Identification of biomarkers associated with the survival of selected *Epichloë* endophytes while in seed storage

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Abstract

Epichloë is a genus of fungal endophytes that form symbiotic associations with cool season grasses in the sub-family Pooideae. In New Zealand, selected strains of asexual *Epichloë* endophytes have been developed into commercial products for pasture persistence and wild-life deterrence where they confer bioprotective traits to their host grasses. As asexual *Epichloë* species are exclusively vertically transmitted, their survival in seed is critical for their dissemination to progeny plants. Endophyte survival declines at a much faster rate than seed viability, however the mechanism responsible for this decline is unknown. This study investigated the effects of seed ageing on mycelial biomass and selected metabolite concentrations in *Epichloë*-infected seed of perennial ryegrass (*Lolium perenne*) with the main objective to identify key metabolites that play a significant role in the viability of endophyte in seed during storage. Fifty-eight seed lines, comprised of three cultivars of perennial ryegrass associated with two economically important endophyte strains (AR1 and AR37), were subjected to an accelerated ageing regime designed to mimic the physical deterioration processes that would occur during natural seed ageing. Selected amino acids, neutral sugars, sugar alcohols, phytohormones and *Epichloë*-derived secondary metabolites were then analysed and their concentrations examined for effect of seed ageing, cultivar, and endophyte strain. As predicted, accelerated ageing had little to no effect on the seed germination rate of the selected ryegrass cultivars but negatively impacted the frequency of endophyte viability within seed. Proline and xylose gave the greatest fold changes in concentration across all grass cultivars after seed ageing but were not correlated to endophyte infection frequencies and therefore not suitable as biomarkers. Ribitol, a sugar alcohol, exhibited concentration fold changes that correlated with endophyte viability and therefore has potential as a biomarker to trace the viability of endophytes in perennial ryegrass seed during storage.

Keywords: accelerated ageing, metabolite, sugar

Introduction

Epichloë (family Clavicipitaceae) is a genus of filamentous fungi that has coevolved with certain cool season grass species within the sub-family Pooideae. *Epichloë*–grass symbioses are enduring, ranging from antagonistic to mutualistic, with the nature of the symbiosis closely linked to the lifecycle of the fungus. Many *Epichloë* species, including all the asexual types, are systemic endophytes, restricted to the intercellular spaces within the aboveground organs of their grass hosts while those capable of sexual reproduction can form stromata on grass reproductive tillers suppressing the development of inflorescences. *Epichloë* mainly confer advantageous traits to their host grasses, including protection from mammalian and invertebrate herbivory, resistance from phytopathogens and tolerance to drought and/or nutrient deficiencies and are therefore regarded as keystone species in many natural grassland ecosystems (Malinowski & Belesky 2000; di Menna et al. 2012; Bastías et al. 2017; Nissinen et al. 2019; Card et al. 2021; Cheng et al. 2021). In New Zealand, selected strains of asexual *Epichloë* endophytes have been developed into commercial products for pasture persistence and wild-life deterrence where they confer bioprotective traits to their host grasses (Pennell et al. 2016). In managed ecosystems, such as pastoral ecosystems in New Zealand, strains of asexual *Epichloë* species have been selected for their invertebrate pest deterrent abilities and their low, or non-existent, toxicity towards grazing

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livestock (Card et al. 2024). These agriculturally safe endophytes, including the strains AR1 and AR37, have proven to be commercially successful, increasing the number of grass products available from many pasture seed companies and contributing millions of dollars to the New Zealand economy (Caradus et al. 2021).

Asexual *Epichloë* species are exclusively transmitted via host seed and are therefore reliant on the success of the plant's reproductive strategy for their dissemination and survival (Zhang et al. 2017). Endophyte survival declines at a much faster rate than seed viability, however the mechanism for this decline is unknown. As endophyte infection frequencies greater than 70% must be assured for marketing endophyte seed products in New Zealand (Johnson & Caradus 2019)`, understanding the survival of the fungus in seed is a critical scientific research area but is largely unexplored due to its biological complexity (Hume & Barker 2005; Rolston & Agee 2007). Mannitol, ribitol and trehalose were found at consistently higher concentrations within endophyte-infected compared to endophyte-free tall fescue seed implicating their importance in endophyte survival in grass seed (Zhang et al. 2019).

This study aimed to further the research on endophyte survival in seed by analysing mycelial biomass, rate of germination and a variety of selected plant and fungal derived primary and secondary metabolites during seed ageing. The study also focused on perennial ryegrass, the most important forage species cultivated in New Zealand.

Materials and Methods

Biological materials

Seed from 58 lines of perennial ryegrass, from three cultivars (Ceres One50, Legion and Platform) harvested from the Canterbury region of New Zealand in the same season, were supplied by PGGW Seeds Ltd. The cultivars are late flowering diploid cultivars with New Zealand and north-west Spanish origin with Ceres One50 being genetically discrete from Platform and Legion (Pers comm. Alan Stewart, PGG Wrightson Seeds Ltd.). Seed lines were colonised by either *Epichloë festucae* var. *lolii* strain AR1 (n=11, n=6, n=4, respectively) or *Epichloë* sp. LpTG-3 strain AR37 (n=13, n=12, n=12, respectively). All seed were stored in paper bags at 0°C and 30% relative humidity in the Margot Forde Genebank, New Zealand's national gene-bank of grassland plants in Palmerston North, New Zealand, until use.

Experimental set-up

Seed from each of the 58 seed lines were separated into six groups each containing 0.5 g of seed. The first group represented an untreated control group of non-aged seed (timepoint 0; T_0), while the remaining 5 groups (T_1-T_5) were subject to an accelerated ageing (AA) regime of varying duration (5, 7, 9, 11 and 15 days respectively), whereby seed was exposed to a temperature of 46°C and relative humidity of 50% (modified from Bylin et al. 2016). Following AA, approximately 100 seeds per group were assessed for seed germination and endophyte infection status as described by Zhang et al. (2019). The remaining seed was frozen at -80°C until mycelial biomass and chemistry analysis could be undertaken.

Sample preparation for mycelial biomass and chemistry analysis

Seeds from T_0 (non-aged) and T_5 (aged seed exposed to 15 days of AA) groups were assessed for *Epichloë* mycelial biomass, targeted *Epichloë*-derived secondary metabolites and selected primary metabolites. Seed samples were transferred to plastic 7 mL screw cap vials (Micro-Analytix NZ Ltd., New Zealand), lyophilised using a FreeZone Plus12 Cascade Console Freeze Dryer (Labconco Corporation, USA) for 48 hours, pulverised to a fine powder using a Bead Ruptor 12 bead mill (Omni International Inc., USA) and stored at -80°C until required.

Analysis of mycelial biomass, neutral sugars, sugar alcohols, amino acids, phytohormones and *Epichloë*-derived secondary metabolites

Epichloë mycelial biomass per timepoint was determined using an enzyme-linked immunosorbent assay (ELISA) developed by AgResearch (Faville et al. 2015). Selected neutral sugars (namely arabinose, fructose, galactose, glucose, lactose, maltose, mannose, raffinose, sucrose, trehalose, and xylose) and sugar alcohols (namely arabitol, galactinol, glycerol, myo-inositol, mannitol, ribitol, sorbitol, and xylitol) were analysed as per the method described by Zhang et al. (2019). Selected amino acids (namely leucine, iso-leucine, tryptophan, proline, glutamic acid, and asparagine), phytohormones (namely jasmonic acid, salicylic acid, abscisic acid, and indole-3-acetic acid) and selected *Epichloë*-derived alkaloidal secondary metabolites including peramine and epoxyjanthitrems (i.e., epoxyjanthitrems I, II, III, IV, and epoxyjanthitriol) were analysed as described by Bastías et al. (2024). AR1 produces the secondary metabolite peramine (responsible for invertebrate pest deterrence) but none of the ergot alkaloids, lolitrems, or epoxyjanthitrems linked to animal toxicosis when associated with its original native grass host or within novel (or artificial) associations. AR37 does not produce peramine, lolitrems or ergot alkaloids, but does produce epoxyjanthitrems, a group of indole-diterpenes.

Preparation and analysis of seed samples to obtain α-tocopherol, δ-tocopherol, γ-tocopherol concentrations

Analysis of tocopherols was modified from the methods of Bao et al. (2020). Standards for α-tocopherol, δ-tocopherol, γ-tocopherol and internal standard tocopherol acetate were obtained from Sigma-Aldrich® (Merck, Germany) while other reagents used in the extraction of tocopherols; ethyl acetate, chloroform, acetone and methanol were obtained from Thermo Fisher Scientific Inc. (Australia). Lyophilised and ground seed samples (0.06 g), stored at -80°C were weighed into 2 mL plastic vials. Two extraction solutions were prepared: extraction solution A consisting of ethyl acetate and chloroform (80:20) and extraction solution B consisting of water, acetone and methanol (75:15:5). To each sample, 180 µL of extraction buffer A, 220 µL of extraction buffer B and 100 µL of tocopherol acetate (2.3 mg/mL in methanol) internal standard was added and mixed thoroughly. The samples were extracted by end-over-end rotation for 1 hour in the dark followed by centrifugation at 10,000 rpm for 10 min (Centrifuge 5427 R, Eppendorf, Germany). Centrifugation at high rpm allowed the formation of a compact pellet and clear phase separation of immiscible liquids in the supernatant. A subsample of the top phase (200 µL) was transferred to an amber HPLC vial and dried under nitrogen gas. The dried sample was re-suspended in 100 µL methanol and transferred to a glass insert and placed into amber HPLC vials for immediate analysis by HPLC coupled to a fluorescence detector.

A 10 µL injection of a set of combined tocopherol calibration standards (serial dilutions from 100 to 0.1 µg/mL in methanol with an internal standard concentration of 230 µg/mL) were analysed alongside samples in each batch. Chromatographic separation of the tocopherols was achieved on a Dionex Ultimate 3000 (Thermo Fisher Scientific Inc., Australia) instrument set at 45°C using a Kinetic XB-C18 2.1 × 150 mm (2.6 µm) column (Phenomenex Inc., USA) and an isocratic gradient of methanol and water (93:7) with a constant flow rate of 0.4 mL/min for 10 min. The tocopherols were detected and quantified by fluorescence (290 nm excitation and 330 nm emission) while Chromeleon v7.2.10 software (Thermo Scientific Inc., USA) was used to process raw data. For δ-tocopherol and γ-tocopherol the limits of detection and quantification were 1.6 µg/g and 5.0 µg/g, respectively and for α-tocopherol were 3.3 µg/g and 6.6 µg/g, respectively.

Statistical analysis

All statistical analyses were carried out using R software (version 4.4.2; http://www.R-project.org, R Foundation for Statistical Computing). In the hypothesis tests, the significance level (alpha) was set at 0.05. To compare endophyte infection frequencies across seed lines, an area under the curve (AUC) was calculated for each seed line using a cubic spline equation for the change in endophyte infection frequency (%) over time. Additionally, to account for the variability in the initial endophyte infection frequency (%) between different seed lines, a normalised AUC for each line was calculated by dividing AUC with its corresponding endophyte infection frequency (%) from the untreated control group (T_0). The AUC function was calculated from the DescTools R package where the cubic spline method was used to calculate area under the curves for each seed line (Signorell, 2025). Welch's t-test and Tukey's HSD was used to test for differences in germination rate (%) and endophyte infection frequency (%). An analysis of variance (ANOVA) was used to compare treatments (timepoint, cultivar and endophyte) for all metabolite values, following which the predictmeans R package was used to obtain mean values and standard errors for each treatment (Luo et al. 2024). Correlation tests were performed using the cor.test function in base R with Pearson correlation coefficients method. The Rstatix R package version 0.7.2 was used to generate means and standard errors of germination and endophyte viability percentages, which were plotted using the ggplot2 R package version 3.5.1.

Results

Seed germination and endophyte infection frequency data

The average germination rates for non-aged seed groups (i.e. T_0) were 89.3, 84.0 and 92.5% for Ceres One50, Legion and Platform, respectively. The germination rates for seed groups exposed to 15 days of AA (i.e. T_5) were 83.2, 81.8 and 88.5%, for Ceres One50, Legion and Platform, respectively (Figure 1). The mean seed germination (%) for all three grass cultivars was not affected by the AA regime even after the maximum of 15 days exposure ($P > 0.05$) (Figure 1).

The average germination rates for non-aged seed groups (i.e. T_0) with AR1 endophyte were 89.3, 84.0 and 93% for Ceres One50, Legion and Platform, respectively, while the average germination rates for seed groups exposed to 15 days of AA (i.e. T_5) were 85.4, 81, and 84.9%, for Ceres One50, Legion and Platform, respectively (Figure 1a). Similarly, the average germination rates for T_0 seed groups with AR37 endophytes were 89.3, 83.8 and 92.4% for Ceres One50, Legion and Platform, respectively and for seed groups exposed to 15 days of AA (i.e. T_5) were 82.1, 82.3 and 89.7%, for Ceres One50, Legion and Platform, respectively (Figure 1b). Overall, there were no significant differences ($P > 0.05$) between AR37 and

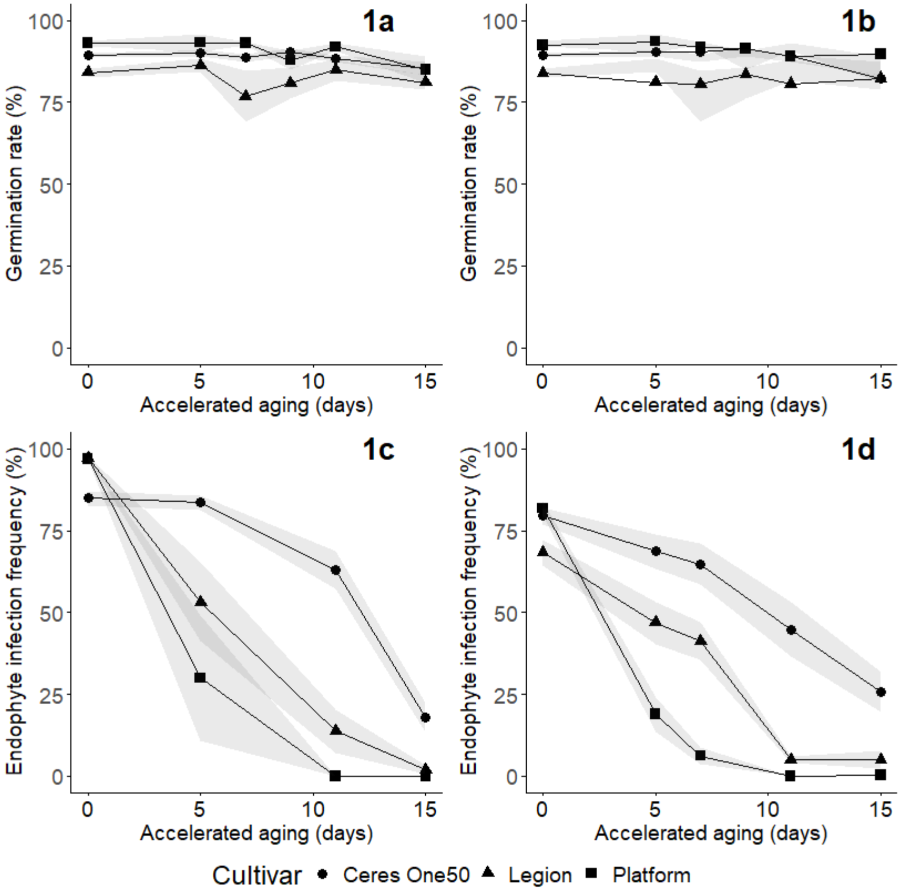

Figure 1 Mean germination rates (%) and endophyte infection frequencies (%) from 58 seed lines of three cultivars of perennial ryegrass (Ceres One50, Legion and Platform), infected with either AR1 or AR37, after exposure to an accelerated ageing (AA) regime. (1a) mean germination rate (%) of AR1, (1b) mean germination rate (%) of AR37 (1c) mean endophyte infection frequencies (%) during AA for seed lines with AR1 and (1d) mean endophyte infection (%) after exposure to AA with AR37. Standard errors are shown as grey ribbons. N.B. A mean value was not included for Legion at days 7 and 9 AA in AR1 and day 9 AA for AR37 seed lines due to a lack of data.

AR1, however, there was a small drop-in germination rate when exposed to 15 days of accelerated ageing with an average germination rate at T_0 of 88.9 to average germination rate at T_5 of 84.3% ($P = 0.03$). There was a significant difference observed in the overall average germination rate between cultivars with Platform that exhibited the highest overall mean germination rate of 90.4%, followed by Ceres One50 with 85.6% and Legion with 83.9% ($P = 0.009$).

The difference in the endophyte infection frequencies (%) between non-aged seed groups (i.e. T_0) and aged seed groups (i.e. those exposed to AA, T_1-T_5) were statistically significant ($P < 0.0001$) with all three factors (timepoint, endophyte strain and cultivar) also statistically significant (all with $P < 0.0001$). Both AR1 and AR37 endophyte infected seed lines showed a decreasing endophyte infection frequency (%) with increasing exposure to AA (Figure 1c and Figure 1d).

The normalised AUC value of a seed line is associated with the changing endophyte infection frequencies for that seed line over the entire duration of AA. A higher normalised AUC value indicates that a higher endophyte infection frequency percentage was maintained for a longer duration during AA by that seed line. Conversely, a lower normalised AUC value indicates that the endophyte infection frequency percentage declined steeply during AA. The normalised AUC values for seed lines from Ceres One50, Legion and Platform cultivars over the AA were significantly different ($P < 0.01$) from each other (3.80, 1.96 and 0.95, respectively).

Comparison of mycelial biomass values in untreated control and accelerated aged seeds

The average mycelial biomass of all the seed lines for non-aged seed groups (i.e. T_0) was 3.25 µg/mg and was significantly ($P < 0.05$) higher compared to the average mycelial biomass for aged seed groups after 15 days of AA (i.e. T_5) at 2.75 µg/mg. An ANOVA of the mycelial biomass for all the seed lines showed that all the factors were significant for timepoint ($P = 0.004$), cultivar ($P < 0.0001$) and endophyte strain ($P < 0.0001$). There was a significant ($P < 0.05$) decline in the average mycelial biomass of AR37 from T_0 to T_5 for Ceres One50 (52%), and Legion (71%), but not for Platform. There was no significant change observed in the average mycelial biomass of AR1 in the three grass cultivars from T_0 to T_5. Interestingly, there was poor correlation between change in mycelial biomass due to AA and normalised AUC (Pearson correlation co-efficient: $r^2 = -0.24$, $P > 0.05$).

Impact of differences in seed weight between cultivars

The average thousand seed weight (TSW) in untreated control samples (T_0) was 1.99 g (± 0.02 g), this was not significantly different when compared to average TSW after accelerated ageing (T_5) at 1.87 g (± 0.02 g) (post-hoc $P < 0.134$). However, the average TSW for cv. Platform was significantly lower ($P < 0.0001$) at 1.78 g (± 0.02 g) compared to Ceres One50 at 1.94 g (± 0.02 g) and Legion at 1.93 g (± 0.03 g) ($P < 0.0002$). To account for the differences in TSW across ryegrass cultivars, the metabolite concentrations obtained for the untreated control groups (i.e. T_0) and groups exposed to 15 days AA (i.e. T_5) were normalised by the average TSW (g) for each accession at the corresponding timepoints.

Comparison of metabolites between non-aged and AA seeds

The $\log_2$ fold-change in normalised concentrations of twenty-six of the forty metabolites analysed were significant ($P < 0.05$) for at least one of the ANOVA factors of timepoint, cultivar or endophyte strain. The strain specific *Epichloë*-derived secondary metabolites, peramine, epoxyjanthitriol and epoxyjanthitrems I-IV were statistically significant ($P < 0.05$) by ANOVA factoring for endophyte strain (AR1 vs AR37). However, these endophyte-derived metabolites were not significantly different between T_0 and T_5 or cultivars and were therefore excluded from further analyses. Table 1 shows the $\log_2$ fold-change in normalised mean concentration of T_5 (final AA timepoint) relative to T_0 (non-aged) for the twenty statistically significant metabolites across the three different grass cultivars.

The $\log_2$ fold-change in concentration of arabitol was significant ($P < 0.05$) for all the ANOVA factors, while the fold-change of many of the analysed metabolites belonging to various metabolite classes (tryptophan, tocopherol-γ, trehalose, xylose and mannitol) were significantly different ($P < 0.05$) for timepoint (between T_0 and T_5) and cultivar. The $\log_2$ fold change concentrations of the remaining metabolites in Table 1 were either significant for timepoint or cultivar ANOVA factors.

Of all the analysed metabolites, the greatest decrease across all cultivars was observed for proline ($\log_2$ fold change -2.9, -1.9 and -2.0 for Ceres One50, Legion and Platform, respectively) while xylose gave the greatest increases across all cultivars ($\log_2$ fold changes 1.8, 1.2 and 1.4 for Ceres One50, Legion and Platform, respectively) (Table 1).

Those metabolites with a statistically significant change in concentration showed a consistent increase or decrease between the cultivars with the only exception being ribitol (Table 1). In Ceres One50, there was an increase in $\log_2$ fold change of ribitol after AA, while in Platform there was a decrease after AA, and there was no change in Legion after AA (Table 1). Further statistical exploration showed that ribitol was positively correlated with normalised AUC (Pearson correlation co-efficient: $r^2 = 0.67$ and $P < 0.0001$). Thus, the $\log_2$ fold change of ribitol increased in seed groups from T_0 to T_5 in lines with a higher normalised AUC and decreased in lines with a lower normalised AUC (Figure 2).

Discussion

This study furthered the research on endophyte survival in seed by analysing a wider range of metabolites than previously described (e.g. Zhang et al. 2019) and by focusing on two economically important endophyte strains (AR1 and AR37) across selected cultivars of perennial ryegrass, the most economically important forage in New Zealand. To investigate the metabolites associated with optimal or increased endophyte survival, a method which compared the metabolite profiles of seeds with and without endophyte infection was modified and expanded from Zhang et al. (2019). Metabolites included amino acids, neutral sugars, phytohormones, sugar alcohols, vitamins and endophyte-derived alkaloidal secondary metabolites. The greatest fold changes observed were associated with proline, that gave decreases in concentration across all cultivars after ageing, and xylose, that gave increases in concentration across all cultivars after ageing. However, only ribitol exhibited fold changes in concentration that correlated with endophyte

Table 1 Statistically significant metabolites (ANOVA $P<0.05$ for at least one factor) and their observed $\log_2$ fold change of normalised means of T_5 relative to T_0 across three perennial ryegrass cultivars (Ceres One50, Legion and Platform). Absence of P-values indicate that the factor was not statistically significant. Negative fold-change values indicate metabolites which decreased in concentration during accelerated ageing, while positive fold change values indicate metabolites which increased in concentration during accelerated ageing. No change in concentration during AA is indicated by 0.0.

Metabolite class and metabolite	**ANOVA factors (P)**			**$\log_2$ fold change (T_5 vs. T_0)**		
	Endophyte	**Timepoint**	**Cultivar**	**Ceres One50 (n = 46)**	**Legion (n = 38)**	**Platform (n = 32)**
Amino acid						
Asparagine		0.03		-1.2	-1.1	-1.3
Glutamic Acid		0.03		-1.2	-0.9	-0.9
Proline		0.04		-2.9	-1.9	-2.0
Tryptophan		0.04	0.00	-0.6	-0.1	-0.5
Neutral sugar						
Mannose			0.01	0.1	0.3	0.6
Sucrose			0.01	-0.1	0.0	-0.1
Trehalose		0.04	0.03	-0.4	-0.3	-0.3
Xylose		0.00	0.01	1.8	1.2	1.4
Phytohormone						
Abscisic acid			0.02	0.2	0.3	0.2
Indole-3 acetic acid			0.00	0.7	0.6	0.4
Jasmonic acid			0.02	0.1	0.2	0.2
Salicylic acid		0.01		0.8	0.6	0.9
Sugar alcohol						
Arabitol	0.04	0.02	0.03	-0.3	-0.1	-0.3
Glycerol			0.02	0.3	0.4	0.7
Inositol			0.02	-0.1	-0.1	0.0
Mannitol		0.00	0.01	-0.3	-0.3	-0.4
Ribitol			0.01	1.1	0.0	-0.8
Sorbitol			0.01	0.5	0.6	1.1
Vitamin						
Tocopherol-δ			0.01	-0.2	-0.2	-0.6
Tocopherol-γ		0.04	0.00	-0.3	-0.1	-0.3

viability and therefore this metabolite has potential as a biomarker to track the viability of AR1 and AR37 in perennial ryegrass during seed storage.

An AA technique was utilised to mimic the physical deterioration processes that would occur during natural ageing of ryegrass seed (Bylin et al. 2016). As predicted, AA had little to no effect on the seed germination rate of the selected ryegrass cultivars but negatively impacted the frequency of endophyte viability within seed. Neil (1940, 1941), more than 80 years ago, described endophyte death in perennial ryegrass and tall fescue seed after 12-18 months storage. This decline in endophyte viability largely fits a logistic relationship whereby a plateau or slow decline is first exhibited, followed by a rapidly increasing decline, and then another plateau as the proportion of seeds with viable endophyte approaches zero (Gundel et al. 2009; Hume et al. 2011; Card et al. 2014). The physiological mechanisms involved in endophyte viability decline within seed have not been determined.

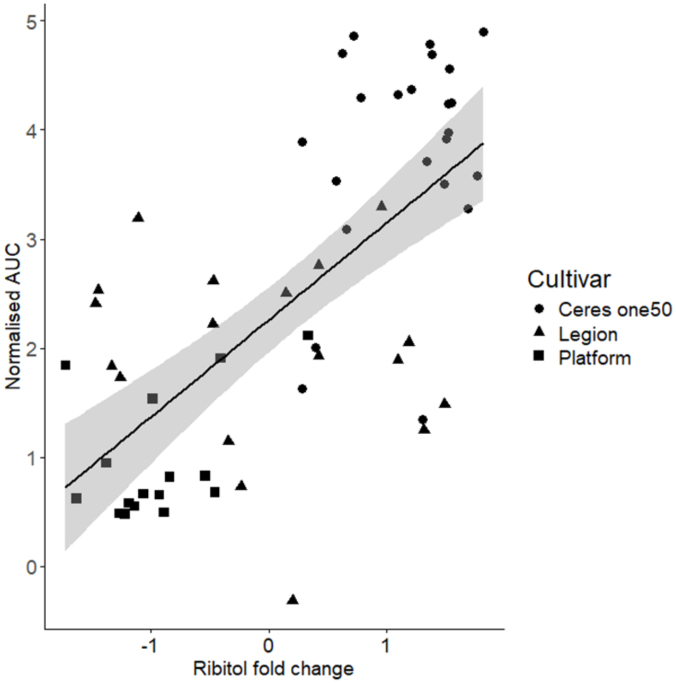

Figure 2 Scatter plot of normalised AUC vs the $\log_2$ fold change in ribitol concentration in T_5 relative to T_0 for the three different grass cultivars (Ceres One50, Legion and Platform). The grey ribbon indicates standard errors and the pattern of positive correlation of ribitol to AUC.

Mycelial biomass has been suggested as being an important factor with respect to endophyte longevity in seed (Card et al. 2014). The current study showed that, for AR37, this was the case with two out of the three cultivars assessed showing reductions in fungal biomass of 52% and 71% in Ceres One50 and Legion, respectively. However, no such relationship between AA and fungal biomass was observed for AR37 associated with the cultivar Platform or for AR1 in any of the three assessed cultivars. It is well known that mycelia are sensitive to heat and that increased temperature can damage fungal cell walls, leading to increased ion leakage and other cellular damage. As there were differences exhibited by the two assessed endophyte strains and the three assessed grass cultivars, endophyte and host genetics are speculated to play a pivotal role here.

Many of the metabolites analysed in the current study recorded significant changes in concentration between the non-aged and aged seed groups. These included the amino acids asparagine, glutamic acid, proline and tryptophan, that all decreased in concentration following seed ageing. The greatest fold changes from an amino acid were recorded for proline. Proline is a proteinogenic amino acid linked to stress response in bacteria, fungi and plants where it accumulates in tissues, such as pollen, that naturally desiccate. In plants, proline functions as an osmoprotectant, inhibiting the denaturation of enzymes, stabilisation of proteins and as a hydroxyl radical scavenger (Hsu et al. 2003), while in seed, proline may have a role in germination keeping the developing embryonic axis in a resting state (Thakur & Sharma 2005). In fungi, proline has been shown to function as a potent antioxidant and inhibitor of programmed cell death protecting cells against various lethal stresses, including UV light, salt, heat, and hydrogen peroxide (Chen & Dickman 2005). In some grasses, the presence of *Epichloë* sp. can increase proline concentration in seedlings and vegetative plants, enhancing establishment in the early stages of plant growth (Ma et al. 2015) and potentially supporting host salt tolerance (Chen et al. 2019), respectively. This study did not set about to determine whether these metabolites were plant or fungal derived or the seed tissues of origin (e.g. embryo vs. endosperm).

Of the analysed neutral sugars, trehalose decreased in concentration following seed ageing while xylose increased in concentration following seed ageing. Trehalose, a disaccharide sugar, is commonly found in bacteria, fungi, plants and insects having multiple functions. In insects, trehalose serves as a blood sugar and is the major energy storage molecule used for flight. In fungi, trehalose functions as a reserve carbohydrate and a stress protectant. Zhang et al. (2019) suggested that trehalose might be a metabolic substrate for endophyte survival during seed storage as lower concentrations of the sugar were found in aged seed compared with nonaged seed, regardless of endophyte strain. The current study also showed that trehalose exhibited a decreasing concentration across all three perennial ryegrass cultivars with AA. However, as the three grass cultivars exhibited significantly different normalised AUC values, any metabolite showing the same fold change trend across cultivars would therefore be unsuitable as a biomarker for endophyte survival in seed. Xylose, a pentose monosaccharide sugar, is the main building block of xylan, the main polysaccharide component of cell walls in grasses and has important functions in plant defence against herbivores and phytopathogens. Although not as prevalent in fungi, Zhang et al. (2019) showed that xylose was present in the seed of some grass-*Epichloë* associations. As observed for trehalose, the increasing xylose concentrations with ageing showed the same trend across all three grass cultivars and was also deemed unsuitable as a biomarker for endophyte survival in seed.

Ribitol, a pentose sugar alcohol, is found naturally in some plants, the embryo of developing seeds, and in bacteria and lichens. Ribitol is not a common fungal metabolite but has been identified in several species including *Puccinia graminis* f. sp. *tritici* and *Candida polymorpha* (Pfyffer & Rast 1980). In lichens ribitol is the main form in which carbohydrate moves from the algal photobiont to the mycobiont (Richardson & Smith 1968). Ribitol is an abundant polyol in the Arctic lichen *Cetraria* sp. and is the sole carbon source for the lichen-associated bacterium *Sphingomonas* sp. PAMC 26621 (Tran et al. 2020). Zhang et al. (2019) showed that ribitol, alongside mannitol, was more abundant in the embryo than the endosperm tissues of endophyte-infected tall fescue seed. Zhang et al. (2019) also showed that the concentration of ribitol was higher in the endophyte-infected than endophyte-free seeds indicating the sugar is related to *Epichloë* spp. mycelia. Within a limited number of seed lines of endophyte-infected tall fescue, Zhang et al. (2019) showed that the concentration of ribitol in endophyte-infected seeds was stable and did not increase or decrease after seed ageing. The current study, however showed that the concentration of ribitol increased in seed lines with a higher normalised AUC following seed ageing and decreased in concentration in seed lines with a lower normalised AUC.

Further work would need to elucidate the host genetics with regard to this finding with the possible aim of determining if high ribitol concentrations (or pathway intermediates leading to the production of ribitol) in selected seed lines offer an optimised environment for selected strains of *Epichloë* spp. and/or to use ribitol as a biomarker in other grass-endophyte associations to determine proof of concept with the overall aim to increase the survival of endophytes in stored grass seed products.

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