



Horizontal transmission and expression of *Epichloë typhina* in orchardgrass (*Dactylis glomerata*)

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Accepted: 18 February 2022 / Published online: 5 May 2022

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Abstract Choke disease, which is caused by *Epichloë typhina*, was first reported in orchardgrass seed production fields in the Willamette Valley, Oregon in 1997 and has continued to persist in the system. The disease can cause up to 30% losses in seed yield. Unlike many *Epichloë* species that are seed transmitted, it is unclear how infection of orchardgrass by *E. typhina* occurs in the field, or how the disease spreads within plants. In this study, the overall incidence of choke on trap plants placed in a choke-infested field was 41% in 2018 and 9% in 2019. Similar incidence of infection occurred when exposed before (39%) or after (43%) swathing in 2018. Contrary to our hypothesis, plants sprayed with an artificial honeydew had a significantly lower incidence of infection (32%) when compared to the controls

(48%). An examination of the spread of *E. typhina* within tillering plants revealed that development of infected tillers was greater than development of uninfected tillers, which could contribute to the gradual increase in choke in fields over time. These studies contribute to a better understanding of how choke is spread within plants and in orchardgrass seed production fields, which is an important step towards developing control methods to reduce choke in orchardgrass seed production fields.

Keywords Choke · *Epichloë typhina* · Orchardgrass · Transmission

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Introduction

Choke disease caused by *Epichloë typhina* (Pers.) Tul. & C. Tul. (Clavicipitaceae, Ascomycota) was first reported in Oregon in 1997 in an orchardgrass (*Dactylis glomerata* L.) stand grown for seed production (Alderman et al., 1997). By 2003 it had spread to 90% of the orchardgrass stands surveyed in Oregon. Yield loss in orchardgrass seed is proportional to choke disease incidence, and in 2004 the estimated regional loss was US \$0.8 million (Pfender & Alderman, 2006). The economic losses can result in fields being removed after only 3–4 years instead of after decades. Despite efforts to find control methods for choke disease (Pfender & Alderman, 2003; Alderman & Rao, 2008a, 2008b; Alderman et al., 2010), there is currently no effective management tool available for orchardgrass seed producers.

Fungi of the genus *Epichloë*, formerly known as *Neotyphodium*, form lifelong and mostly mutualistic associations with cool-season grasses (*Poaceae*). Asexual members of *Epichloë*, generally spread through vertical transmission in the seeds of their host, but some *Epichloë* species can also spread horizontally during their sexual cycle (White, 1988). In species with a sexual cycle, the fungal growth switches from sparse and very regulated to prolific, unregulated growth during flowering of the host plant. As reproductive tillers elongate, and just prior to flower head emergence, a rapid and dense growth of the fungus within and among leaf sheaths forms a sexual reproductive structure (stroma) that destroys, or “chokes”, the host flower (Western & Cavett, 1959; Tadych et al., 2014). Stromata produce conidia that are transported to other stromata by insects, primarily of the genus *Botaniphila* (reviewed in Bultman & Leuchtmann, 2008), or by abiotic means including water or wind, especially in dense plant populations (Rao et al., 2012). The stroma is fertilized when conidia from the opposite mating type land on it, and it subsequently matures to produce sexual ascospores. The ascospores are released by the millions, at night, and under cool, moist conditions, and are produced abundantly from mid-May to early July in the Willamette Valley in Oregon, where much of the commercial orchardgrass seed is produced (Kaser et al., 2009). The ascospores are thought to be the primary source of horizontal transmission in orchardgrass.

While the asexual cycle of the fungus does not visibly impact host fertility, choking of flowers by the

sexual cycle prevents production of seed. A classification of *Epichloë* species and closely related fungi based on the characteristics of their fungus-host relationship has been proposed (White, 1988). *Epichloë typhina* in orchardgrass is the representative for Type I strains (White, 1988). It is considered aggressive and sterilizes most, if not all host flowers. Pith scrapings from the few remaining flowering stems are void of fungal hyphae, and thus the fungus cannot be vertically transmitted (Western & Cavett, 1959; Chung & Schardl, 1997; Leyronas & Raynal, 2008). Thus, *E. typhina* in orchardgrass is spread only through horizontal transmission. Although little research has been done on horizontal transmission of *Epichloë* endophytes, it could occur through three processes: (1) via ascospore infection of ovaries of healthy flowers, (2) through infection of young plants by ascospores present on the seeds or on the seedling, or (3) by infection of mature plants by spores through diverse routes.

In perennial ryegrass, while vertical transmission was not observed, ascospores landing on emerging inflorescences infected developing seeds at a very low rate (1.3%) (Chung & Schardl, 1997). Leyronas (2005) performed a similar experiment on orchardgrass, and observed no transmission, but found *Epichloë* in 42–78% of the seeds of half-choked panicles, although none of the emerging seedlings were infected by *Epichloë*. Because the germination rate was equivalent to seeds from normal panicles, and no mycelia of *Epichloë* grew from seeds placed on potato dextrose agar (PDA), Leyronas (2005) concluded that the viability of hyphae in orchardgrass seeds was low to null.

Leyronas (2005) also examined the possibility of germinating seedlings becoming infected by exposing the seeds to ascospores of *Epichloë*. No infection was observed when seed were dipped in an ascospore solution, but some seedlings were infected when glumes were removed and the seeds were placed in direct contact with ascospores on a glass plate, or when seeds with glumes were exposed to ascospores on water agar (Leyronas, 2005; Leyronas & Raynal, 2008). Similarly, Western and Cavett (1959) explored the possibility of transmitting *Epichloë* by exposing florets, seeds, and/or germinating seedlings to ascospores, but did not observe any infection. Other reports have described successful infection of germinating seeds of *Poa ampla* and *P. secunda* subsp. *juncifolia* using spores of *E. poa* (Tadych et al., 2007; Tadych et al., 2012). Considering the very mixed results of experiments under controlled

environments, these mechanisms are unlikely to contribute to spread of choke in production fields. Therefore, the infection of mature plants needs to be investigated.

Following the early, foundational studies on *Epichloë* species (Sampson, 1933; Ingold, 1948; Large, 1954), Western and Cavett (1959) determined how the disease spreads horizontally. They reported that plants could become infected through the cut end of a flowering stem after swathing, however the results were not reproducible (Leyronas & Raynal, 2008; Alderman, 2013). Furthermore, the weather conditions during harvest in Oregon are generally warm and dry, rendering it unlikely that the fungus would have sufficient time to grow from the cut end of the stalk to the primordia before the cut stem dries out. Alderman (2013) observed some hyphal tips penetrating the epidermis in plants exposed to ascospore release, but the extent of growth in the leaves and the infection status of the plant were not determined. The ascospores germinated better and produced numerous conidia around aphid honeydew, cut wounds and eriophyid mite feeding sites. Leyronas (2005) explored infection through cut stems, cut leaves, and cut young tillers of mature plants, exposed meristems, and by applying spores to the surfaces between the sheath and stem. Ascospores, conidia from white (unfertilized) stromata, and conidia from ascospores were used as inoculum sources. Only 2.1% of inoculated cut leaves and 3.3% of exposed meristems became infected, but the infections could not be sustained more than 10 weeks in the greenhouse. Furthermore, plants were not vernalized to check for stromata formation. Similarly, in a prior study mature plants of orchardgrass could not be infected by brushing ascospores of *E. typhina* on cut stems or leaves (Alderman, unpublished data).

Attempts to understand horizontal spread of choke have not produced results that explain the incidence of disease observed in the field, where a stand of orchardgrass can become less profitable for seed production within 3–4 years, and the disease spreads quadratically within fields (Pfender & Alderman, 2006). Efforts to control choke in production fields by swathing, burning stubble, fungicide applications, or reducing fertilization of the stromata to limit ascospore inoculum using biocontrol or insecticides against *Botaniphila* have not been successful (Pfender & Alderman, 2003; Alderman et al., 2008; Alderman & Rao, 2008a, 2008b). However, burning stubble did reduce the polyetic spread within the field

from ~9.2% in the control and fungicide treated plots to ~2.7% in plots exposed to propane-assisted burning of postharvest stubble (Pfender & Alderman, 2003). Currently, the only management practices to reduce choke is to have a very short rotation (about 3 years), and swathing older fields first during sunny, anticyclonic weather to reduce the inoculum by drying out the stromata and ascospores (Pfender & Alderman, 2003; Alderman & Rao, 2008b).

Since controlling infection is difficult, especially with the limited knowledge of how the disease spreads within the field, Bushman et al. (2016, 2019) screened orchardgrass germplasm for genetic resistance or tolerance to choke. They identified variation in the prevalence of choke within the germplasm, indicating the potential to breed for choke resistant or tolerant orchardgrass. Another way to reduce the impact of choke is to reduce the expression of the disease in infected plants. The effect of fertilization rates and plant growth regulators was explored to determine if they modulated the incidence of choke (Hoffman & Rao, 2012). A downward trend in the proportion of choked tillers was observed in plots that received higher than normal nitrogen fertilization, but the high variance in the data limited the conclusions from the results. Thus, there is limited knowledge available on *E. typhina* infecting orchardgrass, and some of the available knowledge is contradictory. Increasing our understanding of the disease and resolving past inconsistencies will ultimately form a basis for improved management of the disease.

In this study, our objectives were to (1) obtain and observe natural infection of orchardgrass by *E. typhina*, (2) identify parameters that influence transmission of *E. typhina* in a field setting, and (3) examine within-plant spread of *E. typhina*.

Material and methods

Observations of natural orchardgrass field infections by *E. typhina*

In April 2016, the orchardgrass cultivar ‘Profile’ was seeded in a 7.5 m × 38 m field plot at the Oregon State University Hyslop Research Farm (Corvallis, OR). *Epichloë typhina*-infected potted plants (plants exhibiting choke that were removed from orchardgrass seed production fields near Corvallis, OR, and potted in field soil) were placed at regular intervals within the plot

(approximately three infected plants every 2.5 m in every third row) to provide inoculum. In May–June 2018, choke was evaluated by counting healthy and choked heads on 92 orchardgrass plants randomly sampled from the plot. In June of 2018 and 2019, five plants displaying choked heads were removed from the plot and transported to the lab. Each plant was dissected, the flowered and choked heads were counted, and each vegetative tiller was microscopically examined for the presence of *E. typhina* hyphae. Incidence of infection was calculated as the number of choked heads and microscopically positive tillers out of the total number of heads and tillers examined per plant. To determine if analyses could be conducted using data pooled from both years, an analysis of covariance was conducted in R using the ‘stats’ package (R Core Team 2021) comparing the incidence of choked heads versus the incidence of vegetative tillers testing positive. The covariant was year. The analysis of covariance indicated that the results did not differ significantly ($P < 0.05$) between the two years, therefore a global linear regression was performed using the R ‘stats’ package (R Core Team 2021) to compare the incidence of choked heads to the incidence of vegetative tillers testing positive. Results from the model were interpreted using the regression coefficient and associated p value to identify the strength of the correlation between the incidence of choked heads and the incidence of vegetative tillers testing positive.

Staining and examination of tillers for the presence of *E. typhina* hyphae

A small pair of forceps and a razor blade were used to peel the inner epidermis from the leaf sheath, which was placed on a microscope slide in a drop of acidified aniline blue. Excess dye was removed after one minute and the tissue was mounted in a drop of water. Samples were immediately observed using an optical microscope at 200 $\times$ for presence of hyphae characteristic *E. typhina*, which grow intercellularly, exhibit little or no branching and run parallel to the long axis of the leaf (Christensen et al., 2008).

Field trials to investigate natural infection of potted plants (2018)

In 2018, four-month-old potted orchardgrass plants (cultivar ‘Baridana’) grown in Sun Gro Professional Potting

Mix (Sun Gro Horticulture, Agawam, MA) were exposed to naturally released ascospores in a heavily choked orchardgrass field plot at the Oregon State University Hyslop Research Farm (Corvallis, OR). Three different treatments were applied to a total of 220 plants: exposure timing (exposure to heavily choked plants before or after harvest of the orchardgrass field), simulated swathing (plants clipped using clean manual clippers to a height of 15 cm, or not clipped), and presence of artificial honeydew (AH; sprayed or not). Plants were placed in the heavily choked field for two weeks, either between rows before swathing or covered with the fresh cut grass after swathing. Swathing treatment plants were clipped to 15 cm in the evening, just prior to ascospore ejection, and/or sprayed with AH twice during field exposure. The AH, based on the composition of several different aphid honeydews (Lamb, 1959; Sasaki et al., 1990; Fischer et al., 2002), was composed of 83% sugars (glucose 30% [RPI, Prospect, IL], fructose 25% [Now Foods, Bloomington, IL], trehalose 15% [Bulk Supplements, Henderson, NV], melezitose 15% [Sigma, St. Louis, MO], sucrose 10% [Fisher, Fairlawn, NJ], maltose 5% [Fisher, Fairlawn, NJ]), 12% amino acids (35% asparagine [HiMedia Laboratories, Mumbai, India], 30% glutamine [Bulk Supplements, Henderson, NV], 10% glutamic acid [Bulk Supplements, Henderson, NV], 10% aspartic acid [Bulk Supplements, Henderson, NV], 10% cysteine [Sigma, St. Louis, MO]), 5% potassium nitrate (Sigma, St. Louis, MO), 2% citric acid (Sigma, St. Louis, MO) and 3% phosphoric acid (Baker Analyzed, Phillipsburg, NJ); pH 6.5. A confounding factor during the experiment was vole damage to plants, which was scored using an ordinal scale as follows:

- 0 “no sign of feeding”.
- 1 “0 - 33% of the plant damaged, with regrowth”.
- 2 “more than 33% of the plant damaged, some without regrowth”.
- 3 “Completely eaten: dead”.

Following exposure to ascospore release and treatments, plants were removed from the field and placed in a courtyard surrounded by buildings with water applied from above. The plants were exposed to natural weather conditions for the remainder of the summer (June 1–September 15: average high temperatures 27.96 °C and average low temperatures 9.88 °C). At the end of the summer, plants were vernalized in a growth chamber at 8 °C with an 8 h photoperiod for ten weeks, after which the

temperature and day length were increased gradually by 2 °C and 2 h each week to 16 °C and a 16 h photoperiod. The plants were transferred to a greenhouse set at 18 to 20 °C and a 16 h photoperiod, where they started to flower approximately two weeks later. Healthy flowers and choked heads were counted on each plant, and vegetative tillers that were large enough were microscopically examined for the presence of fungal hyphae. All tillers that were mature enough to obtain an epidermal peel from the leaf sheath for microscopic analysis were tested. Among the plants, some had only mature tillers (usually plants with few tillers) and some plants had many immature tillers that were too small for testing (often plants with many tillers). Tillers tested for each plant were representative of all mature tillers across the whole plant. A tiller was rated as positive for choke if microscopic evidence of the pathogen was detected (described above). A head was rated as choked if a stroma was present.

Multiple binomial generalized linear models were conducted in R using the ‘stats’ package to determine the effect of treatments on flowering, positive tiller infection, or stroma development. In all models, the following variables were evaluated using their regression coefficient and associated p-values: timing of exposure (before or after swathing), aphid broth added or not, plants clipped or not clipped, and vole pressure of 0, 1 or 2. Subsequent to model evaluation, individual treatment comparisons for flowering, positive tiller infection, or stroma development were made with chi-square tests. To explore relationships among only the infected plants and treatments, Fisher’s exact tests were used. Analyses were conducted in R.

Field trials to investigate natural infection of potted plants (2019)

In 2019, 219 four-month-old potted orchardgrass plants (cultivar ‘Baridana’) grown in Sun Gro Professional Potting Mix were clipped (as described for clipped plants in 2018) before being exposed in a choked orchardgrass field plot at the Oregon State University Hyslop Research Farm (Corvallis, OR) prior to swathing. Forty plants were clipped, but kept isolated from inoculum in a courtyard surrounded by buildings in Corvallis OR. The following treatments were applied to groups of clipped plants individually as follows:

“Control” n = 40: exposed with no additional treatment applied;

“Comparable to the 2018 treatment” n = 40: Exposed before swathing, clipped, watered from above to allow comparison with plants treated similarly in the 2018 study;

“Clipped 2 weeks” n = 40: clipped to 2–3 cm 2 weeks after exposure in the field;

“Clipped 6 weeks” n = 39: clipped to 2–3 cm 6 weeks after exposure in the field;

“Isolated from inoculum” n = 40: not exposed in the field and kept distant from inoculum sources by placing in a courtyard surrounded by buildings;

“Not vernalized” n = 60: not induced to flower, with plants maintained in a greenhouse set at 18 to 20 °C and a 16 h photoperiod while other plants were vernalized;

After exposure to ascospores and various treatments, plants were removed from the field. One set of 40 plants was watered from above to provide a comparison to the 2018 study, and the remaining plants were watered and fertilized from below all summer in natural weather conditions (June 1 – September 15: average daily high temperatures 27.06 °C and average daily low temperatures 10.76 °C). Sixty plants were not vernalized. The remaining plants were vernalized as described above to induce flowering. The plants were transferred to a greenhouse set at 18 to 20 °C and a 16 h photoperiod, where they started to flower approximately two weeks later. Healthy flowers and choked heads were counted on each plant, and vegetative tillers that were large enough were microscopically examined for the presence of fungal hyphae. Among the plants, some had only mature tillers (usually plants with few tillers) and some plants had many immature tillers that were too small for testing (often plants with many tillers). However, tillers tested for each plant were representative of all mature tillers across the whole plant. A tiller was rated as positive for choke if microscopic evidence of the pathogen was detected. A head was rated positive for choke if a stroma was present.

In 2019, low infection rates made statistical comparisons challenging. The data was non-normal and did not fit the assumptions of a generalized linear model. Between group comparisons were primarily conducted with chi-square tests. Because of low disease incidence in 2019, a Fisher’s exact test to compare each treatment group to the control was conducted but no significant differences were detected. All analyses were conducted in R using the ‘stats’ package.

Spread of *E. typhina* within naturally infected potted plants

To follow the progression of infection within a plant, six partially infected (having both flowers and choked heads) orchardgrass cultivar ‘Baridana’ plants from the field exposure studies in 2018 and 2019 were repotted into larger pots to stimulate tillering. Each tiller was microscopically examined for the presence of *E. typhina* hyphae and tagged accordingly using a rubber band. The rubber band ensured that every new tiller growing out of a “parent” tiller would stay within that rubber band. All new tillers in these plants were examined microscopically for the presence of *E. typhina* hyphae after one and two months of vegetative growth. The plants were vernalized as described above, and transferred to a greenhouse set at 18 to 20 °C and a 16 h photoperiod, where they started to flower approximately two weeks later. New tillers were microscopically examined for the presence of *E. typhina* hyphae after flowering, seven months after being tagged. To compare infection status before and after vernalization, a paired t-test was conducted using the ‘PairedData’ package (Champely & Champely, 2018) in R.

Results

Observations of natural orchardgrass field infections by *E. typhina*

No choke was observed in the spring of 2017 in the field where the choke-infected, mature plants were placed in

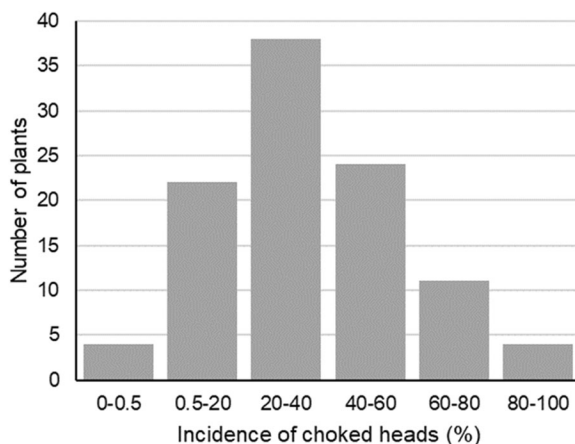


Fig. 1 The frequency of plants ($n = 92$) with different incidence of choked heads after exposure in a field of orchardgrass with natural inoculum of *Epichloë typhina*

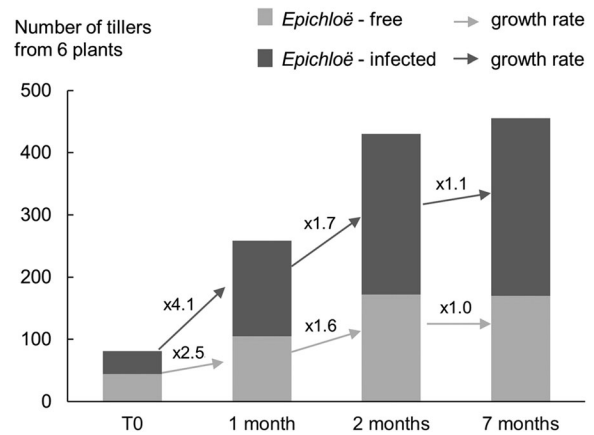


Fig. 2 The number of tillers produced from *Epichloë*-infected and *Epichloë*-free tillers showing the spread of *E. typhina* within the plant. The numbers above the arrows indicate the multiplication rate for infected (above top set of arrows) and non-infected tillers (above bottom set of arrows), respectively

2016, but choke was present in the spring of 2018. Between four and 130 heads (average of 45) on 92 plants were scored as healthy or choked. The mean choke incidence for a plant was 38% (SE = 2.7). Only four plants did not exhibit any choke symptoms, resulting in a 96% incidence of choke in the field. Fifty-five plants (60%) had less than 40% of tillers showing choke, and only four plants had over 80% of their heads choked (Fig. 1).

On ten plants displaying choke symptoms (5 removed in 2018, 5 removed in 2019), a total of 579 heads were scored for the presence of stroma, and 523 vegetative tillers were examined microscopically for the presence of hyphae characteristic of *E. typhina*. The incidence of infected vegetative tillers per plant was 17 to 82%, with a mean of 49% (SE = 6.5) over the two years. The incidence of choked heads ranged from 22 to 79%, with a mean of 49% (SE = 6.2) over the two years. Regression analysis showed that the incidence of choked heads was proportional ($r^2 = 0.5$, $p = 0.03$) to the incidence of vegetative tillers testing positive.

In 2018, stems of healthy-looking flowers were examined for the presence of *E. typhina* hyphae, which were found at least once in the five plants tested. Interestingly, the chance of finding fungal hyphae in apparently healthy flower stems did not correspond with either the incidence of choke or the incidence of infected tillers, as a moderate incidence (33% of the vegetative tillers positive, 22% of heads choked) of infected plants had *E. typhina* hyphae in 38% of their healthy flowering stems, while a plant with twice that incidence of

infection (64% of vegetative tillers positive, 56% of heads choked) had *E. typhina* hyphae in only 7% of the healthy, flowering stems.

Field trials to investigate natural infection of potted plants (2018)

In 2018, the four-month-old orchardgrass plants exposed to natural release of ascospores in a heavily choked orchardgrass field were impacted by vole damage - 24% (68 plants) were lost due to vole damage, most of them after swathing, probably because they were the only green plant material available. Of the surviving plants, 62% flowered after vernalization. Based on the GLM analysis there was a significant effect of vole damage ($p = 0.01$) on incidence of flowering (Table 1). Although numerically more plants flowered in the group that was exposed before harvest compared to those exposed after harvest (67% vs 54%, respectively), the effect was not significant ($p > 0.1$).

Six months after being exposed to ascospores, 2 to 22 tillers from each plant (mean of 9.5, total of 2085 tillers) were examined by microscopy for the presence of *E. typhina* hyphae. A total of 41% of the plants were found to be infected with *E. typhina*. Interestingly, neither

the time of exposure nor clipping had an effect on incidence of infection. The GLM analysis showed that plants treated with AH had a lower incidence of infection (32%) compared to the untreated plants (48%; $p = 0.02$) (Table 1). The chi square analysis further showed that the incidence of infection was dependent on the vole damage score ($p = 0.01$), with the incidence of choked plants being similar for light and medium vole damage (48%, 44%) but much less (21%) when plants suffered severe vole damage (Table 1). The incidence of infected tillers was calculated for each infected plant. Fungal mycelia were detected in 40% of vegetative tillers per infected plant, with incidence ranging from 5 to 100%. It should be noted that not every tiller was microscopically examined, but these values are considered representative. There was a slight numeric increase in spread within the clipped plants (45%) compared to unclipped plants (36%) and within vole damage category (34%, 46% and 42% spread for none/light, moderate and severe damage, respectively), though neither of these were statistically significant based on a Fisher's exact test. Overall, 19 of the 220 plants had choked heads upon flowering in 2018, while 90 of the 220 plants were observed to be infected based on microscopy. Therefore, 78% (71 of 90) of the infected plants would not have been detected based on visual choke symptoms alone.

Table 1 Chi-square analysis to evaluate the effects of different treatments (time of exposure, aphid broth spray, clipping, vole damage) on flowering, infection with *Epichloë typhina*, and choke expression rate in orchardgrass in the 2018 field trial

Variable	Treatment	Number of plants	Number of plants flowering (%)	Number of plants infected (%) ^a	Choke Expressed		Expression rate (%)	
					No ^b	Yes	Global ^c	Infected ^d
Swathing	Before	136	91 (67)	58 (43)	46	12	9	21
	After	84	45 (54)	32 (38)	25	7	8	22
Honeydew	No spray	123	71 (58)	59 (48)	48	11	9	19
	Sprayed	97	65 (67)	31 (32)	23	8	8	26
Clipping	No	113	74 (65)	46 (41)	40	6	5	13
	Yes	107	62 (58)	44 (41)	31	13	12	30
Vole damage	None/light	77	50 (65)	37 (48)	32	5	6	14
	Moderate	100	54 (54)	44 (44)	33	11	11	25
	Severe	43	32 (74)	9 (21)	6	3	7	33

^a Plants infected as determined by microscopy

^b Plants infected as determined by microscopy, but lacking stromata

^c Percent of plants out of total plants in treatment category expressing choke

^d Percent of plants out of infected plants in treatment category expressing choke

* Indicates a p value less than 0.05 and significant treatment effect

Field trials to investigate natural infection of potted plants (2019)

In 2019, the various treatments applied to the 159 plants exposed to ascospores of *E. typhina* in a choke infested field and subsequently vernalized showed no effects on flowering (Table 2). There was a numeric decrease in incidence of flowering for the group clipped 2 weeks after field exposure, with 30% of plants flowering compared to 50% for the control plants, but this was not significant. As expected, the 60 non-vernalized plants did not produce any flowers. Due to the overall low infection rate in 2019, statistical comparisons between the groups were difficult, and no significant differences between the control group or any of the treatment groups were detected using a Fisher's exact test ($p > 0.05$ for all variables analyzed).

In 2019, only 4 of the 22 infected plants that were vernalized and examined microscopically exhibited stomata; in 2018 only 19 of the 90 infected plants had stomata. In both years there was an approximately five times greater incidence of infection based on microscopic examination compared to that observed through expression of stomata.

In 2019, contrary to the 2018 experiment, plants were watered from below until they were transferred to the cold room for vernalization, about 8 weeks after being brought back from the field, in order to more accurately simulate what occurs under field conditions. However, one group of plants (Table 2) was watered from above and thus treated the same way as some of the plants from 2018: clipped, no AH, and exposed to inoculum of *E. typhina* before swathing (2018 $n = 32$; 2019 $n = 40$) which allowed comparison between years. Under the similar experimental conditions, flowering rates were comparable in 2018 (66%) and 2019 (63%); however, there was a much lower incidence of infected plants in 2019 (15.5%) compared to that observed in 2018 (50%).

Spread of *E. typhina* in naturally infected potted plants

From June 2018 to January 2019, the paired t-test showed a significant ($p = 0.03$) increase in the mean percentage of infected tillers of the six tagged plants from 47% (± 7.1) to 69% (± 7.8) (Fig. 2). At each time point, the number of new tillers that lost infection (negative tillers derived from a positive parent tiller) or gained infection (positive tillers

Table 2 Chi-square analysis to evaluate the effects of different treatments (clipping time, exposure, vernalization) on flowering, infection with *Epichloë typhina*, and choke expression rate in orchardgrass in the 2019 field trial

Variable	Number of plants	Number of plants flowering (%)	Number of plants infected (%) ^c	Choke expression		Expression rate (%)	
				No ^d	Yes	Global ^e	Infected ^f
Comparable 2018 results	32	21 (66)	16 (50)	11	5	15.6	31
Control +2018 watering ^a	40	25 (63)	6 (15)	6	0	0	0
Control 2019	40	20 (50)	5 (12.5)	3	2	5	40
Clipped – two weeks ^b	40	12 (30)	6 (15)	6	0	0	0
Clipped – six weeks ^b	39	18 (46)	3 (7.7)	2	1	2.6	33
Exposed in courtyard	40	21 (53)	2 (5)	1	1	2.5	50
Not vernalized	60	0 (0)	2 (3.3)	2	0	0	0

^a Plants were treated under conditions similar to the 32 plants from 2018: clipped, no spray, exposed in the field before harvest and watered overhead

^b Elapsed time after exposure in the field before clipping

^c Plants infected as determined by microscopy

^d Plants infected as determined by microscopy, but lacking stomata

^e Percent of plants out of total plants in treatment category expressing choke

^f Percent of plants out of infected plants in treatment category expressing choke

* Indicates a p value less than 0.05 and significant treatment dependence

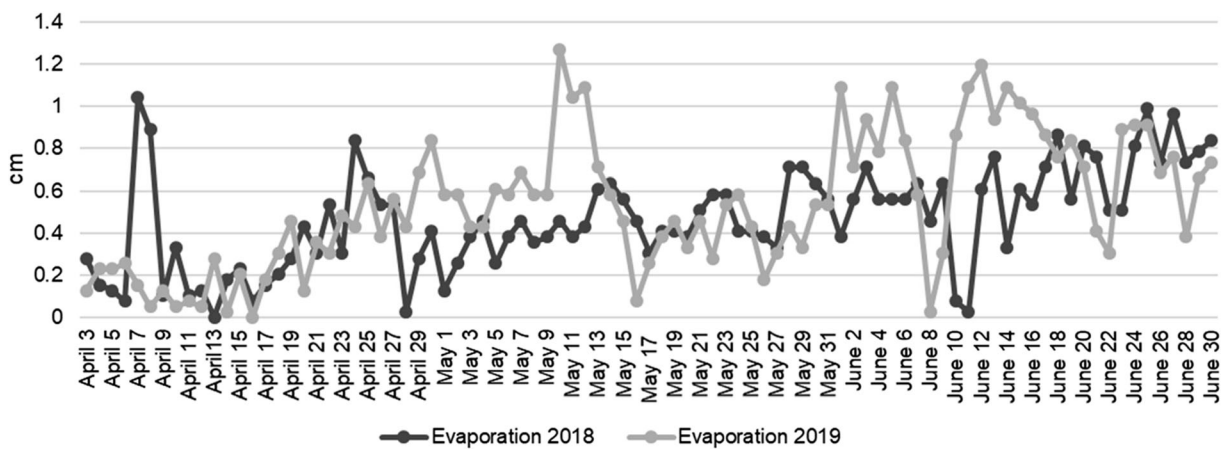


Fig. 3 Graph comparing daily evaporation rates obtained from the nearest weather station (USC00351862, Oregon State University) to the field plot used for experiments in 2018 and 2019. Data for

the period April – June 2017 and 2018 is presented (raw data included in Supplementary Table S1)

derived from a negative parent tiller), were counted, and tagged accordingly. During the experiment, 11 tillers were found to have lost infection, which represented 4.4% of the total new positive tillers, and four tillers derived from negative tillers were found to be infected, representing 3.2% among the total new negative tillers. Considering that a tiller losing or gaining infection could produce several “daughter” tillers before being tested, the rates might be slightly overestimated. Also, all except one of the tillers gaining or losing infection were found after the first month. The multiplication rate of tillers was $3.1 (\pm 0.5)$ over the first month, but only $1.7 (\pm 0.2)$ over the second month, and $1.2 (\pm 0.2)$ during vernalization (Fig. 2). Within 7 months, the number of tillers examined for hyphae of *E. typhina* on the six plants increased from 77 to 455. However, the paired t-test indicated that the multiplication rate was significantly ($p = 0.03$) higher for the positive tillers (9.0 ± 1.7) than for the negative tillers (3.4 ± 0.9). The difference could be due to the higher multiplication of the infected tillers during the first month (4.1 for positive tillers and 2.5 for negative tillers), although after the first month, both positive and negative tillers multiplied at similar rates (Fig. 2).

Discussion

Non-systemic infection and flowers escaping choke result in incomplete floral sterilization

We previously reported that hand-inoculation of orchardgrass seedlings with *E. typhina* causes typical

systemic infection (Merlet et al., 2021) as occurs with plants infected through vertical transmission (Bacon et al., 1977; Hinton & Bacon, 1985). We also showed that natural infection of orchardgrass does not lead to homogeneous infection of all above ground parts of a plant. In our study, even one or two years after infection occurred, plants from a heavily choked field displayed hyphae in only 49% of their vegetative tillers.

Another interesting observation was that even in a field with a choke incidence of 96% based on microscopic examination, choke did not sterilize the orchardgrass to the degree often described in the literature (White, 1988). The lower-than-expected choke incidence can be explained in part by the heterogeneous infection of orchardgrass, as floral meristems that are not infected will not give rise to choked heads. Incomplete sterilization can also be explained by the existence of infected floral stems that give rise to healthy looking flowers, as demonstrated by the occurrence of hyphae in flowered stems, contrary to what is expected from type I strains (White, 1988).

Epichloë infection increases tillering rate

Spread of the fungus within plants was observed for 7 months and through flower induction on six naturally infected plants. As expected, transmission of the infection status from one parent tiller to their daughter tillers is true in most cases, but not always (4.4% of tillers derived from a positive tiller were not infected). The fact that very few tillers emerging from non-infected tillers gained infection suggests that *E. typhina* rarely spreads

into tillers formed prior to infection, which could explain in part the slow spread of infection within a plant. But infection did spread within the plants, with incidence of infection of tillers per plant increasing from 47 to 69%. However, our observations suggests that the increase is mostly driven by an enhanced multiplication of infected tillers. The increased ability of an infected plant to tiller has previously been observed in grass-*Epichloë* interactions, where the endophyte reduces sexual reproduction of its host, but increases its clonal growth (Groppe et al., 1999). Also, *E. typhina* in orchardgrass has been shown to increase photosynthetic ability (Rozpadek et al., 2015). However, it is interesting to speculate that manipulation of host physiology by the fungus could be restricted to the infected tillers rather than being systemic, thus leading to increased growth of only the infected tillers.

Exposure to ascospores in a choked field is sufficient for disease spread

Simply exposing vegetative plants to ascospores in a choked field was sufficient for infection. In June 2018, 41% of the 220 young vegetative plants exposed to natural inoculum were found to be infected six months later. The prevalence of horizontal transmission was similarly observed with sexual strains of *E. sylvatica* in *Brachypodium*, with at least 34% and 17% of plants infected in two natural populations after 2 years of exposure (Brem & Leuchtman, 1999). In that study, infection was confirmed by examining only two tillers per plant, so the incidence of infection may be higher than estimated. In a different study on horizontal transmission using the same pathosystem, almost 500 stromata of *E. sylvatica* were observed on 54 plants that had been exposed for 2 years to symptomatic choked plants, but at least 16% of the vegetative tillers in the plants were not infected (Brem, 2001). Also of interest, was the observation that 62 different strains of *Epichloë* were identified based on AFLP-patterns in the 91 isolates from the newly infected plants, and most plants examined were host to 2 to 12 different strains. Multiple *Epichloë* mating types were also detected in different tillers of infected orchardgrass (Bushman et al., 2019). The results suggest horizontal transmission is very efficient and frequent, as most plants harbor several strains.

While we observed infection to be efficient in 2018, only 15% of the plants were infected during 2019, and no significant differences in incidence of infection were

observed in 2019 between the plants exposed to the different treatments. In June 2019, less inoculum was visible, with fewer stromata observed in the same area of the field, and with many stromata that were not fully fertilized and/or were visibly dry. Weather data from weather station USC00351862 at Oregon State University Hyslop farms for August 2017 through September 2019 is provided (Supplementary Table 1). Graphs comparing 2018 and 2019 average monthly minimum and maximum temperatures (Supplementary Fig. S1a and b, respectively) did not reveal major differences in temperatures between the two years. There were variations in average monthly precipitation between 2018 and 2019 (Supplementary Fig. S1c), with greater overall precipitation in August – December in 2017 (51.4 cm) compared to August – December 2018 (34.5 cm). However, total precipitation was less between January and June in 2018 (47.5 cm) than between January and June in 2019 (53.7 cm). Interestingly, there were several periods with higher evaporation in May and June of 2019 compared to 2018 (Fig. 3), which could have contributed to the drier stromata observed in the field in 2019. The stromata were visually more robust (orange, less shrunk, not dried out) when low in the foliage and protected from desiccation. This observation was also verified in two other heavily choked fields, and similar observations were made by Brem and Leuchtman (1999), with a higher incidence of infection (34%) in the more humid location compared to the drier location (17%). Bushman et al. (2019) suggested a positive association between spring precipitation (humidity) and choke prevalence. This could explain the lower infection rates in 2019 and strongly suggests that reducing inoculum in fields, possibly through the application of fungicides, could effectively reduce infection. However, because the number of choked heads is not directly representative of the previous year's incidence of infection, the effect might only be seen after several years of active inoculum reduction, which would explain why previous attempts to explore inoculum reduction did not show an effect (Pfender & Alderman, 2003). Also, management by inoculum reduction would need to be done when the field is planted, as reducing inoculum on a 2- or 3-year-old stand would have no effect on those plants already infected.

The effect of artificial honeydew and wounding on the incidence of infection

In 2018, our assumption was that the AH treatment would enhance the incidence of infection by facilitating

survival of *E. typhina* on the leaves (Alderman, 2013). However, the significant reduction of infection could be explained by AH stimulating competition from other bacteria or fungi, thereby preventing *E. typhina* from effectively growing and infecting the plant. Since the experiments by Western and Cavett (1959), the common assumption is that infection occurs through cut stems, or more recently through cut leaves (Alderman, 2013; Leyronas & Raynal, 2008). We observed heavy infection on vegetative tissue that was not wounded, and that incidence of infection was the same whether plants were clipped or not. Similarly, plants with severe vole feeding damage (scores of 1 and 2) would be expected to have higher infection rates compared to plants with fewer or no wounds (score 0), which was not the case. Particularly surprising were plants with a vole damage rating of “2”, as they often were eaten to the point of completely exposing the crown, which might be expected to facilitate entry of the fungus to the meristem. This observation suggests that wounding, even close to the crown, does not appear to enhance infection. However, in both cases, the fungus tended to have spread further in clipped plants compared to unclipped plants, and in severely vole-damaged plants compared to lightly damaged plants. Similarly, infected plants were 2.3 times more likely to show symptoms if they were clipped compared to unclipped, or heavily damaged by voles compared to lightly damaged by voles. The expression of more symptoms might be explained by the fact that the fungus had spread further within the plant. It is also possible that the wounds did have an effect, by facilitating multiple infections and thus furthering spread and expression of choke symptoms. Lastly, wounding could have induced metabolic and hormonal changes in the plant, which might increase the probability of *E. typhina* “sensing” its host was stressed, inducing the endophyte to reproduce sexually, independently from the host. Studies by Alderman (personal communication, Pat Boren, Tangent, OR; now Nutrien Ag Solutions) in 2009 were aimed to determine if clipping stubble shorter after harvest could decrease choke incidence by removing new infections before they could reach the crown. Surprisingly, the following year more stromata were observed in plots that were mowed than in those that were not, and an even higher incidence of stromata was observed when fungicide had been applied after mowing. These observations suggest that a stressed host, plus chemical fungicide, may trigger the endophyte to be more aggressive the following year.

Vernalization might play a role in the infection process

We observed that overhead watering after exposure to ascospores was not required to obtain infection by *E. typhina*. Also, clipping the plants two and six weeks after exposure did not appear to reduce infection. These two observations might suggest that penetration and infection of the crown occurs within two weeks after contact with spores, and that atmospheric moisture is sufficient for infection. Alternatively, it is possible that the spores settle close to the crown until sufficiently moist conditions allow them to germinate. Ascospores could be germinated by surface-sterilizing dry stromata kept in the greenhouse for four months and spreading the resulting spore suspension on PDA (Léa Merlet, personal communication). The observations suggest that spores inside the perithecia may have a higher desiccation tolerance than free spores (Alderman, 2013). In our experiment, keeping plants distant from inoculum reduced the incidence of infection, although not significantly, suggesting that infection can occur readily through ascospores dispersed by wind over long distances.

In the 2019 experiment, the treatment having the most effect on incidence of infection was withholding vernalization. It is possible that flower induction plays a role in the infection process, either by enhancing the ability of the fungus to colonize the plant and/or by enhancing its ability to grow into the leaves, where it can be visualized by microscopy. If vernalization does play a role in the infection process, it could explain why some of our infection trials were unsuccessful, and others were sometimes able to detect infection four weeks after exposure, but not after ten or more weeks (Leyronas, 2005).

Future directions for choke research in orchardgrass

Due to the commonly asymptomatic nature of *Epichloë* endophytes in orchardgrass, the link between infection (presence of hyphae in the plant) and expression (visible stroma) is not straightforward. In field trials, lack of visible symptoms on a plant is not sufficient to determine its infection status. For example, in this study, over 75% of the infections would not have been detected by visual observation of stromata. Using inoculated plants bypasses the issue, as the entire above-ground part of the plant is exposed to the fungus, mimicking vertical transmission. In the case of orchardgrass and *E. typhina*,

inoculation is therefore different from natural infection, but can simplify observations. In an inoculated plant, if a flower was produced, it emerged from an infected tiller and was thus able to escape choking. Inoculated plants would be ideal to detect conditions and/or tolerant genotypes that promote flower escape. However, it is important to note that detecting conditions and/or tolerant genotypes that promote flower escape does not equal field resistance, as resistance includes other components that may be bypassed by inoculation.

When *Epichloë* infects mature orchardgrass plants several stages remain uncharacterized, including: (1) germination of spores on the surface of the plant, (2) penetration of the host by the fungus, (3) growth of the fungus toward the meristem, (4) invasion of the meristem, and (5) upward, intercalary growth of the fungus as leaves extend. Disruption of any one of the first three steps renders the infection a failure. Understanding the steps is necessary to determine ways to reduce infection of plants in production fields. Moreover, studies are needed to explore how to reduce expression of the disease in plants that are already infected, as prevention of infection has proven difficult. Although high fertilization may allow the plants to grow faster and possibly escape the fungus (Kirby, 1961; Hoffman & Rao, 2012), the high genetic variability of orchardgrass precluded any significant effect. To overcome the high genetic variability of orchardgrass and to facilitate the examination of various treatments (hormones, fertilization, and/or fungicides) on choke expression, clones of the variety ‘Barlegro’ are currently being used in a series of ongoing experiments to minimize variability between plants (Ruth Martin, personal communication, July 2021).

Recently, the fungicide Almagor® (Triazole + Imidazole) has been shown to interrupt vertical transmission of *E. coenophiala* in tall fescue (Petigrosso et al., 2019) by preventing growth of the fungus in the flowering stems. It is possible that a similar approach could be used to manage choke in orchardgrass seed production fields. Finally, continued screening of orchardgrass germplasm for resistance/tolerance initiated at the USDA (Bushman et al., 2016; Bushman et al., 2019) will facilitate the eventual development of new resistant/tolerant germplasm resources.

Conclusion

Our results show that the interaction between orchardgrass and *E. typhina* is very different from that

between the *Lolium/Epichloë* or fescue/*Epichloë* interactions (Bacon et al., 1977; Schardl et al., 2009), where *Epichloë* is vertically transmitted through the seed. Considering the relatively low success of horizontal transmission through flowers and seeds reported in the literature, and the speed at which the disease spreads between and within production fields in the absence of vertical transmission, we believe that *E. typhina* in orchardgrass relies on horizontal transmission through vegetative tissues, mediated by conidia and in particular, ascospores. Our experiments support this premise, with plants developing an incidence of choke of 41% after only ten days of exposure to ascospore release. Bushman et al. (2019) reported multiple *Epichloë* mating types in different tillers of infected orchardgrass plants. The results suggest horizontal transmission is efficient and very frequent as most plants harbor several strains. The results also indicate that infection by horizontally transmitted *Epichloë* is not systemic, but most likely results from multiple single-tiller infections by different strains that spread into newly formed tillers. The increased tillering rate of infected tillers compared to non-infected tillers from the same plant observed in our studies would also lead to increased incidence of infection within newly infected fields. The knowledge obtained from our results on choke spread within plants and in orchardgrass fields should aid in developing systems for screening for disease resistant/tolerant germplasm and for developing control methods to reduce the expression of choke in infected plants.

Acknowledgements We would like to thank Thomas Lockwood (USDA ARS) for his excellent assistance in maintaining lab, greenhouse and field plots; and Pat Boren, Steve and Tyler Rohner, and Kelly O’Neil at Oregon State University farms for providing field space and plot management support.

Availability of data and material Not applicable.

Code availability Not applicable.

Funding Funding for this research was provided by the United States Department of Agriculture – Agriculture Research Service (USDA-ARS); National Program 215: Grass, Forage and Rangeland Ecosystems, Projects 2072–21000-050-00D and 2072–21000-054-00D.

Declarations This research was supported by the U.S. Department of Agriculture, Agricultural Research Service. USDA is an equal opportunity provider and employer. Mention of trade names

or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

Human and animal rights No human and/or animal participants were involved in this research.

Informed consent All authors consent to this submission.

Conflicts of interest/competing interests The authors declare that they have no conflict of interest.

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