Variation and plasticity in *Epichloë* alkaloid content of *Festuca rubra* across Europe

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Fungal Ecology vol 47, 2020

<https://doi.org/10.1016/j.funeco.2020.100942>

Abstract

Many symbiotic microbes can aid their host plant's defense against herbivores and pathogens either directly or indirectly through plant-mediated mechanisms. Thus, microbes are of interest to agricultural research. In this study, we used vertically transmitted *Epichloë* species because these grass fungal endophytes produce alkaloids that have been shown to protect the host from herbivory. Depending on the amount, type, and profile of alkaloids, *Epichloë* species can promote the host's defense against invertebrate or vertebrate herbivores. Here, we used *Festuca rubra* colonized by *Epichloë festucae* from wild populations of different geographic origins in a reciprocal transplant experiment at three locations across Europe. We sought to determine whether genetic differences among plants colonized by *Epichloë*-colonized plants from different origins or phenotypic plasticity explained variations in alkaloid content and whether the alkaloid content was correlated with the growth and reproduction of plants colonized by *Epichloë*. We found that the geographic origin of the *Epichloë*-colonized genotypes explained differences in ergovaline and peramine production. The ergovaline content also indicated chemotypic plasticity, as profiles changed when transplanted to a new environment, while the concentration of peramine was constant among the transplantation sites. The lack of a strong correlation between alkaloid content and plant performance (number of reproductive stems and total biomass) indicated that the costs of alkaloid production were too low to have consequences for fitness in terms of those parameters. Our findings emphasize the importance of estimating the fungal alkaloid content in multiple field environments in order to determine its potential toxicity and value for breeding programs that are aimed at the improvement of turfgrass and forage cultivars.

Keywords: *Epichloë*; ergovaline; environmental effect; fungal endophyte; peramine; plasticity; red fescue; transplant experiment

1. Introduction

Ranging from antagonistic to mutualistic, symbiotic interactions between microbes and plants are both variable and context-dependent, depending on the interacting species, their life history strategies, and the environmental conditions (Redman et al., 2001; Saikkonen et al., 2004; Thompson, 2005). The advantages provided by beneficial microbes to their hosts include increased growth and reproduction, and resistance to abiotic and biotic stresses. In turn, the microbes receive nutrients, shelter, and in some cases vertical transmission to the next generation of hosts. The benefits for partners are context-dependent and rarely symmetric, and these microbe–plant symbioses are dynamic in both ecological and evolutionary time (Muller and Kraus, 2005; Gundel et al., 2008; Leinonen et al., 2019). Nonetheless, symbioses involving the vertical transmission of a microbe are generally presumed to fall closer to the mutualistic end of the interaction continuum because the fitness of the host and the symbiont are tightly linked (Saikkonen et al., 1998).

Because vertically transmitted microbes can affect plant communities and associated trophic interactions, they drive ecosystem functions (Rudgers et al., 2004; Omacini et al., 2012; Saikkonen et al., 2013b; Gundel et al., 2016). For example, many widespread cool-season grasses (Pooideae) are commonly associated with vertically transmitted (via host seeds) endophytic fungi of the genus *Epichloë* (Ascomycota; Clavicipitaceae), which systemically colonize aerial organs. These endophytes can produce alkaloids, thus contributing to the capacity of grasses to cope with herbivore pressure (Vicari and Bazely, 1993; Siegel and Bush, 1996; Bush et al., 1997; Clay and Schardl, 2002). *Epichloë* species also promote the resistance to abiotic stress as well as plant growth and reproduction (Malinowsky and Belesky, 2000; Hahn et al., 2008; Sabzalain and Mirlohi, 2010; Hume and Sewell, 2014; Song et al., 2015). However, alkaloid-based defensive mutualism appears to provide the best framework to explain the dynamics of *Epichloë*–grass symbioses in natural habitats and agroecosystems (Clay, 1988). Their importance for agriculture is evident, as alkaloids of fungal origin are notorious for their harmful effects on livestock (Fletcher and Harvey, 1981; Lyons et al., 1986; Bacon, 1995). Although grass–*Epichloë* symbioses are generally considered as beneficial for the host plant, studies on natural populations have demonstrated that a symbiotic relationship not only can increase or decrease host fitness, but also be apparently neutral (Saikkonen et al., 1998; Ahlholm et al., 2002; Faeth, 2002; Gundel et al., 2008; Leinonen et al., 2019).

Alkaloids produced by *Epichloë* species include ergot alkaloids (e.g. ergovaline), indole-diterpenes (e.g. lolitrem B), lolines (aminopyrrolizidines), and the pyrrolopyrazine alkaloid peramine. Recently, other pyrrolopyrazines produced by allelic variants of the *ppzA* gene,

responsible for peramine production, have been discovered (Berry et al., 2019). Ergovaline and lolitrem B are toxic to vertebrate grazers, and peramine and lolines are active against invertebrates (Saikkonen et al., 2013a; Schardl et al., 2013a). Ergovaline is the ergopeptine group alkaloid most commonly found in *Epichloë*-infected grasses. It is associated with fescue toxicosis, a syndrome that has detrimental effects on mammalian livestock health and reproduction, causing important economic losses in animal production (Lyons et al., 1986; Schmidt and Osborn, 1993). Lolitrem B has strong tremorgenic activity in mammals and is responsible for ryegrass staggers in livestock (Gallagher et al., 1984; di Menna et al., 2012). Peramine and lolines can protect plants against invertebrate herbivores, but no toxic effects on mammals are known (Rowan, 1993; Wilkinson et al., 2000). The qualitative and quantitative production of alkaloids vary markedly among *Epichloë* species and strains (Vázquez de Aldana et al., 2010; Schardl et al., 2013a; Charlton et al., 2014; Takach and Young, 2014; Saikkonen et al., 2016; Soto-Barajas et al., 2019). For example, *Epichloë festucae* strains differ in their ability to produce different combinations of the four types of alkaloids, but only some strains of *E. coenophiala* are known to simultaneously produce all four types (Schardl et al., 2014).

Although the potential of a fungal strain for alkaloid production can be readily screened using molecular markers related to biosynthetic genes (e.g. Young et al., 2015), the resulting alkaloid content in the grass host is difficult to predict. Alkaloid production is known to be highly affected by environmental conditions (Lane et al., 2000; Brosi et al., 2011; Repussard et al., 2014; Helander et al., 2016). For example, in common garden experiments with tall fescue (*Schedonourus arundinaceus*) at two field sites, alkaloid levels depended on the growth environment, and in general, they were higher in wild populations than in the cultivar Kentucky-31 (Helander et al., 2016). Specific environmental factors, such as drought, can also affect alkaloid concentration (Belesky et al., 1989; Hahn et al., 2008; Vázquez de Aldana et al., 2013; McCulley et al., 2014). Furthermore, the synthesis of alkaloids requires nitrogen, and the production of fungal alkaloids could be limited when nitrogen availability is low. In tall fescue, ergovaline production is known to increase with increasing amounts of nitrogen (Lyons et al., 1986), but under field conditions, the responses can also differ between environments and plant and fungal genotypes (Arechavaleta et al., 1992; Helander et al., 2016). Although equally high levels of alkaloids have been recorded in wild and agronomic grasses (Jensen et al., 2007; Helander et al., 2016), agronomic grasses are the most studied and evidence of alkaloid-based defensive mutualism is commonly reported for such systems (Saikkonen et al., 2010).

Here, we studied the natural variation in alkaloid content of endophyte infected *Festuca rubra* L., which is a host of *Epichloë festucae*, a mostly seed-transmitted species (Leuchtman et al., 2014). The perennial *F. rubra* inhabits a variety of environments including high latitudes,

and is an important forage and turf grass. The incidence of *E. festucae* in natural populations of *F. rubra* varies, with frequencies close to 100% in some populations while absent in others (Bazely et al., 1997; Zabalgoieazcoa et al., 1999; Jensen et al., 2007; Dirihan et al., 2016; Pereira et al., 2019). *F. rubra* infected by *E. festucae* is known to produce ergovaline and peramine alkaloids (Siegel and Bush, 1996; Leuchtmann et al., 2000; Vázquez de Aldana et al., 2010). To test whether variations in ergovaline and peramine content are due to genetic differences among infected plants from different geographic origins and/or chemotypic plasticity, we determined the content of these alkaloids in plants of *F. rubra* colonized by *Epichloë festucae* from natural populations, and subsequently grown in a reciprocal transplant experiment conducted in northern Finland, southern Finland and western Spain. Because alkaloid production is costly in suboptimal environments, we also recorded the biomass and the number of inflorescences of the plants to estimate whether alkaloid production has fitness consequences for the host grass.

2. Materials and methods

2.1. Plant material and field experiments

This work was conducted as a part of a research project in which we first examined the frequencies of *E. festucae* in wild populations of *F. rubra* across Europe (Dirihan et al., 2016). Plants of *F. rubra* were collected at four geographic regions: northern and southern Finland, Faroe Islands, and western Spain (Fig. 1). In northern Finland, collection sites were two subarctic meadows in Utsjoki, in Finnish Lapland (MS1K: 69°38'5.6"N, 27°5'0.9"E; MS2K: 69°43'56.4"N, 27°1'11.6"E). At the Faroe Islands, plants were collected at the islands of Mykines (FAS1: 62°5'50.7"N, 7°40'55.9"W) and Viðoy (FAS2: 62°22'3.4"N, 6°32'31.8"W). In southern Finland, the three sampled populations originated from natural meadows near the shore of the Baltic Sea in Hanko (HA1: 59°50'27"N, 23°13'15"E; HA2: 59°50'23"N, 23°13'40"E; HA3: 59°53'0"N, 23°5'52"E). In Spain, two of the populations were from semiarid oak grasslands (dehesas) near Salamanca (SPLV: 40°56'20.2"N, 6°7'6.6"W; SPPOR: 40°58'24.2"N, 5°57'33.7"W) and one from a Mediterranean oak forest in Garganta de los Infernos (SPGD: 40°12'1.1"N, 5°45'11.0"W). The number of plants collected at each of the four regions was about 40.

The plants were collected in the summer of 2010 and then grown in 250 ml pots filled with a 1:1 (v:v) mixture of peat and sand in a greenhouse at Turku University Botanical Garden (Dirihan et al. 2016). Plants were split into tillers to obtain vegetative clones of each genotype for the transplantation experiment, which was established in 2012. The presence of *Epichloë* in

F. rubra plants was verified by the isolation of the fungus from surface-sterilized stem and leaf sheaths on potato and dextrose agar, and has been documented by Dirihan et al. (2016).

A similar number of plants with and without *Epichloë* from each region was selected for the transplant experiment, except from southern Finland where none of the plants were colonized by *Epichloë*. Then, we established a four-way reciprocal transplant experiment with *Epichloë* infected (E+) and uninfected (E-) *F. rubra* genotypes at each of the four home environments: (i) the Faroe Islands at the Agricultural Center (Búnaðarstovan) (62°06'N, 6°57'W); (ii) northern Finland at the Kevo Subarctic Research Station in Utsjoki (69°45'N, 27°01'E); (iii) southern Finland at an experimental field in Ruissalo Botanical Garden of University of Turku (60°26'N, 22°10'E); (iv) in Spain at Muñovela Research Farm of IRNASA-CSIC in Salamanca (40°54'N, 5°46'W) (Leinonen et al., 2019). Three clonal replicates of each genotype were transplanted at each transplantation site. Planting was done in a fully randomized design at each site, with a distance of 30 cm between plants. Plants were watered when needed until they were well rooted in the first growing season. Competing vegetation was removed periodically during the course of experiment. Experimental areas were fenced to exclude large vertebrate herbivores.

For this study, we only used genotypes of *F. rubra* colonized by *Epichloë* (from now on FR +E genotypes) collected from the Faroe Islands, northern Finland, and Spain (a total of 45 FR +E genotypes) that were grown in northern Finland, southern Finland and Spain (Fig. 1). Plants from south Finland were not included because none of them was colonized by *Epichloë*.

Soil samples were collected at each transplantation site and analyzed for pH, C, N, K, Ca, and Mg by Eurofins Viljavuuspalvelu, a certified commercial laboratory (Table 1).

2.2. Phenotypic and chemotypic measurements

To estimate the variance in plant fitness under the experimental conditions, the aboveground plant parts were harvested in a reproductive stage before seed ripening. This was mid-June in Spain, beginning of August in southern Finland and mid-August in northern Finland. The number of flowering culms was counted, and the aboveground biomass was weighed (Leinonen et al., 2019). At the same time, a subsample of leaves and stems without inflorescences was taken for alkaloid analysis and transported immediately to a deep-freezer (–80°C). The samples were lyophilized and ground to powder. In general, three clonal replicates of each E+FR genotype at each site were analyzed; however in some cases only one or two clonal replicates were available for chemical analysis due to insufficient amounts of biomass. The number of E+FR genotypes per site and geographic origin is shown in Table 2.

The concentration of ergovaline was quantified by high performance liquid chromatography (HPLC) following a modification of the methods described by Hill et al. (1993) and Yue et al. (2000). A plant sample of 0.5 g was extracted in 10 ml of CHCl_3 and 0.5 ml of 0.5 mM NaOH for two hours. One hundred microliters of an internal standard solution of ergotamine ditartrate ($10 \mu\text{g ml}^{-1}$, Sigma-Aldrich) were added to the sample prior to the extraction. The mixture was vacuum filtered through Whatman no. 2 filter paper and then passed through a preconditioned column packed with 500 mg of Ergosil (Analtech, Newark USA). Plant pigments were extracted with 4–5 ml of chloroform:acetone (1:3). The sample was eluted with 2 ml of methanol, vacuum concentrated, redissolved in 1 ml of methanol, and filtered through a $0.22 \mu\text{m}$ nylon filter. Ergovaline quantification in extracts was performed in an HPLC system (Waters 2690) using an Agilent Poroshell 120 EC-C18 column ($4.6 \times 150 \text{ mm}$; $2.7 \mu\text{m}$) and a fluorescence detector (Waters 2475): $\lambda_{\text{exc}} = 250 \text{ nm}$ and $\lambda_{\text{em}} = 420 \text{ nm}$. In the mobile phase, acetonitrile and 0.01 M ammonium acetate with a gradient flow of 0.8 ml min^{-1} were used. The limit of ergovaline quantification was $0.02 \mu\text{g g}^{-1}$. The ergovaline standard was purchased from Forrest Smith (Auburn University, USA).

Peramine was determined using variations of the HPLC method described by Barker et al. (1993) and Yue et al. (2000). Briefly, a freeze-dried and ground 100-mg plant sample was extracted in 3 ml of 30% isopropanol for 30 min at 90°C . The mixture was centrifuged, and the extract was passed through a preconditioned Varian Bond Elut carboxylic acid (CBA) column packed with 100 mg of adsorbent. The extract was filtered through a $0.22\text{-}\mu\text{m}$ nylon filter and chromatographed in a HPLC system (Waters 2690) using a Nova Pak C18 Waters column ($3.9 \times 150 \text{ mm}$). The isocratic mobile phase consisted of 18% (v/v) acetonitrile in a guanidine carbonate (10 mM) formic acid buffer. The detection was performed using a Photodiode Array Detector (Waters 2996) set at $\lambda = 280 \text{ nm}$. The limit of quantification was $0.8 \mu\text{g g}^{-1}$. The peramine standard was a gift from G. Lane (AgResearch, New Zealand).

2.3. Statistical analyses

To determine whether the environmental responses in alkaloid production differed according to geographic origin, we conducted likelihood ratio tests of generalized linear models. We included the transplant site and geographic origin. Their interactions were fixed factors in the models, and we used alkaloid production as a binary response variable (i.e., produced or not). To test the differences between geographic origin in the ergovaline and peramine content at each site, we applied the nonparametric Kruskal–Wallis test because the data were not normally distributed due to the excess number of zeroes.

We examined the relationship between alkaloid production and plant performance (i.e., shoot biomass and the number of flowering culms) using likelihood ratio tests with linear models. The transplant site and geographic origin were included in the models to account for their effects. Spearman correlation tests were used to determine the correlations between the number of flowering culms and alkaloid content in the geographic origins at the study sites. All analyses were performed using R statistical software version 3.5.1 (R Core Team 2018).

3. Results

3.1. Ergovaline

Across all transplantation sites, ergovaline was detected in 63%, 94%, and 50% of the FR +E genotypes from the Faroe Islands, northern Finland, and Spain, respectively. Nine FR +E genotypes did not produce ergovaline at any of the three transplantation sites (4, 1, and 4 genotypes from the Faroe Islands, northern Finland, and Spain, respectively).

Ergovaline was detected in plants from all three regions, but concentrations varied significantly among different FR +E genotypes, and profiles changed when transplanted to new environments (Fig. 2A). For example, FR +E genotypes from northern Finland produced less ergovaline in Spain than at the region of origin, and FR +E genotypes from Spain that produced minimal amounts of ergovaline at the region of origin had considerable concentrations in southern Finland (Figs. 2A and 3A). In addition, FR +E genotypes from Faroe that produced similar ergovaline contents in northern and southern Finland did not produce detectable amounts in Spain (Fig. 2A).

The concentrations of ergovaline in FR +E genotypes varied among the transplantation sites (Figs. 2A and 3A). In the northern Finland transplantation site, ergovaline was detected in 88% of the FR +E genotypes originally collected in that region (Table 2). On average, the concentrations of ergovaline were high (mean = 305 ng g⁻¹). However, only 27% of the Faroese FR +E genotypes produced ergovaline, with a relatively low concentration (mean = 83 ng g⁻¹) and none of the Spanish FR +E genotypes contained detectable amounts of the alkaloid (Fig. 2A).

In southern Finland, ergovaline was detected in 88%, 45% and 50% of the FR +E genotypes from northern Finland, Faroe and Spain respectively (Table 2). Higher concentrations of ergovaline were detected in genotypes from northern Finland (mean = 312 ng g⁻¹) than in those from Faroe or Spain (Figure 2).

In Spain, 76% of the FR +E genotypes from northern Finland produced ergovaline and again they had a greater concentration than genotypes from the other origins (Table 2, Fig. 2A). None of the Faroese FR +E genotypes produced detectable levels of ergovaline. Only 7% of

the Spanish FR +E genotypes contained ergovaline, and their mean concentration was very low (Fig. 2A).

Ergovaline content above the critical level in diets for livestock ($0.40 \mu\text{g g}^{-1}$) (Hovermale and Craig, 2001) were recorded in nine, two, and two FR +E genotypes from northern Finland, the Faroe Islands, and Spain, respectively. However, the ergovaline concentration varied among transplantation sites; for example, in a FR +E genotype from northern Finland the concentration was above the toxic level found in southern Finland but not in Spain (Fig. 3).

3.2. *Peramine*

Peramine production differed among the geographic origins of FR +E genotypes but not among the transplantation sites (Table 3). None of the FR +E genotypes from northern Finland or the Faroe Islands produced peramine at any of the transplantation sites (Fig. 3B); in contrast, peramine was found in 64% of the Spanish genotypes in at least one transplanting site. Peramine was detected in 50%, 57% and 57% of the Spanish FR +E genotypes growing in northern Finland, southern Finland and Spain sites respectively (Table 2), and the mean concentration ranged between 1.00 and $1.63 \mu\text{g g}^{-1}$ across the transplantation sites (Fig. 2B).

Three of the Spanish FR +E genotypes that produced the insect-feeding deterrent peramine alkaloid did not produce ergovaline at any transplantation site.

3.3. *Alkaloids and plant performance*

Neither the ergovaline nor the peramine content correlated significantly with plant biomass when the effects of plant origin and transplantation site were considered (Table 4). However, the ergovaline content did significantly correlate with the number of flowering culms in the plant (Table 4). When the relationship between ergovaline and reproductive fitness, measured as number of flowering culms, was compared separately for each plant origin in the transplantation sites, a positive correlation was found only in the FR +E genotypes from northern Finland that were growing in southern Finland (Fig. 4).

4. Discussion

Our results demonstrate chemotypic plasticity in wild plants of *Festuca rubra* colonized by *Epichloë festucae*. In alignment with previous studies, we detected differences in alkaloid profiles among colonized genotypes that originated in different geographic areas within the species distribution range (Jensen et al., 2007; Helander et al., 2016). This result supports

previous studies, suggesting that genetic variation among *Epichloë* strains could explain the differences in the amounts and the diversity of alkaloids (Vázquez de Aldana et al., 2010; Charlton et al., 2014; Takach and Young, 2014; Young et al., 2015; Berry et al., 2015). However, the chemotypic plasticity observed, which was remarkable for ergovaline, suggests that the alkaloid diversity in grasses colonized by *Epichloë* could be driven by the interactive effects of plant and fungal genotypes as well as environmental conditions. This finding highlights the importance of quantifying alkaloids under various environmental conditions to better understand the mediation of chemical ecology by *Epichloë* species in grasses.

The genetic basis of alkaloid production in *Epichloë* largely explains the detected differences in chemotypic diversity and plasticity. Peramine is synthesized by a single multifunctional enzyme encoded by the *ppzA* gene (Berry et al. 2019). The enzyme, a nonribosomal peptide synthetase, has a C-terminal reductase domain (R) that is missing in variants encoded by some *ppzA* alleles. These truncated variants synthesize a range of novel pyrrolopyrazine products, but no peramine. Such alleles are commonly found in *E. festucae* (Berry et al. 2019).

In contrast to peramine, which is the final product of a single gene, ergovaline is the final product of a biosynthetic pathway that involves enzymes codified by 11 different genes (Schardl et al., 2013b). Therefore, environmental and genetic influences are more likely to affect the latter biosynthetic pathway, which is much more complex because it is dependent on more genes than the peramine pathway is. Previous studies on *F. rubra* in semiarid grasslands revealed that at the population level, plants symbiotic with genetically close strains of *E. festucae* tended to be similar in terms of peramine content, whereas the ergovaline content of symbiotic plants was less dependent on fungal genotype, possibly due to greater plant genotype and environmental control (Vázquez de Aldana et al., 2010).

Overall, in the present study, ergovaline was more commonly detected in plants than peramine. Eighty percent of all FR +E genotypes produced ergovaline at at least one of the transplantation sites, whereas peramine was produced only by the Spanish genotypes. Both ergovaline and peramine production differed among FR +E genotypes originating in different geographic areas. We assumed that the *E. festucae* strains associated with *F. rubra* that did not contain ergovaline or peramine at any transplantation site were missing the functional genes necessary for the pathway. None of the FR +E genotypes from northern Finland and Faroe Islands produced peramine. These isolates likely carry one of the *ppzA* allelic variants not coding the reductase domain required for the synthesis of peramine, but perhaps producing other pyrrolopyrazine compounds (Berry et al. 2019).

Although peramine was reported as the most common alkaloid among *Epichloë*-colonized grass species (Leuchtmann et al., 2000), its absence has been shown to be common in *F. rubra* (Vázquez de Aldana et al., 2007). Also in line with our results, variability in ergovaline content was detected in *F. rubra* from Mediterranean ecosystems. For example, ergovaline was detected in 50% of the *F. rubra* plants, including their seeds, in Spain (Vázquez de Aldana et al., 2003, 2010), and in a much lower proportion in Italian *F. rubra* plants (Jensen et al., 2007).

Chemotypic differentiation among symbiotic grass ecotypes has been commonly assumed to result from prevailing and/or past abiotic and biotic selection pressures and environmental conditions, such as herbivory or soil characteristics (Lyons et al., 1986; Belesky et al., 1988; Arechavaleta et al., 1992; Boning and Bultman, 1996; Malinowski et al., 1998; Bultman et al., 2004; Rasmussen et al., 2007; Sullivan et al., 2007; Schardl et al., 2012; Helander et al., 2016). Defensive mutualism might partly explain the ergovaline production detected in our study, as the sampled populations in all geographic regions were experiencing heavy vertebrate grazing by reindeer in northern Finland, by sheep in the Faroe Islands, and by cattle in Spain. However, the highest ergovaline content was detected in FR +E genotypes from northern Finland, which suggests that other environmental factors could also modulate ergovaline production.

Similar to the higher variability in ergovaline production compared with peramine production, we detected higher chemotypic plasticity in ergovaline content, while the concentration of peramine in most FR +E genotypes was constant among the transplantation sites. Furthermore, differences in alkaloid content among FR +E genotypes of the same geographic origin revealed that some of them showed higher plasticity than others to produce ergovaline, depending on the environmental conditions. Our experimental sites were located in contrasting habitats from northern to southern Europe, and they differed in environmental factors such as temperature, light intensity, length of the growing season, precipitation, and soil characteristics, which affect alkaloid expression in clonal genotypes. For instance, the availability of nitrogen in the soil can directly affect the potential for the synthesis of ergovaline (e.g. Lyons et al., 1986; Arechavaleta et al., 1992; Vázquez de Aldana et al., 2010). However, the opposite was also observed (Helander et al., 2016), and other studies failed to detect an association between peramine content and fertilization or nitrogen content in plants (Roylance et al., 1994; Faeth et al., 2002; Vázquez de Aldana et al., 2010). We found indications of a positive relationship between mean ergovaline levels in the FR +E genotypes from northern Finland and nitrogen content in the soil across the transplantation sites. The lowest concentrations were observed in the Spanish site, and the highest ones in northern Finland.

This relationship was not detected for soil nitrogen and peramine content, even considering that typical peramine levels were several-fold greater than typical ergovaline levels, and that peramine is about twice as rich in nitrogen than ergovaline.

As precipitation decreases, the frequency of ecotypes containing ergovaline can increase (Zurek et al., 2017). Other greenhouse studies also showed that ergovaline content increased with drought stress (Belesky et al., 1989; Hahn et al., 2008; Vázquez de Aldana et al., 2013), whereas peramine production did not vary according to soil moisture (Roylance et al., 1994). Among our study sites the lowest annual precipitation was recorded in northern Finland (Table 1). However, at higher latitudes, early in the growing season melting water may play a significant role in plant growth. Similarly, in Mediterranean ecosystems, the distribution of precipitation in the growing season appears to be more critical than the total seasonal precipitation. We did not observe visual symptoms of water stress in our experimental plants at harvest time, which suggests that they did not experience drought stress. However, the examination of precipitation and alkaloid levels showed that ergovaline was highest in the northern Finland transplantation site, which had the lowest annual and growth season precipitation. Other contrasting environmental factors among the transplantation sites, which may explain the observed chemotypic plasticity in ergovaline production, are the extreme temperatures and seasonal changes in day length. In Spain, plants should be adapted to periodic heat and drought in summer, and in Finland to long cold winters. Furthermore, environments inside the Arctic and Antarctic circles, such as the transplantation site in northern Finland, are characterized by seasonally limited solar energy for plants because the day length ranges between the extremes of the polar day in summer to the polar night in winter (Saikkonen et al., 2012). How light intensity and day length affect alkaloid expression is unknown. Finally, some studies suggest that increased CO₂ emissions could affect fungal alkaloid production by decreasing metabolite concentrations (Brosi et al., 2011).

Epichloë species are currently used for cultivar improvement in some important agronomic grasses such as *Lolium perenne*, *Schedonorus arundinaceus* or *Festuca rubra*. Endophyte-based cultivar improvement consists of controlling alkaloid type and concentration in symbiotic plants. For forage cultivar improvement, plants containing no alkaloids toxic for mammals, such as lolitrems or ergovaline are sought. At the other extreme there are turfgrass cultivars used in airports, where a high content of alkaloids capable of deterring the presence of small mammals and invertebrates is sought (Johnson et al. 2013; Kauppinen et al., 2016; Pennell et al. 2016). The results of this study point out that unless the absence of genes essential for the synthesis of mammalian toxic alkaloids is verified in *Epichloë* strains used for forage cultivar improvement, a null or low alkaloid profile can change in a different

environment. For example, our results demonstrated that the FR +E genotypes that produced ergovaline concentrations exceeding the $0.40 \mu\text{g g}^{-1}$ safety limit for livestock consumption at one location (Hovermale and Craig, 2001) produced lower amounts at a different one (i.e., northern Finland), and the opposite also occurred, such as in some Spanish FR +E genotypes at the transplantation site in southern Finland. More complex can be the situation where toxic endophytes are sought, like in airport turfgrass cultivars. In those cases a high alkaloid profile in one location might not occur in a different one. Understanding the role of phenotypic plasticity in fungal alkaloid content is crucial in estimating the performance of particular fungal strains planted in different environments as well as in predicting the evolution of these traits in response to climate change.

5. Conclusion

Fungal alkaloid content and profiles vary in wild populations of *Festuca rubra*. In this study, we observed important chemotypic differences among genotypes of *F. rubra* colonized by *Epichloë* that originated in different areas across Europe. We found high variability and plasticity in ergovaline content, while peramine was less common and its content in the plants more constant across transplantation sites. Our results indicate that independently of genetic differences among infected plants, there is a remarkable context-dependency in ergovaline production in *E.festucae*-*F.rubra* associations. This plasticity detected in ergovaline content should be considered in agricultural breeding programs, and the same *Epichloë*-colonized genotype should be tested in different environments.

Acknowledgments

This work was supported by INTERACT (Grant Agreement No. 262693) under the European Community's Seventh Framework Program awarded to KS and MH, Academy of Finland (Projects No. 137909 and 295976) to KS, and Spanish Ministry of Economy, Competitiveness and Innovation, and European Regional Development Fund FEDER (AGL2016-76035-C21R) to BRV and IZ. Thanks are due to Amador Alvarez for his technical help.

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Table 1. Soil characteristics at the three transplantation sites, precipitation at nearby weather stations and global irradiation data.

	Northern Finland (Kevo)	Southern Finland (Turku)	Spain (Salamanca)
Soil parameters			
pH	5.6	6.8	6.9
N (%)	0.172	0.146	0.100
C (%)	2.810	1.498	1.110
P (mg/l soil)	13.4	10.5	39.1
K (mg/l soil)	26.3	198.4	187.4
Ca (mg/l soil)	1242	2696	2231
Mg (mg/l soil)	136.4	313.8	392.4
Meteorological data ^{a,b}			
Annual precipitation from January to December 2013 (mm)	359	557	515
Precipitation in growing season 2013 (mm)	113	201	191
Mean temperature in January (°C)	-13.8	4.0	3.6
Mean temperature in June (°C)	9.0	14.4	18.8
Global horizontal irradiation kWh/m ^{2(c)}	737	956	1687
Growing season	June-August	May-September	March-July

^a Data from the Finnish Meteorological Institute (FMI), at Utsjoki and Turku.

^b Data from the Spanish *Agencia Estatal de Meteorología* (AEMET) at the planting site in Muñovela experimental farm.

Temperature data are the means during the period 1981–2010

^c This value is the annual sum of the solar radiation energy that hits one square meter of a horizontal plane. The average of the period 2012-2014 is presented from data of the European Union - Joint Research Centre, at the coordinates of the experimental transplantation sites. available at: re.jrc.ec.europa.eu/pvg_tools/en/tools.htm

Table 2. Number of *Festuca rubra* genotypes colonized by *Epichloë festucae* from northern Finland, Faroe Islands, and Spain in which alkaloids were analyzed (n) and percentage of them having detectable levels of ergovaline and peramine at each of three transplantation sites: northern Finland, southern Finland, and Spain.

		Transplantation site					
	Region of origin	N Finland		S Finland		Spain	
		n	Percentage	n	Percentage	n	Percentage
Ergovaline	N Finland	17	88	17	88	17	76
	Faroe Islands	11	27	11	45	11	0
	Spain	6	0	14	50	14	7
	total	34		42		42	
Peramine	N Finland	17	0	17	0	17	0
	Faroe Islands	11	0	11	0	11	0
	Spain	10	50	14	57	14	57
	total	38		42		42	

Table 3. Statistical significance (deviance and P value) of differences in ergovaline and peramine production (main effect of region of origin and transplantation site, interaction between origin and site,) using likelihood ratio tests (d.f. = 1) of generalized linear models with a binary response variable (produced or did not produce the alkaloid).

Explanatory variable	Ergovaline		Peramine	
	Deviance	P value	Deviance	P value
Origin	47.00	< 0.0001	59.57	< 0.0001
Site	13.28	0.001	0.04	0.981
Site x origin	8.70	0.069	0.00	1.000

Table 4. Statistical significance (F- and P-values) of differences in biomass and reproductive fitness, evaluated as number of flowering culms, explained by ergovaline or peramine content using likelihood ratio tests between linear models with region of origin and transplantation site as covariates.

Explanatory variable	Biomass		Reproductive fitness	
	<i>F value</i>	<i>P value</i>	<i>F value</i>	<i>P value</i>
Ergovaline	0.02	0.878	6.12	0.015
Peramine	0.30	0.583	0.21	0.651

Fig. 1. Location of the *Festuca rubra* wild plant populations and of the experimental translocation sites.

Fig. 2. Ergovaline (A) and peramine (B) content of genotypes of *Festuca rubra* colonized by *Epichloë festucae* from northern Finland, Faroe Islands, and Spain at the three field transplantation sites in northern Finland, southern Finland, and Spain. Differences between the geographic origins at each transplantation site were determined by Kruskal–Wallis tests.

Fig. 3. Variation of alkaloid concentration of particular *Festuca rubra* – *Epichloë festucae* genotypes from different geographical origins (northern Finland, Faroe Islands, and Spain) planted at different sites (northern Finland, southern Finland, and Spain) in A) ergovaline and B) peramine contents.

Fig. 4. Relationship between ergovaline content and reproductive fitness (number of flowering culms during the growing season) of *Festuca rubra* – *Epichloë festucae* genotypes from northern Finland, Faroe Islands and Spain at the three translocation sites: northern Finland, southern Finland and Spain. Results of Spearman correlation tests are shown (Spearman's correlation coefficient and *P* value).