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Impact of endophytic fungi on seed and seedling characteristics in tall and meadow fescues

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Abstract

Tall fescue and meadow fescue are naturally infected with fungal endophytes, *Neotyphodium coenophialum* and *Neotyphodium uncinatum*, respectively. Seed traits are an important measure of the effects of the endophytic fungi which has been less addressed. In this study we used endophyte-infected (EI) and endophyte-free (EF) clones of tall and meadow fescue genotypes in the field to investigate the effects of endophytes on seed production traits and subsequently on seed germination and seedling emergence. Endophyte infection increased the plant seed weight, the number of seeds per plant and the number of panicles per plant in both plant species. Seed germination was not affected by endophytes in tall fescue, while it was improved in endophyte-infected plants of meadow fescue. A negative endophyte effect was detectable for seedling emergence percentage and emergence rate of tall fescue, whereas EI plants of meadow fescue were positively affected by the endophyte for these characteristics. The better performance of EI plants for seed production traits may increase their relative fitness in populations and cause them to be more stable in different environmental conditions.

Keywords: Fescue; Neotyphodium; Seed yield; Seed germination; Seedling emergence.

Running heading: Impact of endophytic fungi on seed related characteristics.

Introduction

Tall fescue (*Festuca arundinacea* Schreb.) and meadow fescue (*Festuca pratensis* Huds.) are two important cool-season grasses of the genus *Festuca*. The higher drought tolerance of these species compared to other cool-season grasses such as *Lolium perenne* (perennial ryegrass) and *Poa pratensis* (Kentucky bluegrass) makes them particularly appropriate for arid and semi-arid regions (Pirnajmedin et al., 2014). They are also widely grown throughout the temperate regions of the world and have good persistence and productivity in spring and fall (Sleper and West, 1996).

Grasses (family Poaceae) and fungi of the family Clavicipitaceae have a long history of symbiosis ranging in a continuum from mutualisms to antagonisms (Schard et al., 2004; Card et al., 2014). Fungi of the genus *Neotyphodium* are symbionts of cool season grasses, including major turf and fodder species. They grow inter-cellularly, with hyphae distributed in all plant parts except the roots (Karami et al., 2012). The fungal growth is systemic throughout the aboveground tissue but is most abundant in the leaf sheaths and reproductive structures, along with a concentration of hyphae at the base of the tillers. When an endophyte-infected seed germinates, the fungus grows between the cells into the emerging leaf sheaths and follows the culm as it elongates into an inflorescence. The fungus then grows further into the ovaries and infects the seeds that will form the next grass generation (Bylin, 2014; Saikkonen, 2000; Hinton and Bacon, 1985). Tall fescue and ryegrass (*Lolium perenne* L.) are naturally infected with *N. coenophialum* and *N. lolii*, respectively (Hesse et al., 2005; Bacon et al., 1997). These endophytes produce a range of alkaloids (Easton et al., 2002), some of which protect the associations from mammalian, insect and nematode herbivores (Latch, 1993). In contrast, meadow fescue infected with *N. uncinatum* is known to produce only Loline alkaloids, for which toxic effects on livestock have not been demonstrated (Malinowski et al., 1997).

The grass–endophyte symbiosis is a promising interaction that, like those involving nitrogen-fixing bacteria and mycorrhizal fungi, may become an important element of sustainable agriculture (Omacini et al., 2012). Some *Neotyphodium* endophytes may induce an improvement of characteristics of infected plants that increase the competitive ability of the host (Latch, 1993; Mirlohi et al., 2004), Therefore turfgrass breeders generally prefer to develop new cultivars with a high percentage of endophtye infection (Johnson et al., 2000). New developments in *Neotyphodium*-related technology led to forage-type grass varieties infected with endophytes that are not toxic to grazing livestock (Bouton et al., 2002). Endophyte infection in tall fescue may significantly improve drought-stress tolerance of host plants (West, 1994; Mirlohi et al., 2004). In some associations, protection of the plants from insect predation (Funk et al., 1983; Breen, 1994), improved disease resistance (Clarke et al., 2000) and effects on plant morphology (Hill et al., 1990; Mirlohi et al., 2004) have also been reported. Infected meadow fescue has been found to exhibit increased persistency and competitive ability (i.e. stronger shoot and root growth) in both monocultures and intercropping regimes (Takai et al., 2010). Pańka et al. (2011) also reported that the presence of endophyte significantly enhanced higher yields of dry matter compared to the non-infested plants.

Conflicting reports have been given about endophytic effects on seed production and seed yield components. Siegel et al. (1985) found no differences in total seed production in "Kenhy" tall fescue due to the endophyte, while Clay (1987) reported increased percentage of filled seeds and increased seedling emergence in endophyte-infected "Kentucky 31" tall fescue. In both studies, no clonal material was used therefore effects of plant genotypes are confounded with fungal status. Saari et al. (2010) reported that endophyte infection did not affect seed mass of tall fescue. However, seed predation was lower in EI than EF grasses in the two tall fescue populations. Also for meadow fescue, the mean number of seeds from EI plants was higher than EF plants. Rice et al. (1990) reported that endophyte-infected tall fescue plants have much higher relative fitness for seed production than endophyte-free plants. Hesse et al. (2005) showed that seed germination was either improved or not affected by the endophyte in Lolium perenne.In this study, we examined tall fescue and its close relative meadow fescue to (1) determine the effects of the endophyte on seed production and associated traits with variability due to plant genotypic effects removed by using clonal material and (2) determine the effects of the endophyte on seed germination and seedling emergence in genotypes of tall and meadow fescue from Iranian ecotypes.

Materials and Methods

Experiment I

Tall and meadow fescue genotypes, used in this study, were selected from three ecotypes (populations) of plant material collected from different native grassland habitats in Iran. Genotypes in each population were screened for the presence of endophytic fungi by means of histological leaf sheath analysis according to the method of Saha et al. (1988). Eventually, six genotypes (single plants), including four tall fescue and two meadow fescue, were selected for the field experiment. Three of the tall fescue genotypes were selected from an ecotype originally collected from Kordestan Province (West Iran) and one was selected from an ecotype originally collected from Khorasan province (East Iran). The two meadow fescue genotypes were selected from an ecotype originally collected from Charmahal province (Central Iran).

The experiment was conducted in the farm, green houses and laboratories of Isfahan University of Technology during 2010-2011 in Isfahan, Iran. For producing endophyte-free (EF) clonal material, the endophyte-infected (EI) plants were first clonally propagated and grown in a green house. EF clones were obtained by treating half of the clones from each genotype with a mixture solution of two fungicides including 0.2% (W/V) a.i. Propiconazole given as Pilt (250g a.i per liter) and 1 ml per liter Tebuconazol given as Folicur (250 EW, Emulsions oil in water). The fungicide was applied in spray application, twice a week for two weeks. By using this method, no visible injury occurred to growing plants treated with fungicides. EI and EF plants were grown for a period of 60 days after chemical application in the green house. Sheath tissue from EI and EF plants was routinely examined (Saha et al., 1988) to establish endophyte infection status. By this method, the same genotype (clone) was obtained in both infected and non-infected status, making it possible to separate plant genotypic effects from endophytic effects.

Tillers of both EI and EF plants were transplanted to the experimental field (clay-loam soil) of the Isfahan University of Technology (IUT) in the first March. The experimental design was a completely randomized block with a factorial arrangement of the treatments (six genotypes and their infection status) in three replications. Six clones were planted in a plot with an intra-row spacing of 30 cm and 50 cm between rows. Each plot consisted of only EI or EF clone. Fertilizer was applied at the beginning of the growing season at a rate of 70 kg N/ha. Microscopic leaf sheath analysis of the plants during the year and before the experiment was performed to observe the success of endophyte eradication in EF clones as well as the presence of the endophytes in EI clones.

At seed maturity stage all panicles from individual plants were removed and placed in bags. Numbers of panicle per plant and length of panicle were determined before threshing the seed. After threshing and cleaning, total seed yield per plant, total seed numbers and thousand seed weight were recorded. Seed per panicle were calculated as the ratio of total seed number per plant to number of panicles per plant. Panicle fertility as an indirect measurement of fertility was calculated as the ratio of the seed weight per panicle (mg) to the average length of panicles (cm).

Experiment II

Seeds derived from EI and EF clones of the six genotypes in experiment I were used to investigate the effects of endophytes on seed germination and seedling emergence under laboratory and greenhouse conditions, respectively. To confirm the presence or absence of endophytes in half- sib family seeds, 30 seeds per clone were randomly detected for endophytes according to the method of Saha et al. (1988). The experimental design in laboratory was a completely randomized block with a factorial arrangement of six clones (genotype) and two infection statuses in four replications. For each experimental plot 100 seeds were placed in a petri dish on a piece of filter paper and then transferred to an incubator in the dark at 22^{oC}. Germination was recorded everyday for 20 days. The germination percentage and germination rate were calculated by the method of MaGuire (1962) as:

Germination percentage = 100 (S/K)

Germination rate = $\sum (N_i / D_i)$

where S is the total number of seeds germinated at the end of experiment, K is the total number of seeds used for each plot, N_i is the number of seeds germinated on day i^{th} and D_i is the number of days from beginning of the experiment.

For seedling emergence study the experiment was conducted in a green house according to a completely randomized block design with a factorial arrangement of clone (genotype) and infection status in four replications. For each experimental plot 100 seeds were planted in one plastic pot (22 cm in diameter and 16 cm in depth) filled with clay loam soil: sand (2:1, v/v). No fertilizer was supplied during the experiment. Seedling emergence was counted daily and was defined as penetration of the coleoptiles through the soil surface. Seedling vigor was evaluated in terms of the emergence rate index, which is calculated as the sum (from first to final count) of the emerged seedling divided by the number of days from planting (Hay and Gamble, 1987). The equations are similar to those for germination percentage and germination rate given above.

After performing an Arc-sin transformation for germination data and emergence percentage, analysis of variance were performed separately for both species using the ANOVA procedure of SAS issued by the Statistical Analysis Systems institute (SAS). Treatment means were compared using LSD test (Least significance difference). To evaluate the differences in parameters between EI and EF of each genotype, the relative difference between endophyte variants (RDE) was calculated by the following formula: RDE = 100 (EI-EF)/ EI.

Results

Effects of endophytic fungi on tall fescue

The analysis of variance (Table 1) indicated that the fungal effect was significant for plant seed weight, number of seeds per plants and number of panicles per plants, but no significant effects was observed for other traits. The genotype (clone) had significant effect on all traits with the exception of panicle length and thousand seed weight. No interactions between genotype (clone) and fungal status were detected for seed production traits.

Table 1. Results of analysis of variance for seed production traits in endophyte-infected and endophyte-free clones of tall and meadow fescue.

Parameter	Tall fescue			Meadow fescue		
	Endophyte Status (ES)	Clone (CL)	ES×Cl	Endophyte Status (ES)	Clone (CL)	ES×Cl
Panicle per plant	**	***	ns	***	*	ns
Panicle length	ns	ns	ns	ns	ns	ns
Seed per panicle	ns	***	ns	ns	ns	ns
Seed per plant	*	*	ns	**	ns	ns
Thousand seed weigh	ns	ns	ns	ns	ns	ns
Plant seed weight	*	***	ns	**	ns	*
Panicle fertility	ns	***	ns	ns	ns	ns

*, ** and *** show significance at levels of 0.05, 0.01, 0.001, ns - not significant.

For all seed-production traits for which a fungal effect was observed significant, EI clones outperformed EF clones (Table 2). In this way, the increase associated with the fungal presence was 22.9% for plant seed weight, 20.5% for seeds per plant and 6.1% for panicles per plant.

Table 2. Mean values for seed production traits of the endophyte-infected (EI) and the endophyte-free (EF) clones of tall and meadow fescue.

Parameter	Tall fescue		Meadow fescue	
	EI	EF	EI	EF
Panicle per plant	15.77 ^{a}	12.04 ^{b}	61.15 ^{a}	30.25 ^{b}
Panicle length (cm)	21.85 ^{a}	21.87 ^{a}	18.11 ^{a}	16.77 ^{a}
Seed per panicle	66.37 ^{a}	66.88 ^{a}	14.52 ^{a}	15.28 ^{a}
Seed per plant	929.30 ^{a}	771 ^{b}	895.6 ^{a}	462.1 ^{b}
Thousand seed weigh	2.34 ^{a}	2.25 ^{a}	1.85 ^{a}	1.55 ^{a}
Plant seed weight (g)	2.09 ^{a}	1.70 ^{b}	1.66 ^{a}	0.7 ^{b}
Panicle fertility (mg/cm)	6.65 ^{a}	6.94 ^{a}	1.47 ^{a}	1.38 ^{a}

* For each genus, means of EI and EF followed by a different letter are significant at 0.05 level according to LSD test.

The analysis of variance for seedling traits indicated that the fungal effect was significant for emergence percentage and emergence rate. However, endophytic fungi did not affect germination of tall fescue (Table 3). Genotype (clone) effect was significant for all seedling traits. Interactions between genotypes and fungal status were significant only for the emergence rate. The mean values (Table 4) showed that the presence of the fungus resulted in lower means for seedling traits. The relative of reduction associated with fungal presence were -23.2% for emergence percentage and -35.2% for emergence rate.

Table 3. Results of analysis of variance for tall and meadow fescue seed germination and seedling emergence in endophyte-infected and endophyte-free (EF) clones of tall and meadow fescue plant genotypes (clones).

Parameter	Tall fescue			Meadow fescue		
	Endophyte Status(ES)	Clone (CL)	ES×Cl	Endophyte Status(ES)	Clone (CL)	ES×Cl
Germination percentage	ns	***	ns	**	**	*
Germination rate	ns	***	ns	**	**	*
Emergence percentage	***	*	ns	**	ns	ns
Emergence rate	***	***	***	*	ns	*

*, ** and *** show significance at levels of 0.05, 0.01, 0.001, ns - not significant.

Effects of endophytic fungi on meadow fescue

The analysis of variance for seed production traits in meadow fescue (Table 1) indicated that the fungal effect was significant for plant seed weight, number of seeds per plants and number of panicles per plants, but not for other traits. The genotype (clone) effect was significant only for panicle per plant. Significant interactions between genotype (clone) and fungal status occurred for plant seed weight.

Similar to *N. coenophialum* in tall fescue, *N. uncinatum* in meadow fescue increased those seed-production traits for which a significant fungal effect was observed. Furthermore meadow fescue was more affected by fungal endophytes than tall fescue (Table 2). The relative increase associated with fungal presence were 137.1% for plant seed weight, 93.8% for seeds per plant and 101.3% for panicles per plant.

The effects of endophyte infection were significant for all seedling traits in meadow fescue (Table 3). The comparison of means showed that the presence of the *N. uncinatum* resulted in higher means for seedling traits (Table 4). The relative increase associated with fungal presence were 18.8% for germination percentage, 72.1% for germination rate, 31.9% for emergence percentage and 42.1% for emergence rate.

Table 4. Mean values for seed germination and seedling emergence traits of the endophyte-infected (EI) and the endophyte-free (EF) clones of tall and meadow fescue.

Parameter	Tall fescue		Meadow fescue	
	EI	EF	EI	EF
Germination percentage	38.83 [a]	39.83 [a]	52.66 [a]	44.33 [b]
Germination rate	1.63 [a]	1.91 [a]	2.96 [a]	1.72 [b]
Emergence percentage	62.39 [b]	81.30 [a]	84.96 [a]	64.41 [b]
Emergence rate	21.84 [b]	33.72 [a]	35.13 [a]	24.73 [b]

* For each genus, means of EI and EF followed by a different letter are significant at 0.05 level according to LSD test.

Discussion

The potential effects of endophytes on tall and meadow fescue have received relatively little attention in ecological research and agricultural management in Iran. Results showed a significantly better performance of endophyte-infected plants for seed production traits for tall fescue as well as, to an even much greater extent, for meadow fescue. The increase in plant seed production in EI clones was due to an increase in the number of panicles per plant and the number of seeds per plant. However, the one thousand seed weight was not affected by endophyte status. The increased seeds per plant in infected plants may be due to increased percentage of filled seeds, as reported by Rice et al. (1990). Arechavaleta et al. (1989) and Belesky et al. (1987) reported that endophyte infection in tall fescue can increase the number of fertile tillers (panicles). However; Hill et al. (1990) did not find consistent response of tall fescue plants to endophyte infection for tiller production and dry matter production. It has also been reported that endophyte-infected plants featured an increased plant biomass and seed production (Clay, 1994) as well as seed set (Clay, 1987). However, Siegel et al. (1985) found no differences in seed production due to fungal status.

Our results suggest that although endophyte infection benefits tall fescue and meadow fescue host grasses by enhancing reproductive effort, EI meadow fescue plants produced considerably more seeds than EF plants. This result is almost in agreement with Saari et al. (2010) which reported that endophyte infection had less effect on seed production of tall fescue but the mean number of seeds from EI plants was higher than EF plants in meadow fescue. Malinowski et al. (1997) found that infection with *N.uncinatum* increased the competitive ability of meadow fescue due to increased shoot and root biomass production and increased forage yield. Meadow fescue (a diploid spesies) is a close relative of tall fescue and is distinctly different from it. It is a very persistent species because the developed rooting system (Bylin, 2014). More advantages of endophyte infections in meadow fescue relative to tall fescue cultivars may reflect differences in breeding programs for tall fescue and meadow fescue and more intensive use of meadow fescue as a forage grass in pastures.

In natural populations, the distribution of endophytes and infection frequencies not only largely depend on the reproductive success of infected hosts compared to their uninfected conspecifics, but also good potential of germination and establishment is necessary. Results of this study indicated that endophyte had inconsistent effects on germination and emergence stage in tall fescue compared to meadow fescue. Endophyte infection resulted in lower means for emergence in tall fescue. This reduction in seedling emergence could be attributed to the dependency of the fungus on host for energy and nutrients. Hesse et al. (2004) found a negative endophyte effects on plant growth of *Lolium perenne*. They suggested that the endophyte may be a "metabolic cost" for the host under certain environmental conditions. However meadow fescue showed improved seed germination and seedling emergence when endophyte-infected and no adverse symbiotic effects were detected for these parameters. Our results suggest that the effects of endophytes vary among grass species. This can show the different endophyte species that infect tall fescue and meadow fescue may have very different effects on their herbivores (Saari et al., 2010). Also the genetic control of symbiotic relationship in tall fescue and meadow fescue may be different needed to more investigation. Thus, successful breeding programs that aim to maximize yield production and improve forage quality require a comprehensive view of how endophytic fungi and their host grasses co-evolve and respond as a unit.

In conclusion, the results of present study indicated that the endophyte infection may confer much higher relative fitness in plant reproductive stage in tall and – more markedly - meadow fescue. The improved seed production traits of EI clones suggest that plant populations with a low endophyte infection level would be less stable than populations with a high percentage of infected plants. In natural populations, the distribution of endophytes and infection frequencies largely depend on the reproductive success of infected hosts compared to their uninfected conspecifics. An increase in the proportion of infected plants could occur in plant populations during seed multiplication and also on pastures where reseeding occurred. Therefore, if the initial plant population had a low level of infection, a rapid shift towards higher infection percentage could occur in a few generations. Furthermore, infection with *N. uncinatum* may increase the competitive ability of meadow fescue in seed germination and seedling emergence. This advantage suggests that infected plants of meadow fescue can be of interest for turf and forage grass breeding, as well as grass production in dry regions and for erosion protection.

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