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# Isolation of Fungal Endophytes from Grasses by Laser Micro Dissection & Pressure Catapulting

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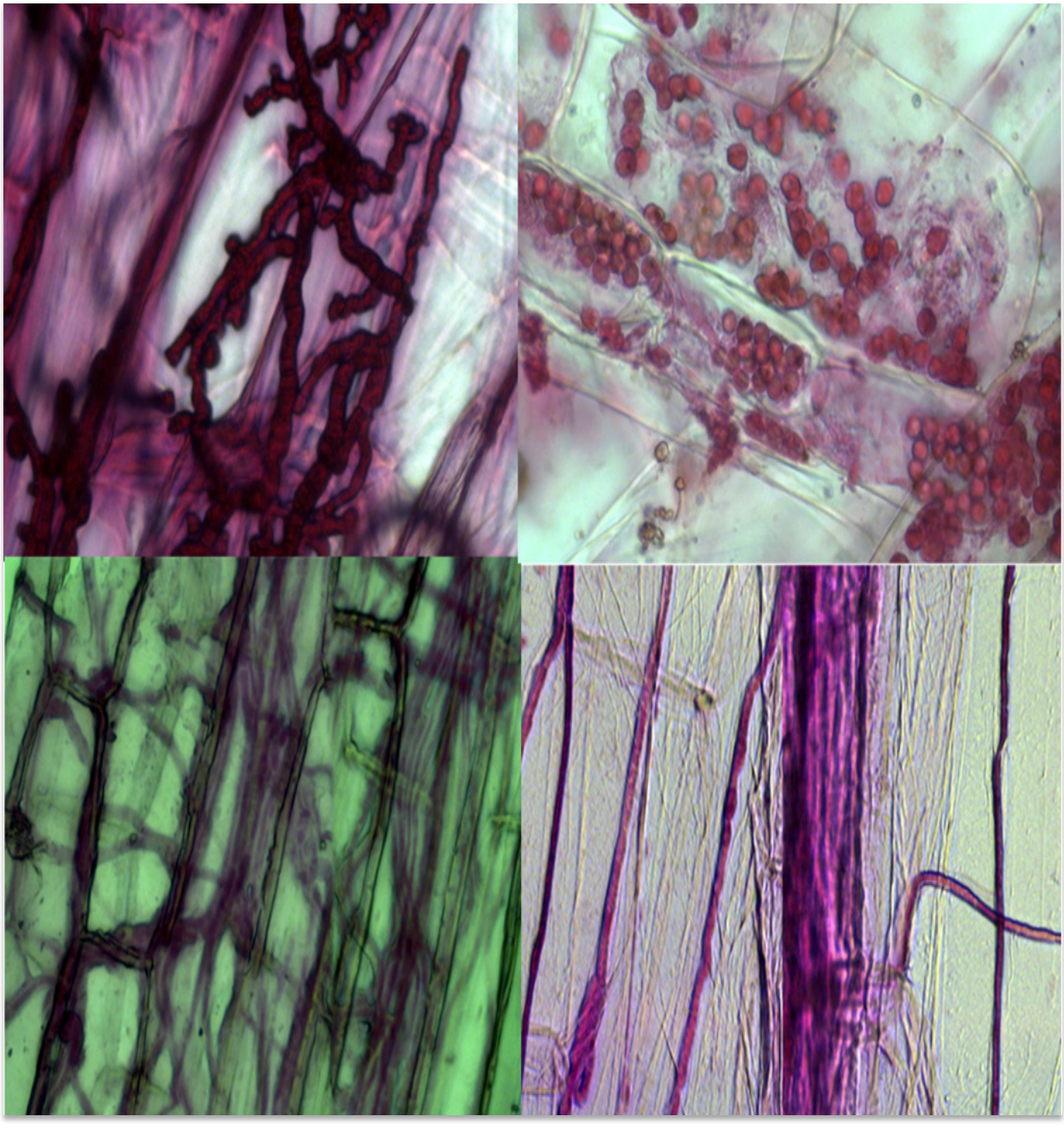
**Xhevahire Jahiri**

*Bio-3950 Master thesis in Biology -- November 2013*



UNIVERSITY OF TROMSØ

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## Acknowledgements

In a successful end, we forget the sacrifices.

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## Abstract

A very diverse group of fungi capable of forming endophytic associations may have profound consequences for natural ecosystems as well as for cultivated plants. Fungal endophytes may benefit their host plants by producing secondary metabolites, and may be an important source for bioactive antimicrobial compounds, used in agriculture, commercial industry, and in medicine. Earlier studies on endophytes using traditional isolation methods were subject to many technical limitations. Molecular approaches available today can overcome some of these technical limitations and provide a more accurate picture of endophytic associations in natural habitats.

In this study, 48 grass individuals were examined for the presence of fungal endophytes. Microscope was used to visualize hyphae morphology and estimate the hyphal load, whereas Laser Micro dissection and Pressure Catapulting (LMPC) technique was used as the first stage for their species determination. With LMPC, hyphae are catapulted and collected directly from the host tissues into the caps of microfuge tubes after which they are identified using molecular techniques.

The grasses belong to the species of *Calamagrostis phragmitoides, Anthoxanthum nipponicum,* and *Festuca* sp. and were collected in meadows along river valleys in eastern Finnmark, northern Norway, during July and August 2008. All grass individuals were found to have several morphologies of hyphae present. From a total of 384 hyphal samples that were catapulted from the three grass species, 36 DNA sequences were successfully isolated by molecular techniques.

The success of retrieving hyphal DNA sequences was similar in all three grass species. The DNA sequences retrieved were shown to belong to six classes of fungal endophytes, namely *Eurotiomycetes, Dothideomycetes, Sordariomycetes, Leotiomycetes* and *Pezizomycetes*, and *Exobasidiomycetes*.

Hyphal load or hyphal morphology was not found to be important for the success of catapulting and PCR amplification. In addition, hyphal morphology was not predictive of endophyte classification, as morphology type was associated with several classes and genera of fungal endophytes.

The conclusion from this study is that although the LMPC technique enables isolation of fungal endophytes directly from grass, other processes, such as the extraction of DNA and the PCR technique, still have limitations for successful endophyte isolation.

# 1. Introduction

The study of fungal endophytes, and in particular their diversity and the understanding of the symbiotic interaction between fungal endophytes and their host grasses, is related to several aspects of biology. For example, to investigate the evolutionary origins of these diverse symbiotic fungi, to determine the community structure and metabolic activity of all fungal symbionts associated with plants across landscapes, to learn more about the contribution of endophytes to plant gene expression, and several questions concerning the biology of endophytes (Rodriguez, White et al. 2009).

Considering the limitations that traditional methods have (Hyde and Soytong 2008), the mycologists need to develop efficient methods for the isolation of fungal endophytes from grasses.

The Laser capture micro dissection (LCM) is a method for isolating cells of interest from specific regions of plant tissue sections, made possible by e.g. the Palm Microbeam equipment by Zeiss. Cells of interest are, when viewed under microscope, cut out and isolated with the use of a laser beam. This technique promises to provide significant improvements in the isolation of fungal endophytes directly from plant tissue (Nancy M. Kerk 2003).

## 1.1 Properties of fungi and the information on endophytes their definition and role

Fungi are among the most widely distributed organisms on earth. Fungi are found in an enormous diversity of habitats and these habitats have diverse characteristics that determine the types of fungi found in them.

Fungi are organized into five phyla *Oomycota, Zygomycota, Ascomycota* (the largest phylum of fungi), *Basidiomycota, Deuteromycetes* and then into classes and orders (Guarro, Gene et al. 1999). They possess characteristic properties that are the key to their specialized lifestyle. All are chemoheterotrophic and obtain their nutrients by absorption, excrete a diversity of enzymes that digest the complex compounds outside the thallus “a type of body that is not differentiated into roots, stems, or leaves” (http://www.thefreedictionary.com/thallus), and then absorb the broken down products. The unit component of growth and development of fungi is the hypha. The hyphae can penetrate in media that gives them access to the nutrition that is inaccessible to other microorganisms. Fungal walls consist mostly of polysaccharides,

where most of them have a complex fibrillar structure built primarily on chitin, chitosan, β-glucans and other polysaccharides. In addition, proteins and lipids are found in the walls, usually in very small concentrations (http://bugs.bio.usyd.edu.au/learning/resources/Mycology/contents.shtml 2004).

Fungi play complex and important ecological roles in the ecosystem, as they continue the cycle of nutrients through ecosystems by breaking down dead organic material, and providing nutrients to plants. Saprophytic fungi are the primary decomposers of plants and woody debris. By decay of cellulose and lignified cellulose, they returned carbon to the atmosphere as carbon dioxide.

Mycorrhizal fungi that inhabit plants roots form mutualistic associations, where the fungal hyphae function is to absorb water and minerals from the soil and protect the roots from parasitic fungi, in return, the plants supplies fungus with carbohydrates (Borneman and Hartin 2000). In terms of their role in humans, fungi, except those that have feeder roles, have proven to be effective curative agents and produce numerous secondary metabolites that have valuable therapeutic properties, such as the antibiotic penicillin derived from the fungi *Penicillium notatum* or the polyketides aflatoxin derived from fungi *Aspergillus flavus* and *A. parasiticus,* (Group 2005). Yeast fungus such as *Saccharomyces cereviseae* are the primary agents responsible for the fermentation.

In agriculture, fungi can provide a means to control plant pests (Luis C. Mejíaa 2008). On the other hand, fungi can also cause a number of devastating plant diseases that affect crop yields. An example of that is the fungus *Puccinia triticina*, the cause of wheat leaf rust (Bolton, Kolmer et al. 2008).

## 1.2 Characteristics of Fungal endophytes

A good definition of endophytes is provided by Petrini (1991); “All organisms inhabiting plant organs that at some time in their life can colonize internal plant tissues without causing apparent harm to the host”.

Endophytic fungi are found in all divisions of fungi and the associations have evolved independently on many occasions (Sydney 2004). The most common endophytes are anamorphic (an artificial assemblage of asexual stages of *Ascomycetes* and *Basidiomycetes*)

members of the *Ascomycetes* and some are closely related to fungi known to cause disease in plants or animals (Seifert 2008). Phylogenetic evidence suggests that some endophytes have evolved from pathogens. The mechanisms of host recognition and colonization may be common among closely related endophytic and pathogenic fungi (http://bugs.bio.usyd.edu.au/learning/resources/Mycology/contents.shtml 2004).

Unlike Mycorrhizae that colonize plant roots and grow out into the rhizosphere, endophytes reside entirely within plant tissues and may grow within roots, stems and/or leaves, emerging only to sporulate during plant or host-tissue senescence (Rodriguez 2009).

### 1.2.1 Importance of Fungal endophytes for plants

"All plants in natural ecosystems appear to be symbiotic with fungal endophytes" (Rodriguez 2009). Endophytes occur intercellularly within the leaves, stems, and reproductive organs of plants. A highly diverse group of fungi may offer significant benefits to their host plants by producing secondary metabolites that provide protection and survival value, such as conferring abiotic and biotic stress tolerance, increasing biomass and decreasing water consumption, enhancing insect and disease resistance. In some cases, endophytes decrease fitness by altering resource allocation (Rodriguez 2009).

Fungal endophytes have proved to be an important source for bioactive antimicrobial compounds such as alkaloids, peptides, steroids and phenol, which have a wide range of applications in the medical field (Strobel, Daisy et al. 2004) (Baby Joseph and R. Mini Priya and 2011). For example, naturally bioactive chemicals produced by the endophytic filamentous fungi *Fusarium spp*. and *Acremonium spp* show antimicrobial activity (Pannapa Powthong 2012). Another endophytic fungus *Pestalotiopsis* sp was isolated from the leaves of *Pinus caneriensis* and showed potent antimicrobial activity by inhibiting the growth of all tested gram positive and gram negative bacteria (Bagyalakshmi Thalavaipandian A 2012).

### 1.2.2 Relationship between Fungal endophytes and plants as a research area

Research on fungal endophytes has largely focused on interactions with host plants, characterization of novel metabolites, and other topics related to endophytic symbioses

(Arnold 2007). New questions constantly arise to characterize the interaction between endophytes and plants such as: How do endophytes communicate with hosts? Which mechanisms do they use to confer fitness benefits, and other questions about specific bioactivity of particular lineages of endophytes.

Methodological limitations have limited the study of this potentially very important group of organisms. The major challenge in the study of endophytes is their life style, knowing that the endophytes are internal to plant structures.

Therefore, technical methods play an important role in exploring the potential of fungal endophytes and their special features. In reality, they are the key to the success for such investigations.

## 1.3 Fungal endophytes isolation methods

Detection and isolation of fungal endophytes rely on microscopy using their morphological characters, on cultivation using several different types of media, and the use of molecular diagnostics methods (Affiliation Cetus Corporation 1990).

### 1.3.1 Traditional, cultivation methods

The fungal identification based on the analysis of morphological characters by culture is as follows: Surface sterilization of plant tissues, and maceration of the plant tissue and streaking the macerate onto nutrient agar, or plating small sterilized segments onto nutrient agar or Potato dextrose agar (Figure 1)(Johannes Hallmann 2006).

Results obtained from these existing techniques are not sufficient to provide a complete overview of the endophytes-plant associations. These methods are often time consuming, lack sensitivity and specificity, are slow, labour intensive and difficult to interpret (Hyde and Soytong 2008).

Many fungi are unable to grow in culture (Duong, McKenzie et al. 2008) Some fungi are very slow growing (Zhu, Yu et al. 2008), while others require specific media (M. van Wyk 2007). Thus, when we isolate endophytes by traditional methodology, some or perhaps numerous endophytes may remain unisolated (Hyde and Soytong 2008). These limitations make the

traditional methods less efficient (Hawksworth 1985). Another approach used to identify fungal endophytes in plants is microscopic visualization, where hyphae morphology are used for identification. Identification of fungal communities using morphology have limitations, as morphological characters are unstable and can change with environmental changes (Sydney 2004).


**Figure 1** Growth of endophytes a few weeks after stems from grass were placed on Potato Dextrose Agar (PDA) with chloramphenicol. Bar lines = 10 mm. (Hellequin, Nicolas Patrick Antoine June 2008).

### 1.3.2 Staining methods and Laser Microdissection and Pressure Catapulting (LMPC) technique

In order to observe and to classify the presence of fungal endophytes in plants, staining methods have been used where endophytes are defined on the basis of morphological characteristics and ideally by the differences among them. Several different staining methods have been used to stain fungal endophytes. Lacto phenol or Cotton Blue and Gentian violet are among these staining methods.

In the Lacto Phenol method, fungi stain blue for easier visualization and examination (see Figure 2)(Clay and Jones 1984) (Harvey, Fletcher et al. 1982). Cotton blue stains the protoplasm of the fungus but does not stain the cell walls (Sampson 1933). Consequently, the septa of spores and mycelium stand out very distinctly because the entire protoplast is stained; the cytoplasm and nuclei are indistinguishable. Gentian violet method does not provide differential staining. It is used as a general histological stain because of its simplicity and its

clear-cut staining (Sampson 1933). Lacto phenol Trypan blue(see Figure 2) is the most used method for staining vesicular - arbuscular Mycorrhizae and provides good results within tree root section (Funk, Halisky et al. 1983, Barrow 2003). Aniline blue (see Figure 2) is also used to stain mycelium (Bacon, Porter et al. 1977).

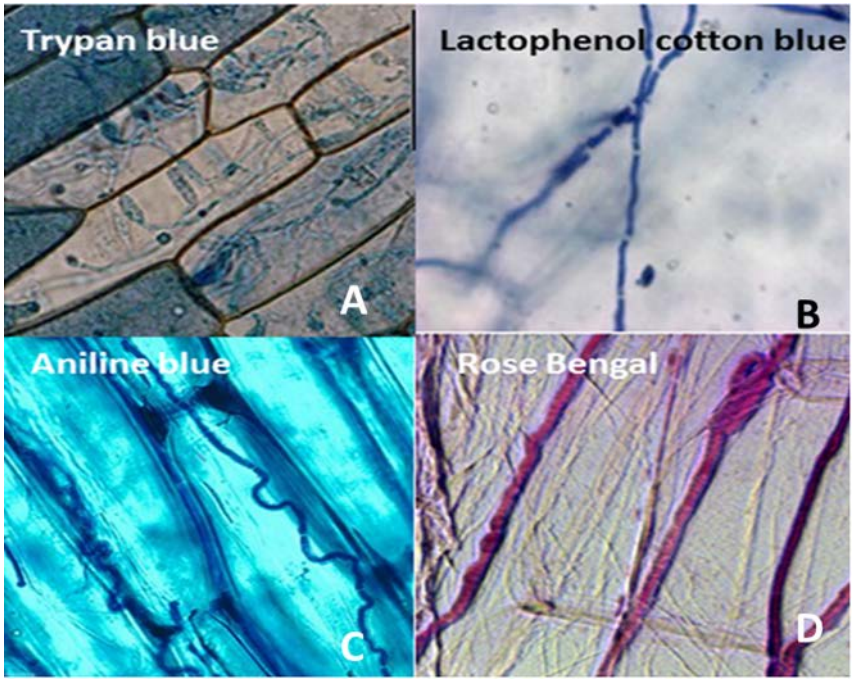


**Figure 2.** Images showing the presence of fungal endophytes stained with four different stains; (A) endophytes stained with Trypan blue (www.sciencedirect.com). (B) endophytes stained with Lactophenol cotton blue (www.springerimages.com). (C) endophytes used Aniline blue to stain endophytes (www.forages.oregonstate) and (D) used Rose Bengal to stain endophytes (Xhevahire Jahiri).

These staining methods offer poor visualization of the fungus, due to extended boiling or staining times. These stains that contain hazardous materials, such as Lactophenol cotton blue, due to the presence of phenol (a caustic, poisonous, white crystalline compound) (http://www.thefreedictionary.com/phenol) that may be potential threats to the health of the researchers and the environment. In particular, they also cause loss of considerable sections of cells during staining procedure, because some methods require careful boiling of the plant tissue (Saha, Jackson et al. 1988).

To overcome the weaknesses of the aforementioned staining methods, Saha et al. have described, a simple, safe, and rapid method (Saha, Jackson et al. 1988). Rose Bengal stain offers better visualization, is fast (30-60 sec) compared to trypan blue (3-5 min), is safer to use, and with the use of a green filter it provides enhanced contrast. An additional advantage

with this stain is that it dried as well as fresh grass could be stained, which means that grass samples can be stored and processed later (Saha, Jackson et al. 1988).

Even though microscopic visualization (Richard J Howard 2004) is a widely used approach for assessing the occurrence of fungal endophytes, it is not precise enough to confirm the species, without the involvement of molecular methods such as PCR and DNA sequence analysis (Consuelo Ferrer1 2001).

Laser capture micro dissection pressure catapulting (LMPC) is an additional promising tool. LMPC allows selective procurement of the targeted cells, under direct microscopic visualization and permits rapid one-step procurement of selected fungal cell populations from a section of complex, heterogeneous, e.g., plant tissue (Balestrini and Bonfante 2008). When used for biological tissues, LMPC is based on wavelengths in the infrared or in the ultraviolet regions, admitting a high-energy laser that allows for very focused and precise cutting, and where the high concentration of photons destroys the chemical bonds in the tissue. The laser of this system has a wavelength of 355 nm. It allows working on living cells or tissues without causing significant artefacts on DNA (Richard J Howard 2004).

### 1.3.3 Molecular methods and phylogenetic analyses

The application of molecular techniques is used to estimate the occurrence of fungal endophytes inside the grass and their species diversity. Fungal endophytes classification and identification is based on DNA sequence analysis, using polymerase chain reaction (PCR) process to amplify internal transcribed spacer (ITS) region, the part of non-functional ribosomal DNA, as a universal DNA barcode marker for fungi (Schoch CL 2012). PCR is a laboratory technique designed to amplify a specific DNA sequence from a starting template, and the technologies provide multiple copies from one single copy using DNA polymerase enzymes under controlled conditions.

To separate DNA fragments by size after the PCR, agarose gel electrophoresis is used. In this process, electrical power is used to make DNA fragments move through an agar gel medium. As the DNA is negative, it will navigate towards the positive electrode of the gel box, and identically sized fragments of DNA will travel at the same distance. The DNA products are stained with ethidium bromide at loading and visualized using of UV-light.

When the targeted DNA fragment has been amplified by PCR, and visualized after agarose gel electrophoresis, they are isolated and sequenced for further investigation. DNA sequencing is the process of determining the precise order of the nucleotide bases in a strand of DNA molecule. To read DNA sequences, computer software is used. In order to investigate genetic relationships based on the obtained sequences, they are compared for a similarity to DNA sequences in non-redundant databases such as GenBank of National Center for Biotechnology Information (NCBI). Basic Local Alignment Search Tool (BLAST) is a bioinformatics software applied to find regions of local similarity between sequences, and the software can infer functional and evolutionary relationships between these sequences (Rajesh Jeewon 2013).

Phylogenetic analyses are used to gain information about the organisms and their evolutionary relationships, where the DNA sequences are used mainly.

> "The most convenient way to build hypotheses, or models, of life's history, phylogenetic trees are built, and presented as evolutionary relationships among a group of organisms. A phylogenetic tree is composed of nodes, that represents a taxonomic unit (species, populations, individuals), and branches, which define the relationship between the taxonomic units in terms of descent and ancestry." (http://www.ncbi.nlm.nih.gov/About/primer/bioinformatics.html)

## 1.4 Aim of this study

The main aim of this thesis was to test Laser Microdissection and Pressure Catapulting (LMPC) as a potential tool for fungal endophytes isolation and identification from grass tissues. Developing a reliable protocol for fungal DNA extraction and PCR amplification was an ancillary goal of this project.

In addition, the combination of hyphal morphology and amount (load) will be investigated, for their role in the success of using LMPC and PCR amplification of ITS fungal regions.

# 2. Materials and methods

## 2.1 Source of plant material:

Three common perennial grass species were selected to study fungal endophytes infection, *Calamagrostis phragmitoides* (17 individuals), *Anthoxanthum nipponicum* (18 individuals) and *Festuca sp*. (13 individuals). The grasses were collected in meadows along river valleys in eastern Finnmark, northern Norway.Two of them, Komagdalen (KO) and Vestre Jakobselv (VJ), were at Varanger peninsula (70 – 71 ° N, 28 – 31 ° E) and one at the Ifjordfjellet (IF) mountain plateau about 100 km further west (71 ° N, 27 ° E), during summer July and August in 2008; see (Soininen, Brathen et al. 2013) for sampling methodology. This region where these grass species were collected has been found to host a range of endophytes species (John Beck Jensen 2011)**.**

The grass samples were placed in folded newspaper, air dried and stored in paper bags at room temperature for one month. Stems and leaf sheaths of the three grasses were used to investigate for occurrence of the fungal endophytes.

## 2.2 Sample preparation

Selected stems or leaf sheaths from 48 individuals of the three grass species were cut in parts approximately 5 cm in length and treated with 25% acetic acid ($CH_3COOH$) for 24-48 hours to eliminate epiphytes (Abd-El-Kareema 2009), soften grass material and to remove the green colour of chlorophyll. Afterwards, the segments were rinsed three times with distilled water, and placed in new tubes with distilled water to avoid drying.

## 2.3 Fungal detection procedure

In order to make microscope slides with preparations from the grass stems or leaf sheaths, we gently took samples from the tube with distilled water and used the following procedure:

First we opened the stem or leaf sheath and peeled of a thin layer on the inside, pulled the most possible thin layer down the stem or leaf sheath. Three to four peeled sections were placed on a glass slide, and one to two drops of rose Bengal solution applied to the samples

(0.5% rose Bengal dissolved in 5% aqueous ethyl alcohol (Saha, Jackson et al. 1988)). The stain must cover all sections. This procedure must be carried out fast since the samples can curl up. 30 seconds is sufficient for a successful staining procedure. After staining, a cover glass is placed over the stained samples where it is pressed down to remove the solution in excess after staining. This staining procedure was carried out for each stem or leaf sheath of all grass individuals. All preparations/slides were examined at 63X magnification under light microscope using a green filter for better contrast (Saha, Jackson et al. 1988).

As key for the identification of fungal endophytes within the grass, eight images of fungal hyphae morphology marked with labels from H1 to H8 and four images of fungal spores morphology marked with labels S1 to S4 that are shown at Figure 3, were used. These eight hyphae morphology are described in Table 1 based on their microscopic images.

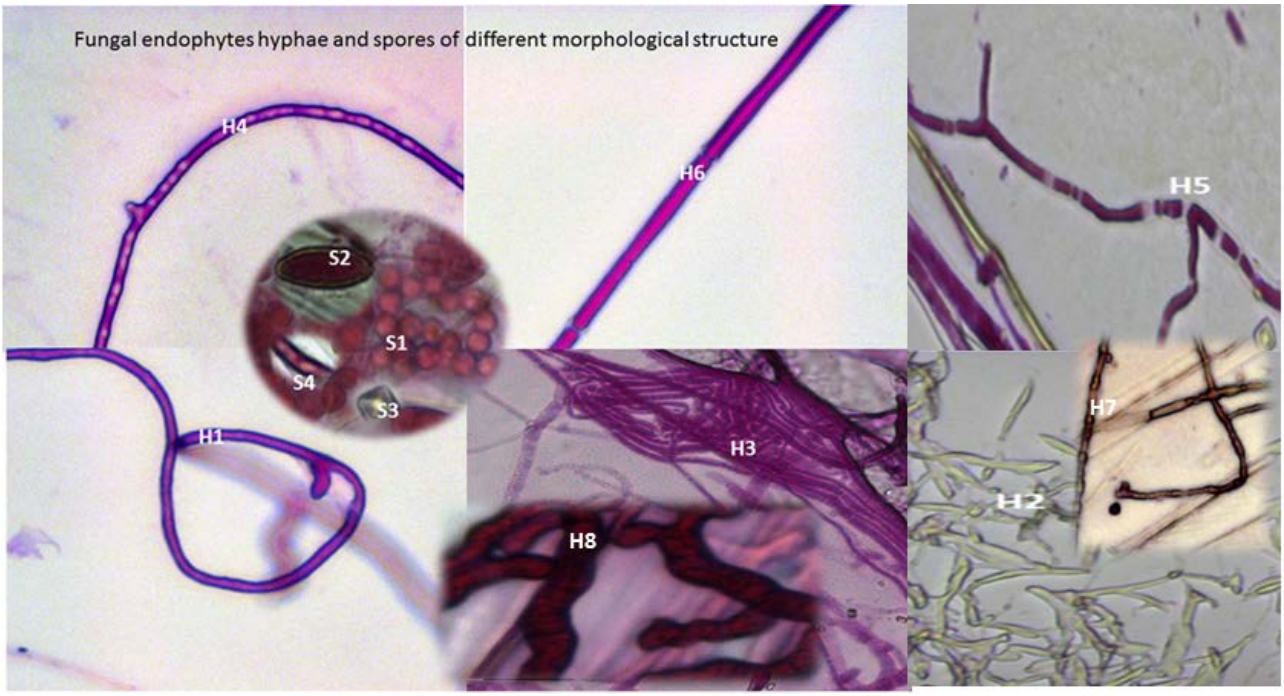


**Figure 3.** Eight images of fungal hyphae with different morphology (marked with labels from H1 to H8) and four images of fungal spores with different morphology (marked with labels S1 to S4) used as key for the identification of fungal endophytes within grasses. Examination of fungal endophytes was done at 63X magnification under light microscope with a green filter. All images of fungal hyphae are obtained from *Calamagrostis phragmitoides*, *Anthoxanthum nipponicum* and *Festuca sp*. Table 1 below has more detailed descriptions for all eight different hyphae types.

**Table 1.** Description of eight fungal endophytes hyphae types with different morphology found by microscope inspection (see figure 3).

| |
|---|
| **H1 -** Hyphae does not contain septets, walls are well pronounced after staining, their cytoplasm completely stained with rose Bengal, when these elongate and penetrate, they branched little or elongate unbranched. |
| **H2 -** Hyphae are short and do not contain septets, walls are well pronounced, their cytoplasm is completely stained/or not with rose Bengal, they are curved and slightly branched. |
| **H3 -** Hyphae do not contain septets, walls are not well pronounced after staining, they are not branched and parallel follow each other, and cytoplasm is completely stained. |
| **H4 -** Hyphae do not contain septets, walls are well pronounced, after staining their cytoplasm is not completely stained; one part is stained, and one part is not, when these elongate and penetrate branched little or unbranched. |
| **H5** - Hyphae contain very short septae, are very thin, and they branched in the form of the letter V. |
| **H6** - Hyphae contain septets that separate the hypha in equal long parts, branched to some degree or not at all, walls are well pronounced after staining and cytoplasm is completely stained/or not with rose Bengal. |
| **H7 -** Hyphae are short strands, containing septets that do not separate the hypha in equal long parts. They are well pronounced after staining where the cytoplasm is not stained in its entirety. |
| **H8** - Hyphae contain septets that separate the hypha in equal short parts, are very strong stained with rose Bengal, are highly branched and look like Mycorrhizae. |

## 2.4 Fungal isolation

### 2.4.1 Laser Micro dissection and Pressure Catapulting (LMPC)

Eight samples were catapulted from each of the 48 individuals of three grass species, i.e. a total of 384 samples (tubes). The experimental procedure of fungal endophytes catapulting from inside of grasses is shown; see Figure 4 and the text below that explains the procedure for catapulting from a microscope slide.

- Placed collection tube cap over tissue section.
- Selected the hyphae boundaries or areas with hyphae (see Figure 4, where green line indicates selected area and white line indicates cut area) for laser micro dissection.
- Selected laser pulse point.
- Laser pulse catapulted dissected area to collected cap (see Figure 4, where the blue arrow indicates for catapulted area) filled with 70 µl lysis buffer.
- Looked into the collection cap, for catapulting effectiveness (10X magnification).

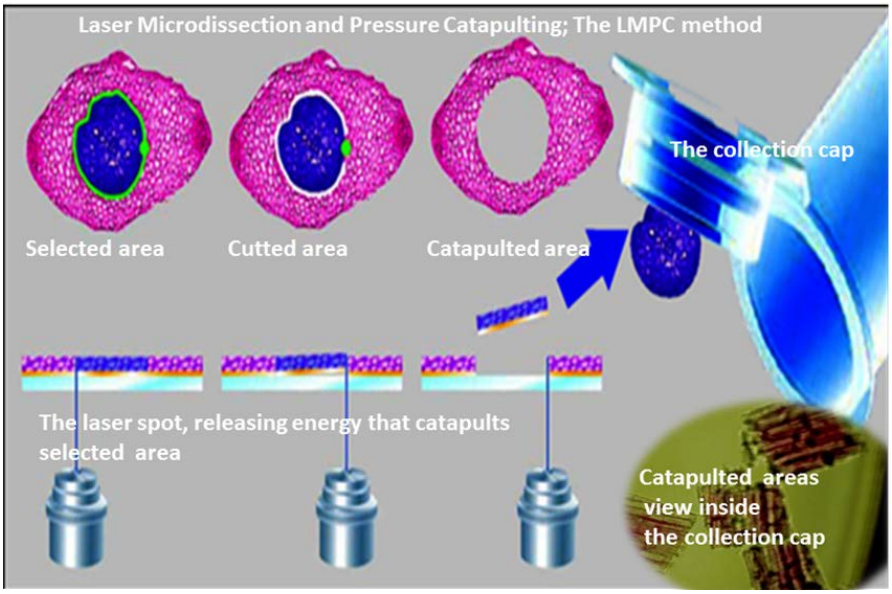


**Figure 4**. The process of isolation of fungal endophytes directly from the inside of grasses by use of Laser Micro dissection and Pressure catapulting. The figure is modified from (http://www.u-picardie.fr/plateforme/icap/images/Palm%20MicroBeam.pdf).

After the catapulting step, DNA was isolated from the collected samples. This procedure is outlined in Figure 5.

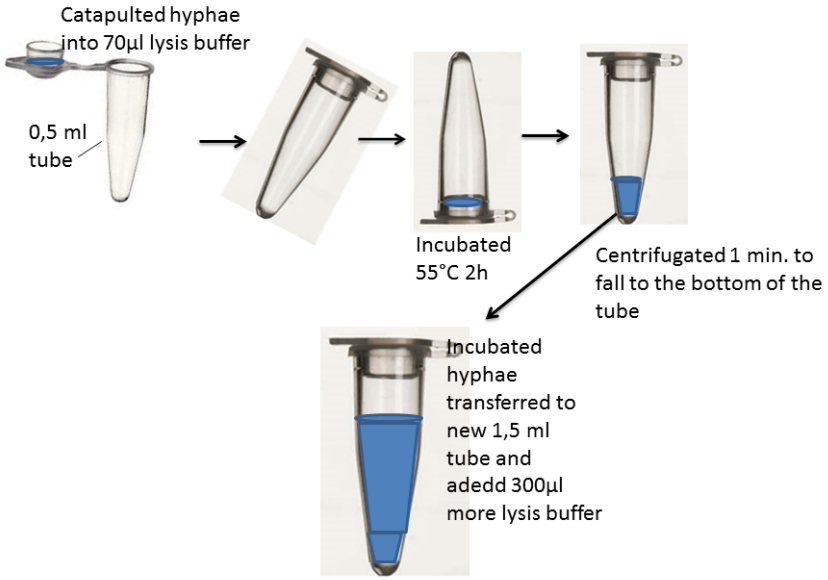


**Figure 5** Schematic illustrations of the catapult proceedings up to the next technique for DNA extraction. Modified figure from us.vwr.com, www.fishersci.com.

## 2.5 DNA extraction

We used DNA extraction to obtain DNA in pure form intended for further analysis to identify the fungal endophytes species.

DNA was extracted from catapulted hyphae that was catapulted into 70µl lysis buffer (see the contents of the buffer below) using a modified method of the protocol described in PCR protocols : A guide to methods and applications (Affiliation Cetus Corporation 1990, Ward 2008).

**Lysis buffer**
1.4M NaCl, 0,1M Tris hydrochloride, 20 mM
ethylenediaminetetraacetate (EDTA),
2% Cetyltrimethylammonium bromide (CTAB)
pH 8.0)

The samples were frozen and thawed twice at -80°C (20 min) and 65°C (10-45 min) respectively. They were incubated for 45 min, and the suspensions were extracted twice with 1 vol. (300 µl) of chloroform for 15 min centrifugation at 10000 g. The aqua phase was removed and the DNA was precipitated with one vol. (300 µl) of ice-cold isopropanol for 10 min. DNA was harvested by centrifugation for 20 min 13000 g and the pellet was washed with 1 ml cold 70% ethanol, and finally re-suspended in 60 µl $dH_2O$ (Affiliation Cetus Corporation 1990).

We also tried two other DNA extraction techniques described in Table 12, see Appendix III.

## 2.6 Polymerase chain reaction (PCR)

Extracted and purified DNA from catapulted grass tissues infected with fungal endophytes is used as a template for polymerase chain reaction (PCR). First, a mixture is created (see table 2) consisting of:

- DNA template to be copied;
- Polymerase enzyme to synthesize new DNA;
- Primers, as a starting point that matches exactly the beginning and end of the DNA template;

- Deoxyribonucleotide triphosphates (dNTPs), as the building blocks from which the DNA polymerase can synthesize new DNA; and
- Magnesium chloride salt solution (MgCl2) a buffer to create an optimal environment for the reaction.

The reaction is performed in an automated machine (thermo cycler), which is capable of rapidly increasing and decreasing the temperature. For steps of PCR, see Table 3.

We know that DNA is a double stranded molecule linked together by weak hydrogen bonds. The DNA needs to be separated into single strands to be able to copy.

By heating the PCR mixture at 94°C the double helix of DNA is denatured. When temperature is lowered to 52°C, the primers anneal to their matching sequence on the original DNA strand. When the sample is reheated to 72°C, DNA polymerase adds complementary nucleotides to elongate DNA. During elongation, DNA polymerase uses the original single strand of DNA as a template to add complimentary dNTPs to the 3' ends of each primer. Repeated heating and cooling rapidly amplifies the DNA segment of interest, in our case the ITS region.

**Table 2**. The reagents and their volumes in the 25 µl DyNAzyme $^{TM}$ DNA Polymerase PCR reaction.

| Reagent | Volume (µl) |
|---|---|
| 10X DyNAzyme$^{TM}$ buffer | 2,5 |
| 10 mM dNTPs | 0,5 |
| 25 mM $MgCl_2$ | 0,5 |
| 25 pmol/µl ITS-5 forwardprimer | 0,5 |
| 25 pmol/µl ITS-4 reverseprimer | 0,5 |
| DyNAzymeTM DNA Polymerase | 0,5 |
| Template | 10 |
| MQ water | 10 |

**Table 3**. PCR program; to generate the desired amount of amplification, steps are repeated 35 times*

| Step | Temperature | Duration |
|---|---|---|
| First denaturation | 94°C | 3 min |
| Denaturation * | 94°C | 30 sec |
| Annealing * | 52°C | 30 sec |
| Extension* | 72°C | 1 min |
| Finish extension | 72°C | 10 min |
| Hold | 4°C | ∞ |

The ITS regions have been regarded as non-functional sequences, and regions that are often highly variable among fungal species. The multi copy characteristic of the rDNA repeat makes the ITS regions easy to amplify from small DNA samples (Gardes and Bruns 1993). Because the internal transcribed spacer (ITS) region is a convenient target region for molecular identification of fungi, we have used ITS4 and ITS5 primers to amplify this region. The primers ITS4-ITS5 (T.M. White 1990) have been described schematically in Figure 6, whereas the length and order of primers nucleotides is shown in table 4; the ITS4-ITS5 primers amplify 600bp-800bp at fungal ITS regions (Gardes and Bruns 1993).

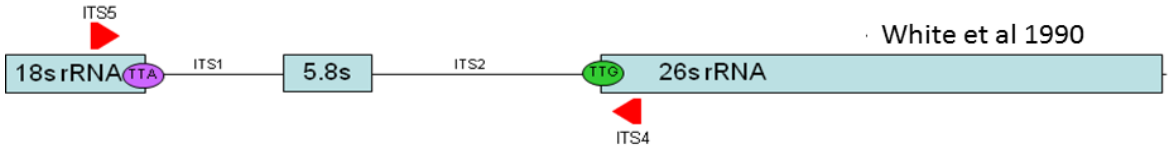


**Figure 6.** Schematic illustration of the internal transcribed spacers (ITS) numbered from 5' end. Red arrows indicate orientation and approximate position of primer sites. (Images modified from www.phytophthoradb.org - 960 × 720.)

**Table 4.** The primers used for amplification of rDNA by PCR process.

| Primer | Sequence (5`→3`) |
|---|---|
| ITS-4 Reverse primer | TCCTCCGCTTATTGATATGC |
| ITS-5 Forward primer: | GGAAGTAAAAGTCGTAACAAGG |

## 2.7 Agarose Gel Electrophoresis

To perform gel elctrophoresis, 0.8% agarose powder is solubilized in electrophoresis buffer (1xTAE)(http://bioinfoweb.com), and then heated in a microwave oven until completely melted. Subsequently, the mixture is poured into a gel frame and seven µl Ethidium Bromide is added to the gel to facilitate visualization of DNA after electrophoresis. One µl 6X Loading buffer was added to the samples (10 µl) to ensure that the samples sink to the bottom of the wells and allow for possible monitoring of their movement through the gel. In order to estimate DNA product sizes, DNA molecular weight markers GeneRuler$^{TM}$ DNA Ladder Mix are included in all gel runs. Agarose gels are run at 60 V for approximately one hour. DNA products are visualized using UV-light and photographed.

## 2.8 DNA Sequencing

DNA sequencing was done to find out which of fungal endophytes species was isolated.

To obtain the accurate base sequence from a PCR amplified piece of DNA, excess primers, nucleotides, and also residual alcohol, salts and phenol must be removed. It is important to have pure DNA, because impure template preparations can inhibit the cycle sequencing reaction, and provide incorrect results.

The purification of PCR products made using ExoSAP-IT, an enzymatic clean up method, prepares PCR products for sequencing application, see Table 5 (Corporation 2000).

**Table 5** Reagent and respective conditions needed to perform ExoSAP-IT method.

| Reagent | Volume | Incubation temp/ time |
|---|---|---|
| ExoSAP-IT (A) | 1µl | 37°C for one hour and 85 °C for 15 minutes. |
| PCR product(B) | 15 µl | |
| Mix (A)+(B) | 16 µl | |

After the ExoSAP-IT treatment of the PCR products, cycle sequencing was carried out in a thermal cycler. It is only a single primer (ITS-4 primer) used complementary to the 3`end of the strand, and only one strand is copied during sequencing.

BigDye Terminator v3.1 is suitable for performing fluorescence-based cycle sequencing reactions on single-stranded Sequencing buffer used to stabilize the reagents and products in the sequencing reaction. The list of sequencing mixture and cycle sequencing can be found in Table 6 and Table 7, respectively.

**Table 6.** Sequencing mixture

| Reagent | Volume |
|---|---|
| BigDye Terminator v3.1 | 1µl |
| sequencing buffer (5X) | 3µl |
| 25 pmol/ µl ITS-4 primer | 1µl |
| PCR product | 3µl |
| MQ water | 12 µl |

**Table 7** PCR program for BigDye Terminator v3.1; Sequencing Cycle

| Step | Temperature | Duration |
|---|---|---|
| First denaturation | 96°C | 1min |
| Denaturation * | 96°C | 10 sec * |
| Annealing * | 50°C | 10 sec * |
| Extension* | 60°C | 4 min * |
| Hold | 4°C | ∞ |

*The steps; denaturation, annealing and extension are repeated 25 times

After sequencing, the nucleotide sequences were determined at the DNA Sequencing Core Facility (Universitetssykehuset Nord Norge).

## 2.9 Phylogenetic analyses

MEGA (Molecular Evolutionary Genetics Analysis) is used as a tool for conducting sequence alignment. These sequence alignment are used to make phylogenetic trees.

The analyses used to construct phylogenetic tree were conducted with the following specifications: Nucleotides were used as substitutions type, maximum likelihood was used as the statistical method, bootstrap method with 2000 bootstrap replications was used to test phylogeny, and finally the model method used was the Kimura 2-parameter model with the tree inference options Heuristic method - Close-Neighbor-Interchange.

Sequence similarity searches were performed for each of the 36 representative fungal sequences against the non-redundant database maintained by the National Center for Biotechnology Information (NCBI) using the "Local Alignment Search Tool" (BLAST) as the tool to infer functional and evolutionary relationships between sequences as well as help to identify members of gene families http://www.ncbi.nlm.nih.gov/About/primer/bioinformatics.html.

In order to test the success of catapulting and PCR according to grass species, hyphal morphology and hyphal load, we made three microscopic slides for each of three sections per individual grass sample (Figure 11 and Appendix I), with three tissue samples per section per slide. We scored morphology and counted number of hyphae for each section/slide at a magnitude of 180 µm.

# 3. Results

The process of isolating fungal endophytes directly from the grass carried out using LMPC and all other methods and techniques that we have described above, provided the following results**.**

## 3.1 Fungal detection

All the stems or leaf sheaths from the perennial grasses *Calamagrostis phragmitoides*, *Anthoxanthum nipponicum* and *Festuca sp*., showed the occurrence of fungal endophytes, see Figures 7, 8 (and Figures 12, 13, 14 in Appendix II). These images show the ability of Rose Bengal to infiltrate the inside of the plant tissue, to stain hyphae of fungal endophytes, and provide image contrast when using green filter in microscopic examination. The staining enabled us to show differences of fungal hyphae and that their morphology is different not only between grass species, but also within the same grass species (see images in Figures 7, 8).

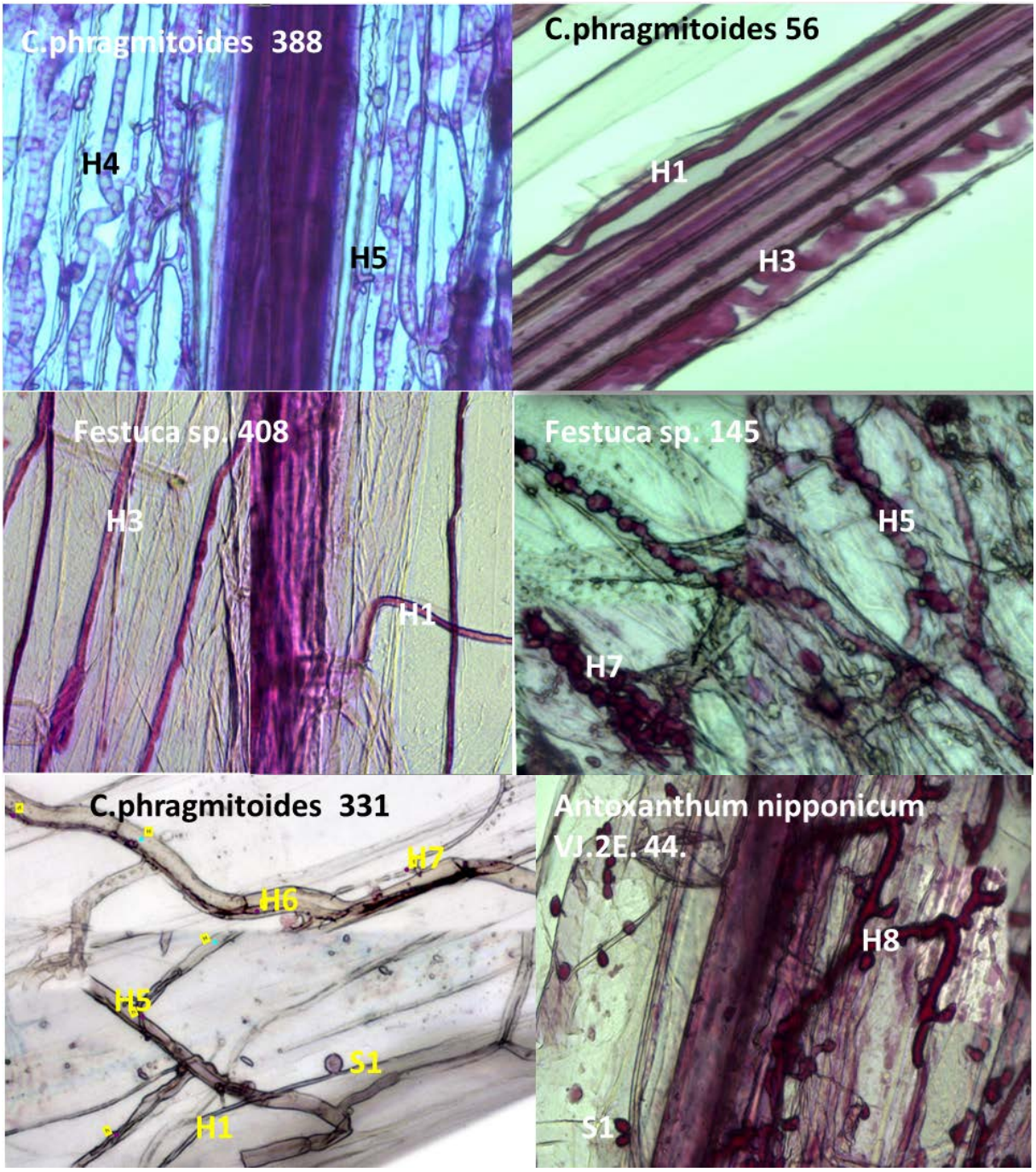


**Figure 7.** Images taken from three perennial grasses examined at 63X magnification under a light microscope with a green filter. The occurrence of fungal endophytes, their hyphae- and spore morphologies and distribution are shown. The observed fungal hyphae morphology within the grasses are categorized and labelled according to Table 1. *Calamagrostis phragmitoides* 388 have these hyphae (H4, H5), *Calamagrostis phragmitoides* 56 (H1, H3), *Festuca sp*. 408 (H3, H1), *Festuca sp*. 145 (H5, H7), *Calamagrostis phragmitoides* 331 (H1, H5, H6, H7 and spore S1), *Antoxanthum nipponicum (VJ.2E. 44).* **(**H8 and S1) the presences of hyphae marked with H8 are present only in this individual **(**388, 331, 408, 145, 44)*- names of areas where the collection is made, Vestre Jakobselv (70$^{o}$18´ N, 29$^{o}$16´ E) and Komagdalen (70$^{o}$19´ N, 30$^{o}$01´ E) Ifjordfjellet (70$^{o}$25´ N, 27$^{o}$20´ E) and grass treatments

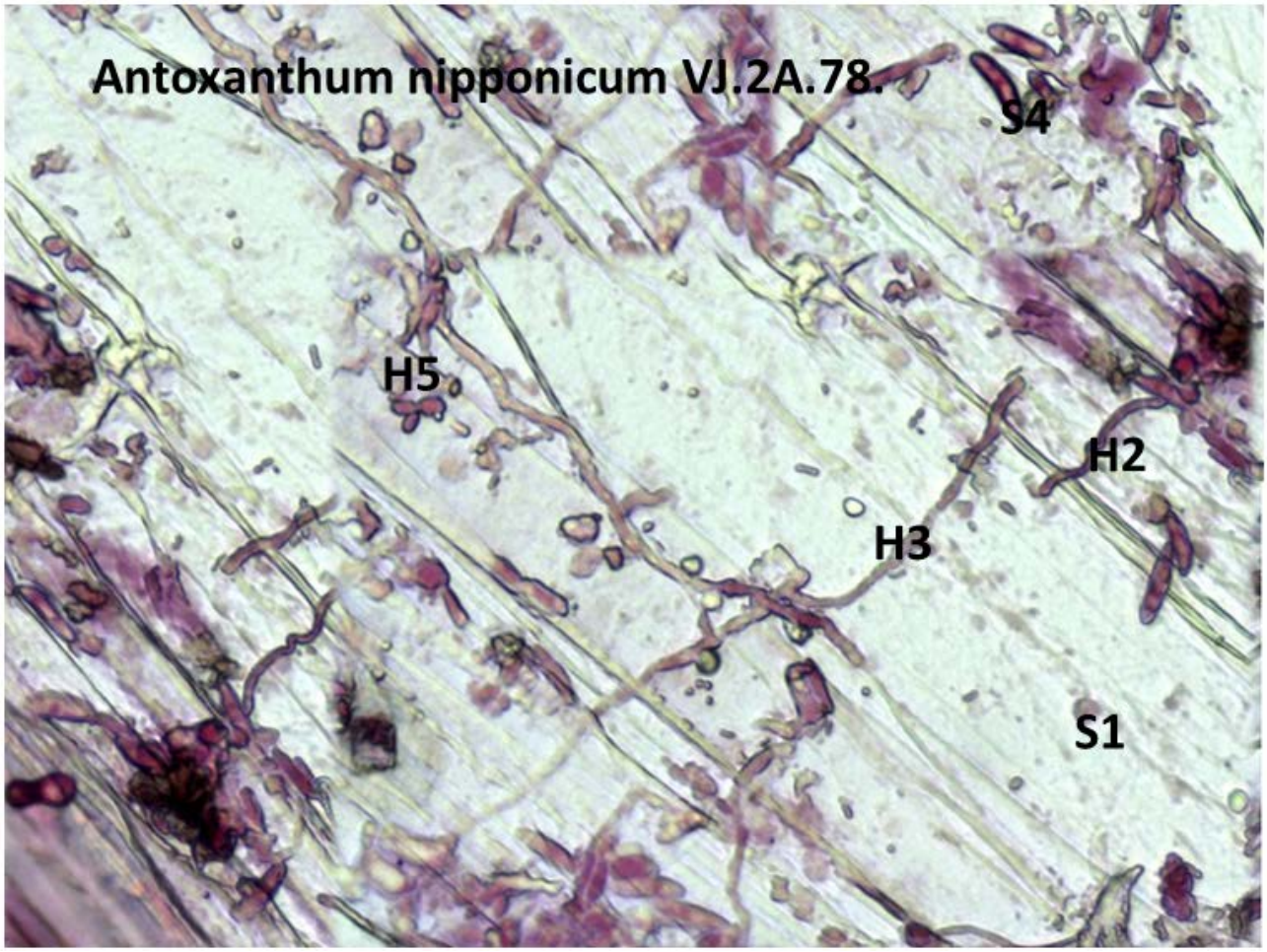


**Figure 8**. Occurrence of fungal endophytes within *Antoxanthum nipponicum*, collected from Vestre Jakobselv (VJ) in Finnmark, northeastern Norway. These fungal hyphae of different morphology marked with (H2, H3, H5) and spores marked with (S1, S2), were examined at 63X magnification under a light microscope with a green filter. Characteristic of this grass individual is the presence of the two different spores and three different hyphae. (VJ.2A.78)* Means the study area Vestre Jakobselv ($70^0$18´ N, $29^0$16´ E) and grass treatments.

The hyphae labelled with H1 are present in 99% of the individuals of the investigated three grass species (Table 8. Hyphae labelled with H2, H3, H 4, H5 and H6, are also present in nearly all the investigated samples of the three grass species, whereas H7 and H8 are more seldom occurring (Table 8).

**Table 8.** Results in the table indicate that hyphae types of morphology do not seem to be significant to the success of catapulting or PCR process.

| Grass species | PCR result | Count of *H1 | Count of *H2 | Count of *H3 | Count of *H4 | Count of *H5 | Count of *H6 | Count of *H7 | Count of *H8 | Number of Hyphae types present |
|---|---|---|---|---|---|---|---|---|---|---|
| ***A. nipponicum*** | Yes | 4 | 4 | 3 | | 2 | | 1 | | 5 |
| ***C. phragmitoides*** | Yes | 3 | 3 | 3 | 2 | 3 | 2 | 2 | | 7 |
| ***Festuca* sp.** | Yes | 2 | 1 | 1 | 2 | | | | | 4 |
| ***A. nipponicum*** | No | 11 | 11 | 3 | | 2 | | | 1 | 5 |
| ***C. phragmitoides*** | No | 6 | 6 | 9 | 3 | 2 | 1 | | | 6 |
| ***Festuca* sp.** | No | 7 | 3 | 2 | 5 | 5 | | | | 5 |

***the H1-H8 abbreviation refers to the fungal hyphal morphology described in Table 1.

## 3.2 Laser Micro dissection and Pressure Catapulting

In order to verify if the catapulted parts have reached the objective, i.e. cap tube, microscopy was performed into the tube cap, as described at (2.4.1).

The catapulting process was successfully completed in all 384 samples (tube) made from each of the 48 individuals of grass species *A. nipponicum, C. phragmitoides, Festuca sp.* Pictures of catapulted parts after microscopy (10X magnification) are shown in Figure 15 Appendix V.

## 3.3 DNA extraction and Polymerase chain reaction (PCR)

Based on microscopic observations, all 48 grass samples showed occurrence of fungal endophytes. The effectiveness of fungal DNA extraction from 384 samples laser catapulted is low, because of the 384 samples, only 36 ITS sequences of fungal endophytes were isolated.

In order to amplify the ITS regions of extracted fungal DNA, PCR was performed using forward ITS5 and reverse ITS4 primers (see Table 4). The ITS PCR products after size separation by agarose gel electrophoresis are shown in Figure 9.

In Figure 9, it can be seen that the PCR products give the expected sizes of 600bp-800bp, indicating that the ITS4 and ITS5 primers successfully amplified fungal DNA internal transcribed spacer (ITS).

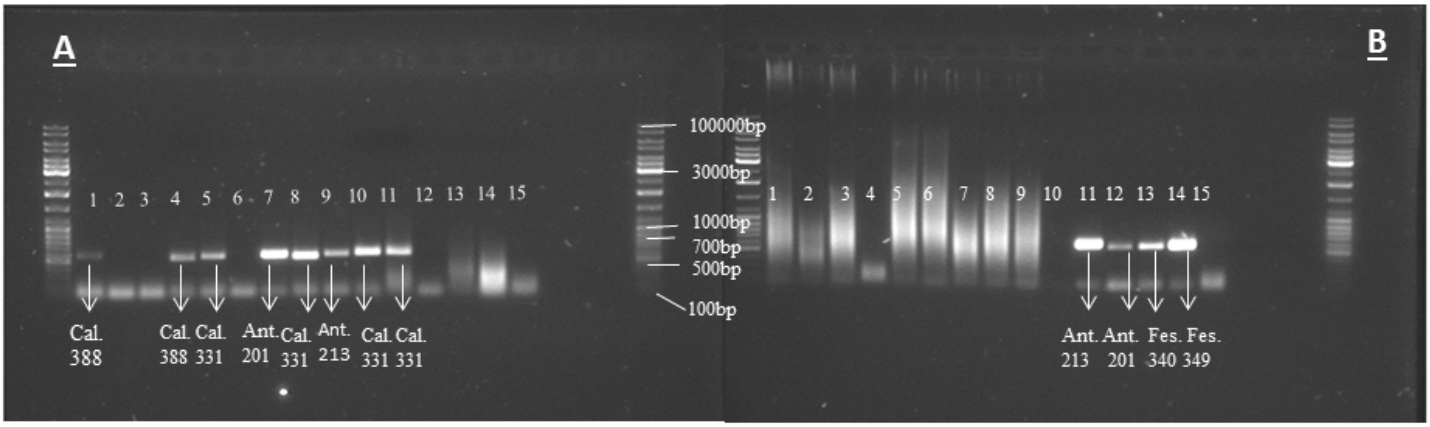


**Figure 9**. Images A and B shown the size of the PCR products, gained by the amplification of the ITS regions of the fungal endophyte, which simultaneously also confirms the occurrence of endophytes fungi in all three grass species, *Calamagrostis phragmitoides, Anthoxanthum nipponicum, Festuca sp*. Abbreviations under each PCR product shown by the arrow in figure tells about the grass species where the fungus was isolated and where and what treatment each grass individuals had. More description about that is available in table 11. Numbers in figures that do not have arrows show absence of product in these samples. The samples were catapulted from these grass species as follows: image A: 2- Ant 201;3- Cal.331; 6-Ant.213;13-Cal.331;14-Cal.332;15-Ant.201; andimage B:1-Cal.388;2-Ant.201;3- Cal 538;4- Cal.388;5-Cal.56;6-Ant.44;7-Fes 349;8- Cal.331; 9-Ant. 44; 10-Cal.331;

In Table 9, the results of PCR efficiency of all 48 individuals from the three grass species can be seen.

**Table 9**. In the table are presented the results of PCR efficiency; number of individuals from the three grass species, which are examined for occurrence of fungal endophytes, as well as results obtained from all individuals.

| **Grass species** | **Grass individuals** | **PCR products** | **%PCR efficiency** |
|---|---|---|---|
| ***A. nipponicum*** | 17 | 6 | 35 % |
| ***C. phragmitoides*** | 18 | 8 | 44 % |
| ***Festuca* sp.** | 13 | 4 | 31 % |
| **Grand total** | 48 | 18 | 38 % |

In order to test the importance of the hyphal amount and their morphology to the success of PCR, it was found that the hyphal amount or types of morphology do not seem to be important to the success of PCR process (see Table 10).

**Table 10.** Results in the table indicate that hyphae amount do not seem to be important to the success of PCR results; average hyphae amount was independent of PCR result.

| Grass species | PCR result | Average of Hyphae amount (3x180 µm) | Minimum of Hyphae amount (3x180µm) | Maximum of Hyphae amount (3x180µm) |
|---|---|---|---|---|
| ***A. nipponicum*** | Yes | 287.8 | 150 | 417 |
| ***C. phragmitoides*** | Yes | 105.4 | 29 | 227 |
| ***Festuca* sp.** | Yes | 144.8 | 66 | 202 |
| ***A. nipponicum*.** | No | 278.9 | 84 | 425 |
| ***C. phragmitoides*** | No | 171.2 | 90 | 275 |
| ***Festuca* sp.** | No | 91.4 | 43 | 168 |

## 3.4 DNA sequencing and Phylogenetic analyses

The DNA sequences obtained was compared with all of the DNA sequences in the default database of BLAST and shows a presence of six classes of fungal endophytes; *Eurotiomycetes, Dothideomycetes, Sordariomycetes, Leotiomycetes* and *Pezizomycetes,* that belong to phylum *Ascomyceta* and *Exobasidiomycetes,* which belong to phylum *Basidiomyceta* (see Table 11).

Identification of fungal endophytes species based on morphology of the same or different hyphae structure do not seem to be predictive of endophytes class or of endophytes genus; one morphology type is related to several classes and genera (see Table 11). Within one individual grass sample *C. phragmitoides* 188, four different genera and two classes of endophytes were found (see Table 11).

| *Grass species | *Plot | *H1 | *H2 | *H3 | *H4 | *H5 | *H6 | *H7 | *H8 | H. amount | *Catap/ PCR | Genus | Class |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***A. nipponicum*** | 376 | | 1 | | | | | 1 | | | Yes | ***Fusarium*** | ***Sordariomycetes*** |
| ***A. nipponicum*** | 332 | | 1 | | | 1 | | | | | Yes | ***Fusarium*** | ***Sordariomycetes*** |
| ***A. nipponicum*** | 213 | 1 | 1 | 1 | | | | | | 417 | Yes | ***Phoma, penicillium, Phaeosphaeria*** | ***Dothideomycetes, Eurotiomycetes*** |
| ***A. nipponicum*** | 201 | 1 | | 1 | | | | | | 297 | Yes | ***Penicillium*** | ***Eurotiomycetes*** |
| ***A. nipponicum*** | 78 | 1 | | 1 | | 1 | | | | 287 | Yes | ***Meira*** | ***Brachybasidiaceae*** |
| ***A.nipponicum*** | 67 | 1 | 1 | | | | | | | 150 | Yes | ***Penicillium*** | ***Eurotiomycetes*** |
| ***A. nipponicum*** | 151 | 1 | 1 | | | | | | | 331 | No | | |
| ***A. nipponicum*** | 10 | 1 | 1 | | | | | | | 425 | No | | |
| ***A. nipponicum*** | 103 | 1 | 1 | 1 | | 1 | | | | 407 | No | | |
| ***A. nipponicum*** | 83 | 1 | 1 | | | | | | | 225 | No | | |
| ***A. nipponicum*** | 207 | 1 | 1 | | | | | | | 224 | No | | |
| ***A. nipponicum*** | 160 | 1 | 1 | | | | | | | 276 | No | | |
| ***A. nipponicum*** | 44 | 1 | 1 | | | | | | 1 | 237 | No | | |
| ***A. nipponicum*** | 116 | 1 | 1 | | | | | | | 84 | No | | |
| ***A. nipponicum*** | 153 | 1 | 1 | | | 1 | | | | 245 | No | | |
| ***A. nipponicum*** | 28 | 1 | 1 | 1 | | | | | | 252 | No | | |
| ***A. nipponicum*** | 55 | 1 | 1 | 1 | | | | | | 362 | No | | |
| ***C. phragmitoides*** | 681 | 1 | | 1 | 1 | | | | | 108 | Yes | ***Penicillium*** | ***Eurotiomycetes*** |
| ***C. phragmitoides*** | 389 | | | | | 1 | | 1 | | | Yes | ***Alternaria*** | ***Dothideomycetes*** |
| ***C. phragmitoides*** | 388 | | 1 | | | 1 | 1 | 1 | | | Yes | ***Davidella, Cladosporium, Phaeosphaeria, Giberella*** | ***Dothideomycetes, Sordariomycetes*** |
| ***C. phragmitoides*** | 331 | | 1 | | | 1 | 1 | | | 108 | Yes | ***Cladosporium, Phaeosphaeria, Fusarium*** | ***Dothideomycetes, Sordariomycetes*** |
| ***C. phragmitoides*** | 316 | | | 1 | | | | | | 55 | Yes | ***Penicillium*** | ***Eurotiomycetes*** |
| ***C. phragmitoides*** | 188 | 1 | 1 | | | | | | | 227 | Yes | ***Peziza*** | ***Pezizomycetes*** |
| ***C. phragmitoides*** | 56 | | | 1 | 1 | | | | | 29 | Yes | | ***Leotiomycetes*** |
| ***C. phragmitoides*** | 35 | 1 | | | 1 | | | | | | Yes | ***Thelebolus*** | ***Leotiomycetes*** |
| ***C. phragmitoides*** | 462 | 1 | 1 | 1 | 1 | | | | | 96 | No | | |
| ***C. phragmitoides*** | 428 | 1 | 1 | 1 | | | | | | 220 | No | | |
| ***C. phragmitoides*** | 190 | 1 | | 1 | | | | | | 115 | No | | |
| ***C. phragmitoides*** | 90 | | | 1 | | | | | | 227 | No | | |
| ***C. phragmitoides*** | 63 | 1 | 1 | 1 | | | | | | 114 | No | | |
| ***C. phragmitoides*** | 454 | | 1 | 1 | | | | | | 258 | No | | |
| ***C. phragmitoides*** | 84 | 1 | | 1 | | | | | | 90 | No | | |
| ***C. phragmitoides*** | 422 | | 1 | 1 | | 1 | | | | 211 | No | | |
| ***C. phragmitoides*** | 469 | 1 | 1 | 1 | | 1 | | | | 106 | No | | |
| ***C. phragmitoides*** | 535 | | | | 1 | | 1 | | | 275 | No | | |
| ***Festuca sp.*** | 622 | | 1 | | 1 | | | | | 124 | Yes | ***Thelebolus*** | ***Leotiomycetes*** |
| ***Festuca sp.*** | 349 | 1 | | | | | | | | 66 | Yes | ***Phoma*** | ***Dothideomycetes*** |
| ***Festuca sp.*** | 332 | 1 | 1 | 1 | 1 | 1 | | | | 202 | Yes | ***Phoma*** | ***Dothideomycetes*** |
| ***Festuca sp.*** | 340 | 1 | | 1 | 1 | | | | | 187 | Yes | ***Phoma*** | ***Dothideomycetes*** |
| ***Festuca sp.*** | 672 | 1 | 1 | | | | | | | 89 | No | | |
| ***Festuca sp.*** | 405 | 1 | | | 1 | 1 | | | | 168 | No | | |
| ***Festuca sp.*** | 204 | 1 | 1 | | | | | | | 88 | No | | |
| ***Festuca sp.*** | 408 | 1 | | | 1 | 1 | | | | 89 | No | | |
| ***Festuca sp.*** | 145 | 1 | | | 1 | 1 | | | | 72 | No | | |
| ***Festuca sp.*** | 678 | | | 1 | 1 | | | | | 164 | No | | |
| ***Festuca sp.*** | 506 | | | 1 | | | | | | 51 | No | | |
| ***Festuca sp.*** | 144 | | 1 | | | 1 | | | | 43 | No | | |
| ***Festuca sp.*** | 359 | | | | 1 | 1 | | | | 59 | No | | |

**Table 11**. Overview of data from the three grass species examined for occurrence of fungal endophytes: The hyphal morphology type identified (H1-H8), the hyphal load (H.amount), whether the catapulting/PCR was successful (Catap/PCR) and the fungal species identified (Genus and Class). Plot refer to the locality of grass sample.

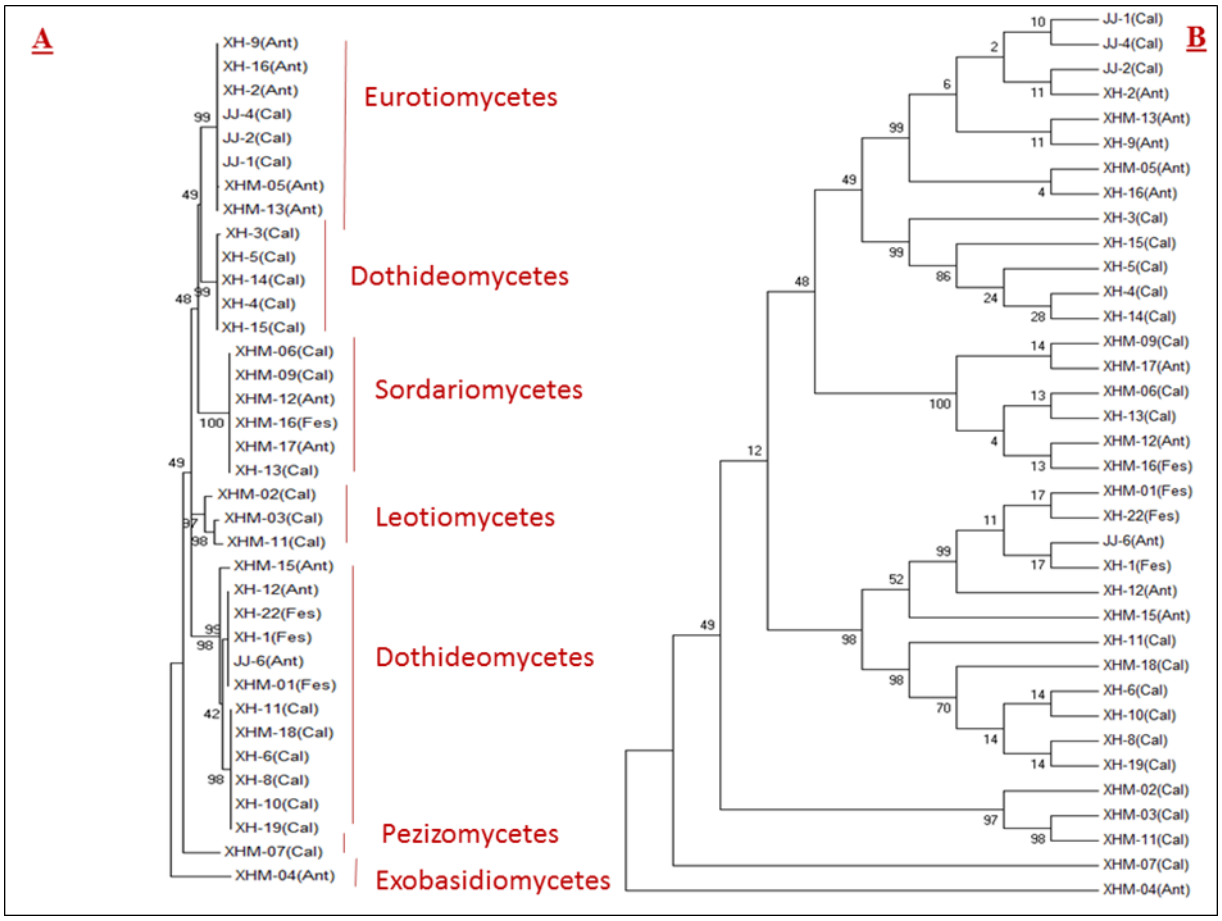


**Figure 10.** The trees **A**- Original tree, and **B**- Bootstrap tree (created for testing the reliability of the dataset), show the difference of fungal endophytes and phylogenetic relationships among species, assembled at the classes' level; Each of the sequence codes, that are seen in the each branch of the phylogenetic trees corresponding to a fungal species, are isolated from stems or leaf sheath of all three grass species (see table 9). As shown here, fungal endophyte species are classified in six classes of fungi, of which five belong to phylum *Ascomycota* (*Eurotiomycetes*, *Dothideomycetes*, *Sordariomycetes*, *Leotiomycetes* and *Pezizomycetes)*, only the class *Exobasidiomycetes* belongs to phylum *Basidiomycota.*

The DNA sequences are visualized in the form of a phylogenetic tree, for organizing knowledge of fungal endophytes diversity, and for their structuring classifications, see Figure 10.

# 4. Discussion

The methods used by mycologists to detect the presence and identification of endophytes in plants, have predominantly been based on cultivation methods. Due to the fact that endophytes do not cause visually recognizable symptoms, their presence and identification have been based on cultivation of the fungi from small surface sterilized plant segments. Identification have been based only on their morphology, i.e. by characteristic structures that can be used in making a visual identification (Johannes Hallmann 2006). These cultivation methods give biased results, because not all endophytes are able to grow in culture. Thus, it is probable that numerous fungal endophytes never have been isolated and that the biodiversity of isolated endophytes is likely to be much lower than that actually presented in nature.

Second, the identification of fungi cannot be made based on morphology alone. Morphological characters are unstable and can change with environmental changes. Therefore, molecular methods are advantageous and potentially more accurate; They can help to identify the fungal endophytes isolated from their plant hosts and to investigate their phylogenetic relationships (Huang, Cai et al. 2009). However, molecular analyses so far are primarily made on fungal endophytes that can be grown and isolated under controlled laborious conditions.

In this study, we used the Laser Micro dissection and Pressure Catapulting technique to investigate its possible application in fungal endophytes isolation directly from the grass host, in order to avoid using the biased method based on endophyte cultivation.

The staining method and microscopy examination enabled us to detect endophytes inside grass tissues. All samples were possible to catapult, but less than 10% resulted in successful identification of the fungal endophytes from the DNA ITS region.

A simplified description for all the techniques that were used during this study, their advantages and limitations, will be presented in the following discussion.

## 4.1 Fungal detection procedure

The staining method with rose Bengal and microscopy examination under 63X magnification enabled us to detect fungal endophytes inside grass tissues. These techniques have a

morphological concept, where endophytes are defined based on morphological characteristics and ideally by the differences among them.

Rose Bengal as an easily water-soluble vital stain has shown ability for infiltrating the inside of the plant tissue to stain endophytes and to provide image contrast when green filter is used at microscopy examination (Norn 1962) (see Figures 7, 8 and Appendix II Figures 12,13,14). One limitation of the rose Bengal stain may be that the solution dries out fast in contact with air, and the resulting stain can become spotty and disturb the microscopic examination of the sample.

## 4.2 Laser Microdissection and Pressure Catapulting (LMPC)

In the introduction, it was mentioned that LMPC permits procurement of selected hyphae of fungal endophytes from a section of grass tissue (Balestrini and Bonfante 2008).

Laser Micro dissection and pressure catapulting succeeded beyond our expectations. The catapulting technique provided selected and crushed hyphae for further analysis.

The detected hyphae were marked, and the cutting and catapulting was done one after the other. Laser energy was manipulated individually for each dissection, regardless of the type of the grasses or thickness of the sections.

Some hyphae were easy to separate from grasses because they do not branch, and are mostly spread in one layer in the grass (see Figure 7, the hyphae labelled with H1 and H3), whereas, some hyphae were cut together with several grass layers, because they were very branched. Branching is not essential for endophytes to spread across grass cell layers, because also hyphae that do not branched can penetrate across the several grass cell layers. However, no-branched hyphae are easy to follow and easy to select.

The catapulting process was successfully completed, although it was possible to lose some piece of tissue under catapulting into the collection cap, due to the distance between the slides and cap collection. This may be the only disadvantage of LMPC.

In this study, we demonstrated that the LMPC is well suited for the first step in isolating fungal endophytes, i.e. dissecting the endophytes of interest from its host.

## 4.3 DNA extraction, Polymerase chain reaction (PCR) and DNA sequencing

Although LMPC holds the potential to yield important information, the success is dependent on the molecular methods following the dissection (Richard J Howard 2004). In this study, less than 10% of the dissected endophytes were successfully identified from their DNA sequence.

### 4.3.1 DNA extraction

Based on the results of low DNA extraction from fungal endophytes, a possible explanation of the failure may be the complex composition of hyphae and their wall construction. The fungal walls that consist of fibrillar material embedded in polysaccharide may be highly protective; therefore, it is difficult to extract DNA. Likewise, the possibility of DNA extraction from the hyphae varies according to the environment where hyphae grow, as well as age. Old hyphae have more rigid walls. In addition, hyphae that do not have sufficient food can have even greater protection from environment (Sydney 2004).

This clearly represents the major hurdle to high-efficiency extraction of fungal DNA. The incapability of techniques to successfully extract the DNA from fungi is a challenge for mycologists.

The most efficient method to extract DNA used in this study is described under section 2.5. Using this method, DNA could be isolated from less than 10% of the catapulted samples and used for PCR amplification.

In the case of LMPC used to catapult fungal hyphae, most likely the power of laser energy during catapulting has crushed the hyphae, by cutting those into the small pieces.

The effectiveness of chloroform to remove proteins and isopropanol to precipitate and to concentrate the DNA (Affiliation Cetus Corporation 1990, Ward 2008) was successful, according to the early studies, therefore the crushed hyphae after catapulting process now were ready to have their DNA extracted using the same process. Based on the result discussed above where less than 10% of the dissected endophytes were successfully identified from their DNA sequence, we can conclude that LMPC has successfully helped to isolated endophytes directly from grass tissue.

We have also used two other methods to extract DNA, which are described in Table 12 in Appendix III.

One method was the based on freezing the catapulted material at - 80°C/or in liquid nitrogen freezing in order to make the cell wall more fragile. This method proved to have very little efficiency in extracting DNA.

The second DNA extraction method was based on microwave radiation of the catapulted material, a potential fast method breaking up cell walls and lysing cells. This method also proved to have very little efficiency in extracting DNA.

Examples of other methods used to break open fungal cells are varied; some of them are laborious in procedure, and imply careful sterilization of the material, may be hazardous, and do not show satisfactory results (McCartney, Foster et al. 2003).

### 4.3.2 Polymerase chain reaction (PCR)

Although the efficiency of PCR amplification of fungal DNA ITS region was to some extent successfull, there may be several reasons why catapulted plant/endophytes tissue do not provide better results.

One of the reasons may be the bias of primers, because they may amplify ITS region towards certain group of fungi before others. Based on our results ITS 4-5primers were amplified most efficiently from the phylum *Ascomycetes* with six classes, while phylum *Basidiomycetes* is presented only with one class (see Table 11).

Some ITS primers, such as ITS1 and ITS5, are biased towards amplification of *Basidiomycetes*, whereas others, e.g. ITS2, ITS3 and ITS4, are biased towards *Ascomycetes* (Bellemain, Carlsen et al. 2010).

Another reason may be that the grass DNA was present together with fungal DNA, because in principle we do not know exactly which DNA is extracted more, the DNA of the plant or the fungi, because it is likely that both DNA are extracted considering that the plant and fungal cells were present after catapulting.

The question is, is it possible that DNA of the plant inhibits the DNA amplification of the fungal ITS regions? Maybe plant DNA was the predominant template in the PCR because plant tissue was the main constituent of the extracted material (Gardes and Bruns 1993).

The high sensitivity of the PCR process, where the method relies on thermal cycling, is also of a reason for spurious results. The PCR process consists of cycles of repeated heating and cooling, where a consequence can be a nonspecific amplification, by that primers bind nonspecifically to the template.

### 4.3.3 DNA sequencing

The 40 PCR products were obtained during PCR amplification of fungal ITS regions. The DNA sequencing procedures yielded thirty-six readable sequences that enable comparing them into database for identification. These 36 sequences can be seen in Table 13, Appendix IV.

In the case of the other four PCR products, that were not readable sequences, some errors may have occurred during the sequencing process, considering that this process is sensitive as with PCR. The other reasons may be weaker products gained during the PCR amplification procedure.

## 4.4 Phylogenetic analyses

A search in the National Center for Biotechnology Information (NCBI) nucleotide database for comparison of thirty six ITS region sequences using BLAST program revealed the presence of fungal endophytes within all three grass species. These isolated fungal endophytes fell into six different classes and 10 genera (see table 11).

The useful properties of the ITS region, combined with growing ITS database, are likely to make this region more usable among mycologists (Huang, Cai et al. 2009). Moreover, for taxonomic considerations, the sequences can be used to include related species into phylogenetic trees.

However, the uses of ITS sequences also have limitations in phylogenetic analysis, because the noncoding ITS sequence is fast evolving and with more variation than other genetic

regions of rDNA. Therefore, these sequences have may not achieve a perfect sequence alignment at high taxonomic levels. But a major limitation is described by Huang and Cai, where they say that it is the limited number of sequences in GenBank and EMBL; also, it has been shown that 20-30% of sequences downloaded from GenBank for comparative analysis may not be accurate in their identification (Huang, Cai et al. 2009).

## 4.5 The hyphal morphology and hyphal load, to the success of PCR amplification

Identification of endophytes based on morphology of different hyphae structures did not seem to be predictive for endophytes class or genus. In fact, one morphology type was related to several classes and genera (see Table 11).

Hyphae amount or types of morphology do not seem to be important to the success of PCR amplification and identification. Slightly higher success rates have been seen in grasses species *Calamagrostis* than in *Anthoxanthum* and *Festuca* (see Tables 8, 10).

A single mycelium is heterogeneous in nature, i.e. the mycelium having different parts in different stages of response that will be responding to available nutrients. Therefore, although their morphology of hyphae look different, this does not mean that the hyphae belong to different species. Also, hypha that seems to have same morphology may not belong to same species. Rather the life form that hyphae follow, as a response to the nutrients in the environment, result in variable hyphae structures as a cause of differences in available food.

Also, senescence (ageing) may be a reason for various hyphae structures, because limited growth increases the diversity of hyphal morphology and structure (http://bugs.bio.usyd.edu.au/learning/resources/Mycology/contents.shtml 2004).

## 4.6 Outlook

Based on the results obtained in this study, as well the assumption that the extraction of DNA could possibly be an obstacle to the satisfactory results, there are a number of additional experiments that should be executed in order to find DNA extraction methods that ensure better results. It would be very interesting to test effects of chemical and enzymatic agents that are not harmful to our health and environment on DNA extraction from fungal endophytes.

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## APPENDIX I

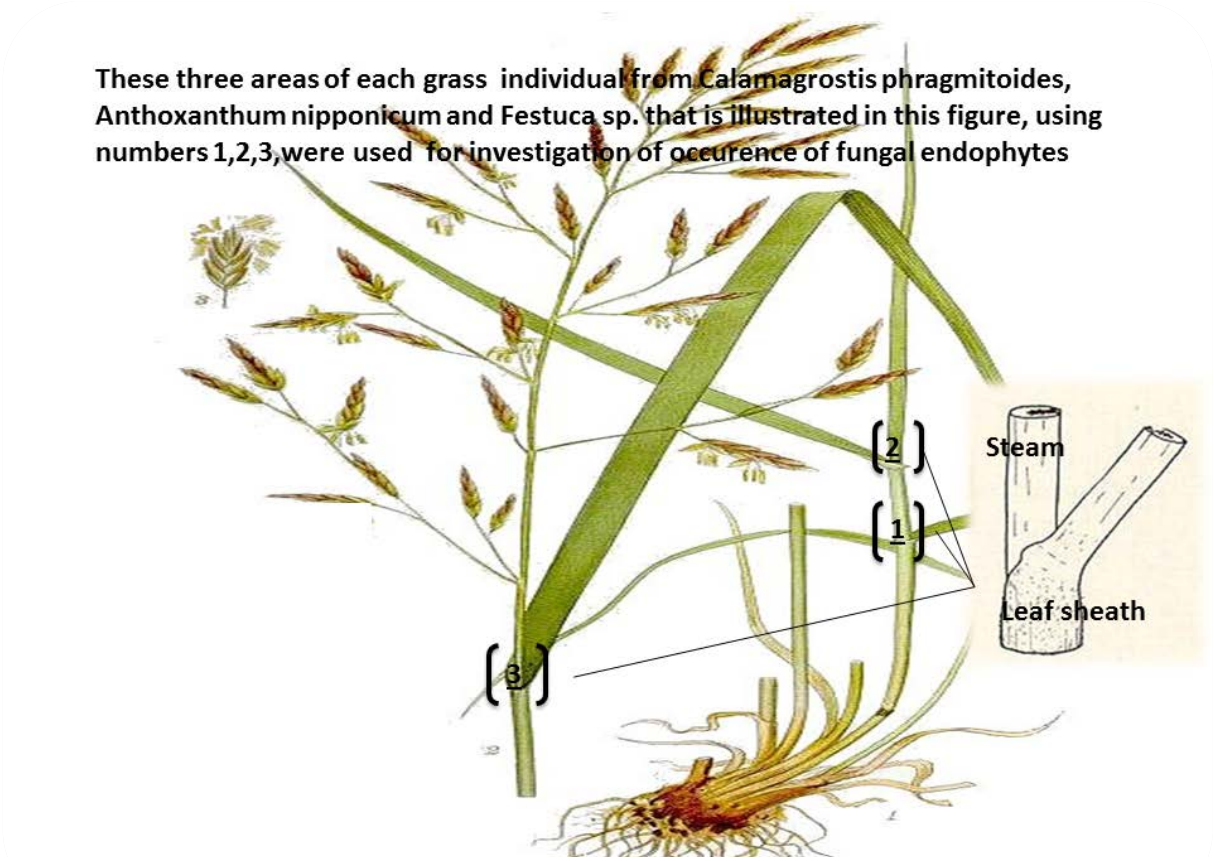


**Figure 11**. describes which the part of a grass individual was used to investigate for the occurrence of fungal endophytes. Three slides were made from these three (1, 2, 3) grass are as ranging from the lower part of the grass toward the top. This is a modified figure from these sources www.caf.wvu.edu - 230 × 210 fr.academic.ru - 445 × 843

# APPENDIX II

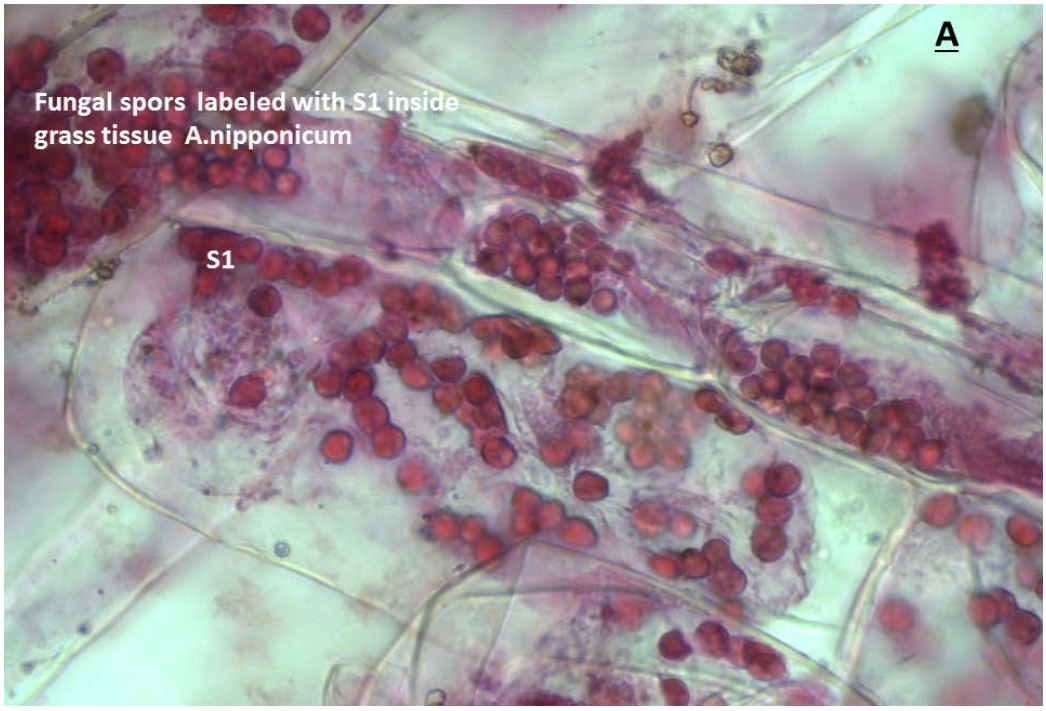


**Figure 12**.The image of fungal spores labelled with S1 within grass specie *A.nipponicum.*

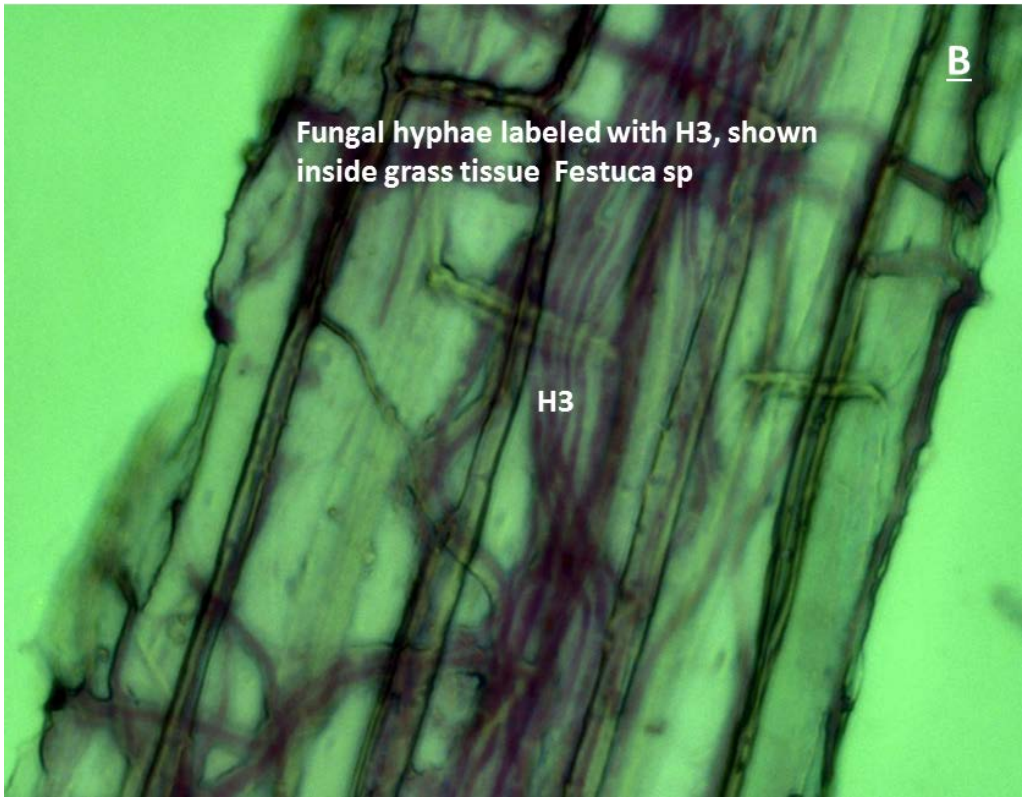


**Figure 13**.The image of hyphae labelled with H3 within grass specie *Festuca sp*.

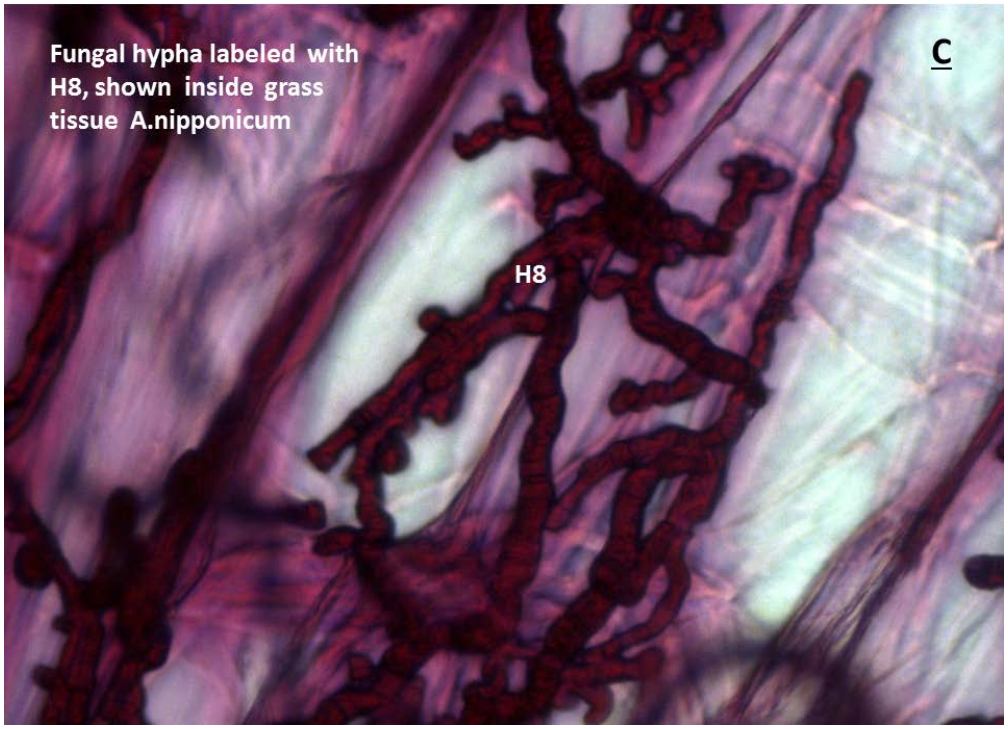


**Figure 14.** The image of hyphae labelled with H8 within grass specie *A. nipponicum.*

# APPENDIX III

**Table 12.**The table contains descriptions of methods 1 and 2 used to extract DNA from fungal endophytes. Method 1 showed only some of the results, while method 2 has not provided any results.

**1- DNA extraction based**: on thefreezing at - 80°C/or liquid Nitrogen

Harvested fungal endophytes into 70 µl MQ water / or TE (10 mM Tris/HCl buffer, pH 8, 100 mM EDTA pH 8) on PCR tube lysed this way :

- Centrifuged for 5 min at 500 g;
- Freeze at - 80°C for 45 min. or in liquid nitrogen;
- Heated at 65°C for 45 min. or 90°C for 10 min.;
- Repeat 3 X;
- Used Savant SC210ASpeedVac ®evaporate liquids system to dry solutes

and then PCR reaction;

- Adds 10 µl MQ water to each tube +15 µl PCR mixture (10 µl MQ water + 5 µl other PCR components in total; or
- Adds 25 µl PCR mixture (20 µl MQ water +5µl other PCR components in total.

**2- DNA extraction based:** on the use of microwave radiation.

Catapulted fungal endophytes into 70 µl of TE (10 mM Tris/HCl buffer, pH 8, 100 mM EDTA pH 8) in micro centrifuge tubes followed by microwave treatment at 2450 MHz frequency in a microwave oven of 230 V output at 28 °C for 30 s. The treated biomass was incubated at 28 °C for 5 min and centrifuged at 10,000 × g for 5 min. The supernatant contained DNA and was used directly for PCR (Batliboi & Co, Mumbai, India),[21]

# APPENDIX IV

**Table 13**.The specific order of nucleotides in thirty-six internal transcribed spacer (ITS) regions of fungal DNA.

| **Fungal DNA sequences of ITS regions isolated from three grass species *Calamagrostis phragmitoides, Anthoxanthum nipponicum* and *Festuca sp.*** |
|---|
| **>JJ-1**<br>GAGGTCACCTGAAAAGGATGATTGGTTGTCGGCTGGCGCCGGCCGGGCCAACAGAGCGGGTGACAAAGCCCCATACGCTCGAGGACCGGACTCGGTGCC<br>GCCGCTGCCTTTCGGACCCGTCCCCGGGGGGACGGAGCCCAACACACAAGCCGTGCTTGAGGGCAGCAATGACGCTCGGACAGGCATGCCCCCCGGAAT<br>ACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAATTCTGCAATTCACATTAGTTATCGCATTTCGCTGCGTTCTTCATCGATGCCGGAACC<br>AAGAGATCCGTTGTTGAAAGTTTTAACTTATTTAGTTTATGCTCAGACTGCAATCTTCAGACAGAGTTCAATAGTGTCTCCGGTGCGCGCGGACCCGGGGG<br>CAGAAGCCCCCCGGCGGCCGTGAGGCGGGCGCACCGAAGCAACAAGGTACAATAAACACGGGTGGGAGGTTGGACCCAGAGGGCCCTCACTCAGTAAT<br>GATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGACTTTTTACTTC<br>**>JJ-2**<br>GAGGTCACCTGAAAAAGGATGATTGGTTGTCGGCTGGCGCCGGCCGGGCCTACAGAGCGGGTGACAAAGCCCCATACGCTCGAGGACCGGACTCGGTGC<br>CGCCGCTGCCTTTCGGACCCGTCCCCGGGGGGACGGAGCCCAACACACAAGCCGTGCTTGAGGGCAGCAATGACGCTCGGACAGGCATGCCCCCCGGAA<br>TACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAATTCTGCAATTCACATTAGTTATCGCATTTCGCTGCGTTCTTCATCGATGCCGGAAC<br>CAAGAGATCCGTTGTTGAAAGTTTTAACTTATTTAGTTTATGCTCAGACTGCAATCTTCAGACAGAGTTCAATAGTGTCTCCGGTGCGCGCGGACCCGGGG<br>GCAGAAGCCCCCCGGCGGCCGTGAGGCGGGCGCACCGAAGCAACAAGGTACAATAAACACGGGTGGGAGGTTGGACCCAGAGGGCCCTCACTCAGTAA<br>TGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGATTTTTTACTTCCA<br>**>JJ-4**<br>GAGGTCACCTGAAAAAGGATGATTGGTTGTCGGCTGGCGCCGGCCGGGCCTACAGAGCGGGTGACAAAGCCCCATACGCTCGAGGACCGGACTCGGTGC<br>CGCCGCTGCCTTTCGGACCCGTCCCCGGGGGGACGGAGCCCAACACACAAGCCGTGCTTGAGGGCAGCAATGACGCTCGGACAGGCATGCCCCCCGGAA<br>TACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAATTCTGCAATTCACATTAGTTATCGCATTTCGCTGCGTTCTTCATCGATGCCGGAAC<br>CAAGAGATCCGTTGTTGAAAGTTTTAACTTATTTAGTTTATGCTCAGACTGCAATCTTCAGACAGAGTTCAATAGTGTCTCCGGTGCGCGCGGACCCGGGG<br>GCAGAAGCCCCCCGGCGGCCGTGAGGCGGGCGCACCGAAGCAACAAGGTACAATAAACACGGGTGGGAGGTTGGACCCAGAGGGCCCTCACTCAGTAA<br>TGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGATTTTTTACTTCCA<br>**>JJ-6**<br>GAGGTCAGAGTGTAAAAAAGTACTTATGGACGTCGTCGTTATGAGTGCAAAGCGCGAGATGTACTGCGCTCCGAAATCAATACGCCGGCTGCCAATTGTT<br>TTAAGGCGAGTCTACACGCAAAGGCGAGACAAACACCCAACACCAAGCAAAGCTTGAAGGTACAAATGACGCTCGAACAGGCATGCCCCATGGAATACCA<br>AGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACACTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGA<br>GATCCGTTGTTGAAAGTTGTAACTATTAAGTTTTTCAGACGCTGATTACAAAACTGCAATGGGTTTAAGTGGTCCAATCGGCGGGCGAACCCACCGAGGAA<br>ACGTAAGTGCTCAAAAAACATGGGTAAGAGATAGCGGGCAAAGCCCACTGACTCTAGGTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGT<br>TT<br>**>XH-1**<br>ATGAGTGCAAAGCGCGAGATGTACTGCGCTCCGAAATCAATACGCCGGCTGCCAATTGTTTTAAGGCGAGTCTACACGCAAAGGCGAGACAAACACCCAA<br>CACCAAGCAAAGCTTGAAGGTACAAATGACGCTCGAACAGGCATGCCCCATGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGA<br>ATTCTGCAATTCACACTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTGTAACTATTAAGTTTTTCAGACG<br>CTGATTACAAAACTGCAATGGGTTTAAGTGGTCCAATCGGCGGGCGAACCCACCGAGGAAACGTAAGTGCTCAAAAAACATGGGTAAGAGATAGCGGGC<br>AAAGCCCACTGACTCTAGGTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGACTTTTTACTTCC<br>**>XH-2**<br>ACCGGACTCGGTGCCGCCGCTGCCTTTCGGACCCGTCCCCGGGGGGACGGAGCCCAACACACAAGCCGTGCTTGAGGGCAGCAATGACGCTCGGACAGG<br>CATGCCCCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAATTCTGCAATTCACATTAGTTATCGCATTTCGCTGCGTTCTTC<br>ATCGATGCCGGAACCAAGAGATCCGTTGTTGAAAGTTTTAACTTATTTAGTTTATGCTCAGACTGCAATCTTCAGACAGAGTTCAATAGTGTCTCCGGTGCG<br>CGCGGACCCGGGGGCAGAAGCCCCCCGGCGGCCGTGAGGCGGGCGCACCGAAGCAACAAGGTACAATAAACACGGGTGGGAGGTTGGACCCAGAGGG<br>CCCTCACTCAGTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGACTTTTACTTAMCT<br>**>XH-3**<br>CCAWCCTCCGCTTATTGATATTTTTTGGAATAGTTTCCACTTYTATTAKGGGACAGAAGACCCAGCCGGACGATTTGAGGCACGCGGCGGACCGCGTTGCC<br>CAATACCAAGCGAGGCTTGAGTGGTGAAATGACGCTCGAACAGGCATGCCCCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCAC<br>TGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTAAAAGTTTTAATTTATTAATTAAGTTTA<br>CTCAGACTGCAAAGTTACGCAAGAGTTTGAAGTGTCCACCCGGAGCCCCCGCCCGAAGGCAGGGTCGCCCCGGAGGCAACAGAGTCGGACAACAAAGGG<br>TTATGAATAACCAGGCCGAAGCCCGGGCGTTCTTGTAATGATCCCTCCGCAGGTTCACCTACGGAGACCTTGTTACGACTTTTACTTCCC<br>**>XH-4**<br>ACTTTTACTTCCATCCTCCGCTTCTTGATATGGGTTGGMACTGCTTTTAATTTTATCTTCCGCCTYYTGATATTKSTTGTCGCTTTGAGGCACGCGGAGGACCG<br>CGTTGCCCAATKKAAKSGAGGCRKGAGTGGTGAAATGACGCTCGAACAGGCATGCCCCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGATTCGATG<br>ATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCRAGAGATCCGTTGTTAAAAGTTTTAATTTATTAATTA<br>AGTTTACTCAGACTGCAAAGTTACGCAAGAGTTTGAAGTGTCCACCCGGAGCCCCCGCCCGAAGGCAGGGTCGCCCCGGAGGCAACAGAGTCGGACAAC<br>AAAGGGTTATGAACATCCCGGTGGTTAGACCGGGGTCACTTGTAATGATCCCTCCGCAGGTTCACCTACGGAGACCTTGTTACGACTTTTACTTCCAA<br>**>XH-5**<br>TTTATGGAATAGTTTCCACTTGCTTAKGGGACAGAAGACCCAGCCGGTCGATTTGAGGCACGCGGCGGACCGCGTTGCCCAATACCAAGCGAGGCTTGAG<br>TGGTGAAATGACGCTCGAACAGGCATGCCCCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTAC<br>TTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTAAAAGTTTTAATTTATTAATTAAGTTTACTCAGACTGCAAAGTTACGCA<br>AGAGTTTGAAGTGTCCACCCGGAGCCCCCGCCCGAAGGCAGGGTCGCCCCGGAGGCAACAGAGTCGGACAACAAAGGGTTATGAACATCCCGGTGGTTA<br>GACCGGGGTCACTTGTAATGATCCCTCCGCAGGTTCACCTACGGAGACCTTGTTACGACTTTTACTTCCCGCATCCGGTGC<br>**>XH-6** |

GGCTGCCAATTGCTTTAAGGCGAGTCCAAACACARAGGAGAGGACAAACACCCAACACCAAGCAGAGCTTGAGGGTACAAATGACGCTCGAACAGGCAT
GCCCCATGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACACTACTTATCGCATTTCGCTGCGTTCTTCATC
GATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTGTAATTATTAAGTTTTTTCAGACGCTGATTGAAAATTAAAAAGGTTATAGTTTTGTCCAATCGGCGGG
CAAGCCCGCCGAGGAAACATGAGTGCGCAAAAGACAAGGGTACAGACAGAGGGCCTGCCGCTCATCAGTAATTAAACTATGCGGGCTTACAGCACCTCCC
GACAGTAGCAAGCTACTGAATTGTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGACTTTTACTTCCAA

**>XH-8**

GGCTGCCAATTGCTTTAAGGCGAGTCCAAACACWRAGGASAGGACAAACACCCAACACCAAGCAGAGCTTGAGGGTACAAATGACGCTCGAACAGGCAT
GCCCCATGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACACTACTTATCGCATTTCGCTGCGTTCTTCATC
GATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTGTAATTATTAAGTTTTTTCAGACGCTGATTGAAAATTAAAAAGGTTATAGTTTTGTCCAATCGGCGGG
CAAGCCCGCCGAGGAAACATGAGTGCGCAAAAGACAAGGGTACAGACAGAGGGCCTGCCGCTCATCAGTAATTAAACTATGCGGGCTTACAGCACCTCCC
GACAGTAGCAAGCTACTGAATTGTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGACTTTTACTTCCCW

**>XH-9**

TGTCRGCTGGCGCCGGCCGGGCCTMCAKAGSGGGTGACAAAGCCCCATACGCTCGAGGACCGGACTCGGTGCCGCCGCTGCCTTTCGGACCCGTCCCCGG
GGGGACGGAGCCCAACACACAAGCCGTGCTTGAGGGCAGCAATGACGCTCGGACAGGCATGCCCCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAA
GACTCGATGATTCACTGAATTCTGCAATTCACATTAGTTATCGCATTTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCCGTTGTTGAAAGTTTTAAC
TTATTTAGTTTATGCTCAGACTGCAATCTTCAGACAGAGTTCAATAGTGTCTCCGGTGCGCGCGGACCCGGGGGCAGAAGCCCCCCGGCGGCCGTGAGGC
GGGCGCACCGAAGCAACAAGGTACAATAAACACGGGTGGGAGGTTGGACCCAGAGGGCCCTCACTCAGTAATGATCCTTCCGCAGGTTCACCTACGGAA
ACCTTGTTACGACTTTTACTTMCA

**>XH-10**

GTGCTGCGCTTCAWTACMAAAACACTGGCTGCCAATTGCTTTAAGGCGAGTCCAAACACAGAGGAGAGGACAAACACCCAACACCAAGCAGAGCTTGAG
GGTACAAATGACGCTCGAACAGGCATGCCCCATGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACACTAC
TTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTGTAATTATTAAGTTTTTTCAGACGCTGATTGAAAATTAAAAA
GGTTATAGTTTTGTCCAATCGGCGGGCAAGCCCGCCGAGGAAACATGAGTGCGCAAAAGACAAGGGTACAGACAGAGGGCCTGCCGCTCATCAGTAATT
AAACTATGCGGGCTTACAGCACCTCCCGACAGTAGCAAGCTACTGAATTGTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGACTTTTACTT
CMC

**>XH-11**

GTGCTGCGCTTAAWTACCAAAACACTGGCTGCCAATTGCTTTAAGGCGAGTCCAAACWCWAAGGASAGGACAAACRCCCRACACCAAGCAGAGCTTGAG
GGTACAAATGACGCTCGAACAGGCATGCCCCATGGAATACCAARGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACACTAC
TTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTGTAATTATTAAGTTTTTTCAGACGCTGATTGAAAATTAAAAA
GGTTATAGTTTTGTCCAATCGGCGGGCAAGCCCGCCGAGGAAACATGARTGCGCAAAAGACAAGGGTACAGACAGARGGCCTGCCGCTCAKCARTAATTA
AACTATGCGGGCTTACARCACCTCCCGACAGTAGCAAGCTACTGAATTGTAATGATCCTTCCGCAGGTTCACCTACKGAAACCTTGTTACCACTTTTACTTCC
AAY

**>XH-12**

ACTGCGCTCCGAAATCAATACGCCGGCTGCCAATTGTTTTAAGGCGAGTCTACACGCAAAGGCGAGACAAACACCCAACACCAAGCAAAGCTTGAAGGTA
CAAATGACGCTCGAACAGGCATGCCCCATGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACACTACTTAT
CGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTGTAACTATTAAGTTTTTCAGACGCTGATTACAAAACTGCAATGGG
TTTAAGTGGTCCAATCGGCGGGCGAACCCACCGAGGAAACGTAAGTGCTCAAAAAACATGGGTAAGAGATAGCGGGCAAAGCCCACTGACTCTAGGTAA
TGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGACTTTTACTTCMCGA

**>XH-13**

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TTGATTTATTTGTTTGTTTTACTCAGAAGTTACAATAAGAAACATTAGAGTTTGGGTCCTCTGGCGGGCCGTCCCGTTTTACGGGGCGCGGGCTGATCCGCC
GAGGCAACATTAAGGTATGTTCACAGGGGTTTGGGAGTTGTAAACTCGGTAATGATCCCTCCGCTGGTTCACCAACGGAGACCTTGTTACGATTTTTTACTT
CCYT

**>XH-14**

AYCCTCCCGAACACCCTTTAKCGAATAGTTTCCACAACGCTTAGGGGACAGAAGACCCAGCCGGTCGATTTGAGGCACGCGGCGGACCGCGTTGCCCAATA
CCAAGCGAGGCTTGAGTGGTGAAATGACGCTCGAACAGGCATGCCCCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAAT
TCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTAAAAGTTTTAATTTATTAATTAAGTTTACTCAG
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**>XH-15**

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ACTTCC

>**XH-16**

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**>XH-19**

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**>XH-22**

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AGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACACTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGA
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**>XHM-01**

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**>XHM-02**

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**>XHM-03**

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**>XHM-04**

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**>XHM-05**

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**>XHM-06**

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**>XHM-07**

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**>XHM-09**

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**>XHM-11**

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**>XHM-12**

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**>XHM-13**

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**>XHM-15**

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TGTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTAC

**>XHM-16**

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CGGAGACCTTGTTAC

**>XHM-17**

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**>XHM-18**

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# APPENDIX V

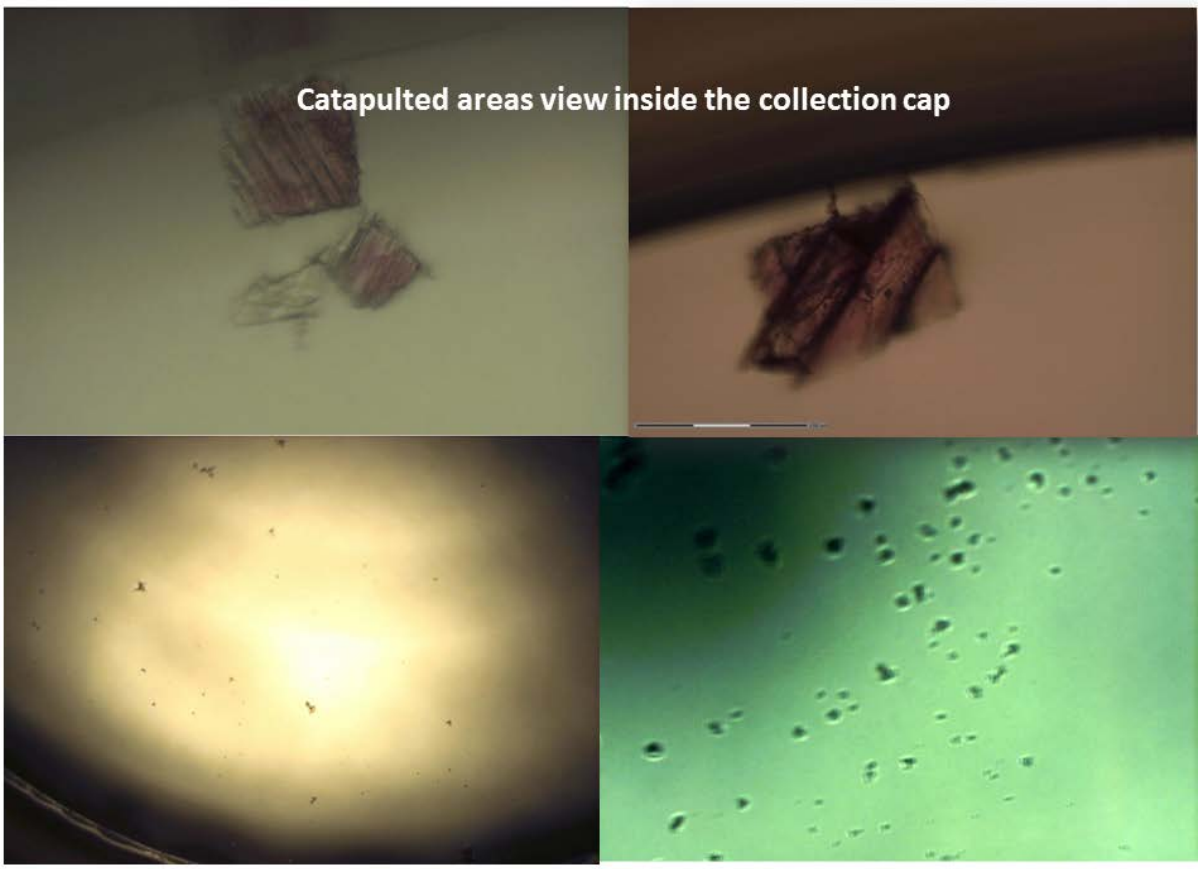
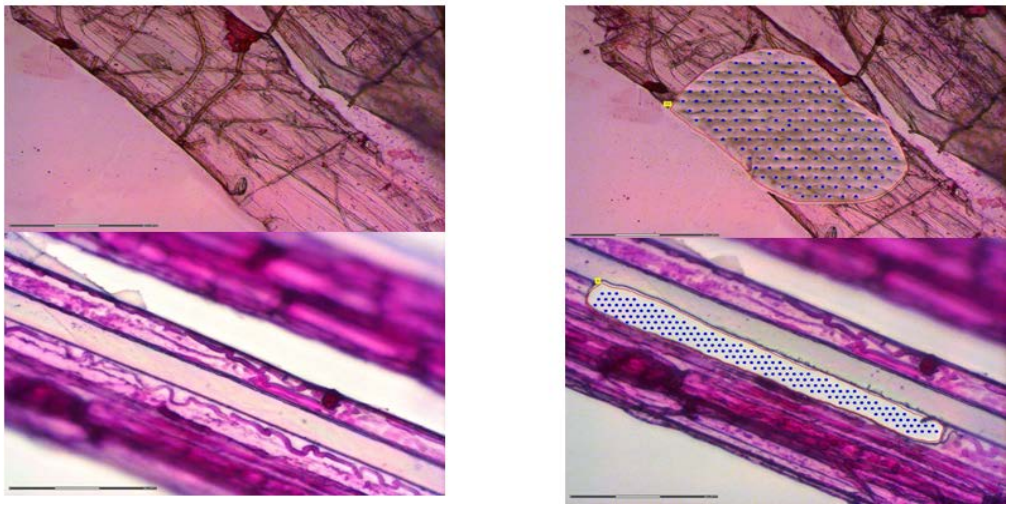



**Figure 15.** Microscope images of catapulted parts viewed inside of collection cap taken at 10x magnification**.**



**Figur 16.** Microscope images of catapulted parts viewed at 63x magnifications.