Investigating Tri-trophic Interactions between Pasture Aphids, Perennial Ryegrass and Fungal Endophytes

Submitted by
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SUMMARY

Perennial ryegrass is the most important forage grass in the Australian and New Zealand dairy industries. The fungal endophyte *Epichloë festucae* var. *lolii* forms a symbiotic relationship with perennial ryegrass, producing various alkaloid compounds that provide protection against invertebrate pests, including aphids. This project investigated this tri-trophic interaction by evaluating the insecticidal activity of five *Epichloë*–perennial ryegrass symbiota (SE, AR1, AR37, NEA2 and NEA6) against four common pasture aphids, *Rhopalosiphum padi*, *Diuraphis noxia*, *Aploneura lentisci* and *Metopolophium dirhodum*. This project developed a high-throughput *in-planta* bioassay to evaluate the effects of *Epichloë*–perennial ryegrass symbiota on aphid life history (mortality and fecundity); profiled the alkaloids produced by *E. festucae* var. *lolii in-planta* to determine their effects on aphid life history and the effects of aphid feeding on alkaloid production; and assessed the mode of action of *Epichloë*–perennial ryegrass symbiota using electrical penetration graph (EPG) to monitor their effect on aphid feeding behaviour. From the bioassay, all aphid species experienced high mortality when feeding on *Epichloë*–perennial ryegrass symbiota, specifically SE and NEA2, followed by NEA6 and AR37, then AR1. *Rhopalosiphum padi* and *A. lentisci* showed significantly decreased fecundity on all endophytes. The alkaloids that correlated with the highest mortality were lolitrem B and ergovaline, produced by SE and NEA2. Alkaloid production was enhanced by aphid feeding highlighting the strong mutualistic symbioses between *E. festucae* var. *lolli* and perennial ryegrass, however the response was different for each endophyte and alkaloid. The mode of action analysis indicated that AR37, NEA2 and NEA6 had a feeding deterrence effect on *R. padi*, whereas SE and AR1 had no effect on feeding. No major alkaloid could be attributed to the feeding deterrance, however it was postulated that an indole-diterpene precursor (e.g. paspaline) or novel compound may be involved. Interestingly, SE had no effect on feeding, yet had the highest mortality on *R. padi*, which may relate to other chemistries produced by SE that suppress deterrent effects or are greater attractants (e.g. volatile organic compounds). The study consistently showed the wild-type endophyte SE provided the most

broad-spectrum insecticidal activity, providing support for the commercial use of this endophyte providing the toxicity can be managed (e.g. through mycotoxin binding agents or combining with less toxic endophytes). Overall this project sheds new light on the tri-trophic interaction between endophytes, perennial ryegrass and aphids, and more clarity on how specific endophytes benefit pasture production with respect to pest management, particularly aphids.

STATEMENT OF AUTHORSHIP

This thesis consists primarily of work by the author that has been published or submitted for publication as described in the text. Except where reference is made in the text of the thesis, this thesis contains no other material published elsewhere or extracted in whole or in part from a thesis submitted for the award of any other degree or diploma. No other person's work has been used without due acknowledgment in the main text of the thesis. This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution.

Nicholas Paul Collinson

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THESIS PREFACE

This thesis is composed of five chapters, with the original experimental content presented in the form of one published peer-reviewed journal article and two papers that are presented in journal article format, one submitted and one in preparation for submission in scholarly journals. Chapter 1 provides a general overview of the literature in this area of research. The manuscript that has been published is presented in Chapter 2, the manuscript that has been submitted for peer-review is presented in Chapter 3, and the manuscript that is complete and awaiting submission for peer-review is presented in Chapter 4. Each of these chapters contains its own detailed introduction, methodology, results, and discussion sections. Each experimental chapter is prefaced by a summary of the work, the publication details of that manuscript, the contribution of co-authors and a statement from the co-author confirming the authorship contribution of the PhD candidate. Chapter 5 provides a general discussion that integrates the major themes from each of these manuscripts, as well as providing suggestions for future research directions. Because each experimental chapter corresponds to an independently published or submitted manuscript, or manuscript in preparation for submission, some redundancy of content has arisen between the introduction and materials and methods sections of the respective journal articles. In addition, the individual experimental chapters employ the respective distinct referencing, citation and formatting styles of the corresponding journals. In contrast, a single referencing and citation style has been employed for chapters 1 and 5, and the bibliography is provided at the end of the thesis.

LIST OF ABBREVIATIONS

BYDV	Barley yellow dwarf virus
CYDV	Cereal yellow dwarf virus
EFN	Extrafloral nectar
EPG	Electrical penetration graph
JA	Jasmonic acid
LC–MS	Liquid chromatography – mass spectrometry
NZ	New Zealand
r_m	Intrinsic rate of increase
RMV	Ryegrass mosaic virus
SA	Salicylic acid
SE	Standard endophyte
VOC	Volatile organic compound
WE	Without endophyte

CHAPTER 1

Introduction

1.1 Tri-trophic interactions

Plant growth, whether for agriculture, horticulture or in the wild, is dependent on interactions with other organisms (White and Torres, 2009). Almost all species of plants are vulnerable to feeding by herbivores or infection by bacteria, fungi or viral pathogens (Clarke and Eagling, 1994; Jones, 2013; Oerke, 2006). However, many interactions between plants and other organisms can be beneficial (Clay, 1988; Landis et al., 2000). Symbiotic mutualism is one such beneficial relationship, whereby a plant is host to another organism (the symbiont) and provides an environment in which it may grow and reproduce, while in turn, the symbiont confers the plant with resistance to certain abiotic or biotic stresses (Clay, 1988). Many plant mutualisms confer tolerance to feeding damage by insect herbivores, one of the main biotic stresses affecting plant growth (Paini et al., 2016). Such interactions, where defensive responses in the plant symbiont are triggered or mediated by a third organism, are referred to as tri-trophic interactions (Agrawal, 2000). Tri-trophic interactions commonly occur between plants and beneficial microorganisms that confer tolerance to a microbial pathogen or a vertebrate or invertebrate herbivore (Bush et al., 1997; Heil, 2008). They can also involve a mutualism between two species of invertebrates, to provide defence against a predator (Birch et al., 1999; Bristow, 1991; Flatt and Weisser, 2000; Heil, 2008).

One example of a tri-trophic interaction is seen in plants that release volatile organic compounds (VOCs) or secrete extrafloral nectar (EFN) as a response to being fed upon (Heil, 2008; Sullivan et al., 2007). These compounds are attractive to natural enemies of insect herbivores (Girling et al., 2008); for example, VOCs are attractive to predatory mites and parasitoid wasps (Heil, 2008). Boland et al. (1998) found that plants that produced the hormone jasmonic acid (JA) in response to herbivory by lepidopteran pests attracted parasitoid wasps; and in another study, applications of JA to gerbera and lima bean plants, designed to simulate the feeding-induced JA production, resulted in the production of VOCs

that attracted natural enemies of herbivorous mites (Gols et al., 1999). The chemical profiles of these VOCs were similar, but not identical, to those emitted by natural herbivory (Gols et al., 1999). Bristow (1991) stated that the interactions between Argentine ants, *Iridomyrmex humilis* (Mayr), Oleander aphids, *Aphis nerii* (Boyer de Fonscolombe), and the aphids' preferred host, oleander plants, *Nerium oleander* L., may be considered tri-trophic due to the mutualism between the ants and the aphid. Bristow (1991) hypothesized that this mutualistic relationship was influenced by host plant factors such as feeding site, as *I. humilis* as more likely to tend colonies of *A. nerii* on floral tips rather than colonies on leaves (Bristow, 1991).

Understanding tri-trophic interactions is beneficial to agriculture, as these mutualistic relationships are often advantageous to crop plants. Cultivating these plant–symbiont interactions has the potential to increase yields, decrease energy expenditure or insecticide use, and improve plant tolerance to adverse climatic conditions (Agrawal, 2000; Birch et al., 1999; Heil, 2008).

1.2 *Epichloë*–perennial ryegrass symbiota

Perennial ryegrass is the most widely used grass species in dairy pastures in the temperate regions of both Australia and New Zealand (NZ) (Dairy Australia, 2019). It is favoured over other species for its high nutritional value and digestibility for dairy cattle, its high tolerance for grazing and its ability to remain productive for up to four years, unlike annual ryegrass, *Lolium multiflorum* Lam., (Wilkins, 1991). Perennial ryegrass has been bred for many decades in order to provide fodder for livestock over multiple years (Wilkins, 1991). Whereas annual ryegrass can have greater herbage yields, perennial ryegrass often has a more extensive root mass, allowing for greater persistence in fields (Wilkins, 1991). This offers the advantage of higher resistance to harsh climate conditions, grazing or trampling, the ability to grow in more compacted soils, and the ability to reduce soil erosion (Wilkins, 1991).

Despite these advantages, perennial ryegrass is susceptible to feeding damage by several types of invertebrates, including sap-sucking hemipterans such as aphids (Popay and

Gerard, 2007; Thom et al., 2014), as well as abiotic factors such as drought (Hesse et al., 2003). Perennial ryegrass shares a symbiotic mutualism with the fungal endophyte *Epichloë festucae* var. *lolii*, which confers the plant with insect tolerance properties (Hume and Sewell, 2014), drought or salt tolerance (Hesse et al., 2003; Sabzalian and Mirlohi, 2010), as well as an increase in both root mass and herbage yield (Schardl et al., 2004). *Epichloë* endophytes exist asymptomatically within the intercellular spaces of the host plant tissue for their entire life cycle (Schardl et al., 2004). These endophytes have no sexual reproductive phase; they remain permanently in the vegetative phase, where they occupy the internal tissues of the plant via continual hyphal growth (Schardl and Leuchtmann, 2005). They colonise the developing spike and spread into the seeds of the host plant, enabling transmission into the subsequent generation of host plants – a transmission method known as vertical transmission (Schardl, 1996). This mutualistic relationship has evolved over millions of years to benefit both perennial ryegrass and the endophyte (Clay, 1993). The benefits of *E. festucae* var. *lolii* in perennial ryegrass are most apparent in the presence of insect herbivory, where the most significant advantages over uninfected plants are seen (Schardl et al., 2004): *Epichloë* endophytes have been shown to decrease overall aphid population size on host plants by up to 64% compared to populations on endophyte-free plants, and individual aphid fecundity has been found to reduce by up to 50% (Bastias et al., 2017b).

Fungal endophytes can also have an indirect effect on host plant resistance to herbivory. Omacini et al. (2001) found that the presence of fungal endophytes negatively affected aphid performance, but also the rate of parasitoid attack on aphids. Similarly, Fuchs et al. (2013) found that alkaloids were present not only in aphids that fed on endophyte-infected grasses, but also in predatory insects that fed on the aphids. Endophytes can also effect the herbivore-induced emission of VOCs that can act as attractants to predators of aphids and other pests (Li et al., 2014).

The key component in *E. festucae* var. *lolii*-induced direct insect tolerance is the production of bioprotective alkaloid compounds (Bush et al., 1997). Alkaloids are chemical

compounds, often toxic or unpalatable to herbivores, and those produced by *Epichloë* endophytes are grouped into the following four categories: indole diterpenes, ergopeptides, pyrrolizidines, and pyrrolopyrazines. Indole diterpenes include tremorgenic neurotoxins called lolitrems, of which the most commonly produced by *E. festucae* var. *lolii* is lolitrem B, which is linked to the condition known as ryegrass staggers in cattle and sheep (Fletcher and Harvey, 1981). Ergopeptides include several alkaloids derived from lysergic acid, most commonly ergovaline (Bush et al., 1997). Ergovaline is a vasoconstrictor and, as such, is also toxic to livestock – it is linked to the condition commonly called fescue foot (Klotz et al., 2007; Tor-Agbidye et al., 2001). Pyrrolizidines include the loline alkaloids such as N-formyl-loline and N-acetyl-loline, both of which are strong, broad-spectrum insecticides, but are not produced by *E. festucae* var. *lolii* (Wilkinson et al., 2000). To date, only one pyrrolopyrazine has been isolated from *Epichloë*–grass symbiota, and that is the feeding deterrent peramine. This alkaloid has a strong anti-feedant effect, as demonstrated on the argentine stem weevil, *Listronotus bonariensis* (Kuschel), which is common in NZ dairy pastures (Rowan et al., 1986; Rowan and Gaynor, 1986). Many alkaloids have varying insecticidal properties on a range of insect pests of perennial ryegrass. This has led to the development of several commercial endophyte symbiota, producing a range of alkaloid profiles, for use as a form of biological control in dairy pastures in Australia and NZ (Thom et al., 2014).

1.3 Invertebrate pest species in perennial ryegrass pastures

There are many species of invertebrates found in pasture grasses (Tscharntke and Greiler, 1995). Some pose no threat to pasture grasses, and others can be beneficial, acting as natural enemies of pest insects (Landis et al., 2000) or as pollinators (Potts et al., 2009). However, many invertebrates are pests of pasture grasses and can result in significant damage to productivity (Henderson and Clements, 1977). Perennial ryegrass is fed upon by a number of insect pests, including *L. bonariensis*; scarab beetles such as the African black beetle,

Heteronychus arator (Fabricius), and the red-headed cockchafer, *Adoryphorus couloni* (Burmeister); the red-legged earth mite, *Halotydeus destructor* (Tucker); and several species of aphids (Pennell et al., 2005).

Insect pests of pastures can have significant effects on production and can potentially lead to a 100% reduction in productivity when left unchecked (Breen, 1993; Chapman et al., 2017). In Australia, invertebrate pests cause an estimated $4.7 billion of damage to agricultural production annually (Hoffmann and Broadhurst, 2016), and in NZ, insect pests cause an average annual loss of $1.4 billion in dairy pastures specifically (Ferguson et al., 2019). Economic impact studies have largely focused on root-feeding insects of pastures (e.g. *H. arator* and *A. couloni*), however, these figures do not take into account the effects of plant viruses, often transmitted by aphids and mites, which contribute further to crop losses and the overall economic cost of invertebrate pests (Walls et al., 2019).

1.3.1 Aphid impacts on pasture and other grass species

Aphids are responsible for significant yield loss in cereal crops and pasture grasses, either as vectors of viral pathogens or via direct feeding and phytotoxic saliva (Hughes and Maywald, 1990). Aphid populations can infest crops rapidly and prove difficult to control (Minks and Harrewijn, 1988). This is largely due to their parthenogenetic method of reproduction, which allows rapid population increase in a short span of time (Kindler et al., 1991; Leather and Dixon, 1982), and their short generation time, which allows rapid development of resistance to insecticides (Edwards et al., 2008).

Several aphid species, most notably the bird cherry-oat aphid, *Rhopalosiphum padi* (L.), are vectors for barley yellow dwarf virus (BYDV). BYDV is considered the most prevalent and economically important virus in cereal crops and pasture grasses worldwide (Banks et al., 1995; McKirdy et al., 2002; Walls et al., 2019): it has been found to reduce yields of wheat and oat by up to 49% (Choudhury et al., 2019), and can also stunt herbage growth and decrease total yield in perennial ryegrass (Catherall, 1966). Catherall (1987) found that BYDV caused

greater yield loss in perennial ryegrass than ryegrass mosaic virus (RMV), a common disease of pasture grasses spread by mites (Mulligan, 1960). In perennial ryegrass, BYDV can cause stunting, increased tillering and leaf yellowing (Jones, 2013). It can also decrease herbage yield by 20–22% compared to healthy swards (Catherall, 1966; Latch, 1980), and can cause a reduction in root dry weight of 30–40% (Eagling et al., 1989b). A survey of Tasmanian pastures discovered several incidences of BYDV in perennial ryegrass: of 1048 samples, 140 contained BYDV (13%), and the highest level of infection in a single pasture was 70%, in a four-year-old pasture in Forth, Tasmania (Guy et al., 1986). A similar survey in South Australia found BYDV present in all 24 examined sites, and a maximum incidence of 86% in a single site (Eagling et al., 1989a), further confirming BYDV's widespread infection in perennial ryegrass.

In addition to acting as virus vectors, many aphids cause significant damage through direct feeding (Harris and Maramorosch, 2014; Parry et al., 2012). The Russian wheat aphid, *Diuraphis noxia* (Kurdjumov), is one such species that can cause significant damage to crops through feeding, due to its phytotoxic salivary secretions (Nicholson et al., 2012). *Diuraphis noxia* is also a pest of high importance in pastures due to its broad host range of cool- and warm-season grasses, allowing it strong over-winter and over-summering potential respectively (Kindler et al., 1991; Kindler and Springer, 1989). Likewise, *R. padi* feeds and reproduces on pasture grasses and can increase in population size at a rapid rate (Hales et al., 2013). Aphid infestations are among the main threats to dairy pasture production that endophytes such as *Epichloë* can offer effective protection against (Thom et al., 2014).

1.3.2 Aphids (Hemiptera: Aphididae) of pasture grasses

The most common aphid pests found in pasture grasses (Poaceae) include *R. padi* (Dixon, 1971); *D. noxia* (Clement et al., 1992); the rose grain aphid, *Metopolophium dirhodum* (Walker) (CABI, 2020a); the English grain aphid, *Sitobion avenae* (Fabricius) (M. C. Johnson et al., 1985); the Indian grain aphid, *Sitobion miscanthi* (Takahashi); the root aphid, *Aploneura*

lentisci (Pennell et al., 2005); the greenbug, *Schizaphis graminum* (Rondani) (Breen, 1992); and the corn leaf aphid, *Rhopalosiphum maidis* (Fitch) (CABI, 2016a). Other aphids, such as the rusty plum aphid, *Hysteroneura setariae* (Thomas), and the mealy plum aphid, *Hyalopterus pruni* (Geoffroy), have also been recorded on pasture grasses but are less prevalent (Blackman and Eastop, 2000, 2007).

Aphids are small, soft-bodied hemipterans of the family Aphididae. They feed on phloem sap from plant hosts and secrete a sticky, sugary fluid (commonly known as honeydew) as a waste product (Auclair, 1963). Individuals feed using specially adapted piercing, sucking mouthparts collectively known as a stylet: a flexible tube that pierces the plant epidermis and probes into the phloem to extract sap (Auclair, 1963; Pollard, 1973). Aphids average 2–8 mm in length (Chakrabarti, 2018; Gerson and Applebaum, 2015) and are most commonly green in colour, but can range from yellow to black, brown or red in some species (Gerson and Applebaum, 2015). Most species possess characteristic rear dorsal tubules known as siphunculi, as well as a posterior tail-like protrusion known as the cauda, both of which are unique to aphids (Fig. 1) (Blackman and Eastop, 2007; Gerson and Applebaum, 2015). Siphunculi produce a defensive fluid, and while the function of the cauda is unknown, it is believed to play a role in flicking honeydew droplets away from the aphid (Gerson and Applebaum, 2015).

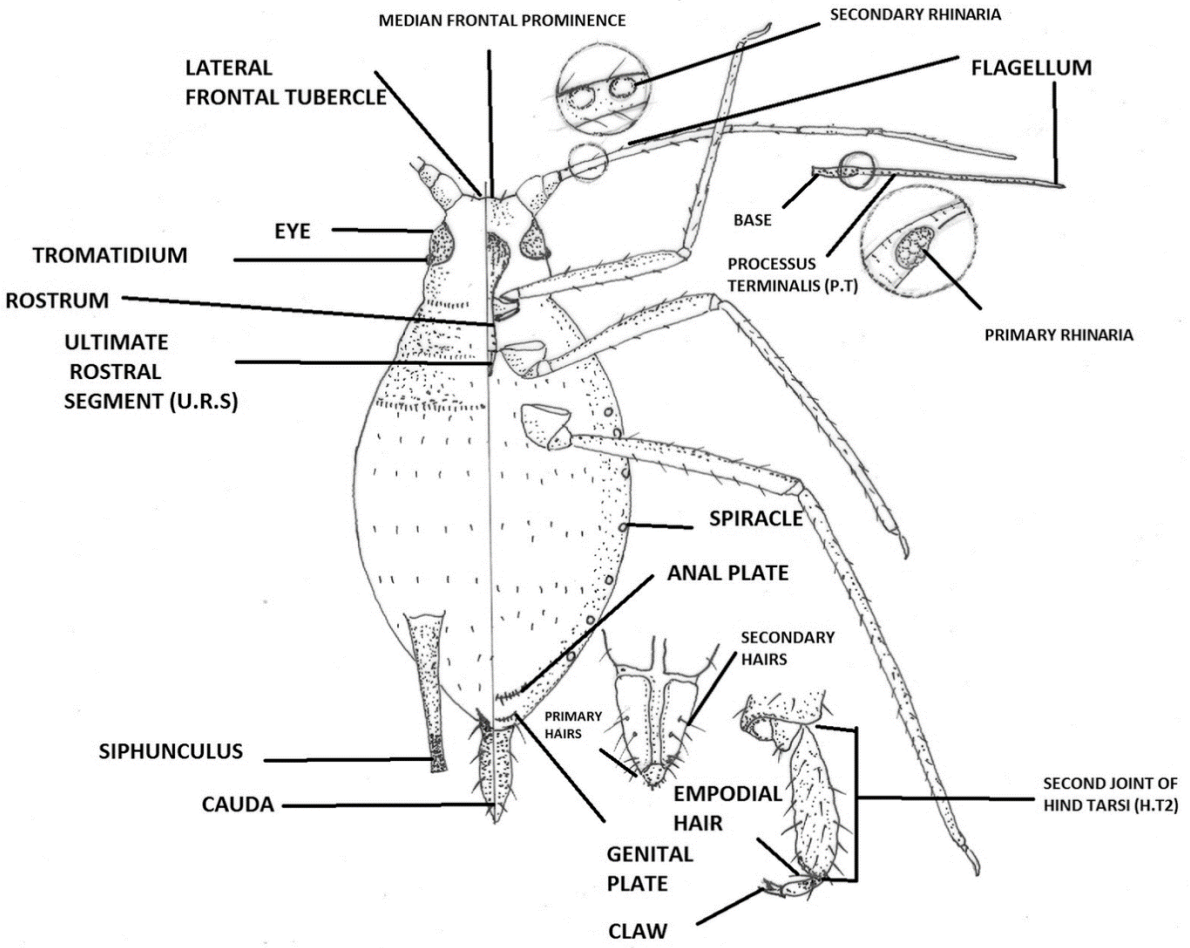

Figure 1: Dorsal and ventral morphology of an aphid showing unique aphid characteristics: siphunculus and cauda. Image modified from Chakrabarti (2018).

There are currently an estimated 4800 extant species of aphids worldwide (Blackman and Eastop, 2007) and the majority of these alternate between sexual and asexual reproduction on corresponding primary and secondary host plants (Fig. 2) (Dixon, 1971; Shufran and Puterka, 2011; Williams and Dixon, 2007). In their native range, sexual aphid morphs mate and lay overwintering eggs on perennial woody or herbaceous plants, and asexual morphs spend the warmer months of the year on annual herbaceous plants, undergoing rapid parthenogenetic reproduction to take advantage of increased plant growth (Williams and Dixon, 2007). However, outside of a species' native range, many aphids (e.g.

R. padi outside of Northern Europe) are anholocyclic, and forego the overwintering host and live entirely on herbaceous plants as asexual morphs, reproducing solely through parthenogenesis (Fig. 2) (Eastop, 1966). This process leads to lower levels of genetic diversity within and between populations outside of their native range, but can also lead to more rapid population growth (Dixon, 1985).

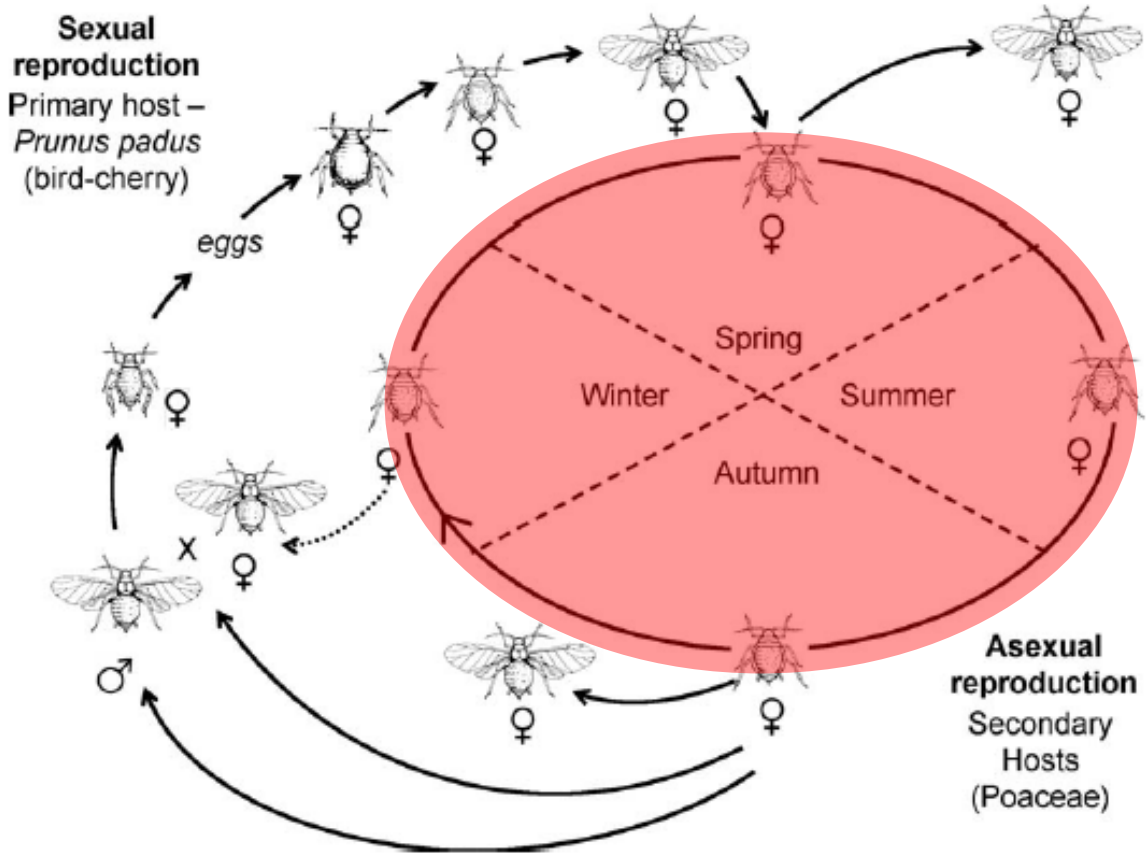

Figure 2: Holocyclic life cycle of Rhopalosiphum padi, found typically in the native range in northern Europe, showing winter sexual reproductive cycle with female and male aphid morphs. Highlighted: Anholocyclic life cycle showing no sexual reproduction and populations that are majority female. Cycles are typical of all aphids investigated in this study. Image modified from Finlay and Luck (2011).

Aphids are short-lived, often living for only one to two months unless overwintering (Williams and Dixon, 2007). In parthenogenetically-reproducing populations, nymphs are born

live and then undergo four moults, progressing through four instars before reaching the adult life stage, when they begin to reproduce (Williams and Dixon, 2007). Nymphs are born with the embryo of the next generation nymph already developing inside them – a process known as telescopic generations (Minks and Harrewijn, 1988). Depending on species, nymphal development typically takes one to two weeks, but can be prolonged by adverse climatic conditions or by feeding on unfavourable host plants, such as endophyte-infected ryegrass (Merrill et al., 2009). There are several species of aphid found in Australian dairy pastures. Detailed below are four of the most common and most significant to pasture production.

1.3.2.1 *Rhopalosiphum padi*

Rhopalosiphum padi is an established pest of broadacre cereal grains and pasture grasses (Pettersson et al., 2007). Its status as a pest is predominantly due to its vectoring of plant viruses, most notably BYDV (Blackman and Eastop, 2007; Parry et al., 2012).

Apterous *R. padi* are on average 2.5 mm long, broadly oval in shape and vary in colour from dark green to black to light yellow/green with a rust-red coloration around the siphunculi and cauda (Eastop, 1966; Emden and Harrington, 2007). *Rhopalosiphum padi* have six antenna segments, where the terminal process of the sixth segment is 3.1–5.2 times as long as the base of that segment (Eastop, 1966).

The common hosts of *R. padi* include: oats, *Avena sativa* L.; barley, *Hordeum vulgare* L.; ryegrass, *Lolium* spp., including perennial ryegrass; rice, *Oryza sativa* L.; wheat, *Triticum aestivum* L.; and maize, *Zea mays* L. (CABI, 2016b). *Rhopalosiphum padi* has a worldwide distribution, being found in Europe, Asia, North America, Central America, South America, Africa, Australia and NZ (Fig. 3) (CABI, 2020b).

Figure 3: Worldwide distribution of Rhopalosiphum padi showing its recorded presence in orange markers for each country, state or territory where recorded. Image from CABI datasheet on Rhopalosiphum padi: www.cabi.org/isc/datasheet/47321 (CABI, 2020b).

In Europe and North America, *R. padi* is holocyclic and reproduces via parthenogenesis in the warmer months of the year on Poaecae secondary hosts, with an annual sexual reproductive stage on a primary host (the bird cherry, *Prunus padus* L.) during the winter (Puterka et al., 1993). In Australia, due to the milder winters and absence of the primary host necessary for sexual reproduction, *R. padi* is most often anholocyclic and lives and reproduces parthenogenically solely on Poaceae species (Valenzuela et al., 2010). As a result, individuals are able to maintain a higher reproductive rate through a greater part of the year (Dixon, 1971), and overwinter from June to September as hibernating third or fourth instar nymphs (Valenzuela et al., 2010). This results in a lower level of genetic diversity in Australian populations compared to European populations (27 distinct genotypes compared to 287 respectively), yet this does not appear to have any negative effect on *R. padi'*s ability to colonise new areas where potential host plants are present (Valenzuela et al., 2010).

The symptoms of *R. padi* infestation include the presence of honeydew or sooty mould, necrotic areas, wilt, and the symptoms commonly associated with BYDV such as yellowing of leaves and early senescence (Banks et al., 1995; Chongrattanameteekul et al., 1991). *Rhopalosiphum padi* is a vector of both BYDV and cereal yellow dwarf virus (CYDV) in perennial ryegrass, both of which are major diseases of grain crops and pastures and can result in overall productivity losses of up to 20% (Catherall, 1966; Jones, 2013). *Rhopalosiphum padi* can also cause damage to host plants through direct feeding (Parry et al., 2012; Valenzuela and Hoffmann, 2015).

1.3.2.2 *Diuraphis noxia*

Diuraphis noxia, or the Russian wheat aphid, is one of the most destructive pests in grains and pasture grasses globally (Clement et al., 1992). It can survive and reproduce in a wide temperature range and on a broad range of host plants, and the salivary proteins it produces cause foliar damage and physiological alterations in plants (Nicholson et al., 2012). This combination of factors makes *D. noxia* one of the more serious threats to cereal crops and pastures (Hughes and Maywald, 1990). Apterous *D. noxia* are ovoid in shape, roughly 2 mm long and light green-yellow in colour (Emden and Harrington, 2007).

Diuraphis noxia feeds on a wide variety of pasture grass and cereal species, including perennial ryegrass; oats; barley; rye, *Secale cereale* L.; Johnson grass, *Sorghum halepense* L.; wheat and durum wheat, *Triticum turgidum* L. While it is predominantly a pest of cereals, *D. noxia* is polyphagous and will readily feed on perennial ryegrass when it is present (Clement et al., 1992). *Diuraphis noxia* can exist in a wide temperature range and can survive on crops in temperatures from -37°C to +45°C, but reproduction and development only occur at temperatures between +2°C and +25°C (Hughes and Maywald, 1990). *Diuraphis noxia* is therefore considered one of the world's most invasive cereal and grass pests (Zhang et al., 2014b). While its native range extends from Southern Russia to the Middle East and Central Asia, *D. noxia* has since been introduced to South Africa, North and South America, Europe and Australia (Fig. 4) (Yazdani et al., 2018; Zhang et al., 2014b). This invasive species

has only recently been detected in Australia (Yazdani et al., 2018) and its full impact on Australian pasture grass systems is not yet fully understood, but it has the potential to cause severe damage to cereal crops and pasture grasses in Australia (Clement et al., 1992; Hughes and Maywald, 1990; Yazdani et al., 2018).

Figure 4: Worldwide distribution of Diuraphis noxia showing its recorded presence in orange markers for each country, state or territory where recorded. Image from CABI datasheet on Diuraphis noxia: www.cabi.org/isc/datasheet/9887 (CABI, 2020c).

Diuraphis noxia exhibits both holocyclic and anholocyclic reproductive cycles, depending on location and climatic conditions (Dolatti et al., 2005). In its native range of southern Russia, the Middle East and Central Asia, *D. noxia* is predominantly holocyclic, with most populations alternating between sexual reproduction and parthenogenesis on wheat and other grains (Zhang et al., 2014a, 2001). However, much like *R. padi,* in many locations where *D. noxia* is not native it is primarily anholocyclic, reproducing mainly through parthenogenesis on multiple Poaceae hosts, resulting in rapid population growth but low genetic diversity (Ricci et al., 2011; Zhang et al., 2014a). This is evidenced by the low genetic diversity between populations in the Americas, which share close similarities

to a single population from South Africa that is believed to be the progenitor of the populations in North and South America (Zhang et al., 2014a).

Diuraphis noxia is almost unique in its feeding behaviour in that its saliva contains phytotoxic secretions, which cause further damage to plants following direct feeding damage (Hughes and Maywald, 1990). The only other species known to have this trait is *S. graminum* (Ma et al., 1990). This toxic compound causes white or pink streaks on leaves and causes leaves to roll into tube-like structures that *D. noxia* colonies use as protection from predators or adverse weather conditions, such as rain, wind or low temperatures (Hughes and Maywald, 1990). This biotic advantage can make control of *D. noxia* through the use of natural enemies or topical insecticide applications difficult, as this species is more physically protected from insecticide sprays or predatory insects (Hughes and Maywald, 1990). Coupled with *D. noxia*'s rapid invasion of new geographic ranges, this trait has resulted in *D. noxia* becoming one of the more economically important aphid pests of Poaceae and grain crops (Avila et al., 2019).

1.3.2.3 *Metopolophium dirhodum*

Metopolophium dirhodum, also known as the rose grain aphid or the rose grass aphid, is a common pest on a wide range of grass and cereal species worldwide.

Apterous *M. dirhodum* are elongate and spindle-shaped, light green or yellow-green in colour with a pronounced darker green dorsal stripe (Jalalizand et al., 2012). Antennae are brightly coloured with darker colouration on the ends of the third, fifth and final antennae segments (Jalalizand et al., 2012).

Metopolophium dirhodum feeds primarily on hosts of the genus *Rosa* and on a number of secondary grass hosts including barley, oat, wheat and perennial ryegrass (Farrell and Stufkens, 1988). It has a worldwide distribution, being found on all continents except Antarctica (Fig. 5). It was first detected in Australia in 1984, in New South Wales (Carver, 1984).

Figure 5: Worldwide distribution of Metopolophium dirhodum showing its recorded presence in orange markers for each country, state or territory where recorded. Image from CABI datasheet on Metopolophium dirhodum: www.cabi.org/isc/datasheet/33625 (CABI, 2020a).

Metopolophium dirhodum is holocyclic in most environments, including where it is invasive. Its sexual stage overwinters on primary hosts of the genus *Rosa*, and asexual morphs reproduce parthenogenetically on various Poaceae species including perennial ryegrass, though barley is their preferred host (Carver, 1984). In climates with milder winters, anholocyclic populations have also been found existing solely on Poaceae (Farrell and Stufkens, 1988).

Symptoms of *M. dirhodum* infestation include discolouration and malformation of leaves, the presence of honeydew and sooty mould, and early senescence of host plants (Carver, 1984).

1.3.2.4 *Aploneura lentisci*

The root aphid, *Aploneura lentisci* (Passerini), is a common pest of pasture grasses throughout Australia and NZ. It is a subterranean species that feeds on the roots of perennial ryegrass

and other Poaceae. Due to its predominantly subterranean life-cycle, *A. lentisci* is relatively difficult to control compared to foliar species, as it is largely protected from foliar insecticide sprays (Wool and Manheim, 1986)

Adult *A. lentisci* are small compared to other aphid species (1–2 mm in length) and light cream-yellow in colouration. They are spindle shaped and have visibly segmented, bulbous abdomens and relatively small legs in relation to overall body size. They have no visible siphunculi (Paul, 1977).

Aploneura lentisci is native to the Mediterranean region and infests the mastic tree, *Pistacia lentiscus* L., as a primary host, and the roots of Poaceae species as a secondary host (Popay and Cox, 2016). It is also widespread in Australia and NZ, where it is found exclusively on the roots of pasture grasses, specifically perennial ryegrass and tall fescue, *Festuca arundinacea* (Schreb) (Popay and Cox, 2016).

In its native range, *A. lentisci* cycle through sexual and asexual forms throughout a two-year cycle, which includes a gall-forming stage on the primary host and parthenogenetic reproduction on the secondary host (Popay and Cox, 2016; Wool and Manheim, 1986). Outside its native range and with no access to its primary host, *A. lentisci* is strictly anholocyclic, and reproduces solely through parthenogenesis on pasture grass hosts throughout most of the year (Popay and Cox, 2016; Wool and Manheim, 1986).

Aploneura lentisci secretes a white, flocculent, waxy substance while feeding on roots, resulting in an easily identifiable mass of this substance around large infestations. This substance has waterproofing properties and protects the aphids in damp soil (Cheng, 1976). The symptoms of root-feeding by *A. lentisci* include reductions in plant growth and changes in biomass allocation, as well as negative effects on nutrient and water acquisition (Pennell et al., 2005). All of these symptoms may also decrease plant competitiveness (Popay and Cox, 2016).

1.3.2.5 Aphid life history

Aphid life history is often quantified by many different metrics including fecundity, length of the pre-reproductive period (time before first reproduction), adult longevity, nymphal development time, adult body size or weight, growth rate and intrinsic rate of increase (r_m) (Flatt and Weisser, 2000; Vorburger, 2005; Wyatt and White, 1977). Of these, growth rate, fecundity and adult body weight are the most commonly researched, as they are relatively easy to quantify and compare across treatments. Growth rate and fecundity in particular can be directly indicative of an aphid population's capacity for growth (Perng, 2002).

Of these metrics, r_m is accepted as the most accurate indicator of the health and overall fitness of individual aphids in a population. The r_m is designed to quantify the relationship between the nymphal development and reproductive potential of an invertebrate (Birch, 1948; Wyatt and White, 1977). In aphids, it is calculated using the formula $r_m = 0.74(\log_e M_d)/d$, where d = the length of the pre-reproductive period and M_d = the number of progeny produced in a subsequent period of time equal to d (Wyatt and White, 1977). Using r_m as a metric in studies of aphids provides greater insight into the ability of individual aphids to grow and reproduce, and therefore the ability of a population to increase in numbers and the rate at which it does so (Wyatt and White, 1977). As such, r_m is often used in entomological studies to determine the potential severity of infestation by pest species (Merrill et al., 2009) or to determine the efficacy of insecticides at controlling infestations (Kerns and Stewart, 2000).

In studies of insecticide treatments, the effects on individual aphid life history are most often quantified by fecundity, growth rate or r_m. Most studies, however, will also observe whole-population effects, such as the number of aphids in a particular population following exposure to insecticide treatments (Harper, 1961), or the effects of treatments on the levels of aphid damage to the host plant, as seen in indicators such as herbage yield (Nettleton and Hain, 1982). These metrics are used to give a more complete understanding of the ability of insecticides to reduce the harm caused by aphids to crops or other plants.

1.3.3 Tri-trophic interactions between aphids, perennial ryegrass and *E. festucae* var. *lolii*

There is a need for greater understanding of the tri-trophic interaction between pasture aphids, perennial ryegrass and *E. festucae* var. *lolii*, as this interaction has strong benefits for pasture production in the dairy and other livestock industries where aphids present a threat to productivity. However, there are currently specific aspects of this interaction that are not well understood. The insecticidal activity of *E. festucae* var. *lolii* is well known; however, the mode of action of this activity is not well understood (Breen, 1994). Similarly, many of the alkaloids produced by *E. festucae* var. *lolii* are known to have either directly insect-toxic effects (e.g. ergovaline) (Popay and Bonos, 2008), or are indirectly insecticidal through deterrence of insect feeding (e.g. peramine) (Rowan et al., 1986). These effects have been tested using a range of methods, but there is so far no standardised test to assess them *in-planta*, nor is there much research on the effects of endophytes on the feeding behaviour of individual aphids. Furthermore, little is currently known about the effects of aphid feeding on endophytes, such as whether feeding affects endophyte alkaloid profiles or the production levels of specific alkaloids.

This investigation into the nature of this tri-trophic interaction will use a range of novel and established methods, to allow a greater understanding of the potential uses of this interaction in producing perennial ryegrass pastures from the aphid species *D. noxia, A. lentisci, M. dirhodum* and *R. padi* using the commercial endophyte symbiota SE, AR1, AR37, NEA2 and NEA6.

1.4 Bioassays for studying tri-trophic interactions (*Epichloë*–ryegrass–aphids)

There is currently no standardised means of assessing the tri-trophic interaction between aphids, perennial ryegrass and fungal endophytes using a lab-based, *in-planta*, high-throughput and rapid methodology. Most current methods used to assess endophyte insecticidal activity include the use of artificial diets containing endophyte-produced alkaloids

(Hennessy et al., 2016; Thakur et al., 2013), cut-leaf assays (Bastias et al., 2017b; Potter et al., 2008; Shymanovich et al., 2019), or glasshouse pot trials (Ruppert et al., 2017). However, these methods have certain disadvantages or limitations that prevent them from being more broadly applicable as standardised tests. Artificial diets allow for the exact quantification and control of alkaloids or other chemicals present in a sample, but they often do not account for other *in-planta* factors such as secondary metabolites or hormones, or the interactions of these chemicals that would be present in living plants (Mittler and Dadd, 1964). Likewise, cut-leaf assays may provide more accurate insights into the effects of the interactions between endophytes, alkaloids and other plant metabolites, but, as these studies do not use whole living plants, the levels of alkaloids produced may not be representative of those in living plants with continuous alkaloid production (Bastias et al., 2017b). Finally, glasshouse trials, while using live plants, often require large areas of space to achieve an adequate number of replicates. Glasshouse trials also have longer experimental periods, which may not meet the required timeframes of the development of commercial endophytes for use in pastures. For these reasons, it was determined that a high-throughput, *in-planta* bioassay that is simple, rapid, reproducible and scalable is required as a standardised method for the accurate assessment of the insecticidal activity of *Epichloë*–perennial ryegrass symbiota, as such a bioassay would provide a more comprehensive overview of the tri-trophic interaction between these organisms. This bioassay could additionally be deployed as part of the Forage Value Index to add insecticidal activity to the assessed metrics, as this index is currently based only on the metrics of yield and heading date (Chapman et al., 2012, 2017; Leddin et al., 2018).

It was proposed that the bioassay employed in this study should be based on an aphid-rearing method developed by Ridland et al. (1988) originally developed for use in BYDV transmission studies as a means to breed populations of *R. padi*, and has since been used for assessing the effects of different host plants and aphid genotypes on aphid life history (Valenzuela et al., 2010). BYDV is not transmitted directly from parent to offspring; rather, it is acquired by the aphid as it feeds on infected plants (Eagling et al., 1989b). This method of infection means that if a newborn aphid nymph is removed and placed on a new, virus-free

host plant immediately after birth and before initiating feeding, it will not acquire the virus (Ridland et al., 1988). The rearing method was developed to take advantage of this, and involves rearing single aphids in small cups so individuals can be monitored easily and effectively, and newborn nymphs can be transferred readily to fresh plants immediately after birth (Ridland et al., 1988). This method's focus on individual aphids allows the level of precision required for a bioassay designed to assess endophyte effects on aphid life history on an individual and daily scale. Furthermore, Valenzuela et al. (2010) used a similar cup-based method to assess the fecundity, longevity and intrinsic rate of increase (r_m) of *R. padi* from different clonal lineages on seedlings of wheat, barley and triticale. This study found significant differences in all aphid life history metrics between different clonal lineages, temperatures and host plants (Valenzuela et al., 2010), indicating this assay method is suitable for assessing differences in aphid life history resulting from different treatments. The proposed bioassay design would assess similar metrics; therefore, based on these results, it was determined that this cup-based design suits all the requirements of a high-throughput *in-planta* bioassay to assess the effects of fungal endophytes on aphid life history. This novel bioassay design is intended to fill a role in the developmental pathway from endophyte discovery and development to utilization in a pasture setting. It is designed to be a simple, rapid, reproducible and scalable method for determining the insecticidal activity of novel endophyte symbiota.

1.5 Alkaloid profiling of perennial ryegrass seedlings

Epichloë festucae var. *lolii* strains produce a range of alkaloids including lolitrem B, ergovaline, janthitrem I and peramine (Ekanayake et al., 2017). Each endophyte strain, including commercial endophytes, produces a unique alkaloid profile consisting of different combinations of these alkaloids. It is important to understand these alkaloid profiles, and how they relate to the effects an endophyte has on the life history of insect pests or the health of grazing livestock.

1.5.1 Alkaloid biosynthesis by *E. festucae* var. *lolii*

The alkaloids produced by *E. festucae* var. *lolii* are synthesised through a number of different pathways within the endophyte, where production is regulated by specific genes depending on the alkaloid class (Fig. 6). Lolitrem B is an indole diterpene alkaloid that consists of a partial diterpene structure of four isoprene units derived from geranylgeranyl diphosphate, and an indole moiety derived from tryptophan (Young et al., 2006). These are combined via initial biosynthetic reactions regulated by the *ltmC*, *ltmM* and *ltmB* genes to generate paspaline (Saikia et al., 2012). The pathway controlled by the *TerQ* and *TerP* genes synthesises terpindole I from paspaline (Saikia et al., 2012). Lolitrems are synthesised from terpindole I through a pathway mediated by the LTM gene cluster, including the *ltmQ, ltmF, ltmK, ltmE* and *ltmJ* genes (Fleetwood et al., 2008; Saikia et al., 2012). This includes lolitrem B as the final product of this pathway (Young et al., 2009). Janthitrem I is also an indole-diterpene and thus follows a similar biosynthetic pathway to lolitrem B. It is also synthesised from paspaline, but with the intermediary precursor paxilline (Fleetwood et al., 2008; Ludlow et al., 2019). Paxilline is synthesised from paspaline by the *PaxP* and *PaxQ* genes, from which epoxy-janthitrems are synthesised by gene clusters at the JTM locus (Ludlow et al., 2019). This gene cluster includes a number of the same genes as the LTM cluster, as well as the genes *jtmD, jtmO, jtm01* and *jtm02*, which are unique to epoxy-janthitrem-producing endophytes such as AR37 and NEA12 (Ludlow et al., 2019).

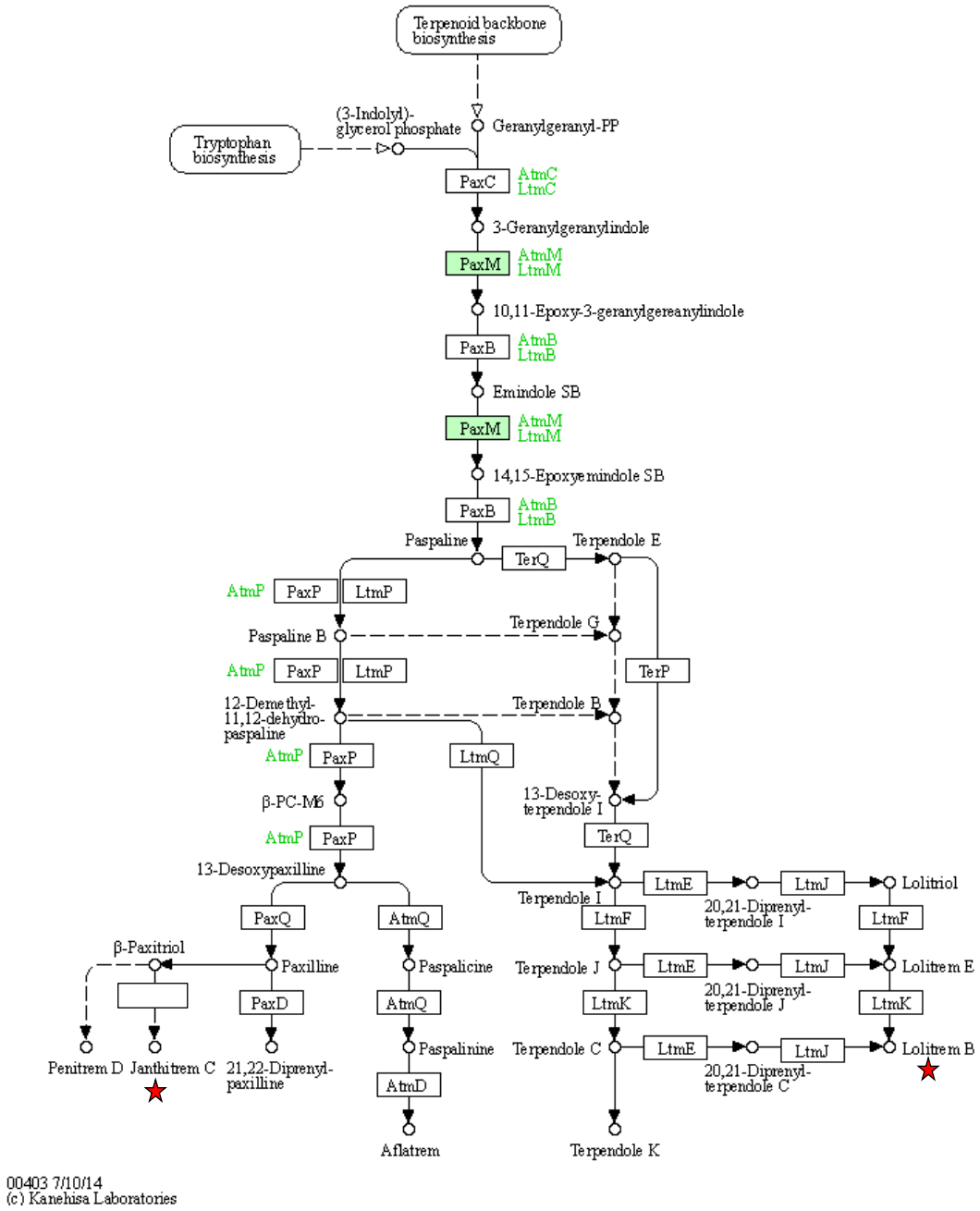

Figure 6: Indole diterpene biosynthetic pathway showing precursors, intermediaries and regulatory genes responsible for the biosynthesis of lolitrem B and epoxy-janthitrems (including janthitrem I). Red stars indicate endpoints in the pathways for janthitrem I and lolitrem B. Image modified from 'KEGG PATHWAY: Indole diterpene alkaloid biosynthesis - Aspergillus nidulans,' (2014).

Ergovaline and other ergot alkaloids are all synthesised from dimethylallyl tryptophan (DMAT) through a pathway controlled mainly by the EAS gene cluster (Fig. 7) (Fleetwood et

al., 2008; Schardl et al., 2013). The first gene in this pathway is *DmaW*, which combines L-tryptophan with dimethylallyl pyrophosphate to generate DMAT. The EAS gene cluster, consisting of the *easF, easE, easC, easD, easA* and *easG* genes, then synthesises numerous clavines such as agroclavine and elymoclavine from DMAT (Schardl et al., 2013). At this point, the *cloA* gene synthesises D-lysergic acid from elymoclavine, from which the *easH* gene synthesises the ergopeptines, including ergovaline and ergotamine, depending on the endophyte genotype (Gerhards et al., 2014; Schardl et al., 2013).

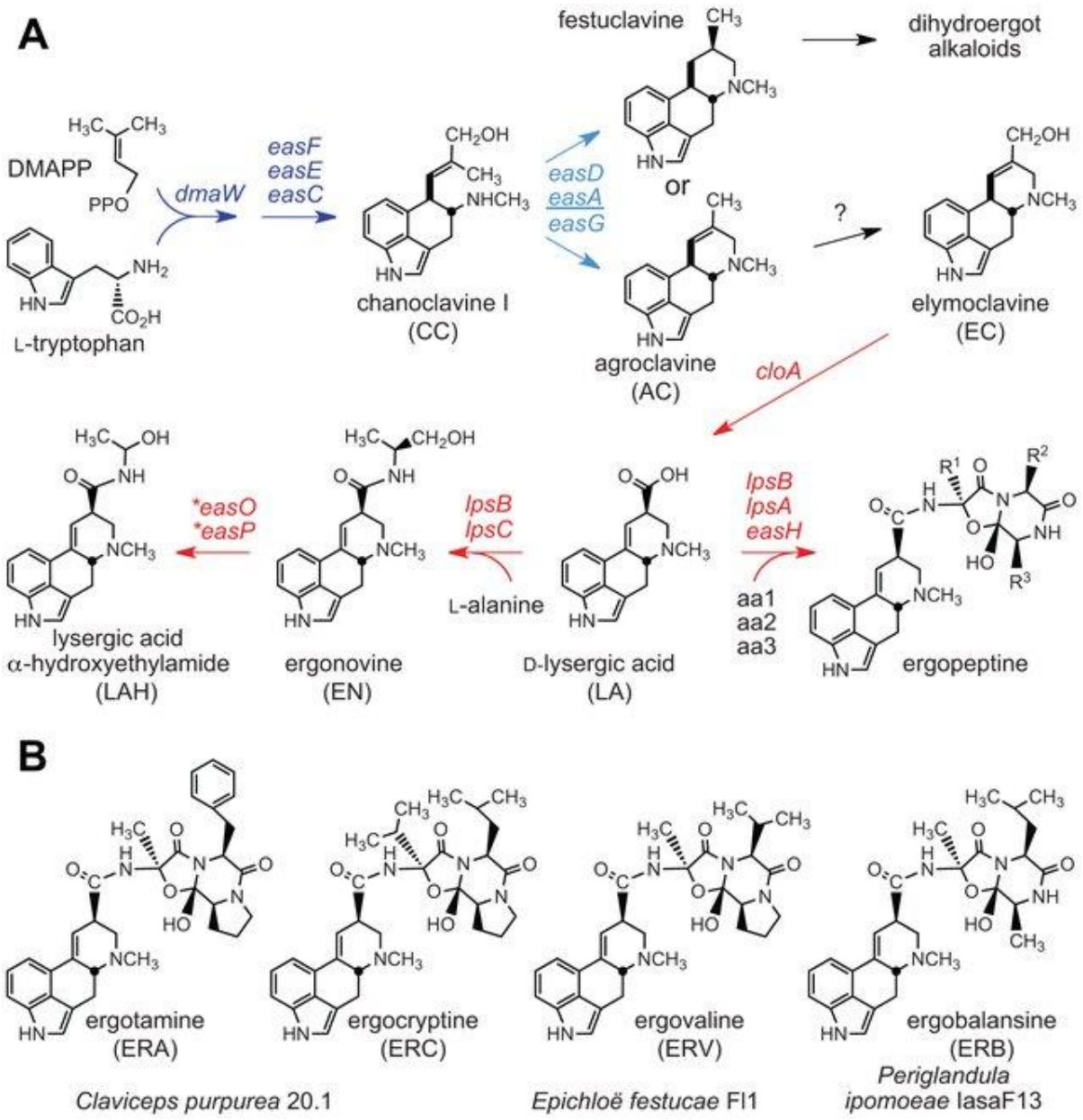

Figure 7: (A). Ergot alkaloid biosynthetic pathway showing precursors, intermediaries and regulatory genes responsible for the biosynthesis of ergopeptines. (B). Various ergopeptines produced by different endophyte genotypes. Image from Schardl et al. (2013).

Peramine is synthesised from the precursors L-arginine and (*S*)-1-pyrroline-5-carboxylate, by a multifunctional, non-ribosomal peptide synthase gene, *PerA*, of which peramine is the only known product in *Epichloë* endophytes (Fig. 8) (Fleetwood et al., 2008; Hettiarachchige et al., 2019). *PerA* has a two-module structure, where module 1 activates and binds (*S*)-1-pyrroline-5-carboxylate and module 2 activates and binds L-arginine (Tanaka et

al., 2005). The condensation domain of *PerA* then forms the peptide bonds between (*S*)-1-pyrroline-5-carboxylate and L-arginine (Tanaka et al., 2005). Following this, the L-arginine moiety is methylated by a methylation domain of module 2, while a reductase domain carries out reduction, cyclisation and release of the peramine ion (Tanaka et al., 2005). It is postulated that the peramine ion then undergoes spontaneous oxidation and rearrangement to form the final product of the pathway (Tanaka et al., 2005).

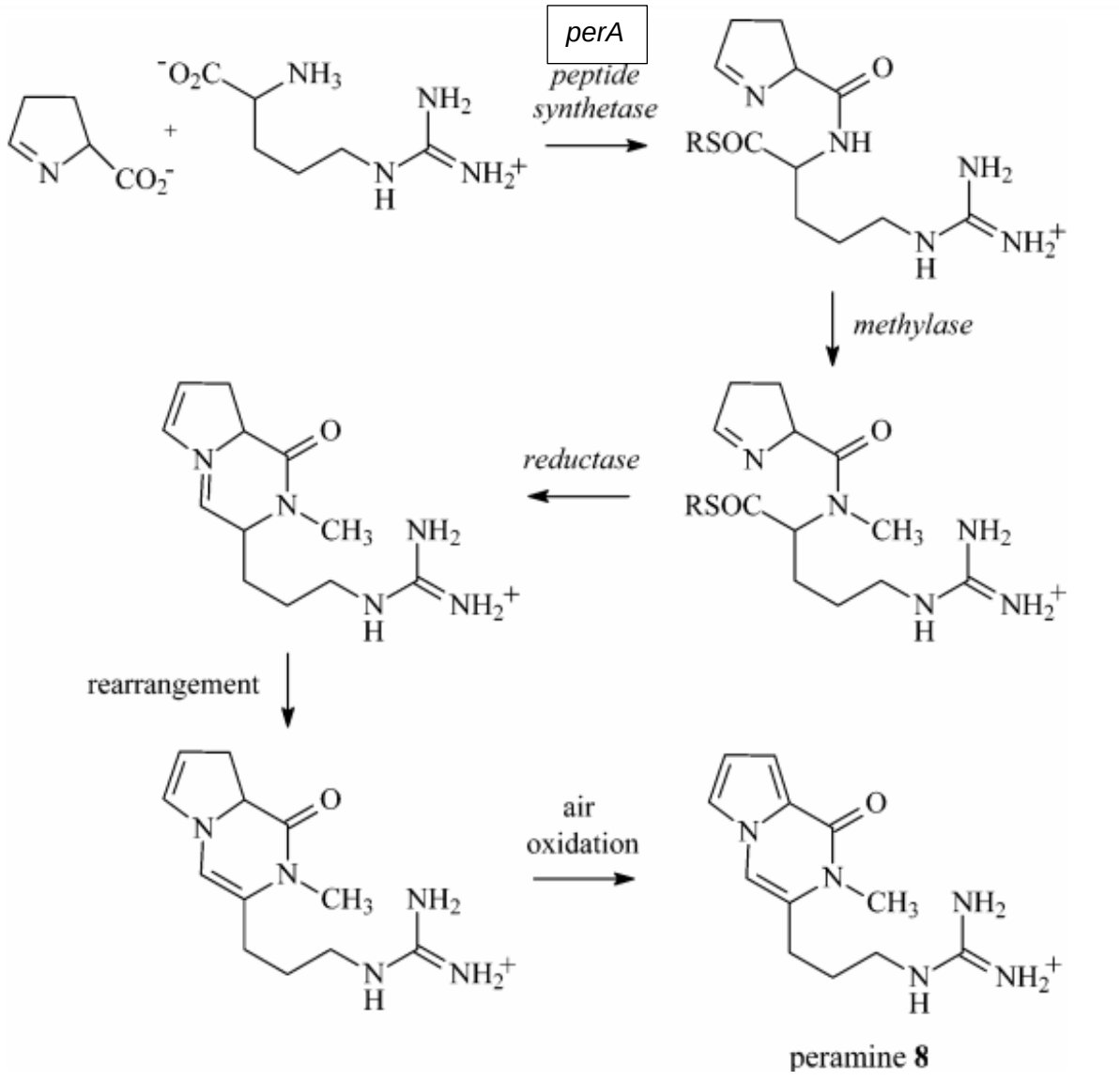

Figure 8: Peramine biosynthetic pathway showing precursors, intermediaries and regulatory genes involved. Image modified from Wei Zhang et al. (2006).

These different and complex biosynthetic pathways can be influenced by a number of genetic and environmental factors. The genome of a particular *Epichloë* strain has the most marked influence on the alkaloids produced, as genes and gene clusters that play a role in these alkaloid biosynthetic pathways may or may not be present within different genomes. As such, selective breeding of pasture grasses containing different endophyte strains has given rise to the range of commercial endophytes with different alkaloid profiles that is available to farmers today (Ekanayake et al., 2017). In addition to genetic factors, environmental stresses and reactions also play a role in alkaloid biosynthesis (Bultman et al., 2018; Hennessy et al., 2016) Bultman et al. (2018) found that cutting perennial ryegrass leaves to simulate grazing by livestock stimulated a higher production of alkaloids by endophytes. Simons et al. (2008) and Sullivan et al. (2007) found that increased production of JA and salicylic acid (SA) both resulted in decreased production of loline alkaloids by the endophyte *Epichloë coenophialium*. Navarro-Meléndez and Heil (2014) also found a decrease in loline production when endophytes were exposed to high levels of JA; however, when only low levels of JA were present, feeding by aphids was shown to increase the production of loline. Many of the factors affecting alkaloid biosynthesis in *Epichloë* endophytes are still not fully understood; but regardless, multiple strains producing different alkaloid profiles are regularly used in a commercial setting to improve pasture performance.

1.5.2 Endophyte insecticidal activity

The alkaloids present in *Epichloë*–perennial ryegrass symbiota have demonstrated a range of insecticidal properties ranging from broad spectrum insect toxicity to targeted effects on the feeding behaviour or life history of certain insect species. For example, lolines (produced naturally by the tall fescue endophyte *E. coenophialium*, which can be artificially cultured in perennial ryegrass) have broad-ranging insect toxicity effects, whereas peramine is a strong feeding deterrent against *L. bonariensis* (Popay and Wyatt, 1995), and ergovaline has been seen to have strong feeding deterrent effects on *H. arator* (Ball et al., 1997). Lolitrem B is not

widely known for its insecticidal properties; however, the related indole diterpene precursor paxilline has been shown to protect perennial ryegrass from *L. bonariensis* (Bush et al., 1997; Rowan and Latch, 1994).

It is important to understand each alkaloid's unique properties in order to identify and select for the most advantageous alkaloid profile for pasture improvement in the development of novel endophytes (Bush et al., 1997). In addition to their insecticidal properties, some of the alkaloids produced by *E. festucae* var. *lolii* – specifically, lolitrem B and ergovaline – have toxic effects on livestock. As such, one significant challenge in endophyte discovery and development is the selection of novel endophytes with alkaloid profiles that maximise insecticidal activity while reducing or eliminating negative effects on grazing livestock (Tapper and Latch, 1999).

1.5.3 Commercial endophyte alkaloid profiles

The commercial endophytes available to farmers produce a broad range of alkaloid profiles, which have varying effects on the host plant's fitness, insect tolerance, and toxicity to livestock. Some commercial endophytes produce a range of different alkaloids with varying insecticidal, feeding-deterrent or antimammalian properties (Siegel et al., 1990). Specifically, SE produces lolitrem B, ergovaline and peramine all in relatively high concentrations; this confers strong insecticidal and antimammalian properties and thus makes it undesirable in pasture grass systems, as it can cause various health problems in livestock. NEA2 produces the same alkaloids as SE but with lolitrem B produced in only trace amounts, so this endophyte is considered safer for livestock consumption. NEA6 produces only ergovaline and peramine, with ergovaline in lower concentrations than in NEA2. NEA2 and NEA6 are often sown together by farmers in an effort to create a pasture system with bioprotective alkaloids at sufficient levels to control invertebrate pests, while maintaining an overall alkaloid

concentration in the pasture that is low enough to be safe for livestock consumption (Barenbrug Agriseeds, 2019).

Other commercial endophytes may produce only one type of alkaloid, allowing for a more targeted approach to invertebrate pest management. This is seen with AR1, which produces the feeding deterrent peramine, which is used to combat infestations of *L. bonariensis* in NZ dairy pastures (Popay and Wyatt, 1995; Rowan and Gaynor, 1986). It is also seen with AR37, which produces janthitrem I, and has been shown to control *A. lentisci* populations in dairy pastures, though its mode of action is unclear (Hume et al., 2007).

1.5.4 Detection of alkaloid profiles in endophyte-infected perennial ryegrass seedlings

Given the variation in insecticidal activity and mammalian toxicity arising from variations in alkaloid profiles, a simple quantitation technique has been developed to measure combined alkaloids *in-planta* using liquid chromatography and mass spectrometry (LC-MS) (Vassiliadis et al., 2019). This method can be used to identify the alkaloid profiles of individual seedlings, which can then be compared to aphid life history data to determine the individual alkaloid profile linked to specific effects on aphid mortality or fecundity. This method is also well suited to investigating the effects of aphid feeding on the production of alkaloids in *Epichloë*–perennial ryegrass symbiota, as it can be used to compare the alkaloid profiles of seedlings that have been fed on with seedlings that have not been fed on. This technique can improve understanding of which endophyte alkaloids provide the greatest protection against pasture aphids. Furthermore, in terms of wider application, the use of this technique in this context can help future studies to determine how other plant–endophyte symbiota react to feeding from aphids or other invertebrates.

1.6 Analysis of aphid feeding behaviour using electrical penetration graph (EPG)

1.6.1 Origins of EPG technique

The Electrical Penetration Graph (EPG) technique is an electrophysiological assay developed to monitor the feeding behaviour of phloem-feeding hemipterans (Tjallingii, 1978). It is based on research by McLean and Kinsey (1964), who demonstrated that aphid stylet penetration could be electrically recorded by attaching a thin wire to an aphid's thorax and recording fluctuations in an electrical signal in a circuit created when the stylet penetrates the plant tissue. Tjallingii (1985, 1978) expanded on this research to identify specific waveform patterns indicative of the stylet movements associated with different aphid feeding and probing behaviours. The EPG technique was originally developed for use with aphids, but has since been adapted for use with other phloem-feeding hemipterans such as psyllids (Bonani et al., 2010) and greenhouse whitefly, *Trialeurodes vaporariorum* (Westw.) (Janssen et al., 1989).

1.6.2 Use of EPG in aphid feeding studies

The EPG technique allows for a high-resolution assessment of the effects of different factors such as drought-stress, insecticide treatments, plant cultivars and endophytes on aphid feeding behaviour (Bastias et al., 2017b; Bonani et al., 2010; Garzo et al., 2016). As such, it provides a comprehensive overview of the mode of action of insecticidal treatments by showing the manner and extent to which they affect aphid feeding or probing.

The EPG technique has been used in past research to assess aphid reactions to different insecticides, including in studies of the evolution of insecticide resistance (Garzo et al., 2016), and in studies of the effects of loline-producing endophytes on *R. padi* feeding on annual ryegrass (Bastias et al., 2017b). It was for this reason that the EPG technique was determined to be ideal for assessing the effects of *E. festucae* var. *lolii* on the feeding behaviour of aphids on *Epichloë*–perennial ryegrass symbiota.

1.6.3 Methodology of EPG experimentation

The EPG technique involves affixing an electrode to the dorsum of an aphid and attaching that electrode to a probe connected to a monitoring device (EPG Systems, Wageningen, Netherlands). A second probe is placed into the soil of a plant to ground the electrical circuit. The aphid is then placed onto the plant and allowed to feed. The penetration of the aphid's stylet into the plant tissue creates a circuit through which a mild electrical current can flow. Fluctuations in the electrical potential of this current can be observed and correlated with different feeding behaviours.

In the majority of EPG experiments, an aphid is left connected to an EPG device that is actively recording feeding behaviour for 8–12 hours (Bonani et al., 2010; Garzo et al., 2016). This is necessary because aphids often feed continuously for several hours on preferred host plants, thus an extended time period is required to provide an accurate portrayal of the broad effects of an insecticidal treatment on feeding and probing behaviour (Tjallingii, 1985).

1.6.4 Analysis of EPG waveforms

Aphid feeding and probing behaviour recorded by EPG can be observed as a continuous waveform on a readout using a specialised software package. The Stylet D package (EPG Systems, The Netherlands) can be used to observe recorded waveforms and quantify specific patterns representing intercellular probing, phloem ingestion, xylem ingestion, electrical potential drops resulting from cellular puncture, and stylet derailment. The analysis process involves the manual identification and assignment of waveforms by the researcher, who categorises segments of the pre-recorded waveform into different feeding and probing behaviours based on the recognisable patterns identified by Tjallingii (1985). The researcher assigns labels to specific time points in the recording at which a behaviour pattern occurred, and the number, duration and timing within the recording period of each behaviour can be exported as numerical data for quantification (Sarria et al., 2009).

Quantification packages such as that developed by Sarria et al. (2009) can be used to quantify the waveform pattern data into up to 102 different metrics. These include the number of instances of a particular behaviour throughout a recording, the total duration of a particular type of behaviour, and the percentage of aphids exhibiting a particular probing behaviour (Sarria et al., 2009). The many quantification metrics available allow for aphid feeding behaviour to be categorised and analysed on multiple levels and in many different ways. With these behaviours quantified numerically, any significant differences between treatments can be calculated using a standard ANOVA or Kruskall-Wallis test (Garzo et al., 2016; Sarria et al., 2009).

1.6.5 Effects of *E. festucae* var *lolii* on aphid feeding behaviour

The EPG technique can be used to assess whether endophyte treatments correlate with reduced phloem-feeding by aphids or if they correlate with delays in the initiation of phloem-feeding, both of which would indicate feeding deterrence (Tjallingii, 1985). The EPG technique can also indicate whether intercellular probing by aphids is being interrupted, which may suggest the presence of a physical barrier to stylet penetration by endophytes (Goggin, 2007; Peñalver-Cruz et al., 2019; Tjallingii, 1985). Understanding the feeding-deterrent mode of action of different endophytes is useful in the selection of existing endophytes for aphid pest control in pastures, as well as the selection of novel endophyte symbiota developed for specific pest management uses.

Endophyte treatments that function as feeding deterrents often result in a delayed initiation of phloem-ingestion by aphids or no feeding at all, while directly toxic endophytes may not have significant effects on feeding behaviour, as aphids are not deterred from feeding but suffer higher mortality (Bastias et al., 2017b; Tjallingii, 1988, 1985). Research by Bastias et al. (2017) determined that *R. padi* showed no change in feeding behaviour when feeding on annual ryegrass infected with the endophyte *Epichloë occultans* producing the alkaloid

loline when compared to an endophyte-free control; however, *R. padi* did experience a reduction in population size on endophyte-infected plants. However, given the potential feeding-deterrent properties of other alkaloids such as peramine, the potential for *E. festucae* var. *lolii* to have an effect on *R. padi* feeding behaviour is considered likely (Rowan et al., 1986). As such, conducting EPG experiments to investigate the specific effects of multiple commercial *E. festucae* var. *lolii* varieties on *R. padi* feeding behaviour will provide further insights into the mode of action of these endophytes, and how they can be applied in aphid pest management schemes in perennial ryegrass pastures.

1.7 Research Plan

This PhD project is part of a research program aimed at pasture improvement and pest management in dairy pastures through the discovery, utilisation and development of novel fungal endophytes. The PhD project will investigate the tri-trophic interaction between aphids, perennial ryegrass and the fungal endophyte *E. festucae* var. *lolii* by determining the effects of five *Epichloë*–perennial ryegrass symbiota and their associated alkaloid profiles on the life history and feeding behaviour of four aphid pest species, as well as the effect of aphid feeding on endophyte alkaloid production. This will allow for a more complete understanding of this tri-trophic interaction and how it can be used to advantage in the development of novel endophyte symbiota for use in pest management in dairy pastures. This PhD project will have three main components: the development and implementation of a high-throughput, *in-planta* bioassay to assess the insecticidal activity of *E. festucae* var. *lolii* against pasture aphids; the identification of the alkaloid profiles of the five endophyte treatments and an assessment of their different effects on aphid life history, coupled with an investigation of the effects of aphid feeding on alkaloid production; and an investigation into the effects of *E. festucae* var. *lolii* endophyte symbiota on the feeding behaviour of *R. padi* using EPG assays.

The development of a high-throughput *in-planta* bioassay is necessary, as there is currently no standardised, lab-based, high-throughput, rapid, simple and easily replicable method for assessing the insecticidal activity of endophyte–grass symbiota. This bioassay will be designed to assess the insecticidal effects of five different strains of *E. festucae* var. *lolii* on the life history of four aphid species, and will be conducted by cultivating single aphids in 40 ml plastic cups with a single seedling of endophyte-infected perennial ryegrass (or endophyte-free perennial ryegrass or barley as controls). The bioassay will assess aphid life history by measuring the fecundity, r_m and mortality of aphids feeding on endophyte-infected perennial ryegrass seedlings. The aphid species to be tested using this method are *R. padi*, *D. noxia*, *M. dirhodum* and *A. lentisci*. These species were selected for this study as they represent common foliar and root pests of perennial ryegrass. All experiments will be carried out using perennial ryegrass of the cultivar Alto, and will include four commercial endophyte strains (AR1, AR37, NEA2 and NEA6) and one wild type, or standard, endophyte (SE). Controls will include perennial ryegrass cultivar Alto without an endophyte (WE) and barley cultivar Hindmarsh, which is included as an additional control to confirm the overall health of aphid colonies on a preferred host (Carver, 1984; Pettersson et al., 2007; Ponder et al., 2001). The simple bioassay design will utilize small plastic cups arrayed on trays, with each cup containing a single perennial ryegrass seedling and a single aphid. Aphids will be monitored daily to record and assess their life history. This experimental process will be rapid in comparison to larger scale trials that require mature plants, or experiments in multiple field plots. It is easily reproducible and scalable due to the ready availability and low cost of the materials. The results of this bioassay will indicate the effectiveness of the five endophyte treatments tested as control measures against pasture aphids, and will also provide an indication of its suitability for the stated purpose as an assessment tool in the endophyte development process.

In addition to this, alkaloid profiling of seedlings used in the bioassay will further examine the tri-trophic interaction of aphids, perennial ryegrass and *E. festucae* var. *lolii* by

investigating the effects of aphid feeding on the alkaloid profiles of *Epichloë*–perennial ryegrass symbiota, as well as the effects of alkaloid presence and alkaloid concentration on the life history of *M. dirhodum*. This will involve using the same methods as per Vassiliadis et al. (2019) to profile the alkaloids in 16 seedlings from each endophyte treatment and the WE control. This profiling will be performed using LC–MS and will quantify the concentration of the different alkaloids in individual perennial ryegrass seedlings. These concentrations will be compared to the aphid life history data for the corresponding seedling in order to correlate alkaloid profile and concentration with effects on aphid life history. These results will serve to provide a deeper understanding of the insecticidal effects of *E. festucae* var. *lolii*. Results will also provide greater insight into the interaction between the endophyte and its host plant by determining if a defence response to aphid feeding, involving an increase in alkaloid production when host plants are fed upon by aphids, exists in endophyte-infected plants.

Finally, the EPG technique will be employed to investigate the effects of *E. festucae* var. *lolii* on the feeding behaviour of *R. padi* – specifically, to determine the feeding deterrent effects of *Epichloë* endophytes, which would potentially cause delayed initiation of phloem ingestion and fewer sustained ingestion events overall. EPG assays will be performed on 24 individual *R. padi* across each of the five endophyte treatments (SE, AR1, AR37, NEA2 and NEA6), plus two controls (WE and barley). Feeding behaviour will be recorded for 12 hours for each replicate, and will be quantified manually to determine the length of probing before initiating feeding; the number, duration and frequency of phloem ingestion events; and the number and duration of xylem ingestion and stylet derailment events. Results will provide a greater understanding of the mode of action of insecticidal endophytes by indicating if observed effects on aphid life history can be correlated with delayed feeding or other feeding-deterrence effects, or if such life history effects are due to more directly toxic or anti-fecundity effects that do not deter feeding.

1.8 Aims

- Develop a high-throughput *in-planta* bioassay for assessing the insecticidal activity of novel endophyte symbiota on pasture aphids. The method must be simple, rapid, reproducible and scalable.
- Investigate the insecticidal activity of the endophytes SE, AR1, AR37, NEA2 and NEA6 on the life history of the aphids *R. padi, D. noxia, M. dirhodum* and *A. lentisci*. Life history is assessed according to the metrics: fecundity, r_m and mortality.
- Identify the alkaloid profiles of *Epichloë*–perennial ryegrass symbiota using liquid LC–MS, and determine if there is a correlation between these profiles and *M. dirhodum* life history data.
- Determine the effects of aphid feeding on alkaloid production by comparing the alkaloid profiles of seedlings that have been fed on by *M. dirhodum* with those of seedlings that have not been fed on.
- Assess the effects of *E. festucae* var. *lolii* on *R. padi* feeding behaviour using the Electrical Penetration Graph (EPG) technique.

CHAPTER 2

Novel bioassay to assess antibiotic effects of fungal endophytes on aphids

2.1: Chapter Preface

There is a need for a standardised, lab based method for the high-throughput assessment of the insecticidal activity of fungal endophytes in grasses. This method needs to be simple, rapid, reproducible and scalable. This chapter represents the first published paper of this thesis by publication, and details the development of a high-throughput *in-planta* bioassay to fill this role. This bioassay was used to assess the insecticidal activity of four commercial fungal endophytes (AR1, AR37, NEA2 and NEA6), as well as one standard endophyte (SE), on the life history of *Diuraphis noxia* and *Aploneura lentisci.* This bioassay was also conducted on *Metopolophium dirhodum* and *Rhopalosiphum padi* and the results of these are detailed in the subsequent two chapters. Both *D. noxia* and *A. lentisci* exhibited negative life history effects relating to mortality and fecundity when feeding on *Epichloë*–perennial ryegrass symbiota. *Diuraphis noxia* suffered significantly increased nymphal mortality on all endophyte treatments, and *A. lentisci* suffered significantly increased mortality and decreased fecundity on SE and NEA2. These endophytes both produce the alkaloids lolitrem B and ergovaline in varying quantities, so it is likely that the insecticidal activity may be due to the presence of these alkaloids.

This chapter is presented in published format.

2.2: Publication details

Title: Novel bioassay to assess antibiotic effects of fungal endophytes on aphids

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Authors: Nicholas Paul Collinson, Ross Cameron Mann, Khageswor Giri, Mallik Malipatil, Jatinder Kaur, German Spangenberg, Isabel Valenzuela

2.3: Statement of contribution of joint authorship

NPC performed all work relating to maintaining colonies of aphids, and conducting aphid bioassays. NPC generated all figures and drafted the majority of the manuscript. NPC and JK conducted genetic analysis of aphid specimens and KASP assay of endophyte presence in perennial ryegrass seed batches. NPC and KG conducted all statistical and data analysis. KG assisted in drafting the statistical sections of the manuscript. RM, GS, IV, MM, JK and NPC all conceptualised the project and assisted in drafting the manuscript.

2.4 Statement from the co-author confirming the authorship contribution of the PhD candidate

"As co-author of the manuscript 'Collinson, N.P., Mann, R.C., Giri, K., Malipatil, M., Kaur, J., Spangenberg, G., Valenzuela, I., 2020. Novel bioassay to assess antibiotic effects of fungal endophytes on aphids. PLOS ONE 15, e0228813. https://doi.org/10.1371/journal.pone.0228813' I confirm that Nicholas Collinson made the following contributions,

- Survey, collection, taxonomical verification and rearing of colonies of aphid species (*D. noxia* and *A. lentisci*)
- Conducted aphid bioassays
- Bioassay data analysis
- Generated all figures
- Writing the manuscript, critical appraisal of content and response to reviewers"

Associate Professor Mallik Malipatil

Date: 28/06/2020

PLOS ONE

RESEARCH ARTICLE

Novel bioassay to assess antibiotic effects of fungal endophytes on aphids

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Data Availability Statement: All relevant data are within the manuscript.

Abstract

Perennial ryegrass is an important feed base for the dairy and livestock industries around the world. It is often infected with mutualistic fungal endophytes that confer protection to the plant against biotic and abiotic stresses. Bioassays that test their antibiotic effect on invertebrates are varied and range from excised leaves to whole plants. The aim of this study was to design and validate a "high-throughput" in-planta bioassay using 7-day-old seedlings confined in small cups, allowing for rapid assessments of aphid life history to be made while maintaining high replication and treatment numbers. Antibiosis was evaluated on the foliar and the root aphid species; *Diuraphis noxia* (Mordvilko) and *Aploneura lentisci* (Passerini) feeding on a range of perennial ryegrass–*Epichloë festucae* var. *Lolii* endophyte symbiota. As expected, both *D. noxia* and *A. lentisci* reared on endophyte-infected plants showed negatively affected life history traits by comparison to non-infected plants. Both species exhibited the highest mortality at the nymphal stage with an average total mortality across all endophyte treatments of 91% and 89% for *D. noxia* and *A. lentisci* respectively. Fecundity decreased significantly on all endophyte treatments with an average total reduction of 18% and 16% for *D. noxia* and *A. lentisci* respectively by comparison to non-infected plants. Overall, the bioassay proved to be a rapid method of evaluating the insecticidal activity of perennial ryegrass–endophyte symbiota on aphids (nymph mortality could be assessed in as little as 24 and 48 hours for *D. noxia* and *A. lentisci* respectively). This rapid and simple approach can be used to benchmark novel grass–endophyte symbiota on a range of aphid species that feed on leaves of plants, however we would caution that it may not be suitable for the assessment of root-feeding aphids, as this species exhibited relatively high mortality on the control as well.

Introduction

Perennial ryegrass, *Lolium perenne* (L.), is one of the most important pasture grass species globally [1]. It is favoured over other grass species, particularly in southern Australia, as it is highly nutritious, can grow with minimal soil moisture during dry conditions and can remain

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Competing interests: NPC received a salary from DairyBio for the duration of this study, as part of a stipend for a PhD project. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

productive for up to four years [1,2]. Like any domesticated crop, however, perennial ryegrass is prone to a range of biotic and abiotic stresses that reduce its yield and persistence [3].

Nematoda, Mollusca, Collembola, Acari, and Insecta (Coleoptera, Lepidoptera, Orthoptera, Diptera, Thysanoptera and Hemiptera) are among the main invertebrate groups of significance that negatively impact perennial ryegrass crops in Australia and around the world predominantly through feeding injuries and the transmission of plant diseases [4,5]. The damage caused by invertebrate pests is mostly quantified in terms of the loss of dry matter or seed yield the extent of which varies depending on the pest type and abundance, as well as the grass species, its growth stage and the part of the plant that is consumed [6]. The most common metric used to assess crop damage by pests is the resultant economic loss [7]. For instance, in New Zealand, grass grubs, *Costelytra zealandica* (White), have been reported to cause productivity losses of $140–380 million NZD on dairy farms, while Argentine stem weevil, *Listronotus bonariensis* (Kuschel), is estimated to cause annual productivity losses of up to $200 million NZD [7]. Often, farmers attempt to offset this high economic cost through the increased use of insecticides and/or increased sowing frequency. Depending on the severity of the damage, farmers may need to supplement herds' diets with silage or grains, further increasing the costs. As a result, the overall economic impact that invertebrate pests have on dairy pastures in New Zealand has recently been estimated to reach up to $1.4 billion NZD [7]. This study found that the bulk of the losses were caused by scarab beetles (up to $600 million approx.), nematodes (up to $300 million approx.) and weevils ($200 million approx.) [7]. The overall economic loss caused by invertebrates on dairy pastures in Australia is unknown.

One group of invertebrates that causes serious damage to arable and pasture crops are aphids, due to direct feeding injuries and virus-associated injuries [8]. Common aphid species found in pasture grasses in Australia and around the world are *Rhopalosiphum* spp., *Sitobion* spp., *Metopolophium* spp., *Schizaphis graminum* (Rondani), *Diuraphis noxia* (Mordvilko) and *Aploneura lentisci* (Passerini) [9]. All these aphids have the potential to cause serious damage to pasture crops in Australia except for *S. graminum* that is not found in Australia. *Rhopalosiphum* spp., *Sitobion* spp. and *Metopolophium* spp. are vectors of Luteoviruses that can cause significant yield losses in perennial ryegrass [10], while *D. noxia*, a recent arrival to Australia, and *A. lentisci* have the potential to cause significant damage due to direct feeding injuries [11]. Farmers use systemic insecticides or spray to control aphids particularly when the crop is at the seedling stage when it is most at risk of virus infection. Despite the relative success of insecticides in controlling foliar aphids, the control of root-feeding aphids such as *A. lentisci* is more difficult. Farmers are also consciously moving away from using insecticides, due to concerns related to increased resistance in aphid populations, increased mortality of natural enemies, along with general health and environmental concerns [12,13]. Hence, there is global interest in the development of innovative pest control solutions, particularly the use of endophytic fungi (e.g. *Acremonium* spp., *Epichloë* spp.) that occur naturally in grasses and are known to protect them from biotic and abiotic stresses [14].

Fungal endophytes have beneficial effects on the host plant as they play an important role in its survival, protecting it from invertebrates and grazing animals through the production of certain chemical compounds, and providing resistance to environmental extremes such as drought or high temperatures [14,15]. However, the same fungal endophytes can be harmful to grazing livestock as these chemical compounds can cause a range of toxicoses such as ryegrass staggers in cattle and sheep, costing the livestock industry millions of dollars in productivity losses [14,16]. These compounds, that confer resistance to vertebrates and invertebrates, are alkaloids of various classes consisting of: ergopeptines (e.g. ergovaline), indole diterpenes (e.g. lolitrem B and epoxy-janthitrems), pyrrolizidines (e.g. lolines), and polyketides (e.g. peramine) [17–19]. These alkaloids have various degrees of toxicity toward vertebrates and

Table 1. Aphid species collection details and GenBank accession numbers.

Aphid species	GenBank Accession number	Locality	GPS	Date of collection	Host plant
Diuraphis noxia	MN066606	Horsham, Victoria	36˚43'17.8"S 142˚10'26.9"E	1/06/2016	Barley
Aploneura lentisci	MN066607	Bundoora, Victoria	37˚43'05.2"S 145˚02'49.6"E	10/06/2017	Perennial ryegrass (cv. Impact 04)

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invertebrates [18–20]. Developing endophyte strains that produce favourable quantities and types of alkaloids that remain harmless to grazing animals but retain the toxicity towards invertebrates has been the focus of recent research, particularly in Australia with *Epichloë festucae* var. *lolii* in perennial ryegrass [21–23].

Numerous studies have been carried out since the 1980s to determine what grass–endophyte association has the greatest negative effect on aphids but has minimal effect on vertebrates. In these studies, aphids' settling preferences, host acceptance, feeding, body size and life history parameters were assessed under a range of conditions [24–28]. A common trend across many studies has emerged that showed varying responses depending on the aphid–grass–endophyte combination. For instance, Siegel, et al. [29] assessed a range of grasses, cultivars and endophytes and showed an increase in mortality for *R. padi* and *S. graminum*, which was associated with the presence of lolines, whilst the presence of peramine increased the mortality of *S. graminum* only. Furthermore Meister, et al. [28] found that *R. padi* life span and fecundity (assessed individually) were significantly reduced when feeding on *Epichloë festucae* var. *lolii* infected perennial ryegrass while *M. dirhodum* showed no response. And finally Popay and Cox [30] found certain endophytes decreased *A. lentisci* population size more than others.

There is an additional factor that can influence aphid life history in response to grass endophyte symbiota, namely the methodology used to assess the insecticidal effect on aphids, which makes comparisons between studies difficult. This is not surprising as methods range from detached leaf bioassays to whole plant bioassays, density-dependent to density-independent bioassays, and different bioassay durations [19,24,28,30]. From a routine analytical perspective, it is important to develop a bioassay that is fast and easy to conduct with reproducible results. To date, there are no standard *in-planta* protocols developed to assess the insecticidal effects of grass–endophyte symbiota on aphids, such as those available for synthetic insecticide testing [31,32].

Thus, our study aimed at evaluating the insecticidal effects of grass–endophyte symbiota on life history parameters of aphids using a single seedling bioassay, as a first step towards developing a standard protocol that is rapid, simple, reproducible and scalable, for high throughput screening of many grass–endophyte symbiota combinations and aphid species. Our study also aimed at characterising, for the first time in Australia, the effects of five grass–endophyte symbiota (four commercial and one standard/wild type) on the life history of two species of aphid, *D. noxia* and *A. lentisci* (foliar and root feeding species respectively), and the timeframe in which these symbiota show the strongest effect.

Materials and methods

Aphids

Diuraphis noxia and *A. lentisci* were collected in South Eastern Australia from a range of locations and host plants (Table 1). *Diuraphis noxia* were collected in June 2016 from barley *Hordeum vulgare* (L.), while *A. lentisci* were collected in August 2017 from glasshouse populations on perennial ryegrass cv. Impact 04 (Table 1). These species were selected because they both represent potential or established foliar and root pests of perennial ryegrass in Victoria, Australia [30,33,34].

Ten *D. noxia* individuals were isolated from a field plant and reared on separate barley cv. Hindmarsh. One clone was selected, and its progeny maintained on barley and perennial ryegrass cv. Alto (the latter without endophyte). Eight *A. lentisci* individuals were isolated from glasshouse plants and reared on separate perennial ryegrass cv. Alto only (the latter also without endophyte). Colonies were established from one clonal lineage per species, maintained on mature plants of barley and perennial ryegrass (*D. noxia*) and perennial ryegrass (*A. lentisci*). Each aphid colony was reared for 4–6 weeks, at which point twenty individuals from the colony were transferred to a new mature plant in an insect-free cage. These aphids were used for all subsequent life history bioassays. From these colonies, twenty to thirty adult aphids were kept individually on barley and perennial ryegrass (*D. noxia*), sixty on perennial ryegrass (*A. lentisci*) and left to produce young. Each day newborn nymphs (1–2 newborn aphids/aphid/day) were transferred to the cup assays. In total there were up to 60 newborn aphids/day for both species. Of these, 24 (*D. noxia)* and 48 (*A. lentisci)* were used in the life history assays per treatment. These newborn aphids were collected over a period of 1–2 weeks approximately to a total of 24 and 168 *D. noxia* from barley and perennial ryegrass respectively, and 288 *A. lentisci* from perennial ryegrass (this meant that not all the assays started the same day).

To confirm aphid species identification, a molecular-based identification method was carried out using the barcode region of the *cytochrome oxidase subunit 1* (CO1) gene [35]. Aphid DNA was extracted using Bio-Rad Chelex© 100 Resin following the method of Walsh et al. [36], with minor modifications: individual aphids were placed in 1.5ml Eppendorf tubes containing 2 glass beads and 20μl of Proteinase K. The contents of the tubes were crushed in a mixer mill for 1 min at 30hz., then 150μl of 5% Chelex© 100 Resin (BioRad) was added and the extract was incubated at 55˚C for 1hr, then at 85˚C for 8 min. A section of the CO1 gene was amplified using the primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) [35]. The PCR was performed in 25 μL reaction volumes containing: 1x bovine serum albumin (NEB), 10x Immobuffer (Bioline), 2.5 mM dNTP (Qiagen), 10 μM of each primer, 5 units/μL of Immolase DNA polymerase (Bioline) and 5 μL of template DNA. The cycling conditions were: 94˚C for 6 min; 40 cycles of 94˚C for 30 sec, 51˚C for 50 sec and 72˚C for 50 sec; followed by 2 min at 72˚C. PCR products were sequenced by Macrogen Inc. (Seoul, Korea) and sequences were compared to public databases (NCBI BLASTn), determining similarity and coverage with previously identified species. Sequences were submitted to GenBank (Table 1), and specimens were preserved in 70% and 100% ethanol and deposited in the Victorian Agricultural Insect Collection and Victoria Agricultural Insect Tissue Collection (VAIC and VAITC) at AgriBio, Bundoora, Australia.

Plants

Perennial ryegrass cv. Alto seed was sourced from Agriseeds (Christchurch, New Zealand) and barley cv. Hindmarsh seed was sourced from Seednet (Horsham, Australia). Perennial ryegrass WE (without endophyte) and barley seeds were germinated on petri dishes with moistened 90mm filter paper (Whatman™) at room temperature (approximately 23˚C and 63% RH). After seven days, seedlings were transferred to potting mix in pots and grown in a controlled environment room at 20˚±2˚C and 62.0±5% RH, and a photoperiod of 14h light: 10h dark until maturity and placed in an insect-free cage (W24.5 x D24.5 x H63.0 cm; 150 x 150μm mesh size; Bugdorm, MegaView Science, Pty. Ltd. Taiwan). These plants were used for maintaining the aphid colonies.

The same germination method was used to grow seedlings of perennial ryegrass (+/- endophytes) for life history bioassays. Bioassays utilised a cup-based system [37], whereby one-

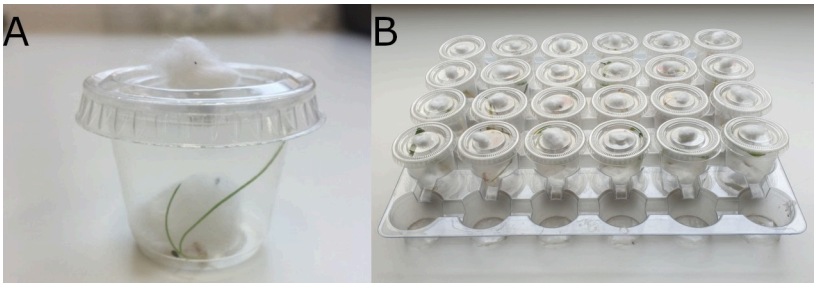

Fig 1. High throughput *in-planta* bioassay equipment. (A) An image of an individual bioassay cup containing a seedling with an aphid and moist cotton wool around the roots. (B) Bioassay tray setup containing multiple bioassay cups.

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week-old seedlings were placed into individual 30mL plastic cups (Olympus Packaging Pty. Ltd.) with a small piece of moistened cotton wool surrounding the root system. The lid of each cup was perforated to create a hole, and a small piece of cotton wool was placed in each hole to allow evaporation of excess humidity (Fig 1A). Cups were placed in trays that fit up to 30 cups (Fig 1B). The seedlings were watered every few days and were replaced every 7 days during bioassays.

Endophytes

Perennial ryegrass seeds (see *Plants* section) were infected with fungal endophytes of the species *Epichloë festucae* var. *lolii*. These included four different commercial endophyte strains (AR1, AR37, NEA2 and NEA6) (Table 2) and one wild type or standard endophyte (SE), which is found naturally in many perennial ryegrass pastures [38]. These *Epichloë* endophytes produce a range of alkaloids [Indole Diterpenes (lolitrem B and janthitrem I), Ergopeptines (ergovaline) and Polyketides (peramine)] (Table 2), resulting in each endophyte having a unique alkaloid profile (Table 2).

The presence of endophytes in seed batches and the identity of endophyte strains were confirmed using a Competitive Allele Specific PCR (KASP) bioassay [39]. KASP primers were previously designed and generated by AgriBio and GeneWorks based on allelic variation of single nucleotide polymorphisms (SNP) that differentiate endophytes from one another (SE, AR1, AR37, NEA2 and NEA6). The SNP allele-specific detection is based on a homogeneous fluorescence bioassay, with each forward primer incorporating one distinct fluorescent dye (HEX and FAM) specific for each SNP. The PCR reaction was performed in 10.14 μL reaction volumes containing: 5 μL of 2 x KASP master mix (GeneWorks), 0.14 μl of KASP bioassay mix (GeneWorks) and 5 μl of template DNA. The cycling conditions were: 94˚C for 15 minutes; 9 cycles of 94˚C for 20 seconds and 61˚C for 60 seconds (drop—0.6˚C / cycle); 26 cycles of 94˚C

Table 2. *Epichloë festucae* var. *lolii* endophyte strains used in this study and associated alkaloid profiles.

Alkaloid class	Alkaloid type	SE	AR1	AR37	NEA2	NEA6
Ergopeptide	Ergovaline	P	*	*	P	P
Polyketide	Peramine	P	P	*	P	P
Indole Diterpene	Janthitrem I	*	*	P	*	*
	Lolitrem B	P	*	*	T	*

The host plant for all endophytes was perennial ryegrass cv. Alto. SE = standard endophyte; AR1, AR37, NEA2, NEA6 = commercial endophytes. P = alkaloid present * = alkaloid absent; T = alkaloid present only in trace levels.

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20 seconds and 55˚C for 60 seconds; followed by 37˚C for 60 seconds. The data was visualised and analysed using Bio-Rad CFX manager 3.1 software to detect fluorescence and discriminate allelic variation between endophytes.

Aphid life history parameters

A total of 168 new-born *D*. *noxia* nymphs and 288 new-born *A*. *lentisci* nymphs were collected (24 newborn nymphs x 1 aphid species x 5 endophyte treatments and 2 controls; 48 newborn nymphs x 1 aphid species x 5 endophyte treatments and 1 control = 456 newborn nymphs total) and placed individually into new bioassay cups with 7-day-old perennial ryegrass seedlings with and without endophytes (Table 2). *Diuraphis noxia* were placed on to leaves, while *A*. *lentisci* were placed onto roots. Barley was included as an additional control for *D*. *noxia* as it is a common host plant for *D*. *noxia*, where it exhibits high levels of fecundity and survival [40]. This provided assurance of the health of the *D*. *noxia* clone selected. Life history data of *D*. *noxia* on barley was not included in statistical analyses. Every 7 days (up to 28 days) new 7-day old seedlings were provided to the aphids. When replacing seedlings, aphids were gently transferred to the new seedlings by lightly brushing the aphid dorsum with a fine paintbrush until they withdrew their stylets. Aphids were then picked up using the same paintbrush and placed near the leaf or the root of the new seedling.

The parameters investigated in this study were mortality and fecundity. In order to calculate these parameters, data was collated on development (whether aphids were nymphs or adults), achieved by recording the number of moults and, time to reproduction (number of days from birth to first reproduction) used to calculate the intrinsic rate of increase (r_m). Fecundity was recorded as the number of nymphs born every 24 hours, which were also removed daily, and mortality as the number of aphids that died each day. The intrinsic rate of increase (r_m) was calculated using the following formula:

$$r_m = 0.738 \times \{ln(FD)/(D)\}$$

Where FD is the number of nymphs produced during a time equal or greater than the pre-reproductive period and D is the pre-reproductive period (number of days from birth to first reproduction) [41]. Aphids were transferred onto a new seedling, every 7 days. All experiments were carried out in a controlled environment room at 20˚±2˚C and 62.0±5%RH, and a photoperiod of 14h light: 10h dark. All experiments were carried out over a 28 day period from the moment of birth; 28 days was chosen for the duration of the bioassay as it represents a major portion of the average reproductive lifespan of *D*. *noxia* [40] and *A*. *lentisci* on perennial ryegrass [30] at 20˚C.

Statistical analyses

Diuraphis noxia nymph mortality was grouped into four time periods of 0–24 (24 hrs), 24–48 (48 hrs), 48–72 (72 hrs) and >72 (>72 hrs) hours, while *A*. *lentisci* nymph mortality was grouped into four time periods of 0–48 (48 hrs), 48–96 (96 hrs), 96–144 (144 hrs), and >144 (>144 hrs) hours. Adult mortality was grouped into time periods of 14, 21 and 28 days for *D*. *noxia* and *A*. *lentisci*. Daily observed fecundity data was grouped into time periods of 14 and 21 days for *D*. *noxia* and 14, 21 and 28 days for *A*. *lentisci*. Fecundity and r_m were both assessed twice. First considering all aphids in each treatment and then considering only aphids that survived to reproduction. Data on fecundity and r_m was analysed using a one-way analysis of variance. Differences between treatment means were examined using the least significant difference (LSD) at a 5% level of significance. The unit of analysis was individual aphids in plastic cups (up to 24 and 48 replicates for *D*. *noxia* and *A*. *lentisci* respectively). Residuals

versus fitted values plots were examined to determine any need for data transformation to ensure the normality of residuals with constant variance. The difference in mortality of aphid nymphs and adults between treatments at each time period was analysed using logistic regression models, where the number of aphid deaths at each time period was the response variable (success) and logit was the link function. The cumulative mortality of nymphs was also analysed using logistic regression models, where the number of nymphs dead was response and Treatment*Day was the full model, with logit as link function. These models were used to compute the probability of aphid mortality in each time period on each treatment and compare between treatments and time periods. The Wald chi-squared test was used to include/exclude a term in the model. All data in this study was analysed using GenStat version 18 [42].

Results

Molecular results

Aphid species identification using the barcode region of the CO1 gene confirmed the identity of *D. noxia* and *A. lentisci* with 100% similarity to previously observed and catalogued DNA sequences.

Endophyte presence in seed batches was confirmed using strain-specific Competitive Allele Specific PCR (KASP) assay, with results showing high incidence in all seed batches. Of approximately 150 seedlings tested for each treatment all were shown to have 100% incidence of the expected endophyte, indicating that endophyte presence is very high in the entire seed batch.

Effects of *Epichloë festucae* var. *lolii* endophyte symbiota on life history of Diuraphis noxia

Epichloë festucae var. *lolii* endophyte symbiota had a significant effect on the mortality of *D. noxia*, most notably at the nymphal stage (Table 3). The average mortality at the nymphal stage was 91% across all endophyte treatments. The total average nymph mortality was highest on aphids tested on SE and NEA2 (both caused 100% nymph mortality) followed by AR1 (96%), NEA6 (88%) and AR37 (71%). The endophyte treatments SE, NEA2 and AR1 resulted

Table 3. Mortality of *Diuraphis noxia* on all endophyte and endophyte-free treatments observed at the nymphal stage (24, 48, 72 and >72 hrs) and the adult stage (14, 21 and 28 days).

Mortality	Barley	WE	SE	AR1	AR37	NEA2	NEA6	Total mortality [a]	LSD	P-value[b]
Nymph mortality	4/21 (0.19)	8/22 (0.36)	24/24 (1.00)	23/24 (0.96)	17/24 (0.71)	24/24 (1.00)	21/24 (0.88)	0.91	0.17	**<0.001**
Nymph mortality (24 hrs)	0/21 (0.00)	0/22 (0.00)	4/24 (0.17)	7/24 (0.29)	4/24 (0.17)	9/24 (0.38)	7/24 (0.29)	0.26	0.22	**0.004**
Nymph mortality (48 hrs)	0/21 (0.00)	2/22 (0.09)	7/24 (0.29)	9/24 (0.38)	3/24 (0.12)	10/24 (0.41)	8/24 (0.33)	0.31	0.24	**0.029**
Nymph mortality (72 hrs)	2/21 (0.10)	2/22 (0.09)	6/24 (0.25)	5/24 (0.21)	5/24 (0.21)	2/24 (0.08)	2/24 (0.08)	0.17	0.2	0.337
Nymph mortality (>72 hrs)	2/21 (0.10)	4/22 (0.18)	7/24 (0.29)	2/24 (0.08)	5/24 (0.21)	2/24 (0.08)	4/24 (0.17)	0.17	0.21	0.509
Adult mortality	17/21 (0.81)	16/22 (0.67)	*	01/24 (0.04)	7/24 (0.29)	*	3/24 (0.12)	0.15	0.17	**<0.001**
Adult mortality (14 days)	3/21 (0.14)	1/22 (0.04)	*	0/24 (0.00)	0/24 (0.00)	*	0/24 (0.00)	0	0.03	0.606
Adult mortality (21 days)	7/21 (0.33)	6/22 (0.25)	*	0/24 (0.00)	4/24 (0.17)	*	3/24 (0.13)	0.1	0.14	**<0.001**
Adult mortality (28 days)	7/21 (0.38)	9/22 (0.38)	*	1/24 (0.04)	3/24 (0.13)	*	0/24 (0.00)	0.05	0.12	**<0.001**

Results are shown as proportions.

[a] Average mortality on endophyte treatments (excluding WE).

[b] p values were calculated using a logistic regression analysis.

* No aphids survived. WE = without endophyte; SE = standard endophyte; AR1, AR37, NEA2, NEA6 = commercial endophytes. Barley data is shown but was not included in the statistical analysis. All bioassays were carried out for 28 days from the moment of birth.

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in significantly higher mortality than AR37 ($P < 0.001$). All endophyte treatments resulted in significantly higher nymph mortality than the control (36%).

The total average nymph mortality was highest at 24 and 48 hrs (26% and 31% respectively) and lowest at 72 and >72 hrs (17% in both cases) (Table 3). At 24 and 48 hrs, NEA2, NEA6 and AR1 caused the highest mortality by comparison to all other endophyte treatments and the control WE while at 72 hrs and >72 hrs there was no significant difference between treatments. The rate of mortality (cumulative mortality at 24, 48, 72, >72 hrs) was assessed to determine the time period (early or late nymphal stage) at which treatments had the greatest mortality (Fig 2). Observations of the rate of mortality indicated that gradients of NEA2, NEA6 and AR1 followed a linear-logarithmic gradient (higher mortality at earlier time periods), whereas SE and AR37 followed a linear-exponential gradient (higher mortality at later time periods). Statistically there was no significant difference in the rate or mortality between treatments (Wald statistics $P = 0.269$).

The average mortality at the adult life stage was 15% across all endophyte treatments (Table 3). There was no adult mortality calculated for SE or NEA2 as no aphid survived to the adult life stage on these treatments. At 14 days, there was no significant difference between treatments. At 21 and 28 days, the highest mortality was observed on WE (25% and 38% for each time period respectively) and was significantly different from all other endophyte treatments at 28 days ($P < 0.001$).

The average fecundity was 1.3 nymphs per adult per day across all endophyte treatments, when assessing all aphids in each treatment (Table 4). There was no fecundity calculated for SE or NEA2 as no aphid survived to the adult life stage on these treatments. At 14 and 21 days AR1 exhibited the strongest reduction in fecundity (0.04 and 0.2 nymphs per adult respectively), followed by AR37 (1.5 and 2.8 respectively) and NEA6 (1.4 and 2.0 respectively). All endophyte treatments exhibited significantly reduced fecundity, compared to the control ($P < 0.001$), but there was no significant difference between endophyte treatments. In addition, we carried out a statistical analysis considering the aphids that survived to reproductive age with results showing no significant differences in fecundity between treatments (Table 4). The fecundity rate (cumulative fecundity at 7–21 days) was assessed to determine the time

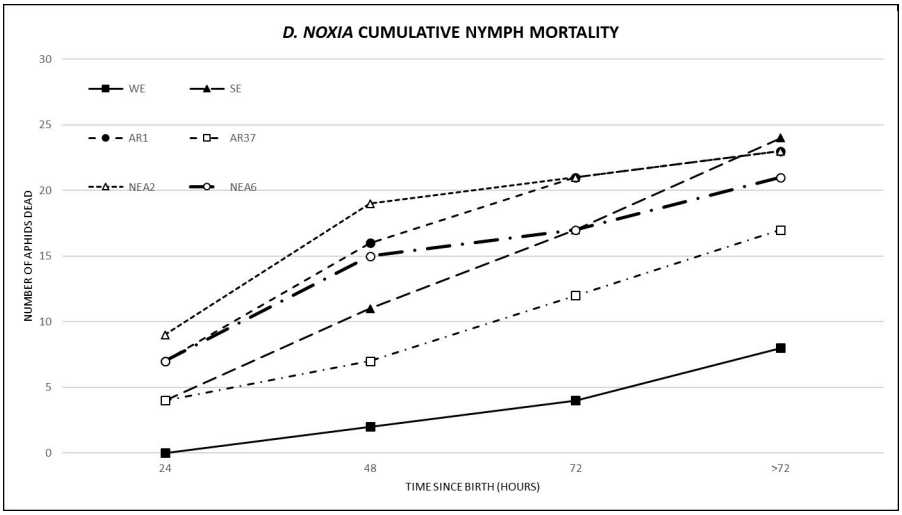

Fig 2. Cumulative nymph mortality of *Diuraphis noxia* on all endophyte and endophyte-free treatments observed at 24, 48, 72 and >72 hrs. Results are shown as the cumulative total number of nymphs dead for each time period. WE = without endophyte; SE = standard endophyte; AR1, AR37, NEA2, NEA6 = commercial endophytes.

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Table 4. Fecundity of *Diuraphis noxia* on all endophyte treatments.

Fecundity (all aphids)	Barley	WE (n = 22)	SE (n = 24)	AR1 (n = 24)	AR37 (n = 24)	NEA2 (n = 24)	NEA6 (n = 24)	Total fecundity[a]	Standard Error	LSD	P-value[b]
Total fecundity	23.2	7.1	*	0.1	2.1	*	1.7	1.3	n/a	n/a	n/a
Fecundity (14 days)	18.1	5.1 (±0.66)	*	0.04 (±0.63)	1.5 (±0.63)	*	1.5 (±0.63)	1.0	±0.9	1.8	**<0.001**
Fecundity (21 days)	28.3	10.0 (±1.11)	*	0.2 (±1.06)	2.8 (±1.06)	*	2.1 (1.06)	1.7	±1.5	2.9	**<0.001**
r_m	0.29	0.14 (±0.02)	*	0.003 (±0.01)	0.05 (±0.01)	*	0.03 (±0.01)	0.03	±0.02	0.04	**<0.001**
Fecundity (reproducing aphids)	**Barley (n = 17)**	**WE (n = 14)**	**SE**	**AR1 (n = 1)**	**AR37 (n = 7)**	**NEA2**	**NEA6 (n = 3)**	**Total fecundity[a]**	**Standard Error**	**LSD**	**P-value[b]**
Total fecundity	28.7	11.2	*	2.5	7.4	*	13.7	7.8	n/a	n/a	n/a
Fecundity (14 days)	22.4	8.1 (±1.25)	*	1.0 (±4.68)	5.0 (±1.77)	*	12.3 (±2.70)	5.8	±3.94	8.19	0.092
Fecundity (21 days)	35	15.6 (±2.00)	*	4.0 (±7.49)	9.7 (±2.83)	*	17.0 (±4.33)	9.9	±6.30	13.11	0.192
r_m	0.36	0.21 (±0.02)	*	0.07 (±0.08)	0.19 (±0.03)	*	0.25 (±0.05)	0.17	±0.07	0.14	0.296

Results are shown as means.

[a] Average fecundity per female per day on endophyte treatments (excluding WE).

[b] p values were calculated using a one-way analysis of variance.

* No aphids survived. WE = without endophyte; SE = standard endophyte; AR1, AR37, NEA2, NEA6 = commercial endophytes. Barley data is shown but was not included in the statistical analysis.

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period at which treatments had the greatest effect on fecundity (Fig 3). Observations of the fecundity rate indicated that AR1 had a low gradient (low no. of nymphs born) over the 7-21-day period, whereas AR37 and WE had a medium gradient (medium no. of nymphs born), and NEA6 had a high gradient (high no. of nymphs born). The fecundity rate was delayed in aphids on AR1 as reproduction began at 13 days, compared to AR37, NEA6 and WE, where

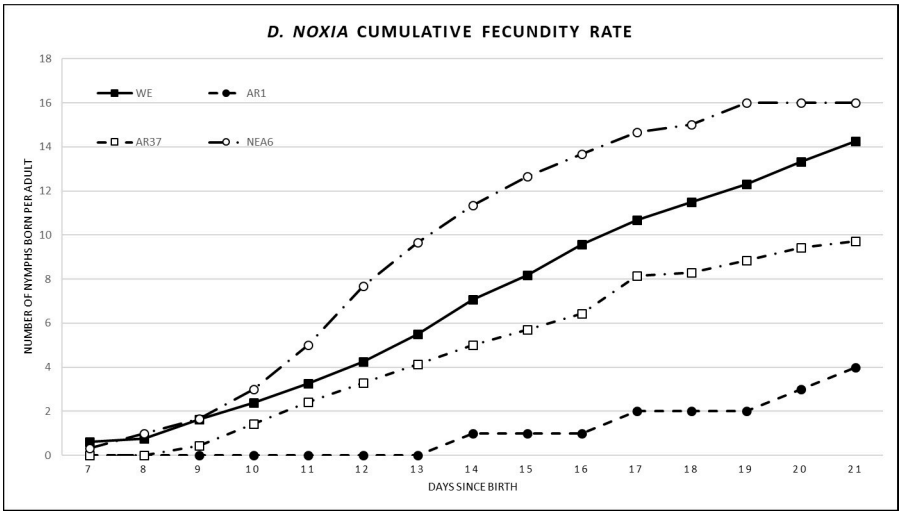

Fig 3. Cumulative fecundity of *Diuraphis noxia* on all endophyte and endophyte-free treatments observed over 7–21 days. Results are shown as the average of newborn nymphs per female per day based on aphids that reproduced. Sample sizes are: WE (n = 16), AR1 (n = 1), AR37 (n = 7) and NEA6 (n = 3). Averages of fecundity based on aphids that reproduced is shown in Table 4. WE = without endophyte; AR1, AR37, NEA6 = commercial endophytes.

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reproduction began at 7 days. The fecundity rate was increased in aphids on NEA6, particularly between 11–19 days, as the number of nymphs born was consistently above WE.

The average intrinsic rate of increase (r_m) was 0.03 across all endophyte treatments, when assessing all aphids in each treatment (Table 4). There was no r_m calculated for SE or NEA2 as no aphid survived to the adult life stage on these treatments. AR1 exhibited the strongest reduction in r_m (0.003), followed by NEA6 (0.03) and AR37 (0.05). All endophyte treatments exhibited significantly reduced r_m compared to the control ($P < 0.001$). There was no significant difference between endophyte treatments. There was no significant difference in r_m between treatments when assessing only the aphids that survived to reproductive age (Table 4).

Effects of *Epichloë festucae* var. *lolii* endophyte symbiota on life history of Aploneura lentisci

Epichloë festucae var. lolii endophyte symbiota significantly affected the mortality of *A. lentisci*, and as in *D. noxia*, especially affected the nymphal stage (Table 5). A total of 89% of aphids died during the nymphal stage on all endophyte treatments. The total average nymph mortality was highest on aphids tested on NEA6 and SE (both causing 94% nymph mortality) followed by AR37 and NEA2 (both 88%) and AR1 (83%). All endophyte treatments had significantly higher nymph mortality than the control (69%) ($P = 0.008$), but there was no significant difference between endophyte treatments.

The total average nymph mortality was highest at 96 hrs and 144 hrs (26% and 28% respectively) and lowest at 48 hrs and >144 hrs (17% and 19% respectively) (Table 5). The mortality rates differed over time depending on endophyte treatment. At 48 and 96 hrs NEA6 caused the highest mortality (33% and 40%), although at 48 hrs this was not different from the control. At 144 hrs AR1, caused the highest mortality (50%) while at >144 hrs AR37 showed the highest mortality (29%) of the endophyte group but not of all treatments as the control WE showed the highest mortality (44%). The rate of mortality (cumulative mortality at 48, 96, 144 and >144 hrs) was assessed to determine the time period (early or late nymphal stage) at which treatments had the greatest effect on mortality (Fig 4). Observations of the rate of mortality indicated that gradient of NEA6 followed a linear-logarithmic gradient (higher mortality at earlier time periods), whereas NEA2, SE and AR37 followed a linear gradient (consistent mortality across all time periods), and AR1 and WE followed a linear-exponential gradient (higher mortality at later time periods). Statistically there was no significant difference in the rate or mortality between treatments (Wald statistics $P = 0.060$).

Adult mortality was low in comparison to nymph mortality (11%) (Table 5). At 14 days, mortality was not significantly different between treatments but there were significantly higher mortality rates for WE at 21 and 28 days compared to many endophyte treatments ($P < 0.001$ and $P = 0.007$ respectively).

Average fecundity of *A. lentisci* was significantly reduced to 0.6 nymphs per adult per day across all endophyte treatments, when assessing all aphids (Table 6). For all observation times i.e., 14, 21 and 28 days, endophyte associated mortality was significantly different from the control (except for AR1 at 14 days) ($P < 0.001$) (Table 6). AR37, NEA2 and NEA6 exhibited the strongest reduction in fecundity by comparison to AR1 but also SE showed a strong reduction in fecundity. The fecundity rate (cumulative fecundity at 7–21 days) was assessed to determine the time period at which treatments had the greatest effect on fecundity (Fig 5). Observations of the fecundity rate indicated that NEA2, SE, AR37 and NEA6 all had a low gradient (low no. of nymphs born) over the 7-21-day period, whereas AR1 had a medium gradient (medium no. of nymphs born), and WE had a high gradient (high no. of nymphs born).

Table 5. Mortality of *Aploneura lentisci* on all endophyte and endophyte-free treatments observed at the nymphal stage (48, 96, 144 and >144 hrs) and the adult stage (14, 21 and 28 days).

Mortality	WE	SE	AR1	AR37	NEA2	NEA6	Total mortality [a]	LSD	P-value[b]
Nymph mortality	33/48 (0.69)	45/48 (0.94)	40/48 (0.83)	42/48 (0.88)	42/47 (0.88)	45/48 (0.94)	0.89	0.13	**0.008**
Nymph mortality (48 hrs)	11/48 (0.23)	6/48 (0.13)	1/48 (0.02)	10/48 (0.20)	8/47 (0.17)	16/48 (0.33)	0.17	0.15	**<0.001**
Nymph mortality (96 hrs hrs)	0/48 (0.00)	14/48 (0.29)	8/48 (0.17)	9/48 (0.19)	11/47 (0.23)	19/48 (0.40)	0.26	0.15	**<0.001**
Nymph mortality (144 hrs)	1/48 (0.02)	14/48 (0.29)	24/48 (0.50)	9/48 (0.19)	17/47 (0.35)	3/48 (0.06)	0.28	0.15	**<0.001**
Nymph mortality (>144 hrs)	21/48 (0.44)	11/48 (0.23)	7/48 (0.15)	14/48 (0.29)	6/47 (0.13)	7/48 (0.15)	0.19	0.16	**0.002**
Adult mortality	15/48 (0.31)	3/48 (0.06)	8/48 (0.17)	6/48 (0.125)	6/47 (0.125)	3/48 (0.06)	0.11	0.13	**0.008**
Adult mortality (14 days)	2/48 (0.04)	3/48 (0.06)	0/48 (0.00)	1/48 (0.02)	3/47 (0.06)	0/48 (0.00)	0.03	0.07	0.115
Adult mortality (21 days)	8/48 (0.17)	0/48 (0.00)	6/48 (0.13)	1/48 (0.02)	0/47 (0.00)	2/48 (0.04)	0.04	0.08	**<0.001**
Adult mortality (28 days)	5/48 (0.10)	0/48 (0.00)	0/48 (0.00)	3/48 (0.06)	1/47 (0.02)	0/48 (0.00)	0.02	0.06	**0.007**

Results are shown as proportions.

[a] Average mortality on endophyte treatments (excluding WE).

[b] p values were calculated using a logistic regression analysis. WE = without endophyte; SE = standard endophyte; AR1, AR37, NEA2, NEA6 = commercial endophytes. All bioassays were carried out for 28 days from the moment of birth.

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The fecundity rate was delayed in aphids on AR37, NEA2 and NEA6 as reproduction began at 9–11 days, compared to WE, SE and AR1, where reproduction began at 7 days. The fecundity rate was slightly increased in aphids on AR1 between 11–14 days, as the number of nymphs born was above that of WE.

The average intrinsic rate of increase (r_m) was 0.01 across all endophyte treatments, when assessing all aphids (Table 6). SE and NEA6 exhibited the strongest reduction in r_m (both 0.005), followed by AR37 and NEA2 (both 0.01) and AR1 (0.03). All endophyte treatments exhibited significantly reduced r_m compared to the control, and SE and NEA6 exhibited significantly reduced r_m compared to AR1 ($P < 0.001$). The r_m was 0.11 across all endophyte treatments, when assessing only the aphids that survived to reproductive age (Table 6). NEA6 exhibited the strongest reduction in r_m (0.08), followed by AR37 and NEA2 (0.10), SE (0.11) and AR1 (0.17). Only NEA6 exhibited significantly reduced r_m compared to the control ($P < 0.001$). There was no significant difference between endophyte treatments.

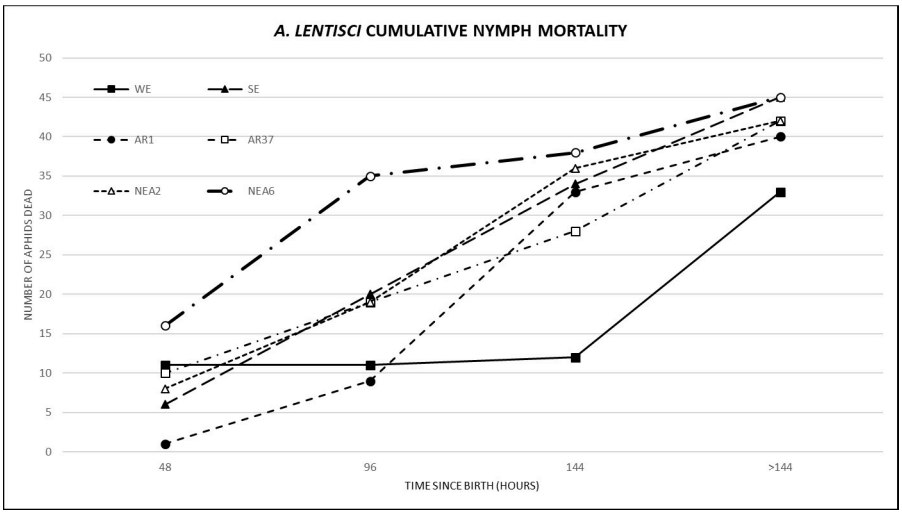

Fig 4. Cumulative nymph mortality of *Aploneura lentisci* on all endophyte and endophyte-free treatments observed at 48, 96, 144 and >144 hrs. Results are shown as the cumulative total number of nymphs dead for each time period. WE = without endophyte; SE = standard endophyte; AR1, AR37, NEA2, NEA6 = commercial endophytes.

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Table 6. Fecundity of *Aploneura lentisci* on all endophyte and endophyte-free treatments.

Fecundity (all aphids)	WE (n = 48)	SE (n = 48)	AR1 (n = 48)	AR37 (n = 48)	NEA2 (n = 47)	NEA6 (n = 48)	Total fecundity[a]	Standard Error	LSD	P-value[b]
Total fecundity	3.7	0.2	1.6	0.6	0.4	0.2	0.6	n/a	n/a	n/a
Fecundity (14 days)	1.9 (±0.4)	0.2 (±0.4)	1.1 (±0.4)	0.1 (±0.4)	0.1 (±0.4)	0.1 (±0.4)	0.3	±0.4	0.8	**<0.001**
Fecundity (21 days)	4.5 (±0.8)	0.2 (±0.8)	1.7 (±0.8)	0.6 (±0.8)	0.5 (±0.8)	0.3 (±0.8)	0.6	±0.8	1.58	**<0.001**
Fecundity (28 days)	4.6 (±0.9)	0.2 (±0.9)	1.9 (±0.9)	1.0 (±0.9)	0.5 (±0.9)	0.3 (±0.9)	0.8	±0.9	1.8	**<0.001**
r_m	0.05 (±0.01)	0.004 (±0.01)	0.03 (±0.01)	0.01 (±0.01)	0.01 (±0.01)	0.005 (±0.01)	0.01	±0.011	0.02	**<0.001**
Fecundity (reproducing aphids)	**WE (n = 15)**	**SE (n = 3)**	**AR1 (n = 8)**	**AR37 (n = 6)**	**NEA2 (n = 6)**	**NEA6 (n = 3)**	**Total fecundity[a]**	**Standard Error**	**LSD**	**P-value[b]**
Total fecundity	11.7	3.3	9.4	4.7	3	3.4	4.8	n/a	n/a	n/a
Fecundity (14 days)	6.0 (±0.98)	3.0 (±2.20)	6.5 (±1.35)	1.0 (±1.55)	1.2 (±1.55)	1.0 (±2.20)	2.6	±2.4	4.8	**0.014**
Fecundity (21 days)	14.3 (±1.57)	3.0 (±3.52)	10.0 (±2.16)	4.7 (±2.49)	3.7 (±2.49)	4.7 (±3.52)	5.3	±3.8	7.7	**0.002**
Fecundity (28 days)	14.9 (±1.89)	3.0 (±4.23)	11.6 (±2.59)	8.3 (±2.99)	4.2 (±2.99)	4.7 (±4.23)	6.4	±4.6	9.2	**0.02**
r_m	0.17 (±0.02)	0.064 (±0.05)	0.17 (0.03)	0.08 (±0.03)	0.06 (±0.03)	0.08 (±0.05)	0.11	±0.05	0.10	**0.019**

Results are shown as means.

[a] Average fecundity per female per day on endophyte treatments (excluding WE).

[b] p values were calculated using a one-way analysis of variance. WE = without endophyte; SE = standard endophyte; AR1, AR37, NEA2, NEA6 = commercial endophytes.

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Discussion

Development of a standardised protocol to determine the insecticidal activity of grass–endophyte symbiota against aphid pests

The bioassay successfully determined the insecticidal activity of grass–endophyte symbiota against *D. noxia* and *A. lentisci*, as results showed aphid life history was significantly negatively

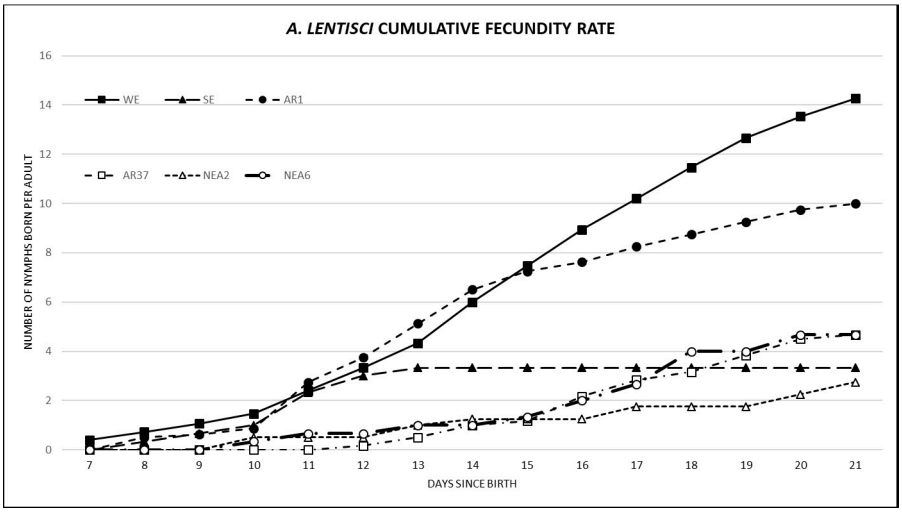

Fig 5. Cumulative fecundity of *Aploneura lentisci* on all endophyte and endophyte-free treatments observed over 7–21 days. Results are shown the average of newborn nymphs per female per day based on aphids that reproduced. Sample sizes are: WE (n = 15), SE (n = 3), AR1 (n = 8), AR37 (n = 6), NEA2 (n = 6) and NEA6 (n = 3). Averages of fecundity based on aphids that reproduced is shown in Table 6. WE = without endophyte; AR1, AR37, NEA2, NEA6 = commercial endophytes; SE = standard endophyte.

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impacted by all endophyte treatments compared to the control (WE). The bioassay was also able to benchmark endophyte treatments, a key requirement for the establishment of a standardised protocol for comparing grass–endophyte symbiota. The bioassay was also simple, rapid, reproducible, scalable and inexpensive. The simple design consisted of small plastic cups arrayed in a tray, with each cup containing a 7-day-old perennial ryegrass seedling fed on by a single aphid. The assessment procedure was also simple, involving regular aphid life history observations (1 every 24 hours) that were conducted with minimal disturbance by removing the cup lid and visually assessing aphid development, reproduction and mortality. The bioassay was rapid, as a duration of 14 and 21 days was optimal to make meaningful assessments of endophyte insecticidal activities on *D. noxia* and *A. lentisci* respectively. The design was highly reproducible, given there were minimal variables (1 symbiota, 1 aphid) and environmental conditions were kept constant to ensure consistent aphid life history. Contamination by common glasshouse pests that can also influence aphid life history, such as thrips, mites or parasitoids, was avoided through the cup-based design. The compact design of the bioassay was highly scalable, predominantly due to the use of seedlings as opposed to whole plants; this offered the advantage of increasing the number of treatments and replicates while keeping space requirements to a minimum. Finally, the design was also inexpensive, as the materials used were low-cost and readily available. A further advantage of this bioassay is its potential to be deployed as part of the Forage Value Index, which aims to compare perennial ryegrass cultivars in Australia and New Zealand based on yield and heading date, since insecticidal activity is not currently evaluated [43–45]. While the bioassay was developed for perennial ryegrass–*Epichloë* symbiota, it could also potentially be applied to grass–endophyte symbiota across all major pasture species grown in Australia and New Zealand (e.g. Annual ryegrass: *Lolium rigidum–Epichloë occultans*, Tall fescue: *Lolium arundinaceum–Epichloë coenophiala*) [23]. Application of the bioassay could also easily be extended to seed-associated microbes other than *Epichloë* spp., which would offer major benefits considering that biological seed treatments are speculated to capture as much as 20% of the global seed treatment market [46,47]. The bioassay could also be extended to many other species of aphids and possibly other root- and foliar-feeding pasture pests, which are the primary cause of yield loss in pasture systems [48]. Finally, the bioassay could be used to investigate the effects of endophytes on the transmission of plant diseases, given the cup-based assay was originally designed to study aphid-transmitted Luteoviruses [37], as well as to test the effects of other insecticidal seed treatments. Studies are already in progress using this cup-based method and a modified version of it with *A. lentisci* and *R. padi* to investigate the effects of loline-producing alkaloids and entomopathogenic bacterial seed treatments of perennial ryegrass for each species respectively. The versatility of this cup-based assay means it can be used in other areas of research such as life history studies as seen in [49] where the life history of five *R. padi* genotypes was tested on four different host plants and two temperatures.

All *D. noxia* and *A. lentisci* used in this study were sourced from the same clonal populations and therefore lacked genetic variation; however, this bioassay can be applied to multiple aphid populations of different genotypes to determine both inter- and intra-specific differences of endophyte effects on life history [17,50]. In contrast to the clonal aphid populations, the perennial ryegrass cultivars consisted of a mixture of genotypes [1], thus adding an extraneous variable to the study. Previous studies have shown that plant genotype is one of the main factors contributing to both endophyte effectiveness and the alkaloid concentration in a plant [18,20,51–54]. It is therefore possible that individual aphids were exposed to different concentrations of alkaloids throughout their life history, as the seedlings that aphids were reared on were replaced every 7 days. However, the high-replicate design of this bioassay (24–48 replicates) mitigated the potential effect of alkaloid variability caused by plant genotype.

Alkaloid presence was validated from the outset of the study using a strain-specific diagnostic KASP assay, which indicated high incidences of endophytes in seed batches. As such, every seedling evaluated in the bioassay was highly likely to contain an endophyte producing the desired alkaloids. It is also worth noting that although this study involved replacing seedlings every 7 days, observations of seedling health indicated that seedlings in this bioassay design could have been used for 14 days, which would further reduce variability in the alkaloid levels that individual aphids are exposed to throughout their life history. Results also suggest that an experimental period of 14 days, rather than 28 days, would be sufficient to gather significant data on aphid mortality, thus completely eliminating alkaloid variability associated with replacing seedlings.

One final point of note of this study design is its varying suitability to different aphid species. *Diuraphis noxia* reared on the WE control exhibited natural mortality rates, with higher mortality at the adult stage (67%) than at the nymphal stage (36%). This indicated that the design–including the plant species and cultivar–was particularly suited to this aphid species. By contrast, *A*. *lentisci* reared on the WE control experienced higher mortality at the nymphal stage (69%) than at the adult stage (31%), indicating that the population's life cycle was negatively impacted by a variable or variables other than endophyte treatment, such as the design, plant or cultivar. It is possible that the cup-based design left *A*. *lentisci* individuals too exposed compared to their natural subterranean habitat, leading to decreased survival at the nymphal stage [30]. Additionally, while every effort was made to transfer aphids gently, the transferral process may still have had a negative effect on aphid health, so eliminating this process may be advantageous. This needs to be tested further. Current research is ongoing into adapting this design to better suit *A*. *lentisci* life history assays.

Insecticidal activities of endophytes: Mortality

Mortality was highest in the nymphal stage (i.e. <7 days) for both species, with very few nymphs reaching the adult stage on all endophyte treatments. The nymphal stage has been reported to be more susceptible to endophytes than the adult stage in other aphid species, such as *R*. *padi* [24]. This finding is of economic importance as early-acting insecticides that are most effective against nymphs will more successfully reduce aphid population growth, due to fewer aphids surviving to reproductive age. The endophyte that showed the strongest effect on nymph mortality in both aphid species was SE, followed by NEA2 (*D*. *noxia*) and NEA6 (*A*. *lentisci*). SE produces ergovaline and peramine, as well as high levels of lolitrem B [55,56]. The point of difference between these endophytes is their lolitrem B production: SE produces high levels of lolitrem B, whereas NEA2 only produces trace amounts and NEA6 produce none [17]. The high mortality in *D*. *noxia* observed on both SE and NEA2 (100%) indicated that aphid mortality occurred regardless of whether lolitrem B was present in high or trace amounts. Lolitrem B is an indole diterpene alkaloid that is most commonly associated with the condition known as ryegrass staggers that affects cattle and sheep when present in high concentrations [57]. The insecticidal mode of action of lolitrem B is not well understood; it is proposed to have either an antibiotic or antixenotic effect [58]. There is evidence of other indole diterpenes (e.g. nodulisporic acids) having insecticidal effects on Diptera [59] and Coleoptera [60], but to date their effect on aphids is unknown. Given that NEA2 produces only trace amounts of lolitrem B and is therefore considered safe for animal consumption, this endophyte may prove an important insecticide against *D*. *noxia*. For *A*. *lentisci*, the most insecticidally active endophytes were SE and NEA6, which both produce peramine and ergovaline [61]. Peramine is known to have feeding deterrent effects on certain insects, most notably the Argentine stem weevil, *Listronotus bonariensis* (Kuschel), but these are less potent against aphids

[62]. Conversely, the effect on ergovaline on aphid populations or life history is not well known [17,29,63]. Peramine has been found to have little to no effect on *A*. *lentisci* from studies with the endophyte AR1 (peramine only) [30]. As such, the insecticidal effect of SE and NEA6 is likely to be derived from ergovaline. Ergovaline is an ergopeptide that is associated with Fescue Foot, a vasoconstrictive condition in grazing livestock that leads to lameness and limb necrosis when ergovaline is present in high concentrations [64]. While the insecticidal activity of ergovaline is known, the mode of action of ergovaline is not well understood [51]. Commercially, NEA6 is regularly sold in conjunction with NEA2 to ensure the pasture contains the insecticidal effects of ergovaline and lolitrem B, but at diluted concentrations to limit any effect on livestock [61]. SE may also act as a viable commercial endophyte under high aphid pressure or when combined with an endophyte that produces no lolitrem B.

Insecticidal activities of endophytes: Fecundity

Fecundity was reduced by all endophyte treatments. The endophytes that caused the greatest reduction in *A*. *lentisci* fecundity were SE, NEA2 and NEA6, whereas the endophyte that caused the greatest reduction in *D*. *noxia* fecundity was AR1. In *D*. *noxia*, no fecundity was observed on SE and NEA2 as no aphids survived to reproductive age. The common alkaloids associated with these endophyte treatments are lolitrem B, ergovaline and peramine. Other studies have also observed the negative effect of endophytes on aphid fecundity, predominantly endophytes producing lolitrem B and peramine [40, 46]: Meister et al. [28] saw a significant decrease in fecundity of *Rhopalosiphum padi* on perennial ryegrass infected with peramine and lolitrem B-producing endophytes.

Given the high mortality and the low numbers of aphids that reached reproductive maturity, it is likely that the effect of endophytes on fecundity was due to adults experiencing lower general health. The adult and nymph embryo may suffer from malnutrition caused by antixenosis, or the nymph may experience antibiotic effects during gestation within the adult. Our data has already shown that endophytes had a strong effect on nymph mortality; however, it is also worth noting that stillborn aphids were observed on occasion throughout the bioassay–a likely indicator of antibiotic effects during gestation. This is supported by Clement et al. [52], who found *D*. *noxia* population densities were significantly decreased on four accessions of wild barley infected with endophytic fungi, which they determined was the result of either antibiosis or starvation due to antixenosis. Similarly, when studying *A*. *lentisci*, Popay & Thom [65] and Popay & Hume [66] found that aphid infestation and population size were consistently lowest on perennial ryegrass infected with certain strains of *Epichloë*. However, in both of these studies it is unclear whether negative effects on adults or nymphs were responsible for these changes in population.

Translation to field performance

Results of this study indicated that endophyte-free perennial ryegrass is susceptible to infestation by *D*. *noxia* and *A*. *lentisci*, highlighting the importance of utilising endophytes in pasture systems under aphid pressure. This study also demonstrated that endophytes have a strong but varying effect on the survival and fecundity of aphids, and this effect is dependent on both endophyte strain and aphid species–a conclusion similar to that of Clement et al. [26,52,67] and Meister et al. [28]. This suggests that a tailored approach to endophyte selection would be required to achieve targeted aphid control, whereas the use of multiple endophytes producing a wide range of alkaloid profiles would be required for effective broad-spectrum control of multiple aphid species. Of the endophytes tested, SE was consistently the most effective at controlling both *A*. *lentisci* and *D*. *noxia*, however, it is unsafe for animal consumption and

therefore has limited options for use in pasture production. Nevertheless, it could have commercial potential provided that strategies are used to mitigate lolitrem B toxicity in grazing livestock; for example, through the use of mycotoxin binding agents [68], or by combining SE with other endophytes that produce no lolitrem B (e.g. NEA6). SE is the dominant endophyte profile in unmanaged pasture systems, which suggests it has a clear ecological advantage over other endophyte types. If the risk of ryegrass staggers due to lolitrem B toxicity is too great, a combination of NEA2 and NEA6 would provide adequate broad spectrum control of aphids in pasture systems.

Conclusions

This study has demonstrated that both *D. noxia* and *A. lentisci* are susceptible to endophytes producing the alkaloids lolitrem B, ergovaline and peramine, and these endophytes have the potential to be used in sustainable pasture management systems to increase production and decrease the use of insecticide sprays. The bioassay we designed can be used as a simple, rapid, reproducible, scalable and inexpensive method of assessing the insecticidal activity of novel endophyte symbiota, and can therefore be used to benchmark commercial and pre-commercial symbiota for use in perennial ryegrass pasture systems. While it may not, in its current form, be entirely suitable for assessment of root-feeding aphids, it is suitable for use with foliar-feeding species and as such, it may significantly accelerate the developmental pipeline from endophyte discovery to endophyte utilisation in dairy farming systems.

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References

1. Wilkins PW. Breeding perennial ryegrass for agriculture. Euphytica. 1991; 52: 201–214. https://doi.org/10.1007/BF00029397
2. Dairy Australia. Perennial ryegrass management. 2019 [cited 1 Apr 2019]. Available: https://www.dairyaustralia.com.au/farm/feedbase-and-animal-nutrition/pasture/perennial-ryegrass-management
3. Plumb RT. The effects of pests and diseases on grasses. In: Jones MB, Lazenby A, editors. The Grass Crop: The Physiological basis of production. Dordrecht: Springer Netherlands; 1988. pp. 277–309. https://doi.org/10.1007/978-94-009-1187-1_8
4. Cunningham PJ, Blumenthal MJ, Anderson MW, Prakash KS, Leonforte A. Perennial ryegrass improvement in Australia. N Z J Agric Res. 1994; 37: 295–310. https://doi.org/10.1080/00288233.1994.9513068
5. Curry JP. The composition of the invertebrate fauna. Grassland Invertebrates: Ecology, Influence on Soil Fertility and Effects on Plant Growth. Department of Environmental Resource Management, University College Dublin, Ireland: Chapman & Hall, London; 1994. p. 33.
6. Curry JP. Effects of herbivores on grassland productivity. Grassland Invertebrates: Ecology, Influence on Soil Fertility and Effects on Plant Growth. Department of Environmental Resource Management, University College Dublin, Ireland: Chapman & Hall, London; 1993. p. 303.
7. Ferguson CM, Barratt BIP, Bell N, Goldson SL, Hardwick S, Jackson M, et al. Quantifying the economic cost of invertebrate pests to New Zealand's pastoral industry. N Z J Agric Res. 2019; 62: 255–315. https://doi.org/10.1080/00288233.2018.1478860
8. Van Emden HF, Harrington R, editors. Aphids as crop pests. Wallingford: CABI; 2007.
9. Curry JP. Hemiptera. Grassland Invertebrates: Ecology, influence on soil fertility and effects on plant growth. Department of Environmental Resource Management, University College Dublin, Ireland: Chapman & Hall, London; 1994. p. 95.
10. Jones RAC. Virus diseases of pasture grasses in Australia: incidences, losses, epidemiology, and management. Crop Pasture Sci. 2013; 64: 216. https://doi.org/10.1071/CP13134
11. Mustafa TM, Akkawi M. The occurrence, economic importance and control of wheat root aphid (Aploneura lentisci Passerini, Homoptera, Aphididae) on wheat in Jordan. Dirasat Jordan. 1987; 14: 83–88.
12. Edwards OR, Franzmann B, Thackray D, Micic S. Insecticide resistance and implications for future aphid management in Australian grains and pastures: a review. Aust J Exp Agric. 2008; 48: 1523. https://doi.org/10.1071/EA07426
13. Devine GJ, Furlong MJ. Insecticide use: Contexts and ecological consequences. Agric Hum Values. 2007; 24: 281–306. https://doi.org/10.1007/s10460-007-9067-z
14. Siegel MR, Latch GCM, Johnson MC. Fungal Endophytes of Grasses. Annu Rev Phytopathol. 1987; 25: 293–315. https://doi.org/10.1146/annurev.py.25.090187.001453
15. Hesse U, Schöberlein W, Wittenmayer L, Förster K, Warnstorff K, Diepenbrock W, et al. Effects of Neotyphodium endophytes on growth, reproduction and drought-stress tolerance of three Lolium perenne L. genotypes. Grass Forage Sci. 2003; 58: 407–415. https://doi.org/10.1111/j.1365-2494.2003.00393.x
16. Hoveland CS. Importance and economic significance of the Acremonium endophytes to performance of animals and grass plant. Agric Ecosyst Environ. 1993; 44: 3–12. https://doi.org/10.1016/0167-8809(93)90036-O
17. Bush LP, Wilkinson HH, Schardl CL. Bioprotective Alkaloids of Grass-Fungal Endophyte Symbioses. Plant Physiol. 1997; 114: 1–7. https://doi.org/10.1104/pp.114.1.1 PMID: 12223685
18. Reed KFM, Walsh JR, Cross PA, McFarlane NM, Sprague MA. Ryegrass endophyte (Neotyphodium lolii) alkaloids and mineral concentrations in perennial ryegrass (Lolium perenne) from southwest Victorian pasture. Aust J Exp Agric. 2004; 44: 1185. https://doi.org/10.1071/EA03242
19. Wilkinson HH, Siegel MR, Blankenship JD, Mallory AC, Bush LP, Schardl CL. Contribution of Fungal Loline Alkaloids to Protection from Aphids in a Grass-Endophyte Mutualism. Mol Plant Microbe Interact. 2000; 13: 1027–1033. https://doi.org/10.1094/MPMI.2000.13.10.1027 PMID: 11043464

20. Faeth SH, Bush LP, Sullivan TJ. Peramine Alkaloid Variation in Neotyphodium-Infected Arizona Fescue: Effects of Endophyte and Host Genotype and Environment. J Chem Ecol. 2002; 28: 1511–1526. https://doi.org/10.1023/a:1019916227153 PMID: 12371807

21. Hettiarachchige IK, Elkins AC, Reddy P, Mann RC, Guthridge KM, Sawbridge TI, et al. Genetic modification of asexual Epichloë endophytes with the perA gene for peramine biosynthesis. Mol Genet Genomics. 2019; 294: 315–328. https://doi.org/10.1007/s00438-018-1510-x PMID: 30443676

22. Karpyn Esqueda M, Yen AL, Rochfort S, Guthridge KM, Powell KS, Edwards J, et al. A Review of Perennial Ryegrass Endophytes and Their Potential Use in the Management of African Black Beetle in Perennial Grazing Systems in Australia. Front Plant Sci. 2017;8. https://doi.org/10.3389/fpls.2017.00008

23. Kauppinen M, Saikkonen K, Helander M, Pirttilä AM, Wäli PR. Epichloë grass endophytes in sustainable agriculture. Nat Plants. 2016; 2: 15224. https://doi.org/10.1038/nplants.2015.224 PMID: 27249195

24. Bastias DA, Ueno AC, Machado Assefh CR, Alvarez AE, Young CA, Gundel PE. Metabolism or behavior: explaining the performance of aphids on alkaloid-producing fungal endophytes in annual ryegrass (Lolium multiflorum). Oecologia. 2017; 185: 245–256. https://doi.org/10.1007/s00442-017-3940-2 PMID: 28879573

25. Breen JP. Enhanced Resistance to Three Species of Aphids (Homoptera: Aphididae) in Acremonium Endophyte-Infected Turfgrasses. J Econ Entomol. 1993; 86: 1279–1286. https://doi.org/10.1093/jee/86.4.1279

26. Clement SL, Lester DG, Wilson AD, Pike KS. Behavior and Performance of Diuraphis noxia (Homoptera: Aphididae) on Fungal Endophyte-Infected and Uninfected Perennial Ryegrass. J Econ Entomol. 1992; 85: 583–588. https://doi.org/10.1093/jee/85.2.583

27. Johnson MC, Dahlman DL, Siegel MR, Bush LP, Latch GCM, Potter DA, et al. Insect Feeding Deterrents in Endophyte-Infected Tall Fescue. Appl Environ Microbiol. 1985; 49: 568–571. PMID: 16346751

28. Meister B, Krauss J, Härri SA, Victoria Schneider M, Müller CB. Fungal endosymbionts affect aphid population size by reduction of adult life span and fecundity. Basic Appl Ecol. 2006; 7: 244–252. https://doi.org/10.1016/j.baae.2005.06.002

29. Siegel MR, Latch GCM, Bush LP, Fannin FF, Rowan DD, Tapper BA, et al. Fungal endophyte-infected grasses: Alkaloid accumulation and aphid response. J Chem Ecol. 1990; 16: 3301–3315. https://doi.org/10.1007/BF00982100 PMID: 24263431

30. Popay AJ, Cox NR. Aploneura lentisci (Homoptera: Aphididae) and Its Interactions with Fungal Endophytes in Perennial Ryegrass (Lolium perenne). Front Plant Sci. 2016; 7. https://doi.org/10.3389/fpls.2016.01395 PMID: 27695470

31. Holland PT, Boyd AJ, Malcom CP. Performance validation of a multi-residue method for 170 pesticides in kiwifruit. In: Fajgelj A, Ambrus A, editors. Special Publications. Cambridge: Royal Society of Chemistry; 2000. pp. 29–40. https://doi.org/10.1039/9781847551757-00029

32. Food and Agriculture Organization of the United Nations, World Health Organization, Joint Meeting on Pesticide Specifications. Manual on development and use of FAO and WHO specifications for pesticides. 2016.

33. Yazdani M, Baker G, DeGraaf H, Henry K, Hill K, Kimber B, et al. First detection of Russian wheat aphid Diuraphis noxia Kurdjumov (Hemiptera: Aphididae) in Australia: a major threat to cereal production. Austral Entomol. 2018; 57: 410–417. https://doi.org/10.1111/aen.12292

34. Hughes RD, Maywald GF. Forecasting the favourableness of the Australian environment for the Russian wheat aphid, Diuraphis noxia (Homoptera: Aphididae), and its potential impact on Australian wheat yields. Bull Entomol Res. 1990; 80: 165. https://doi.org/10.1017/S0007485300013389

35. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. 1994; 3: 294–299.

36. Walsh P, Metzger D, Higuchi R. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. BioTechniques. 1991; 10: 506–513. PMID: 1867860

37. Ridland PM, Sward RJ, Tomkins RB. A simple rearing system for cereal aphids, especially suited to transmission studies with barley yellow dwarf viruses. 1988; 17: 3.

38. Clay K, Hardy TN, Hammond AM. Fungal Endophytes of Grasses and Their Effects on an Insect Herbivore. Oecologia. 1985; 66: 1–5. https://doi.org/10.1007/BF00378545 PMID: 28310805

39. Graves H, Rayburn AL, Gonzalez-Hernandez JL, Nah G, Kim D-S, Lee DK. Validating DNA Polymorphisms Using KASP Assay in Prairie Cordgrass (Spartina pectinata Link) Populations in the U.S. Front Plant Sci. 2016;6. https://doi.org/10.3389/fpls.2016.00006

40. Kindler SD, Springer TL. Alternate Hosts of Russian Wheat Aphid (Homoptera: Aphididae). J Econ Entomol. 1989; 82: 1358–1362. https://doi.org/10.1093/jee/82.5.1358

41. Wyatt IJ, White PF. Simple Estimation of Intrinsic Increase Rates for Aphids and Tetranychid Mites. J Appl Ecol. 1977; 14: 757–766. https://doi.org/10.2307/2402807

42. VSN International. Genstat for Windows 19th Edition. Hemel Hamstead, UK: VSN International; 2015. Available: genstat.co.uk

43. Chapman DF, Bryant JR, Olayemi ME, Edwards GR, Thorrold BS, McMillan WH, et al. An economically based evaluation index for perennial and short-term ryegrasses in New Zealand dairy farm systems. Grass Forage Sci. 2017; 72: 1–21. https://doi.org/10.1111/gfs.12213

44. Leddin CM, Jacobs JL, Smith KF, Giri K, Malcolm B, Ho CKM. Development of a system to rank perennial ryegrass cultivars according to their economic value to dairy farm businesses in south-eastern Australia. Anim Prod Sci. 2018; 58: 1552. https://doi.org/10.1071/AN17815

45. Chapman D, Bryant J, McMillan W, Thorrold B, Edwards G, Willocks M, et al. An economic forage value index for New Zealand dairy farmers. Australas Dairy Sci Symp 2012 Proc 5th Australas Dairy Sci Symp Melb Aust 13–15 Novemb 2012. 2012; 422–423.

46. New Ag International. Biocontrol in Asia: Gaining Momentum! Seed treatments with biologicals: a new and promising frontier. 2015 [cited 25 Jun 2019]. Available: http://dunhamtrimmer.com/wp-content/uploads/2012/07/Biocontrol-in-Asia-Gaining-Momentum.pdf

47. O'Callaghan M. Microbial inoculation of seed for improved crop performance: issues and opportunities. Appl Microbiol Biotechnol. 2016; 100: 5729–5746. https://doi.org/10.1007/s00253-016-7590-9 PMID: 27188775

48. Bailey PT. Pests of Field Crops and Pastures: Identification and Control. CSIRO Publishing; 2007.

49. Valenzuela I, Ridland PM, Weeks AR, Hoffmann AA. Patterns of Genetic Variation and Host Adaptation in an Invasive Population of Rhopalosiphum padi (Hemiptera: Aphididae). Ann Entomol Soc Am. 2010; 103: 886–897. https://doi.org/10.1603/AN09113

50. Shufran KA, Puterka GJ. DNA Barcoding to Identify All Life Stages of Holocyclic Cereal Aphids (Hemiptera: Aphididae) on Wheat and Other Poaceae. Ann Entomol Soc Am. 2011; 104: 39–42. https://doi.org/10.1603/AN10129

51. Roylance JT, Hill NS, Agee CS. Ergovaline and peramine production in endophyte-infected tall fescue: Independent regulation and effects of plant and endophyte genotype. J Chem Ecol. 1994; 20: 2171–2183. https://doi.org/10.1007/BF02033195 PMID: 24242799

52. Clement SL, Wilson AD, Lester DG, Davitt CM. Fungal endophytes of wild barley and their effects on Diuraphis noxia population development. Entomol Exp Appl. 1997; 82: 275–281. https://doi.org/10.1046/j.1570-7458.1997.00141.x

53. Spiering MJ, Lane GA, Christensen MJ, Schmid J. Distribution of the fungal endophyte Neotyphodium lolii is not a major determinant of the distribution of fungal alkaloids in Lolium perenne plants. Phytochemistry. 2005; 66: 195–202. https://doi.org/10.1016/j.phytochem.2004.11.021 PMID: 15652576

54. Ryan GD, Rasmussen S, Parsons AJ, Newman JA. The effects of carbohydrate supply and host genetic background on Epichloë endophyte and alkaloid concentrations in perennial ryegrass. Fungal Ecol. 2015; 18: 115–125. https://doi.org/10.1016/j.funeco.2015.07.006

55. Gallagher RT, White EP, Mortimer PH. Ryegrass Staggers: Isolation of Potent Neurotoxins Lolitrem a and Lolitrem B from Staggers-Producing Pastures. N Z Vet J. 1981; 29: 189–190. https://doi.org/10.1080/00480169.1981.34843 PMID: 6950333

56. van Zijll de Jong E, Dobrowolski MP, Sandford A, Smith KF, Willocks MJ, Spangenberg GC, et al. Detection and characterisation of novel fungal endophyte genotypic variation in cultivars of perennial ryegrass (Lolium perenne L.). Aust J Agric Res. 2008; 59: 214. https://doi.org/10.1071/AR07270

57. Fletcher LR, Harvey IC. An Association of a Lolium Endophyte with Ryegrass Staggers. N Z Vet J. 1981; 29: 185–186. https://doi.org/10.1080/00480169.1981.34839 PMID: 6950332

58. Breen JP. Acremonium Endophyte Interactions with Enhanced Plant Resistance to Insects. Annu Rev Entomol. 1994; 39: 401–423. https://doi.org/10.1146/annurev.en.39.010194.002153

59. Ostlind DA, Felcetto T, Misura A, Ondeyka J, Smith S, Goetz M, et al. Discovery of a novel indole diterpene insecticide using first instars of Lucilia sericata. Med Vet Entomol. 1997; 11: 407–408. https://doi.org/10.1111/j.1365-2915.1997.tb00431.x PMID: 9430124

60. Gaynor DL, Rowan DD. Insect resistance, animal toxicity and endophyte-infected grass. Proc N Z Grassl Assoc. 1986; 47: 115–120.

61. Tian P, Le TN, Ludlow EJ, Smith KF, Forster JW, Guthridge KM, et al. Characterisation of novel perennial ryegrass host–Neotyphodium endophyte associations. Crop Pasture Sci. 2013; 64: 716. https://doi.org/10.1071/CP13067

62. Rowan DD, Latch GCM. Utilization of Endophyte-Infected Perennial Ryegrass for Increased Insect Resistance. In: Bacon CW, White JF, editors. Biotechnology of Endophytic Fungi of Grasses. 1994. pp. 169–183.

63. Hunt MG, Newman JA. Reduced herbivore resistance from a novel grass-endophyte association: Herbivore resistance of novel endophytes. J Appl Ecol. 2005; 42: 762–769. https://doi.org/10.1111/j.1365-2664.2005.01061.x

64. Klotz JL, Bush LP, Smith DL, Shafer WD, Smith LL, Arrington BC, et al. Ergovaline-induced vasoconstriction in an isolated bovine lateral saphenous vein bioassay. J Anim Sci. 2007; 85: 2330–2336. https://doi.org/10.2527/jas.2006-803 PMID: 17504952

65. Popay AJ, Thom ER. Endophyte effects on major insect pests in Waikato dairy pasture. Proc N Z Grassl Assoc. 2009; 71: 121–126.

66. Popay AJ, Hume DE. Endophytes improve ryegrass persistence by controlling insects. Pasture Persistance—Grassl Res Pract Ser. 2011; 15: 149–156.

67. Clement SL, Elberson LR, Bosque-Perez NA, Schotzko DJ. Detrimental and neutral effects of wild barley-Neotyphodium fungal endophyte associations on insect survival. Entomol Exp Appl. 2005; 114: 119–125. https://doi.org/10.1111/j.1570-7458.2005.00236.x

68. Mann SP, Parfitt D. Mycotoxin-reducing composition. US20110150853A1, 2011. Available: https://patents.google.com/patent/US20110150853A1/en

CHAPTER 3

Effects of fungal endophyte alkaloids on the life history of *Metopolophium dirhodum* (Walker) on perennial ryegrass

3.1: Chapter Preface

Epichloë festucae var. *lolii* produces a range of bioactive alkaloid compounds with various detrimental effects on vertebrates and invertebrates. The presence and concentration of these alkaloids can be quantified in endophyte-infected plant tissue using Liquid Chromatography–Mass Spectrometry (LC–MS) analysis. Perennial ryegrass seedlings containing the endophytes SE, AR1 AR37, NEA2 or NEA6, and a control without endophyte (WE) were analysed using this method to determine the alkaloid profiles (presence and concentration) of seedlings that were fed on by the aphid *Metopolophium dirhodum*. These profiles were compared to aphid life history results determined using the same high-throughput bioassay method detailed in Chapter 2, to correlate alkaloid profiles with aphid mortality. Seedlings that were not fed on by *M. dirhodum* were also analysed using LC-MS to determine the effect of aphid feeding on the production of alkaloids by different endophytes. *Metopolophium dirhodum* mortality was significantly increased when feeding on the endophytes SE and NEA2, compared to the control. The alkaloid profiling experiments confirmed that endophytes producing lolitrem B in high levels and any level of ergovaline resulted in the highest and most rapid nymphal mortality. Furthermore, the production of alkaloids increased when the host plant had been fed on by an aphid, particularly for lolitrem B by NEA2. This result demonstrates the tri-trophic interaction between *Epichloë*, perennial ryegrass and aphids.

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Title: Effects of fungal endophyte alkaloids on the life history of *Metopolophium dirhodum* (Walker) on perennial ryegrass

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3.3: Statement of contribution of joint authorship

NPC performed all work relating to maintaining colonies of *M. dirhodum*, and conducting bioassay experiments. NPC and SV prepared all perennial ryegrass samples for alkaloid profiling analysis. SV performed alkaloid profiling using LC-MS instrumentation. NPC and SV performed all analysis of alkaloid profiling data. NPC generated all figures and drafted the majority of the manuscript. NPC and KG conducted all statistical analysis of bioassay and alkaloid profiling data. KG assisted in drafting the statistical sections of the manuscript. RM, GS, IV, MM, SV and NPC all conceptualised the project and assisted in drafting the manuscript.

3.4 Statement from the co-author confirming the authorship contribution of the PhD candidate

“As co-author of the manuscript ‘Collinson, N.P., Valenzuela, I., Giri, K., Malipatil, M., Vassiliadis, S., Spangenberg, G., Mann, R.C., 2020. Effects of fungal endophyte alkaloids on the life history of *Metopolophium dirhodum* (Walker) on perennial ryegrass’ I confirm that Nicholas Collinson made the following contributions,

- Survey, collection, taxonomical verification and rearing of colonies of aphid species (*M. dirhodum*)
- Conducted aphid bioassays
- Conducted metabolite extractions
- Bioassay and alkaloid data analysis

- Generated all figures
- Writing the manuscript and critical appraisal of content”

Associate Professor Mallik Malipatil

Date: 28/06/2020

Effects of fungal endophyte alkaloids on the mortality and longevity of *Metopolophium dirhodum* (Walker) on perennial ryegrass

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Endophyte alkaloids control pasture grass aphids

Abstract

The fungal endophyte *Epichloë festucae* var. *lolii* produces a range of alkaloids, including lolitrem B, ergovaline, peramine and epoxy-janthitrem I, with each strain producing differing combinations and concentrations in the pasture grass, perennial ryegrass (*Lolium perenne*). These alkaloids contribute to pasture grass tolerance against insect pests, including the rose grain aphid, *Metopolophium dirhodum*. Perennial ryegrass is the most widely used forage grass in the Australian dairy industry, therefore a clearer understanding of how alkaloids protect against insects is of great importance. The aim of this study was to profile the alkaloid diversity and concentration of five commercial endophyte strains (SE, AR1, AR37, NEA2 and NEA6) found in perennial ryegrass and to investigate their effect on *M. dirhodum* mortality through the use of a high-throughput *in-planta* bioassay. Seedlings containing the endophyte SE produced lolitrem B (3.19–26.34 ppm), ergovaline (0.35–19.60 ppm) and peramine (8.87–57.97 ppm); AR1 produced peramine (8.45–68.60 ppm); NEA2 produced lolitrem B (1.08–4.40 ppm),

ergovaline (0.05–0.58 ppm) and peramine (5.80–16.09 ppm); and NEA6 produced ergovaline (3.17–138.49 ppm) and peramine (6.53–68.13 ppm). AR37 produced epoxy-janthitrem I, however, due to lack of a chemical standard, its abundance was measured as a response of the peak area (9678-56895051, arbitrary units). Lolitrem B was associated with the highest mortality (80% chance of nymph death in *M. dirhodum* reared on plants producing lolitrem B versus 48% when reared on plants that did not produce lolitrem B), followed by ergovaline (76% versus 44%). Aphid longevity decreased as levels of lolitrem B increased but was not affected by changes in the levels of ergovaline. Neither peramine nor epoxy-janthitrem I had a significant effect on aphid mortality or longevity. The endophyte treatment with the strongest negative effect on *M. dirhodum* mortality was SE, followed by NEA2 (both producers of lolitrem B and ergovaline), with nymphal mortality rates of 98%, and 83% respectively. A trend indicated that alkaloid concentrations were higher in perennial ryegrass fed on by *M. dirhodum*, suggesting the existence of a tri-trophic interaction where aphid feeding on the plant triggers a defensive response from the endophyte to produce more alkaloids. The commercial implications of these results are that endophytes producing both ergovaline and higher concentrations of lolitrem B in pastures would allow the most effective control of *M. dirhodum*, providing strategies were in place to mitigate toxicity towards livestock.

Key Words

Endophytes; Aphids; Perennial ryegrass; LC-MS; Alkaloids; Pasture grass; Rose-grain aphid; *Metopolophium dirhodum*; dairy; integrated pest management

INTRODUCTION

Several wild and cultivated grass species have evolved symbiotic, mutualistic relationships with fungal endophytes that provide the grasses with resistance to multiple biotic and abiotic stresses (Kauppinen et al., 2016; Shymanovich et al., 2015). Perennial ryegrass, *Lolium perenne* L., is commonly infected with the endophyte *Epichloë festucae* var. *lolii*, (Latch, M.J. Christensen & Samuels) C.W. Bacon & Schardl, which provides it with enhanced fitness through increased drought tolerance and insect tolerance (Clement et al., 1990; Hesse et al., 2003; Hume & Sewell, 2014). Perennial ryegrass is one of the most widely sown pasture grass species in dairy production worldwide, favoured over other grasses because of its persistence in fields for at least four years, its high nutritional value for livestock and its resistance to certain insect pests (Wilkins, 1991). While there are clear benefits, endophyte-infected perennial ryegrass can also result in health conditions such as ryegrass staggers in grazing livestock, caused by the production of the alkaloid lolitrem B by the endophyte (Tor-Agbidye et al., 2001). As such, the mutualistic relationship between perennial ryegrass and *E. festucae* var. *lolii* has been extensively studied for close to 40 years (Moate et al., 2012; Popay & Thom, 2009; Shymanovich et al., 2015) to maximise the biocidal effects on invertebrate pests while minimising toxic effects on livestock (Breen, 1993; Clement et al., 1990; Johnson et al., 1985).

One of the most well-studied traits of grass-endophyte symbiota is their production of bioactive alkaloids, which is the key cause of both endophyte-induced resistance to invertebrate pests, and toxic effects in livestock (Kauppinen et al., 2016). The alkaloids produced by *E. festucae* var. *lolii* are grouped into four classes: indole-diterpenes (lolitrem B, epoxy-janthitrems), ergopeptides (ergovaline, ergotamine) and polyketides (peramine) (Schardl, 2001). Alkaloids from all of these classes are present in various concentrations and combinations in different commercial endophytes currently available to farmers (Barenbrug Agriseeds, 2019). Some endophytes – such as NEA2 (peramine, ergovaline and lolitrem B) and

NEA6 (peramine and ergovaline) – produce multiple alkaloids, whereas others – such as AR1 (peramine) and AR37 (epoxy-janthitrem I) – are generally stated to produce only one alkaloid (Barenbrug Agriseeds, 2019; Shymanovich et al., 2015). Indole-diterpenes, most notably lolitrem B, are known for varying levels of toxicity to grazing livestock such as cattle, as lolitrem B is the tremorgenic causative agent of the condition ryegrass staggers, while some indole-diterpenes (e.g. nodulisporic acids) also have some level of insecticidal activity (Fuchs et al., 2013; Gallagher et al., 1981; Ondeyka et al., 1997). Furthermore, ergovaline is a vasoconstrictor that, when present in high concentrations in tall fescue, is the causative agent of a condition known as fescue foot in grazing livestock (Tor-Agbidye et al., 2001). It can cause heat stress effects when present in lower concentrations in other grasses (Klotz et al., 2007; Tor-Agbidye et al., 2001), and has also been shown to have broad ranging insecticidal properties (Potter et al., 2008; Shymanovich et al., 2015). Peramine is not known to cause health conditions for livestock and has been shown to have feeding deterrence effects on certain invertebrates, most notably the Argentine stem weevil, *Listronotus bonariensis* (Kuschel) (Popay & Wyatt, 1995). Loline alkaloids (produced by *Epichloë coenophialium* and *Epichloë uncinata*) have also been shown to have broad ranging insecticidal activity, and minimal or no toxicity to vertebrates, including livestock (Wilkinson et al., 2000). The toxicity of endophyte-infected perennial ryegrass can vary based on alkaloid concentration (Guerre, 2015). Alkaloids such as lolitrem B or ergovaline can be present in concentrations that are toxic to invertebrates, but not harmful to vertebrates (Fuchs et al., 2013; Hovermale & Craig, 2001; Potter et al., 2008).

Alkaloid production by endophytes can be influenced by several factors including plant genotype (Faeth et al., 2002), endophyte strain (Latch, 1993), and plant damage from grazing (Bultman et al., 2018). Feeding by aphids and other insects can induce the production of plant volatiles and hormones such as salicylic or jasmonic acids in grasses (Girling et al., 2008), which can protect the plant by attracting natural enemies of insect herbivores, and also influence

alkaloid production by fungal endophytes including *Epichloë* (Girling et al., 2008; Saikkonen et al., 2013). Though there is little research into the influence of aphid feeding on alkaloid production, current data suggests that it may play a part in the pathways relating to alkaloid production in grasses (Moore et al., 2015; Zhang et al., 2011).

Endophyte-free perennial ryegrass is susceptible to feeding by a range of insect pests, including foliar feeding aphids such as the rose grain aphid, *Metopolophium dirhodum* (Walker), which is a common pest on several poaceae species in temperate dairy pastures around the world (Guy, 1988; Meister et al., 2006). *Metopolophium dirhodum* is commonly detected on perennial ryegrass, where it can cause damage through direct feeding and by vectoring plant luteoviruses such as barley yellow dwarf virus (BYDV) (Guy, 1988). It has been observed to develop on perennial ryegrass as proficiently as it does on cereal grasses such as barley (Dean, 1974). It was first detected in Australia by Carver (1984) on barley in New South Wales and has since spread to other Australian states including Victoria, where it is regularly trapped in surveillance programs (Valenzuela, personal communication). Control measures range from foliar applications of broad-spectrum insecticides (Stribley et al., 1983) to biological control using parasitoid wasps (Schmidt et al., 2003). The use of fungal endophytes in pasture grasses to control aphid pests has shown potential (Clement et al., 2005) and other studies have shown that *M. dirhodum* is susceptible to the insecticidal effects of endophyte-produced alkaloids (Corcuera, 1984; Meister et al., 2006).

The current study aimed to characterise, for the first time in Australia, the effects of five grass–endophyte symbiota (four commercial and one standard/wild type) and their associated alkaloid profiles on the mortality and longevity of *M. dirhodum.* The approach involved using a high-throughput *in planta* bioassay to determine mortality effects while simultaneously quantifying alkaloid concentrations using a liquid chromatography-mass spectrometry (LC-

MS) technique. The effect of aphid feeding on the production of alkaloids by *E. festucae* var. *lolii* was also investigated.

MATERIALS AND METHODS

Grass–endophyte symbiota

Both endophyte infected and endophyte free perennial ryegrass (cv. Alto) seed batches were sourced from Barenbrug Agriseeds (Christchurch, New Zealand). Endophytes included a wild-type or standard endophyte (SE) and the commercial endophytes AR1, AR37, NEA2 and NEA6. Perennial ryegrass without an endophyte (WE) was used as a control, and barley (cv. Hindmarsh) was used as a further control in the mortality bioassays, as it is a preferred host of *M. dirhodum* (Dean, 1974; Farrell & Stufkens, 1988), and was sourced from Seednet (Horsham, Victoria). All seeds were germinated on petri dishes with filter paper moistened with tap water and allowed to grow for seven days at room temperature.

To maintain aphid colonies, seedlings of perennial ryegrass (Alto-WE) and barley (Hindmarsh) that had been germinated and then grown for seven days, were transferred to plastic pots containing potting mix (Bio Gro, Mount Gambier, Australia), supplemented with vermiculite, perlite, Macrocote slow-release fertiliser, nitrogen slow-release fertiliser, water-holding granules, trace elements and garden lime. Seedlings were left to grow for four–six weeks before adding 20 aphids (approximately) to each plant (Collinson et al. 2020).

For mortality bioassays and alkaloid profiling seven-day-old seedlings were placed in small plastic cups. Seedlings were replaced in the bioassay every seven days, up to 28 days as per methods described in Collinson et al., (2020).

To confirm endophyte presence and strain identity in seed batches, a Kompetitive Allele Specific PCR (KASP) assay was performed on 100 seven-day-old seedlings. KASP assays and

analyses were carried out as per methods described in Graves et al. (2016), with modification as per methods described in Collinson et al. (2020).

Alkaloid profiling

The alkaloid classes produced by the endophytes used in this study consist of indole diterpenes (lolitrem B and epoxy-janthitrem I), ergopeptides (ergovaline) and polyketides (peramine). Each endophyte strain had a unique alkaloid profile (Table 1).

The alkaloid concentration was profiled for 16 perennial ryegrass seedlings (cv. Alto) from each endophyte treatment (SE, AR1, AR37, NEA2, NEA6) and the control (96 seedlings in total), with seedling dry weight ranging from 2 – 6 mg. Eight of these seedlings were fed on by *M. dirhodum* as part of the mortality bioassay and a further eight seedlings were grown without the presence of aphids, all under controlled conditions at 20±2°C and 62.0±5%RH, and a photoperiod of 14 hrs light-10 hrs dark. After seven days each seedling was removed in its entirety (including the root system) snap frozen in liquid nitrogen and stored at –80°C (Ekanayake et al., 2017). Alkaloid profiling of all endophyte-infected perennial ryegrass seedlings were carried out as per methods described in Vassiliadis et al., (2019) with the following modifications: the frozen plant material was freeze-dried for approximately 48 hours, weighed (~1 mg), ground in a TissueLyser Mixer Mill at 30 hz for 5 min (Qiagen, mixer 400, Germany) and extracted twice with 500 µl of methanol:water (80:20, v:v). The supernatants were dried under nitrogen gas and reconstituted in 100 µl methanol:water (80:20, v:v) containing ergotamine D-tartrate as an internal standard.

Quality control perennial ryegrass samples with standard toxic endophyte (SE) were used to compare the presence or absence of targeted compounds as per methods described in Vassiliadis et al. (2019). All LC-MS parameters and the use of chemical standards were

employed as per Vassiliadis et al. (2019). All other standards used were prepared and analysed according to Vassiliadis et al. (2019).

Due to instability of the epoxy-janthitrem I, a chemical standard was not available for the accurate quantitation of the compound at the time of this study. However, previous work has reported the relative quantitation of epoxy-janthitrems I by utilising the peak area and confirming the compound to mass fragmentation patterns via LS-MS (Ludlow et al., 2019). The results were reported as relative quantitation expressed as a peak area in arbitrary units. Absolute quantitation, reported as concentration in parts per million (ppm), is presented for lolitrem B, ergovaline and peramine.

Aphids

Metopolophium dirhodum individuals were isolated from wild grass in Yea, Victoria (Table 2) and reared on barley seedlings for three generations. Nymphs were removed and transferred to new seedlings immediately after birth to eliminate any possible transmission of aphid-borne plant viruses to our experiments (Ajayi & Dewar, 1983; Ridland et al., 1988). One aphid clone was selected to start each of the colonies reared on barley (cv. Hindmarsh) and endophyte-free perennial ryegrass (cv. Alto-WE). Aphids maintained on barley were assayed on barley, while aphids maintained on perennial ryegrass (cv. Alto-WE) were assayed on perennial ryegrass (cv. Alto-WE, -SE, -AR1, -AR37, -NEA2, and -NEA6). Colonies of *M. dirhodum* on both barley and perennial ryegrass were maintained as per methods described in Collinson et al. (2020).

Metopolophium dirhodum species identification was confirmed with a molecular based identification method, using the barcode region of the cytochrome oxidase subunit 1 (CO1) gene with primers LCO1490 and HCO2198 (Folmer et al., 1994). Aphid DNA was extracted using Bio-Rad Chelex© 100 Resin as per methods described in Walsh et al., (1991), and PCR

was carried out as per methods described in Folmer et al. (1994) with minor modifications, as per methods described in Collinson et al. (2020). PCR products were sequenced by Macrogen Inc. (Seoul, Korea) and sequences were compared to public databases (NCBI), determining similarity and coverage with previously identified specimens. Sequences were submitted to GenBank (Table 2), and specimens were preserved in 70% and 100% ethanol and deposited in the Victorian Agricultural Insect Collection (VAIC) at AgriBio, Bundoora, Australia.

Aphid mortality bioassay

For the mortality bioassay, perennial ryegrass (+/– endophytes) and barley seedlings were prepared following the same germination method and conditions used to germinate seedlings for colony plants. A cup-based system developed by Ridland et al. (1988) was adapted as per methods described in Collinson et al. (2020) to study changes in aphid life history over a period of 28 days. In brief, 30 individual adult *M. dirhodum* reared on endophyte-free perennial ryegrass were placed in plastic cups containing a single perennial ryegrass (cv. Alto-WE) seedling and monitored daily for newborn nymphs. For each treatment, a total of 24 new-born *M. dirhodum* nymphs were collected over a span of 24 hours (24 newborn nymphs x 5 endophyte treatments and 2 controls = 168 newborn nymphs total) and placed individually into new bioassay cups with seven-day-old perennial ryegrass seedlings with and without endophytes (Table 1). In addition, 24 new-born *M. dirhodum* nymphs born on barley (cv. Hindmarsh) seedlings were placed into bioassay cups containing seven-day old barley seedlings. The use of barley as an additional control provided assurance of the health of the *M. dirhodum* clone selected; mortality data of *M. dirhodum* on barley was not included in statistical analyses. Aphids were transferred onto a new seedling every seven days throughout the bioassay for up to 28 days.

The parameters investigated in this study were mortality and longevity. Mortality was determined as per methods described in Collinson et al. (2020), whereby *M. dirhodum* were observed every 24 hours over a period of 28 days to record the number of aphids that died every 24 hours. Daily mortality recordings were used to determine the longevity (number of days before death) of each individual aphid. All experiments were carried out over a 30-day period from the moment of birth, with recordings taken up to 28 days. All aphids that were still alive after the 28-day period were recorded as dying at >28 days for the purposes of statistical analysis. A period of 28 days was chosen for the duration of the bioassay as it represents the average lifespan of *M. dirhodum* on perennial ryegrass at 20°C (Meister et al., 2006). All experiments were carried out in a controlled environment room at 20±2°C and 62.0±5%RH, and a photoperiod of 14 hrs light-10 hrs dark.

Statistical analysis

Metopolophium dirhodum nymphal mortality was categorised by time into four periods: 0–48 (48 hrs), 48–96 (96 hrs), 96–144 (144 hrs), and >144 (>144 hrs) hours. Adult mortality was grouped into time periods of 14, 21 and 28 days. The unit of analysis was individual aphids in plastic cups (up to 24 replicates).

The difference in mortality of aphid nymphs and adults between treatments at each time period was analysed using logistic regression models, where the number of aphid deaths at each time period was the response variable (success) and logit was the link function. The cumulative mortality of nymphs was also analysed using logistic regression models, where the number of nymphs dead was the response and Treatment*Day was the full model, with logit as link function. These models were used to compute the probability of aphid mortality in each time period on each treatment and compare between treatments and time periods. The Wald chi-

squared test was used to include/exclude a term in the model. All data in this study was analysed using GenStat version 18 (VSN International, 2015).

The effect of aphid presence or absence on alkaloid concentration was analysed using a standard one-way analysis of variance. Differences between treatment means were examined using the least significant difference (LSD) at a 5% level of significance. The effect of alkaloid presence or absence on aphid mortality was analysed using a logistic regression analysis with the number of aphids surviving each day as the response (success) variable and alkaloid presence or absence (for each individual alkaloid) being the full model and logit as the link function. This model provides the odds of an aphid surviving when exposed to each alkaloid. The Wald test was used to include/exclude a term in the model. A general linear regression model was fitted to study the relationship between alkaloid concentration and aphid longevity.

RESULTS

Alkaloid profiling

Alkaloid profiles were confirmed for seven-day old perennial ryegrass seedlings that contained the endophyte: SE producing lolitrem B (3.19–26.34 ppm), ergovaline (0.35–19.60 ppm) and peramine (8.87–57.97 ppm); AR1 producing peramine (8.45–68.60 ppm); AR37 producing epoxy-janthitrem I (9678–56895051, expressed as peak are in arbitrary units); NEA2 producing lolitrem B (1.08–4.40 ppm), ergovaline (0.05–0.58 ppm) and peramine (5.80–16.09 ppm); and NEA6 producing ergovaline (3.17–138.49 ppm) and peramine (6.53–68.13 ppm).

Endophyte confirmation

Endophyte presence in seed batches was confirmed using strain-specific Competitive Allele Specific PCR (KASP) assay, with results showing high incidence in all seed batches. Of

approximately 150 seedlings tested for each treatment all were shown to have 100% incidence of the expected endophyte, indicating that endophyte presence is very high in the entire seed batch.

Effects of M. dirhodum presence on alkaloid profile

The presence or absence of an aphid (*M. dirhodum*) on a seedling was associated with a higher concentration of alkaloids (Table 3). The trend was that a total of 50% of treatments had higher alkaloid concentrations when fed on by an aphid, 30% had lower alkaloid concentrations and 20% remained unchanged. For NEA2, lolitrem B concentrations increased by 222.2%, peramine concentrations increased by 106.1%, and ergovaline concentrations remained unchanged. For NEA6, ergovaline concentrations increased by 58.2% and peramine concentrations increased by 34.1% when fed on by an aphid. For SE, peramine concentrations increased by 20.4%, lolitrem B concentrations remained unchanged and ergovaline concentrations decreased by 22.7%. For AR1, peramine concentrations decreased by 32.5%. For AR37, epoxy-janthitrem I concentrations decreased by 41.5%. Of these treatments, lolitrem B concentrations were significantly higher in NEA2 seedlings that had been fed on (2.9 ppm), compared to those that had not (0.9 ppm) ($P = 0.010$). Overall, the effect observed was related to endophyte treatment and not alkaloid, as alkaloid concentrations did not consistently change in the presence or absence of an aphid, irrespective of endophyte treatment.

Effects of alkaloid concentration on M. dirhodum longevity

Alkaloid concentration was shown to affect *M. dirhodum* longevity, with higher concentrations of lolitrem B resulting in shorter aphid life spans. (Fig. 1). For lolitrem B there was a negative correlation between concentration and the longevity of aphids reared on plants containing

endophytes producing lolitrem B (P = 0.027). Higher concentrations of lolitrem B (11.4–21.2 ppm, 4 samples, SE only) were observed in comparison to the average (10.6 ppm), which affected aphid longevity, with aphids surviving for 2 days. The analysis determined that for each unit increase (ppm) in lolitrem B concentration, aphid longevity was decreased by an average of 1.2 days. For ergovaline there was no correlation between concentration and aphid longevity (P = 0.831). Higher concentrations of ergovaline (32.6–138.5 ppm, 3 samples) were observed in comparison to the average (14.8 ppm), however this did not affect aphid longevity, as aphids under high concentrations survived for 11–15 days. For peramine there was no confirmed correlation between concentration and aphid longevity (P = 0.122). Higher concentrations of peramine (58.0–68.3 ppm, 2 samples) were observed in comparison to the average (20.4 ppm), however, this did not affect aphid longevity, as aphids under high concentrations survived for 2 and 11 days. For epoxy-janthitrem I there was no significant correlation between concentration and aphid longevity (P = 0.154), with concentrations remaining relatively consistent across all samples, and aphids surviving for 11–28 days. However, there was a trend across the four data points indicating a slight positive correlation.

Effects of alkaloid presence on M. dirhodum mortality.

Endophytes that produced lolitrem B and ergovaline were shown to significantly increase *M. dirhodum* nymphal mortality (Table 4). For lolitrem B, there was a 32% increase in nymphal mortality in aphids reared on plants containing endophytes that produced lolitrem B (80%), compared to plants that did not contain endophytes producing lolitrem B (48%) (P = 0.049). For ergovaline, there was a 32% increase in nymphal mortality in aphids reared on plants containing endophytes that produced ergovaline (76%), compared to plants that did not contain endophytes producing ergovaline (0.44) (P = 0.031). For peramine, there was no significant difference in mortality when peramine was present compared to when it was not (P = 0.517),

however, there was a trend indicating a 9% increase in nymphal mortality when peramine was present. For epoxy-janthitrem I, there was no significant difference in mortality when epoxy-janthitrem I was present compared to when it was not ($P = 0.808$).

Effects of Epichloë festucae var. lolii endophyte symbiota on mortality of Metopolophium dirhodum

Epichloë festucae var. *lolii* endophyte symbiota significantly affected the mortality of *M. dirhodum*, most notably at the nymphal stage (Table 5). The average nymphal mortality was highest for aphids reared on plants containing the endophyte SE (92% nymphal mortality), followed by NEA2 (83%), AR37 (63%), NEA6 (58%) and AR1 (38%), while plants containing no endophyte (WE) had a nymphal mortality of 67%. Plants with the endophyte SE had significantly higher nymphal mortality in comparison to all other treatments, except NEA2 ($P < 0.001$). While plants with the endophyte NEA2 had significantly higher nymphal mortality in comparison to NEA6, AR37 and AR1. Nymphal mortality on plants with the endophyte AR1 was similar to the barley control and survival was increased, compared to WE, suggesting little to no insecticidal activity and a life-history similar to the barley control.

The average nymphal mortality was highest at 24–48 hrs (39%) and lowest at 48–96 hrs (1%), across all endophyte treatments. At 24–48 hrs, nymphal mortality was highest on plants containing SE (75%), followed by NEA2 (50%), AR37 (42%), AR1 (21%) and NEA6 (8%). Plants containing SE had significantly higher mortality compared to the WE control ($P < 0.001$). At 96 hrs and 144 hrs there was no significant difference between treatments. At >144 hrs, nymphal mortality was highest on plants containing NEA6 (50%), followed by AR37 (17%), SE (13%), NEA2 (13%) and AR1 (8%). Plants containing NEA6 had significantly higher nymphal mortality compared to all other endophyte treatments ($P = 0.005$), but not the WE control (38%).

The average adult mortality was highest for aphids reared on plants containing the endophyte AR1 (63%), followed by NEA6 (42%), AR37 (38%), NEA2 (17%) and SE (8%), while plants containing no endophyte (WE) had an adult mortality of 33%. Plants with the endophyte AR1 had significantly higher mortality in comparison to all other treatments except NEA6 ($P < 0.001$). While plants with the endophyte NEA6 had significantly higher adult mortality in comparison to NEA2 and SE. Adult mortality on plants with the endophyte AR1 was similar to the Barley control, suggesting the mortality followed the natural aphid life cycle (Farrell & Stufkens, 1988).

The average adult mortality was highest at 28 days and >28 days (both 17%), and lowest at 14 days (4%), across all endophyte treatments. At 14 and 21 days there was no significant difference between treatments. At 28 days, adult mortality was highest on plants containing AR1 (29%), followed by AR37 (8%), NEA6 (8%), NEA2 (4%) and SE (0%). Plants containing AR1 showed significantly higher adult mortality than all other treatments including the WE control (17%). At >28 days, adult mortality was highest on plants containing NEA6 (33%), followed by AR1 (21%), AR37 (21%), NEA2 (8%) and SE (0%). Plants containing NEA6 showed significantly higher adult mortality compared to plants containing NEA2 or SE but not the WE control (13%), and plants containing AR1 and AR37 showed significantly higher adult mortality compared to plants containing SE but not the WE control.

DISCUSSION

Effects of alkaloids on M. dirhodum mortality and longevity.

In this study, the presence of both lolitrem B and ergovaline in perennial ryegrass seedlings were associated with a significant increase in aphid nymphal mortality. However, the presence of peramine caused no significant difference in aphid mortality compared to treatments where

these alkaloids were not present. Similarily, epoxy-janthitrem I showed no significant difference in aphid mortality, however the number of replicates was low in this treatment (n=4). Furthermore, variation in lolitrem B concentration had a significant effect on *M. dirhodum* longevity, where higher concentrations of lolitrem B resulted in significantly shorter aphid lifespans. These results are evidence that *M. dirhodum* is susceptible to the insecticidal effects of both lolitrem B and ergovaline, and that in the case of lolitrem B, those effects become more rapid with higher alkaloid concentrations. It is also possible that the increased insecticidal effect seen on SE is due, in part, to other alkaloids or metabolites that were not tested for in this study. Other research (Collinson et al. unpublished data) has found a reduction in *R. padi* feeding behaviour on certain endophyte infected perennial ryegrass seedlings that could not be attributed to a common alkaloid. It was concluded that a common precursor, or other endophyte metabolite or plant metabolite may have been causing a reduction in *R. padi* feeding. It is possible that a similar metabolite or unknown alkaloid or precursor is responsible for the increased mortality seen here, however the common endophytes between the most effective endophytes are evidence of a lolitrem B and/or ergovaline effect. Studies have shown several aphid species, including *M. dirhodum* to be negatively affected by the presence of a number of different alkaloids (Corcuera, 1984; Siegel et al., 1990; Wilkinson et al., 2000). Corcuera (1984) found that *M. dirhodum, Schizaphis graminum* (Rondani) and *Rhopalosiphum maidis* (Fitch) population densities were all reduced by the presence of hydroxamic acid in wheat and maize. Meister et al. (2006) found that both *R. padi* (L.) and *M. dirhodum* populations were reduced when feeding on perennial ryegrass infected with *Epichloë* endophytes producing lolitrem B, with *R. padi* being more strongly affected than *M. dirhodum*. While other research has shown that loline alkaloids produced by certain *Epichloë* endophytes have a negative effect on the survival of *R. padi* and *S. graminum* (Siegel et al., 1990). Seigel et al identified that peramine had a negative effect on the survival of *S. graminum*, however in the current study peramine

showed no activity, suggesting that the activity of peramine may affect specific species (Siegel et al., 1990). Given that lolitrem B, ergovaline, loline and peramine have all been shown to provide differing insecticidal activity against a variety of aphid species (Corcuera, 1984; Siegel et al., 1990; Wilkinson et al., 2000) this suggests that for broad spectrum aphid control, a diverse array of alkaloids would be required.

Effects of aphid feeding on alkaloid production

To explore the concept of tri-trophic interactions this study also assessed whether the presence of aphids may illicit a response from the plant, that in turn triggers the endophyte to produce higher levels of alkaloids as a defence response. NEA2 produced lolitrem B in significantly higher concentrations in host plants that had been fed on by *M. dirhodum* compared to plants that were not fed on, indicating increased production of alkaloids as a defensive response. This increase in lolitrem B could be due to a direct endophyte response to the presence of aphid feeding damage, which has been observed in tall fescue and other crop species (Sullivan et al., 2007; Züst & Agrawal, 2016). However, AR1 produced peramine in lower concentrations in host plants that had been fed on compared to those that had not, indicating a potential suppression of alkaloid production in this endophyte by the aphid. Studies have shown that the level of alkaloid production in ryegrass by *Epichloë* endophytes can be influenced by several factors including cutting or feeding damage by grazing livestock (Bultman & Bell, 2003; Moore et al., 2015; Zhang et al., 2011). Cutting of perennial ryegrass to simulate grazing has been found to induce the production of insecticidal alkaloids that in turn decreased feeding by weevils (Bultman et al., 2018). This could be extrapolated to incorporate feeding damage from other sources, including aphids.

409 The increase in lolitrem B levels following aphid feeding was originally believed to
410 have been caused by the production of plant hormones that regulate biotic stress tolerance (e.g.
411 jasmonic acid – JA, salicylic acid – SA) which are elicited following exposure to stress
412 (Rahman et al., 2013; Zarate et al., 2007). Feeding by sap-sucking hemipterans such as
413 Silverleaf Whitefly, *Bemisia tabaci* (Gennadius), and aphids has been shown to affect plant
414 production of both JA and SA (Girling et al., 2008; Heidel & Baldwin, 2004; Zarate et al.,
415 2007). Specifically, Girling et al. (2008) found that *Arabidopsis thaliana* plants increased
416 production of SA in response to feeding by Green Peach Aphid, *Myzus persicae* (Sulzer). There
417 is evidence that SA has an effect on the production of loline alkaloids by *Epichloë occultans* in
418 *L. multiflorum* plants (Bastías et al., 2018). However, the study found that increased levels of
419 SA decreased the production of lolines by endophytes. Likewise, Simons et al. (2008) found
420 that increased levels of JA suppressed loline production by endophytes. This could explain the
421 decrease in production of peramine by AR1 when seedlings were fed on, but the increase in
422 lolitrem B production by NEA2 could be influenced by another pathway. When JA levels are
423 not increased, herbivory by insect herbivores has been shown to result in increased loline
424 production (Simons et al., 2008; Sullivan et al., 2007). Furthermore, certain fungal endophytes
425 can supress the JA and SA pathways in their host plants, but this effect is variable, which could
426 explain the increased production of alkaloids by some endophytes and not others (Navarro-
427 Meléndez & Heil, 2014). These and the current study provide further evidence that aphid
428 feeding can increase alkaloid production, though the pathway responsible for this increase is
429 still uncertain. Despite observing an effect of aphid feeding on alkaloid production, it was only
430 observed in selected endophytes and with weak statistical support. As such, further research is
431 required to validate the effect. Furthermore, the level of alkaloids in plant tissue can also be
432 dependent on other factors such as endophyte hyphae density (Bastías et al., 2018), which
433 would need to be controlled for in future research.

Effects of endophyte treatment on M. dirhodum mortality

The endophyte that showed the strongest effect on nymphal mortality was SE, followed by NEA2, both of which produce lolitrem B, ergovaline and peramine. The key difference between these endophytes being that SE produces high levels (4.6–21.2 ppm) of lolitrem B, whereas NEA2 only produces trace amounts (1.9–4.4 ppm). Endophytes that had no significant effect on nymphal mortality (NEA6 and AR37) produced ergovaline, peramine or epoxy-janthitrem I, but did not produce lolitrem B, indicating that lolitrem B is an important insecticidal compound against aphids. This correlates with the alkaloid profiling results that showed endophytes producing high levels of lolitrem B and ergovaline would be the most toxic towards *M. dirhodum*. This result also correlates with results from two other aphid species, *Diuraphis noxia* and *Aploneura lentisci*, both of which showed similarly increased nymphal mortality on SE and NEA2 (Collinson et al. 2020). In addition, Clement et al. (2005) determined that *M. dirhodum* population densities were lower on wild barley, *Hordeum brevisubulatum,* infected with *Epichloe* (*Neotyphodium*) endophytes, compared to endophyte-free wild barley. Likewise, Meister et al., 2006 observed a reduction in *M. dirhodum* population density on endophyte-infected perennial ryegrass compared to endophyte-free plants. In both of these studies the presence of endophytes was not as effective at reducing populations of *R. padi*. However, AR1 (peramine) was associated with a significant decrease in *M. dirhodum* mortality. This is a similar result to Popay & Cox (2016), who found a similar decrease in *A. lentisci* mortality on AR1. It is clear that presence of an endophyte is important for aphid control, however the responses may vary depending on the aphid species. Furthermore, the genotype of the endophyte will affect the insecticidal activity of the symbiota. As such, for broad spectrum aphid control a diverse range of endophyte strains would also be required to achieve a desired alkaloid combination.

Previous research has established that perennial ryegrass seed casings contain high alkaloid concentrations (Hewitt et al., 2020), which aphids would not have been exposed to while feeding on leaves. However, as whole seedlings were used in all replicates in this study, the variation in alkaloid profiles between seedlings would still be reflective of the varying concentrations of alkaloids to which aphids were exposed.

Translation to field performance

This study showed that lolitrem B and ergovaline are the alkaloids with the strongest toxic effect on *M. dirhodum*. However, when present in high concentrations in fields, these endophytes are also toxic to livestock such as cattle and sheep. Our results showed that these alkaloids remain toxic to *M. dirhodum* even in low concentrations, so endophytes producing levels of lolitrem B and ergovaline that are considered safe for animal consumption could still be effective at controlling *M. dirhodum*. However, our results determined that higher concentrations of lolitrem B will result in faster control of *M. dirhodum* populations. Therefore, additional factors, such as mycotoxin binding agents, could be considered to allow for the use of higher lolitrem B concentrations without the associated risk to livestock (Mann & Parfitt, 2011; Merrill, 2007). Endophytes producing lolitrem B and ergovaline in lower concentrations are available and do still have an advantage over endophyte-free ryegrass, but further experimentation should be undertaken to determine the exact concentration of these alkaloids that maintains control of insect pests without causing harm to livestock (Blythe et al., 2007).

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REFERENCES

Ajayi BO, Dewar AM. 1983. The effect of barley yellow dwarf virus on field populations of the cereal aphids, *Sitobion avenae* and *Metopolophium dirhodum*. *Annals of Applied Biology* **103**: 1–11. DOI: 10.1111/j.1744-7348.1983.tb02734.x

Barenbrug Agriseeds. 2019. Barenbrug Agriseeds - Superior pastures // Dairy > cultivars > Ryegrass endophyte. Available from: https://www.agriseeds.co.nz/dairy/cultivars/endophyte-options.htm (Accessed 3 July 2019)

Bastías DA, Martínez-Ghersa MA, Newman JA, Card SD, Mace WJ, Gundel PE. 2018. The plant hormone salicylic acid interacts with the mechanism of anti-herbivory conferred by fungal endophytes in grasses. *Plant, Cell & Environment* **41**: 395–405. DOI: 10.1111/pce.13102

Blythe L, Estill C, Males J, Craig AM. 2007. Determination of the toxic threshold of lolitrem B in cattle eating endophyte-infected perennial ryegrass. In *Proceedings of the 6th international symposium on fungal endophytes of grasses. New Zealand Grassland Association, Christchurch, New Zealand* (pp. 399-402).

Breen JP. 1993. Enhanced resistance to three species of aphids (Homoptera: Aphididae) in *Acremonium* endophyte-infected turfgrasses. *Journal of Economic Entomology* **86**: 1279–1286. DOI: 10.1093/jee/86.4.1279

Bultman TL, Bell GD. 2003. Interaction between fungal endophytes and environmental stressors influences plant resistance to insects. *Oikos* **103**: 182–190. DOI: 10.1034/j.1600-0706.2003.11574.x

509 Bultman TL, McNeill MR, Krueger K, De Nicolo G, Popay AJ, Hume DE, Mace WJ, Fletcher
510 LR, Koh YM, Sullivan TJ. 2018. Complex interactions among sheep, snsects, grass, and
511 fungi in a simple New Zealand grazing system. *Journal of Chemical Ecology* **44**: 957–
512 964. DOI: 10.1007/s10886-018-0993-6

513 Carver M. 1984. *Metopolophium dirhodum* (Walker) (Homoptera: Aphididiae) newly recorded
514 from Australia. *Australian Journal of Entomology* **23**: 192–192. DOI: 10.1111/j.1440-
515 6055.1984.tb01944.x

516 Clement SL, Elberson LR, Bosque-Pérez NA, Schotzko DJ. 2005. Detrimental and neutral
517 effects of wild barley–*Neotyphodium* fungal endophyte associations on insect survival.
518 *Entomologia Experimentalis et Applicata* **114**: 119–125. DOI: 10.1111/j.1570-
519 7458.2005.00236.x

520 Clement SL, Pike KS, Kaiser WJ, Wilson AD. 1990. Resistance of endophyte-infected plants
521 of tall fescue and perennial ryegrass to the Russian Wheat Aphid (Homoptera:
522 Aphididae). *Journal of the Kansas Entomological Society* **63**: 646–648.

523 Collinson NP, Mann RC, Giri K, Malipatil M, Kaur J, Spangenberg G, Valenzuela I. 2020.
524 Novel bioassay to assess antibiotic effects of fungal endophytes on aphids. *PLOS ONE*
525 **15**: e0228813. DOI: 10.1371/journal.pone.0228813

526 Corcuera LJ. 1984. Effects of indole alkaloids from gramineae on aphids. *Phytochemistry* **23**:
527 539–541. DOI: 10.1016/S0031-9422(00)80376-3

528 Dean GJ. 1974. Effect of temperature on the cereal aphids Metopolophium dirhodum (Wlk.),
529 *Rhopalosiphum padi* (L.) and *Macrosiphum avenue* (F.) (Hem., Aphididae). *Bulletin of*
530 *Entomological Research* **63**: 401. DOI: 10.1017/S0007485300040888

Ekanayake PN, Kaur J, Tian P, Rochfort SJ, Guthridge KM, Sawbridge TI, Spangenberg GC, Forster JW. 2017. Genomic and metabolic characterisation of alkaloid biosynthesis by asexual *Epichloe* fungal endophytes of tall fescue pasture grasses. *Genome* DOI: 10.1139/gen-2016-0173

Faeth SH, Bush LP, Sullivan TJ. 2002. Peramine alkaloid variation in *Neotyphodium*-infected Arizona fescue: effects of endophyte and host genotype and environment. *Journal of Chemical Ecology* **28**: 1511–1526. DOI: 10.1023/A:1019916227153

Farrell JA, Stufkens MW. 1988. Abundance of the rose-grain aphid, *Metopolophium dirhodum*, on barley in Canterbury, New Zealand, 1984–87. *New Zealand Journal of Zoology* **15**: 499–505. DOI: 10.1080/03014223.1988.10422630

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates - Google Scholar. **3**: 294–299.

Fuchs B, Krischke M, Mueller MJ, Krauss J. 2013. Peramine and lolitrem B from endophyte-grass associations cascade up the food chain. *Journal of Chemical Ecology* **39**: 1385–1389. DOI: 10.1007/s10886-013-0364-2

Gallagher RT, White EP, Mortimer PH. 1981. Ryegrass staggers: isolation of potent neurotoxins lolitrem A and lolitrem B from staggers-producing pastures. *New Zealand Veterinary Journal* **29**: 189–190. DOI: 10.1080/00480169.1981.34843

Girling RD, Madison R, Hassall M, Poppy GM, Turner JG. 2008. Investigations into plant biochemical wound-response pathways involved in the production of aphid-induced plant volatiles. *Journal of Experimental Botany* **59**: 3077–3085.

553 Graves H, Rayburn AL, Gonzalez-Hernandez JL, Nah G, Kim D-S, Lee DK. 2016. Validating
554 DNA polymorphisms using KASP assay in prairie cordgrass (*Spartina pectinata* Link)
555 Populations in the U.S. *Frontiers in Plant Science* **6** DOI: 10.3389/fpls.2015.01271

556 Guerre P. 2015. Ergot alkaloids produced by endophytic fungi of the genus *Epichloë*. *Toxins* **7**:
557 773–790. DOI: 10.3390/toxins7030773

558 Guy PL. 1988. Pasture ecology of barley yellow dwarf viruses at Sandford, Tasmania. *Plant*
559 *Pathology* **37**: 546–550. DOI: 10.1111/j.1365-3059.1988.tb02113.x

560 Heidel AJ, Baldwin IT. 2004. Microarray analysis of salicylic acid- and jasmonic acid-
561 signalling in responses of *Nicotiana attenuata* to attack by insects from multiple feeding
562 guilds. *Plant, Cell & Environment* **27**: 1362–1373. DOI: 10.1111/j.1365-
563 3040.2004.01228.x

564 Hesse U, Schöberlein W, Wittenmayer L, Förster K, Warnstorff K, Diepenbrock W, Merbach
565 W. 2003. Effects of *Neotyphodium* endophytes on growth, reproduction and drought-
566 stress tolerance of three *Lolium perenne* L. genotypes. *Grass and Forage Science* **58**:
567 407–415. DOI: 10.1111/j.1365-2494.2003.00393.x

568 Hewitt, K.G., Mace, W.J., McKenzie, C.M., Matthew, C., Popay, A.J., 2020. Fungal Alkaloid
569 Occurrence in Endophyte-Infected Perennial Ryegrass during Seedling Establishment.
570 *J. Chem. Ecol.* 46, 410–421. https://doi.org/10.1007/s10886-020-01162-w

571 Hovermale JT, Craig AM. 2001. Correlation of ergovaline and lolitrem B levels in endophyte-
572 infected perennial ryegrass (*Lolium perenne*). *Journal of Veterinary Diagnostic*
573 *Investigation* **13**: 323–327. DOI: 10.1177/104063870101300407

574 Hume DE, Sewell JC. 2014. Agronomic advantages conferred by endophyte infection of
575 perennial ryegrass (*Lolium perenne* L.) and tall fescue (*Festuca arundinacea* Schreb.)
576 in Australia. *Crop and Pasture Science* **65**: 747. DOI: 10.1071/CP13383

577 Johnson MC, Dahlman DL, Siegel MR, Bush LP, Latch GCM, Potter DA, Varney DR. 1985.
578 Insect feeding deterrents in endophyte-infected tall fescue. *Applied and Environmental*
579 *Microbiology* **49**: 568–571.

580 Kauppinen M, Saikkonen K, Helander M, Pirttilä AM, Wäli PR. 2016. *Epichloë* grass
581 endophytes in sustainable agriculture. *Nature Plants* **2**: 15224. DOI:
582 10.1038/nplants.2015.224

583 Klotz JL, Bush LP, Smith DL, Shafer WD, Smith LL, Arrington BC, Strickland JR. 2007.
584 Ergovaline-induced vasoconstriction in an isolated bovine lateral saphenous vein
585 bioassay. *Journal of Animal Science* **85**: 2330–2336. DOI: 10.2527/jas.2006-803

586 Latch GCM. 1993. Physiological interactions of endophytic fungi and their hosts. Biotic stress
587 tolerance imparted to grasses by endophytes. *Agriculture, Ecosystems & Environment*
588 **44**: 143–156. DOI: 10.1016/0167-8809(93)90043-O

589 Ludlow, E.J., Vassiliadis, S., Ekanayake, P.N., Hettiarachchige, I.K., Reddy, P., Sawbridge,
590 T.I., Rochfort, S.J., Spangenberg, G.C., Guthridge, K.M., 2019. Analysis of the Indole
591 Diterpene Gene Cluster for Biosynthesis of the Epoxy-Janthitrems in Epichloë
592 Endophytes. Microorganisms 7, 560. https://doi.org/10.3390/microorganisms7110560

593 Mann SP, Parfitt D. 2011. Mycotoxin-reducing composition

Meister B, Krauss J, Härri SA, Victoria Schneider M, Müller CB. 2006. Fungal endosymbionts affect aphid population size by reduction of adult life span and fecundity. *Basic and Applied Ecology* **7**: 244–252. DOI: 10.1016/j.baae.2005.06.002

Merrill ML. 2007. The ability of a yeast-derived cell wall preparation to minimize toxic effects of high-alkaloid tall fescue straw in beef cattle

Moate PJ et al. 2012. Effects of wild-type, AR1 and AR37 endophyte-infected perennial ryegrass on dairy production in Victoria, Australia. *Animal Production Science* **52**: 1117–1130. DOI: 10.1071/AN12126

Moore JR, Pratley JE, Mace WJ, Weston LA. 2015. Variation in alkaloid production from genetically diverse *Lolium* accessions infected with *Epichloë* species. *Journal of Agricultural and Food Chemistry* **63**: 10355–10365. DOI: 10.1021/acs.jafc.5b03089

Navarro-Meléndez AL, Heil M. 2014. Symptomless endophytic fungi suppress endogenous levels of salicylic acid and interact with the jasmonate-dependent indirect defence traits of their host, lima bean (*Phaseolus lunatus*). *Journal of Chemical Ecology* **40**: 816–825. DOI: 10.1007/s10886-014-0477-2

Ondeyka, J.G., Helms, G.L., Hensens, O.D., Goetz, M.A., Zink, D.L., Tsipouras, A., Shoop, W.L., Slayton, L., Dombrowski, A.W., Polishook, J.D., Ostlind, D.A., Tsou, N.N., Ball, R.G., Singh, S.B., 1997. Nodulisporic Acid A, a Novel and Potent Insecticide from a Nodulisporium Sp. Isolation, Structure Determination, and Chemical Transformations. J. Am. Chem. Soc. 119, 8809–8816. https://doi.org/10.1021/ja971664k

615 Popay AJ, Cox NR. 2016. *Aploneura lentisci* (Homoptera: Aphididae) and its interactions with
616 fungal endophytes in perennial ryegrass (*Lolium perenne*). *Frontiers in Plant Science*
617 **7**: DOI: 10.3389/fpls.2016.01395

618 Popay AJ, Thom ER. 2009. Endophyte effects on major insect pests in Waikato dairy pasture.
619 *Proceedings of the New Zealand Grasslands Association* **71**: 121–126.

620 Popay AJ, Wyatt RT. 1995. Resistance to Argentine stem weevil in perennial ryegrass infected
621 with endophytes producing different alkaloids. *Proceedings of the Forty Eighth New*
622 *Zealand Plant Protection Conference, Angus Inn, Hastings, New Zealand, August 8-10,*
623 *1995.* : 229–236.

624 Potter DA, Stokes JT, Redmond CT, Schardl CL, Panaccione DG. 2008. Contribution of ergot
625 alkaloids to suppression of a grass-feeding caterpillar assessed with gene knockout
626 endophytes in perennial ryegrass. *Entomologia Experimentalis et Applicata* **126**: 138–
627 147. DOI: 10.1111/j.1570-7458.2007.00650.x

628 Rahman A, Kuldau GA, Uddin W. 2013. Induction of salicylic acid–mediated defense response
629 in perennial ryegrass against infection by *Magnaporthe oryzae*. *Phytopathology*^{TM} **104**:
630 614–623. DOI: 10.1094/PHYTO-09-13-0268-R

631 Ridland PM, Sward RJ, Tomkins RB. 1988. A simple rearing system for cereal aphids,
632 especially suited to transmission studies with barley yellow dwarf viruses. *Australasian*
633 *Plant Pathology* **17**: 17–19.

634 Saikkonen K, Gundel PE, Helander M. 2013. Chemical ecology mediated by fungal endophytes
635 in grasses. *Journal of Chemical Ecology* **39**: 962–968. DOI: 10.1007/s10886-013-0310-
636 3

637 Schardl CL. 2001. *Epichloë festucae* and related mutualistic symbionts of grasses. *Fungal*
638 *Genetics and Biology* **33**: 69–82. DOI: 10.1006/fgbi.2001.1275

639 Schmidt MH, Lauer Andreas, Thies Carsten, Schaefer Matthias, Tscharntke Teja. 2003.
640 Relative importance of predators and parasitoids for cereal aphid control. *Proceedings*
641 *of the Royal Society of London. Series B: Biological Sciences* **270**: 1905–1909. DOI:
642 10.1098/rspb.2003.2469

643 Shymanovich T, Saari S, Lovin ME, Jarmusch AK, Jarmusch SA, Musso AM, Charlton ND,
644 Young CA, Cech NB, Faeth SH. 2015. Alkaloid variation among Epichloid endophytes
645 of sleepygrass (*Achnatherum robustum*) and consequences for resistance to insect
646 herbivores. *Journal of Chemical Ecology* **41**: 93–104. DOI: 10.1007/s10886-014-0534-
647 x

648 Siegel MR, Latch GCM, Bush LP, Fannin FF, Rowan DD, Tapper BA, Bacon CW, Johnson
649 MC. 1990. Fungal endophyte-infected grasses: alkaloid accumulation and aphid
650 response. *Journal of Chemical Ecology* **16**: 3301–3315. DOI: 10.1007/BF00982100

651 Simons L, Bultman TL, Sullivan TJ. 2008. Effects of methyl jasmonate and an endophytic
652 fungus on plant resistance to insect herbivores. *Journal of Chemical Ecology* **34**: 1511.
653 DOI: 10.1007/s10886-008-9551-y

654 Stribley MF, Moores GD, Devonshire AL, Sawicki RM. 1983. Application of the FAO-
655 recommended method for detecting insecticide resistance in *Aphis jabae* Scopoli,
656 *Sitobion avenae* (F.), *Metopolophium dirhodum* (Walker) and *Rhopalosiphum padi* (L.)
657 (Hemiptera: Aphididae). *Bulletin of Entomological Research* **73**: 107–115. DOI:
658 10.1017/S0007485300013845

659 Sullivan TJ, Rodstrom J, Vandop J, Librizzi J, Graham C, Schardl CL, Bultman TL. 2007.
660 Symbiont-mediated changes in *Lolium arundinaceum* inducible defenses: evidence
661 from changes in gene expression and leaf composition. *The New Phytologist* **176**: 673–
662 679.

663 Tor-Agbidye J, Blythe LL, Craig AM. 2001. Correlation of endophyte toxins (ergovaline and
664 lolitrem B) with clinical disease: fescue foot and perennial ryegrass staggers. *Veterinary*
665 *and Human Toxicology* **43**: 140–146.

666 Vassiliadis S, Elkins AC, Reddy P, Guthridge KM, Spangenberg GC, Rochfort SJ. 2019. A
667 simple LC–MS method for the quantitation of alkaloids in endophyte-infected perennial
668 ryegrass. *Toxins* **11**: 649. DOI: 10.3390/toxins11110649

669 VSN International. 2015. *Genstat for Windows 19th Edition*. VSN International: Hemel
670 Hamstead, UK.

671 Walsh P, Metzger D, Higuchi R. 1991. Chelex 100 as a medium for simple extraction of DNA
672 for PCR-based typing from forensic material. *BioTechniques* **10**: 506–513.

673 Wilkins PW. 1991. Breeding perennial ryegrass for agriculture. *Euphytica* **52**: 201–214. DOI:
674 10.1007/BF00029397

675 Wilkinson HH, Siegel MR, Blankenship JD, Mallory AC, Bush LP, Schardl CL. 2000.
676 Contribution of fungal loline alkaloids to protection from aphids in a grass-endophyte
677 mutualism. *Molecular Plant-Microbe Interactions* **13**: 1027–1033. DOI:
678 10.1094/MPMI.2000.13.10.1027

679 Zarate SI, Kempema LA, Walling LL. 2007. Silverleaf whitefly induces salicylic acid defenses
680 and suppresses effectual jasmonic acid defenses. *Plant Physiology* **143**: 866–875.

681 Zhang X, Li C, Nan Z. 2011. Effects of cutting frequency and height on alkaloid production in
682 endophyte-infected drunken horse grass (*Achnatherum inebrians*). *Science China Life*
683 *Sciences* **54**: 567–571. DOI: 10.1007/s11427-011-4181-y

684 Züst T, Agrawal AA. 2016. Mechanisms and evolution of plant resistance to aphids. *Nature*
685 *Plants* **2**: 1–9. DOI: 10.1038/nplants.2015.206
686

687 **Supporting Information**

688 ***Tables***

689 **Table 1. *Epichloë festucae* var. *lolii* endophyte strains used in this study and their**

690 **associated alkaloid profiles.**

Alkaloid class	Alkaloid type	SE	AR1	AR37	NEA2	NEA6
Ergopeptide	Ergovaline	P	-	-	P	P
Polyketide	Peramine	P	P	-	P	P
Indole Diterpene	Epoxy-janthitrem I	-	-	P	-	-
	Lolitrem B	P	-	-	T	-

691 The host plant for all endophytes was perennial ryegrass cv. Alto. SE = standard endophyte;

692 AR1, AR37, NEA2, NEA6= commercial endophytes. P = alkaloid present; - = alkaloid absent;

693 T = alkaloid present only in trace levels.

694 **Table 2. Aphid collection details and GenBank accession number.**

Aphid species	GenBank accession number	Locality	GPS	Date of collection	Host plant
Metopolophium dirhodum	MN066606	Yea, Victoria	37°12'23.7"S 145°25'46.6"E	18/03/2017	Wild grass

695

Table 3: Average alkaloid concentrations (in ppm) in each endophyte treatment including samples that were fed on (Aphid+) and samples that were not (Aphid-)

Alkaloid type		SE	AR1	AR37	NEA2	NEA6	Total sample size
Ergovaline	Aphid+	5.8	n/a	n/a	0.2	31.0	20
	Aphid-	7.5	n/a	n/a	0.2	19.6	20
Peramine	Aphid+	25.4	19.7	n/a	10.1	18.1	29
	Aphid-	21.1	29.2	n/a	4.9	13.5	28
Lolitrem B	Aphid+	10.6	n/a	n/a	2.9	n/a	15
	Aphid-	10.7	n/a	n/a	0.9	n/a	13
Epoxy-janthitrem I	Aphid+	n/a	n/a	1.45E+07	n/a	n/a	4
	Aphid-	n/a	n/a	2.48E+07	n/a	n/a	7

699 **Table 4: Nymphal mortality of *Metopolophium dirhodum* associated with the presence (P)**
700 **or absence (A) of the alkaloids lolitrem B, ergovaline, peramine and epoxy-janthitrem I.**

Alkaloid	Presence/Absence	Endophyte	Mortality (proportion) [a]	P - value[b]
Lolitrem B	P	SE, NEA2	0.80	**0.049**
	A	AR1, AR37, NEA6	0.48	
Ergovaline	P	SE, NEA2, NEA6	0.76	**0.031**
	A	AR1, AR37	0.44	
Peramine	P	SE, NEA2, NEA6, AR1	0.62	0.517
	A	AR37	0.53	
Epoxy-janthitrem I	P	AR37	0.50	0.808
	A	SE, AR1, NEA2, NEA6	0.59	

701 [a] Average mortality (%) with alkaloids present or absent.

702 [b] p-values were calculated using a logistic regression analysis.

703 **Table 5: Mortality of *Metopolophium dirhodum* on all endophyte (SE, AR1, AR37,**
704 **NEA2, NEA6) and endophyte-free (WE) treatments observed at the nymphal stage (48,**
705 **96, 144 and >144 hrs) and the adult stage (14, 21 and 28 days).**

Mortality	Barley[c]	WE	SE	AR1	AR37	NEA2	NEA6	LSD	P-value[b]
Nymphal mortality	8/24 (0.33)	16/24 (0.67)	22/24 (0.92)	9/24 (0.38)	15/24 (0.63)	20/24 (0.83)	14/24 (0.58)	0.20	**<0.001**
Nymphal mortality (24–48 hrs)	3/24 (0.13)	5/24 (0.21)	18/24 (0.75)	5/24 (0.21)	10/2 (0.42)	12/24 (0.50)	2/24 (0.08)	0.30	**<0.001**
Nymphal mortality (48–96 hrs)	1/24 (0.04)	0/24 (0.00)	0/24 (0.00)	0/24 (0.00)	0/24 (0.00)	1/24 (0.04)	0/24 (0.00)	0.28	0.606
Nymphal mortality (96–144 hrs)	0/24 (0.00)	2/24 (0.08)	1/24 (0.04)	2/24 (0.08)	1/24 (0.04)	4/24 (0.17)	0/24 (0.00)	0.28	0.233
Nymphal mortality (>144 hrs)	4/24 (0.17)	9/24 (0.38)	3/24 (0.13)	2/24 (0.08)	4/24 (0.17)	3/24 (0.13)	12/24 (0.50)	0.27	**0.005**
Adult Mortality	16/24 (0.67)	8/24 (0.33)	2/24 (0.08)	15/24 (0.63)	9/24 (0.38)	4/24 (0.17)	10/24 (0.42)	0.24	**<0.001**
Adult Mortality (14 days)	4/24 (0.17)	0/24 (0.00)	1/24 (0.04)	0/24 (0.00)	1/24 (0.04)	1/24 (0.04)	0/24 (0.00)	0.07	0.518
Adult Mortality (21 days)	6/247 (0.25)	1/24 (0.04)	1/24 (0.04)	3/24 (0.13)	1/24 (0.04)	0/24 (0.00)	0./24 (0.00)	0.09	0.232
Adult Mortality (28 days)	3/24 (0.13)	4/24 (0.17)	0/24 (0.00)	7/24 (0.29)	2/24 (0.08)	1/24 (0.04)	2/24 (0.08)	0.11	**0.016**
Adult Mortality (>28 days)	3/24 (0.13)	3/24 (0.13)	0/24 (0.00)	5/24 (0.21)	5/24 (0.21)	2/24 (0.08)	8/24 (0.33)	0.20	**0.011**

706 [a] Average mortality on endophyte treatments (excluding WE).

707 [b] p values were calculated using a logistic regression analysis.

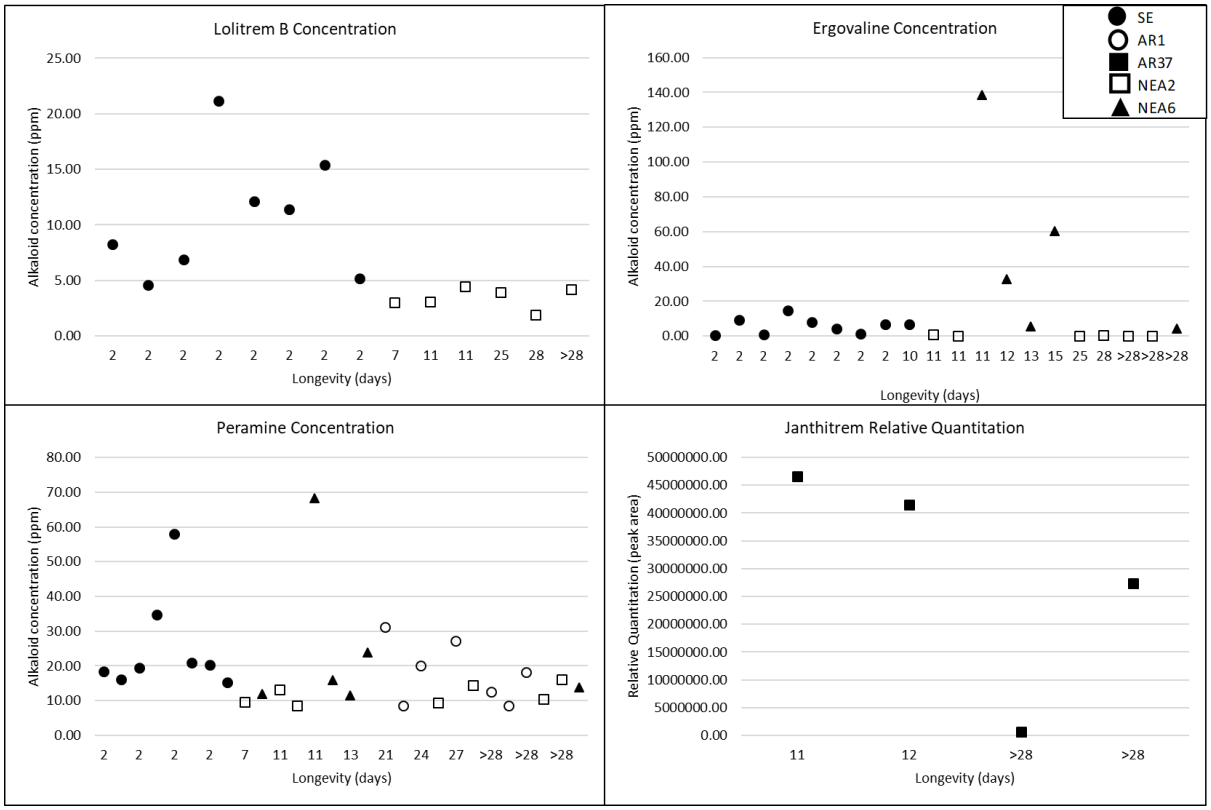
708 [c] barley was included as a control, but not in the statistical analysis

Figure Legends

Fig. 1: Correlation between alkaloid concentration and *Metopolophium dirhodum* longevity. Results are shown as the alkaloid concentration in parts per million (ppm) for lolitrem B (a), ergovaline (b) and peramine (c), and relative quantitation, shown as peak area, for epoxy-janthitrem I (d); correlated with the longevity (measured as number of days until death) of aphids reared on each replicate. The number of data points in each graph represents the number of successful alkaloid profiles of individual samples conducted. As such, some graphs show more data points than others.

Figures

Fig. 1



CHAPTER 4

Effects of fungal endophytes on the feeding behaviour of *Rhopalosiphum padi* on perennial ryegrass seedlings

4.1: Chapter Preface

Epichloë fungal endophytes have feeding deterrent properties against some invertebrate pests, such as the Argentine stem weevil, based on the activity of key alkaloids they produce (e.g. peramine and lolines). The Electrical Penetration Graph (EPG) technique is used to assess the feeding behaviour of phloem-feeding hemipterans by creating a circuit through the insect and the plant they feed on, and measuring fluctuations in the electrical potential flowing through that circuit as changes in feeding behaviour. This experiment assessed the feeding behaviour of the bird cherry-oat aphid, *Rhopalosiphum padi* (L.), on five different *Epichloë* fungal endophyte treatments (SE, AR1, AR37, NEA2 and NEA6) and a control without endophyte (WE) in order to determine the effects of these endophytes on aphid feeding and probing. Some endophytes (AR37, NEA2 and NEA6) exhibited a feeding deterrence effect with a reduced number and duration of probing and feeding events, while others had no effect (SE, AR1). Bioassays were also conducted with *R. padi* as per the methods outlined in Chapter 2. *Rhopalosiphum padi* showed significantly reduced fecundity, and a trend towards increased mortality on all endophyte treatments. The feeding deterrence observed in the study could not be attributed to a major alkaloid (lolitrem B, ergovaline, peramine or janthitrem I), however it was postulated that an indole-diterpene precursor (e.g. paspaline) or novel compound may be involved. The absence of any feeding deterrence from the endophyte with the highest nymph mortality and most diverse alkaloid profile (SE – lolitrem B, ergovaline, peramine) suggested this endophyte may be producing an attractant that supersedes any deterrence effects, such a volatile organic compound. This study provides new insights into the mode of action of *Epichloë* endophytes against aphids and their importance in pasture management.

This chapter is presented in submission-ready format for the journal Oecologia.

4.2: Publication details

Title: Evaluating the effects of *Epichloë* fungal endophytes of perennial ryegrass on the feeding behaviour and life history of *Rhopalosiphum padi*

Journal details: Oecologia

Stage of publication: Pre-submission

Authors: Nicholas Paul Collinson, Isabel Valenzuela, Khageswor Giri, Mallik Malipatil, Jatinder Kaur, German Spangenberg, Ross Cameron Mann

4.3: Statement of contribution of joint authorship

NPC performed all work relating to maintaining colonies of aphids, conducting aphid bioassays and performing EPG bioassays. NPC generated all figures and drafted the majority of the manuscript. NPC, JK and IV conducted genetic analysis of aphid specimens. NPC, IV and KG conducted all statistical and data analysis. KG assisted in drafting the statistical sections of the manuscript. RM, GS, IV, MM, JK and NPC all conceptualised the project and assisted in drafting the manuscript.

4.4 Statement from the co-author confirming the authorship contribution of the PhD candidate

“As co-author of the manuscript ‘Collinson, NP, Valenzuela, I, Giri, K, Malipatil, M, Kaur, J, Spangenberg, G, & Mann, RC. 2020. Effects of fungal endophytes on the feeding behaviour

of *Rhopalosiphum padi* on perennial ryegrass seedlings' I confirm that Nicholas Collinson made the following contributions,

- Taxonomical verification and rearing of colonies of aphid species (*R. padi*)
- Feeding behaviour experimentation on EPG
- Conducted aphid life history bioassays
- Feeding behaviour and life history data analysis
- Generated all figures
- Writing the manuscript and critical appraisal of content"

Associate Professor Mallik Malipatil

Date: 28/06/2020

Evaluating the effects of *Epichloë* fungal endophytes of perennial ryegrass on the feeding behaviour and life history of *Rhopalosiphum padi*

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Abstract

The bird cherry-oat aphid, *Rhopalosiphum padi* (L.), is one of the most widespread and economically significant pests of pasture grasses in Australian dairy farming regions. Measuring aphid feeding behaviour can provide insights into the effectiveness and mode of action of different insecticidal fungal endophytes that exist in pasture grasses. The electrical penetration graph (EPG) technique is an accurate and reliable method of measuring aphid feeding behaviour on a range of plants. This study investigated the effects of different *Epichloë*–perennial ryegrass symbiota on the feeding behaviour of *R. padi* using EPG, while also assessing aphid life history using a cup-based bioassay. Aphids on perennial ryegrass infected with the commercial endophytes NEA6, NEA2 and AR37 were deterred from probing (31–55% less) and phloem-feeding (29–49% less) with significantly fewer events, compared to the commercial endophyte AR1, the wildtype endophyte SE and endophyte-free perennial ryegrass. Aphids feeding on perennial ryegrass infected with any endophyte showed significantly lower fecundity than aphids reared on endophyte-free perennial ryegrass, while there was also higher nymph mortality on all endophyte treatments compared to endophyte-free perennial ryegrass, although not significant. The feeding deterrence observed in the study could not be attributed to a major alkaloid (lolitrem B, ergovaline, peramine or janthitrem I), however it was postulated that an indole-diterpene precursor (e.g. paspaline) or novel compound may be involved. The absence of any feeding deterrence

from the endophyte with the highest nymph mortality suggested this endophyte may be producing an attractant that supersedes any deterrence effects, such a volatile organic compound. Overall, this study sheds new light on the mode of action of *Epichloë* endophytes against aphids and highlights the importance of selecting an *Epichloë* endophyte to best manage insect pests in dairy pastures.

Key words

Endophytes; perennial ryegrass; aphids; *Rhopalosiphum padi*; electrical penetration graph

Introduction

Perennial ryegrass, *Lolium perenne* (L.), is the most commonly used pasture grass species in Australian and New Zealand dairy pastures (Wilkins, 1991). It has several advantages over other pasture grasses, including strong persistence in fields for up to four years, high nutrient content and a level of resistance to drought and certain pests and diseases (Wilkins, 1991). Despite these advantages, perennial ryegrass is still susceptible to biotic stresses including some insect pests such as the Argentine stem weevil, *Listronotus bonariensis* (Kuschel), the African black beetle, *Heteronychus arator* (Fabricius), and certain aphid species (Rogers et al., 2019; Smith, 1977). To defend against these stresses, perennial ryegrass has evolved a mutualistic relationship with endophytic fungi (Heeswijck and McDonald, 1992). Perennial ryegrass is commonly infected with a fungal endophyte known as *Epichloë festucae* var. *lolii* (Heeswijck and McDonald, 1992), which confers the host plant with further resistance to vertebrate and invertebrate herbivores, as well as a higher tolerance to drought and saline conditions (Sabzalian and Mirlohi, 2010). Endophytes, such as *Epichloë,* protect plants from insect herbivory, predominantly through the production of bioactive alkaloids that can be toxic or unpalatable to insect pests (Ball et al., 1997). The alkaloid peramine in particular is known to have feeding deterrent properties against *L. bonariensis* (Popay and Wyatt, 1995; Rowan et al., 1986), and loline alkaloids are known to have direct toxic insecticidal properties (Eichenseer et al., 1991; Wilkinson et al., 2000). Indole diterpene alkaloids produced by *Epichloë* species are more commonly associated with neurotoxic effects on livestock, however other indole diterpenes produced by fungi have been demonstrated to have insecticidal activity (e.g. nodulisporic acids) (Ondeyka et al., 1997). The alkaloid ergovaline

produced by *Epichloë* species also has detrimental effects on grazing livestock (Klotz et al., 2007). As endophytes grow in the intercellular plant tissue, the presence of endophytes within the plants may in itself present a physical barrier to feeding by phloem-feeding hemipterans, such as aphids. This barrier could impede hemipteran stylet movement and feeding behaviour, thus resulting in feeding difficulties or deterrence (Christensen et al., 2002).

Rhopalosiphum padi (L.) is a common phloem-feeding pest of grains and pasture grasses (Leather and Dixon, 1982). It is often found in perennial ryegrass dairy pastures, where it can cause significant damage through direct feeding and by vectoring viral plant diseases such as barley yellow dwarf virus (BYDV) (Debarro and Maelzer, 1993; Latch, 1977). *Rhopalosiphum padi* reproduce asexually through parthenogenesis, and a single female can produce an average of 2–3 nymphs per day when well-nourished (Taheri et al., 2010).This results in very rapid population growth under favourable climatic conditions when temperatures range from 20–25°C (Dean, 1974). Aphids such as *R. padi* feed by inserting anatomically adapted mouthparts (stylets) into plant tissues and probing and exploring plant tissue for nutrient-rich phloem sap (Auclair, 1963; Pollard, 1973). This feeding behaviour can be negatively affected by different insecticide treatments that deter aphids from the phloem (Johnson et al., 1985). Monitoring changes in aphid feeding behaviour can be an effective way to identify the mode of action of different insecticides.

One of the most effective methods for monitoring and quantifying changes in aphid feeding behaviour is the Electrical Penetration Graph (EPG) technique (Tjallingii, 1978). The EPG technique was developed for the purpose of studying hemipteran feeding behaviour (McLean and Kinsey, 1964), and has been used and improved upon in the years since to study the feeding behaviour of aphids (Tjallingii, 1988) and psyllids (Bonani et al., 2010). The technique is used to study the movement of an aphids' stylet within the plant tissue through the creation of an electrical circuit between an electrode attached to the dorsum of a feeding aphid and an electrode inserted into the soil of the plant the insect is feeding on. Variations in the location of the stylet tip in the plant tissue result in fluctuations in electrical potential in this circuit, which are represented as different recognisable waveform patterns using a detection device and specialised software (Giordanengo, 2014; Tjallingii, 2020). Specific waveform patterns correlate to different specific feeding and probing behaviours, such as phloem-ingestion or xylem-ingestion (Kimmins and Tjallingii, 1985; Tjallingii, 1988, 1985; Tjallingii and Esch, 1993). Therefore, changes in the electrical potential in the circuit can be monitored to assess stylet movements

within plant tissue (Walker, 2000). The use of this technique allows for the quantification of the frequency and duration of aphid feeding behaviour on different host plants or when aphids are exposed to different treatments (Tjallingii and Esch, 1993). This has practical uses in the study of insecticides as, for example, insecticides that cause feeding deterrence effects may result in waveforms showing fewer phloem ingestion events or events of a shorter duration from aphids feeding on treated plants (Garzo et al., 2016).

The main aim of this study was to investigate the effects of five different *Epichloë*–perennial ryegrass symbiota on *R. padi* feeding behaviour and life history. These treatments included four commercial endophytes (AR1, AR37, NEA2 and NEA6), as well as the wild type (Standard Endophyte – SE) and two controls (perennial ryegrass without endophyte – WE – and barley). An additional aim of this study was to assess the effectiveness of the EPG technique for differentiating between endophyte treatments, specifically with regards to their effects on aphid feeding behaviour, as part of a wider study to determine effective methods for benchmarking novel endophyte symbiota (Collinson et al., 2020). This technique could provide useful insights into the potential antifeedant mode of action of insecticidal fungal endophytes in perennial ryegrass. There is little specific research on the effects of endophytes on *R. padi* feeding behaviour using EPG, and what research there is has mostly investigated only a single endophyte strain (Bastias et al., 2017).

Methods

Aphids, endophytes and plants

Rhopalosiphum padi were collected in South Eastern Australia on oat, *Avena sativa* (L), in June 2009 (Table 1). Populations were previously used in experimentation on transmission of BYDV, so in order to ensure BYDV was not present in experimental populations ten individuals of *R. padi* were isolated and reared on separate barley and perennial ryegrass seedlings for a minimum of three generations, with nymphs removed to new seedlings immediately after birth to eliminate any possible transmission of aphid-borne plant viruses (Ajayi and Dewar, 1983; Ridland et al., 1988). Nymphs were removed and transferred using a fine paintbrush, by gently touching the aphid until it withdrew its stylet, then picking it up with the paintbrush and placing it onto the new seedling. One clonal lineage was selected for

further experiments. Colonies were maintained on barley and perennial ryegrass in 24.5x24.5x63.0cm BugDorm 42260F insect rearing cages with a mesh size of 150x150µm (Megaview Science, Taiwan), at 20±2° C and 62.0±5 % RH, and a photoperiod of 14 hrs light–10 hrs dark.

To confirm aphid species identification, a molecular based identification method was carried out using the barcode region of the cytochrome oxidase subunit 1 (CO1) gene (Folmer et al., 1994). Aphid DNA was extracted using Bio-Rad Chelex© 100 Resin following the method of Walsh et al., (1991), with minor modifications: individual aphids were placed in 1.5 ml Eppendorf tubes containing 2 glass beads each, and 20 µl of Proteinase K. Aphids were crushed in a mixer mill for 1 min at 30 hz., then 100 µl of 5% Chelex© 100 (BioRad) was added and the extract was incubated at 55°C for 1 hr, then at 85°C for 8 min. Extractions were centrifuged, and the supernatant used for PCR. A portion of the CO1 gene was amplified using the primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al., 1994). The PCR was performed in 25 µL reaction volumes containing: 1x bovine serum albumin (NEB), 10x Immobuffer (Bioline), 2.5 mM dNTP (Qiagen), 10 µM of each primer, 5 units/µL of Immolase DNA polymerase (Bioline) and 5 µL of template DNA. The cycling conditions were: 94°C for 7 min, 40 cycles of 94°C for 30 sec, 51°C for 50 sec, 72°C for 50 sec, followed by 2 min at 72°C. PCR products were sequenced by Macrogen Inc (Seoul, Korea) and sequences were compared to public databases (NCBI BLASTn), determining 100% similarity and coverage with previously identified species. Sequences were submitted to GenBank (Table 1) and specimens preserved in 70% and 100% ethanol were deposited in the Victorian Agricultural Insect Collection (VAIC) at Agribio, Bundoora. Aphids were confirmed as *R. padi*, Rp1 haplotype, based on 100% sequence similarity with previously sequenced populations from Australia (Valenzuela et al., 2010).

Plants were germinated as per methods described in Collinson et al. (2020). Perennial ryegrass seeds infected with fungal endophytes of the species *Epichloë festucae* var. *lolii* were sourced from Agriseeds (Christchurch, New Zealand). These included four different commercial endophyte varieties (AR1, AR37, NEA2 and NEA6) and one wild type or Standard endophyte (SE), which are found naturally in many perennial ryegrass pastures (Clay et al., 1985), and a control of perennial ryegrass without endophyte (WE) (Table 2). These *Epichloë* endophytes produce a range of alkaloids [Indole Diterpenes (Lolitrem B and Janthitrem I), Ergopeptides (Ergovaline) and Polyketides (Peramine)], resulting in each endophyte having a unique alkaloid profile (Table 2).

Feeding behaviour bioassays

EPG setup and methodology

The Electrical Penetration Graph (EPG) technique was used to monitor the feeding behaviour of adult *R. padi* on seven-day old perennial ryegrass seedlings both with and without endophytes (SE, AR1, AR37, NEA2, NEA6 and WE).

To create the circuit for EPG recordings, electrodes were constructed by attaching a 10–20 mm length of 18 µm thick gold wire to a 20 mm length of copper wire using a water based glue with suspended conductive silver particles. The copper wire was attached at the opposite end to the head of a copper nail using lead solder. The plant and the aphid were made part of an electrical circuit by first attaching the gold wire electrode to the aphid dorsum using the same conductive glue. This was done by securing the aphid to a plastic pipette tip attached to a mild vacuum device (Javac Pty. Ltd., Australia) and applying a small drop of the glue to the aphid dorsum and holding the tip of the gold wire in place until the glue cured. The first fully unfurled leaf of each seedling was affixed to a plastic tag using a metal clamp and foam padding to limit movement (Fig. 1). The copper nail ends of the electrodes were connected to the EPG Giga8 device via the input of the head stage amplifier with a 1 giga-ohm input resistance and 50× gain (EPG Systems, The Netherlands). To complete the circuit copper soil probes, 2 mm thick x 10 cm long copper rods, were inserted into the soil of seven-day old perennial ryegrass seedlings and connected to the plant voltage output plug of the EPG device. The aphid inserting its stylet into the plant (probing) closed the circuit, and the resulting electrical signals were recorded. A total of eight probes were run at any one time to create a total of 20–24 replicates per treatment. Measurements were performed during the first 12 hours of contact between aphids and the test plants. Experiments were conducted in a Faraday cage and maintained under laboratory conditions (20 ± 2 °C and constant light).

EPG waveforms and variables.

Seven different EPG waveforms were identified to relate to different aphid probing, feeding and non-probing activities. For probing, C represents the first electrical stylet contact with the epidermis, and

intercellular sheath salivation in epidermis and mesophyll, pd represents the drop in electrical potential observed when brief intracellular punctures by the stylet occur, and F represents stylet derailment – where the stylet components becoming separated from each other – or other stylet penetration difficulties. For feeding, E1 represents the sieve element salivation directly prior to phloem ingestion, E2 represents phloem sap ingestion (i.e. feeding), and G represents the ingestion of xylem sap as opposed to phloem. For non-probing, Np represents the non-probing period when the stylet is withdrawn and probing is not taking place (Tjallingii, 2000). Waveforms were recorded using the Stylet-D software package, and identification and analysis were conducted using the Stylet-A software package (EPG Systems, The Netherlands), and compared to known examples representing different aphid feeding behaviours (Tjallingii, 2000).

Life history bioassays

Apterae aphids reared on endophyte-free perennial ryegrass (WE) were individually placed in 4 ml clear plastic cups (Olympus Packaging, Pty. Ltd. Australia), each with a single WE perennial ryegrass seedling and monitored every day to collect new-born nymphs as per the methods in Collinson et al. (2020). Bioassays were conducted to assess *R. padi* life history on different endophyte treatments as per the methods in Collinson et al. (2020). In brief, 24 new-born nymphs per treatment were collected using a fine paintbrush and placed individually into new plastic cups with seven-day old seedlings of endophyte free (WE) or endophyte-treated (SE, AR1, AR37, NEA2, NEA6) perennial ryegrass. Barley (cv. Hindmarsh) was included as an additional control and followed the same germination and monitoring protocols as for perennial ryegrass (data not shown). The parameters investigated in this study were a) fecundity – number of nymphs born over a 30-day period, b) mortality – life expectancy up to 30 days, and c) intrinsic rate of increase (r_m). Fecundity was measured by counting the number of nymphs born every 24 hours for 30 days from the moment of birth, mortality was measured by recording the number of aphids that died each day up to 30 days, and r_m was calculated using the formula $r_m = 0.74(\log_e M_d)/d$, where d = the length of the pre-reproductive period and M_d = the number of progeny produced in a period of time equal to d (Wyatt and White, 1977). Aphid life history was monitored every day for 30 days and aphids were transferred onto a new seedling every seven days. All experiments were carried out in a controlled environment room at 20±2°C and 62.0±5%RH, and a

photoperiod of 14 hrs light–10 hrs dark. Thirty days was chosen for the duration of the assay as it represents a major portion of the average reproductive lifespan of *R. padi* on barley at 20°C (Auad et al., 2009).

Data analyses

To analyse EPG data aphid feeding behaviour was recorded using the Stylet+ software package (EPG Systems, The Netherlands), and quantified by 102 separate metrics to define the seven major waveforms using a specialised quantification package in Microsoft Excel (CSIC, Spain). Numerical data categorised by these metrics was analysed using a one-way analysis of variance. Differences between treatment means were examined using the least significant difference (LSD) at a 5% level of significance. The unit of analysis was individual aphids connected to EPG electrodes (up to 24 replicates per treatment). Residuals versus fitted values plots were examined to determine any need for data transformation to ensure the normality of residuals with constant variance. When data transformation was required due to non-normally distributed data, a log transformation was performed, and transformed data was analysed using a one-way analysis of variance as above.

For aphid life history analysis, *R. padi* nymph mortality was grouped into four time periods: 0–24 (24 hrs), 24–48 (48 hrs), 48–72 (72 hrs) and >72 (>72 hrs) hours. Adult mortality was grouped into three time periods: 14, 21 and 28 days. Daily observed fecundity data was grouped into three time periods: 14, 21 and 28 days. Fecundity and r_m were both calculated using only aphids that survived to reproduction. The difference in mortality of aphid nymphs and adults between treatments at each time period was analysed using logistic regression models, where the number of aphid deaths at each time period was the response variable (success) and logit was the link function. The Wald chi-squared test was used to include/exclude a term in the model. Data on fecundity and r_m was analysed using a one-way analysis of variance. Differences between treatment means were examined using the least significant difference (LSD) at a 5% level of significance. The unit of analysis was individual aphids in plastic cups (up to 24 replicates). Residuals versus fitted values plots were examined to determine any need for data transformation to ensure the normality of residuals with constant variance. All data in this study was analysed using GenStat version 18 (VSN International, 2015).

Correlation coefficients were determined using the Data Analysis function in Microsoft Excel (Microsoft, USA) to conduct a Regression Analysis.

Results

Feeding behaviour bioassays

The feeding behaviour of *R. padi* showed a significant difference between endophyte treatments and the control (WE) for eight of the 102 metrics describing the seven major waveforms (Table 3). For probing, the number of potential drops (pd) was significantly lower for AR37, NEA6 and NEA2, compared to AR1 and WE, with 31–49% fewer events. Likewise, the total time of pd was significantly lower for AR37, NEA6 and NEA2, compared to AR1 and WE, with 49%–55% less time spent in pd. Additionally, the percentage of probe time spent in intercellular probing (C) was significantly lower on NEA6, NEA2 and AR37 compared to AR1 and WE, with a 32–38% reduction. Furthermore, the total C time was significantly lower for NEA6, NEA2 and AR37, with 31–39% less time spent in C. No difference in stylet derailment (F) was observed in any treatment.

For feeding, the number of sieve element salivations directly prior to phloem ingestion (E1) was significantly lower for NEA2, NEA6 and AR37 compared to WE, with 32–49% fewer events. The number of phloem sap ingestion (E2) events was significantly lower for NEA2, NEA6 and AR37, with 33–48% fewer events. Furthermore, the average duration of the first E1 event was significantly longer for AR37, NEA2, NEA6 and SE compared to WE, with 29–42% more time spent in the first E1 event. No difference in xylem ingestion (G) was observed in any treatment.

For the non-probing period (Np), the median Np time was significantly longer for NEA6 compared to SE, AR37 and WE, with 46–65% more time spent in Np.

Life history bioassays

The average mortality of *R. padi* at the nymphal stage was 26% on endophyte-infected plants compared to 8% on the control (Table 4). The total nymph mortality was highest on SE and AR37 (33% mortality), followed by NEA6 (25%), AR1 (21%) and NEA2 (17%). While there was no significant difference in

overall nymph mortality (P = 0.223) there was a trend showing higher nymph mortality on endophyte treatments compared to WE, with the largest difference (0.24) observed between both SE and AR37 and WE. Average nymphal mortality on endophyte treatments was highest >72 hours (15%) and lowest at 72 hours (0%). The only significant difference in nymph mortality was observed at 48 hours, where SE and AR37 (both 13%) showed significantly (P = 0.042) higher mortality than the control (0%).

The average adult mortality was 74% across all endophyte treatments compared to 92% on the control. At 14, 21 and 28 days there was no significant difference in mortality between treatments. There was a significant (P = 0.031) difference in adult mortality at >28-days with the highest mortality observed on WE (0.75) and the lowest on SE (0.29).

The average fecundity was 16.7 nymphs per adult aphid per day across all endophyte treatments compared to 21.4 on the control (Table 5). Fecundity at 14 days was significantly reduced on all endophyte treatments (P = 0.003) compared to the control, with AR37 and NEA2 both exhibiting the strongest reduction in fecundity (both 8.8), followed by SE (9.1), AR1 (10.2) and NEA6 (10.3). Fecundity at 21 days was significantly reduced on all endophyte treatments (P = 0.041) compared to the control (20.6), with NEA2 exhibiting the strongest reduction in fecundity (14.2), followed by SE (14.3), AR37 (14.6), AR1 (15.2) and NEA6 (16.8). There was no significant difference between the control and any endophyte treatments at 28 days, however the trend continued showing higher fecundity on the control. There was no significant difference between endophyte treatments at any time period, however, NEA2 had consistently the lowest fecundity at all time periods (Table 5).

The average intrinsic rate of increase (r_m) was 0.27 across all endophyte treatments compared to 0.33 on the control. AR37 exhibited the strongest reduction in r_m (0.24), followed by SE and AR1 (both 0.26), NEA2 (0.28) and NEA6 (0.30). No endophyte treatments exhibited significantly reduced r_m compared to the control (P = 0.057), however, all endophyte treatments exhibited lower average r_m than the control (Table 5).

Correlation between feeding behaviour and life history

There was a strong positive correlation between both the probing metric (Percentage of probe time in C, R = 0.79) and the feeding metric (No. of E2 events, R = 0.72), with fecundity (Table 6). There was no strong positive or negative correlation between any other EPG metric with fecundity. There was no

strong positive or negative correlation between nymphal mortality, adult mortality or r_m with any EPG metrics.

Discussion

Overview

There was a clear endophyte effect on *R. padi* feeding behaviour and life history. Fungal endophytes are known to have insecticidal properties, but the mode of action of their effect is still elusive. The electrical penetration graph technique was used to investigate whether endophyte infection in perennial ryegrass affected *R. padi* feeding behaviour, indicating some form of feeding deterrence. Analysis of aphid feeding behaviour on endophyte-infected perennial ryegrass using EPG has not been reported before, though this technique has been used to investigate the feeding behaviour of *R. padi* on annual ryegrass infected with endophytes (Bastias et al., 2017), and to investigate the effects of other factors such as nitrogen, water-stress, insecticides or host plant on *R. padi* feeding behaviour (Daniels et al., 2009; Nam and Hardie, 2012; Ponder et al., 2001).

Feeding deterrence

Assessments of endophyte mode of action using EPG experiments showed that *R. padi* reared on perennial ryegrass infected with the endophytes AR37, NEA2 or NEA6 spent less time probing (C) and feeding (E2) within plant tissue, and more time in the non-probing phase (Np), with their stylets withdrawn, compared to the control, SE and AR1. These results indicate a level of feeding deterrence induced by AR37, NEA2 and NEA6, as they all point to both delayed and reduced feeding by *R. padi*. The endophytes assessed in this study have been shown to have varying insecticidal effects on other aphid species, which have been predominantly linked to the alkaloids lolitrem B and ergovaline, produced by SE and NEA2 (Collinson et al., 2020). However, the endophytes that showed the strongest effect on aphid feeding behaviour, AR37, NEA2 and NEA6, had alternate alkaloid profiles, with NEA2 producing lolitrem B, ergovaline and peramine, NEA6 producing ergovaline and peramine, and AR37 producing janthitrem I (Ekanayake et al., 2017; Gerhards et al., 2014; Ludlow et al., 2019). Furthermore, given that SE produces the same alkaloids as NEA2 at higher concentrations (Collinson et al.

unpublished data), but did not have a significant effect on *R. padi* feeding behaviour, this implies that a compound other than the known alkaloids may be contributing to aphid feeding deterrence effects. A possibility could be elevated concentrations of an indole diterpene precursor, given that AR37, NEA2 and NEA6 have the genes required for certain sections of the indole diterpene alkaloid biosynthesis pathway (data not shown). A common precursor between these endophytes could be paspaline, which has been shown to have insecticidal activity (Reddy et al., 2019b; Saikia et al., 2006). Paspaline could be a good candidate for further investigation into its insecticidal properties and importance for pasture production, given that there have been no neurotoxic effects associated with this compound (Reddy et al., 2019b, 2019a). Another possibility could be a completely unknown insecticidal compound shared by these three endophytes, which would also warrant further investigation.

Interestingly, despite *R. padi* having high mortality on SE, this endophyte did not affect feeding behaviour. As mentioned, SE produces the same alkaloids as NEA2, but in higher concentrations (Collinson et al. unpublished data). This implies that SE may be producing additional factors that suppress any feeding deterrent effects associated with the alkaloids produced (Molyneux and Ralphs, 1992). This may be a compound within the endophyte or bound to the alkaloid, that masks its unpalatability (Jani et al., 2010), or a structural change to the alkaloid molecule that alters the palatability while retaining toxic effects (Molyneux and Ralphs, 1992)

Another possibility could be that the endophyte produces an attractant to aphids that has a stronger affect than the deterrent effects of the alkaloids. Fungi have been shown to produce volatile organic compounds (VOCs) that act as strong attractants to insects, including aphids (Boucias et al., 2012; Davis and Landolt, 2013). Such compounds include Methl(Z)-3-methyldodec-2-enoate, chokol K, which attracts *Botanophila* flies; or phenyl ethyl alcohol, which attracts Dipteran pollinators (Boucias et al., 2012). Studies have also shown that fungi can improve the host plant production of VOCs that often act as attractants to aphid predators, which has also been demonstrated in *Epichloë*-grass symbiota (Fuchs and Krauss, 2019). Despite the effects of SE on *R. padi* feeding behaviour remaining unclear, there is evidence to suggest that this endophyte plays a pivotal role in the fitness of perennial ryegrass, and the tri-trophic interaction with aphids.

The utilisation of EPG to study feeding deterrence in aphids is justified, given that the technique was designed to investigate feeding behaviour (McLean and Kinsey, 1964). Many EPG studies have used mature plants (Bastias et al., 2017; Bonani et al., 2010; Garzo et al., 2016), however, the current

study demonstrated that seedlings can be used as well to investigate endophyte effects on aphid feeding. Other research has determined that *R. padi* feeding was deterred when feeding on tall fescue infected with *Epichloë coenophialum* endophytes (Guy and Davis, 2002) and *R. padi* feeding behaviour has also been delayed on water and nitrogen stressed barley plants, where aphids spent longer in the salivatory period prior to feeding (Ponder et al., 2001). Bastias et al. (2017b) investigated feeding behaviour of *R. padi* on *Epichloë*-infected annual ryegrass; however, they found minimal endophyte effects on stylet movement and phloem ingestion, but did see significant effects on *R. padi* life history, particularly fecundity, consistent with our findings.

Life history effects

Rhopalosiphum padi fecundity was significantly reduced on all endophyte treatments particularly in the early–mid stages of reproduction. This indicates not only a reduction in overall aphid fecundity, but also a slowing of reproduction, which could lead to slower population growth. This was observed by Meister et al. (2006) who found that *R. padi* showed reduced population growth and reduced fecundity when reared on endophyte-infected perennial ryegrass compared to those reared on endophyte-free perennial ryegrass. In another study, *R. padi* showed similarly reduced population growth when reared on tall fescue infected with fungal endophytes (Züst et al., 2008). The reduction in overall aphid fecundity, coupled with the reduced feeding seen in the feeding behaviour experiments indicate that adult *R. padi* may not have been receiving sufficient nutrition in order to reproduce at their optimal rate, which has been observed in *R. padi* feeding on barley (Ponder et al., 2000). The reduced fecundity could also reflect direct endophyte toxicity on unborn aphid nymphs, resulting in stillborn nymphs (Collinson et al., 2020). Bastias et al. (2017) also showed that *R. padi* fecundity is negatively affected by the presence of endophytes in annual ryegrass, and postulated that this activity was due to direct toxicity. The direct negative effect of *Epichloë* endophytes on aphid fecundity clearly demonstrates its importance in managing aphid populations and broader insect control in pastures.

Rhopalosiphum padi nymphs showed higher mortality on endophyte-infected perennial ryegrass compared to control plants at all time periods. While these results were not statistically significant at all time periods, they do show a trend that conforms with previous research with *R. padi* on endophyte-infected grasses (Bastias et al., 2017; Eichenseer et al., 1991). Furthermore, nymph

mortality was significantly higher on SE and AR37 at 48 hours, similar to the results in Collinson et al. (2020), where SE was shown to result in the highest nymph mortality in the Russian wheat aphid, *Diuraphis noxia* (Kurdjumov), and the root aphid *Aploneura lentisci* (Passerini). Multiple studies have shown that *R. padi* experience higher mortality on different varieties of *Epichloë* endophytes, implying that each endophyte delivers a different insecticidal activity (Bastias et al., 2017; Eichenseer et al., 1991; Wilkinson et al., 2000). The results of this study also showed a significant difference in mortality of adult *R. padi* at 28 days, where mortality was significantly higher on control plants, indicating a normal *R. padi* life span, as opposed to the endophyte treatments where mortality was greatest in the nymphal stage (Auad et al., 2009; Descamps and Chopa, 2011).

Translation to field performance

The results of the feeding behaviour bioassays indicate that *R. padi* were significantly deterred from feeding on perennial ryegrass infected with the alkaloids AR37, NEA2 and NEA6. Likewise, the results from the life history bioassays indicated that *R. padi* showed significantly reduced fecundity on these same endophyte treatments. There was a strong correlation between these metrics, indicating that fecundity is strongly influenced by nutritional intake and therefore a reduction in feeding will directly result in reduced reproduction. Furthermore, there was an increase in *R. padi* nymphal mortality on *Epichloë*–perennial ryegrass symbiota, indicating a direct toxic effect in addition to feeding deterrence. These effects cannot be directly attributed to any known alkaloid profile associated with AR37, NEA2 or NEA6, as there are no known alkaloids shared by all three endophytes. This indicates that an additional, unknown factor is resulting in increased feeding deterrence. Nevertheless, the feeding deterrence and life history effects of these endophytes have practical benefits in dairy pastures where they can be used to reduce population growth of *R. padi* in fields, decreasing pest pressure on pasture systems thus allowing for a reduction in the use of other insecticides. These results show that these endophytes can play a pivotal role in the broader pest management scheme of *R. padi* in dairy pasture production.

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References

Ajayi, B.O., Dewar, A.M., 1983. The effect of barley yellow dwarf virus on field populations of the cereal aphids, *Sitobion avenae* and *Metopolophium dirhodum*. Annals of Applied Biology 103, 1–11. https://doi.org/10.1111/j.1744-7348.1983.tb02734.x

Auad, A.M., Alves, S.O., Carvalho, C.A., Silva, D.M., Resende, T.T., Veríssimo, B.A., 2009. The Impact of Temperature on Biological Aspects and Life Table of *Rhopalosiphum padi* (Hemiptera: Aphididae) Fed with Signal Grass. Florida Entomologist 92, 569–577. https://doi.org/10.1653/024.092.0406

Auclair, J.L., 1963. Aphid Feeding and Nutrition. Annual Review of Entomology 8, 439–490. https://doi.org/10.1146/annurev.en.08.010163.002255

Ball, O.J.-P., Miles, C.O., Prestidge, R.A., 1997. Ergopeptine Alkaloids and *Neotyphodium lolii*-Mediated Resistance in Perennial Ryegrass Against Adult Heteronychus arator (Coleoptera: Scarabaeidae). J Econ Entomol 90, 1382–1391. https://doi.org/10.1093/jee/90.5.1382

Bastias, D.A., Ueno, A.C., Machado Assefh, C.R., Alvarez, A.E., Young, C.A., Gundel, P.E., 2017. Metabolism or behavior: explaining the performance of aphids on alkaloid-producing fungal endophytes in annual ryegrass (*Lolium multiflorum*). Oecologia 185, 245–256. https://doi.org/10.1007/s00442-017-3940-2

Bonani, J.P., Fereres, A., Garzo, E., Miranda, M.P., Appezzato-Da-Gloria, B., Lopes, J.R.S., 2010. Characterization of electrical penetration graphs of the Asian citrus psyllid, *Diaphorina citri*, in sweet orange seedlings. Entomologia Experimentalis et Applicata 134, 35–49. https://doi.org/10.1111/j.1570-7458.2009.00937.x

Boucias, D.G., Lietze, V.-U., Teal, P., 2012. Chemical Signals That Mediate Insect-Fungal Interactions, in: Witzany, G. (Ed.), Biocommunication of Fungi. Springer Netherlands, Dordrecht, pp. 305–336. https://doi.org/10.1007/978-94-007-4264-2_20

Christensen, M.J., Bennett, R.J., Schmid, J., 2002. Growth of Epichloë / Neotyphodium and p-endophytes in leaves of *Lolium* and *Festuca* grasses. Mycological Research 106, 93–106. https://doi.org/10.1017/S095375620100510X

Collinson, N.P., Mann, R.C., Giri, K., Malipatil, M., Kaur, J., Spangenberg, G., Valenzuela, I., 2020. Novel bioassay to assess antibiotic effects of fungal endophytes on aphids. PLOS ONE 15, e0228813. https://doi.org/10.1371/journal.pone.0228813

438 Collinson, N.P., Valenzuela, I., Giri, K., Malipatil, M., Vassiliadis, S., Spangenberg, G., Mann, R.C.,
439 2020. Effects of fungal endophyte alkaloids on the life history of *Metopolophium dirhodum*
440 (Walker) on perennial ryegrass. Manuscript submitted for publication.

441 Daniels, M., Bale, J.S., Newbury, H.J., Lind, R.J., Pritchard, J., 2009. A sublethal dose of thiamethoxam
442 causes a reduction in xylem feeding by the bird cherry-oat aphid (*Rhopalosiphum padi*), which
443 is associated with dehydration and reduced performance. Journal of Insect Physiology 55, 758–
444 765. https://doi.org/10.1016/j.jinsphys.2009.03.002

445 Davis, T.S., Landolt, P.J., 2013. A Survey of Insect Assemblages Responding to Volatiles from a
446 Ubiquitous Fungus in an Agricultural Landscape. J Chem Ecol 39, 860–868.
447 https://doi.org/10.1007/s10886-013-0278-z

448 Dean, G.J., 1974. Effect of temperature on the cereal aphids Metopolophium dirhodum (Wlk.),
449 *Rhopalosiphum padi* (L.) and *Macrosiphum avenue* (F.) (Hem., Aphididae). Bulletin of
450 Entomological Research 63, 401. https://doi.org/10.1017/S0007485300040888

451 Debarro, P.J., Maelzer, D.A., 1993. Influence of High-Temperatures on the Survival of Rhopalosiphum-
452 Padi (L) (Hemiptera, Aphididae) in Irrigated Perennial Grass Pastures in South-Australia. Aust.
453 J. Zool. 41, 123–132. https://doi.org/10.1071/zo9930123

454 Descamps, L.R., Chopa, C.S., 2011. Population growth of *Rhopalosiphum padi* L. (Homoptera:
455 Aphididae) on different cereal crops from the semiarid Pampas of Argentina under laboratory
456 conditions. https://dx.doi.org/10.4067/S0718-58392011000300007

457 Eichenseer, H., Dahlman, D.L., Bush, L.P., 1991. Influence of endophyte infection, plant age and
458 harvest interval on *Rhopalosiphum padi* survival and its relation to quantity of N-formyl and N-
459 acetyl loline in tall fescue. Entomologia Experimentalis et Applicata 60, 29–38.
460 https://doi.org/10.1111/j.1570-7458.1991.tb01519.x

461 Ekanayake, P.N., Kaur, J., Tian, P., Rochfort, S.J., Guthridge, K.M., Sawbridge, T.I., Spangenberg,
462 G.C., Forster, J.W., 2017. Genomic and metabolic characterisation of alkaloid biosynthesis by
463 asexual Epichloe fungal endophytes of tall fescue pasture grasses. Genome.
464 https://doi.org/10.1139/gen-2016-0173

465 Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R., 1994. DNA primers for amplification of
466 mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates 3, 294–299.

Fuchs, B., Krauss, J., 2019. Can *Epichloë* endophytes enhance direct and indirect plant defence? Fungal Ecology, From antagonism to mutualism: the chemical basis of insect-fungus interactions 38, 98–103. https://doi.org/10.1016/j.funeco.2018.07.002

Garzo, E., Moreno, A., Hernando, S., Mariño, V., Torne, M., Santamaria, E., Díaz, I., Fereres, A., 2016. Electrical penetration graph technique as a tool to monitor the early stages of aphid resistance to insecticides. Pest Management Science 72, 707–718. https://doi.org/10.1002/ps.4041

Gerhards, N., Neubauer, L., Tudzynski, P., Li, S.-M., 2014. Biosynthetic Pathways of Ergot Alkaloids. Toxins 6, 3281–3295. https://doi.org/10.3390/toxins6123281

Giordanengo, P., 2014. EPG-Calc: a PHP-based script to calculate electrical penetration graph (EPG) parameters. Arthropod-Plant Interactions 8, 163–169. https://doi.org/10.1007/s11829-014-9298-z

Guy, P.L., Davis, L.T., 2002. Variation in the incidence of Barley yellow dwarf virus and in the ability of Neotyphodium endophytes to deter feeding by aphids (*Rhopalosiphum padi*) on Australasian tall fescue. Australasian Plant Pathology 31, 307–308.

Heeswijck, R. van, McDonald, G., 1992. *Acremonium* endophytes in perennial ryegrass and other pasture grasses in Australia and New Zealand. Aust. J. Agric. Res. 43, 1683–1709. https://doi.org/10.1071/ar9921683

Jani, A.J., Faeth, S.H., Gardner, D., 2010. Asexual endophytes and associated alkaloids alter arthropod community structure and increase herbivore abundances on a native grass. Ecology Letters 13, 106–117. https://doi.org/10.1111/j.1461-0248.2009.01401.x

Johnson, M.C., Dahlman, D.L., Siegel, M.R., Bush, L.P., Latch, G.C.M., Potter, D.A., Varney, D.R., 1985. Insect Feeding Deterrents in Endophyte-Infected Tall Fescue. Appl. Environ. Microbiol. 49, 568–571.

Kimmins, F.M., Tjallingii, W.F., 1985. Ultrastructure of sieve element penetration by aphid stylets during electrical recording. Entomologia Experimentalis et Applicata 39, 135–141. https://doi.org/10.1111/j.1570-7458.1985.tb03554.x

Klotz, J.L., Bush, L.P., Smith, D.L., Shafer, W.D., Smith, L.L., Arrington, B.C., Strickland, J.R., 2007. Ergovaline-induced vasoconstriction in an isolated bovine lateral saphenous vein bioassay. Journal of Animal Science 85, 2330–2336. https://doi.org/10.2527/jas.2006-803

496 Latch, G.C.M., 1977. Incidence of barley yellow dwarf virus in ryegrass pastures in New Zealand. New
497 Zealand Journal of Agricultural Research 20, 87–89.
498 https://doi.org/10.1080/00288233.1977.10427308
499 Leather, S.R., Dixon, A.F.G., 1982. Secondary host preferences and reproductive activity of the bird
500 cherry-oat aphid, *Rhopalosiphum padi*. Annals of Applied Biology 101, 219–228.
501 https://doi.org/10.1111/j.1744-7348.1982.tb00816.x
502 Ludlow, E.J., Vassiliadis, S., Ekanayake, P.N., Hettiarachchige, I.K., Reddy, P., Sawbridge, T.I.,
503 Rochfort, S.J., Spangenberg, G.C., Guthridge, K.M., 2019. Analysis of the Indole Diterpene
504 Gene Cluster for Biosynthesis of the Epoxy-Janthitrems in Epichloë Endophytes.
505 Microorganisms 7, 560. https://doi.org/10.3390/microorganisms7110560
506 McLean, D.L., Kinsey, M.G., 1964. A Technique for Electronically Recording Aphid Feeding and
507 Salivation. Nature 202, 1358. https://doi.org/10.1038/2021358a0
508 Meister, B., Krauss, J., Härri, S.A., Victoria Schneider, M., Müller, C.B., 2006. Fungal endosymbionts
509 affect aphid population size by reduction of adult life span and fecundity. Basic and Applied
510 Ecology 7, 244–252. https://doi.org/10.1016/j.baae.2005.06.002
511 Molyneux, R.J., Ralphs, M.H., 1992. Plant toxins and palatability to herbivores. Journal of Range
512 Management 45, 13–18.
513 Nam, K.J., Hardie, J., 2012. Host acceptance by aphids: Probing and larviposition behaviour of the bird
514 cherry-oat aphid, *Rhopalosiphum padi* on host and non-host plants. Journal of Insect
515 Physiology 58, 660–668. https://doi.org/10.1016/j.jinsphys.2012.01.017
516 Ondeyka, J.G., Helms, G.L., Hensens, O.D., Goetz, M.A., Zink, D.L., Tsipouras, A., Shoop, W.L.,
517 Slayton, L., Dombrowski, A.W., Polishook, J.D., Ostlind, D.A., Tsou, N.N., Ball, R.G., Singh,
518 S.B., 1997. Nodulisporic Acid A, a Novel and Potent Insecticide from a *Nodulisporium* Sp.
519 Isolation, Structure Determination, and Chemical Transformations. J. Am. Chem. Soc. 119,
520 8809–8816. https://doi.org/10.1021/ja971664k
521 Pollard, D.G., 1973. Plant penetration by feeding aphids (Hemiptera, Aphidoidea): a review. Bulletin of
522 Entomological Research 62, 631–714. https://doi.org/10.1017/S0007485300005526
523 Ponder, K.L., Pritchard, J., Harrington, R., Bale, J.S., 2001. Feeding behaviour of the aphid
524 *Rhopalosiphum padi* (Hemiptera: Aphididae) on nitrogen and water-stressed barley (Hordeum

vulgare) seedlings. Bulletin of Entomological Research 91, 125–130. https://doi.org/10.1079/BER200072

Ponder, K.L., Pritchard, J., Harrington, R., Bale, J.S., 2000. Difficulties in location and acceptance of phloem sap combined with reduced concentration of phloem amino acids explain lowered performance of the aphid *Rhopalosiphum padi* on nitrogen deficient barley (Hordeum vulgare) seedlings. Entomologia Experimentalis et Applicata 97, 203–210. https://doi.org/10.1046/j.1570-7458.2000.00731.x

Popay, A.J., Wyatt, R.T., 1995. Resistance to Argentine stem weevil in perennial ryegrass infected with endophytes producing different alkaloids. Proceedings of the Forty Eighth New Zealand Plant Protection Conference, Angus Inn, Hastings, New Zealand, August 8-10, 1995. 229–236.

Reddy, P., Deseo, M.A., Ezernieks, V., Guthridge, K., Spangenberg, G., Rochfort, S., 2019a. Toxic Indole Diterpenes from Endophyte-Infected Perennial Ryegrass *Lolium perenne* L.: Isolation and Stability. Toxins 11, 16. https://doi.org/10.3390/toxins11010016

Reddy, P., Guthridge, K., Vassiliadis, S., Hemsworth, J., Hettiarachchige, I., Spangenberg, G., Rochfort, S., 2019b. Tremorgenic Mycotoxins: Structure Diversity and Biological Activity. Toxins 11, 302. https://doi.org/10.3390/toxins11050302

Ridland, P.M., Sward, R.J., Tomkins, R.B., 1988. A simple rearing system for cereal aphids, especially suited to transmission studies with barley yellow dwarf viruses. Australasian Plant Pathology 17, 17–19.

Rogers, M.E., Lawson, A.R., Kelly, K.B., 2019. Summer production and survival of perennial ryegrass (*Lolium perenne*) and tall fescue (*Festuca arundinacea*) genotypes in northern Victoria under differing irrigation management. Crop Pasture Sci. https://doi.org/10.1071/CP18542

Rowan, D.D., Hunt, M.B., Gaynor, D.L., 1986. Peramine, a novel insect feeding deterrent from ryegrass infected with the endophyte *Acremonium loliae*. J. Chem. Soc., Chem. Commun. 0, 935–936. https://doi.org/10.1039/C39860000935

Sabzalian, M.R., Mirlohi, A., 2010. *Neotyphodium* endophytes trigger salt resistance in tall and meadow fescues. Journal of Plant Nutrition and Soil Science 173, 952–957. https://doi.org/10.1002/jpln.200900345

553 Saikia, S., Parker, E.J., Koulman, A., Scott, B., 2006. Four gene products are required for the fungal
554 synthesis of the indole-diterpene, paspaline. FEBS Letters 580, 1625–1630.
555 https://doi.org/10.1016/j.febslet.2006.02.008
556 Smith, T.J.R., 1977. Effects of Root-Feeding by Scarabaeid Larvae on Growth of Perennial Ryegrass
557 Plants. Journal of Applied Ecology 14, 73–80. https://doi.org/10.2307/2401828
558 Taheri, S., Razmjou, J., Rastegari, N., 2010. Fecundity and development rate of the bird cherry-oat
559 aphid, *Rhopalosiphum padi* (L) (Hom.: Aphididae) on six wheat cultivars. Plant Protection
560 Science 46, 72–78. https://doi.org/10.17221/10/2009-PPS
561 Tjallingii, F.W., 2020. Measuring Systems. EPG Systems. URL https://www.epgsystems.eu/measuring-
562 systems (accessed 5.6.20).
563 Tjallingii, W.F., 2000. Comparison of AC and DC Systems for Electronic Monitoring of Stylet Penetration
564 Activities by Homopterans, in: Principles and Applications of Electronic Monitoring and Other
565 Techniques in the Study of Homopteran Feeding Behaviour. Entomological Society of America,
566 Department of Entomology, Wageningen Agricultural University, The Netherlands, pp. 41–69.
567 Tjallingii, W.F., 1988. Electrical recording of stylet penetration activities., in: Minks, A.K., Harrewijn, P.
568 (Eds.), Aphids, Their Biology, Natural Enemies and Control. Elsevier Science Publishers,
569 Amsterdam, pp. 95–108.
570 Tjallingii, W.F., 1985. Electrical nature of recorded signals during stylet penetration by aphids.
571 Entomologia Experimentalis et Applicata 38, 177–186. https://doi.org/10.1111/j.1570-
572 7458.1985.tb03516.x
573 Tjallingii, W.F., 1978. Electronic Recording of Penetration Behaviour by Aphids. Entomologia
574 Experimentalis et Applicata 24, 721–730. https://doi.org/10.1111/j.1570-7458.1978.tb02836.x
575 Tjallingii, W.F., Esch, T.H., 1993. Fine structure of aphid stylet routes in plant tissues in correlation with
576 EPG signals. Physiological Entomology 18, 317–328. https://doi.org/10.1111/j.1365-
577 3032.1993.tb00604.x
578 Valenzuela, I., Ridland, P.M., Weeks, A.R., Hoffmann, A.A., 2010. Patterns of Genetic Variation and
579 Host Adaptation in an Invasive Population of *Rhopalosiphum padi* (Hemiptera: Aphididae).
580 Annals of the Entomological Society of America 103, 886–897.
581 https://doi.org/10.1603/AN09113
582 VSN International, 2015. Genstat for Windows 19th Edition. VSN International, Hemel Hamstead, UK.

583 Walker, G.P., 2000. A Beginner's Guide to Electronic Monitoring of Homopteran Probing Behavior, in:
584 Principles and Applications of Electronic Monitoring and Other Techniques in the Study of
585 Homopteran Feeding Behavior. Entomological Society of America, pp. 14–40.
586 Wilkins, P.W., 1991. Breeding perennial ryegrass for agriculture. Euphytica 52, 201–214.
587 https://doi.org/10.1007/BF00029397
588 Wilkinson, H.H., Siegel, M.R., Blankenship, J.D., Mallory, A.C., Bush, L.P., Schardl, C.L., 2000.
589 Contribution of Fungal Loline Alkaloids to Protection from Aphids in a Grass-Endophyte
590 Mutualism. Molecular Plant-Microbe Interactions 13, 1027–1033.
591 https://doi.org/10.1094/MPMI.2000.13.10.1027
592 Wyatt, I.J., White, P.F., 1977. Simple Estimation of Intrinsic Increase Rates for Aphids and Tetranychid
593 Mites. Journal of Applied Ecology 14, 757–766. https://doi.org/10.2307/2402807
594 Züst, T., Härri, S.A., Müller, C.B., 2008. Endophytic fungi decrease available resources for the aphid
595 *Rhopalosiphum padi* and impair their ability to induce defences against predators. Ecological
596 Entomology 33, 80–85. https://doi.org/10.1111/j.1365-2311.2007.00940.x
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Tables

Table 1. Aphid species collection details and GenBank accession number.

Aphid species	GenBank accession number	Locality	GPS	Date of collection	Host plant
Rhopalosiphum padi	MT119781	Horsham, Victoria	36°43'16.7"S 142°10'26.2"E	1-Jun-09	Oat

Table 2: *Epichloë festucae* var. *lolii* endophyte strains used in this study and associated alkaloid profiles.

Alkaloid class	Alkaloid type	SE	AR1	AR37	NEA2	NEA6
Ergopeptide	Ergovaline	P	*	*	P	P
Polyketide	Peramine	P	P	*	P	P
Indole Diterpene	Janthitrem I	*	*	P	*	*
	Lolitrem B	P	*	*	T	*

The host plant for all endophytes was perennial ryegrass cv. Alto. SE= standard endophyte; AR1, AR37, NEA2, NEA6= commercial endophytes. P = alkaloid present; * = alkaloid absent; T = alkaloid present only in trace levels.

Table 3: Significant feeding behaviour metrics of *R. padi* on all endophyte and endophyte-free treatments observed using EPG. P-value and LSD determined through log transformation to normalise results for ANOVA.

			WE (n/a)	SE (LEP)	AR1 (P)	AR37 (J)	NEA2 (LEP)	NEA6 (EP)	LSD	Chi-square
	Sample Size		24	24	20	24	24	24		
Probing	**Number of Potential Drops**	Mean	99.88	84.00	103.10	71.63	53.00	65.71		
		Log(10)	1.94 ab	1.84 abc	1.96 a	1.70 c	1.68 c	1.72 c	0.20	0.018
	Sum of Potential Drop Time (minutes)	Mean	8.01	6.80	8.55	5.53	4.38	5.39		
		Log(10)	0.85 ab	0.75 abc	0.88 a	0.60 c	0.61 c	0.64 c	0.20	0.016
	Percentage of Probe Time in C	Mean	33.01	24.42	25.91	22.60	21.02	20.63		
		Log(10)	1.45 a	1.22 b	1.33 ab	1.14 b	1.21 b	1.14 b	0.22	0.045
	Sum of C Time (minutes)	Mean	195.07	155.13	149.78	134.96	131.73	118.82		
		Log(10)	2.23 a	2.04 ab	2.11 ab	1.94 b	2.01 b	1.93 b	0.21	0.040
Feeding	**Number of E1 Events**	Mean	10.29	7.58	8.65	6.96	5.29	6.63		
		Log(10)	0.96 a	0.75 ab	0.85 ab	0.69 b	0.63 b	0.68 b	0.22	0.042
	Number of E2 Events	Mean	10.13	7.46	8.60	6.75	5.25	6.46		
		Log(10)	0.95 a	0.74 ab	0.84 ab	0.67 b	0.63 b	0.67 b	0.23	0.045
	Average Duration of 1st E1 (minutes)	Mean	0.34	0.43	0.42	0.48	0.50	0.59		
		Log(10)	-0.50 a	-0.38 bc	-0.41 ab	-0.36 bcd	-0.33 bcd	-0.27 d	0.09	<0.001
Non-probing	**Median Np Time (minutes)**	Mean	8.99	6.86	12.61	10.67	18.17	19.80		
		Log(10)	0.69 b	0.75 b	0.90 ab	0.65 b	0.80 ab	1.07 a	0.28	0.035

EPG feeding behaviour signals are categorised by the following waveform patterns: Np = non probing; C = intracellular probing phase; pd = potential drop resulting from cell puncture; E1 = pre-phloem ingestion salivatory phase; E2 = active phloem ingestion. Endophytes produced the following alkaloids: L = lolitrem B, E = ergovaline, P = peramine, J = janthitrem I.

Table 4: Mortality of *R. padi* on all endophyte and endophyte-free treatments observed at the nymphal stage (24, 48, 72 and >72 hours) and the adult stage (14, 21, 28 and >28 days).

Mortality	Barley	WE	SE	AR1	AR37	NEA2	NEA6	Average mortality [a]	LSD	P-value[b]
Nymph mortality	4/24 (0.17)	2/24 (0.08)	8/24 (0.33)	5/24 (0.21)	8/24 (0.33)	4/24 (0.17)	6/24 (0.25)	0.26	0.23	0.223
Nymph mortality (24 hrs)	1/24 (0.04)	0/24 (0.00)	1/24 (0.04)	0/24 (0.00)	1/24 (0.04)	3/24 (0.13)	1/24 (0.04)	0.05	0.10	0.232
Nymph mortality (48 hrs)	3/24 (0.13)	0/24 (0.00)	3/24 (0.13)	1/24 (0.04)	3/24 (0.13)	0/24 (0.00)	0/24 (0.00)	0.06	0.10	**0.042**
Nymph mortality (72 hrs)	0/24 (0.00)	0/24 (0.00)	0/24 (0.00)	0/24 (0.00)	0/24 (0.00)	0/24 (0.00)	0/24 (0.00)	0.00	0.00	1.000
Nymph mortality (>72 hrs)	0/24 (0.00)	2/24 (0.08)	4/24 (0.17)	4/24 (0.17)	4/24 (0.17)	1/24 (0.04)	5/24 (0.21)	0.15	0.19	0.477
Adult mortality	20/24 (0.83)	22/24 (0.92)	16/24 (0.67)	19/24 (0.79)	16/24 (0.67)	20/24 (0.83)	18/24 (0.75)	0.74	0.23	0.223
Adult mortality (14 days)	1/24 (0.04)	0/24 (0.00)	3/24 (0.13)	5/24 (0.21)	3/24 (0.13)	2/24 (0.08)	3/24 (0.13)	0.13	0.17	0.163
Adult mortality (21 days)	2/24 (0.08)	2/24 (0.08)	4/24 (0.17)	2/24 (0.08)	1/24 (0.04)	4/24 (0.17)	3/24 (0.13)	0.12	0.18	0.658
Adult mortality (28 days)	10/24 (0.42)	2/24 (0.08)	2/24 (0.08)	3/24 (0.13)	2/24 (0.08)	2/24 (0.08)	0/24 (0.00)	0.08	0.15	0.472
Adult mortality (>28 days)	7/24 (0.29)	18/24 (0.75)	7/24 (0.29)	9/24 (0.38)	10/24 (0.42)	12/24 (0.50)	12/24 (0.50)	0.42	0.27	**0.031**

Results are shown as proportions, reflecting the number of dead aphids vs. the number tested (percent mortality represented in parentheses). [a] Average mortality on endophyte treatments (excluding WE). [b] p values were calculated using a logistic regression analysis. WE= without endophyte; SE= standard endophyte; AR1, AR37, NEA2, NEA6= commercial endophytes. Barley data is shown but was not included in the statistical analysis. All bioassays were carried out for 28 days from the moment of birth.

Table 5: Fecundity of *R. padi* on all endophyte and endophyte-free treatments.

Fecundity (all aphids)	Barley	WE (n=21)	SE (n=16)	AR1 (n=20)	AR37 (n=18)	NEA2 (n=20)	NEA6 (n=18)	Average fecundity[a]	Standard Error	LSD	P-value[b]
Fecundity (14 days)	34.5	14.3	9.1	10.2	8.8	8.8	10.3	9.4	1.6	3.4	**0.003**
Fecundity (21 days)	42	20.6	14.3	15.2	14.6	14.2	16.8	15	2.4	4.7	**0.041**
Fecundity (28 days)	42	21.4	15.9	17.1	16.8	15.5	18.0	16.7	2.6	5.2	0.207
r_m	0.54	0.33	0.26	0.26	0.24	0.28	0.30	0.27	0.03	0.06	0.057

Results are shown as means. Only data from aphids that survived to reproduction was analysed. [a] Average fecundity per female per day on endophyte treatments (excluding WE). [b] p values were calculated using a one-way analysis of variance. WE= without endophyte; SE= standard endophyte; AR1, AR37, NEA2, NEA6= commercial endophytes. Barley data is shown but was not included in the statistical analysis.

Table 6: Coefficients of correlation between significant EPG metrics and life history metrics of *R. padi*.

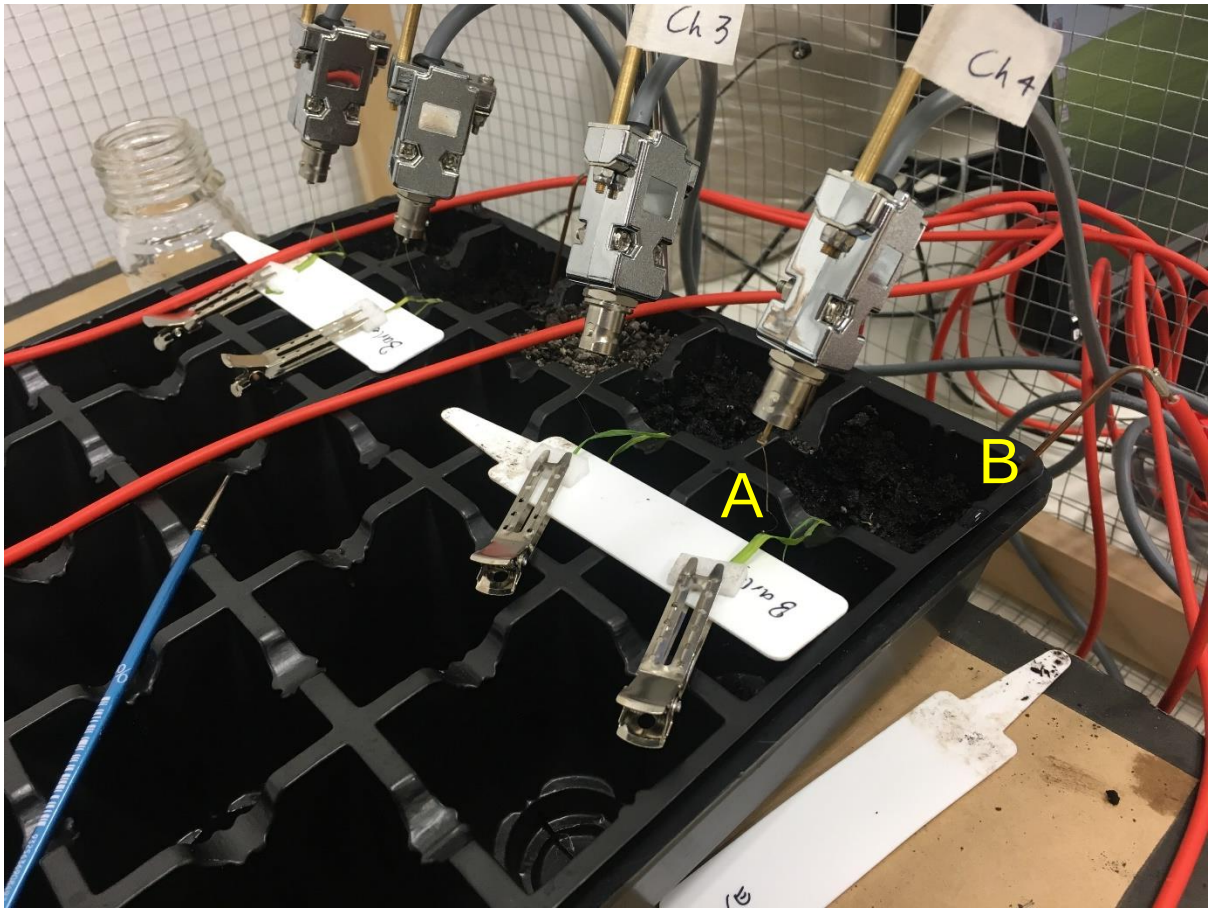
EPG Metric	Nymph Mortality	Adult Mortality	Fecundity	r_m
Probing				
Number of Potential Drops	-0.25	0.25	*0.50*	0.19
Sum of Potential Drop Time (minutes)	-0.28	0.28	0.49	0.20
Percentage of Probe Time in C	-0.60	*0.60*	**0.79**	0.53
Sum of C Time (minutes)	*-0.55*	*0.55*	*0.69*	0.46
Feeding				
Number of E1 Events	-0.31	0.31	*0.60*	0.31
Number of E2 Events	-0.44	0.44	**0.72**	0.44
Average Duration of 1st E1 (minutes)	0.32	-0.32	-0.48	-0.12
Non-probing				
Median Np Time (minutes)	-0.19	0.19	-0.07	0.22

Correlations shown as coefficient between -1 and +1, where -1 represents a perfect negative correlation, 0 represents no correlation and +1 represents a perfect positive correlation. Coefficients greater than +/-0.7 (in bold) are considered strong correlations, and coefficients between +/-0.5 and +/-0.7 (in italics) are considered correlations.

Figure legends

Fig 1: Image of EPG circuits with aphids attached to electrodes (A), and soil probes (B). Perennial ryegrass seedlings secured to plastic planter tabs to prevent movement due to plant growth.

Figures



CHAPTER 5

Discussion

5.1 Context of research

This thesis investigated the tri-trophic interaction between the fungal endophyte *Epichloë festucae* var. *lolii*, its host plant (perennial ryegrass) and pasture aphids. This interaction involves a mutualism that is beneficial to both the endophyte and the plant, as the endophyte is provided with an environment in which to live and reproduce, and the plant is provided with a level of tolerance to herbivores such as aphids, as well as a level of tolerance to drought and salinity. Furthermore, feeding by aphids has been shown to induce a defensive response on the part of either the plant or the endophyte that involves the increased production of certain insect-toxic alkaloids. This defensive response demonstrates that this relationship constitutes a tri-trophic interaction. Five different strains of *E. festucae* var. *lolii* were investigated in this study (SE, AR1, AR37, NEA2 and NEA6), each with different alkaloid profiles and potentially different insecticidal properties and modes of action. Similarly, four species of aphid were investigated, including three common foliar-feeding aphid species (*Rhopalosiphum padi*, *Diuraphis noxia* and *Metopolophium dirhodum*) and one root-feeding species (*Aploneura lentisci*). The interactions between these endophytes, aphids and the host plant were investigated in order to determine which endophytes and associated alkaloid profiles proved most effective at controlling each of these aphid species, and to determine the primary mode of action of the endophytes.

These tri-trophic interactions were investigated through three main research activities: (i) the development of a novel, high-throughput, *in-planta* bioassay method, to assess the insecticidal activity of fungal endophytes; (ii) the profiling of alkaloids using LC–MS to determine the alkaloids associated with the strongest insecticidal effects; and (iii) the use of EPG bioassays to assess the effects of endophytes on aphid feeding, to further understand the mode of action of endophyte-induced insecticidal activity and feeding deterrence. To

achieve these research activities there was a requirement to: (i) research, conceptualise and test a high-throughput, *in-planta* bioassay suitable for assessing the insecticidal activity of fungal endophytes on aphids; (ii) assess LC–MS and alkaloid quantification methodology to review its suitability for investigating the effects of alkaloids on aphid life history and the effects of aphid feeding on alkaloid production by *E. festucae* var. *lolii*; and (iii) research, source, build and test an EPG system suitable for studying aphid feeding behaviour.

5.1.1 *In-planta* bioassay to assess the effect of *Epichloë* endophytes on aphid life history

Aphids are a major pest of perennial ryegrass pastures, due to their ability to cause damage through direct feeding and the transmission of viruses. As such, there is a need to study the interaction between aphids and *Epichloë*–perennial ryegrass symbiota, which have the potential to control invertebrate pests through the production of insecticidal alkaloid compounds (Breen, 1993; Bultman et al., 2006; Clement et al., 1990). To study this interaction it was necessary to develop an assay to benchmark the insecticidal activity of novel *Epichloë*–perennial ryegrass symbiota for use in the Australian and New Zealand (NZ) dairy industries (Bastias et al., 2017a; Hennessy et al., 2016; Ruppert et al., 2017). A high-throughput *in-planta* bioassay was developed as a simple, rapid, reproducible and scalable method for assessing the effects of *Epichloë*–perennial ryegrass symbiota on aphid life history. All four aphid species were assessed on all five endophyte treatments using this method. Experiments involved observing individual aphids on single perennial ryegrass seedlings (with and without endophytes) inside 4 ml plastic cups (Olympus Packaging Pty. Ltd.). Aphids were observed daily for 28 days and individuals' growth rate (time from birth to adulthood), fecundity (the number of new nymphs born each day) and their mortality (the number of aphids that died each day) were recorded. These results were then quantified as the percentage mortality of both nymphs and adults at specific time intervals (24, 48 and 72 hours for *R. padi* and *D. noxia* nymphs; 48, 96 and 144 hours for *A. lentisci* and *M. dirhodum* nymphs; and 14, 21 and 28

days for all adults); the fecundity at specific time intervals (14, 21 and 28 days); the rate of mortality over time; and the intrinsic rate of increase (r_m).

The bioassay was an effective tool for determining endophyte effects on the mortality and fecundity of aphids, and it allowed a level of precision sufficient to differentiate between the five endophyte treatments for varying effects on aphid life history. These experiments demonstrated that all aphids were predominantly susceptible to endophytes that produced the alkaloids lolitrem B and ergovaline, with the most effective endophyte being SE, followed by NEA2. *Diuraphis noxia* was the aphid most susceptible to endophyte insecticidal activity, as it showed the highest mortality on all endophyte treatments, as well as the highest mortality within the first 24 hours. *Aploneura lentisci* and *M. dirhodum* both showed high mortality on the specific endophyte treatments SE and NEA2, especially within the first 24–48 hours, again indicating a strong effect on nymph mortality. Finally, *R. padi* exhibited no significant change in overall nymph mortality, but there was a trend of increased mortality on all endophyte treatments, with SE and AR37 showing the highest mortality at 33%, compared to 8% on the WE control. Furthermore, *R. padi* exhibited significantly decreased fecundity on all endophyte treatments at 14 days, compared to the WE control.

These results indicate that *E. festucae* var. *lolii* endophytes have a strong negative effect on aphid life history. Particularly, the alkaloids lolitrem B and ergovaline had the most significant effect on aphid life history. These alkaloids both have deleterious effects on livestock health, but their potential benefit for aphid control should not be overlooked, as negative effects on livestock can be mitigated through other means such as mixing seed batches with less toxic endophytes, or using mycotoxin binding agents to mask toxicity or unpalatability (Mann and Parfitt, 2011; Molyneux and Ralphs, 1992).

The bioassay design was well suited to studying the three different species of foliar aphids, but less suited for use with *A. lentisci*, as suggested by the high mortality observed in this species on both the WE control and the endophyte treatments. However, with some

modifications, this bioassay has the potential to be well suited to studying root-feeding species as well. Given its broad applicability, this bioassay could be used to assess endophyte effects on a broad range of foliar-feeding and possibly root-feeding invertebrate pests, such as other aphid species, psyllids or mites. This meets the stated aim of developing this bioassay as a standardised test to assess the insecticidal activity of fungal endophytes in pasture grasses.

This bioassay has the potential to be deployed as part of the Forage Value Index, which is used to compare perennial ryegrass cultivars in Australia and NZ based on yield and heading date (Chapman et al., 2012, 2017; Leddin et al., 2018). This index does not yet include insecticidal activity as a metric for benchmarking perennial ryegrass cultivars, so including this metric could greatly benefit farmers attempting to select new seed for pastures under stress from insect pests (Chapman et al., 2012, 2017; Leddin et al., 2018).

5.1.2 *Epichloë* alkaloids effects on aphid life history

Epichloë festucae var. *lolii* produces four main alkaloids: lolitrem B, ergovaline, peramine and janthitrem I (Schardl, 1996). These alkaloids have been reported to provide insecticidal activity, while some have also been reported to cause detrimental health conditions in livestock (Gallagher et al., 1981). Even within the same endophyte strain, alkaloid production can vary depending on the genotype of the host plant (Ekanayake et al., 2017), so alkaloid profiling experiments were conducted to determine whether there was a link between alkaloid concentration and the severity of insecticidal effects. A number of endophytes have the ability to synthesise multiple alkaloids (e.g. SE produces lolitrem B, ergovaline and peramine), whereas others can synthesise only one (e.g. AR37 produces only janthitrem I) (Ekanayake et al., 2017). The specific effects of the five endophytes – SE, AR1, AR37, NEA2 and NEA6 – and their alkaloid profiles were assessed against the aphid species *M. dirhodum*. These alkaloid profiling experiments were conducted in conjunction with the bioassay experiments to

determine which specific alkaloids or alkaloid profiles were the most effective in controlling *M. dirhodum*, and whether alkaloid concentration had an effect on aphid longevity.

These experiments utilised the *in-planta* bioassay established in Chapter 2 to assess the effects of the five endophytes on the life history of *M. dirhodum* for a period of seven days. Following this period, the seedlings were removed and alkaloids and broader metabolome were freeze dried, extracted in MtOH, and analysed using LC–MS as per Vassiliadis et al. (2019) to establish the alkaloid profile and concentration. In conjunction, the alkaloid profile and concentration were also established for a set of 7-day-old seedlings that had not been fed on.

These experiments demonstrated that the alkaloids lolitrem B and ergovaline – both present in SE and NEA2 in varying concentrations – had the strongest effect on aphid mortality, and that there was a negative correlation between lolitrem B concentration and *M. dirhodum* longevity. Ergovaline showed no such correlation between concentration and *M. dirhodum* longevity: concentration was relatively constant, and longevity ranged 2–28 days with an average lifespan of 12 days, indicating that even low concentrations of ergovaline had a strong toxic effect on *M. dirhodum*.

These experiments also showed that certain endophytes produced higher concentrations of some alkaloids in host plants that were fed on by aphids compared to those that were not fed on. In particular, the endophyte NEA2 produced lolitrem B in significantly higher quantities in host plants that were fed on by *M. dirhodum*, as well as slightly increased concentrations of peramine. Likewise, NEA6 and SE both produced increased levels of peramine when the host plant had been fed on. This is indicative of an endophyte-related defence response to aphid feeding, and is further evidence of the tri-trophic interaction between aphids, perennial ryegrass and *E. festucae* var. *lolii*.

The levels at which endophytes produce alkaloids within plant tissue are influenced by several different factors, including plant and endophyte genetics (Ekanayake et al., 2017)

temperature or other abiotic factors (Huizing et al., 1991; Malinowski and Belesky, 2000), and damage to the plant by cutting, grazing or insect feeding (Bultman et al., 2018; Navarro-Meléndez and Heil, 2014). Of these, feeding by aphids has been found to increase the production of certain alkaloids such as lolines (Simons et al., 2008; Sullivan et al., 2007). Based on the results of this study and others, it is possible that only certain alkaloid production pathways are affected or up-regulated by aphid feeding, as only some alkaloids appeared to be produced in greater levels – and only by some endophytes – as a result of aphid feeding (Navarro-Meléndez and Heil, 2014; Simons et al., 2008).

5.1.3 Electrical penetration graph (EPG) assays to assess aphid feeding behaviour

The mode of action of *Epichloë* endophytes and their alkaloids have been postulated to include deterrence of feeding behaviour; however, studies into these feeding-deterrence effects have thus far been conducted predominantly using artificial diets when investigating coleopteran pests (Rowan et al., 1990), or cut leaf assays and choice-or-no choice studies when investigating aphids (Eichenseer and Dahlman, 1992; M. C. Johnson et al., 1985). While these methods may indicate the extent to which endophytes deter insect feeding, they do not assess endophyte effects on the mechanics of aphid feeding behaviour. The EPG technique is useful for assessing this particular aspect of endophyte mode of action, as it produces data on aphid feeding behaviour *in-planta* and provides detailed insights into endophyte effects on the mechanics of feeding behaviour, such as stylet movement and duration of phloem ingestion (Salvador-Recatalà and Tjallingii, 2015; Tjallingii, 1988). The EPG experiments were performed to determine the effects of *E. festucae* var. *lolii* on *R. padi* feeding behaviour. This was done to determine the mode of action of insecticidal endophytes – specifically, if their insecticidal effects were related to any form of feeding deterrence or alternative hindrance of aphid feeding behaviour.

Results of the EPG experiments revealed that *R. padi* feeding initiation was significantly delayed, and the number of feeding events significantly reduced, on the AR37, NEA2 and NEA6 endophyte treatments, indicating that these endophytes have deterrent properties against aphid feeding. This feeding deterrence could directly contribute to the anti-fecundity effects of these endophytes on *R. padi*, given the importance of nutrition to aphid reproduction (Leather and Dixon, 1981). AR37, NEA2 and NEA6 do not share a common alkaloid profile, but do produce similar alkaloids that share common precursors (Ludlow et al., 2019). It is therefore possible that this shared effect is due to one of these precursors (e.g. paspaline), or an additional chemical factor that is currently unknown. Furthermore, SE, the endophyte treatment that resulted in the highest mortality in all aphid species, showed no effect on *R. padi* feeding behaviour, despite producing the same alkaloids as NEA2, and in greater quantities. This suggests that the high mortality seen in aphids feeding on SE is due to the direct toxicity of the alkaloids produced, and that another factor may be present in SE-infected perennial ryegrass that potentially negates the feeding deterrent effects seen in NEA2 – for example, a binding agent or structural change to the alkaloids, which could mask unpalatability (Molyneux and Ralphs, 1992). This merits further study, as it suggests a possible advantage of using commercial endophytes such as NEA2 over SE (Schardl, 1996), and may potentially have uses in improving the palatability and masking the toxicity of SE towards livestock (Mann and Parfitt, 2011).

There was also a decrease in *R. padi* fecundity on all endophyte treatments, with the strongest effects observed on AR37, NEA2 and NEA6, directly correlating with the decrease in feeding. As aphids require adequate nutrition from phloem feeding to maintain a high reproductive rate, these results suggest that the reduction in feeding caused a decrease in nutritional intake significant enough to negatively affect *R. padi* reproductive fitness. Thus, these endophyte treatments – AR37, NEA2 and NEA6 – could potentially be used to slow population growth of *R. padi* in pastures as part of a broader integrated pest management

strategy alongside other insecticidal treatments, which could be used in lower quantities than without the presence of endophytes.

5.2 New research since this thesis commenced

Since this thesis was initially undertaken in 2016, a number of studies in Australia and overseas have been published that have further investigated the interactions between insect pests, perennial ryegrass and fungal endophytes or other symbiotic microorganisms of pasture grasses. These include studies into the development of novel endophytes for use in pastures (Hettiarachchige et al., 2019; Reddy et al., 2019), and the effects of endophytes that produce different classes of alkaloids on the life history of root- or foliar-feeding aphids, or other insect pests (Fuchs and Krauss, 2019; Popay and Cox, 2016; Ruppert et al., 2017). Other studies have investigated different beneficial aspects of the perennial ryegrass microbiome (Chen et al., 2016), and the influence of additional trophic factors, including the production of plant hormones (Bastias et al., 2017a) and the effects of grazing by livestock (Bultman et al., 2018), on the tri-trophic interaction between aphids, pasture grasses and endophytes.

5.2.1 Development of novel endophytes

It is believed that most, if not all, plant species are host to some form of asymptomatic endophyte, and capitalising on this symbiosis may be advantageous when developing new biological controls of pests and diseases in agriculture (Lugtenberg et al., 2016). Endophyte discovery and development research is ongoing, with researchers working to develop more effective endophyte treatments for the improvement of pest management and pasture grasses in dairy farming systems (Popay and Hume, 2020; Reddy et al., 2019). A major focus of this research has been the discovery and isolation of novel endophytes that produce potent alkaloids with targeted toxicity towards invertebrate pests (Adhikari et al., 2016; Cagnano et

al., 2020; Reddy et al., 2019). Loline alkaloids have been identified as highly effective broad spectrum insecticides, but are not naturally produced by *E. festucae* var. *lolii* and are therefore more difficult to implement in perennial ryegrass pastures (Wilkinson et al., 2000). However, certain loline-producing endophytes from other grasses, such as tall fescue, can be transferred to perennial ryegrass hosts (Ball and Tapper, 1999). Research into their effectiveness at protecting perennial ryegrass pastures from invertebrate pests is ongoing.

5.2.2 Ongoing research on root aphids

Aploneura lentisci and other root-feeding pests pose a significant threat to dairy pastures, as they are often more difficult to detect, identify and control than foliar-feeding pests, due to their predominantly subterranean life cycle (Pennell et al., 2005). In spite of the threat *A. lentisci* poses, there is little research on the life history and management of this species in dairy pastures, and it is not well understood compared to foliar aphid pests of grasses. Recently, however, more research has been conducted to determine effective control strategies for *A. lentisci* on pasture grasses, especially in NZ and Australia (Popay and Cox, 2016).

In recent years, a number of studies into the effects of novel endophyte symbiota on the life history of *A. lentisci* have been published by researchers in NZ, working alongside the NZ dairy industry and seed companies (Müller, 2019; Popay and Cox, 2016). For example, Popay and Cox (2016) determined that the endophyte AR1 – widely used in NZ dairy pastures due to its effective control of Argentine stem weevil populations (Rowan et al., 1990) – had little effect on *A. lentisci* life history or population size; however, the endophyte AR37 significantly reduced *A. lentisci* numbers in glasshouse trials, suggesting this endophyte has potential for *A. lentisci* control. Similar results were seen in field trials in Victoria, Australia, where *A. lentisci* numbers were significantly lower in soil samples collected from pastures growing AR37-infected perennial ryegrass (Moate et al., 2012). There have been relatively few studies on *A. lentisci* conducted in Australia in recent years, but a number of projects are

being conducted by Australian researchers with Agriculture Victoria to assess the effects of novel fungal endophytes and their associated alkaloids on *A. lentisci*. This research is being conducted using a range of techniques, including modifications of the bioassay method developed for this thesis to improve its suitability for use with root-feeding invertebrates, and the use of artificial diets – adapted from Wille and Hartman (2008) – containing specific alkaloids in order to determine their effects on *A. lentisci* life history.

5.2.3 Further study into the perennial ryegrass microbiome

There is a clear tri-trophic interaction between *E. festucae* var. *lolii*, perennial ryegrass and aphids; however, it is unclear what other microbes may interact within this system. The microbiomes of plants are made up of fungal and bacterial epiphytes (microorganisms that live on the plant surface) and endophytes. These microorganisms often function either to protect the plant from biotic or abiotic factors, or to support host plant nutrition through the fixing of nitrogen, phosphorus or other chemical nutrients (Bacon and White, 2016). New studies have identified that perennial ryegrass supports a diverse microbiome within the plant tissue, on the surface of roots and leaves, and in the surrounding soil environment, all of which constitute vastly different microbial communities (Chen et al., 2016). While the specific role of these microorganisms is unclear, it has been postulated that they have a beneficial effect on the growth, nutrition and insect tolerance of perennial ryegrass (Chen et al., 2016). Furthermore, within the perennial ryegrass microbiome, rhizobial and other bacterial communities can be influenced by the presence of *E. festucae* var. *lolii* fungal endophytes, as evidenced by significant differences observed between the microbial diversity of endophyte-infected and endophyte-free perennial ryegrass (Wakelin et al., 2015). Much is still unknown about the full species diversity of these microbiomes, and their full effects on the growth, health and productivity of perennial ryegrass, so it is important to understand how this interaction can be best used to improve the performance of dairy pasture.

5.3 Future research

5.3.1 Use of bioassay to assess other endophytes and microbes

The bioassay developed as part of this thesis was designed as a tool for assessing the insecticidal activity of endophyte symbiota in perennial ryegrass seedlings. The design is well suited to this purpose and can be extended to assess multiple novel endophytes in different host grasses. This bioassay can potentially be used to assess other systemic insecticide or seed treatments as well. For example, there are plans for future research by Agriculture Victoria to use this bioassay to assess different bacterial seed treatments on barley to determine the insecticidal activity of these treatments against aphids.

5.3.2 Adaptation of bioassay for use with *Aploneura lentisci*

While the bioassay was well suited to assessing the insecticidal activity of endophyte-infected perennial ryegrass on foliar aphids, it was less suited to making the same assessments on *A. lentisci.* Research is underway at Agriculture Victoria to modify this bioassay to better suit root-feeding species. As *A. lentisci* is a predominantly subterranean species, it is likely that the design of the cup bioassay may have left the aphids more exposed than in their natural habitat, which may have affected growth and development (Müller, 2019). Mortality was proportionally high on all treatments including the control, which suggests that the difference in growth conditions between *A. lentisci'*s natural habitat and the bioassay cup had a far more detrimental effect on survival than in foliar-feeding species. The high *A. lentisci* mortality may have been caused by the increased light levels the aphids were exposed to, as well as the decreased levels of surrounding moisture (InfluentialPoints, 2020). Another factor that may have contributed to the high mortality is the cotton wool that surrounded the roots of seedlings, which may have impeded *A. lentisci* feeding or caused aphids to become entangled. *Aploneura lentisci* was also difficult to cultivate in lab colonies compared to foliar-feeding

species due to its lower population growth in potted plants and the difficulties in extracting individuals from the roots of colony plants, where they are protected by the dense root mass, soil and flocculence (InfluentialPoints, 2020).

Adapting the bioassay for use with *A. lentisci* has centred around creating an environment where perennial ryegrass roots are more accessible to researchers, and where the environment within the bioassay containers is closer to the natural habitat of *A. lentisci*. Two major challenges that new designs would need to address in order to create an optimal environment for *A. lentisci* include the creation of a dark and moist environment within the bioassay containers to better replicate *A. lentisci*'s natural subterranean habitat; and the positioning of roots in a more loose and open arrangement, allowing researchers better access to individual *A. lentisci* and preventing aphids from becoming entangled in the cotton wool commonly used in standard bioassay design. These requirements have resulted in several design concepts, but as yet, none have been tested in full-scale trials.

With minor modifications, this bioassay could also be applied for use with a wider range of root-feeding insect pests, including pasture mealybugs and potentially chewing insects such as scarab beetles. Modifications to address these aims and to expand the scope of this bioassay to include other insect species could include using opaque containers to better replicate the subterranean habitat of root-feeding insects, and increasing the size of bioassay containers to accommodate larger insects or greater quantities of plant material, and to allow better access to plant roots. Modifying this bioassay in such a way would expand its applicability to a wider range of pest species and enable a more comprehensive assessment of novel endophyte symbiota for use in pasture systems.

5.3.3 Aphid gut bacteria research (*Buchnera*)

A potential avenue for future research involves investigating the effects of fungal endophytes on the aphid endosymbiont *Buchnera aphidicola,* a type of bacteria that makes up the majority

of the gut microbiome in most aphid species (Douglas, 1998). *Buchnera aphidicola* is an obligate mutualist that plays a vital part in aphid metabolism, as it contains genes that synthesise amino acids necessary for aphid survival, but which are not present in the aphid diet of phloem sap (Chong et al., 2019; Douglas, 1998). It has been postulated that *Epichloë* alkaloids affect not only the aphid, but also the aphid gut microbiome and *B. aphidicola*. If *B. aphidicola* populations were reduced in the gut, this would limit the production of key amino acids in the aphid, negatively affecting growth and development (Machado-Assefh et al., 2015). Other endosymbiotic bacteria, such as those of genus *Hamiltonella*, while not considered vital, also have an effect on aphid fitness (Degnan et al., 2009), which could also be influenced by endophytes. Identifying the effect of endophytes on *B. aphidicola* and other gut bacteria could provide a greater understanding of the insecticidal activity and mode of action of *Epichloë* fungal endophytes against aphids.

5.4 Final conclusions

This project demonstrated that the different *E. festucae* var. *lolii* endophytes have a diverse range of effects on the life history of the four aphid species, but effects were universally negative, as evidenced by the increased mortality, reduced fecundity and reduced feeding observed.

Diuraphis noxia exhibited the greatest increase in overall mortality when exposed to endophytes, while *A. lentisci* and *M. dirhodum* showed significantly increased mortality on specific endophytes that shared similar alkaloid profiles producing lolitrem B and ergovaline, but not on others. *Rhopalosiphum padi* did not exhibit a significant increase in mortality on endophyte-infected plants, but did show a trend towards an endophyte insecticidal effect. Additionally, *R. padi* and *A. lentisci* exhibited significantly reduced fecundity on AR37, NEA2 and NEA6. An assessment of endophyte effects on *R. padi* feeding behaviour using the EPG technique indicated that aphids were deterred from feeding on the same endophytes that saw

the most reduced fecundity in *R. padi* and *A. lentisci*, establishing a correlation between feeding behaviour and reproductive fitness.

The increased mortality seen in all aphid species can most likely be attributed to the alkaloids lolitrem B and ergovaline, as endophytes that consistently resulted in the highest aphid mortality, SE and NEA2, produced these alkaloids. This was confirmed by alkaloid profiling of *Epichloë*–perennial ryegrass symbiota, which revealed that the presence of lolitrem B and ergovaline in seedlings resulted in high *M. dirhodum* mortality, and there was a direct correlation between higher concentrations of lolitrem B and shorter *M. dirhodum* lifespans. Furthermore, *M. dirhodum* feeding behaviour was found to result in increased concentrations of certain alkaloids in some endophytes. Most notably, the production of lolitrem B by NEA2 was significantly increased in response to aphid feeding. This increased alkaloid concentration implies a defensive response on the part of the endophyte to protect the host plant from aphid herbivory. Such a defence response is evidence of the tri-trophic interaction between endophyte, perennial ryegrass and aphid. Interestingly, the endophytes linked to the strongest reduction in feeding behaviour (AR37, NEA2 and NEA6) do not share similar alkaloid profiles, suggesting that another factor – such as a common precursor or other unknown metabolite – was responsible for deterring *R. padi* from feeding.

Nevertheless, the alkaloids lolitrem B and ergovaline (produced by SE and NEA2) were determined to have the strongest overall negative effect on aphid life history, indicating that they would provide the most effective control over aphid infestations within dairy pastures. However, these alkaloids are toxic to grazing herbivores, such as cattle and sheep, in high concentrations. As such, extra measures would need to be taken to counteract these toxic effects on livestock, such as limiting alkaloid concentrations to non-toxic levels (Reddy et al., 2019), or employing mycotoxin binding agents to mitigate toxicity and unpalatability towards livestock (Mann and Parfitt, 2011; Molyneux and Ralphs, 1992).

The targeted use of novel *Epichloë* endophytes within the tri-trophic interaction between pasture aphids, perennial ryegrass and *E. festucae* var. *lolii* has great potential in aphid pest management in perennial ryegrass dairy pastures in Australia. Though originally designed for use with aphids, the bioassay technique developed and used in this study can be applied more widely to assess a range of insecticidal treatments on different pasture pests, and may be used in the development of novel endophyte symbiota and other novel insecticide treatments for pasture grasses. While more research is required to perfect a standardised testing method for benchmarking novel endophyte symbiota, the work presented in this thesis provides a strong foundation for future research and advances the development of effective novel endophytes to improve dairy pasture performance.

References

Adhikari, K.B., Boelt, B., Fomsgaard, I.S., 2016. Identification and quantification of loline-type alkaloids in endophyte-infected grasses by LC-MS/MS. J. Agric. Food Chem. 64, 6212–6218. https://doi.org/10.1021/acs.jafc.6b02616

Agrawal, A.A., 2000. Mechanisms, ecological consequences and agricultural implications of tri-trophic interactions. Curr. Opin. Plant Biol. 3, 329–335.

Auclair, J.L., 1963. Aphid Feeding and Nutrition. Annu. Rev. Entomol. 8, 439–490. https://doi.org/10.1146/annurev.en.08.010163.002255

Avila, G.A., Davidson, M., Helden, M. van, Fagan, L., 2019. The potential distribution of the Russian wheat aphid (*Diuraphis noxia*): an updated distribution model including irrigation improves model fit for predicting potential spread. Bull. Entomol. Res. 109, 90–101. https://doi.org/10.1017/S0007485318000226

Bacon, C.W., White, J.F., 2016. Functions, mechanisms and regulation of endophytic and epiphytic microbial communities of plants. Symbiosis 68, 87–98. https://doi.org/10.1007/s13199-015-0350-2

Ball, O.J.-P., Miles, C.O., Prestidge, R.A., 1997. Ergopeptine alkaloids and *Neotyphodium lolii*-mediated resistance in perennial ryegrass against adult *Heteronychus arator* (Coleoptera: Scarabaeidae). J. Econ. Entomol. 90, 1382–1391. https://doi.org/10.1093/jee/90.5.1382

Ball, O.J.-P., Tapper, B.A., 1999. The production of loline alkaloids in artificial and natural grass/endophyte associations. Proc. N. Z. Plant Prot. Conf. 52, 264–269. https://doi.org/10.30843/nzpp.1999.52.11583

Banks, P., Davidson, J., Bariana, H., Larkin, P., 1995. Effects of barley yellow dwarf virus on the yield of winter wheat. Aust. J. Agric. Res. 46, 935–946.

Barenbrug Agriseeds, 2019. Endophyte Options. Barenbrug Agriseeds - Super. Pastures Dairy Cultiv. Ryegrass Endophyte. URL https://www.agriseeds.co.nz/dairy/cultivars/endophyte-options.htm (accessed 7.3.19).

Bastias, D.A., Martínez-Ghersa, M.A., Ballaré, C.L., Gundel, P.E., 2017a. *Epichloë* fungal endophytes and plant defenses: not just alkaloids. Trends Plant Sci. 22, 939–948. https://doi.org/10.1016/j.tplants.2017.08.005

Bastias, D.A., Ueno, A.C., Machado Assefh, C.R., Alvarez, A.E., Young, C.A., Gundel, P.E., 2017b. Metabolism or behavior: explaining the performance of aphids on alkaloid-producing fungal endophytes in annual ryegrass (*Lolium multiflorum*). Oecologia 185, 245–256. https://doi.org/10.1007/s00442-017-3940-2

Birch, A.N.E., Geoghegan, I.E., Majerus, M.E., McNicol, J.W., Hackett, C.A., Gatehouse, A.M., Gatehouse, J.A., 1999. Tri-trophic interactions involving pest aphids, predatory 2-spot ladybirds and transgenic potatoes expressing snowdrop lectin for aphid resistance. Mol. Breed. 5, 75–83.

Birch, L.C., 1948. The intrinsic rate of natural increase of an insect population. J. Anim. Ecol. 17, 15–26. https://doi.org/10.2307/1605

Blackman, Roger L., Eastop, V.F., 2007. Taxonomic Issues, in: Aphids as Crop Pests. CABI International, pp. 1–29.

Blackman, R.L., Eastop, V.F., 2000. Aphids on the World's Crops, Second Edition. ed. John Wiley & Sons with the Natural History Museum.

Bonani, J.P., Fereres, A., Garzo, E., Miranda, M.P., Appezzato-Da-Gloria, B., Lopes, J.R.S., 2010. Characterization of electrical penetration graphs of the Asian citrus psyllid, *Diaphorina citri*, in sweet orange seedlings. Entomol. Exp. Appl. 134, 35–49. https://doi.org/10.1111/j.1570-7458.2009.00937.x

Breen, J.P., 1994. *Acremonium* endophyte interactions with enhanced plant resistance to insects. Annu. Rev. Entomol. 39, 401–423. https://doi.org/10.1146/annurev.en.39.010194.002153

Breen, J.P., 1993. Enhanced resistance to three species of aphids (Homoptera: Aphididae) in Acremonium endophyte-infected turfgrasses. J. Econ. Entomol. 86, 1279–1286. https://doi.org/10.1093/jee/86.4.1279

Breen, J.P., 1992. Temperature and seasonal effects on expression of *Acremonium* endophyte-enhanced resistance to *Schizaphis graminum* (Homoptera: Aphididae). Environ. Entomol. 21, 68–74. https://doi.org/10.1093/ee/21.1.68

Bristow, C.M., 1991. Are ant-aphid associations a tritrophic interaction? Oleander aphids and Argentine ants. Oecologia 87, 514–521.

Bultman, T.L., McNeill, M.R., Krueger, K., De Nicolo, G., Popay, A.J., Hume, D.E., Mace, W.J., Fletcher, L.R., Koh, Y.M., Sullivan, T.J., 2018. Complex interactions among sheep, insects, grass, and fungi in a simple New Zealand grazing system. J. Chem. Ecol. 44, 957–964. https://doi.org/10.1007/s10886-018-0993-6

Bultman, T.L., Pulas, C., Grant, L., Bell, G., Sullivan, T.J., 2006. Effects of fungal endophyte isolate on performance and preference of bird cherry-oat aphid. Environ. Entomol. 35, 6.

Bush, L.P., Wilkinson, H.H., Schardl, C.L., 1997. Bioprotective alkaloids of grass-fungal endophyte symbioses. Plant Physiol. 114, 1–7. https://doi.org/10.1104/pp.114.1.1

CABI, 2020a. *Metopolophium dirhodum* (rose-grass aphid). URL https://www-cabi-org.ez.library.latrobe.edu.au/isc/datasheet/33625 (accessed 4.9.20).

CABI, 2020b. *Rhopalosiphum padi* (grain aphid). URL https://www-cabi-org.ez.library.latrobe.edu.au/isc/datasheet/47321 (accessed 4.9.20).

CABI, 2020c. *Diuraphis noxia* (Russian wheat aphid). URL https://www-cabi-org.ez.library.latrobe.edu.au/isc/datasheet/9887 (accessed 4.9.20).

CABI, 2016a. *Rhopalosiphum maidis* (green corn aphid). URL http://www.cabi.org/isc/datasheet/47321 (accessed 11.2.16).

CABI, 2016b. *Rhopalosiphum padi* (grain aphid). URL http://www.cabi.org/isc/datasheet/47321 (accessed 11.2.16).

Cagnano, G., Vázquez-de-Aldana, B.R., Asp, T., Roulund, N., Jensen, C.S., Soto-Barajas, M.C., 2020. Determination of loline alkaloids and mycelial biomass in endophyte-infected *Schedonorus pratensis* by near-infrared spectroscopy and chemometrics. Microorganisms 8, 776. https://doi.org/10.3390/microorganisms8050776

Carver, M., 1984. *Metopolophium dirhodum* (Walker) (Homoptera: Aphididae) newly recorded from Australia. Aust. J. Entomol. 23, 192–192. https://doi.org/10.1111/j.1440-6055.1984.tb01944.x

Catherall, P.L., 1966. Effects of barley yellow dwarf virus on the growth and yield of single plants and simulated swards of perennial rye-grass. Ann. Appl. Biol. 57, 155–162. https://doi.org/10.1111/j.1744-7348.1966.tb06876.x

Chakrabarti, S., 2018. Aphids, in: Omkar (Ed.), Pests and Their Management. Springer, Singapore, pp. 871–908. https://doi.org/10.1007/978-981-10-8687-8_24

Chapman, D., Bryant, J., McMillan, W., Thorrold, B., Edwards, G., Willocks, M., Green, D., Kerr, G., Judson, G., Cookson, T., 2012. An economic forage value index for New Zealand dairy farmers. Australas. Dairy Sci. Symp. 2012 Proc. 5th Australas. Dairy Sci. Symp. Melb. Aust. 13-15 Novemb. 2012 422–423.

Chapman, D.F., Bryant, J.R., Olayemi, M.E., Edwards, G.R., Thorrold, B.S., McMillan, W.H., Kerr, G.A., Judson, G., Cookson, T., Moorhead, A., Norriss, M., 2017. An economically based evaluation index for perennial and short-term ryegrasses in New Zealand dairy farm systems. Grass Forage Sci. 72, 1–21. https://doi.org/10.1111/gfs.12213

Chen, L., Brookes, P.C., Xu, J., Zhang, J., Zhang, C., Zhou, X., Luo, Y., 2016. Structural and functional differentiation of the root-associated bacterial microbiomes of perennial ryegrass. Soil Biol. Biochem. 98, 1–10. https://doi.org/10.1016/j.soilbio.2016.04.004

Cheng, L. (Ed.), 1976. Marine insects. North-Holland Publ. Co, Amsterdam.

Chong, R.A., Park, H., Moran, N.A., 2019. Genome Evolution of the Obligate Endosymbiont *Buchnera aphidicola*. Mol. Biol. Evol. 36, 1481–1489. https://doi.org/10.1093/molbev/msz082

Chongrattanameteekul, W., Foster, J.E., Shukle, R.H., Araya, J.E., 1991. Feeding behavior of *Rhopalosiphum padi* (L.) and *Sitobion avenae* (F.) (Horn., Aphididae) on wheat as affected by conspecific and interspecific interactions. J. Appl. Entomol. 111, 361–364.

Choudhury, S., Larkin, P., Meinke, H., Hasanuzzaman, M.D., Johnson, P., Zhou, M., 2019. Barley yellow dwarf virus infection affects physiology, morphology, grain yield and flour

pasting properties of wheat. Crop Pasture Sci. 70, 16–25. https://doi.org/10.1071/CP18364

Clarke, R.G., Eagling, D.R., 1994. Effects of pathogens on perennial pasture grasses. N. Z. J. Agric. Res. 37, 319–327. https://doi.org/10.1080/00288233.1994.9513070

Clay, K., 1993. The ecology and evolution of endophytes. Agric. Ecosyst. Environ. 44, 39–64. https://doi.org/10.1016/0167-8809(93)90038-Q

Clay, K., 1988. Fungal endophytes of grasses: a defensive mutualism between plants and fungi. Ecology 69, 10–16. https://doi.org/10.2307/1943155

Clement, S.L., Lester, D.G., Wilson, A.D., Pike, K.S., 1992. Behavior and performance of *Diuraphis noxia* (Homoptera: Aphididae) on fungal endophyte-infected and uninfected perennial ryegrass. J. Econ. Entomol. 85, 583–588. https://doi.org/10.1093/jee/85.2.583

Clement, S.L., Pike, K.S., Kaiser, W.J., Wilson, A.D., 1990. Resistance of endophyte-infected plants of tall fescue and perennial ryegrass to the Russian wheat aphid (Homoptera: Aphididae). J. Kans. Entomol. Soc. 63, 646–648.

Dairy Australia, 2019. Perennial ryegrass management. URL https://www.dairyaustralia.com.au/farm/feedbase-and-animal-nutrition/pasture/perennial-ryegrass-management (accessed 4.1.19).

Degnan, P.H., Yu, Y., Sisneros, N., Wing, R.A., Moran, N.A., DeLong, E.F., 2009. Hamiltonella Defensa, Genome Evolution of Protective Bacterial Endosymbiont from Pathogenic Ancestors. Proc. Natl. Acad. Sci. U. S. A. 106, 9063–9068.

Dixon, A.F.G., 1985. Structure of Aphid Populations. Ann. Rev. Entomol. 30, 155–174.

Dixon, A.F.G., 1971. The life-cycle and host preferences of the bird cherry-oat aphid, *Rhopalosiphum padi* L., and their bearing on the theories of host alternation in aphids. Ann. Appl. Biol. 68, 135–147. https://doi.org/10.1111/j.1744-7348.1971.tb06450.x

Dolatti, L., Ghareyazie, B., Moharramipour, S., Noori-Daloii, M.R., 2005. Evidence for regional diversity and host adaptation in Iranian populations of the Russian wheat aphid. Entomol. Exp. Appl. 114, 171–180. https://doi.org/10.1111/j.1570-7458.2005.00247.x

Douglas, A.E., 1998. Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. Annu. Rev. Entomol. 43, 17–37. https://doi.org/10.1146/annurev.ento.43.1.17

Eagling, D.R., Cunningham, P.J., Sward, R.J., Halloran, G.M., 1989a. The incidence of barley yellow dwarf virus isolates in perennial ryegrass (*Lolium perenne*) in south-western Victoria. Plant Pathol. 38, 408–413. https://doi.org/10.1111/j.1365-3059.1989.tb02160.x

Eagling, D.R., Sward, R.J., Halloran, G.M., 1989b. The effect of barley yellow dwarf virus on early plant growth in ryegrass (*Lolium* spp.). Crop Pasture Sci. 40, 293–299. https://doi.org/10.1071/AR9890293

Eastop, V.F., 1966. A taxonomic study of Australian Aphidoidea (Homoptera). Aust. J. Zool. 399–592.

Edwards, O.R., Franzmann, B., Thackray, D., Micic, S., 2008. Insecticide resistance and implications for future aphid management in Australian grains and pastures: a review. Aust. J. Exp. Agric. 48, 1523. https://doi.org/10.1071/EA07426

Eichenseer, H., Dahlman, D.L., 1992. Antibiotic and deterrent qualities of endophyte-infected tall fescue to two aphid species (Homoptera: Aphididae). Environ. Entomol. 21, 1046–1051. https://doi.org/10.1093/ee/21.5.1046

Ekanayake, P.N., Kaur, J., Tian, P., Rochfort, S.J., Guthridge, K.M., Sawbridge, T.I., Spangenberg, G.C., Forster, J.W., 2017. Genomic and metabolic characterisation of alkaloid biosynthesis by asexual *Epichloë* fungal endophytes of tall fescue pasture grasses. Genome. https://doi.org/10.1139/gen-2016-0173

Emden, H.F.V., Harrington, R., 2007. Aphids as Crop Pests. CABI Publishing.

Farrell, J.A., Stufkens, M.W., 1988. Abundance of the rose-grain aphid, *Metopolophium dirhodum*, on barley in Canterbury, New Zealand, 1984–87. N. Z. J. Zool. 15, 499–505. https://doi.org/10.1080/03014223.1988.10422630

Ferguson, C.M., Barratt, B.I.P., Bell, N., Goldson, S.L., Hardwick, S., Jackson, M., Jackson, T.A., Phillips, C.B., Popay, A.J., Rennie, G., Sinclair, S., Townsend, R., Wilson, M.,

2019. Quantifying the economic cost of invertebrate pests to New Zealand's pastoral industry. N. Z. J. Agric. Res. 62, 255–315. https://doi.org/10.1080/00288233.2018.1478860

Finlay, K.J., Luck, J.E., 2011. Response of the bird cherry-oat aphid (*Rhopalosiphum padi*) to climate change in relation to its pest status, vectoring potential and function in a crop–vector–virus pathosystem. Agric. Ecosyst. Environ. 144, 405–421. https://doi.org/10.1016/j.agee.2011.08.011

Flatt, T., Weisser, W.W., 2000. The effects of mutualistic ants on aphid life history traits. Ecology 81, 3522–3529. https://doi.org/10.1890/0012-9658(2000)081[3522:TEOMAO]2.0.CO;2

Fleetwood, D.J., Scott, B., Voisey, C.R., Johnson, R.D., 2008. Insights into the molecular biology of *Epichloë* endophyte alkaloid biosynthesis. Proc. N. Z. Grassl. Assoc. 70 217–220.

Fletcher, L.R., Harvey, I.C., 1981. An association of a *Lolium* endophyte with ryegrass staggers. N. Z. Vet. J. 29, 185–186. https://doi.org/10.1080/00480169.1981.34839

Fuchs, B., Krischke, M., Mueller, M.J., Krauss, J., 2013. Peramine and Lolitrem B from Endophyte-Grass Associations Cascade Up the Food Chain. J. Chem. Ecol. 39, 1385–1389. https://doi.org/10.1007/s10886-013-0364-2.

Fuchs, B., Krauss, J., 2019. Can *Epichloë* endophytes enhance direct and indirect plant defence? Fungal Ecol., From antagonism to mutualism: the chemical basis of insect-fungus interactions 38, 98–103. https://doi.org/10.1016/j.funeco.2018.07.002

Gallagher, R.T., White, E.P., Mortimer, P.H., 1981. Ryegrass staggers: isolation of potent neurotoxins lolitrem a and lolitrem B from staggers-producing pastures. N. Z. Vet. J. 29, 189–190. https://doi.org/10.1080/00480169.1981.34843

Garzo, E., Moreno, A., Hernando, S., Mariño, V., Torne, M., Santamaria, E., Díaz, I., Fereres, A., 2016. Electrical penetration graph technique as a tool to monitor the early stages

of aphid resistance to insecticides. Pest Manag. Sci. 72, 707–718. https://doi.org/10.1002/ps.4041

Gerhards, N., Neubauer, L., Tudzynski, P., Li, S.-M., 2014. Biosynthetic pathways of ergot alkaloids. Toxins 6, 3281–3295. https://doi.org/10.3390/toxins6123281

Gerson, U., Applebaum, S., 2015. Aphididae. Plant Pests Middle East. URL http://www.agri.huji.ac.il/mepests/entry/Aphididae/ (accessed 4.8.20).

Girling, R.D., Madison, R., Hassall, M., Poppy, G.M., Turner, J.G., 2008. Investigations into plant biochemical wound-response pathways involved in the production of aphid-induced plant volatiles. J. Exp. Bot. 59, 3077–3085.

Goggin, F.L., 2007. Plant–aphid interactions: molecular and ecological perspectives. Curr. Opin. Plant Biol., Special Issue on Biotic Interactions 10, 399–408. https://doi.org/10.1016/j.pbi.2007.06.004

Gols, R., Posthumus, M., Dicke, M., Ludeking, D., 1999. Jasmonic acid induces the production of gerbera volatiles that attract the biological control agent *Phytoseiulus persimilis*. Entomol. Exp. Appl. 93, 77–86. https://doi.org/10.1046/j.1570-7458.1999.00564.x

Guy, P.L., Johnstone, G.R., Duffus, J.E., 1986. Occurrence and identity of barley yellow dwarf viruses in Tasmanian pasture grasses. Crop Pasture Sci. 37, 43–53. https://doi.org/10.1071/AR9860043

Hales, D.F., Tomiuk, J., Woehrmann, K., Sunnucks, P., 2013. Evolutionary and genetic aspects of aphid biology: A review. EJE 94, 1–55.

Harper, A.M., 1961. Effect of insecticides on the sugar-beet root aphid, *Pemphigus betae*. J. Econ. Entomol. 54, 1151–1153. https://doi.org/10.1093/jee/54.6.1151

Harris, K.F., Maramorosch, K., 2014. Aphids as Virus Vectors. Elsevier.

Heil, M., 2008. Indirect defence via tritrophic interactions. New Phytol. 178, 41–61. https://doi.org/10.1111/j.1469-8137.2007.02330.x

Henderson, I.F., Clements, R.O., 1977. Grass growth in different parts of England in relation to invertebrate numbers and pesticide treatment. Grass Forage Sci. 32, 89–98. https://doi.org/10.1111/j.1365-2494.1977.tb01418.x

Hennessy, L.M., Popay, A.J., Finch, S.C., Clearwater, M.J., Cave, V.M., 2016. Temperature and plant genotype alter alkaloid concentrations in ryegrass infected with an *Epichloë* endophyte and this affects an insect herbivore. Front. Plant Sci. 7. https://doi.org/10.3389/fpls.2016.01097

Hesse, U., Schöberlein, W., Wittenmayer, L., Förster, K., Warnstorff, K., Diepenbrock, W., Merbach, W., 2003. Effects of *Neotyphodium* endophytes on growth, reproduction and drought-stress tolerance of three *Lolium perenne* L. genotypes. Grass Forage Sci. 58, 407–415. https://doi.org/10.1111/j.1365-2494.2003.00393.x

Hettiarachchige, I.K., Elkins, A.C., Reddy, P., Mann, R.C., Guthridge, K.M., Sawbridge, T.I., Forster, J.W., Spangenberg, G.C., 2019. Genetic modification of asexual *Epichloë* endophytes with the perA gene for peramine biosynthesis. Mol. Genet. Genomics 294, 315–328. https://doi.org/10.1007/s00438-018-1510-x

Hoffmann, B.D., Broadhurst, L.M., 2016. The economic cost of managing invasive species in Australia. NeoBiota 31, 1–18. https://doi.org/10.3897/neobiota.31.6960

Hughes, R. d., Maywald, G. f., 1990. Forecasting the favourableness of the Australian environment for the Russian wheat aphid, *Diuraphis noxia* (Homoptera: Aphididae), and its potential impact on Australian wheat yields. Bull. Entomol. Res. 80, 165–175. https://doi.org/10.1017/S0007485300013389

Huizing, H.J., Molen, W.V.D., Kloek, W., Nijs, A.P.M.D., 1991. Detection of lolines in endophyte-containing meadow fescue in The Netherlands and the effect of elevated temperature on induction of lolines in endophyte-infected perennial ryegrass. Grass Forage Sci. 46, 441–445. https://doi.org/10.1111/j.1365-2494.1991.tb02405.x

Hume, D.E., Ryan, D.L., Cooper, B.M., Popay, A.J., 2007. Agronomic performance of AR37-infected ryegrass in northern New Zealand. Proc. N. Z. Grassl. Assoc. 69, 201–205.

Hume, D.E., Sewell, J.C., 2014. Agronomic advantages conferred by endophyte infection of perennial ryegrass (*Lolium perenne* L.) and tall fescue (*Festuca arundinacea* Schreb.) in Australia. Crop Pasture Sci. 65, 747. https://doi.org/10.1071/CP13383

InfluentialPoints, 2020. *Aploneura lentisci* (Mealy grass root aphid) identification, images, ecology, control. URL https://influentialpoints.com/Gallery/Aploneura_lentisci_mealy_grass_root_aphid.htm (accessed 4.1.20).

Jalalizand, A.R., Mirhendi, H., Karimi, A., Modaresi, M., Mahmoodi, E., 2012. Morphological and molecular identification aphids of Rosae. APCBEE Procedia 4, 12–15. https://doi.org/10.1016/j.apcbee.2012.11.003

Janssen, J. a. M., Tjallingii, W.F., Lenteren, J.C. van, 1989. Electrical recording and ultrastructure of stylet penetration by the greenhouse whitefly. Entomol. Exp. Appl. 52, 69–81. https://doi.org/10.1111/j.1570-7458.1989.tb01250.x

Johnson, M. C., Dahlman, D.L., Siegel, M.R., Bush, L.P., Latch, G.C.M., Potter, D.A., Varney, D.R., 1985. Insect feeding deterrents in endophyte-infected tall fescue. Appl. Environ. Microbiol. 49, 568–571.

Jones, R.A.C., 2013. Virus diseases of pasture grasses in Australia: incidences, losses, epidemiology, and management. Crop Pasture Sci. 64, 216–233.

KEGG PATHWAY: Indole diterpene alkaloid biosynthesis - *Aspergillus nidulans*, 2014. URL https://www.genome.jp/kegg-bin/show_pathway?ani00403 (accessed 5.4.20).

Kerns, D.L., Stewart, S.D., 2000. Sublethal effects of insecticides on the intrinsic rate of increase of cotton aphid. Entomol. Exp. Appl. 94, 41–49. https://doi.org/10.1046/j.1570-7458.2000.00602.x

Kindler, S.D., Breen, J.P., Springer, T.L., 1991. Reproduction and damage by Russian wheat aphid (Homoptera: Aphididae) as influenced by fungal endophytes and cool-season turfgrasses. J. Econ. Entomol. 84, 685–692. https://doi.org/10.1093/jee/84.2.685

Kindler, S.D., Springer, T.L., 1989. Alternate hosts of Russian wheat aphid (Homoptera: Aphididae). J. Econ. Entomol. 82, 1358–1362. https://doi.org/10.1093/jee/82.5.1358

Klotz, J.L., Bush, L.P., Smith, D.L., Shafer, W.D., Smith, L.L., Arrington, B.C., Strickland, J.R., 2007. Ergovaline-induced vasoconstriction in an isolated bovine lateral saphenous vein bioassay. J. Anim. Sci. 85, 2330–2336. https://doi.org/10.2527/jas.2006-803

Landis, D.A., Wratten, S.D., Gurr, G.M., 2000. Habitat management to conserve natural enemies of arthropod pests in agriculture. Annu. Rev. Entomol. 45, 175–201. https://doi.org/10.1146/annurev.ento.45.1.175

Latch, G.C.M., 1980. Effects of barley yellow dwarf virus on simulated swards of Nui perennial ryegrass. N. Z. J. Agric. Res. 23, 373–378. https://doi.org/10.1080/00288233.1980.10425370

Leather, S.R., Dixon, A.F.G., 1982. Secondary host preferences and reproductive activity of the bird cherry-oat aphid, *Rhopalosiphum padi*. Ann. Appl. Biol. 101, 219–228. https://doi.org/10.1111/j.1744-7348.1982.tb00816.x

Leather, S.R., Dixon, A.F.G., 1981. Growth, survival and reproduction of the bird-cherry aphid, *Rhopalosiphum padi*, on its primary host. Ann. Appl. Biol. 99, 115–118. https://doi.org/10.1111/j.1744-7348.1981.tb05136.x

Leddin, C.M., Jacobs, J.L., Smith, K.F., Giri, K., Malcolm, B., Ho, C.K.M., 2018. Development of a system to rank perennial ryegrass cultivars according to their economic value to dairy farm businesses in south-eastern Australia. Anim. Prod. Sci. 58, 1552. https://doi.org/10.1071/AN17815

Li, T., Blande, J.D., Gundel, P.E., Helander, M., Saikkonen, K., 2014. Epichloë Endophytes Alter Inducible Indirect Defences in Host Grasses. PLOS ONE 9, e101331. https://doi.org/10.1371/journal.pone.0101331

Ludlow, E.J., Vassiliadis, S., Ekanayake, P.N., Hettiarachchige, I.K., Reddy, P., Sawbridge, T.I., Rochfort, S.J., Spangenberg, G.C., Guthridge, K.M., 2019. Analysis of the indole diterpene gene cluster for biosynthesis of the epoxy-janthitrems in *Epichloë* endophytes. Microorganisms 7, 560. https://doi.org/10.3390/microorganisms7110560

Lugtenberg, B.J.J., Caradus, J.R., Johnson, L.J., 2016. Fungal endophytes for sustainable crop production. FEMS Microbiol. Ecol. 92, fiw194. https://doi.org/10.1093/femsec/fiw194

Ma, R., Reese, J.C., Black, W.C., Bramel-Cox, P., 1990. Detection of pectinesterase and polygalacturonase from salivary secretions of living greenbugs, Schizaphis graminum

(Homoptera: Aphididae). J. Insect Physiol. 36, 507–512. https://doi.org/10.1016/0022-1910(90)90102-L

Machado-Assefh, C.R., Lopez-Isasmendi, G., Tjallingii, W.F., Jander, G., Alvarez, A.E., 2015. Disrupting *Buchnera aphidicola*, the endosymbiotic bacteria of *Myzus persicae*, delays host plant acceptance. Arthropod-Plant Interact. 9, 529–541. https://doi.org/10.1007/s11829-015-9394-8

Malinowski, D.P., Belesky, D.P., 2000. Adaptations of endophyte-infected cool-season grasses to environmental stresses: mechanisms of drought and mineral stress tolerance. Crop Sci. 40, 923–940. https://doi.org/10.2135/cropsci2000.404923x

Mann, S.P., Parfitt, D., 2011. Mycotoxin-reducing composition. US20110150853A1.

McKirdy, S.J., Jones, R. a. C., Nutter, F.W., 2002. Quantification of yield losses caused by barley yellow dwarf virus in wheat and oats. Plant Dis. 86, 769–773. https://doi.org/10.1094/PDIS.2002.86.7.769

McLean, D.L., Kinsey, M.G., 1964. A Technique for electronically recording aphid feeding and salivation. Nature 202, 1358. https://doi.org/10.1038/2021358a0

Merrill, S.C., Holtzer, Thomas.O., Peairs, F.B., 2009. *Diuraphis noxia* reproduction and development with a comparison of intrinsic rates of increase to other important small grain aphids: a meta-analysis. Environ. Entomol. 38, 1061–1068. https://doi.org/10.1603/022.038.0413

Minks, A.K., Harrewijn, P., 1988. Aphids: Their Biology, Natural Enemies and Control. Elsevier.

Mittler, T.E., Dadd, R.H., 1964. An improved method for feeding aphids on artificial diets. Ann. Entomol. Soc. Am. 57, 139–140. https://doi.org/10.1093/aesa/57.1.139a

Moate, P.J., Williams, S.R.O., Grainger, C., Hannah, M.C., Mapleson, D., Auldist, M.J., Greenwood, J.S., Popay, A.J., Hume, D.E., Mace, W.J., Wales, W.J., 2012. Effects of wild-type, AR1 and AR37 endophyte-infected perennial ryegrass on dairy production

in Victoria, Australia. Anim. Prod. Sci. 52, 1117–1130. https://doi.org/10.1071/AN12126

Molyneux, R.J., Ralphs, M.H., 1992. Plant toxins and palatability to herbivores.

Müller, J.L., 2019. A study of root aphid *Aploneura lentisci* Pass. biology and root aphid-host interactions with perennial ryegrass/endophyte associations in New Zealand : a thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Entomology at Massey University, Manawatū, New Zealand (Thesis). Massey University.

Mulligan, T.E., 1960. The transmission by mites, host-range and properties of ryegrass mosaic virus. Ann. Appl. Biol. 48, 575–579. https://doi.org/10.1111/j.1744-7348.1960.tb03559.x

Navarro-Meléndez, A.L., Heil, M., 2014. Symptomless endophytic fungi suppress endogenous levels of salicylic acid and interact with the jasmonate-dependent indirect defense traits of their host, lima bean (*Phaseolus lunatus*). J. Chem. Ecol. 40, 816–825. https://doi.org/10.1007/s10886-014-0477-2

Nettleton, W.A., Hain, F.P., 1982. The life history, foliage damage, and control of the balsam twig aphid, *Mindarus abietinus* (Homoptera: Aphididae), in Fraser fir Christmas Tree plantations of western North Carolina. Can. Entomol. 114, 155–165. https://doi.org/10.4039/Ent114155-2

Nicholson, S.J., Hartson, S.D., Puterka, G.J., 2012. Proteomic analysis of secreted saliva from Russian Wheat Aphid (*Diuraphis noxia* Kurd.) biotypes that differ in virulence to wheat. J. Proteomics 75, 2252–2268. https://doi.org/10.1016/j.jprot.2012.01.031

Oerke, E.C., 2006. Crop losses to pests. ResearchGate 144, 31–43. https://doi.org/10.1017/S0021859605005708

Omacini, M., Chaneton, E.J., Ghersa, C.M., Muller, C.B., 2001. Symbiotic fungal endophytes control insect host-parasite interaction webs. Nature 409, 78-81.

Paini, D.R., Sheppard, A.W., Cook, D.C., De Barro, P.J., Worner, S.P., Thomas, M.B., 2016. Global threat to agriculture from invasive species. Proc. Natl. Acad. Sci. U. S. A. 113, 7575–7579.

Parry, H.R., Macfadyen, S., Kriticos, D.J., 2012. The geographical distribution of Yellow dwarf viruses and their aphid vectors in Australian grasslands and wheat. Australas. Plant Pathol. 41, 375–387. https://doi.org/10.1007/s13313-012-0133-7

Paul, R.G., 1977. Aspects of the biology and taxonomy of British myrmecophilous root aphids.

Peñalver-Cruz, A., Garzo, E., Prieto-Ruiz, I., Díaz-Carro, M., Winters, A., Moreno, A., Fereres, A., 2019. Feeding behavior, life history, and virus transmission ability of *Bemisia tabaci* Mediterranean species (Hemiptera: Aleyrodidae) under elevated CO2. Insect Sci. n/a. https://doi.org/10.1111/1744-7917.12661

Pennell, C.G.L., Popay, A.J., Ball, O.J.-P., Hume, D.E., Baird, D.B., 2005. Occurrence and impact of pasture mealybug (*Balanococcus poae*) and root aphid (*Aploneura lentisci*) on ryegrass (*Lolium* spp.) with and without infection by Neotyphodium fungal endophytes. N. Z. J. Agric. Res. 48, 329–337. https://doi.org/10.1080/00288233.2005.9513663

Perng, J.J., 2002. Life history traits of *Aphis gossypii Glover* (Hom., Aphididae) reared on four widely distributed weeds. J. Appl. Entomol. 126, 97–100. https://doi.org/10.1046/j.1439-0418.2002.00613.x

Pettersson, J., Tjallingii, W.F., Hardie, J., 2007. Host-plant selection and feeding., in: Emden, H.F. van, Harrington, R. (Eds.), Aphids as Crop Pests. CABI, Wallingford, pp. 87–113. https://doi.org/10.1079/9780851998190.0087

Pollard, D.G., 1973. Plant penetration by feeding aphids (Hemiptera, Aphidoidea): a review. Bull. Entomol. Res. 62, 631–714. https://doi.org/10.1017/S0007485300005526

Ponder, K.L., Pritchard, J., Harrington, R., Bale, J.S., 2001. Feeding behaviour of the aphid *Rhopalosiphum padi* (Hemiptera: Aphididae) on nitrogen and water-stressed barley (Hordeum vulgare) seedlings. Bull. Entomol. Res. 91, 125–130. https://doi.org/10.1079/BER200072

Popay, A.J., Bonos, S.A., 2008. Biotic responses in endophytic grasses, in: *Neotyphodium* in Cool-Season Grasses. John Wiley & Sons, Ltd, pp. 163–185. https://doi.org/10.1002/9780470384916.ch7

Popay, A.J., Cox, N.R., 2016. *Aploneura lentisci* (Homoptera: Aphididae) and Its Interactions with Fungal Endophytes in Perennial Ryegrass (*Lolium perenne*). Front. Plant Sci. 7. https://doi.org/10.3389/fpls.2016.01395

Popay, A.J., Gerard, P.J., 2007. Cultivar and endophyte effects on a root aphid *Aploneura lentisci* in perennial ryegrass. N. Z. Plant Prot. 60, 223–227. https://doi.org/10.30843/nzpp.2007.60.4624

Popay, A.J., Hume, D.E., 2020. Endophytes for improving ryegrass performance: Current status and future possibilities. Ecol. Control Vertebr. Invertebr. Pests Grass Forage 1625–1626.

Popay, A.J., Wyatt, R.T., 1995. Resistance to Argentine stem weevil in perennial ryegrass infected with endophytes producing different alkaloids. Proc. Forty Eighth N. Z. Plant Prot. Conf. Angus Inn Hastings N. Z. August 8-10 1995 229–236.

Potter, D.A., Stokes, J.T., Redmond, C.T., Schardl, C.L., Panaccione, D.G., 2008. Contribution of ergot alkaloids to suppression of a grass-feeding caterpillar assessed with gene knockout endophytes in perennial ryegrass. Entomol. Exp. Appl. 126, 138–147. https://doi.org/10.1111/j.1570-7458.2007.00650.x

Potts, S.G., Woodcock, B.A., Roberts, S.P.M., Tscheulin, T., Pilgrim, E.S., Brown, V.K., Tallowin, J.R., 2009. Enhancing pollinator biodiversity in intensive grasslands. J. Appl. Ecol. 46, 369–379.

Puterka, G., Black, W., Steiner, W., Burton, R., 1993. Genetic variation and phylogenetic relationships among worldwide collections of the Russian wheat aphid, *Diuraphis noxia* (Mordvilko), inferred from allozyme and RAPD-PCR markers. Heredity 70, 604–618.

Reddy, P., Deseo, M.A., Ezernieks, V., Guthridge, K., Spangenberg, G., Rochfort, S., 2019. Toxic indole diterpenes from endophyte-infected perennial ryegrass *Lolium perenne* L.: Isolation and Stability. Toxins 11, 16. https://doi.org/10.3390/toxins11010016

Ricci, M., Tocho, E., Dixon, A.F.G., Castro, A.M., 2011. *Diuraphis noxia*: reproductive behaviour in Argentina. Bull. Insectology 64, 235–241.

Ridland, P.M., Sward, R.J., Tomkins, R.B., 1988. A simple rearing system for cereal aphids, especially suited to transmission studies with barley yellow dwarf viruses 17, 3.

Rowan, D.D., Dymock, J.J., Brimble, M.A., 1990. Effect of fungal metabolite peramine and analogs on feeding and development of argentine stem weevil (*Listronotus bonariensis*). J. Chem. Ecol. 16, 1683–1695. https://doi.org/10.1007/BF01014100

Rowan, D.D., Gaynor, D.L., 1986. Isolation of feeding deterrents against argentine stem weevil from ryegrass infected with the endophyte *Acremonium loliae*. J. Chem. Ecol. 12, 647–658. https://doi.org/10.1007/BF01012099

Rowan, D.D., Hunt, M.B., Gaynor, D.L., 1986. Peramine, a novel insect feeding deterrent from ryegrass infected with the endophyte *Acremonium loliae*. J. Chem. Soc. Chem. Commun. 0, 935–936. https://doi.org/10.1039/C39860000935

Rowan, D.D., Latch, G.C.M., 1994. Utilization of endophyte-infected perennial ryegrass for increased insect resistance, in: Bacon, C.W., White, J.F. (Eds.), Biotechnology of Endophytic Fungi of Grasses. pp. 169–183.

Ruppert, K.G., Matthew, C., McKenzie, C.M., Popay, A.J., 2017. Impact of Epichloë endophytes on adult Argentine stem weevil damage to perennial ryegrass seedlings. Entomol. Exp. Appl. 163, 328–337. https://doi.org/10.1111/eea.12584

Sabzalian, M.R., Mirlohi, A., 2010. *Neotyphodium* endophytes trigger salt resistance in tall and meadow fescues. J. Plant Nutr. Soil Sci. 173, 952–957. https://doi.org/10.1002/jpln.200900345

Saikia, S., Takemoto, D., Tapper, B.A., Lane, G.A., Fraser, K., Scott, B., 2012. Functional analysis of an indole-diterpene gene cluster for lolitrem B biosynthesis in the grass endosymbiont Epichloë festucae. FEBS Lett. 586, 2563–2569. https://doi.org/10.1016/j.febslet.2012.06.035

Salvador-Recatalà, V., Tjallingii, W.F., 2015. A new application of the electrical penetration graph (EPG) for acquiring and measuring electrical signals in phloem sieve elements. J. Vis. Exp. https://doi.org/10.3791/52826

Sarria, E., Cid, M., Garzo, E., Fereres, A., 2009. Excel Workbook for automatic parameter calculation of EPG data. Comput. Electron. Agric. 67, 35–42. https://doi.org/10.1016/j.compag.2009.02.006

Schardl, C., Leuchtmann, A., 2005. The *Epichloë* endophytes of grasses and the symbiotic continuum, in: Dighton, J., White, J., Oudemans, P. (Eds.), The Fungal Community, Mycology. CRC Press, pp. 475–503. https://doi.org/10.1201/9781420027891.ch24

Schardl, C.L., 1996. Epichloë Species: Fungal symbionts of grasses. Annu. Rev. Phytopathol. 34, 109–130. https://doi.org/10.1146/annurev.phyto.34.1.109

Schardl, C.L., Leuchtmann, A., Spiering, M.J., 2004. Symbioses of grasses with seedborne fungal endophytes. Annu. Rev. Plant Biol. 55, 315–340. https://doi.org/10.1146/annurev.arplant.55.031903.141735

Schardl, C.L., Young, C.A., Hesse, U., Amyotte, S.G., Andreeva, K., Calie, P.J., Fleetwood, D.J., Haws, D.C., Moore, N., Oeser, B., Panaccione, D.G., Schweri, K.K., Voisey, C.R., Farman, M.L., Jaromczyk, J.W., Roe, B.A., O'Sullivan, D.M., Scott, B., Tudzynski, P., An, Z., Arnaoudova, E.G., Bullock, C.T., Charlton, N.D., Chen, L., Cox, M., Dinkins, R.D., Florea, S., Glenn, A.E., Gordon, A., Güldener, U., Harris, D.R., Hollin, W., Jaromczyk, J., Johnson, R.D., Khan, A.K., Leistner, E., Leuchtmann, A., Li, C., Liu, JinGe, Liu, Jinze, Liu, M., Mace, W., Machado, C., Nagabhyru, P., Pan, J., Schmid, J., Sugawara, K., Steiner, U., Takach, J.E., Tanaka, E., Webb, J.S., Wilson, E.V., Wiseman, J.L., Yoshida, R., Zeng, Z., 2013. Plant-symbiotic fungi as chemical engineers: multi-genome analysis of the clavicipitaceae reveals dynamics of alkaloid loci. PLoS Genet. 9. https://doi.org/10.1371/journal.pgen.1003323

Shufran, K.A., Puterka, G.J., 2011. DNA Barcoding to identify all life stages of holocyclic cereal aphids (Hemiptera: Aphididae) on wheat and other Poaceae. Ann. Entomol. Soc. Am. 104, 39–42. https://doi.org/10.1603/AN10129

Shymanovich, T., Musso, A.M., Cech, N.B., Faeth, S.H., 2019. *Epichloë* endophytes of *Poa alsodes* employ alternative mechanisms for host defense: insecticidal versus deterrence. Arthropod-Plant Interact. 13, 79–90. https://doi.org/10.1007/s11829-018-9635-8

Siegel, M.R., Latch, G.C.M., Bush, L.P., Fannin, F.F., Rowan, D.D., Tapper, B.A., Bacon, C.W., Johnson, M.C., 1990. Fungal endophyte-infected grasses: Alkaloid accumulation and aphid response. J. Chem. Ecol. 16, 3301–3315. https://doi.org/10.1007/BF00982100

Simons, L., Bultman, T.L., Sullivan, T.J., 2008. Effects of methyl jasmonate and an endophytic fungus on plant resistance to insect herbivores. J. Chem. Ecol. 34, 1511. https://doi.org/10.1007/s10886-008-9551-y

Sullivan, T.J., Rodstrom, J., Vandop, J., Librizzi, J., Graham, C., Schardl, C.L., Bultman, T.L., 2007. Symbiont-mediated changes in *Lolium arundinaceum* inducible defenses: Evidence from changes in gene expression and leaf composition. New Phytol. 176, 673–679.

Tanaka, A., Tapper, B.A., Popay, A., Parker, E.J., Scott, B., 2005. A symbiosis expressed non-ribosomal peptide synthetase from a mutualistic fungal endophyte of perennial ryegrass confers protection to the symbiotum from insect herbivory. Mol. Microbiol. 57, 1036–1050. https://doi.org/10.1111/j.1365-2958.2005.04747.x

Tapper, B.A., Latch, G.C.M., 1999. Selection against toxin production in endophyte-infected perennial ryegrass. Grassl. Res. Pract. Ser. 7, 107–111.

Thakur, A., Singh, V., Kaur, A., Kaur, S., 2013. Insecticidal potential of an endophytic fungus, *Cladosporium uredinicola*, against *Spodoptera litura*. Phytoparasitica 41, 373–382. https://doi.org/10.1007/s12600-013-0298-9

Thom, E.R., Popay, A.J., Waugh, C.D., Minneé, E.M.K., 2014. Impact of novel endophytes in perennial ryegrass on herbage production and insect pests from pastures under dairy cow grazing in northern New Zealand. Grass Forage Sci. 69, 191–204. https://doi.org/10.1111/gfs.12040

Tjallingii, W.F., 1988. Electrical recording of stylet penetration activities., in: Minks, A.K., Harrewijn, P. (Eds.), Aphids, Their Biology, Natural Enemies and Control. Elsevier Science Publishers, Amsterdam, pp. 95–108.

Tjallingii, W.F., 1985. Electrical nature of recorded signals during stylet penetration by aphids. Entomol. Exp. Appl. 38, 177–186. https://doi.org/10.1111/j.1570-7458.1985.tb03516.x

Tjallingii, W.F., 1978. Electronic recording of penetration behaviour by aphids. Entomol. Exp. Appl. 24, 721–730. https://doi.org/10.1111/j.1570-7458.1978.tb02836.x

Tor-Agbidye, J., Blythe, L.L., Craig, A.M., 2001. Correlation of endophyte toxins (ergovaline and lolitrem B) with clinical disease: fescue foot and perennial ryegrass staggers. Vet. Hum. Toxicol. 43, 140–146.

Tscharntke, T., Greiler, H.J., 1995. Insect communities, grasses, and grasslands. Annu. Rev. Entomol. 40, 535–558. https://doi.org/10.1146/annurev.en.40.010195.002535

Valenzuela, I., Hoffmann, A.A., 2015. Effects of aphid feeding and associated virus injury on grain crops in Australia: Economic loss of grains by aphids. Austral Entomol. 54, 292–305. https://doi.org/10.1111/aen.12122

Valenzuela, I., Ridland, P.M., Weeks, A.R., Hoffmann, A.A., 2010. Patterns of genetic variation and host adaptation in an invasive population of *Rhopalosiphum padi* (Hemiptera: Aphididae). Ann. Entomol. Soc. Am. 103, 886–897. https://doi.org/10.1603/AN09113

Vassiliadis, S., Elkins, A.C., Reddy, P., Guthridge, K.M., Spangenberg, G.C., Rochfort, S.J., 2019. A simple LC–MS method for the quantitation of alkaloids in endophyte-infected Perennial Ryegrass. Toxins 11, 649. https://doi.org/10.3390/toxins11110649

Vorburger, C., 2005. Positive genetic correlations among major life-history traits related to ecological success in the aphid *Myzus perisicae*. Evolution 59, 1006–1015. https://doi.org/10.1111/j.0014-3820.2005.tb01039.x

Wakelin, S., Harrison, S., Mander, C., Dignam, B., Rasmussen, S., Monk, S., Fraser, K., O'Callaghan, M., 2015. Impacts of endophyte infection of ryegrass on rhizosphere metabolome and microbial community. Crop Pasture Sci. 66, 1049–1057. https://doi.org/10.1071/CP14321

Walls, J., Rajotte, E., Rosa, C., 2019. The past, present, and future of barley yellow dwarf management. Agriculture 9, 23. https://doi.org/10.3390/agriculture9010023

Wei Zhang, H., Chun Song, Y., Xiang Tan, R., 2006. Biology and chemistry of endophytes. Nat. Prod. Rep. 23, 753–771. https://doi.org/10.1039/B609472B

White, J.F., Torres, M.S., 2009. Defensive mutualism in microbial symbiosis. CRC Press.

Wilkins, P.W., 1991. Breeding perennial ryegrass for agriculture. Euphytica 52, 201–214. https://doi.org/10.1007/BF00029397

Wilkinson, H.H., Siegel, M.R., Blankenship, J.D., Mallory, A.C., Bush, L.P., Schardl, C.L., 2000. Contribution of fungal loline alkaloids to protection from aphids in a grass-endophyte mutualism. Mol. Plant. Microbe Interact. 13, 1027–1033. https://doi.org/10.1094/MPMI.2000.13.10.1027

Wille, B.D., Hartman, G.L., 2008. Evaluation of artificial diets for rearing *Aphis glycines* (Hemiptera: Aphididae). J. Econ. Entomol. 101, 1228–1232. https://doi.org/10.1093/jee/101.4.1228

Williams, I.S., Dixon, A.F.G., 2007. Life cycles and polymorphism., in: Emden, H.F. van, Harrington, R. (Eds.), Aphids as Crop Pests. CABI, Wallingford, pp. 69–85. https://doi.org/10.1079/9780851998190.0069

Wool, D., Manheim, O., 1986. Population ecology of the gall-forming aphid, *Aploneura lentisci* (Pass.) in Israel. Res. Popul. Ecol. 28, 151–162. https://doi.org/10.1007/BF02515543

Wyatt, I.J., White, P.F., 1977. Simple estimation of intrinsic increase rates for aphids and Tetranychid mites. J. Appl. Ecol. 14, 757–766. https://doi.org/10.2307/2402807

Yazdani, M., Baker, G., DeGraaf, H., Henry, K., Hill, K., Kimber, B., Malipatil, M., Perry, K., Valenzuela, I., Nash, M.A., 2018. First detection of Russian wheat aphid *Diuraphis noxia* Kurdjumov (Hemiptera: Aphididae) in Australia: a major threat to cereal production. Austral Entomol. 57, 410–417. https://doi.org/10.1111/aen.12292

Young, C.A., Felitti, S., Shields, K., Spangenberg, G., Johnson, R.D., Bryan, G.T., Saikia, S., Scott, B., 2006. A complex gene cluster for indole-diterpene biosynthesis in the grass

endophyte *Neotyphodium lolii*. Fungal Genet. Biol. 43, 679–693. https://doi.org/10.1016/j.fgb.2006.04.004

Young, C.A., Tapper, B.A., May, K., Moon, C.D., Schardl, C.L., Scott, B., 2009. Indole-diterpene biosynthetic capability of *Epichloë* endophytes as predicted by ltm gene analysis. Appl. Environ. Microbiol. 75, 2200–2211. https://doi.org/10.1128/AEM.00953-08

Zhang, B., Edwards, O., Kang, L., Fuller, S., 2014a. A multi-genome analysis approach enables tracking of the invasion of a single Russian wheat aphid (*Diuraphis noxia*) clone throughout the New World. Mol. Ecol. 23, 1940–1951. https://doi.org/10.1111/mec.12714

Zhang, B., Ma, C., Edwards, O., Fuller, S., Kang, L., 2014b. The mitochondrial genome of the Russian wheat aphid *Diuraphis noxia*: Large repetitive sequences between trnE and trnF in aphids. Gene 533, 253–260. https://doi.org/10.1016/j.gene.2013.09.064

Zhang, R., Liang, H., Ren, L., Zhang, G., 2001. Induced life cycle transition from holocycly to anholocycly of the Russian wheat aphid (Homoptera: Aphididae). Sci. China C Life Sci. 44, 1. https://doi.org/10.1007/BF02882066