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Accumulation of Alkaloids in Different Tall Fescue KY31 Clones Harboring the Common Toxic *Epichloë coenophiala* Endophyte under Field Conditions

Randy D. Dinkins [1,*], Brenda L. Coe [1], Timothy D. Phillips [2] and Huihua Ji [3]

[1] USDA-ARS, Forage-Animal Production Research Unit, Lexington, KY 40546, USA
[2] Department of Plant and Soil Sciences, University of Kentucky, Lexington, KY 40546, USA
[3] Kentucky Tobacco Research and Development Center, University of Kentucky, Lexington, KY 40546, USA
* Correspondence: randy.dinkins@usda.gov

Abstract: Tall fescue (*Lolium arundinaceum*) is a highly adaptable forage, pasture and turf grass that is grown on over 14 M ha in the eastern half of the United States and in other temperate regions of the world. A significant factor in adaptability, productivity and stand persistence is in part due to the presence of an intercellular, seed-transmissible, endophytic fungus, *Epichloë coenophiala*. *Epichloë* endophytes have been shown to produce a number of alkaloid compounds only *in planta*, some that are beneficial in repelling insects, while others are toxic to animals. The goal of this work was to monitor the level of the ergot and loline (classified as pyrrolizidine) alkaloid accumulation in individual plants to determine the plant genotype contribution to alkaloid concentrations. The experimental design consisted of sixteen tall fescue KY31 clones in a space-planted, replicated trial over three years. Our results demonstrated that while changes in the alkaloid concentrations for each plant/endophyte genotype were observed over the three years, the overall alkaloid levels remained relatively constant when compared to other plant/endophyte genotypes combinations in the field. Additionally, overall levels of the ergot and loline alkaloid accumulation did not vary in the same way over the three years. Since the *E. coenophiala* endophyte genotype was the same across all clones, our results indicate that it is the plant genotype that is responsible for determining alkaloid levels in each plant, and suggest that the signal(s) from the plant to the endophyte may not be the same for ergot and loline alkaloid production.

Keywords: tall fescue; endophyte; *Epichloë coenophiala*; ergot alkaloids; loline alkaloids; plant persistence

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1. Introduction

Tall fescue (*Lolium arundinaceum* = *Schedonorus arundinaceus* = *Festuca arundinacea*) is an extremely adaptable forage, pasture and turf grass that is grown on over 14 M ha in the eastern half of the United States. Originating from Europe, tall fescue is now considered an invasive species, especially in the conditions found in the temperate transition zone of the United States [1]; its spread was aided by the release of the KY31 cultivar in the 1940s for pasture and forage production by the University of Kentucky [2]. A significant factor in adaptability, productivity and stand persistence under biotic and abiotic stress conditions is in part due to the presence of a systemic, seed-transmissible, endophytic fungus *Epichloë coenophiala* [(Morgan-Jones et W. Gams) C. W. Bacon et Schardl]. The fungal endophytes of Epichloë genus have been the subject of intense research interest due to their symbiosis with a number of agriculturally important cool-season grasses (subfamily Poöideae) as they are documented to contribute benefits to the host plant (reviewed in [3–8]). These benefits have been additionally highlighted in the discovery of fugally produced compounds that are insect deterrents, such as peramine and loline (classified as pyrrolizidine) alkaloids [9,10], and anti-mammalian toxins, ergot alkaloids and indole-diterpines [11,12] are some of the compounds produced by *Epichloë* endophytes that

are beneficial for plant response to biotic pressures. Most of the Epichloë species are plant host specific, although some form associations with more than one plant species [13], and the different Epichloë endophytes produce different alkaloids described above [7,14–16]. The most intensely studied relationships have been the association of perennial ryegrass (*Lolium perenne*) with *Epichloë festucaea* var. *lolii* (formerly *Neotyphodium lolii*) and tall fescue with *E. coenophiala* (formerly *Neotyphodium coenophialum*) due to the ill effects of the lolitrems (indole diterpene alkaloids) and ergovaline (ergot alkaloids) on sheep and cattle from grazing perennial ryegrass and tall fescue, respectively.

E. coenophiala is an interspecific hybrid between three sexual progenitors *E. festucae*, *E. typhina* and *E. baroni* species belonging to the *Lolium*-associated endophyte (LAE) clade [17,18]. The alkaloids associated with the continental European-derived *E coenophiala* strains primarily contain genes encoding the ergot, loline and peramine alkaloids, but lack indole diterpenes, while Mediterranean-derived *E coenophiala* strains can contain some of the indole diterpene genes and many have lost the ergot alkaloid producing genes. As European-derived tall fescue cultivars are heavily utilized in pasture-based animal production settings, the endophyte contributions to host fitness and the toxic effects on animal production have been well documented [19]. The presence of ergot alkaloids has been shown to result in a number of negative effects with respect to animal production, a phenomenon generally known as fescue toxicosis [16,20,21]. While tall fescue does not require the endophyte for survival, and a number of varieties have been released that are endophyte-free, the presence of the endophyte increases plant fitness and results in more persistent stands under field settings [22,23]. Adcock et al. [24] were able to select for high and low ergot alkaloid lines starting from the common variety Jesup, demonstrating that selection for different alkaloid levels in the succeeding generations is possible. The production of the alkaloids is only found when the endophyte is in association with its plant host, strictly accumulating in planta [25,26]. This suggests that the endophyte perceives some plant derived signal that leads it to the initiation of transcription of the genes involved in the biosynthesis of the alkaloids. Previous research has shown that accumulation of alkaloids is dependent on environmental factors as well as plant genetic differences [27–29]. The goal of this work was to monitor alkaloid profiles and content in individual tall fescue clones over the course of three years in order to analyze the plant genotype contribution to alkaloid production.

2. Materials and Methods

2.1. Plant Materials: Clone Pair Development

The clone pairs were developed from the tall fescue (*Lolium arundinaceum* = *Schedonorus arundinaceus* = *Festuca arundinacea*) Kentucky 31 (KY31) cultivar, harvested at the University of Kentucky Experimental North (Spindletop) Farm. The KY31 cultivar is symbiotic with the endophyte, *Epichloë coenophiala* (Morgan-Jones et W. Gams) C. W. Bacon et Schardl. Seeds were germinated and plants grown in the greenhouse until large enough to separate individual tillers. Half of the tillers of each individual plant were then transferred to a hydroponic solution (1X Hoagland) where half were treated with Folicur 3.6F (tebuconazole-((RS)-1-p -chlorophenyl-4,4-dimethyl-3-(1H -1,2,4-triazol-1-yl-methyl) pentane-3-ol), a foliar fungicide (Bayer Crop Science, Whippaty, NJ, USA), at a final concentration of 43 mg ai/L, was used to kill the fungal endophyte. The remaining tillers were grown hydroponically without the fungicide (control plants). After three weeks, plants were transferred to soil and allowed to grow. New tillers were tested for the presence of the endophyte by tissue print immunoblot (Agrinostics, Watkinsville, GA, USA) and PCR [30], and each of those original clones was subsequently divided and new ramets propagated. The plants treated with Folicur 3.6F were verified to no longer have the endophyte, while the control-treated plants were expected to still have the endophyte. Following two more rounds of testing to verify that the fungicide-treated plants did not harbor the endophyte and the cognizant genotype did harbor the endophyte, plants were designated as a respective E− and E+ clone pair and numbered accordingly. In total, 25 E−/E+ clone pairs originating from

the KY31 CTE+ plants following the Folicur treatment were obtained for further study. Thus, one individual of each clone pair harbored the common toxic endophyte (CTE+) and one was aposymbiotic (E−). The plants were continually divided and maintained in the greenhouse with periodic testing to confirm the endophyte status via immunoblot and PCR.

2.2. Field Study

For the current study, clones were multiplied by separating ramets and each (3/pot) transferred into new pots. Sixteen CTE clones and 34 E+ and E− clones harboring eight different non-ergot-producing strains, also known as novel or non-toxic *E. coenophiala* (NTE) strains, were randomly selected for transplanting based on sufficient number of tillers and the growth of the plants in the greenhouse. Ramets were transplanted into the field at the University of Kentucky Spindletop Farm (38.1245516, −84.490415), in a field with Huntington silt loam soil with pH = 6.7 at establishment on September 20, 2014. The field plot consisted of six replications of 100 genotype/symbiont combinations (50 E+/E− clones) randomly distributed over the 10 × 10 grid at a 30 × 30 cm spacing. Two similarly spaced rows were planted surrounding the experiment plots with random ramets derived from the excess plants. Plants were irrigated after transplanting to ensure successful establishment. Plots were fertilized by annual applications of 67 kg/ha nitrogen (in the form of urea) in early March. Weed control consisted of applications of 2,4-D amine at a rate of 1.06 kg a.i./ha in early March. Areas between plants were mowed with a lawn mower at 3 cm height as needed during the summer for control of weed species not effectively managed by herbicide use. The plot area was mowed in August and September, and in February before spring green-up, to remove senesced material at 5 cm height. In order to not confound the analysis with comparisons of different endophyte strains, only the CTE clones were analyzed as only the CTE clones produced the ergot alkaloids. The plants were allowed to grow for two years prior to data collection and data were collected at seed harvest, as the plants were being used for seed collection. Plants were harvested on 8 June 2017, 18 June 2018 and 11/12 June 2019. Ambient temperature and precipitation data were collected on site hourly and are available at: http://weather.uky.edu/php/fam_www.php (Accessed: 16 September 2022). Plant biomass was determined as the weight of the harvested plant cut to 5 cm at ground level, air dried and weighed.

2.3. Alkaloid Measurements

Ergot alkaloid concentrations were measured using a modified method based on high-performance liquid chromatography (HPLC) developed by Yates and Powell [31]. The ground fescue sample was extracted with 80% methanol by mechanical shaking for two hours. The extract solution was cleaned up through a solid phase extraction (SPE) C18 column and a 0.2 µm polytetrafluoroethylene filter (PTFE filter) before being injected onto HPLC. The quantitative analysis was performed on an HPLC (PerkinElmer series 200) equipped with an autosampler and fluorescence detector. The elution was made with solutions comprising (A) 0.1 M ammonium acetate:acetonitrile, 97:3 *v/v* and (B) 100% acetonitrile. The samples were separated using a reversed-phase Kinetex XB-C18 column (100 mm × 4.6 mm with 2.6 µm particle size (Phenomenex, Torrance, CA, USA)) at a flow rate of 1.2 mL/min. The gradient condition was applied: an initial 22% mobile phase B was increased linearly to 35% in 20 min and further increased linearly to 58% B in 8 min before it was increased to 100% B and held for 5 min. Later, it was decreased to 22% B and held for 9 min for re-equilibration. The ergot alkaloids were detected with excitation at 310 nm and emission at 420 nm.

Loline alkaloid concentrations were measured using a modified gas chromatography (GC) protocol based on Blankenship et al. [32]. The extraction was achieved by adding sodium bicarbonate and methylene chloride containing quinoline (15 µg/mL) as an internal standard to samples and shaking for one hour. The extract solution was filtered by a KimWipe paper and transferred into an amber vial. The analysis was performed using a GC

(PerkinElmer Clarus 500; PerkinElmer, Shelton, CT, USA) equipped with an autosampler, an FID detector, and an SPB-1 fused silica capillary column (15 m × 0.53 m, 0.5 µm film thickness) from SUPELCO, USA. The GC temperature program was set as follows: the temperature was ramped from 80 to 160 °C at 20 °C/min, held for 2 min, then ramped at a rate of 45 °C/min to 290 °C and held for 5 min. The injector and detector temperature were set at 250 and 275 °C, respectively.

2.4. Statistical Analysis

Analysis of the data was performed using JMP (JMP, Cary, NC, USA) within the JMP Genomics (ver 9.1) software. Analysis and graphing were undertaken where each variable (alkaloids, plant height, plant biomass, seed yield) was analyzed across the clones. The standard errors depicted in the figures denote significant differences observed at $\alpha = 0.05$. Bivariate fit Y by X—contextual model was used to analyze the relationship between loline and ergot alkaloid levels within each clone and time point.

3. Results

The overall levels of the total ergot and loline alkaloids found in the seed across all the genotypes are presented in Figure 1a,b. Ergot alkaloid levels across all genotypes were 1.72, 6.81 and 6.27 ppm in 2017, 2018 and 2019, respectively. The combined loline levels were 2954, 2407 and 3427 ppm in 2017, 2018 and 2019, respectively. The role of local climatic conditions on alkaloid accumulation is not known, although it was observed that 2017 was the driest year (1875 mm precipitation) and 2018 the wettest (2385 mm precipitation), while 2019 had 1936 mm (Supplemental Figure S1). Significant differences in ergot alkaloid concentrations were observed between genotypes where CTE27, CTE46, CTE52 and CTE59 had the lowest levels and CTE23 the highest (Figure 2a). Although significant differences were observed across years, and some variability between genotypes over the different years, the overall pattern of ergot alkaloid concentrations remained fairly constant between genotypes over the course of the three years (Figure 2b–d). Measurements of the individual ergot alkaloids that are observed to accumulate to the highest levels in tall fescue, namely ergovaline and ergovalanine, also followed the same pattern across the genotypes (Supplemental Figures S2 and S3).

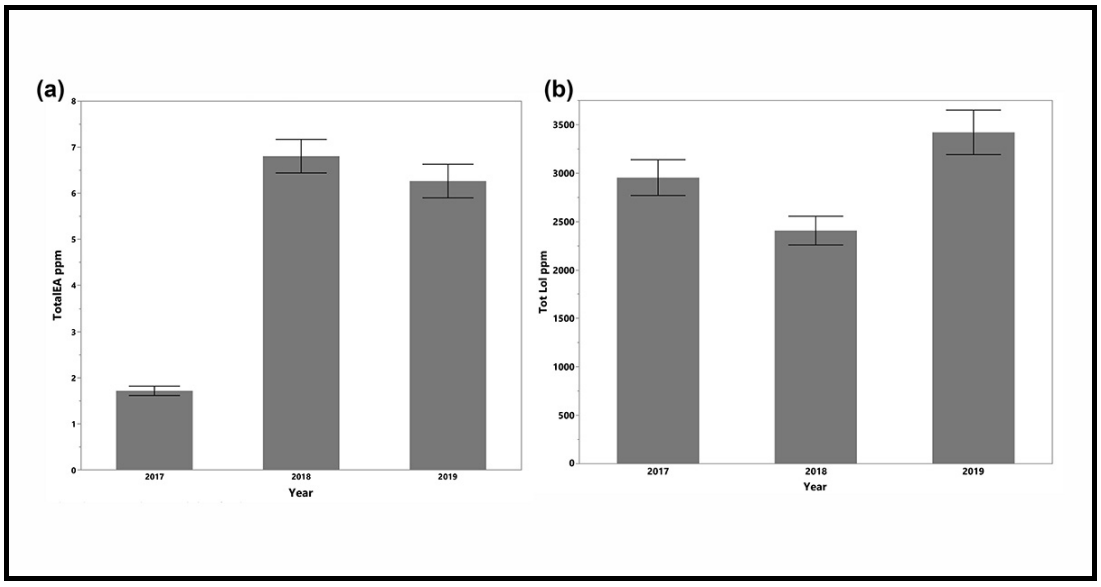

Figure 1. Levels of (**a**) ergot (Total EA) and (**b**) loline (Total lol) alkaloid accumulation across all clones for 2017, 2018 and 2019. Data are means +/− 1 s.e.

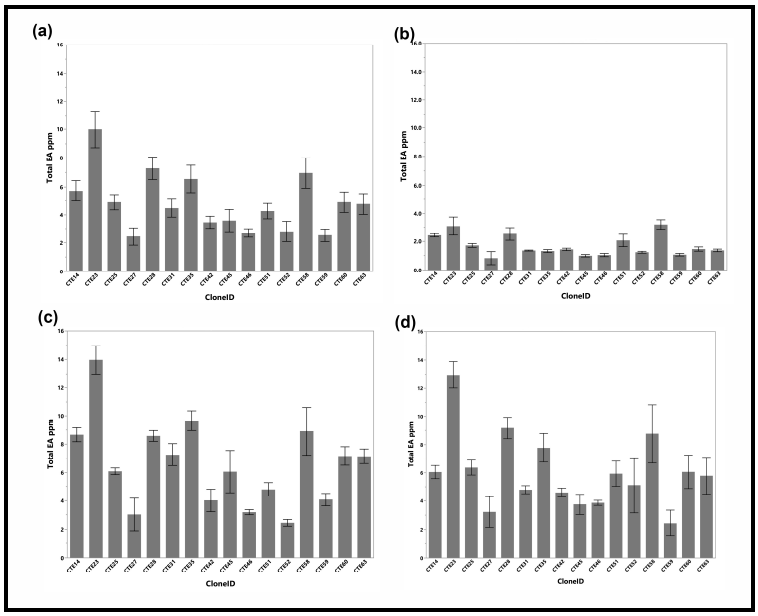

Figure 2. Seed head ergot alkaloid (Total EA) levels in 16 CTE tall fescue clones (**a**) averaged over three years of study, and for (**b**) 2017, (**c**) 2018 and (**d**) 2019 individually. Data are means +/− 1 s.e.

A similar genotypic difference was observed in the accumulation of loline alkaloids (Figure 3). Total loline levels across all years were seen to be lowest in CTE27, CTE46 and CTE63 and highest in CTE23, CTE28 and CTE58 (Figure 3a). This pattern was consistent across each year (Figure 3b–d). The levels of the different lolines varied based on genotype (Figure 4a–c). Interestingly, CTE28 appears to have significantly lower levels of the loline alkaloid N-acetylloline (NAL) (Figure 4c). While the biosynthetic pathway from N-acetylnorloline (NANL) to NAL is still not known, it is postulated not to be fungal-derived, but produced via a plant acetyltransferase [33].

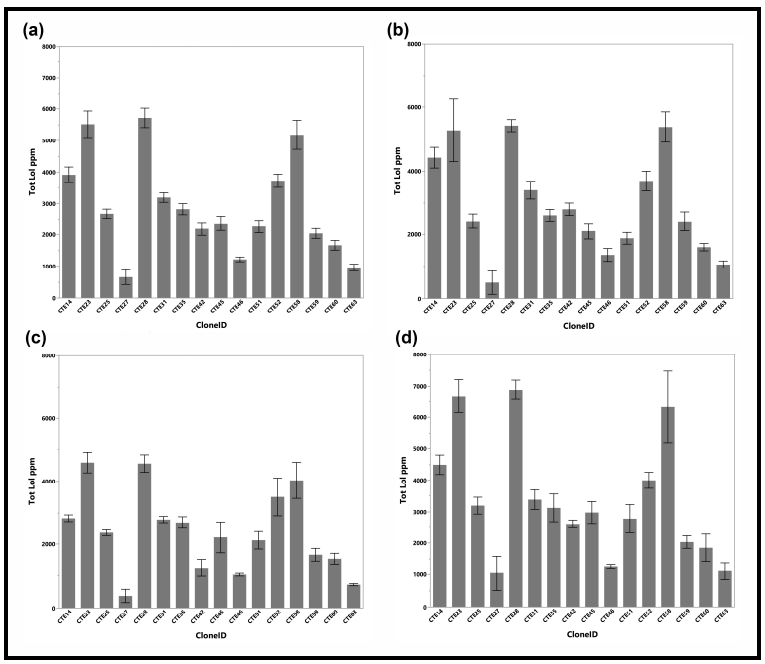

Figure 3. Seed head loline (Total lol) alkaloid levels in 16 CTE tall fescue clones (**a**) averaged over three years of study and for (**b**) 2017, (**c**) 2018 and (**d**) 2019 individually. Data are means +/− 1 s.e.

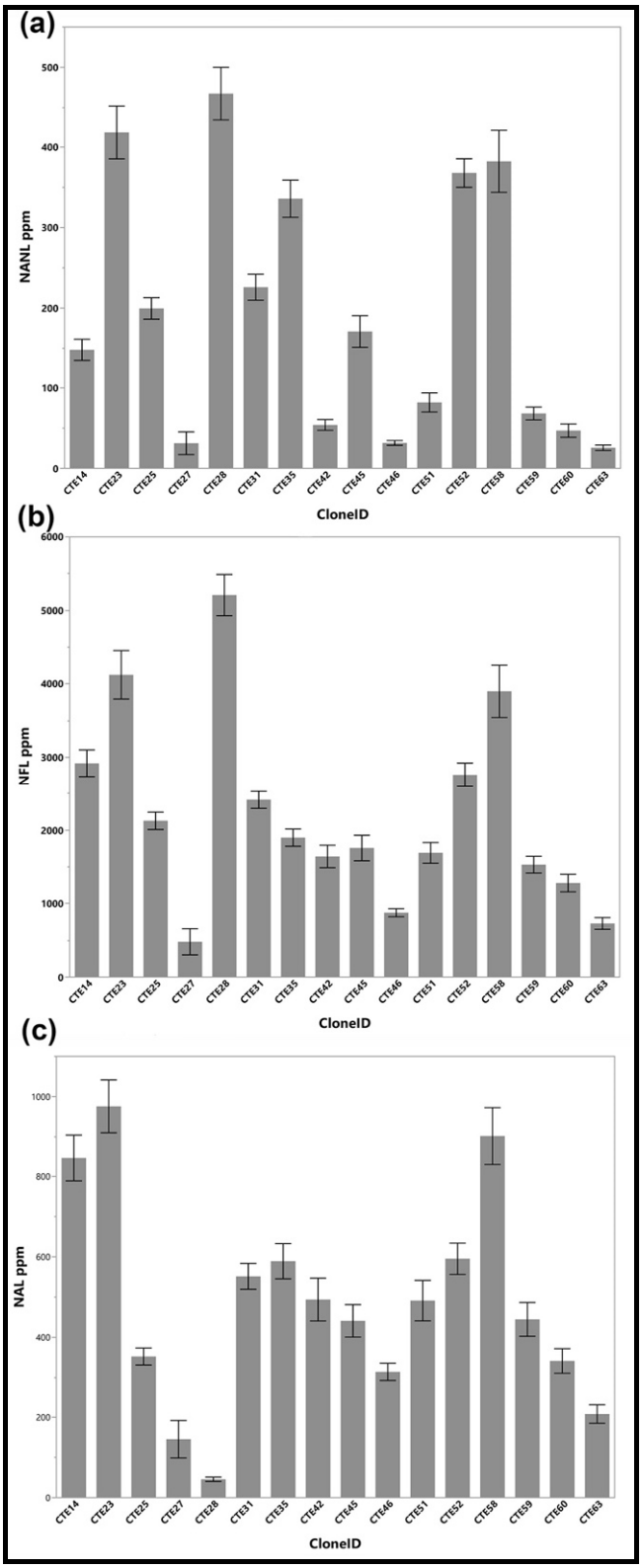

Figure 4. Average accumulation of the different lolines (**a**) N-acetylnorloline (NANL), (**b**) N-formal loline (NFL), and (**c**) N-acetylloline (NAL) in seed heads of 16 CTE tall fescue clones from 2017 to 2019. Data are means +/− 1 s.e.

While tall fescue clones that tended to have the highest levels of ergot alkaloids were also the clones that had the highest levels of loline alkaloids (CTE23, CTE28, CTE35 and

CTE58), and clone CTE27 had the lowest levels of both, the production of loline and ergot alkaloids across all 16 genotypes appears to be weakly correlated (r^2 = 0.20; Figure 5a). Likewise, CTE27 was also the least productive clone with respect to tiller number and aboveground biomass (Figure 6). However, no correlation was observed when comparing the accumulations of ergot or loline alkaloids with overall tiller number (not shown) or aboveground biomass (Figure 5b,c).

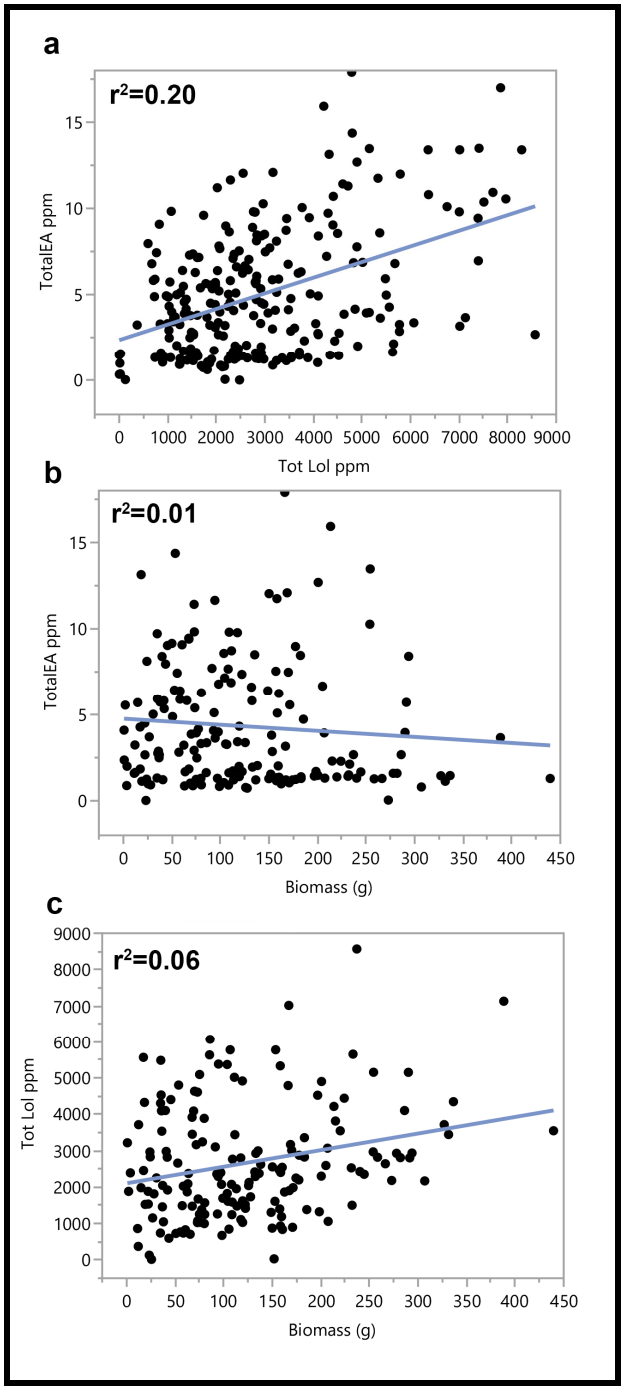

Figure 5. Correlation between accumulation of ergot and loline alkaloids (**a**); correlation between ergot alkaloid (**b**) and loline (**c**) accumulation and biomass over the 2017–2019 period.

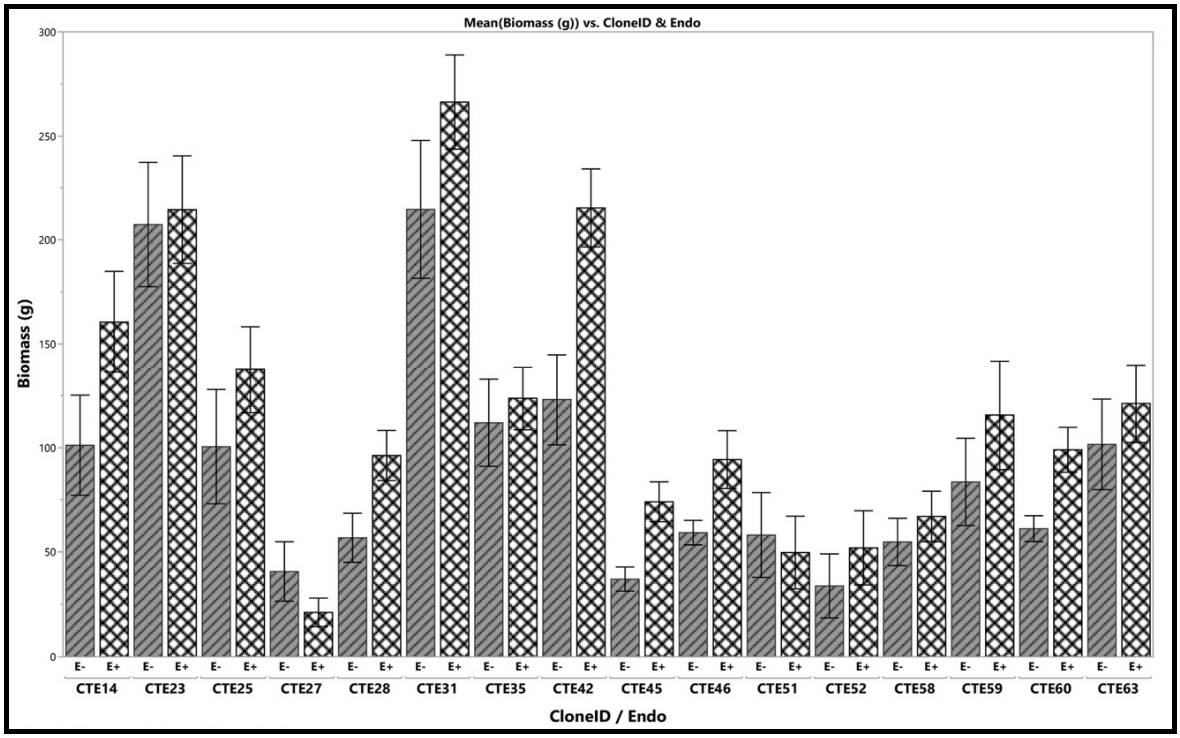

Figure 6. Average aboveground biomass for 16 tall fescue clones that were endophyte-free (E–; diagonal line bars) or endophyte-infected (E+; crosshatch bars) from 2017–2019. Data are means +/− 1 s.e.

When comparing the E+ vs. E− clone biomass and tiller number, the results showed that there were no differences between the E+ and E− plants, or that in some clone pairs the E+ plants produced slightly more biomass (Figure 6). This is in agreement with previous results that suggest that the presence of the endophyte provides some benefit over E− plants.

4. Discussion

Tall fescue, and other related Poaceae species, do not require the presence of an endophytic fungus for survival, although the presence of endophytes that produce different alkaloid compounds has persisted in their populations. This suggests that presence of an endophyte has increased the fitness of populations of these grasses in their respective environments. Under a managed agricultural setting, where tall fescue is normally found in North America, the continued spread of "infected" grasses, namely those harboring an endophyte, suggests that the endophyte has served a purpose in maintaining host population fitness [34]. However, there have been general discussions in the literature regarding the benefit/cost of the tall fescue/endophyte symbiosis and whether this symbiosis is mutualistic or runs on a mutualistic-parasitism continuum [16,35,36].

It might not be unexpected that the lines utilized in the current study may be more "predisposed" to the presence of an endophyte, as they were derived from the KY31 genetic background that has persisted in Kentucky's environment over a long time period [1]. Overall, the KY31 background has shown to provide very good fitness and persistence, although a significant amount of clone-to-clone (i.e., genotype) variability was observed in the current study. This was seen both in regard to the accumulation of different alkaloids and with respect to aboveground production.

It is highly unlikely that *E. coenophiala* is not detected by the host plant given the observations that *Epichloë* spp. tend to be highly host-specific, and in instances where they have been transferred mechanically between host species, they elicit a variety of defense-like responses in these non-native hosts [37–39]. In addition, mutations in the fungus that

affect fungal response and growth within the plant have been shown to be detrimental to the plant [40,41]. The production of alkaloids is only found when the endophyte is in association with its plant host [25], suggesting that the endophyte perceives some plant-derived signal that leads it to initiate transcription of the genes involved in the biosynthesis of these compounds. While the specific signaling between host and endophyte has not been identified, several studies have shown that growth of the fungus appears to parallel the plant growth, responding to cues in the plant [25,42–44]. We have previously shown that the presence of the endophyte has little effect on overall plant transcription, although we have also shown that the presence of the endophyte does suggest that it might prime the plant for abiotic stress tolerance [45,46]. The overall effect of the presence of the different strains appears to be based on the host plant genotype [47], while differences in fungal gene expression appear to be minimal [48].

Local environmental factors were found to affect the level of different alkaloids over the course of the experiment, and some of the changes in the overall rank between genotypes resulted in a statistical genotype x year interaction. However, the relative levels of both ergot and loline alkaloids remained relatively constant when comparing plant clonal genotypes over the course of the three years. Thus, by selecting, as conducted by Adcock et al., [24], it should be possible to identify tall fescue genotypes that will have high and low alkaloid levels. Additionally, we observed that the factors affecting the overall level of the ergot alkaloids and loline alkaloids appeared to be controlled differently, as the environmental factors that affected the overall levels of ergot alkaloid accumulation were different for the accumulation of loline alkaloids, particularly in 2017. It is unclear how environmental factors affect the production of the different alkaloids. While general climatic conditions affect the overall fitness of the genotypes, specific environmental factors likely condition the yearly alkaloid levels, as seen in the ergot alkaloid levels observed between years in the current study. It is well documented that abiotic factors such as pasture management, fertilization and water availability influence the accumulation of the different alkaloids [49–54]. Simultaneously, the presence of the endophyte has also been demonstrated to change the physiological and metabolic profile of plants harboring endophytes when compared to endophyte-free plants [51,55–59]. In the current research, we expanded this finding to demonstrate that the interaction of the fungus regarding the accumulation of the different alkaloids is specific within each plant genotype, or generally speaking, to each symbiotic genotype, or symbiotum. While differing levels of alkaloids have been characterized for different tall fescue cultivars [22,24], the plant genotype effect, in relation to alkaloid levels and growth and transmission of the *Epichloë* endophytes, has been better characterized in *L. perenne* symbiotic with *E. festucae* var. *lolii* [60–62]. Additionally, the plant genotype association for endophyte transmission, and levels of the pyrrolizidine NFL, were mapped to specific regions of the *L perenne* genome by quantitative trait loci (QTL) mapping, further suggesting plant control [61]. Further study is needed to confirm these findings, and hopefully elucidate the genes of interest as molecular tools refined for these grass species.

It is also possible that the differential accumulation of ergot and loline alkaloids during the 2017 growing season occurred because the environmental conditions during 2017 affected the growth of the fungus during the colonization of the seeds. Temperature has been shown to affect fungal growth and seed transmission in tall fescue [63,64]. While fungal biomass was not quantified in the current study, it is possible that the lower levels of ergot alkaloids might be the result of lower rates of infection of the seed. The levels of ergot alkaloids tend to be associated with the presence of the endophyte in the different tissues, although translocation of the ergovaline into the leaves has been demonstrated [65]. The pyrrolizidine (loline) alkaloids NFL and NAL are found distributed throughout the plant, with the highest levels also observed in the seed, and levels are also positively correlated with the amount of fungal mycelium in the plant [66]. Thus, even though year-to-year differences are observed, it is the plant genotype that, in all likelihood, regulates fungal

growth, thereby determining the levels of the alkaloid observed in the seed since the growth pattern remained the same across years.

Under the strict vertical maternal transmission observed for *E. coenophiala*, only in plants where the maternal plant genotype provided a good "environment" for the endophyte, or where the endophyte and plant genotypes were compatible, would the transmission be successful. Although the seed heads of tall fescue are known to be the sites where the highest alkaloid levels are found, the accumulation of ergot and loline alkaloids in the seed heads of tall fescue over the course of the three years is indicative of the overall alkaloid levels of this subset of plants, and suggests that these would be the observed alkaloid levels were this a tall fescue cultivar [27,67]. In nature, transmission of tall fescue genes as well as the endophyte genes would continue. What is lost, due to outcrossing, is the specific plant/endophyte combination that is not maintained. Clonal populations provide for a fixed genotype x fungus interaction, specifically plant genotype since the fungal genotype is common to all plant genotypes. Considering the current experiment, genes associated with propagation of Plant 27 CTE+ would have less probability of populating the following generation compared to Plant 23 CTE+ or Plant 25 CTE+. In fact, from the current study, it would seem that Plant 27 E– appears to have a higher "fitness" than Plant 27 CTE+ based on the overall biomass and seed set in the field.

In summary, we document that while environmental variability affects the yearly levels of alkaloid concentrations, the overall relative plant/endophyte combination (symbiotum) levels tend to remain the same relative to other genotypes in the population over time. Different environments lead to selection based on the local biotic and abiotic stresses, resulting in the propagation of select plant/endophyte combinations [3,27]. While there must be a plant signal/component that determines how the fungus will respond within that genotypic environment, it has not currently been identified. Thus, this work demonstrates that plant genotype, either directly or via plant genotype in combination with the endophyte genotype, is responsible for the levels of alkaloid accumulation. From the current work, this signal, or signals, involved in establishing the internal symbiotum environment leading to the production of ergot alkaloids, does not appear to be the same for loline alkaloid production. Future work is needed to identify the plant-derived signal(s), and how the plant and fungus communicate to produce these different compounds that aid in the fitness of these grasses.

Supplementary Materials: The following information can be downloaded at journal website: https://www.mdpi.com/article/10.3390/agronomy13020356/s1. Supplemental_Figures.pdf contains: Figure S1: Daily average temperatures (in Celsius) and precipitation (in mm) events during the course of the study beginning in 1 January 2016 through 18 June 2019; Figure S2: Seed head ergot alkaloid ergovaline (EGV ppm) levels in 16 CTE tall fescue clones (a) averaged over three years of study, and for (b) 2017, (c) 2018 and (d) 2019 individually. Data are means +/− 1 s.e.; Figure S3: Seed head ergot alkaloid ergovalanine (EGVN ppm) levels in 16 CTE tall fescue clones (a) averaged over three years of study, and for (b) 2017, (c) 2018 and (d) 2019 individually. Data are means +/− 1 s.e.

Author Contributions: R.D.D. and T.D.P. planned and obtained funds for the experiment. R.D.D., T.D.P., B.L.C. and H.J. performed the experiments and collated the data. R.D.D., B.L.C. analyzed the data and R.D.D. wrote the first draft of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The clone pairs described in this manuscript are available upon request.

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Conflicts of Interest: The authors declare no conflict of interest.

Author's Statement: 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 or the University of Kentucky. USDA and the University of Kentucky are equal opportunity providers and employers.

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