Isolation and Characterization of New Neotyphodium Infected Tall Fescue in Iranian Rangelands

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Abstract: Tall fescue (Festuca arundinacea Schreb.) which is the prominent Iranian pastures grass used as forage and lawn, was infected by the endophytic fungus, Neotyphodium coenophialum Morgan-Jones & Gams specifically. Persistence and competitiveness of tall fescue is superior when infected with the endophyte. The objective of our research was to detect endophytic fungus Neotyphodium in native Iranian pasture. We found 6 new endophyte infected seeds was confirmed by histochemical (Detection with microscope) method and specific PCR in seeds. Histochemical examination of this accession showed the presence of endophytic fungi in their seeds. Then one pair specifically primer designed from tub2 (tubulin2) gene of Neotyphodium sp used for sensitive endophyte detection.

Key words: Festuca arundinacea, Symbiosis, Grass, PCR

INTRODUCTION

Endophytic fungi of the genus Neotyphodium live in symbiosis with many cool-season grasses. The endophyte colonizes the intercellular spaces of all aerial plant parts and depends entirely on the host for nutrition and dissemination via seeds. These fungi do not produce infectious propagules and complete their entire life-cycle within the plant (Siegel and Bush, 1994).

Furthermore, endophyte infection in tall fescue can significantly benefit from the association in several ways, including enhancement of vigor, greater drought tolerance and increased resistance to attack by insects and other herbivores (Omacini et al., 2001; West, 1994). Insect resistance is conferred by the presence of toxic alkaloids produced by the fungus that are present in the grass foliage (Rowan and Latch, 1994).

Tall fescue, Festuca arundinacea Schreb., an important forage, pasture, and turf grass, is sometimes infected with endophyte fungi that grow symptomatically within the grass foliage (Hill et al., 2005). These fungi, formerly placed in the genus Acremonium, have recently been assigned to the new genus Neotyphodium, (Glenn et al., 1996) based on DNA sequence analysis. Molecular analysis has demonstrated that they are descended from fungi of the genus Epichloë (Ascomycotina, Clavicipitaceae) (Leuchtmann and Clay, 1990).

The aim of this study was detection of new Neotyphodium coenophialum isolate in native Iranian tall fescue seeds using histochemical, molecular and tissue culture techniques.

MATERIALS AND METHODS

Seed Source:
The tall fescue accessions used were from the germplasm collection at the Agricultural Biotechnology Research Institute Central region of Iran (ABRICI). Seeds of these accessions were collected from Iranian rangelands and store at 4-5°C and 30-35% relative humidity since 13 October 2007 for no longer than 1 year.

Histochemical Detection:

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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) (Saha et al., 1988). Then, individual seeds were placed on a microscope slide and squashed under a cover slip, and observed microscopically for fungal hyphae. In this way, the percentage of infected seeds was determined.

**Tissue Culture Technique:**
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⁻¹) and streptomycin (76.3 units ml⁻¹). 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 (Bacon et al. 1994).

**Molecular Detection:**

**DNA extraction:**
Seed and fungal DNA extractions were performed according to the modified method reported by (Groppe et al., 1997).

Fresh mycelium and seed (200 mg) was transferred to a sterilized mortar and ground with liquid nitrogen, then transferred in 1.5-mL Eppendorf tube containing 500 ml of CTAB (hexadecyltrimethylammonium bromide), extraction buffer (0.7 M NaCl, 10 mM EDTA, 50 mM 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-isooamyl 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).

Primer Selection, PCR Protocol and detection of products
PCR primers were designed to amplify the intron region of the tubulin 2 gene (tub2) for Neotyphodium spp. (3). Sequences of the primers are as follows: IS-tub2w-5'-GTG A GT TCA ACC TCT CTG TTT GTC TTG-3' and 5'-GT T GT T 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. A standard three step PCR was used, starting at 94°C for 25 s, annealing at 65°C for 1 min, and extension at 72°C for 2 min for 32 cycles.

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

**RESULTS AND DISCUSSION**
In this study detected 6 new ecotype of Neotyphodium infected tall fescue from 4 different ecological zones consisting, Honjan (FaHa, FaHb), Abayanch (FaAa, FaAb), Keshe (FaKa), Margon (FaMa) districts. Each plants were hexaploid (2n=6x=42) and their seeds were cultivated in field located in Isfahan.

During our initial analysis to detect Neotyphodium spp. in seed of tall fescue, we utilized previously described histochemical method (Figure 1). Fungi myceliums were detected in all ecotypes seeds but amount of mycelium were different.

Seeds of infected samples were cultured in PDA media (Figure 2) for selected vital endophyte infected seeds and then planted in the field. All endophytic fungi grew in PDA media with different growth speed (Table 1). For instance, mycelium of ecotype FaHa seed (Isolate FaHa) emerged after 35 days and ecotype FaKa seed (Isolate FaKa) emerged after 60 days.

Detection endophyte base PCR performed with previously described primers and was indicated that these fungi belong to Neotyphodium genus (Figure3).
Fig. 1: *Neotyphodium* infected FaHa seed (A) *Neotyphodium* free FaHa seed (B) according to rose Bengal method (400X zoom with Nikon light microscope). 1- Amyloplast, 2-Endophyte mycelium's.

Fig. 2: *Neotyphodium* fungi isolation in PDA media from FaAa seed (A) subcultured colony of FaHa (B), FaHb (C), FaAa (E) FaAb (F), FaKa (G), FaMa (H)

Fig. 3: An ethidium bromide-stained agarose gel shows 536 bp band from amplification of 6 Neotyphodium -infected tall fescue DNA extracts with IS-tub2w-5 primers. 1 to 6 tall fescue infected seed with 1- FaHa, 2- FaHb, 3- FaAa, 4- FaAb, 5- FaKa, 6- FaMa and 7 to 12 fungi isolate of 7- FaHa, 8- FaHb, 9- FaAa, 10- FaAb, 11- FaKa, 12- FaMa, 13 and 14 respectively Neotyphodium free FaKa and FaMa, M- molecular weight marker (kb).

Presence of the endophytic fungus Neotyphodium coenophialum in tall fescue (Festuca artuidinacea Schreb) enhances host persistence in drought prone environments. However, the physiological mechanism is not well understood. (West et al, 1994) reported that Neotyphodium endophyte infection affect on water deficit on osmotic adjustment, stomatal conductance, tiller survival and leaf elongation. Also endophyte infected tall fescue was found in arid pasture of Iran.

In this study tall fescue germplasm collection were examined originated from seeds collected in the wild pasture of Iran. Histochemical examination of this accession showed the presence of endophytic fungi, as did a subsequent tissue culture test. To survey endophyte infections in seeds and plants efficiently it is critical to have a simple, fast, and reliable method of detection. Microscopic examination has been shown to be a reliable approach for this purpose. However, it does require specific expertise and training to be able to identify fungal infections in tissue and seed. In addition, histochemical staining of fungal hyphae is a nonspecific process that could result in false positives or conversely.

Microscopic examination may not be as sensitive as PCR method and can miss endophyte infected plant and seed samples with sparse hyphae. PCR has been shown to provide a reliable, sensitive and rapid approach to fungal detection in seed and plant tissues (Choicchetti et al., 2001; Dongyi and Kelemu, 2004; Doss and Welty, 1995; Groppe and Boller, 1997). Our results indicate that by using 1 ng of isolated DNA template, this PCR strategy has sufficient sensitivity to detect Neotyphodium spp. Furthermore this primer, based on the intervening sequence of a B-tubulin gene (tub2) that was present in all of Neotyphodium spp. isolates.

Since histochemical and PCR methods couldn’t distinguish alive endophyte infected seed from dead, there’s a need for a method to show which seeds have infected with alive endophyte. Seed culture in field and microscopically investigation the leaf sheath and seed culture in PDA media are the best methods for this aim. The seeds were cultured in PDA media to distinguish alive endophytes according to the fungal growth on the media (Table 1). All seeds harvested concurrently at autumn but the speed of apparent mycelium in PDA media were different.

| Table 1: Characteristics of new Neotyphodium isolate in PDA media. |
|----------------|----------------|----------------|----------------|
| Isolate | Days to emerge from seed | Colony diameter (mm) | Colony color | villus |
| FaHa | 40 | 20-25 | Cream | + |
| FaHb | 35 | 25-30 | Light cream | + |
| FaAa | 45 | 8-15 | White | + |
| FaAb | 50 | 5-10 | White | + |
| FaKa | 60 | 5-10 | Dark cream | + |
| FaMa | 55 | 25-30 | Light cream | + |

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2547


