# Acremonium endophyte viability in seeds and the effects of storage.

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## Abstract

The effects of the fungal endophyte *Acremonium lolii* in perennial ryegrass (*Lolium perenne*) and *A. coenophialum* in tall fescue (*Festuca arundinacea*) are reviewed. A five year seed storage trial compared the effects of four storage conditions (-15°C/90%RH, 0°C/30%RH, +5°C/60-70%RH and ambient warehouse 5-25°C), four initial seed moisture levels (8.6, 10.0, 12.1, 13.8%), and three packaging types (calico, polyethylene-aluminium (PAL), polyethylene films, 9 to 140 microns). Endophyte viability declined rapidly in seed stored for 6 months in ambient conditions at high seed moistures, but was maintained for 5 years at temperatures of 0 and -15°C; and at low seed moistures in PAL packs at all temperatures. Endophyte viability was considerably more sensitive than seed germination to the effects of high temperature and seed moisture. The maintenance of high endophyte levels will require storage at low temperatures (5°C or less) and/or storage of seed of low moisture content in moisture proof bags.

The removal of existing endophytes to allow inoculation of seed with improved endophyte strains and the maintenance of viable endophyte in seed lots is important. Accelerated loss of endophyte viability can be achieved in seed stored for 21 d at 37°C and at high humidity.

***Additional key words:*** Acremonium coenophialum, A. lolii, Lolium perenne, *perennial ryegrass*, Festuca arundinacea, *tall fescue*.

## Introduction

*Acremonium* endophyte fungi have a wide distribution in many grass species including *Bromus, Festuca, Lolium, Poa* and *Stipa* (Siegel *et al.*, 1987). The term endophyte (Greek: *endo* = within + *phyte* = plant) has been defined as an organism contained or growing entirely within the substrate of a plant, whether parasitic or not. In this paper endophytes are fungi living entirely within a plant in the intercellular spaces of the host tissue. The relationship of *Acremonium* endophytes and their host grasses is described as mutualistic symbiosis (Siegel *et al.,* 1987).

As well as the *Acremonium lolii* endophytes of perennial and hybrid ryegrass (*Lolium perenne,* x *L. bucheanum*), other endophytes include *Acremonium*-like species of annual ryegrass (*L. multiflorum*), *Gliocladium*-like species of perennial, annual and hybrid ryegrass, *Epichloe typhina* in perennial ryegrass (PRG) (Latch and Tapper, 1988), and *Acremonium coenophialum* in tall fescue (*Festuca arundinacea*). The distribution of *A. lolii* is widespread. Plants from 53 out of 64 old pastures of PRG from eight European countries were found to be infected with *A. lolii* (Siegel *et al.*, 1987).

*Acremonium* endophytes are seed borne and cause both beneficial effects to the host and detrimental effects to grazing livestock and insect herbivores. Reviews on fungal endophytes in grasses by Siegel *et al.* (1987), and Fletcher *et al.* (1990), and 69 papers reported at the recent Second International Symposium on *Acremonium* (Hume *et al.*, 1993) give detailed background to the subject including taxonomic classification, incidence, methods of detection in seeds and plant tissue, methods of culture, toxicities of grazing animals and insect deterrence.

**Animal toxicities**

Ryegrass staggers is a neurological disorder common in late summer in sheep, cattle, deer and horses and is caused by the indole alkaloid lolitrem B in endophyte-infected ryegrass herbage. Concentrations of 2 ppm (dry weight) are sufficient to cause staggers and concentrations of lolitrem B can peak in autumn at 3-5ppm (Rowan, 1993). Lolitrem B (C42H55N07) is the major lolitrem found in endophytic ryegrass.

Poor cattle performance associated with *A. coenophialum*-infected tall fescue include summer syndromes of reduced weight gain, decreased milk production, excessive salivation, increased respiration rate and high rectal temperature. The winter syndrome is called fescue foot - symptoms are the loss of hooves and tail in severe cases. Several alkaloids have been suggested to contribute to fescue toxicosis (Siegel *et al.*, 1987).

**Insect tolerance**

The tolerance of perennial ryegrass to Argentine stem weevil (*Listronotus bonariensis*) (Prestidge *et al.*, 1982) is considered to be determined by the feeding deterrent peramine, a novel guanidinium alkaloid, effective at concentrations as little as 0.1 ppm (Latch and Tapper, 1988). Peramine occurs in endophyte-infected ryegrass at concentrations between 10 and 30 ppm dry weight (Tapper *et al.*, 1989). Endophytes of perennial ryegrass and tall fescue have also been reported to affect 22 other insect species including sod webworms (*Crambus* spp.) (Latch, 1993).

**Plant growth benefits**

A number of changes in plant physiology and patterns of growth in high endophyte grass genotypes (when compared to non-infected) have been reported, including greater rates of photosynthesis, and increased tiller numbers and resistance to drought (Siegel *et al.*, 1987).

**Clover suppression**

Ryegrass pastures containing *A. lolii* have a lower white clover content compared with endophyte-free ryegrass (Sutherland and Hoglund, 1989, 1990; Stevens and Hickey, 1990). Approximately 50% of the reduction in clover production could be accounted for by increased competition from the more vigorous endophyte ryegrass. Unexplained residual variation could be caused by an allelopathic effect of *A. lolii.*

**Novel endophytes**

The identification of low and zero lolitrem producing endophytes in ryegrass (Latch and Tapper, 1988) and the development of a method to inoculate endophyte-free seedlings with endophyte (Latch and Christensen, 1985) has led to the development of Endosafe® ryegrass (i.e., endophyte that does not cause ryegrass staggers). The endophyte strain 'Premier' was patented in 1990.

**Seed infection process**

Seed becomes infected with endophyte during the reproductive process. As the plant enters the reproductive phase the endophyte in the vegetative apex enters the developing inflorescence primordium from where it penetrates the ovary and ovule tissues (Philipson and Christey, 1986). Entry into the embryo probably occurs soon after fertilisation. The endophyte hyphae are widespread in the embryo of mature seed and are concentrated between the cells of the aleurone layer.

Nitrogen fertiliser (100 kg N/ha) applied at spikelet initiation reduced the amount of mycelium, but not the percentage of seeds with endophyte in perennial ryegrass (Stewart, 1986). Fungicides (including propiconazole and triadiamefon) applied to control stem rusts (*Puccinia graminis*) at 125 g ai/ha did not reduce the level of endophyte in the resulting seed crop. Endophyte is not transmitted in pollen, and Siegel *et al.* (1987) state that it is believed that the only means of dissemination of *Acremonium* endophytes is through maternal transmission in infected seed.

**Seed quality**

Using seed from 10 endophyte-free and 10 endophyte-infected tall fescue clones, the presence of *Acremonium* enhanced the germination by 5% at 16 and 24°C at 0.0, 0.5 and 0.75 MPa osmotic pressure (Pinkerton *et al.*, 1990). They attributed the response to a physiological effect of the endophyte or its metabolic products on the germination process.

**Removal of endophyte with fungicides**

Endophyte mycelium of perennial ryegrass and tall fescue have been killed by applying to seed ergosterol biosynthesis inhibiting fungicides such as propiconazole and prochloraz at 0.25 g ai/ha, while carboxim + thiram and $SP^2$ + $SP^2$ had no effect (Harvey *et al.*, 1982).

**Endophyte viability in storage**

Neill (1941) reported that endophyte-free plants are produced when infected seed is stored for 12 months or more after harvest. Siegel *et al.* (1985) reported most endophyte-infected seed that has been stored in seed warehouses for 2 years contains little or no viable endophyte. They noted that the loss of endophyte is retarded by low temperature and 'low humidity', with perennial ryegrass seed stored at 0-5°C and near zero humidity containing living endophyte, and tall fescue stored for 27 months at 10, 6, -20°C having 30, 90, and 90% viable endophyte respectively.

Rolston *et al.* (1986) reported that to maintain endophyte in perennial ryegrass for 12 months, storage at 5°C or less was required, or ambient storage at seed moisture content below 11.5%. Welty *et al.* (1987) found a rapid decline in the viable endophyte of perennial ryegrass and tall fescue with increasing seed moisture and increasing temperatures (10, 20 and 30°C).

Endophyte viability is a major issue in the seed industry and developing methods to maintain viability of novel endophytes in seed is important. The trial reported here was initiated to allow the development of effective long term storage of endophyte infected seed.

## Methods

### Storage trial

The methods used in this trial have been previously reported (Rolston *et al.*, 1986; Hare *et al.*, 1990). In brief, the trial was established in 1984 using perennial ryegrass cv. 'Grasslands Nui' which had 92% germination and 84% viable *A. lolii*. Seed samples were each of 100 g. Treatment variables were storage conditions, seed moisture and package type.

Storage conditions were 1) ambient (5-25°C) in a seed warehouse; 2) refrigerated at 5°C at 60-70% RH; 3) 0°C/30% RH; 4) subfreezing -15°C/90% RH. Initial seed moisture contents were 13.8, 12.1, 10.0 and 8.6%. Package types were calico, heat sealed aluminium polyethylene (PAL), polyethylene film bags of 9, 35 or 70 µm thickness, single or double layered.

Treatments were sampled at 0.5, 1, 2, 3, 4 and 5 years, with germination and viable endophyte in 6 wk old seedlings being determined.

### Killing endophyte in seed

Trials investigating heat treatments to kill endophyte in perennial ryegrass seeds were carried out. Seeds were surface sterilised for five hours with 1% sodium hypochlorite or dusted with the fungicides captan or thiram. The seeds were held in Petri dishes which were placed in bell jars containing water to give high humidity and stored at 30°C or 37°C. Samples of seed were taken weekly, 48 seeds per treatment, and grown in sand trays to determine germination and presence of endophyte.

## Results and Discussion

### Seed moisture

In short term storage from 12 to 24 months, high seed moisture content (SMC) resulted in a rapid decline of viable endophyte in ambient storage (Fig. 1). Storage for 12 months required a final SMC of 11.5% or less, and for 24 months of 8.5% to maintain a high percentage of viable endophyte. The rate of viable endophyte decline at ambient storage was 29% for every 1% increase in seed moisture content above the minimum SMC required to maintain endophyte. The decline in endophyte was more rapid than reported by Welty *et al.* (1987), who report a linear decline in endophyte over 16 months. After 12 months storage they reported that the viable endophyte in perennial ryegrass seeds held at 10% SMC was 80% at 10°C, and 40% at 20°C; while for tall fescue, viability was 62% at 10°C and 53% at 20°C. From data given by Welty *et al.* (1987) we have calculated the rate of decline of viable endophyte at 20°C as 8.5 and 5.9% for perennial ryegrass and tall fescue, respectively for every 1% increase in SMC. The results suggest that differences in endophyte vigour between seed lots could exist. This could be similar to differences in seed vigour that occur in grass seed lots resulting in variability in the rate of decline of germination during seed storage (Hampton and Hill, 1990).

Seed maintained viable endophyte for 5 years when stored at ambient with a SMC of 8.6%, at 5°C with 12.1% SMC (Table 1) and at 0 and -15°C with 13.7% SMC.

### Temperature

Temperature has a marked influence on endophyte viability in seed (Fig. 2) with a decline occurring after 0.5 and 3 years respectively in ambient and 5°C storage. There was a small decline (11%) after 5 years at 0 and -15°C. With subfreezing there was a trend to a lower endophyte viability at higher SMC; 72 ± 1.8% at 13.7% SMC, versus 79 ± 1.4% at 8.6% SMC. Siegel *et al.* (1984) reported that tall fescue stored at -20°C for 27 months maintained 90% viable endophyte while Welty *et al.* (1987) reported a 1% decline in viable endophyte per week at -18°C.

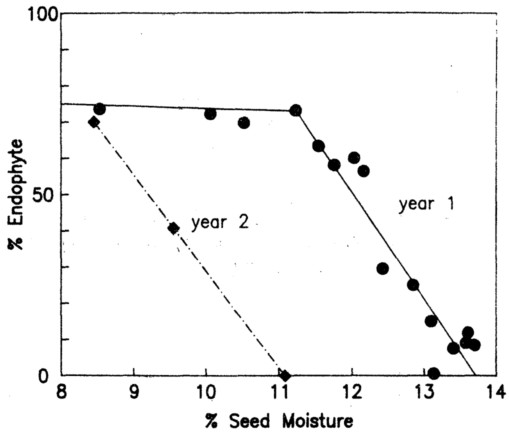


**Figure 1. Effect of seed moisture content on percentage seeds of perennial ryegrass with viable endophyte.**

**Table 1. Effect of seed moisture content (SMC) on endophyte viability of perennial ryegrass seed stored under ambient conditions in polyethylene aluminium bags for up to 5 years.**

| | | Number of years of storage | | | | | |
|---|---|---|---|---|---|---|---|
| Temperature | SMC | 0.5 | 1 | 2 | 3 | 4 | 5 |
| ambient | 8.6 | 82 | 72 | 68 | 64 | 74 | 10 |
| | 10.0 | 87 | 70 | 40 | 40 | 11 | 0 |
| | 12.1 | 73 | 54 | 0 | 0 | 0 | 0 |
| | 13.7 | 14 | 0 | 0 | 0 | 0 | 0 |
| 5°C | 8.6 | 85 | 82 | 80 | 78 | 76 | 80 |
| | 10.0 | 78 | 78 | 80 | 76 | 86 | 64 |
| | 12.1 | 82 | 80 | 78 | 78 | 78 | 72 |
| | 13.7 | 86 | 79 | 68 | 68 | 66 | 34 |

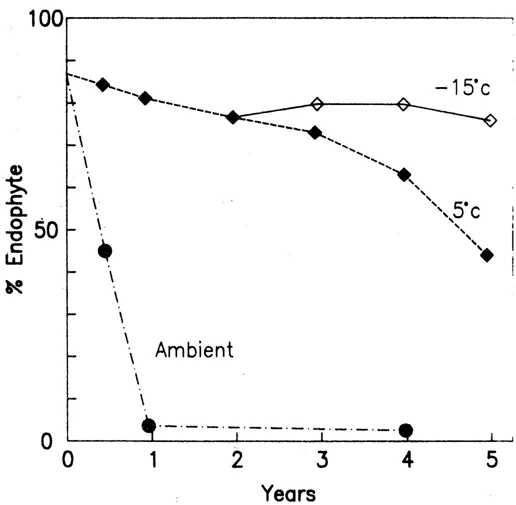


**Figure 2. Effect of storage temperature on percentage seeds of perennial ryegrass with viable endophyte.**

**Package type**

Different types of package material had a large influence on endophyte viability in ambient storage (Table 1). This response was due to the effectiveness of the packaging material in maintaining the SMC of the seed lot.

**Killing endophytes in seeds**

The killing of all endophyte mycelium in ryegrass seeds without significantly damaging seed germination could be achieved by holding seeds at high humidity for 3 wk at 37°C. Germination of these seeds averaged 74% whereas germination of the controls averaged 83%. A storage temperature of 30°C was unsatisfactory for killing mycelium. Even after 5 wk storage 75% of seeds still contained viable endophyte, whereas 93% of the control seeds had viable endophyte.

Treatment of seed to prevent growth of saprophytic fungi was necessary, the best treatment being surface sterilisation with 1% sodium hypochlorite. Dusting seeds with captan or thiram was unsatisfactory.

**Germination**

In these trials seed germination was much more tolerant of seed storage conditions than endophyte viability but was reduced by higher temperatures and high seed moistures (Table 2). The loss of endophyte viability was a least four times as rapid as the decline in germination. Seed germination in calico bags at ambient conditions had not declined after 2 years (91% germination), with a small decline of 10% after 3 years (83% germination) while endophyte viability had declined 51% in 0.5 years so that only 43% of seeds had viable endophyte. At 5°C a similar pattern was developing, but over a longer time period (Table 3).

In the storage trials of Welty *et al.* (1987), under conditions where germination was declining steadily (but not rapidly, i.e., more than 50% germination after 12 months), the rate of loss of endophyte viability was 1.4 to 2.4 times faster than the rate of germination loss for tall fescue and perennial ryegrass.

**Table 2. Effect of package type on endophyte viability and seed moisture content (SMC) after 12 months in ambient storage.**

| Package type | | % seeds with viable endophyte | SMC (%)[1] |
|---|---|---|---|
| calico | | 14 | 13.6 |
| polyethylene | 9µ | 24 | 12.8 |
| | 18µ | 28 | 12.4 |
| | 35µ | 58 | 12.0 |
| | 70µ | 72 | 11.8 |
| | 140µ | 78 | 10.6 |
| polyethylene/aluminium | | 72 | 10.1 |

[1] Initial SMC = 10%

**Table 3. Change in seed germination and viable endophyte in seed stored in calico bags at ambient or 5°C for 5 years.**

| Years | Germination % Ambient | 5°C | Endophyte % Ambient | 5°C |
|---|---|---|---|---|
| 0 | 94 | 94 | 84 | 84 |
| 0.5 | 95 ± 0.6[1] | 94 ± 0.8 | 46 ± 9.7 | 84 ± 1.6 |
| 1 | 92 ± 1.5 | 91 ± 1.1 | 8 ± 0.5 | 77 ± 2.3 |
| 2 | 91 ± 0.8 | 92 ± 0.8 | 0 | 70 ± 4.1 |
| 3 | 83 ± 1.6 | 92 ± 1.3 | 0 | 60 ± 7.6 |
| 4 | 57 ± 2.0 | 92 ± 0.9 | 0 | 45 ± 5.8 |
| 5 | 38 ± 1.8 | 90 ± 0.7 | 0 | 19 ± 7.3 |

[1] ± s.e.m.

## Conclusion

Endophyte viability can be maintained by seed storage at 5°C for 3 years and for more than 5 years at 0°C or -15°C. Packaging seed dried to 8.5% seed moisture content in appropriate moisture-proof bags will maintain high endophyte for more than 2 years in ambient warehouse conditions.

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