Effects of Storage Conditions on Endophyte and Seed Viability in Pasture Grasses

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Abstract. Several important temperate pasture grasses have co-evolved with mutualistic *Epichloë* fungal endophytes. These endophytes impart beneficial attributes to their host as they enhance the fitness of the grass when under biotic and abiotic stresses. The asexual species of these fungi (formerly classed as *Neotyphodium*) are obligate symbionts, and efficiently colonise newly formed tillers and infect seed by direct colonisation of the embryo. These endophytes are strictly seed transmitted. Survival of the fungus in this seed is therefore critical for the dissemination of endophyte-infected seed to grassland farmers. Longevity of endophyte in stored seed is primarily determined by the length of storage, temperature, and relative humidity as this is in equilibrium with seed moisture. Elevated temperature and relative humidity both reduce endophyte viability. The relative importance of each of these environmental parameters is unclear. Longevity may be further modified by grass species, cultivar, seed lot, and endophyte strain. Valuable seed requiring long term storage can utilise controlled storage facilities where temperature is preferably ≤5 °C and relative humidity≤30% (seed moisture <8%). For large quantities of commercial seed, moisture barrier packaging can be used.

Keywords: *Festuca arundinacea, Lolium perenne, Lolium hybridum, Lolium multiflorum, Epichloë, Neotyphodium.*

Introduction

Many temperate forage grasses have co-evolved with endophytic fungal symbionts of *Epichloë*, including asexual morphs formerly classified in the form genus *Neotyphodium* (Schardl *et al.* 1997). Of greatest significance to agriculture have been the asexual species that infect important pastoral grasses such as tall fescue (*Festuca arundinacea* Schreb.), meadow fescue (*F. pratensis* Huds.), and ryegrasses [perennial, annual/Italian, perennial x Italian hybrids (*L. perenne* L., *L. multiflorum* Lam., *L. boucheanum* Kunth syn. *L. hybridum* Hausskn.)]. When these grass species are infected with *Neotyphodium* endophytes, mutualistic, non-symptomatic associations are formed (Schardl 2001). The fungus (obligate symbionts) obtains a suitable habitat, nutrition from the plant and an efficient means of dissemination to successive host generations as it infects newly formed tillers and seed. The grass host benefits through increased tolerance to biotic and abiotic stresses, notably insect predation and drought stress (Malinowski and Belesky 2000; Popay and Bonos 2005). While such mutualistic associations are critical in many pastoral systems for plant productivity, some strains of endophyte produce secondary metabolites that cause ill health and productivity losses in livestock grazing these pastures. To mitigate the effects on livestock, but still gain the agronomic advantages of endophyte infection, 'novel' or 'selected' naturally occurring beneficial endophytes have been incorporated into elite cultivars of tall fescue and perennial and hybrid ryegrasses, and marketed as novel associations (Fletcher 2012). Where endophyte-infected grasses are purposely utilised in pastoral systems (New Zealand, Australia and USA) seed is the only path to deploying these endophytes. The survival of endophyte in this seed is critical when cultivars are developed and marketed as conveying beneficial endophyte-derived traits. In this review, we describe the seed-endophyte biology for these important grass-endophyte associations, focusing on the factors determining endophyte survival in seed.

Seed-endophyte biology

Neotyphodium grass endophytes are truly seed-transmitted, being totally reliant on the success of the plants reproductive strategy for their dissemination and survival. Their growth is fully synchronised and highly regulated with that of their grass hosts (Christensen *et al.* 2002; Christensen *et al.* 2008). Lineages of single fungal genotypes colonise the floret through the base of the ovary. Here the fungus has been observed colonising the apoplastic spaces of many flower tissues including the lodicules, stamens and stigmas. There is no evidence of these fungi colonising the pollen grains. The fungus travels to and colonises the nucellus tissue that surrounds the megagametophyte (Sampson 1933; Philipson and Christey 1986). In this area, where the grass embryo sac will develop after fertilisation, the endophyte grows with a high degree of hyphal branching that is seen in other plant tissues with undifferentiated, developing cells, such as the apical meristem. The nucellus is used as a source of nutrition for the developing embryo and as this structure differentiates and expands, the endophyte is left in the nucellus remnants, now a thin membranous layer of crushed cells. At seed maturity, the fungus is found in two main locations; within the remnants of the nucellus tissue, which now lies between the seed coat (pericarp) and the aleurone layer; and between the cells of the embryo/ scutellum, an area that has been labelled 'the infection layer' (Freeman 1904). The endophyte is not found within the triploid endosperm. Seeds from the true grasses (Poaceae) contain a unique structure; a modified enzyme-secreting cotyledon called the scutellum that transfers nutrients from the endosperm to the developing embryo. Contrasting evidence exists on the timing of embryo colonisation by these endophytes and no consensus has yet been reached. Two of the most recent reports include Philipson and Christey (1986) who show by transmission electron microscope investigations the presence of endophyte within the very early embryo, before the differentiation of the scutellum in both *Lolium* and *Festuca* spp., while Majewska-Sawka and Nakashima (2004), who used immuno-detection, report that young embryos are never colonised by the fungus and therefore endophyte infection must occur at a late stage in seed formation. Where the authors do agree is at embryo maturity, hyphae are found in the main components of the embryo, namely the plumule apex. At the onset of seed germination, fungal colonisation proceeds via systemic invasion through the apical meristem. Hyphae within the shoot apical meristem then start to colonise leaf primordia and axillary buds, advancing systemically throughout the above ground parts of the plant via intercalary hyphal extension (Christensen *et al.* 2008) and the vertical transmission cycle is completed. Many sexually producing *Epichloë* species can also behave antagonistically to their hosts in some instances, transmitting horizontally (contagiously) through the dissemination of ascospores produced on stromata formed on host inflorescences, manifesting in the disease known as choke (Leuchtmann and Schardl 1998).

Endophyte survival in stored seed

It has been well established that endophyte dies when seed is stored. This is dependent on a range of factors, particularly the length of storage, and conditions of temperature and humidity. The effect of length of storage at ambient conditions was first described some 70 years ago for perennial ryegrass and tall fescue, with Neill (1940, 1941) reporting seed of these grasses had no viable endophyte after 12-18 months storage. In more recent studies, similar responses have been reported for meadow fescue (Mika and Bumerl 1995), annual ryegrass (Gundel *et al.* 2009) and rigid ryegrass (*L. rigidum* Gaud.) (Kirkby *et al.* 2011) although the length of time may vary between species and studies. While Welty *et al.* (1987) for tall fescue and perennial ryegrass found a linear decline in viable endophyte with increasing time in storage, we repeatedly find these declines fit a logistic relationship (Hume *et al.* 2011) as did Gundel *et al.* (2009) for annual ryegrass. That is, a plateau or slow decline at first, then a rapidly increasing decline, followed by a plateau as the proportion of seeds with viable endophyte approaches zero. Elevated temperature and humidity are detrimental to both endophyte viability in stored seed (Rolston *et al.* 1986, Gundel *et al.* 2009) and the host grass seed (Copeland and McDonald 1995) and the effects are additive. Endophyte viability however, generally decreases at a faster rate than seed viability, so seed may still have high germination but no live endophyte (Hume *et al.* 2011). The effect of relative humidity on longevity of endophyte occurs through changes in seed moisture content, as this is in equilibrium with the air surrounding the seed. The physiological mechanisms involved in the loss of endophyte viability have not been elucidated.

Several additional factors may affect length of endophyte storage, including host grass species, cultivar, seed lot and endophyte strain. USA and New Zealand seed studies report tall fescue-endophyte associations are less robust than perennial ryegrass-endophyte associations in terms of endophyte viability (Neill 1940, 1941, Welty *et al.* 1987) although this is confounded by each grass hosting a different species of *Neotyphodium* endophyte. Within a grass species, cultivars may differ in endophyte longevity (Wheatley *et al.* 2007), and this may be a trait which is amenable to selection pressure in a plant breeding programme, as has been achieved for transmission of endophyte to seed (Easton 2007). Endophyte strains within a cultivar may differ in endophyte longevity, with selected endophytes generally being less robust than the native animal-toxic endophyte within the same cultivar (Easton and Tapper 2005). All such studies need to consider confounding effects of potential differences in endophyte vigour between seed lots (Rolston *et al.* 1994, Hume and Rolston unpublished data) similar to seed lot differences for seed vigour, which are determined by management and environmental conditions prior to and immediately post-harvest.

Commercialisation of endophyte-infected seed

Marketing endophyte-infected seed has added an extra layer of complexity and quality assurance to the seed production and supply chain (Rolston and Agee 2007). Viability of endophyte is a key parameter, which under licence agreements for selected endophytes such as AR1, AR37 and AR542 is required to be >70% (Hume and Barker 2005). It has been advocated that seed production companies and the whole supply chain, including farmers, treat endophyte-infected seed as a *perishable* high-value product and so take appropriate measures to ensure the product meets endophyte specifications therefore avoiding product failure when sown in pastures (Rolston and Agee 2007). Storage of harvested seed at ambient conditions close to points of sale can result in rapid declines in endophyte viability to below this 70% level (Wheatley *et al.* 2007; Hume *et al.* 2011). Research has therefore focussed on ways to maintain viable endophyte levels within specifications until seed is sold.

As outlined previously, temperature and humidity are the key environmental parameters that drive the viability of endophyte in stored seed. While specialised controlled condition storage facilities (*i.e.* low temperature and humidity) can be used for high value commercial seed (nucleus/breeders seed) and valuable genetic resources (grass and fungal) (Rolston *et al.* 2002; Hume *et al.* 2011) this significantly increases storage costs for large quantities of seed in commercial production. For these large quantities, geographic location can be a feasible option for short-term seed storage. For example, in New Zealand, the further south seed is stored the greater the longevity of endophyte, corresponding closely with reduced ambient temperatures and humidity (Hume *et al.* 2011).

The relative importance of controlling temperature and humidity in storage facilities is a critical issue, particularly as the thermodynamic properties of moist air mean that lowering temperature increases the relative humidity. Effects on endophyte longevity will therefore be positive as a result of a lower temperature but negative as a result of higher relative humidity. These thermodynamic properties of air are not simple linear relationships, with changes dependent on levels of each parameter. Using multiple regression analysis, Welty *et al.* (1987) in the USA reported linear models to predict endophyte survival from time (length of storage) (0-18 months), temperature (10, 20, 30^{o}C), and seed moisture content (5-24%). For tall fescue, time had the greatest influence, while seed moisture content had more influence than time for perennial ryegrass. For both grasses, seed moisture content had up to a 2-fold greater effect on endophyte survival than temperature. Our interpretation of the data from Welty *et al.* (1987) is that curvilinear models may provide a better fit and therefore prediction. Increases in endophyte longevity were most responsive when temperature was below 20^{o}C and seed moisture contents kept below 10-12%. At least for seed moisture content, similar effects occur for annual ryegrass (Gundel *et al.* 2009). In contrast, for perennial ryegrass in New Zealand, Rolston *et al.* (1994) reported that endophyte longevity was not responsive to seed moisture below 11.5% after 12 months storage but after 24 months this threshold had dropped to 8.5% seed moisture. At ambient conditions the greatest losses in endophyte occur over the summer and early autumn (Hume and Rolston unpublished data). At this time of the year, relative humidity is low compared with winter, so the high temperatures of summer/autumn are likely to be the critical driver of endophyte loss.

In commerce, temperature is relatively cheap to control compared with controlling relative humidity, so research has investigated maintaining seed moistures at low levels through the use of moisture barrier packaging. For perennial ryegrass, aluminium-polyethylene laminated bags can effectively maintain seed at low moisture content and maintain endophyte viability for up to 15 years when combined with cold storage (Rolston *et al.* 2002). While expensive, such packaging is used on a commercial scale in the USA for tall fescue infected with the selected endophyte AR542 (MaxQ®). Polyethylene bags are a lower cost option but are semi-permeable reducing their overall effectiveness, although bags as thick as 140 µm can be effective for up to 4 years at ambient storage conditions (Hare *et al.* 1990).

Conclusion

For pastoral farmers to capture the beneficial traits of *Neotyphodium* endophytes in temperate grasses, it is crucial that we understand the biological processes involved in endophyte viability within stored seed. Determining the genetic, management, and environmental factors involved, and their relative importance, is critical for the production and distribution of endophyte-infected grasses.

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