophyte infection. Growth and survival of a major New Zealand pasture pest, *Costelytra zealandica*, can also be reduced by endophyte infection of tall fescue (Popay et al. 1993) and meadow fescue (Popay et al. 2003b). Endophyte infection of meadow fescue also greatly reduces infestations by the root aphid, *Aploneura lentisci* (Pass.) (Schmidt and Guy 1997).

In ryegrass there are few reports of endophyte influencing populations of soil invertebrates. Numbers of phyto-nematodes (mainly the ecto-parasitic species *Paratylenchus*) were higher on roots of endophyte-free than on endophyte-infected ryegrass in field and pot experiments (Eerens et al. 1998b) and fewer galls and female *Meloidogyne naasi* were found in roots of endophyte-infected ryegrass than in endophyte-free (Stewart et al. 1993). Ball et al. (1997d) found that a certain strain of endophyte in ryegrass reduced populations of the root-knot nematode (*Meloidogyne marylandi*). Other studies, however, have failed to clearly demonstrate adverse effects of endophyte infection on plant parasitic nematodes (Yeates & Prestidge 1985; Watson et al. 1995). No major effects of endophyte-infected ryegrass on grass grub larvae have been shown (Prestidge and Ball 1993).

The impact of endophyte infection in grasses is not limited to direct effects on herbivores. Interactions between the *Neotyphodium* fungi which are endophytic in shoots and the arbuscular mycorrhiza (AM) that are endophytic to roots of plants illustrate the complexity of ecological interactions that can occur. The resistance to Argentine stem weevil conferred by *Neotyphodium* endophyte infection is lessened when plants are inoculated with the VAM fungus *Glomus fasciculatum* (Barker 1987). This VAM fungus had no effect on Argentine stem weevil in the absence of *Neotyphodium* infection. Vicari et al. (2002) in a similar study using larvae of the noctuid moth, *Phlogophora meticulosa*, concluded that an adverse effect of *N. lolii* in ryegrass on larval feeding was moderated but not eliminated by the presence of the mycorrhizal fungus, *G. mossae*. Another study has shown decreased colonisation of roots of young endophyte-infected ryegrass plants by an inoculated AM fungus (*Sclerocystis* sp.) and that in some cases this was associated with a negative plant growth response (Müller 2003). Colonisation of tall fescue roots by AM fungi is also reported to be inhibited by *Neotyphodium* infection (Chu-Chou et al. 1992; Guo et al 1992).

Other indirect effects of endophyte infection on soil and litter communities have been reported. An oribatid mite species (*Galumna* sp.) was found in fewer numbers in an endophyte-infected tall fescue field than in endophyte-free, and assemblages of collembolan species also differed between these same fields (Bernard et al. 1997). Abundance of predatory invertebrates measured by pitfall trapping and comprised mainly of species of spiders, carabids and staphylinids was higher in endophyte-infected ryegrass pastures than in endophyte-free (Prestidge & Marshall 1997). Some of these differences may be related to the presence of greater plant biomass where endophytic plants are dominant. In addition, a recent study has shown reduced rates of decomposition of litter from endophyte-infected *L. multiflorum* by comparison with endophyte-free plant litter (Omacini et. al. 2004). Biomass of tall fescue was reduced when grown in soil previously dominated by conspecifics, by comparison with its biomass when grown in soil previously dominated by *Poa pratensis*, possibly as a result of soil-mediated feed-back mechanisms or build-up of parasites that are specific to endophyte-infected tall fescue (Matthews & Clay 2001).

Endophyte-mediated abiotic effects in roots have only been demonstrated in tall fescue. Phosphorous (P) uptake and specific root length are higher under conditions of P deficiency in endophyte-infected tall fescue than in endophyte-free (Malinowski and Belesky 1999; Malinowski et al. 1999). Low P availability also generated greater reducing activity in roots of infected plants and this was associated with greater amounts of phenolic-like compounds in roots and shoots (Malinowski et al. 1998). When grown in nutrient solution root morphology appeared to be altered by endophyte status regardless of P level, when endophyte-infected plants produced roots with smaller diameter and longer root hairs than endophyte-free isolines (Malinowski et al. 1999).

1.2 ROOTS AND ROOT ECOLOGY

1.2.1 Root Growth

The geometry of the root system has been described as "fundamental to its function" (Fitter 1996). In perennial ryegrass each tiller develops its own set of

adventitious roots that arise from nodes close to the soil surface. Together the roots from each tiller form the fibrous root system which is typical of grasses (Langer 1973). The roots end in very fine terminal branches described as an effective minimum diameter capable of accommodating the root structures needed to provide transport (Fitter 1996). These fine roots give root systems a high specific root length (= length per unit weight) which, together with a high length and density of root hairs and the presence of mycorrhiza, optimise uptake of nutrients, the primary function of the root system. On the other hand roots with larger diameter have a greater ability to penetrate soil (Williams et al. 1983; Crush et al. 2002) and a higher level of drought resistance (Torbet et al. 1990) than fine roots. Fine roots may also be more vulnerable to grazing, physical damage and pathogens than larger roots (Fitter 1987). In addition plants may incur greater carbon costs to the plant in construction and maintenance of fine roots (Fitter 1996; Eissenstat & Yanai 1997).

Abiotic factors that affect root growth include the availability of nutrients, soil structure, temperature and seasonal factors (Fitter 1996). In grasses root growth is greatest in the upper parts of the soil profile where nutrient concentrations are highest. The ability of roots to proliferate in these nutrient rich patches in soil has also been well documented (Robinson 1994). Root growth is also related to shoot growth. According to a functional equilibrium concept (Brouwer and de Wit 1969) the plant allocates its resources to achieve a balance between the assimilation of carbon in the shoots and uptake of nitrogen in the roots. Plant genotypic differences between above- and below-ground biomass are related to levels of plant hormones.

1.2.2 Mycorrhiza

The presence of endo-, ecto and endoectomycorrhiza are another very important element for consideration in the study of roots. AM fungi substantially increase the ability of roots to forage for nutrients (Smith et al. 1992; Wilcox 1996), may protect roots from specific soil-borne pathogens (Dehne 1982; Smith & Read 1997) and can also induce changes in secondary compounds (Piepp et al. 1997; Hause et al. 2002). In general plants with fine, highly branched, rapidly-growing and relatively short-lived roots, such as grasses, have low levels of

endomycorrhizal infection (Wilcox 1996). Grasses have been shown to have little reliance on mycorrhiza for nutrient absorption except under severe phosphorus-limiting conditions (van der Heijden et al. 1998; Müller 2003). AM fungi may also interact with plants to affect herbivory by changing the nutrient status of their hosts (Gange et al. 1999). Mycorrhizal hyphae in soil may be an important food source for collembola and other soil fungivores (Lussehnhop 1992; Fitter & Gabaye 1994), although this is disputed by Gange (2000).

1.2.3 Herbivory

Above-ground herbivory can affect root growth by altering the partitioning of carbon and nitrogen between roots and shoots to compensate for the damage. The plants capacity to compensate depends on their being vascular connections between the undamaged (source) and damaged (sink) plant parts (Whitham et al. 1991) and may be compromised by a lack of nutrients (Wilson 1988) and water (Trumble 1993). In general defoliation results in translocation of assimilates to the shoots at the expense of the roots (eg. Detling et al. 1979; Chapin and Slack 1979; Ryle & Powell 1975; Polley & Detling 1989; Masters & Brown 1992). In contrast to these studies, however, Holland et al. (1996) used carbon labelling to demonstrate an increase in root carbon after grazing of maize (*Zea mays*) by grasshoppers (*Romalea guttata*) reduced leaf area by 25 – 50%. These authors suggested that there was short-term storage of the carbon in the roots where it was not accessible to further herbivory. Such reserves may be important for the ability of plants to compensate for damage. It has been estimated that a small portion of daily assimilated carbon in perennial ryegrass is stored in stem bases for later allocation to regrowth after defoliation (Danckwerts & Gordon 1987). Concomitant with the changes in carbon allocation, there are also changes in nitrogen levels within plants following above-ground herbivory. Again partitioning patterns vary, with both increases in root nitrogen accumulation (Ruess et al. 1983; Jaramillo and Detling 1988; Polley and Detling 1989) and leaf nitrogen content (Jaramillo and Detling 1988) being reported.

Root herbivory and its effects on plant growth have not been given the same degree of research attention as above-ground herbivory (Brown & Gange 1990; Hunter 2001). Consequences for the plant will depend on the type of

herbivory and its severity and in grazed plants will also depend on frequency of defoliation. As for foliar herbivory, the plant compensates for root herbivory by diverting resources to the increased demand of roots, resulting in increased respiration and depletion of carbohydrates (Goldson 1988) and changes in partitioning of carbohydrate and soluble nitrogen (Mattson 1980; Gange & Brown 1989; Masters & Brown 1995; Dunn & Frommelt 1998). Herbivore damage to roots may also allow invasion of root pathogens which further damage the plants (Brown & Gange 1990). A combination of insecticide and fungicide applications can synergistically increase root longevity compared with either pesticide applied alone (Eissenstat et al. 2000). Root pruning reduces root weight or increases it, depending on its severity and environmental conditions. Ridsdill-Smith (1977) found that root biomass decreased even at low densities of the root pruning scarabaeid (*Sericesthis nigrolineata*). In contrast to this study, reduced root growth and turnover was recorded in insecticide-induced absence of herbivory by tipulid larvae (*Tipula* spp.) (Dawson et al. 2003). The reasons for this are unclear, but low levels of herbivory may enhance plant growth as found in white clover (*Trifolium repens*) with low level infestations of the clover cyst nematode (*Heterodera trifolii*) (Bardgett et al. 1999; Yeates et al. 1978). Other studies have also noted that the root damage may cause water stress and that the impact of herbivory on the plant is therefore exacerbated under conditions of low water and nutrient availability (Ridsdill-Smith 1977; Gange & Brown 1989; Masters 1995). Root herbivory can also be a significant factor in plant mortality (Maron 1998). Root feeding by tipulid larvae on perennial ryegrass decreased the proportion of the root system present as laterals although there was no reduction in total root biomass or root length (Dawson et al. 2002) indicating that root feeding may also alter root morphology.

Plants, particularly grasses, are often exposed to more than one herbivorous species at any one time. There are complex interactions between above and below-ground feeding, and between different guilds of insects (eg. chewing or sucking) exploiting the same plant part, that impact on both the plant and the herbivore. Root pruning by scarabaeid (*Sericesthis nigrolineata* (Boisd.)) larvae reduced root weight regardless of whether plants were defoliated or not but only reduced green yield when plants were also defoliated (Ridsdill-Smith 1977).

Effects of root and foliar herbivory on plant fitness of bush lupine (*Lupinus arboreus*) were found to be additive with no interactions between the two (Maron 1998). Vertebrate foliar herbivory may increase densities of below-ground herbivores, depending on its intensity, by increasing root nitrogen and thereby the quality of the food source (Seastedt et al. 1988a). Alternatively where foliar herbivory reduces root biomass it will reduce the feeding niches available to root herbivores and adversely affect their performance (Masters & Brown 1992). For instance whereas feeding by the root aphid, *Pemphigus betae*, on *Chenopodium album* had no measurable effect on its leaf galling counterpart, *Hayhurstia atriplicis*, presence of the leaf galling aphid reduced numbers of the root aphid by 91% (Moran & Whitham 1990). Conversely root feeding by a scarabaeid larvae, *Phyllopertha horticola*, increased growth rate, fecundity and longevity of a foliar aphid, *Aphis fabae*, on an annual herb, *Capsella bursa-pastoris*, but only under a low watering regime (Gange & Brown 1989). Low watering and root pruning by *P. horticola* caused water stress in the plants which reduced vegetative biomass but improved food quality by increasing total soluble nitrogen.

1.2.4 Soil Biota

There is now a considerable body of evidence showing that both above- and below-ground herbivory promotes the activity of soil biota involved in the detrital food web. Herbivory increases root exudation of carbon and/or nitrogen which stimulates soil microbial activity (eg. Holland et al. 1996; Denton et al 1999; Yeates et al. 1998; Bardgett et al.1998, 1999; Grayston et al. 2001; Tu et al. 2003). Microfaunal populations may also differ according to whether plants are grazed or not. Nematode numbers are higher under grazed compared to ungrazed annual grasslands (Freckman et al. 1979; Ingham & Detling 1984; Bardgett et al. 1997) and a similar response has also been found for collembola (Bardgett et al. 1993). Changes in population dynamics of these soil fauna are due to dung and urine inputs, improvements in litter quality, increases in soil temperature and moisture and a larger soil microbial community in grazed systems. An increase in components of detrital foodwebs resulting from grazing will not necessarily result in an increase in available nutrients for the plant since the plant and the soil biota are competing for the same resource. A recent study, however, demonstrated that a sequence of events occurred after grazing of *Poa pratensis* which ultimately

resulted in greater soil organic nitrogen and uptake of nitrogen by the plants and increased photosynthesis (Hamilton & Frank 2001). These authors propose the existence of feed-back mechanisms in which the plant participates actively to promote rhizosphere processes that facilitate uptake of a growth-limiting resource.

CHAPTER TWO

INTRODUCTION

Plant roots are the framework on which a multiplicity of interactions is built. Their primary functions are resource acquisition and anchorage but they also carry, store and synthesise secondary metabolites, release exudates that enhance microbial populations, interact with ecto- and endo-mycorrhiza and provide a substrate for root herbivores and detrital feeders. Root growth is modified by the allocation of resources used in construction and maintenance, by soil structure, the environment, temperature, moisture and the availability of nutrients. Root quality impacts on the amount of root herbivory which in turn affects plant performance by inducing physiological changes in the plant that alter partitioning of carbon and nitrogen between roots and shoots. Intricate foodwebs are created in specialised habitats provided by the roots which benefit plants by enhancing the nutrient supply. Root exudation which increases in response to herbivory provides a valuable substrate for microbial growth and this in turn supports a greater abundance of soil fauna such as Collembola and nematodes. These invertebrates can disrupt the mycelial network of mycorrhizal fungi which have a key role in acquiring nutrients for many plant species.

Infection of a plant with a mutualistic fungal endophyte that reduces herbivory is bound to have consequences for root ecology even when that endophyte is located in aerial parts of the plant. The biotrophic clavicipitaceous fungal endophytes belonging to the genus *Neotyphodium* that infect grasses represent such a system. These seed-borne fungi infect two economically important pasture species, tall fescue (*Festuca arundinacea*) and perennial ryegrass (*Lolium perenne*). They produce secondary metabolites that moderate feeding by insect and mammalian herbivores, and the presence of these endosymbionts can also alter the physiology of the plant in ways that mitigate against the effects of drought and change the mineral composition. As a result, endophyte-infected ryegrass and tall fescue have increased survival, growth and persistence compared with their endophyte-free counterparts. While this gives them an advantage in agricultural systems, the *Neotyphodium* endophytes can also

cause significant toxicity in grazing mammals. This dual effect of endophytes has been the focus of much research attention in the last 25 years, most of it on the above-ground consequences of infection.

In recent years it has become more apparent that the endophyte can have profound effects below-ground, at least in tall fescue. Because plants allocate their resources according to demand, even at the simplest level, a reduction in herbivory above ground mediated by the endophyte will affect root growth. Given that the endophyte may also be able to reduce below-ground herbivory, modify root morphology and uptake of minerals, and interact with mycorrhiza, then it becomes apparent that there are likely to be significant consequences of infection for root ecology. The effects of *Neotyphodium* endophyte infection below-ground have mainly been demonstrated in the fescues and have focussed largely on abiotic effects. Ryegrass has been less well studied than tall fescue with respect to endophyte effects in roots and there is no evidence that the response of ryegrass would be the same as that in tall fescue. Indeed, while the outcomes of infection with endophyte are broadly similar for tall fescue and ryegrass, there is an apparent disparity in the mechanisms involved. Much of the improved host fitness as a result of endophyte infection is attributed to relief from abiotic stress in tall fescue whereas protection from herbivory appears to be the main factor conferring an advantage in ryegrass.

For both tall fescue and ryegrass, in order to resolve the problem of mammalian toxicity caused by the endophyte while also exploiting its beneficial properties, New Zealand researchers sought other naturally occurring endophytes with different alkaloid profiles. This led to the discovery of a range of endophytes including one that infected perennial ryegrass, AR37, which had a unique metabolic profile. Personal observations made on plants containing this endophyte prior to undertaking this study suggested that it, unlike other ryegrass endophytes, may protect the plant from infestations by a root aphid, *Aploneura lentisci*. Based on this observation and current knowledge of endophytes in ryegrass, the following hypotheses were formulated for consideration in this thesis:

1. That certain strains of *Neotyphodium* endophytes in ryegrass would affect root herbivory and that this is mediated by the presence of alkaloids in roots.
2. That members of decomposer food-webs in the soil would not be directly affected by the presence of different strains of *Neotyphodium* (eg. by toxic compounds produced by the endophyte) in perennial ryegrass but may be affected as a result of trophic interactions resulting from differential herbivory.
3. That occurrence of arbuscular mycorrhiza and other fungi in the roots would not be affected by *Neotyphodium* infection.
4. That root growth, root biomass accumulation, root/shoot ratios and root morphology would be affected by differential herbivory resulting from *Neotyphodium* infection but would not be altered by the presence of the fungus in the absence of herbivory.
5. That occurrence of alkaloids in roots of endophyte-infected ryegrass would vary according to the type of alkaloid and seasonal and environmental factors and that it would be a function of the amount in the leaf sheath.

To investigate these hypotheses, three major experiments were carried out in which comparisons were made between individual ryegrass plants infected with three different endophytes, AR1, AR37 and Wild-type, and ryegrass without endophyte (Nil). In the first two experiments, insecticide was used to manipulate the amount of herbivory. Plant growth and populations of soil invertebrates were monitored over a 2 year period in the first trial and similar measurements were taken on plants under a high and low nutrient regime over a shorter time period in the second trial. In the third trial, root biomass and root aphid populations were measured destructively three times over a period of 9 months. The occurrence of alkaloids and of arbuscular mycorrhiza and other endophytic fungi in the roots was investigated in all trials. Root morphology of plants infected with different endophytes was determined in the first and third trial.

In addition to the above trials, two further trials were conducted to investigate the relationship between endophyte and the occurrence of root aphid. A pot trial

compared root aphid populations on ryegrass infected with endophytes that gave a range of different alkaloid profiles. As part of that same trial certain endophytes were also tested in three different cultivars to ascertain if there were any plant genotype/endophyte interactions affecting the insect. Four separate bioassays were conducted to investigate behaviour of root aphid and mechanisms by which endophytes affected them (ie. toxicity and/or deterrency). One other experiment utilised natural differences in $\delta^{13}C$ between a C_3 and a C_4 plant to determine if any of the endophytes were deterrent to the root-chewing larvae of the grass grub (*Costelytra zealandica*).

CHAPTER THREE

METHODS AND MATERIALS

3.1 GENERAL METHODS

The following are methods and materials that apply to all trial work.

3.1.1 Endophytes

The list of *Neotyphodium* endophyte strains used in the main trials are given in Table 1. The endophyte strains are distinguished from each other by their geographic origins and the spectrum of alkaloids they are known to produce. Endophyte-free plants are referred to as Nil.

Table 3.1 *Neotyphodium* fungal endophytes used in the main trials, their geographic origin and chemical profile.

Endophyte	Origin	Alkaloids			
		Peramine	Ergovaline	Lolitrem B	Janthitrems
Wild-type	Naturalised in NZ	+	+	+	–
AR1	Italy	+	–	–	–
AR37	France	–	–	–	+

3.1.2 Plant Growth and Maintenance

All plants used in these trials were grown from seed obtained from the Margot Forde Germplasm Centre, AgResearch, Palmerston Nth, New Zealand.

Plants grown from seed were germinated by placing seed on damp filter paper in petri dishes and keeping them in the dark in a controlled environment room at 20°C for 5 – 7 days. The germinated seed was then planted into a growing medium appropriate for each trial in polystyrene trays or individual pots which were then transferred to a shadehouse where they were kept under ambient light

and temperature conditions. Plants were watered by an automatic overhead sprinkler system for 30 minutes each watering period with the frequency dependent on the time of the year.

The growing medium used in the trials consisted of 2 parts of an unsterilised silt loam field soil with one part of washed river sand mixed thoroughly together in a wheelbarrow. On occasions plants were grown in a commercial potting mix before transplanting into the soil/sand growing medium.

Nutrients were supplied to plants in two forms. At planting Osmocote® slow release fertiliser (containing 19% nitrogen, 2.6% phosphorous, 10% potassium) was incorporated into the top 50 mm of the planting medium at a rate of approximately 2.0 g per plant. Once plants became established after approximately 2 months they received a nutrient solution comprised of a commercially available nutrient mix, Thrive™, prepared at the recommended rate (approximately 8 g per 4.5 L of tap water) and additional nitrogen (approximately 5 g per 4.5 L) in the form of urea (46% nitrogen). Thrive™ contains: 27% nitrogen, 5.5% phosphorous, 9% potassium, 0.15% magnesium, 0.005% copper, 0.02% zinc, 0.005% boron, 0.04% manganese, 0.18% iron and 0.002% molybdenum. Growth rate of white clover supplied fortnightly with a half rate of Thrive™ has been shown to be comparable to growth using an inorganic fertiliser recipe specifically for white clover, and in addition had no adverse effects on development of the nematode *Heterodera trifolii* (Grant & Mercer 1993).

3.1.3 Assessment of Endophyte Infection

The endophyte infection status of plants used in all trials was determined by staining and microscopic examination or by an immunoblot procedure. For the former, a tiller was removed at the base of each plant and a small piece (approx. 2 mm x 5 mm) of epidermis was removed from the leaf sheath, placed on a glass slide and stained *in situ* with aniline blue (0.1 g aniline blue in 875 mL lactic acid and 125 mL tap water). After 20 minutes at ambient temperature the material was examined for the presence of hyphae typical of *Neotyphodium* infection (Fig. 3.1) at 200x magnification. The tissue immunoblot procedure uses polyclonal antibodies developed against *Neotyphodium* protein to give a colour reaction to

the fungus (Hahn et al. 2003). The procedure involves dabbing or crushing the cut end of the base of a tiller onto nitrocellulose membranes which are then developed overnight in an antibody mix. A red stain is indicative of fungal presence. Where tillers gave unexpectedly negative or ambiguous readings a second tiller was taken and checked for fungal hyphae by staining and microscopic examination.

Fig. 3.1 Stained hyphae of *Neotyphodium* spp.

3.1.4 Insecticide

Herbivory was manipulated in some trials using a granular insecticide, Confidur® containing 5% imidacloprid. This is a systemic insecticide which is recommended for use in ornamental growing media for control of whiteflies, aphids, sciarid larvae and black vine weevil. Confidur® was mixed with sand and applied to the soil surface at the rate of 80 – 100 mg per plant. Applications were made following planting of the trials and after each sampling occasion.

3.1.5 Sample drying and weighing

Herbage samples were oven dried at 60 °C for 36 – 48 h and roots at 80 °C for 48 – 60 h except for those that were required for chemical analysis. These samples were initially frozen at – 25 °C and then later freeze dried at ambient temperature and −0.4 mbar vacuum. All samples were weighed immediately after drying.

3.2 TRIAL METHODS

Three trials form the basis of this thesis, a Plant Growth Trial, a Nutrient Trial and a Root Biomass Trial. The results for these trials are reported in: Chapter 4: Effect of *Neotyphodium* endophytes on root herbivores; Chapter 5: Effect of *Neotyphodium* endophytes on soil biota and interactions with root herbivory; Chapter 6: Effect of *Neotyphodium* endophytes on plant growth and root morphology and interactions with herbivores; Chapter 7: Chemistry of roots and interactions with *Neotyphodium*, herbivory and plant growth. The set-up and design of these trials is described below and further details pertaining to each trial method are given in the relevant chapters.

3.2.1 Plant Preparation

Perennial ryegrass plants, cv. Grasslands Samson, that were endophyte-free (Nil), or contained the wild-type, AR1 or AR37 endophyte strains, were grown from seed. The germinated seed was planted into plastic pots (120 mm diam.) on 10 September 1999, individually labelled, and transferred to a shadehouse. There were 70 plants in the Nil treatment, and 50, 49 and 50 in the Wild-type, AR1 and AR37 treatments respectively. The plants were maintained under regular automatic overhead watering, were trimmed as necessary to maintain vegetative growth and fed with 30 mL of nutrient solution each time they were trimmed.

On December 14, 1999, a tiller was taken from each plant and checked for the presence of endophyte by immunoblot procedure. Two plants found to be infected with endophyte in the Nil treatment were discarded as were 10 Wild-type, 2 AR1 and 5 AR37 plants that were found to have no endophyte. Plants continued to be maintained in the shadehouse as described above

3.2.2 Plant Growth Trial

In this trial, root herbivores and their impact on root and foliar growth were monitored over a period of 2 years on individual perennial ryegrass plants without endophyte infection or infected with the Wild-type, AR1 or AR37 endophytes. Populations of soil invertebrates were also monitored regularly. At the completion

of the trial mycorrhizal colonisation, other root fungi, endophyte hyphal concentration, root morphology and root chemistry were also determined.

For each of the four endophyte treatments, 20 individual plant genotypes were used with each plant cloned once to give 40 plants altogether. One of each clonal pair was treated with insecticide to reduce herbivory which allowed the impact of insect feeding on plant growth to be determined. Cloned plants were used to eliminate the major effect that plant genotype and plant genotype/endophyte interactions have on growth and herbivory of plants (Easton et al. 2000).

Cloned plants were obtained by splitting plants, grown as described in 3.2.1 above, into two ramets of 6 tillers each. These were planted individually into a sand/soil growing medium in polythene planter bags (90 x 90 x 200 mm). To enable root growth to be measured periodically without disturbing the plant, additional pairs of holes (5 mm diam., 25 mm apart) had been made in each planter bag at 30 mm, 70 mm and 110 mm from the top of the planter bag and aligned with existing holes (Fig. 3.2 & 3.3).

Fig. 3.2 Ryegrass in planter bag showing root outgrowth through holes made in the side of the bag.

Each replicate consisting of eight plants (four cloned pairs) was set up in a split-plot design (insecticide-treated (TR) or not treated (UN)) in black plastic tubs, internal dimensions of 485 x 875 mm with a depth of 300 mm (Fig. 3.3). Plants were arranged randomly within each half of the tub on a sand base in each tub, with a plastic barrier placed in the middle to reduce leaching of insecticide into neighbouring plants.

Fig. 3.3 Arrangement of plants in tubs

Initially sand was placed around the planter bags until it was level with the planting medium in the bags. After the first root sampling in August 2000, each plant was isolated from others in the trough by placing the small planter bag inside a larger one (160 x 160 x 370 mm), with the area between each bag filled with sand (Fig. 3.4). Biomass of the root outgrowth emerging from the small planter bag into the sand within the large planter bag was measured at subsequent samplings.

The trial was planted on April 14, 2000. At the end of June 2000, insecticide was applied to one plant of each clonal pair in each tub. Thereafter insecticide was applied after each plant growth sampling.

Fig. 3.4 Small planter bag with plant inside larger planter bag filled with sand

Sampling of root and foliar growth was carried out on five occasions, August and December, 2000, March/April and September/October 2001, and in January and April/May 2002. Invertebrates were sampled at both samplings in 2001 and 2002. Each sampling took approximately a fortnight to complete and so a group of replicates (generally 5) were fully sampled, replanted into the bags and placed back in the tubs before sampling of the next group of replicates proceeded.

The endophyte status of all plants was checked again at the beginning of November 2001 by immunoblot. Both AR37 plants in Rep 2 were found to have lost their endophyte and were therefore excluded from all analyses. One of the AR37 plants in Rep 13 also tested negative for endophyte and these plants were also excluded from data analyses. All other plants were found to have the appropriate endophyte status.

Osmocote® slow release fertiliser was initially incorporated into the growing medium for each plant. Subsequently, nutrient solution was applied to all plants immediately after each sampling and thereafter at least monthly with additional applications in spring and autumn during periods of rapid growth.

Initially each plant received 30 mL of nutrient solution but this was increased to 70 mL in December 2000. Effects of herbivory on plants are less apparent under high soil moisture. Irrigation of this trial was therefore deliberately kept to the minimum needed to prevent the plants from wilting and dying during prolonged dry weather (approximately once per week). Plants were watered by hand with a hose held for 4 seconds over each plant, or using a sprinkler which was left on for 2 hours.

3.2.3 Nutrient Trial

This trial investigated the effect of nutrient and endophyte status of perennial ryegrass on root growth, root chemistry, invertebrates and mycorrhiza. The trial was 4 x 2 x 2 factorial with four endophyte treatments (Wild-type, AR1, AR37, and Nil), low and high nutrient status and with and without insecticide to give 16 treatments in all. For each endophyte treatment, 10 individual plant genotypes were used and these plants were cloned across nutrient and insecticide treatments. The 10 replicates of each treatment were arranged in a randomized block design.

Plants used in this trial were 10-months-old and were from the same set of plants used for the Plant Growth Trial. The trial was planted in the same way as in the Plant Growth Trial using two different sized planter bags as described above. In July, 2000, four single tillers were taken from each plant for each endophyte treatment and planted individually into four polythene planter bags filled to within 25 mm of the top with the soil/sand growing medium. The four cloned plants were randomly assigned to a high or low nutrient treatment, and a TR or UN insecticide treatment. The trial was set up outside with plants randomly arranged within each replicate in four rows of four.

Two weeks after the trial was set up each of the high nutrient treatments received 30 mL of nutrient solution and insecticide. High nutrient treatments received fortnightly applications of nutrient solution with the amount increased to 70 mL in December, after the first sampling of the trial. Because of very different water requirements of the plants due to the size differences under the two nutrient regimes, high soil moisture was maintained throughout the period of the trial by regular hand watering. Root and foliar growth were sampled in December 2000

and the trial was terminated in March 2001, when invertebrates and plant growth were sampled, root samples were taken to assess for mycorrhiza and roots were frozen for later alkaloid analysis.

3.2.4 Root Biomass Trial

This trial was designed to supplement the information on the effect of endophyte on root aphid numbers and root growth using a destructive harvest method to determine root biomass rather than regular severing of root outgrowth which, in itself, may have affected the outcomes of the Plant Growth Trial. The trial consisted of four endophyte treatments (AR1, AR37, Wild-type and Nil) grown in different sized containers (ie. two soil volumes). Root aphid colonies are often prevalent on roots that accumulate at the interface between the potting medium and the container. It could be expected, therefore, that root aphid numbers may be relatively high in small containers where root growth on the outer surface of the growing medium becomes very dense. This trial also provided an opportunity to further investigate root morphology and root aphid population dynamics for the different treatments and changes in these with time.

Germinated perennial ryegrass seed cv. Samson without endophyte or infected with AR1, AR37 or Wild-type endophyte was planted into commercial potting mix in polystyrene trays in August 2001. In November all plants were checked for the presence of endophyte using the immunoblot method. Plants that were not of the appropriate endophyte status were discarded.

At the end of January 2002, 15 healthy plants of each treatment were selected for use in the trial. Each plant was split to give two cloned ramets of six tillers each. One of each cloned pair was planted into a small planter bag (120 x 120 x 230 mm containing 2484 cm^3 of growing medium) and the other into a large bag (140 x 140 x 280 mm containing 4900 cm^3 of growing medium) so that plant genotype was not a factor in these comparisons, with 15 replicate pairs for each endophyte treatment.

Plants were initially arranged in a shadehouse in two rows with cloned pairs of plants adjacent to each other in separate rows and treatments randomly

arranged within each replicate. They were watered daily for 30 minutes via an automatic overhead watering system. A square of black weed mat was placed underneath each planter bag so that any roots which grew through the base of the bag could be sampled. In late June 2002, plants were transferred from the shadehouse to an outdoor area and arranged in the same way. Thereafter plants were watered with a hand held hose (4 seconds per plant) only when they were very dry. Plants were fed with 70 mL of nutrients each time a foliar sample was taken. This trial was destructively sampled on three occasions (September 2002, January 2003 and June 2003), with five replicates taken down each time.

CHAPTER FOUR

THE EFFECT OF *NEOTYPHODIUM* ENDOPHYTES ON ROOT HERBIVORES

4.1 INTRODUCTION

There is an abundance of literature on the interactions between above-ground herbivores and their host plants, but comparatively little on root herbivores (Brown & Gange 1990; Hunter 2001), despite the profound effects that the latter can have on plant growth and physiology, and on the determination and regulation of soil communities (Anderson 1987; Brown & Gange 1990; Hunter 2001; Wardle 2002). The consequences that root herbivory have for individual plants, depending on the type of feeding and its severity, include reductions in above and below-ground plant growth, changes in biomass allocation, and effects on nutrient acquisition, water relations and physiological and morphological parameters of the plant (Brown & Gange 1990; Hunter 2001; Wardle 2002). At the community level, root herbivory may alter plant competitiveness and diversity and the rate and direction of succession (Brown & Gange 1990; Hunter 2001; Wardle 2002). As a major component of the soil foodweb, root herbivory also has major repercussions for soil microbial and invertebrate populations (Bardgett et al. 1999; Denton et al. 1999; Wardle 2002)

Species of *Lolium* and *Festuca* are often infected with clavicipitaceous endophytic fungi belonging to the genus *Neotyphodium*. These endophytes are obligate biotrophs and form, in most cases, a mutualistic relationship with their hosts in which they produce secondary metabolites that are deterrent or toxic to herbivorous insects (Popay & Rowan 1994). Much of the research into the effects of the *Neotyphodium* infection of grasses on insect herbivores has focused on those that feed above-ground. In part this relates to the location of endophyte infection mainly in the meristematic and basal leaf sheath tissue and the alkaloids that they produce

which are concentrated in above-ground tissues (Ball 1997 b & c; Lane et al. 1997a). Above-ground herbivores, such as Argentine stem weevil larvae (*Listronotus bonariensis*) and black beetle (*Heteronychus arator*) adults are strongly deterred by endophyte infection in ryegrass (Prestidge & Ball 1993).

In New Zealand pastures, there is a high frequency of endophyte infection of ryegrass by strains of the fungus (referred to as Wild-type) that share a common chemical profile (Easton 1999). Of the alkaloids they produce, ergovaline and lolitrem B are toxic to grazing mammals (Fletcher & Easton 1997) as well as having effects on insect herbivores (Popay & Rowan 1994), while a third alkaloid, peramine, is a powerful deterrent to Argentine stem weevil (Rowan et al. 1990) with no known effect on mammals (Fletcher 1999; Tapper & Latch 1999). In order to resolve the animal health problems associated with Wild-type infection of ryegrass while retaining the anti-insect properties that infection provides, endophytes with different metabolic profiles have been investigated (Tapper & Latch 1999). One of these, AR1, which produces peramine but not the mammalian toxins lolitrem B and ergovaline, is now available to New Zealand farmers. In the course of this research, strains which lack the ability to produce peramine, ergovaline or lolitrem B were also identified by the endophyte research team in AgResearch, New Zealand. One of these is known as AR37.

Clearly the distribution and concentration of alkaloids within plants are critical factors in determining endophyte-mediated resistance to herbivores. The particular alkaloids produced by *Neotyphodium* fungi are a characteristic of each different strain (Lane et al. 2000), although several factors moderate the quantities that are produced. These factors include plant genotype (Ball et al. 1995a & b), nutrient status (Lyons et al. 1986; Azevedo et al. 1993; Rottinghaus et al. 1991), and environmental and seasonal factors (Easton et al. 1993; Ball et al. 1995a,b). Location of alkaloids within plants, however, appears to be mainly an attribute of the compounds themselves (Ball et al. 1995b, 1997c,d; Keogh et al. 1996; Lane et al.

1997a) although this may also be modified to a degree by plant genotype (Popay et al. 2003a).

The limited amount of literature on effects of infection on root-feeding arthropods indicates endophytes can reduce feeding by some species but the effects are often inconsistent and the mechanisms poorly understood. *N. coenophialum* infection of tall fescue and/or *N. uncinatum* in meadow fescue can affect a variety of root-feeding invertebrates, including plant parasitic nematodes (West et al. 1988; Elmi et al. 2000), coleopteran scarab larvae (Popay et al. 2003b) and root aphids (*Aploneura lentisci* Homoptera:Aphidoidea) (Schmidt & Guy 1997). These particular endophytic fungi produce loline alkaloids in high concentrations which are translocated throughout the plant including into the roots (Bush et al. 1993). Lolines deter scarab larvae (Patterson et al. 1991; Popay & Lane 2000) and foliar-feeding aphids (Wilkinson et al. 2000). *Neotyphodium* endophytes in perennial ryegrass (*L. perenne*) do not produce loline alkaloids, but there is a variety of chemotypes which produce a diverse array of chemicals (Lane et al. 2000), although none are generally known to occur in significant quantities in the roots (Ball et al. 1997c,d). Nevertheless, the Wild-type endophyte in New Zealand ryegrass can reduce populations of plant parasitic nematodes (Eerens et al. 1998b; Stewart et al. 1993) albeit not consistently (Watson et al. 1995; Yeates & Prestidge 1996). Similar equivocal effects of endophyte-infected ryegrass on another root herbivore, the native grass grub (*Costelytra zealandica* Coleoptera:Scarabaeidae), were reported by Prestidge & Ball (1993). They demonstrated a significant effect of *N. lolii* infection on weight gain of second instar larvae in laboratory experiments but found no effects of infection on growth and survival of third instar grubs in pot trials or on population densities in the field.

Different feeding guilds may have differing effects on plants and will not necessarily be affected by the same allelochemicals. Two root herbivores that are common in New Zealand pastures and that are representative of two types of feeding are the root-chewing larvae of the grass grub, *C. zealandica* and the phloem-feeding

root aphid, *A. lentisci*. Scarab larvae such as grass grub are widespread pests of grassland habitats in many parts of the world (Curry 1994). The root aphid, *A. lentisci*, (Fig. 4.1) is endemic to the Mediterranean region where it is holocyclic, forming galls on its primary host, *Pistacia lentiscus* (Anacardiaceae) and alternating over a 2 year period between *Pistacia* and secondary hosts, principally species of Graminae (Cottier 1953; Wool & Manheim 1986a). This aphid has a much wider geographical range on its secondary hosts, on which it exists as permanent, anholocyclic, parthenogenetic populations (Wool & Manheim 1986b). It is reported to be abundant in grassland in Britain (Purvis & Curry 1981) and occurs throughout New Zealand (A.J. Popay, C. Pennell, D.E. Hume, unpublished observations). *A. lentisci* has been reported to cause severe damage to young wheat plants (Mustafa & Akkawi 1987) but Cottier (1953) considered the aphid to be of no economic importance on Graminae in New Zealand. There is, however, little published information on the biology or ecology of the root-living forms.

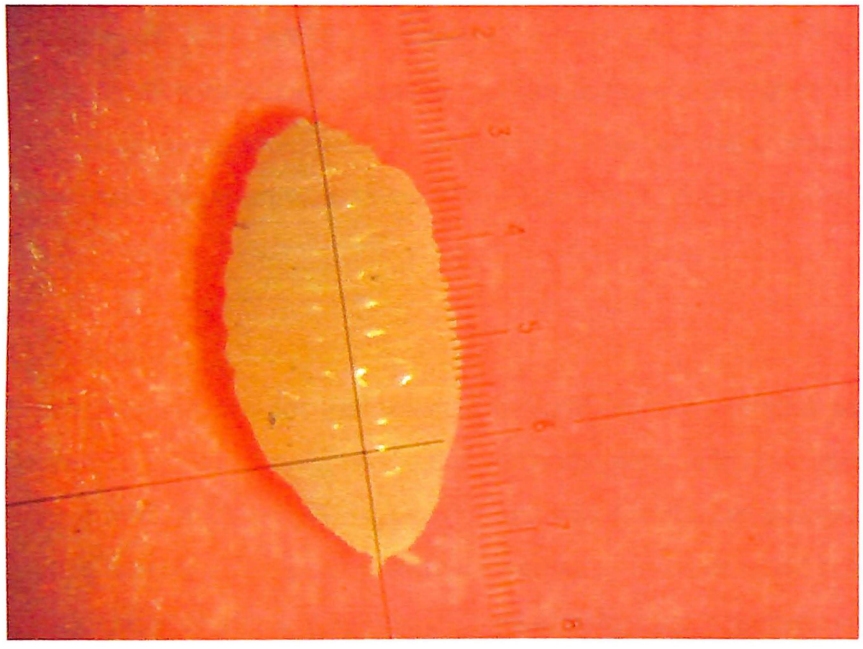

Fig. 4.1 Dorsal view of adult *Aploneura lentisci* (1.6 graticules = 1 mm)

Prior to undertaking the research reported in this thesis, casual observations of potted ryegrass indicated plants that were infected with one particular *Neotyphodium*

strain, AR37, were rarely infested by *A. lentisci*. In this chapter root aphid numbers on perennial ryegrass infected with different strains of endophyte have been measured over time, under different growth conditions and on plants under two contrasting nutrient regimes. Insecticide was used to manipulate aphid numbers in two trials in order to determine the impact of the aphid on plants. In addition the mechanisms of aphid response to ryegrass with or without endophyte were investigated in a series of trials in petri dishes. Finally, one trial was conducted to determine the effect of two endophyte strains on root chewing larvae of the grass grub.

4. 2 ROOT APHID POT TRIALS

The effects of *Neotyphodium* infection of ryegrass on root aphid populations were determined in four pot trials, namely a Plant Growth Trial, a Nutrient Trial, a Root Biomass Trial and an Endophyte/Cultivar Trial. The first three of these trials compared root aphid populations on ryegrass without endophyte (Nil) or infected with the endophytes AR1, AR37 or Wild-type. In the Endophyte/Cultivar Trial a range of endophytes with differing alkaloid profiles in three different cultivars were tested for their effects on root aphid. In addition to these pot trials, changes in root aphid numbers and behaviour were investigated in a series of four trials carried out in petri dishes.

4.2.1 Methods

Plant Growth Trial. In this pot trial, planted in April 2000, root aphid populations were compared on perennial ryegrass cv. Samson without endophyte or infected with the endophytes AR1, AR37 or Wild-type. The trial was designed to measure the long term impact of root aphid on plant growth as well as to monitor aphid populations. Twenty cloned pairs of plants were used for each endophyte treatment and one of each cloned pair was treated regularly with insecticide. Five months after the trial was set up in April 2001, all plants not treated with insecticide were inoculated with root aphid using small pieces of infested root from potted ryegrass and tall fescue plants.

Each plant was grown in a small planter bag with holes in the sides that was placed inside a larger planter bag with the space between them filled with sand to enable root outgrowth to be monitored and sampled separately from the main roots. Root aphids were sampled on the root outgrowth in the sand medium on four occasions (April and September 2001, January and May 2002) over a 2 year period. At the final sampling in May 2002 root aphid numbers were determined separately on the outgrowth and main roots. The dry weight of roots were obtained at each sampling (see Chapter 6) to enable aphid numbers to be analysed as a function of root weight as well as a total number per plant. Full details of the design of this trial are given in Chapter 3.

Root aphids were extracted by flotation in water and wet sieving. The growing medium and roots were washed and the resulting suspension decanted through three sieves (2.00 mm, 710 μm and 210 μm). The two larger sieves were rinsed thoroughly before all material that had collected on the 210 μm sieve was washed into a 70 mL specimen container. Samples were stored at 4 °C until counting.

For counting, samples were transferred to a beaker and diluted if necessary to give an amount between 30 and 60 mL. The total amount depended on the size of the original sample and the number of aphids present. The sample was stirred thoroughly to disperse the aphids before a 10 mL subsample was removed to a petri dish base (90 mm diam.) in five 2 mL aliquots, using a pipette. The base of the petri dish was marked with a grid (approximately 1 mm^2) to facilitate counting of the aphids in the dish. Counting was carried out under a stereo microscope at 16x magnification.

Nutrient Trial. Root aphid numbers in this trial were measured on perennial ryegrass cv. Samson plants, planted in July 2000, that were either regularly given additional nutrients (high nutrients) or not given any nutrients (low nutrients). The same endophyte treatments were used as in the Plant Growth Trial ie. Nil, AR1, AR37 and Wild-type and insecticide was again used to reduce aphid numbers. Each plant/endophyte treatment was cloned four times across each treatment so that the

same plant genotype was represented in the high/low nutrient and +/− insecticide regimes. As in the previous trial, plants were contained in small planter bags placed inside larger bags to enable separate monitoring of root outgrowth.

All plants not treated with insecticide were inoculated with root aphid in October 2000. Few root aphids were observed in the trial when root growth samples were taken in late November so further inoculations were made in early January.

The trial was sampled twice. At the first sampling in late November 2000 the visible presence of root aphid on the plants was noted but numbers were not counted. At the second sampling in March 2001, invertebrates from the root outgrowth and main roots in the smaller planter bag were sampled separately and counted as described above. As before, root weights were also obtained from each sample.

Root Biomass Trial. This trial, set-up in January 2001, was carried out to provide additional information, including seasonal influences, on the effect of endophytes on root aphid infestation using the same endophyte treatments as in the two previous trials. In addition, the effect of container size on development of root aphid infestations was examined. Root aphid colonies are often prevalent on the roots that accumulate at the interface between the potting medium and the container. It could be expected, therefore, that root aphid numbers may be relatively high in small containers where root growth on the outer surface of the growing medium becomes very dense. Cloned pairs of ryegrass plants were planted individually into two sizes of planter bag so that plant genotype was not a factor in these comparisons with 15 replicate pairs for each endophyte treatment. The trial design is described fully in Chapter 3.

Plants were inoculated with root aphid in late February 2002. Aphid populations were determined on three occasions, September 17 2002, January 8 2003 and June 30 2003, by destructively harvesting five replicates each time. The method

used for sampling and counting root aphid was the same as that described for the Plant Growth Trial.

Endophyte/Cultivar Trial. This trial tested both the effect of a range of endophytes expressing different alkaloids and interactions between cultivar and endophyte on root aphid populations in a pot trial. Three perennial ryegrass cultivars, Grasslands Samson, Grasslands Nui and Grasslands Impact, were used and a range of endophytes chosen to test for the effects of different alkaloid composition on root aphid. The structure of the trial was constrained by the availability of different endophyte strains in different cultivars. The endophytes tested in Nui, with the exception of AR37, produced different combinations of the alkaloids peramine, lolitrem B and ergovaline (Table 4.3). The endophytes, AR12 and AR22, with the same alkaloid profiles as AR1 were tested in cv. Samson. The effect of AR37 was determined in two cultivars and the effect of AR1 and Wild-type in all three cultivars.

Germinated seed was planted into a potting medium of two parts soil and one part washed river sand in 100 mm diam. plastic pots on August 22 2002. Twelve replicate pots were prepared for each plant/endophyte combination and spare seed was planted into polystyrene trays. Plants were supplied with slow release fertilizer and retained in the shadehouse.

In November 2002, all plants were checked for the presence of endophyte by immunoblot. At the end of December, 10 replicate pots of each treatment were arranged in a randomised block design in the shadehouse and each plant was inoculated with root aphid. Three months later pots were destructively harvested, root aphids were extracted by flotation and wet sieving as described earlier and root weight determined.

4.2.2 Statistical Analysis

Root aphid numbers/plant and number/g of root (aphid loading) for each of the pot trials were log transformed after examining residual plot data for homogeneity and

normality. Because of zeros in the data, all log transformations used a constant that was based on the minimum number possible for each data set. Data were analysed using a general analysis of variance in Genstat Release 6.1 and testing for main effects of endophyte and insecticide in the Plant Growth Trial, endophyte, nutrient and insecticide in the Nutrient Trial, and endophyte, container size and harvest date in the Root Biomass Trial. Block strata for the analysis of the Plant Growth Trial was based on the randomised block design for each replicate of endophyte treatments, the split-plot of the plus/minus insecticide treatments and the clonal pairs of plants for each endophyte treatment within a replicate. Similarly in the Nutrient and Root Biomass Trials, the analyses were structured to take into account the randomised block design of the trial and the cloned plants within each replicate. Means were separated using Fisher's protected least significant difference test in Genstat Release 6.1. Within each endophyte treatment in the Plant Growth Trial there was a large range in number of aphids/plant with some apparent consistency over time for different plant genotypes. To determine if these differences between individual plants were significant (ie. if there was a plant genotype effect) an analysis of variance was also carried out on aphid numbers on root outgrowth for each plant in the AR1, Nil and Wild-type treatments using the four sampling times as replicates. Correlation analysis comparing root aphid numbers/ plant and per g of root on cloned pairs of plants grown in the two container sizes was also used to test for a plant genotype effect in the Root Biomass Trial. After analysis, data were back transformed using the SED, number of observations and the constant used in the log transformations. However, the back-transformed data did not always adequately reflect the original data, particularly where there was a high level of variance in the latter. Thus all data are presented as arithmetic means, and measures of variance (SED or SEM) are not given. Log data and statistics are given in Appendix 1.

4.2.3 Results

The Plant Growth and Nutrient Trials included insecticide treatments that were pertinent to determining the effects of herbivory on plant growth. These results will be presented in Chapter 6. The data presented here are for the untreated plants only.

Plant Growth Trial. The most consistent and statistically significant result in this trial is the almost complete inhibition of root aphids on AR37 plants. This is shown for both aphids per plant and aphid loadings (number/g of root) (Fig. 4.2a & b; Appendix 1 – Tables 1 & 2). This result occurred at all sampling times on root outgrowth and also on the main plant roots at the final sampling in May 2002. Conversely, AR1 plants had consistently high infestations of root aphid which on some, but not all occasions, were significantly greater than those on Nil. By comparison with Nil treatments, root aphids tended to be less numerous on Wild-type plants but for most samplings this difference was not significant.

The highest aphid loading occurred on root outgrowth of AR1 plants in April 2001 (Fig 4.2b), while aphid numbers/plant were highest for this treatment in September (Fig 4.2a). The aphid loading on AR1 in March was greater than that on Nil and Wild-type ($P<0.05$), whereas aphid numbers/plant at this time had been similar for these three treatments. Aphid loading on Wild-type in April was less than on Nil ($P<0.05$) and not significantly different from that on AR37 ($P>0.05$). Other relative differences between endophyte treatments generally remained the same as for the number/plant. Aphid loadings on the main plant roots were relatively low by comparison with the loading on outgrowth.

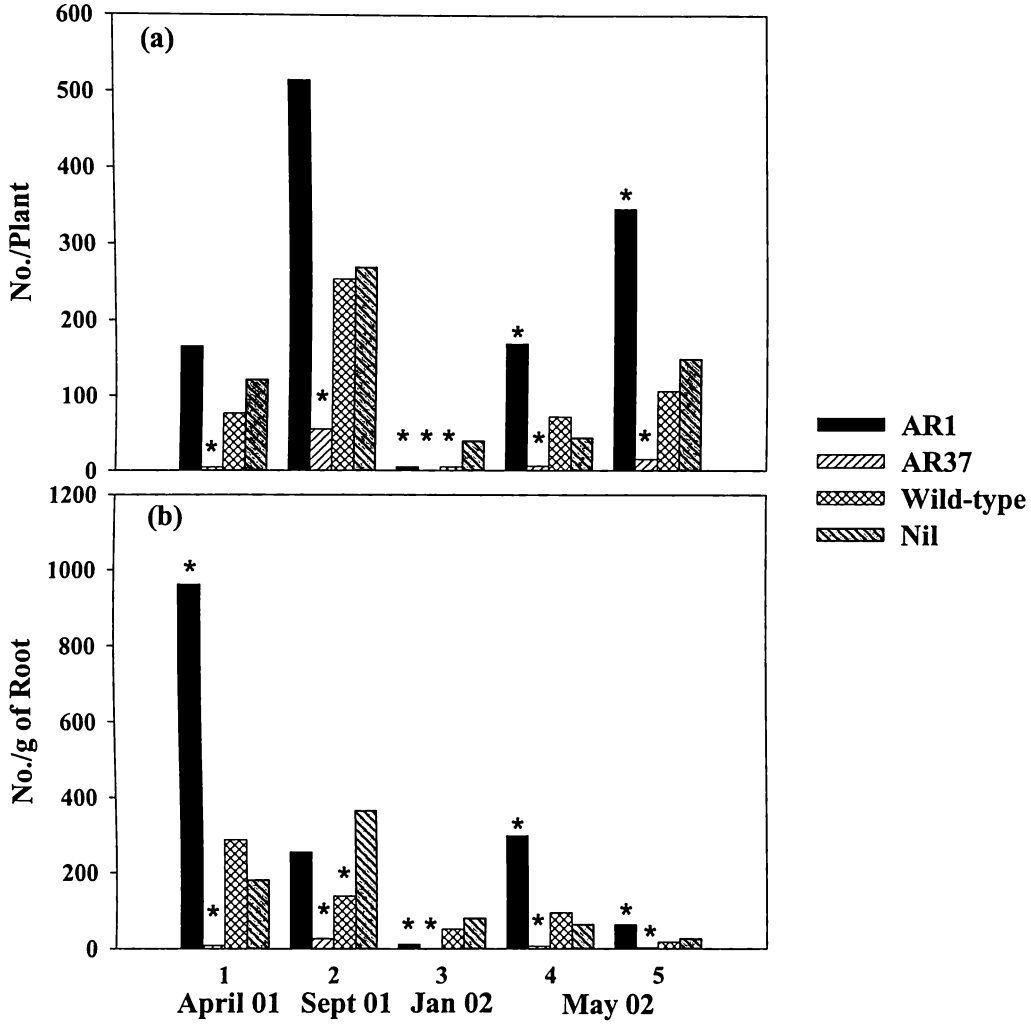

Fig. 4.2 Effect of endophyte on (a) mean number of root aphids/plant and (b) per g of root on root growth sampled four times (1-4) and on main plant roots sampled in May 2002 (5). * Denotes endophyte treatments that are significantly different from Nil (P<0.05) for each sampling.

Root aphid populations varied widely among individual plants of AR1 and Nil, varied less on Wild-type but showed little variation on AR37 plants (Figs 4.3a & b). On AR1 numbers ranged from 3 to over 2000 on the root outgrowth at the

September 2001 sampling, and from 15 to 750 on the main plant roots in May 2002.

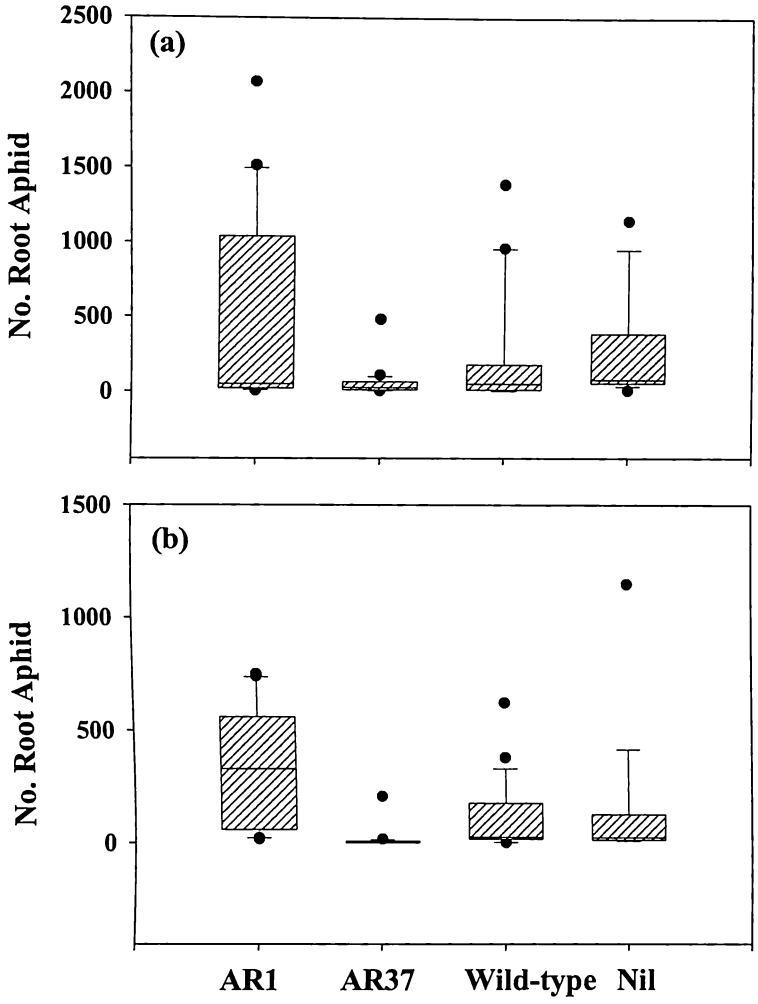

Fig. 4.3 Variability in the number of root aphid on individual plants of each endophyte treatment at (a) the September 2001 sampling of root outgrowth and (b) the May 2002 sampling of the main roots in the Plant Growth Trial. The boundary of the box closest to zero = the 25th percentile, the line within the box = the median and the upper boundary of the box = the 75th percentile. The error bars represent the 10th and 90th percentiles and points (•) are outliers.

The role of plant genotype in this variability was investigated by analysing for differences between individual plants within AR1, Wild-type and Nil treatments using the data for the number of root aphid/plant and number/g of root at each sampling of outgrowth. There were highly significant differences between plants within the AR1 and Wild-type treatments for both the number of aphids/plant and number/g of root ($P<0.01$) indicating that some of the variability in aphid numbers was linked to plant genotype. The number of aphids on individual plants within the Nil treatments also varied significantly ($P<0.05$) but this was less apparent for the number/g ($P=0.089$).

Nutrient Trial. Results of this trial confirmed those of the previous trial with root aphid numbers consistently low on AR37 and high on AR1. Aphid numbers/plant (total for outgrowth and main roots and for high and low nutrients) were lower on AR37 (2/plant) than all other endophyte treatments (295, 103 and 141/plant respectively for AR1, Wild-type and Nil) ($P<0.001$ for AR1 and Nil, $P<0.05$ for Wild-type). There were more aphids on AR1 than on Nil ($P<0.05$) which in turn had more aphids than Wild-type ($P<0.05$). As in the Plant Growth Trial, root aphid numbers on individual plants varied widely, ranging in AR1 from 0 to 4800.

High nutrient plants had more root aphid and higher root aphid loadings than low nutrient plants for all endophyte treatments ($P<0.001$) except AR37 (Fig. 4.4a & b; Appendix 1 – Tables 3 & 4). The relative differences in root aphid numbers between endophyte treatments did not change under the low nutrient regime. Under high nutrient supplements, total aphid numbers on root outgrowth of AR1 were higher than on Wild-type and Nil ($P<0.05$) and these treatments were in turn higher than on AR37 ($P<0.05$) (Fig. 4.4a) but aphid loading on Nil and AR1 were similar (Fig. 4.4b). On low nutrient plants, more root aphids occurred on the root outgrowth of AR1 than on AR37 and Nil ($P<0.05$) both as the total per plant and per g of root but AR1 was not significantly different from Wild-type ($P>0.05$). For the main roots of high nutrient plants, the sequence of significant endophyte treatment differences was AR1>Nil>Wild-type>AR37 for total root aphid numbers but aphid loading on

AR1 was similar to Nil. Both AR1 and Nil had more root aphid/plant and per g of root than Wild-type and AR37 in the low nutrient plants ($P<0.05$).

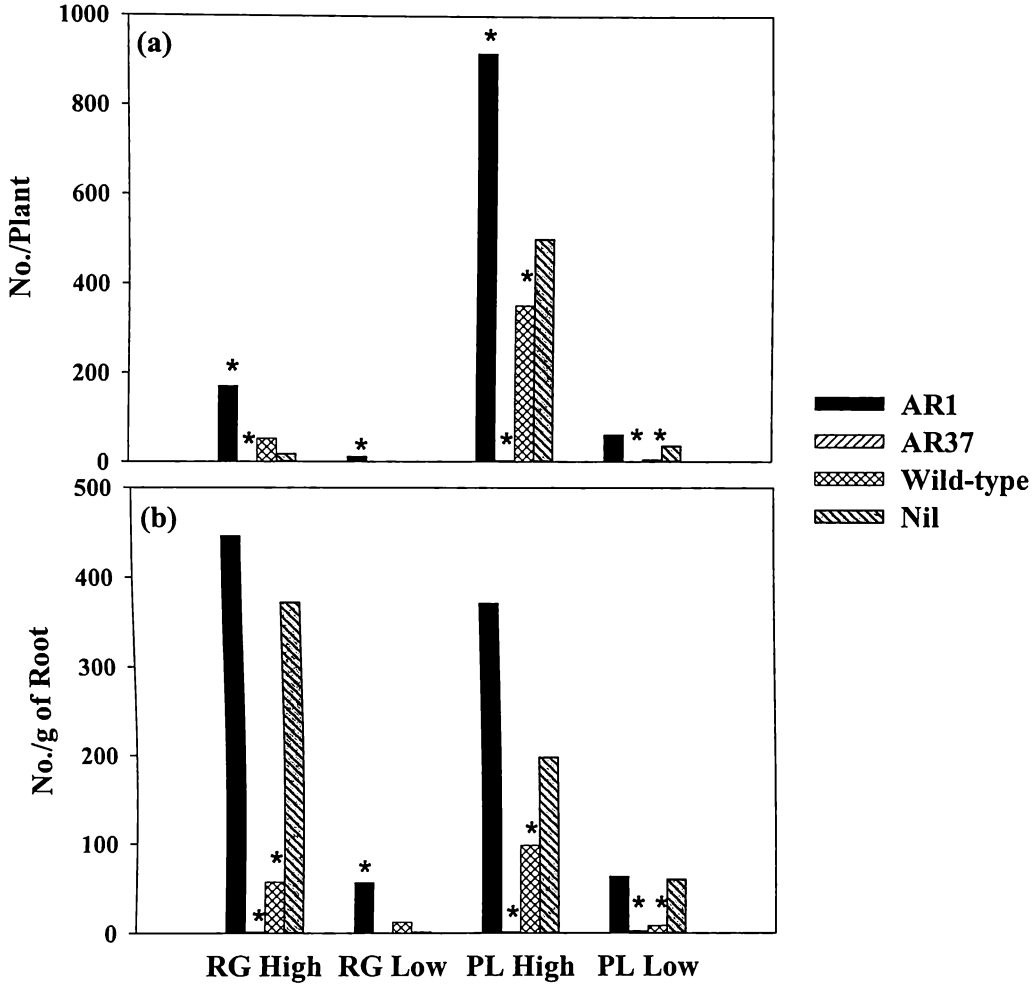

Fig 4.4 Endophyte effects on root aphid under high and low nutrient treatments: mean number of root aphids (a) per plant and (b) per g of root for root outgrowth (RG) and main plant roots (PL) of ryegrass plants under a high and low nutrient regime. * Denotes endophyte treatments that are significantly different from Nil ($P<0.05$)

Root Biomass Trial. AR37 again had fewer total aphids/plant than all other endophyte treatments ($P<0.05$) and there were more aphids on AR1 and Nil than on

Wild-type ($P<0.05$) (Table 4.1). Aphid loadings, however, were greater overall on AR1 than on Nil ($P<0.05$).

Container size had no significant effect on root aphid populations either for individual endophyte treatments or overall ($P>0.05$). In a significant interaction between endophyte and container size, aphid loadings for AR1 plants only were greater in small containers than in the large ones for ($P<0.05$) (Table 4.1; Appendix 1 Tables 5 & 6).

Table 4.1 Effect of endophyte treatment and container size on aphid populations: mean number of root aphid/plant and per g of root over all treatments and harvest dates (Mean), for large and small containers and for the three different harvest dates in the Root Biomass Trial.

	AR1	AR37	Wild-type	Nil
No./plant				
Mean (total)	406	6***	137*	382
Large	396	9***	99*	411
Small	416	3***	174*	353
September 02	315	11***	19***	555
January 03	605**	3***	258	172
June 03	296	3***	133*	419
No./g of root				
Mean (total)	173*	2***	21**	102
Large	144**	2***	13**	86
Small	203*	1***	30*	118
September 02	234	4***	5***	214
January 03	198*	1***	44	35
June 03	88	1***	16*	57

*, **, *** Significantly different from Nil treatment at $P<0.05$, $P<0.01$, $P<0.001$

There was no consistent pattern in root aphid populations over the different harvest dates. There were more aphids on AR1 and Wild-type plants sampled in January 2003 than at other harvests, although aphid loadings showed less variation for these two treatments. In Nil plants the highest aphid populations and loadings occurred in September 2002. Over all treatments aphid loadings were lowest in June.

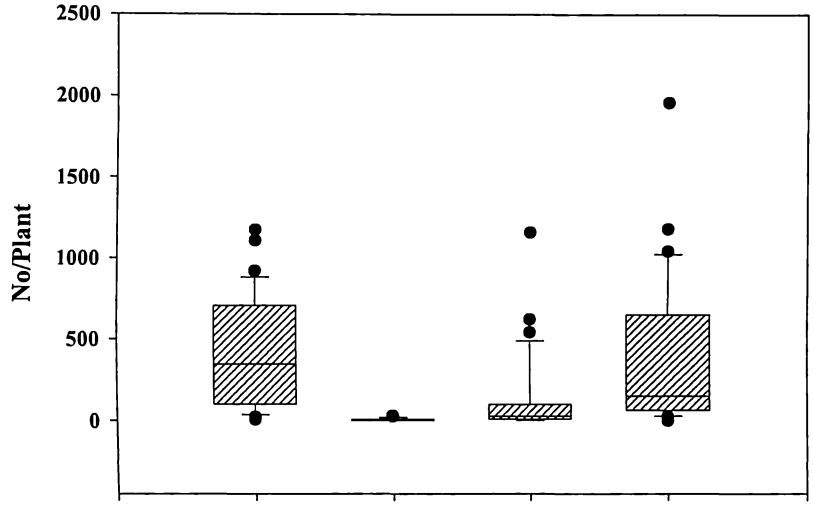

Fig 4.5 Variability in total root aphid numbers/plant among individual ryegrass plants without endophyte (Nil) or infected with AR1, AR37 or Wild-type in the Root Biomass Trial. For an explanation of the box plots see Fig. 4.3.

The extreme variability in root aphid numbers per plant which characterised the Plant Growth and Nutrient Trials was also evident in this trial for AR1 and Nil plants (Fig 4.5). Individual plants within each treatment had been cloned between the large and small containers. A correlation analysis was carried out of log transformed aphid numbers/plant and per g of root for each clonal pair of plants within each treatment to investigate the role of plant genotype in the variance in root aphid numbers in AR1, Wild-type and Nil treatments. There was a significant correlation between the cloned pairs of Wild-type -infected plants for aphid number/plant and for aphids/g of root. Aphid loading was also significantly correlated in AR1-infected plants, whereas neither parameter was correlated in Nil plants (Table 4.2).

Table 4.2 Pearson's correlation coefficient for the log number of root aphid/plant and per g of root for cloned pairs of plants in large and small containers in three endophyte treatments in the Root Biomass Trial.

Endophyte	No. aphids/plant		No. aphids/g of root	
	Correlation	P	Correlation	P
AR1	0.47	0.089	0.76	0.002
Wild-type	0.81	0.000	0.77	0.001
Nil	0.30	0.300	0.42	0.140

Endophyte/Cultivar Trial Among the endophyte treatments producing different combinations of peramine, ergovaline and lolitrem B in the cultivar Nui, the highest root aphid numbers occurred on AR1 (peramine only) and AR23 (peramine and lolitrem B) and the lowest on AR6 (peramine and ergovaline) (Table 4.3; Appendix 1 – Table 7). Numbers on Wild-type (peramine, ergovaline and lolitrem B) were similar to those on Nil. Relative differences were the same for aphid loading (data not presented).

In the cultivar Samson, there were no significant differences between aphid numbers (Table 4.3) or aphid numbers/g of root on AR1, AR12 and AR22, which all produce peramine only and these treatments were not significantly different from Nil. In Impact and Samson, Wild-type had fewer aphids than Nil plants ($P<0.05$) but in Nui aphid numbers were similar in these two treatments. AR1 numbers were particularly high in the cultivar Samson and were higher than Nil plants in all cultivars but not significantly so.

Both cultivars infected with the AR37 endophyte were highly resistant to root aphid. In Nui number of aphids on AR37 treatments were the same as those on AR6 and significantly less than those on all other treatments.

Table 4.3 Effect of a range of endophytes with different alkaloid profiles and in different ryegrass cultivars on mean number of root aphid/plant.

Endophyte	Alkaloid[1]				Cultivar		
	Pe	Er	Lo	Ja	Nui	Samson	Impact
Nil	–	–	–	–	355	367	505
Wild-type	+	+	+	–	312	72	26
AR1	+	–	–	–	648	1691	574
AR23	+	–	+	–	1597	-	-
AR6	+	+	–	–	2	-	-
AR12	+	–	–	–	-	519	-
AR22	+	–	–	–	-	1205	-
AR37	–	–	–	+	11	12	-
SEM					278.2	349.0	210.3

[1]Pe = peramine; Er = ergovaline; Lo = Lolitrem B; Ja = janthitrems

- not tested

4.3 MECHANISMS OF RESISTANCE TO *APLONEURA LENTISCI*

As a follow-up to the results obtained in 4.2 above, four experiments (A – D) were conducted on rooted plants in petri dishes to enable continuous observations to be made of root aphid behaviour and population dynamics on perennial ryegrass, without endophyte (Nil) or with the endophytes AR1, AR37 or Wild-type. In addition to the effects of endophyte treatment, the effect of plant genotype was investigated by using cloned plants in Trials A and B which were tested for their effects on root aphid at different times, and then using cloned plants again in Trial C which were tested concurrently.

4.3.1 Methods

For each trial, the base of a 90 mm diam. petri dish was firmly packed with a 60 mL volume of perlite mixed with 25 mL of tap water and approximately 2.0 g of Osmocote® slow release fertiliser. Plants or tillers from plants were placed in the petri dishes so that the base of the tiller was level with a hole (approx 10 mm wide) cut in the side of the base and top of each dish (Fig 4.6). Roots were splayed out on the surface of the perlite before the lid was put in place and sealed with a 20 mm wide piece of parafilm. Replicate groups of petri dishes were placed together in random order and fastened with a rubber band. A piece of black polythene with a slit in the centre where the tillers emerged was placed over each group of petri dishes and fastened in place with another rubber band. Each replicate was then partially buried in potting mix in a polystyrene planter box and kept outside under ambient light and temperature conditions.

Fig. 4.6 Plant growing in petri dish

Mature and immature root aphids taken from potted plants in the shadehouse were transferred with a fine paint brush onto, or in the immediate vicinity of, roots of the plants in the petri dishes. Maturity was arbitrarily based on size (immature < 1 mm > mature) because it was difficult to distinguish adult aphids from mature nymphs. Aphids were later checked and replaced if damaged in any way before lids and parafilm were replaced.

To count and observe root aphid in each trial, lids were removed from the petri dishes and the surface of the perlite and roots were inspected under a stereo microscope (16x magnification) every 3 – 4 days (Fig. 4.7). The number of live and dead aphids and their stage of maturity were recorded and dead aphids were removed. Location of the aphids on or off roots (recorded as off when on perlite and not in contact with roots) was noted at each inspection in all trials, and their preference for new (ie. roots grown since planting) or old roots in Trials A and B. In Trial A, 5 – 10 mL of water was added to each petri dish at every second inspection, which kept the perlite damp. In subsequent trials water was added only as necessary to maintain the perlite in a moist condition. Watering was more frequent for those plants which were more actively growing.

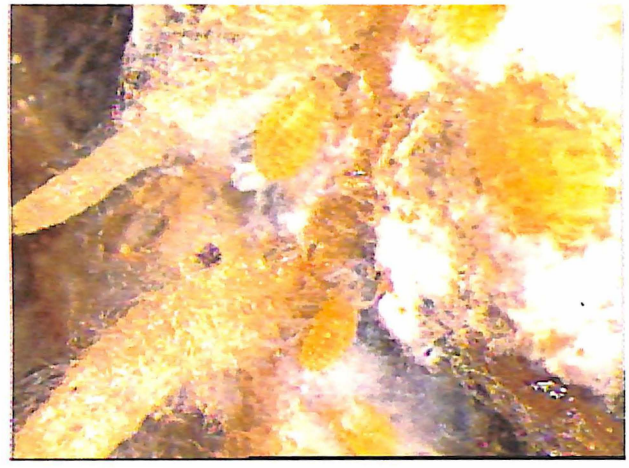

Fig. 4.7 Root aphid, *Aploneura lentisci* clustered on roots in a petri dish

At the completion of each trial root aphid numbers on the roots and perlite throughout the petri dishes were counted. The number of tillers on each plant was recorded in Trials A-C and the endophyte status of at least one tiller from each plant was confirmed by staining and microscopic examination.

Four trials were carried out in this way using perennial ryegrass cv. Samson and comparing aphid performance on plants without endophyte or infected with the endophytes AR1, AR37 and Wild-type.

Trial A: A single healthy tiller was removed from each of five 1-year-old plants of each treatment and planted into separate petri dishes to give five replicates of each endophyte treatment. One week after planting, 10 mature and five immature aphids were released onto each plant. The trial was terminated after 35 days.

Trial B: This was planted at the same time as Trial A using clones of the same plants with five replicates of each endophyte treatment. Plants were inoculated with 10 mature and five immature aphids 4 weeks after planting. Petri dishes were inspected regularly for 25 days.

Trial C: For each endophyte treatment, five cloned pairs of plants were tested by taking two ramets of two tillers, matched for root size, from five individual one-year-old plants and planting them separately into petri dishes. Four weeks after planting five mature and five immature root aphids were released into each petri dish. The experiment was assessed for 21 days.

Trial D: This trial tested the effects of the different endophyte treatments in 10 week-old ryegrass plants. Plants were initially grown from germinated seed in potting mix for 6 weeks. They were tested for endophyte before 20 plants of each endophyte treatment were planted into petri dishes. Four weeks after planting, the ten healthiest plants of each treatment were inoculated with 12 root aphids, of which at least five were mature and five immature. The petri dishes were checked regularly for 21 days and then left without checking for a further month during which time they were watered individually. At the completion of the trial the number of aphids present was recorded and a dry weight of foliage and root material was obtained.

4.3.2 Results

On plants infected with AR37, root aphid survival declined to very low levels in all four trials, after an initial phase of 5 – 10 days in which numbers were similar in all treatments (Fig. 4.8a-d). In Trials A and C root aphid numbers on Wild-type plants followed a similar pattern of decline to those on AR37 whereas in Trials B and D aphid performance on Wild-type was similar to that on AR1 and Nil treatments.

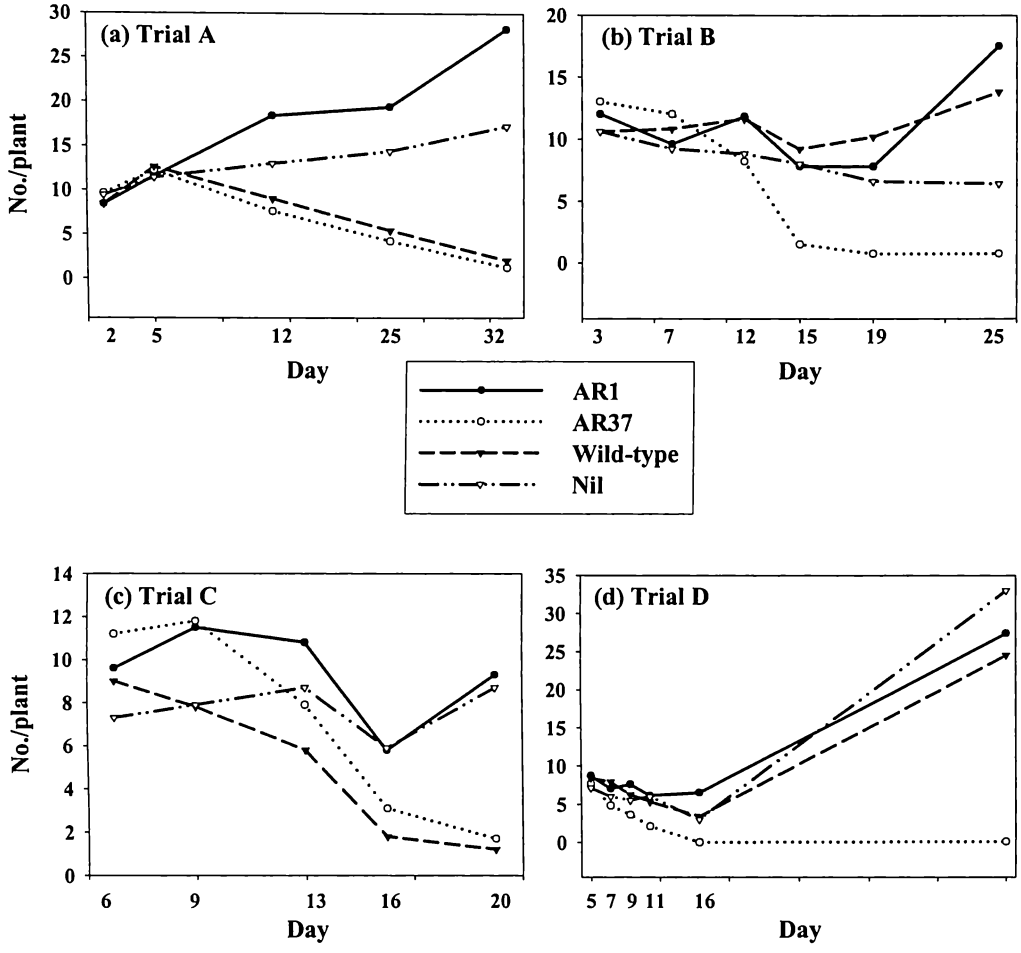

Fig. 4.8 Numbers of root aphid/plant on ryegrass without endophyte or infected with AR1, AR37 or Wild-type in petri dish Trials A – D.

The role of plant genotype was considered by comparing aphid performance on cloned plants in Trials A and B and again in Trial C. For AR1, final numbers/plant were highly correlated between individual cloned plants in Trials A and B and again between the cloned plants in Trial C (Table 4.4). For Wild-type-infected plants the strongly contrasting differences in aphid performance between Trials A and B showed no evidence of a plant genotype effect while in Trial C plant genotype effects could not be tested for when aphid numbers fell to low levels on all five plants. Aphid

numbers on Nil plants were not correlated between either Trials A and B (-0.44) or in Trial C (-0.22).

A low percentage of the root aphids observed in the petri dishes were recorded away from roots with little difference between treatments overall (Table 4.5) or between different assessment dates. Nymphs displayed a marked preference for new roots in all treatments where this was noted in Trials A and B. Mature aphids showed a similar preference in Trial B but not in Trial A. Aphids on AR37 plants displayed less of a preference for new roots than those on other plants.

Table 4.4 Effect of plant genotype on root aphid: final number of aphids/plant for cloned pairs of plants tested at two different times in Trials A and B and at the same time in Trial C.

Rep	Trials A & B – Cloned Pairs		Trial C – Cloned Pairs	
	A	B	A	B
1	2	0	0	1
2	59	35	38	23
3	46	15	7	6
4	27	10	12	0
5	1	2	0	6
Correlation[1]	0.96		0.87	

[1]Pearson's correlation coefficient

At the completion of Trials A – C the number of tillers on each plant was similar for all treatments (Table 4.5). In Trial D dry weights of the roots and foliage of each live plant were recorded. Observations suggested that more vigorous plants often supported more root aphids than those that were not actively growing. In Trial D root weight at the end of the trial was plotted against root aphid numbers/plant (Fig. 4.9) revealing an apparent relationship between root aphid numbers and dry weight of roots when aphid numbers were low. The main outlier in this relationship

was a Wild-type plant which had good growth but no aphids. When root aphid numbers were high (93, 87 and 85/plant for AR1, Wild-type and Nil respectively) the corresponding root weights were well below the apparent trend of increasing growth at low aphid densities. Mean foliage dry weights for AR1, Wild-type and Nil plants were 165, 265 and 265 mg respectively. For plants containing AR37, the mean root dry weight was 40 mg and foliar weight was 192 mg.

Table 4.5 Total percentage of root aphids recorded away from roots and the percentage of immature and mature aphids found on new roots during the petri dish trials and the number of tillers/plant at the final assessment.

	Trial	AR1	AR37	Wild-type	Nil
% Aphids	A	17.4	23.5	28.3	15.3
off roots	B	14.0	22.8	15.4	12.9
	C	7.2	8.1	8.6	8.1
	D	2.2	12.1	9.0	4.7
% Aphids on	A - Imm	83.2	61.0	78.1	83.6
new roots	A - Mat	55.0	36.4	50.0	61.7
	B - Imm	97.9	83.5	89.0	89.3
	B - Mat	85.7	80.8	84.2	68.3
No. Tillers/	A	2.6	3.0	2.6	3.0
plant	B	8.5	7.8	7.8	6.2
	C	6.8	7.7	6.5	10.3

On Day 7 in Trial B, two aphids on separate AR37 plants were noticed to be trembling quite violently. Both were still shaking when observed twice more over the ensuing 24 h period and after 36 h they had died. Over that period both aphids remained stationary, one with its stylet inserted into the root throughout. Following this, aphids in all treatments were closely observed and others were also found to be trembling and their movements uncoordinated but only in AR37 treatments. No

aphids were subsequently found with tremors as severe as those first observed. Trembling aphids were recorded at Day 5 in Trial D but not until Day 13 in Trial C.

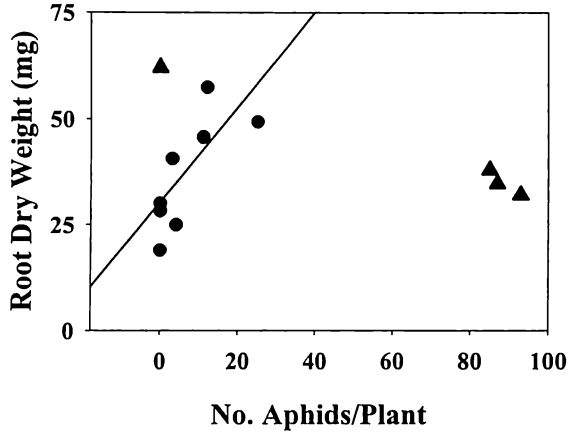

Fig 4.9 Relationship between number of aphids/plant and root dry weight in Trial D of the petri dish experiments. • = points, relating to low aphid numbers only, on which the regression is based; ▲= points not included in the regression: a Wild-type plant with no aphids but high root dry weight and three plants that had high root aphid numbers.

4.4 THE EFFECT OF *NEOTYPHODIUM* ENDOPHYTE ON *COSTELYTRA ZEALANDICA*

In this trial the effects of endophyte-free ryegrass and ryegrass infected with the endophytes, AR22 (an endophyte similar to AR1) and AR37, on survival and feeding by grass grub, a root-chewing insect, were investigated in a choice and a no-choice trial. In the choice trial the feeding preferences of larvae for maize or ryegrass with and without endophyte infection was investigated by utilising the natural $\delta^{13}C$ difference in a C_4 grass (maize) and a C_3 grass (ryegrass). Since animals generally reflect the stable isotope composition of their diet (Neilson et al. 1998) differences in the $\delta^{13}C$ composition of the larvae should reflect the proportions of maize and

ryegrass in their diet and can be used to determine if an endophyte treatment is deterrent to the larvae.

4.4.1 Methods

One-year-old perennial ryegrass cv Samson plants were used in this trial. Plants were tested for the presence of endophyte by immunoblot in November 2000. In late January 2001, a week before transplanting the ryegrass into the trial, all plants were trimmed, dead material was removed and plants were given 70 mL of nutrients.

For the choice trial, 10 individual plants of each of the ryegrass endophyte-treatments were paired with a maize plant (experimental line 38G43) (Fig 4.10). Black plastic rectangular containers (320 x 140 x 120 mm) containing a 20 mm base of washed river sand were two-thirds filled with coarsely sieved field soil. Ryegrass plants were transplanted into the soil at one end of the container and a germinated maize seed was planted at the other end.

Fig. 4.10 Paired maize and ryegrass plants used in the choice trial

In the no-choice trial single plants of each of the ryegrass treatments and maize were planted in 150 mm diameter plastic pots filled with the same soil as used in the choice trial. Four replicate pots were set up for each treatment.

Each trial was arranged in a randomized block design in the shadehouse, under automatic overhead watering. A week prior to the introduction of grass grub to the trial, ryegrass plants were trimmed and all plants were given 30 mL of nutrients.

On February 20, healthy second instar grass grub larvae field-collected at Lincoln, Canterbury, were selected for use in the trial. A random sample of 20 of these grass grub was weighed and had an individual mean weight of 43 mg. Five larvae were placed on the soil surface in the middle of each container of the choice trial and two larvae placed at the base of plants in the no-choice trial. Any larvae that failed to bury themselves in the soil were replaced. The trial continued to be maintained in the shadehouse but was only watered with a hand-held hose as necessary to prevent over watering.

After 8 weeks the trial was taken down by hand-sorting the soil and recording the number of grass grub and their instar. All larvae taken from the trial were kept at ambient temperatures for 24 h to allow evacuation of the gut and were then frozen. They were later oven dried at 60°C for 24 h before being sent to Waikato University Stable Isotope Unit for $\delta^{13}C$ analysis. All plant roots were washed thoroughly and then oven dried at 80°C for 48 h. Roots of each plant in two replicates of the no-choice trial were also sent for $\delta^{13}C$ analysis.

In preparation for $\delta^{13}C$ analysis the grass grub from each replicate and samples of the washed roots from the plants in the no-choice trial were again oven dried at 80°C for 2-3 days. Samples were cooled in a desiccator and then ground finely (<200 μm) and stored in vials. Weighed samples of ground material between 2.6 and 3.1 mg were encapsulated and then loaded onto a Dumas Elemental Analyser interfaced to an Isotope Mass Spectrometer for determination of $\delta^{13}C$.

The proportion of maize consumed in each choice treatment was estimated using the following formula:

$$(\delta^{13}Crg - \delta^{13}Cgg/ \delta^{13}Crg- \delta^{13}ma)*100$$

where: rg = grass grub fed ryegrass only

gg = grass grub from each replicate from the choice trial

ma = grass grub fed maize only

Data for the proportion of maize consumed were examined for normality and homogeneity before being analysed by ANOVA in Genstat Version 6.1.

4.4.2 Results

Survival of grass grub in all treatments in the choice trial and in the ryegrass treatments in the no-choice trial was greater than 75% but there was a lower survival on maize plants in the latter (Table 4.6). There was no indication that treatments affected larval development since only one of the surviving grass grub at the end of the trial had not developed through to the third instar.

Table 4.6 Percent survival, $\delta^{13}C$ content of ryegrass with different endophyte treatments (AR22, AR37 and Nil) and maize; $\delta^{13}C$ content of grass grub larvae feeding on different combinations of ryegrass/endophyte and maize treatments; and estimated percent consumption of maize in the choice feeding trial.

	No Choice				Choice with maize		
	Nil	AR22	AR37	Maize	Nil	AR22	AR37
% Survival	75	75	75	38	80	80	80
$\delta^{13}C$ Plant	28.68	29.20	29.84	11.8			
$\delta^{13}C$ Larva	27.54	27.61	27.80	16.27	22.41	22.37	21.53
% Maize					45.5	46.2	54.4
						SED = 7.78	

An estimate of maize consumption based on the isotopic enrichment of the grass grub in the choice trial showed similar amounts of maize and ryegrass were consumed by these larvae. Larvae given a choice between AR37-infected ryegrass and maize showed a slight but not significant increase ($P>0.10$) in the proportion of maize in their diet compared with those larvae given a choice between maize and Nil endophyte ryegrass or maize and AR22-infected ryegrass.

4.5 DISCUSSION

Interactions between insect herbivores and their host plants at any one time depend on host quality, defined by Leather (1994) as "those plant attributes, chemical or physical, that contribute either negatively or positively to the fitness of the insect population or individual insect that feeds upon the plant's tissues". Insect performance is therefore governed by a balance between those chemical factors that positively influence its fitness and those that have a negative effect while other elements of host quality include resource availability. *Neotyphodium* endophyte infection of grasses changes the host quality in terms of its chemistry for those insects that utilise the infected plant as a food source. The response of any one insect species can vary from negative, where the presence of alkaloids impair the performance of the insect, to neutral, where the insect is not affected (Popay & Rowan 1994), to positive, where insect fitness appears to be better on infected plants than on uninfected (Saikkonen et al. 1999; Bultman & Bell 2003). Effects may be endophyte-strain specific and be transitory rather than stable.

The effects of host quality on insect performance are exemplified in the results of the trials reported here for the root aphid, *A. lentisci*. Populations of this aphid have exhibited a marked response to host ryegrass plants ranging from negative to positive that have been largely driven not only by the presence or absence of *Neotyphodium* infection but also by the strain of endophyte. At the negative end of the scale, ryegrass infected with AR37 is highly resistant to *A. lentisci*. The effect is

stable, showing only minor seasonal variation with some increases in populations in spring and little variation in the level of resistance among individual plants. At the other end of the spectrum, ryegrass infected with AR1 is often more susceptible to root aphid than endophyte-free plants. Aphid populations were highly variable on AR1 both on individual plants and over time. In addition, ryegrass infected with other endophytes similar to AR1 show similar levels of vulnerability to root aphid. For Nil plants there was considerable inter-plant and temporal variation in the number of root aphids/plant, and overall aphid performance on this treatment could be considered to range from neutral to positive. Aphids tended to be less numerous on Wild-type than on Nil plants but not always significantly so. Thus aphid performance on ryegrass with Wild-type endophyte is mostly neutral with what appears to be transient negative effects. Inter-plant and temporal variations in number of aphids on Wild-type was much less than on Nil and AR1.

Root availability may provide one explanation for differences between treatments but aphid loadings generally reflected the numbers/plant and did not change relative differences between endophyte treatments suggesting that this was not a limiting factor. As a measure of resource availability, however, root weight is not sufficient because it takes no account of differences in root morphology and age which may be equally, if not more important, for aphid performance. This is evident from the petri dish experiments in which aphids demonstrated a strong preference for new roots suggesting that the availability of new roots, rather than the total root weight *per se,* is more important for development of root aphid populations. In this regard, the design of the pot trials in allowing separate sampling of new root growth was useful.

If habitat is not limiting aphid populations then plant chemistry is the most likely basis for the differences observed among endophyte treatments. The effects of AR37 on root aphid are most likely attributable to the production of a metabolite by the fungus that is toxic to the aphid. The tremors induced when the aphid feeds on plants infected with AR37 indicate that the compound is a neurotoxin. In all the petri

dish trials there was an initial phase lasting up to 10 days after aphids were released on to the plants in which the aphid behaviour, feeding and reproduction appeared normal. The delayed effect suggests that the toxin is either a slow-acting constitutive compound or one that is inducible. The proportion of aphids recorded on roots provided no evidence that deterrency was a factor in aphid response to AR37.

One can be less confident that the response of aphids to infection of ryegrass by Wild-type is also due to the presence of an allelochemical. In Trials A and C in petri dishes, the rapid decline in aphid numbers on Wild-type was symptomatic of the presence of a toxin but there was no indication of this in Trials B and D. Plants in Trials B and C were from the same source, had been in the petri dishes for a similar length of time prior to inoculation with aphids and were kept under similar ambient conditions. Trial C was conducted a month after Trial B in the spring when temperatures were warmer but there was no indication in the pot trials that aphid performance on Wild-type varied with seasons or temperature. The alkaloids produced by Wild-type endophyte with known anti-insect activity are lolitrem B, ergovaline and peramine (Popay & Rowan 1994). Peramine is ruled out as affecting aphids since it is the only one of the three compounds that is also produced by AR1. In the trial comparing endophytes with a range of metabolic profiles, AR23, which produces peramine and lolitrem B but not ergovaline was highly susceptible to *A. lentisci*. In contrast to this, aphid numbers were extremely low on AR6, an endophyte which produces peramine and ergovaline but not lolitrem B. This would suggest that ergovaline is responsible for the low root aphid populations on plants infected with Wild-type or AR6. It must be noted, however, that considerable numbers of root aphid have been observed on plants infected with AR6 previously (A.J. Popay unpublished). This observation is consistent also with the contrasting performance of root aphid on Wild-type in Trials A and B in the petri dishes. Ergovaline concentrations in plants vary seasonally and with environmental conditions (Ball et al. 1995a; Lane et al. 1997b) and are also linked to plant genotype (Easton et al. 2002).

There was evidence of a strong host plant genotype influence on aphid fitness on Wild-type and AR1-infected plants in both the Plant Growth and Root Biomass Trials and in addition for AR1 in the petri dish experiments. In Nil the link between plant genotype and aphid performance was less marked than for the endophyte-infected plants suggesting that a host plant genotype/endophyte interaction may be moderating aphid performance more than plant genotype itself. A similar high degree of variability associated with inter-plant genotypic differences has been found in the amount of damage inflicted on AR1-infected plants by black beetle adults (Easton et al. 2000), due possibly to an unidentified metabolite produced by AR1 which is assumed to have some deterrent effect on this insect (Popay & Baltus 2001). Alkaloid production is linked to endophyte concentration in the plant and is markedly influenced by host plant/endophyte interactions (Ball et al. 1995 a & b; Easton et al. 2002). Other aspects of plant growth and mineral uptake have also been shown to vary according to interactive effects of endophyte and host plant genotype (Malinowski & Belesky 1999; Malinowski et al. 2000; Cheplick & Cho 2003).

Composition and concentration of amino acids and concentration of sucrose in the phloem are important determinants of aphid performance (Douglas 1993; Karley et al. 2002) and levels of soluble nitrogen are often causally linked to inter- and intra-plant differences in aphid fitness, site preferences, host alternating behaviour and seasonality (Leather 1994). Differences in some of these chemical factors may account for not only the apparent differences in aphid performance between AR1 and Nil, but also the extreme variability between individual plants. In the Nutrient Trial aphid performance was reduced on nutrient-stressed plants, presumably because their nutritional requirements were not being met. The roots of low nutrient plants contained lower concentrations of nitrogen than did high nutrient plants while concentrations of two other major ions, phosphorus and potassium, were not reduced (Chapter 7). Despite this, the relative differences between endophyte treatments were maintained. If high populations of root aphid on AR1 were due to an increased supply of nitrogen in these plants then it could be expected that a lack of nutrients would eliminate this advantage but this was not the case. Thus the factors enhancing host

quality for the aphids in AR1-infected plants are also able to operate under conditions of low nutrient supply, albeit to a more limited extent. In addition, nutrient stress did not reduce the ability of AR37 and Wild-type to mediate resistance to root aphids. A lack of phosphorus and nitrogen reduces ergovaline production by *Neotyphodium* fungi in tall fescue (Arechavaleta et al. 1992; Lyons et al. 1990) but effects of nutrient status on production of other alkaloids are not consistent (Lane et al. 2000).

The preference root aphid show for new roots may be explained by changes in chemistry as roots age but equally may be due to physical factors such as increasing lignification that may make it difficult for the aphid to probe older roots. Respiration rates are higher and uptake of nutrients and water more efficient in new than in old roots (Eissenstat & Yanai 1997; Bouma et al. 2001) but there is little other information on physiological changes in maturing roots that may explain aphid preference. Graham (1995) found that young roots in citrus were more susceptible to parasitism and herbivory than older roots which were protected by a thick secondary cell wall.

In Trial D of the Petri Dish trials, there were indications of a positive relationship between low root aphid numbers and root growth which may suggest that actively growing roots are important for aphid fitness or, alternatively, may support findings showing low amounts of root herbivory can stimulate root growth (Quinn and Hall 1992; Bardgett et al. 1999). An observation made in the petri dish trials, however, that aphid survival and reproduction were severely compromised if plant growth was poor, even though the plant remained alive throughout the trial, supports the contention that active growth is a requirement for aphid fitness. Growth will affect the quantity and quality of phloem, both of which are factors that contribute to aphid performance (Whitham 1978). As the high variability in aphid numbers indicates, however, a healthy plant with abundant new root growth did not always result in high aphid numbers either in the petri dish trials or in the pot trials for any of the treatments.

Unlike many foliar-feeding aphid species, there was no discernible pattern in aphid numbers over time or season. In the Plant Growth Trial, numbers were highest in March 2001 but then fell to very low levels in January 2002. Seasonality was not the cause since aphid populations were generally high in January 2003 in the Root Biomass Trial. Anderson (1987) noted that root herbivores are often chronic pests and this would seem to be true for *A. lentisci*. Like many root herbivores, however, *A. lentisci* are highly aggregated in their distribution, forming sometimes large colonies on roots where they cocoon themselves in wax secretions. They show no preference for a particular depth in the soil profile but exploit large pore spaces in the soil structure where there is often a proliferation of roots; hence their apparent prevalence at the interface between the growing medium and container. Thus root distribution may be important in determining populations and was probably the reason for the higher aphid loadings on AR1 plants in the small growing containers, compared with the larger ones. This effect was negated by greater root growth in the Nil and Wild-type treatments (see Chapter 6). At times in the field and in potted plants, aphids have been observed feeding at the soil surface, clustered around the base of tillers. Since no alate aphids have been seen at any time during the course of this study, dispersal mechanisms are unknown but may involve the highly mobile nymphs moving from plant to plant and perhaps also being wind-dispersed. As the aphids mature they become more sedentary. Considerable phenotypic variation in aphid size was observed which may be related to their reproductive capacity (Dixon & Kundu 1998).

While AR37 has a strongly adverse effect on root aphid, there was little indication that it also affects the root-chewing grass grub larvae. Survival was not affected by AR37 in the no-choice pot trial and the small increase in consumption of maize in the choice trial compared with the other treatments was not significant. Popay et al. (2003b), using the same stable isotope method, recorded a 30% increase in feeding on maize for grass grub larvae given a choice between maize and meadow fescue infected with *N. uncinatum* compared with larvae given a choice between maize and endophyte-free meadow fescue.

CHAPTER FIVE

THE EFFECT OF *NEOTYPHODIUM* ENDOPHYTES ON SOIL BIOTA AND INTERACTIONS WITH A ROOT HERBIVORE

5.1 INTRODUCTION

The soil environment surrounding plant roots is a highly complex and competitive environment within which interactions between microfauna and microorganisms have a pivotal role. Complex food webs are built as simple trophic interactions between two organisms multiply as the number of species within a community increases (Moore et al. 1988).

In terms of biomass, saprophytic fungi are often the predominant microorganism in many soil environments. They are major components of decomposer food webs with a capacity to rapidly exploit changes in resources by high rates of sporulation and growth. Community analyses, based on those species which are readily cultured, suggest certain suites of fungi are characteristic of certain vegetative types and geographic areas (Thorn 1997). Though not direct participants in decomposer food webs, arbuscular mycorrhizal (AM) fungi are also important contributors to plant/soil communities with some plants dependant on the ability of these fungi to sequester and transport nutrients to maintain their competitiveness. Mycorrhiza can also benefit plants by mitigating against the effects of herbivory (Gange et al. 2002), moisture stress (Dodd 2000) and fungal pathogens (Dehne 1982).

Invertebrates in the soil environment have an integral role in structuring communities and in doing so contribute significantly to decomposition and nutrient cycling (Moore et al. 1988). They have the capacity to alter the functioning of fungal-based food webs by selective grazing and dissemination of fungal propagules (Moore et al. 1988; Lussenhop 1992). Their activities can change soil structure and stimulate microbial activity by adding mineral nutrients in the form of urine and faeces (Moore et al. 1988; Lussenhop 1992; Filser 2002).

Major fungivorous taxa in the soil include species of Collembola, Acari and Nematoda. Collembola have been shown to preferentially graze certain species of saprophytic fungi and mycorrhiza (Warnock et al. 1982; Finlay 1985; Visser 1985) to such an extent that the composition of communities is changed (Visser 1985). Indeed the apparent ineffectiveness of mycorrhiza in the field has been attributed to disruption of hyphal networks by invertebrate activity (Finlay 1985; Fitter & Gabaye 1994). Gange (2000), however, contends that there is little evidence that collembola preferentially graze mycorrhiza and are more likely to consume saprophytic species.

Plants are at the heart of soil community interactions, providing food, habitats and a physico-chemical environment that impact on community composition. Arthropod and microbial populations respond to the quality and quantity of vegetative (Wardle et al. 1999a & b) and carbon inputs from roots (Holland & Detling 1990; Holland et al. 1992; Todd et al. 1992; Tu et al. 2003). Herbivory increases those inputs directly through increases in litter and decaying roots or indirectly in the form of dung and urine and root exudation (Holland et al. 1996). In reciprocal interactions, the plant benefits directly from nitrogen mineralization resulting from activities of the soil biota (Moore et al. 1988; Bardgett & Chan 1999; Bardgett et al. 1999) but can also be adversely affected by invasion of pathogenic fungi.

In grassland environments, the infection of some species of Graminae by biotrophic *Neotyphodium* endophytic fungi has the potential to alter the structure of soil communities either directly via allelochemicals or indirectly by reducing herbivory. Few studies have addressed this issue, although changes in non-herbivorous arthropod and microbial communities as a result of *Neotyphodium* infection of ryegrass or tall fescue have been reported. Bernard et al. (1997) found that assemblages of collembolan species in litter were altered by *Neotyphodium* infection of tall fescue. Similarly, composition of surface-dwelling arachnid species differed between ryegrass paddocks with a high frequency of endophyte infection compared to those with low infection (Prestidge & Marshall 1997) while earthworm numbers in these same paddocks were similar (Prestidge et al. 1997). Differences in decomposition rates of litter from endophyte-infected and

endophyte-free *L. multiflorum* suggest there are also consequences of endophyte infection for detrital food webs (Omacini et al. 2004).

Mycorrhizal colonisation of roots may be disrupted by *Neotyphodium* infection in both ryegrass (Müller 2003) and tall fescue (Chu-Chou et al. 1992; Guo et al. 1992). Conversely the effectiveness of endophyte-mediated defence mechanisms against herbivores may be reduced by mycorrhiza (Barker 1987; Vicari et al. 2002). In an example of the complexity of the interactions that are possible, Matthews and Clay (2001) reported soil-mediated inhibition of endophyte-infected tall fescue grown in soil previously dominated by conspecifics possibly due to the accumulation of organisms specifically antagonistic to that plant/endophyte association.

In this study the response of soil invertebrates, root fungi and mycorrhiza to infection of ryegrass by different *Neotyphodium* strains and the interactions between these and changes in herbivory resulting from endophyte infection are investigated.

5.2 METHODS

5.2.1 Invertebrates

Numbers of invertebrates belonging to three taxanomic groups, Collembola, Nematoda and Acari were determined in two trials, a Plant Growth Trial and a Nutrient Trial.

Plant Growth Trial. In this trial invertebrate populations surrounding perennial ryegrass cv. Samson roots without endophyte or infected with Wild-type, AR1 or AR37 endophytes were monitored for 2 years. There were 20 cloned pairs of plants of each ryegrass/endophyte combination, with one of each pair of plants treated regularly with insecticide. Each plant was contained in a sand/soil growing medium in a small planter bag with additional holes in the sides through which roots could grow. The smaller planter bag was then placed inside a larger bag and the enclosed space filled with sand. A full description of the trial design is given in Chapter 3.

In order to replicate the invertebrate fauna that ryegrass plants would be exposed to in the field, a range of different species were introduced to plants in this trial in the spring of 2000. In addition, a ryegrass/white clover pasture was sampled using a vortex suction machine in early November and 5 mL of the litter collected from this sampling was scattered around the base of each plant not treated with insecticide. The composition of the sample was not quantified but was comprised mainly of Collembola and aphid species, but also included mites, spiders, Diptera and coleopteran adults and larvae. Plants were also inoculated with root aphid.

Invertebrates associated with the root outgrowth were extracted from the sand medium surrounding the small planter bag on four occasions: March and September 2001, January and May 2002. At the completion of the trial, invertebrates were also sampled from the soil/sand medium in the small planter bag. In the flotation and wet sieving process used to extract the invertebrates, the growing medium and roots were washed and the resulting suspension decanted through three sieves (2.00 mm, 710 μm and 210 μm). Any macroarthropods that collected on the 710 μm sieve were noted, while all the material that collected on the 210 μm sieve was washed into a 70 mL container. Each sample was diluted if necessary before a single 10 mL subsample was taken in 5 x 2mL aliquots and the microarthropods counted in a marked petri dish under 16x magnification.

Nutrient Trial This trial compared invertebrate numbers among roots of perennial ryegrass plants, without endophyte or infected with Wild-type, AR1 or AR37, under a high nutrient regime (high nutrients) or one where no additional nutrients were supplied (low nutrients). As for the Plant Growth Trial cloned plants were used across treatments with insecticide also used to reduce herbivory in half the plants. Thus each plant/endophyte treatment was cloned four times so that the same plant genotype was represented in the high/low nutrients and +/– insecticide treatments. As for the Plant Growth Trial, plants were contained in a soil/sand medium in smaller planter bags which were then placed inside larger bags and surrounded with sand. A full description of the trial set-up and design is given in Chapter 3.

A 5 mL vacuum sample of litter was placed on the soil surface of the plants not treated with insecticide in early November 2000 and these plants were also inoculated with root aphid. Microarthropods were sampled separately from the root outgrowth in the sand medium and the main plant roots in a soil/sand mix in March 2001, 8 months after the trial commenced using the wet sieving method described above.

5.2.2 Mycorrhiza and Root Fungi

Root samples were taken from five replicates of the Plant Growth Trial (Reps 2, 5, 6, 11 and 19) in May 2002 and all replicates of the Nutrient Trial in March 2001 for examining for the presence of mycorrhiza. A third trial, a Root Biomass Trial, was also sampled (details given in Chapter 3). In this trial, cloned pairs of perennial ryegrass cv. Samson plants with Nil AR1, AR37 and Wild-type endophyte treatments were grown in two different sized containers to determine the effect of this on populations of the root aphid, *Aponeura lentisci*. There were 15 replicates of each container size for each endophyte treatment and five of those replicates were destructively harvested on three occasions. Samples for assessment of mycorrhiza were taken from each plant at the first two harvests in September 2002 and January 2003.

A sample from each plant consisted of at least four segments, 10 – 25 mm long, of seminal roots cut at random from the main root system. In the Plant Growth and Nutrient Trials samples were taken from the main roots in the sand/soil growing medium and not from the root outgrowth. After sampling, root segments were washed and then placed together in 5 mL vials and stored at 4°C until staining.

Prior to staining, roots were immersed in 10% KOH and heated in a waterbath at 60°C for 4 h. Roots were rinsed in tap water and then immersed in 0.05% solution of hydrogen peroxide for 20 minutes before rinsing again in tap water. Initially roots were stained in Acid Fuchsin and aniline blue and the results compared. Both stains were prepared by dissolving 0.1 g stain in 875 mL of lactic acid, 63 mL of glycerol and 63 mL of tap water. Since there appeared to be no difference between the stains in the amount of mycorrhiza visible, aniline blue

was used in order to ascertain if hyphae of *Neotyphodium*, which also readily take up this stain, were present in roots. Following this, roots were stained in aniline blue in a waterbath at 60°C for 2 h and then preserved in lactoglycerol (875 mL lactic acid, 63 mL glycerol, 63 mL tap water).

Four root segments from each plant were measured and then placed in lactoglycerol on a glass slide. These were examined under a microscope at 200x magnification. In the Plant Growth and Nutrient Trial samples, only the presence or absence of mycorrhiza was noted. In the Plant Biomass Trial a visual estimate of the amount of infection in each root segment was made and given a score on a scale of 0 – 5 where 0 = no mychorriza and 5 = mycorrhizal infection in 80 – 100% of the length of the root segment.

A second batch of root segment subsamples was taken at the same time and from the same replicates of the Plant Growth Trial as were sampled for mycorrhiza to determine root colonisation by saprophytic and other fungi. From each sub-sample taken, 40 root pieces were harvested, surface sterilised in 10% Janola® for 3 minutes, rinsed four times in sterile distilled water and plated onto potato dextrose agar and water agar with antibiotics added to inhibit bacterial growth. Five root pieces were placed in each plate. Plates were incubated at 25°C for 3 days and fungal colonies identified and counted.

5.2.3 Statistical Analysis

Residual plots of count data for Collembola, nematodes and mites were examined for homogeneity and normality, before data were $\log_{10}$-transformed to normalise variances. Where the data included values of zero, the constant used in the log transformation was based on the minimum value possible which depended on the dilution of the count samples. A general analysis of variance was carried out in Genstat Release 6.1 to compare the main effects of different endophyte treatments and insecticide on populations of these invertebrates in the Plant Growth Trial and endophytes, insecticide and nutrients in the Nutrient Trial. Similar comparisons were made in ANOVA for untransformed data on colonisation by mycorrhiza. In the Plant Growth Trial, block strata in the analyses took account of the randomised block design for endophyte replicates, the split-plot for the

insecticide/no insecticide treatments and the cloned pairs of plants. In the Nutrient Trial the split-plot block strata was not used in the trial design and therefore omitted from the analyses. Simple linear regression analysis was carried out to determine if relationships existed between groups of invertebrates.

Means were separated by calculating Fisher's protected least significant difference test in Genstat Release 6.1. Only arithmetic means are presented where data were log transformed because back-transformations based on standard error of the difference, number of observations and the constants used in the log transformation did not always adequately reflect the original data. Hence standard errors are not included in presentation of these data. Log transformed data on which analyses were based are presented in Appendix 2.

5.3 RESULTS

5.3.1 Invertebrates

The three major taxonomic groups of invertebrates present in the Plant Growth and Nutrient Trials were species of Collembola, Nematoda and Acari. Enchytraeids were also common but their numbers were highly variable. Numbers of enchytraeids were highly variable and because of this could not be analysed alone. Their numbers were therefore combined with that of nematodes, with enchytraeids representing between 1 and 15% of the combined populations. The collembolan fauna was dominated by two species of Poduroidea while the Acari encountered were all oribatid mites. The 210 μm sieve size used to capture the invertebrates would have been too large to capture many of the major nematode species including those that are plant parasitic. A subsample of nematodes from several sieve samples taken at final sampling of the Plant Growth Trial was found to contain mainly genera belonging to the order Dorylaimida (Dr Nigel Bell pers. comm.)

The information on invertebrate populations in each trial presented below applies only to those plants not treated with insecticide.

Plant Growth Trial Populations of Collembola, the most abundant of the microarthropods in this trial, were significantly higher on AR1 than on the other endophyte treatments in the samples taken from the main plant roots in May 2002 (Fig. 5.1a; Appendix 2 – Table 1). There was no effect of endophyte treatment on collembola in the root outgrowth samples at any time. Collembola populations varied considerably over time, reaching a peak in the September 2001 root outgrowth sample.

There were more mites on AR1 root outgrowth samples than on Wild-type samples in March 2001 ($P<0.05$) but there was no other significant effect of endophyte treatment on mite populations in this trial (Fig. 5.1b; Appendix 2 – Table 2). Though differences were not significant, mite populations among the main plant roots in the May 2002 sample followed the same pattern as those of Collembola sampled at the same time (Sample 5, Fig 5.1b cf. Fig 5.1a). Mite numbers were stable throughout this trial.

Fewer nematodes were found on AR37 than on Wild-type treatments ($P<0.05$) in the September 2001 root outgrowth sample but there were no other significant effects of endophyte on nematode populations (Fig. 5.1c; Appendix 2 – Table 3). Nematode numbers among the main roots in the May 2002 sample showed a similar pattern to those of Collembola and mites for each endophyte treatment sampled at the same time although differences were very small (Sample 5 Fig. 5.1c cf. Figs 5.1a & b). Nematode populations fluctuated over time and were highest in the main plant root sample.

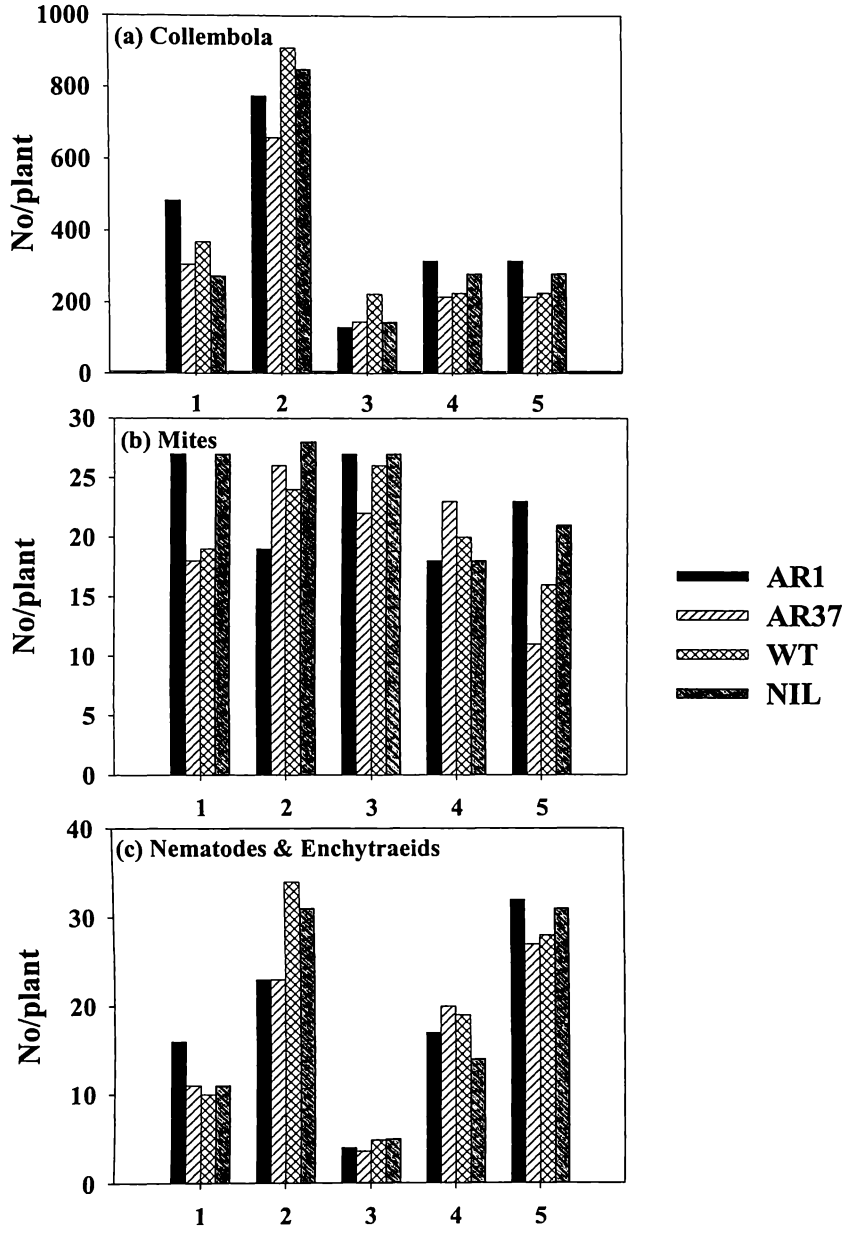

Fig. 5.1 Mean numbers/plant of (a) Collembola (b) mites and (c) nematodes and enchytraeids from root outgrowth samples taken on four occasions (1-4) and from main root samples taken in May 02 (5) in the Plant Growth Trial.

Nutrient Trial. There were no effects of endophyte status on populations of Collembola, mites or nematodes on either the root outgrowth or main root samples taken in this trial (Fig. 5.2a & b; Appendix 2 – Table 4). Total populations on plants (combined data for root outgrowth and main roots) also showed no differences between endophyte treatment.

Low nutrient plants had fewer Collembola and mites among the root outgrowth and the main plant roots in comparison with high nutrient plants ($P<0.001$) (Fig. 5.2a & b; Appendix 2 – Tables 4 & 5). Similar numbers of nematodes occurred among the root outgrowth of the high and low nutrient plants but, in contrast to this, nematode numbers in the main root samples were considerably higher under low nutrient plants than under high nutrient plants ($P<0.001$) (Fig. 5.2c; Appendix 2 – Table 6). There were no significant interactions between nutrient status and endophyte treatment for any of the invertebrate groups.

Collembola were relatively less abundant and mites more abundant in the Nutrient Trial compared with the Plant Growth Trial. Nematode populations were generally similar except for the high numbers in the low nutrient main root samples.

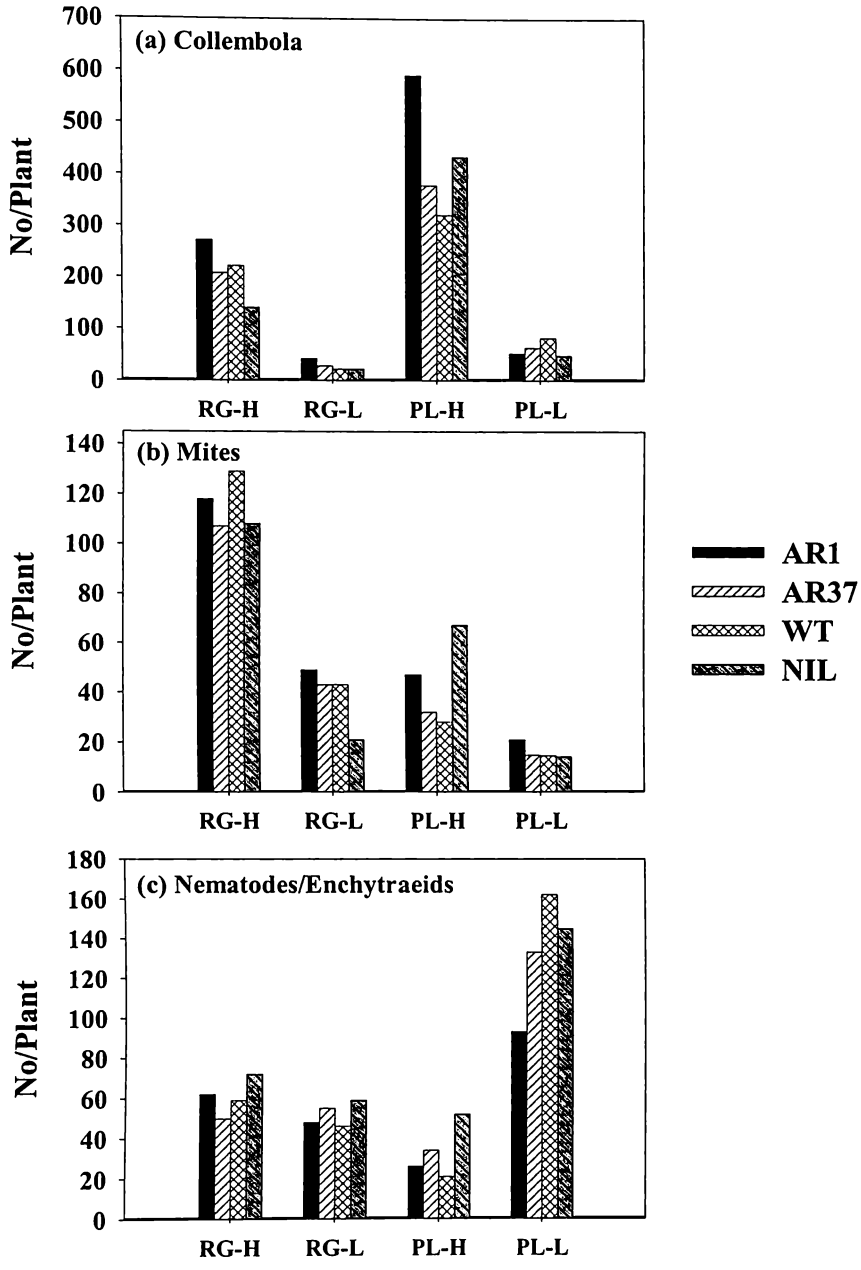

Fig. 5.2 Mean number/plant of (a) Collembola (b) mites and (c) nematodes and enchytraeids on root outgrowth (RG) and main root (PL) samples form high (H) and low (L) nutrient plants.

Effect of Insecticide. Insecticide severely reduced numbers of Collembola in both the Plant Growth and Nutrient Trials (Table 5.1). Mites were similarly affected except at the final sampling of root outgrowth in the Plant Growth Trial when mite numbers were the same for insecticide-treated and untreated plants.

In the Plant Growth Trial, fewer mites were recorded in the pooled root outgrowth samples from the insecticide-treated Nil plants than in the equivalent endophyte-infected treatments although the difference was significant only between Nil and AR37 ($P<0.05$) (Table 5.1). The pooled result reflects differences between samples from Nil and endophyte-infected plants taken in March 2001 and January 2002 from root outgrowth. Differences were significant only between Nil and AR1 in March but were significant between Nil and all endophyte-infected treatments in January 2002. This result may reflect species differences between treatments.

Nematode numbers associated with root outgrowth in the Plant Growth Trial tended to be higher under insecticide-treated than untreated. This was significant for all endophyte treatments in the September 2001 sampling ($P<0.05$) (data not presented) and when numbers for all root outgrowth samplings were pooled ($P<0.05$) (Table 5.1). In contrast to this, nematode numbers in the main root systems tended to be lower in insecticide-treated than untreated plants although this was not significant ($P=0.074$). The insecticide was applied to the surface of the soil/sand medium of the inner planter bag and not to the sand where root outgrowth occurred. These differences in nematode response to insecticide between root outgrowth and main roots may reflect more direct contact with insecticide for the latter. In the Nutrient Trial, however, insecticide had no effect on nematode numbers in either the root outgrowth or in the main root system ($P>0.05$).

Table 5.1 Mean and standard error of the number of Collembola, mites and nematodes per insecticide-treated (TR) and untreated (UN) plant in the Plant Growth and Nutrient Trials. Data for root outgrowth samples in the Plant Growth Trial are the total from samples taken on four occasions.

Trial	Endophyte	Collembola		Mites		Nematodes	
		UN	TR	UN	TR	UN	TR
Plant Growth Trial							
Root growth	AR1	1641	161	90	53	62	75
	AR37	1315	126	90	58	54	74
	Wild-type	1804	132	88	53	70	84
	Nil	1611	99	101	41	67	88
	SEM	207.2	16.6	9.05	4.92	7.48	4.92
Main roots	AR1	787	17	25	12	35	31
	AR37	341	9	14	8	46	20
	Wild-type	492	11	19	7	39	21
	Nil	652	8	24	8	34	31
	SEM	129.2	2.56	4.24	1.83	11.64	7.74
Nutrient Trial							
Root growth	AR1	112	12	80	39	51	51
	AR37	95	8	78	34	40	42
	Wild-type	79	10	76	34	46	37
	Nil	59	5	50	44	71	79
	SEM	18.9	1.99	13.7	7.2	11.35	11.39
Main roots	AR1	180	12	26	12	70	43
	AR37	196	11	23	9	81	50
	Wild-type	165	8	16	16	68	88
	Nil	172	7	33	9	109	63
	SEM	44.99	2.35	4.80	2.25	19.49	14.52

5.3.2 Mycorrhiza and Root Fungi

Colonisation of roots by mycorrhiza was significantly affected by endophyte treatment, insecticide, harvest time and soil volume. AR1 had fewer roots colonised by mycorrhiza than other endophyte treatments in the Plant Growth

Trial samples taken both from insecticide-treated and untreated plants ($P<0.05$) (Table 5.2). Similarly, roots sampled from AR1 plants grown in small containers in the Root Biomass Trial had a lower infection score than AR37 ($P<0.05$). Conversely, AR37 and Wild-type had lower colonisation rates than Nil and AR1 in the September harvest of the latter trial. Mean colonisation rate over all treatments increased from 69% to 91% between September and January while the mean infection score increased from 1.14 to 3.12.

Insecticide-treated plants had a lower proportion of roots infected with mycorrhiza in both the Plant Growth and Nutrient Trials ($P<0.05$) (Table 5.2). Roots in the smaller soil volumes had a lower infection score (mean = 1.91) than roots from larger volumes (mean = 2.36) over all endophyte-treatments ($P<0.05$) but infection rates were similar (76% ands 84% for small and large volumes respectively).

Table 5.2 Proportion of roots colonised by mycorrhiza and mycorrhizal infection score in three trials as affected by endophyte (AR1, AR37, Wild-type, Nil), insecticide (UN = untreated and TR = treated), nutrient supplements (High) or no nutrients (Low), harvest time (September 02, January 03) and soil volume (Large and Small) on

Trial	Treatment	AR1	AR37	Wild-type	Nil	Mean
Plant Growth	UN	0.50 b[1]	0.90 a	1.00 a	0.85 a	0.81 x[2]
	TR	0.45 b	0.70 a	0.90 a	0.65 a	0.68 y
Nutrient Trial	High UN	0.40 a	0.35 a	0.23 a	0.48 a	0.36 x
	High TR	0.24 a	0.35 a	0.35 a	0.26 a	0.30 x
	Low UN	0.45 a	0.44 a	0.48 a	0.55 a	0.48 x
	Low TR	0.23 a	0.38 a	0.33 a	0.45 a	0.34 y
Root Biomass	Sept -Propn	0.82 a	0.58 b	0.53 b	0.84 a	0.69 y
	Jan -Propn	0.85 a	1.00 a	0.95 a	0.85 a	0.91 x
	Sept - Score	1.37 a	1.07 a	0.80 a	1.32 a	1.14 y
	Jan - Score	2.82 b	4.02 a	3.20 ab	2.45 b	3.12 x
	Large - Propn	0.92 a	0.83 a	0.80 a	0.82 a	0.84 x
	Small - Propn	0.75 a	0.75 a	0.68 a	0.88 a	0.76 x
	Large - Score	2.85 a	2.53 ab	2.23 ab	1.84 b	2.36 x
	Small – Score	1.35 b	2.58 a	1.78 ab	1.93 ab	1.14 y

[1] Numbers in rows without the letters *a* and *b* in common are significantly different ($P<0.05$) for comparisons between different endophyte treatments

[2] Means without the letters *x* and *y* in common are significantly different between each pair of treatments in the column.

There were no significant differences in the number of colonies of any of the main fungal species cultured from roots of the different endophyte treatments

or in the total numbers of all species (Table 5.3). There was a slight but significant ($P<0.05$) increase in the total number of fungal colonies from all species cultured from roots from insecticide-treated plants (Mean number of colonies: 17.5 and 18.8 for no insecticide treatment and insecticide treatment respectively).

Table 5.3. Mean number of colonies of the main fungal taxa cultured from surface sterilised roots of perennial ryegrass without endophyte (Nil) or infected with the endophytes AR1, AR37 and Wild-type.

	AR1	AR37	Wild-type	Nil
Acremonium	1.0	0.2	0.8	1.0
Codinea	2.2	0.6	2.2	0.8
Fusarium oxysporum	1.2	2.0	0.8	1.2
All *Fusarium* spp.	1.8	3.4	1.4	2.2
Penicillium	0.0	1.4	0.8	0.8
Periconia	0.2	2.0	0.2	0.2
Trichoderma	1.2	0.6	0.6	0.6
Sterile	7.6	3.6	7.6	8.2
Total All[1]	18.0	18.2	17.6	16.2

[1] Includes other fungal species not listed in the table.

5.3.3 Interactions between Invertebrates

There were significant positive linear relationships between the number of Collembola and numbers of root aphids. In the Plant Growth Trial the relationship between root aphid and Collembola was significant for the total numbers/plant from all outgrowth samples ($y = 1257 + 0.686x$, $R^2 = 0.24$, $P<0.001$) (Fig. 5.3a) and for the sample from the main roots ($y = 359 + 0.653x$, $R^2 = 0.15$, $P<0.01$) (Fig. 5.3b). Similar significant linear regressions occurred between root aphid and Collembola for root outgrowth from the high nutrient plants in the Nutrient Trial ($y = 179 + 0.535x$, $R^2 = 0.23$, $P<0.001$) and for main plant roots ($y = 313 + 0.267x$, $R^2 = 0.19$, $P<0.01$) but not for low nutrient plant samples.

Nematodes were negatively correlated with the number of root aphids for the main root sample taken in the Plant Growth Trial ($y = 1.60 - 0.250x$, $R^2 =$

0.13, P<0.01) and in the Nutrient Trial ($y = 1.98 - 0.241x$, $R^2 = 0.17$, P<0.001). In the latter trial the relationships between nematodes and root aphid were significant for both the high and low nutrient plants.

There were no significant relationships between populations of the different invertebrate taxa.

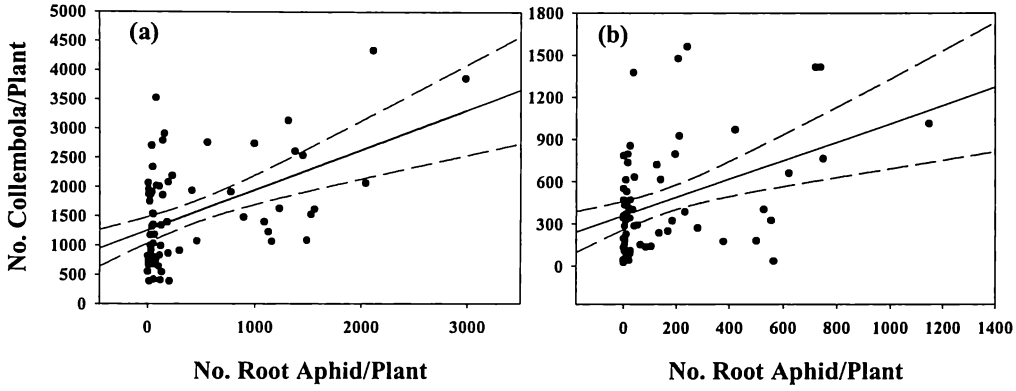

Fig. 5.3 Relationships between the occurrence of Collembola and root aphid for (a) the total numbers sampled from four root outgrowth samples and (b) the numbers from the main root samples in the Plant Growth Trial. Dashed line indicates 95% confidence limits.

5.4 DISCUSSION

These trials have not shown there to be any direct deleterious effect of *Neotyphodium* infection of ryegrass on members of the microarthropod community in soil, but indicate that changes to this community may occur as a result of multitrophic interactions stemming from endophyte effects on herbivory. Thus increased susceptibility to root aphid is associated with increases in abundance of Collembola and a decrease in the abundance of dorylaimid nematodes. In addition there have been significant time-related changes in levels of root colonisation by AM fungi which can be both directly and indirectly attributed to presence of *Neotyphodium* infection.

Below-ground food webs are thought to be dominated by fauna that are polyphagous and/or omnivorous (Setälä 2002) and Collembola are no exception.

They are considered to be mainly fungivorous but have also been observed feeding on detritus, carrion, directly on roots, root hairs and root tips (Petersen 2002) and also predating nematodes (Gilmore & Potter 1993). In laboratory choice trials Collembola have exhibited feeding preferences for certain types of fungi whereas analysis of gut contents suggest they are not specialised feeders (Peterson 2002). The increase in Collembola numbers associated with root aphid here is most likely a response to increases in food resources as microbial populations respond to the substrates provided by the honeydew excretions of the aphid and/or root exudation and senescence. It is also conceivable that Collembola feed directly on honeydew which is rich in sugars.

Dorylaimid nematodes are thought to be mainly omnivorous, consuming fungal hyphae and bacteria and predating other organisms (Yeates et al. 1993) but relative to other nematode taxa little is known of their feeding habits. Here their numbers were negatively correlated with root aphid for reasons that are not clear. Competitive displacement seems likely since both mites and Collembola have suppressed nematode populations in microcosm studies (eg. Huhta et al. 1998). Populations of these omnivorous nematodes, however, have been positively correlated with those of bacterial-feeding nematodes (Neher et al. 1999) and with numbers of aerobic bacteria (Yeates 1973) suggesting, perhaps, that in the absence of high root aphid populations there is a shift in microbial populations towards a greater dominance of bacteria. Further to this, root mortality which is likely to increase as a result of herbivory, has been suggested as the reason for increases in the abundance of fungivorous nematodes associated with increasing intensity of defoliation found in a mixed grassland community (Mikola et al. 2001). The nematodes sampled here responded most favourably to conditions in the soil/sand medium under the low nutrient plants. Fiscus & Neher (2002) found that nutrient/chemical enrichment reduced populations of the dorylaim *Eudorylaimus*, although this species may not be representative of the species sampled in the trials reported here.

The differences in extent of mycorrhizal colonisation between endophytes and between different treatments are an intriguing aspect of this study but, without further research, are also difficult to interpret. In particular the low colonisation

rates of AR1-infected plants relative to other endophyte treatments in the 2-year-old plants in the Plant Growth Trial cannot easily be explained. Gange et al. (2002) have found that mycorrhizal infection rates are lower in plants affected by herbivory and has attributed this to insufficient carbon resources in the plant to support infection. This is unlikely to be the case for AR1-infected plants here since, in the absence of herbivory (ie. in insecticide-treated plants), these plants also had lower infection levels than other endophyte treatments. Furthermore, a similar effect did not occur in endophyte-free plants which were also severely affected by insects. Plant genotype influences mycorrhizal colonisation but this is unlikely here given the high initial rates of colonisation in the Root Biomass Trial. Because of the apparent decline in colonisation, it would seem that there may be time-related factors peculiar to the physiology of the plant/AR1 endophyte interaction that culminates in it becoming less hospitable to AM fungi. Death of the root cortex which is associated with low levels of mycorrhizal infection in mature plants that have previously supported high levels of AM fungi (Smith et al. 1993) is one possible explanation.

Where the development of mycorrhizal infections of roots is reliant on fungi indigenous to the soil as in these experiments, colonisation takes time and is dependent on the natural infectivity of the soil (Smith et al. 1992), largely defined by the availability of propagules to roots. Thus the low colonisation rate of plants infected with AR37 and Wild-type in September, compared with that in AR1 and Nil at the same time, may be directly attributable to a limited supply of spores in relation to the higher root biomass of these two treatments (Chapter 6). The low colonisation rates of roots in small soil volumes where there was a high root density compared to that in larger volumes tends to support this idea. Spore dissemination to AR37 and Wild-type plants may also be indirectly reduced compared to AR1 and Nil by less microarthropod activity resulting from fewer root aphid and may also be the reason for lower rates of mycorrhizal infection in plants treated with insecticide. One study, however, has suggested that AM spores are too large to be dispersed by microathropods (Warner et al. 1987). The possibility of a direct effect of the insecticide on AM fungi cannot be excluded, but seems unlikely given that populations of other culturable fungi in roots were not affected. Gange et al. (2002) found insecticide increased AM colonisation of

roots, when they used an insecticide which targeted herbivores but had little activity against non-target organisms.

If spore concentrations in soil were a factor initially limiting mycorrhizal colonisation then this should be rectified over time as was the case here, when there were substantial increases in AM colonisation of AR37 and Wild-type plant roots between September 2002 and January 2003. It therefore seems unlikely that *Neotyphodium* provides a physical or chemical barrier to AM fungal colonisation as hypothesised by Müller (2003). This author found less mycorrhiza in ryegrass plants infected with *N. lolii* or *E. typhina* than in endophyte-free plants after inoculating soil with mycorrhiza. It is, however, possible that a chemical response that is antagonistic to mycorrhizal infection is elicited in endophyte-infected plants when they are challenged by large amounts of fungi. Another alternative explanation is that mycorrhizal colonisation is being regulated by P demands of the plant (eg. Schubert & Hayman 1986; Muller & Hofner 1991) which may differ according to endophyte status.

Concomitant with the large increase in colonisation of AR37 and Wild-type plants by AM fungi between September and January, there was a much smaller increase in colonisation in AR1 and Nil plants. Several studies have demonstrated that, at low densities, Collembola enhance mycorrhizal colonisation of plants with an associated benefit to plant growth whereas high densities have an adverse effect (Warnock et al. 1982; Finlay 1985; Lussenhop 1992; Bakonyi et al. 2002). Regrettably numbers of Collembola were not recorded in this particular experiment but there were high numbers of root aphid present on AR1 and Nil plants (Chapter 4). Given the positive relationship between root aphid and Collembola, populations of the latter are likely to have been higher in the Nil and AR1 treatments and, hence, may account for the reduced rate of mycorrhizal colonisation in these treatments during this period. Equally however, the carbon cost to the plant as a result of root aphid herbivory, which was reflected in low root growth at this time (Chapter 6), may be the reason for the lower rate of mycorrhizal colonisation (Gange et al. 2002).

Studies of subterranean environments are constrained by their very nature and interpretation is often limited by the organisms that are not measured. The size range of invertebrates sampled was limited to those larger that 210 μm and this has ignored a very large part of the nematode community. Fungivorous and bacterivorous nematodes interacting with other soil organisms are major components of food-webs (Moore et al. 1988) and are sensitive to environmental factors in a way that makes them useful indicator species of soil quality (Fiscus & Neher 2002). The effects of different endophyte strains on microbivorous nematodes and their interaction with other soil biota in ryegrass are unknown. Moreover, root herbivory by plant parasitic nematodes also impacts on soil microbial dynamics. This study is also limited by the lack of measurements of microbial biomass which provide the foundations on which decomposer networks in soil are based (Wardle et al. 1999a). The diversity of fungi cultured from the roots will in part reflect the composition of fungi in the soil but this too ignores many species that are not readily cultured. There were slight but not significant differences between treatments in abundance of some fungal species (eg. *Acremonium*, *Codinea* and *Penicillium*) that may be due to interactions between root aphid and Collembola but a further investigation would be needed to confirm this. Similarly there may also have been differences in species composition among the major microarthropod taxa. The Collembola fauna was dominated by two species but there were several species of mites and the considerable size variation among the nematodes suggested also that several species of these were present. Differences in species composition may alter the process of litter decomposition and nutrient release as Cragg and Bardgett (2001) demonstrated for individual species of fungal-feeding Collembola.

Neotyphodium infection of grasses provides an excellent model system on which to base food web studies because they modify both root and foliar herbivory without the non-target effects of insecticide treatment. The positive response of Collembola populations to root aphid and the converse response in the particular nematode fauna sampled warrants further investigation, as do the changes in AM fungal colonisation of the different treatments.

CHAPTER SIX

THE EFFECT OF *NEOTYPHODIUM* ENDOPHYTES ON ROOT GROWTH AND MORPHOLOGY IN *LOLIUM PERENNE* AND INTERACTIONS WITH HERBIVORES

6.1 INTRODUCTION

In agriculture the advantage of using grasses infected with *Neotyphodium* endophytes lies in the ability of the fungus to enhance plant vegetative growth and persistence. These improvements in growth in part result from the production of secondary metabolites by the fungus which leads to a reduction in insect herbivory. In addition, however, endophyte infection in tall fescue may mitigate against various abiotic stresses including drought (West 1994) and mineral deficiency (Malinowski and Belesky 1999).

In New Zealand improved growth of perennial ryegrass infected with *Neotyphodium* is attributed mainly to fungal-mediated reductions in insect herbivory, mainly by Argentine stem weevil (*Listronotus bonariensis*) and black beetle (*Heteronychus arator*) (Popay et al. 1999). Several studies have failed to show consistent increases in growth of endophyte-infected ryegrass or any degree of abiotic stress tolerance in the apparent absence of insect pests (Hume 1993; Hume et al. 1993; Barker et al. 1997; Eerens et al. 1998b). Notwithstanding these results, Latch et al. (1985) recorded a 38% increase in dry matter yield of ryegrass infected with Wild-type endophyte compared with endophyte-free clones under ideal growing conditions in a controlled environment room with no insects present.

The effect that endophyte infection may have on root growth that is independent of effects on shoot growth has been given little attention in the literature. Yet the ability of the plant to sustain high vegetative growth relies on the efficiency of the root system in accessing and distributing essential water and nutrients. Recent studies have shown that uptake of phosphorus, root growth and

root morphology are altered by *N. coenophialum* infection of tall fescue (Malinowski & Belesky 1999; Malinowski et al. 2000). The questions addressed in this chapter are whether or not similar endophyte-induced changes in root growth and morphology also occur in perennial ryegrass infected with different endophyte strains, and if they do, to what extent those changes are related to differences in insect herbivory, in particular by the root aphid *Aploneura lentisci*.

There is close coordination of root and shoot growth in plants, in part resulting from source/sink relationships which determine growth allocation processes. Plant investment in root growth is governed primarily by light and nutrients but is also affected by edaphic factors such as temperature, moisture, seasonality and soil structure, as well by herbivory. Response to insect herbivory depends on the type (eg. sucking vs chewing) and extent of damage, and the plant part on which the insect feeds. Damage to roots, in general, results in allocation of resources to root growth at the expense of vegetative growth as the plant attempts to compensate for the damage which the insect inflicts (Anderson 1987; Brown & Gange 1990; Murray et al. 2002; Bardgett et al. 1999). The converse happens if the damage is to above-ground parts of the plant. In grasses, increased plant allocation to shoot growth also occurs after the plant is defoliated by grazing mammals (Polley & Detling 1989; Holland & Detling 1990). There are, however, cases where allocations in response to herbivory are the opposite of that predicted in, for instance, plants that are not tolerant of grazing (Jaramillo & Detling 1988; Holland & Detling 1992).

Although partitioning of growth to roots and shoots is partly dependent on plant genotype, in a highly heterogeneous environment like soil roots also display a certain amount of phenotypic plasticity (Robinson 1994). In addition, investment in roots may not be measurable simply in terms of root biomass but may require measurements of root morphology such as total length of the root system, root diameter, branching and surface area (Box 1996). Changes in root morphology as a consequence of herbivory will alter the ability of roots to access water and nutrients and to penetrate soil, which in turn will also have a bearing on foliar growth. Thinner roots are likely to be more vulnerable to invertebrate damage and are more short-lived than larger roots (Eissenstat & Yanai 1997)

Biomass allocation to foliar and root growth of endophyte-free perennial ryegrass or ryegrass infected with one of three different strains of *Neotyphodium* was measured here in three trials, and in two of those trials aspects of root morphology were also investigated. Insecticide was used in two of the trials to manipulate herbivory so that the impact of insects on foliar and root growth could be measured in the different plant-endophyte associations. This also allowed any direct effects of endophyte status on plant growth to be determined.

6.2 METHODS

6.2.1 Plant Growth Trial

This trial was designed to compare root growth of perennial ryegrass infected with Wild-type, AR1 and AR37 endophytes with that of an endophyte-free control, and with and without herbivory, over a period of 2 years. At the completion of the trial total root mass was determined and samples were taken for investigating root morphology.

In each of the 20 replicates there were two cloned plants for each endophyte treatment (= 8 plants in total), one of which was regularly treated with insecticide to reduce herbivory. Plants were arranged in tubs in a split-plot design with insecticide-treated plants (TR) in one half and untreated plants (UN) in the other. The method used to determine root growth was a modification of that described by Lund et al. (1970) as the "implanted soil mass technique" or more simply as the ingrowth technique (Rosario et al. 2000). Ryegrass plants were individually planted into a soil/sand medium in small planter bags with holes in the sides and were surrounded by sand. Roots growing from the small planter bag into the sand were removed on each sampling occasion to obtain the dry weight of root growth (termed outgrowth here). The trial was set up in April 2000 and a full description of the methodology is given in Chapter 3.

Growth measurements were first taken from this trial in August, 2000, four months after the trial was set up. Initially roots were allowed to grow freely into sand surrounding each small planter bag with no barrier between adjacent

plants in each split plot. A large amount of root growth resulted in considerable intermingling of roots of neighbouring plants. Each planter bag was levered from the sand with as much of the roots as possible intact. Some roots were broken in this process but were not recovered because of the difficulties in distinguishing root growth of one plant from that of its neighbour. This led inevitably to some inaccuracies in determining the amount of the root growth for this sampling. Subsequently the smaller planter bag was placed in a larger planter bag as described in Chapter 3. The root growth into the sand in the larger planter bag was then more easily and more accurately measured. Further sampling of this trial took place in December, 2000, March/April and September/October 2001, and in January and April/May 2002.

Root outgrowth was measured by severing roots from the plant where they exited the smaller planter bag and capturing them in a three-stage washing process that was also designed to remove invertebrates from the samples. The bulk of the roots were removed by hand when root material and sand were stirred in a bucket to release the invertebrates. The suspension containing the invertebrates was then decanted through sieves and the remaining sand washed through a net screen (later a wire sieve was used) with 2.5 mm^2 mesh size. Roots were retrieved from both the mesh screen and sieves.

Roots were later washed more thoroughly under running water over a sieve (mesh size 1 mm) to remove any further sand and debris. Roots destined to be analysed for alkaloids or nutrient content were immediately frozen and later freeze-dried before weighing while those not required for chemical analysis were oven-dried at 80°C.

At all samplings, live and dead tillers were counted and foliar growth above a height of 50 mm was harvested, oven-dried and weighed. Dead tillers were removed and discarded from the plants after counting and some of the dead outer sheath material which accumulated between samplings was also stripped away. In the summer samplings in 2001 and 2002, reproductive tillers were also counted. Dead and reproductive tillers were removed at the base on each occasion.

After counting reproductive tillers, some aftermath heading occurred in some plants and this was removed to maintain the plants in a vegetative condition.

At the final assessment during late April and early May 2002 the root and foliar growth of each plant was harvested as described above. In addition, herbage (mainly leaf sheath material), below the 50 mm cutting height, was severed from the base of the plant at ground level and kept separate from the foliar growth samples. Dead tillers were discarded as before and all live tillers were immediately frozen for later freeze drying and weighing. Main plant roots were washed in the same way as root outgrowth and all samples were frozen for later freeze drying. Prior to freezing sub-samples of roots were taken from 10 replicates for root morphology studies (see below).

6.2.2 Nutrient Trial

This trial investigated the response of ryegrass with and without endophyte to a high and a low nutrient supply in the presence or absence of herbivory using the same endophyte treatments as in the Plant Growth Trial; ie. Nil, Wild-type, AR1 and AR37 in perennial ryegrass. This gave 16 treatments in total with plants for each endophyte treatment cloned across high/low nutrients and +/- insecticide. High nutrient plants were regularly given nutrient supplements while low nutrient plants were given no additional nutrients after the trial was planted in July 2000. As for the Plant Growth Trial ryegrass was planted into a small planter bag which was then placed inside a larger planter bag to enable root growth to be measured. A full description of the trial design is given in Chapter 3.

Plant growth in the Nutrient Trial was sampled in the same way as that described above for the Plant Growth Trial. The number of live and dead tillers and samples of foliage and root growth were taken at the end of November 2000 and again at the completion of the trial in March 2001.

In addition to measuring regrowth at the final sampling in March, all tillers were cut from the base of the plant, live tillers were dried and weighed and the roots in the small planter bag were sampled in the same way as that described for

the Plant Growth Trial. These roots were frozen and later freeze dried before they too were weighed.

6.2.3 Root Biomass Trial

This trial was designed to further investigate the effect of endophyte status and different endophyte strains on root growth and root morphology by measuring these in cloned ryegrass plants grown in two soil volumes. Root outgrowth was not measured in this trial as it was in the Plant Growth and Nutrient Trials to allow root response to be investigated where new growth was not being removed. Instead five replicates from the trial were destructively harvested three times over a period of 9 months. Full details of the trial methods are given in Chapter 3.

Foliage samples above a height of 50 mm were harvested from each plant every 6 – 8 weeks, and oven-dried before weighing.

Five replicates of the trial were destructively harvested on three separate occasions (September 17 2002, January 8 2003 and June 30 2003) to obtain measurements of total root and vegetative biomass and number of live and dead tillers. After harvesting foliar growth and recording the number of tillers, plants were cut at the base, dead tillers were discarded and live tillers were frozen. Roots were washed as described above for the final sampling of the Plant Growth Trial and were also frozen. Later both tillers and roots were freeze dried and weighed. A sub-sample of roots was also taken for root morphology studies after which it was oven-dried, weighed and the amount added to the weight of the freeze-dried sample to give a total root biomass.

6.2.4 Root Morphology and Colour

Subsamples of roots were taken for root morphology studies from all plants in Reps 1, 2, 5, 6, 7, 11, 14, 16, 17 and 19 of the Plant Growth Trial and from all plants in the Root Biomass Trial. The sub-sample was taken by severing a group of roots from the crown of the plant and gently pulling these away from the remaining root system. Roots were rinsed carefully in water and then stored in sealed plastic bags at 4°C until their root morphology was investigated.

Roots from the Plant Growth Trial were stained before scanning by immersing each sub-sample for 30 – 60 seconds in methylene blue stain (125 mg methylene blue dissolved in 250 mL water) in a beaker. Roots from the Root Biomass Trial were not stained prior to scanning. Roots were rinsed with tap water and then spread out in a perspex tray containing approximately 5 mm of water. The tray was placed on a WinMac Rhizo Scanner (Std 1600+ Reagent Instrument) and scanned at 400 dpi. Images were stored on computer and later analysed using the WinRhizo programme in 10 x 0.25 mm diameter size classes. After scanning roots were retrieved and later oven-dried and weighed. The weight of each sub-sample was used to calculate the total length of roots of each plant.

Roots sampled from the Nutrient Trial in November 2000 and from the Plant Growth Trial in September 2001 varied considerably in colour and were scored on a scale of 1 -5, where a score of 1 meant most roots were very light in colour and 5 that all roots were dark brown.

6.2.5 Statistical Analysis

All data were examined for homogeneity and normality using residual plots. Data for the total length of roots of plants in the Plant Growth Trial, were $\log_{10}$-transformed to normalise variances prior to analysis. All other data were not transformed. A general analysis of variance was carried out in Genstat Release 6.1 on all data using main effects of endophyte and insecticide in the Plant Growth Trial, endophyte, nutrient level and insecticide in the Nutrient Trial and endophyte, soil volume and harvest date in the Root Biomass Trial. Each analysis of variance took account of trial structure as described in Chapter 4. Means were separated using Fishers protected least significant difference test in Genstat Release 6.1. Linear regression analyses were used to determine if differences in plant growth response could be predicted by populations of root aphid and the proportion of tillers infested with an unidentified mealybug.

6.3 RESULTS

In all three pot trials both foliar and root growth and root:shoot ratios of UN AR37 plants were higher than those of UN AR1 and Nil during periods of biotic

stress from herbivory and particularly where this coincided with abiotic stresses. Thus there were few significant growth differences in the Nutrient Trial which was well watered by comparison with the Plant Growth and Root Biomass Trials. In the Plant Growth Trial AR37–infected plants showed no growth responses to insecticide treatment whereas other TR endophyte treatments had increased root and foliar growth and root:shoot ratios. TR AR37 plants tended to have lower root growth than other TR endophyte treatments during summer, but maintained a high cumulative root biomass in UN plants because of the absence of any apparent herbivory by either the root aphid, *A. lentisci* or the pseudococcid mealybug. In the Root Biomass Trial there were differences in root:shoot ratios and root growth of AR37 between the September to January and January to June periods which contrasted with patterns of root growth in Wild-type. These differences in root growth suggest that plant phenology of AR37 differs from that of other endophyte treatments.

Foliar but not root growth of Wild-type infected plants in the Plant Growth Trial responded significantly to insecticide treatment which virtually eliminated the root aphid, *A. lentisci* from plants. Cumulative foliar growth in UN Wild-type plants was less than that of UN AR37 in this trial. In the Nutrient and Root Biomass Trials, however, growth of Wild-type matched and occasionally exceeded that of AR37. Root aphid loadings on Wild-type plants were lower in the latter two trials than they were in the Plant Growth Trial. Root:shoot ratios were stable for each harvest period in the Root Biomass Trial.

Herbivore pressure was highest on plants infected with AR1 and on endophyte-free plants. This was reflected in low root and foliar growth, low root:shoot ratios and high tiller and plant mortality of UN plants in the Plant Growth Trial. All growth parameters in AR1 and Nil were significantly improved by insecticide treatment. Similar growth reductions in these two treatments were recorded in the Root Biomass Trial but were less apparent in the Nutrient Trial.

6.3.1 Plant Growth Trial

Roots For plants not treated with insecticide (UN) AR37 had higher cumulative root outgrowth compared with other endophyte treatments in this trial (Fig 6.1a).

Differences between AR37, AR1 and Wild-type were not significant but root outgrowth on AR37 plants exceeded that on Nil ($P<0.05$) in September 2001 and January 2002. At individual sampling times (Fig. 6.2), Nil had higher root outgrowth than AR1 in April 2001, whereas AR37 and Wild-type had higher outgrowth than Nil in September 2001.

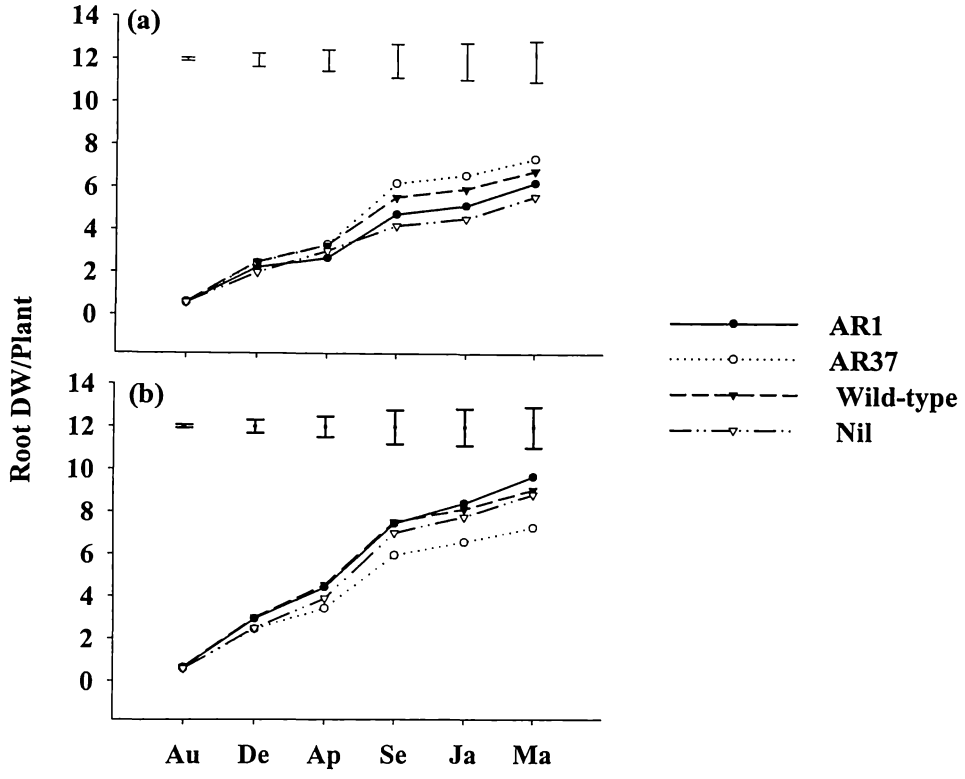

Fig. 6.1 Cumulative root outgrowth (g/plant) on (a) plants not treated with insecticide and (b) treated with insecticide for ryegrass without endophyte or infected with AR1, AR37 or Wild-type endophytes. Sampling times were: August and December 2000; April and September 2001; January and May 2002. Error bars = LSD (5%)

Insecticide substantially increased root outgrowth in all endophyte treatments except AR37. At no time did AR37 outgrowth on insecticide-treated (TR) plants exceed that on UN for either cumulative growth (Fig. 6.1b) or individual samplings (Fig 6.2b). In contrast to this, cumulative root outgrowth on AR1, Nil and Wild-type was higher on TR than UN plants by December 2000 and this pattern continued until the end of the trial. Generally differences between TR

and UN at individual sampling times were less for Wild-type-infected ryegrass than for Nil and AR1 (Fig. 6.2 & 6.3; Appendix 3 – Table 1).

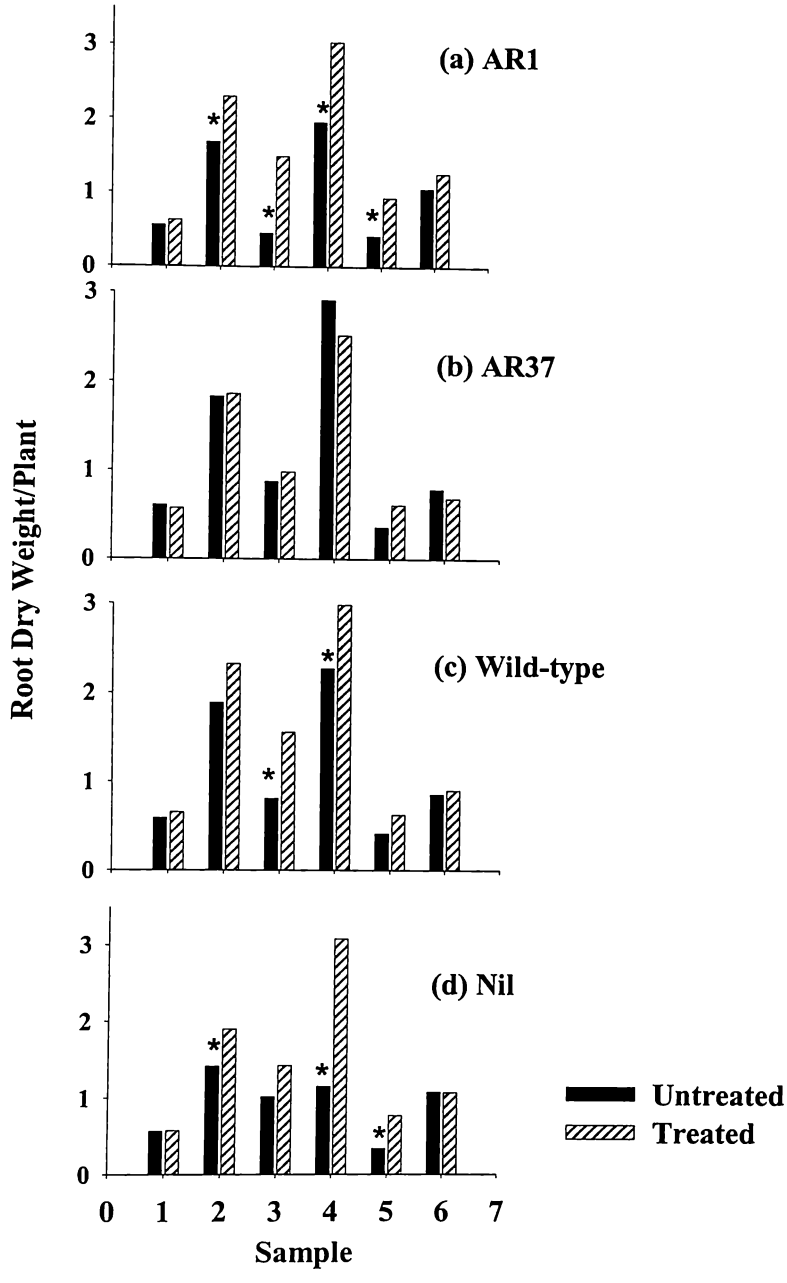

Fig. 6.2 Root outgrowth (g/plant) of ryegrass without endophyte (Nil) or infected with AR1, AR37 or Wild-type, and treated or not treated with insecticide, at six sampling times: 1- August 2000; 2 –December 2000; 3 – April 2001; 4 – September 2001; 5 – January 2002; 6 – May 2002.

*** = significant differences between treated and untreated plants (P<0.05).**

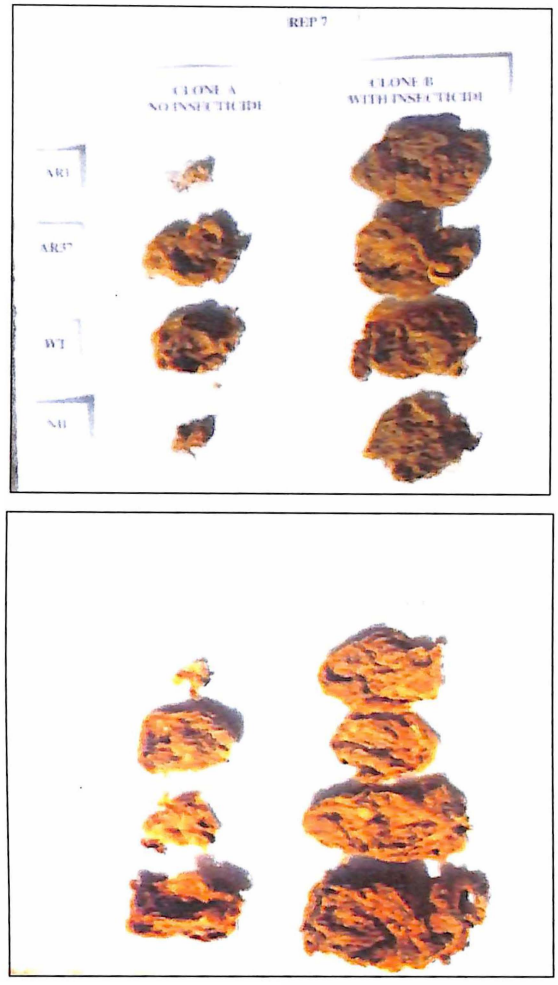

Fig. 6.3 Examples of differences in root growth between insecticide-treated (R) and untreated (L) plants in two replicates of the Plant Growth Trial in April 2001. Treatments from top to bottom are: AR1, AR37, Wild-type and Nil.

In TR plants AR37 accumulated less root outgrowth than other endophyte treatments (Fig. 6.1b). Differences between AR37 and Wild-type were significant ($P<0.05$) in April 2001 and between AR37 and AR1 in April 2001 and January and May 2002.

AR37 had the highest weight of main plant roots for UN plants but this did not differ significantly from other endophyte treatments (Table 6.1). Insecticide

significantly increased the weight of main roots of AR1, Wild-type and Nil but not of AR37. Among the TR plants AR1 had a higher root dry weight than both Wild-type and AR37 treatments ($P<0.05$).

Table 6.1 Dry weight of main plant roots and live leaf sheath material at the final sampling of ryegrass plants in the Plant Growth Trial.

Endophyte	Main Roots (g)			Leaf Sheath (g)		
	UN	TR		UN	TR	
AR1	6.94	13.37	LSD[1](5%)	2.35	3.08	LSD[1](5%)
AR37	8.78	9.26	= 2.43	2.59	2.54	= 0.46
Wild-type	7.52	10.14	SED	1.95	2.33	SED
Nil	6.69	10.54	= 1.24	2.16	2.80	= 0.314
SED	1.567			0.314		
LSD[2] (5%)	3.11			0.72		

[1]LSD for differences between treated and untreated plants

[2]LSD for differences between endophyte treatments within each insecticide stratum

Foliage Cumulative foliar growth on UN plants was significantly higher on AR37 than on all other endophyte treatments at all sampling dates except December 2000 (Fig. 6.4a). At individual sampling times (Fig. 6.5), growth of AR37 plants exceeded that of the other endophyte treatments only in April and September 2001 ($P<0.05$).

Insecticide treatment did not increase foliar growth of AR37 but did in other endophyte treatments. Differences in cumulative growth between TR and UN became significant in December 2000 for AR1, April 2001 for Nil and September 2001 for Wild-type and then persisted until the end of the trial (Fig. 6.4a & b; Appendix 3 – Table 2). On TR plants cumulative foliar growth did not differ significantly between endophyte treatments (Fig. 6.4b). Growth of AR1, Wild-type and Nil was improved by insecticide treatment at the April and

September 2001 and January 2002 samplings (Figs. 6.5, 6.6). Insecticide also increased the growth of AR1 in December 2000.

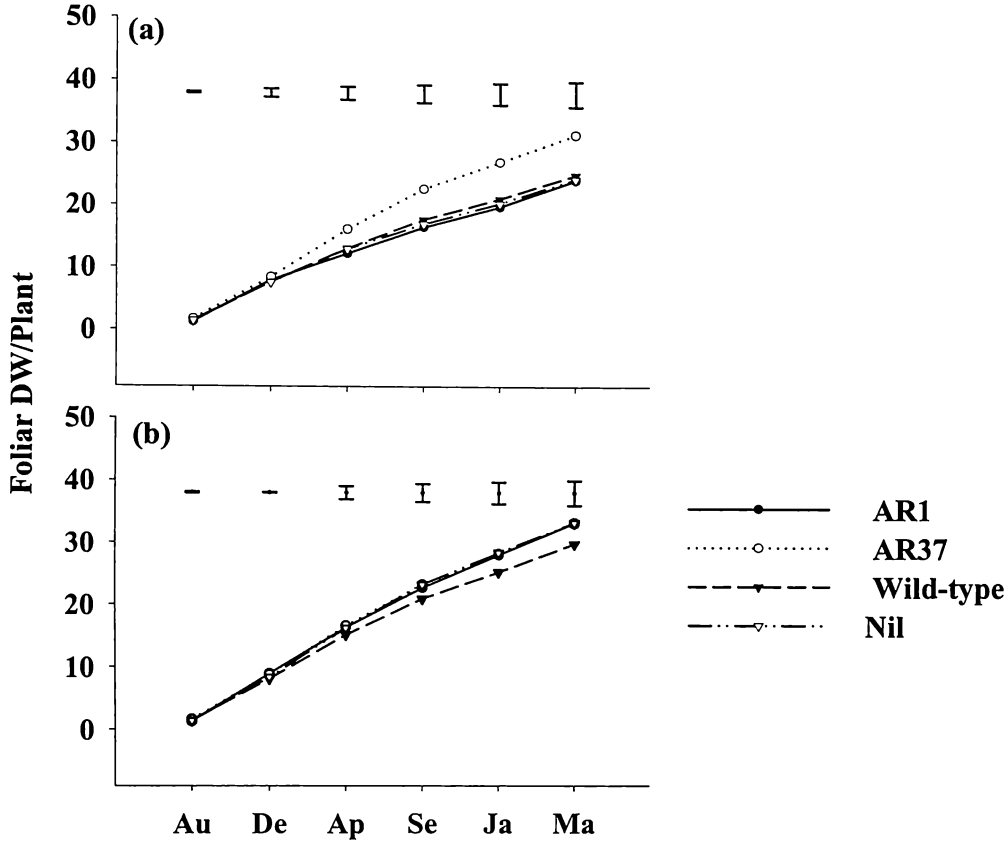

Fig. 6.4 Cumulative foliar growth (g/plant) on (a) plants not treated with insecticide and (b) treated with insecticide for ryegrass without endophyte or infected with AR1, AR37 or Wild-type endophytes. Sampling times were: August and December 2000; April and September 2001; January and May 2002. Error bars = LSD (5%)

The amount of leaf sheath material on plants did not differ significantly between endophyte treatments but increased significantly on AR1 and Nil plants as a result of insecticide treatment (Table 6.1).

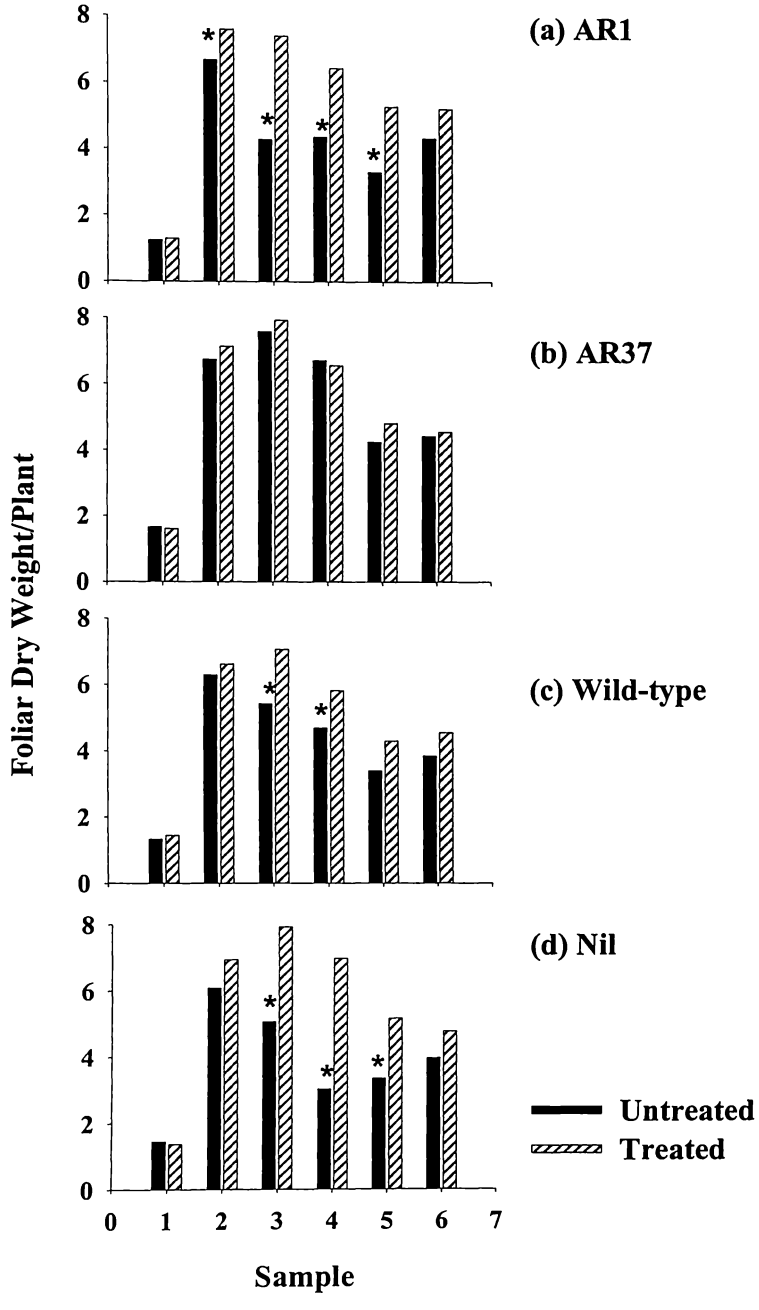

Fig. 6.5 Foliar growth (g/plant) of ryegrass without endophyte (Nil) or infected with AR1, AR37 or Wild-type, and treated or not treated with insecticide, at six sampling times. 1- August 2000; 2 –December 2000; 3 – April 2001; 4 – September 2001; 5 – January 2002; 6 – May 2002.

*** = significant differences between treated and untreated plants ($P<0.05$)**

Fig. 6.6 Difference in foliar growth between insecticide-treated (bottom) and untreated (top) in one replicate of the Plant Growth Trial. Endophyte treatments are clockwise starting from top left: Top - AR1, Wild-type, Nil and AR37; Bottom – Wild-type, AR37, AR1 and Nil.

Root:shoot In UN plants this ratio was significantly less for AR1 than for Nil in April 2001 but did not differ significantly between endophyte treatments at other sampling times or for an overall root:shoot ratio calculated for the combined totals of root outgrowth and foliar growth (Table 6.2).

Root:shoot ratios of AR1 were more responsive to insecticide treatment than other endophyte treatments. Insecticide increased root:shoot ratios of AR1–infected ryegrass in December 2000, April 2001 and January 2002 (Table 6.2). In contrast to this, differences between UN and TR plants were significant for Nil plants only in December 2000 and for Wild-type only in April 2001. At no time was root:shoot ratios of AR37 altered by insecticide treatment.

AR37 had the lowest root:shoot ratio among the endophyte treatments in TR plants. This was significantly less than the ratio for Wild-type and AR1 in April 2001 and also less than the ratio for these two treatments over the total growth (Table 6.2).

Table 6.2 Root:shoot ratios of ryegrass without endophyte or infected with AR1, AR37 or Wild-type endophytes for plants treated (TR) and untreated (UN) with insecticide. Ratios are calculated from root outgrowth and foliar growth at a 50 mm cutting height at different sampling times

Sample	AR1		AR37		Wild-type		Nil		LSD[1] (5%)
	UN	TR	UN	TR	UN	TR	UN	TR	
Aug 00	0.47	0.49	0.38	0.39	0.47	0.46	0.38	0.42	0.104
Dec 00	0.25	0.31*	0.30	0.27	0.30	0.36	0.23	0.30*	0.088
Apr 01	0.08	0.20*	0.12	0.12	0.13	0.21*	0.15	0.17	0.055
Sep 01	0.46	0.48	0.44	0.39	0.50	0.52	0.35	0.45	0.135
Jan 02	0.12	0.19*	0.09	0.12	0.11	0.15	0.10	0.14	0.051
May 02	0.24	0.24	0.18	0.16	0.22	0.22	0.24	0.20	0.087
Mean	0.26	0.30*	0.24	0.22	0.22	0.26*	0.27	0.31*	0.06

[1]LSD is for comparison between endophyte treatments within each insecticide stratum and not for comparisons between UN and TR plants within each endophyte treatment.

* Indicates significant difference between treated and untreated plants within each endophyte treatment

Tiller and Plant Mortality Tiller death in UN AR37 and Wild-type plants was very low compared with that on AR1 and Nil plants in April 2001 and September 2001 (Table 6.3). On TR plants tiller mortality remained low and did not differ significantly between endophyte treatments. In January and May 2002 tiller mortality increased on all plants and there was no difference between endophyte treatments or between UN and TR plants (data not presented). Several UN AR1 and Nil plants died (Table 6.3) after the April 2001 sampling. Given the

differences between TR and UN plants, both tiller and plant mortality are likely to represent losses to herbivory.

Table 6.3 Tiller and plant mortality on perennial ryegrass plants without endophyte (Nil) or infected with AR1, AR37 or Wild-type endophytes, and treated with insecticide (TR) or not treated (UN)

	Tiller Mortality (%)						Plant Mortality	
	Dec 2000		April 2000		Sept 2001		(%)	
	UN	TR	UN	TR	UN	TR	UN	TR
AR1	5.0	1.2	14.8	2.9	27.0	7.8	20	0
AR37	1.5	0.2	4.1	1.8	3.3	6.8	0	0
Wild	1.6	0.5	5.0	1.9	7.9	8.2	0	0
Nil	2.7	0.3	24.9	1.8	45.7	7.9	30	0
SED	1.01		4.11		6.75			

Insects The root aphid, *A. lentisci* was the major insect pest present on plants throughout the trial. This aphid was almost completely absent on UN AR37 plants while populations were highest on AR1 (Chapter 4). Insecticide reduced root aphid numbers/g of root at each individual sampling occasion on all endophyte-treatments ($P<0.001$) with the exception of AR37 (Table 6.4).

Table 6.4 Mean number of root aphid/g of root on ryegrass infected with different endophytes and treated (TR) or untreated (UN) with insecticide at different sampling times in the Plant Growth Trial.

	AR1		AR37		Wild-type		Nil		SED
	UN	TR	UN	TR	UN	TR	UN	TR	
Apr 01	466	9	10	7	78	11	190	11	66.8
Sept 01	163	6	21	6	53	6	221	5	30.2
Jan 02	5	2	2	2	13	1	40	2	1.1
May 02[1]	163	3	8	3	50	4	53	3	25.7
May 02[2]	48	1	2	1	10	1	14	1	9.3

[1]Root growth; [2] Main roots

In addition to root aphid, there were minor infestations of an unidentified species of mealybug (Pseudococcidae) in the leaf sheaths of UN AR1 and Nil plants in August and December 2000 which became severe in April 2001 (Table 6.5). Insecticide treatment applied to TR plants in June had little effect on mealybug occurrence in August but virtually eliminated these insects from TR plants thereafter. Minor black beetle adult damage to tillers was also recorded in 20% of UN AR1 plants and 15% of UN Nil plants in December 2000. An unidentified species of sod webworm (Crambidae:Lepidoptera) became a problem in all plants, regardless of endophyte status or insecticide treatment, in January and May 2002.

Table 6.5 Infestations of an unidentified mealybug (Pseudococcidae) species in ryegrass plants with different endophyte treatments and treated (TR) or untreated (TR) with insecticide, recorded as percentage of plants infested in August and December 2000 and percentage of tillers/plant infested in April 2001.

	August 2000 % Plants		December 2000 % Plants		April 2001 % Tillers/plant	
	UN	TR	UN	TR	UN	TR
AR1	30	30	35	0	26	0.25
AR37	10	0	0	0	1	0.04
Wild-type	10	20	0	0	2	0.04
Nil	45	20	20	0	34	0.06
LSD (5%)					6.04	

Relationships between root aphid and foliar growth were generally weak with low correlation coefficients (Table 6.6). Regressions using the difference in foliar growth between untreated and treated plants which were intended to remove any confounding effect of plant genotype on growth, did not improve the strength of the relationships. Among AR1-infected plants mealybug and root aphid appeared to contribute almost equally to reducing foliar growth in April and September 2001 whereas in Nil the effect of mealybug alone was highly

significant (Table 6.6). Neither root aphid nor mealybug could account for growth differences among plants infected with Wild-type endophyte or differences between treated and untreated plants. Where they occurred, relationships between root growth and the incidence of mealybug and/or root aphid were very weak.

Table 6.6 Relationships between plant growth (foliar and root) and root aphid (RA) and mealybug (MB) in April and September 2001 in the Plant Growth Trial

Endophyte treatment	Date	Response	Predictor	DF	P	R^2
All	April	FG TR – UN	Log (L) RA/g	75	<0.001	0.15
		FG TR –UN	L RA/g + %MB	75	<0.001	0.27
		Foliar Growth	L RA/g + %MB	75	<0.001	0.27
		RG TR – UN	L RA/g	75	0.006	0.11
		RG TR - UN	L RA/g + % MB	75	0.003	0.10
AR1	April	Foliar growth	L RA/g	19	0.028	0.20
		Foliar growth	L RA/g + % MB	19	0.018	0.30
		Root growth	L RA/g	19	0.039	0.17
		Root growth	L RA/g + % MB	19	0.015	0.32
	Sept	Foliar growth	L RA/g	15	0.04	0.21
		Foliar growth	L RA/g + %MB	15	0.009	0.44
Nil	April	Foliar growth	% MB	17	<0.001	0.64
		Foliar growth	% MB + L RA/g	17	<0.001	0.63
		Root growth	% MB	17	0.018	0.26
		Root growth	% MB + L RA/g	17	NS	
	Sept	Foliar growth	% MB	12	NS	
		Foliar growth	% MB + L RA/g	12	NS	

6.3.2 Nutrient Trial

Roots Root outgrowth for the combined high and low nutrient treatments in the November sampling was similar for all endophyte treatments (data not presented) but by March Nil plants had less root outgrowth (0.30 g) than AR1 (0.59 g) and Wild-type (0.77 g) ($P<0.05$). AR37 (0.49 g) was intermediate between these

treatments with significantly less outgrowth than Wild-type ($P<0.05$). There was no significant effect of endophyte on dry weight of main plant roots (data not presented).

Nutrient supplementation increased root weight of all endophyte treatments ($P<0.001$) for both the November and March root outgrowth samples and for the main root sample (Table 6.7). At the March sampling root outgrowth from high nutrient Wild-type, AR1 and AR37 plants were similar and Wild-type and AR1 were greater than Nil. Root outgrowth from low nutrient plants was similar for all endophyte treatments.

Table 6.7 Root and foliar growth (g/plant), root/shoot ratios and tiller mortality of ryegrass with different endophyte treatments given additional nutrients (high) or no nutrients (low).

Parameter[1]	AR1		AR37		Wild-type		Nil		LSD
	High	Low	High	Low	High	Low	High	Low	(5%)
RGNov	1.49	0.51	1.36	0.54	1.24	0.45	1.24	0.46	0.44
RGMar	0.94	0.24	0.79	0.19	1.15	0.39	0.43	0.16	0.37
RMain	3.39	0.85	3.41	0.84	3.34	1.00	2.82	0.71	0.79
FGNov	4.39	0.99	4.39	0.82	3.72	0.71	4.57	1.09	0.77
FGMar	5.18	0.54	6.12	0.71	6.74	0.86	4.81	0.57	1.06
Sheath	3.12	0.55	3.36	0.71	3.97	1.09	3.02	0.65	0.63
R/SNov	0.38	0.69	0.35	0.80	0.41	0.90	0.35	0.56	0.29
R/SMar RG	0.16	0.43	0.13	0.28	0.18	0.38	0.07	0.25	0.12
R/SMar[2]	0.53	1.08	0.46	0.81	0.43	0.72	0.38	0.77	0.18
%TM Nov	0.3	2.0	0.1	3.4	0.2	0.3	0.4	1.5	2.5
Mar	1.9	7.7	1.1	8.1	2.9	6.6	10.0	11.5	7.6

[1]RG = root outgrowth; RMain = main plant roots; FG = foliar growth; Sheath = leaf sheaths; R/S = root:shoot ratio; %TM = % tiller mortality; Nov = November; Mar = March

[2]Root:shoot ratio is for whole plant

Insecticide application did not increase root outgrowth or size of main plant roots for any endophyte treatment or for the nutrient treatments (data not presented).

Foliage Foliar growth (mean of TR and UN and high and low nutrients) in November was similar for all endophyte treatments (data not presented) but by March growth of Wild-type-infected ryegrass (3.8 g/plant) exceeded that on AR1 (2.9 g/plant) and Nil plants (2.7 g/plant) ($P<0.05$) with growth on AR37 (3.4 g/plant) intermediate and not significantly different from these.

Foliar growth on high nutrient plants was much greater than on low nutrient plants at both the November and March samplings ($P<0.001$) (Table 6.7). High nutrient plants with AR37 and Wild-type had more growth than Nil plants ($P<0.05$) and growth of Wild-type plants also exceeded that of AR1 ($P<0.05$). Foliar growth was similar for all low nutrient plants. Insecticide did not increase growth of plants for any endophyte or nutrient treatment at either date (data not presented). Differences in leaf sheath dry weight were similar to the differences in foliar dry weight (Table 6.7).

Tiller mortality was higher on UN Nil than on AR1, AR37 and Wild-type plants in the March sampling. Fewer tillers died on TR plants than on UN and, overall, low nutrient plants had higher tiller mortality than high nutrient plants (Table 6.7).

Root:Shoot Root/shoot ratios on high nutrient plants did not differ significantly among endophyte treatments for the two root growth samplings or for the whole plant ratio (Table 6.7). In the low nutrient treatment in March, AR1 and Wild-type had higher root/shoot ratios than Nil ($P<0.01$) for root outgrowth and AR1 was also higher than AR37. For the whole plant AR1 had a higher ratio than all other endophyte treatments. All root/shoot ratios increased significantly in response to nutrient stress ($P<0.001$) although the response in Nil plants was smaller than in other endophyte treatments in November.

Insects Root aphid loadings were highest on UN high nutrient AR1 and Nil plants and very low on UN AR37 (Table 6.8). Root aphid populations were low on low nutrient plants. Insecticide treatment significantly reduced root aphid numbers on high nutrient AR1, Wild-type and Nil plants and on AR1 and Nil low nutrient plants. Argentine stem weevil contributed to some of the tiller mortality on five of 20 Nil plants and three of 20 AR1 plants but this was not quantified. Minor infestations of mealybug were also noted on three AR1 and nine Nil plants.

Table 6.8 Root aphid loading (No./g of root) on ryegrass without endophyte or infected with AR1, AR37 or wild-type endophytes. Plants were given additional nutrients (high nutrient) or no additional nutrients (low nutrient) and were treated with insecticide (TR) or untreated (UN).

	High Nutrient		Low Nutrient	
	UN	TR	UN	TR
AR1	363 a[1]	6 ***[2]	62 a	3 *
AR37	1 b	0	1 b	0
Wild-type	91 c	2 *	8 b	0
Nil	177 a	4 ***	53 a	7 *

[1] Numbers with no letter in common within a column represent a significant difference between endophyte treatments

[2] *, ** represents a significant difference between insecticide treated (TR) and untreated (UN) at $P<0.05$ and $P<0.001$.

6.3.3 Root Biomass Trial

Roots Root weights were affected by endophyte treatment, root volume and harvest date. AR1 had lower root weights than other endophyte treatments ($P<0.05$) at all harvest times and regardless of soil volume (Fig. 6.7). Root weight was consistently highest in Wild-type and AR37 treatments in both large and small soil volumes and at all harvest dates. Over all treatments Wild-type root mass exceeded that of Nil ($P<0.05$) while Nil was not significantly different from AR37 ($P>0.05$). Root growth between harvest periods varied among the endophyte treatments. AR37 had very low root growth between the September

and January harvests followed by a much larger increase in root mass in the January – June period relative to other endophyte treatments.

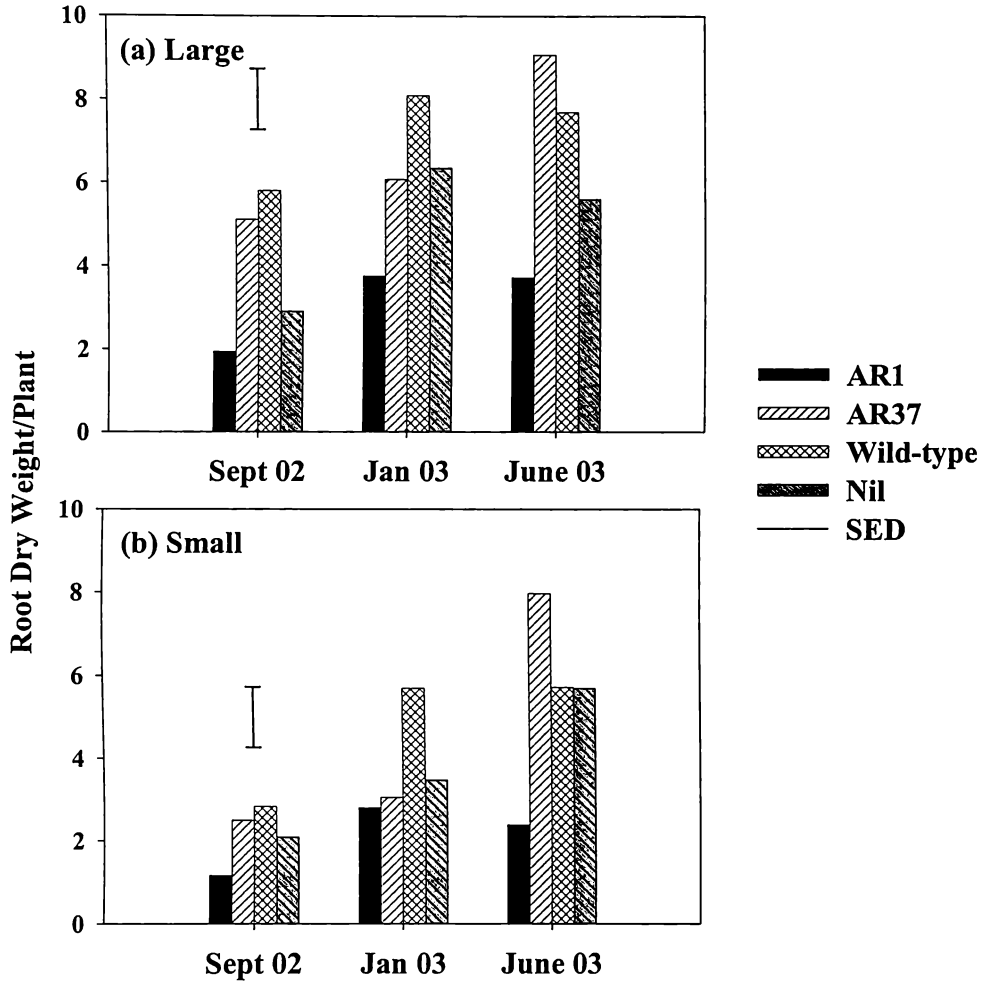

Fig. 6.7 Root biomass (g/plant) of ryegrass without endophyte or infected with AR1, AR37 or wild-type grown in two soil volumes (a) large and (b) small and harvested on three occasions.

Plants in low soil volumes had a lower root dry weight than those in the large soil volumes over all ($P<0.001$) with AR1 the only endophyte treatment for which this was not significant (Fig. 6.7a cf b). Root density, however, was higher in the small than in the large soil volumes over all (1.52 and 1.24 mg/cm^3 respectively) and was higher at each sampling with the difference between these treatments increasing with each successive harvest: September – 0.86 & 0.80;

January – 1.51 and 1.24; June 2.19 and 1.33 mg/cm^3 respectively for small and large).

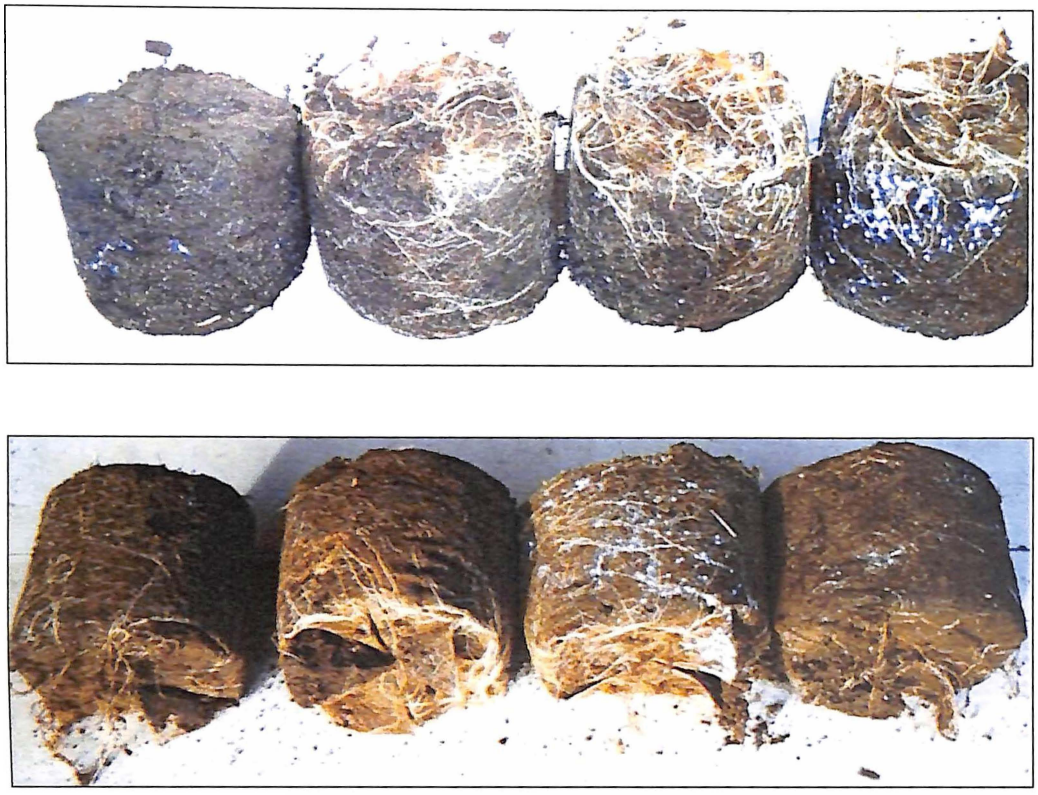

Fig. 6.8 Differences in amount of roots for plants in large soil volumes in two replicates of the Root biomass Trial. White markings on surface are root aphid colonies. Endophyte treatments from L - R are: Top – AR1, AR37, Wild-type and Nil; Bottom – Nil, AR37, Wild-type and AR1

Foliage There were significant differences in foliar growth between endophyte treatments by the first sampling of the trial, 10 weeks after it was set up, when growth of both AR37 and Wild-type exceeded that of AR1 ($P<0.05$). At the first cut, growth on Nil plants was not significantly less than on AR37 but became so by the second cut. For cumulative growth this pattern of differences (ie. AR37 and Wild-type > Nil and AR1) persisted throughout the trial (Fig. 6.9). For individual cuts the only time where significant effects of endophyte on growth did not occur were in January and June 2003 (Table 6.9). Dry weight of leaf sheath material cut from the base of each plant at the final harvest in June, also did not differ between endophyte treatments. Plants yielded less dry matter in the first harvest period (up to and including September 2002) when plants were younger than at the two other

harvest times, with no interaction with endophyte treatment. The proportion of dead tillers at each harvest time did not differ between endophyte treatments.

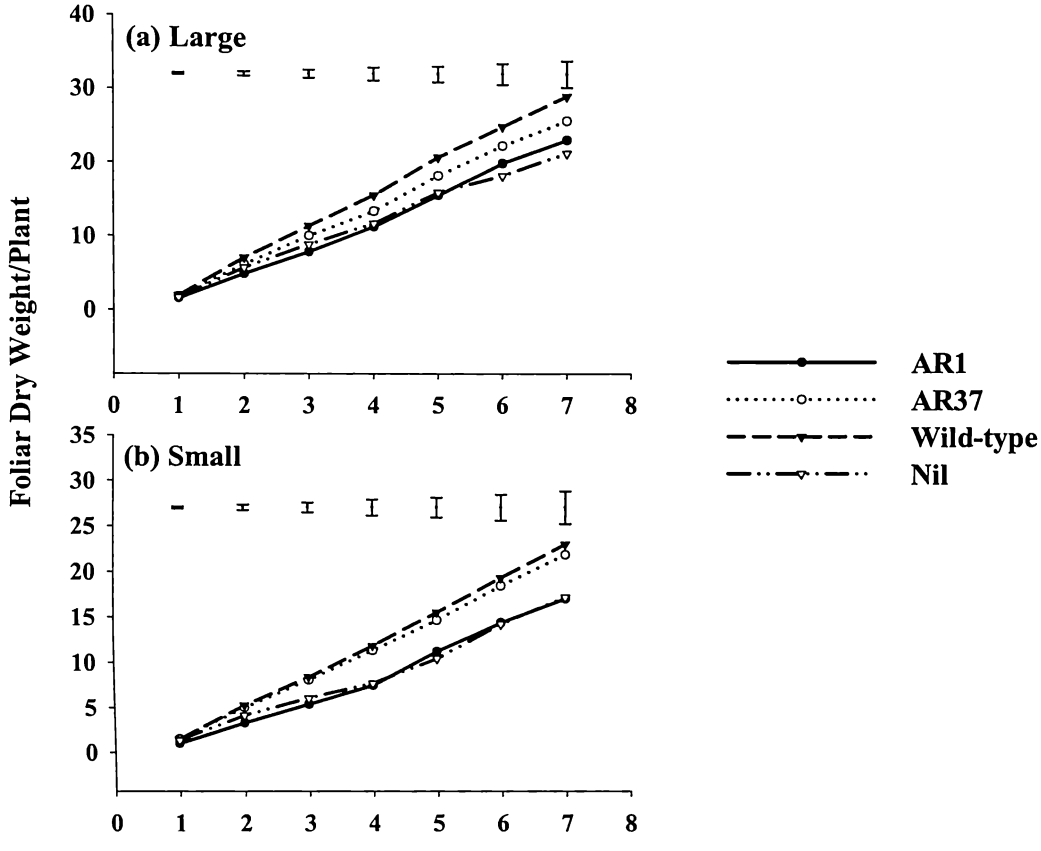

Fig. 6.9 Effect of endophyte treatment in ryegrass on cumulative foliar growth (g/plant) of plants grown in two soil volumes, (a) large and (b) small, cut on seven occasions (see Table 6.12) between April 2002 and June 2003. Error bars = LSD (5%)

Foliar growth of plants in large soil volumes exceeded that in the smaller volumes at the first sample ($P<0.001$), and at every individual sample taken after that date until the final foliar cut in June 2003 when growth in both soil volumes was similar (Table 6.9). Wild-type accumulated more growth than AR37 ($P<0.05$) in the large soil volumes but not in the small ($P>0.05$).

Table 6.9 Foliar dry weights at different sampling times for ryegrass plants without endophyte or infected with AR1, AR37 or Wild-type and grown in two soil volumes.

Date	Large soil volume				Small soil volume				LSD
	AR1	AR37	Wild-type	Nil	AR1	AR37	Wild-type	Nil	(5%)
Ap 02	1.50	2.00	2.28	1.85	0.97	1.48	1.46	1.32	0.25
Ju 02	3.35	4.18	4.77	3.84	2.30	3.45	3.74	2.77	0.44
Se 02	3.00	3.84	4.30	3.16	2.06	3.09	3.15	1.90	0.66
No 02	3.08	3.26	3.70	2.76	2.15	2.95	2.65	1.68	0.75
Ja 03	4.16	4.73	5.00	4.05	3.89	3.44	3.84	2.88	0.93
Ap 03	2.96	4.15	4.98	3.18	2.69	3.42	3.75	3.08	1.02
Ju 03	3.15	3.39	4.15	3.07	2.64	3.42	3.71	2.95	0.99

Table 6.10 Root:shoot ratios for perennial ryegrass without endophyte or infected with endophytes, AR1, AR37 or Wild-type, at different harvest times and for two soil volumes.

	Overall Mean	Large soil volume			Small soil volume		
		September	January	June	September	January	June
AR1	0.525	0.522	0.497	0.746	0.430	0.429	0.524
AR37	0.889	0.828	0.736	1.294	0.668	0.489	1.316
Wild-type	1.015	1.037	1.089	0.988	0.937	1.153	0.883
Nil	0.890	0.656	0.808	1.070	1.035	0.669	1.099
LSD	0.185		Endophyte:	0.373	Soil volume:	0.286	
SED	0.0909		Endophyte:	0.187	Soil volume:	0.142	

Root:Shoot The differences in root mass were largely reflected in similar differences between endophyte treatments in root:shoot ratios. Over all AR1 had a lower root/shoot than Wild-type, AR37 and Nil ($P<0.05$) (Table 6.10). Root:shoot for AR37 plants increased significantly between the January and June harvests

whereas root:shoot of Wild-type and AR1 plants did not differ significantly between harvest times (P>0.05). Soil volume did not affect root:shoot ratios.

Insects All Nil plants and all but one AR1 plant were infested with low numbers of mealybug in April 2002, but this pest was not present thereafter. Root aphid numbers/g of root were highest on AR1 plants and least on AR37 (Chapter 4).

There were significant negative relationships between aphid loading and foliar dry weight for the large soil volume but much weaker relationships for the small soil volume (Table 6.11). For plants in the large soil volumes the strongest correlation occurred between root aphid and mean foliar dry weight per cut in September. In contrast to this, no significant relationships between foliar dry weight and aphid loading were apparent in the small soil volumes until the final harvest in June. At the same time that significant relationships between root aphid and foliar dry weight occurred there were often also relationships between root mass and root aphid.

Table 6.11 Relationships between root aphid/g of root and growth of perennial ryegrass (mean foliar dry weight/cut and root dry weight determined at harvest) without endophyte or infected with AR1, AR37 or Wild-type endophytes in the Root Biomass Trial.

Response	Harvest	Large soil volume			Small soil volume		
		DF	P	R^2	DF	P	R^2
Foliar DW/cut	All	57	<0.001	0.27	59	0.007	0.11
	September	19	<0.001	0.52	19	NS	
	January	19	0.039	0.17	19	NS	
	June	17	NS		19	0.031	0.19
Root DW	All	57	0.006	0.11	59	NS	
	September	19	0.002	0.39	19	NS	
	January	19	0.021	0.22	19	NS	
	June	17	NS		19	NS	

6.3.4 Root Morphology and Colour

Plant Growth Trial Specific root length (SRL) (ie. root length/g) of the main roots was highest on AR1 and lowest on Nil plants but this difference was not significant (P = 0.072) (Table 6.12). Insecticide treatment significantly increased SRL over all endophyte treatments (P<0.05) but for individual treatments this was significant only for AR37.

Table 6.12 Specific root length, estimated total length and proportion of roots in two diameter size categories for ryegrass plants without endophyte or infected with AR1, AR37 or Wild-type, and treated (TR) or untreated (UN) with insecticide.

Endophyte	Insecticide treatment	SRL cm/mg	Total length[1] (m)	Average Diam. (mm)	Diam <0.25 mm (%)	Diam 0.25 – 0.50 mm (%)
AR1	UN	24.1	1636	0.306	59.2	26.0
	TR	29.0	3109	0.285	63.6	23.3
	Mean	26.5	2303	0.296	61.4	24.7
AR37	UN	21.6	2215	0.307	59.2	24.9
	TR	28.0	2875	0.302	60.5	24.9
	Mean	24.9	2603	0.305	59.9	24.9
Wild-type	UN	22.0	1651	0.303	58.7	26.5
	TR	24.3	2766	0.313	59.0	25.2
	Mean	23.2	2130	0.308	58.8	25.9
Nil	UN	19.8	1172	0.305	57.9	27.1
	TR	22.7	2350	0.304	60.3	24.8
	Mean	21.2	1741	0.305	59.1	25.9
SED		2.92	430.9	0.0147	2.34	1.13
P	Endophyte	0.072	0.073	0.70	0.46	0.24
	Insecticide	0.02	0.02	0.48	0.06	0.04

[1] Back transformed data

On average 60% of the root system was comprised of roots less than 0.25 mm in diameter while a further 25% of roots had diameters between 0.25 and 0.50 mm. Endophyte treatment did not affect the proportion of roots in any of the size categories. Insecticide treatment slightly increased the proportion of roots with a diameter less than 0.25 mm from 59% in UN to 61% in TR ($P=0.073$) and significantly decreased the proportion of roots with diameters between 0.25 and 0.50 mm ($P<0.05$).

Estimated total length of main roots was highest in AR37 and lowest in Nil treatments ($P = 0.073$) (Table 6.12). Insecticide treatment did not increase total length of roots in AR37 but did in AR1, Wild-type and Nil ($P<0.01$).

Root Biomass Trial Specific root length was higher in AR1 plants than in AR37 ($P<0.05$) and Nil ($P<0.01$) but was not significantly different from Wild-type ($P>0.05$) (Table 6.13). Neither soil volume nor harvest time significantly affected SRL (data not presented).

The total length of roots calculated from the SRL largely reflected significant differences in root weight except that whereas root weight of Nil plants had often exceeded that of AR1, root length of these two treatments was similar ($P>0.05$) (Table 6.13).

Average diameter of AR1 roots was less than on Nil and AR37 plants ($P<0.01$), and less than on Wild-type ($P<0.05$) (Table 6.13). Average diameter was less in January than in September or June ($P<0.01$) but there were no interactions between endophyte and harvest time or between endophyte and soil volume.

The differences between endophyte treatments in SRL and average diameter were a result of some small but significant differences in the proportion of roots in different size categories. The greatest proportion of roots (mean 43%) had mean diameters between 0.25 and 0.50 mm with a further 34% having diameters less than 0.25 mm. AR1 had the highest proportion of roots in both these size categories relative to other endophyte treatments (Table 6.13) resulting

in almost 80% of AR1 roots having diameters less than 0.50 mm, compared with 75% for Nil, (significantly less than AR1 P<0.001), 77% for AR37 and 78% for Wild-type (significantly less than AR1 P<0.05).

There were significant endophyte*harvest time interactions for roots in the <0.25 and in the 0.25 – 0.50 diameter size classes. The most obvious of these was the higher proportion of AR1 and Wild-type roots with diameters <0.25 mm in January compared with AR37 and Nil (P<0.05). There was neither a direct effect of soil volume nor any interaction between endophyte and soil volume that affected root diameter.

Table 6.13 Effect of endophyte treatments in ryegrass plants on root morphology in the Root Biomass Trial.

	AR1	AR37	Wild-type	Nil	LSD
SRL	20.43	18.38	18.95	16.81	1.75
Total Length (m)	560	1026	1079	782	245
Mean Diam. (mm)	0.407	0.446	0.433	0.451	0.03
% <0.25 mm	35.7	31.8	34.6	33.1	2.56
% 0.25-0.50 mm	44.0	44.8	43.0	41.7	1.97
%<0.50 mm	79.8	76.6	77.5	74.8	2.16
%<0.25 Sept	31.5	26.7	32.4	31.6	4.44
%<0.25 Jan	38.2	33.7	39.4	33.2	4.44
%<0.25 June	37.5	35.1	32.1	34.6	4.44
% 0.25-0.50 Sept	44.6	46.0	42.1	39.0	3.42
% 0.25-0.50 Jan	44.5	46.3	41.2	45.3	3.42
% 0.25- 0.50 June	43.0	42.0	45.6	40.8	3.42

Root Colour In a visual scoring of root colour in September 2001 in the Plant Growth Trial, UN AR37 had more light-coloured roots (mean score – 2.7) than both Wild-type (3.5) and Nil (3.3) (SED 0.2731, d.f. 100, P<0.05). The score for UN AR1 (2.9) was not significantly different from any endophyte treatment. Mean scores for TR plants were 2.8, 2.8, 3.1 and 2.9 for AR1, AR37, Wild-type and Nil respectively with no difference between endophyte treatments.

Roots from high nutrient plants, with an overall mean score of 3.9 for colour, were significantly darker than roots from low nutrient plants (mean score 2.1) ($P<0.001$). Roots from AR37 plants had the lowest mean colour score (2.8) but this was not significantly less than the highest score (3.3) for Nil roots ($P=0.06$).

6.4 DISCUSSION

These trials have indicated that endophyte status of ryegrass modifies different aspects of plant growth as a result of differential herbivory. In addition, strain-specific host plant-endophyte interactions have given rise to differences in root morphology and plant phenology that appear to be independent of insect damage.

According to Whitham et al. (1991) "To unequivocally demonstrate the impact of herbivores on plants, whether negative or positive, requires that the grazed plants exhibit a significant change in fitness relative to ungrazed controls. In reality this turns out to be difficult data to obtain". In these trials, AR37 has represented an ungrazed control and there is no doubt that the fitness of AR1 and Nil relative to AR37 is dramatically reduced in terms of plant growth and survival. Furthermore the fitness of both AR1 and Nil treatments has been substantially increased by insecticide treatment. Nonetheless, it has proven to be very difficult to directly correlate changes in root and shoot growth with insects present, particularly for root aphid. This may, in part, be due to temporal changes in root aphid populations associated with fluctuations in plant quality. Thus measurements at one time may show little relationship to total populations over a period of time. In addition, in the Plant Growth Trial the aphid numbers on root outgrowth may not have accurately reflected those on the main roots as appears to be the case for the final sampling of the Plant Growth Trial when the correlation between aphid loading on root outgrowth with that on main roots was only 0.53. The severe mealybug infestations in leaf sheaths in the Plant Growth also contributed to a decline in plant fitness particularly in Nil treatments. In the Root Biomass Trial, however, root aphid was the predominant insect pest present on AR1 and Nil with low tiller mortality across all endophyte treatments suggesting that above-ground herbivory was not of great importance. Root aphid would seem

therefore to have had a significant impact on root and foliar growth of AR1 and, to a lesser extent, Nil in this trial and this is reflected in stronger correlations between aphid loading and plant growth parameters than found in the Plant Growth Trial.

The most compelling evidence for the effects of root aphid on growth is the impaired performance of Wild-type plants compared to that of insecticide-treated counterparts and AR37 in the Plant Growth Trial, together with the lack of response of AR37 to insecticide. Mealybug was not present in either treatment, whereas root aphids were found on occasions in high numbers on Wild-type but not on AR37. Moreover growth of Wild-type was similar to, or occasionally exceeded that, of AR37 in both the Nutrient and Root Biomass Trial when aphid populations on this treatment were generally low. The apparently better growth of Wild-type in relation to AR37 could perhaps be due to compensatory responses to low levels of herbivory in the former. Cases of overcompensation leading to improved performance relative to plants free of herbivory are recognised in the literature (eg. McNaughton 1983). It is notable here, however, that whereas in the large soil volumes Wild-type overall had better growth than AR37 plants, these two treatments were equal in low soil volumes. If a compensatory response to low herbivory in Wild-type has increased its performance then this has not been apparent where growth was impeded by other factors. A similar reasoning lies behind the failure to demonstrate any effects of herbivory on plant performance in the Nutrient Trial. Unlike the other two trials, soil moistures were kept high in the Nutrient Trial and it seems likely that this has allowed plants to better compensate for insect damage. Cox & McEvoy (1983) found that the ability of *Senecio jacobaea* to compensate for damage by the cinnabar moth (*Tyria jacobaeae*) was positively correlated with water availability. In addition root feeding pests such as *A. lentisci* can have a detrimental effect on water relations in the plant (Dunn & Kempton 1974, cited in Brown & Gange 1990; Gray & Steffey 1998) and would thereby exacerbate any moisture stress plants were already under.

It is difficult to assess here what impact herbivory has had on plant allocation to vegetative and root growth because, in general, reductions in foliar growth have been mirrored by similar reductions in root growth. Lower root/shoot

ratios relative to insecticide-treated counterparts, and to other endophyte treatments less affected by insects, suggest that herbivory has resulted in a diversion of resources away from roots to shoots. This would be expected when mealybug were present (eg. in April in the Plant Growth Trial) but it has also occurred on occasions and in treatments where mealybug were absent (eg. for Wild-type in April 2001 and for AR1 in January 2002 in the Plant Growth Trial; for the September harvest of the Root Biomass Trial). The comparatively greater allocation to shoots as a result of root herbivory is contrary to the general rule that root damage results in greater investment in root growth. Root aphids, however, do not remove plant tissue as such but withdraw carbon from the phloem which may reduce the C:N ratio resulting in the plant partitioning its resources in order to redress that balance.

In the field, AR37 attains a higher root biomass than AR1, Wild-type and Nil (Bell, 1999; D.E. Hume unpublished data) and this is associated with superior vegetative growth (D.E. Hume unpublished data). Apart from its insect-resistant properties, the factor that set AR37 apart from the two other endophyte strains in the trials here was its relatively low investment in root growth in summer – early autumn. This appears to be a direct effect of an endophyte/host plant interaction, rather than mediated by herbivory, and was particularly evident for root growth of insecticide-treated plants in the Plant Growth Trial when it was associated with a comparatively low root/:shoot ratio. Similarly, a low root:shoot value relative to Wild-type was recorded for AR37 plants in January in the Root Biomass Trial and was followed by a much greater investment in root growth over the late autumn and early winter period. The ability of AR37 plants to sustain a high rate of vegetative growth during the summer despite an apparently low rate of root growth suggests it has a more efficient root system than other endophyte treatments. With respect to this, root colour, thought to be indicative of new root growth because it lacks the pigmentation and darkening of roots associated with invasion by pathogenic fungi, was lighter for AR37 on the two occasions that it was scored.

AR1 had the highest SRL of all endophyte treatments whether plants were treated with insecticide or not suggesting that this is an attribute of the interaction

between the endophyte strain and its host rather than a consequence of herbivory. Nil plants, which supported insect populations at similar levels to those on AR1, had the lowest SRL in both trials where this was measured. Root morphology measurements were determined on only a small proportion of the root system in these trials so the results need to be interpreted with some caution. They were, however, consistent between trials and with other reports showing endophyte-associated differences in root distribution in ryegrass (Crush et al. in press) and smaller root diameters in endophyte-infected tall fescue than in endophyte-free (Malinowski et al. 1999). The mechanisms by which such changes come about are unknown but root morphology and growth is to some extent under the control of plant growth regulators including indole acetic acid which has been produced by *N. coenophialum* in culture (de Battista et al. 1990).

While fine roots may give the plant greater access to nutrients and water, they do not necessarily maximise plant growth. There is a carbon cost to the plant associated with maintenance and construction of the root system and these costs increase with increasing SRL (Eissenstat & Yanai 1997; Fitter 1996). On the other hand a low SRL limits the ability of the plant to forage for nutrients which may also impact on plant growth. Insecticide increased SRL in all treatments including AR37, even though the latter showed no other response to insecticide. Fine roots may be more vulnerable to herbivory (Eissenstat & Yanai 1997) but it is possible that activity of other invertebrates such as Collembola, mites and nematodes may also damage or even feed directly on these roots.

CHAPTER SEVEN

CHEMISTRY OF ROOTS OF *LOLIUM PERENNE* INFECTED WITH DIFFERENT STRAINS OF *NEOTYPHODIUM* ENDOPHYTE

7.1 INTRODUCTION

Alkaloids synthesised by *Neotyphodium* fungi in their host grasses are the basis for array of biological activity displayed by each endophyte strain against insect and mammalian herbivores. In the *Neotyphodium lolii*/ryegrass association much of that activity has been attributed to three classes of compounds and specifically to three alkaloids within those classes, namely peramine, ergovaline and lolitrem B.

The pyrrolopyrazine alkaloid, peramine appears to be unique to the *Neotyphodium* and *Epichloe* spp. complex and is a potent feeding deterrent to Argentine stem weevil (Rowan et al. 1990) but has not been shown to have strong activity against other insects (Popay & Rowan 1994). Peramine is water soluble and translocated freely in the above-ground parts of the plant with concentrations usually higher in leaf blades than in the leaf sheath (Keogh et al. 1996; Ball et al. 1997b). Concentrations of peramine are generally at their highest in the plant between December and May and decline over winter, and are associated with seasonal changes in endophyte concentration measured by ELISA (Ball et al. 1995a,b).

Ergovaline is one of a group of ergot alkaloid derivatives of lysergic acid that are produced not only by *Neotyphodium* and *Epichloe* spp. but also by *Claviceps.* This compound is concentrated in basal leaf sheath, developing inflorescences and seed (Davies et al. 1993; Lane et al. 1997a). Ergot alkaloids are toxic and/or deterrent to a range of insects (Yates et al. 1989; Clay & Cheplick 1989; Popay & Rowan 1994; Ball et al. 1997a) but are also believed to be the causal agent of fescue toxicosis in cattle grazing *N. coenophialum* tall fescue (Stuedamann & Thompson 1993) and similar symptoms observed in sheep

the factors such as plant genotype and seasonality (Ball et al. 1995b, 1997b & c; Easton et al. 2002) that affect the quantity of alkaloids that are produced in a plant, nothing is known of the factors that affect their translocation to roots.

Plants depend on the ability of roots to acquire sufficient nutrients to meet their growth requirements. Nitrogen (N) is not only essential for plant growth but the amounts available can affect the partitioning responses of plants. In addition N is a major factor affecting performance of insects feeding on plants (Mattson 1980), with roots tending have lower levels of this element than foliar plant parts (Seastedt et al. 1988b). Root length, distribution and morphological plasiticity, which allow roots to exploit nutrient-rich patches in the soil, are important for the capture of immobile ions such as phosphorus (P) and potassium (K) as well as for the more mobile N. No major effects of endophyte infection on the nitrogen economy of ryegrass have been detected (Cheplick et al. 1989; Lewis et al. 1996) but increased nitrogen has been shown in above-ground parts of endophyte-infected tall fescue (Lyons et al. 1990). Also in tall fescue, P levels are higher in endophyte-infected plants than in endophyte-free under phophorus-deficient conditions (Malinowksi & Belesky 1999).

Strain-specific effects of endophyte-infection of perennial ryegrass on a root aphid, *Aploneura lenitisci*, have been shown to range from strong resistance in AR37, low susceptibility or transient resistance in Wild-type to increased susceptibility in AR1 (Chapter 4). The presence of root aphid reduces plant growth and alters biomass allocation (Chapter 6) but it also appears that endophyte infection may modify growth in other ways. Root morphology and root distribution differ between endophyte-infected and endophyte-free plants (Chapter 6; Crush et al. 2004) and seasonal biomass allocation in AR37-infected plants appears to differ from AR1, Wild-type and endophyte-free plants (Chapter 6). Thus alkaloid levels in roots and leaf sheaths were monitored here in three pot trials to gain some initial information on the occurrence of alkaloids in roots and associate that with biological activity of endophytes against the root aphid. In addition to the alkaloids, concentration of N, P and K in roots was also determined to enable a better understanding of plant growth responses to be made and to

determine if host plant quality factors may be responsible for the high inter-plant variation in susceptibility to root aphid noted in Chapter 4.

7.2 METHODS

Plant material (roots and herbage) sampled from three trials, the Plant Growth, Nutrient and Root Biomass Trials, was analysed for alkaloids and/or nitrogen (N) potassium (K) and phosphorus (P) content. Each of these trials was comprised of the same four endophyte treatments; viz. perennial ryegrass (*Lolium perenne*) cv. Samson, that was free of endophyte (Nil), or infected with AR1, Wild-type or AR37 endophytes. Each of these endophytes has a different chemical profile. Of the known alkaloids, AR1 produces peramine, Wild-type peramine, ergovaline and lolitrem B and AR37 four epoxy janthitrem fractions. Details of the design of each of these trials are given in Chapter 3 and sampling of plant material in Chapter 6.

In the Plant Growth Trial, plant growth and root aphid (*A. lentisci*) populations had been monitored for 2 years on cloned pairs of plants, one of which was treated regularly with insecticide. At the final sampling of this trial in May 2002 leaf sheath, main plant roots and root outgrowth were taken separately from each of 20 replicate plants that had been either insecticide-treated or untreated. All material was frozen immediately after sampling and then later freeze-dried. Of 13 replicates where all plants had survived during the trial, 10 were chosen at random for chemical analysis. Samples of root outgrowth, main roots and leaf sheaths were analysed for the presence of the alkaloids, peramine and ergovaline (AR1 and Wild-type), lolitrem B (Wild-type only) and janthitrems (AR37 only). Samples from main roots and green leaf lamina (cut at a height of 50 mm) were also analysed for percent composition of N, P and K. In addition, root weight for each plant had been determined and root morphology (ie. specific root length and total root length) was also measured on subsamples from the main plant roots (Chapter 6). Concentration of fungal hyphae in leaf sheaths was also estimated for all endophyte-infected plants.

In the Nutrient Trial, the effect of endophyte treatment on plant growth and root aphid numbers were determined on ryegrass plants with (high nutrients) and without (low nutrients) nutrient supplementation and treated or not treated with insecticide. There were 10 replicate plant genotypes with each plant genotype cloned across treatments. At the final sampling in March 2001, 8 months after the trial was set up, only the main roots from the last five replicates of each treatment were retained for chemical analysis. Root samples from each plant were analysed for peramine, ergovaline, lolitrem B or janthitrem content depending on endophyte treatment. Owing to the small size of the low nutrient plants, remaining root samples from each replicate were pooled to ascertain the N, P and K composition for each treatment. The pooled samples were comprised of the same weight of root material from each plant.

Plant growth and root aphid numbers were again monitored in the Root Biomass Trial in which 15 ryegrass plants were grown in cloned pairs in large and small soil volumes for each endophyte treatment. Five replicates from each treatment were destructively harvested 8, 12 and 16 months after the trial was set up in September 2002, January 2003 and June 2004 respectively. Root material from each plant harvested at each sampling time was analysed for both alkaloids and N, P and K. Alkaloid content of leaf sheaths was also determined. In addition root morphology measurements were made on each plant.

All root material was washed thoroughly and frozen (−25 °C) soon after sampling. Herbage was also frozen immediately after sampling. Prior to analysis, plant material was freeze dried and then ground in a mill (John Wiley Scientific Instruments) using a 1.00 mm screen.

7.2.1 Alkaloid Analysis

Alkaloid analysis was carried out on weighed samples (approximately 50 mg) of ground plant material. Peramine and ergovaline were extracted with propanol, water and lactic acid (50:50:1) using a method similar to that described in Spiering et al. (2002). Peramine was separated by HPLC on a silica column with an aqueous solvent and detected by UV absorption using homoperamine, a synthetic analogue of peramine, as an internal standard. After extraction,

ergovaline was determined by reverse phase HPLC with fluorescence detection and measured as the sum of ergovaline and ergovalinine using and compared to an ergotamine internal standard. Lolitrem B and the epoxy janthitrems were extracted from separate samples. After extraction and separation by HPLC using a silica column, lolitrem B was measured against a reference standard by fluorescence detection. The janthitrems were also measured using reverse phase HPLC with fluorescence detection. The concentrations of the four janthitrem fractions are relative to each other but have not been determined against an internal standard using purified compounds. Measurements are derived from an indirect comparison with lolitrem B determined by normal phase HPLC, and referenced against a sample derived from an AR37-infected plant and used in each batch that is anlaysed.

The accuracy of the detection methods are ± 15%, with approximate limits of detection for ergovaline and lolitrem B of 0.1 μg/g and for peramine 1 μg/g.

7.2.2 N, P, K Analysis

Analysis of finely ground root and herbage for N, P and K content was carried out by *e*-lab Ltd, Ruakura Research Centre Hamilton. Nitrogen content of dry matter was determined using Kjeldahl digestion followed by colorimetric analysis. K and P analysis was carried out by nitric perchloric digest followed by ICP determination.

7.2.3 Hyphal Density

Hyphal density was determined on three tillers taken from each plant in the Plant Growth Trial using the method described in di Menna & Waller (1986). The outermost leaf sheath associated with a green leaf was removed from each tiller and stained in lactophenol-cotton blue. Stained sheaths were mounted, inner side uppermost and examined under an Olympus BH2 microscope at 400x magnification. Hyphal counts were made across the breadth of the leaf sheath as close as possible to the attachment of the tiller. Counts made are twice the average number of hyphae seen/microscope field of 0.5 mm to give the number of hyphae/mm breadth of leaf sheath.

7.2.4 Statistical analysis

Comparisons of alkaloid content of leaf sheaths were made among the different endophytes and between insecticide-treated and untreated plants in the Plant Growth Trial, using an analysis of variance of untransformed data after examining data for homogeneity and normality. Concentration and total N, P and K content of roots and leaf sheaths were also compared among endophyte treatments and between insecticide-treated and untreated plants. The general analysis of variance in Genstat Release 6.1 was structured to take into account the randomised block design of the endophyte treatments, the split-plot design for insecticide treatments and the cloned pairs of plant used. Data for alkaloid and N, P and K content of plants in the Nutrient Trial were not analysed because samples were bulked to give overall values for the different treatments. In the Root Biomass Trial an ANOVA was carried out on the concentrations of N, P and K in plants using endophyte treatment, soil volume and harvest date as main effects.

Simple linear regression analysis was used to determine if hyphal density could be used to predict alkaloid concentration. Relationships between N, P and K content of plants and root weight or length were also investigated using linear regressions.

7.3 RESULTS

7.3.1 Alkaloids

Two of the four janthitrem fractions identified in AR37 samples, peak A3 and peak B, were consistently found in roots of most infected plants in all three trials, but only in 'possible trace' amounts. Of 18 infected plants tested in the Plant Growth Trial, main plant roots of 17 contained peak *A3* and 14 peak *B* (Table 7.1). These fractions were also found in the roots of the majority of plants tested in the Nutrient and Root Biomass Trials, with the exception of the September harvest of the latter. Also notable was that these fractions were found in roots of low nutrient plants, albeit in fewer plants and at lower concentrations than found in the high nutrient plants. Fractions *A1* and *A2* were also recorded in the main roots of a high proportion of plants in the Plant Growth Trial but were almost completely absent in root outgrowth samples taken at the same time and in root

samples from the other trials. Peak *B* was the dominant fraction in the leaf sheath samples. The Plant Growth Trial had lower concentrations of all these alkaloids compared with the Root Biomass Trial, and, in the latter, lowest levels were recorded in September. Insecticide had no significant effect on concentration of janthitrems in leaf sheath.

Concentration of ergovaline, lolitrem B and peramine in roots was low and sporadic in all three trials. Ergovaline was not found in the main plant roots of the Plant Growth Trial but was found in root outgrowth (Table 7.2) in 60% of the plants sampled at concentrations that averaged 17% of that in the leaf sheath tissue. Root outgrowth was not tested for ergovaline in the Nutrient trial and none was found in the main plant roots. In the Root Biomass Trial ergovaline was only found consistently in plant roots in the June harvest when it occurred in nine of ten Wild-type plants (Table 7.2). Concentrations found in roots ranged from trace amounts of 0.03 to 0.22 μg/g. There was no indication of a relationship between the amount of ergovaline in the leaf sheath and the amount in the root; ie. high ergovaline in the leaf sheath did not correspond with ergovaline being found in root outgrowth. Of eight cloned pairs of plants for which data were complete in the Plant Growth Trial, six had either no ergovaline (2) or some ergovaline (4) in both root outgrowth samples from the cloned pairs. This suggests there may be a plant genotype effect on distribution of ergovaline but more data is needed to confirm this. Ergovaline concentrations in the leaf sheath were not affected by insecticide treatment in the Plant Growth trial or by harvest date in the Root Biomass Trial.

Table 7.1 Concentrations (μg/g) of four janthitrem fractions found in roots, root outgrowth and leaf sheath of individual perennial ryegrass plants infected with AR37 in three trials. Concentrations in leaf sheath are the mean for the total number of plants tested (n) and in roots and root outgrowth are the mean only for plants in which the fraction was found (number in parenthesis).

Treatment		Fraction			
		A1	*A2*	*A3*	*B*
Plant Growth Trial					
UN[1]	Root	0.08 (5)	0.05 (6)	0.06 (9)	0.02 (7)
n = 9	Outgrowth	0	0.01 (2)	0.03 (6)	0.03 (6)
	Sheath	0.78	0.43	0.93	1.55
TR	Root	0.08 (7)	0.05 (7)	0.08 (8)	0.03 (7)
n = 9	Outgrowth	0	0	0.01 (5)	0.02(7)
	Sheath	0.94	0.54	1.12	1.71
Nutrient Trial					
High[2] n = 5	Root	0.02 (1)	0.07 (2)	0.07 (7)	0.04 (9)
Low n = 5	Root	0	0.02 (1)	0.03 (5)	0.03 (5)
Root Biomass Trial					
September	Root	0	0.01 (1)	0	0.02 (2)
n = 10	Sheath	0.34	0.20	0.65	0.60
January	Root	0.01 (1)	0	0.02 (8)	0.03 (7)
n = 10	Sheath	1.24	2.07	1.92	4.25
June	Root	0.02 (1)	0.03 (2)	0.03 (5)	0.04 (9)
n = 10	Sheath	1.72	0.82	1.67	3.16

[1] UN – plants not treated with insecticide; TR – plants treated with insecticide

[2] High – plants given additional nutrients; Low – plants given no nutrients

Table 7.2 Concentration (μg/g) of the alkaloids, peramine, ergovaline and lolitrem B in leaf sheaths, main plant roots and root outgrowth of ryegrass infected with AR1 and Wild-type in two trials. Concentrations in leaf sheath are the mean for the total number of plants tested (n) and in roots and root outgrowth are the mean only for plants in which the alkaloid was found (number in parenthesis). In the Plant Growth Trial plants were treated (TR) or not treated (UN) with insecticide and samples were taken in May 2002. In the Root Biomass Trial plants were grown in two soil volumes (large and small) and harvested on three occasions.

		Peramine		Ergovaline		Lolitrem B	
Plant Growth Trial		UN	TR	UN	TR	UN	TR
AR1	Sheath	38.0	29.1*	0	0	-	-
n = 10	Outgrowth	2.73 (2)	2.35 (3)	0	0	-	-
	Root	0	0	0		-	-
Wild-type	Sheath	20.1	21.7^{ns}	0.71	0.89^{ns}	1.55	2.21*
n = 10	Outgrowth	4.26 (2)	2.59 (3)	0.07 (7)	0.11 (5)	0	0
	Root	0	0	0	0	0	0
Root Biomass Trial		Large	Small	Large	Small	Large	Small
AR1	September	0	0	0	0	-	-
Root	January	0	0	0	0	-	-
n = 5	June	1.2 (1)	2.4 (2)	0	0	-	-
AR1	September	21.1	22.2	0	0	-	-
Sheath	January	34.8	37.0	0	0	-	-
n = 5	June	22.9	31.8	0	0	-	-
Wild-type	September	3.9 (1)	0	0.15 (1)	0.06 (1)	0	0
Root n = 5	January	0	0	0.09 (2)	0	0.12 (1)	0.17 (2)
	June	0	0	0.13 (4)	0.09 (5)	0.17 (1)	0.08 (1)
Wild-type	September	22.0	20.9	0.75	0.83	1.39	1.15
Sheath	January	30.7	32.9	1.24	1.38	6.00	3.94
n = 5	June	29.9	29.7	1.28	1.00	2.51	2.41

* Denotes a significant difference between insecticide-treated and untreated plants.

Peramine, like ergovaline, was only found in the root outgrowth of the Plant Growth Trial and not in the main plant roots. Peramine occurred in root outgrowth of 25% of AR1 and Wild-type plants at concentrations that were, on

average, 9% of those in the leaf sheaths. No peramine was found in the plant roots in the Nutrient Trial and it occurred very sporadically in roots in the Root Biomass Trial (Table 7.2). In the Plant Growth Trial, AR1-infected plants had higher peramine concentrations than Wild-type plants ($P<0.001$) in the leaf sheath and also higher concentrations in UN than in TR plants ($P<0.05$) whereas insecticide had no effect on concentrations of this alkaloid in Wild-type (Table 7.2). Similarly mean peramine concentration in the leaf sheaths of AR1 plants in small soil volumes (30.4 μg/g) was higher than the concentrations in equivalent plants in large soil volumes (26.3 μg/g) ($P<0.01$).

7.3.2 Hyphal Density

Number of fungal hyphae in the leaf sheaths for AR37, Wild-type and AR1 respectively was 13.8, 8.9 and 5.9/mm breadth of leaf sheath (d.f. 68, SED 1.35). AR37–infected plants had higher hyphal densities than Wild-type and AR1 ($P<0.01$) and Wild-type was also greater than AR1 ($P<0.05$). There was no effect of insecticide treatment on mean hyphal concentration and no correlation between cloned pairs of TR and UN plants for each endophyte strain.

7.3.3 Root Aphid, Alkaloids and Hyphal Density

Peramine is not considered likely to affect root aphid since this alkaloid is produced by the AR1 endophyte and plants containing this strain are often highly susceptible to root aphid. Root aphid populations on plants infected with Wild-type endophyte which produces ergovaline and lolitrem B as well as peramine tend to be less susceptible and at times apparently resistant to root aphid relative to plants without endophyte (Chapter 4). When lolitrem B was consistently present in roots of Wild-type plants in the March sampling of the Nutrient Trial, there were significantly fewer aphids on these plants than on Nil plants. There was no relationship, however, between the concentrations of lolitrem B found in the roots (range 0.13 to 0.48 μg/g) and populations of root aphid which ranged from 13 to over 2000/plant on individual plants. In addition there were other occasions when root aphid numbers were significantly less on Wild-type plants than on Nil such as in the September and June samplings of the Root Biomass Trial when this alkaloid was not detected consistently in roots. Similarly, when ergovaline was consistently found in root outgrowth in the Plant Growth Trial 2002, root aphid

numbers on root outgrowth of Wild-type plants were not significantly different from Nil. In addition, inter-plant variation in aphid numbers was unrelated to ergovaline concentrations. Levels of these alkaloids in the leaf sheath were also not related in any way to root aphid populations found on plants.

Because of the very low populations of root aphid on all AR37 plants no attempt was made to correlate these with root concentrations of the janthitrem fractions.

Hyphal density was not correlated with concentration of any of the alkaloids in AR1 or Wild-type or with populations of root aphid on either the outgrowth or the main plant roots sampled at the same time. A strong plant genotype/endophyte interaction resulted in alkaloid expression in cloned pairs of plants used in the both the Plant Growth Trial (cloned TR and UN plants) and the Root Biomass Trial (cloned pairs in large and small soil volumes) being highly correlated (coefficients ranging from 0.73 to 0.92 for each alkaloid). Hyphal density in AR37 was also not correlated with janthitrem expression but like ergovaline, peramine and lolitrem B there were generally strong correlations in alkaloid production between cloned pairs of plants. Between treated and untreated plants in the Plant Growth Trial correlation coefficients were > 0.90, and between cloned plants in the large and small soil volumes coefficients were > 0.80 for all fractions except A3 for which the coefficient was 0.55.

7.3.4 Nutrient Analysis

Endophyte-free plants consistently had lower concentrations of K in their roots than infected plants (Fig. 7.1a). These differences were significant for the Plant Growth Trial ($P<0.001$) but not in the Root Biomass Trial ($P>0.05$). Low nutrient plants in the Nutrient Trial had, on average, 64% more K in their roots than plants in the high nutrient treatment (Table 7.3). AR1 showed the smallest response to low nutrients with a 42% increase in K concentration in the roots compared with a 90% increase in Wild-type.

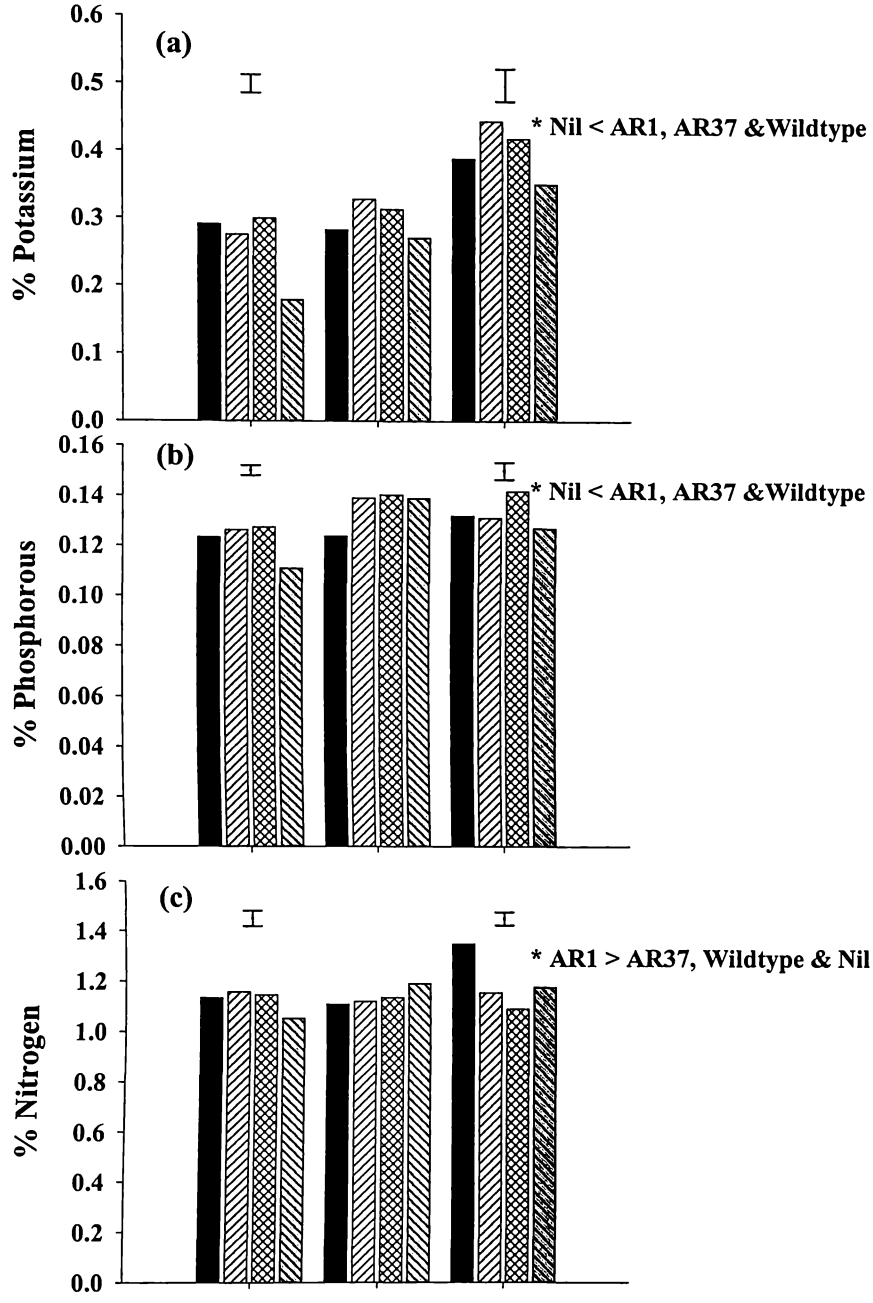

Fig. 7.1 Mean concentrations of (a) potassium, (b) phosphorus and (c) nitrogen in roots of perennial ryegrass plants without endophyte (Nil) or infected with AR1, AR37 or Wild-type, in three trials. Error bars = SED

Concentrations of P in plants followed similar patterns to that of K with lower concentrations in endophyte-free plant roots than in endophyte-infected in

the Plant Growth Trial ($P<0.001$) but no significant differences in the Root Biomass Trial ($P>0.05$) (Fig. 7.1b). In the latter trial, however, Wild-type plant roots in the September harvest contained more %P than other endophyte treatments ($P<0.05$) (Table 7.3). Concentrations of P in roots of low nutrient plants were higher than concentrations in high nutrient plants with a 2% increase in AR1 and a 31% increase in Wild-type and Nil plants.

Table 7.3 Percent concentration of elements N, P and K in roots of ryegrass without endophyte or infected with endophytes AR1, AR37 or Wild-type in two trials. In the Nutrient trial plants plants were given additional nutrients (High) or no nutrients (Low) and in the Root Biomass trial samples were taken on three different occasions.

Trial	Element	Treatment	AR1	AR37	Wild-type	Nil	LSD
Nutrient	K	High	0.23	0.25	0.22	0.20	
		Low	0.33	0.41	0.41	0.34	
	P	High	0.12	0.13	0.13	0.12	
		Low	0.13	0.15	0.15	0.16	
	N	High	1.33	1.27	1.36	1.40	
		Low	0.90	0.97	0.91	0.98	
Root	K	September	0.47	0.61	0.57	0.45	0.17
Biomass		January	0.20	0.23	0.19	0.20	0.17
		June	0.49	0.50	0.50	0.41	0.17
		LSD (5%)	0.19	0.19	0.19	0.19	
	P	September	0.12	0.13	0.16	0.12	0.024
		January	0.12	0.13	0.11	0.11	0.024
		June	0.17	0.14	0.16	0.15	0.024
		LSD (5%)	0.025	0.025	0.025	0.025	

Nitrogen, like P and K, was less concentrated in roots of endophyte-free plants in the Plant Growth Trial but differences were not significant ($P>0.05$) (Fig. 7.1c). In the Root Biomass Trial, however, %N in roots of AR1 plants exceeded that of other treatments ($P<0.001$) (Fig. 7.1c). There were also significant effects

of endophyte treatment on changes in %N with time in this trial. In AR37 %N increased substantially between September and January whereas there was little change in roots of other endophyte treatments (Fig. 7.2). Conversely, between January and June %N increased significantly in roots of AR1, Nil and Wild-type but not in AR37. AR1 had higher levels of N than Wild-type at all harvests and more N than AR37 in September and June. Plants of all endophyte treatments had a higher %N in the smaller soil volume than in the larger soil volume. For individual treatments this effect was significant for AR37 and Wild-type ($P<0.05$) but not for AR1 and Nil treatments.

Concentrations of K and P in leaf blades in the Plant Growth Trial did not reflect the significant differences seen in the roots. Concentrations were, respectively, for AR1, AR37, Wild-type and Nil: 2.5, 2.4, 2.6, 2.5 %K and 0.32, 0.32, 0.35, 0.33 %P. Leaf blade concentrations of N (2.4, 2.5, 2.6, 2.5 % for AR1, AR37, Wild-type and Nil respectively) were also very were very similar across endophyte treatments.

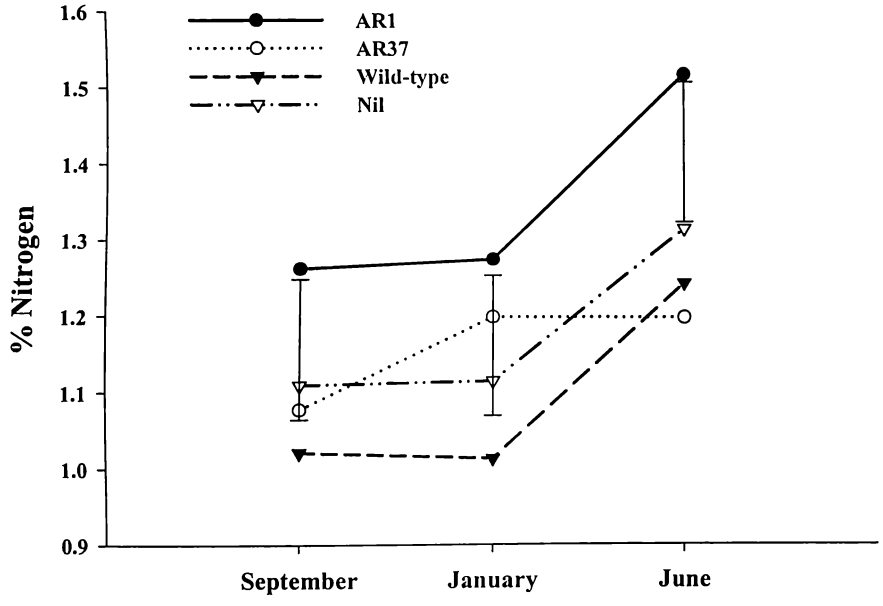

Fig. 7.2 Changes in percent nitrogen in roots of perennial ryegrass without endophyte or infected with AR1, AR37 or Wild-type endophytes over three harvest periods. Error bars represent the LSD (5%) for comparison between endophyte treatments at each harvest date.

In the Plant Growth Trial, Nil plants had less K in their roots than all endophyte-infected plants and less N and P than AR37, calculated on the basis of weight (Table 7.4). Differences were apparent for both TR and UN plants (Fig. 7.3). N, P and K content of leaf blades did not differ significantly between endophyte treatments but AR1 consistently had the highest levels of all elements in UN plants (Fig. 7.4; Table 7.4). At the time this trial was sampled there were no significant differences between endophyte treatments in root or foliar dry weights.

Differences in weight of N, P and K in roots in the Root Biomass Trial were lowest in AR1 and highest in AR37 and Wild-type (Table 7.4). These differences largely reflected significant differences in root dry weights at the time the samples were taken.

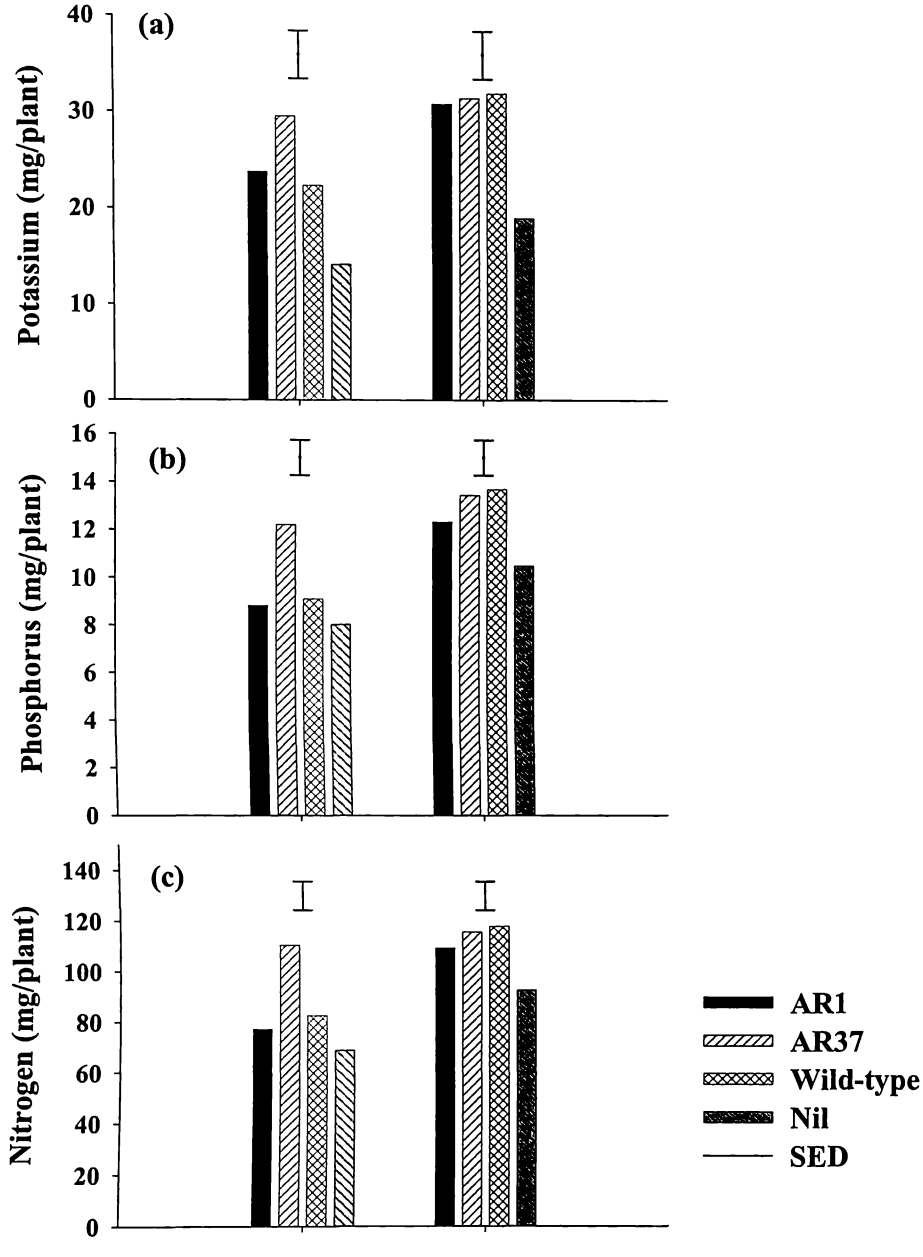

Fig. 7.3 Differences in amounts of (a) potassium, (b) phosphorus and (c) nitrogen in roots of ryegrass plants without endophyte or infected with AR1, AR37 or Wild-type endophytes and treated or untreated with insecticide.

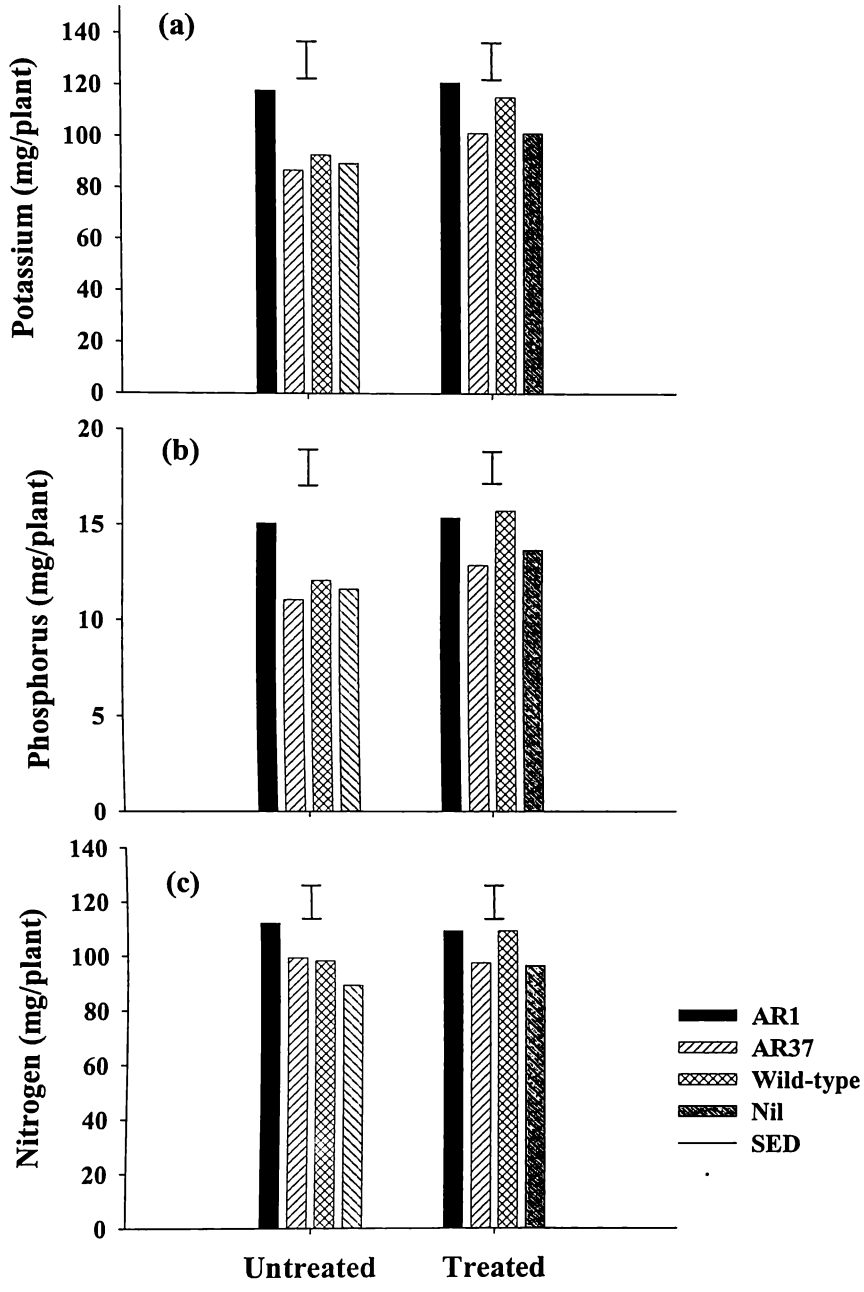

Fig 7.4 Differences in amounts of (a) potassium, (b) phosphorus and (c) nitrogen in leaf blades in ryegrass plants without endophyte or infected with AR1, AR37 or Wild-type endophytes and treated or untreated with insecticide.

Table 7.4 Effect of endophyte treatment on mean weight (mg/plant) of potassium, nitrogen and phosphorus in perennial ryegrass main roots and leaf blades for pooled data from treated and untreated plants in the Plant Growth Trial sampled in May 2002 and in roots only for pooled data for plants in large and small soil volumes in the Root Biomass Trial sampled in September, January and June.

		AR1	AR37	Wild-type	Nil	LSD
Plant Growth						
Root	K	27.4	30.5	27.2	16.6	10.0
	N	93.4	113.2	100.4	80.9	22.8
	P	10.6	12.8	11.4	9.3	2.9
Leaf	K	119.8	94.5	104.5	95.9	28.8
	N	110.9	98.6	103.9	93.0	24.6
	P	15.2	12.0	13.9	12.6	3.8
Root Biomass						
September	K	8.0	27.8	26.6	12.0	10.9
January		7.0	11.5	13.6	11.3	10.9
June		16.2	45.2	35.2	27.6	10.9
LSD (5%)		7.9	7.9	7.9	7.9	
September	N	19.1	37.2	41.0	26.7	12.2
January		38.4	50.7	67.8	51.4	12.2
June		49.4	93.7	79.0	77.6	12.2
LSD (5%)		8.8	8.8	8.8	8.8	
September	P	1.9	5.2	6.6	3.1	2.04
January		3.6	5.6	7.6	5.3	2.04
June		5.3	11.9	10.2	9.0	2.04
LSD (5%)		1.5	1.5	1.5	1.5	

7.4.5 Relationships between concentration of elements, root biomass and morphology and occurrence of root aphid

No significant relationships were found between the concentrations of P and K in the roots and occurrence of root aphid. In the Plant Growth Trial there was a positive linear relationship between concentration of peramine in the leaf sheath

and K in the roots for AR1 (R^2 = 0.78; P<0.001). No similar relationships were found in AR1 in the Root Biomass Trial or for peramine and K concentrations in Wild-type.

Concentrations of N, P and K in roots were correlated in different ways with root biomass and to a lesser extent root length. The most significant of these were negative linear correlations between %N and root weight in the Root Biomass Trial (Table 7.5). For the first harvest in September, P was weakly but positively correlated with root dry weight (R^2 = 0.16; P<0.05). Thereafter, however, negative relationships between root weight and P composition of roots were found in the January harvest (R^2 = 0.28 for large soil volume and 0.38 for small; P<0.01) and also for the June harvest for the small soil volume only (R^2 = 0.61, P<0.001). Percent K content of roots, in contrast to %P, was positively correlated with root weight for the September sampling for both small and large soil volumes and for the June sampling of the large soil volume (R^2 = 0.35 in each case, P<0.01).

Table 7.5 Relationships between root dry weight and percent N for each harvest in large and small soil volumes in the Root Biomass Trial.

Treatment	Harvest	Equation	R^2	P
Large	September	$y = 1.35 - 0.078x$	0.86	<0.001
	January	$y = 1.53 - 0.075x$	0.86	<0.001
	June	$y = 1.70 - 0.057x$	0.79	<0.001
Small	September	$y = 1.38 - 0.091x$	0.59	<0.001
	January	$y = 1.58 - 0.097x$	0.67	<0.001
	June	$y = 1.70 - 0.072x$	0.84	<0.001

Due to the high correlation between root dry weight and percent nitrogen in the Root Biomass Trial, data on %N in roots of ryegrass with the different endophyte treatments were reanalysed using root dry weight as a covariate. AR1 and AR37 contained the highest concentrations of N (1.22% for each one) but this was not significantly different from concentrations in Wild-type and Nil (1.18 and 1.17% respectively) (P= 0.06).

7.4 DISCUSSION

Two of the four janthitrem fractions produced in AR37-infected ryegrass were detected consistently in root samples, even in plants suffering nutrient stress. The only time when these fractions were largely absent was in the September sampling of the Root Biomass trial. Previous results have indicated that resistance to root aphid in AR37-infected plants is lower in September than at other times of the year (Chapter 4). While this may infer a relationship, clearly further work is needed to provide conclusive evidence that one or both of these compounds is responsible for the observed toxicity of AR37 infection to root aphid. The concentrations at which these alkaloids were found in root tissues cannot be interpreted with any confidence, but are likely to be higher in the phloem itself.

The alkaloids, peramine, ergovaline and lolitrem B were found in roots only occasionally and at very low levels in these trials which is in agreement with previous work (Ball et al. 1997 b,c; Lane et al. 1997a). In these trials, however, the consistent appearance in roots of ergovaline on two occasions and lolitrem B on one indicates that each of these alkaloids is transported to roots only under certain circumstances. Detection of ergovaline only in new roots (ie. root outgrowth) in the Plant Growth Trial and again in June in the Plant Biomass Trial when there was abundant new root growth suggests this alkaloid may be preferentially supplied to actively growing tissues. This is consistent with the observations made by Lane et al. (1997a) that ergovaline concentrations are highest in the crown, basal tissues from which new tillers emerge, and developing inflorescences. Lolitrem B, on the other hand was only found in roots of the majority of plants tested in the Nutrient Trial sampled in March. This coincides with the period when concentrations of lolitrem B are highest in the leaf sheaths (di Menna et al. 1992; Ball et al. 1997c; Eerens et al. 1998a) although in individual plants there was no indication that high concentrations recorded in the leaf sheaths was correlated with the appearance of either alkaloid in roots.

The measurement of ergovaline and lolitrem B in these experiments has done little to shed light on the reasons for the observed occasional resistance of the Wild-type endophyte strain to root aphid. Both these alkaloids remain as

possible candidates for conferring resistance since their appearance in roots has coincided with some of the times when the Wild-type strain was exhibiting significant resistance to root aphid in comparison with endophyte-free plants. It is reported earlier in this thesis that another endophyte strain that produces ergovaline but not lolitrem B also reduces aphid populations whereas the converse (ie. a strain producing lolitrem B but not ergovaline) had no resistance (Chapter 4). This together with the fact that root aphid show a preference for feeding on new root growth (Chapter 4) and the apparent association of ergovaline with these tissues favours ergovaline being the more likely of the two alkaloids to be responsible for resistance. On the other hand lolitrem B is structurally related to the janthitrems which are the most likely cause of aphid resistance in AR37. Furthermore, Ball et al. (1997d) comparing several endophyte strains in perennial ryegrass which produce lolitrem B but not ergovaline found one that showed resistance to the root-knot nematode (*Meloidogyne marylandi*). With regard to both alkaloids, however, it should be noted that perennial ryegrass infected with the Wild-type endophyte does not affect the foliar-feeding aphid, *Rhopolasiphum padi* (Latch et al. 1985; Siegel et al. 1990) although it has some effects on another foliar-feeding aphid, the greenbug (*Schizaphis graminum*) (Siegel et al. 1990).

Concentrations of all alkaloids in leaf sheaths were highly correlated with plant genotype for cloned plants, an effect that has been reported before for ergovaline (Adcock et al. 1997; Hiatt & Hill 1997; Easton et al. 2002), lolitrem B and peramine (Ball et al. 1995b) but not for the janthitrems. There are, however, other factors including nutrient concentration in plants that may affect alkaloid production. Here for instance peramine concentration in leaf sheath was well correlated with potassium in roots on one occasion in AR1, although this relationship was not apparent in other samples. In addition, peramine levels were higher in leaf sheaths of AR1-infected plants that had not been treated with insecticide than in treated plants. Since this same effect was not obtained with peramine in Wild-type plants, this suggests that herbivore stress on AR1 may be responsible. Conversely lolitrem B concentrations were less in untreated than in treated Wild-type plants which may be due to a nutrient effect given the higher weight of N, P and K found in the latter.

No relationship between hyphal density and alkaloid concentration were found in these trials. In other studies significant correlations between endophyte concentration determined by ELISA and production of peramine, ergovaline and lolitrem B have been obtained over the annual cycle of endophyte but not for each individual sampling (Ball et al. 1995a & b). Hyphal density in the tiller may not be a good measure of total endophyte in the plant since a considerable mass of mycelium occurs below the level at which the tiller is severed although seasonal changes in hyphal mass measured this way are similar to those measured by ELISA (di Menna et al. 1992 cf. Ball et al. 1995a & b) and have correlated in a general way with seasonal changes in lolitrem B concentration (di Menna et al. 1992).

Increased plant quality resulting from endophyte infection was thought to be the most likely basis of the increased susceptibility to root aphid shown by certain plant genotypes hosting AR1 (Chapter 4). The higher N, P and K content of roots and leaf blades of AR1-infected plants in the Plant Growth Trial, compared to Nil, particularly in insecticide-treated plants provide some support for this theory. Concentrations of N, P and K also tended to be higher in roots of AR1 plants in the Root Biomass Trial. The role that nutrient status may have in aphid performance is indicated in a study of two *Myzus* species on *Plantago lanceolata* by Gange et al. (1999) who found that aphid fitness increased as a result of mycorrhiza infection and P supplementation. For the ryegrass/AR1 association, however, further, more targeted, research will be needed before conclusions can be drawn as to the reasons for the high susceptibility of these plants to root aphid. Aphids in general are responsive to a variety of plant constituents including sucrose and amino acids. In this context, increased amino acid synthesis which resulted in higher levels of glutamine and asparagine in tall fescue infected with *N. coenophialum* compared with endopyte-free fescue (Lyons et al. 1990) may be an aspect worth further investigation. Differences in the performance of the aphid *Myzus persicae* on potato plants at different stages of development were attributed to differences in glutamine levels (Karley et al. 2002). Non-structural carbohydrates may also be important. Effects of endophyte infection on total non-structural carbohydrates are markedly host genotype specific (Cheplick and Cho 2003) and it has been noted here (Chapter 4) that there

is also a strong host plant genotype component in the performance of root aphid on AR1-infected plants.

Nutrient uptake in plants is influenced by a variety of factors including root length, diameter, surface area and root hairs and root age. In tall fescue, endophyte infection can increase P uptake in P-deficient soil (Malinowski and Belesky 1999) possibly as a result of reduced root diameter and longer root hairs in infected plants (Malinowski et al. 1999). Here, the increased concentrations of P and K found in endophyte-infected ryegrass plants were also in line with smaller root diameters and longer root lengths recorded in these plants (Chapter 6). At least for K there were also positive correlations between root dry weight and to a lesser extent root length on several occasions. P concentration, however, was more likely to be negatively correlated with root dry weight and root length, while percent N was strongly negatively correlated with root dry weight. There are three possible explanations for this. The first, that N and P in the growing medium was limiting, and therefore concentrations were lower where root biomass is higher, is rejected because small soil volumes with a higher root density than large soil volumes had higher levels of these ions, as did insecticide-treated compared with untreated plants. The second more likely explanation is that N and P have become more dilute as total root biomass has increased relative to the availability of sites which are actively taking up these ions. A third possible explanation is that carbon:nitrogen balances have been altered by removal of carbon by root aphid feeding as indicated by the reductions in root dry weight and root:shoot ratios (Chapter 6). This situation is analogous to those reported by Seastedt et al. (1988b) and Polley & Detling (1989) who showed increases in N content of roots and reductions in root biomass as a result of defoliation. In the trials reported in this thesis, however, the relationship between root dry weight and N also holds true for insecticide-treated plants and for AR37 plants that are resistant to root aphid. It therefore seems unlikely that root aphid is a major cause of the increases in N although it has clearly played a role in the differences noted between endophyte treatments insofar as they have resulted in lower root weights and lower root/shoot ratios.

There were clear differences between AR37 and the other endophyte treatments in the concentrations of N at the different harvest times In the Root Biomass Trial. The marked increase in percent N in roots of AR37 between September and January compared with the relatively stable levels in the other endophyte treatments is compatible with the relatively low root growth and root:shoot ratios of AR37 during this time (Chapter 6). Between January and June there was a large increase in root mass of AR37 plants and an increased root:shoot ratio. The ability of these plants to sustain this increase in root mass while maintaining foliar growth that was equal to that of Wild-type may in part be due to the accumulation of N in the roots in the preceding period. This supports the notion put forward in Chapter 6 that seasonal partitioning of root and shoot growth responses of AR37 differ from that of the other endophyte treatments.

CHAPTER EIGHT

SUMMARY AND GENERAL DISCUSSION

8.1 SUMMARY

At the beginning of this thesis five hypotheses were nominated for consideration and these are now revisited with a summary of results given in relation to each one.

1. **That certain strains of *Neotyphodium* endophytes in ryegrass would affect root herbivory and that this is mediated by the presence of alkaloids in roots.**

Strain-specific effects on a root aphid, *Aploneura lentisci*, ranging from increased susceptibility with AR1 to complete resistance with AR37 were found. Results were similar for all three pot trials. In contrast, no effects of endophyte infection by AR37 or AR22 (an endophyte similar to AR1) on feeding preferences and survival of scarab larvae, *Costelytra zealandica*, were detected.

Resistance to root aphids in plants containing AR37 was stable throughout the year except for a slight increase in populations in spring and showed little interplant variation within two cultivars tested. Antibiosis rather than antixenosis was the basis of the resistance with affected aphids displaying symptoms of neurotoxicity before dying. Within the detection limits of the method used only possible trace amounts of two janthitrem fractions were found in the roots but the consistency with which they were found suggests they may be responsible for the observed toxicity. More direct methods of investigation involving analysis of phloem or bioassay of fractions would be required to prove this.

AR1-infected plants showed a high degree of interplant variation in susceptibility to root aphid, a key part of which was associated with plant genotype or a plant genotype/endophyte interaction. AR1 tended to have higher

levels of phosphorus, potassium and nitrogen in both roots and shoots, suggesting that plant quality may be a factor influencing susceptibility but whether this is related to plant genotype/endophyte interactions could not be determined from these trials.

Root aphids were less numerous on plants infected with Wild-type than on endophyte-free plants and, on occasions, these plants appeared to be significantly resistant to this insect. A chemical factor in the roots is the most likely reason for this transient resistance. Both lolitrem B and ergovaline are possible candidates since both alkaloids occasionally occurred in roots. There was less inter-plant variability in aphid numbers on plants infected with Wild-type than those on AR1-infected plants. Like AR1, however, a component of that variability was related to plant genotype or a plant genotype/endophyte interaction.

Endophyte-free plants also showed a range in susceptibility to root aphids but the plant genotype component of this was less strong than for endophyte-infected plants.

Nutrient deficiency did not alter the relative susceptibility or resistance properties of the different endophyte strains.

2. That members of decomposer food-webs in the soil would not be directly affected by the presence of different strains of *Neotyphodium* (eg. by toxic compounds produced by the endophyte) in perennial ryegrass but may be affected as a result of trophic interactions resulting from differential herbivory.

No direct effects of endophyte infection on populations of Collembola, oribatid mites or nematodes (predominantly Dorylaimida) were detected. This conclusion is based on the absence of any consistent differences in populations of these organisms associated with root outgrowth of the different endophyte treatments. In the final main root sampling, however, AR1-infected plants supported more Collembola than AR37-infected plants. This is attributed to positive correlations between root aphid populations and Collembola rather than

direct negative effects of AR37 on Collembola. Increasing populations of Collembola were associated with increasing numbers of root aphid in both trials where these were monitored and is believed to be mediated via trophic interactions relating to increases in saprophytic fungi that utilise honeydew or root exudates as a substrate. Infestations of root aphid were also negatively correlated with combined numbers of dorylaimid nematodes and enchytraeids but the latter were not correlated with Collembola. The mechanisms behind these apparent changes in faunal composition of soil associated with endophyte infection are not understood but may relate to changes in microbial biomass and competitive interactions within the soil community arising directly from root aphid infestations or indirectly via its effects on plants.

3. That occurrence of arbuscular mycorrhiza and other fungi in the roots would not be affected by the *Neotyphodium* infection.

Factors in the AR1/host interaction resulted, over time, in these plants becoming less hospitable to AM mycorrhiza. In addition mycorrhizal infection of AR37 and Wild-type infected plants were initially slower to develop than in AR1 and endophyte-free plants. These effects seem unlikely to be a direct response to *Neotyphodium* infection in the sense that the latter has created a chemical or physical barrier to infection and are more likely to be mediated via other effects the endophyte has on plants. The slower development of mycorrhizal infection in spring in AR37 and Wild-type is tentatively attributed to reduced availability of propagules to these plants because of their high root biomass, or alternatively because of lower microarthropod activity associated with these plants. In addition, mycorrhizal colonisation is moderated by P availability to the plant and this cannot be ruled out as a possible cause of differences given the higher P concentrations in roots of endophyte-infected plants. No effects of endophyte infection on the incidence of other root-inhabiting fungi were found.

4. That root growth, root biomass accumulation, root/shoot ratios and root morphology would be affected by differential herbivory resulting from *Neotyphodium* infection but would not be altered by the presence of the fungus in the absence of herbivory.

Herbivory by the root aphid, *A. lentisci*, and on occasions by a pseudococcid mealybug in tillers was a major factor in consistently lowering root growth and biomass accumulation in AR1 and to a lesser extent in Nil relative to AR37 and Wild-type. In addition root morphology of plants was modified by AR1 infection and plant phenology by AR37. These latter consequences of endophyte infection on plant growth parameters were not apparently mediated by endophyte effects on herbivory but are difficult to quantify in terms of plant benefit.

The differences between endophyte treatments in terms of plant growth became apparent through manipulation of herbivory with insecticide. Thus biomass of AR37-infected plants did not increase as a result of insecticide treatment, whereas treated endophyte-free plants and those infected with AR1 or Wild-type had higher root and foliar biomass compared with untreated. In addition *A. lentisci* and pseudococcid infestations on occasions coincided with decreases in root/shoot ratios. The changes suggested preferential investment in foliar growth as a result of insect damage regardless of whether the insect was an above-ground or below-ground feeder. Biomass allocation to foliar growth in response to root aphid feeding is contrary to the theory that root feeding will generally result in compensatory root growth. Aphid feeding withdraws carbon from the plant and it is postulated that resulting decreases in the carbon:nitrogen ratios lead to increased investment in foliar growth to redress this balance. The inverse relationship between nitrogen concentration and root biomass found in this study also supports this theory.

For plants treated with insecticide, growth parameters were generally similar for all endophyte-infected treatments except that Wild-type accumulated slightly less foliar biomass and AR37 less root biomass. This latter difference provided the first clues that certain strains of endophyte modified plant growth habits in the absence of insect damage. Changes in root/shoot ratios in the Root

Biomass Trial provided further evidence of differences in growth allocation processes in AR37-infected plants that appeared to be independent of herbivory. Conservative root growth relative to foliar growth of these plants during summer were followed by large investments in root growth in autumn and early winter suggesting that there are mechanisms altering plant phenology in the interaction between this strain and its host. The reason for such changes will be discussed further but may lie in the cost to the plant of accommodating the endophyte.

AR1 had lower SRL and finer roots than endophyte-free plants with AR37 and Wild-type consistently intermediate between these treatments. This meant that, while AR1 root biomass was often less than that of Nil, root length of these two treatments were similar or greater in AR1. Insecticide treatment increased SRL and root length of all endophyte treatments including AR37 but did not change the relative differences between endophyte treatments, providing evidence that this too was a direct effect of endophyte infection on the plant and not one mediated by herbivory. Both this effect on root morphology and the changes in plant phenology suggest hormonal balances within the plant may be modified by *Neotyphodium* infection.

5. That occurrence of alkaloids in roots of endophyte-infected ryegrass would vary according to the type of alkaloid and seasonal and environmental factors and that it would be a function of the amount in the leaf sheath.

Alkaloids were mostly found in very low quantities in roots relative to amounts in leaf sheaths. The conditions under which they were consistently found differed according to the compound and were not necessarily functions of the amount in the leaf sheath. There is insufficient information to draw conclusions as to the chemical factors that are responsible for the effects on root aphid observed in AR37 and Wild-type.

In AR37-infected plant, two of four janthitrem fractions were consistently found in roots of AR37-infected plants at all times of sampling. Given the limits of detection of the analytical method, however, is cannot be concluded with any confidence that these janthitrems are the causal agent of toxicity to root aphid.

Similarly in Wild-type-infected plants, ergovaline and lolitrem B were found in roots of most plants on occasions but the low concentrations make it difficult to draw meaningful conclusions as to their possible effects on root aphid. The occurrence of ergovaline was associated with new root growth and did not reflect amounts in leaf sheaths. On the other hand, lolitrem B was only recorded in roots of plants under high nutrient and moisture conditions sampled in March when levels in the leaf sheath could be expected to be high. Peramine was found sporadically in roots of few AR1 and Wild-type plants. Concentrations of these alkaloids within phloem are likely to be higher than in the surrounding tissues since *Neotyphodium* hyphae do not extend into roots.

In addition to the alkaloids, levels of N, P and K in roots and, on one occasion, in shoots were investigated primarily to determine if they were indicators of plant quality factors that may have influenced susceptibility to root aphid. Concentrations of P and K were significantly higher in roots of endophyte-infected plants than in endophyte-free plants in the Plant Growth Trial and consistently, but not significantly, higher in the other trials. Increasing K concentration was associated with increasing root biomass and root length. A similar association was not apparent for P levels which were more likely to be negatively correlated with root biomass. There was also no evidence that P concentration in roots was related to mycorrhizal infection.

Concentration of N in roots was not significantly affected by endophyte treatment in the Plant Growth Trial but in the Root Biomass Trial %N in roots of AR1 was greater than in endophyte-free, Wild-type and AR37 plants. Percent N in roots was inversely related to root biomass in all endophyte-treatments. Thus high N concentration in roots of AR1-infected plants was the consequence of a reduction in root biomass as a result of herbivory rather than being directly attributable to root aphid. In addition, there were changes in %N in roots with time which have implications for the nitrogen economy of the plant in relation to growth. Concentration of N in roots of AR37 increased substantially between September and January but changed little between January and June whereas the converse occurred in other endophyte treatments. Changes in %N in roots

matched differences in growth patterns between AR37 and other endophyte treatments noted above.

Endophyte-free plants had lower K levels (=weight) in their roots than did endophyte-infected plants, and less N and P than AR37 in the Plant Growth Trial at a time when differences in root biomass between treatments were not significant. There were no significant differences between endophyte treatments in concentrations or weight of N, P and K in the leaf blades but a consistently higher weight of these elements in AR1 relative to endophyte-free plants suggests that plant quality factors may contribute to the high susceptibility of AR1 to root aphid.

8.2 GENERAL DISCUSSION

This study has shown that *Neotyphodium* infection of perennial ryegrass has consequences below-ground not only for plants and their herbivores but also for the wider soil community. Equally apparent is that these effects are strain specific, mediated to a large extent by differential effects on herbivory but also affected to a degree by differences in root morphology and plant phenology. Within the relationship between plant and endophyte strain, host genotype/fungal interactions also played a role in determining susceptibility to root aphid. Clearly, while there is evidence that *Neotyphodium* endophytes have evolved from a single *Epichlöe* strain (*E. festucae*) evolution has taken different paths to create different strains (and/or species) and these differences have consequences both in agricultural and in evolutionary contexts.

8.2.1 Significance for agriculture

Between 1996 and 1999 a series of field trials at five North Island and two Canterbury sites evaluated the performance of ryegrass infected with a range of different endophytes relative to endophyte-free ryegrass (Popay et al. 1999). These trials showed significant plant yield advantages to all endophyte-infected plants particularly during the summer-autumn period, with the extent of the advantage depending on infection levels and on endophyte strain (Popay et al. 1999). Thus there were occasions when Wild-type-infected ryegrass was more

productive than AR1. AR37 was included in these trials but results pertaining to this endophyte were not reported in Popay et al. (1999) for intellectual property reasons. The productivity of ryegrass infected with AR37 in these trials was outstanding, with yields of these plants exceeding that of all other treatments in 22 of 38 samples in summer and 28 of 38 samples taken in autumn (D.E. Hume pers. comm). At no time was the productivity of AR37 significantly less than that of other treatments. The question was – what made AR37 better than the rest?

Differential herbivory by the major insect pests, Argentine stem weevil (*Listronotus bonariensis*), black beetle (*Heteronychus arator*) larvae and pasture mealybug (*Balanococcus poae*) were thought to be mainly responsible for the differences in plant productivity observed in these trials but could not explain the yield advantages of AR37-infected plants. For instance plants with AR1, AR37 and Wild-type endophytes are equally resistant to Argentine stem weevil (Popay & Wyatt 1995; Popay et al. 1999) and pasture mealybug (Popay et al. 2000) while both Wild-type and AR37 give a similar resistance to black beetle, a major ryegrass pest in northern areas (Popay unpublished). Thus a very significant finding of the study reported in this thesis is that not only is AR37 resistant to root aphid, a ubiquitous insect in New Zealand pastures, but also that root aphid is capable of significantly reducing plant productivity. Undoubtedly there are differences in demonstrating this in pot trials as opposed to in the field but this aphid has been recorded in high numbers in field trials when there has also been an associated decrease in yield (C. Pennell et al. in prep.; Popay unpublished data). Furthermore, in the field, the chronic nature of this insect, the likelihood of concurrent herbivore pressure in summer and autumn together with the edaphic stresses on plants at this time are all factors likely to contribute to yield losses associated with aphid feeding.

The ability of ryegrass plants infected with AR37 to conserve root growth during the stressful summer period, along with a concomitant increase in nitrogen concentrations in roots may also allow plants infected with this endophyte to maximise their growth. Furthermore the strong resistance to herbivory means that the plant is not penalised in absolute terms by having a low root biomass. On the

contrary, root growth of AR37-infected plants has often exceeded that of others except when application of insecticide has 'levelled the playing field'.

The trials reported in this thesis have suggested that AR1–infected plants may be at a selective disadvantage compared with endophyte-free plants due their increased susceptibility to root aphid. A wider range of natural pest pressures in the field, however, results in enhanced productivity of AR1-infected ryegrass relative to endophyte-free (Popay et al. 1999). Nevertheless root aphid may be the cause of unexpected reductions in productivity of AR1 in relation to ryegrass with Wild-type endophyte. In the trials reported here, a strong host-plant genotype interaction with the AR1 endophyte played a major role in inter-plant variability in aphid populations on individual plants. This should allow selection of plants infected with this strain that are less vulnerable to root aphid and which may be more robust in the field. In addition to aphid susceptibility, however, the finer root system in AR1 plants may not be advantageous in high fertility pastures where access to nutrients is generally not limiting, and may instead result in an additional cost to the plant in root construction and maintenance.

Trials conducted in this study were all carried out on individual plants whereas, in reality, no plant exists in isolation. Competitive interactions in which plants physically interfere with one another or compete for a limited resource have an important role in structuring plant communities, both in agriculture and nature, and are major determinants of plant productivity and persistence. Allelopathy is one mechanism by which plants may directly interfere with one another and has been purported to reduce the competitiveness of clover in interactions with ryegrass infected with Wild-type endophyte (Sutherland & Hogland 1989; Sutherland et al. 1999). Such effects, however, may also be due to the greater ability of the endophyte-infected plant to access light and nutrients when it is protected from herbivory. The competitive abilities of AR1 and AR37 are largely unknown but one could predict on the basis of this study that ryegrass infected with AR37 is likely to be highly competitive and ryegrass with AR1 less so.

On a wider spatial scale competitive interactions also arise where communities are made up of plants that are resistant to herbivory and those that

are not. Thus, depending on their mobility and host selection capability with regard to resistant plants, insect pests may do proportionally more damage to non-resistant plants in the presence of resistant ones. This is a well recognised phenomenon in ryegrass pastures where there can be rapid shifts in frequency of endophyte infection from predominantly endophyte-free to almost completely endophyte-infected (eg. Prestidge et al. 1984, 1985; Popay et al. 2003a). Such changes are probably more likely to occur where resistance is based on deterrency that allows the insect to select suitable hosts. The mechanism of herbivore resistance in AR37 appears to be due more to toxicity than deterrency as shown here for root aphid and as has been demonstrated for Argentine stem weevil (Popay & Wyatt 1995; Popay unpublished) whereas AR1 resistance to Argentine stem weevil is entirely based on the deterrent activity of peramine. Plant population dynamics of ryegrass infected with each of these strains may therefore differ when they are in mixtures with endophyte-free ryegrass.

8.2.2 Evolutionary significance

Evolutionary theory predicts that for those organisms that are obligate biotrophic symbionts such as *Neotyphodium* spp., benefits to the host must accrue from the relationship in order that it be maintained. In an evolutionary context, however, plant fitness is not defined by the plant's ability to produce vegetative growth as it is in an agricultural context but rather by its ability to propagate. Furthermore, although survival is a key attribute for success of plants in both contexts, in the agricultural sense survival of individuals is important whereas in the evolutionary sense survival of the species may be more so.

Much of the evidence for mutualism originates from studies of plant/endophyte associations in agriculture that have been artificially selected for plant benefit, albeit unwittingly, for many years. Based on this there is a tendency in the literature for all-encompassing statements to be made with respect to the functional significance of the plant host/*Neotyphodium* relationships (eg. Clay and Schardl 2002; White et al. 2002). There is of course strong evidence for mutualism but should this be construed as meaning that all *Neotyphodium* relationships with their hosts are mutualistic or even strongly mutualistic, if one equates increasing plant benefit with increasing strength of mutualism? Moreover

there is a dearth of good studies on host/endophyte relationships in native habitats, particularly for the strains that are commonly used in agriculture, on which dissenting views can be based. One such study has shown that the presence of *Neotyphodium* in the native grass Arizona fescue (*Festuca arizonica*) appears to be more disadvantageous than advantageous to the host, with researchers concluding on that basis that *Neotyphodium* infections are more likely to be parasitic than mutualistic (Faeth & Sullivan 2003).

Such opposing viewpoints have paid little heed to the diversity of endophyte biotypes or species known to exist in natural and semi-natural habitats and the likelihood of a similar diversity in the nature of the symbiosis. It could be predicted that within such diversity lies a continuum from parasitism to strong mutualism with the functional significance of each relationship determined by selection pressures in each habitat. While there are obvious dangers in interpreting the results of trials conducted here in this context, it could be argued that AR37 represents a strongly mutualistic association (ie. defined here in terms of high plant benefit) whereas AR1 is much less so. In fact this study suggests that AR1 is parasitic rather than mutualistic although there is evidence from other environments that it is the latter with host plants displaying enhanced survival and vegetative productivity relative to those that are endophyte-free (eg. Popay et al. 1999).

On the basis of the differences observed here between AR37 and AR1, it could be argued that the strength of the mutualism, in terms of plant benefit, is inversely related to the variability in host genotype/endophyte relationships and their resultant outcomes. Thus within plant populations where the endophyte mutualism is weak or even parasitic, a high degree of interplant variability in anti-herbivore defences or abiotic stress tolerance is inherent, allowing plasticity in plant response to sporadic selection pressures. On the other hand the strong defensive properties of AR37 are associated with little interplant variation, are strongly mutualistic and have probably arisen in response to continuous severe selection pressures.

In addition to the strength of selection pressures, the form that they take in the original habitats of these two endophytes may be quite different. It could be envisaged for instance that AR1 has been selected in an environment where herbivore pressure is low and where acquisition of nutrients is a high priority. Hence there is investment in a finer root system. Moreover the host may be reliant on the presence of legumes for its nitrogen so the endophyte/host relationship does not produce a highly competitive plant. AR1-infected plants may be relatively short-lived with defences more capable of protecting seeds and seedlings rather than mature plants. Thus a high turnover of plants in this habitat would retain the interplant variation needed to maintain responsiveness to periodic herbivore attack. Conversely, herbivore defence is paramount for AR37 in a strongly competitive environment in which the phenology of the host/endophyte relationship has evolved to maximise nutrient and water acquisition with less reliance on nitrogen fixing capabilities of legumes. Plants with AR37 may be long-lived as constant selection pressures eliminate those with weak defence systems at the seedling stage. Plant turnover and interplant variability is also relatively low in this environment.

The endophyte of course is not altruistic, and its effects in plants have been selected to ensure its own survival in perpetuity. Thus the cost to the plant of maintaining the endophyte should at the very least be equal to the benefit the plant gains from infection. Fungal biomass relative to the whole plant biomass is low but proportional to the production of alkaloids (Ball 1995a & b). In addition, as an exporter of materials, the cost of the endophyte could be expected to exceed that of providing sustenance for fungal growth and maintenance alone. Thus those endophytes that synthesise a range of alkaloids which require a complexity of pathways and/or expensive resources are likely to be more of a drain on the economy of the plant than are those that synthesise a narrow range of alkaloids. Significant differences in hyphal density between endophyte strains, with AR1 having the lowest and AR37 the highest, in this study are relevant to this argument. This result is also not surprising in the context of the preceding discussion as to the functional significance of each endophyte strain. The expectation would be that lower hyphal mass equates with lower cost to the plant and therefore fewer benefits to the host and vice-versa for high hyphal densities.

A similar argument has been used in a model describing the relationship between extent of mycorrhizal colonisation of a plant and the benefit that the plant derives (Gange & Ayres 1999). The model proposed is curvilinear where the plant receives maximum benefit at moderate mycorrhizal densities at which benefits outweigh the costs. At low hyphal densities the benefits are small whereas at high densities the cost of supporting the fungal network exceeds the benefits to plant growth. Further research would be needed to determine if *Neotyphodium* endophyte/plant interactions fitted a similar model although data interpretion would require considerable care, since the nature and extent of the biotic and abiotic challenges in relation to hyphal density to the plant may be the determining factor in any cost/benefit analysis.

Numerous studies have shown that investment in defence costs the plant in resources with a concomitant growth reduction (reviewed in Gershenzon 1994). The question here is not just how much does endophyte cost the plant but whether or not there are mechanisms in the relationship through which costs can be offset, aside from those relating to defence. It could be inferred from data in this thesis (Chapter 6) that there is indeed a cost to maintaining both Wildtype and AR37 since these endophytes had, respectively, lower cumulative foliar and root growth compared with endophyte-free and AR1 plants when all were treated with insecticide. Insecticide-treated AR1 plants on the other hand showed no growth reductions relative to treated endophyte-free plants. The greater growth reductions in untreated AR1 compared with untreated endophyte-free, however, may partly be due to the cost of maintaining the endophyte under stressful conditions rather than entirely due to differences in insect numbers. In addition, low levels of mycorrhiza in older AR1 plants may suggest that over time the infected plant dispenses with these associations because it cannot afford the additional cost of sustaining them. The costs of maintaining Wild-type and AR37, assuming there are any, are more than offset by their defensive properties. It is also tempting to speculate, however, that the ability of endophyte-infected plants in this study to acquire higher concentrations of nutrients in roots than endophyte-free plants is a mechanism designed to compensate for plant expenditure on the symbiont. The fact that high concentrations in roots were not mirrored by high concentrations in leaf blades supports this hypothesis. Furthermore, in relation to the differences in

hyphal densities already noted, although AR37 had the highest mass of N, P, K in the roots compared with AR1 and Wild-type plants it had the lowest concentrations of these elements in the shoots.

8.2.3 Implications for Root Ecology

Below-ground environments are inherently difficult to study. In this research an initial attempt has been made to determine possible consequences of endophyte-infection for soil biota participating in below-ground food-webs, beyond the direct interactions between herbivore and plant. Positive and negative interactions involving Collembola and dorylaimid nematodes were observed and may be founded on the differential effects of endophytes on the occurrence of root aphid. Similarly, multitrophic interactions emanating from interactions between soil biota and herbivory may have resulted in differences in colonisation of roots by mycorrhiza. It also became apparent later in this study that there were differences in nitrogen content of roots, partly associated with root aphid infestation. Nitrogen is an important resource limiting populations of soil fauna (Seastedt et al. 1988a) so the consequences of these differences cannot be ignored. These interactions have only become apparent over time, emphasising the need for long term studies on population dynamics of soil communities.

Whether or not the small but significant changes in soil biota impact on decomposition and nutrient cycling is a matter for further study. At the ecosystem level, biomass of Collembola is thought to represent no more than 15% of the total fauna even in early successional communities and disturbed soils where they are prevalent (Petersen 2002). Some studies have demonstrated increases in nitrogen mineralization in grassland soils in the presence of Collembola (eg. Bakonyi 1989; Bardgett & Chan 1999; Cragg & Bardgett 2001) while others have found no effect (Bardgett et al. 1993). Where effects occur they are thought to be indirect, resulting from changes in microbial populations and interaction with other soil biota (Filser 2002). Population density of Collembola is one factor influencing their contribution to the soil carbon and nitrogen dynamics (Filser 2002) and in the study here the high populations of Collembola are probably a reflection of an increase in microbial populations. Thus, pastures dominated by AR1-infected ryegrass, which is highly susceptible to root aphid, may have a

more active microarthropod and microbial community and higher rates of N mineralization than pastures dominated by AR37 which is resistant to root aphid. Any benefits this may have for AR1 plant growth, however, are likely to be offset by negative effects of the root aphid.

In their native environment and in an agricultural environment the influence these endophytes exert on the soil community may depend as much on the diversity of plants present as on the strain-specific effects of endophyte-infection on herbivory. If, as argued above, plants infected with AR37 are highly competitive, they are likely to be dominant members of the plant community. Under these circumstances their influence on the soil community could be expected to be disproportionately greater than that of the less competitive AR1. There are, however, a variety of factors that influence above and below-ground diversity and the former is not necessarily correlated with the latter (Hooper et al. 2000).

As pointed out in Chapter 5, the study is limited by what has not been measured and has asked more questions than it has answered. Five stand out as being particularly important: (1) What is the environmental significance of the apparent relationships between *A. lentisici* and Collembola and *A. lentisici* and nematodes and what mechanisms are involved in these interactions?; (2) What are the factors which have altered colonisation of plants by mycorrhiza and what are the implications of differences in mycorrhiza levels for plant growth and the plant/endophyte interaction?; (3) Given nitrogen may be a factor limiting soil fauna how do changes in the nitrogen concentrations of roots in relation to their biomass relate to activity of soil organisms? (4) Why did specific root length increase in insecticide-treated plants even in AR37 which was highly resistant to root aphid, and what does this mean for the plant? (5) Do the effects observed in microcosms here in reality have any effect on soil/plant community interactions over a larger spatial and temporal scale?

Possible mechanisms relating to some of these questions have been discussed already in Chapter 5 and will not be further discussed here. Despite increasing recognition of the importance of below-ground food webs, however, much has yet to be learnt on the mechanisms that drive them and the

consequences they have for the root ecology of individual plants and their communities. There is no doubt that the interactions involved are extremely complex but ryegrasses together with their endosymbionts make useful model plants on which to base further research.

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APPENDIX 1

Table 1. Log_{10} (n + 2) number of root aphid per plant on root outgrowth (RG) sampled four times and on main plant roots (MR) sampled once in May 2002 on ryegrass treated (TR) and untreated (UN) with insecticide in the Plant Growth Trial.

Endophyte	Treatment	April 01 RG	Sept 01 RG	Jan 02 RG	May 02 RG	May 02 MR
AR1	UN	1.517	2.016	0.537	1.640	2.253
AR37	UN	0.543	1.389	0.332	0.628	0.731
Wild-type	UN	1.213	1.722	0.615	1.205	1.569
Nil	UN	1.403	2.028	0.994	1.196	1.668
AR1	TR	0.675	0.867	0.321	0.392	0.423
AR37	TR	0.480	0.829	0.326	0.319	0.345
Wild-type	TR	0.725	0.893	0.301	0.404	0.365
Nil	TR	0.652	0.793	0.321	0.349	0.349
SED	End*Ins	0.1634	0.1827	0.0951	0.1629	0.1434
d.f.	End*Ins	128	69	97	90	87
F. prob.	Endophyte	<0.001	0.012	<0.001	<0.001	<0.001
	Insecticide	<0.001	<0.001	<0.001	<0.001	<0.001
	Endo*Ins	0.008	0.009	<0.001	<0.001	<0.001

Table 2. Log_{10} (n + 2) number of root aphid per g of root for root outgrowth (RG) sampled four times and main plant roots (MR) sampled once in May 2002 on ryegrass treated (TR) and untreated (UN) with insecticide in the Plant Growth Trial.

Endophyte	Treatment	April 01 RG	Sept 01 RG	Jan 02 RG	May 02 RG	May 02 MR
AR1	UN	2.095	1.866	0.423	1.710	1.526
AR37	UN	0.483	1.004	0.073	0.441	0.254
Wild-type	UN	1.325	1.388	0.771	1.200	0.867
Nil	UN	1.707	1.999	1.241	1.224	1.020
AR1	TR	0.440	0.475	0.031	0.119	0.054
AR37	TR	0.348	0.476	0.107	0.044	0.033
Wild-type	TR	0.498	0.489	0.000	0.162	0.024
Nil	TR	0.497	0.434	0.037	0.077	0.043
SED	End*Ins	0.2233	0.1738	0.1807	0.2094	0.1192
d.f.	End*Ins	126	75	94	74	97
F. prob.	Endophyte	<0.001	<0.001	<0.001	<0.001	<0.001
	Insecticide	<0.001	<0.001	<0.001	<0.001	<0.001
	Endo*Ins	<0.001	<0.001	<0.001	<0.001	<0.001

Table 3. Log_{10} (n + 2) number of root aphid per plant on root outgrowth and main plant roots for ryegrass plants treated or untreated with insecticide and under high or low nutrient treatments in the Nutrient Trial.

Endophyte	Treatment	Root outgrowth		Main roots	
	Ins/Nutrient	High	Low	High	Low
AR1	UN	1.611	0.724	2.645	1.110
AR37	UN	0.451	0.304	0.496	0.470
Wild-type	UN	0.919	0.403	1.677	0.614
Nil	UN	0.972	0.341	2.122	1.119
AR1	TR	0.618	0.475	1.035	0.540
AR37	TR	0.466	0.301	0.341	0.301
Wild-type	TR	0.469	0.341	0.586	0.341
Nil	TR	0.409	0.396	0.807	0.435
	d.f.	SED	F. prob.	SED	F. prob.
Endophyte	27	0.1027	<0.001	0.1590	<0.001
Nutrient	107	0.0583	<0.001	0.0711	<0.001
Insecticide	107	0.0583	<0.001	0.0711	<0.001
Endo*Nut	66	0.1317	0.195	0.1881	<0.001
Endo*Ins	66	0.1317	0.003	0.1881	<0.001
Nut*Ins	107	0.0824	<0.001	0.1005	<0.001
End*Nut*Ins	119	0.1758	0.108	0.2358	0.060

Table 4. Log_{10} (n + 2) number of root aphid per g of root on root outgrowth and main plant roots for ryegrass plants treated or untreated with insecticide and under high or low nutrient treatments in the Nutrient Trial.

Endophyte	Treatment	Root outgrowth		Main roots	
	Ins/Nutrient	High	Low	High	Low
AR1	UN	1.658	0.980	2.164	1.148
AR37	UN	0.428	0.300	0.402	0.476
Wild-type	UN	0.924	0.566	1.295	0.645
Nil	UN	1.504	0.391	1.892	1.281
AR1	TR	0.675	0.731	0.733	0.544
AR37	TR	0.506	0.301	0.308	0.301
Wild-type	TR	0.470	0.333	0.445	0.338
Nil	TR	0.458	0.650	0.641	0.528
	d.f.	SED	F. prob.	SED	F. prob.
Endophyte	27	0.1334	<0.001	0.1453	<0.001
Nutrient	107	0.0925	0.002	0.0703	<0.001
Insecticide	107	0.0925	<0.001	0.0703	<0.001
Endo*Nut	84	0.1868	0.717	0.1761	0.017
Endo*Ins	84	0.1868	0.096	0.1761	<0.001
Nut*Ins	107	0.1308	0.004	0.0994	0.002
End*Nut*Ins	131	0.2629	0.050	0.2253	0.145

Table 5. Log_{10} (n + 4) number of root aphid per plant on ryegrass infected with different endophytes and grown in two soil volumes and harvested on three different dates in the Root Biomass Trial.

Endophyte	Large soil volume			Small soil volume		
	Sept	Jan	June	Sept	Jan	June
AR1	2.246	2.589	1.832	2.277	2.811	2.276
AR37	1.227	0.843	0.845	0.924	0.733	0.662
Wild-type	1.358	1.684	1.620	1.143	2.115	1.654
Nil	2.312	2.206	2.472	2.240	1.740	2.322

	d.f.	SED	F. probability
Endophyte	36	0.1789	<0.001
Soil Volume	46	0.0774	0.716
Harvest Date	12	0.1871	0.741
Endo*Soil volume	61	0.2097	0.115
Endo*Harvest	46	0.3271	0.109
Soil volume*Harvest	18	0.2098	0.595
Endo *Soil vol*Harv	74	0.3780	0.603

Table 6. Log_{10} (n + 4) number of root aphid per g of root on ryegrass infected with different endophytes and grown in two soil volumes and harvested on three different dates in the Root Biomass Trial.

Endophyte	Large soil volume			Small soil volume		
	Sept	Jan	June	Sept	Jan	June
AR1	2.026	2.097	1.437	2.225	2.418	2.018
AR37	0.866	0.677	0.678	0.775	0.682	0.614
Wild-type	0.883	1.136	1.028	0.920	1.507	1.175
Nil	1.901	1.496	1.741	1.970	1.348	1.630

	d.f.	SED	F. probability
Endophyte	36	0.1297	<0.001
Soil Volume	46	0.0576	0.063
Harvest Date	12	0.1367	0.495
Endo*Soil volume	61	0.1531	0.032
Endo*Harvest	46	0.2377	0.065
Soil volume*Harvest	18	0.1538	0.790
Endo *Soil vol *Harv	74	0.2764	0.718

Table 7. Log_{10} (n+2) mean number of root aphid per plant in different ryegrass cultivars infected with different endophytes.

Endophyte	Culitvar		
	Nui	Samson	Impact
Nil	1.911	1.925	2.063
Wild-type	1.854	1.225	0.804
AR1	2.171	2.587	2.119
AR23	2.562		
AR6	0.631		
AR12		2.075	
AR22		2.440	
AR37	0.458	0.500	
	d.f.	SED	F. probability
	149	Min. rep. 0.3399	Endo: <0.001
		Max-min 0.2775	Endo*Cult 0.103
		Max. rep. 0.1962	

APPENDIX 2

Table 1. Log_{10} (n + 2) number of Collembola per plant on root outgrowth (RG) sampled four times and on main plant roots (MR) sampled once in May 2002 on ryegrass treated (TR) and untreated (UN) with insecticide in the Plant Growth Trial.

Endophyte	Treatment	April 01 RG	Sept 01 RG	Jan 02 RG	May 02 RG	May 02 MR
AR1	UN	2.512	2.812	2.005	2.247	2.676
AR37	UN	2.434	2.706	2.011	2.156	2.315
Wild-type	UN	2.488	2.863	2.095	2.159	2.473
Nil	UN	2.309	2.802	2.089	2.100	2.595
AR1	TR	0.915	1.546	1.097	1.795	1.053
AR37	TR	0.828	1.469	0.930	1.667	0.810
Wild-type	TR	0.821	1.458	0.929	1.782	0.895
Nil	TR	0.757	1.280	0.824	1.602	0.797
SED	End*Ins	0.1108	0.1158	0.1221	0.1318	0.1385
d.f.	End*Ins	96	86	72	87	64
F. prob.	Endophyte	0.099	0.221	0.569	0.218	0.004
	Insecticide	<0.001	<0.001	<0.001	<0.001	<0.001
	Endo*Ins	0.888	0.203	0.103	0.887	0.313

Table 2. Log_{10} (n + 2) number of mites per plant on root outgrowth (RG) sampled four times and on main plant roots (MR) sampled once in May 2002 on ryegrass treated (TR) and untreated (UN) with insecticide in the Plant Growth Trial.

Endophyte	Treatment	April 01 RG	Sept 01 RG	Jan 02 RG	May 02 RG	May 02 MR
AR1	UN	1.295	1.220	1.369	1.219	1.248
AR37	UN	1.135	1.320	1.328	1.315	1.026
Wild-type	UN	1.050	1.338	1.376	1.269	1.134
Nil	UN	1.242	1.424	1.371	1.225	1.239
AR1	TR	0.807	1.104	1.228	1.153	0.975
AR37	TR	0.708	1.050	1.255	1.200	0.810
Wild-type	TR	0.767	1.079	1.157	1.237	0.779
Nil	TR	0.558	1.055	0.898	1.215	0.821
SED	End*Ins	0.1089	0.0995	0.0814	0.1096	0.1258
d.f.	End*Ins	92	109	96	84	92
F. prob.	Endophyte	0.113	0.711	0.010	0.711	0.100
	Insecticide	<0.001	<0.001	<0.001	0.426	<0.001
	Endo*Ins	0.045	0.345	0.002	0.885	0.629

Table 3. Log_{10} (n + 2) number of nematodes (plus enchytraeids) per plant on root outgrowth (RG) sampled four times and on main plant roots (MR) sampled once in May 2002 on ryegrass treated (TR) and untreated (UN) with insecticide in the Plant Growth Trial.

Endophyte	Treatment	April 01 RG	Sept 01 RG	Jan 02 RG	May 02 RG	May 02 MR
AR1	UN	1.014	1.261	0.619	1.156	1.186
AR37	UN	0.938	1.242	0.615	1.162	1.293
Wild-type	UN	0.845	1.463	0.670	1.149	1.228
Nil	UN	0.875	1.441	0.718	1.018	1.167
AR1	TR	0.945	1.492	0.673	1.156	1.127
AR37	TR	0.881	1.384	0.746	1.239	0.959
Wild-type	TR	0.973	1.544	0.713	1.284	0.973
Nil	TR	0.885	1.452	0.670	1.358	1.130
SED	End*Ins	0.1349	0.0967	0.1159	0.1320	0.1831
d.f.	End*Ins	108	112	114	103	112
F. prob.	Endophyte	0.734	0.039	0.933	0.922	0.974
	Insecticide	0.967	0.025	0.432	0.073	0.074
	Endo*Ins	0.694	0.434	0.761	0.276	0.596

Table 4. Log_{10} (n + 2) number of collembola per plant on root outgrowth and main plant roots for ryegrass plants treated or untreated with insecticide and under high or low nutrient treatments in the Nutrient Trial.

Endophyte	Treatment	Root outgrowth		Main roots	
	Ins/Nutrient	High	Low	High	Low
AR1	UN	2.331	1.369	2.502	1.477
AR37	UN	2.205	1.354	2.475	1.579
Wild-type	UN	2.134	1.267	2.228	1.677
Nil	UN	1.956	1.201	2.281	1.660
AR1	TR	1.161	0.740	1.036	0.684
AR37	TR	0.972	0.649	1.046	0.649
Wild-type	TR	1.031	0.737	0.952	0.481
Nil	TR	0.739	0.578	0.865	0.504
	d.f.	SED	F. prob.	SED	F. prob.
Endophyte	27	0.1056	0.086	0.1182	0.697
Nutrient	107	0.0580	<0.001	0.0690	<0.001
Insecticide	107	0.0580	<0.001	0.0690	<0.001
Endo*Nut	64	0.1337	0.568	0.1533	0.677
Endo*Ins	64	0.1337	0.828	0.1533	0.868
Nut*Ins	107	0.0821	<0.001	0.0976	0.007
End*Nut*Ins	117	0.1771	0.997	0.2063	0.447

Table 5. Log_{10} (n + 2) number of mites per plant on root outgrowth and main plant roots for ryegrass plants treated or untreated with insecticide and under high or low nutrient treatments in the Nutrient Trial.

Endophyte	Treatment	Root outgrowth		Main roots	
	Ins/Nutrient	High	Low	High	Low
AR1	UN	1.992	1.516	1.415	1.161
AR37	UN	1.964	1.526	1.430	1.053
Wild-type	UN	1.949	1.514	1.136	1.032
Nil	UN	1.912	1.204	1.610	1.153
AR1	TR	1.702	1.201	1.257	0.692
AR37	TR	1.694	1.091	1.102	0.645
Wild-type	TR	1.536	1.252	1.175	0.997
Nil	TR	1.666	1.333	1.028	0.793
	d.f.	SED	F. prob.	SED	F. prob.
Endophyte	27	0.0901	0.877	0.0834	0.698
Nutrient	107	0.0537	<0.001	0.0601	<0.001
Insecticide	107	0.0537	<0.001	0.0601	<0.001
Endo*Nut	70	0.1179	0.677	0.1191	0.331
Endo*Ins	70	0.1179	0.185	0.1191	0.040
Nut*Ins	107	0.0760	0.436	0.0850	0.616
End*Nut*Ins	123	0.1595	0.321	0.1692	0.483

Table 6. Log_{10} (n + 2) number of nematodes (plus enchytraeids) per plant on root outgrowth and main plant roots for ryegrass plants treated or untreated with insecticide and under high or low nutrient treatments in the Nutrient Trial.

Endophyte	Treatment	Root outgrowth		Main roots	
	Ins/Nutrient	High	Low	High	Low
AR1	UN	1.542	1.505	1.298	1.934
AR37	UN	1.390	1.445	1.355	1.995
Wild-type	UN	1.480	1.484	1.139	2.071
Nil	UN	1.716	1.598	1.498	2.105
AR1	TR	1.371	1.666	1.034	1.781
AR37	TR	1.331	1.548	1.179	1.771
Wild-type	TR	1.213	1.563	1.522	1.902
Nil	TR	1.666	1.744	1.267	1.878
	d.f.	SED	F. prob.	SED	F. prob.
Endophyte	27	0.0999	0.061	0.1070	0.361
Nutrient	107	0.0621	0.092	0.0691	<0.001
Insecticide	107	0.0621	0.909	0.0691	0.058
Endo*Nut	74	0.1330	0.698	0.1449	0.973
Endo*Ins	74	0.1330	0.861	0.1449	0.266
Nut*Ins	107	0.0878	0.039	0.0978	0.382
End*Nut*Ins	126	0.1819	0.934	0.2003	0.339

APPENDIX 3

Table 1. Root outgrowth (g/plant) of ryegrass without endophyte (Nil) or infected with AR1, AR37 or Wild-type and treated (TR) or not treated (UN) with insecticide at six sampling times in the Plant Growth Trial.

Endophyte	Treatment	Sampling Time					
		Aug 00	Dec 00	Apr 01	Sept 01	Jan 02	May 02
AR1	UN	0.556	1.687	0.450	1.960	0.418	1.063
AR37	UN	0.606	1.833	0.877	2.918	0.361	0.788
Wild-type	UN	0.592	1.889	0.810	2.274	0.415	0.855
Nil	UN	0.569	1.421	1.023	1.155	0.342	1.082
AR1	TR	0.627	2.305	1.497	3.039	0.938	1.271
AR37	TR	0.573	1.864	0.985	2.522	0.612	0.688
Wild-type	TR	0.657	2.326	1.559	2.985	0.626	0.902
Nil	TR	0.578	1.904	1.428	3.085	0.774	1.072
SED	Endophyte	0.0572	0.2473	0.2274	0.3158	0.1059	0.2107
(d.f.)		(55)	(55)	(55)	(55)	(56)	(56)
	Insecticide	0.0304	0.1023	0.1087	0.1837	0.0567	0.1067
		(74)	(74)	(73)	(63)	(63)	(62)
	Endo*Ins	0.0716	0.2865	0.2744	0.4089	0.1328	0.2591
		(109)	(91)	(93)	(110)	(107)	(104)
F. prob.	Endophyte	0.838	0.313	0.475	0.253	0.303	0.181
	Insecticide	0.364	<0.001	<0.001	<0.001	<0.001	0.735
	Endo*Ins	0.583	0.215	0.019	<0.001	0.177	0.776

Table 2. Foliar growth (g/plant) of ryegrass without endophyte (Nil) or infected with AR1, AR37 or Wild-type and treated (TR) or not treated (UN) with insecticide at six sampling times in the Plant Growth Trial.

Endophyte	Treatment	Sampling Time					
		Aug 00	Dec 00	Apr 01	Sept 01	Jan 02	May 02
AR1	UN	1.241	6.68	4.29	4.37	3.312	4.34
AR37	UN	1.660	6.74	7.58	6.72	4.258	4.43
Wild-type	UN	1.333	6.29	5.43	4.69	3.401	3.85
Nil	UN	1.448	6.09	5.07	3.03	3.356	3.97
AR1	TR	1.291	7.60	7.40	6.43	5.278	5.22
AR37	TR	1.611	7.14	7.92	6.56	4.823	4.55
Wild-type	TR	1.438	6.62	7.06	5.82	4.303	4.55
Nil	TR	1.370	6.94	7.93	6.98	5.165	4.78
SED (d.f.)	Endophyte	0.1069 (55)	0.562 (55)	0.678 (55)	0.468 (55)	0.4273 (56)	0.517 (56)
	Insecticide	0.0371 (74)	0.230 (74)	0.290 (73)	0.208 (63)	0.1878 (63)	0.253 (62)
	Endo*Ins	0.1190 (81)	0.649 (90)	0.793 (93)	0.553 94)	0.5031 (95)	0.628 (101)
F. prob.	Endophyte	0.010	0.556	0.042	0.005	0.451	0.724
	Insecticide	0.853	0.008	<0.001	<0.001	<0.001	0.016
	Endo*Ins	0.276	0.725	0.004	<0.001	0.026	0.706