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Original Article

Quantification of ergovaline using HPLC and mass spectrometry in Iranian *Neotyphodium* infected tall fescue

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Abstract

Ergovaline, the main ergopeptine alkaloid produced in tall fescue (*Fescue arundinacea* Schreb.) infected with endophyte (*Neotyphodium coenophialum* Morgan- Jones & Gams), is known to cause tall fescue toxicosis. This study was conducted to examine the presence of fungal endophytes in five populations of tall fescue collected from various regions of Iran. The existence of *Neotyphodium* mycelia in the tissues of the samples was confirmed by microscopic examination, and the isolation was performed from leaf tissues of the hosts on potato dextrose agar. All isolates were confirmed as the *Neotyphodium* species by PCR, using specific primers. Mass detection and determination of ergovaline were performed by HPLC at three plant growth stages. Ergovaline was detected in all isolates, with the mean concentrations of 0.24 to 3.48 µg/g dry matter of different populations for the whole three plant growth stages. The differences in ergovaline content between plant populations and sampling time were statistically significant. This is the first report of ergovaline content in endophyte infected *Fescue arundinacea* from natural grasslands in Iran.

Keywords: *Fescue arundinacea*; Alkaloid; Ergovaline; *Neotyphodium coenophialum*; toxicosis

INTRODUCTION

Fescue arundinacea, commonly known as tall fescue, is one of the fescue species. This grass is a cool season perennial C_3 grass species. Tall fescue is adapted to a wide range of soil and climatic conditions and it can grow in most soils including, marginal, acidic, poorly drained soils, low fertility areas, and under the stresses due to drought and overgrazing. These beneficial attributes are now known to be the result of a symbiosis between tall fescue and the fungus *Neotyphodium coenophialum* (Ascomycota, family Clavicipitaceae).

This association between tall fescue and the endophyte fungi is a mutualistic relationship in which both symbionts derive benefits. The fungus remains completely intercellular, growing between the cells of outfield ground parts of its grass host. The fungus is asexual and transmitted to new generations of tall fescue only through seeds, a mode known as vertical transmission. Thus in nature, the fungus does not live outside the plant (1).

The tall fescue endophyte symbiosis confers a competitive advantage to the plant. Endophyte infected tall fescue compared to endophyte free tall fescue deters feeding by insects and mammals, and produces resistance to plant disease and environmental stresses such as soil pH fluctuations (2) and drought (3). Production of several compounds such as various alkaloids, auxin, abscisic acid, kitinase, arabitol, mannitol, and proline is increased in infected grasses by endophytes and some of these are responsible for increased plant fitness (4). The positive effects of the endophyte on the tall fescue host, combined with its deleterious effects on grazing animals, present livestock producers with a biological dilemma (5). If the livestock

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utilize endophyte infected tall fescue of pasture, they suffer toxicosis conditions (6). When the tall fescue is free of endophyte, the risk of the loss of the pasture due to environmental and/or insect stresses (7) will increase.

The alkaloids of endophytic tall fescue include 1-aminopyrrolizidines (lolines), ergot alkaloids (ergovaline), and the pyrrolopyrazine (peramine). Ergopeptines are formed by the nonribosomal peptide synthetase, catalyzing addition of a tripeptide to activated lysergic acid (8). Ergovaline is assumed the main causative agent of fescue toxicosis in livestock (9). Ergovaline causes changes in normal homeostatic mechanisms in animals which may result in reduced weight gain, low forage intake, hormonal imbalances leading to reduced fertility, high respiration rates, elevated core temperatures (with water and shade used to cool body temperature), excessive salivation, restricted blood flow, reduced blood prolactin needed for milk production, and reduced reproductive performance (10).

The ergovaline concentration varies across different endophyte infected tall fescue populations depending on plant and fungus genotypes, plant growth conditions, seasons, plant parts, and environmental conditions such as atmospheric CO_2 concentrations (11).

Due to the possible effect of ergovaline on animal reproduction, information on ergovaline contents in different endophyte infected tall fescue genotypes and seasonal changes of this alkaloid would be required to prevent livestock toxicity. Such information would have a great influence on employment of the symbiotic relationship in crop quality improvement. In this study, we determined the ergovaline concentration in Iranian native endophyte infected tall fescue and examined the differences in ergovaline levels at different developmental stages of the host.

MATERIALS AND METHODS

Plant sources and growth conditions

Twelve genotypes of endophyte infected tall fescues from five different populations, growing in various regions of Iran including,

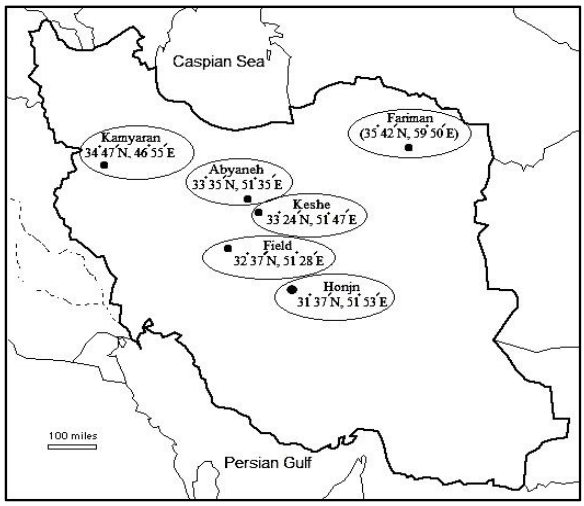

Fig. 1. Geographical distribution (collecting places) of infected tall fescue in Iran.

Fariman in Khorasan province (FaFa, FaFb, FaFc and FaFd), Kamyaran in Kordestan province (FaKa, FaKb and FaKc), Honjan district (FaHa and FaHb), Abyaneh district (FaAa and FaAb) and Keshe district (FaEa) were selected (Fig. 1). The plants were hexaploid (2n=6x=42), and their seeds were transplanted to a field plot on an experimental farm located in Agricultural Biotechnology Research Institute, Central region of Iran (ABRICI, Lat. 32.631943 N, Long. 51.478146) in 2007.

According to the soil experimentation results, field-grown plants were fertilized initially and then each following February with 100 kg/ha of urea fertilizer. The plants were irrigated once every two weeks during their establishment year. The climate of experimental farm is characterized as semi arid continental climate, with the temperature ranging between a minimum mean of 2.1 ºC in the winter and a maximum mean of 28.3 ºC in the summer.

In 2008, the superior part of the plants (herbage) was harvested three times at different stages of growth: first at the end of April (stem elongation stage), second at the end of July (pollination stage), and third at the end of October (tillering stage) (Fig. 2). A particular growth stage was reached when 50% of the plants within the plot had achieved that stage. Maximum and minimum daily temperatures at the Najafabad station were

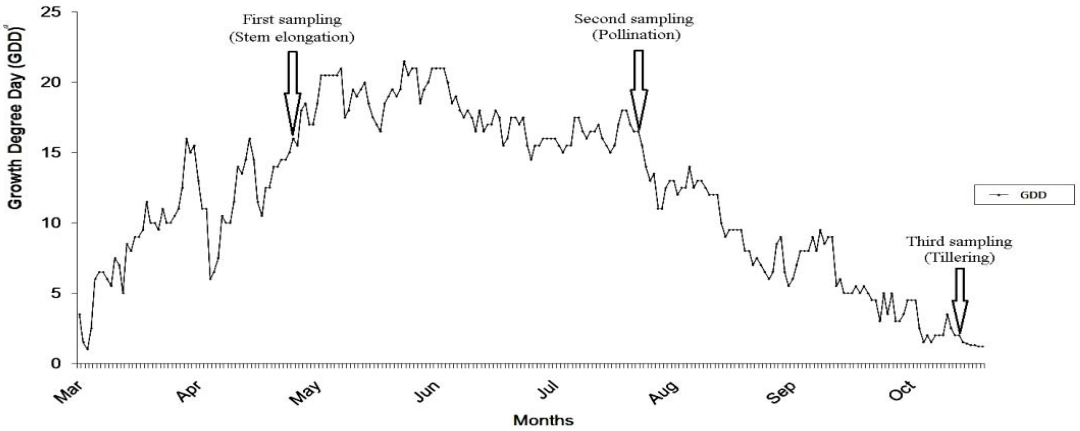

Fig. 2. The trend of growth degree day (GDD) and growth stages of tall fescue in crop year 2008 at the ABRICI, Isfahan. a: GDD are measure of heat accumulation used by horticulturists and gardeners to predict the date that a flower will bloom or crop reach maturity.

used to calculate growing degree days (GDD, a measure of heat accumulation used by horticulturists and gardeners to predict the date that a flower will bloom or a crop reach maturity) with a 5 ºC baseline temperature.

Endophyte detection

Fungal endophytes were identified according to the descriptions given by White and Morgan-Jones (12). The presence of hyphae in plants indicated the presence of the fungal endophyte. Endophyte infection in asymptomatic plants was determined by microscopic examination of the leaf sheaths tissue stained with 5% aqueous ethanolic solution of rose Bengal (Sigma) (13). Infected grasses (100 % infected) were selected for PCR and alkaloid analysis. Pure cultures of endophytic fungi were obtained by culturing infected leaf sheaths pieces in potato dextrose agar.

Molecular detection

Plant and fungal DNA extraction was performed according to a modified method of CTAB (hexacetyldecyltrimethylammonium bromide) extraction (14). Briefly, fresh mycelium or plant (200 mg) were transferred to a sterilized mortar and ground with liquid nitrogen. The resulting powder was transferred to a 1.5 ml micro centrifuge tube containing 500 ml of CTAB. Extracting buffer containing 0.7 M NaCl, 10 mM EDTA, 1% 2-mercaptoethanol, 1% CTAB, and 50 mM Tris-HCl pH 8.0 was then added and the mixture incubated for 30 to 60 min at 65 ºC. An equal volume of SEVAG (chloroform:isoamyl alcohol, 24:1 v/v) was added and samples were gently mixed for 30 min on a rocking platform. The samples were spun in a micro centrifuge for 10 min (15000 *g*) and the aqueous upper phase was transferred to a fresh tube. DNA was precipitated with an equal volume of isopropanol, and the tubes were spun in a micro centrifuge for 10 min. The resultant pellets were washed first with 70% and then 100% ethyl alcohol, dried, and resuspended in TE buffer (10 mM Tris HCl pH 8.0 and 1 mM EDTA). The DNA was re-precipitated with 0.3 M sodium acetate and two volumes of ethanol. The samples were again spun, and the pellets were washed, dried, and resuspended in 200 µl of TE buffer. DNA was quantified by staining with a fluorescent DNA binding dye (Fermentas dye) followed by determination of the fluorescence intensity with a TKO 100 with calf thymus DNA of known concentration used as standard.

PCR primers were designed to amplify the intron region of the tubulin 2 gene (*tub2*) for *Neotyphodium* sp. (14). Sequences of the primers were as follows: IS-tub2w-5 5'-GTG AGT TCA ACC TCT CTG TTT GTC TTG-3' and 5'-GTT GTT GCC AGA AGC CTG TCA-3'. PCR was performed using Hot Star Taq Master Mix (Roche) in a Biometra Thermocycler in 50 µl reaction volumes. Each

reaction contained 20 pmol of each primer and approximately 1 ng of DNA extract as template. The amplification was performed for 32 cycles as follows: starting at 94 ºC for 25 s, annealing at 65 ºC for 1 min, and extension at 72 ºC for 2 min. Products were separated on 1% agarose gels and visualized by staining with 0.2 µg/ml ethidium bromide. Gels were illuminated and photographed using gel documentation equipment (Biometra).

Extraction of alkaloid

Freeze-dried forage samples (50 mg) weighed and transferred into 2.0 ml plastic vials. One ml of 2-propanol/lactic acid mixture containing internal standard, ergotamine tartrate, was added to each vial and the vials were agitated for 90 s at 60000 rpm in a cell disrupter (Heidolph S, Germany). All samples were extracted and maintained in the darkness for 2 h at room temperature. Particles in the extracts were removed by replacing the vial cap with a modified porous cap of 3.2 mm in thickness and 70 µm pore size polyethylene filter (Whatman) fitted onto a second extraction vial and filtering the extracts into the second vial by centrifugation at 6000 *g* for 5 min. The filtrate were transferred into the HPLC vials and were either immediately loaded onto the HPLC or stored at -20 ºC for no longer than 24 h before the analysis (15).

Standard solutions and calibration procedure

A stock solution of ergovaline was prepared by dissolving 10 mg ergovaline standard in 10 ml of distilled water. A series of standard solutions at concentrations of 0.2, 1, 10, 20, 30, 40, and 50 µg/ml were prepared freshly in the morning of each day by diluting the stock solution in distilled water. The prepared standard solutions were kept at 4 ºC until analysis by HPLC.

Blank sample was prepared from whole-extracted samples and stored at -20 ºC. Calibration standards of ergovaline were prepared in blank sample by adding 100 µl of prepared standards at concentrations of 0.2, 1, 10, 20, 30, 40, and 50 µg/ml in 1 ml of blank sample to yield standard ergovaline solutions at concentrations of 0.020-5 µg/ml. A 20 µl aliquot of the final solution was injected into the HPLC column. Calibration curves were constructed by plotting the peak area ratio (y) of ergovaline to the internal standard versus its concentrations (x). Linear regression was used to quantify ergovaline in the sample through determination of its peak area ratio to the internal standard and by reference to the calibration curve.

Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ were calculated from the signal-to-noise ratio by comparing the signal of a test sample with known concentrations of analyte to a blank. As the LOD, analyte concentration with a signal-to-noise ratio of 3:1 was accepted. The LOQ was identified as the lowest plasma concentration of the standard curve that could be quantified with acceptable accuracy, precision and variability.

Liquid chromatography and mass spectrometry (LC-MS) analysis

A Genesis C18 HPLC column (250×4 mm i.d., 4 µm, Alltech) was used for the analyses. The column temperature was maintained at 28 °C. Aliquots of 20 µl of plant extracts were injected into the column through the injection port (S-5111 injector valve, Sykam). The column was developed using a binary gradient of acetontrile (solvent A) and 0.1 M aqueous ammonium acetate (solvent B) as follows: 0 min, 66% v/v solvent A, 34% v/v solvent B; 40 min, 34% v/v solvent A, 66% v/v solvent B. The flow rate was 1 ml/min that controlled by a S-2100 S solvent delivery system (Sykam). Compounds were identified and measured by fluorescent detection at λ_{ex} = 310 nm, λ_{em} = 410 nm using a Sykam RF-10A detector.

Mass spectrometry was carried out by electrospray soft ionization in the positive ion mode using a linear ion trap mass spectrometer (Shimadzu- 2010 EV). Measurements were carried out by selective reaction monitoring of the transition m/z 534 to m/z 268 for ergovaline.

Statistical analysis

A split plots in time assay within a randomized complete block design with

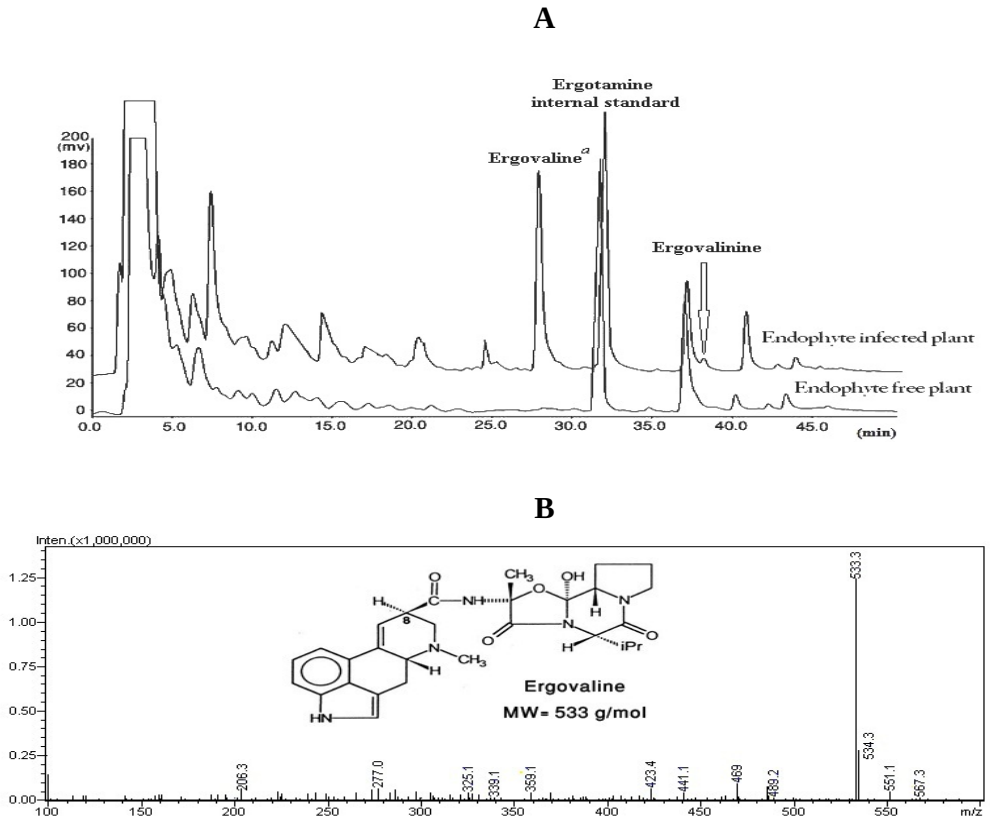

Fig. 3. HPLC analysis of extracts of *Neotyphodium* infected (Upper trace) and free (lower trace) tall fescue (A), and mass spectrum of ergovaline (B).

three replications was used to analyze the effect of plant population and the growth stage (stem elongation, pollination and tillering). Grass samples (cut from the basal regions of the grass plant) were analyzed by HPLC in triplicates. The tests for significant differences between means were performed with Fisher's test of least significant difference (P<0.01). SAS Institute, 2001 was used to perform all statistical analyses.

RESULTS

Endophyte infection

During the initial analysis to detect *Neotyphodium* sp. in the leaf sheaths of tall fescue, a histochemical method, previously described (14), was employed. *Neotyphodium* mycelia was detected in the leaf sheath of all populations. Isolation of fungal isolates was performed by growing *N. coenophialum* out of the infected plant in potato dextrose agar media.

PCR based method for the detection of endophyte was performed using previously described primers (14). The 536-bp fragment size was detected in all the fungi isolates and endophyte infected *F. arundinacea* plants. These results show that all the fungi isolates belong to *Neotyphodium* genus. However, no PCR products were obtained from non-infected grass.

Ergovaline concentrations

Samples were analyzed using LC-MS. A typical chromatogram is presented in Fig. 3. A standard curve with linear regression and a coefficient of 0.997 over the concentration range of 0.020-5 µg/ml was used to determine the quantities of ergovaline in the samples (Table 1). The detection limit for ergovaline was approximately 7 ng/ml at a signal to noise ratio of 3:1 and the limit of quantification was20 ng/ml. The inter- and intra-day precisions and accuracies were within the acceptable range (Table 1).

Table 1. Intra-day and inter-day variability of the HPLC assay for quantification of ergovaline in plant extract.

C (µg/ml)	Intra-day variability				Inter-day variability			
	Mean	SD	CV%	Error%	Mean	SD	CV%	Error%
0.02	0.019	0.001	5.25	5.00	0.022	0.002	9.01	10.0
0.1	0.098	0.005	5.58	0.13	0.1	0.005	5.47	0.06
1	1.026	0.077	7.56	2.66	1.046	0.077	7.42	4.70
2	2.023	0.092	4.59	2.33	2.026	0.133	6.57	2.60
3	3.013	0.112	3.73	1.33	3.046	0.195	6.41	4.70
4	4.046	0.094	2.33	4.66	4.036	0.245	6.08	3.70
5	5.036	0.164	3.26	3.66	5.076	0.222	4.38	7.70

Table 2. Analysis of varied effect of *Neotyphodium* infected genotypes (A) and growth stages (B) on ergovaline levels.

Source of variation	*df*	Sum of squars	Mean squars	*F* ratio	*P*
Block (r)	2	0.057939	0.028969	703.8	<0.0001[a]
Genotype (A)	11	133.2647	12.11497	294327	<0.0001
(eA)	22	0.000928	4.22E-05	1.02	0.4576
Harvesting stage (B)	2	68.17467	34.08734	828134	<0.0001
AB	22	55.21539	2.509791	60974.1	<0.0001
rB	4	0.000189	4.72E-05	1.15	0.347
error	44	0.0018111	4.12E-05		

[a]Significant at the 0.01 level of probability

Table 3. Characteristics of endophytic strains isolated from various hosts and ranking of genotypes based ergovaline concentrations based t-test.

Infected forage	Colony diameter (mm)	Colony color	Mean of ergovaline[a] (µg/g DM)	t-group[b]
FaFa	15-20	White	0.73	G
FaFb	15-20	White	1.05	E
FaFc	15-18	Cream	0.54	H
FaFd	20-25	White	0.36	I
FaKa	15-22	Dark cream	3.48	A
FaKb	15-20	Cream	2.6	C
FaKc	16-22	Light cream	3.13	B
FaHa	20-25	Cream	1.11	D
FaHb	25-30	Light cream	0.86	F
FaAa	15-20	White	0.36	I
FaAb	10-15	White	0.28	J
FaEa	10-15	Dark cream	0.24	K

[a]Values for ergovaline include those of its isomer ergovalinine, [b]Least significant different at the 0.01 level is 0.0063

HPLC analyses showed that no ergovaline was detected in endophyte free plants, while it was detected in all the infected plants. All the infected genotypes produced ergovaline at each of the three harvests, but the content of the alkaloid varied significantly among different endophyte infected genotypes and different growth stages (Table 2). As shown in Table 3 and Fig. 4, for the whole stages, the proportion of plants containing ergovaline ranged from 0.24 µg/g DM in FaEa to 3.48 µg/g dry matter (DM) in FaKa (Values for ergovaline include those of its isomer ergovalinine). The highest concentration was

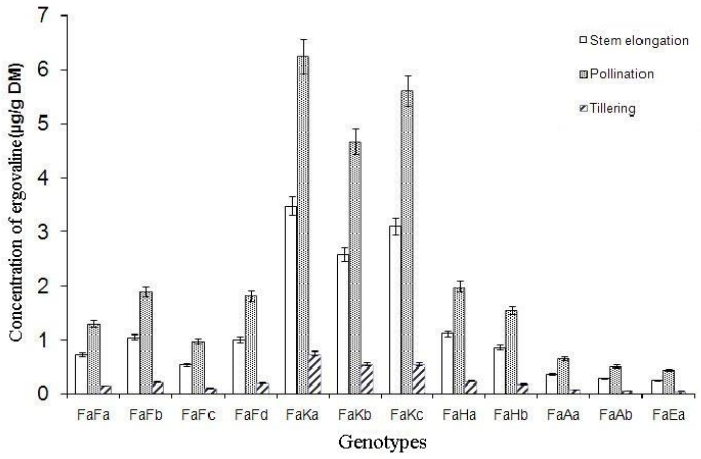

Fig. 4. Variation of ergovaline means in different endophyte infected genotypes of tall fescue and different growth stages. Means of each treatment and control were compared with student t-test (P<0.05).

Table 4. Ranking of growth stages based ergovaline concentrations based t-test.

Growth stage	Mean of ergovaline (µg/g DM)	t Grouping[a]
Pollination	2.20	A
stem elongation	1.22	B
Tillering	0.26	C

[a]Least significant different at the 0.01 level is 0.003

detected in the populations in Kordestan district (FaKa, FaKb, FaKc) and the lowest concentration was detected in the population in Keshe district (FaEa). A t-test revealed that differences in ergovaline concentrations between these genotypes were statistically significant (P<0.05). For whole genotypes, the concentration of ergovaline was lowest (0.26 µg/g DM) in the tillering stage, the highest in the pollination stage (2.20 µg/g DM) and intermediate in the stem elongation stage (1.22 µg/g DM) (Table 4).

DISCUSSION

Tall fescue is an important cool season perennial forage used in the world, including Iran, as a native growth and is an important resource for livestock. Considering that most parts of Iran has a dry climates, drought resistant plants in these areas are very useful. Infection by endophytic fungi symbiosis of tall fescue grants good properties, including resistance to drought to the host. Therefore, endophyte infected tall fescue is desirable to expand vegetation in these regions, providing food for livestock and to prevent soil erosion.

The results presented here show that the presence of *Neotyphodium coenophialum* is necessary for the production of alkaloid ergovaline in Iranian tall fescue. The removal of endophyte from plants would result in no detectable ergovaline production at any conditions. Systemic fungal endophytes in culture can produce ergovaline but at lower concentrations than the infected plant.

Siegel et al. (7) have reported that some endophyte strains in different plant genotypes produce different amounts of alkaloids, suggesting that host plant genotype influences alkaloid production. Our results indicate that the ergovaline quantitie in fescue plant samples hosting endophyte isolates were different. This could be due to genetic variation among the endophytic fungi, or genotypic variation of plant hosts or combination of both factors (16).

An ergovaline concentration of 0.40 µg/g DM in the diet is considered the critical level above which clinical symptoms of fescue

toxicosis may be observed (17). In this study, mean ergovaline concentrations of all genotypes were above this critical value. Furthermore, the results show that ergovaline concentration in endophyte infected populations in Kamyaran region of Kurdistan district (FaKa, FaKb, and FaKc) is more than other populations. In the area where the plants were collected, there are no large grazing herbivores and it seems that the effects of high fungal alkaloid is well known in agronomic grass, *F. arundinacea*, where they are directly linked to livestock toxicosis and neurological disorders in sheep, cattle and horses (18).

Several studies (4,19,20) have addressed the alkaloid resistance to herbivores, not only domestic herbivores but also small mammals, insects and nematodes, of a wide range of both cultivated and wild endophyte infected grasses. This defense against herbivores may provide a selective advantage to infected plants. It is likely that ergovaline production in endophyte infected plants would presumably play a role in plant defense against wild herbivores, and it seems that there is an evolutionary relation in this phenomenon. Another study (20) has suggested that there is a general assumption regarding the positive correlation between the presence of the *lpsA* (lysergyl peptide synthetase) gene and production of ergovaline.

The level of alkaloids was not only dependant on a particular plant fungus association but sometimes also on the time of harvests. The results show that there is a significant difference in the ergovaline concentration with sampling date (Table 4). Ergovaline concentration in *Neotyphodium* infected tall fescue increases in summer when the plants are in reproductive stage. It seems that increasing the temperature at this stage would cause an increase in mycelium growth in tissues and production of more ergovaline. Furthermore, an increase in ergovaline concentration in endophyte infected *F. arundinacea*, challenged by a water deficit, has been reported. This suggests that ergovaline may also provide an advantage in water stress conditions and be related to salinity tolerance. Several studies have shown that endophyte infected grasses are more tolerant to drought and other abiotic stresses (2,3). In reproductive stage, plants usually confront to drought stress for compensation of aforementioned difficulties, by allowing the fungi to increase growth and to produce alkaloids and other compounds to lessen effects of stress on the plant. In the harsh conditions, where the plants were found, the cost of harboring the endophyte and the cost of producing this secondary metabolite should bring benefits for them.

CONCLUSION

Considering the variation in ergovaline concentrations between different growth stages, we would suggest that the harvest time scheduling should be based upon the likelihood of maximum ergovaline level in the plant (Fig. 4). The consumption of all endophyte infected populations (50-100% infected grass) by livestock for more than 10 continuous days, from elongation stage until seed ripening, can be harmful with clinical effects. In these stages, three course of action can be taken for renovation of infected pastures: 1) dilution with a legume; 2) killing the fescue and reseeding a cool season grass; 3) killing the fescue and reseeding a warm season grass.

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