

## Preliminary study of endophytic fungi in timothy (*Phleum pratense*) in Estonia

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Timothy (*Phleum pratense* L.) is an important agricultural grass in Europe and North America, but there is little research into the occurrence and abundance of fungal endophyte species associated with this grass. The aim of this study was to identify fungal endophytes living within *P. pratense* and to determine if additional moisture applied during the growing season increases the diversity of endophytic fungi. We studied 58 isolates obtained from surface-sterilised blades of 60 *P. pratense* plants collected from Rõka Free Air Humidity Manipulation experimental plots (FAHM), Estonia. Morphological and molecular methods were used for isolate identification. As a result, 45 strains from 10 different taxa were identified, all belonging to Ascomycota. Five species were found to be new to *P. pratense*.

**Key words:** agricultural grasses, endophytes, isolate identification, ITS, FAHM

### INTRODUCTION

Plants and fungi commonly have a mutualistic relationship and, in the case of endophytes, they reside inside plant tissue, mostly without causing disease (Carroll 1988; Clay, Schardl 2002). Sometimes impact from the environment can make these fungi parasitic or pathogenic to host plants (Schardl et al. 1997; Meijer, Leuchtman 1999; Schulz et al. 1999; Kogel et al. 2006; Johnson, Oelmüller 2009). Most of them are non-specific regarding their host preference (Petrini 1986; Saikkonen et al. 1998; Arnold et al. 2000) and can live together with other endophytes in the same host (An et al. 1992; Saikkonen et al. 1998; Arnold et al. 2000), holding greater promise for plant growth promotion and increased yield in agriculturally important grasses.

Endophyte presence in plants has also been shown to help them deal with prolonged periods of drought, excess moisture or light (Bouton et al. 1993; Bier 1995; Marks, Clay 1996; Lewis et al. 1997; Cheplick et al. 2000; Malinowski, Belesky 2000).

Increases in biomass and tolerance to attack from herbivores and insects are often ascribed to endophytes (Bacon, Hill 1996; Clay and Holah 1999; Sahay, Varma 1999; Redman et al. 2001; Arnold et al. 2003; Waller et al. 2005; Márquez et al. 2007).

*P. pratense* is a widespread arable crop that is, however, poorly investigated in terms of its endophytic fungal diversity. The purpose of our study was to identify fungal endophytes living inside the leaves of *P. pratense* and to determine if differences in water treatments in the FAHM experimental area affect the diversity of endophytes.

## MATERIALS AND METHODS

**Sampling plant material and endophyte isolation.** Samples were collected on 29 October 2008 from Free Air Humidity Manipulation (FAHM) experimental plots at Rõka, Tartu County, in southeast Estonia (58°14'44"N, 27°17'58"E). Plants were collected from four plots with additional moisture being applied to the plots (H1-H4) and from two control plots without additional water being applied (C1 and C4). In one plot, H3, there appeared a problem with the watering system and it could not be used in comparison. Altogether, 60 *P. pratense* plants were collected.

Immediately after collection, one to two *P. pratense* leaves were separated from the plants and cut into 5 × 5 mm fragments. The surfaces of the fragments were disinfected by rinsing in 96% ethanol for 1 min, followed by repeated rinsing in sterile water. Fragments were placed in Petri dishes (9 cm diam.) on potato dextrose agar (PDA, Merck KGaA Germany) supplemented with tetracycline and streptomycin (50 µl each per ml) for suppression of bacterial growth. Petri dishes were examined for mycelial growth for 30 days and all fungal growth was subcultured on individual PDA plates with antibiotics for subsequent identification. For molecular identification, 7-10 days after inoculation of subcultures, a 5 × 5 mm piece of mycelium with agar was cut and preserved in sodium dodecyl sulphate (SDS).

**Endophyte identification by morphology.** Morphological characterization relied on the colour, appearance, growth rate and microscopical hyphal features of endophytic colonies. Similar cultures were grouped together based on morphotype and the main characteristics were analysed for representatives of these groups. Growth rate was measured for 23 days. Image Pro Plus 6.0 program was used to calculate the area occupied by mycelium.

Colony colour was determined at approximately 17 days using the Methuen Handbook of Colour (Kornerup, Wanscher 1967). Both surface and reverse colours were recorded and in case of more than one dominant colour, all were recorded (data not shown). Colony colour was determined by viewing the isolates in daylight. The colour of the medium and the occurrence of aerial mycelium were also noted if present. Morphological descriptions were made in sterile water using a Zeiss Axioskop 40 FL microscope, an AxioCam MRc camera and the Axio Vison 1.6 program. Species determination followed taxonomic monographs and manuals (Domsch et al. 1980; Ellis, Ellis 1997; Leslie, Summerell 2006). Voucher strains were deposited in the Collection of fungal living cultures [TFC 2010-001 – TFC 2010-035] at the Estonian University of Life Sciences.

**Endophyte identification by molecular methods.** Genomic DNA was extracted from pure cultures using Genomic DNA Purification Kit K0512 (Fermentas) and stored in CTAB buffer according to Gardes and Bruns (1993). ITS regions of rDNA were amplified by PCR using primers ITS1F (5' to 3': CTT GGT CAT TTA GAG GAA GTA A) and ITS4 (5' to 3': TCC TCC GCT TAT TGA TAT GC) using a Biometra TProfessional Basic Thermocycler (Biometra GmbH, Germany) with 35 cycles of 30 s at 95°C (denaturing), 30 s at 55°C (annealing), and 1 min at 72°C (extension).

The resulting PCR product was purified with Exo-Sap (Fermentas) following manufacturer's instructions. Samples were sequenced in Macrogen (Korea) with primers ITS1 5' to 3': TCC GTA GGT GAA CCT GCG G and ITS5 5' to 3': GGA AGT AAA AGT CGT AAC AAG G. Sequences were edited using the program Sequence Scanner 1.0 (Applied Biosystems, Foster City, California, USA). Sequences obtained from 45 cultures were compared with the ones in GenBank using BLASTn algorithm. Sequences that were more than 97% identical were considered to be homospesific. Colonization frequency (CF%, the percentage of plants colonized) was calculated by using the formula:  $CF = (N_{col}/N_t) \times 100$ , where,  $N_{col}$  is the number of segments colonized by each endophyte and  $N_t$  is the total number of segments observed (Hata, Futai 1995).

## RESULTS

In total, 58 endophyte strains were isolated from the 60 plants. Of these, 45 isolates were identified by sequence analysis of the ITS region. These isolates belonged to 10 taxa based on sequence data ITS: *Alternaria arbusti* E.G. Simmons, *Lewia viburni* E.G. Simmons (anamorph *Alternaria viburni* E.G. Simmons), *Apiospora montagnei* Sacc. (anamorph *Arthrinium arundis* (Corda) Dyko et B. Sutton), *Aureobasidium pullulans* (de Bary) G. Arnaud, *Epicoccum nigrum* Link, *Fusarium* sp., *Gibberella avenacea* R.J. Cooke (anamorph *Fusarium avenaceum* (Fr.) Sacc.), *Monographella* sp., *Paraphaeosphaeria michotii* (Westend.) O.E. Erikss. and *Phaeosphaeria herpotrichoides* (De Not.) L. Holm (Fig. 1). Species determinations using GenBank references are given in Table 1. Colonization frequency of isolated fungi is shown on Figure 2.

Morphological studies were performed for most isolates, but only 33% of them resulted in determination of species or genus, while others remained sterile. Morphological studies allowed identification of six species or genera; *A. pullulans*, *E. nigrum*, *F. sporotrichioides*, *F. avenaceum*, *Arthrinium* sp. and *Alternaria* sp. (Fig. 1). A plant sample usually contained only one fungal species, although two of the samples gave two different species each (*E. nigrum* and *A. arbusti*, *E. nigrum* and *P. herpotrichioides*). In morphological analyses, 67% of cultures were sterile and for these ITS analysis and morphotype concept (Guo et al. 2000; Arnold et al. 2003) was used to determine species.

Growth rates had noticeable variation both between species (0.08–0.71 cm/day) and also within species. Growth rate within the *E. nigrum* group was 0.10–0.26 cm/day. Average growth rate for all species combined was 0.25 cm per day, with *A. pullulans* having the lowest (0.08) and *A. montagnei* the highest (0.71) radial growth.

Table 1  
Endophytic fungi isolated based on sequence comparison to GenBank database

Isolate	Species	GenBank accession no.
H4-8-2	<i>Phaeosphaeria herpotrichoides</i>	JN688915
H3-8-3	<i>Apiospora montagnei</i>	JN688916
C1-7	<i>Aureobasidium pullulans</i>	JN688917
C1-6	<i>Epicoccum nigrum</i>	JN688918
C1-4-3	<i>Alternaria arbusti</i>	JN688919
H3-6-1	<i>Fusarium avenaceum</i>	JN688920
H4-4-1	<i>Alternaria viburni</i>	JN688921
C1-9-1	<i>Fusarium sporotrichioides</i>	JN688922
C4-7	<i>Monographella</i> sp.	JN688923
C1-3-3	<i>Paraphaeosphaeria michotii</i>	JN688924

The results of our study showed that the diversity of endophytes was not higher in the experimental plots with additional water given to the plants during the growing season. In fact, both the highest number of isolates (plot H4, 15 isolates) and the lowest number of isolates (plot H1, 5) were obtained from plots with applied additional moisture. In average, 11 isolates were obtained from each control plot and 9 isolates were obtained from each experimental plot. Considering these results, the positive effect of extra moisture on the diversity of endophytic fungi was not established.

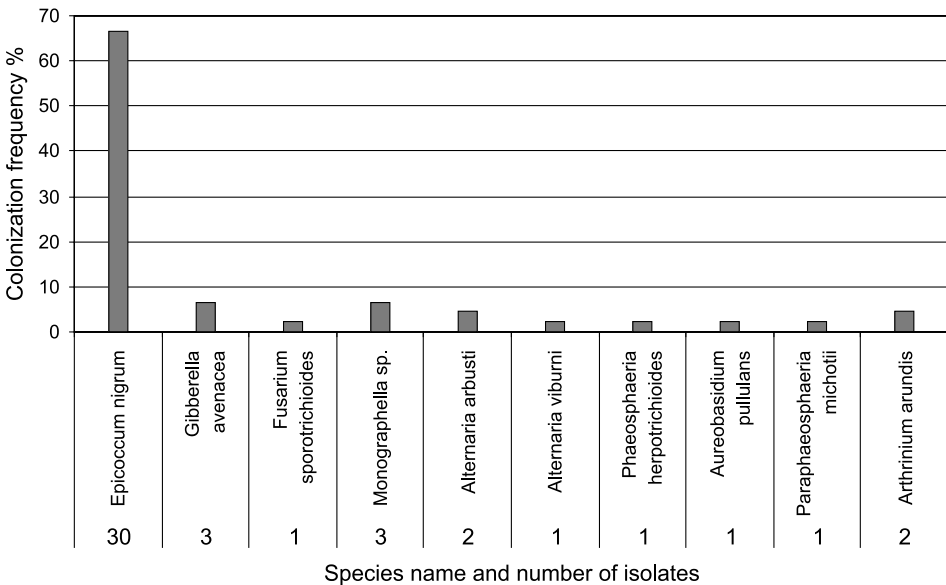


Fig. 2. Identified species and their colonization frequency (CF%).

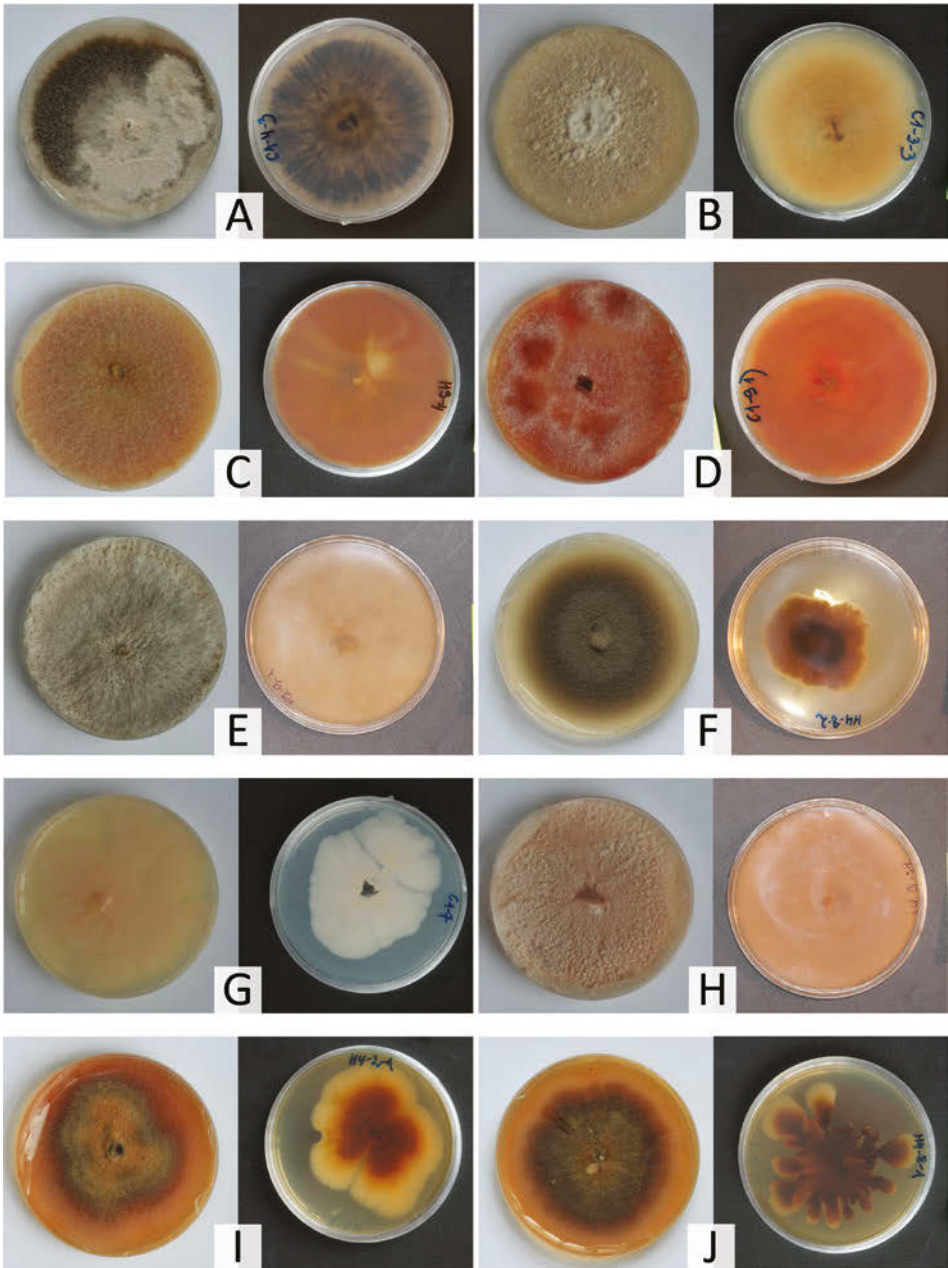


Fig. 1. Photographs of fungal cultures (surface and reverse): A – *Alternaria arbusti*, B – *Phaeosphaeria herpotrichoides*, C – *Gibberella avenacea* (*Fusarium avenaceum*), D – *Fusarium sporotrichioides*, E – *Apiospora montagnei* (*Arthrimum arundis*), F – *Paraphaeosphaeria michotii*, G – *Aureobasidium pullulans*, H – *Monographella* sp., I – *Epicoccum nigrum*, J – *Epicoccum nigrum*, *Lewia viburni* (*Alternaria viburni*) photograph not included.

## DISCUSSION

This study of the endophytes of *P. pratense*, a temperate perennial grass, resulted in the identification of 45 strains from 10 different taxa. According to previous research, the presence of endophytes can be higher in tropical plants (Arnold, Lutzoni 2007), approaching 100%. In plants from colder climates the endophyte diversity is much lower (Rosa et al. 2009). The rate of endophytes is also dependent on environmental conditions. Results from Zamora et al. (2008) suggest that the season, isolation method and tissue health decisively affected the frequency and species distribution of endophytes. Almost all species isolated in Zamora et al. (2008) work were recovered in autumn and nearly half of these exclusively during this season.

The fungal endophytes associated with *P. pratense* have previously been investigated mostly within large scale studies along with other species and in relation to their toxicity to animals, differences in infection rates in natural and cultural communities and diversity (Bacon, Hill 1996; Clay, Holah 1999; Sahay, Varma 1999; Redman et al. 2001; Arnold et al. 2003; Waller et al. 2005; Márquez et al. 2007).

Earlier studies have found, from *P. pratense*, mainly Ascomycota, but also Basidiomycota, Blastocladiomycota, Chytridiomycota and Zygomycota (Waipara et al. 1996; Ellis, Ellis 1997; Sanchez Marquez et al. 2010). These species range from saprobes to pathogens, but some might turn out to be endophytes as well. It is sometimes difficult to differentiate between pathogens and parasites or epiphytes and endophytes. Transitions may occur between these types (Redlin, Carris 1996; Azevedo 1998). Most research concerning *P. pratense* has so far dealt mostly with pathogens, epiphytes and saprobes. For a complete list of previously described fungi connected with *P. pratense* see the following resources: NewCROP ([http://www.hort.purdue.edu/newcrop/duke\\_energy/Phleum\\_pratense.html](http://www.hort.purdue.edu/newcrop/duke_energy/Phleum_pratense.html)), Ellis and Ellis (1997), Ing (1990). Some of these species are known to have only one type of association with the host plant, while others have been found to be either epiphytic, endophytic, saprotrophic or pathogenic, depending on the host plant. This suggests that the type of association is not always constant and depends on many variables.

All of the 10 endophytes identified in this study belong to Ascomycota, four of which were anamorphs for whom teleomorphs connections are unknown or not recorded according to Index Fungorum ([www.indexfungorum.org](http://www.indexfungorum.org)). These are *A. arbusi*, *A. pullulans*, *E. nigrum* and *F. sporotrichioides*. *P. michotii*, *P. herpotrichoides*, and mostly also *Monographella* sp. teleomorphs connections with anamorphs are problematic and mostly unknown or not recorded. Only three of our species have known connections between tele- and anamorphs: *A. montagnei* is associated with *A. arundinis*, *G. avenacea* with *F. avenaceum* and *L. viburni* with *A. viburni*.

In terms of abundance, we found in our study, that *E. nigrum* was the dominating species (colonizing frequency 67% of all isolates (Fig. 2). When one plant sample was shown to have more than one endophyte, *E. nigrum* was always present. Sanchez Marquez et al. (2010) also referred to the high domination rate of *Holcus lanatus* L. by *Epicoccum*, *Alternaria* and *Aureobasidium*.

In terms of fungal diversity based on different water treatments, no definite positive effect of additional water availability was observed.

## CONCLUSIONS

The aim of this study was to identify fungal endophytes living inside the leaves of *P. pratense*.

As a result, 45 strains from 10 different taxa were identified. Half of the taxa isolated in this study – *A. pullulans*, *E. nigrum*, *G. avenacea*, *Monographella* sp., and *P. herpotrichoides* – had been identified from timothy earlier as endophytes (Salkin et al. 1986; Domch et al. 1993; Huang et al. 2008; Osono 2008; Tao et al. 2008; Dodd 2010;). *A. arbusti*, *L. viburni*, *A. montagnei*, *F. sporotrichioides* and *P. michotii* are new endophytic fungi isolated from timothy. Two of these, *A. arbusti* and *L. viburni*, are also new species to Estonia (Varvas, Kullman 2012).

To obtain a better insight into the communities of blade endophytes, *in situ* molecular techniques should be used for unculturable and slowly growing fungal taxa. In particular, 454 sequencing allows to identify of hundreds of species using a large number of samples (Jumpponen, Jones 2009).

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