RESEARCH ARTICLE

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BRIEF COMMUNICATION

Persistence of endophytic fungi in cultivars of *Lolium perenne* grown from seeds stored for 22 years[1]

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PREMISE OF THE STUDY: Genetic resources for forage crops often consist of seeds of specific species and cultivars in cold storage for future use in breeding and selection programs. Temperate grasses such as *Lolium perenne*, used worldwide for forage and turf, produce seeds commonly infected by hyphae of an endophytic fungus (*Epichloë festucae* var. *lolii*). This research determined whether endophytes could persist and infect seedlings of *L. perenne* emerging from seeds stored for over two decades.

METHODS: Endophyte-infected seeds (>90% infected) of four cultivars were obtained in 1994 and stored dry in plastic bags at 4°C. Seed germination was tested after 12 yr (for two cultivars) and after 18 and 22 yr (for all cultivars). Seedling leaf sheaths were excised, stained, and examined at 400× for endophytic hyphae to quantify infection frequency (% plants infected) and intensity (mean number of endophytic hyphae per field of view).

KEY RESULTS: Seed germination after 22 yr depended on cultivar, ranging from 53 to 78%. Between 58 and 73% of plants grown from seeds stored for 22 yr still contained viable endophytic hyphae. Infection intensity remained at original levels for 18 yr in one cultivar; however, in all cultivars, infection intensity declined significantly between 18 and 22 yr.

CONCLUSIONS: Persistence of the grass seed–endophyte symbiosis for over 20 yr surpasses all prior records of endophyte longevity within stored seeds. Storage of germplasm of cool-season grass cultivars that contain potentially beneficial fungal endophytes should be possible for several decades under dry, cold conditions.

KEY WORDS endophytic fungi; *Epichloë festucae* var. *lolii*; forage crop; genetic resources; *Lolium perenne*; perennial ryegrass; Poaceae; seeds; symbiosis persistence

Long-term storage of seeds in refrigerated conditions is needed to provide plant genetic resources for future use in breeding programs, crop improvement, and restoration of degraded land (Batello et al., 2008; Peres, 2016). Seed resources are also important for the preservation of genetic diversity of widespread plant species that are likely to undergo significant evolutionary changes in response to anthropogenic alterations in ecosystems and climate. Viable seeds from the past can be used by future researchers to examine questions germane to the basic evolutionary ecology of plants (Orsini et al., 2013; Etterson et al., 2016). Stored seeds also function as gene banks that preserve valuable cultivars and breeding material of economically important species used for food and forage (Marshall, 1990; Tanksley and McCouch, 1997; Batello et al., 2008; Dwivedi et al., 2016; FAO, 2016; Peres, 2016).

Given the common occurrence of vertically transmitted fungal endophytes within the seeds of economically important, cool-season, forage grass crops (Schardl et al., 2004; Gundel et al., 2013), it is important to consider the longevity of symbionts within seeds stored for significant time periods. The genetic base of forage species used in pastures and rangelands is relatively narrow (Batello et al., 2008), but endophytic fungi in seed bank collections could be useful to plant scientists in forage and turfgrass improvement (Funk et al., 1994; Clement, 2001; Brilman, 2005). In addition, because of their ability to positively affect physiology and growth in some species under stressful environmental conditions (Kuldau and Bacon, 2008; Cheplick and Faeth, 2009), fungal symbionts are expected to modify plant responses to the altered environmental conditions associated with anthropogenic global changes (Kivlin et al., 2013). Efficient interactions with beneficial microbes are also envisioned as an important breeding target for future crop production (Sessitsch and Mitter, 2015).

In nature, it is well known that vertical transmission of endophytes within seeds is not always perfect, and thus infection can be

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lost from sexually reproducing plants (Ravel et al., 1997; Afkhami and Rudgers, 2008). However, despite the potential usefulness to future plant breeders, pasture production, and ecological restoration, there is almost no information available on the persistence of endophytic fungi in the stored seeds of cool-season grasses and the plants that might be grown from them.

This report highlights the frequency and intensity of endophyte infection in four cultivars of *Lolium perenne* L., a globally important forage and turfgrass, examined at intervals between 0 and 22 yr of seed storage. All four cultivars were originally highly infected (>90%; Clay, 1987; Funk et al., 1994) by the asymptomatic, systemic fungal endophyte *Epichloë festucae* var. *lolii* (Latch, M.J.Chr. & Samuels) C.W.Bacon & Schardl (formerly *Neotyphodium lolii*) (Leuchtmann et al., 2014). After seeds were incubated under optimal germination temperatures (Shen et al., 2008), plants were grown in common conditions for several months and then examined microscopically for the presence of viable endophytic hyphae within leaf sheath tissue. Results suggest that both seed germination and viable endophytic hyphae can be maintained at relatively high levels for several decades in *L. perenne* when seeds are stored under cold, dry conditions.

MATERIALS AND METHODS

Seed sources and storage—Several hundred seeds of four endophyte-infected cultivars (Palmer II, Prelude II, Repell II, Yorktown III) were obtained in 1994 from Lofts/Pennington Seed Company (Madison, Georgia, USA). These cultivars were originally developed from field-collected, infected clones and specifically selected for use in turf in temperate regions of the United States (Hurley et al., 1994a–c, 1996; Murphy and Mohr, 2004). The initial infection frequency in seeds of Yorktown III was 94.5% ($N = 55$; see below for details of endophyte assessment) and that of Repell II was 92% (Clay, 1987). The exact infection frequencies of the seeds of Palmer II and Prelude II at the start (in 1994) were not determined, but were likely to also be over 90% as both cultivars were selected from plants that were all infected by fungal endophytes (Hurley et al., 1994b, c).

All seeds were stored dry in rolled plastic bags beginning in 1994, separately by cultivar, and maintained in a refrigerator at 4°C. Note that before storage, the seeds were not dried purposefully using any specific technique, but were allowed to become air-dry at ambient laboratory humidity and temperature. Ordinarily, this procedure will maintain water content in the range of 4–16% of seed fresh mass (Priestley, 1986; McDonald et al., 1996). Periodically, seeds were removed for germination and endophyte assessment of the seedlings as described next.

Assessment of endophyte infection—Standard methods of staining leaf sheath tissues with aniline blue (Bacon and White, 1994), followed by examination at 400× with a light microscope (Cheplick and Faeth, 2009), were used to assess endophyte infection in seedlings that were 3–4 mo old. The medium used to grow seedlings was a mixture of 2 parts commercial topsoil to 1 part perlite. Because of extensive prior research using Yorktown III (Cheplick, 1997, 1998, 2004; Cheplick et al., 2000; Cheplick and Cho, 2003), the greatest effort for endophyte sampling was made with this cultivar (0, 3, 12, 18, and 22 yr storage). Cultivar Palmer II was sampled at 12, 18, and 22 yr; cultivars Prelude II and Repell II were sampled at 18 and 22 yr. The number of plants used to assess endophyte infection varied from 20 to 55 for a cultivar in a sample year (Table 1).

Several photomicrographs were taken of infected leaf sheaths of cultivars Palmer, Prelude, and Yorktown at 22 yr. An EVOS light microscope with an autoimaging system (Thermo Fisher Scientific, Waltham, Massachusetts, USA [formerly Life Technologies]) was used to document hyphal integrity of endophytic fungi that resumed growth within *L. perenne* plants after being dormant within seeds for 22 yr.

Assessment of germination—Because assessment of seed germinability per se was not a primary goal of the original study, data on the proportion of seeds germinating were not recorded in earlier samples taken for endophyte examination. However, data on germination were collected for the Yorktown cultivar at 12 yr and all four cultivars at 22 yr.

For Yorktown at 12 yr, 50 seeds were added to moistened fine vermiculite within shallow plastic pans (16 cm diameter) and covered with 1 cm of additional vermiculite. Three pans were maintained in a heated greenhouse (22–30°C), and seedling emergence was recorded after 2 wk.

Because ryegrass seeds were so old in the final endophyte survey at 22 yr, it was desired to quantify seed germinability more precisely in all cultivars using standardized conditions. In January 2016, 20 seeds of each cultivar were added to each of 10 petri dishes ($N = 200$ seeds per cultivar) containing a 9-cm-diameter disk of moistened filter paper. All dishes were wrapped in a single layer of Parafilm and placed into cold (4°C) storage for 6 wk stratification. Dishes were then moved to an incubator maintained on a daily cycle of 16-h light/30°C and 8-h dark/20°C, optimal conditions for germination of *L. perenne* seeds (Shen et al., 2008). The number of germinated seeds was recorded at 5, 8, 12, 19, 26, and 33 d, after which no further germination occurred. After the experiment, a sample of ungerminated seeds was tested for viability using a 0.5% w/v aqueous 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich, St. Louis, Missouri, USA) at room temperature. Seeds were sliced lengthwise and soaked for 2 h; viability was assessed by the presence of pink-stained embryos.

Statistical analyses—Endophyte infection frequency across sample years was analyzed separately by cultivar using a *G*-test of independence (Gotelli and Ellison, 2004). This statistic tests the null hypothesis that infection frequency does not depend on sample year. Infection intensity, assessed as the number of fungal hyphae

TABLE 1. Endophyte infection frequency (%) in four cultivars of *Lolium perenne* grown from seeds in cold (4°C) storage for varying numbers of years.

	Yorktown III		Palmer II		Prelude II		Repell II	
Year	n	%	n	%	n	%	n	%
0	55	94.5		—		—	25	92.0
3	20	95.0		—		—		—
12	30	80.0	30	86.7		—		—
18	35	77.1	38	73.7	30	73.3	30	90.0
22	26	65.4	22	72.7	24	58.3	30	66.7
G		15.0		2.2		1.3		5.0
Sign.		$P < 0.01$		NS		NS		$P < 0.05$

Notes: N = number of plants sampled for endophyte; Sign. = significance level; NS = not significant. A dash indicates no data for that year and cultivar. The *G* test of independence tests the hypothesis that infection frequency does not depend on sample year. Data for Repell in Year 0 are from Clay (1987).

per microscopic field of view at 400×, was analyzed by two-way ANOVA: fixed factors were cultivar, sample year, and their interaction. To accord with ANOVA assumptions, these count data were square-root transformed before analysis (Gotelli and Ellison, 2004). Final seed germination at 33 d for the 22-yr sample was analyzed with a one-way ANOVA with cultivar as the fixed factor. Least significant differences at $\alpha = 0.05$ were used to distinguish mean germination levels among cultivars. All analyses employed the Statistical Analysis System, version 9.2 (SAS Institute, Cary, North Carolina, USA).

RESULTS

Infection frequency—For the Yorktown cultivar, which was the most intensively sampled, the percentage of seedlings infected declined significantly from 94.5% at the start to 65.4% after 22 yr of storage (Table 1). The Repell cultivar also exhibited a significant decrease from 92.0 to 66.7% after 22 yr. The percentage of infected seedlings for Palmer and Prelude cultivars after 22 yr was 72.7% and 58.3%, respectively (Table 1).

Infection intensity—There was a highly significant effect of sampling year on the intensity of endophyte infection ($F_{4, 280} = 10.84$, $P < 0.0001$). Cultivar had a marginally significant effect on infection intensity ($F_{3, 280} = 2.40$, $P = 0.0682$), while there was no interaction of cultivar with sampling year ($F_{4, 280} = 1.06$, $P = 0.3764$). The Yorktown cultivar showed the highest infection intensity overall, but all cultivars showed a decrease in hyphal density between 18 and 22 yr of storage (Fig. 1).

Seed germination—Yorktown seeds at 12 yr germinated to 84.7 ± 2.4% (mean ± SE). After 22 yr, germination curves for seeds of the four cultivars revealed that Yorktown seeds had the greatest germination (78.0 ± 3.4%), while Prelude seeds had the lowest germination (52.5 ± 4.1%; Fig. 2). Cultivar had a significant effect on the final number of germinated seeds ($F_{3, 36} = 7.44$, $P = 0.0005$).

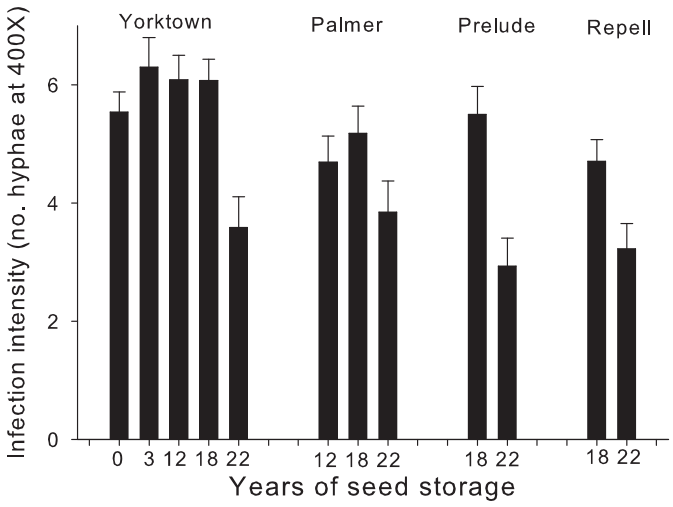

FIGURE 1 Infection intensity (number of hyphae traversing the field of view at 400×) in leaf samples of four cultivars of *Lolium perenne* grown from seeds stored for 0–22 yr. Bars show means ± SE. Sample sizes are in Table 1.

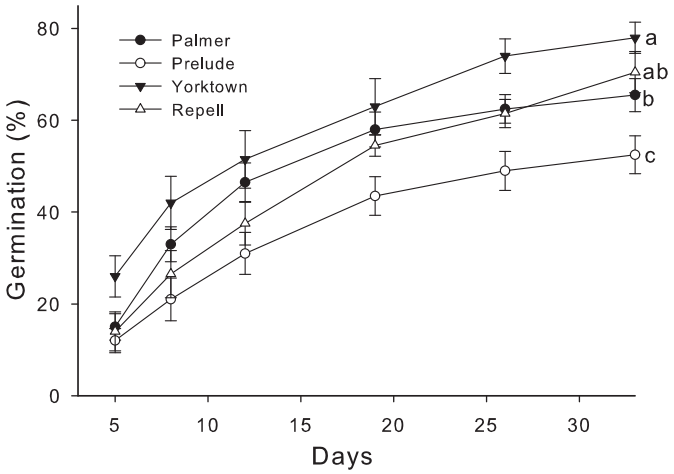

FIGURE 2 Mean (±SE) percentage germination of seeds of four cultivars of *Lolium perenne* after 22 yr of cold storage. Curves depict germination over 33 d. Means at 33 d followed by different letters are significantly different ($P < 0.05$).

Seed germination of Prelude was significantly lower than that of the other three cultivars, while final germination of Yorktown was significantly greater than that of Palmer (Fig. 2). Viability tests revealed that 15–23% of the ungerminated seeds remained alive at the end of the germination trial.

Microscopic assessment of infection—Photomicrographs were taken to document the morphology of endophytic hyphae in leaf samples of cultivar seedlings grown from seeds stored for 22 yr. The hyphae of *Epichloë festucae* var. *lolii* (formerly *Neotyphodium lolii*) were readily identified by their characteristic unbranched growth parallel with the long axis of leaf sheath epidermal cells and were directly comparable in appearance and dimensions with light photomicrographs of this species published by mycologists (Welty et al., 1986; Christensen et al., 2002; Christensen and Voisey, 2007). Intact hyphae stained with aniline blue generally appeared straight when growing within the intercellular spaces between adjacent plant cell walls (right arrow in Fig. 3) or were slightly convoluted when partially extruded from the intercellular space (left arrow in Fig. 3).

DISCUSSION

This research has shown that under cold (4°C), dry conditions, viable endophytes and germinable seeds of *Lolium perenne* cultivars can be maintained for at least 22 yr. Prior work has indicated that the loss of endophytic fungi from seeds stored for 1–10 yr for cool-season, perennial grasses is most rapid under warm, moist conditions (Latch and Christensen, 1982; Rolston et al., 1986; Welty et al., 1987; Clement et al., 2008). In perennial ryegrass, viable endophytes were found in 40% of seedlings from seeds of cultivar Nui stored at 0–5°C for 7 yr and 80% of seedlings of cultivar Ellett seeds stored for 6 yr (Latch and Christensen, 1982). In tall fescue, viable endophytes were found in 50–96% of seedlings from several accessions of seeds stored at 4°C for 8–10 yr (Clement et al., 2008). The current study indicates that germplasm of perennial ryegrass may contain viable endophytes for a much longer time when stored dry at 4°C.

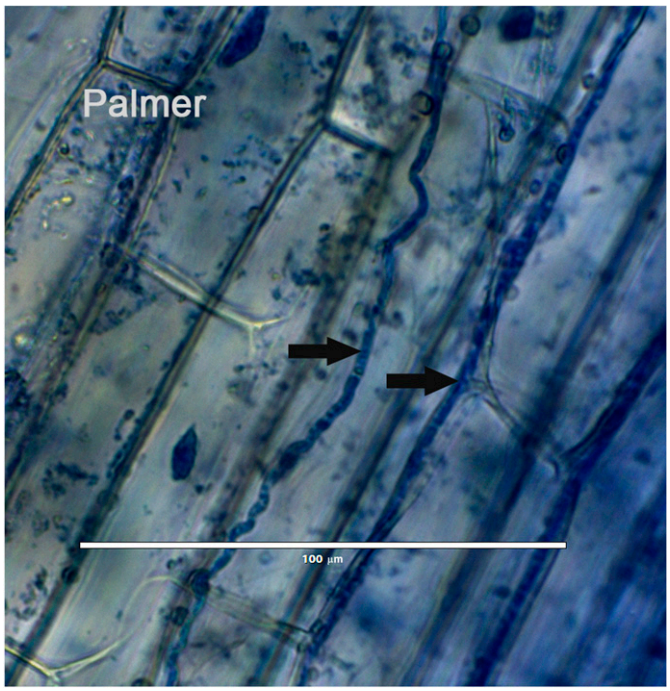

FIGURE 3 Endophytic fungal hyphae (arrows) in a leaf sheath sample from cultivar Palmer II of *Lolium perenne* grown from a 22-yr-old seed.

Endophyte infection is widespread and common in many temperate grass species (Rudgers et al., 2009; Semmartin et al., 2015), and endophytes influence many aspects of their ecology and evolution (reviewed by Cheplick and Faeth, 2009). By establishing seed banks for endophyte-infected grass species, evolutionary potential might be stored for decades, thereby providing a "repository of potentially valuable traits and genotypes available as resources for future users" (Peres, 2016, p. 102). In addition, stored seeds may provide unique opportunities for resurrection studies that aim to better evaluate contemporary evolution of plant populations (Etterson et al., 2016).

Given the potential for endophyte-infected germplasm to be used to improve yield, forage quality, and pest resistance (Gundel et al., 2013) or for more basic studies of contemporary evolution, it is important to recognize the optimal conditions needed to sustain both seed and endophyte viability in germplasm collections (Clement, 2001). Of course, some loss of seed germinability and endophyte infection is to be expected in germplasm collections, even under optimal storage conditions (Latch and Christensen, 1982; Priestley, 1986; Clement et al., 2008). In the current study, seed germination was reduced after 22 yr of cold storage, but still exceeded 50% in all cultivars tested. The proportion of infected seeds generally exceeds 90% in newly developed *L. perenne* cultivars, but after 22 yr, the four cultivars examined here still retained infection frequencies from 58–73%. These results provide an optimistic scenario for the retention of endophyte-infected seeds in germplasm collections of important forage species such as *L. perenne* and perhaps other cool-season grass species as well.

For future improvement of forages and other crops, the "efficient interaction with beneficial microorganisms will be an additional breeding target" (Sessitsch and Mitter, 2015, p. 33). It will be of interest to determine whether aged endophytic fungi retain their ability to act as mutualistic symbionts, improving plant growth and yield under stressful environmental conditions.

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