Increasing endophyte alkaloid expression in tall fescue by selecting for increased endophyte hyphal density

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Abstract

Two AR542-endophyte infected tall fescue breeding pools were subjected to one cycle of selection for increased endophyte hyphal density. Hyphal density was assessed subjectively by microscopic examination of the leaf sheath. Levels of alkaloids produced by both the original and selected populations were subsequently measured during summer. Levels of lolines and peramine alkaloids increased significantly in one breeding pool (by 40% and 29%, respectively), with non-significant increases (by 24% and 2%) in the other breeding pool. The apparent correlation between observed hyphal density and levels of alkaloids supports the hypothesis that levels of lolines and peramine produced by fungal endophytes in their host grass are at least partly due to endophyte hyphal mass in the host sheath tissue.

Measured lolines in one breeding pool were 21 times greater than the other, while sheath hyphal density was less. Possible explanations include contrasting patterns of hyphal colonisation beyond the sheath, and/or variation in the quantity of lolines produced per unit hyphal mass.

Keywords: *Neotyphodium coenophalium,* endophyte, *Festuca arundinacea,* tall fescue, peramine, lolines, hyphal mass

Introduction

Neotyphodium fungal endophytes form mutualistic associations with their grass hosts. Within this association, a number of alkaloids are synthesised *in planta*, which can function as deterrents to micro and macro herbivores, enhancing the survival of the endophyte and its grass partner. Infection of tall fescue with *N. coenophialum* can increase pasture persistence and yield, at least partly because of the anti-herbivory activity of some ergot alkaloids, peramine, and several loline species. While the lolines (Schardl *et al.* 2007) and peramine (Pownall *et al*. 1995) appear to be non-toxic to mammals, sufficient concentrations of ergot alkaloids produced by the association can be toxic to livestock (Bouton *et al*. 2002). An *N. coenophialum* biotype "AR542" (also known as MaxP® and MaxQ®), produces peramine and lolines *in planta*, but no ergot alkaloids. AR542 confers useful levels of micro herbivore protection to its grass host (Pennell & Ball 1999; Popay *et al.* 2005; Jensen & Popay 2007), while livestock performance on AR542 infected pastures is no different to those grazing an uninfected control (Bouton *et al*. 2002).

Loline concentrations in these synthetic associations have been measured at 3-60 times lower than in a meadow fescue (*F. pratensis*) breeding line naturally infected with *N. uncinatum* (M. Norriss, unpublished data). This suggests the possibility of obtaining higher levels of alkaloids in AR542 associations, with further increases in micro herbivore resistance. Our work has shown that endophyte hyphal density in the leaf sheath varies between plant genotypes infected with AR542 (data not shown). Others have reported at least some degree of correlation between hyphal mass and metabolite production (Ball *et al*. 1995; Easton *et al.* 2002; Spiering *et al.* 2005; Faville *et al.* 2007, all in perennial ryegrass infected with *N. lolii*; and Adcock *et al.* 1997 in tall fescue infected with *N. coenophialum*), and that the level of metabolite production was highly heritable (Easton *et al*. 2002; Adcock *et al.* 1997). These observations support the hypothesis that (a) a plant-breeding programme designed to increase hyphal mass will be successful, and (b) that the selected population will produce increased concentrations of at least some endophyte derived alkaloids.

Materials and Methods

Plant breeding

Plant breeding was performed at Kimihia Research Centre, 15 km south west of Christchurch, New Zealand, on a Templeton silt loam. Plant genotypes were subjected to hard rotational grazing by sheep. Once plants were established, no irrigation was applied.

Tall fescue cv. 'Resolute' and cv. 'Quantum' each infected with AR542 endophyte, were both subjected to one cycle of selection for agronomic worth, followed by a second cycle of selection, for both agronomic worth and increased hyphal density. For the second 'Resolute' cycle, 10 000 initial plants were reduced to 1 033 elites over 8 months, by field selection for agronomic worth. From the 1 033 elites, 86 plants with the highest hyphal density were selected; this number was further reduced to nine plants by selecting those plants that gave the least liveweight gain to grass grub (*Costelytra zealandica*) feeding on their roots. The nine plants were inter-pollinated, and seed from seven was combined, forming nucleus seed of 'Resolute II'. For the second

'Quantum'cycle, 7 000 initial plants were reduced to 82 elites over 12 months, by field selection for agronomic worth. From the 82 elites, 12 plants with the highest hyphal density were selected and inter-pollinated. Seed from five of these plants was combined, forming nucleus seed of 'Quantum II'.

Determination of hyphal density in single plants

Four tillers were examined from each plant during April (autumn), and one leaf sheath was removed from each tiller. The 4 mm basal section of the removed leaf sheath was scraped with a scalpel until approximately one cell thick, and removed from the rest of the sheath. The section was then immersed in aniline blue stain (1 g of aniline blue powder dissolved in 100 ml deionised water and added to 200 ml lactic acid) and mounted on a slide. Once the hyphae had absorbed the aniline blue, the basal end of the section was systematically examined at 400x magnification using a blue interference filter, and hyphal density was subjectively determined on the following 1-9 scale: 1 = one strand of hyphae sighted in the section, 2 = ~12% of the section covered with hyphae, 3 = ~25%, 4 = ~37%, 5 = ~50%, 6 = ~62%, 7 = ~75%, 8 = ~87%, and 9 = 100% of section covered with hyphae (no plant cells visible). The scores for each of the four tillers were averaged to give a mean hyphal density score per plant.

Testing of selections in field plots

A three replicate field trial containing 'Resolute', 'Resolute II', 'Quantum', and 'Quantum II', was sown at Kimihia in autumn 2004. Sheep grazed the trial over winter and spring. Herbage was sampled on 20 December 2004, 27 January 2005 and 14 March 2005. Samples were pure tall fescue and were cut at approximately 30 mm above ground level. Samples were kept cold immediately after harvest, were frozen within 2 hours, then freeze-dried and analysed for alkaloids as described below.

Milled plant material was analysed by HPLC for peramine, using procedures based on Spiering *et al.* (2002). Lolines were analysed by GC-FID using modification of the methods of Kennedy & Bush (1983) and Yates *et al*. (1989). Of the three loline species tested for (*N*-acetylnorloline, *N*-formylloline, and *N*-acetylloline), only *N*-acetylnorloline was detected. Results were averaged over the three sampling dates, and analysed by ANOVA.

On 1 April 2005, 25 tillers were sampled at random from replicate one of each cultivar. Tiller hyphal density was determined by the method described above, and cultivar means calculated.

Results and Discussion

Alkaloid concentrations in the field plots showed that selection for increased hyphal density within 'Resolute' was associated with a 40% increase in lolines ($P<0.05$) (Fig. 1a) and a 29% increase in peramine ($P<0.05$) (Fig. 1b). This corresponded with a 74% increase in hyphal density score (Fig. 2). A similar but non-significant ($P>0.05$) trend occurred for the selection within 'Quantum', with a 25% increase in lolines (Fig. 3a), and a 2% increase in peramine (Fig. 3b), associated with 23% higher hyphal density score (Fig. 4). For lolines, the analytical measurement errors (±30 ppm) were relatively high for the lower concentrations measured in 'Quantum' and 'Quantum II' (mean, 76 ppm), compared with the higher concentrations that occurred in 'Resolute' and 'Resolute II' (mean, 1570 ppm). Peramine concentrations in the 'Quantum' population were very low (mean, 2 ppm), and at the limits of analytical detection (±2 ppm).

These data support the hypothesis that an increase in hyphal density in the sheath is at least partly linked to increased metabolite production in the whole plant. However with the lack of statistical significance in the 'Quantum II' data, more work is needed to confirm this correlation.

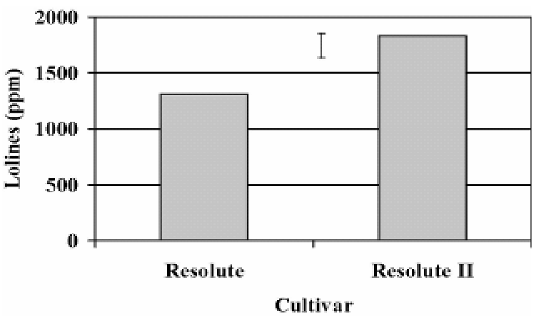
The greater qualitative hyphal density increases achieved within the 'Resolute' population compared with the 'Quantum' population, could indicate that 'Resolute' germplasm was more responsive to selection. However, this is more likely a result of the greater selection pressure applied to the 'Resolute' population (86 high density plants selected from 1 033, or 8%) compared with the 'Quantum' population (12 high density plants selected from 82, or 15%).

Data supporting the hyphal density hypothesis are confounded in at least two ways. Firstly, 'Quantum' and 'Resolute' were subject to agronomic worth selection in each of their selection cycles, before the hyphal density selection in cycle two. Secondly, 'Resolute' germplasm was further selected for grass-grub resistance in cycle two. The agronomic worth selection, and/or the grass grub resistance selection, may have indirectly increased alkaloid concentrations, with high alkaloid plants being more micro-herbivore resistant and therefore of greater agronomic worth. The possible selection for insect resistance may have been the link to increased alkaloid levels, rather than the selection for increased hyphal density. Therefore it cannot be stated with certainty that the association between hyphal density and increased alkaloid levels is causal.

While the degree to which the agronomic and grass grub selection work indirectly influenced metabolite production is unknown, large effects are unlikely, given that no insect activity was observed during agronomic selection. Furthermore, subsequent pot testing of 'Resolute II' for improved grass grub resistance showed no significant effect of the selection work, (A.J. Popay,

Figure 1 Concentration (ppm, ug/g) of (a) loline alkaloids and (b) peramine in herbage of cv. 'Resolute' and cv. 'Resolute II' infected with endophyte AR542. Data are a mean of the three harvests over summer. Error bar is LSD, P≤0.05.

a Lolines



b Peramine

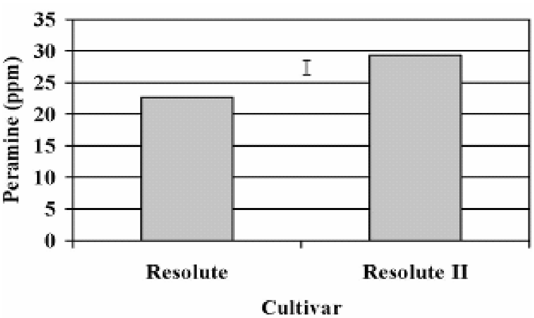
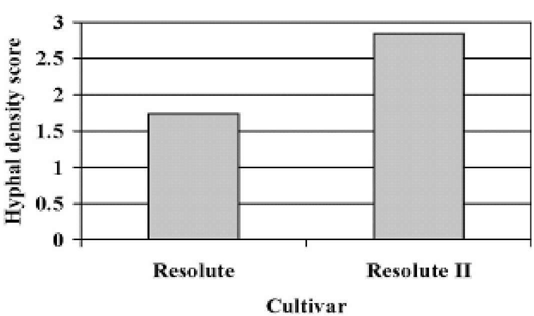


Figure 2 Endophyte hyphal density of leaf sheath material in cv. 'Resolute' and cv. 'Resolute II' in field plots. See text for description of hyphal density score method.

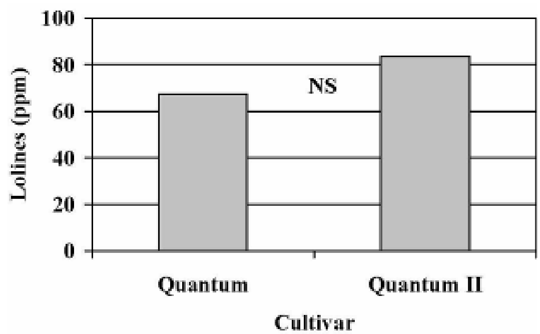


data not shown) although testing of more mature plants is needed to confirm this result.

Under plot harvests, 'Resolute' germplasm produced about 21 times more lolines than 'Quantum' germplasm, while sheath endophyte density was lower in the 'Resolute' germplasm. This implies that factor(s) other than sheath endophyte density are important in metabolite

Figure 3 Concentration (ppm, ug/g) of (a) loline alkaloids and (b) peramine in herbage of cv. 'Quantum' and cv. 'Quantum II' infected with endophyte AR542. Data are a mean of the three harvests over summer. Means were not significantly different (NS) (P>0.05).

a Lolines



b Peramine

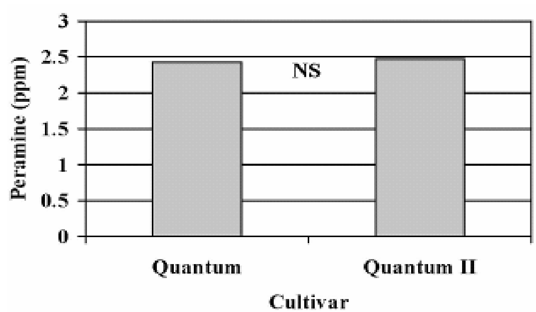
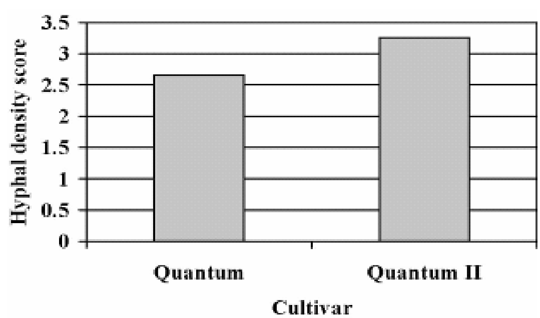


Figure 4 Endophyte hyphal density of leaf sheath material in cv. 'Quantum' and cv. 'Quantum II' in field plots. See text for description of hyphal density score method.



production. Possible explanations include (a) 'Resolute' eliciting more metabolites per unit of hyphal mass, or (b) higher concentrations of hyphae in Resolute leaf blades (and therefore much more hyphal mass per plant), or a combination of both these factors. M. J. Christensen (unpublished data) infected both tall fescue and meadow fescue with the same *N. coenophialum* biotype, and found that the meadow fescue produced several times more ergovaline per unit hyphal mass than tall fescue, supporting hypothesis (a) above. Supporting (b), Christensen *et al*. (1998) examined growth of three endophyte biotypes in both the leaf and sheath of 'Resolute' (tested as KFa949), and in three other commercial tall fescue cultivars. Results showed an average 66% of each 'Resolute' leaf blade was colonised with endophyte, with the other cultivars averaging 11%, 14% and 0% leaf colonisation. 'Resolute' also averaged 340% of the leaf derived ergovaline production of the other three cultivars, but only 28% of the others in the sheath.

The degree of leaf colonisation of AR542 was not measured in the current experiment. However, Christensen's work elucidates two mechanisms that may explain the much greater levels of lolines found in the Resolute germplasm.

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