Effect of temperature on endophyte and plant growth of annual ryegrass, perennial ryegrass and tall fescue

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Summary Annual ryegrass, perennial ryegrass and tall fescue are agronomically important grasses for pasture, amenity and erosion control. These cool season grasses are often infected with a systemic fungal endophyte that increases the tolerance of the grass host to both biotic and abiotic stresses. Transmission of the endophyte is via seeds from infected plants and loss of viable endophyte may occur in the seed, particularly at elevated levels of temperature and humidity. The aim of this experiment was to determine if endophyte remained viable after heating infected seed lots of annual ryegrass, perennial ryegrass and tall fescue, and if so, would this treatment have any effect on germination and the subsequent growth of the resulting plants. A total of five seed lots were stored for 4, 8 and 12 days at temperatures of 4, 25 and 60°C. Seed was then germinated over a period of 14 days and plants maintained for a further 15 weeks. For percentage germination and viable endophyte, there were significant interactions between temperature treatment and seed lot, but plant biomass and tiller number were not significantly affected. Heating seed of tall fescue and perennial ryegrass had no or little effect on germination. Germination of annual ryegrass was affected to a greater extent, but this may have been a reflection of declining seed vigour in the seed lots tested. Viable endophyte was significantly reduced in all seed lots subjected to heat. Relatively high levels of viable endophyte were still found in the perennial and annual ryegrass seed lots after 12 days heat treatment, indicating that heat and humidity treatment may be required to completely kill endophyte in seed. Further studies are needed to determine the optimal conditions for killing endophyte in annual ryegrass seed, without unduly compromising seed germination and early plant growth.

Keywords Annual ryegrass, *Lolium rigidum*, perennial ryegrass, tall fescue, germination, temperature, endophyte.

INTRODUCTION

Annual ryegrass (*Lolium rigidum* Gaud.), later categorised as the ecotype Wimmera, was introduced into Australia in 1887 as a pasture species for the sheep zone (Kloot 1983). Annual ryegrass has adapted to the rotation phases of winter cereal crops and become a widespread weed that has evolved herbicide resistance to multiple modes of action (Heap and Knight 1982, Powles and Howat 1990, Pratley *et al*. 1996, Broster and Pratley 2006). Annual ryegrass is frequently infected with the symptomless fungal endophyte *Neotyphodium occultans* (Moon *et al*. 2000). Fungal hyphae grow between cells of the host grass, primarily in the lower base of leaf sheaths (Forde *et al*. 1988, Hume *et al*. 2001, Latch *et al*. 1984, 1988), and are only discerned microscopically. Endophyte transmission only occurs through the seed, and in Australia, this seed is commonly exposed to high temperatures in the field during summer. It is not known what impact these temperatures have on endophyte viability. The role *N. occultans* plays in the competitive nature of this grass in Australia is the subject of investigation (Kirkby *et al.* 2008).

In comparison, perennial ryegrass (*Lolium perenne*) and tall fescue (*Festuca arundinacea*) are important pasture grasses in the higher rainfall, permanent pasture regions of southern Australia, and commonly have associations with the endophytes *Neotyphodium lolii* and *N. coenophialum,* respectively (Latch *et al.* 1984). The role of these endophytes in these grass species has been well studied and pasture varieties have been bred with improved endophyte associations for enhanced plant and livestock production. Removing viable endophyte from seed by heat treatment has been used for research and breeding purposes for these grasses (Siegel *et al*. 1984, Easton 2007).

This study was undertaken to evaluate, in comparison with perennial ryegrass and tall fescue, the effect of temperature on the survival of *N. occultans* in annual ryegrass seed, and whether such effects could be associated with the growth of the grass species. This is needed for studies comparing uninfected plants (grown from temperature treated seed) with infected plants. Differences in grass growth in such studies must be attributed to infection status and not temperature treatment.

MATERIALS AND METHODS

Three grass species were used in this study: tall fescue (cultivar ‘Quantum’, infected with *N. coenophalium*

strain 'AR542'), perennial ryegrass (cultivar 'Samson', infected with *N. lolii* strains 'AR1' or 'AR37') and annual ryegrass (samples – ARGNSW and ARGWA, both infected with *N. occultans*). Seed-borne endophyte infection frequencies (Latch *et al*. 1987) for these five seed lots (n = 150 per seed lot) were 96, 100, 50, 56 and 58%, respectively. The incidence of infection found using this technique represents the maximum percentage infection at the time of harvest, and not necessarily repeated as viable endophyte incidence in plants grown from stored seed.

Seven temperature treatments were evaluated: ambient temperature as control (temperature ranged between 20 and 30°C); cold treatment (refrigerator at 4°C for 4, 8 and 12 days); and heat treatment (oven at 60°C for 4, 8 and 12 days). Treatments were arranged such that the temperature treatments were completed on the same day. There were three replicates per treatment.

The experiment commenced 17 September 2009 with 100 seeds from each line placed in paper envelopes and subjected to temperature treatments. Seeds from each treatment were then incubated on 29 September 2009 on moist filter paper in Petri dishes and incubated at 22°C with continuous light for 14 days. Viable seed was calculated as percentage of seed that had germinated by day 14 (Anon. 2006). At this time 50 seedlings per seed lot, per temperature treatment, were selected at random and transplanted into plastic trays (2 cm apart) lined with newspaper and filled with 50:50 sand:peat mix. Plants were maintained in a temperature controlled glasshouse (min 10°C, max 25°C) with natural photoperiod at Wagga Wagga, NSW.

Two harvests were conducted – at 11 (20 plants) and 15 weeks after planting (30 plants). At each harvest the number of tillers was recorded before cutting the tillers at the very base of the plant (soil surface). Mean number of tillers per plant was calculated. Plant biomass (mg dry weight) was recorded for each seed lot and temperature treatment by drying the cut herbage at 80°C for 48 h before the mean dry weight per plant was calculated.

Endophyte infection was assessed at the first harvest on two tillers per plant using the tissue print-immunoblot technique (Gwinn *et al*. 1991). Endophyte infection was calculated as the percentage of plants returning positive blots on both tillers.

Statistical analysis Two binomial generalised linear mixed models were fitted using GenStat (11th Edition) (Payne *et al*. 2008), with logit link function. The temperature and seed lot variables were included in the model as fixed effects, percent germination and viable endophyte as response variables, with replicate as a random effect. Tiller number and aboveground biomass were analysed using linear mixed models with temperature and seed lot as treatments, and replicates as blocks.

RESULTS

Germination There was a significant interaction between temperature treatment and seed lot for germination ($P = 0.004$) (Table 1). The seed lot containing AR37 was unaffected by the temperature treatment, while only one of the seven temperature treatments in AR1 and tall fescue had significantly lower germination. Both annual ryegrasses had lower mean germination (<75%) than perennial ryegrass and tall fescue seed lots (≥90%). For annual ryegrass, lower germination occurred in two of the seven temperature treatments (both at 60°C) for ARGWA, and five of the seven treatments for ARGNSW.

Viable endophyte Viable endophyte (% of plants with endophyte) at ambient temperature (Table 2) was similar to seed-borne infection frequencies for AR1, AR37 and ARGWA. Tall fescue and ARGNSW, however, had very low viable endophyte (<10%), considerably lower than the seed-borne infection frequencies of 96 and 56%, respectively.

A significant interaction occurred between temperature treatment and seed lot ($P = 0.022$). In most cases heat treatment of seed (60°C) reduced viable endophyte compared with ambient and cold treatments. Differences between ambient and cold were not consistent.

Number of tillers Seed lots differed in the number of tillers per plant at both harvests ($P < 0.001$) (Table 3). Annual ryegrass seed lots had more tillers than both perennial ryegrasses at harvest 1, but not at harvest 2. There were no within species differences for the two ryegrass species. Tall fescue had the lowest number of tillers at both harvests. Temperature treatments had no significant effect ($P > 0.05$).

Plant biomass The temperature treatment under which the seed was stored prior to incubation had no effect on the biomass produced in any seed lot ($P > 0.05$). Tall fescue produced the most biomass per plant at both harvests, significantly more than all other seed lots at harvest 1 and all but ARGWA at harvest 2 ($P < 0.001$) (Table 4). There were no differences in plant biomass between: both perennial and annual ryegrasses at harvest 1; the two perennial ryegrass seed lots and ARGNSW at harvest 2; and the two annual ryegrasses at harvest 2 (Table 4).

Table 1. Germination (%, back-transformed means) for five seed lots under seven temperature treatments at day 14 (tall fescue – TF; perennial ryegrass – AR1 and AR37; annual ryegrass – ARGNSW and ARGWA). Means within a column with different letters, and within a row with different superscript numbers, differ at P <0.001.

Treatment	TF	AR1	AR37	ARG NSW	ARG WA
Ambient	91[a]	95[a]	93[a]	81[a]	75[a]
4°C–4	91[a]	97[a]	95[a]	82[a]	76[a]
4°C–8	90[a]	97[a]	94[a]	75[b]	78[a]
4°C–12	92[a]	96[a]	96[a]	73[b]	74[a]
60°C–4	84[b]	97[a]	96[a]	72[b]	61[b]
60°C–8	89[a]	96[a]	94[a]	74[b]	62[b]
60°C–12	90[a]	84[b]	95[a]	61[b]	76[a]
Mean	90[2]	95[1]	95[1]	74[3]	72[3]

Table 2. Viable endophyte (%; back-transformed means) for five seed lots under seven temperature treatments (tall fescue – TF; perennial ryegrass – AR1 and AR37; annual ryegrass – ARGNSW and ARGWA). Means within a column with different letters, and within a row with different superscript numbers, differ at P <0.001.

Treatment	TF	AR1	AR37	ARG NSW	ARG WA
Ambient	4[a]	93[b]	56[b]	8[a]	47[b]
4°C–4	2[b]	98[a]	60[bc]	4[b]	68[a]
4°C–8	2[b]	87[c]	46[b]	6[ab]	63[a]
4°C–12	0[c]	93[b]	59[bc]	8[a]	43[b]
60°C–4	0[c]	75[d]	64[a]	7[a]	47[b]
60°C–8	0[c]	67[e]	64[a]	2[c]	45[b]
60°C–12	0[c]	70[e]	47[b]	4[b]	75[c]
Mean	<1[3]	87[1]	57[2]	5[3]	56[2]

Table 3. Number of tillers per plant for five seed lots, mean of all temperature treatments at two harvest dates. Means within a column with different letters differ at P <0.001.

Seed lot	Harvest 1	Harvest 2
TF	2.05[c]	3.30[b]
AR1	3.72[b]	4.57[a]
AR37	4.02[b]	4.58[a]
ARGNSW	5.16[a]	4.39[a]
ARGWA	5.24[a]	4.92[a]

Table 4. Mean plant dry weight (mg) for five seed lots. Means within a column with different letters differ at P <0.001.

Seed lot	Harvest 1	Harvest 2
TF	51.9[a]	530[a]
AR1	35.8[b]	373[c]
AR37	39.9[b]	364[c]
ARGNSW	36.4[b]	416[bc]
ARGWA	36.4[b]	463[ab]

DISCUSSION

Heat treatment generally reduced seed germination, although this effect varied between seed lots. There was little if any effect in perennial ryegrass and tall fescue, and larger but not necessarily consistent effects in the annual ryegrasses. The annual ryegrasses had notably lower germination than the other seed lots, indicating that vigour of the seed at commencement of this study may have been in decline, making them more susceptible to heat treatment. Germination in seed lots may also have been influenced by seed dormancy (not measured in this study).

The large difference observed in viable endophyte in comparison with seed-borne infection frequencies for tall fescue and ARGNSW seed lots may be an artifact of host genetics and/or length and condition of seed storage (Hill and Roach 2009, K.A. Kirkby unpublished data) prior to the commencement of this study. The general reduction in viable endophyte from heat-treated seed is consistent with other published studies (Siegel *et al*. 1984, Kannadan and Rudgers 2008).

Although there was an interaction between temperature and seed lot for viable endophyte, significant levels of viable infection remained in both perennial and annual ryegrass seed lots after 12 days storage at high temperature. The general trend was a lower viable endophyte level after 12 days at 60°C compared to ambient temperature. However, the ARGWA seed

lot results were inconsistent with trends seen in the other seed lots. No viable endophyte was detected in all heat treated tall fescue seed.

The results of this study found the use of heat alone (as may be found in the field on soil surfaces) was insufficient to kill all endophyte in annual ryegrass seed. This is in contrast with results reported by Kannadan and Rudgers (2008) for Grove bluegrass (*Poa alsodes*) infected with *Neotyphodium* endophyte, where the fungus was destroyed after 6 days in an oven at 60°C. Future attempts to remove viable endophyte from annual ryegrass seed should employ both temperature and humidity treatments as done in studies for perennial and tall fescue. The finding that tiller number and plant biomass were not affected by temperature treatments may be important in comparative studies of uninfected plants (where the endophyte has been successfully removed from seed by heat treatment) and infected plants.

Future work is required to determine the optimal number of days, temperature and humidity for successful removal of *N. occultans* from annual ryegrass seed.

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