Relative Epichloë endophyte fungal biomass in ryegrass tillers grown pre and post vernalization

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Abstract

Efficient transmission of Epichloë endophytes from fungal initials in the seed through to the next generation of seed is an important component of New Zealand ryegrass seed production. New Zealand industry standards require that seed sold with proprietary endophyte should contain > 70% of viable endophyte that is true to type. Achieving this through 3-4 multiplication-production cycles is difficult as transmission per generation is often < 95%. One hypothesis for poor transmission is that being asexual, the endophyte symbiont cannot be selectively bred as quickly as the sexual host plant can be. Endophytes from Mediterranean climates are not suited to cool NZ winters, but ryegrass has been heavily selected for growth under such conditions. To test this, endophyte-containing seed was grown at two temperatures (vernalized and non-vernalized), and the relative growth of the endophyte to the ryegrass was measured. Results demonstrate that under cold conditions, the endophyte grew significantly less, and physiological and phenotypic changes in plant tissue following vernalization compromised the ability to compare relative growth rates. This result has important practical implications to produce novel endophyte-containing ryegrass seed production.

Additional keywords: *Epichloë festucae* var *lolii*, *Lolium perenne*, vertical transmission

Introduction

New Zealand (NZ) grasslands, particularly north of Taupo, come under immense insect pressure from species such as the Argentine stem weevil and black beetle (Popay & Rowan, 1994). This is countered by the sowing of ryegrass containing the asexual symbiotic *Epichloë* spp. (family Clavicipitaceae) (Leuchtmann *et al*., 2014). These are fungi that are vertically transmitted from host seed via the vegetative mother plant to the next generation of host seed. During the vegetative life cycle of the host, Epichloë spp. live systemically through the aerial parts of the plant where they confer abiotic and biotic benefits (Popay & Rowan, 1994; Schardl *et al.*, 2004; Johnson *et al.*, 2013), depending upon the strain or subspecies of the fungus. Efficient vertical transmission of the fungus is essential for commercial exploitation (Caradus & Johnson, 2019), as without it, seed companies cannot efficiently produce sufficient endophyte-colonised seed.

Understanding vertical transmission is a key research goal for seed companies. Gagic *et al.* (2018) investigated the effect of host genotype on AR37 endophyte and highlighted that AR37 stability and transmission was affected by host genotype. This study inadvertently highlighted a dichotomy between the genetics of the *Lolium perenne* host, an outcrossing sexual

species, and that of the asexual *Epichloë festucae* var *lolii* fungus. Grass breeders can put extreme selection pressure on the host and rapidly shift its genetic, and therefore phenotypic, make-up, but the asexual fungus cannot be shifted in such a way. However, ryegrass being obligatory outcrossing means that each new seed has only 50% of nuclear DNA from the mother. Thus, the norm is vertical transfer into a genotypically different host. This is not a problem within a geographically-localised ecotype population, but when breeders make crosses outside the normal host range, e.g. between different ecotypes or interspecific crosses (Freitas *et al.*, 2020), then this may introduce host genetic differences that the endophyte cannot tolerate as well. This is perhaps the case for many NZ cultivars which originated from the UK (Stewart, 2006) (though most new cultivars now have some Spanish germplasm integrated into this (Pembleton *et al.*, 2018)), whilst many commercial Epichloë endophytes are from Spain (NEA2) (Hettiarachchige *et al.*, 2015) and France (AR37, AR1) (WO2004/106487) (dairynz.co.nz). Thus, in NZ, warm-climate endophyte strains are often present in cool-climate perennial ryegrasses that have been strongly selected for winter growth in NZ.

Epichloë endophytes have specific cardinal temperatures (Ju *et al.*, 2006), while perennial ryegrass also has optimal temperature growth requirements (McCormick *et al.*, (2014). Freitas, (2017) and Freitas *et al*., (2020) investigated the effect of temperature regimes on vertical transmission of a tall fescue endophyte in a ryegrass and tall fescue host (grasses with different cardinal temperatures). Hillis (2019) investigated *in vivo* management practices to try and identify key environmental variables that might affect the host symbiotic relationship. These and earlier studies (e.g. Bacon & Siegel, 1988; Breen, 1992; Ju *et al.*, 2006) suggest that temperature has a strong influence on the symbiotic relationship. However, none seem to take account of the requirement for ryegrass to undergo vernalization for it to flower; a condition where both ryegrass and endophyte are under short days and cool (< 12°C), sub-optimal, growth conditions. The process of vernalization causes gross physiological and phenotypic changes in the ryegrass (Jokela *et al.*, 2014; Paina *et al.*, 2014). These can compromise the ability to assess endophyte growth, as the compositional and structural plant differences hinder direct comparisons of biomass within the plant tissues.

For this study, it was hypothesised that sowing ryegrass in autumn, going into colder conditions for winter to meet vernalization requirements, and where NZ ryegrass has been selected to continue to grow, could disadvantage the growth of the endophyte. Here, we report an initial investigation into relative fungal biomass in seedlings grown at different temperature regimes and following regrowth after vernalization. Physiological differences between vernalized and non-vernalized ryegrass tillers provide a layer of complexity to determining relative endophyte biomass that warrants further investigation.

Materials and Methods

Plant and endophyte material

For this study, the perennial ryegrass cultivar Governor with *E. festucae* var. *lolii* strains AR1 and AR37, provided by Barenbrug NZ, were investigated. All seed lots contained over 80% viable endophyte according to an endophyte grow-out test

performed within two months of the experiment start date.

For Experiment 1, in September 2019, 420 ryegrass seeds, half containing AR1 and half AR37, were sown into 15 x 7 seedling trays (plug size 2 x 2 x 4 cm) containing a standard potting mix and germinated in a glasshouse. Once seedlings had produced their first true leaf, they were trimmed and placed into a vernalization chamber at 8°C for twelve weeks under an 11/13-hour light/dark regime. Six weeks later, this process was repeated, except that these plants were left to grow in the warm germination glasshouse (with the only intervention being cooling if temperatures exceeded 22°C). All seedlings were watered as required. In mid-November, when seedlings had been growing for twelve weeks in the cold or six weeks in the warm, plants from both treatments looked phenotypically similar. At this point, half the seedlings were assessed for endophyte analysis (see Figure 1) (Experiment 1 'cold' vs 'warm' grown plants).

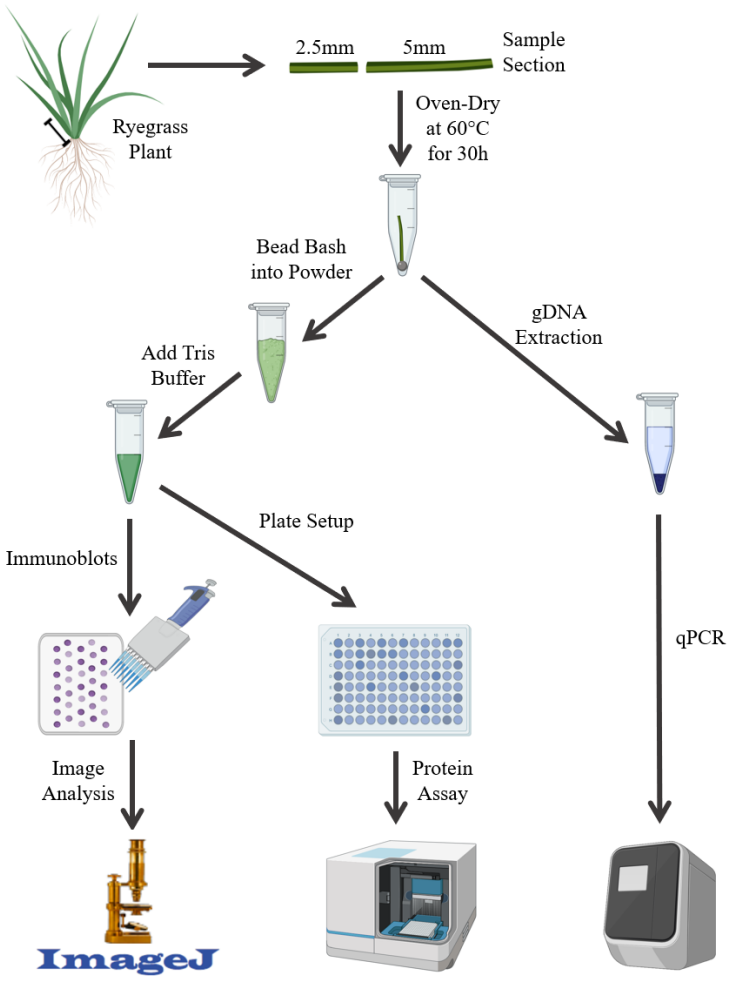

Figure 1: Overview of the sampling and processing of perennial ryegrass samples for endophyte quantification (created using BioRender).

For Experiment 2, the remaining seedlings from both treatments were re-potted into 8.5 x 8.5 x 10.5 cm pots, placed outside on a gravel pad, and left to grow under Canterbury summer conditions for the remainder of November through to January. Plants were watered at 7 am, 3 pm and 11 pm every day using an automated system. After seven weeks, the remaining plants were assessed for endophyte analysis (Figure 1).

Plants grown under these two different temperature regimes, i.e. 'cold to warm' or 'warm to warm', are hereafter referred to as C-W and W-W, respectively.

Sampling for endophyte analysis

Plants were pulled from the pots, and tillers were dissected immediately above the point of root initiation. The tiller sheath from this base was cut into two sections: a lower 2.5 mm section and an upper 5 mm section (Figure 1). For Experiment 1, all tillers were harvested; for Experiment 2, three tillers were selected randomly from each plant. Harvested material was immediately dried for 30h at 60°C and ground to a powder for further analysis (Figure 1).

Analysis of tiller samples

The ground tiller samples were analysed by immunoblot and image analysis to quantify endophyte amount, Bradford's reagent for soluble protein, and qPCR to determine relative endophyte to plant nuclear DNA content (Figure 1 and details below).

Immunoblots

Oven-dried tiller sections were bead-bashed into powder for 2 min using a Spex Sample Prep 1600 MiniG (totallab.co.nz). Then, Tris buffer (pH = 8.2) was added, equivalent to 100 µL per tiller to lyse and stabilise the homogenate. This solution was centrifuged for 5 min at room temperature at 2000g, after which 1.2 µL of each supernatant was blotted onto a nitrocellulose membrane (Amersham Protran 0.45 µm NC, GE Healthcare Life Science). Included on the blot was a serial dilution set from a 'warm' grown, known endophyte positive and negative tiller section (controls).

The membranes were developed as described by Hillis (2019). Briefly, membranes were agitated in blocking solution for 30 min. This was then replaced by 40 mL of blocking solution plus 100 µL of primary anti-endophyte antibody (monoclonal antibody supplied by Cropmark Seeds) and shaken for 1 hour. Membranes were then rinsed twice with blocking solution (~ 50 mL), and then 40 mL of blocking solution plus 15 µL of secondary antibody (goat anti-mouse IgA alkaline phosphatase) were added and agitated for a further hour. Membranes were rinsed twice as above and gently shaken with the developer for 10-30 min until the positive controls developed a dark purple colour. Once developed, the sheets were rinsed with water and digital images were taken with a Samsung Galaxy S9. ImageJ version 1.52s software was used to quantify the colour intensity of each blot. This was done by use of subtracting the background function, followed by lighting the background to a standard 50-pixel section, and then inverting the image. Using an oval selection tool, each blot was analysed using the 'Measure' function to give an average intensity for each blot. Relative endophyte amount was thus represented by an average grey value per blot.

Protein assay

To quantify the amount of protein per sample, the supernatant (as used for the immunoblotting) was analysed using Bradford (1976) reagent. Briefly, triplicates of 10 µL of each sample were assayed, alongside BSA standard dilutions of between 25 - 1500 µg/mL, and analysed using the CLARIOstar^{Plus} with the MARS software to measure and calculate protein levels as per the Coomassie Plus (Bradford) Assay Kit (thermofisher.com).

qPCR analysis

Oven-dried tiller sections were frozen using liquid nitrogen, and bead-bashed at 4°C for 1.5 min. Genomic DNA (gDNA) was extracted as described in Edwards *et al.* (1991), with minor variations to the protocol (i.e. 500 µL extraction buffer rather than 400 µL, and 400 µL of supernatant mixed with 400 µL of isopropanol instead of 300 µL of each). The final gDNA precipitate was also washed with 500 µL of 70% ethanol and centrifuged for 2.5 min. DNA samples were stored at -20°C until needed for qPCR analysis.

Chitinase A (F 5'-aagtccaggctcgaattgtg-3', R 5'-ttgaggtagcggttgttcttc-3', 353 bp) was used as the endophyte-specific primer (Rasmussen *et al*., 2006), and the primer G06_096 (F 5'-gatcttgcaggccgtctaac-3', R 5'-gccagcgtctttatttaggc-3', 160 bp) was used to amplify the ryegrass DNA (Do Canto *et al*., 2018). The qPCR was performed using the LightCycler 480 (Roche, Germany), and the reaction mixture was made up of 5 µL 2x SYBR GREEN I Master Mix, 0.5 µL of each primer, and 3 µL double-distilled water, to which 1 µL of the gDNA template was added. The efficiency and linear (dynamic) range for each primer pair was determined based on standard curves generated using a serially diluted PCR product. The gDNA extract was diluted 1/3 for Experiment 1 and 1/100 for Experiment 2, so values fell within the linear range for both primer pairs. The qPCR conditions were 94°C for 5min, followed by 50 cycles of 94°C for 10 s, 58°C for 8 s and 72°C for 10 s. Relative amount of Epichloë mycelia to host template in the amplification was then calculated using the Roche Light Cycler 480 software version 1.5.1 following the user instruction guide.

Statistical analysis

RStudio version 1.1.423 (R Core Team, 2018) was used for statistical analysis and producing the graphs for this study. For statistical significance, a two-sample t-Test was performed ($\alpha = 0.05$).

Preliminary work

Initially, the endophyte immunoblots (based on measurements in ImageJ) and protein levels were analysed to give a simple ratio of endophyte: soluble protein. As expected, this calculation produced a higher endophyte ratio in 'warm' compared to 'cold' plants, but unexpectedly, it gave a lower endophyte ratio when W-W was compared to C-W plants. The ratio calculation did not account for phenotypice/physiological tissue differences between the treatments. This difference was manifested in the different amount of soluble protein extracted between the treatments (data not shown). To account for the extra soluble protein extracted from the 'warm' treatment (Experiment 1) and the W-W (Experiment 2), the endophyte: soluble protein ratio was multiplied by a factor equal to the multiple of extra soluble protein they contained. This then took account of the phenotypic/physiological difference between the tiller tissue. For Experiment 1, this factor was 1.8 and 1.9 for AR1 and AR37, respectively, for the 'warm' treatment, and for Experiment 2, it was 5.3 and 3.9 for the AR1 and AR37, respectively, for the W-W treatment. This relative 'per unit of tissue' data takes account of the plant tissue differences (Figure 2, 4).

Results

In Experiment 1, the 'cold' grown plants had lower endophyte levels for both AR1

and AR37 per unit of tissue than 'warm' grown plants (Figure 2). This was statistically significant for endophyte AR1 ($P<0.01$) and endophyte AR37 ($P<0.001$). This result was significant, whether measured per tissue or endophyte: protein ratio.

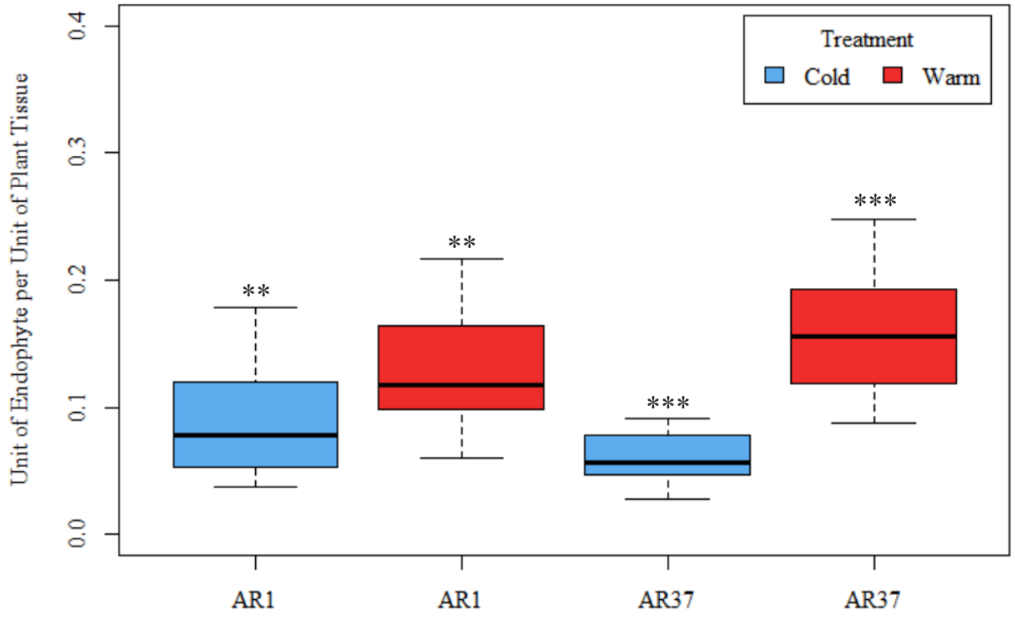

Figure 2: Boxplot showing the unit of endophyte per unit of plant tissue for the upper 5 mm section of tiller sheath section from plants grown at either cold (blue) or warm (red) temperatures and containing endophytes AR1 or AR37 (Experiment 1). ** $P<0.01$ and *** $P<0.001$ based on a two-sample t-Test.

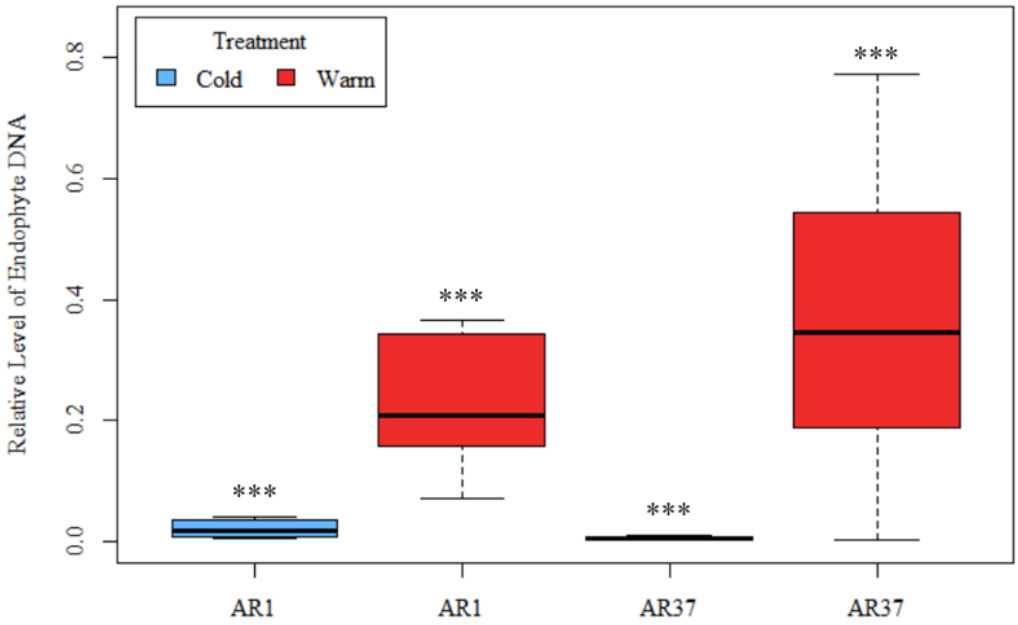

Figure 3: Boxplot showing the relative amount of endophyte (chitinase A) to plant gDNA from the upper 5 mm tiller sheath section of plants grown at either cold (blue) or warm (red) temperatures and containing endophytes AR1 or AR37 (Experiment 1). *** $P<0.001$ based on a two-sample t-Test.

Comparison of the relative gDNA levels also demonstrated that there was relatively more endophyte present in the 'warm' grown tillers than 'cold' grown tillers (Figure 3), with both endophytes showing a highly significant difference in the amount of endophyte DNA present relative to the amount of plant DNA.

In Experiment 2, the C-W grown plants also had a significant ($P<0.01$) decrease in endophyte growth compared to W-W grown plants when presented per unit of plant tissue (Figure 4). However, unlike Experiment 1, when presented on a simple ratio basis, the C-W plants had more endophyte (data not shown). In Experiment 2, the amount of endophyte per unit protein was approximately 15 times greater than in Experiment 1. This was expected, as in Experiment 1, the endophyte was only just starting to colonise the host tiller, but in Experiment 2, it would have been established.

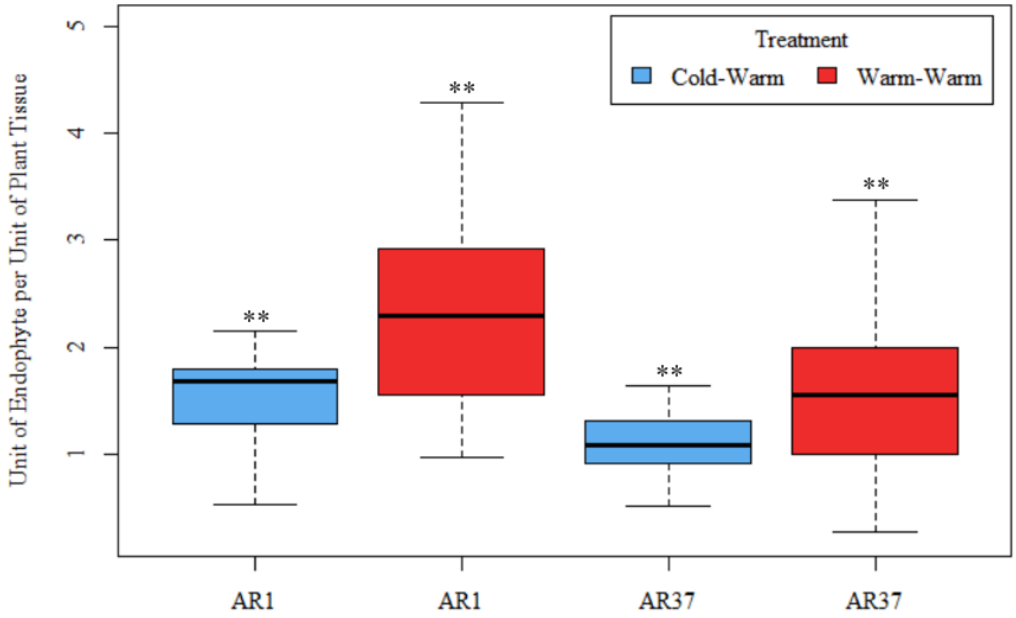

Figure 4: Boxplot showing the unit of endophyte per unit of plant tissue for the upper 5 mm tiller sheath section of plants grown at two different temperature regimes: C-W (blue) or W-W (red), and containing endophytes AR1 and AR37 (Experiment 2). ** $P<0.01$ based on a two-sample t-Test.

The qPCR data in Experiment 2 showed that there was no significant difference between the ratio of endophyte to plant DNA between the C-W and W-W plants (Figure 5).

Data presented in Figures 2 to 5 is for the 5 mm upper tiller sheath section only, as the greater volume of tissue gave lower variances. The lower tiller sheath sections gave similar overall patterns (data not shown).

Phenotypic observations between the plants showed that plants grown under the 'cold' or 'warm' regime for 12 and 6 weeks, respectively, had very similar phenotypes and plant protein levels indicating that phenotypically and physiologically, the tissue was similar for Experiment 1. However, this was not the case for Experiment 2 where the C-W plants were different from the W-W plants. This is because the vernalization response caused

the C-W plants to elongate, have very different protein levels, more woody tillers, and produce seed heads on several tillers. The W-W plants grew wider, had softer (more succulent) tillers and produced no seed heads (Figure 6).

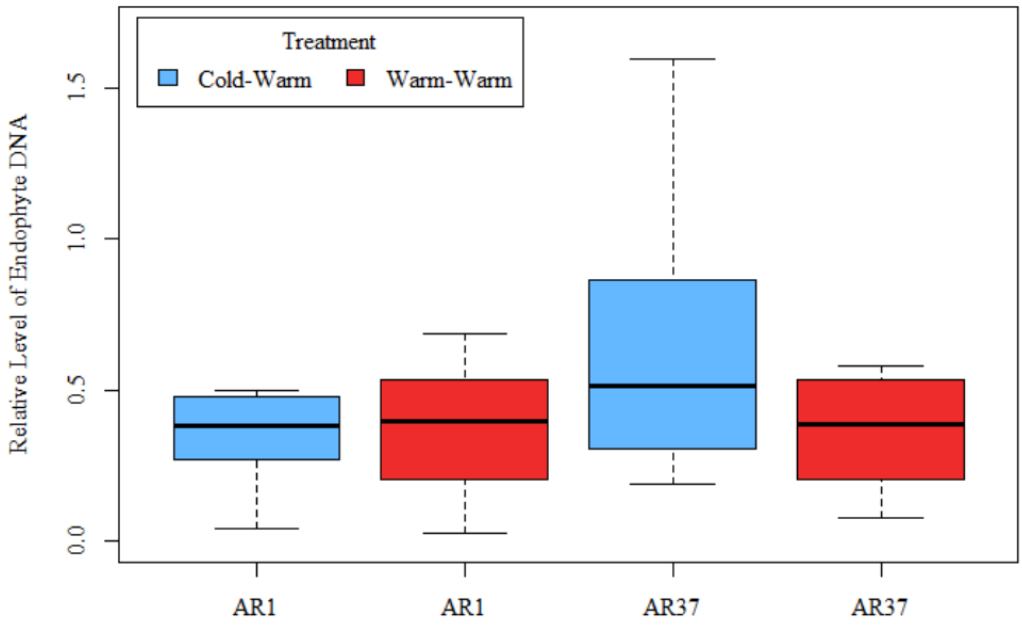

Figure 5: Boxplot showing the relative amount of endophyte (*chitinase A*) to plant gDNA from the upper 5 mm tiller sheath section of plants grown at two different temperature regimes: C-W (blue) or W-W (red), and containing endophytes AR1 or AR37 endophyte (Experiment 2). There are no significant differences based on a two-sample t-Test.

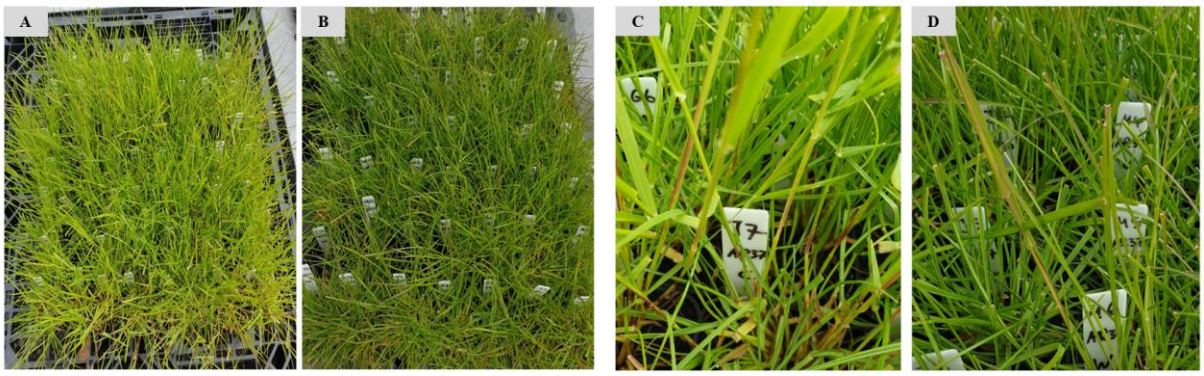

Figure 6: Phenotypic differences between vernalized C-W (A, C) and non-vernalized W-W (B, D) plants from Experiment 2, 17 weeks after the start of the experiment.

Discussion

The aim of this study was to test the hypothesis that sowing ryegrass in autumn, to meet vernalization requirements for flowering, reduces the growth of endophytes not adapted to the cold conditions of NZ winters. To determine the relative amount of endophyte in plants subjected to a cold vernalization treatment, compared with plants grown in warm conditions, a measure of the endophyte present in the different samples was needed. We set out to determine the amount of

endophyte in a sample using either endophyte-specific antibodies or qPCR to measure endophyte DNA levels, relative to the amount of extract, determined by measuring total soluble protein or plant DNA (using qPCR with plant-specific primers).

Preliminary investigations were undertaken to determine how much extract to use for immunoblots, protein analysis and qPCR determination (data not shown). Standard dilutions were blotted on the immunoblots to demonstrate that the intensity of the blots fell within a range that differentiated within a linear fashion (data not shown), likewise samples for protein analysis fell within the linear range of the standard being a BSA dilution series. For the DNA extractions, the more mature plants (C-W and W-W) had to be diluted by 1/100 for the template to fit within the linear range for the primers, whilst the initial samples ('warm' or 'cold' grown) were only diluted 1/3. This reflected the fact that the older tillers samples were larger (girth-wise), so more DNA was extracted per sample.

This investigation into the role of vernalization on endophyte-host growth relationships initially reiterated previous research, and demonstrated that the fungal biomass in growing tillers is much less when grown under cold (~ 8°C) compared with warm (~22°C) conditions (Experiment 1). This was the case for both endophytes under investigation, though the effect was more apparent in the AR37 endophyte strain. Although grown at different temperatures, the data between the 'warm' and 'cold' plants was comparable as plants were physiologically and phenotypically similar. Earlier studies (Bartholomew & Williams, 2005) show that cell growth and physiology of non-vernalized plants is based on a cumulative temperature, which supports the assumption that the longer grown 'cold' plants were physiologically and phenotypically equivalent to the more briefly grown 'warm' plants. Thus, the fungal relationship was as expected, as the intercalary growth of the Epichloë directly links vertical fungal growth to plant growth (Christensen *et al.*, 2008; Voisey, 2010) and a correlation would be expected. However, growth in the crown (shoot apical meristem) of the plant is different as it has to spread horizontally to new apices (Christensen *et al.*, 2008), and it is the amount of this heavily branched mycelia in this stage that might be affected by vernalization that causes gross changes to the meristems.

Experiment 2 endophyte: soluble protein ratio data was unexpected as it indicated that there was greater Epichloë biomass in the C-W plants than in the W-W plants. This was contrary to the proposed hypothesis, but consistent with findings from Freitas *et al.* (2020), who observed a similar increase. However, the phenotypic differences between C-W vs W-W meant that a straight comparison of endophyte amount against soluble protein was misleading. The tillers from C-W had 3.8 to 5.3 times less soluble protein per unit volume than tillers from W-W. Adjusting the data to represent it on a per unit of tissue basis showed the endophyte to be more abundant in the W-W treatment. The DNA data indicated similar amounts of endophyte: plant DNA between the two treatments. No obvious DNA extraction difference was observed between the Experiment 2 treatments, but confounding factors, such as greater cell number in the W-W tillers and more extraction-inhibiting soluble protein, impaired the ability to account for tissue differences.

The effects of vernalization on tillers are well documented. Increases in cell extension (Kemp *et al.*, 1989), gibberellic acid (Barre

et al., 2015), and lignification (Seppänen *et al.*, 2010) are known to occur, along with a decrease in available fructans (Pollock & Jones, 1979). These act to increase growth rate (Kemp *et al.*, 1989), but decrease the nutritive value of the tiller (Seppänen *et al.*, 2010), with nutrients going to the reproductive organ and to the production of lignin to strengthen the elongated tiller. Our findings suggest the following model (Figure 7) for fungal growth in tillers, which is consistent with low temperature causing a decrease in endophyte presence. This is contrary to what has been reported previously (Freitas, 2017; Freitas *et al.*, 2020), but accounts for phenotypic and physiological differences following vernalization.

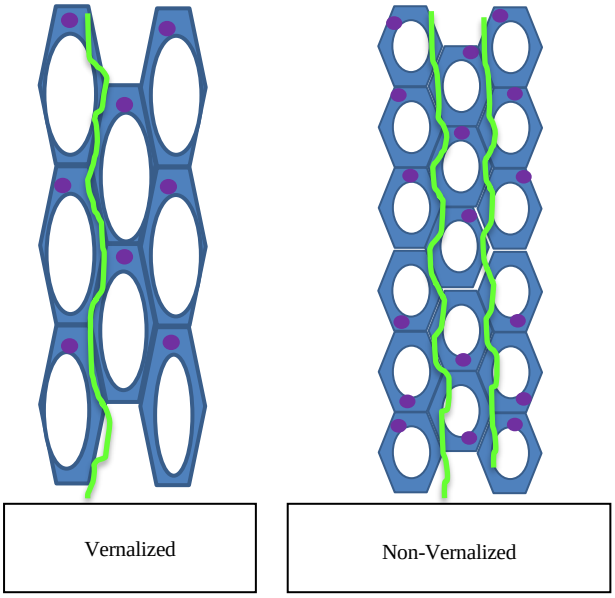

Figure 7: Schematic proposing the difference in endophyte growth between vernalized (C-W; left) and non-vernalized (W-W; right) ryegrass tillers. Each hexagon represents a cell with light blue cytoplasm, a central vacuole, and a purple nucleus. Endophyte hyphae are shown in green. Each section represents the same length of tiller. On a per tiller section basis there is twice as much endophyte in the non-vernalized tiller compared to the vernalized tiller (depicted as 1 and 2 hyphal strands). However, due to the physiological differences between the two tillers, this difference is not observed in an endophyte/plant DNA or endophyte/soluble protein ratio. On a nuclear DNA (●) basis, there is an ~equivalent endophyte: plant ratio of 1 fungal hypha:8 plant nuclei in the vernalized section, and 2:17 ratio on the non-vernalized section (similar to the results in Figure 5). Our measurements indicated an approximately 4x higher amount of soluble protein in the non-vernalised section compared to the vernalized section (indicated by a greater content of cytoplasm in the non-vernalized section). In our model, this would result in an endophyte/protein ratio of 1:1 in the vernalized tiller and 2:4 in the non-vernalized tissue, leading to the misleading suggestions of greater endophyte growth in the vernalized material.

What effect the physiological changes of vernalization have on the host-endophyte relationship is poorly understood. One hypothesis for this is that vernalization differentially affects the growth of the branched Epichloë mycelia within the shoot

apical meristem and the growth of the plant meristem itself. Thus, under colder conditions, less mycelia are available to invade the elongating shoot and grow in a linked intercalary fashion, as demonstrated by Christensen *et al.* (2008). Such decreased colonization could then ultimately affect the number of hyphae in the reproductive tillers and potentially endophyte growth into the seed. The authors can find no research that directly investigates the transition through vernalization on endophyte-generative tiller colonization. The illusion caused by the plant tissue transformation has hidden the observation reported here. Freitas (2017) and Freitas *et al*. (2020) did follow a cold growth period with a warm growth period, but the duration of cold was only three weeks, which was insufficient for vernalization. Freitas observed an increase in mycelial growth for the C-W treatment but took no account of plant tissue differences. Freitas (2017) concluded that "*Epichloid endophyte survival and dissemination depend on plant host success (Johnson et al., 2013). It would therefore be advantageous to have the plant host growing under optimal temperature conditions to stimulate a high concentration of endophyte mycelia, but this will not usually occur in farm systems*". At present, the requirement for vernalization to flower means that this cannot occur in the seed production context. The NZ median minimum winter temperature in seed production regions is 0-2°C (niwa.co.nz), which is much lower than grass Epichloë endophyte cardinal temperatures (Ju *et al.*, 2006; Freitas, 2017). Reducing the negative effect that vernalization has on Epichloë growth may be possible by choosing growth conditions that minimise the duration and severity of the cold temperatures, but still allow vernalization.

Conclusion

Under vernalization conditions, there is a significant reduction in endophyte growth. This study also highlights how the physiological changes that occur in ryegrass following vernalization can impact on the ability to compare endophyte growth under different temperature regimes. Further research into vernalization on endophyte growth and transmission into reproductive tillers is required. Such insights may help improve endophyte transmission during ryegrass multiplication, which would be especially valuable for the NZ ryegrass seed industry.

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