Synergistic defensive effects of silicon and *Epichloë* endophytes on insect pests of agricultural grasses in New Zealand

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Abstract

New Zealand has several severe insect pasture pests which cause economic losses of between $1.7 B and $2.3 B annually in the dairy, and 'sheep and beef' industries. Research on grass species defence provides insights into methods to reduce this economic impact. *Epichloë* endophytes and silicon accumulation are two well-studied mechanisms of reducing phytophagous insect damage in grass species, but their potential synergies have not been investigated. Previous research has hypothesised that plants infected with endophyte may accumulate more silicon than their non-endophyte infected counterparts, suggesting multi-tiered defences. Of specific interest to this thesis is the potential impact of silicon supplementation on the alkaloid profiles of novel grass-endophyte associations. This research aimed to investigate changes to silicon concentration over time, endophyte growth and alkaloid concentration in two cool-season grass species from the sub-family *Pooideae* and subsequent effects of these variables on major New Zealand insect pests. This interaction was investigated through the use of whole plant glasshouse trials, excised root bioassays, and artificial diet experiments using a range of above- and below-ground phytophagous pasture pests (*Listronotus bonariensis, Wiseana copularis* and *Costelytra giveni*) as well as a generalist herbivore model study organism (*Epiphyas postvittana*). The two grass species included; *Lolium perenne* infected with a novel association with an endophyte naturally found in *Festuca arundinacea* (*Epichloë coenophiala*), and *Festuca pratensis* infected with its naturally occurring endophyte (*Epichloë unicatum*).

Results from this research do not indicate that, in the grass-endophyte associations studied, endophyte infection is linked to an increase in plant silicon content. There is evidence to suggest that the herbage material of *L. perenne* endophyte-infected plants has less silicon than endophyte-free plants. There were no direct correlations between plant silicon content and the production of bioactive secondary metabolite alkaloids (lolines) or endophytic mycelial mass in *F. pratensis* and *L. perenne*. Although silicon supplementation was not linked to a direct increase in plant silicon content,

there was an increase in loline production in the herbage of *L. perenne*, indicating a potential role of silicon in either modulating the soil environment or influencing plant biochemical reactions potentially leading to a change in production of lolines. This study also found negative effects of endophyte alkaloids on insect performance and feeding which correlate to previous literature but was not able to accept nor reject the initial hypothesis of synergistic effects of the two defences studied. Interestingly, results suggested differential allocation of silicon between plant species and lolines within individual plant tissues. Silicon was higher in the root material of *L. perenne* than *F. pratensis* and the opposite was true for the herbage material. Also, the proportion of each loline in the root and herbage of *F. pratensis* differed. In summary, this research has provided insights into the temporal interactions between silicon and endophyte infection. However, further research is required to investigate the multi-tiered effects of these two plant defences on economically important phytophagous insects.

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1. Introduction

Grass species are of economic importance to the New Zealand pastoral sector, which contributes to a significant portion of the annual gross domestic product (GDP) (Ferguson *et al.,* 2019). It has been estimated that ryegrasses, the primary plant species in our pasture systems, are worth $14.6 B to the economy and insect herbivores have a significant negative impact on this value. Ferguson *et al.* (2019) estimates that the most common pasture insect pests cause losses of between $1.7 and $2.3 B per annum through herbivorous feeding and subsequent production loss. Recent changes to public acceptance and regulations of pesticide use highlights the need for increased research into utilising naturally occurring plant defences to reduce the economic impact of insect pests (Ricciardi *et al.*, 2017).

Silicon accumulation and *Epichloë* endophyte infection are two plant defences against insect herbivore feeding that are commonly found in grass species. Although a non-essential element in plants, silicon uptake by plants is known to protect from a range of biotic and abiotic stresses (Ma & Yamaji, 2008) and silicon is often present in quantities more than that of other essential elements (Epstein, 1999). In some species, known as hyper-accumulators, silicon content can be up to 10% of the dry weight of the plant. Plant silicon research commonly focusses on grasses from the *Poaceae* family (a common hyper-accumulator) and the consequences silicon has on the feeding of agricultural insect pests. Recent research has shown an association between elevated plant silicon and negative effects on a range of insects (Garbuzov *et al.*, 2011; Hunt *et al.*, 2008; Massey & Hartley, 2009; Ryalls *et al.*, 2017), including a below-ground herbivore (Frew *et al.*, 2017a; Frew *et al.*, 2016).

Grasses within the Poaceae family commonly form symbiotic relationships with endophytic fungi from the genus *Epichloë* (Schardl *et al.*, 1997). The production of bioactive alkaloids by the endophyte provides another line of defence from insect herbivores either through anti-feedant or toxic

properties (Clay, 1987; Johnson *et al.*, 2013). The defensive interactions between these two defences (silicon accumulation and endophyte infection) are poorly understood (Huitu *et al.*, 2014) and will be the focus of this thesis. This work focussed on the herbivory of common New Zealand pasture pests; particularly the below-ground feeding scarab beetle larva, *Costelytra giveni* (grass grub). Previous work indicated that plants infected with endophyte accumulated 16% more silicon, suggesting multi-tiered defences in grass species (Huitu *et al.*, 2014).

This thesis will investigate the synergistic defensive properties of endophyte infection and silicon supplementation in two grass species, *Lolium perenne* and *Festuca pratensis*. The *L. perenne* (perennial ryegrass; breedling line GPT12011) cultivar used in this study was infected with a loline-producing endophyte (*E. coenophiala*) that naturally occurs in *Festuca arundinacea* (tall fescue). The *F. pratensis* (meadow fescue; breeding line M1S9) cultivar was infected with a naturally-occurring loline-producing endophyte (*E. uncinata*). The first part of this thesis will examine whether the two defences have synergistic negative effects on a range of New Zealand pasture pests, including *Listronotus bonariensis* (Argentine Stem Weevil; ASW), *Wiseana copularis* (porina) and a specific focus on grass grub larvae. This information will provide insights into the potential use of a combination of the defences to improve current methods of control of insect pests, the cause of large economic losses in the New Zealand agricultural industry (Ferguson *et al.*, 2019). The second part of this thesis will explore the relationship between endophytes and silicon in grass species, investigating temporal changes in silicon concentration, endophyte growth, and alkaloid production. These results will provide insight into the interaction between the two defences over time and potential effects on insect herbivores.

1.1 Plant defences

Anti-herbivore plant defences are generally categorised into three classes; chemical, physical, and mutualistic (Vicari & Bazely, 1993). The resource availability hypothesis states that the defence strategies adopted by plants are determined by their growth rate (Massey *et al.*, 2007a). This hypothesis suggests that fast-growing plants typically invest in minimal anti-herbivore mechanisms whereas slow-growing plants, that would struggle to recover after herbivore damage, invest heavily in pest-resistant defences to prevent damage from occurring (Massey *et al.*, 2007a; Vicari & Bazely, 1993). Plant defences can be defined as being inducible or constitutive, the latter being beneficial in environments where the chance of attack by herbivores is high (van Dam, 2009). It was previously thought that grass species, which are adapted to grazing, rely on properties such as basal meristems and tillering that allow them to regrow and recover following herbivore damage, rather than investing in energetically demanding defence mechanisms (Huitu *et al.*, 2014).

Vicari and Bazely (1993) however, discuss a range of defences that grass species are known to employ. These defences are from all the plant defence categories and include silicon deposition, production of alkaloids, phenolics and hydroxamic acids, and interactions with endophytic fungi. Massey *et al.* (2007a) studied the defence mechanisms of 18 grass species. They found that different species allocated energy to different defence strategies, including silicon, phenolics and plant toughness. Of the plant species studied, silicon content was determined as the best predictor for feeding preference of *Microtus agretis* (voles), a mammalian herbivore. This study determined that cumulative defence scores were negatively correlated to plant relative growth rates, supporting the resource availability hypothesis (Massey *et al.*, 2007a).

1.1.1 Plant-herbivore interactions in a changing climate

Half of the described insect species in the world are phytophagous (Price *et al.*, 2011), and consequently plant-herbivore interactions are an important aspect of agriculture. It is estimated that worldwide production loss in the agricultural industry from insect feeding is equivalent to production required to feed more than 1 billion people (Birch *et al.*, 2011). Climate change is expected to alter insect pest feeding, increasing the damage they cause and consequent production losses. Changes to average temperatures, carbon dioxide levels (CO_2), and weather patterns are all expected to change plant-herbivore interactions (Riegler, 2018).

Deutsch *et al.* (2018) discuss two characteristics of insect pests that are relevant in a warming climate; metabolic rate and population growth. Increases in temperature cause an increase in metabolic rates of insects and hence an increase in consumption, as well as an increase in population growth. These characteristics are expected to have a more pronounced effect on insects present in temperate regions than those in tropical regions (Deutsch *et al.*, 2018). Other experiments show enhanced CO_2 typically alters the carbon to nitrogen ratio of plant material (Frew *et al.*, 2017a). This causes plants to be of lower nutritional quality to insects due to the dilution of nitrogen and consequently increases herbivory. Anticipated rises in CO_2 levels are predicted to cause a 40% increase in feeding per herbivore (Coley, 1998). Increased herbivory, alongside reductions in pesticide use, and a rapidly growing human population will collectively exacerbate the challenges associated with achieving global food security and sustainable agriculture (Riegler, 2018). These factors influence a need for increased insect pest research, investigating solutions for continued and improved productive, sustainable agriculture in a changing climate (Birch *et al.*, 2011; Coley, 1998; Gregory *et al.*, 2009; Thomas *et al.*, 2019).

1.2 Silicon

Silicon is the second most abundant element in the Earth's crust (Epstein, 1994). Within the plant kingdom, there is variability in silicon content (between species as well as within species) ranging from 0.1% to 10% dry weight of plants. Epstein (1999) notes that even values as low as 0.1% are comparable to values of other essential plant nutrients such as sulfur, phosphorus and magnesium. Except for diatoms and plants in the order Equisetales (horsetails), however, silicon is not regarded as an essential nutrient (Epstein, 1994). This is based on the guidelines of plant nutrient essentiality described in Arnon and Stout (1939). One of the three guidelines is that the nutrient is involved in plant metabolism (Arnon & Stout, 1939), for which there is currently no evidence with silicon (Ma, 2004). Epstein (1994) argues that it is not possible to determine if silicon is essential because silicon is difficult to remove completely from nutrient solutions. There is, however, a large amount of research demonstrating the benefits of increased silicon uptake (Coskun *et al.*, 2018; Epstein, 1994; Frew *et al.*, 2018; Ma, 2004). Epstein (1994) defined silicon as a 'quasi-essential' element.

Plants are commonly categorised into three broad groups based on their silicon content. Plants with silicon concentrations above 1% are considered accumulators, below 0.5% silicon content are categorised as non-accumulators and those in between are grouped as intermediates (Guntzer *et al.*, 2012). Differences do arise among genotypes of the same species (Deren, 2001; Epstein, 1999) and the availability of silicon in the soil determines the quantity absorbed by the plant (Henriet *et al.*, 2006). Silicon has been associated with protection from several biotic and abiotic stresses including drought, heat stress, salt stress, heavy metal toxicity, radiation damage, pathogens, and both mammalian and invertebrate herbivores (Epstein, 1994; Frew *et al.*, 2017b; Guntzer *et al.*, 2012; Ma, 2004). Silicon's role in providing plants with improved resistance from insect herbivores is a key feature of this thesis.

1.2.1 The silicon cycle

The global silicon cycle is an interaction of biological, chemical and geological processes. Silicates make up 90% of the Earth's crust and silicon is second only to oxygen in abundance (Epstein, 1994; Struyf *et al.*, 2009). Plants play a major role in the cycling of silicon, through both the uptake and storage of amorphous silica and the subsequent release into the soil from plant decomposition, and the weathering of silicate rock (Raven, 2003). Biological movement of CO_2 from the bulk atmosphere into the soil atmosphere during photosynthesis increases the soil's CO_2 concentration. The elevated concentration of CO_2 increases the rate that silicate rock is weathered and silicic acid ($Si(OH)_4$; the form in which plants are able to take up silicon) is then solubilised into the soil environment (Raven, 2003; Struyf *et al.*, 2009). $Si(OH)_4$ can either be absorbed by terrestrial plants or ultimately reach the ocean through transport in the soil water solution. In the ocean and other water bodies, silicon sustains the growth of diatoms, which use it to build their cell walls.

The silicon cycle is an important component in aquatic primary production as silicon concentrations determine the proportion of diatoms and phytoplankton (Struyf *et al.*, 2009). It was previously thought that the terrestrial silicon cycle was minimal compared with the oceanic. Conley (2002), however, estimated the global average uptake of silicon by terrestrial plant species (60-200 Tmol Si yr^{-1}) as potentially in the range of the global oceanic/diatom cycle (~240 Tmol Si yr^{-1}).

1.2.2 Uptake and storage of silicon in plants

Silicon is absorbed from the soil by plants in the form of $Si(OH)_4$ (Kumar *et al.*, 2017). Even though silicon is abundant in soils, it is commonly not in the soluble form required for plants to absorb it (Richmond & Sussman, 2003). The amount of $Si(OH)_4$ in soil is dependent on several factors including soil type, age, parent rock, pH, soil biota, and climate. Typical concentrations of $Si(OH)_4$ are between 0.1 and 0.6 mM in soil solution (Cornelis & Delvaux, 2016; Epstein, 1994). Once absorbed, silicon is deposited irreversibly

throughout all plant organs as amorphous silica (in the form of phytoliths) (see *Fig. 1.1*), including within cell walls (Richmond & Sussman, 2003).

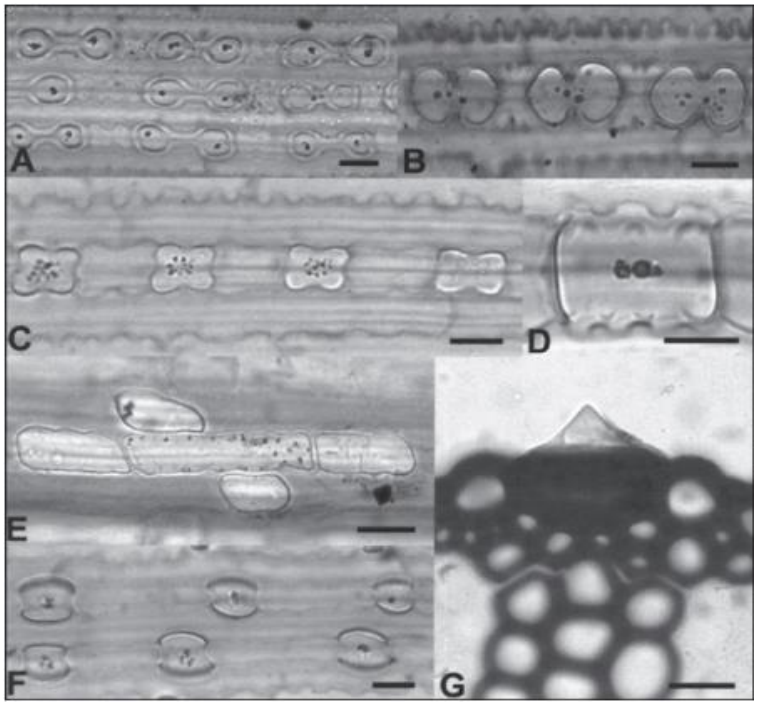

Figure 1.1: Variety of silica bodies found in the epidermal cells of Poaceae (Prychid & Rudall, 2003). *A, B, C, D and F bar = 10 µm, E and G bar = 20 µm.*

Kumar *et al.* (2017) determined that the most intensely silicified parts of grass species were the leaf epidermis, root endodermis, and the abaxial endodermis of inflorescence bracts. The morphology of silica bodies is highly variable throughout plant taxa (Prychid & Rudall, 2003), and the functional significance of these differences is not yet understood (Cooke & Leishman, 2011; Garbuzov *et al.*, 2011). Passive silicon deposition is often linked to transpiration (Trembath-Reichert *et al.*, 2015). Silicon deposition can also be linked to biological factors such as herbivore damage which elicits an active uptake of silicon (Guntzer *et al.*, 2012; Massey *et al.*, 2007b). Kumar *et al.* (2017) theorise that, in grass species, there is likely a combination of active and passive silicification, dependent on cell type.

The mechanisms of silicon uptake have primarily been studied in *Oryza sativa* (rice: a hyper-accumulator). $Si(OH)_4$ can move into the plant through both active and passive transport (Guntzer *et al.*, 2012). Deshmukh and Belanger (2016) discuss two key silicon transporters found in rice plants. The first is Lsi1 which belongs to a subfamily of aquaporins known as

nodulin-26-like proteins (NIPs) (Ma *et al.*, 2006) and secondly Lsi2, a transmembrane efflux transporter (Ma *et al.*, 2007). Lsi1 passively transports $Si(OH)_4$ whereas Lsi2 actively transports it, driven by a proton gradient. These transporters are found in the exodermis and endodermis of rice roots. Lsi1 transporters are on the distal side and Lsi2 on the proximal. The coupling of the two transporters within the same cell allows for the transport of $Si(OH)_4$ across the casparian strip and either into the cortex apoplast or the stele (see *Fig. 1.2*) (Ma & Yamaji, 2008). Once within the xylem of the stele, $Si(OH)_4$ can be transported throughout the rest of the plant (Deshmukh & Belanger, 2016; Ma & Yamaji, 2008; Ma *et al.*, 2007). Silicon is unloaded from the xylem through Lsi6 transporters, but the exact mechanisms for further transportation and deposition are not known (Ma & Yamaji, 2015).

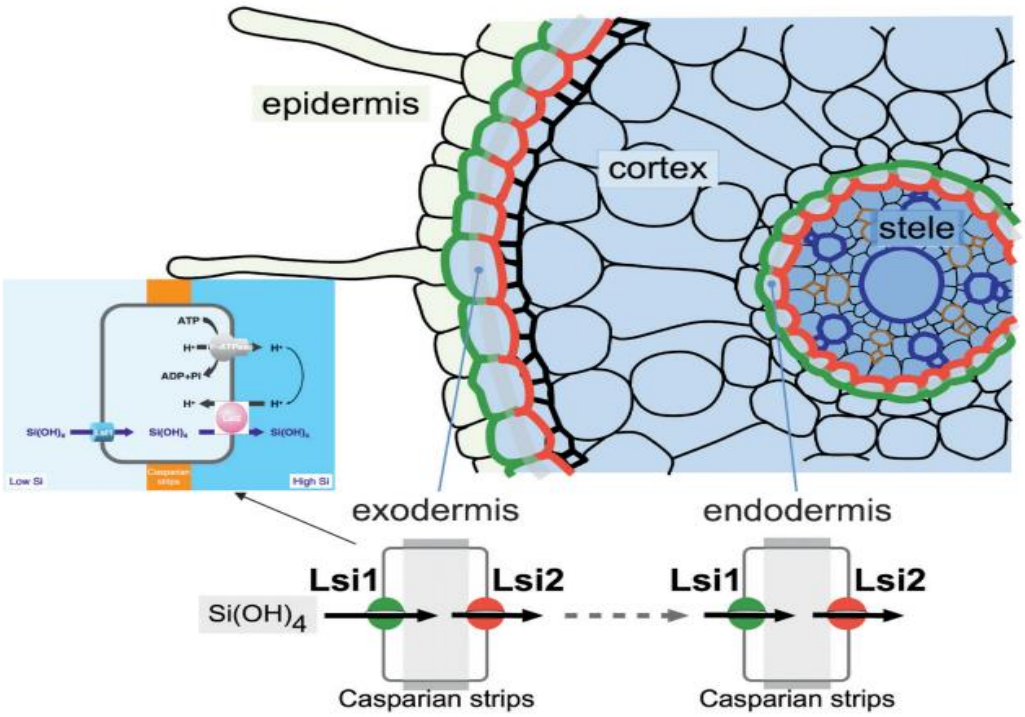

Figure 1.2: Model of silicic acid transporters in rice (Ma and Yamaji, 2008)

1.2.3 Silicon's role in the alleviation of abiotic and biotic stress

Silicon has been associated with the alleviation of both biotic and abiotic stresses in many plant species (Coskun *et al.*, 2018; Frew *et al.*, 2018;

Guntzer *et al.*, 2012; Ma, 2004). Coskun *et al.* (2018) discuss the apoplastic obstruction hypothesis, a mechanism for how silicon alters plant resistance to multiple stress sources. This hypothesis theorises that the deposition of amorphous silica in plant material leads to the suppression and promotion of numerous biological processes, leading to beneficial impacts. Biotic stresses include attack from fungal and bacterial pathogens, viruses, nematodes, parasites, and insect and mammalian herbivores. The physical barrier formed by phytolith deposition affects the ability of pathogens to invade plant tissues (Frew *et al.*, 2018).

However, research suggests that the physical deposition of silicon does not fully explain this mechanism of protection (Coskun *et al.*, 2018). Silicon may also affect the ability of effector molecules from pathogens and herbivores to reach their targets and subsequently affect plant chemical defences and/or the ability of the pathogenic organism to recognise the plant as a suitable host (Coskun *et al.*, 2018; Frew *et al.*, 2018). Some research shows that silicon acts as a signal to increase other plant defences, for example phenolics and jasmonic acid (Ma, 2004). Additionally, silicon can alleviate the severity of abiotic stresses including drought, salinity, radiation, extreme temperatures, metal toxicity and nutrient imbalances (Coskun *et al.*, 2018). The presence and production of reactive oxygen species is a key indicator of stress in plants (Ma, 2004). Studies have found an increase in antioxidant enzymes in plants supplemented with silicon, reducing the damage caused by stress-induced reactive oxygen species (Guntzer *et al.*, 2012).

The role of silicon in unstressed plants is under debate. It has previously been hypothesised that silicon does not affect plant metabolism in stress-free conditions (Ma, 2004). Brunings *et al.* (2009) however, investigated changes in gene expression of unstressed silicon-supplemented rice plants. The study found that silicon supplemented plants differentially expressed 221 genes compared to control plants. Of these 221 genes, 28 were involved in plant defence or stress pathways. The remainder were involved in primary metabolism, indicating that silicon is involved in more than just a stress response in rice (Brunings *et al.*, 2009). Frew *et al.* (2018) argue that stress-free environments for plants are uncommon and ‘stress’ is often

associated with basic metabolic processes. For example, reactive oxygen species are produced as a by-product during respiration and photosynthesis, indicating that silicon may have a role in basic metabolic functions (Frew *et al.*, 2018).

1.2.4 Silicon herbivore defence

A well-documented benefit of elevated plant silicon is improved protection from mammalian and invertebrate herbivores. Previous studies, as discussed below, have demonstrated this effect on a range of plant and herbivore species. Silicon was first suspected to be responsible for increased pest resistance in 1923 when authors hypothesised that wheat plants were resistant to the Hessian fly due to an accumulation of silicon (McColloch & Salmon, 1923). In recent years, there has been an increase in silicon defence research. Silica phytoliths are a hard material and elevate the abrasiveness of plant tissue, affecting the palatability to herbivores and having the potential to damage the mandibles, reducing their ability to continue feeding (Deren, 2001). However, in some insect species where the mandibles are replaced with each moult, damage to mouthparts cannot fully explain the negative impact that silicon has on their performance (Massey & Hartley, 2009). Sanson *et al.* (2007) found that silica phytoliths in grass species were softer than tooth enamel, rejecting the previously postulated hypothesis that silica bodies damage the teeth of grazing mammals.

The digestibility of plant material is affected by silicon, altering herbivores' ability to acquire essential nutrients and affecting the overall performance of the insect (Massey & Hartley, 2006). Nitrogen is often the limiting nutrient in insect diets and multiple studies have determined that silicon further reduces the availability of nitrogen for absorption by herbivores (Massey & Hartley, 2006, 2009). Massey *et al.,* (2009) found that insects acquired 34% less nitrogen when fed high-silicon diets. This study also demonstrated that the effects of a high silicon diet on an armyworm larva (*Spodoptera exempta*) were progressive and irreversible, even when the larvae were changed to a low silicon diet. Barker (1989) found that feeding by ASW was reduced in

two ryegrass cultivars as the silicon content increased. Deposition of silicon also negatively impacted the ability of female weevils to oviposit in leaf sheaths (Barker, 1989). Silicon can also affect the behaviour of insects' natural enemies. Kvedaras *et al.* (2010) demonstrated through olfactometer and field studies that natural enemies of *Helicoverpa armigera* were more attracted to high silicon supplemented plants previously infested by *H. armigera* than non-silicon supplemented infested plants.

Similar silicon concentrations in different species can result in varying herbivore deterrence properties. Garbuzov *et al.* (2011) studied feeding of a locust insect on two grass species. The authors found that silicon addition altered the consumption of *Poa annua* and *L. perenne* by a desert locust. In non-silicon supplemented plants, herbivory was higher on *L. perenne* than *P. annua*. The reverse was true when plants were supplemented with silicon, which resulted in a 4-fold increase in silicon for both species, even though silicon levels were similar between the species under the two treatments. The authors hypothesised that the form in which phytoliths are present in plants may contribute to the differences in herbivore resistance and more research is required to determine the functional importance of these differences (Cooke & Leishman, 2011; Garbuzov *et al.*, 2011).

Silicon accumulation is not just a passive process but an inducible plant defence that is upregulated following above-ground herbivore damage (Hartley & DeGabriel, 2016; Massey *et al.*, 2007b; Massey & Hartley, 2006). This suggests that silicon accumulation has an energetic cost to plants (Massey *et al.*, 2007a). Results from Massey *et al*., (2007b) showed that high silicon accumulators increased active transport in response to foliage damage. Induction was dependent on not only the type of damage but also the severity. Plants with continued damage every 3 to 4 weeks over the period of a year (16 damage events in total) had higher silicon concentrations than plants only treated with one damage event over the same period (Massey *et al.*, 2007b). In another study, Power *et al.* (2016) demonstrated that root-herbivory induced silicon accumulation. A limitation of this study was that only foliage silicon concentrations were recorded and not root concentrations. It has been observed several times that mechanical

damage does not induce the same silicon accumulation response as herbivore damage (Hartley & DeGabriel, 2016; Massey *et al.*, 2007b).

1.2.5 Evolution of silicon defences

Silicon accumulation is a cheaper alternative for structural support in plants compared to carbon-based components, such as lignin (Cooke & Leishman, 2011). Raven (1983) estimated that assimilation of carbon into lignin was ten to twenty times more energetically expensive than accumulation and deposition of silicon. It has been hypothesised that silicon accumulation evolved as an alternative to carbon during periods of low atmospheric CO_2 (for example during the Miocene) (Cooke & Leishman, 2011). Silicon-accumulating plants had a competitive advantage for structural support over non-accumulating plants which rely on carbon-based mechanisms. Stromberg *et al.* (2016) analysed published plant silicon records through comparative phylogenetic methods and found that all major clades of vascular plants contained taxa which were hyper-accumulators of silicon. The authors concluded that silicon accumulation has evolved independently in different lineages.

Stromberg *et al.* (2016) also determined that there is no evidence for grass-grazer co-evolution during the Cenozoic era (Cooke *et al.*, 2016; Stromberg *et al.*, 2016), contrary to discussions by McNaughton and Tarrants (1983). It was during the Cenozoic that high accumulators such as grasses came to ecological prominence (Trembath-Reichert *et al.*, 2015). The phytolith diversity seen in grasses today is theorised to be an adaptation to insect herbivores rather than mammalian grazers (Stromberg *et al.*, 2016). These authors also suggest that to understand differences in silicon accumulation and functional significance, phylogeny, as well as ecological information, and phytolith morphology/distribution need to be considered. The exact evolutionary history of silicon accumulation in plants is still unclear.

1.3 *Epichloë* Endophytes

Endophyte is the generic name for an organism living within a plant (Clay & Shardl, 2002). Fungal species of the genus *Epichloë* form endophytic symbiotic relationships with grasses from the *Poaceae* family, including perennial ryegrass, tall fescue and meadow fescue (Esqueda *et al.*, 2017; Sampson, 1933). The relationship is mutually beneficial for the two organisms through the exploitation of the partner (Saikkonen *et al.*, 2004). The endophyte gains nutrients, a habitat, and a mode of reproduction, while the plant benefits through increased growth, reproduction, and resistance to a range of abiotic and biotic factors (Saikkonen *et al.*, 2004). Specifically, resistance to herbivory is associated with the production of fungal alkaloids (Malinowski & Belesky, 2019). This is beneficial in the control of insect pests, although there are also potential health implications for grazing animals.

1.3.1 Growth and lifecycle

Epichloë endophytes grow intercellularly, parallel to plant cells in the leaf and stem tissue of the host (Clay, 1987; Easton, 2007). As the leaf grows and extends from the leaf primordia, the fungal hyphae also extend. There are two distinct life cycles of *Epichloë* endophytes, sexual and asexual (Bush et al., 1997; Johnson et al., 2013) (see *Fig. 1.3*). Species of endophytic *Epichloë* fungi were previously classified separately based on their reproductive strategy. Those that reproduce sexually were classified under *Epichloë* and asexual species under *Neotyphodium.* Recent changes to fungal nomenclature have classified all species under a single genus, *Epichloë* (Leuchtmann *et al.*, 2014). The asexual life cycle results in the transmission of the endophyte to the seed and the subsequent seedlings are infected with endophyte. The sexual lifecycle relies on the production of spores on a stroma which arrests development of the inflorescence on the affected tiller. This is often referred to as ‘choke disease’. There are some incidences where the endophyte utilises a mixture of both reproductive strategies (Bush et al., 1997; Clay & Shardl, 2002). *Epichloë* endophytes, both sexual and asexual, are not able to survive without the host plant

(Easton, 2007). The endophyte strains used in this research reproduce asexually.

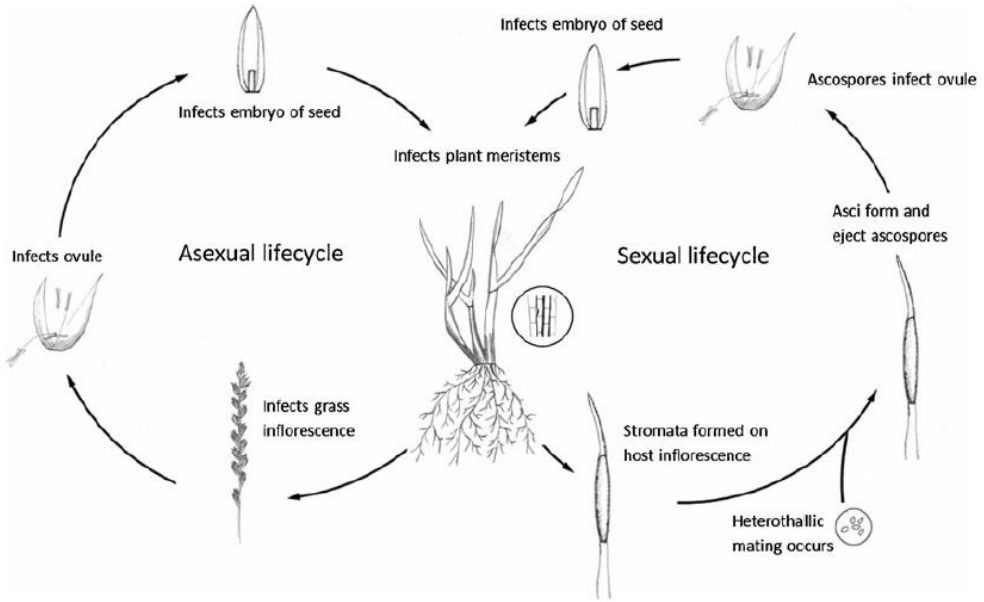

Figure 1.3: Diagram of Epichloë endophyte sexual and asexual lifecycles (Johnson et al., 2013)

1.3.2 Alkaloids

Alkaloids are nitrogenous secondary metabolites. There are several known bioactive alkaloid groups produced in grass-*Epichloë* associations, which have detrimental effects on the herbivory of both invertebrates and vertebrates (Malinowski & Belesky, 2019). These include ergot, loline, pyrrolizidine, and diterpene alkaloids (see *Fig. 1.4*) (Bush *et al.*, 1997; Johnson *et al.*, 2013). Synthesis of these secondary metabolites is controlled by the fungal genome. The complete profile of alkaloids produced is determined by the endophyte strain and host plant genotype association (Bush et al., 1997). The alkaloid composition produced is controlled by the strain of endophyte, and the concentrations are determined by the host plant genotype, hypha concentration, and environmental conditions (Malinowski & Belesky, 2019). As well as effects on invertebrates, some alkaloids are associated with stock health issues; for example, ergovaline is associated with tall fescue toxicosis and lolitrems, specifically lolitrem B, cause ryegrass staggers (Bush *et al.*, 1997; Fletcher, 1999). Lolines and peramine (pyrrolizidine alkaloid) have not been associated with mammalian toxicity

but protect from insect feeding (Bush *et al.*, 1997; Cooper, 1996; Johnson *et al.*, 2013).

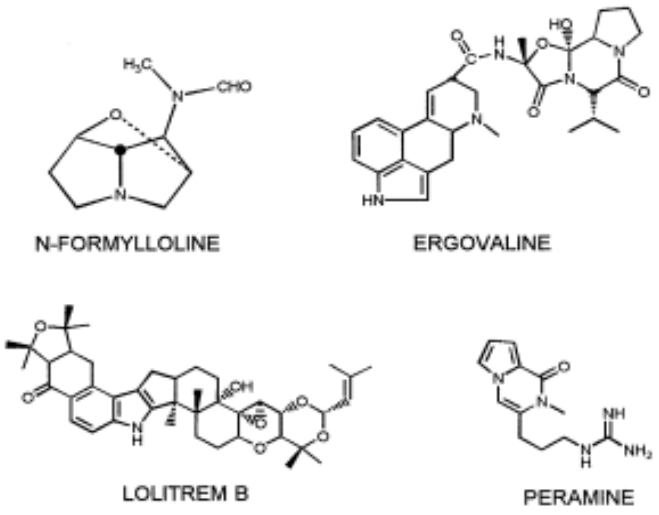

Figure 1.4: Representative alkaloid structures of each group (Bush et al., 1997). N-formylloline (loline), ergovaline (ergot), lolitrem B (diterpene), and peramine (pyrrolizidine).

1.3.3 Loline alkaloids

Loline alkaloids (see *Fig. 1.5*) are the alkaloid group of interest in this thesis and are associated with reduced insect herbivory in grasses (Popay & Tapper, 2007; Riedell *et al.*, 1991). Meadow fescue and tall fescue, infected with the endophyte strains *Epichloë uncinata* (formerly *Neotyphodium uncinatum*) and *Epichloë coenophiala* (formerly *N. coenophialum*) respectively, are known to produce loline alkaloids that protect from insect feeding (Malinowski & Belesky, 2019), including against grass grub (Patchett *et al.*, 2011b). Typically, there are four loline alkaloids produced; *N*-formyl loline, *N*-acetyl loline, *N*-acetyl norloline and *N*-methyl loline. Lolines are present not only in the foliage but are also transported to the roots, although concentrations are much lower in roots than herbage (Barker *et al.*, 2015; Bush *et al.*, 1997). Previous research has shown that plants differ in their loline concentrations and this appears to be dependent on the plant-endophyte combination (Patchett *et al.*, 2011b). Loline alkaloids are of interest to the pastoral industry of New Zealand because they are not toxic to livestock and have broad-spectrum activity on pests, particularly root-feeders for which there is a current lack of effective control. The

presence of lolines in root tissue has the potential to improve control of economically important pests such as grass grub. Patchett *et al.,* (2008) reported that loline concentrations in roots increased after feeding by grass grub, indicating that lolines are an inducible defence and are transported to the area of the plant under attack (Patchett *et al.*, 2008b).

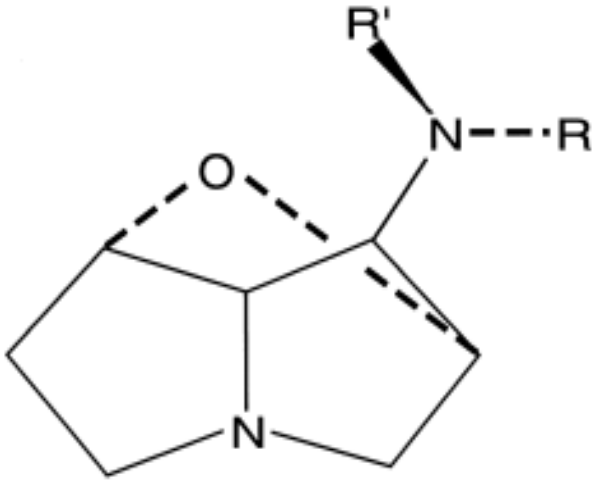

Figure 1.5: Basic chemical structure of loline alkaloids (Schardl et al., 1997)

1.3.4 History of endophytes in New Zealand

New Zealand pastures are primarily made up of ryegrasses (*Lolium* spp.) and clover species (*Trifolium* spp.) (Charlton & Stewart, 1999; Ferguson *et al.*, 2019). Perennial ryegrass (*L. perenne*) is the most widely used grass within New Zealand and grows well in a range of fertile, moist conditions but it does not perform well in drought (Charlton & Stewart, 1999). It was estimated by Nixon (2016) that ryegrasses contribute $14.6 billion to New Zealand's GDP annually and provide 75% of the nutritional needs for livestock in the agricultural industry. Hence, interactions with fungal endophytes are an important component when considering ryegrasses for pasture due to the effects on stock health and pest insect feeding (Johnson *et al.*, 2013; Rowan, 1993; Tapper & Latch, 1999).

Historically, stock health issues such as ryegrass staggers and heat stress (Easton *et al.*, 1996; Fletcher *et al.*, 1999) caused by the naturalised wildtype endophyte (also known as Standard or Common toxic endophyte)

in perennial ryegrass led farmers to plant pasture with seed containing no endophyte (Johnson *et al.*, 2013; Latch & Christensen, 1982). It was quickly apparent that pastures were failing, due to pressure from insect pests. The alkaloids produced in endophyte associations responsible for activity against insects were identified in the 1980s (Clay, 1987; Rowan, 1993; Siegel *et al.*, 1987). Research began to find endophyte strains with reduced mammalian toxicity effects while retaining the pest-resistant properties.

The introduction of two new *E. festucae* var. *lolii* strains of endophyte, AR1 and AR37, in ryegrass that are less toxic than the wildtype contributes $200 million to the New Zealand economy each year (Johnson *et al.*, 2013). AR1 produces only peramine, but not the two mammalian toxins, ergovaline and lolitrem B, and was quickly adopted after its release in 2001. By 2007 80% of perennial ryegrass seed sold was infected with AR1 endophyte. It soon became apparent that AR1 was not persisting throughout the country, namely the northern North Island, due to pests such as the African black beetle (*Heteronychus arator*) and root aphid (*Aploneura lentisci*). Another strain, AR37, was discovered and provided resistance from ASW, black beetle, root aphid, pasture mealybug (*Balanococcus poae*) and porina but occasionally resulted in ryegrass staggers. The staggers were less severe and did not last as long as those caused by wildtype endophyte (Popay & Hume, 2013; Thom *et al.*, 2012). The only identified bioactive compounds present in AR37 are epoxy-janthitrems. Due to the need for a strain that provided more resistance than AR1, AR37 was commercially released in 2007 (Johnson *et al.*, 2013). It is now the endophyte species most commonly planted in ryegrass cultivars by farmers. There is continued research to develop novel plant-endophyte combinations to provide broad-spectrum insect control with no effects on livestock (Easton, 2007; Gundel *et al.*, 2013). Meadow fescue, the other species of interest in this thesis, is not commonly used as a pasture grass as it performs poorly in many New Zealand agricultural systems (Cooper, 1996). Meadow fescue, however, forms a symbiotic relationship with the fungal endophytic strain *E. uncinata,* resulting in the production of loline alkaloids, and negative effects on insect herbivores investigated in this thesis (Jensen *et al.*, 2009; Patchett *et al.*,

2008a; Popay *et al.*, 2003). These effects include below-ground activity which is not found in *L. perenne* associations.

1.3.5 Novel loline-producing endophyte-grass associations

Endophyte research frequently focusses on the discovery of endophyte/grass associations which have little to no production of harmful alkaloids, while retaining insect deterrence properties (Malinowski & Belesky, 2019). These are referred to as novel associates or symbiotically modified organisms (SMOs) (Gundel *et al.*, 2013). Gundel *et al.* (2013) provide a meta-analysis of the literature on novel associations. The authors concluded that an improved understanding of these associations is required in order to fully exploit their beneficial use in agricultural systems (Gundel *et al.*, 2013).

Novel endophyte-grass associations (between grass species and fungi that do not naturally occur) typically have lower concentrations of beneficial alkaloids compared to native associations (Gundel *et al.*, 2013; Malinowski & Belesky, 2019). Ball and Tapper (1999) experimented with ryegrass inoculated with *E. coenophiala* (which naturally occurs in tall fescue). They found that only one of the loline alkaloids, *N*-formyl loline (NFL), was produced and it was present in lower concentrations than typically found in meadow fescue and tall fescue in the field (Ball & Tapper, 1999). Another study found that loline concentrations in these novel associations were approximately one-third of what would be expected to be found in tall fescue plants at the same time of year (Easton *et al.*, 2007). Continued research is required to identify novel-associations beneficial to the New Zealand agricultural industry.

This thesis investigates two grass species; meadow fescue (breeding line M1S9) and perennial ryegrass (breeding line GPT12011). The meadow fescue was infected with its naturally occurring endophyte (*E. uncinata*) and the ryegrass had been infected by an endophyte that is typically found in tall fescue plants (*E. coenophiala;* AR501); both produced loline alkaloids. Studies on loline alkaloids in meadow fescue have found that NFL is

consistently present in the highest concentrations of all the loline alkaloids (Patchett *et al.*, 2011c). The authors observed an increase in root loline concentration coupled with a decrease in shoot concentration, indicating that plants can mobilise and relocate loline alkaloids (Patchett *et al.*, 2011c). Meadow fescue infected with endophyte is known to be deterrent to the range of insect pests studied in this thesis (Jensen *et al.*, 2009; Patchett *et al.*, 2008b; Patchett *et al.*, 2011b; Popay & Lane, 2000), hence is used as a reference to compare against the novel association with ryegrass.

1.4 Invertebrate New Zealand pasture pests

The pastoral agricultural sector is of high economic importance in New Zealand. In 2011, it was estimated that the sector had a gross annual production value of $19.6 B and in 2016 accounted for 4% of the country's GDP (Anon, 2016; Ferguson *et al.*, 2019). The productivity of the industry is determined by a range of abiotic and biotic factors. One of the key influences is the damage caused by invertebrate pasture pests. It is estimated by Ferguson *et al.* (2019) that in an average year, these pests cause damage equivalent to between $1.7 B and $2.3 B. The majority occurs on dairy farms ($1.4 B) and the remainder on sheep and beef farms ($0.9 B). Included in this estimate is the impact of three major pests researched in this thesis; grass grub, ASW, and porina. Grass grub, a native scarab, is estimated to cause damage worth $140-380 M on dairy farms and $75-205 M on sheep and beef farms. This is New Zealand's most economically damaging pastoral pest. Porina is estimated to cause losses of $84 M and $88 M respectively. ASW, an exotic pest, results in damage of up to $200 M per annum total for both dairy and sheep and beef farms. Other pests included in the Ferguson *et al.* (2019) analysis are manuka beetle and black beetle, Tasmanian grass grub, clover root weevil, slugs, and parasitic root nematodes. Both black beetle and manuka beetle larvae are root feeders like grass grub. All contribute to economic losses in New Zealand's pastoral industry.

1.5 Thesis aims

The overall aims of this thesis are to:

(a) explore potential synergistic plant defensive effects of endophyte and silicon supplementation on New Zealand pasture pests;

(b) investigate temporal changes in silicon concentrations, endophyte growth, and alkaloid production.

Epichloë endophyte infection (Johnson *et al.*, 2013; Malinowski & Belesky, 2019) and silicon accumulation (Coskun *et al.*, 2018; Frew *et al.*, 2018) are two well-studied means of defence against insect herbivores in grass species. There is limited research investigating the potential synergies between them. Huitu *et al.* (2014) found that meadow fescue plants infected with endophyte had 16% higher silicon than those without endophyte. This study investigated the feeding of a mammalian herbivore (field vole) but did not specifically investigate the effects of silicon and endophyte in a multifactorial study design.

The first part of this thesis aims to investigate the synergistic interactions of endophyte and silicon on three major pasture pests in New Zealand; ASW, porina, and grass grub and specifically, the relationship with loline alkaloid producing endophytes.

Grass grub is the most economically damaging pasture pest in New Zealand and there is currently a lack of effective long-term control (Barratt *et al.*, 1990; Ferguson *et al.*, 2019). Loline alkaloids are known to have negative effects on grass grub larvae (Patchett *et al.*, 2011b). In some novel ryegrass-endophyte associations lolines are present in the roots but in low concentrations and therefore do not affect larval feeding. A current area of research is the association of tall fescue endophytes inoculated into perennial ryegrass cultivars with the goal of distributing lolines into the root tissue. The first part of this thesis will investigate the effect of these associations, in conjunction with silicon supplementation, on grass grub root feeding.

The second part of this thesis aims to investigate temporal changes in endophyte growth, loline alkaloid concentration, and silicon concentration. It is hypothesised that there is an interaction between endophytes and silicon in grass species, although very little is known about the relationship (Huitu *et al.*, 2014). This study aims to investigate how changes to both endophyte growth (mycelial mass) and silicon accumulation interact with the production of loline alkaloids in foliage and roots.

The hypotheses for this thesis are:

- Silicon and endophyte will have synergistic negative effects on the herbivory and performance of insect pasture pests;
- Silicon concentration will increase in root and foliage tissue over time, alongside an increase in endophyte mycelial mass, leading to the increased concentration of loline alkaloids in root tissue.

2. General Methods and Materials

Materials and methods described in this chapter are common to all experiments in this thesis.

2.1 Plant Maintenance

2.1.1 Soil

Top soil as the potting medium was purchased from Complete Landscape Supplies in Hamilton, New Zealand on 06/08/2018. A sub-sample of the soil was sent to Hill Laboratories for a basic soil test on 30/08/2018. For results refer to Appendix 8.1.

2.1.2 Accelerated ageing to produce endophyte free seed

Seed for each grass species (breeding lines GPT12011 and M1S9), containing their respective endophytes was set up for accelerated aging, a process which kills the endophyte within the seed, rendering it a 'nil' or endophyte free (EF) seed and thus the subsequent seedling will not be infected with endophyte (Card *et al.*, 2014). This allows plants of the same cultivars with (E+) and without (EF) endophyte to be utilised in experiments. Seeds were placed into labelled 60 mm Petri dishes, with the lid removed, and placed in a glass desiccator. The bottom of the desiccator contained a solution of glycerol and water (40:60) to maintain humidity at approximately 80% (Phil Rolston pers.comm). The desiccator was incubated at 40°C for 10 days. (see *Fig. 2.1*).

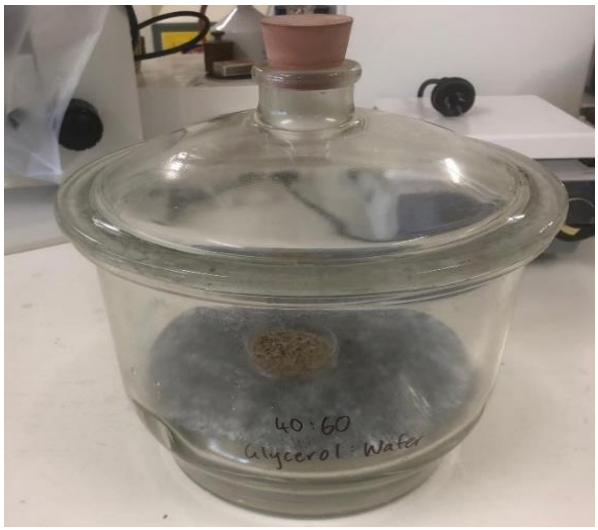

Figure 2.1: Seed set up (in 60 mm Petri dish) in desiccator containing glycerol and water for accelerated aging

2.1.3 Seed germination

Seeds, for each cultivar and endophyte status, were set up for germination in 90 mm Petri dishes on a piece of filter paper dampened with 1 mL of distilled water (see *Fig. 2.2*). Each Petri dish contained approximately fifty seeds and was sealed with Parafilm, then placed in a sealed container in a 20°C controlled environment room. Seeds were left to germinate for seven days, during which time they were monitored for moisture. Additional water was applied if the filter paper appeared to be drying out.

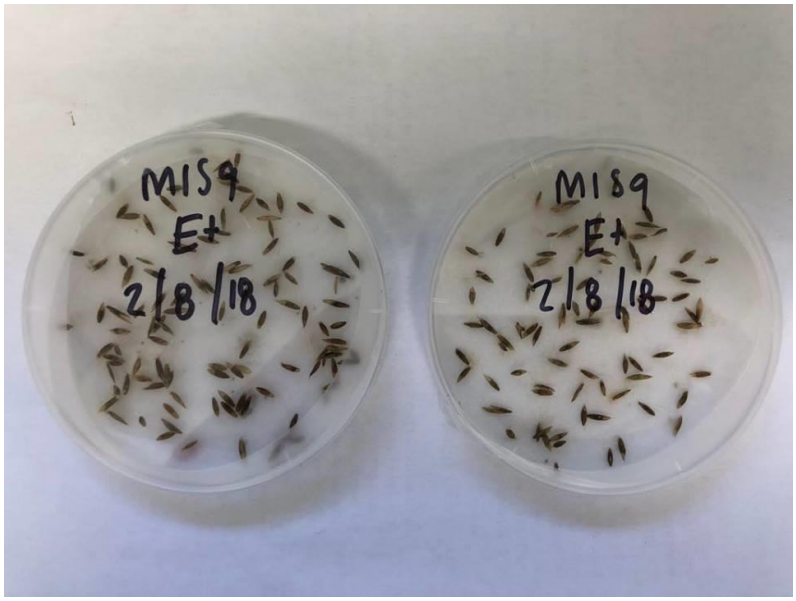

Figure 2.2: 90 mm Petri dishes set up with seed for germination. ~50 seeds in each dish

2.1.4 Establishment of plants from seed

After germination, seedlings were transferred to polystyrene trays filled with top soil (see *2.1.1*) in numbered positions. Each tray contained fifty plants and were left in a glasshouse to establish. Plants were watered, trimmed, and fertilised (see section *2.1.9*) as required to maintain strong plant growth.

2.1.5 Immunoblotting

Once plants had established and had several tillers (between 6 and 8 weeks of growth), they were tested for endophyte infection through a tissue print-immunoblot assay (Gwinn *et al.*, 1991; Simpson *et al.*, 2012). One tiller per plant was excised at the base of the plant, where fungal mycelium is concentrated. Any necrotic material was removed, and the moist freshly cut end of the tiller was pressed against a nitrocellulose membrane paper, leaving a circular mark. Immunoblots were developed by Jan Sprosen at AgResearch Ruakura using a method adapted from Simpson *et al.* (2012). The presence of mycelium is indicated by a bright pink result, while a light pink indicates that the blotted tiller is not infected with endophyte (see *Fig. 2.3*). These results were used to assign plants to treatments in subsequent experiments.

Figure 2.3: Developed immunoblot nitrocellulose paper. Dark pink and light pink results indicate presence and absence of endophyte in blotted tiller respectively

2.1.6 Cloning and potting of plants

Plants were split into two or three individual plants (cloned) to create plants with the same genotype either across or within experiments. Plants for cloning were selected based on immunoblot results and number of tillers (at least 10). Each clonal plant was of equal size and root mass, trimmed to 5 cm and potted into individual square pots (height – 180 mm, width – 90 mm) filled with soil, leaving a gap of 1 cm between the top of the pot and the soil surface. Plants were appropriately labelled with cultivar, endophyte status, and experimental assignment and placed in a screenhouse to establish (see *Fig. 2.4*). Temperatures were recorded with a data logger over the course of all experiments. Plants were watered, trimmed, and fertilised as required (see *section 2.1.9*).

Figure 2.4: Recently cloned potted plants. Coloured tags indicate plants to be supplemented with silicon

2.1.7 Treatments

There were eight treatments across all experiments (see *Table 2.1*). However, exact treatments in each insect trial varied slightly and are detailed in the corresponding chapter methods.

Table 2.1: List of plant treatments utilised throughout this project in two-way factorial design experiments. E+ = endophyte infected plants, EF = plants not infected with endophyte

Species	Endophyte	Silicon Supplementation
Ryegrass	E+	Y
		N
	EF	Y
		N
Meadow Fescue	E+	Y
		N
	EF	Y
		N

2.1.8 Silicon application

Silicon was applied to assigned plants three times weekly for 4-10 weeks, dependent on the experiment. Sodium metasilicate nonahydrate ($Na_2SiO_3 \cdot 9H_20$) was purchased from Sigma-Aldrich and was combined with tap water to make a solution of 500 mgL^{-1}. As required, 10 g of $Na_2SiO_3 \cdot 9H_20$ was weighed on an analytical balance (wearing safety glasses, a lab coat, gloves and a respiratory mask) and placed in a 20 L container which was then filled with tap water. Tap water likely already contained some silicon (Tulagi, 2011). Each plant that required silicon supplementation had 50 mL of this solution added to the soil surface three times weekly. All other plants (no additional silicon) had 50 mL of tap water, from the same source, added to the soil surface in the same intervals.

2.1.9 General maintenance

Plants were regularly trimmed, at least every 4 weeks, to 5 cm with scissors. Between plants, scissors were dipped in 70% ethanol to prevent the spread of any fungal or bacterial pathogens. Plants were also fertilised as required with 50 mL of solution containing Thrive™ fertiliser (9.4 g) and urea (9.8 g) dissolved in 9 L of tap water, added to the soil surface. Trimming and fertilising generally occurred on the same day. In the screenhouse,

automatic overhead watering occurred three times weekly. Plants were observed for any dry patches and watered additionally as required.

2.2 Harvest and preparation of plant material

2.2.1 Harvesting plant material

This section outlines the method used for harvesting plant material for further analyses (see *section 2.3*) in experiments throughout this thesis. Tillers were severed from the base of the plant and necrotic tissue was removed. Pseudostems were separated from leaf material (see *Fig. 2.5*) and placed in separate labelled plastic bags. Root material was extracted from the soil and rinsed under cold tap water to remove excess soil. Samples were squeezed and patted dry with paper towels to remove excess moisture before being placed in a labelled plastic bag. All samples were stored in a -20°C freezer to await further processing.

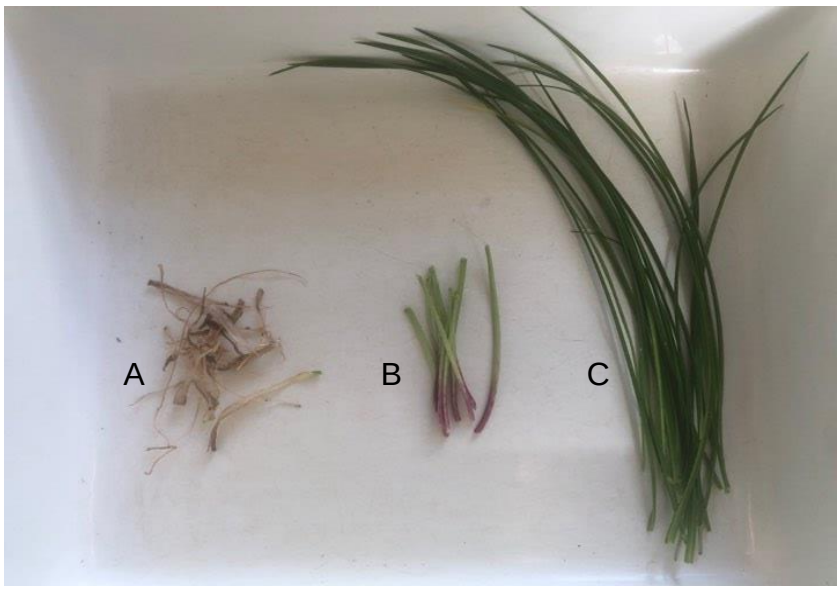

Figure 2.5: Grass plant split into; A) necrotic tissue, B) pseudostems and C) leaves

2.2.2 Preparation of plant material

Pre-frozen samples were freeze dried (John Morris Scientific Alpha-1-2-LDplus) over a period of 2-3 days until all moisture was removed. Samples were later ground into a fine powder using a ball mill (Retsch MM400) (see *Fig. 2.6*), for 60 seconds (27.5 Hz). If samples were not adequately ground, they were placed in the ball mill for an additional 30 seconds (27.5 Hz).

Freeze-dried and ground samples were stored in individual labelled plastic bags in a -20°C freezer until required for analysis.

Figure 2.6: Procedure for grinding plant material. A) Ball mill, B) Root material prior to grinding, C) Pseudostems prior to grinding and D) Pseudostems post grinding

2.3 Analysis of plant material

After plant material was ground into a fine powder, several analyses were performed, dependent on the sample type and experiment. The general methods for these analyses are outlined below. Analyses were performed on ground tissue of roots and pseudostems.

2.3.1 Silicon analysis

Silicon analysis was performed on both root and herbage material of bulked endophyte infected and endophyte free plants. All measurements were carried out using a PANalytical Epsilon 3 EDXRF spectrometer. The silicon standard used was a certified reference material from China National Analysis Centre for Iron and Steel (NCS ZC73018 – Citrus leaves), which has a silicon content of 0.41% ± 0.08. For the full output of the XRF analysis, refer to Appendix 8.2. Each XRF run had a silicon standard and nine samples (see *Fig. 2.7*). Plant material was placed in the small mass holder and evenly spread across the bottom surface, then pressed down with a small metal spatula. Care was taken to ensure that the entire bottom surface

was covered in plant material. Hiltpold *et al.* (2016) describes the method used.

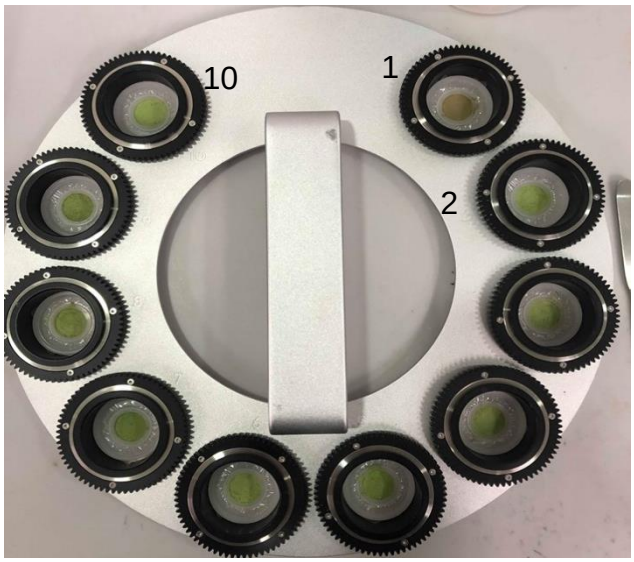

Figure 2.7: XRF sample holder. Position 1 - silicon standard, Positions 2-10 - herbage samples

2.3.2 Mycelial Mass

A subset of samples (herbage infected with endophyte) was analysed for mycelial mass by Jan Sprosen at AgResearch Ruakura. Initially, 20 mg of sample was extracted in a glass tube with 10 mL of phosphate buffered saline with tween (1%), samples were inverted to mix and incubated for three hours at 37°C. Test tubes were stored in the fridge overnight (4°C). An aliquot of 150 µL was taken for mycelial mass analysis through an indirect ELISA. Duplicate samples were run for a subset of the samples, and each sample had two dilutions analysed. The ELISA is a quantification of mycelium in the plant tissue and indicates endophytic growth (Ball *et al.*, 1995; Faville *et al.*, 2015).

2.3.3 Loline alkaloid analysis

A subset of samples (root and herbage infected with endophyte) were analysed by Wade Mace at AgResearch Grasslands for loline alkaloids through gas chromatography (GC-FID). The method for loline analysis is outlined in Bastias *et al.* (2018).

3. Initial screening of insect feeding on silicon-supplemented grasses using two above-ground herbivores

3.1 Introduction

Porina and Argentine Stem Weevil (ASW) are both above-ground herbivores that are economically important in the New Zealand pastoral industry. Collectively they are estimated to cause $372 M in lost production annually (Ferguson *et al.*, 2019). They both feed on pastoral plants, including perennial ryegrass (Goldson, 1982; Harris, 1969; Prestidge *et al.*, 1991) and are affected by the presence of loline alkaloids (Jensen *et al.*, 2009; Popay & Lane, 2000).

3.1.1 Porina

Porina is the common name given to a group of endemic moths from the genus *Wiseana* that are present in New Zealand pastures (Jensen & Popay, 2004). They are most prevalent in the South Island and lower North Island (Barratt *et al.*, 1990) (see *Fig. 3.1*), but their presence at damaging population levels is sporadic. The group consists of seven species; *Wiseana cervinata, W. copularis, W. fuliginea, W. jocosa, W.mimica, W. umbraculata,* and *W. signata.* Differentiation between the species on a morphological basis is difficult and instead is often based on molecular techniques (Brown

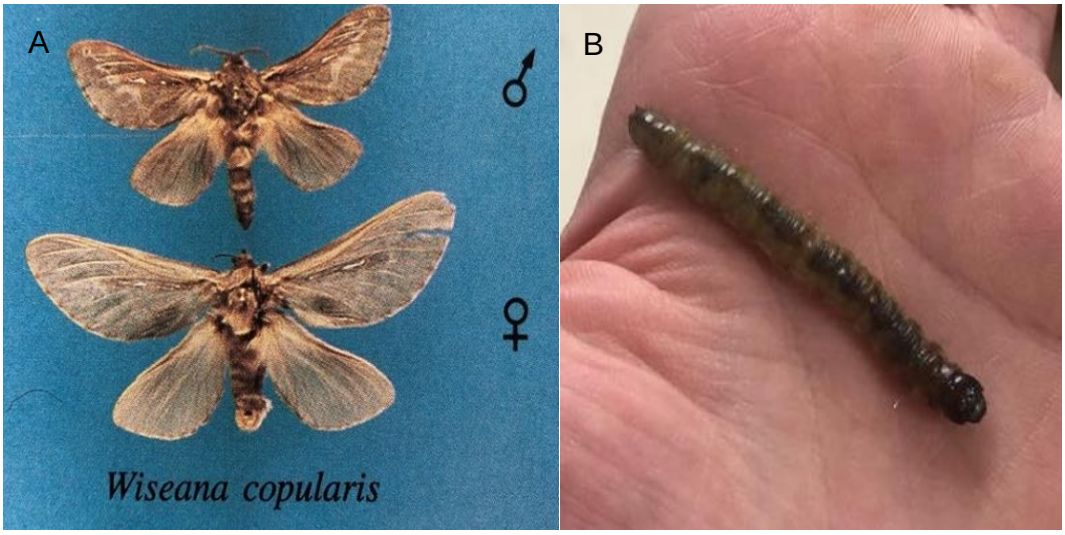

Figure 3.1: Porina A) Male and female adult W. copularis (Barratt et al., 1990). B) Large porina larva reared in colony at AgResearch Ruakura

et al., 1999; Richards *et al.*, 2017). The most abundant and damaging species are *W. cervinata* and *W. copularis* (Jensen & Popay, 2004); the latter is used in this experiment. The developmental biology of the various *Wiseana* species is similar (see *Fig. 3.2*), but they differ in phenology (Ferguson *et al.*, 2019). Therefore, the time of year that populations reach damaging levels is dependent on the specific species present (Barlow *et al.*, 1986).

Porina caterpillars are grazers at low densities (less than 40/m^2), but at high densities (more than 40/m^2) over-grazing results in plant death or 'denuding' (complete removal of plant cover so that soil is exposed). This further exacerbates production loss and the bare soil is susceptible to weed establishment. Ferguson *et al.* (2019) estimated the economic cost of porina/m^2 for dairy, and sheep and beef farms based on population density (see *Fig. 3.3*).

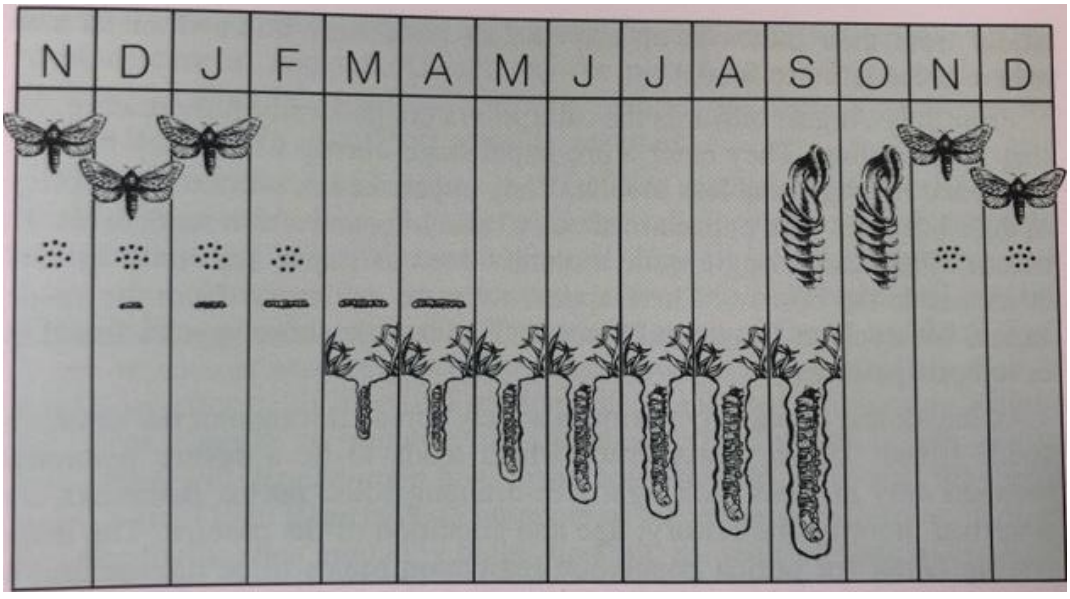

Figure 3.2: Diagram of general Wiseana spp. lifecycle (Barratt et al.,1990)

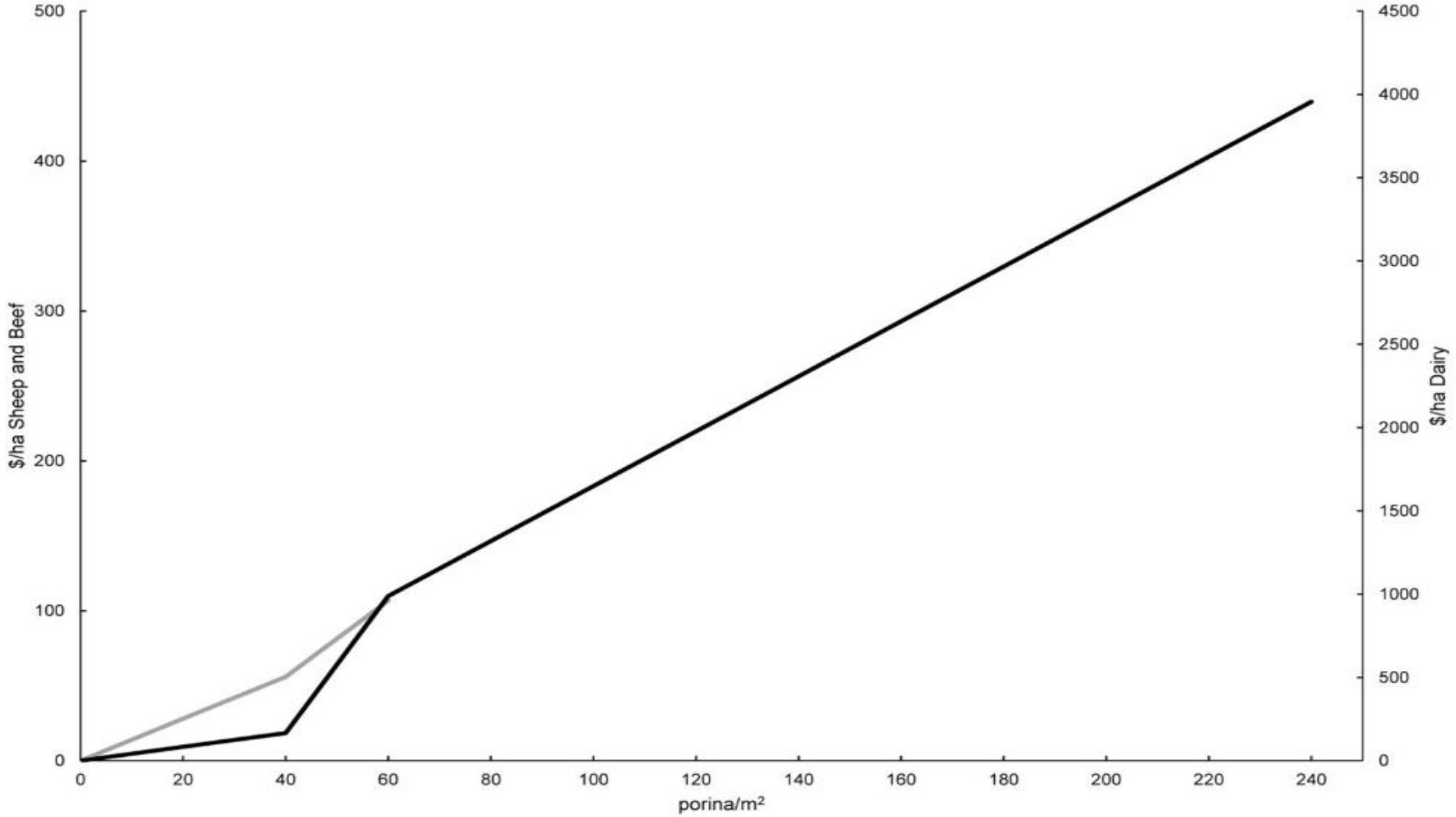

Figure 3.3: The cost of porina to sheep and beef, and dairy farms (based on 2012 prices). Change in slope between 20 and 40 porina/m^2 represents change from grazing to denuding (Ferguson et al., 2019).

3.1.1.1 General lifecycle

The *Wiseana* spp. life cycle takes one year to complete (see *Fig. 3.2*). Adult moths are present during spring, and summer depending on the species. The adult stage does not feed and is short lived, during which time they mate and females are able to disperse over 3,000 eggs through pasture (Barlow *et al.*, 1986; Ferguson *et al.*, 2019). Larvae hatch 4 to 6 weeks later and are present on the soil surface for several weeks in silk webbing (Stewart, 2001). The larvae then burrow into the soil to a depth of 10 to 30 cm, where they remain for the rest of their lifecycle, growing to a length of between 70 to 100 mm before pupation. During the damaging larval stage, each caterpillar will moult between 6 and 10 times over 7 to 8 months. Larvae feed at night by severing tillers at the base of the plant and dragging them back into their burrows to consume (Barlow *et al.*, 1986).

3.1.1.2 Methods of control

The main methods used to reduce damage caused by porina larvae are outlined below. Methods include use of fungal endophytes, naturally occurring pathogens, knowledge and disruption of species phenology, and insecticide application.

Porina larvae are affected by several bioactive compounds produced in grass-endophyte symbioses. Popay and Lane (2000) experimented with crude loline extracts incorporated into artificial diets. They found that loline concentrations of 500 and 1000 µg/g significantly increased larval mortality. Larvae also fed significantly less on loline containing diets than controls (Popay & Lane, 2000). The ryegrass endophyte AR37 was commercially released in 2007. The bio-actives in AR37 are known as epoxy-janthitrems and are also effective against porina larvae (Hennessy *et al.*, 2016; Jensen & Popay, 2004; Popay *et al.*, 2012).

There are naturally occurring pathogens, mostly viruses, which regulate porina populations. Host-pathogen interactions are disrupted by cultivation or sudden decreases in larval populations (Ferguson *et al.*, 2019). Young

pastures (2 to 4 years) are most susceptible to outbreak populations of porina when there is little control from natural pathogens.

Alternative methods of control require knowledge of the porina lifecycle as well as the *Wiseana* species present. For example, high pasture cover at the time of egg laying influences larval survival. When there is low cover, the eggs and newly-hatched larvae are prone to desiccation (Stewart, 2001). High stocking rates while young larvae are on the soil surface can also reduce populations. Knowledge of when larvae are likely to be present allow farmers to plan grazing schedules and reduce populations in particularly vulnerable pastures (Ferguson *et al.*, 2019).

Another common method of control is the use of insecticides. Diflubenzuron is a mimic insect hormone that prevents the caterpillars from moulting. Application is most effective in early stages because caterpillars moult more frequently (Ferguson, 2000). Diflubenzuron is a cost effective and relatively safe method but requires knowledge of insect life cycles to be most efficient. Because of this, farmers often use broad-spectrum organophosphate insecticides, which have larger safety risks, environmental consequences, and a higher cost (Ferguson *et al.*, 2019).

3.1.2 Argentine stem weevil

Argentine stem weevil (ASW) was introduced to New Zealand from South America in the early nineteenth century and is now present in pastures throughout the country (Prestidge *et al.*, 1991). It was not until the 1950s that the damage caused by ASW in pastures was recognised (Goldson *et al.*, 2005). The adult weevil feeds on the leaves of grasses (see *Fig. 3.4A*) but the larval stage is the most damaging. Larvae mine the central part of the grass stem (see *Fig. 3.4B*) often resulting in death of the tiller (Ferguson *et al.*, 2019). Newly sown seedlings in pasture are the most susceptible to damage by existing populations of ASW (Prestidge *et al.*, 1991).

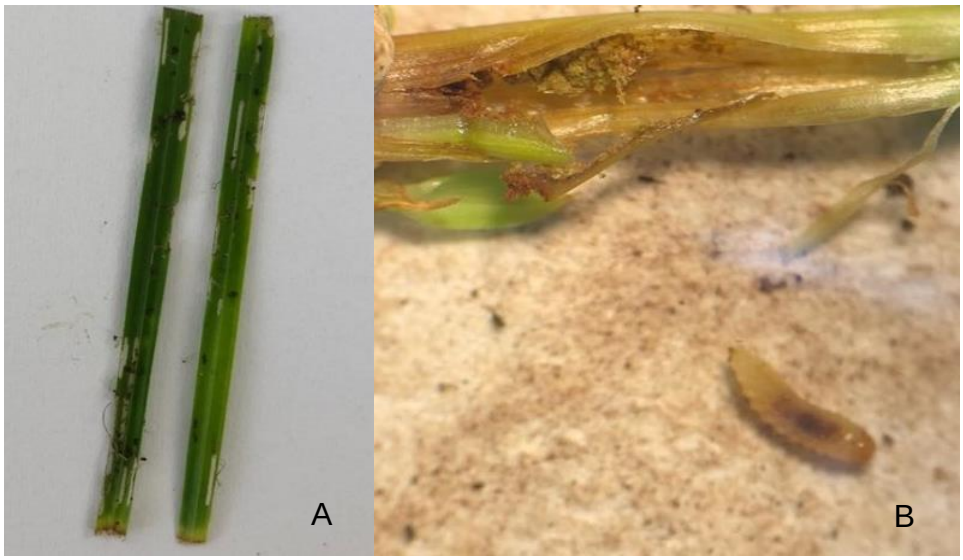

Figure 3.4: A) Windowing damage on ryegrass tillers caused by adult ASW. B) Ryegrass stem mined by ASW larva. Larva pictured below stem

3.1.2.1 Lifecycle

Adult ASW are present in pastures throughout the year but overwinter in a state of reproductive diapause (Barker & Pottinger, 1986; Goldson, 1981). The number of generations that the weevil completes each year depends on the region. In the warmer northern North Island the weevil can complete three generations, but this is reduced to two in Canterbury and Otago due to lower average temperatures (Ferguson *et al.*, 2019). The number of generations completed influences the pest status of ASW in specific regions, because more generations result in more damage. Female adults lay eggs under the leaf sheath on grass tillers (see *Fig. 3.5*) and the emerging larvae

Figure 3.5: ASW eggs under leaf sheath. Photo credit: L. M. Hennessy

mines through the tiller, often causing damage to the basal meristem resulting in no regrowth of the tiller. A larva completes four instars and is capable of destroying 3 to 8 tillers before pupation (Ferguson *et al.*, 2019). Interestingly, Barker (1989) found that the number of eggs laid by ASW was negatively correlated to the number of silica deposits per mm^2 in two cultivars of *Lolium multiflorim.*

3.1.2.2 Methods of control

Control of ASW in New Zealand is an example of integrated pest management (IPM). There are two biocontrol agents currently utilised, endophyte-infected grass species and a parasitic wasp (*Microctonus hyperodae*) (Ferguson, 2000), albeit in some areas ASW continues to be an economically important pest (Popay *et al.*, 2011).

The use of novel endophyte strains in ryegrass and their associated alkaloids aid in the control of ASW in New Zealand. Peramine concentration has a significant negative relationship (as peramine increases, feeding decreases) with adult feeding. There is also a less pronounced effect on reducing larval damage (Popay & Wyatt, 1995). AR37, which produces epoxy-janthitrems, is not active against adults but has a strong effect on larvae. Loline alkaloids reduce larval damage and adult oviposition but have little effect on adult feeding (Jensen *et al.*, 2009; Popay *et al.*, 2009).

The biocontrol agent *M. hyperodae* was successfully introduced to New Zealand to control ASW in 1991 (Goldson *et al.*, 1994). The adult wasp attacks the adult weevil, laying an egg within the weevil. The parasitoid larva develops inside the weevil and eventually kills the weevil host when it emerges (Loan & Lloyd, 1974; Popay *et al.*, 2011). In areas where ASW were previously most damaging, the release of *M. hyperodae* significantly reduced egg and larval populations quickly after release. Barker and Addison (2006) reported parasitism rates between 75 and 90% in overwintering adults in the North Island three years after release of the parasitoid. Establishment was slower in other areas such as Canterbury (Goldson *et al.*, 1998). The stability of the parasitoid-stem weevil association

is currently under question as parasitism rates across the country have declined (Goldson & Tomasetto, 2016; Goldson *et al.*, 2014a; Goldson *et al.*, 2014b; Tomasetto *et al.*, 2017).

Another major concern with ASW is that populations cause severe damage to newly-sown pastures. To combat this insecticides are often coated on to seed which helps to supress populations during initial pasture development (Ferguson *et al.*, 2019). This method increases the cost of re-sowing pastures and there is an ongoing decline in the public acceptance of insecticide use which may limit the use of this method in the future (Ricciardi *et al.*, 2017).

3.2 Methods

Leaf material for both experiments was collected from plants that had been supplemented with silicon for a period of four weeks (see section *2.1.8*). The first application was on 11/02/2019 and the final on 08/03/2019, totalling 12 silicon applications. Both ryegrass and meadow fescue treatments were used in the porina bioassay and only ryegrass treatments in the ASW experiment (see *Table 2.1*).

3.2.1 Porina artificial diet bioassay

3.2.1.1 Porina larvae collection and sorting

Porina larvae were collected from a colony maintained at AgResearch Ruakura (see *Fig 3.1B*). Adult female moths were caught near Mosgiel in Otago and identified as *W. copularis* by Colin Ferguson (AgResearch, Invermay). Eggs were collected and transported to Ruakura where they were surface sterilised (Carpenter, 1983) and set up in 90 mm Petri dishes with damp filter paper to hatch at 20°C (08/11/2018). Once hatched (23/11/2018), first instar larvae were placed in plastic containers half filled with bark and fed a semi-artificial diet which was replaced weekly (Popay, 2001). Containers were kept in a 15°C controlled environment room until late February when they were transferred to a 10°C incubator to slow the growth of larvae.

On 26/03/2019, 150 larvae were removed from colony containers and individually placed in 30 mL specimen containers. These were covered in a damp paper towel to maintain humidity and stored in a cool box for 24 hours in a 10°C incubator. The following day larvae were weighed individually on an analytical balance (Mettler Toledo XS204). Any soil or frass was removed prior to weighing. Larvae weighed between 114 and 511 mg. Larvae were assigned to treatments and replicates based on weight. They were allocated so that all treatments in a replicate had larvae of a similar weight. The larvae within a replicate were then randomly assigned to a treatment to ensure that the lightest larvae were not always in the same treatment. There were 15 replicates for each treatment, including a negative control group of larvae that were subjected to the same conditions but received no diet throughout the experiment. Once assigned to a treatment, larvae were placed into a corresponding labelled 70 mL specimen vials ¾ filled with bark (Bloom Decor Bark, Grade: Fine) (see *Fig. 3.6*). The vials were arranged by treatment and stored in polystyrene trays at 10°C for the remainder of the experiment.

Figure 3.6: Example 70 mL specimen vial 3/4 filled with bark

3.2.1.2 Diet Preparation

Herbage was collected on 12/03/2019 from a set of 10 plants for each treatment and freeze-dried and ground (see *section 2.2*). Samples for each treatment were separated into two vials containing 3 g each and stored in a -20°C freezer until required for diet preparation.

A semi-synthetic diet like that described in Popay (2001) was made and fed to the porina larvae over a period of two weeks. Fresh carrot (250 g) was chopped into small cubes and blended with 500 mL Milli-Q water until the mixture was homogenous. This was then strained using a sieve and 375 mL of 'carrot water' was obtained, to which 6.25 g agar was added and heated in a microwave to boiling point in a 1 L beaker. The diet was left to cool to 70°C, stirring occasionally.

Diet was weighed (27 g) into 100 mL beakers labelled for each treatment. Beakers had been warmed on a hot plate to ensure the agar did not set too quickly. To this, 3 g of the corresponding herbage was added and thoroughly mixed before being evenly spread into a 90 mm Petri dish. Diets were stored in a cool box until plugs of diet were taken using a 1 cm cork borer, weighed, and placed in the 70 mL specimen vials with porina larvae. Remaining diet was sealed with Parafilm, wrapped in tinfoil and placed in a refrigerator (4°C). A set of three diet replicates for each treatment, without larvae, was set up to look at moisture gain/loss of the diet in the bark and vial environment. Diet was made fresh for each week, on 27/03/2019 and 03/04/2019 and was enough for a diet change mid-way through the week. Once the larvae and diet were both in the 70 mL vial, the polystyrene trays were covered in dark cloth to block out light and placed in a 10°C incubator.

3.2.1.3 Experiment Assessment

During the experiment there were four assessment dates (31/03/2019, 03/04/2019, 06/04/2019 and 10/04/2019). On each assessment day any remaining diet was cleaned of debris and weighed to determine how much the larvae had fed. Fresh diet (either from the fridge or made that day) was weighed and added to the vial, except on the final assessment (10/04/2019).

For this, remnant diet was weighed, specimen vials were emptied, and the larvae were returned to the vial for 24 hours in the 10°C incubator and subsequently weighed on 11/04/2019. Samples of fresh and remnant diet were collected and stored in -20°C freezer for potential loline analysis during each assessment. The vials containing larvae and no diet were checked for survival at each assessment. All weights for this experiment were done on an analytical balance (Mettler Toledo XS204).

3.2.2 ASW leaf blade experiment

3.2.2.1 ASW collection and sorting

ASW adults were collected, using a modified blower-vac with a mesh collection bag inserted in the pipe, from Ruakura Research farm on 06/03/2019. A total of 60 weevils were collected from the suction sample and were placed into individual 30 mL containers with no food. Weevils were kept in a cool box for 24 hours in a 20°C controlled environment room and then weighed using an analytical balance on 07/03/2019 (Mettler AT20 FACT).

Due to the small number of weevils obtained from suction samples, a limited number of treatments and replicates (n=10) were set up. Those selected included all four treatments for ryegrass (see *Table 2.1*) and a negative control to assess change in weevil weight with no plant material. ASW were assigned to replicates and treatments based on weight. The lightest were in replicate one and the heaviest in replicate 10. The weevils within each replicate were then randomised across treatments to ensure that the lightest of that replicate was not always assigned to the same treatment.

3.2.2.2 Experiment set-up and assessment

ASW were placed into labelled plastic vials (height: 5 cm, diameter: 1.5 cm) along with two sections of leaf (4 cm in length) of the appropriate treatment (see *Fig. 3.7*). Leaf sections were taken from second oldest leaf (first being the outermost) and from the middle of the length of the leaf. All vials were placed in a larger plastic container in a randomised block design, covered

in tinfoil to prevent light penetration and kept in a 20°C controlled environment room for the course of the experiment.

There were two assessments of this experiment. The first assessment after 4 days (11/03/2019) involved removing leaf sections from vials and visually scoring them for damage by counting the number of leaf scars. Preliminary studies compared the use of scanning leaves and assessing leaf area damaged on computer software, and visual scoring. This experiment determined that the two methods were significantly correlated ($R^2 = 0.934$) (Popay & van Amsterdam; unpublished, 2018). Therefore, the application of visual scoring is a valid and accurate method. The plant material was replaced with fresh material from the same plant and any excess moisture in the vial was wiped away with a tissue. Negative control tubes were opened, and any moisture was removed for consistency across treatments. The same method for scoring damage was repeated on 14/03/2019, no fresh material was added and the ASW were left in the empty vials, covered in tinfoil, for 24 hours in a 20°C controlled environment room. ASW were then reweighed on 15/03/2019 using the same analytical balance. Preliminary studies found significant differences in ASW weight change when fed leaves with and without endophyte (Popay & van Amsterdam; unpublished, 2018).

Figure 3.7: ASW adult pictured in plastic vial with two 4 cm ryegrass leaf segments

3.2.3 Silicon and alkaloid analysis

Plant material for both experiments was sourced from the same plants. Pseudostem material from these plants was analysed for silicon and loline concentrations (see *section 2.3*). Plant material was bulked together (n=3) to allow for enough material for analysis.

3.2.4 Statistical analyses

Data were analysed using GenStat (v.19). Consumption (porina), feeding scars (ASW), insect weight change and silicon concentrations were analysed by general ANOVA and treatment blocked by species, endophyte status, and silicon supplementation. Data for insects that died during the experiments were removed from analysis.

3.3 Results

3.3.1 Porina artificial diet bioassay

Larval survival throughout the experiment was relatively equal across treatments (see *Table. 3.1*). There did not appear to be a toxicity effect of any of the treatments. Data from larvae that died during the experiment was removed before analysis. There was no significant difference between moisture loss/gain of control diets between treatments (*P = 0.871*).

Table 3.1: Number of dead porina larvae per treatment out of 15 larvae per treatment

Species	Endophyte	Silicon Supplementation	Number of dead larvae
Ryegrass	E+	Y	2
		N	1
	EF	Y	0
		N	0
Meadow Fescue	E+	Y	1
		N	3
	EF	Y	1
		N	2
Negative control			2

There was no interactive effect of endophyte infection and silicon supplementation on the total diet consumption of porina larvae. Additionally, there was no effect of silicon supplementation on diet consumption (see *Table 3.2*). There were significant differences between species and endophyte status. Larvae fed the least on endophyte-infected (E+) meadow fescue and the most on endophyte-free (EF) meadow fescue, 397 mg and 982 mg respectively, with feeding on perennial ryegrass with and without endophyte not significantly different from EF meadow fescue. There was no significant difference between average consumption for larvae fed E+ and EF ryegrass diets (see *Fig. 3.8*).

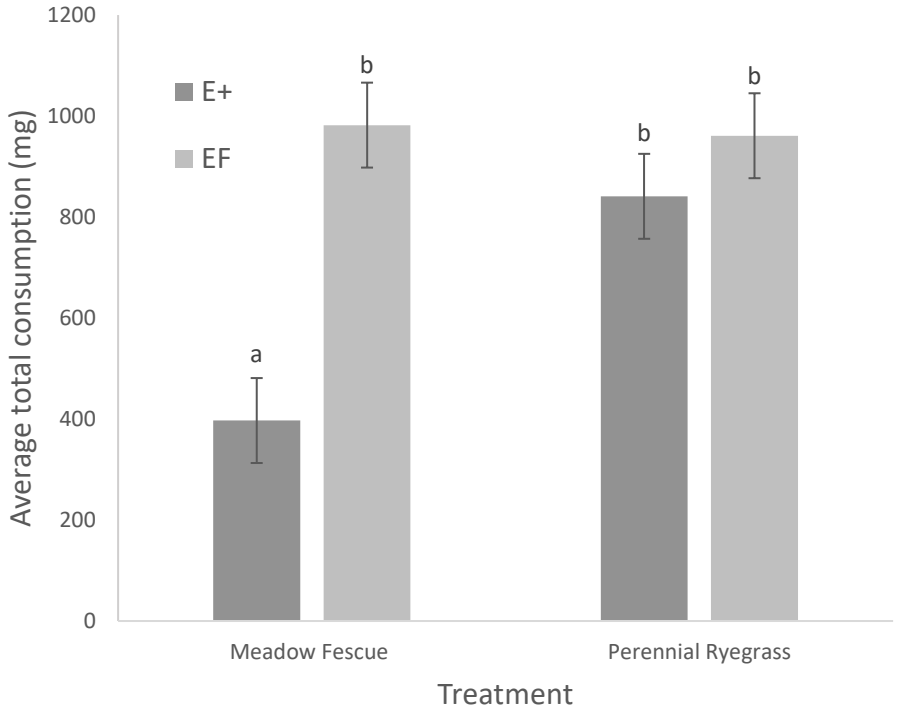

Figure 3.8: Average total consumption (mg) of semi-artificial diet by porina larvae. Bars with different letters above differ in Fishers unprotected test (P < 0.05). Error bars are SE.

Table 3.2: ANOVA results for average total diet consumption by porina larvae. Significant values in bold, residual degrees of freedom = 87.

Source of Variation	F-statistic	P-value
Species	6.32	**0.014**
Endophyte	17.56	**<0.001**
Silicon	0.05	0.825
Species.Endophyte	7.64	**0.007**
Species.Silicon	0.02	0.875
Endophyte.Silicon	1.63	0.204
Species.Endophyte.Silicon	0.67	0.416

At every assessment larvae fed significantly less on meadow fescue E+ than the EF (*P < 0.05*) whereas there was no difference between the feeding on E+ and EF ryegrass at any assessment point (*P > 0.05*). There was no effect of silicon in all assessments and treatments (*P > 0.05*).

Consumption was generally highest during the first assessment and remained relatively stable throughout the remainder of the experiment across treatments (see *Fig. 3.9*).

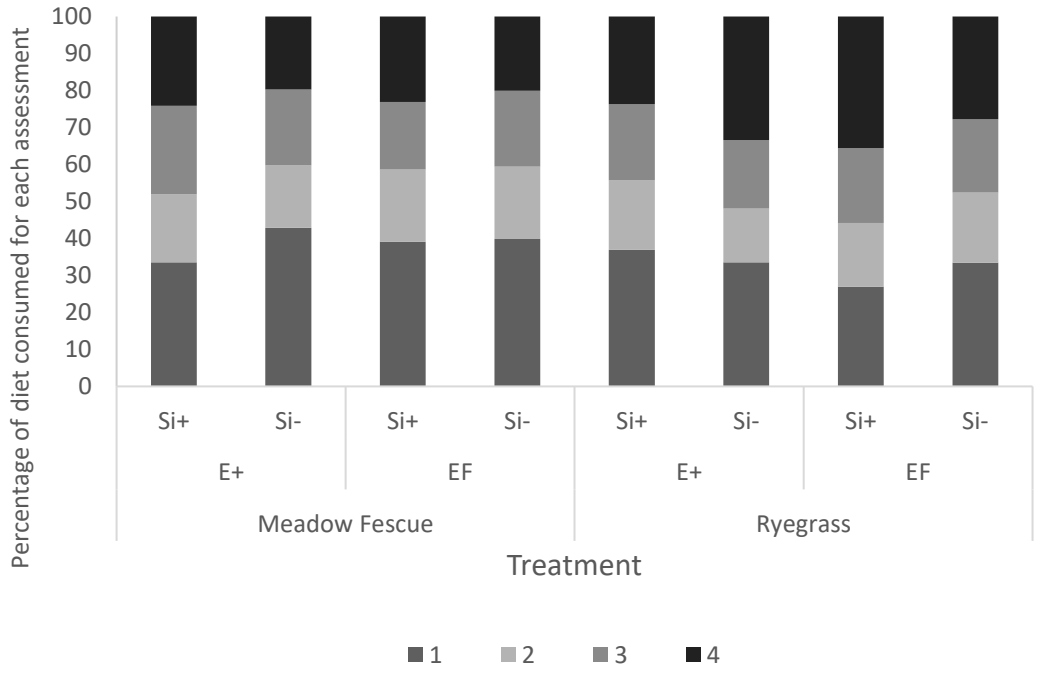

Figure 3.9: Percentage of the total amount of diet consumed for each treatment that was eaten at each assessment (1-4), represented as average feeding per day of each assessment. Each colour represents different assessment.

Differences in diet consumption between treatments were not reflected in the change in weight of porina larvae (see *Fig. 3.10*). Larvae fed meadow fescue E+ gained less weight than those meadow fescue EF, but this was not statistically different. There was a significant difference between the two species, with those fed meadow fescue and ryegrass gaining 61.6 mg and 84.0 mg ($P = 0.035$) respectively on average (see *Table 3.3*). There was also an overall effect of endophyte, larvae fed E+ diets gained 60.2 mg compared to those fed EF diets which gained 85.4 mg on average ($P = 0.018$). Larvae that were in the starved group on average lost 28.4 mg. This was significantly different from all other treatments (see *Fig. 3.10*).

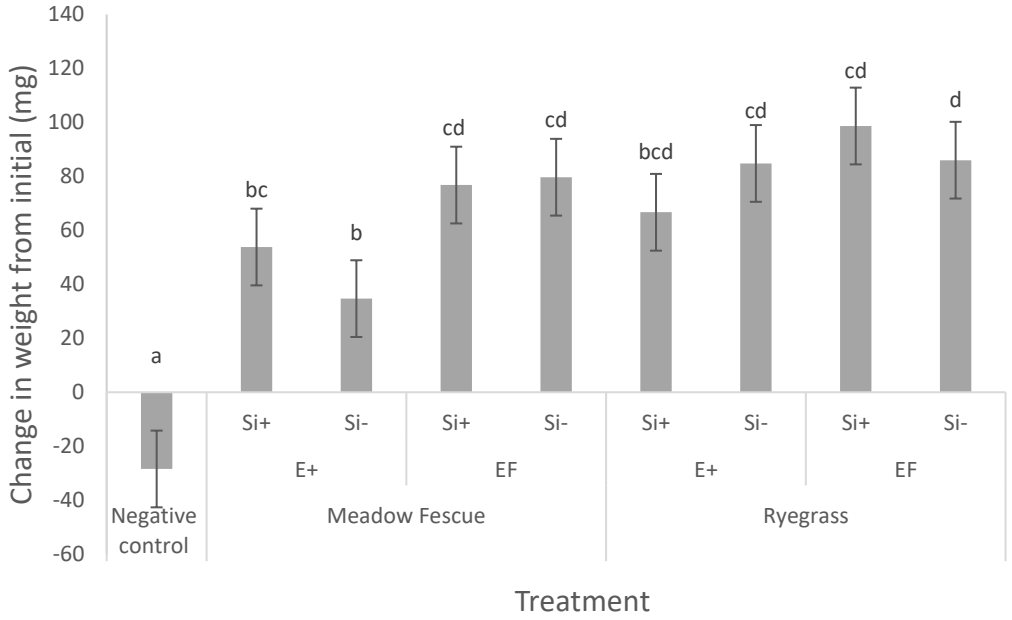

Figure 3.10: Average change in porina larvae weights from start of experiment to end. Bars with different letters above differ in Fishers unprotected test (P < 0.05). Error bars are SE

Table 3.3: ANOVA results for average weight change of porina larvae. Significant values in bold, residual degrees of freedom = 87.

Source of Variation	F-statistic	P-value
Species	4.58	**0.035**
Endophyte	5.82	**0.018**
Silicon	0.05	0.830
Species.Endophyte	0.66	0.420
Species.Silicon	0.29	0.594
Endophyte.Silicon	0.05	0.816
Species.Endophyte.Silicon	1.69	0.198

3.3.2 ASW ryegrass leaf blade experiment

There was no interaction between silicon supplementation and endophyte status on the total number of ASW feeding scars and no effect of silicon supplementation (see *Table 3.4*). There was a significant effect of endophyte infection on number of leaf scars for assessment 1 (*P = 0.023*), assessment 2 (*P = 0.002*), and overall (*P = 0.004*) (see *Fig. 3.11*). Total average number of feeding scars for E+ plants was 15.8 and 40.4 for EF plants. A total of 3 weevils died in the duration of the experiment and were excluded from analysis. One replicate was removed due to suspicion of incorrect initial weight recorded.

Table 3.4: ANOVA results for total number of ASW leaf scars. Significant values in bold, residual degrees of freedom = 23.

Source of Variation	F-statistic	P-value
Endophyte	10.34	**0.004**
Silicon	0.01	0.923
Endophyte.Silicon	1.3	0.266

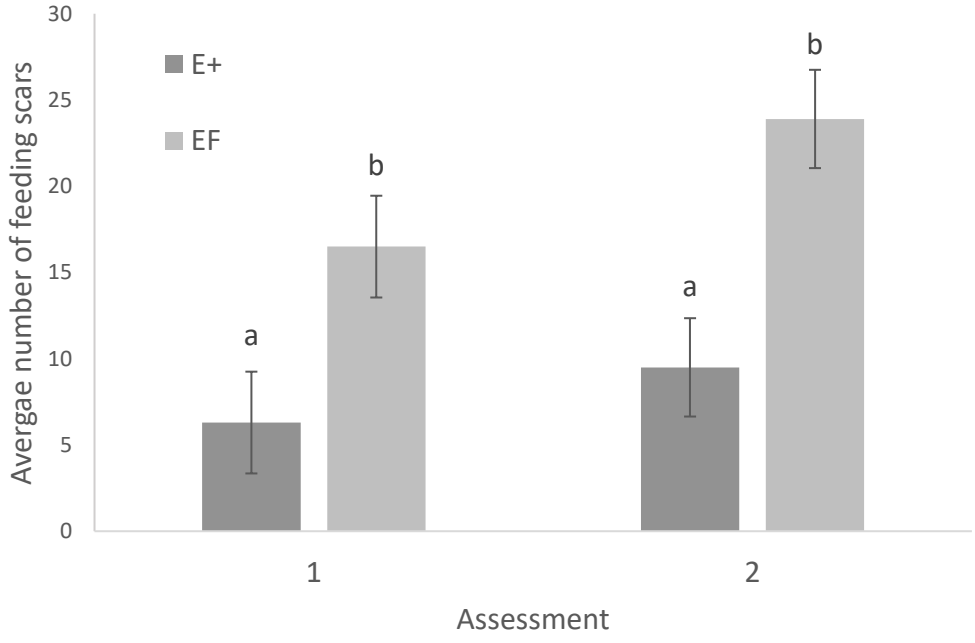

Figure 3.11: Average number of feeding scars (n = 9) for each assessment based on endophyte status. Bars with different letters above differ in Fishers unprotected test (P < 0.05). Error bars are SE.

There was a significant difference in change of weevil weights dependent on treatment (*P < 0.001*) (see *Fig 3.12*). Weevils in the negative control group on average lost 15.8% of their original body weight. There was a significant overall effect of endophyte on weevil weight change. Those fed E+ lost 0.0048 mg on average and those fed EF plant material gained 0.0351 mg (*P = 0.027*). There was no overall effect of silicon or an interaction between endophyte infection and silicon supplementation on changes to weevil weights (see *Table 3.5*).

Table 3.5: ANOVA results for percentage weight change of ASW adults. Significant values in bold, residual degrees of freedom = 23.

Source of Variation	F-statistic	P-value
Endophyte	5.55	**0.027**
Silicon	0.07	0.800
Endophyte.Silicon	0.00	0.964

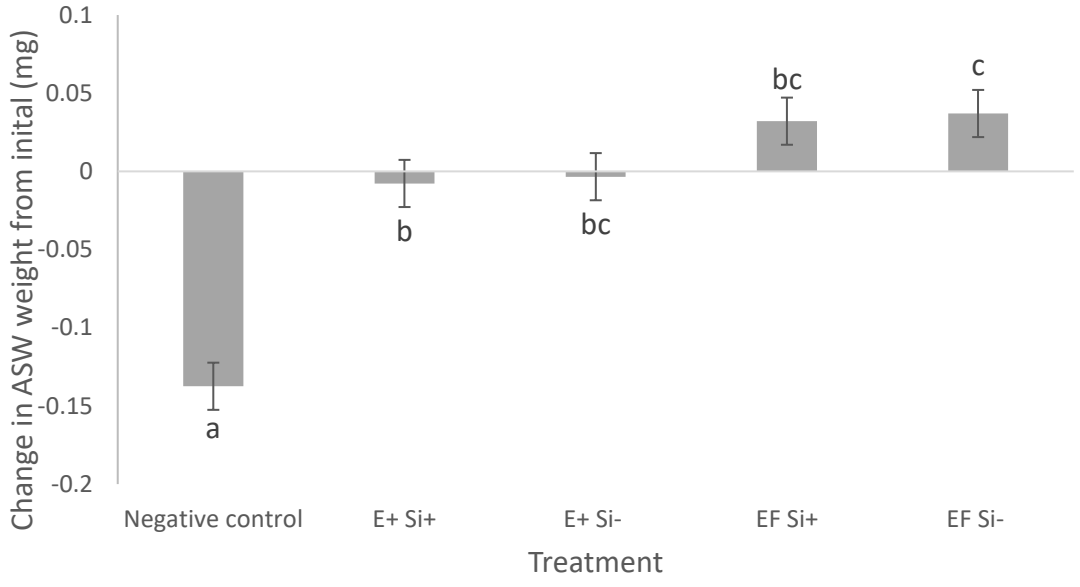

Figure 3.12: Average weight change of ASW adults (n = 9) from start to finish of leaf blade experiment. Bars with different letters above differ in Fishers unprotected test (P < 0.05) Error bars are SE

3.3.3 Post-hoc analysis of plant material for silicon

Post-hoc analysis of the pseudostem material from plants used in these experiments revealed that there was no significant difference in the silicon concentrations between treatments, based on silicon supplementation (*P = 0.099*). There was a significant interaction between species and endophyte infection, indicating that ryegrass EF plants had higher herbage silicon concentrations than E+ ryegrass. There was no significant difference in meadow fescue silicon content based on endophyte infection (see *Fig. 3.13*).

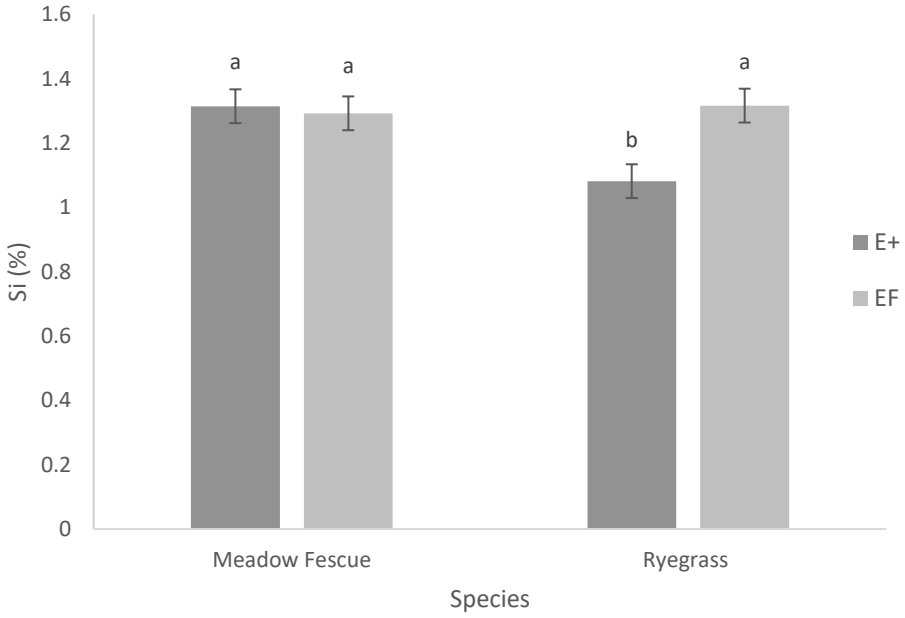

Figure 3.13: Silicon concentrations (%) of pseudostem plant material (n = 3) after four weeks of silicon supplementation. Leaf material (as well as pseudostems) from these plants used in ASW and Porina experiments. Bars with different letters above differ in Fishers unprotected test (P < 0.05), within species. Error bars are SE.

3.3.4 Post-hoc analysis of plant material for lolines

Analysis of pseudostem material from plants used in both the porina and ASW (just ryegrass) experiment revealed that meadow fescue had significantly higher total lolines than ryegrass (*P < 0.001*). There were no significant differences between the total loline concentrations for either species, based on silicon supplementation (see *Fig. 3.14*). Results were the same for NFL and NANL. In comparison, there was an interactive effect of plant species and silicon supplementation on the production of NAL

(*P = 0.002*). Ryegrass supplemented with silicon produced significantly more NAL (78.9 µg/g) than ryegrass not supplemented with silicon (*P = 0.027*), which had nil detection of NAL in any samples. The opposite was true for meadow fescue; silicon supplemented plants produced significantly less NAL than non-silicon supplemented (*P = 0.005*). Meadow fescue had significantly more NAL, whether or not silicon supplemented, than ryegrass treatments (*P < 0.001*).

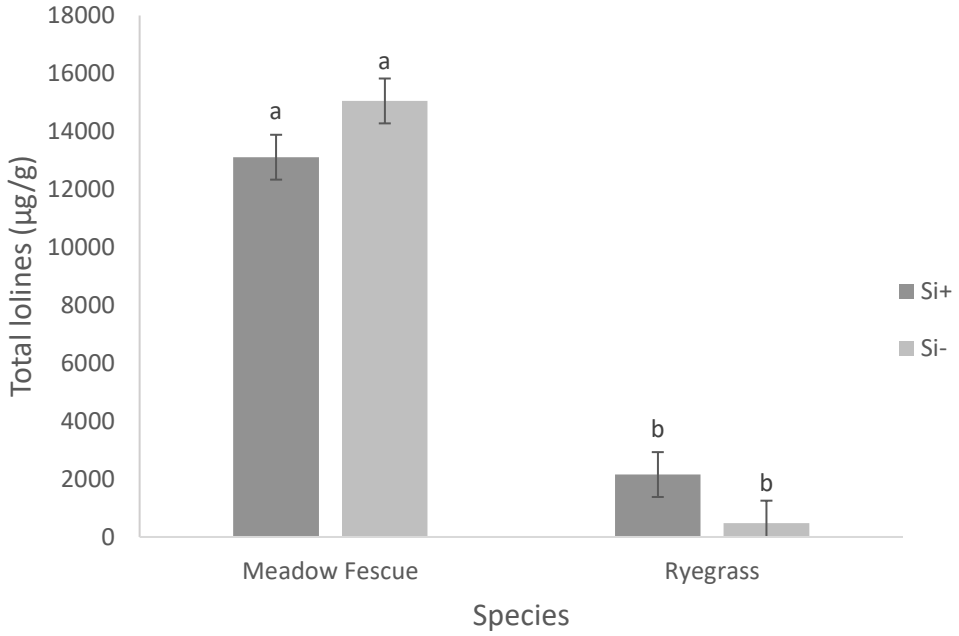

Figure 3.14: Total loline concentration of plant pseudostems (n = 3) after four weeks of silicon supplementation. Leaf material from these plants used in ASW and Porina (as well as pseudostems) experiments. Bars with different letters above differ in Fishers unprotected test (P < 0.05), within species. Error bars are SE.

3.4 Discussion

Feeding of both ASW adults and porina larvae was, to some extent, affected by the presence of endophytes in this study but not silicon supplementation. Endophyte in meadow fescue but not perennial ryegrass significantly reduced the feeding of porina larvae. In contrast, endophyte-infected ryegrass significantly reduced the number of feeding scars from ASW adults. These endophyte results correlate with previously published work.

3.4.1 Porina larvae bioassay

Popay and Lane (2000) conducted an artificial diet bioassay with porina larvae, like the one reported in this chapter, using crude loline extracts which contained NAL and NFL. The results indicated that porina feeding was reduced at a loline concentration of 250 µg/g through to 1000 µg/g. This indicates that the total lolines present in the meadow fescue treatment, and perhaps the ryegrass, in this study were an effective concentration to reduce feeding. Porina larvae are not affected by the alkaloid peramine (Jensen & Popay, 2004), thus production of peramine in the ryegrass treatment is not expected to have influenced results in this experiment. Popay and Lane (2000) also observed increased mortality in treatments with 500 µg/g and 1000 µg/g total lolines compared to their respective controls. There was no mortality effect observed in this study, but this experiment was run for a week less than that in Popay and Lane (2000) because of a shortage of plant material. The continuation of the experiment may have resulted in increased mortality due to longer exposure to loline alkaloids. Porina feeding was significantly reduced in meadow fescue E+ compared to EF, but not in ryegrass treatments. Those fed E+ diets gained less weight than the respective EF treatments in both species, but these were not significant differences. The bioassay could only be run for a period of two weeks, which may have altered the ability to detect differences in larval weight change.

The silicon concentration of EF ryegrass plants was higher than E+ plants, but this difference was lower than differences previously reported to alter herbivore feeding (Massey *et al.*, 2006; Massey *et al.*, 2007a). Massey *et*

al. (2006) reported increases in perennial ryegrass silicon concentration from 0.54% to 4.68% (a 766% increase) with supplementation and this difference influenced the feeding of two folivorous insects. In contrast, the difference in silicon concentrations of EF and E+ perennial ryegrass in this study are 1.08% and 1.32% (a 22% difference) respectively. These differences are marginal in comparison (Massey *et al.*, 2006), although the authors did not mention the endophyte status of plants in this study. Therefore, there is no strong evidence that the silicon concentrations reported here would have influenced the feeding of porina in either EF or E+ plants.

Due to the lack of difference in plant silicon based on supplementation, further experiments are required to determine if there is a synergistic effect of silicon and endophyte on the feeding of porina larvae. Potentially, this could involve hydroponics and longer-term silicon supplementation in two-way factorial plant experiments. The use of hydroponics allows for increased differences in silicon because plants not supplemented with silicon only receive nutrient solution and water, meaning plants cannot absorb already available silicon in soil, as is likely to have occurred in this study. It is of note that water itself will contain a level of silicon (Tulagi, 2011) and this is difficult to remove completely (Epstein, 2009). Further studies on porina larvae could involve the assessment of mandible wear (Mir *et al.*, 2019), to assist in assessing the impact that silicon has on the performance of porina larvae. Mir *et al.* (2019) found progressively more microwear to mandibles of *Oxya grandis* (grasshopper) after feeding on *Bromus catharticus* (rescuegrass) with increasingly high levels of silicon.

3.4.2 ASW leaf blade experiment

Patchett *et al.* (2008a) determined, in field studies, that ASW adult feeding was consistently reduced when loline concentrations were above 400 µg/g. In contrast, Jensen *et al.* (2009), found that ASW adult feeding was not affected by the presence of two loline alkaloids, NFL and NANL, in separate artificial diets. Concentrations of lolines in the diets in Jensen *et al.* (2009)

experiments were in the range of what would be expected to be found in plants in the field (Justus *et al.*, 1997). Jensen *et al.* (2009) also found increased mortality of ASW in these treatments but there was no mortality effect in the leaf blade experiment reported here. However, ASW were only exposed to loline alkaloids for a week, whereas the mortality effect in Jensen *et al.* (2009) did not become significant until after more than 20 days of exposure. The reduced feeding by ASW adults in this experiment is unlikely to be due to the presence of lolines in the ryegrass, but instead peramine, which is known to be an effective deterrent to ASW adult feeding (Johnson *et al.*, 2013; Popay *et al.*, 1990; Rowan *et al.*, 1990). Samples are awaiting peramine analysis to determine how much peramine the insects were exposed to.

ASW adults fed significantly less on the leaf material of E+ ryegrass plants, irrespective of silicon supplementation treatment. This shows that the alkaloid profile of this plant species and endophyte strain association is successful at reducing ASW adult feeding, but there was no influence of silicon supplementation. This difference in feeding was also reflected in changes to weevil weights. However, since the experiment, the accuracy of the analytical balance utilised for weevil weights in the study reported here has come into question. Previous results by Popay & van Amsterdam (2018; unpublished) found detectable differences in ASW weights dependent on the endophyte status of their diet in a leaf blade bioassay like the one conducted in this thesis.

Like the porina experiment, differences in the silicon concentration of E+ and EF ryegrass are not likely to have altered the feeding of ASW adults based on the silicon levels required to change herbivore feeding reported in other studies (Massey *et al.*, 2006).

A limitation of this study is that the exact loline and silicon levels for individual leaves/plants fed to the ASW are unknown and these can be variable between tillers in individual plants (Hartley *et al.*, 2015; Mace *et al.*, 2014; Soininen *et al.*, 2012). Had this information been able to be obtained,

it would have provided more insight into the effects of both defences on ASW feeding.

Further experiments to determine the effects of both silicon and loline-producing endophytes, alone and in combination, on the feeding and weight change of ASW are required. Research investigating long term synergistic effects of silicon and endophyte on ASW populations would aid in the understanding of potential new methods of control to reduce the economic impact of this pest. Silicon, like lolines in Jensen *et al.* (2009), may have adverse effects on ASW populations over an extended period of time. For example, Massey *et al.* (2006) found reductions in pupal mass in response to elevated silicon. Reduced pupal mass adversely affects the dispersal and fecundity of emerging adults, thus having detrimental effects on the resulting populations. Another recommendation for future experiments is to quantify the different defences of individual plants, in order to best interpret the effects of each defence (silicon, alkaloids, phenolics etc). This is important because as reported in Massey *et al.* (2007a) there is large variability in the different defence mechanisms that grass plants/species allocate resources to and this influences the feeding of insect herbivores in experiments.

3.4.3 Relationship of endophyte infection and silicon supplementation

Overall, results indicate that there was no interactive effect of silicon supplementation and endophyte effect on above-ground herbivore feeding after four weeks of silicon supplementation. There was no effect of silicon supplementation treatments on the percentage silicon levels in both species of grass. Overall perennial ryegrass EF plants had significantly more silicon than E+ plants of the same species/cultivar, indicating that there may be a negative association between silicon accumulation and endophyte infection in perennial ryegrass. This is contrary to results reported in Huitu *et al.* (2014) who found a 16% greater silicon concentration of meadow fescue infected with endophyte compared to the EF. The meadow fescue used in this study did not have a similar pattern of silicon accumulation in relation to endophyte

infection. A limitation of this study is that a shortage of plant material meant plant replicates had to be bulked together (n=3) to allow enough material for analysis. This reduced the variability in the results and decreased the potential to identify differences in treatments. Levels of silicon in perennial ryegrass reported in other studies (Massey *et al.*, 2006) are much lower than those found in this experiment. In contrast to this study Massey *et al.* (2006) also grew plants in an inert growth medium and supplemented with silicon for 12 to 15 weeks. Silicon accumulation in the present study may have been influenced by the short period of time that plants were supplemented for or the abiotic conditions they were grown in. Continued research is needed to investigate the synergistic effects of silicon and endophyte infection on insect herbivore performance.

4. Feeding of a below-ground herbivore, grass grub, in root bioassays and pot trials on silicon supplemented grasses

4.1 Introduction

4.1.1 Below-ground herbivores

Root herbivores are severe pests in agriculture and are often difficult to detect and control (Brown & Gange, 1990; Johnson *et al.*, 2016a; Moore & Johnson, 2017). Previous research shows that root-feeders have drastic effects on plant performance and physiology (Blossey & Hunt-Joshi, 2003; Meyer *et al.*, 2009; van Dam, 2009). Damage has detrimental consequences on the functioning of roots, and this affects water and nutrient acquisition as well as transport, storage, and synthesis of secondary compounds. The consequences of root-herbivory can be so severe that in some cases root-herbivores have been introduced as a biological control for invasive weed species (Brown & Gange, 1990). Root damage typically occurs less frequently than foliage damage, however, root-herbivores remain within the environment and damage is ongoing. Damage to roots interacts with other stressors, such as drought, and results in increased negative effects of the drought compared to that for an undamaged plant. As a consequence, root-herbivory can have disastrous effects for plants (Johnson *et al.*, 2016a; Moore & Johnson, 2017). Studies have shown that loss of biomass from the roots is more harmful to the plant than from the shoots (Hunter, 2001). One study found that plants with 25% of root-mass removed produced significantly less total biomass than plants that had 25% of foliage removed (Reichman & Smoth, 1991). Despite this, below-ground herbivory is often not prioritised in plant-insect interactions research (Brown & Gange, 1990; Hunter, 2001; van Dam, 2009).

The presence of soil-dwelling herbivores often goes undetected until damage has already occurred (Moore & Johnson, 2017). The soil environment limits mobility and herbivores are unable to readily disperse

between host plants. This means that damage is less frequent but when it does occur, it is prolonged and severe without intervention (Johnson *et al.*, 2016b). In comparison to above-ground herbivores, those below-ground have a more complex and diverse habitat because of the heterogeneous soil environment. Challenges can include variable nutrient distribution and a wide range of microbial interactions (Johnson *et al.*, 2016b). Below-ground herbivore populations also tend to take longer to reach damaging levels. For example, grass grub populations typically become damaging in pasture 2 to 4 years after sowing (Popay & Thom, 2009; Zydenbos *et al.*, 2011). The above factors contribute to the difficulty in studying and implementing controls against root-feeding insect pests.

4.1.2 Silicon defence and root-herbivores

Silicon research primarily focusses on plant resistance to aboveground herbivores (Hunter, 2001; Johnson *et al.*, 2010). It is hypothesised that like foliage, the accumulation of silicon strengthens plant roots. Recently, Frew *et al.,* (2017) demonstrated that increased root silicon negatively affected a root-feeding herbivore, greyback canegrub (*Dermolepida albohirtum*). This study reported a 65% reduction in overall feeding when the canegrubs were fed roots with a high silicon content and a negative correlation between canegrub change in mass and silicon content (Frew *et al.*, 2017a). This suggests that silicon accumulation in grass roots has the potential to be effective at reducing feeding by other root feeding larvae. Studies have also observed that root-applied silicon can result in higher concentrations in the roots than in foliage (Moore & Johnson, 2017). This is important when considering pasture plant species because high silicon concentrations in the foliage has the potential to impact the digestibility of the plant material not only for insect herbivores but also grazing stock. Massey *et al.* (2009) found that increased silicon did not alter sheep feeding preferences, however this study did not look at long-term consumption and digestability of material.

4.1.3 Grass grub in New Zealand

Grass grub, *Costelytra giveni* (formerly *C. zealandica*) (Coca-Abia & Romero-Samper, 2016), is a member of the scarab family and is endemic to New Zealand. The native habitat of this species is typically tussock grasslands. However, the larval stage has become a significant pest in introduced pasture plant systems (Barratt *et al.*, 1990) (see *Fig. 4.1*). Recently, Ferguson *et al.* (2019) estimated that New Zealand's most common pasture insect pests cause losses of between $1.7 B and $2.3 B during an average year. Of this, grass grub is responsible for $140-380 M on dairy farms and $75-205 M on sheep and beef farms. Thus, grass grub is the most economically important pasture pest in the agricultural industry (Ferguson *et al.*, 2019).

Figure 4.1: 3rd instar grass grub larva in soil

The establishment of native insects as invasive pests is a consequence of the replacement of native ecosystems with introduced agriculturally-beneficial plant species (Jackson & Klein, 2006; Lefort, 2013). Lefort *et al.* (2015b) found that grass grub larvae performed better on white clover roots when the jasmonic acid pathway had been induced compared to control diets, whereas there was no such difference for a closely related species that had not invaded pastures. The authors proposed that the grass grub larvae were pre-adapted to overcome and benefit from defence pathways of an introduced host, contributing to their success and establishment as a

pest species. Studies have also suggested that there is considerable genetic diversity among grass grub populations across regions (Richards *et al.*, 1997). Lefort *et al.* (2015a) discusses the potential for differences between populations found in native tussock regions compared to those in intensive agriculture systems, and this potentially alters their performance in the two habitats.

4.1.3.1 Life cycle

In most areas of New Zealand, the grass grub lifecycle takes one year to complete (see *Fig.4.2*). Adults emerge from the soil in spring and live for four to six weeks, in which time they mate and lay eggs. Females typically lay eggs in a single clump of 20-30 eggs. After two to three weeks the eggs hatch and the first instar larvae emerge to feed on the fine roots of pasture plants. By autumn the larvae typically have reached the third instar and are at their most damaging stage. The larvae over-winter and pupation occurs in spring once the larvae have reached an appropriate size (Ferguson *et al.*, 2019). There are instances where the insect undergoes a two-year lifecycle. A biennial life cycle is more common in Otago and Southland and is normally

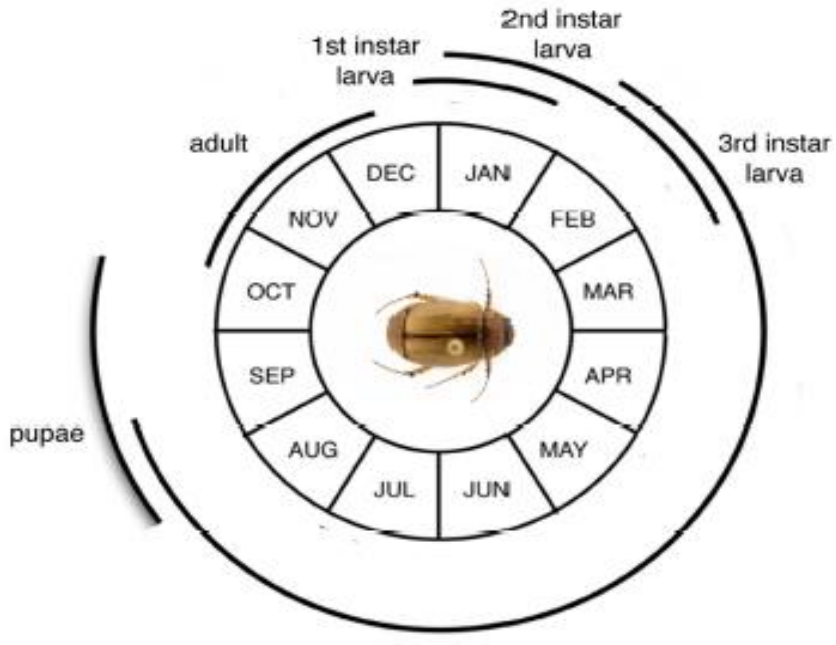

Figure 4.2: Univoltine lifecycle of New Zealand grass grub. Note a biennial lifecycle often occurs in the south of the South Island. (Lefort, 2013).

associated with low temperatures or environmental stresses such as drought. Larval growth is reduced, and they are not an appropriate size to pupate in spring. This means that larval damage to plant roots continues through spring and summer as well as the typical autumn period, affecting methods of control that can be used (Barratt *et al.*, 1990).

4.1.3.2 Impacts

Grass grub adult beetles are often seen feeding on the foliage of trees and shrubs (Barratt *et al.*, 1990). Due to the short life-span of the adult beetles, resulting damage to trees is minor compared caused to grass species by larval stages. First instar grubs tend to feed between 15 to 20 cm soil depth on the fine roots of white clover plants and grasses. By the time the larvae are third instar, they are typically found in the top 5 cm of the soil profile, feeding on the larger roots. At high grub densities, they will cause significant damage to pasture resulting in it being easily pulled by stock and rolled back (Barratt *et al.*, 1990). van Toor and Dodds (1994) estimated that a density of 100 grubs/m^2 results in 6% pasture loss.

4.1.3.3 Methods of control

Currently there is no long-term effective control for grass grub and there are limited options for reducing populations in the short term. These include natural pathogens, methods of early detection, and physical methods to disrupt populations.

There are several naturally-occurring pathogenic organisms that have been isolated from grass grub which assist in population control (Jackson & Klein, 2006). Two of these are milky disease and amber disease, both caused by bacteria (Barratt *et al.*, 1990). Milky disease is the result of infection by *Bacillus* spp. (Steinkraus & Tashiro, 1967) which causes grubs to turn a milky white colour before death (Barratt *et al.*, 1990). Amber disease is caused by two groups of *Serratia* spp. of bacteria living in the soil (Stucki *et al.*, 1984). Infection causes cessation of feeding within 2 to 5 days and eventual death several weeks later (Barratt *et al.*, 1990; Jackson *et al.*,

1993). These bacteria have been utilised as a biopesticide under the name Bioshield™ (Glare *et al.*, 2012; Glare & O'Callaghan, 2017). Despite grass grub being a major pest in New Zealand, the market for the product is small and this has affected its continued availability (Glare & O'Callaghan, 2017).

Other methods rely on early detection and suppression of populations. One technique of early detection is to apply an insecticide to a plot in January. If surrounding pasture appears yellow in subsequent weeks it is an indication of a root-herbivore and sampling can confirm the presence of grass grub (Stewart *et al.*, 1988). This technique allows farmers to recognise areas susceptible to damage in the coming months and account for it in their management plan. Another method of control is cultivation, which reduces larval populations but it also has adverse effects on natural disease levels, resulting in the reoccurrence of grass grub populations and is not effective when second instar biennial grubs are present (Barratt *et al.*, 1990). Barratt *et al.,* (1990) discuss the use of heavy rolling, which is effective short term. This method has the same issues with biennial populations and does not result in lower grub densities in the following year (Stewart & van Toor, 1983). Some population regulation occurs as a result of 'larval combat' when grub densities are high but unfortunately at this point pasture has already been damaged (Barratt *et al.*, 1990).

4.1.3.4 Effects of Epichloë endophytes

Loline alkaloids reduce grass grub larval feeding and hence damage to pasture is also reduced (Patchett *et al.*, 2011b; Popay & Lane, 2000; Popay & Tapper, 2007). Lolines are produced in tall fescue and meadow fescue infected with their naturally-occurring endophytes but not in natural ryegrass associations with *E. festucae* variant *lolii* (formerly *N. lolii*). However, ryegrass is the most common pasture grass species used in New Zealand and inoculation of loline-producing endophytes into ryegrass has potential to aid in control of grass grub. One study found that these novel associations produce loline alkaloids but at much lower concentrations than the natural associations (Ball & Tapper, 1999).

Popay and Lane (2000) incorporated crude loline extracts into artificial diets and found that larvae fed diets with a loline concentration of 100 µg/g weighed significantly less than control diet larvae. Larval weight declined progressively as loline concentration increased through to 2000 µg/g. Popay and Tapper (2007) found that meadow fescue and tall fescue seed infected with loline producing endophytes was fed on less by third instar grass grub than their EF counterparts. There was a slight effect of AR37 infected perennial ryegrass at the beginning of the trial, but this was not significant overall. Another study found no differences in grass grub populations in a field trial with perennial ryegrass pastures with AR1, AR37, common toxic and EF (Popay & Thom, 2009), none of which produce loline alkaloids.

4.2 Methods

This chapter reports the results of experiments investigating the effect of silicon on grass grub larval feeding and performance. Experiments include a whole plant pot trial and two bioassays with excised root material. Plants were germinated on 12/11/2018 and grown in polystyrene trays (see *sections 2.1.3* and *2.1.4*). Plants were split into three daughter plants (see *section 2.1.6*) between 05/02/2019 and 09/02/2019 and assigned to treatments based on results of immunoblots (see *section 2.1.5*). Genotype clones were used across the experiments in this chapter. Plants were watered, trimmed and fertilised as required and kept in a screenhouse for the duration of experiments (see *section 2.1.9*).

All plants supplemented with silicon (see *section 2.1.8*) had silicon applied 3 times per week for a period of ten weeks. The first application was on 18/02/2019 and the final on 26/04/2019, totalling 30 applications. All eight treatments were used in the whole plant experiment and bioassay I, and only ryegrass treatments in bioassay II (see *Table 2.1*). Silicon concentration and loline analysis was performed on the remaining plant material from bioassay I. It was assumed that because plants in the other two experiments were genotypic clones of these plants and were treated and supplemented in the same way over the same period, silicon accumulation would be similar.

4.2.1 Collection, storage, and sorting of grass grub larvae

Grass grub larvae were collected from a dairy farm transitioning to organic status north of Taupo in the North Island of New Zealand on 08/05/2019. This farm had been converted from forestry ~5 years previously and grass grub populations had reached damaging levels in the last two years (see *Fig. 4.3*). Third instar larvae were collected as they are the most damaging stage of the life cycle and most suitable to use in experiments.

Figure 4.3: Site of grass grub larvae collection. Bare patches in pasture indicate areas of high grub densities

Larvae were stored in individual wells of a 24-well cell culture plate (see *Fig. 4.4A*) to prevent larval combat and covered in soil. Plates were wrapped in damp paper towels, placed in a cool box and transported back to AgResearch Ruakura. All larvae were stored within the cool box in a cool store (4°C) until required for experiments. A sub-sample of larvae were viewed under a microscope to confirm the correct species (*C. giveni*) were collected by looking at the raster (see *Fig. 4.4B*) (Lefort *et al.*, 2013).

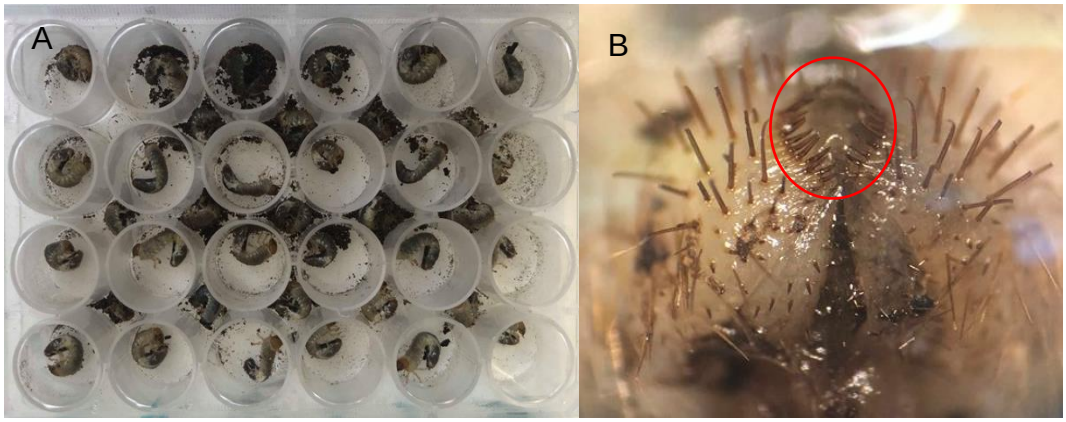

Figure 4.4: A) Storage of larvae in 24-well cell culture plate. B). Raster of grass grub viewed under a microscope

As required, larvae were removed from the cool store and sorted into new labelled 24-well culture plates. Prior to all experiments, larvae were left for 24 hours in an 18°C controlled environment room (whole plant and bioassay I) or a 15°C incubator (bioassay II). They were subsequently weighed on an analytical balance (Mettler Toledo XS204) and assigned to treatments and replicates based on weight. All treatments within a replicate had larvae of a similar weight and the larvae within a replicate were randomly assigned to treatments. This ensured that one treatment did not always receive the lowest weight larvae across replicates.

4.2.2 Whole plant experiment

Grass grub larvae were removed from the cool store on 13/05/2019, left for 24 hours with no food, weighed and assigned to plants. Plants were arranged in a split-plot-block design in a screenhouse, based on silicon supplementation regime (i.e. pair of plants of each species and endophyte status one supplemented and one non-supplemented with silicon), with 20 plants for each treatment. Each plant had three larvae added to the soil surface on 14/05/2019. Larvae on each plant were all near equal weight and collective grub weight on each plant across a replicate was similar. Initial weights of grubs were between 70 and 143 mg. Larvae were watched to ensure that they buried into the soil. Plants were trimmed and fertilised (see *section 2.1.9*) on 30/05/2019 and watered by automatic watering 3 times weekly in the screenhouse.

The experiment was run for a period of five weeks. Plants were destructively harvested, and larvae retrieved over the course of a week (between 17/06/2019 to 21/06/2019; due to time limitation). One tiller from each plant was immunoblotted to re-confirm endophyte status. Remaining root material was washed with cold water to remove soil. Herbage and roots were placed in paper bags and oven dried at 80°C for 48 hours and subsequently weighed. Grubs were re-weighed using the same analytical balance, noting any sick or dead grubs (data of which were removed from analysis). Grubs were determined to be sick if they were of a yellowish colouring. A score of

damage severity (‘sever score’) based on attachment of roots to the herbage was assigned to each plant. Those that were completely attached were recorded as zero, those which were half detached were scored a 1, and those that were completely severed were scored a 2.

4.2.3 Root bioassay I

There was a total of 20 replicates set up for each of the eight treatments. Set up was staggered across 16/05/2019, 17/05/2019, 19/05/2019 and 20/05/2019, with five replicates set up each day, due to time constraints. Root material was removed from soil and washed under cold water the day before required. Roots were patted dry with a paper towel, placed in a labelled plastic bag and stored in the fridge (4°C) until the next day when a subset of roots (100 mg ± 5 mg) was weighed and placed in a labelled 60 mm Petri dish, with the assigned larva (see *section 4.2.1*) (see *Fig.* 4.5). Each replicate had root material from a different plant. Root material was returned to the fridge until day four, when additional root material was added to each Petri dish. The remaining root material was freeze-dried and ground for further analyses (see *section 2.3*).

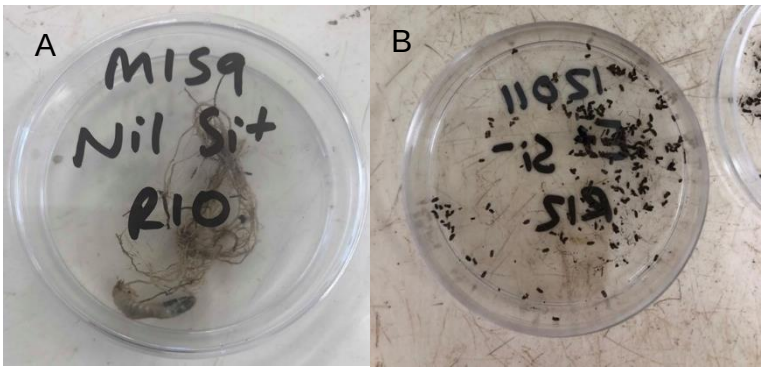

Figure 4.5: A) Grass grub larva in 60mm Petri dish with ~100 mg fresh root material for bioassay I. B) Remaining frass from grass grub

All treatments for a replicate were wrapped in a damp paper towel together, placed in a sealed plastic container, and left in an 18°C controlled environment room. Containers were checked daily to ensure paper towels were remaining damp. Additional root (100 mg ± 5 mg) was added on day four and grubs were weighed. All remaining root was weighed on day seven,

and grubs were placed into 24-well plates wrapped in damp paper towels and left for 24 hours at 18°C before being re-weighed. Any frass from these larvae was added to the original Petri dish. Frass was left in open Petri dishes to dry for 48 hours in a 25°C controlled environment room and then weighed.

4.2.4 Root bioassay II

Inconclusive results in bioassay I prompted slight alterations to methods in bioassay II. On 15/07/2019, larvae were removed from the cool store and any healthy/live grass grubs were placed individually in a 24-well plate well with a cube of fresh carrot (see *Fig. 4.6A*), to check if larvae were still actively feeding. Plates were wrapped in a damp paper towels in a sealed plastic container and left for 24 hours in a 15°C incubator. The following days grubs that had fed (see *Fig. 4.6B*) were kept and left with no food overnight. The grubs were weighed and assigned to treatments on 17/07/2019 (see *section 4.2.1*).

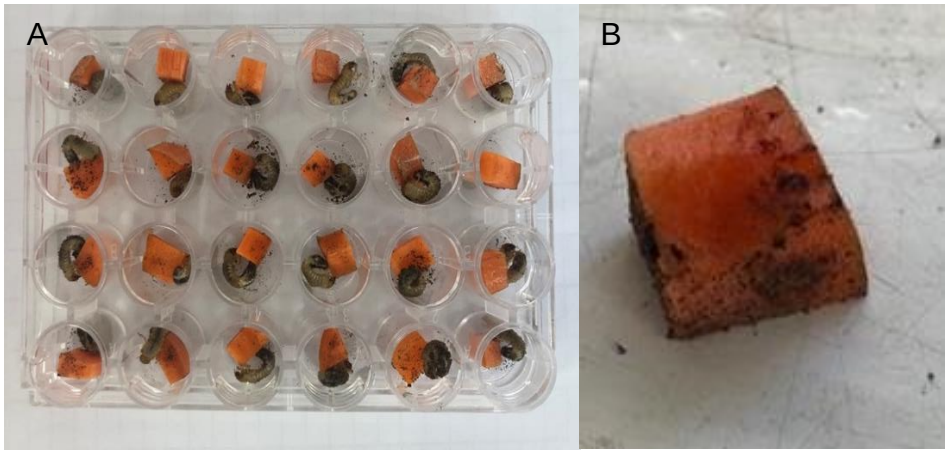

Figure 4.6: A) Grass grub larvae in 24-well plate with cubes of carrot. b) Evidence of grass grub larval feeding on cube of carrot

Roots were collected on 16/07/2019 using the same methods as bioassay I. Roots and larvae were placed in sealed plastic 30 mL specimen containers, arranged in a randomised block design in polystyrene trays and kept in a 15°C incubator (see *Fig. 4.7*). On day three (20/07/2019) any remaining roots were removed and weighed, and fresh roots collected the day before were added. Each replicate did not have roots from an individual plant but

a subset from a bulk sample of root for each treatment. On day seven (24/07/2019), all grubs and any remaining roots were weighed, grubs were left overnight with no food and re-weighed the next day. Frass was left to dry for 48 hours in a 25°C controlled environment room and weighed. All weights were recorded on an analytical balance (Mettler Toledo XS204).

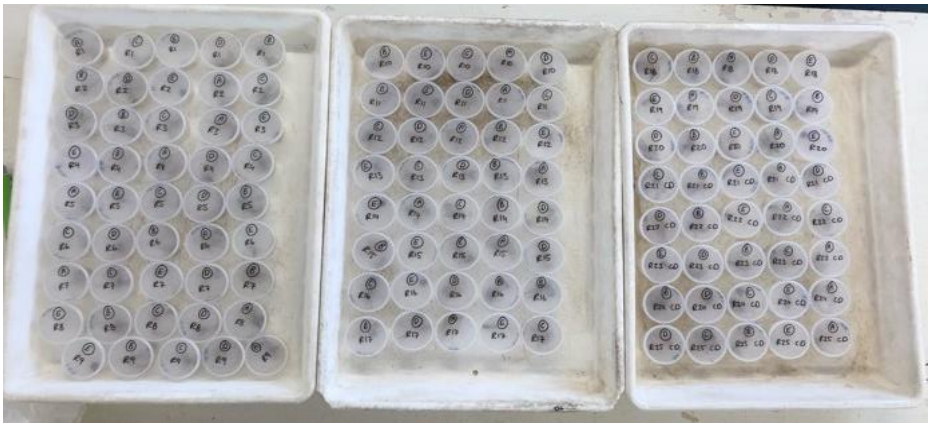

Figure 4.7: Set up of grass grub bioassay II, arranged in a randomised block design

There was a total of 25 replicates for each treatment. Only ryegrass treatments were used (see *Table 4.1*) with a starvation control (larva with no roots), totalling five treatments, due to limiting numbers of surviving and actively feeding larvae. Of the 25 replicates, five were weighed daily to track changes in larval weight across the entire experiment. There were also five replicates of root only controls for each of the treatments, to monitor moisture loss from roots.

4.2.5 Statistical analyses

Data were analysed using GenStat (v.19). Data were analysed by general ANOVA and treatment blocked by species, endophyte status, and silicon supplementation to investigate any potential interactions. Regression analysis of root consumption and frass production was carried out for both bioassays. Regression analysis was also performed to compare the start and finish weight of root only controls to investigate moisture loss throughout the experiment. Results from dead larvae and larvae determined as unwell were removed from analysis.

4.3 Results

4.3.1 Whole plant experiment

Of the larvae that were put on the plants initially, 81.7% were able to be recovered. Of those recovered 90.9% were healthy; this varied between individual treatments but there was not a significant difference (see *Table 4.1*). There was a significant difference between plant species (P = 0.005), with 95.9% and 85.8% healthy recovered larvae in the perennial ryegrass and meadow fescue treatments respectively. Larvae that were not able to be recovered were assumed to have died during the experiment.

Table 4.1: Total number and percentage of healthy grass grub larvae recovered from whole plant experiment

Species	Endophyte	Silicon Supplementation	Number of recovered larvae	Percentage healthy
Meadow Fescue	E+	Y	44	84.2
		N	44	81.7
	EF	Y	50	93.3
		N	46	84.2
Perennial Ryegrass	E+	Y	49	97.5
		N	53	93.3
	EF	Y	54	94.6
		N	52	98.3

There was a significant difference in the mean sever damage scores of plants based on endophyte, on average E+ plants had a score of 0.312 and EF had a score of 0.637 (P = 0.002) (see *Table 4.2*). However, this difference was not significant for ryegrass plants overall, whereas meadow fescue plants had a significant difference based on endophyte status. E+ and EF meadow fescue plants had an average score of 0.05 and 0.725 respectively. There were no meadow fescue E+ plants with a sever score of 2 (see *Fig. 4.8*). There was no effect of silicon supplementation on the

sever score of plants, nor an interactive effect of silicon supplementation and endophyte status (see *Table 4.2*).

Table 4.2: ANOVA results for sever score of plants in whole plant grass grub experiment. Significant values in bold. Residual degrees of freedom = 133.

Source of Variation	F-statistic	P-value
Species	2.97	0.087
Endophyte	10.24	**0.002**
Silicon	0.06	0.806
Species.Endophyte	11.87	**<0.001**
Species.Silicon	0.97	0.327
Endophyte.Silicon	0.24	0.623
Species.Endophyte.Silicon	2.97	0.087

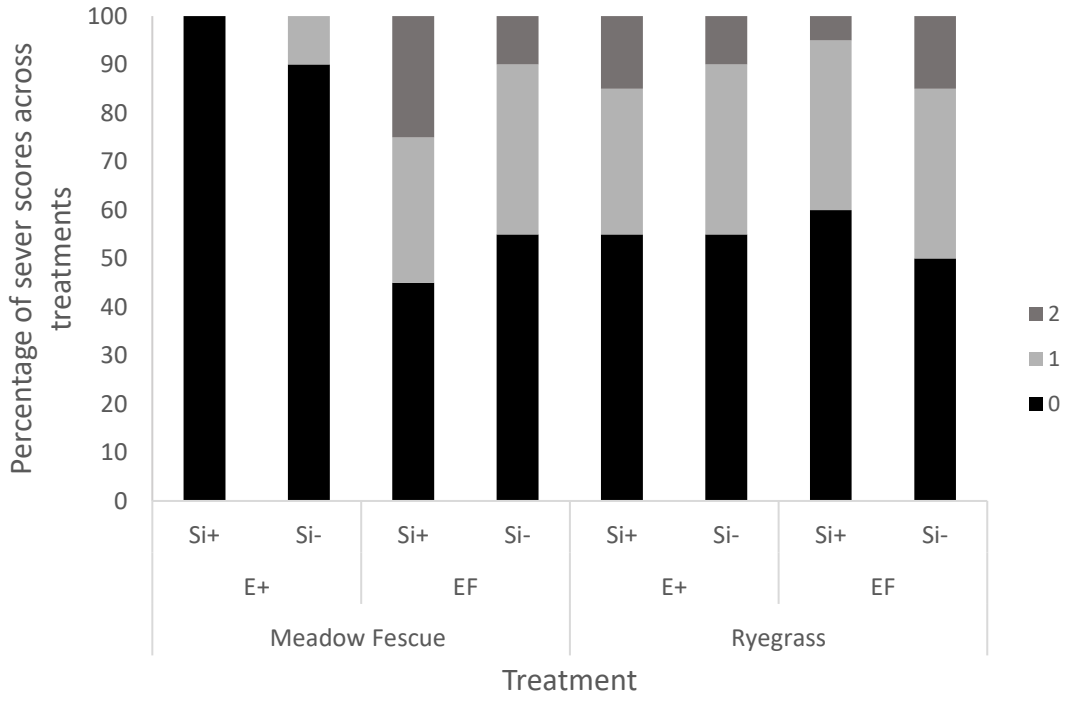

Figure 4.8: Percentage of sever scores for each treatment in grass grub whole plant experiment. 0 = completely attached, 1 = half detached and 2 = completely severed.

There was a significant interaction between plant species and endophyte on larval change in weight ($P < 0.001$). Larvae in the meadow fescue E+ treatments gained significantly less weight compared to meadow fescue EF, 1.15 mg and 7.24 mg respectively (see *Fig. 4.9*). The effect was the opposite for ryegrass E+ and EF (however, not a significant difference), 6.49 mg and 3.72 mg respectively. There was no significant effect of silicon supplementation on larval weight gain, nor an interaction between endophyte and silicon ($P > 0.05$).

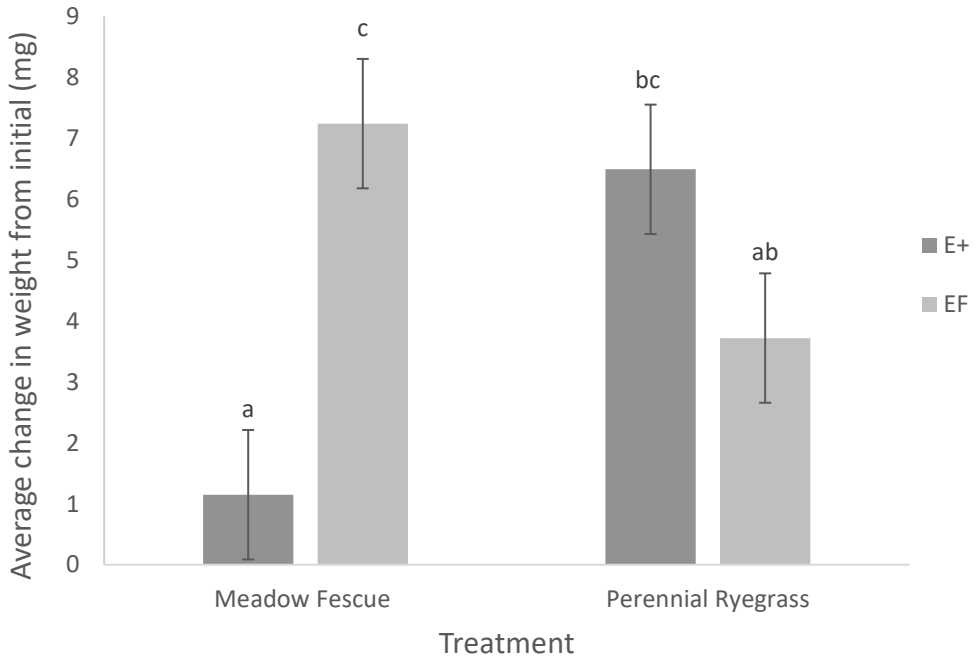

Figure 4.9: Average weight increase of grass grub larvae in whole plant experiment based on average initial weights for the 3 larvae on each plant. Values are means ±SEM. Letters above bars denote significant differences between treatments (Fishers Unprotected test P < 0.05).

Remaining biomass of both herbage and roots following grass grub larval feeding was not affected by silicon supplementation ($P = 0.609$ and $P = 0.435$ respectively) and there was no interactive effect of silicon supplementation and endophyte status ($P = 0.520$ and $P = 0.983$, respectively). However, there was a significant effect of endophyte ($P < 0.001$) (see *Fig. 4.10*). There were significant differences in the weight of remaining herbage and roots for E+ and EF meadow fescue plants. The biomass of EF ryegrass plants was less than that of E+, but this was not statistically significant.

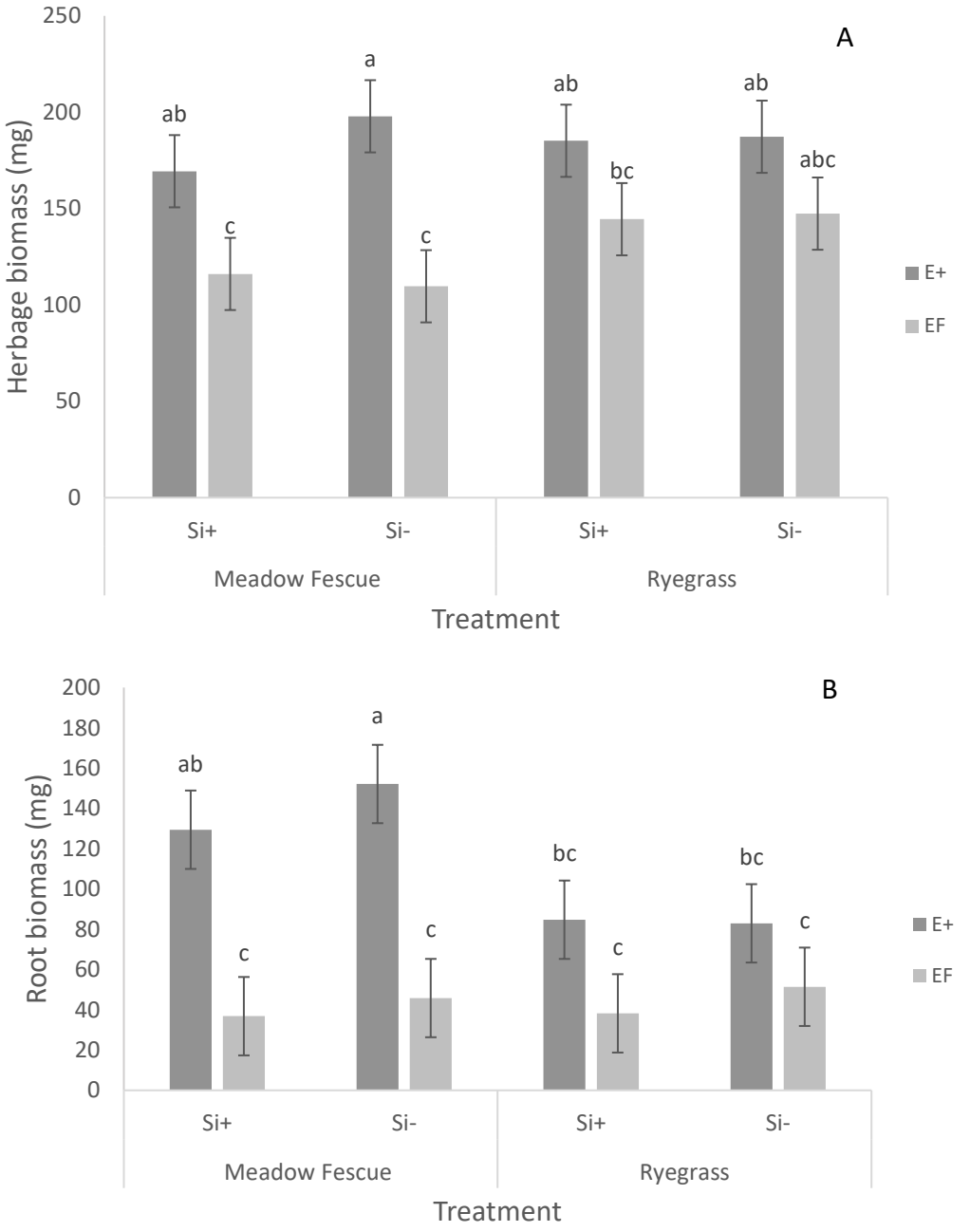

Figure 4.10: Biomass (dry weight) of plant material after five weeks of grass grub larval feeding in whole plant grass grub experiment. A) Herbage weights and B) Root weights. Values are means ±SEM. Letters above bars denote significant differences between treatments (Fishers Unprotected test $P < 0.05$).

4.3.2 Root bioassay I

Across the eight treatments and 20 replicates, there were eight larvae that died during the experiment (5%) and five (3.13%) which appeared to be unwell, but this was not influenced by treatment (P = 0.567). Midway through the experiment grub weight gain ranged between 4.12% and 6.73% from their initial weight across all treatments and there were no significant differences between treatments (P = 0.813). There was no effect or interactive effects of plant species, endophyte status, or silicon supplementation on grub weights at day four (P > 0.05). At the end of the experiment, larvae were starved for 24 hours prior to being weighed. In most treatments, on average larvae lost weight from their initial weight, except for ryegrass E+ non-silicon supplemented. These final weights had significant differences between treatments (P < 0.001) (see *Fig.4.11*) and there was an interactive effect of silicon supplementation and endophyte infection (P < 0.001). For both plant species grubs in the E+ silicon supplemented and the EF non-supplemented lost significantly more weight than those in the other two treatments (EF supplemented and E+ non-supplemented).

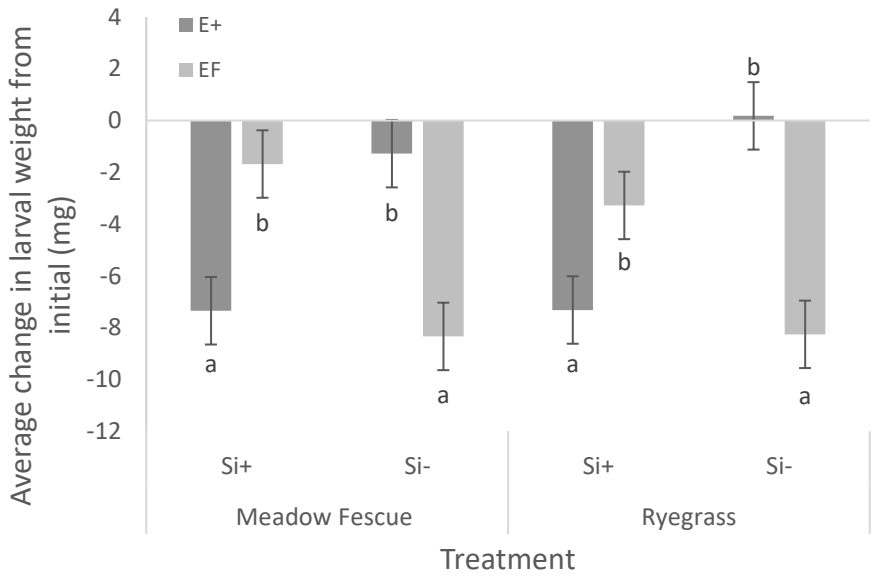

Figure 4.11: Average change in weight from initial weight of grass grub larvae from bioassay I for each treatment. Values are means ±SEM. Letters above bars denote significant differences between treatments (Fishers Unprotected test P < 0.05).

There was a significant difference in the consumption of roots based on plant species. On average, consumption in meadow fescue and ryegrass treatments was 114 mg and 98.4 mg respectively (P = 0.036). There were no significant differences between treatments within a plant species (P = 0.260) (see *Fig. 4.12A*). Production of frass was consistent with consumption results (see *Fig. 4.12B*), with no significant differences between treatments within a plant species (P = 0.405), nor an effect of plant species on frass production (P = 0.163). There was a strong linear relationship between root consumption and frass production (P < 0.001, R^2 = 0.85, F-statistic = 53.88) (see *Fig. 4.13*).

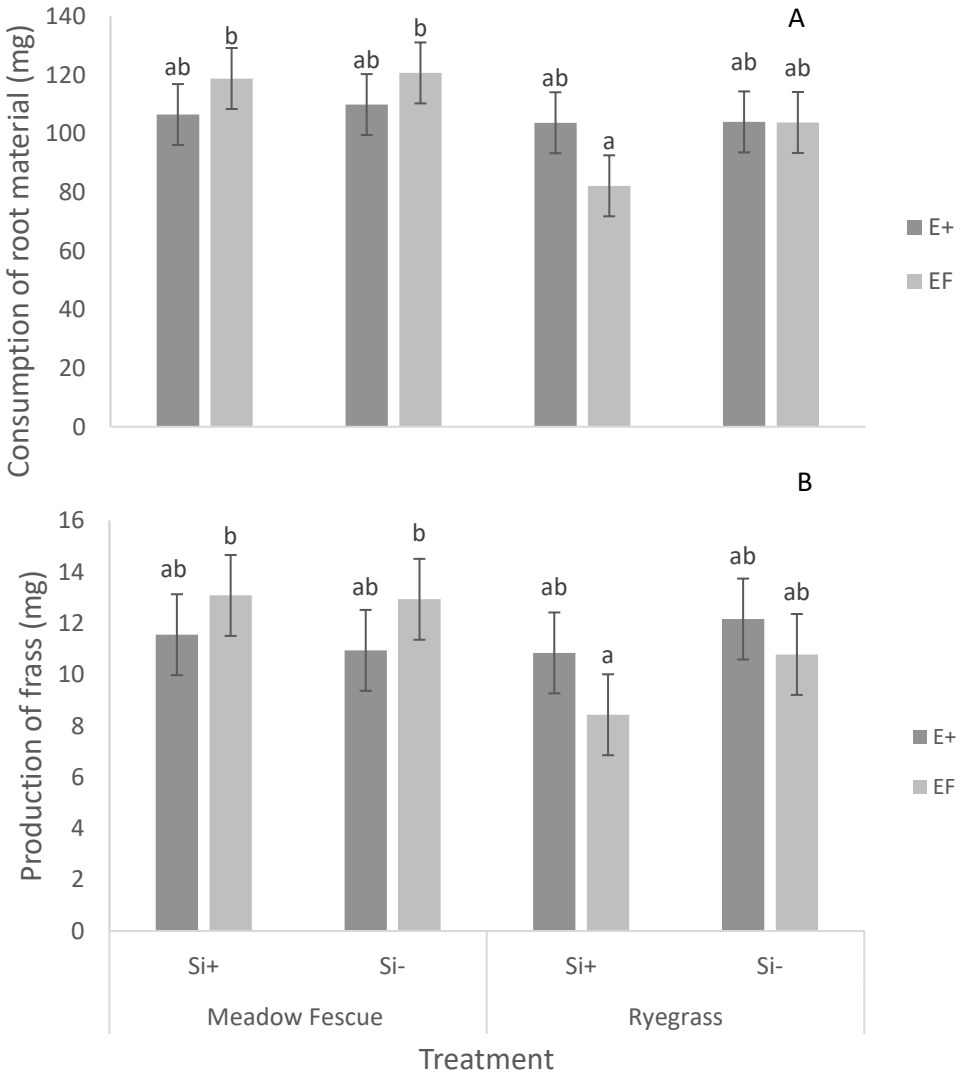

Figure 4.12: A) Total consumption of roots (mg). B) total frass production (mg) for grass grub larvae bioassay I. Values are means ±SEM. Letters above bars denote significant differences between treatments (Fishers Unprotected test P < 0.05).

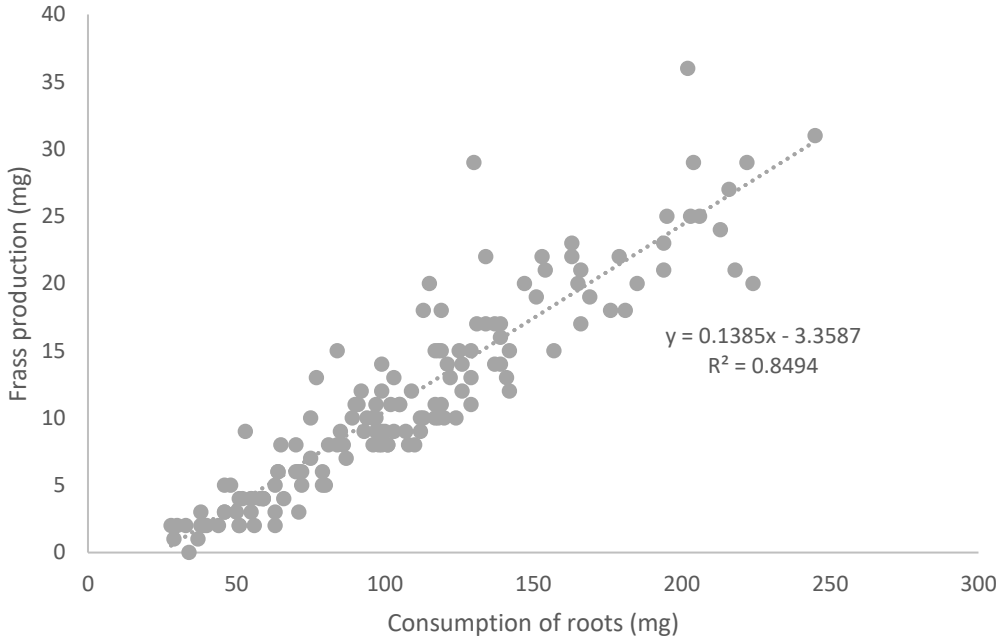

Figure 4.13: Regression analysis comparing total consumption of roots and production of frass in grass grub larvae bioassay I

4.3.3 Post-hoc analysis of plant material – Bioassay I

The remaining root material from Bioassay I was analysed for silicon, by bulking together groups of five replicates so that there was enough plant material (n = 4). There were significant differences between some treatments ($P < 0.001$) (see *Fig. 4.14*). EF silicon supplemented ryegrass roots had significantly more silicon than all other treatments except for E+ silicon supplemented ryegrass. An overall significant effect of species on silicon concentration was apparent with roots from meadow fescue having a lower silicon content of 2.38% compared with ryegrass at 3.63% silicon ($P < 0.001$). Overall plants supplemented with silicon had a higher silicon concentration than non-supplemented, 3.34% and 2.67% respectively ($P = 0.012$). There were no interactive effects between species, endophyte status or silicon supplementation (see *Table 4.3*).

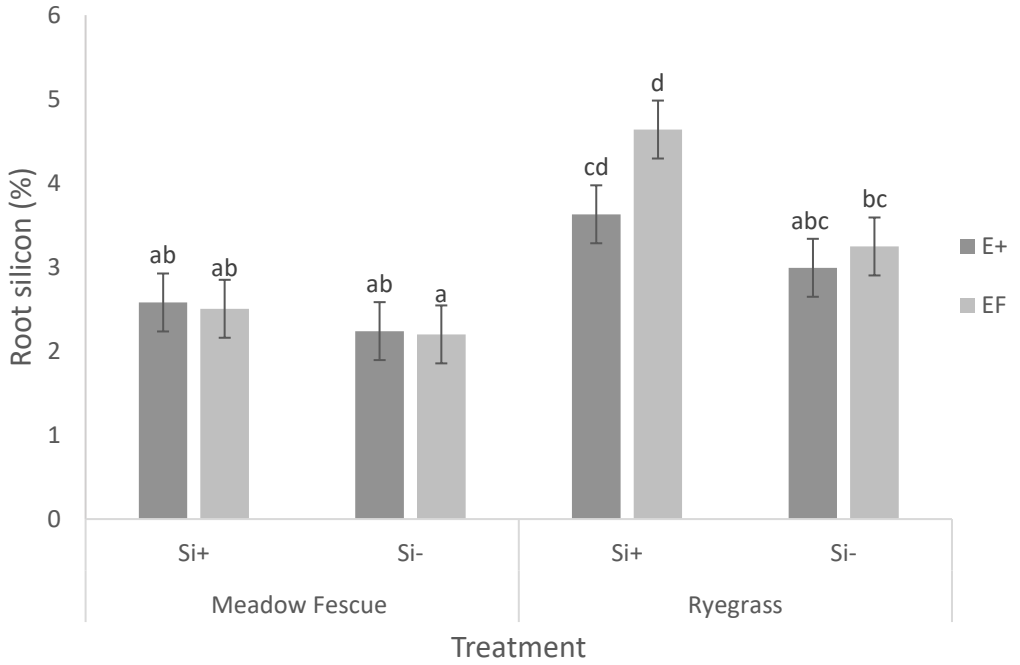

Figure 4.14: Root silicon concentrations for Bioassay I plant material. Values are means ±SEM. Letters above bars denote significant differences between treatments (Fishers Unprotected test P < 0.05).

Table 4.3: ANOVA results for silicon root content from grass grub larvae bioassay I. Significant values in bold. Residual degrees of freedom = 21

Source of Variation	F-statistic	P-value
Species	26.16	**<0.001**
Endophyte	1.39	0.252
Silicon	7.53	**0.012**
Species.Endophyte	1.99	0.173
Species.Silicon	2.01	0.171
Endophyte.Silicon	0.54	0.469
Species.Endophyte.Silicon	0.66	0.427

The same root material samples were also analysed for loline alkaloid concentration. Meadow fescue roots had significantly more total lolines than ryegrass (P < 0.001), 248 µg/g and 9 µg/g respectively. Silicon supplementation did not significantly affect total lolines concentration in either species (see *Fig. 4.15*). There were no lolines detected in ryegrass root material except for NFL in plants that had not been supplemented with silicon. Lolines in meadow fescue silicon supplemented material were

higher than in non-supplemented but this was not a significant difference ($P > 0.05$), except for NANL which was not detected in non-supplemented plants ($P = 0.049$).

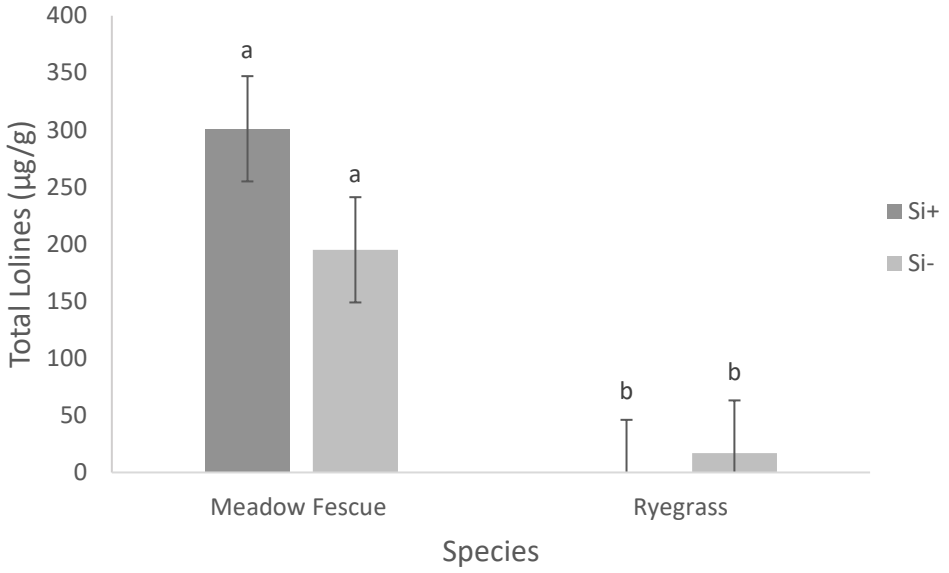

Figure 4.15: Total lolines (µg/g) in roots of endophyte infected plants used in Bioassay I. Values are means ±SEM. Letters above bars denote significant differences between treatments (Fishers Unprotected test P < 0.05).

4.3.4 Root bioassay II

Across the five treatments, 25 replicates in each, there was a total of 22 larvae deemed as unwell at the end of the experiment (17.6%) and two had died (1.6%). There was no treatment effect on larval health (P = 0.524). There was no significant difference in the moisture lost from root only controls between treatments (P = 0.158) and there was a strong linear relationship between the initial weights of the roots and the final weight of root ($P < 0.001$, $R^2 = 0.93$, F-statistic = 37.97). On average root only controls lost 25.88% of their initial weight.

The five replicates that were checked daily for fluctuations in weight change all followed the same pattern across treatments (see *Fig. 4.16A*). Larvae in

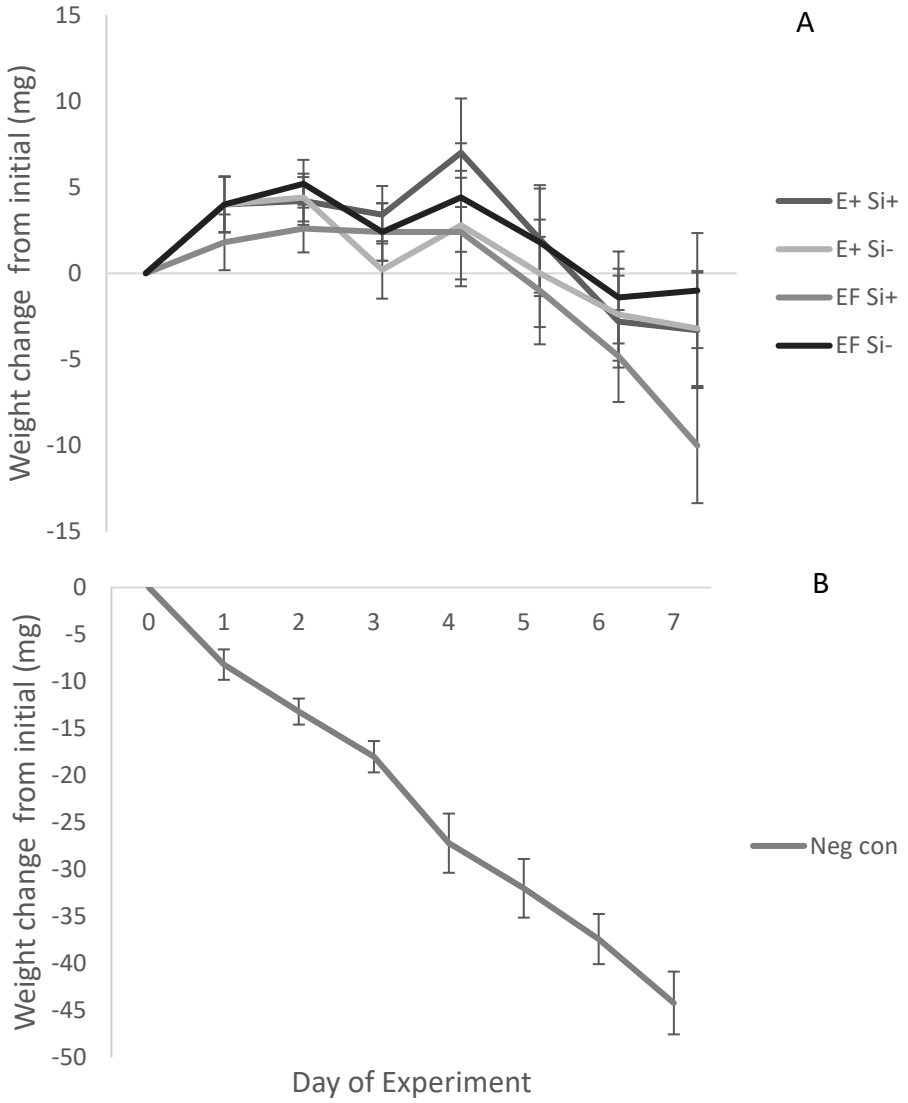

Figure 4.16: Daily change in larval weights (n = 5) from initial weight (mg) over the course of bioassay II; A) treatments containing root B) no root control

the root-free treatment lost more weight than those that were fed during the experiment ($P < 0.001$) (see *Fig. 4.16B*), however, there was no difference between the root treatments ($P > 0.05$). On day three of the experiment, all larvae were beginning to lose weight compared with the previous day. Roots were replaced in the pottles and the following day larval weights had increased again but then declined steadily over the remaining three days. On average at the end of the experiment larvae checked daily had lost weight from their initial weights (see *Fig. 4.16*).

Analysis including all 25 replicates found that on average larvae did not lose weight from the initial weight (see *Fig. 4.17*), except in the no root control, where on average they lost 34.7% body weight. There was no interactive effect of endophyte and silicon supplementation, nor a significant effect of silicon on its own to changes in grub weights. There was, however, a significant effect of endophyte ($P = 0.022$). Larvae fed E+ and EF roots gained 3.07 and 1.21 mg respectively.

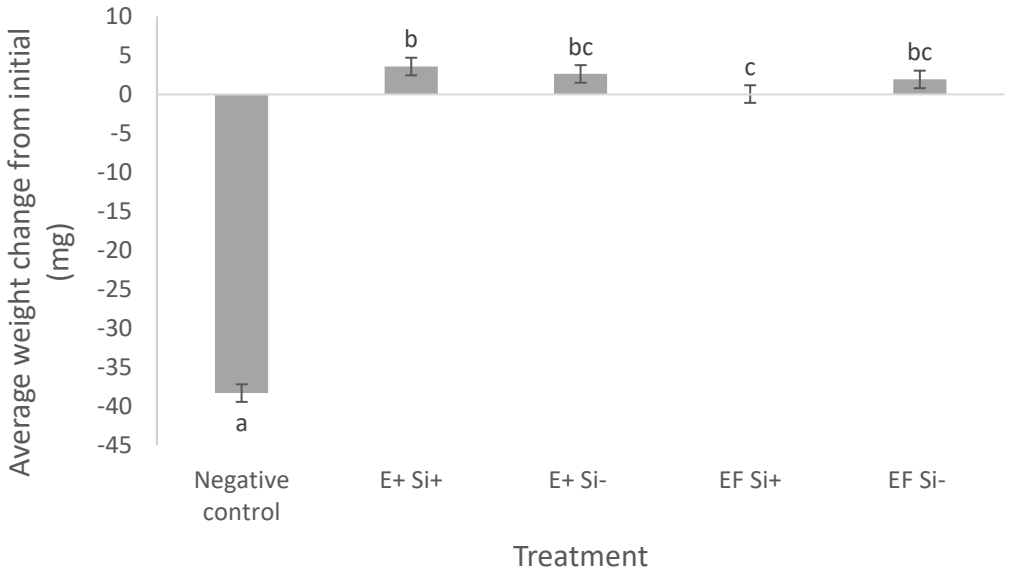

Figure 4.17: Average change in weight from initial weight to final of grass grub larvae from bioassay II. Values are means ±SEM. Letters above bars denote significant differences between treatments (Fishers Unprotected test P < 0.05).

Root consumption did not differ between treatments (P = 0.327) (see *Fig. 4.18A*). There was also no effect of endophyte status or silicon supplementation alone on the feeding of larvae (P = 0.592 and P = 0.454 respectively). Larvae which fed on E+ and silicon supplemented material produced significantly more frass than those fed EF also supplemented with silicon, and E+ not supplemented with silicon (see *Fig. 4.18B*). There was a strong linear relationship between root consumption and frass production (P < 0.001, R^2 = 0.87, F- statistic = 536.53).

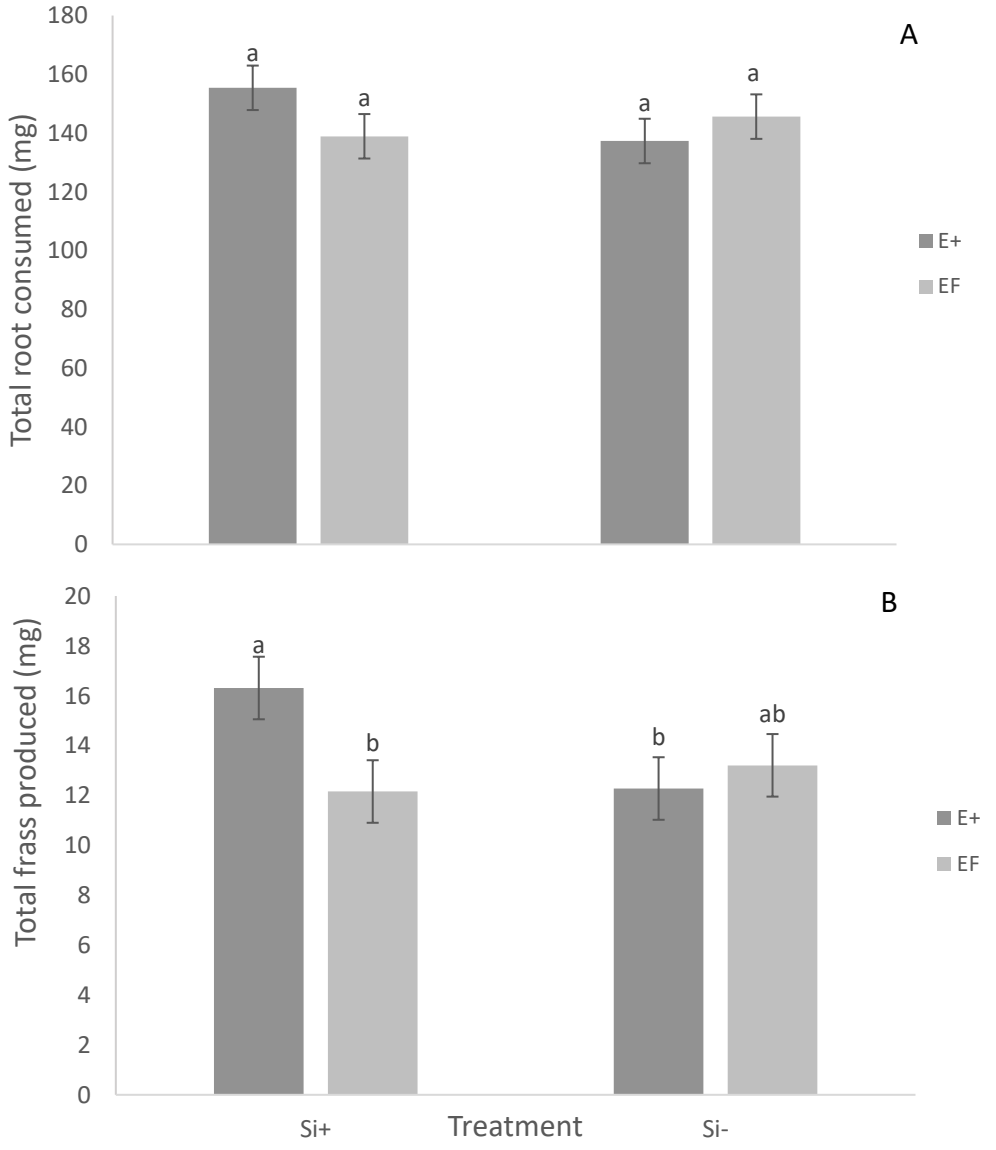

Figure 4.18: A) Total consumption of roots (mg). B) total frass production (mg) for grass grub larvae bioassay II. Values are means ±SEM. Letters above bars denote significant differences between treatments (Fishers Unprotected test P < 0.05).

4.4 Discussion

4.4.1 Whole plant experiment

The feeding of grass grub larvae was influenced by the presence of endophyte in meadow fescue. Larvae placed on E+ plants gained significantly less weight than those on EF plants. Meadow fescue E+ plants had significantly more remaining root material than the EF counterparts and grew more herbage after plants were trimmed to 5 cm. These results suggest that the elevated root herbivory on EF meadow fescue plants was reducing the ability of plants to regrow above-ground. Previous studies have found that in the absence of stress there is no difference in the root mass of E+ and EF plants (Cheplick & Cho, 2003; Cheplick *et al.*, 2000). Therefore, the differences in remaining root biomass are unlikely to have been influenced by an inherent difference in plants based on endophyte status but were due to the effects of endophyte on grass grub herbivory. The sever score of meadow fescue plants was reduced by endophyte. Collectively, out of the E+ plants, there were no plants that were completely severed, and only one out of the forty was partially severed. This aligns with previous studies which reported a defensive effect of endophytes in meadow fescue plants against grass grub larvae (Patchett *et al.*, 2011b).

There was also a significant difference in the larval percentage weight gain on the ryegrass plants. Unexpectedly, larvae on ryegrass E+ plants gained significantly more weight than those on EF. It was initially anticipated that the endophyte would have negative effects on larval performance. However, the endophyte strain in this ryegrass cultivar is naturally found only in tall fescue plants. These plant-endophyte associations have previously been found to have lower alkaloid levels than in their natural tall fescue hosts (Ball & Tapper, 1999; Easton *et al.*, 2007; Malinowski & Belesky, 2019). The loline levels found in the root material of genotypic clones of plants used in the whole plant experiment ranged from 0 to 37 µg/g total lolines. This is not a concentration which is known to be effective against grass grub larvae (Popay & Lane, 2000), suggesting that alkaloids would not have influenced grub weight change in this instance.

Although not significant, there was some evidence that EF ryegrass plants supplemented with silicon had higher root silicon concentrations than other ryegrass treatments. A higher concentration of silicon in the EF roots may have contributed to the reduced weight gain of the larvae (Frew *et al.*, 2017a), whereas the E+ roots lacked silicon in comparison and did not have high enough alkaloid levels to alter larvae performance. Previous studies such as Ryalls *et al.* (2017) reported an increase in silicon concentration of 0.3% was sufficient to reduce herbivore damage. Additionally, Frew *et al.* (2017a) reported similar differences in root silicon which led to the reduced performance of a soil-dwelling root-feeding herbivore. This suggests that the difference in silicon reported here may have altered the performance of grass grub larvae in ryegrass treatments and not the presence of loline alkaloids.

When plants were harvested, all remaining root material of the plant was collected. In retrospect, collection of only the roots still attached to the plant would have been a more appropriate method. Roots that have been detached from the plant no longer have an influence on the growth of the plant and therefore consumption of them by larvae is not relevant. For example, a plant may have had a sever score of 2, which would leave the plant severely damaged and chances of survival would be low but there may have been detached roots remaining in the soil. The method of root collection used may therefore have masked the true differences in relevant remaining root biomass between treatments.

Another observation made in this experiment was that grubs were potentially able to move between plants. There were two plants out of the 160 that contained four grubs rather than the original three upon assessment. A layer of weed mat was placed in the bottom of the pots, to prevent the movement of grubs through the holes in the bottom of the pots and into a new pot. However, plants were potted so that there was a 1 cm clearance between the top of the soil and the top of the pot so there was still potential for movement of grubs. Several larvae were noticed on the soil surface during the experiment. It is unusual that larvae come to the surface, but some patches of the trial set up were damper than others because of

the automatic watering. Damp soil has potentially influenced the behaviour of larvae in this whole plant experiment. Future experiments should aim to minimise saturation of pots and monitor automatic watering to ensure all plants are appropriately watered.

4.4.2 Root bioassays

Results from root bioassay I found no significant differences in root consumption between treatments, which may have been influenced by moisture loss from roots. Moisture loss from roots potentially altered the ability to detect differences in feeding. However, there was a drastic difference in changes to larval weights, most of which were negative. This bioassay was carried out using 60 mm Petri dishes, which are not airtight. Although a damp paper towel was wrapped around each of the replicates and checked daily that it was remaining damp, it is hypothesised that changes to larval weights were due to moisture loss. The extreme differences between treatments is puzzling and is potentially influenced by the position of each Petri dish. Petri dishes within a replicate were randomly stacked together in two stacks of four, but this was not a randomly generated design and bias has potentially been inadvertently introduced causing vast differences in moisture loss between treatments.

Interestingly, there were significant differences in both silicon concentration and consumption between plant species in bioassay I ($P < 0.001$ and $P = 0.036$, respectively). Meadow fescue plants had lower silicon on average (2.38% compared to 3.63%) and higher consumption (114 mg compared to 98.4 mg) than ryegrass plants. These results suggest that a higher silicon concentration on average in ryegrass plants may have reduced the consumption of this plant material. This pattern was not reflected in frass results. Additionally, the treatment with the highest silicon concentration was ryegrass EF supplemented with silicon (4.64% compared to 3.00% as the overall average across all treatments) and this was also the treatment with the lowest consumption (82.2 mg compared to the overall

average of 106.2 mg). However, consumption in this treatment was not significantly different from the other treatments.

The unclear results from bioassay I prompted alterations to methodology in a second bioassay. Instead of being contained in Petri dishes larvae were kept in 30 mL airtight specimen vials, and a subset were weighed daily to assess changes in weight over the course of the experiment. Overall, there was not a decrease in grub weights as seen in bioassay I. This further suggested issues with moisture loss in the previous experiment. Only ryegrass treatments were assessed in bioassay II and there was no significant interactive effect of silicon and endophyte infection on changes to larval weights. There was an effect of endophyte, with those fed E+ gaining significantly more weight than those fed EF. There was no significant differences in the quantity of root consumed and slight differences in the amount of frass produced. As mentioned above, there was some indication that EF roots contain more silicon than E+. Previous studies report changes in the ability of insect herbivores to convert food into biomass in relation to silicon content (Massey *et al.*, 2006), this may be the case in this experiment. Thus, larvae fed EF roots were exposed to higher levels of silicon and did not gain as much weight as those fed E+ for the same consumption of root mass. As previously mentioned, the loline concentrations found in the root material used in this experiment were not sufficient to affect grass grub larval feeding.

Of the larvae that were checked daily, all treatments had lost weight from their initial by the end of the experiment. There were no significant differences between treatments. These results suggest that increasing handling of larvae (i.e. daily) negatively affected them, leading to a reduced performance. In future experiments, handling of larvae should be minimised to ensure that this does not influence results, albeit, daily checking did provide some interesting results. It appears that with fresh roots larvae feed and gain weight but when roots aged after harvest and presumably their condition deteriorated, grass grub fed on them less and began to lose weight. With the addition of fresh roots, larvae gain weight again and then the same pattern reoccurs but to a greater extent in roots that had been

stored for a period. Bioassays carried out over a shorter period may be beneficial to detect differences between treatments without introducing the large variability that occurs in an artificial environment over an extended time period. For example, Frew *et al.* (2017a) detected feeding differences in a bioassay run over 24 hours.

Another factor that potentially influenced the feeding results seen in bioassay II was the age of the larvae. At the time of the experiment the larvae had been kept in small containers of soil in a cool store without additional food for close to 10 weeks. Although only actively feeding larvae were used, storage likely affected the health of the grubs. This is reflected in the higher sickness/mortality rate in bioassay II compared to bioassay I. It is possible that this also affected their feeding and results may not be reflective of newly collected grubs from pasture.

4.4.3 Further studies and recommendations

This study was not able to successfully determine if there is an interactive effect of silicon and endophyte on the feeding of a below-ground herbivore (grass grub) in a pasture grass species. This was due to a number of factors including grass-endophyte associations studied, lack of silicon differences between supplemented and unsupplemented plants, and limitations of experimental design due to time restrictions and plant growth. However, these experiments have provided a basis for future studies in this area.

Future experiments to assess the effect of silicon on the feeding of herbivores should make use of inert growth media (such as perlite/vermiculite) or hydroponics to grow plants (Massey & Hartley, 2006). It is likely that, in this study, there was already a high level of bioavailable silicon ($Si(OH)_4$) in the soil which plants were grown in. This led to limited additional accumulation of silicon following supplementation. The variability seen in silicon concentrations in this study is likely to be more reflective of plant genotypes than it is silicon treatments. The use of inert growth media would result in meaningful differences in silicon concentrations between the different silicon treatments. Due to the variability in silicon accumulation

within a cultivar, experiments utilising cloned plants (one supplemented and one not) would further reduce this variability.

Another limitation was the bulking of replicate plants together for silicon and loline analysis. This was done because plant growth was not as high as anticipated and there was not enough material for analysis from single plants. Also, both the preparation and analysis of these plant samples is very time consuming and would not have been possible for all the samples within the time available.

It was originally hypothesised that the addition of silicon may alter the alkaloid production of endophytes, hence the use of a ryegrass-endophyte association which potentially does not produce high concentrations of lolines in the roots (Ball & Tapper, 1999). However, because of this selection, experiments conducted here did not detect an effect of endophyte infection on herbivore feeding in ryegrass treatments. It may be beneficial to examine the effects of silicon on grass-endophyte associations which are known to have efficient anti-insect herbivore properties, such as AR1 and AR37 (Johnson *et al.*, 2013). However, this could only be studied in above-ground herbivore interactions as alkaloids are not translocated to the roots in these associations.

There was a high correlation between root consumption and frass production for both bioassay I and II ($P < 0.001$, $R^2 = 0.85$ and $P < 0.001$, $R^2 = 0.87$ respectively). This indicates that measurement of just one of these variables in future experiments may be sufficient. For example, incorporation of a soil environment in these bioassays would limit the ability to measure frass production but would still enable quantification of root consumption. Adapting the method in this way would be beneficial as it would reduce how artificial the environment is for soil-dwelling larvae. However, because silicon is known to influence digestibility of plant material and potentially influence frass production, for experiments incorporating silicon it would be best to measure both consumption and frass.

5. Temporal changes in grass-endophyte associations with silicon supplementation and effects on a generalist herbivore

5.1 Introduction

This chapter aims to assess the temporal interactions of silicon and endophyte through the measurement of endophytic mycelial mass, production of loline alkaloids and silicon in a two-way factorial design using two grass species. Known levels of silicon in spare root material from this initial experiment prompted the use of this material in an artificial diet to assess the development of a generalist herbivore moth larva, *Epiphyas postvittana* (Light brown apple moth or LBAM). It was hypothesised that the known levels of silicon would enable assessment of developmental differences due to silicon, which was not able to be achieved in previous experiments outlined in chapters three and four.

5.1.1 Interactions of endophyte infections and silicon in grass species

There are very limited studies investigating the interactions between silicon and *Epichloë* endophytes in pasture grass species. The investigation by Huitu *et al.* (2014) is the only known study to report differences in silicon content based on *Epichloë* endophyte infection. The authors investigated vole feeding preferences and measured silicon concentrations in response to endophyte infection in meadow fescue as well as different intensities of herbivore grazing. They found that silicon increased with grazing intensity and E+ plants had approximately 16% higher silicon than EF at all levels of grazing (Huitu *et al.*, 2014). Several other recent publications mention both silicon and endophytes as defences in grass species but do not specifically investigate the potential synergies (Helander *et al.*, 2016; Reynolds *et al.*, 2016; Saikkonen *et al.*, 2016). This chapter outlines an experiment assessing the silicon concentrations, mycelial mass, and loline alkaloid concentration in plants supplemented with silicon for four, seven or ten weeks.

5.1.2 Seasonal interactions of endophyte growth and alkaloid production

Studies have shown that endophytic alkaloid concentrations vary seasonally. For example, Patchett *et al.* (2011a) measured loline alkaloid concentrations in ten meadow fescue lines in a New Zealand field trial. The study found that total loline concentrations were highest in mid spring (October), followed by a sharp decline in late spring (November) and a further decline in autumn (March to May). There was a highly significant interaction between the meadow fescue breeding line and time of harvest. However, this study only measured herbage loline concentrations and not root concentrations. Another study has suggested that loline alkaloids are redistributed through the plant seasonally rather than a total change in concentration, specifically in response to herbivore attack (Patchett *et al.*, 2008b). Other factors such as nutrient availability and water stress can also influence alkaloid production (Malinowski & Belesky, 2000). It is hypothesised that low temperature reduces endophytic growth and may contribute to seasonal changes in alkaloid production (Ju *et al.*, 2006; Patchett *et al.*, 2011a). di Menna and Waller (1986) visually assessed changes to mycelium levels in perennial ryegrass grown in New Zealand. The authors found that mycelium counts were greatest in summer and autumn and concluded that this was related to temperature (di Menna & Waller, 1986). However, there is some evidence to suggest that endophytic growth (mycelial mass) does not determine the alkaloid concentration and distribution in plants (Spiering, 2000).

5.1.3 LBAM description and lifecycle

LBAM is used by the Endophyte research team at AgResearch Ruakura as a model organism, due to easy accessibility year-round and the polyphagous feeding habits of the larvae. However, LBAM is not a current pest in pastures. LBAM is an Australian native which has been introduced to New Zealand as well as the United Kingdom, New Caledonia and Hawaii (Danthanarayana, 1975) and more recently the United States (Brown *et al.*, 2010). LBAM is a leafroller moth and is known to feed on more than 500

plant species from 121 plant families, including important crops such as apples and citrus (Brown *et al.*, 2010; Mo *et al.*, 2006).

LBAM is able to complete two to four generations per year, dependent on both temperature and latitude (Brown *et al.*, 2010). The optimal temperature for development is 20°C. In New Zealand, there are typically three generations annually but occasionally four (Collyer & van Geldermalsen, 1975). Laboratory studies have found that the upper and lower thresholds of development are 31°C and 7.5°C respectively (Danthanarayana, 1975), indicating the LBAM has the potential to be widespread across New Zealand regions.

Female moths deposit egg masses of between 2 and 150 eggs on the smooth surfaces of foliage including the leaves, stems and fruit. Females are able to produce up to 1500 eggs (Danthanarayana, 1983). First instar larvae are ~1.5 mm long with a dark coloured head and light body. The larvae go through five (male) to six (female) instars and growth continues through winter, albeit much slower. Early instar larvae feed on the abaxial side of leaves in self-made silk webs. However, later instars fold individual leaves, cluster leaves together or create a web of leaves which connect to fruit and larvae feed on the fruit surface. Pupation occurs within this nest over ~10 days and the adult moth emerges (Danthanarayana, 1975). Some control of populations occurs due to natural predators and pathogens, including larval parasitoids. Other methods of control include the application of insecticides, and sex pheromones which disrupt mating (Collyer & van Geldermalsen, 1975).

5.2 Methods

5.2.1 Temporal changes to endophyte and silicon

Plants were germinated on 02/08/2018 and grown in polystyrene trays (see *sections 2.1.3* and *2.1.4*). Plants were assigned to treatments based on immunoblot results (see *section 2.1.5*) conducted on 08/10/2018. All eight treatments were used in this experiment (see *Table 2.1*). There were 10 plants per treatment and each plant was split into three even-sized daughter plants 10 weeks after germination (see *section 2.1.6*). Plants were supplemented with silicon for a period of either four, seven or ten weeks. One of each genotype clone was randomly assigned to each harvest time point (T1, T2 or T3). Silicon supplementation began on 11/02/2019. Plants were trimmed to 5 cm and fertilised (see *section 2.1.9*) on 18/02/2019, 12/03/2019 and 02/04/2019. Plants were kept in a screenhouse for the duration of the experiment (see *Fig. 2.4*) arranged in a randomised split-split-plot design. Harvesting of the first set of plants (T1) took place between 11/03/2019 and 13/03/2019, the second set of plants (T2) was harvested on 01/04/2019 and 02/04/2019, and the final set (T3) was harvested on 23/04/2019 and 24/04/2019.

Upon harvest, root material was separated from herbage and washed under cold water to remove excess soil. Herbage was split into pseudostems and leaf blades (see *section 2.2.1* and *Fig. 2.5*) and necrotic tissue was removed. Roots and pseudostems were placed into individual plastic bags and stored until required (see *section 2.2.2*). For each harvest point there were ten replicate plants. Plant material was bulked together to ensure there was enough material for silicon, loline, and mycelial mass analysis (see *section 2.3*). Replicates one to three, four to six, and seven to ten were bulked together. Silicon analysis was conducted on all samples (see *section 2.3.1*). Loline analysis was carried out for E+ samples only (see *section 2.3.3*) and mycelial mass analysis was only measured for E+ herbage samples (there is no mycelium in roots) (sees *section 2.3.2*).

5.2.2 LBAM artifical diet rapid bioassay

The known silicon concentrations of root material from the previous experiment was used as an indicator to select remaining root material to include in an artificial diet rapid bioassay with LBAM. For each species and endophyte status, the roots with the highest and lowest silicon concentration were selected (see *Table 5.1*), ground into a fine powder, and incorporated into a diet.

Table 5.1: Approximate silicon concentrations of selected root material for LBAM rapid bioassay based on analysis of the same plant genotypes used in experiment described in section 5.2.1

Species	Endophyte	Silicon treatment	Approximate silicon (%)
Meadow Fescue	E+	High	4.67
		Low	2.47
	EF	High	4.30
		Low	1.88
Perennial Ryegrass	E+	High	4.78
		Low	2.38
	EF	High	5.20
		Low	2.60

5.2.2.1 LBAM diet preparation

A semi-synthetic diet was made on 14/08/2019 and fed to newly hatched LBAM larvae for a period of two weeks. Ingredients included; 4.68 g agar, 153 mL Milli-Q water, 0.31 g sorbic acid, 0.2 g methyl parahydroxybenzoate, 2.5 g casein, 0.94 g Wesson's salts, 2.81 g finely ground wheatgerm, 5 g yeast, 3.75 g finely ground freeze-dried carrot, 0.47 mL linoleic acid, 0.047 g cholesterol, 0.43 g ascorbic acid and 1.5 g of freeze-dried ground root material for each treatment. This diet has previously been successfully used the by AgResearch Ruakura team with LBAM.

Agar, water, sorbic acid, methyl parahydroxybenzoate, casein, Wesson's salts and wheatgerm were heated in a microwave until boiling point in a 1 L

beaker. The diet was left to cool to 70°C, stirring occasionally. To the cooled mixture, the remaining ingredients (except root material). were added and thoroughly mixed through.

Diet was weighed (8.5 g) into 50 mL beakers labelled for each treatment. Beakers had been warmed on a hot plate to ensure the agar did not set too quickly. To this, 1.5 g of the corresponding root material was added and thoroughly mixed before being evenly spread into a 60 mm Petri dish. Diets were left at room temperature to set and then placed in the fridge overnight.

5.2.2.2 Setup of LBAM rapid bioassay

LBAM eggs were received at AgResearch Ruakura from Anne Barrington of Plant and Food Research Auckland on 14/08/2019. Eggs were left overnight in a 70 mL specimen vial at approximately 20°C. The following morning (15/08/2019) enough larvae had hatched to set up the bioassay. There was a total of 25 replicates for each of the eight treatments (totalling 200 larvae).

To each 1.5 mL Eppendorf tube, a circular piece of diet (~ 0.5 cm diameter) was added. A freshly hatched LBAM larva was carefully picked up with the tip of a fine paintbrush and placed on the side of the Eppendorf tube next to the diet (see *Fig. 5.1A*). The Eppendorf tubes were arranged in wooden blocks in a randomised design blocked by replicate (see *Fig. 5.1B*). Wooden blocks were wrapped in aluminium foil to prevent light penetration and placed in a 20°C controlled environment room.

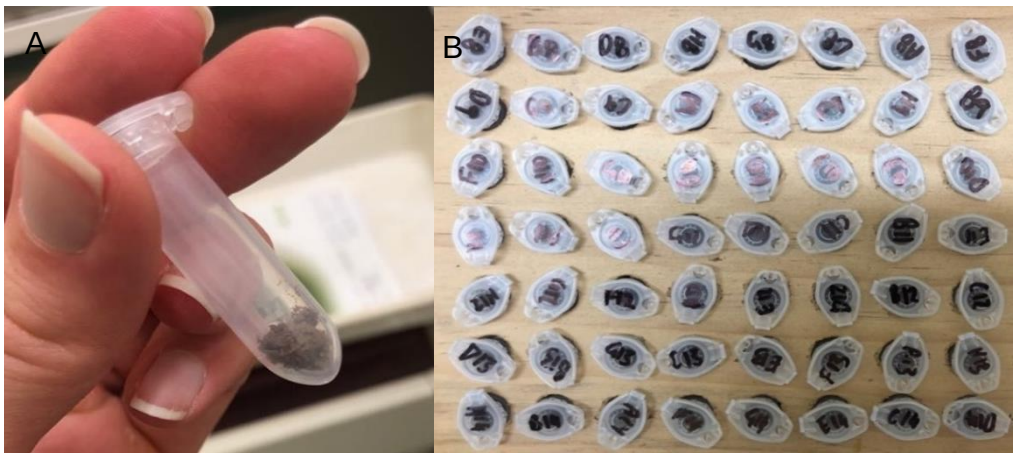

Figure 5.1 LBAM rapid bioassay setup. A) Piece of diet in Eppendorf tube with a single LBAM larva. Larva not visible. B). LBAM Eppendorf tubes arranged in a randomised block design in wooden block (Replicates 8 – 14).

5.2.2.3 LBAM experiment assessment

Larvae were assessed within the Eppendorf tubes under a stereo microscope at 63x magnification. The first assessment, on 16/08/2019, involved checking the survival, and whether the larva had started to produce webbing (see *Fig. 5.2*). The production of webbing indicated that the larva had established on the diet. On 20/08/2019, 21/08/2019, and 22/08/2019 the larva was checked to see whether or not it had undergone its first moult, and the amount of webbing was scored. A score of 3 indicated the larva had produced webbing all around the diet, 2 indicated webbing covering a large portion of the diet, 1 indicated a small amount of webbing, and 0 no webbing. The final assessment on 28/08/2019 involved checking larval instar, web scoring, and larval weight (Mettler AT261 Delta Range). Larval instar was based on the appearance and size of the head capsule. Larvae were left in empty Eppendorf tubes and placed in a -20°C freezer until 01/10/2019 when head capsule width was measured.

Figure 5.2: First instar LBAM viewed through plastic Eppendorf tube under a stereo microscope at 63x magnification. Webbing is visible above and to the right of larva.

5.3 Results

5.3.1 Correlations between silicon, lolines, and mycelial mass

There were no significant correlations between silicon, total lolines, and mycelial mass in herbage from either species. Nor were there significant correlations between silicon and total lolines in meadow fescue root material. Tests for correlations between silicon and loline contents in ryegrass root material were not possible because a large proportion of samples contained no detectable levels of loline alkaloids. There were significant correlations between the production of the individual lolines (NFL, NANL, and NAL) in the herbage material of both species (see *Table 5.2*).

Table 5.2: Correlation coefficients (r) of individual loline alkaloid production in meadow fescue and ryegrass herbage

	Meadow Fescue		**Ryegrass**	
NAL	0.91		0.94	
NANL	0.92	0.82	0.95	1.00
	NFL	NAL	NFL	NAL

5.3.2 Loline concentrations

Total loline concentrations in the herbage of meadow fescue plants declined throughout the experiment (see *Table 5.3*); this pattern was similar across the 3 individual lolines measured (NAL, NANL, and NFL). Total loline concentrations were significantly less at T3 (10 weeks supplementation) compared to T1 (4 weeks) and T2 (7 weeks) ($P < 0.05$), declining by 42.2 % between T1 and T3. There was no significant difference between total lolines at T1 and T2. NANL and NFL showed a similar pattern, reducing by 28.7 % and 43.8 % between T1 and T3 respectively. There was no significant difference between T1 and T2. Results for NAL varied slightly, there was a significant difference between T1 and T2, as well as T2 and T3. Overall the concentration of NAL reduced by 32.1 % between the first and last harvest date. In contrast, there was no significant decline in the root loline concentrations for meadow fescue plants (see *Table 5.4*), although

Table 5.3: Meadow fescue herbage loline levels over 10 weeks of silicon supplementation. Different letters next to loline concentration indicate significant differences between harvest time for that loline (Fishers Unprotected LSD, P < 0.05).

Loline alkaloid	Harvest Time	Mean Concentration (µg/g)	SD	Range	Percentage change from T1 (%)
NAL	T1	557 a	69.9	458 - 649	-
	T2	474 b	78.3	335 - 565	- 14.9
	T3	378 c	20.0	345 - 349	- 32.1
NANL	T1	1057 a	138.4	858 - 1204	-
	T2	1013 a	268.1	513 - 1320	- 4.2
	T3	754 b	63.1	703 - 876	- 28.7
NFL	T1	12455 a	1737.9	9732 - 14393	-
	T2	10882 a	2179.5	6756 - 13125	- 12.6
	T3	7000 b	545.1	6473 - 7970	- 43.8
Total Lolines	T1	14080 a	1922.1	11047 - 16152	-
	T2	12368 a	2505.3	7605 - 14948	- 12.2
	T3	8132 b	607.1	7578 - 9233	- 42.2

Table 5.4: Meadow fescue root loline levels over 10 weeks of silicon supplementation. Different letters next to loline concentration indicate significant differences between harvest time for that loline (Fishers Unprotected LSD, P < 0.05). ND = not detectable, concentrations were below the detectable limit.

Loline alkaloid	Harvest Time	Mean Concentration (µg/g)	SD	Range	Percentage change from T1 (%)
NAL	T1	5.99 a	14.7	ND - 36	-
	T2	ND a	-		- 100
	T3	ND a	-		- 100
NANL	T1	14.68 a	23.8	ND - 55	-
	T2	ND a	-	-	- 100
	T3	ND a	-	-	- 100
NFL	T1	317 a	188.3	109 - 631	-
	T2	225 a	98.9	133 – 352	- 29.1
	T3	243 a	153.0	74 - 454	- 23.3
Total Lolines	T1	344 a	220.5	109 – 722	-
	T2	229 a	106.0	133 – 369	- 33.4
	T3	247 a	159.3	74 - 477	- 28.3

total lolines reduced by 28.3% between T1 and T3. There was no detection of either NAL or NANL in T2 or T3, and only low levels in T1 (detected in 3 and 6 samples total respectively). Root loline concentrations for meadow fescue were significantly lower than herbage concentrations for each loline and overall ($P < 0.05$). The percentage of NFL out of total lolines in meadow fescue roots (96.59%) was higher than in the herbage (87.83%) ($P < 0.001$), while the opposite was true for NANL and NAL was (4.15% in herbage and 1.17% in roots, and 8.02% in herbage and 2.23% in roots, respectively) ($P < 0.001$). There was no effect of silicon supplementation on the proportions of lolines in roots and herbage.

Ryegrass plants had a different pattern of lolines compared to meadow fescue. Ryegrass herbage had significantly lower loline concentrations than those found in meadow fescue ($P < 0.05$). There was a significant decrease in total lolines and NFL in ryegrass herbage between T1 and T3, 120.3 % and 66.2 % respectively (see *Table 5.5*). Concentrations from T2 were not different from either T1 or T3. There was also a reduction in NAL and NANL (detected only in silicon supplemented samples) between T1 and T3, but these were not significant differences. Ryegrass roots were the only samples to show an increase in loline concentrations over time (see *Table 5.6*), although increases were not significant ($P > 0.05$). Total loline concentrations were low in ryegrass root material, ranging from non-detectable to 292 µg/g. NAL and NANL were not detected in any T1 or T3 root samples, and levels were low in T2 (only detected in two samples total). There was no difference in the proportions of each of the lolines between the roots and the herbage.

There was no effect of silicon supplementation on the production of total or individual lolines in meadow fescue herbage or ryegrass roots ($P > 0.05$). There were differences in the presence of lolines in meadow fescue roots based on silicon supplementation. Roots supplemented with silicon had half the total lolines of those not supplemented ($P = 0.006$) and significantly less NFL ($P = 0.005$). There was no effect of silicon supplementation on NAL or NANL in meadow fescue roots.

Over the whole experiment, silicon supplementation significantly increased the production of each loline in ryegrass herbage (NFL (*P = 0.006*), NANL (*P = 0.004*), and NAL (*P = 0.005*)). There was also a significant interaction between silicon supplementation and harvest date on total loline production (*P = 0.037*) and each of the individual lolines (NFL (*P = 0.035*), NANL (*P = 0.042*), and NAL (*P = 0.05*)) (see *Fig. 5.3*). This effect was due to the significantly higher lolines detected in silicon supplemented plants at T1 (see *Fig. 5.3*). No NAL or NANL was found in any samples that were not supplemented with silicon.

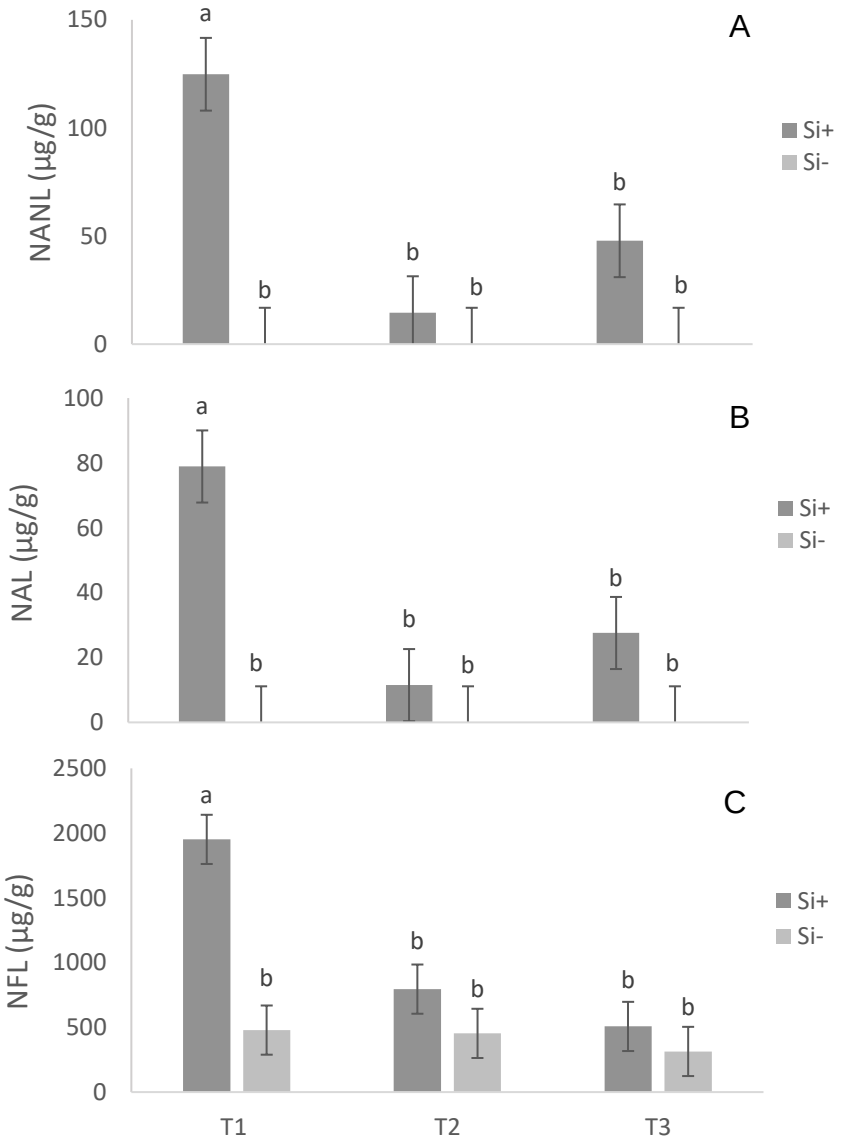

Figure 5.3: Concentration of individual lolines in ryegrass herbage with and without silicon supplementation over a 10 week period; A) NANL B) NAL C) NFL. Error bars are ± SEM. Different letters above bars denote a significant difference LSD (Fishers Unprotected (P < 0.05)).

Table 5.5: : Ryegrass herbage loline levels over 10 weeks of silicon supplementation, values are not based on silicon supplementation i.e. include both supplemented and not supplemented. Different letters next to loline concentration indicate significant differences between destruction dates for that loline (Fishers Unprotected LSD, P < 0.05). ND = not detectable, concentrations were below the detectable limit.

Loline alkaloid	Harvest Time	Mean Concentration (µg/g)	SD	Range	Percentage change from T1 (%)
NAL	T1	39.45 a	48.6	ND – 117	-
	T2	5.74 a	14.1	ND – 34	- 85.4
	T3	13.72 a	21.7	ND – 47	- 65.2
NANL	T1	62.42 a	79.0	ND – 193	-
	T2	7.28 a	17.8	ND – 44	- 88.3
	T3	23.91 a	30.2	ND – 73	- 61.7
NFL	T1	1215 a	938.0	446 – 2808	-
	T2	624 ab	234.1	345 – 1022	- 48.6
	T3	411 b	149.2	276 – 695	- 66.2
Total Lolines	T1	1317 a	1065.1	446 – 3118	-
	T2	644 ab	264.3	345 – 1100	- 51.1
	T3	542 b	197.3	276 – 815	- 120.3

Table 5.6: Ryegrass root loline levels over 10 weeks of silicon supplementation, values are not based on silicon supplementation i.e. include both supplemented and not supplemented. Different letters next to loline concentration indicate significant differences between destruction dates for that loline (Fishers Unprotected LSD, P < 0.05). ND = not detectable, concentrations were below the detectable limit.

Loline alkaloid	Harvest Time	Mean Concentration (µg/g)	SD	Range	Percentage change from T1 (%)
NAL	T1	ND a	-	ND	-
	T2	6.3 a	15.5	ND - 38	-
	T3	ND a	-	ND	-
NANL	T1	ND a	-	ND	-
	T2	8.39 a	20.6	ND - 50	-
	T3	ND a	-	ND	-
NFL	T1	11.29 a	17.9	ND - 40	-
	T2	33.99 a	83.3	ND – 204	+ 201.1
	T3	36.7 a	44.9	ND – 109	+ 225. 1
Total Lolines	T1	11.29 a	17.9	ND – 40	-
	T2	48.73 a	119.4	ND – 292	+ 331.6
	T3	40.44 a	52.4	ND – 131	+ 258.2

5.3.3 Mycelial mass

Average mycelial mass in ryegrass (5.32 µg/mg) was significantly higher than in meadow fescue plants (3.62 µg/mg) (*P < 0.001*). In a significant interaction between plant species and silicon supplementation (*P < 0.001*) (see *Fig. 5.4*) ryegrass with silicon had significantly more mycelial mass than those not supplemented (*P = 0.002*), whereas the opposite was true for meadow fescue (*P = 0.006*).

Meadow fescue non-silicon supplemented plants at T3 had significantly lower mycelial mass than the same plant genotypes sampled at T1 *(P = 0.016)* and T2 (*P = 0.026*). There was not an overall effect of harvest time, however, on the mycelial mass of E+ treatments (*P = 0.176*) (see *Table 5.7*) or other significant differences with any other treatments.

Table 5.7: ANOVA results of mycelial mass. Significant effects and interactions in bold. Residual degrees of freedom = 35.

Source of Variation	F-statistic	P-value
Harvest time	2.36	0.176
Species	41.56	**< 0.001**
Silicon	0.13	0.725
Species.Silicon	23.53	**< 0.001**
Species.Harvest time	3.37	0.057
Silicon.Harvest time	0.3	0.747
Species.Silicon.Harvest time	0.85	0.444

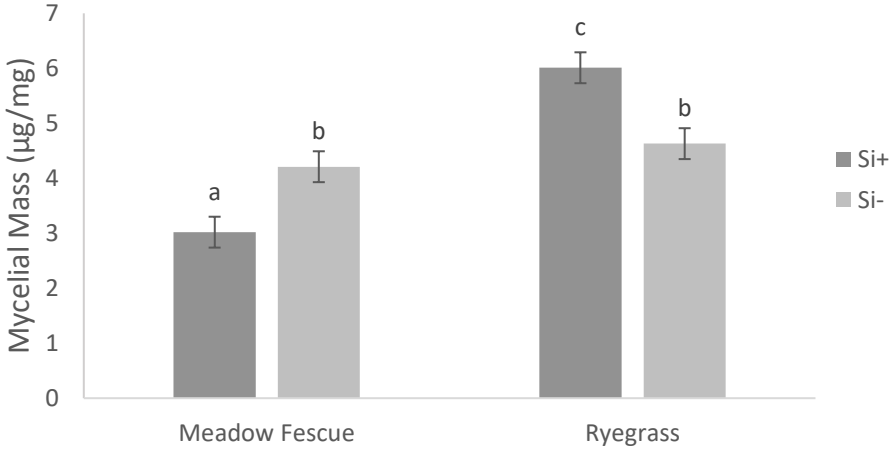

Figure 5.4: Mycelial mass (µg/mg) of meadow fescue and ryegrass based on silicon supplementation. Error bars are ± SEM. Different letters above bars denote a significant difference LSD (Fishers Unprotected (P < 0.05)).

5.3.4 Silicon levels

There were no significant correlations between the root and herbage silicon for either species, endophyte status, or silicon supplementation.

5.3.4.1 Herbage material

Meadow fescue had significantly higher silicon content in herbage than ryegrass (*P < 0.001*), 1.40% and 1.19% respectively. There was a significant effect of both harvest date (T1 = 1.30%, T2 = 1.65%, T3 = 1.26%; *P = 0.007*) and silicon supplementation (Si+ = 1.48%, Si- = 1.33%; *P = 0.031*) on silicon levels of meadow fescue herbage, but no effect of endophyte infection (*P = 0.337*). There were no interactive effects. In contrast, ryegrass herbage silicon levels (see *Table 5.8*) were significantly different based on harvest date (T1 = 1.20%, T2 = 1.40%, T3 = 0.96%; *P = 0.004*), and endophyte infection (E+ = 1.12%, EF = 1.25%; *P = 0.031*) but not silicon supplementation (*P = 0.154*). There were no interactive effects for ryegrass herbage.

5.3.4.2 Root material

Ryegrass root material had significantly higher silicon levels than meadow fescue (*P < 0.001*); 3.66 % and 2.90 % respectively. There was no effect of harvest date, endophyte, or silicon supplementation on ryegrass or meadow fescue root silicon levels, nor were there any interactive effects for ryegrass (*P > 0.05*). There was an interactive effect of silicon, endophyte infection, and silicon supplementation on the root silicon levels in meadow fescue (*P = 0.034*). This was due to a significant difference between T1 and T2 (*P = 0.03*) and T1 and T3 (*P = 0.005*) EF non-supplemented plants. No other treatments had significant differences between harvest times (see *Fig. 5.5*).

Table 5.8: Herbage silicon (%) levels over 10 weeks of silicon supplementation. Different letters next to silicon level indicates significant differences between other values for that plant species (Fishers Unprotected LSD, P < 0.05).

Treatment	Harvest Time	Silicon level (%)	SD	Range	Percentage change from T1 (%)
Meadow Fescue Si+	T1	1.32 ab	0.12	1.20 – 1.47	-
	T2	1.78 c	0.28	1.45 – 2.25	+ 34.2
	T3	1.35 ab	0.12	1.22 – 1.51	+ 1.8
Meadow Fescue Si-	T1	1.28 ab	0.09	1.16 – 1.41	-
	T2	1.52 b	0.23	1.27 – 1.92	+ 18.2
	T3	1.17 a	0.22	0.90 – 1.45	- 8.8
Ryegrass E+	T1	1.08 a	0.12	0.96 – 1.23	-
	T2	1.34 b	0.25	1.03 – 1.69	+ 23.6
	T3	0.94 a	0.14	0.73 – 1.13	- 13.3
Ryegrass EF	T1	1.32 b	0.20	1.10 – 1.69	-
	T2	1.46 b	0.11	1.31 – 1.62	+ 10.7
	T3	0.98 a	0.14	0.81 – 1.15	- 25.9

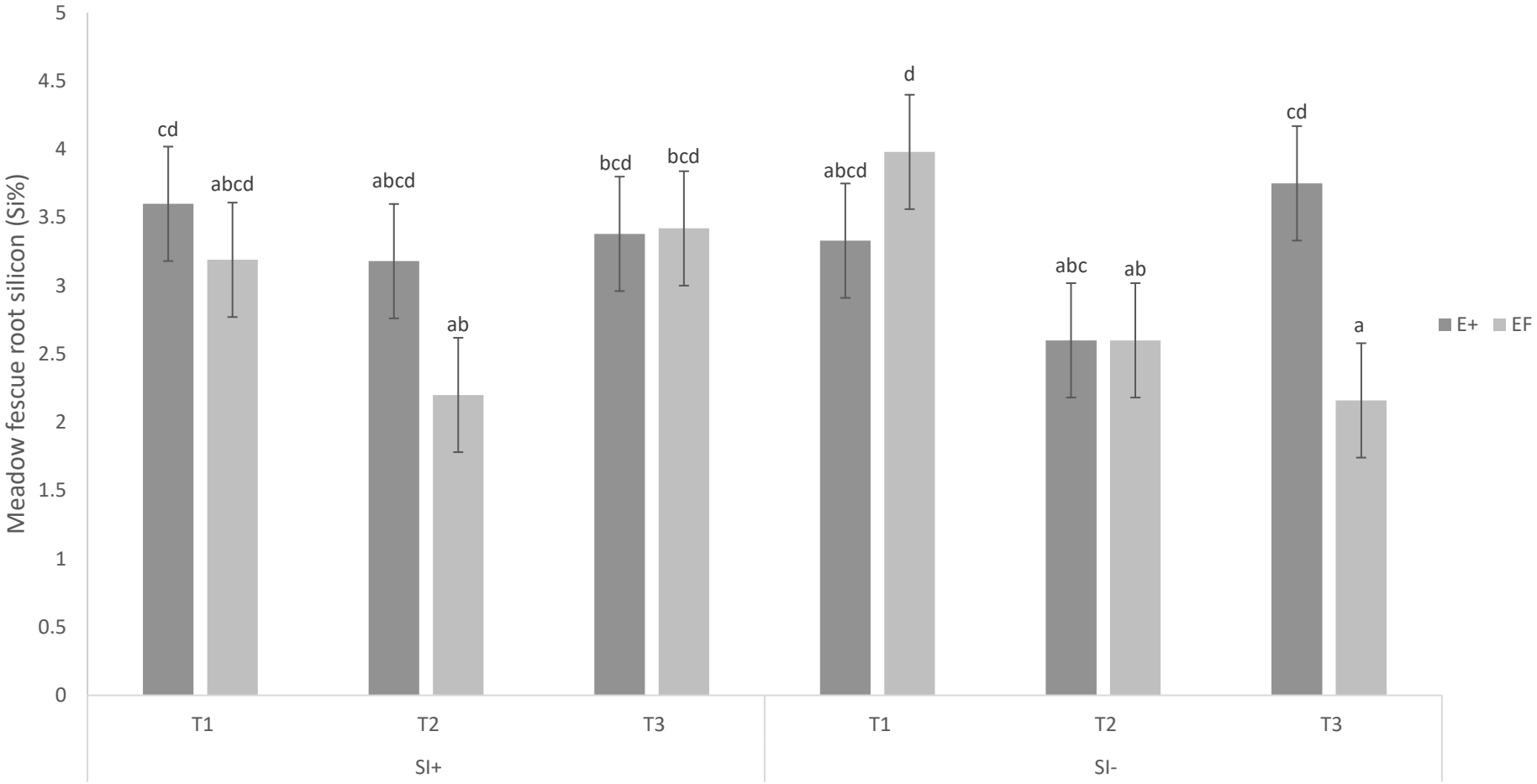

Figure 5.5: Meadow fescue root silicon level (%) at 3 time points. Error bars are ± SEM. Different letters above bars denote statistically significant difference (Fishers Unprotected LSD, P < 0.05).

5.3.5 LBAM bioassay

At the first assessment (20/08/2019) the number of larvae that had moulted in ryegrass E+ high silicon was significantly lower than ryegrass E+ low silicon (*P = 0.032*) and ryegrass EF high silicon (*P = 0.032*), but not significantly different from ryegrass EF low silicon (*P = 0.538*). There were no significant differences between any meadow fescue treatments (*P > 0.05*). By the following day (21/08/2019) there were no significant differences in larval instar for any treatments. Results were the same on 22/08/2019. By the final assessment, larval instar for ryegrass EF low silicon was significantly less than all other treatments (*P < 0.05*).

There was no significant difference between the web scores of treatments on 20/08/2019 (*P > 0.05*). By the final assessment (28/08/2019) the web score of ryegrass EF high silicon was significantly lower than that of ryegrass E+ low silicon (*P = 0.023*). There were no other significant differences between web scores of treatments.

There were significant differences in the final weight and head capsule size of larvae between treatments (*P < 0.001*) (see *Fig. 5.6*). There was a significant effect of plant species and of silicon status (i.e. high or low; see *Table 5.1*) on final larval weights and head capsule size (*P < 0.001*). Larvae in meadow fescue treatments were heavier and had larger head capsules than ryegrass treatment larvae, and those fed high silicon roots were larger than those fed roots with low silicon. Endophyte infection was a significant variable for larval weights (*P = 0.001*) but not head capsule size (*P = 0.828*). Larvae in E+ treatments weighed significantly less than those in EF; this effect was significant across all treatments and for meadow fescue (*P < 0.001*) but not ryegrass (*P = 0.103*). Of the 200 larvae, seven did not survive throughout the experiment (3.5%) but there was no treatment effect on mortality.

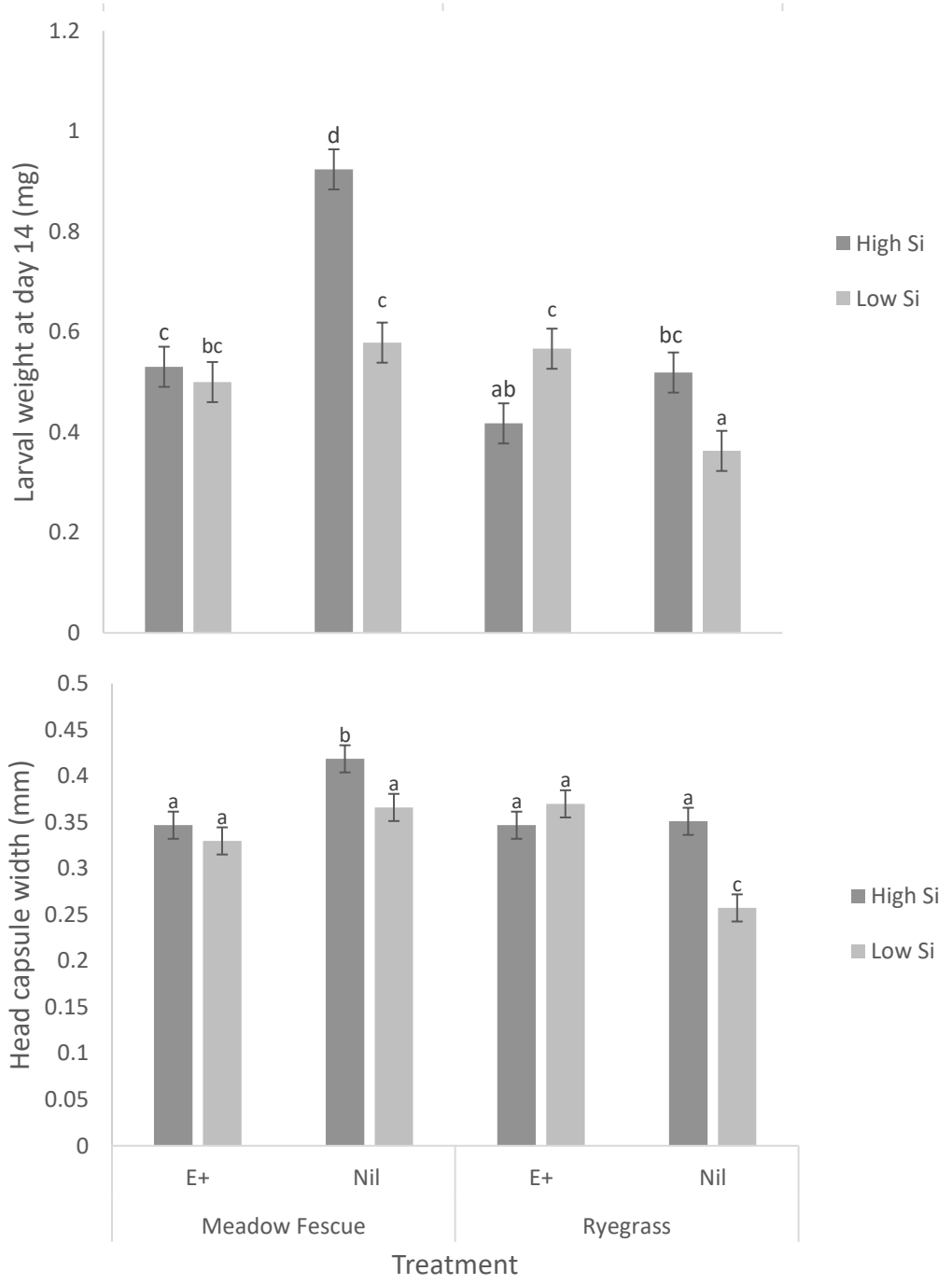

Figure 5.6: Weight and head capsule size of LBAM larvae after being fed meadow fescue or ryegrass roots with different levels of silicon. A) Larval weight (mg) B) Head capsule width (mm). Error bars are ± SEM. Different letters above bars denote statistically significant difference (Fishers Unprotected LSD, P < 0.05).

5.4 Discussion

5.4.1 Temporal changes to silicon, lolines and mycelial mass

This study found no correlations between silicon levels and endophyte mycelial mass or loline alkaloid concentrations in either root or herbage material of ryegrass and meadow fescue. This suggests that the presence of one of these defences does not directly influence the presence of the other. There was no increase in any of the measured variables over time, contrary to what was expected. These results may have been affected by seasonality and/or plant trimming regimes used in this experiment. Interestingly, there was nil detection of both NANL and NAL in ryegrass not supplemented with silicon, while there was a low concentration detected in silicon supplemented. Mycelial mass results did not indicate any significant change over time in either species, apart from a decrease between T1 and T3 in meadow fescue non-silicon supplemented plants. This supports results from Spiering (2000), that mycelial mass or endophytic growth does not appear to be proportional to alkaloid production. Overall these results reject the original hypothesis of this thesis, that silicon concentration would increase over time and this would be associated with an increase in endophytic mycelial mass and subsequently loline alkaloids.

This study also did not find an anticipated increase in silicon concentration in E+ plants compared to EF. There was no difference in meadow fescue silicon levels based on endophyte infection, and ryegrass EF plants had 11.61% more silicon on average than E+ plants. In contrast, Huitu *et al.* (2014) observed an average increase of 16% in silicon levels of E+ meadow fescue plants, however, this was a different cultivar than the one used in this experiment. In the grass-endophyte associations studied here, the hypothesis that endophyte infection is linked to increased silicon concentration (Huitu *et al.*, 2014) is rejected.

Interestingly, meadow fescue had higher herbage silicon levels than ryegrass and the opposite was true for root material. This suggests that the two species differentially allocate silicon to their tissues. Hodson *et al.*

(2005), Hartley *et al.* (2015), and Massey *et al.* (2007a) discuss differences in the accumulation of silicon between and within species but only report shoot silicon concentrations. There are numerous other studies which compare shoot silicon concentrations between species, but few studies report shoot and root silicon concentrations and compare allocations of silicon between species. This study demonstrates that between two pasture grass species, the accumulation and storage of silicon in below- and above-ground portions of the plant is variable.

Herbage silicon results were potentially influenced by the sampling method and plant trimming regime carried out in this experiment. Remaining plants (for subsequent harvests), were trimmed after each harvest, ensuring that all plants had equal time to re-grow before harvest. This approach was used to simulate typical grazing that would occur in a pastoral agriculture setting. However, trimming and removal of plant material did not allow for an accumulation of silicon in herbage over time. Also, pseudostem tissue was used for analysis because this is where mycelial mass is concentrated. Similar experiments investigating silicon analysed both the leaf and pseudostem bulked together, or just leaf material which is known to accumulate high levels of silicon (Hall *et al.*, 2019; Kumar *et al.*, 2017). Ideally, both leaf and pseudostem tissue would have been analysed separately in this experiment to gain further insight into the distribution of silicon and alkaloids throughout plants. However, because sample preparation and analysis is labour intensive, this was not possible.

This study also found a decrease in ryegrass herbage loline levels alongside an increase in root lolines in response to silicon supplementation. Root loline concentrations in this novel-association were minimal and other work has shown an absence of lolines in the roots of novel associations between ryegrass hosts and tall fescue endophytes (Popay, A. J. unpublished). Although the same effect was not observed in meadow fescue in this study, in previous work by Patchett (2007) a shift in lolines from pseudostems to root material had been observed, during a similar time of year, in various meadow fescue breeding lines. The authors suggested that this shift was aligned with decreases in soil temperature and implied that rather than

producing additional alkaloids, plants are able to mobilise and relocate them, which aligns with the timing of the experiment reported here. They also proposed that this shift coordinated with when roots were most susceptible to attack from belowground feeders, such as grass grub, and lolines would be relocated to herbage in spring to protect new growth from folivore herbivores (Patchett, 2007).

Results for all chemical analyses were also likely impacted by low replication. During experimental design, it was anticipated that plants would grow large enough that there would be enough material for analysis of each individual plant. However, plant growth was less than expected and therefore replicates had to be bulked together. Additionally, sample preparation was more labour intensive than predicted. The low replication for each treatment limited the ability to detect differences between and within treatments and did not allow for comparison of clonal plants between harvest times. It is recommended that future studies allow plants to grow larger (more tillers) prior to beginning silicon supplementation. Based on these results, and lack of correlations between the measured variables, analysis of all of the variables may not be required and so less plant material would be needed.

5.4.2 LBAM larval development bioassay

Root tissue incorporated into artificial diets was selected based on known values of silicon from the previous experiment and would therefore only be approximate. Larvae fed roots from E+ ryegrass with high silicon gained less weight than the low silicon E+, while results from meadow fescue E+ treatments were not significantly different. In contrast, in both species, larvae fed EF high silicon gained more weight than EF low silicon. This suggests that LBAM larvae perform better on high silicon diets when there is no endophyte present. The reason for this is unknown. There is some evidence to suggest that silicon may alter the carbon to nitrogen ratio of the plant (Frew *et al.*, 2019) which may affect the development of larvae (Moise *et al.*, 2019). Moise *et al.* (2019) studied the effect of silicon and nitrogen

application on *Zea mays* (maize) and the performance of an armyworm (*Pseudeletia unipuncta*). The authors found that the deleterious effects of silicon on the insect were partially mitigated with the application of nitrogen. There is also evidence that nitrogen and other elements such as carbon and phosphorus, alongside silica, are occluded in phytolith structures as they are formed (Alexandre *et al.*, 2016; Laue *et al.*, 2007). This potentially changes the nutritional quality of plant material and influences the development of insects. Analysis of plant carbon to nitrogen ratios in the future would enable further disentanglement of the underlying causes of differences in larval development between treatments. Another factor that may have influenced results is the unknown effect that grinding plant tissue has on the structure of phytoliths and the importance this may have on feeding deterrence properties. The functional significance of structural diversity in phytoliths is unknown (Cooke & Leishman, 2011), suggesting that structure, as well as silicon content itself, may have a role in alleviating herbivore damage.

Silicon levels in the roots used in this experiment appear to be much higher than those observed in other root-herbivore studies. Frew *et al.* (2017a) found that root silicon levels in sugarcane fell between 0.25% and a maximum of 2.5%. This study found a negative effect on insect herbivore mass increase with increasing silicon concentrations. In the present study, root silicon concentrations were estimated to fall between 1.88% and 5.20%. It is possible that the lowest silicon levels used here are higher than what would cause negative effects on herbivores. Therefore, the differences seen in herbivore performance are likely due to underlying factors such as nutrient balance rather than silicon content. However, there are limited studies investigating the effect of silicon in root tissue on root-herbivores and further research is required to better understand the mechanism behind root-herbivore feeding and silicon defences.

Overall, larvae in E+ treatments gained significantly less weight than those in EF treatments. This effect was only significant for meadow fescue and not ryegrass. This suggests that the loline alkaloid levels present in meadow fescue root material were sufficient to reduce the performance of LBAM

larvae, but not in ryegrass. This is not unexpected as alkaloid concentrations in the novel endophyte association are typically low (Malinowski & Belesky, 2019) and the results presented here show that they were lower than those which have been previously been known to reduce insect feeding (Jensen *et al.*, 2009; Popay & Lane, 2000).

Results from this study indicate some areas of improved methodology to be used in the future. Larval weights were more effective at detecting differences in the development of larvae than measurement of head capsules. Head capsule measurement was limited as it is strongly correlated to larval instar, and the majority of larvae were second or third instar by the end of the experiment. However, larval weight, even within an instar, is more variable and provides more information on the overall development of the larvae. This suggests that in the future only measurement of final larval weight is required. The measurement of both variables does however provide an indication of relative development of the larvae between treatments i.e. larvae may gain weight but not develop to the next instar (noted through head capsule measurement). Additionally, when the first assessment of larval instar was conducted the majority of larvae had already developed to the second instar. In future, the development of larvae should be checked sooner and more frequently in order to detect any differences in initial larval development.

6. Final conclusions

The initial aims of this thesis were to:

a) investigate the potential synergistic defensive effects of silicon accumulation and *Epichloë* endophyte infection in pasture grass species on the performance of common New Zealand pasture pests;

b) determine temporal changes to silicon levels, loline concentrations, and endophytic mycelial mass in two species of grass infected with different endophyte.

6.1 Key findings

Initial experiments found that there was no effect of silicon supplementation on the feeding of either ASW adults or porina larvae, but their feeding was affected by the presence of endophytes. Presence of endophyte in the ryegrass did not reduce porina feeding but reduced the number of ASW feeding scars. Subsequent analysis of silicon content found no difference between silicon supplemented and non-supplemented plants. Later studies on the root-herbivore grass grub, with plant material supplemented with silicon for ten weeks, also found no effect of silicon and no significant differences in silicon content based on supplementation. There was a negative effect of meadow fescue endophytes on grass grub performance in whole-plant experiments but not in excised root assays, and no negative effects of ryegrass endophyte in either. Due to study limitations, discussed below, this study was not able to resolve the hypothesis that silicon and endophyte would have synergistic negative effects on the herbivory and performance of insect pasture pests. Further research is needed to be able to accept or reject this hypothesis.

There was not a clear temporal pattern of increases in silicon, loline alkaloids or mycelial mass in the two breeding lines. Nor were there any strong correlations between any of these variables. Interestingly, silicon supplementation of ryegrass did not result in an increase in plant silicon

content but did cause a significant increase in the production of loline alkaloids in the herbage. Overall, this study can reject the original hypothesis that silicon concentration would increase in both root and herbage material over time and that this increase would occur alongside an increase in mycelial mass and increased concentration of loline alkaloids in root tissue.

6.2 Study limitations

The main limitation of this study was the inability to 'produce' significant silicon differences between silicon supplemented and non-supplemented treatments. This meant that the synergies of silicon and endophyte were not able to be studied in a two-way factorial design as anticipated. It is hypothesised that bioavailable silicon in the soil utilised for plant growth was high, meaning that plants, even in non-supplemented treatments, were accumulating as much silicon as they were genotypically capable of. Consequently, the application of additional silicon did not have an effect on overall plant silicon content, and silicon content in this study was likely to be more strongly influenced by genotypic variability than silicon application regimes. Also of note is the potential increase in silicon accumulation with increasing moisture content reported by Ryalls *et al.* (2018). This study found that an Australian pasture grass, *Microlaena stipoides*, accumulated more silicon in herbage material when grown in elevated soil moisture conditions. In this experiment, the silicon supplementation regime and automatic watering of plants in the screenhouse meant that the soil moisture in which plants were grown was high. This may have further impacted the lack of differences in silicon content between treatments.

Another limitation of this study was the need to bulk replicate samples together for chemical analysis. This was both because of time pressure in sample preparation and analysis, and lack of plant material available from individual plants. Downstream effects of this were that, statistically, the low number of replicates reduced the ability to detect differences between treatments. Had each plant been analysed separately it would have been

possible to directly compare the silicon/loline values of each plant with the feeding and performance of the individual insects that fed on that plant material. This would have provided greater insight into the interactions between silicon and endophyte and subsequent effects on insect herbivores in individual plants.

6.3 Future research directions and suggestions

Results from this study have opened up further research questions that require investigation and suggestions for such research are discussed below.

Firstly, studies aiming to investigate the effects of silicon in pot trials should make use of inert growth media or hydroponics. This will optimise the potential to have differences in silicon content between treatments, while also reducing the variability of factors in other growth media such as soils. Researchers should also ensure the nutrient and moisture content is optimal for plant growth so that plants grow large enough that there is sufficient material for individual plant silicon analysis. Alternatively, researchers could employ analytical techniques for silicon analysis that require less plant material and sample preparation, such as high-resolution continuum source graphite furnace atomic absorption spectrometry (HR-GS SS-GF AAS) (Boschetti *et al.*, 2015) or inductively coupled plasma mass spectrometry (ICP-MS) (Pohl *et al.*, 2010). Another recommendation is to have genotypic clones of each plant (one supplemented and one not supplemented with silicon) and subsequently compare herbivore performance on these plants. This would reduce the effects of genotypic variability between plants and allow the study of direct effects of silicon supplementation. For example, in the summer of 2019/2020 genotypic clones will be grown in inert growth medium and subjected to varying silicon supplementation regimes. The clonal plants will then be re-potted into the same pot and ASW adults will be given a choice between the two plants for feeding and oviposition. This method will allow for the detection of differences in feeding and oviposition based on silicon supplementation treatment of the plant. Future experiments

investigating effects on plants/herbivores should utilise similar methods to optimise the study of silicon.

A further recommendation is to measure other relevant variables such as carbon to nitrogen ratios (Frew *et al.*, 2019) and phenolics (Massey *et al.*, 2007a) of plant material. These variables are known to have effects on insect herbivores (Frew *et al.*, 2016; Loranger *et al.*, 2012), and therefore would enable the researcher to have a more holistic understanding of plant-herbivore interactions in their studies. Analysis of all of these variables may lead to the ability to breed plant lines which are naturally highest in both defences; for instance, both high silicon content (Johnson *et al.*, 2016a) and alkaloid production.

Another potential factor affecting plant-herbivore interactions in these studies is the occlusion of other nutrients such as carbon and nitrogen within silica phytolith structures (Alexandre *et al.*, 2016; Laue *et al.*, 2007). Further studies are needed to understand the effect that this may have on experimental outcomes. Specifically, the effect when plant material is ground into a fine powder and incorporated into an artificial diet for insect feeding assessment may alter the phytolith structure. This might therefore alter the available carbon and nitrogen within plant material thereby influencing herbivore performance. Also, because silicon is concentrated within phytoliths, effects on insect mandibles and/or intestinal tracts may be less obvious to observe after material is ground.

A key area of future research is the need for a better understanding of the potential impact that silicon fertilisation may have in pastoral New Zealand systems. There is an abundance of research indicating that increased silicon increases resistance from insect herbivores (Frew *et al.*, 2017a; Hall *et al.*, 2019; Massey *et al.*, 2007b; Ryalls *et al.*, 2017). However, the bioavailable silicon content of our pastoral soils is not known. Potentially, some soil may already have a high concentration of silicic acid, such that supplementation of silicon would not influence the silicon content of plants. A survey of New Zealand soils, as well as plants, in different environments, would increase understanding of the potential impact that silicon application

would have in a field setting. Additionally, recent research has indicated that the application of nitrogen fertiliser (a common practice in New Zealand pastures) partially mitigates the negative effects of silicon on insect herbivore performance (Moise *et al.*, 2019). This suggests that the application of silicon in New Zealand may not be a practical or economical means of reducing damage to pastures caused by insect herbivores.

There is also potential that silicon not only plays a structural role in defence, but may also have a metabolic role (Leroy *et al.*, 2019), or influence changes to soil nutrients and microbial communities which consequently alters soil ecosystem properties and potentially plant performance (Frew *et al.*, 2019). For example, in the present study, there was no difference in herbage silicon content, but there was some evidence of an increase in loline alkaloid production in ryegrass plants supplemented with silicon. This suggests that silicon supplementation may be influencing plant performance through mechanisms other than silicon accumulation. Further research into the effects of silicon on soil properties as well as expression of genetic and metabolic properties of plants will aid in increasing our understanding of these possible mechanisms (Zargar *et al.*, 2019).

Another area that requires further research is knowledge of an effective level of silicon to reduce herbivore performance. In the literature, there is large variability in silicon contents which are reported to influence herbivory. For example, Moise *et al.* (2019) reported an effective increase from ~0.3% silicon to ~0.9% in maize. However, Massey *et al.* (2006) reported an average increase in ryegrass silicon content from 0.54% to 4.68% with supplementation and this influenced the feeding of two folivorous insects. A better understanding of the silicon content required to alter feeding of insects, across both plant and herbivore species, would aid in the potential implementation of silicon fertilisation as an effective means to reduce damage by pest insects in field settings.

Overall, this area requires further research to understand potential real-world applications of silicon, specifically in New Zealand pastoral systems, where the use of *Epichloë* endophytes is prevalent. Continued

investigation in this area and increased understanding of the interactions between silicon and endophytes may aid in reducing the economic impact of the most severe New Zealand pasture pests in the future.

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8. Appendices

8.1 Hill Laboratories soil test results

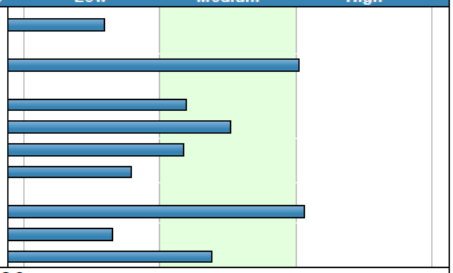
Summary of Methods

The following table(s) gives a brief description of the methods used to conduct the analyses for this job. The detection limits given below are those attainable in a relatively clean matrix. Detection limits may be higher for individual samples should insufficient sample be available, or if the matrix requires that dilutions be performed during analysis.

Sample Type: Soil			
Test	**Method Description**	**Default Detection Limit**	**Sample No**
Sample Registration*	Samples were registered according to instructions received.	-	1
Soil Prep (Dry & Grind)*	Air dried at 35 - 40°C overnight (residual moisture typically 4%) and crushed to pass through a 2mm screen.	-	1
pH	1:2 (v/v) soil:water slurry followed by potentiometric determination of pH.	0.1 pH Units	1
Olsen Phosphorus	Olsen extraction followed by Molybdenum Blue colorimetry.	1 mg/L	1
Potassium	1M Neutral ammonium acetate extraction followed by ICP-OES.	0.01 me/100g	1
Calcium	1M Neutral ammonium acetate extraction followed by ICP-OES.	0.5 me/100g	1
Magnesium	1M Neutral ammonium acetate extraction followed by ICP-OES.	0.04 me/100g	1
Sodium	1M Neutral ammonium acetate extraction followed by ICP-OES.	0.05 me/100g	1
CEC	Summation of extractable cations (K, Ca, Mg, Na) and extractable acidity. May be overestimated if soil contains high levels of soluble salts or carbonates.	2 me/100g	1
Total Base Saturation	Calculated from Extractable Cations and Cation Exchange Capacity.	5 %	1
Volume Weight	The weight/volume ratio of dried, ground soil.	0.01 g/mL	1

Sample Name: GG exp 1 **Lab Number:** 2040856.1
Sample Type: SOIL General, Outdoor (S10)

Analysis		Level Found	Medium Range	Low / Medium / High
pH	pH Units	5.5	5.8 - 6.3	
Olsen Phosphorus	mg/L	31	20 - 30	
Potassium	me/100g	0.56	0.50 - 0.80	
Calcium	me/100g	9.1	6.0 - 12.0	
Magnesium	me/100g	1.35	1.00 - 3.00	
Sodium	me/100g	0.16	0.20 - 0.50	
CEC	me/100g	27	12 - 25	
Total Base Saturation	%	41	50 - 85	
Volume Weight	g/mL	0.75	0.60 - 1.00	
Base Saturation %		K 2.1	Ca 34	Mg 5.0 Na 0.6
MAF Units		K 9	Ca 9	Mg 23 Na 5



The above nutrient graph compares the levels found with reference interpretation levels. NOTE: It is important that the correct sample type be assigned, and that the recommended sampling procedure has been followed. R J Hill Laboratories Limited does not accept any responsibility for the resulting use of this information. IANZ Accreditation does not apply to comments and interpretations, i.e. the 'Range Levels' and subsequent graphs.

8.2 Summarised XRF element output

Table 8.1: Herbage material summarised XRF output. All values in average (of all plants in specified treatment throughout all experiments in this thesis) ppm ± standard deviation, except silicon which is percentage ± standard deviation. MF = meadow fescue. RG = ryegrass.

Treatment	Si	Na	Mg	Al	P	K	Ca
MF E+ Si+	1.44 ± 0.32	378 ± 162	2644 ± 384	410 ± 31	4152 ± 580	40594 ± 6965	3713 ± 366
MF E+ Si-	1.30 ± 0.21	370 ± 242	2837 ± 298	397 ± 52	3944 ± 706	43819 ± 5942	3984 ± 401
MF EF Si+	1.37 ± 0.22	454 ± 230	3053 ± 379	402 ± 32	4041 ± 649	42615 ± 6004	4037 ± 311
MF EF Si-	1.31 ± 0.20	355 ± 128	2981 ± 414	398 ± 32	3894 ± 823	42452 ± 7065	4173 ± 533
RG E+ Si+	1.08 ± 0.23	717 ± 410	2287 ± 410	432 ± 33	3381 ± 469	34097 ± 7576	3184 ± 286
RG E+ Si-	1.08 ± 0.23	802 ± 514	2359 ± 563	412 ± 28	3321 ± 632	37082 ± 7280	3652 ± 247
RG EF Si+	1.22 ± 0.26	927 ± 256	2771 ± 551	438 ± 33	3731 ± 520	36638 ± 8362	3642 ± 283
RG EF Si-	1.15 ± 0.23	850 ± 332	2855 ± 424	417 ± 29	3405 ± 501	41123 ± 7515	4089 ± 369

Treatment	Mn	Fe	Cu	Zn	Ag	S	Cl
MF E+ Si+	69.4 ± 15.8	372 ± 280	4.86 ± 0.78	59.2 ± 13.4	290 ± 85	1854 ± 364	28544 ± 4485
MF E+ Si-	37.4 ± 13.7	377 ± 261	4.78 ± 0.65	65.5 ± 11.8	269 ± 82	2021 ± 234	28904 ± 3619
MF EF Si+	63.3 ± 12.0	368 ± 193	4.85 ± 0.61	64.3 ± 14.3	264 ± 65	2019 ± 258	29288 ± 3786
MF EF Si-	73.0 ± 15.8	451 ± 302	5.06 ± 0.60	61.5 ± 13.9	286 ± 52	2038 ± 298	28603 ± 3555
RG E+ Si+	81.7 ± 27.4	349 ± 173	5.36 ± 0.90	58.0 ± 14.6	346 ± 138	2086 ± 328	33209 ± 3910
RG E+ Si-	82.5 ± 19.4	319 ± 189	5.26 ± 0.75	63.6 ± 20.6	300 ± 109	2127 ± 262	33693 ± 6681
RG EF Si+	73.6 ± 21.8	518 ± 235	5.20 ± 0.79	61.8 ± 10.9	279 ± 63	2316 ± 322	36130 ± 5725
RG EF Si-	77.2 ± 15.8	506 ± 236	5.47 ± 0.83	62.1 ± 12.4	287 ± 47	2239 ± 275	36666 ± 6186

Table 8.2: Root material summarised XRF output. All values in average (of all plants in specified treatment throughout all experiments in this thesis) ppm ± standard deviation, except silicon which is percentage ± standard deviation. MF = meadow fescue. RG = ryegrass.

Treatment	Si	Na	Mg	Al	P	K	Ca
MF E+ Si+	3.14 ± 0.73	204 ± 62	726 ± 130	4007 ± 874	1351 ± 208	9467 ± 4277	4201 ± 654
MF E+ Si-	2.92 ± 0.92	160 ± 118	756 ± 155	4018 ± 1116	1504 ± 166	12052 ± 5168	4441 ± 861
MF EF Si+	2.80 ± 0.65	181 ± 84	613 ± 147	3678 ± 840	1383 ± 211	9830 ± 4368	4052 ± 718
MF EF Si-	2.69 ± 0.98	169 ± 94	637 ± 187	3915 ± 1510	1447 ± 257	11499 ± 5507	4395 ± 729
RG E+ Si+	3.55 ± 0.72	177 ± 70	710 ± 101	4434 ± 1011	1357 ± 198	8051 ± 2106	4293 ± 862
RG E+ Si-	3.36 ± 0.81	170 ± 70	696 ± 181	4166 ± 965	1496 ± 238	9011 ± 2907	4126 ± 421
RG EF Si+	4.26 ± 1.04	174 ± 45	714 ± 111	5104 ± 1312	1305 ± 151	6488 ± 2530	4037 ± 523
RG EF Si-	3.46 ± 0.87	148 ± 96	793 ± 212	4110 ± 993	1315 ± 213	7445 ± 3772	3949 ± 706

Treatment	Mn	Fe	Cu	Zn	Ag	S	Cl
MF E+ Si+	79.3 ± 23.2	3732 ± 1142	15.0 ± 4.8	61.9 ± 17.8	302 ± 53	1090 ± 340	3704 ± 1749
MF E+ Si-	82.0 ± 24.8	4101 ± 3321	15.4 ± 4.8	60.8 ± 7.5	306 ± 80	1315 ± 294	4142 ± 1726
MF EF Si+	65.7 ± 15.8	3087 ± 856	16.3 ± 2.9	51.3 ± 6.3	316 ± 58	1215 ±347	3614 ± 1470
MF EF Si-	75.9 ± 16.8	3370 ± 1031	17.2 ± 4.1	70.5 ± 29.3	328 ± 49	1258 ± 303	3666 ± 1755
RG E+ Si+	103.3 ± 20.6	4580 ± 1653	20.8 ± 8.5	71.7 ± 11.9	386 ± 82	962 ± 139	3157 ± 1418
RG E+ Si-	93.1 ± 25.5	3768 ± 1052	18.4 ± 7.5	91.4 ± 42.9	369 ± 116	1039 ±180	3210 ± 1486
RG EF Si+	95.7 ± 18.9	4564 ± 1345	20.2 ± 9.7	65.4 ± 19.5	378 ± 95	919 ± 191	2387 ± 1265
RG EF Si-	92.2 ± 20.6	4401 ± 2263	18.4 ± 4.6	64.3 ± 17.3	356 ± 58	948 ± 189	3205 ± 1930