Spontaneous *in planta* changes in fungal endophytes impact symbiosis

W.R. SIMPSON[1], M.J. CHRISTENSEN[1], R.D. JOHNSON[1] and J.SCHMID[2]
[1] *Forage Improvement Section, AgResearch Limited, Grasslands Research Centre, Tennent Drive, Private Bag 11008, Palmerston North, New Zealand*
[2]*IMBS, Turitea Campus, Massey University, Palmerston North*
wayne.simpson@agresearch.co.nz

Abstract

The symbiosis between the asexual Ascomycetous fungi of the genus *Neotyphodium* and host grasses is considered to be symptomless. Symbioses involving *Epichloë* can show symptoms but this is restricted to the formation of stroma on floral structures. Fungal mycelium of both *Epichloë* and *Neotyphodium* endophytes occupy the intercellular space of plant tissues with no breach of host cell walls. Research involving these endophytes and their host grass symbioses relies on staining of host tissues and microscopic examination, immuno-detection or *in-vitro* isolation to determine infection status. This work reports on the observation of two independent instances of spontaneous change in the endophyte fungus that manifests as changes in the host grass vegetative morphology; one involving a genetically modified *Epichloë festucae* inoculated into a perennial ryegrass (*Lolium perenne*) population and the other a resident wild type *Neotyphodium lolii*. The relationship between wild type and variant fungus is confirmed as is the connection between variant fungus and host morphology. This work demonstrates that spontaneous *in-planta* changes in fungal endophytes occur and that these can impact on fungus/host grass symbioses.

Keywords: *Neotyphodium*, *Epichloë*, symbiosis, endophyte, perennial ryegrass

Introduction

Fungal endophytes of grasses, like all organisms, are subject to change both at a genetic and epigenic level and yet they maintain mutually advantageous symbioses with their hosts. First principles tell us that any changes in the endophyte that adversely affect the symbiosis will be selected against and therefore the evidence of such changes in the population is likely to be ephemeral. Here we describe the host plant effects of two examples of spontaneous *in-planta* change of fungal endophytes, one in *Neotyphodium lolii* and one in *Epichloë festucae*.

The sexual *Epichloë* species exhibit overt signs of their presence within the host when they produce stromata on reproductive tillers (Schardl *et al.* 2004). Asexual *Neotyphodium* do not produce stromata on reproductive tillers and infected plants are in no way observably different from uninfected notwithstanding any effects of biotic and abiotic stresses in the field. To positively ascertain infection status of grasses forming symbioses with *Neotyphodium* endophytes such as the perennial ryegrass endophyte *N.lolii* and *E. festucae*, it is necessary either to stain tissue and observe mycelium directly or to use an immunological technique to observe the presence of fungus indirectly.

The 'invisibility' of these endophytic fungi reflects the level of compatibility of the union of these symbionts.

A previous report described a deviation from this asymptomatic presence of *Neotyphodium* endophyte where a cultured novel *N.lolii* strain, AR5, induced a change in host grass phenotype (Simpson *et al.* 2004). Work performed in this study demonstrates that the host effects in this case are not due to a variant of the novel AR5 endophyte but to a variant, P1f, of the resident endemic *N.lolii*, P41f, that is extant in the population. An implication of this finding is that the change in the fungus has occurred *in-planta* with no opportunity for change within the unnatural environment of a Petri plate.

We also report on one other independent observation of spontaneous *in-planta* change in the endophytic fungus that results in a perturbation of the symbiosis involving a genetically modified *E.festucae*, Fl1 Nc25 (Johnson *et al.* 2007), and show that this change is stable in the fungus allowing transfer of the host altering effect to other populations via artificial infection.

Methods

Fungal isolation, seedling preparation and inoculation

Fungus was isolated from endophyte-infected plants by surface-sterilising plant tissue and culturing on antibiotic potato dextrose agar (ABPDA). Seed was surface-sterilised and germinated in the dark on 4% water agar and resulting seedlings inoculated as described by Latch and Christensen (1985).

Identification of infected plants

Inoculated seedlings were incubated in the dark for 7 days then placed under white fluorescent tubes for 7 days before being removed from Petri plates and planted into potting mix. Plants were grown for approximately 6 weeks before being assayed for endophyte infection using an immuno-assay as described by Hahn *et al.* (2003).

Some plants were examined by removing epidermal strips and staining with aniline blue and examining using a compound microscope at 100X magnification.

Endophyte elimination

Intact tillers, including roots, were removed from infected plants and washed thoroughly to remove potting soil then immersed in a Benlate solution (2g/L) for 3+ hours. Tillers were planted in river sand in pots without drainage and watered to saturation with the same Benlate solution; watered pots were weighed and regularly watered to weight with fresh water only (to maintain original Benlate concentration) over a period of approximately 6 weeks.

SSR

For each analysis two tillers cut at soil level were trimmed to ca. 3cm length and transferred to Q-BIOgene DNA extraction vials. Extracted DNA was amplified by PCR using primers flanking simple sequence repeats (SSRs) that are known to be polymorphic across strains of *Neotyphodium* endophyte (Moon *et al.* 1999).

Alkaloid determination

Herbage samples were cut to 5mm of soil level and after removal of dead material divided into leaf blade and pseudostem portions prior to freeze drying. Samples were milled in a modified volume reduced domestic coffee grinder.

Ergovaline, combined with its isomer ergovalinine, was estimated by minor modifications of the method of Spiering et al. (Spiering *et al.* 2002) using 50mg samples extracted with 1ml of solvent. Solid residue was removed from the extract by

Figure 1 Separated tillers of perennial ryegrass. Left: infected with endemic *N. lolii* showing normal phenotype. Right: infected with *N. lolii* variant fungus showing small tillered, fine leaved phenotype.

Figure 2 Small stunted tillers develop on one side of a perennial ryegrass plant infected with *Epichloë festucae* strain FL1 NC25.

Figure 3 Mycelium of *N.lolii* P41f emerging from normal phenotype perennial ryegrass tissue on ABPDA.

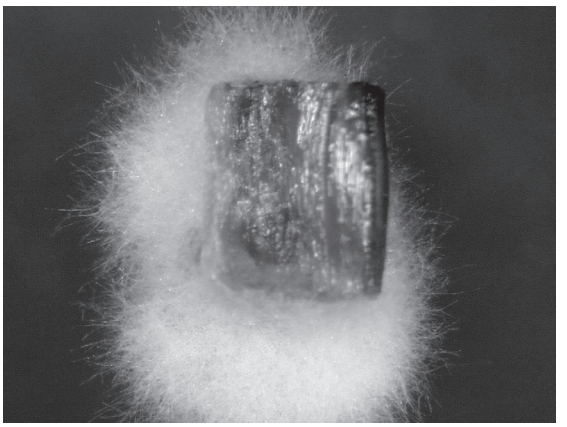
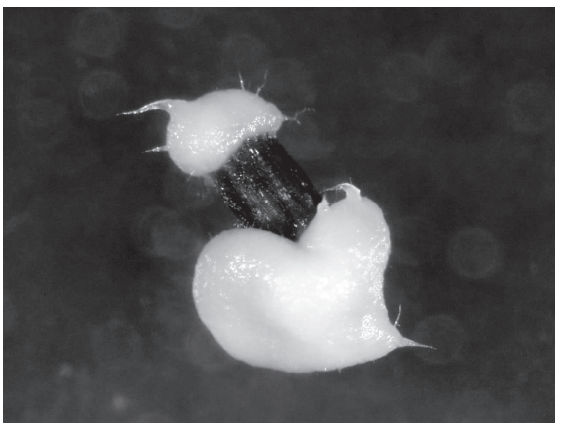


Figure 4 Mycelium of *N.lolii* variant fungus P1f from atypical morphology perennial ryegrass on ABPDA.



centrifugation. Peramine was estimated on the same extract using a different HPLC system also based upon Spiering et al. (Spiering *et al.* 2002).

Lolitrem B was estimated by a simplification of the method of Gallagher et al. (Gallagher *et al.* 1985). Samples (50mg) were extracted with 1ml of dichloroethane – methanol (9:1 by volume) for 1hr with mixing at ambient temperature and then centrifuged to remove solid residues prior to direct HPLC. Peak areas were compared to an external standard of lolitrem B.

Results and Discussion

***N. lolii* fungus P41f is wild-type of P1f variant**

Alkaloid analysis of the parent plant of *N.lolii* AR5 (AR5pp) shows expression of peramine and ergovaline but not lolitrem B. Plant P41 hosting the endophyte P41f was positive for peramine, ergovaline and lolitrem B. Plant P1 hosting the variant fungus P1f was positive for peramine and ergovaline only, suggesting that AR5 is the wild-type of the variant fungus, however, SSR analysis shows that this is not the case.

Analysis of plant material representing AR5pp, P1 and P41 all showed a 177 peak at the B10 locus. At the B11 locus AR5pp showed a 238 peak while both P1 and P41 showed a 177 peak. SSR data indicate that P41f is the wild-type of P1f but no lolitrem B has been detected in a P1f symbiosis.

Endophyte-free populations infected with P1f and variant Fl1 Nc25 show altered phenotype

P1f was isolated and used in reinfection studies using both a heterogeneous out-crossing population and clonal plantlets from a single perennial ryegrass genotype to demonstrate a link between the fungus and the effects on the host grass. All seedlings from the endophyte-free population infected with P1f displayed the fine leaved phenotype (Fig. 1) previously described (Simpson *et al.* 2004) and the only clone infected with P1f was also fine leaved.

Plants infected with cultured AR5 or with P41f had a normal phenotype that did not differ from uninfected individuals from the same host population.

The seedlings of perennial ryegrass infected with the genetically modified *E. festucae* strain Fl1 Nc25 were initially all normal phenotype. After a period of time one of the infected plants developed stunted tillers to one side (Fig. 2). Both normal and stunted tillers were examined and confirmed to be endophyte-infected. Fungal isolates from normal and stunted tillers were re-infected into an endophyte-free population of perennial ryegrass. Plants infected from these inoculations reproduced the effects: fungus from normal tillers gave rise to infected seedlings with a typical phenotype and fungus from stunted tillers gave rise to infected seedlings with a stunted phenotype.

In addition, the fine-leaved P1 plants were treated with fungicide to eliminate the endophyte fungus P1f; the endophyte-free clones obtained all had a normal phenotype. It is clear from these results that the *N.lolii* fungus is responsible for the change in host phenotype.

P1f shows altered morphology in culture, Fl1 Nc25 variant similar to source fungus

Fungus isolated from AR5pp and P41 was typical in culture with a proliferation of aerial hyphae (Fig. 3) but P1f was not, with hyphae forming an aggregated mass with a smooth moist appearance (Fig. 4). Fungus isolated from Fl1 Nc25-infected normal tillers and that isolated from stunted tillers, produced colonies within the range of what might be considered typical for *E.festucae* Fl1.

Variant Fl1 Nc25 and P1f have *in planta* phenotypes different from their respective wild-types and from each other

Epidermal strips from variant Fl1 Nc25 plants stained with aniline blue show a proliferation of branched hyphae that contrasts with the moderate levels of largely unbranched hyphae in wildtype Fl1 Nc25.

Stained epidermal strips of P1 revealed extremely sparse distribution of otherwise typical hyphae. This result is interesting in that it demonstrates that it is not necessary to have a proliferation of mycelium to effect a change in host plant architecture.

Plants infected with P1f or Fl1 Nc25 have not produced seed

When plants infected with P41 f and P1f were vernalised with a view to harvesting seed only the P41f-infected plants produced seed, P1f-infected plants did not produce any floral structures at all. There has not been any evidence of flowering or seed production in the Nc25 stunted plants, indeed the plants are very weak vegetatively and tend to undergo decline in the glasshouse.

P1f and Fl1 Nc25 variant a result of *in planta* change

Both the *N.lolii* P41f and the *E. festucae* Fl1 Nc25 have undergone a change *in planta* that has resulted in a change in the phenotype of hosts infected with these variant fungi. This effect persists over time within the host and is a characteristic of the fungi that is maintained through culturing and re-infection of new hosts.

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