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Occurrence of Fungal Endophytes in Species of Wild *Triticum*

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ABSTRACT

Seedborne, nonpathogenic, fungal endophytes are commonly found in symbiotic relationships with many members of the cool-season grass subfamily *Pooideae*. The beneficial effects on plants possessing fungal endophytes, and the detrimental effects on consumers of fungal endophyte-infected plants are widely known. The objective of our research was to determine if fungal endophytes exist in indigenous, wild *Triticum* (wheat) species from Turkey. From the *Triticum* species collected, we found two different fungal endophytes. Fungi identified morphologically as members of the genus *Neotyphodium* were found in the diploid *Triticum* species *T. dichasians* (Zhuk.) Bowden and *T. tripsacoides* (Jaub. & Spach) Bowden. The second endophyte, an *Acremonium* species, was found in *T. columnare* (Zhuk.) Morris & Sears, *T. cylindricum* Ces., *T. monococcum* L., *T. neglecta* Morris & Sears, *T. recta* Morris & Sears, *T. triunciale* (L.) Raspail, *T. turgidum* L. , and *T. umbellulatum* (Zhuk.) Bowden. No fungal endophytes were found in *T. kotschyi* (Boiss.) Bowden, *T. ovatum* (L.) Raspail, *T. peregrinum* Morris & Sears, *T. speltoides* (Tausch) Gren. ex Richter, and *T. tauschii* (Coss.) Schmal., although the number of samples tested was small for some of these species. Both *Acremonium* endophyte-infected and *Acremonium* endophyte-free plants of *T. triunciale* were found to occur at different frequencies at four collection sites on the Anatolian Plateau. Through two selfed generations of the plants, it was found that the *Neotyphodium* endophyte was transmitted to 100% of the progeny of *T. dichasians* and *T. tripsacoides*. However, the *Acremonium* endophytes were not transmitted in all plants that originally possessed them. We concluded that fungal endophytes of the genera *Neotyphodium* and *Acremonium* inhabit some wild wheat species grown indigenously in Turkey. These endophytes may influence the ecology and distribution of *Triticum* species, and may also serve as a source of biological control agents of pests or abiotic stress factors in wheat.

To economically feed an increasing world population, it is important that food production be increased while the cost of producing the food be decreased. The strategic use of naturally occurring organisms to control pest populations and increase production of major crops represents a viable option to host-plant resistance and pesticide-based pest control. One group of biological control agents that provide a source for novel pest control are the mutualistic fungal symbionts belonging to the genus *Epichloë* (Clay, 1989). These fungi, in association with their host grass plant, produce a range of deterrence to various insects and some plant diseases (Latch, 1993). In addition, improved growth and drought tolerance are characteristic of some plants possessing fungal endophytes (West, 1994). The basis of much of the pest deterrence in grasses possessing endophytes is the production of alkaloids by the endophytes (Siegel et al., 1991). Endophyte-infected grasses have caused toxicity-related problems in livestock, such as cattle, sheep, and horses, that graze on the infected pastures (Hoveland, 1993). Humans ingesting food products derived from endophyte-infected grasses probably would also suffer similar toxicity, because the fungal endophytes involved are relatives of the sclerotial (ergot) forming *Clavicipiteae*, whose toxicity effects are widely known (Groger, 1972). Thus, if fungal endophytes are to be safe and effective biological control agents of food crops, the means must be found to either eliminate any potentially harmful toxic compounds or to select toxic-specific fungal strains.

The systemic, seedborne, nonpathogenic, fungal endophytes of most interest as biological control agents belong to the genus *Neotyphodium* Glenn, Bacon, Price, and Hanlin (formerly *Acremonium* section *Albolanosa* Morgan-Jones and Gams) (Glenn et al., 1996). These fungi are conidial anamorphs of *Epichloë* spp. (Persoon:Fries) Tulasne (Schardl and Phillips, 1997). Another group of fungal endophytes of grasses that have been identified are the p-endophytes, which as a group are closely related to each other, and have been found to sometimes coexist in plants with *Neotyphodium* endophytes (An et al., 1993). However, the biology and ecology of the p-endophytes are relatively unknown. Fungal

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endophytes belonging to the genus *Acremonium*, such as *A. chilense* Morgan-Jones, White, and Piontelli (1990), could represent a third endophytic grouping based on the apparent unrelatedness to the p-endophytes and *Neotyphodium*.

In the *Gramineae* family, fungal endophytes have been found in several tribes of the cool-season grass subfamily *Pooideae*. The most intensively studied are from the *Poeae* tribe in the genera *Festuca*, *Lolium*, and *Poa* (Bacon, 1995). Other grass tribes having members that possess fungal endophytes include the *Bromeae* (White, 1987), *Stipeae* (White and Morgan-Jones, 1987; Bruehl et al., 1994; Kaiser et al., 1996), *Meliceae* (White, 1987), *Aveneae* (White, 1994), and *Triticeae* (Wilson et al., 1991). In the *Triticeae* tribe, fungal endophytes have been found in *Elymus* (White, 1987) and *Hordeum* (Wilson et al., 1991). A member of the *Triticeae* tribe of paramount importance to global food resources is the genus *Triticum,* which contains the wild and cultivated forms of wheat. Cultivated wheat has been found to be free of beneficial, seedborne, nonpathogenic fungal endophytes (Marshall, 1991, unpublished data). However, by examining seeds of *Triticum* species from the USDA National Small Grains Collection, we observed some evidence of fungal hyphae associated with the aleurone cells of some accessions but were never able to find endophytic fungal hyphae in living plants, nor were we able to isolate endophytic-fungal cultures (Marshall, 1991, unpublished data). We then hypothesized that endophytic fungi may be found in freshly collected seed of wild *Triticum* from indigenous habitats. We selected Turkey as the collection area because more diverse species of naturally occurring *Triticum* exist within Turkey's borders than any country (Kimber and Feldman, 1987). In the research presented here, we have used the classification of Morris and Sears (1967) that combines the two former genera *Triticum* and *Aegilops*, into the single genus *Triticum*.

MATERIALS AND METHODS

Plant Material

Seeds of *Triticum* species as well as other cereals and grasses were collected by the authors during July 1992 from more than 80 sites in Turkey, mainly on the Anatolian Plateau, ranging from approximately 36°30′ to 41°30′ N and from 30° to 35° E. At each site, ripe or near-ripe seeds were collected into envelopes, and assigned an accession number. Additional data recorded at each site included collection date, geographic location, altitude, and a general description of the physical environment of the site. Seeds from other indigenous cereals and temperate grasses were also collected at each site. A complete, descriptive listing of the accessions collected can be obtained from the senior author.

Microscopic Detection, Isolation, and Taxonomic Grouping of Endophytic Fungi

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 (Saha et al., 1988). Later, we found it more expedient to detect endophytic fungal hyphae in squashed seeds by following the sodium hydroxide soaking and water washing with a 60- to 90-s boiling in 0.4% aqueous aniline blue stain. After staining, individual seeds were placed on a microscope slide in a drop of 0.2% aqueous aniline blue stain, squashed under a cover slip, and observed microscopically for fungal hyphae.

In addition to seed examination, leaf sheaths from living plants were also examined for endophytes. Here, seeds were surface sterilized in 50% aqueous sodium hypochlorite (NaOCl) for about 5 min, rinsed three times in sterile water, then planted into steam-sterilized potting medium in a greenhouse. After 4 to 5 wk, a leaf sheath was removed, and an epidermal strip from the inside surface of the sheath was placed in a drop of 0.2% aniline blue on a microscope slide, covered with a slip, passed briefly over a flame, and microscopically observed. Leaf sheaths were examined in a minimum of ten plants from each accession.

For endophyte isolation, several methods of seed decontamination were tested (White et al., 1993; Bacon, 1988; Bacon and White, 1994); however, these techniques were inconsistent for subsequent seed germination or adequate decontamination. A technique that was more effective in removing contaminants while maintaining seed and endophyte viability was to surface sterilize dry seeds by placing them in porous tissue paper and suspending the seeds over a mixture of full-strength bleach (6.25% NaOCl; 100 mL) with 5 mL of HCl in a desiccator placed in a fume hood. This technique produces chlorine gas, to which the seeds were exposed for about 2 to 3 h prior to plating onto Difco malt extract agar. Endophytic fungi were visible from the seed of some species after 6 d, while other endophytes took nearly 3 wk to grow out. Endophytic fungi were also isolated from leaf sheath and stem material. Plants were grown in the greenhouse as mentioned above. After 4 to 5 wk of growth, some sheath and stem material was removed from the plants and surface decontaminated by chlorine gas (as mentioned above), or by the method described by Bacon and White (1994).

Classification of the endophytes into taxons was based on phialide and conidia morphology, and growth in culture. All of the endophytes classified as *Neotyphodium* had solitary phialides, arising from aerial hyphae, with no basal septa. The conidia were fusiform in shape and >10 mm in length. All those endophytes classified as *Acremonium* produced small conidia (2–6 mm in length), which were collected in slimy heads.

Multiple Generation Testing

Plants possessing endophytes were designated E+, and those plants lacking endophytes were designated E−. In order to determine if the endophytic fungi observed in the originally collected seeds were transmitted through successive generations, seeds from both E+ and E− plants were collected from the original self-pollinated plants that had been grown in the greenhouse for fungal detection and isolation. Some of the selfed seeds were squashed and examined for fungal endophytes as described above. Other selfed seeds were decontaminated by chlorine gas, germinated on 3% water agar plates, then planted into a sterilized soil mix and placed in a growth chamber set at 10-h days of 23°C and 14-h nights of 18°C for 3 to 4 wk. Leaf sheath samples were taken for endophyte detection and isolation as described above. Plants were vernalized at 3°C for 4 wk, then transferred to the greenhouse until the plants were mature, and selfed for a second generation.

Seeds produced from the second generation of selfed plants were processed and examined for endophytes in the same manner as the first generation seeds. All of the species tested over multiple generations were self-pollinated, except for *T. tripsacoides*, which required cross-fertilization. Here, we allowed four E+ plants from each *T. tripsacoides* accession to intermate for each of the two generations tested. The multiple generation testing for fungal endophytes in *Triticum* species was conducted during a 4-yr period of time from 1993 to 1996.

RESULTS

Endophyte Occurrence

Of the 787 cereal and grass accessions collected, 411 belonged to the genus *Triticum*. The remaining accessions belonged to genera of other cereals (*Avena*, *Hordeum*, and *Secale*) and some temperate grasses (*Agropyron*, *Bromus*, *Dactylis*, *Digitaria*, *Festuca*, *Lolium*, *Poa*, and *Taeniathrum*). Within *Triticum*, we collected 16 species, 10 of which were E+ for hyphae detected in seeds and plants (Table 1). From the growth characteristics of the hyphae in the plants, and subsequently, from fungi isolated from within the plants, we identified two different endophytes. The first was a *Neotyphodium* species that exhibited convoluted hyphae in association with the seed aleurone cells and in the leaf sheath. Isolates of *Neotyphodium* were slow growing and produced solitary conidia that were reniform in shape and >10 mm in length. The second type was an *Acremonium* species that generally had straight-shaped hyphae in seeds and plants. The *Acremonium* produced cylindrically shaped conidia, which varied from 2 to 6 mm in length, and which collected in slimy heads in culture.

The *Neotyphodium* endophyte was found only in the two diploid wheats, *T. dichasians* and *T. tripsacoides*. Four of the five accessions of *T. dichasians* and 15 of 20 accessions of *T. tripsacoides* contained the *Neotyphodium* endophyte (Table 1). The species of *Triticum* that contained the *Acremonium* endophyte were *T. columnare*, *T. cylindricum*, *T. monococcum*, *T. neglecta*, *T. recta*, *T. triunciale*, *T. turgidum*, and *T. umbellulatum* (Table 1). No fungal endophytes were evident in the one accession of *T. kotschyi*, the two accessions of *T. ovatum*, the three accessions of *T. peregrinum*, the five accessions of *T. speltoides*, or the 12 accessions of *T. tauschii*. Similarly, no endophytes were found in the 40 accessions of *T. aestivum*. However, all of the *T. aestivum* accessions were from wheat cultivars that had been planted as crops, and therefore did not represent indigenous *T. aestivum*.

All of the *Triticum* species collected had some accessions that were endophyte-free (Table 1). However some of the species that were sparsely collected, such as *T. columnare*, *T. dichasians*, and *T. recta*, had high percentages of accessions that were E+ (Table 1). Two of the more numerously collected species, *T. cylindricum* and *T. monococcum*, each had about 64% E+ accessions. At all collection sites, we observed no evidence of "choke" symptoms (stromata) on any of the *Triticum* species in nature. Similarly, no choke symptoms were found on any of the plants grown in the greenhouse.

Table 1. Endophytic fungi in 16 species of indigenous *Triticum* from Turkey.

Triticum species†	Ploidy level†	Genome†	Total accessions	Number of accessions with fungal endophyte	
				Neotyphodium	*Acremonium*
aestivum‡	6*x*	*ABD*	40	0	0
columnare	4*x*	*UM*	6	0	4
cylindricum	4*x*	*CD*	76	0	49
dichasians	2*x*	*C*	5	4	0
kotschyi	4*x*	*US*	1	0	0
monococcum	2*x*	*A*	48	0	31
neglecta	4*x*	*UM*	18	0	9
ovatum	4*x*	*UM*	3	0	0
peregrinum	4*x*	*US*	2	0	0
recta	6*x*	UMU_N	6	0	5
speltoides	2*x*	*S*	5	0	0
tauschii	2*x*	*D*	12	0	0
tripsacoides	2*x*	M_T	20	15	0
triunciale	4*x*	*UC*	133	0	73
turgidum	4*x*	*AB*	16	0	12
umbellulatum	2*x*	*U*	20	0	7

† Nomenclature, ploidy level (*x* = 7 chromosomes), and genome type follows that of Kimber and Feldman (1987).
‡ All accessions of *T. aestivum* were from wheat cultivars.

Endophyte Distribution in *Triticum triunciale*

The most numerous and widely distributed species collected was *T. triunciale*. Across all collection sites, we found that 53% of the accessions of *T. triunciale* were E+ with the *Acremonium* endophyte. We did not find the *Neotyphodium* endophyte in *T. triunciale* (Table 1). At four collection sites, populations of *T. triunciale* were particularly numerous, and we determined the percent of the plants that contained endophytes at each of the four locations. At Afyon and Gölbaşi, the total percentage of E+ plants was similar (54% at Afyon and 52% at Gölbaşi) (Table 2). However, at Kalecik, we found that 76% of the *T. triunciale* plants were E+ and only 24% were E−. A shift toward endophyte-free plants occurred at Kaman, where 40% of the plants were E+ and 60% were E− (Table 2).

Transmission of Fungal Endophytes through Successive Seed Generations

For a period of 4 yr, we tested the selfed progeny of the originally collected E+ seeds for two generations in order to determine if the fungi were transmitted through successive generations. For the *Neotyphodium* endophytes in *T. dichasians* and *T. tripsacoides*, the fungus

Table 2. Distribution of *Acremonium* endophyte-containing plants and endophyte-free plants of *Triticum triunciale* populations at four locations in Turkey.

Collection site†	Plants with endophytes (E+)	Plants without endophytes (E−)
	%	
Afyon	54	46
Gölbaşi	52	48
Kalecik	76	24
Kaman	40	60

† The collection sites were Afyon = 20 km northeast of Afyon at 1150-m altitude; Gölbaşi = 1 km southwest of Gölbaşi at 1000-m altitude; Kalecik = 2.5 km southwest of Kalecik at 910-m altitude; and Kaman = 5 km west of Kaman at 1190-m altitude.

was transmitted in all of the progeny plants after both generations of testing (Table 3). However, this was not the case for the *Acremonium* endophyte. For example, in *T. columnare*, 93% of the selfed original E+ plants were also E+ with *Acremonium*. In the second generation of testing, 94% of the selfed F_1 E+ plants were also E+ (Table 3). However, in *T. cylindricum*, only 74% of the plants produced from the original E+ plants transmitted the *Acremonium* endophyte, yet all of those F_1 plants produced F_2 plants that retained the fungus (Table 3).

DISCUSSION

The results of this study indicate that fungal endophytes occur in different species of the genus *Triticum* at the evolutionary center of the plant genus. Two endophyte species (*Neotyphodium* and *Acremonium*) were found to occur within the host plants. During the collection expedition in Turkey, we attempted to gather seed from as many diverse plant sources as possible. Thus, the number of accessions of each species was sometimes small, dependent upon the relative occurrence of each species at each collection site. The fact that we were able to find endophytic fungi in the few collected accessions of *T. columnare*, *T. dichasians*, and *T. recta*, suggests that the fungi may be widespread in occurrence in these species. A more systematic survey for fungal endophytes within each species throughout the habitation range would be needed to better estimate endophyte distribution. We were able to make a preliminary estimate of distribution of *Acremonium* endophyte within one species, *T. triunciale*, because of the common occurrence of the plant species across the collection area. *T. triunciale* has the largest distribution of all wild *Triticum* species in Turkey (Kimber and Feldman, 1987). There was variation in the occurrence of fungal endophytes in *T. triunciale* at four selected collection sites. At Afyon and Gölbaşi, the E+ and E− plants were nearly equal in occurrence, while at Kalecik, 76% of the *T. triunciale* plants contained endophytes. At Kaman, only 40% of the plants were E+. This indicates that wild *T. triunciale* plants can apparently persist without *Acremonium* endophytes under the same environmental conditions as do *Acremonium*-infected plants. Presumably, the relative occurrence of E+ and E− plants within a species at a given location will fluctuate over time, dependent on the selection stresses encountered and the relative contribution of the endophyte to plant survival. In the other *Triticum* species, we observed similar occurrences of E+ and E− plants at individual collection sites, but the numbers of plants collected were too few to allow dependable estimates. Although the role these *Neotyphodium* and *Acremonium* endophytes play in *Triticum* evolution is unknown, their occurrence in indigenous plants may have contributed to the genetic diversity and persistence of the *Triticum* species. Endophytic fungi have been found to be involved with the evolution, distribution and, phenotypic plasticity of other grasses such as *Danthonia* (Clay, 1994), *Festuca* (Leuchtmann, 1994), and *Lolium* (Cheplick, 1997). However, a close association between the nonpathogenic, seedborne, fungal endophytes and their hosts does not necessarily imply coevolution between the fungus and host. Evidence within *Festuca* suggests that the mutualistic, symbiotic relationship between the host and *Neotyphodium* species may have occurred quite late in *Festuca* speciation (Schardl and Siegel, 1993).

This study represents an exploratory survey with a limited sampling of wild *Triticum* species and may not be representative of a more detailed study of the species. Obviously, the biological, genetic, and environmental history of a location have had their cumulative effects on their present status, and therefore, the status of the seed collected. Thus, an in situ study of endophyte persistence is needed, as it would aid in understanding the role these endophytes play in *Triticum* ecology, evolution, and diversity. We did not find endophytes in the accessions of collected cultivated wheat (*T. aestivum*). Possibly, fungal endophytes have inadvertently been removed from cultivated wheat because of selection against adverse human effects, poor seed storage conditions, or the use of wild species principally as pollen donors in crosses. Similarly, Latch (1987), examined *Lolium* and *Festuca* grasses from their center of origin in Italy and southern France, and found endophyte infection in 86% of the plants, whereas commercial lines

Table 3. Percentage of transmission of *Neotyphodium* and *Acremonium* fungal endophytes in 10 species of *Triticum* during two generations of selfing.

Triticum species†	Infected initial progeny (selfed original) with endophyte type		Infected successive progeny (selfed initial) with endophyte type	
	Neotyphodium	*Acremonium*	*Neotyphodium*	*Acremonium*
	%			
columnare	0	93	0	94
cylindricum	0	74	0	100
dichasians	100	0	100	0
monococcum	0	88	0	94
neglecta	0	85	0	95
recta	0	94	0	90
tripsacoides	100	0	100	0
triunciale	0	91	0	97
turgidum	0	76	0	85
umbellulatum	0	90	0	95

† Nomenclature follows that of Kimber and Feldman (1987).

from the same area had just 19% infection. Thus, endophyte-free ryegrass and fescue plants have only become common since the development of modern agricultural practices and the storage of seed prior to planting (Latch, 1987).

Within the tribe *Triticeae*, seedborne nonpathogenic fungal endophytes of the genus *Neotyphodium* have been found in the genera *Elymus* (White, 1987) and *Hordeum* (Wilson et al., 1991). Our study is the first accounting of the *Neotyphodium* endophyte in the genus *Triticum*. Even though some of the *Triticum* species we observed were represented by few accessions, a total of six diploid, eight tetraploid, and two hexaploid species were examined (Table 1). *Neotyphodium* endophytes were found only in the diploid species, *T. dichasians* and *T. tripsacoides*. The affinity of the *Neotyphodium* endophytes from *T. dichasians* and *T. tripsacoides* to those from other grasses has yet to be elucidated. The diploids *T. monococcum* and *T. umbellulatum* contained the *Acremonium* endophyte, but not *Neotyphodium*. Two other diploids, *T. speltoides* and *T. tauschii*, appeared to be free of fungal endophytes. Within the tetraploids, *T. columnare*, *T. cylindricum*, *T. neglecta*, *T. triunciale*, and *T. turgidum* contained the *Acremonium* endophyte. However, three other tetraploid species, *T. kotschyi*, *T. ovatum*, and *T. peregrinum* were apparently endophyte-free. The only wild hexaploid collected, *T. recta* contained the *Acremonium*, but not the *Neotyphodium* endophyte. This indicates that the genome size of the plant may have an effect on the type of symbiotic association established with fungal endophytes. The genome type may also have an effect. The endophytes were found in all the collected species containing the *C* genome, whereas plants from *S* genome species did not contain endophytes (Table 1). An endophyte–wheat genome relationship could lend evidence as to which parent was female when hybridization occurred in the evolution of *Triticum* polyploids, but more plants would need to be collected and analyzed in order to get a better estimate of the effect of genome type and ploidy level.

Seedborne transmission of the *Acremonium* endophyte decreased somewhat during two generations of testing. On the average across all species, 84% of the original plants that were infected with the *Acremonium* endophyte transmitted the endophyte on to the initial progeny. From the initial progeny to the next generation, the *Acremonium* endophyte was transmitted to an average of 95% of the plants. However, the *Neotyphodium* endophyte was always transmitted to the progeny of *Neotyphodium* E+ plants. This may indicate that the relationship of the *Acremonium* endophyte to the plant host is not as dependent as is the *Neotyphodium–Triticum* relationship. It could be that the *Acremonium* endophyte in this study may be endophytic only under certain conditions, but under other circumstances may live outside the plant. We could find no evidence of pathogenesis of either of the endophytes on any of the host species. Thus, the purpose these endophytes have in the plants has yet to be elucidated. In *Festuca* and *Lolium*, it was speculated that the cosymbiotic endophytes (*Neotyphodium* and p-endophytes) may have synergistic activities in biological protection and other aspects of host fitness (An et al., 1993). The role of the p-endophytes in *Festuca* and *Lolium* may be less ecologically important to their hosts than the *Neotyphodium* endophytes because the p-endophytes are not as commonly disseminated by seed as is *Neotyphodium* (Siegel et al., 1995).

Classification of the endophytes in this study into either *Neotyphodium* or *Acremonium* was simply based on morphology of the phialides and conidia, conidial size, and cultural characteristics. Differences between the two groups were definitive and consistent during the 4 yr of this study. More detailed phyletic studies need to be conducted to determine the relationship of these endophytes to other members of the *Neotyphodium* or *Acremonium* genera. It is possible the endophytes we have identified as being members of *Neotyphodium*, may be a new taxonomic grouping.

The finding of nonpathogenic, fungal symbionts in wild wheat relatives under natural, indigenous conditions could lead to new methods and strategies of controlling pests and subsequently increasing yields in cultivated wheat. In addition, the evolution of *Triticum* species and the domestication of wheat may have been influenced by *Neotyphodium* and/or *Acremonium* endophytes. However, much work needs to be done, in particular concerning the relatedness of the *Triticum* endophytes to those of other grasses, as well as concerning the pest-deterrent effect (if any) of the *Triticum* endophytes.

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