CHOKE EXPRESSION IN *EPICHLOË TYPHINA* SEEDLING-INFECTED ORCHARDGRASS (*DACTYLIS GLOMERATA*) GERMPLASM

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Introduction

Choke disease caused by *Epichloë typhina* (Pers.) Tul. & C. Tul. was first reported in Oregon in 1997 in an orchardgrass (*Dactylis glomerata* L.) stand grown for seed production (Alderman et al., 1997), and by 2003 it was reported to be present in 90% of the orchardgrass seed production fields surveyed in Oregon (Pfender and Alderman, 2006). While the fungus is innocuous most of the year, as the reproductive tillers elongate just prior to flower head emergence, a rapid and dense growth of fungus within and among leaf sheaths forms a sexual reproductive structure (stroma) that destroys, or "chokes," the host flower. This disease is estimated to cause up to 30% losses in seed yield (Pfender and Alderman, 2006).

Efforts to control this disease have focused on insect control and fungicide application to prevent fertilization/growth of the fungal stromata or to slow the growth of the fungus, stubble removal by propane burning after straw removal, and fertility management and plant growth regulators to help plants outgrow the fungus (Alderman et al., 2008). While most of these methods had no significant effect for controlling choke, burning stubble after straw removal and nitrogen fertilization efforts seemed to show some benefits. However, burning stubble did not significantly reduce choke incidence, possibly due to the high variability present in those studies.

With the high prevalence of choke and lack of control methods for this disease, Bushman and coworkers (2019) initiated efforts to identify choke-resistant orchardgrass germplasm to provide an alternative long-term approach to disease management. In the current study, we focused on screening progeny of their most promising choke-resistant germplasm using traditional methods and supplemental trials using preinoculated seedlings. Progeny from 23 of the most resistant maternal lines and two susceptible and resistant control cultivars were inoculated with *E. typhina* at a very early seedling stage (Feekes 1.0). These inoculated seedlings were used to search for tolerant germplasm lines that may be able to escape expression of choke symptoms even when infected with *E. typhina*. The noninoculated plants were used to establish flowering times for the accessions.

Materials and Methods

Plant and fungal materials

Hand-inoculated seedlings of 23 accessions identified in previous trials as having better resistance/tolerance to choke, plus 'Potomac' and 'Baraula' as susceptible cultivars and 'Killarney' and 'Barlegro' as resistant cultivars, were utilized in this study. Accessions were assigned an ID number from #1 to #28 (#13 missing) to facilitate tracking. Based on earlier data (Bushman et al., 2019), parental lines 1–5 are early-heading lines, 6–18 are medium-heading lines, and 19–24 are late-heading lines. Of the reference varieties used, 'Baraula' (#25), 'Barlegro' (#27), and 'Killarney' (#28) are late heading, and 'Potomac' (#26) is an early-heading cultivar. Three infected plants (G4, G5, and G6) removed from two commercial orchardgrass fields were used as sources of *E. typhina* for inoculating seedlings. *Epichloë typhina* was isolated each week by placing surface-sterilized leaves from each infected source plant onto potato dextrose agar (PDA) plates. Cultures were transferred 9 days later and were used for inoculations the following week only in order to provide fresh cultures for all inoculations.

Inoculation protocol

For seedling inoculation, 15–20 surface-sterilized seeds were placed on 3% water agar in 100 mm square x 15 mm Petri dishes and were germinated at 24°C in the dark on vertically positioned plates. When seedlings were 2–5 cm long and the meristem at the junction of the mesocotyl and hypocotyl was visible under a stereo microscope, they were inoculated using a modified version of Latch and Christensen (1985). A sterile scalpel was used to make a superficial 2–3 mm longitudinal slit above the meristem. A small piece of mycelia scraped from a fresh *E. typhina* culture was introduced carefully into the slit near the meristem, trying to not tear the tissue. Plates were placed vertically in the dark at 24°C for at least 48 hours to facilitate growth of the fungi and were then transferred to 24-hour light/24°C conditions for about 2 days. Seedlings were transferred to Cone-tainers (Stuewe and Sons, Albany, OR) prefilled with moist Sun Gro Professional potting mix (Sun Gro Horticulture, Hubbard, OR). Seedlings were watered from below and

lightly fertilized with Technigro 20-18-20 all-purpose fertilizer (Sun Gro Horticulture, Hubbard, OR) every other week.

Examination for presence of *Epichloë typhina*

When plants were 6–7 weeks postinoculation, one of the older sheaths was examined microscopically for the presence of hyphae. Small forceps and a razor blade were used to peel a strand of the inner epidermis, which was put on a microscope slide in a drop of acidified aniline blue. Samples were mounted in a drop of water and immediately examined for presence of *E. typhina* hyphae using a binocular microscope at 200x magnification. Plants were transplanted into the field in October 2018 when they were between 2 and 3.5 months old.

Variety trial using hand-inoculated plants (ongoing study)

A field trial was planted to evaluate the susceptibility of progeny from select genetic accessions that were previously identified as more resistant to choke. Two locations (A and B) near Corvallis, OR, were used, and each plot was divided into one section for hand-inoculated plants and one section for noninoculated plants. Within each section, there were three replicates of 23 genetic accessions and 4 control cultivars randomly arranged. In total, there were 18 preinoculated plants and 30 noninoculated plants for each of the 27 genotypes, duplicated at two locations. The two plots were planted in October 2018 and maintained following the recommended cultural practices for orchardgrass seed production.

Field assessment

Scoring for choke expression was performed on June 5, 2019, when all noninoculated plants were flowering. Each plant was scored (+/-) for choke incidence and anthesis: "choked" if stromata were observed on at least one tiller and "flowered" when at least one flower that would produce some seeds, either perfectly healthy or partially choked, was observed. Each plant was categorized as belonging to one of four groups: only flowered, both flowers and choked heads, only choked, or not headed.

Results

Infection of hand-inoculated plants

In February 2018, seven 'Potomac' plants were hand-inoculated with *E. typhina* and transferred to the greenhouse. One month later, stem sheaths of inoculated plants were stained, and microscopically examined for the presence of hyphae. All seven plants were confirmed positive for the presence of *E. typhina*. Six months later, all tillers were examined by microscopy for the presence of hyphae. In total, 107 tillers were tested, 12–17 per plant. All tillers were positive for the presence of hyphae. Four months later, after vernalization in a cold chamber, all seven plants headed and produced only choked heads.

Hand-inoculation success varied depending on genetic background

Overall, 2,017 out of 2,775 inoculated seedlings were still alive 6–7 weeks postinoculation, with an average survival rate of 73%. For the 27 accessions, the survival rate ranged from 55% to 94%. Among the survivors, fungal hyphae were observed in 1,228 plants when examined microscopically, which represents a 44% infection rate on average, ranging from 23% to 85% among accessions. An overview of the response of different lines to inoculation is shown in Figure 1. The infection rate among the surviving plants ranged from 34% to 90%, with an average of 61%. These plants were maintained in Cone-tainers in the greenhouse until field plots were planted.

Characteristics of field trial locations

In October 2018, 907 inoculated plants, instead of the 972 planned (36 x 27 = 972), were planted at two locations, as some accessions were recalcitrant to infect and not enough plants were obtained in time. In spring 2019, 843 plants had survived and were scored. Because not enough plants could be obtained in time, some were missing at location B. Out of the 162 plants that should have been planted in each replicate, 21 were missing from the second replicate (R5) and 40 from the third replicate (R6). Coincidently, location B was flooded in April 2019, and R6 was the most affected replicate, with 40 plants in that replicate dying (a 33% mortality rate). The other 24 plants that died were distributed evenly across the remaining 5 replicates, leading to a low mortality rate (3%) in absence of the prolonged flood.

Heading date of noninoculated plants and heading defect in inoculated plants

Most genetic lines were classified the same as their progenitors for heading class, except for lines 8, 9, 14, and 18, which were classified as early instead of medium, and 22, which was classified medium instead of late. In spring 2019, 15% of the inoculated plants showed no sign of heading. Although 91–92% of the plants headed in the first two replicates at each location,

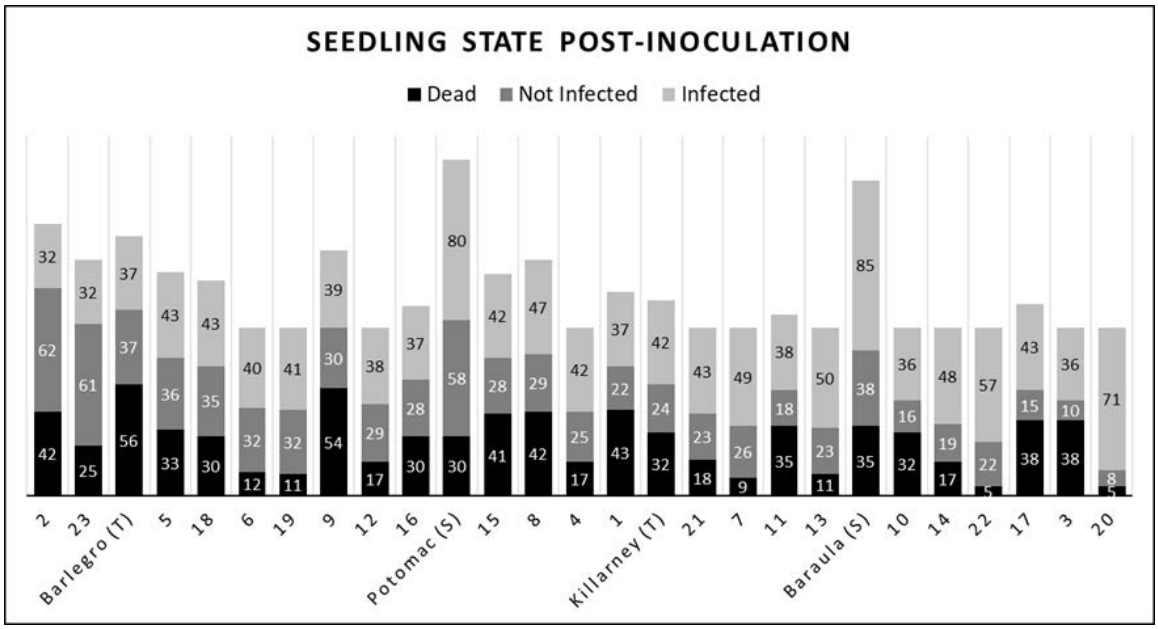

Figure 1. Response of seedlings to inoculation with *Epichloë typhina*. Number of seedlings that died (black), number of seedlings that survived but were not infected (dark gray), and number of surviving seedlings that were infected.

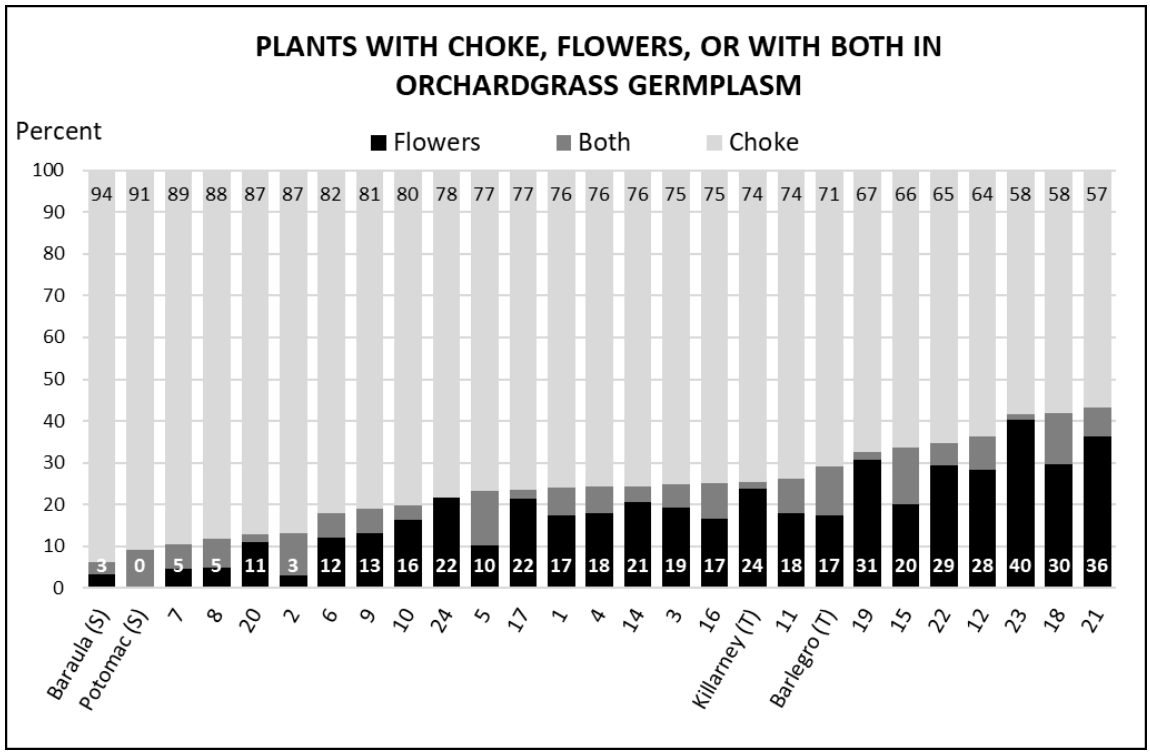

Figure 2. Percentage of hand-inoculated plants producing flowers only (values in white at the base of the columns), flowers and choke, or only choke (values in black at top of the columns) for each genetic accession (average over 2019 and 2020).

only 66% headed at location A replicate 3 (driest area of the plot) and 72% at location B replicate 6 (in the flooded area). The probability of heading was also dependent on the time of heading. Twelve percent of the plants classified as "early" did not head, while that proportion was 20% for plants from the "late" group. It is possible that some late-flowering accessions may not have finished flowering at the time of scoring in the driest (R3) and the flooded (R6) replicates.

Choke expression in artificially inoculated plants with true systemic infection over 2 years

The overall distribution of plants that exhibited only choke, only flowers, or both is depicted in Figure 2. Among the plants that headed, 85% showed at least some sign of choke, and 24% had at least one flower head with potential for seed production. Overall, an average of 76% of all plants were completely choked, with a range of 57–94% of plants in the different accessions. Six percent of the plants, overall, showed both healthy flowers and choked heads, ranging from 0–14% in the different accessions. Interestingly, over the 2 years, an average of 18% of the plants showed only healthy flowers with no sign of stromata, with a range of 0–40% in the different germplasm lines. Early-flowering genotypes were more fully choked (81%), included more plants with both choke and flowers (9%), and had significantly fewer fully flowered heads (10%) than the medium-flowering (77%, 6%, and 17%, respectively) and late-flowering genotypes (75%, 5%, and 20%, respectively). As expected, the two susceptible cultivars were among the lines having plants with the most choked tillers, while the tolerant checks were among lines that had fewer plants with choked heads and more plants with only flowers.

Discussion

Because of the asymptomatic nature of the *E. typhina* endophyte in orchardgrass infections, the link between infection (whether hyphae are present in the plant) and expression (whether stromata are visible) is not straightforward. In a field trial, lack of visible symptoms on a plant is not sufficient to determine its infection status. Using artificially inoculated plants bypasses this issue, as it creates plants that have fungi in all of their above-ground parts, similar to vertical transmission. In the case of orchardgrass and *E. typhina*, although this artificial infection is different from natural infections, it simplifies observations. If a flower appears, it emerged from an infected tiller and was able to escape choking. This technique is ideal to detect conditions and/or tolerant genotypes that promote flower escape. However, it is important to note that this does not necessarily equal field resistance, as overall resistance likely includes other parameters that are bypassed by hand inoculation. As an example, germination and survival of spores on the plant may differ in a greenhouse setting compared to field conditions, as well as penetration or growth of the fungus in the plant toward the meristem. In this greenhouse trial, both the hand-inoculation failure rate, which may represent the resistance of plants to fungal infection, and the appearance of fertile flowers in the field, which represents the resistance/tolerance to the fungi starting its sexual cycle, were dependent on the genetic line. This confirms that an artificial inoculation approach can be used to complement the field resistance/tolerance trials by identifying traits impacting symptom expression in different genotypes.

The appearance of flowers in artificially infected plants was dependent on both flowering class, with more escaped flowers on later-flowering groups, and location ($P = 0.06$), with more escaped flowers at location B. It is possible that moist environments and later-flowering types improve the ability for flowering. Since the early 1960s, the hypothesis has been that lack or presence of choke in infected plants depends on whether or not they are able to escape infection (Kirby, 1961). The speed of growth of the fungus and its host during a precise time period was suggested to determine the probability of escape. In this experiment, plants were confirmed positive about a month before planting. However, it is possible that some plants managed to completely suppress the fungus in the year that followed. Interestingly, the area with the most escaped flowers was R6, which was the most affected by the flood. It is possible that adverse abiotic stresses (e.g., flooding) led to loss of the fungus in some plants.

When plants were artificially inoculated to have the fungus in all their tillers, most of the plants (78%) had no sign of normal flowering. This suggests that each tiller's status as choked or nonchoked depends mainly on its infection status and that conditions may have only a marginal impact on choke expression. However, the probability to choke did depend on the genetic line, with the percentage of escape ranging from 4 to 42% for the best genetic lines. This indicates that there may be a genetic basis for tolerance to *E. typhina* and the ability to flower despite its presence in orchardgrass. If these results are consistent over the years, these tolerant genotypes will prove useful toward breeding for tolerance to choke.

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