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Original Contribution

ERGOVALINE LEVELS IN IRANIAN ECOTYPES OF *FESTUCA ARUNDINACEA* SCHREB

Ah. Sobhani Najafabadi[1], M. Reza Mofid[2*], M. Solouki[3], R. Mohammadi[1]

[1]Department of secondary metabolite, Agricultural Biotechnology Research Institute Central region of Iran (ABRICI), Isfahan, Iran
[2]Dept. of Biochemistry, School of Pharmacy and Isfahan Pharmaceutical Sciences Research Center, Isfahan University of Medical Sciences, Isfahan, Iran
[3]University of Zabol (UOZ), Zabol, Sistan and Balochestan, Iran

ABSTRACT

Ergovaline is an alkaloid toxic to livestock, produced by fungal endophyte infecting cool season grass. The presence of ergovaline in seeds and percentage of seeds infected with the fungal endophyte *Neotyphodium coenophialum* Morgan- Jones & Gams in 12 ecotypes of tall fescue (*Festuca arundinacea* Schreb.) were determined. Ergovaline was analysed by HPLC with fluorescence detection in all ecotypes. Ergovaline contents in seed lots of endophyte-infected *F. arundinacea* Schreb. ranged from 1.23 to 8.11 µg/g (average 4.95 µg/g). The percentage of infection determined by histochemical and ergovaline contents method.

Key words: *Neotyphodium* endophyte, symbiosis, seed, grass, alkaloid, fungi

INTRODUCTION

Since drought is the important problem in the world, finding the resistant mechanisms for plant is vital. Nonpathogenic, seedborne, fungal endophytes are commonly found in symbiotic relationships with many members of the cool season grass subfamily *Pooideae.* These endophytes have been shown to convey to the host grasses certain advantageous physiological and ecological characteristics such as increased vigor, tolerance, and resistance to drought and pests (1-2). These fungi improve their host growth by producing several compounds such as various alkaloids, oxine, abscisic acid, kitinase, arabitol, mannitol, and proline which have been previously investigated. Other fungal-derived alkaloids, however, can be harmful to livestock, reducing the forage value of endophyte-infected grass, for example ergovaline (3, 4, 5, 6). Ergovaline is the most prevalent ergopeptine alkaloid in endophyte-infected plants (7), and acts as a strong vasoconstrictive and dopaminegic molecule.

Tall fescue, *Festuca arundinacea* Schreb., an important forage, pasture, and turf grass, is sometimes infected with endophytic fungi that grow asymptomatically within the grass foliage (8). These fungi, formerly placed in the genus *Acremonium*, have recently been assigned to the new genus *Neotyphodium* (9), based on DNA sequence analysis. Molecular analysis has demonstrated that they are descended from fungi of the genus *Epichlöe* (10).

The endophyte infection of *F. arundinacea* Schreb. has caused important economic losses in animal production in the world. Hence, from an agronomic perspective, whether or not a tall fescue genotype is infected with endophyte is an important determinant of end use (11). Apparently, during the breeding process endophyte strains were selected which could significantly improve plant persistence.

The aim of this study was determined ergovaline amount in seeds of tall fescue and investigated the relationship between

***Correspondence to:** *Mohammad Reza Mofid,Dept. of Biochemistry, School of Pharmacy and Isfahan Pharmaceutical Sciences Research Center, Isfahan University of Medical Sciences, Isfahan, Iran. Hezarjerib str., 81746-73461, Isfahan, Iran. mrezamofid@yahoo.de. Ph (+98)-311-79225Fax (+98)-0311-6680011*

ergovaline amount and the speed of seed mycelium emergence in PDA media.

MATERIALS AND METHODS

Seed source

In this study was used of 12 ecotypes of *Neotyphodium*-infected tall fescue from 6 different ecological zones consisting, 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). Each plant was hexaploid (2n=6x=42) and the seeds were cultivated in field located in Agricultural Biotechnology Research Institute Central region of Iran (*ABRICI*).

The seeds of accessions store at 4-5˚ C and 30-35% relative humidity since 13 October 2007 for no longer than 1 year.

Histochemical detection

Initial screening for the detection of fungal endophytes was done by squashing 15 to 20 seeds of each accession and examining the aleurone layer and adjoining seed coat for fungal hyphae. At first, the seeds were soaked in 5% NaOH for 16 h at room temperature, washed thoroughly in sterile deionized water, and stained for 36 to 48 h in 5% aqueous ethanol, rose Bengal (Sigma) (12). Then, individual seeds were placed on a microscope slide and squashed under a cover slip, and observed microscopically (400X zoom) for fungal hyphae. In this way, the percentage of infected seeds was determined.

Tissue culture techniques

Seeds were sterilized in 50-ml Costar tubes with 70% ethanol for 1 min, followed by rinsing with sterile water, then soaked for 5 min in 5 % sodium hypochlorite and plated on potato dextrose agar (39 g per liter of PDA, Merck KGaA) with penicillin (167 units/ ml) and streptomycin (76.3 units/ ml). The plates were held in dark at 25˚ C. Speed of fungi growth measured. After 30-60 days, fungal mycelium growth occurred. Then, 5 pieces of fungal mycelium grown out (approximately 0.5 cm) of the plant material was removed from the agar and sub cultured in new PDA media to growth (13).

Molecular detection

Isolation of genomic DNA

Seed and fungal DNA extractions were performed according to the modified method reported by Groppe et al. (14):

Fresh mycelium and seed (200 mg) were transferred to a sterilized mortar and grinded with liquid nitrogen, then transferred in 1.5-mL Eppendorf (micro centrifuge) tube containing 500 ml of CTAB (hexacetyldecyltrimethylammonium bromide), extraction buffer (0.7 M NaCl, 10 mM EDTA, 50mM Tris-HCl [pH 8.0], 1% 2-mercaptoethanol, 1% CTAB) and incubated for 30 to 60 min at 65˚C. An equal volume of SEVAG (chloroform-isoamyl alcohol [24:1, vol/vol]) 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 (15,000 × *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 with 70 and 100% ethyl alcohol, dried, and resuspended in TE (10 mM Tris HCl [pH 8.0], 1 mM EDTA). The DNA was reprecipitated with 0.3 M sodium acetate and 2 volumes of ethanol. Samples were again spun, and the pellets were washed, dried, and resuspended in TE (200 ml). DNA was quantified by staining with a fluorescent DNA-binding dye (Fermentas dye) followed by determination of fluorescence with a TKO 100 with calf thymus DNA of known concentration as a standard.

Primer Selection, PCR Protocol and detection of products

PCR primers were designed to amplify *PerA* gene for *Neotyphodium* spp. (15). Sequences of the primers are as follows: PerA- F: 5'- ATC AAC AAC CgA CCT ATC AgT CCT gAA gCT -3' and PerA- R: 5'- CCg ACC Cgg gTg gCC ggA AAC ACC ACg CAA -3'. PCR was performed in a Biometra Thermocycler in 15 µL reaction volumes. Each reaction contained 10 pmol of each primer and approximately 1 ng of DNA extract as template. A standard three step PCR was used, starting at 95˚C for 30 s, annealing at 58˚C for 30 s, and extension at 72˚C for 1 min for 30 cycles.

Products were separated on 1.2% agarose gels and visualized by staining with 0.2 µg ml^{-1} ethidium bromide. Gels were illuminated and photographed on a Biometra imager.

Ergovaline analysis

Sample Extraction

Dried seed samples (50 mg) were weighed into 2.0 ml plastic vials and extracted in 1 ml of

aqueous 2-propanol (50% v/v) with 1% (w/v) lactic acid, unless indicated otherwise. 2-propanol-lactic acid containing the internal standard ergotamine tartrate was added to each vial, and the vials were agitated for 90 s at setting 60000 rpm in a cell disrupter (Silent crusher S- Heidolph). All samples were extracted standing in darkness for 2 h at room temperature. Particulate material in the extracts were removed by replacing the vial cap with a modified cap containing a porous 3.2 mm thick, 70µm 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 (18 Centrifuge- Microfuge). The filtered extracts were transferred into HPLC vials. The extracts were either immediately loaded onto the HPLC or stored at -20 °C for no longer than 24 h before loading (16). Handling of extracts in this way ensured that no detectable degradation of the ergopeptines occurred between collection and analysis.

Ergovaline Analysis by HPLC

The column was a 250×4 mm i.d., 4µm, Genesis C18 (Alltech), and the column temperature was 28˚C. Sample extracts (volumes between 20µL) were injected with injector (S-5111 injector valve breacket, Sykam) onto the column. Multilinear binary gradients for the separation of ergovaline in the HPLC were run as follows: 0 min, 66% (v/v) solvent A, 34% (v/v) solvent B; 40 min, 34% solvent A, 66% solvent B. Solvent A was acetonitrile and solvent B was aqueous ammonium acetate (0.1 M). The flow rate was 1 ml/min, controlled by a S-2100 S solvent delivery system (Sykam). Each compound was identified and measured by fluorescent detection λ_{ex} = 310 nm, λ_{em} = 410 nm, Sykam RF-10A detector.

RESULTS

During our initial analysis to detect *Neotyphodium* spp. in seed of tall fescue, we utilized previously described histochemical method **(Figure 1).** Fungi mycelium concentration and percentage of infected seeds were different between ecotypes **(Table 1)**. However, all ecotypes infected with endophytic fungi.

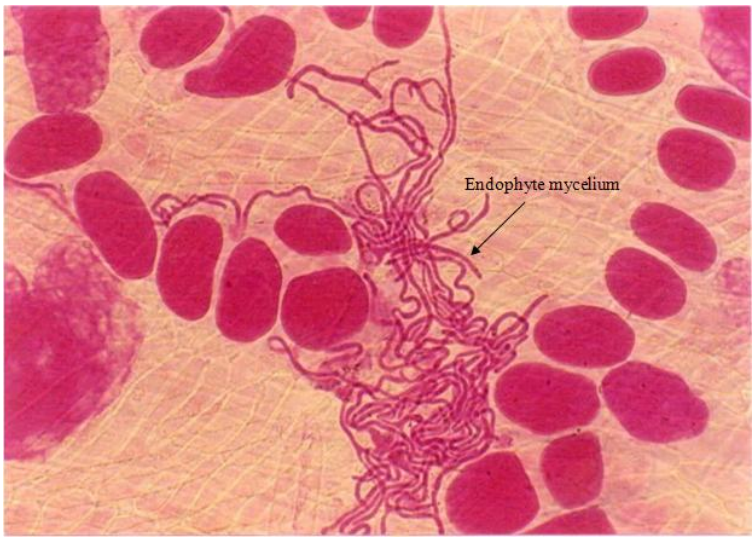

Figure 1. *Neotyphodium*-infected FaFa seed according to rose Bengal method (400X zoom with Nikon light microscope). 1- Amyloplast, 2- Endophyte mycelium's.

Seeds of infected samples were cultured in PDA media for selected vital endophyte-infected seeds and then planted in the field **(Figure 2)**. All endophytic fungi grew in PDA media with different growth speed. For instance, mycelium of ecotype FaKa seed (Isolate FaKa) advent after 21 days in the case ecotype FaEa seed it was after 60 days.

Table 1. *Characteristics and ergovaline amount of Neotyphodium isolate.*

Isolate	Infection %	Days to emerge from seed	Colony diameter (mm)	Colony color	Ergovaline content (µg/g)
FaFa	90	55	15-20	White	4.10
FaFb	75	55	10-15	White	3.90
FaFc	80	60	15-20	Cream	2.86
FaFd	90	50	15-20	White	4.02
FaKa	90	30	15-25	Dark cream	8.11
FaKb	95	37	15-20	Dark cream	6.84
FaKc	90	35	12-16	Light cream	6.50
FaHa	90	40	20-25	Cream	5.80
FaHb	90	35	25-30	Light cream	6.78
FaAa	80	45	8-15	White	4.52
FaAb	85	50	5-10	White	5.10
FaKa	85	60	5-10	Dark cream	1.23

Figure 2. *Neotyphodium* fungi isolation in PDA media from FaAa seed.

Detection endophyte base PCR performed with previously described primers. Using the primer pair PerA-F/R, the 243-bp fragment **(Figure 3)** was amplified from all fungi isolates and endophyte-infected *F. arundinacea* plans. Therfore, all fungi isolate belong to *Neotyphodium* genus **(Figure 3)**. However, no PCR products were obtained from non-infected grass.

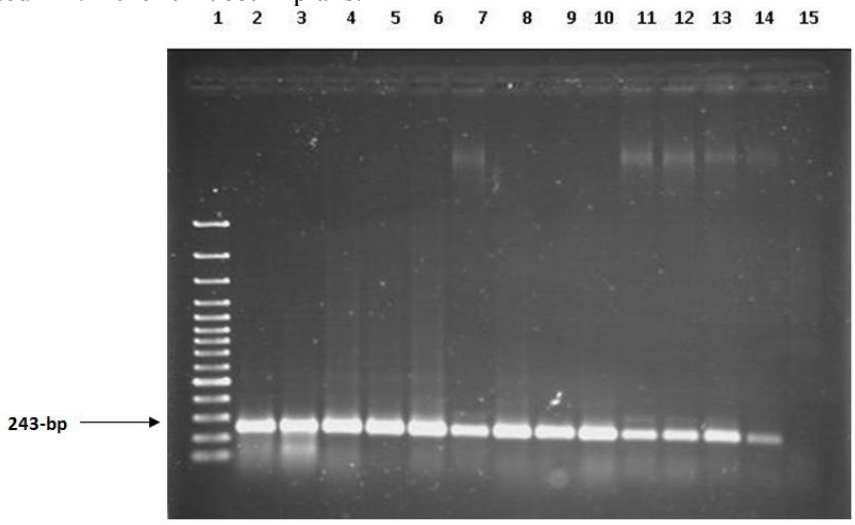

Figure 3. An ethidium bromide-stained agarose gel shows 243-bp band from amplification of 12 *Neotyphodium*-infected tall fescue DNA extracts with PerA primers. 1: molecular weight marker (kb), 2 to 13 mycelium of Neotyphodium Isolate: 2- FaFa, 3- FaFb, 4- FaFc, 5- FaFd, 6- FaKa, 7- FaKb, 8- FaKc, 9- FaHa, 10- FaHb, 11- FaAa, 12- FaAb, 13- FaEa, 14- tall fescue infected FaFa and 15- Non- endophyte-infected tall fescue.

Ability to produce ergovaline was investigated in all endophyte-infected *F. arundinacea* collected in Iran. Ergovaline was detectable in HPLC method with fluorescent detector. Retention time (RT) of ergovaline is 28 min **(Figure 4a)**. Ergovalin mass is 533.1 g/mol that was showed with LC-MS **(Figure 4b)**. Very low levels of ergovaline were detected in seeds of the FaEa ecotype (1.23 µg/g). The highest content of ergovaline was detected in seeds of FaKb ecotype (8.11 µg/g). The mean ergovaline concentration analyzed in seeds of the endophyte-infected tall fescue ecotypes was 4.95 µg/g.

DISCUSSION

In this study tall fescue germplasm collection were examined originated from seeds collected in the wild pasture of Iran. Histochemical and PCR examination of this accession showed the presence of endophytic fungi. Production of the 747-bp band by PCR, originating from genomic DNA of fungal isolates, provided an indication of the existence of the PerA gene sequence in endophytes isolated from plants endogenous to Iran. Tall fescue infected by *N. coenophialum* is desirable because the fungus increase the plant resistance against abiotic and biotic stresses. However, when livestock producers use it, management practices should be considered to reduce the toxic effects of endophyte-infected forage.

All endophyte-infected *F. arundinacea* plants contained ergovaline. The ergovaline quantities in fescue seed samples hosting endophyte isolates found to be different. This can be caused by genetic variation among the endophytic fungi for ergovaline production or genotypic variation of plant hosts or a combination of both factors (17-18-19). Because the mycelium was not quantified no statistic analysis was performed on the quantitive data.

Ergovaline was also detected in forage samples, but concentrations were lower than those detected in seeds. Richardson et al (20) reported, *Neotyphodium*-infected grass seeds have 10% biomass rather than non-infected seeds and germination of infected seed is better than non-infected seeds. This difference indicates that higher ergovaline levels seem to accumulate in plant parts which are essential for fungus propagation.

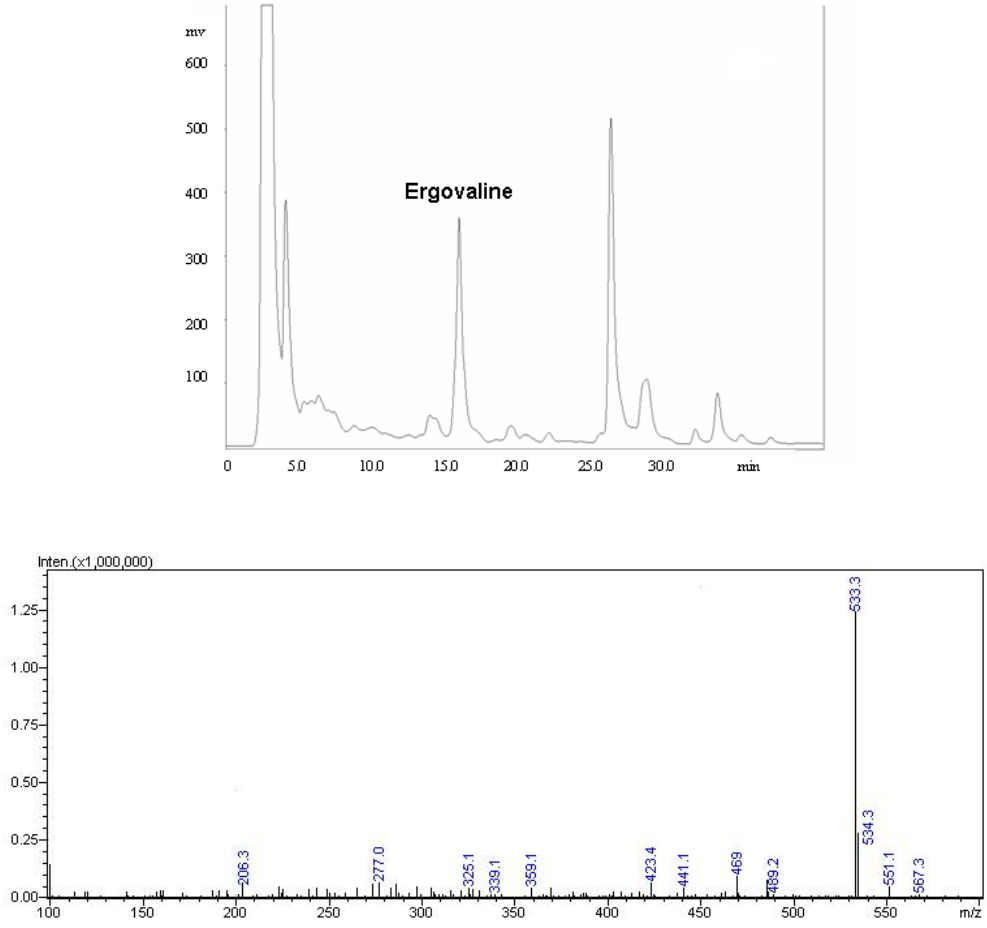

Figure 4. HPLC chromatograms (ChromStar light software version 6.3) of *Neotyphodium*-infected tall fescue seeds (A) and spectrogram of ergovaline (B).

CONCLUSION

Our results indicated that the endophyte mycelium advent earlier in infected seeds with more ergovaline content in PDA media. The results suggest that amount of ergovaline have positive relation with endophyte growth speed. The speed of mycelium advented in PDA media may be related with mycelium density in seed or with ergovaline content. In other words endophytic ergovaline could be benefit for supplied nitrogen for mycelium growth.

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