Influence of Moisture Content, Temperature, and Length of Storage on Seed Germination and Survival of Endophytic Fungi in Seeds of Tall Fescue and Perennial Ryegrass

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Cooperative investigations of the USDA-ARS and Oregon Agricultural Experiment Station. Technical Paper 7896 of the Oregon Agricultural Experiment Station.

The use of trade names in this publication does not imply endorsement by the U.S. Department of Agriculture of the products named, or criticism of similar ones not mentioned.

The assistance of Kathy L. Cook with microscopy, R. G. Petersen with statistical analysis, and A. Haunold with translation of articles from German to English is gratefully acknowledged.

Accepted for publication 21 November 1986 (submitted for electronic processing).

ABSTRACT

Welty, R. E., Azevedo, M. D., and Cooper, T. M. 1987. Influence of moisture content, temperature, and length of storage on seed germination and survival of endophytic fungi in seeds of tall fescue and perennial ryegrass. Phytopathology 77:893-900.

Seeds of tall fescue and perennial ryegrass were stored 18 mo at 10–30 C and 11.5–95% relative humidity to evaluate the effect of these conditions on germination and viable endophyte. It was found that temperature, moisture content of seed, and time interact to influence germination and endophyte survival in both grass species. Moisture contents of tall fescue seed that resulted in the most rapid decrease in endophyte viability, while maintaining the highest level of germination, were 19.4, 9.6, and 8.2% at 10, 20, and 30 C, respectively. Moisture contents that resulted in decreases in viable endophyte and germination at 10, 20, and 30 C were 24, 15.2, and 14.1%, respectively. The results for endophyte survival and germination in seeds of perennial ryegrass were generally similar to those for seeds of tall fescue; however, in moisture contents that maintained germination, endophyte viability decreased more slowly in ryegrass than in tall fescue. Conditions that favored rapid decreases in endophyte viability in ryegrass seeds also resulted in loss of germination. When seeds of either species are stored at or above 15% moisture content and 20 or 30 C, loss of germination is likely to occur after 2 or 3 mo of storage. Field emergence of stored seed was lower than expected by blotter germination.

Additional key words: *Acremonium coenophialum, Acremonium loliae, Epichloë typhina*, fescue toxicosis, *Festuca arundinacea, Lolium perenne*, ryegrass staggers, seed pathology.

Choke diseases in cool-season grasses, caused by *Epichloë typhina* (Pers. ex Fr.) Tul., have been known since 1881 (23). Detailed descriptions of the disease in red fescue, *Festuca rubra* L., were published in 1933 (24) and in species of *Lolium* and *Festuca* in 1941 (20). Since then, the known host range of *E. typhina* has increased to 48 species, including 5 species of *Festuca* (12).

Recent interest has focused on *E. typhina* and its anamorph *Acremonium coenophialum* Morgan-Jones & Gams in tall fescue (*F. arundinacea* Schreb.) and *A. loliae* Latch, Christensen, & Samuels in perennial ryegrass (*L. perenne* L.) (1,6,9–11,15,17, 25–27,30). Interest was stimulated by the discovery that *A. coenophialum* growing endophytically in tall fescue was associated with fescue toxicosis in cattle, whereas *A. loliae* in perennial ryegrass was associated with ryegrass staggers. Fescue toxicosis costs livestock producers an estimated $50–200 million annually (2,9), and ryegrass staggers results in stock losses ranging from 2 to 10% (5). Both fungi are commonly referred to as endophytes because they grow entirely within the grass host, rarely showing external signs or symptoms (1,14,19,20).

A beneficial aspect of endophyte infection was discovered in New Zealand (21) and confirmed in New Jersey (7). Prestidge et al (21) discovered that insect feeding was reduced in endophyte-infected plants when compared with endophyte-free plants. A review of this topic was recently published (26).

The only known mode of spreading the endophytic fungi is by sowing infected seed (18,24–26). Resowing pastures with endophyte-free seed or with seed having low levels of endophyte (<5%) is the least expensive method of control. Seedborne infection can be reduced by treatment of the seed with fungicides (8,13,14,27,30,31) or heat (13,14,27,30). Although survival of the endophytes in stored seed has been studied (13,14,19,27,30), seed moisture was not controlled, sample sizes were limited (usually 20 seeds or seedlings), and neither the seed source nor the age of seed was specified.

The first objective of this study was to evaluate the influence of seed moisture content, temperature, and length of storage on seed germination and endophyte survival for tall fescue and perennial ryegrass seed. Storage conditions were selected to simulate those encountered in commerce or in farm storage. The second objective was to sow seeds in pasteurized soil and field soil to compare seedling emergence of tall fescue and perennial ryegrass seeds stored 18 mo at several temperatures and relative humidities. The third objective was to evaluate the effects of relatively high temperatures of storage (40, 50, and 60 C) on seed germination and endophyte survival since it has been reported that storing tall fescue seed at 49 C for 4 wk eliminated viable endophyte (27). A portion of these results was reported earlier (28).

MATERIALS AND METHODS

Seeds of tall fescue strain G1-307 and perennial ryegrass cultivar Repell were harvested in the summer of 1983, air-dried to about 14% moisture content, and stored 3–6 mo at −18 C until used in this study. In the first experiment, seeds of both species were stored in the dark at 10, 20, and 30 C above saturated aqueous solutions of lithium chloride (LiCl), magnesium chloride ($MgCl_2$), sodium chloride (NaCl), potassium chloride (KCl), and glucose ($C_6H_{12}O_6$). The saturated solutions at these temperatures provide relative humidities (RH) that range from 11.5 to 88%, respectively (32). Based on the equilibrium moisture content of the seeds, the saturated glucose solution at these temperatures provided a higher RH than the expected 55% as published (32). Saturated solutions were placed in individual round, plastic containers fitted with a snap-lip, and aluminum screens were set on rubber stoppers about 1 cm above the solutions. Open petri dishes (9 cm in diameter)

containing about 80 g of seed were set on the screens. Before the storage test was started, samples of 100 seeds were assayed for germination and level of infection by viable endophyte.

The study was arranged as a three-temperature × five-RH factorial with sampling serving as replication in time. At monthly intervals for the first 12 mo, and every 3 mo for the remaining 6 mo, seeds were evaluated for germination, viable endophyte, and moisture content. At each sampling, the original sources of seed stored at −18 C were also evaluated as the control. At every sampling interval, 16 seed samples were tested for each species for germination and endophyte level. At each sampling, seed samples were mixed and about 0.5 g of seed was removed and soaked 1 hr in captafol [4F (*N*-(1,1,2,2-tetrachloroethyl)thio-4-cyclohexene-1,2-dicarboximide)] (7,390 μg/ml) and air-dried. Captafol, which was applied to control seed saprophytes, does not reduce germination or endophyte growth in seedlings (29). Fifty seeds were placed on blotter paper moistened with 0.1% KNO_3 in plastic boxes measuring 11.5 × 11.5 × 3.3 cm and incubated 7 days at 5 C in the dark to break dormancy. Boxes were then moved to an incubator providing 16 hr of dark at 15 C and 8 hr of light (50 microeinsteins per square meters per second) at 25 C. After 2 wk, the seedlings were counted and the percentage germination determined. A seed was considered germinated if a normal-appearing root and shoot emerged. This combination of temperature and light is used for determining germination of tall fescue seeds (4).

To detect endophytic fungi, the lemma and palea were gently peeled from seedlings, and leaves were cut above the leaf sheath and discarded. Roots were cut within 2–5 mm of the root-shoot interface and also discarded. The remaining root-shoot pieces were stained in 5% NaOH and 0.1% trypan blue for about 16–18 hr. Pieces were then washed in distilled water and boiled gently for 20 min in lactophenol 1:1:1:5 (v/v; 85% lactic acid:glycerine:phenol:water) containing 0.1% trypan blue. This was followed by rinsing in distilled water, squash-mounting in two to three drops of glyerine:water (1:3, v/v), and examination at × 100–1,000 using bright-field microscopy. Endophyte hyphae were present in leaf sheaths and meristems as previously reported (29).

To isolate the endophytic fungi, seedling pieces were surface sterilized with 1% NaOCl for 1 min and cultured on cornmeal agar or potato-dextrose agar. The identification of *A. coenophialum* from tall fescue and *A. loliae* from perennial ryegrass was based on their cultural characteristics (15,17).

At each sampling, seeds were mixed and 2 g of seed dried at 100–103 C for 24 hr. Moisture content is reported on a wet-weight basis.

When germination decreased below 100% or when 50 seedlings were not available for the endophyte assay, an adjusted value (X) was used to account for losses in germination, thus allowing a direct comparison for each given set of storage conditions. The adjusted value (X = percentage of seedlings with viable endophyte) consisted of number of seedlings with endophyte hyphae in the leaf sheath or meristem ÷ number of seedlings examined × number of germinated seedlings × 100.

For the second experiment, about 100 g of seeds from both species was put into separate cloth bags and incubated at 40, 50, and 60 C. As before, the original seed stored at −18 C was included as the control. No attempt was made to control moisture content or atmospheric humidity. At weekly intervals for the first 4 wk and then biweekly from weeks 10 to 18 of storage, seeds were evaluated as previously described for germination, viable endophyte, and seed moisture content. Sample size was 100 seeds for germination and 50 seedlings for viable endophyte detection. The adjusted value was not needed in this study because at least 50 seedlings were always available for testing.

For the third experiment, 100 seeds of tall fescue and perennial ryegrass stored 18 mo at nine combinations of temperature and RH were sown in a pasteurized mixture of 1:1:1 (v/v/v) soil:sand:perlite in flats placed outdoors or planted in the field near Corvallis, OR. Seeds from six combinations of temperature and RH storage conditions were not included because they were dead. Plants in the pasteurized soil or in the field were watered to maintain vigorous growth. Fifty seeds from each storage condition were also germinated on blotters as previously described. Seedling emergence for pasteurized soil and in field plots was counted 6 wk after sowing; seedlings in the blotter-germination tests were counted after 3 wk.

Seeds stored at moisture contents above 13% are frequently invaded by storage fungi, particularly species of *Aspergillus*, which cause a decrease in germination and a loss of quality (3). After 6 mo of storage at 10, 20, or 30 C above the saturated glucose solution, seeds of both grass species were surface sterilized in 1% NaOCl for 1 min, rinsed in sterile distilled water, and cultured on Czapek's-Dox broth agar or Czapek's-Dox broth agar plus 20% sucrose (22), media known to permit vigorous growth of most species of *Aspergillus*. Twenty-five seeds from each of the storage conditions were cultured on agar and incubated at 25 C for 8 days in the dark. *Aspergillus* species were identified without subculturing (22).

RESULTS

Seeds of both species used in the storage studies were germinated and analyzed for viable endophyte content before being stored. For tall fescue, germination and viable endophyte content were 98 and 99%, respectively, and for perennial ryegrass were 96 and 91%, respectively. Moisture content of seeds stored in each humidity chamber at each temperature was determined when sampled. The mean and standard deviation (14 measurements) are presented in each figure.

Average moisture contents for the tall fescue seeds stored at 20 C above saturated solutions of LiCl, $MgCl_2$, NaCl, KCl, and glucose were 6.2, 9.6, 15.2, 17.6, and 21.3%, respectively. The moisture contents for tall fescue seeds above these saturated solutions at 10 and 30 C were slightly higher and slightly lower than at 20 C, respectively (Fig. 1). The moisture contents for perennial ryegrass seeds stored in these same containers were generally similar (Fig. 2) to those for tall fescue seeds. Relative humidity of the air space above the saturated solutions was not measured.

When tall fescue seed was stored at 10 C and <20% moisture content for 18 mo, germination generally remained high and near the initial level (98%) (Fig. 1A–D); in contrast, endophyte survival generally decreased during storage at 10% moisture content and above (Fig. 1B–E). The storage condition that provided the most rapid decrease in *A. coenophialum* while maintaining a high level of seed germination (98%) was 10 C and 19.4% moisture content ± 0.7 (Fig. 1D). At 24% moisture content ± 0.9 and 10 C (Fig. 1E), germination and endophyte decreased at about the same rate, with germination decrease lagging behind endophyte decrease by about 2–5 mo.

When seed was stored at 20 C (Fig. 1F–J), germination remained above 90% at moisture contents <9.6% ± 0.9 while endophyte survival decreased at these and higher moisture contents. The moisture content at 20 C that maintained the highest level of germination and provided the fastest decline in viable endophyte was 9.6% ± 0.9 (Fig. 1G). As none of the saturated solutions at 20 C provided moisture contents between 9.6 and 15.2%, these were not evaluated. At 15.2% moisture content and above, germination and endophyte survival decreased more rapidly, with germination lagging endophyte survival by about 1–10 mo (Fig. 1H–J). At 17.6% moisture content and above, germination and endophyte survival decreased rapidly and at about the same rate.

When seed was stored at 30 C, germination remained above 90% at moisture contents <8.2% ± 0.8 (Fig. 1K and L), but *A. coenophialum* survival decreased. The moisture content that maintained the highest germination and with the most rapid decrease in endophyte was 8.2% ± 0.8 (Fig. 1L). At 14.1% moisture content and above, conditions provided for rapid decreases in both germination and endophyte survival (Fig. 1M–O).

The data for endophyte survival and germination were fitted to a multiple-regression model to explain the contribution of each storage variable (temperature of storage, time of storage, and seed moisture content). Each variable was a highly significant source of variation (P = >0.001). The model to predict endophyte survival is

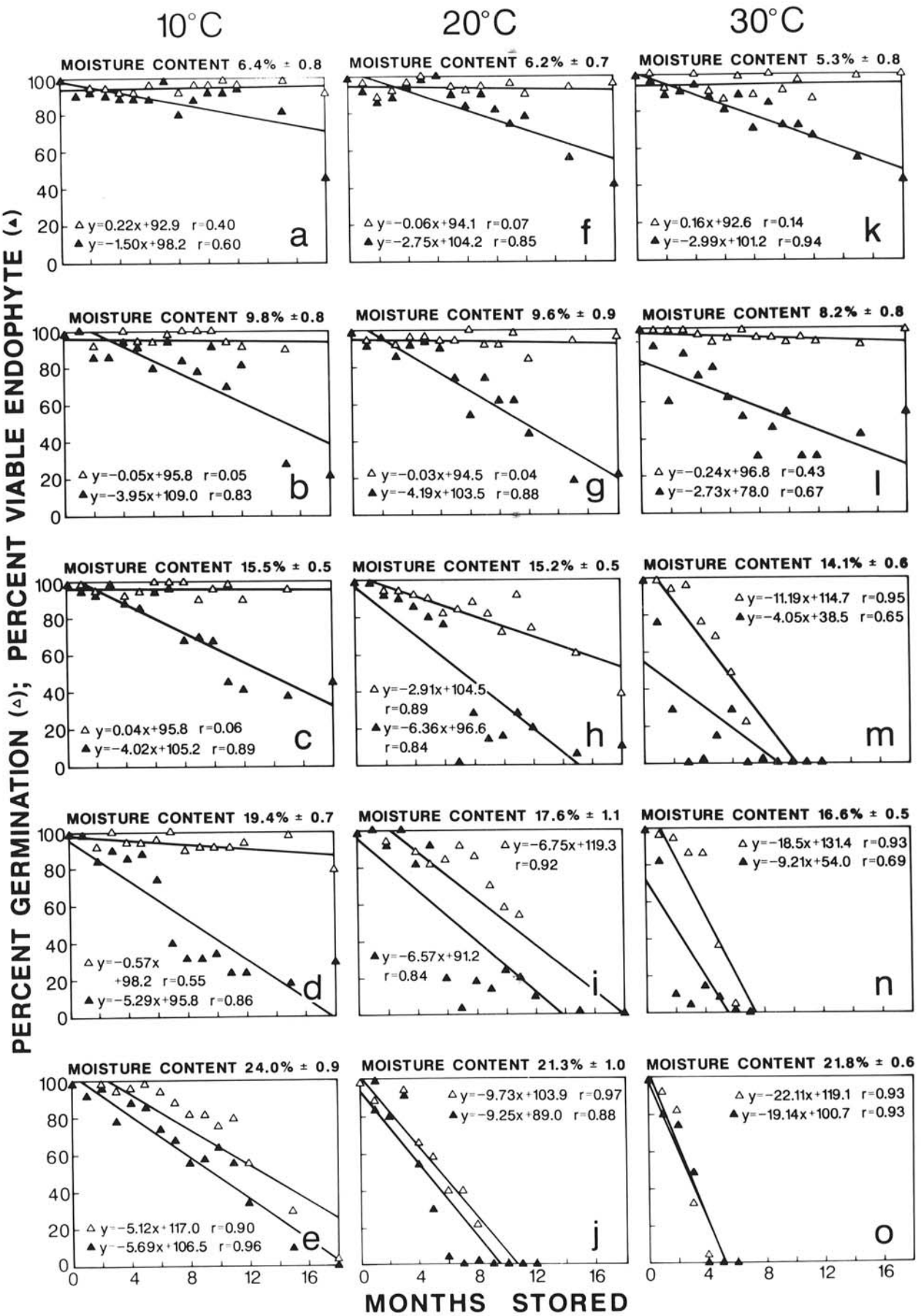

Fig. 1. Relation of survival of viable endophyte and germination to time for seed of tall fescue stored at five nominal relative humidities (RH) above saturated aqueous solutions. At 10 C: **A–E** = 13.5, 34, 76.5, 88, and 55% RH, respectively; at 20 C: **F–J** = 12.5, 33, 76, 85, and 55% RH, respectively; and at 30 C: **K–O** = 11.5, 32.5, 75.5, 84.5, and 55% RH, respectively.

$Y = 85.76 - 1.08$ (temperature) $- 2.10$ (time) $- 1.53$ (moisture content), $r^2 = .63$; the model to predict germination is $Y = 79.27 - 0.78$ (temperature) $- 0.98$ (time) $- 1.23$ (moisture content), $r^2 = .38$. Substituted values for temperature, time, and moisture content within the limits of this test can predict endophyte survival and germination.

Changes in ryegrass seed germination and endophyte survival when seeds are stored at 10, 20, and 30 C (Fig. 2) were very similar to the results for tall fescue seed (Fig. 1). However, the endophyte *A. loliae* usually survived at higher levels in perennial ryegrass than did *A. coenophialum* in tall fescue seed; the rate of *A. loliae* decrease was also generally less than that of *A. coenophialum*. None of the perennial ryegrass seed storage conditions in which germination was maintained at 90%+ caused a decrease in endophyte to 0% after 18 mo of storage. Generally, when temperatures and moisture content were favorable for reductions in endophyte, germination also decreased. The single exception occurred at 10 C and 19% moisture content ± .5 (Fig. 2D), in which germination was maintained at about 96% and endophyte decreased to about 30%. In seeds of tall fescue under these same conditions (Fig. 1D), endophyte survival had decreased to about 0%.

The data for endophyte survival and germination were fitted to a multiple-regression model to explain the contribution of each storage variable (temperature of storage, time of storage, and seed moisture content). Each variable was a highly significant source of variation ($P = >0.001$). The model to predict endophyte survival is $Y = 85.81 - 0.87$ (temperature) $- 1.65$ (time) $- 1.8$ (moisture content), $r^2 = .58$; the model to predict germination is $Y = 84.11 - 0.85$ (temperature) $- 0.91$ (time) $- 1.62$ (moisture content), $r^2 = .45$. Substituted values for temperature, time, and moisture content within the limits of this test can predict endophyte survival and germination.

When tall fescue seed was stored at high temperature, germination decreased to about 80% after storage for 18 wk at 60 C but was unaffected at −18, 40, and 50 C (Fig. 3A). Endophyte viability decreased during storage at all four temperatures, with the rate of decrease faster at the higher temperatures (Fig. 3B). Viable endophyte was found in seedlings at all temperatures after 18 wk of storage. Moisture loss from seeds during storage was greatest at the higher temperatures (Fig. 3C). Seed moisture content for zero storage time was not included in the linear regression equations in Figure 3C and F. Since most of the moisture was lost from the seeds during the first few hours of storage on the first day, we assumed that this value would distort the equation.

When ryegrass seed was stored at high temperature, germination decreased to about 80% after storage for 18 wk at 60 C (Fig. 3D), a result similar to that for tall fescue. Germination was unaffected at the other three temperatures. As with tall fescue, endophyte decreased during storage at all four temperatures, with the rate of decrease greatest at the higher temperatures (Fig. 3E). After 18 wk at 60 C, viable endophyte was reduced to about 50%. Moisture content decreased during storage, with the loss greatest at the higher temperatures (Fig. 3F).

In the planting study, germination measured by the blotter test was generally greater than seedling emergence from soil (Table 1). This was true for both species for all nine storage conditions. Seedling emergence was greater in pasteurized soil than at field locations. Averages (18 treatments) for germination on blotters and emergence from pasteurized soil and field locations were 85, 78, and 64%, respectively.

Percentage seedling emergence within field plantings (for 18 of the 30 storage treatments) was dependent on storage treatment. Seedling emergence in field plots and pasteurized soil was almost always less than seedling germination on moistened blotters.

Aspergillus species were associated with stored seed, with *A. versicolor* group species most frequently isolated from seeds stored for 6 mo above the saturated glucose solution at 20 and 30 C. At the lowest storage temperature (10 C), a species of *Alternaria* was the most common isolated (Table 2). Storage fungi (especially *A. versicolor*) increased as germination decreased.

TABLE 1. Percentage germination (blotter test) and seedling emergence (in soil) of tall fescue and ryegrass seeds after storage for 18 mo at 10–30 C and 11.5–88% relative humidity

Species	Temp (C)	Relative humidity (%)[a]	EMC (%)[b]	Seedling emergence (%)[c] Germination[d]	Field plots[e]	Pasteurized soil[f]
Tall fescue	10	13.5	6.4	92	89	95
		34.0	9.8	96	75	93
		76.5	15.5	100	86	87
		88.0	19.4	80	63	78
	20	12.5	6.2	96	80	97
		33.0	9.6	98	65	92
		76.0	15.2	38	10	11
	30	11.5	5.3	100	81	93
		32.5	8.2	98	60	92
Perennial ryegrass	10	13.5	6.7	98	88	91
		34.0	10.0	92	71	91
		76.5	15.7	94	79	94
		88.0	19.0	78	28	51
	20	12.5	6.3	78	51	51
		33.0	9.8	94	73	86
		76.0	15.4	12	10	9
	30	11.5	5.2	94	66	94
		32.5	8.8	92	71	95

[a] Maintained by saturated solutions of LiCl, $MgCl_2$, NaCl, and KCl.
[b] Equilibrium moisture content of seed (wet-weight basis).
[c] 100 seeds for each storage treatment.
[d] Seeds from each storage condition were placed on blotter paper moistened with 0.1% KNO_3 and incubated 1 wk at 5 C in dark, followed by 2 wk at alternating 25 C, 8-hr light and 15 C, 16-hr dark.
[e] Planted at Botany Field Lab near Corvallis, OR (25 September to 6 November 1985).
[f] Planted in pasteurized 1:1:1 (v/v/v) soil:sand:perlite in flats and grown outside (23 September to 4 November 1985).

DISCUSSION

Viability of seeds and endophyte survival were influenced by temperature, length of storage, and seed moisture content. These results confirm earlier findings (27,30) that germination and endophyte viability decrease with length of storage and that the rate of decrease is greatest at higher temperatures. This study

TABLE 2. Fungi growing from stored tall fescue or perennial ryegrass seeds incubated 8 days on two culture media at 25 C in dark[a]

Culture media	Fungi	Perennial ryegrass 10 C	20 C	30 C	Tall fescue 10 C	20 C	30 C
Czapek's broth agar	*Alternaria* sp.	10[b]	2	0	21	0	0
	Aspergillus flavus, group	0	0	0	0	1	0
	A. niger, group	0	0	0	0	1	0
	A. ochraceus, group	0	0	6	0	1	0
	A. versicolor, group	0	15	18	0	21	22
	Unknown (not sporulating)	0	6	5	4	0	1
Czapek's broth agar plus 20% sucrose	*Alternaria* sp.	6	1	0	5	0	0
	Aspergillus flavus, group	0	2	3	0	0	0
	A. glaucus, group	0	2	0	0	0	0
	A. niger, group	0	0	0	0	0	4
	A. ochraceus, group	0	0	2	0	0	0
	A. versicolor, group	0	14	18	2	18	25
	Unknown (not sporulating)	0	10	2	3	1	0

[a] Seeds stored 6 mo above saturated glucose solution; average seed moisture content for ryegrass was 24.2, 21.1, and 19.9% at 10, 20, and 30 C, respectively.
[b] Number of seeds yielding fungus based on culturing 25 surface-sterilized seeds from each storage condition on each medium.

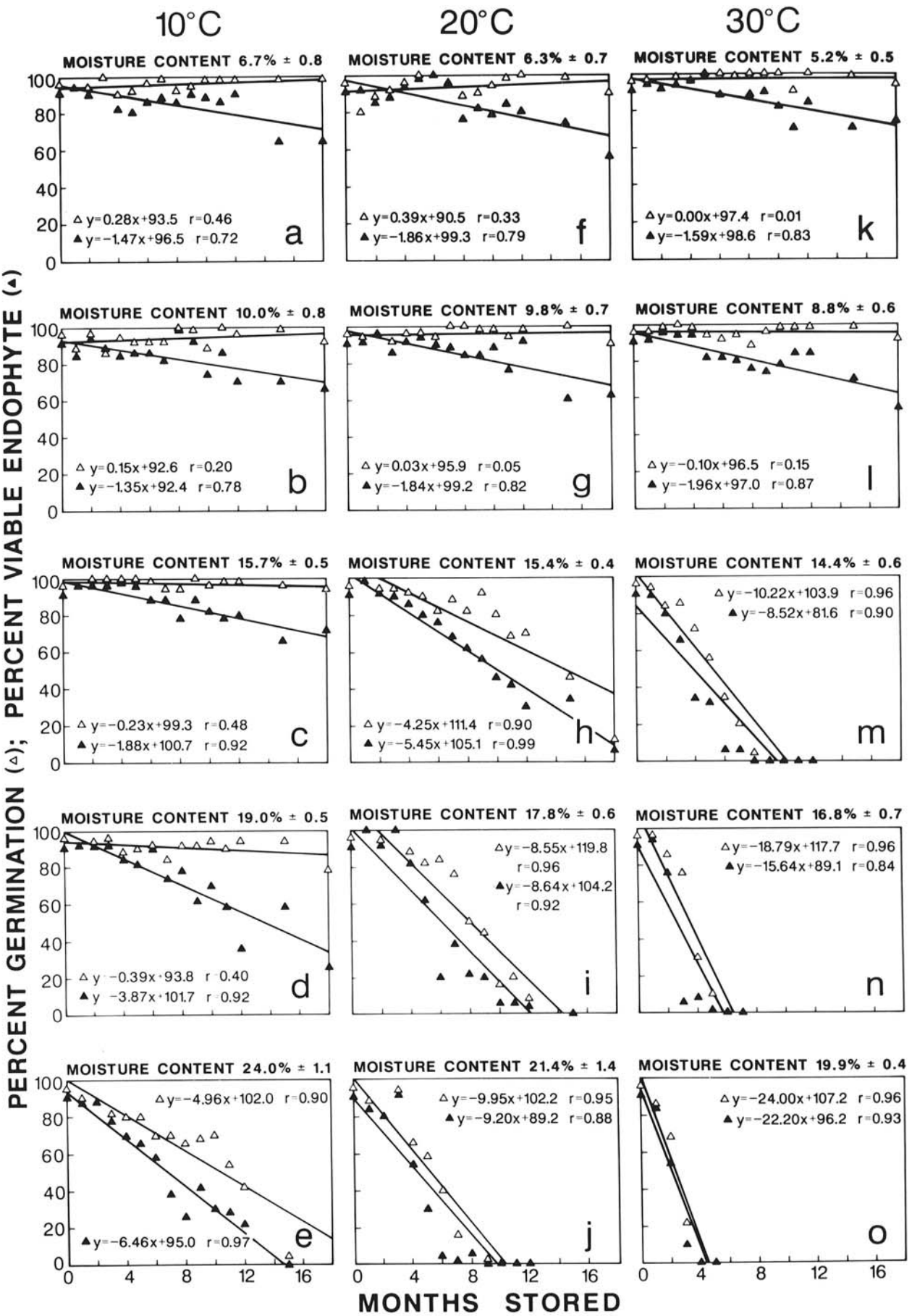

Fig. 2. Relation of survival of viable endophyte and germination to time for seed of perennial ryegrass stored at five nominal relative humidities (RH) above saturated aqueous solutions. At 10 C: **A–E** = 13.5, 34, 76.5, 88, and 55% RH, respectively; at 20 C: **F–J** = 12.5, 33, 76, 85, and 55% RH, respectively; and at 30 C: **K–O** = 11.5, 32.5, 75.5, 84.5, and 55% RH, respectively.

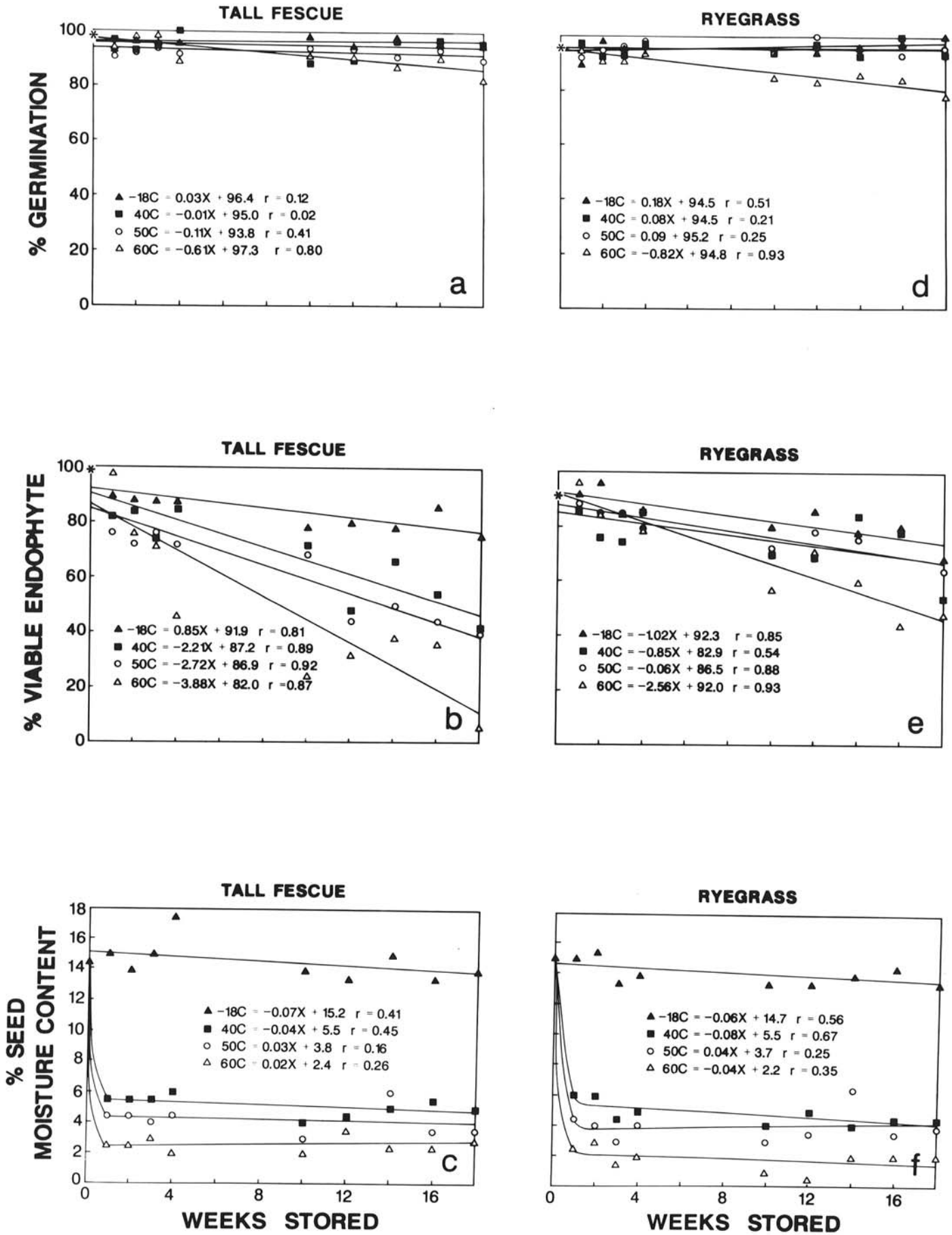

Fig. 3. Relation of germination, endophyte survival, or moisture content to time in seed of tall fescue (**A–C**) or perennial ryegrass (**D–F**) stored 18 wk at −18, 40, 50, or 60 C.

extends the earlier reports by demonstrating that seed moisture content interacts with temperature and time. The study also has revealed that when seeds of tall fescue and perennial ryegrass are stored at the same temperature and RH, changes in viability of endophyte are different for the two grass species. The practical outcome of this investigation is the identification of conditions that will maintain high germination while reducing viable endophyte. However, these conditions—particularly those affecting seed moisture—must be carefully controlled. For example, for tall fescue seed stored under controlled temperature and moisture content to maintain the highest original germination, the greatest reduction in viable endophyte at 10, 20, or 30 C occurred when seed moisture contents were 19.4, 9.8, or 8.2%, respectively.

Losses in germination and endophyte viability were similar for perennial ryegrass seed and tall fescue. However, most conditions that resulted in a loss of endophyte viability to 0% after 18 mo storage also resulted in a loss of germination. Optimum reduction of endophyte in tall fescue with least reduction of germination occurred at 10 C and 19% moisture content. In perennial ryegrass stored in these conditions, germination was maintained above 95%, while the endophyte decreased by 50%. In general, temperatures that favored high germination also maintained endophyte viability.

Short-term storage (up to 18 wk) at high temperatures (40–60 C) did not eliminate viable endophyte from tall fescue or ryegrass seeds, which conflicts with earlier reports (27). This conflict could arise from the low seed-moisture content (2–5%) used during storage, the application of dry rather than moist heat, use of different grass cultivars, or age of seed before exposure to high-temperature storage. Cloth bags were chosen for storing seeds during exposure to 40–60 C because this system would be more available to farmers and commercial concerns. The experiment on high-temperature storage was originally planned to last 4 wk; when viable endophyte remained in the seed after this period of storage, however, the length of storage was extended to 18 wk. The experiment was terminated because of equipment failure. However, if the storage period were longer, it appears that exposing small batches of tall fescue seed to dry heat at 60 C for 20 or more weeks may eliminate viable endophyte, although this would be accompanied by reductions in germination of 20% or more. More research is needed before the method can be used to heat-treat seeds to reduce viable endophytes.

Emergence of both grass species in the field is likely to be less than would be expected by moistened-blotter germination tests, a result that has been reported among other field crops (16). Thus, increased seeding rates to compensate for lower emergence are advised.

The increase of species of *Aspergillus* in seeds stored above 13% moisture content is consistent with what is known about deterioration of seeds in storage (3). However, increased seed respiration may also have contributed to germination loss.

These data indicate that tall fescue seed stored below about 10% moisture content and 10–30 C for 18 mo will maintain initial levels of seed germination, with a gradual decline in viable endophyte. However, when seed moisture content exceeds 10%, temperature and moisture content will interact to influence seed and endophyte survival. Generally, storing bulks of tall fescue seed at moisture contents exceeding 10% to reduce endophyte and maintain germination either should be avoided or storage conditions should be carefully controlled. Further, seed should be evaluated every few months for germination and viable endophyte content. These results support earlier reports (27,30) that seeds of tall fescue can be stored to eliminate viable endophyte and to maintain germination. However, attaining the proper temperature and seed moisture content that allow this to occur routinely in on-farm storage (30) appears to have been fortuitous. Differences in the results of the current investigation from earlier studies most likely arise from the lack of control of seed moisture content in the latter. Finally, other factors that could influence seed and endophyte survival in storage include grass and cultivar, seed source, races of endophyte, and conditions of handling before storage—for example, freezing at high moisture contents.

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