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Methodological Advances

Plant age and seasonal timing determine endophyte growth and alkaloid biosynthesis

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ABSTRACT

The systemic grass endophyte *Epichloë festucae* var. *lolii* produces alkaloids which can protect the host grass *Lolium perenne* from herbivory. Alkaloid concentrations depend on genetic predisposition of grass and endophyte, and are affected by the environment. However, the role of plant age and seasonal timing remains unknown. We monitored monthly endophyte and alkaloid concentrations in endophyte infected perennial ryegrass over 29 months in a common garden experiment in Germany. Climatic conditions in spring and summer enhanced endophyte growth and alkaloid production, explaining a dominant role of seasonal timing. Alkaloid concentrations also increased with plant age and exceeded the toxic threshold for invertebrates in the first summer and for livestock in the third summer. Our results highlight the key role of plant age and seasonal timing in affecting the toxicity of systemic fungal endophytes of grasses. Endophyte mediated livestock intoxications may increase on European grasslands with global warming.

1. Introduction

Fungal grass endophytes of the genus *Epichloë* affect their host grasses and alter their interactions with above- and belowground species (Kauppinen et al., 2016). Effects of grass endophytes can range from changes in physiological plant processes such as growth promotion (Krauss et al., 2007), higher seed production (Saari et al., 2010), and enhanced drought stress tolerance (Hesse et al., 2003), to alterations in symbiosis with root associated fungi (Liu et al., 2011) and changes in the whole plant community composition (Rudgers and Clay, 2007). The benefit of fungal plant infection is thought to be most pronounced under extreme abiotic conditions such as low nutrient availability and water deficiency (Saikkonen et al., 1998; Malinowski and Belesky, 2000).

The cool-season grass *Lolium perenne* (perennial ryegrass) is globally distributed with high agronomic value. *L. perenne* can be associated with the endophytic fungus *Epichloë festucae* var. *lolii*, a vertically transmitted fungus of the Clavicipitaceae family (Cheplick and Faeth, 2009). *E. festucae* var. *lolii* can negatively affect vertebrate and invertebrate herbivores feeding on the host grass (Shymanovich et al., 2014; Guerre, 2015), indicating a defensive mutualistic relationship between grass and endophyte (Clay, 2014), but see (Cheplick and Faeth, 2009). Herbivore toxicity is caused by several endophyte - derived alkaloids and these are of great agronomic and scientific importance (Lane et al., 2000).

E. festucae var. *lolii* produces three main alkaloids affecting herbivore fitness (Schardl et al., 2004). The pyrrolizidine alkaloid peramine has toxic effects on invertebrate herbivores and confers resistance to insect pests such as the Argentine stem weevil, which is a serious pasture pest species in New Zealand (Rowan et al., 1990). The indole-diterpene alkaloid lolitrem B and the ergot alkaloid ergovaline are tremorgenic mycotoxins for vertebrate herbivores, causing diseases such as ryegrass staggers and fescue toxicosis, respectively, to grazing livestock (Guerre, 2015).

Meta-analyses of the effects of grass endophytes on trophic and community interactions have displayed wide variation in results

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(Saikkonen et al., 2006; Larimer et al., 2010), which may be due to varying alkaloid concentrations. Alkaloids vary in quantity depending on grass and endophyte genotype combination (Schardl et al., 2013; Ryan et al., 2015), and are affected by abiotic factors such as plant nutrition (Malinowski et al., 1998; Helander et al., 2016), temperature (Salminen et al., 2005; McCulley et al., 2014) and drought (Bush et al., 1993, 1997). Years with enhanced endophyte mediated livestock diseases in Australia correlated with rainy spring and dry-warm summer conditions (Reed et al., 2011). Herbivore performance is indirectly determined by abiotic factors affecting grass endophytic fungi, shown by the decreased performance of fall armyworm at higher temperatures, indicating higher alkaloid levels in the host grass (Salminen et al., 2005). In addition to temperature, age of the host grass-endophyte association may determine alkaloid concentrations. Survival of aphids was greater on young plants (Eichenseer et al., 1991) and alkaloid concentrations were lower in endophyte infected young (6 weeks) grass than in older (2 y) grass (Fuchs et al., 2013). Low alkaloid concentrations may derive from low fungal concentration in the plant, as alkaloid and fungal concentrations are correlated under ambient CO_2 conditions (Ryan et al., 2014).

To be toxic to herbivores, alkaloid concentrations must reach a certain threshold in the plant. For example, larval mortality of the Argentine stem weevil increased when feeding on a diet containing a peramine concentration of 2 $\mu g\ g^{-1}$ (Rowan et al., 1990; Siegel and Bush, 1996). Ergovaline caused symptoms in vertebrates at concentrations between 0.3 and 0.8 $\mu g\ g^{-1}$, and lolitrem B caused symptoms at concentrations from about 1.8 $\mu g\ g^{-1}$ (Hovermale and Craig, 2001; Tor-Agbidye et al., 2001). Studies on the ecological role of endophyte infected grass in multi-species interactions have often ignored the impact of plant age and seasonal timing, which together may affect alkaloid concentrations and, therefore, species interactions.

We determined endophyte growth and alkaloid production of the endophytic fungus *E. festucae* var. *lolii* in the grass *L. perenne* over a growth period of three summers and two winters after sowing. We addressed the question of whether plant age and season affect endophyte and alkaloid concentrations.

Our hypotheses were:

(1) Endophyte growth and alkaloid concentrations fluctuate seasonally, corresponding to differing climatic conditions of the four seasons in a temperate region;
(2) Alkaloid concentrations correlate with endophyte concentration independent of plant age;
(3) Alkaloid and fungal concentrations increase with plant age.

2. Materials and methods

2.1. Study design

We sowed 350 seeds of *L. perenne* infected with the endophytic fungus *E. festucae* var. *lolii* (cv. Grasslands Samson 'common toxic' (Johnson et al., 2013)) on 15 April 2013 into round propagation trays (5 × 5 cm), and repotted the germinated plants in June 2013 into bigger pots (18 × 18 × 18 cm) filled with common garden soil (Einheitserde classic CL ED73, Profi Substrat). Pots were placed in a garden outside the University of Würzburg, Germany for the entire study period, from April 2013 through September 2015. Pots were physically separated from natural soil with two layers of plastic sheet.

The grass - endophyte association (cv. Grassland Samson 'common toxic') has been used in previous studies and its effects on aphids and their enemies have been frequently reported (e.g. (Meister et al., 2006; de Sassi et al., 2006; Härri et al., 2008; Fuchs et al., 2013). Plants were watered daily when necessary, to prevent drought effects on the grass and the endophytic fungus. We did not water plants when temperatures were below 0 °C. NPK fertilizer was added at the beginning of March 2014 and 2015 (Compo 20-5-10, equivalent to 200 N kg ha^{-1} yr^{-1}). In March 2014 and March 2015 all senesced tillers were manually removed from the pots to provide space and light for fresh tillers to grow before addition of fertilizer. On the 21st of each month 10 plants were randomly sampled by cutting all aboveground herbage to soil level with sharp gardening scissors. Only green plant material was considered for further analysis. Sampling started 1 month after seeds germinated. We never sampled the same plant twice. Immediately after harvesting, aboveground plant material was frozen in liquid nitrogen. Plant material was subsequently ground and circa 50 mg plant material was used each for qPCR and UPLC-MS analysis.

2.2. Climate data

Climate data were provided by the 'Deutscher Wetterdienst' (DWD Climate Data Cente, 2017), recorded hourly at approximately 1 km distance from the study site at a similar altitude (Latitude: 49.77°, Longitude: 9.96° absolute altitude: 272 m above sea level). We used a 'growing degree day' (GDD) model to show the influence of seasonal timing on fungal and alkaloid concentrations, using published protocols for cool-season grass species (Kreuser and Soldat, 2011; Repussard et al., 2014). GDD was calculated for cool-season grass by summing mean daily air temperatures (°C) with a base temperature of 0 °C. We calculated GDD from 01 January through 31 August (plant maturity with fully developed seeds) in 2014 and 2015 (Saiyed et al., 2009). Seasons were assigned meteorologically for the northern hemisphere; winter including December–February, spring including March–May, summer including June–August, autumn including September–November.

2.3. Endophyte and alkaloid analysis

Endophyte concentration was determined separately for each of the 10 monthly harvested plant replicates by quantitative PCR (qPCR) analysis (for detailed protocol see Fuchs et al. (2017b) modified after Rasmussen et al. (2007)). Genomic DNA (gDNA) was extracted from circa 50 mg powdered grass material. Exact scaling was not necessary because we quantified the amount of endophytic gDNA in relation to amplified grass gDNA. Results represent the ratio of amplified fungal gDNA per 10,000 copies of amplified grass gDNA. For quantification of the endophyte gDNA during qPCR we used a fungal specific primer (for the *Chitinase A* gene) and for plant gDNA quantification we used a grass specific primer (for the *β tubulin* gene) (Fuchs et al., 2017b).

Alkaloids were detected and quantified with ultra-high performance liquid chromatography – mass spectrometry (UPLC-MS) (for detailed protocol see Fuchs et al., 2013). Extraction was performed with dichloromethane and methanol from about 50 mg powdered grass material. Exact scaling was performed with a micro scale (Mettler-Toledo Intl. Inc., Columbus, OH, USA) and results from the UPLC-MS analysis were referred to sample weight ($\mu g\ g^{-1}$). The alkaloids peramine and lolitrem B were both quantified with reference to the internal standard homoperamine. Ergovaline was quantified with reference to the internal standard compound ergotamine. Five hundred ng of each internal standard compound was added in the first step of alkaloid extraction. Detection limit for all alkaloids was 5 ng.

In plant extracts where we neither detected endophytic DNA nor alkaloids, plants were considered as endophyte free and removed from the analysis. Loss of endophyte infection can occur due to seed