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Animal Feed Science and Technology 93: 169-176 (2001)
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Abstract

Ergovaline is an alkaloid toxic to livestock, produced by fungal endophytes infecting grasses. The percentage of seeds infected with the fungal endophyte *Neotyphodium coenophialum* and the presence of ergovaline in seed and forage samples of ten commercial cultivars of tall fescue (*Festuca arundinacea*) were determined. Infection with endophytic fungus was detected in seeds of eight cultivars. The percentage of infection in seeds of these cultivars ranged from 1 to 72%. Ergovaline was analysed by HPLC with fluorescence detection and was detected in the eight cultivars of *Festuca arundinacea* infected by the endophyte *N. coenophialum*. Ergovaline contents in commercial seed lots of endophyte infected *Festuca arundinacea* ranged from 0.02 and 3.71 µg g\(^{-1}\) (average 1.9 µg g\(^{-1}\)). Analysis of ergovaline could be a better method in determining the presence of endophytic fungi in seed samples when the percentage of infection is very low. There was an exponential relationship between the percentage of infection of the seed lot and ergovaline concentration across all cultivars (R\(^2\) = 0.876). The standard error of estimation was 0.618. The ergovaline contents detected in vegetative plant tissue samples of individual plants could cause cattle toxicity problems regarding critical levels reported in literature.

Keywords: endophyte, festucosis, *Neotyphodium*, mycotoxin, tall fescue

Introduction

Tall fescue (*Festuca arundinacea*) is a perennial grass originate from Western Europe, like all species of the tribe *Festuceae* (Buckner, 1985). Its
preferred natural habitats are damp grasslands, river banks and sea-shores (Tutin et al., 1980). Tall fescue is drought resistant and winter hardy, tolerant to poor soils and variable soil pH and is well adapted to the humid temperate areas (Holmes, 1989). It is distributed through most of Europe (but absent from most Russia), N.W. Africa and temperate Asia. The species was introduced in North and South America, Australia, Japan, New Zealand, and South and East Africa. During the late 1940s the cultivar “Kentucky 31” became widely used in the USA and nowadays is grown on more than 14 million ha for forage, turf and soil conservation (Buckner, 1985). In Spain and other European countries, where the grass species is native, tall fescue is widely distributed in natural grasslands. In Spain, sales of tall fescue seed comprises a 5.3% of the total grass seed market (Piñeiro, 1994). This grass species is cultivated for forage in the humid areas of the North, in mixtures with other grass species (Dactylis glomerata, Lolium perenne) and legumes.

The endophytic fungus Neotyphodium coenophialum infects Festuca arundinacea plants. This fungi lives enterely within the host grass, in the intercellular spaces of leaves, stems and reproductive organs; and is transmitted through the seed to the next generation. This grass-fungus association is advantageous to infected plants because they are more resistant to drought and pests that non infected plants. On the other hand, infected plants can cause health problems in livestock due to the production of alkaloids toxic to herbivores (Siegel and Bush, 1996). Fescue toxicosis is a syndrome occurring in grazing livestock and caused by the ingestion of Festuca arundinacea infected by the endophyte Neotyphodium coenophialum. Reduced weight gain, lower feed intake, rough hair coat, high rectal temperatures and
decreased milk production are among the characteristic symptoms of fescue toxicosis (Hemken et al., 1984; Schmidt and Osborn, 1993). These disorders are attributed to the presence of ergopeptine alkaloids. Ergovaline is the most prevalent ergopeptine alkaloid in endophyte infected plants (Yates et al., 1985), and acts as a strong vasoconstrictive and dopaminergic molecule. The endophyte infection of *F. arundinacea* has caused important economic losses in animal production in the USA. These facts promoted that much of the research on the grass-fungus association has been focused on tall fescue, and more precisely on the “Kentucky 31” cultivar.

The incidence of endophyte infection in wild and cultivated tall fescue grasses has been described in several European countries (Oliveira and Castro, 1997; Zabalgogeazcoa et al., 1999; Saikkonen et al., 2000). However, information about the occurrence of toxic alkaloids is scarce. The purpose of this research was to determine the presence of ergovaline in seed and vegetative plant tissue samples of several commercial cultivars of *Festuca arundinacea*. Since the ergovaline alkaloid can cause health problems in livestock, this will help to quantify the importance of endophytic infections in commercial cultivars.

**Material and Methods**

**Plant material**

Seeds of ten commercial cultivars of *Festuca arundinacea* were used in this study. Seed samples were obtained from The Spanish Registry of Cultivated Varieties and seed companies. Infected seeds of the ecotype Her-1 of *F. arundinacea* were used as a positive control. It is a wild ecotype obtained
in Northern Spain. The country of registration and use of each cultivar are listed in Table 1 (MAPA, 1998; OECD, 1998).

Seeds of “Kilimanjaro” and “El Dorado” cultivars were germinated in pots in a mixture of peat, perlite and sand (1:1:1). Separated plants were transplanted and maintained in an experimental farm for one year before harvest. For each cultivar, part of these plants were harvested at the end of May at heading stage. The seeds produced by the remaining plants were collected in July. In this case, a seed sample consisted of seeds produced by 10 to 15 individual plants. A forage sample consisted of the aboveground biomass (leaves, stems and inflorescence) of an individual one year old plant. Seeds were stored in cloth bags and allowed to dry at ambient air temperature. Dry seeds were hand-threshed, cleaned and stored at 4°C. Forage samples were lyophilised and stored at -20°C.

Detection of endophytes

Endophyte infection was determined by microscopic examination of a set of 100 seeds of each cultivar according to the procedure of Latch et al. (1987). Seeds were soaked overnight in a 5% aqueous solution of sodium hydroxide at room temperature. They then were washed with tap water to remove most of the alkali. Seeds were deglumed, mounted on microscope slides, stained with aniline blue (1%) and examined with a microscope. The presence of blue stained hyphae in the aleurone layer indicated that the endophyte was present. The percentage of infection of each cultivar was expressed as the percentage of infected seeds.
Pure cultures of endophytic fungi were obtained from surface disinfected seeds of each cultivar, and fungal endophytes were identified as *Neotyphodium coenophialum* based on the descriptions given by White and Morgan-Jones (1987).

**Analysis of ergovaline**

Ergovaline content was determined in ground seed and forage samples by a modification of the chromatographic method of Shelby et al. (1997) as follows. Samples (5.0 g) were spiked with 50 μl of an internal standard of ergotamine tartrate (Sigma Aldrich) in methanol solution at 100 ppm. Fifty millilitres of methanol/water (70:30 v/v) containing 0.3 % (v/v) ammonium hydroxide were then added to plant material and allowed to stand in the ultrasound bath for 4 hours. After filtering, 25 ml of the filtrate was evaporated at room temperature overnight to a volume of approximately 10 ml. The pH was adjusted to 8.5 with ammonium hydroxide and the filtrate was extracted 3 times with 5 ml of chloroform in a centrifuge tube. The chloroform extract was evaporated at room temperature and the residue was dissolved in 1 ml of methanol/water (70:30 v/v) containing 0.3% (v/v) ammonium hydroxide. Precipitates were removed by centrifugation and samples were then filtered through a 0.22 μm nylon filter. Chromatography of sample extracts was conducted on a Waters 501 HPLC system with a NovaPak C₁₈ (Waters Assoc.) column (150 mm × 3.9 mm) with a guard column of the same material. Detection was by fluorescence on a Waters 474 Scanning Fluorescence Detector (excitation at 310 nm and emission at 415 nm). The mobile phase was methanol:water (40:60 v/v) plus 0.03 % (v/v) ammonium hydroxide (A) and
methanol:water (80:20) plus 0.03 % (v/v) ammonium hydroxide (B). The flow rate was 1 ml min$^{-1}$ in a linear gradient from 100% A to 100% B in 45 min, holding 100% B for 10 min. Samples were prepared in duplicate and the amount of ergovaline was determined from calibration standards. The ergovaline standard used was a gift from Dr. Forrest Smith, Auburn University (USA).

Results

Endophyte infection

The percentage of infection in the cultivars of tall fescue analysed is shown in Table 1. Part of these results were previously published by Zabalgogeazcoa et al. (1998). The presence of the fungal endophyte Neotyphodium coenophialum was observed in eight of the ten cultivars analysed and was not detected in “Houndog” and “Ondine” cultivars. The “Amelie” and “Cadi AX13” cultivars had the highest percentage of infected seeds. In contrast, very low levels of infection were found in seeds of the “Demeter” and “Fawn” cultivars.

The percentage of infection was also determined in seeds produced by the plants grown from commercial seeds of “El Dorado” and “Kilimajaro” cultivars (Table 1). The results show that “El Dorado” had a higher percentage of infection in the commercial seed lot than in the seeds produced by plants in our farm. However, in the “Kilimanjaro” cultivar there were no differences in the percentage of infection between equivalent seed lots.

Ergovaline content
The results of the ergovaline analysis in seeds of *F. arundinacea* are presented in Table 1. Ergovaline was detected in eight of the ten cultivars of tall fescue considered in this study. Ergovaline was not detected in the “Houndog” or “Ondine” cultivars. Very low levels of ergovaline were detected in seeds of the “Demeter” and “Fawn” cultivars. The highest content of ergovaline was detected in the “Cadi AX13”, “Amelie”, “Melba” and “Azteca” cultivars. The mean ergovaline concentration in seeds of the endophyte infected tall fescue cultivars analysed was 1.9 μg g\(^{-1}\).

The ergovaline contents of tall fescue forage grown from commercial seeds were 0.12 μg g\(^{-1}\) in “El Dorado”, 0.11 μg g\(^{-1}\) in “Kilimanjaro” and 1.24 μg g\(^{-1}\) in “Her-1”. Plants of “El Dorado” and “Kilimanjaro” cultivars had similar ergovaline concentrations and lower than that of the “Her-1” reference cultivar.

**Discussion**

Eighty per cent of the *F. arundinacea* cultivars were infected by *Neotyphodium coenophialum*. The percentage of infection in seeds of “Demeter” and “Fawn” cultivars originally was reported to be zero (Zabalgogeazcoa et al., 1998). However, very low levels of ergovaline were detected in seed samples of both cultivars, suggesting the presence of the endophytic fungi. Thus, a new set of 100 seeds of each cultivar was microscopically examined for the presence of endophytes. The presence of endophytic fungi was detected in 1 of the 100 seeds of both “Demeter” and “Fawn” cultivars (Table 1). These results suggest that analysis of ergovaline could be a better method in determining the presence of endophytic fungi in seed samples when the percentage of infection is very low. This is in part due to
the large differences between the sample size of microscopic and ergovaline analysis: a seed sample used for ergovaline analysis includes 5.0 g of seeds, which means 1800-2000 seeds approximately. Therefore, sample size is critical and should be large enough (200 seeds) to establish a confident decision if microscopical examination is the only method used to identify endophyte-free seeds.

Compared with the commercial seed lot, a decrease in the percentage of infection was observed in seeds produced by plants of “El Dorado”. The viability of Neotyphodium in seeds declines during storage (Williams et al., 1984). Thus, some loss of viability may have occurred during the storage of “El Dorado” seed lot; however the percentage of infected seeds in the “Kilimajaro” cultivar did not change suggesting that the endophytic fungus viability in these seeds was not affected by the storage conditions.

Within a cultivar, seed samples of the commercial lot and those seeds produced by the plants in our farm were compared for the ergovaline content. In the “El Dorado” cultivar, the highest concentration of ergovaline was found in the commercial lot and was related to the higher proportion of infected seeds. It seems that storage conditions of seeds can produce loss of fungus viability but the ergovaline mycotoxin prevails in some extent. However, the difference in the percentage of infection between seed lots, seems to be proportionally much greater than difference in the ergovaline content, suggesting that greater levels of ergovaline could be expected in the commercial seed lot with adequate storage conditions to maintain alive the endophyte.

In the case of the “Kilimanjaro” cultivar, seeds of the commercial lot had higher ergovaline concentration than seeds produced by plants. The percentage
of infection was the same in both cases and therefore it does not seem to be a
loss of fungal viability, and consequently a decrease in the ergovaline content.
Such a difference could be related to the variation between years in the
production of ergovaline and has been found in seeds and straw of *F.
arundinacea* (Welty et al., 1994).

The relationship between percentage of infection and ergovaline content
showed the best fitting to an exponential curve of the form, %infection = 2.034
e^{0.92\text{ergovaline}}, R^2=0.876 (Figure 1). The percentage of endophyte infection in a
seed lot of tall fescue could give an approximate estimation of the ergovaline
content (standard error of estimation of 0.618).

The ergovaline seed contents were within the range reported in other
cultivars (Rottinghaus et al., 1991; TePaske et al., 1993; Welty et al., 1994) and
in seeds of *Festuca arundinacea* from natural Mediterranean grasslands
(Vazquez de Aldana et al., 2000). Ergovaline was also detected in forage
samples, but concentrations were lower than those detected in seeds. Such a
difference has been previously reported by Rottinghaus et al. (1991) and
TePaske et al. (1993) and indicates that higher ergovaline levels seems to
accumulate in plant parts which are essential for fungus propagation.

Feeding cattle with diets containing 0.05 µg g^-1 of ergovaline has been
reported to cause measurable physiological effects (increase of body
temperature) in cattle stressed by heat (Cornell et al., 1990). According to
Aldrich-Markham and Pirelli (1995) the thresholds levels for ergovaline in the
diet that produce clinical disease are described in the USA within the range 0.4-
0.7 µg g^-1 in cattle, 0.3-0.5 µg g^-1 in horses and 0.8-1.2 µg g^-1 in sheep.
Threshold levels vary because environment also plays a role in the
development of clinical signs (Aldrich et al., 1993). Concentrations of 0.2-0.3 µg g\(^{-1}\) however have caused measurable changes in physiological functions such as reduced milk production, reduced weight gain (Peters et al., 1992) and are considered likely to cause sub-clinical effects on livestock performance. Our results on ergovaline levels indicate that endophyte infected plants obtained from commercial seeds can reach values potentially toxic for cattle. The effect of dilution of total toxin intake will depend on the percentage of infected plants in the sown meadow. Forage samples were harvested at spring time, and ergovaline concentration can increase in autumn (Belesky et al., 1988). Concentration of ergovaline varies with the season and amount of nitrogen fertilisation, and these factors should be considered.

The use of tall fescue infected by \textit{N. coenophialum} is desirable because it increases the resistance of the plant against abiotic and abiotic stresses. However when livestock producers use it, management practices to reduce the toxic effects of endophyte infected forage should be considered. Since ergovaline is concentrated in the seedhead, any factor reducing seed head formation like grazing swards at young stages before heading, or increasing grazing pressure to reduce formation of seeds, will help to minimize the effect of endophyte infected tall fescue on cattle performance.

Conclusions

Infection of tall fescue by the fungal endophyte \textit{Neotyphodium coenophialum} and production of the ergovaline mycotoxin are common in commercial cultivars. The results suggest that analysis of ergovaline could be a better method in determining the presence of endophytic fungi in seed samples.
when the percentage of infection is very low (1-2 %). On the other the
relationship between percentage of infection and ergovaline content across all
cultivars showed the best fitting to an exponential curve.

Due to the toxic amounts of ergovaline (for livestock grazing) produced
by the fungal endophyte of tall fescue in commercial cultivars, the percentage of
endophyte infection in a seed lot must be a parameter to be considered in
nutritional quality if cultivar is used for forage.

Acknowledgements

This work was funded by the “Junta de Castilla y León “ (CSI3/98).
Beatriz R. Vazquez de Aldana was supported by a Research Contract from the
Spanish “Ministerio de Ciencia y Tecnología”. We are grateful to M. Hernandez
and L.G. Criado for technical assistance in the field and to Dr. Forrest Smith
(Auburn University, USA) for the ergovaline reference sample.
References


Table 1. Cultivar characteristics, percentage of seeds infected by *Neotyphodium coenophialum* and ergovaline content (µg g⁻¹) in commercial seeds of cultivars of *Festuca arundinacea*.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Country of registration</th>
<th>Type</th>
<th>% infection</th>
<th>Ergovaline (µg g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amelie</td>
<td>France</td>
<td>forage</td>
<td>72*</td>
<td>3.71</td>
</tr>
<tr>
<td>Azteca</td>
<td>Spain</td>
<td>turf</td>
<td>23*</td>
<td>2.08</td>
</tr>
<tr>
<td>Cadi AX-13</td>
<td>forage</td>
<td></td>
<td>65*</td>
<td>4.62</td>
</tr>
<tr>
<td>Demeter</td>
<td>Australia, Portugal</td>
<td>forage</td>
<td>1</td>
<td>0.04</td>
</tr>
<tr>
<td>El Dorado</td>
<td>Netherlands, USA</td>
<td>turf</td>
<td>13</td>
<td>0.95</td>
</tr>
<tr>
<td>El Dorado**</td>
<td></td>
<td></td>
<td>4</td>
<td>0.52</td>
</tr>
<tr>
<td>Fawn</td>
<td>USA</td>
<td>forage</td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td>Houndog</td>
<td>Netherlands, USA</td>
<td>turf</td>
<td>0*</td>
<td>nd</td>
</tr>
<tr>
<td>Kilimanjaro</td>
<td>Spain</td>
<td>turf</td>
<td>8</td>
<td>1.26</td>
</tr>
<tr>
<td>Kilimanjaro**</td>
<td></td>
<td></td>
<td>8</td>
<td>0.81</td>
</tr>
<tr>
<td>Melba</td>
<td>forage</td>
<td></td>
<td>17*</td>
<td>2.26</td>
</tr>
<tr>
<td>Ondine</td>
<td>France</td>
<td>forage</td>
<td>0*</td>
<td>nd</td>
</tr>
<tr>
<td>Her-1</td>
<td>reference</td>
<td>forage</td>
<td>98*</td>
<td>4.06</td>
</tr>
</tbody>
</table>

nd = not detected

* Data published by Zabalgogeazcoa et al. (1998)

** Seed samples produced by plants
Figure 1. Relationship between ergovaline content and percentage of endophyte infection in seeds of several cultivars of *Festuca arundinacea*.