The University of South Bohemia in České Budějovice
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Root-Colonization by Arbuscular Mycorrhizal Fungi and Dark Septate Endophytes in Himalayan Plants: A Comparison between 2015 and 2022

Bachelor thesis

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Bibliographic details, Annotation, and Declaration

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Annotation:
Climate change is an undeniable reality that impacts biological systems worldwide. As temperature rises, plant species are forced to adapt, extinct, or migrate to higher elevations. In harsh environments like the Himalayan mountains, fungi such as arbuscular mycorrhizal fungi (AMF) and dark septated endophytes (DSEs) support the majority of herbaceous plants by providing nutrients and protecting them from pathogens. This study evaluates root colonization rates of AMF and DSE in diverse plant species sampled from the Ladakh range in 2015 and 2022. Significant findings include a notable increase in AMF infection rates and a decrease in DSE colonization rates between these years. This is possibly linked to climate change-induced temperature rises and prolonged vegetation seasons. Furthermore, AMF were found to be abundant in such stressed environments. Additionally, deeper investigations into the roles of AMF and DSE in different plant species are needed for a more nuanced understanding of their ecological implications.

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

České Budějovice, 04. May 2024

Table of Contents

List of Abbreviations

Abbreviation	Definition
a.s.l.	Above sea level
AMF	Arbuscular Mycorrhizal Fungi
C	Carbon
CO_2	Carbon dioxide
cm	Centimeter
DSE	Dark Septate Endophytes
°C	Degree Celsius
demi-water	Demineralized water
g	Gram
HCl	Hydrochloric acid
L	Liter
m	Meter
mL	Milliliter
mm	Millimeter
min	Minute
N	Nitrogen
%	Percent
P	Phosphorus
KOH	Potassium hydroxide

Keywords

Arbuscular mycorrhizal fungi, dark septate endophytes, climate change, Himalayas, Ladakh range, root colonization rate

1 Introduction

1.1 Climate Change and the Himalayas

1.1.1 Differences in climate

Climate change is a present topic, an indisputable reality, and influences biological systems. Excessive release of carbon dioxide (CO_2), changes in land covers, and nutrient availability are just a few of the alterations. Furthermore, the impacts of global warming, like major surge in annual temperature or melting of glaciers and snowpacks, can be seen all over the world (Bhutiyani, et al., 2009). Mountains are the most fragile environments and recover the slowest after harsh disturbances (Allen, et al., 1987). Moreover, they reposit endangered species, water, biodiversity (Nogués-Bravo, et al., 2007), and are important indicators of climate change.

1.1.2 Ladakh range

In this study, the focus lies on the great Himalayas. It is the highest mountain chain in the world, located in Asia, and comprised of five different ranges. The Ladakh range, which is a cold desert located in the north-westernmost part of India, was studied. The landscape is vast barren and unglaciated, the climate is arid-cold, and the water is scarce (Chevuturi, et al., 2018). Furthermore, in higher altitudes, the annual temperature rises by a greater amount than in tropical zones because of the snow-ice albedo feedback (HilleRisLambers, et al., 2012).

1.2 Changes in vegetation

Earlier studies suggested that the warming trend along with reduced precipitation may lead to an irreversible disruption of the fragile balance in vegetation in the area of Ladakh (Chevuturi, et al., 2018). The distribution of plant species depends on factors like mutualism, competitors, consumers, temperature, or water supply. Since all these factors are influenced by the climate, a change can therefore affect the plant ranges (HilleRisLambers, et al., 2012).

In mountains, plant species are being pushed to higher elevations as mean temperatures rise by both abiotic constraints and increasing competition with species migrating from lower elevations. Plants therefore face the choice to either spread to new habitats, go extinct, or adapt (Wilson, et al., 2005). If climate change occurs faster than the plants can adapt (Dobrowski, et al., 2013), fungi could support them with nutrients until the scarcity is over, therefore have a large impact on ecosystems (Bennett & Classen, 2020). Furthermore, plants must sustain more extreme events and conditions, such as storing more water and nutrients, and for that purpose, the plant-fungal symbiosis is crucial (Chevuturi, et al., 2018).

1.3 Arbuscular Mycorrhizal Fungi

1.3.1 AMF symbiosis

The majority (80-90%) of herbaceous plant species coalesce with fungi to form a symbiotic relationship known as mycorrhiza. Especially the arbuscular mycorrhizal fungi (AMF; sub-phylum Glomeromycotina) are the most prevalent type in roots. They have their origin 400 million years ago in the early Devonian (Martin, et al., 2017). AMF receive photosynthates (Pietikäinen, et al., 2007) and in exchange increase AMF the inorganic nutrient (Phosphorus (P) or Nitrogen (N)) and water uptake of the plant, enhancing plant growth and protecting the plants from pathogens and herbivores. Furthermore, AMF have host-specific effects on the host plant growth and this fungal-plant-symbiosis is considered mutualistic (Duarte & Maherali, 2022).

1.3.2 AMF colonization

Hyphae, vesicles, and arbuscules are formed within or between the cells and extraradical mycelium. The range of plants which get infected is large and depends on temperature, water supply, environment, nutrients, and light. Some plants might be selective for a special fungal symbiosis (Smith & Read, 2008), while others are generalists. The mycorrhiza colonization rate is larger where host plants form guilds (Blaschke, 1991) and in seedlings or juvenile plants (Dhillion, 1994) compared to adult plants. AMF colonization rates are not directly related to the effectiveness of nutrient uptake (Onipchenko & Zobel, 2000) and may depend on the presence of neighboring plants, as mycorrhiza colonization is positively correlated with plant density. AMF can also survive in cold environments and without a host plant according to Pietikäinen et al. (2007). The roots must be stained to identify the fungi under the microscope.

1.3.3 AMF in higher altitudes

Fungi inhabit nearly every environment, regardless of altitude, but the extent and type of colonization vary. (Haselwandter & Read, 1980). Plants in alpine habitats need to withstand harsh climates like short growing seasons, strong wind, solar radiation, low air pressure, and temperature. Some of them adapted and formed a symbiosis with AMF (Cripps & Eddington, 2005). In cold environments, AMF colonization plays a crucial role because mycorrhizae are influenced by temperature change or vegetation coverage, the rates are variable and range from 60 to 75%. Read and Haselwandter (1981) found 20% of AMF-root infections at about 1600 m above sea level (a.s.l.) and only 7% at 3200 m a.s.l. extreme sites of the Austrian Alps. In 1900–2500 m a.s.l. documented Gardes and Dahlberg (1996) the highest level of AMF. But, in higher

mountain ranges like the Himalayas, Kotilínek et al. (2017) found the greatest AMF colonization rates (30-40%) at mid-elevation (4500 m a.s.l.), while the infection rate in higher altitudes decreased.

1.3.4 AMF in disturbed habitats

It is stated, that with increasing altitude the host plant diversity and thus the percentage of AMF colonized vascular plants decreases, and other nonmycorrhizal plants are in abundance. Plants in higher elevations are not limited by N and P. Thus, they might not need mycorrhiza (Väre, et al., 1997). The infection rates are the highest in undisturbed areas, changes in the climate might lead to a loss of AMF. Therefore, in revegetated (youngest disturbed) habitats only 46% of the plants are colonized and in currently highly disturbed ones the AMF diversity is the lowest. If there is a lack of inter-plant contact, AMF rates could be lower (Haselwandter & Read, 1980) Yearly and seasonal fluctuations of the infection rate were found in other ecosystems (Allen, et al., 1987).

1.4 Dark Septate Endophytes

1.4.1 DSE symbiosis and colonization

Dark septate endophytes (DSE) are fungi which colonize plants in addition to AMF, and they are predominantly found in higher elevation species like alpine plants. DSE are capable of degrading organic matter and increasing N and P uptake with their fine endophytes. Moreover, they favor plant growth and are facultative symbionts. DSE penetrate the cortical cells and form intra- and intercellular (along the main axis) septate, melanized, dark hyphae, and microsclerotia, which appear brown under the microscope. Compared to AMF are DSE less studied and much of their symbiotic function, ecology, physiology, mutualistic status, and taxonomy remain unclear (Bueno de Mesquita, et al., 2018).

1.4.2 DSE in higher altitudes

In increasingly stressed environments like high-altitude alpine habitats, AMF might not be well adapted and are facultative. Under these conditions, the DSE might become more important and abundant and can partially or completely replace the AMF (Read & Haselwandter, 1981). For example, Kotilínek et al. (2017) documented a rise in the DSE infection (30%) above 4500 m a.s.l. In contrast, Ruotsalainen et al. (2004) argued that there is no significant shift in the DSE colonization rate with altitude change.

1.5 Fungal colonization differences

1.5.1 Environmental impact

The host-fungal type specifies if the symbiosis is mutualistic or antagonistic. Moreover, the availability of vegetation cover, soil moisture and propagules affect the root colonization (Gardes & Dahlberg, 1996). Intense competition leads to nutrient stress because of too intensive root-to-root contact. This was observed in the border between forest and snow thus, high fungal infection rates were found (Read & Haselwandter, 1981).

1.5.2 Snow and frost influences

In higher altitudes, there is less plant competition and root contact because of the sparsity of plants. Moreover, nutrient stress is not the limiting factors for plant growth. Consequently, it has been argued that lower levels of fungal infections have been found because plants do not suffer from nutrient limitation. After and during the snowmelt an intensive root growth was recognized, probably thanks to the water availability or due to the increased sunlight and temperatures, thus higher fungal infection rates (Read & Haselwandter, 1981). The frost in alpine systems leads to slow mineralization and thus the nutrients needed are stored in the soil (Väre, et al., 1997).

1.5.3 Lower altitudes

In lower altitudes, the rising temperature due to climate change might have a modest positive effect on plants and lead to a higher colonization rate of fungi in plants. Especially AMF, because they are less cold-tolerant than DSE (Duarte & Maherali, 2022).

1.5.4 Higher altitudes

Schmidt et al. (2008) stated that in lower elevations, less DSE and more AMF are present. However, at 5391 m were more DSE found, and AMF were absent. This was partly proven by Kotilínek et al. (2017). In this paper, a rise in AMF and a decrease in DSE until 5000 m a.s.l. is postulated. Beyond this altitude, the AMF colonization rate declines drastically, and the DSE infection rate surges. But this mutualistic lifestyle has a maximum and is only possible to a certain degree because those fungal structures might not be able to form in extremely high mountain ranges (6150 m a.s.l.) anymore. However, the average colonization rate differs between the species (Ruotsalainen, et al., 2004). It appears that the altitudinal distribution of fungi found in European highlands might not hold for higher, drier ranges such as the

Himalayas. Compared with the Alps, the Himalayas have a far higher altitude and drier conditions. Due to climate change, plant growth is changing because of other soil conditions, precipitation, and temperature differences. General warming leads to a glacial retreat and thus new environments open up for plants. The fungi, as secondary colonists, move with them upwards (Schmidt, et al., 2008).

1.5.5 Elevated atmospheric CO_2 levels

Other factors might also play a significant role in root colonization than climate change (Zubek, et al., 2009). For example, more CO_2 leads to more carbon (C) fixation. This is then transferred to the roots and can support fungal growth, and they can increase the P and N uptake. Functions like photosynthesis get better because they are determined by the P level. On the other hand, mycorrhiza influences the C-cycling of soil to plants so if the soil warms due to climate change, the fungal colonization grows, and the C demand increases. This might lead to less plant growth resulting in fewer fungal infections (Fitter, et al., 2000).

1.6 The aim of this study

The study aimed to investigate the level of root colonization rate of AMF and DSE of various plant species, collected in 2015 and 2022 by a research group from the Ladakh range. It is further hypothesized, that the possible differences in AMF and DSE quantities, which might have arisen in those seven years, can be explained by climate change.

1.6.1 Questions of this work

How high are the colonization rates by AMF and DSE for the probed plant species? Are AMF or DSE abundant in these elevated altitudes and is it consistent with previous works? Do AMF and DSE influence each other? Are there any differences between the years 2015 and 2022, and if so, can they be explained? How much do the infection rates fluctuate amongst the species? And to what extent does climate change can influence those variations in fungal infection rates? To answer these questions, the sampled roots from the Himalayan plant were stained and examined under the microscope. Moreover, the fungi were differentiated into four types: AMF hyphae, AMF vesicles, DSE hyphae, and DSE microsclerotia. The gathered values were evaluated with Excel and R, compared to previous studies, and discussed.

2 Materials and Methods

2.1 Study site

The examined samples were collected by a research group of the Biology Centre and Institute of Botany and came from different locations in the Ladakh range of the Himalayan mountains (India). Roots from 46 plant species were gathered in triplicates in August 2015 at 17 locations along 4 sampling tracks in elevations from 4000 m to 5600 m above sea level. Specimens of the same plant species were then re-collected at the same sites within a 100 m radius in August 2022. A map of the exact sampling locations is displayed in Figure 1.

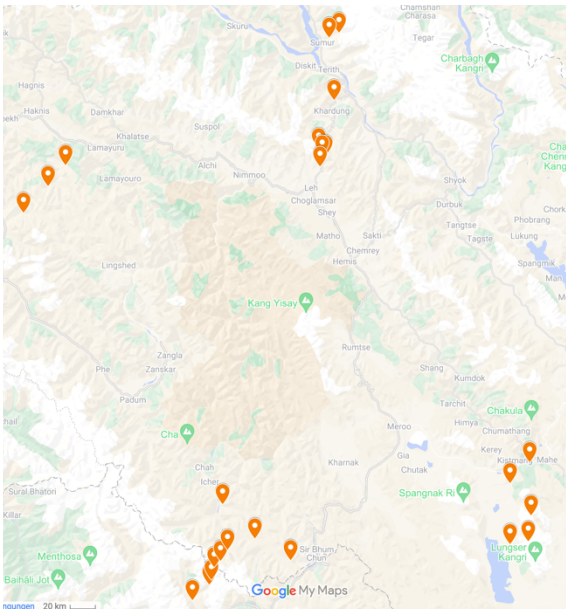

Figure 1: Map of the study sites in the Ladakh range of the Himalayas

2.2 Samples

To assess the degree of colonization by AMF and DSE in each plant at each place, triplicates of root samples were pooled and analyzed collectively. Each sample was preserved individually using silica gel to prevent biological degradation as done previously (Kotilínek, et al., 2017). For brevity purposes, the letter- and number codes were used to denote each plant species instead of the full name. For example, for *Anaphalis nubigena* the code H2/1/3 was used. H2 stands for a specific location, 1 is the plant-species code and 3 is the number of the replicate. A full list detailing the codes and their corresponding plant names is given in Tables 2 and 3 (page 15). The amount of each sample varied considerably, some had many roots to choose from and others only a few. To analyze the roots, they first were stained and then examined under a microscope.

2.3 Root staining

2.3.1 Soaking in water

First, the hard, wooden roots were soaked one day before staining in a 50 mL tube containing demineralized water (demi-water) to increase their permeability for the dye. Fine roots were soaked for only two hours.

2.3.2 Clearing

After a few hours or a day of soaking, the roots were cleared. For this purpose, the white, thin samples were treated with 2.5% potassium hydroxide solution (KOH, 25 g solid KOH in 1 L demi-water) (Kormanik & McGraw, 1982) and dark, thick roots with 10% KOH (100 g solid KOH in 1 L demi-water), this harsh method was recommended by Phillips and Hayman (1970). The strength of the applied KOH varies from species to species and depends on the texture, e.g., the thickness of the root. To clear the samples, the proper KOH was added to the tubes containing the roots, which were then placed in a water bath set to 80 °C for one hour. This step was important because the KOH removes the cytoplasm and background material from the root cell while leaving the fungi and root structure intact. Only the vascular cylinder of the root and existing fungi were afterward visible under the microscope, as shown in Figure 2 on the right.

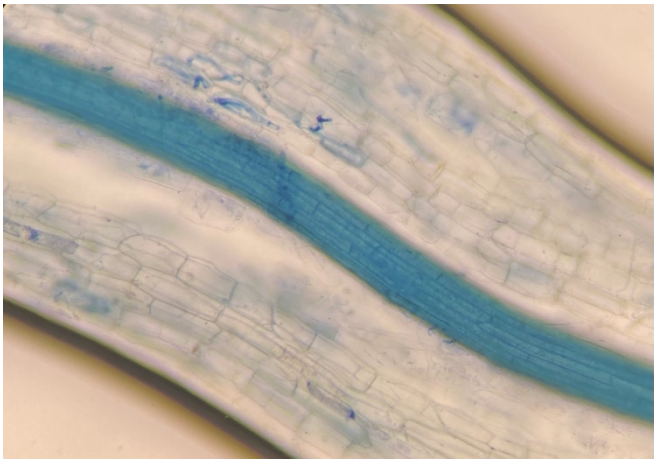

Figure 2: Stained root with vascular cylinder (blue) in the middle

2.3.3 Acidification

After the KOH treatment, the roots were washed with demi-water. Then, they were acidified with hydrochloric acid. For this purpose, 4% hydrochloric acid (60 mL HCl (35%) and 440 mL demi-water) was added to the tubes containing the samples and soaked for 30 min. This step was necessary because the roots are alkaline and for the dye (trypan blue) to better bind to the fungal structures, they should be acidic (Koske & Gemma, 1989).

2.3.4 Staining with trypan blue

The acidified samples were briefly rinsed with demi-water. Then, the 0.05% trypan blue staining solution was added to the tubes containing the roots. This solution consisted of 1 L acidic glycerol and 0.5 g solid trypan blue. For the acidic glycerol, 500 mL glycerol, 450 mL demi-water, and 50 mL 1% HCl were mixed. The glycerol was necessary for the stain to attach evenly along the hyphae of the infected roots. The samples in the tubes with the dye were then heated to 80 °C in a water bath for 15 min. Since no cytoplasm was present anymore due to the KOH treatment, only the fungi were stained blue.

2.3.5 Destaining

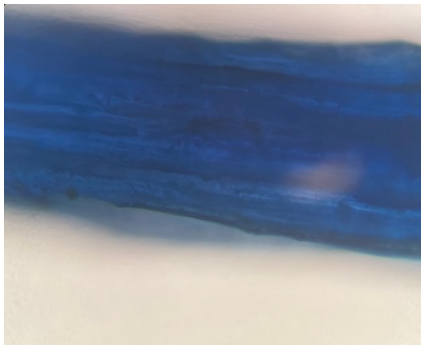

Figure 3: Root too dark (blue)

After the staining, the roots were washed again with demi-water. In the final step, the samples were destained with acidic glycerol (500 mL glycerol, 450 mL demi-water, and 50 mL 1% HCl), otherwise, the roots would appear too blue under the microscope, as shown in Figure 3. For this, the destaining solution was poured into the tubes with the samples and heated at 80 °C in a water bath for 15 min, to enhance the destaining of the root cells.

2.4 Microscopy

2.4.1 Preparation

After the roots had been stained, they were examined under a light microscope (Arsenal LP3000i). Using tweezers, the desired samples from the destaining solution were mounted onto a microscope slide (76x26 mm) parallel to the longitudinal direction (long axis). The ideal length of the roots was 4 cm but occasionally they were slightly shorter. In such cases, several were placed next to each other. The roots of the same sample were aligned in 5 to 7 rows parallel down to the long axis of the microscope slide. Furthermore, tangles should be avoided and finally, a 40x22 mm cover slip was placed on top of the samples on the slide.

2.4.2 Scanning

For the quantification, the microscope slide with the roots was placed on the stage, clamped, and magnifications of 10x, 40x, and 100x were used, depending on the wanted accuracy. Next, the samples were systematically scanned (McGonigle, et al., 1990). This was done by starting with the root at the top left corner and observing the field of view to see whether fungi were present. For detailed information, the type of the seen fungal was noted down in the laboratory journal as a systematic table. Then, the stage was moved vertically (perpendicular to the long axis) down to the next root. This field of view was also examined, fungi noted, and the stage was shifted down to the next. At the lowest root, the stage was moved horizontally 2 mm to the left, and the field of view was again checked for fungi. Afterward, the stage was shifted vertically upwards again. With this method, the parallel-arranged specimens were scanned systematically, and the shown fungi were noted down. It should be managed to get around 80 fields of view per sample (average was 86), but since the number of roots available varied considerably, this could not always be adhered to (range from 37 to 131).

2.4.3 Differentiation

The first distinction was whether fungi were present or absent (marked in the table as N). If one was visible, a differentiation between four categories was made: blue AMF hyphae (A); blue, globular AMF vesicles (V); brown DSE hyphae (D); brown, globular DSE microsclerotia (P). Occasionally, several fungal types could be seen in one field of view, which were then all listed. At other times the samples were too dark or there was a gap between two roots on the microscope slide, this field of view was noted in the table with a slash (and did not count towards the total number). Occasionally the samples were overall too woody to be examined properly. An example (sample M8/2 2022) of such a systematic table can be seen in Table 1.

Table 1: Systematic table of the different fungi, numbers are the mm distances written on the microscope stage, sample M8/2

64	**62**	**60**	**58**	**56**	**54**	**52**	**50**	**48**	**46**	**44**	**42**	**40**	**38**	**36**	**34**
AD	N	A	N	V	AD	A	N	N	N	N	A	A	N	AV	AD
A	A	N	A	N	N	A	N	A	N	AV	N	N	AD	AD	AD
AD	AD	AD	AD	A	N	A	A	A	N	N	A	AP	/	/	/
AV	AV	AF	AV	AV	AV	AV	A	A	AV	A	N	N	A	N	D
AD	AD	AD	AD	AD	AD	AV	AV	AD	AV	AD	AD	AD	A	N	A
AV	AV	A	AV	AV	AV	AV	AV	A	AV	A	A	AV	A	A	AV

2.5 Data analysis

2.5.1 Creating Spreadsheets

After the samples had been examined under the microscope in the laboratory and all the data had been entered into tables in the laboratory journal, the notes were transferred to Microsoft Excel 365 for MacBook (Version 16.83, 24031120). For every probed year (2015 and 2022) a separate spreadsheet was created, and each plant species was entered in a new row, with the corresponding location (e.g. H_1) and name (*Bistorta affinis*). The differentiation letters of the observations (A, D, V, P) for the specific plant were entered in the rows to the right.

2.5.2 Counting and calculations

Then, the following counting and calculations were carried out and listed in separate columns for each plant species: To count the noted letters in the rows the function “COUNTIF” was used in Excel and subsequently, the following calculations were made: ALL (N+A+D+V+P),

Observations (A+D+V+P), Percentage of observations (Observation divided by ALL), AMF hyphae (A), Percentage of AMF hyphae (all A/ALL), AMF Vesicles (V), Percentage of AMF vesicles (all V/ALL), AMF (A+V), Percentage of AMF ((A+V)/ALL), DSE hyphae (D), Percentage of DSE hyphae (D), Percentage of DSE hyphae (all D/ALL), DSE microsclerotia (P), Percentage of DSE microsclerotia (all P/ALL), DSE (D+P), Percentage of DSE ((D+P)/ALL), fields of view and uncountable fields. Subsequently, the plants in the columns and rows of the spreadsheet 2022 were arranged in the same order as in the spreadsheet of 2015, and species, which were not sampled in both years were deleted to achieve a direct comparison of the years. Finally, the net changes and the total overall percentage change of the determined AMF- and DSE percentages from 2015 to 2022 were calculated for each plant species separately.

2.5.3 Colonization rates in R and Excel

First, the percentages of the AMF and DSE colonization rates were read into the application R (RStudio for MacBook, Version 2023.12.1+402, Console R 4.2.3) and plotted with the package ggplot2 (Version 3.5.0) using the function “ggplot(data, aes+geom_point+geom_smooth)”. All values of AMF and DSE of the two years together (2015 and 2022) were summarized in one graph. Furthermore, a linear trend line and the confidence interval were added with the function “method=lm”. Moreover, in Excel, the mean colonization rates by AMF and DSE for 2015 and 2022 separately were calculated and a bar chart was plotted.

2.5.4 Boxplot and heatmaps in R

Afterward, a boxplot was created in R with the function “boxplot(y~x, xlab, ylab, col+points)”, using the net differences of AMF and DSE, and the mean value was added.

For the heatmaps of the AMF and DSE net colonization rate differences, the following function was used: “ggplot(data, aes+geom_tile+scale_fill_gradient2)”. First, empty rows and columns (no measurement data, just filling) were created with the functions “expand.grid” and “left_join”, representing plant species, which were not collected in certain areas. Subsequently, the sample names (e.g. *Bistorta affinis*) and the corresponding locations (e.g. H1) were plotted. The used values were the net changes of the colonization rates by AMF and DSE separately, with a color gradient.

3 Results

3.1 Identification

3.1.1 AMF hyphae

The AMF formed predominantly hyphae in plant roots (Tables 2 and 3). Hyphae usually ran intercellular parallel to the vascular cylinder (Figure 4) but could also be perpendicular, branched, tangled, and crisscrossed (Figures 5 and 6, brown woody roots). Furthermore, the AMF hyphae were thick, unseptated, and appeared (dark) blue under the microscope.

Figure 4: Parallel aligned AMF hyphae

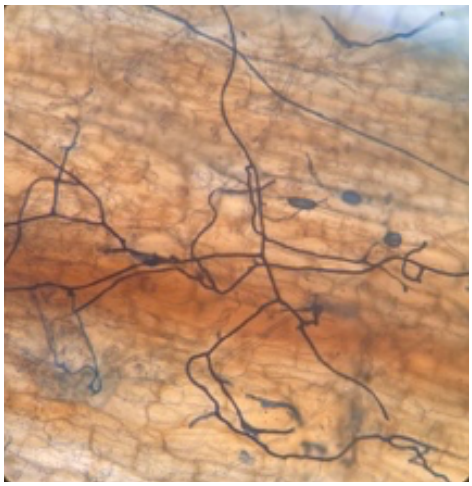

Figure 5: Perpendicular, branched AMF hyphae,

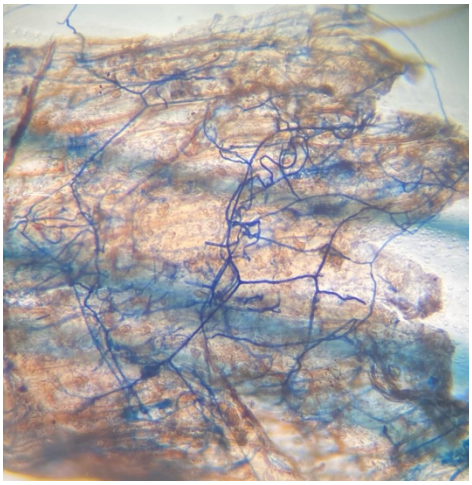

Figure 6: Tangled, crisscrossed AMF hyphae

3.1.2 AMF vesicles

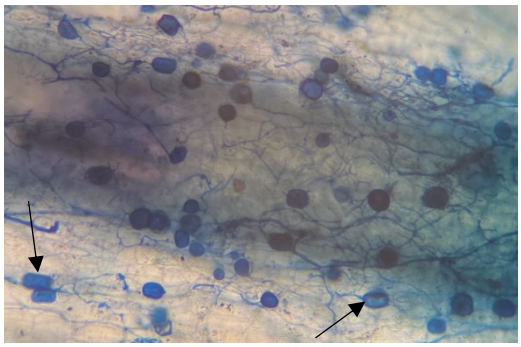

Figure 7: Intracellular, globular, not segmented AMF

Besides hyphae, AMF could form vesicles in plant roots. Vesicles were globular storage structures, which were located near the vascular cylinder. They occurred in a non-clustered form, intracellular, and not segmented (Figure 7, arrows).

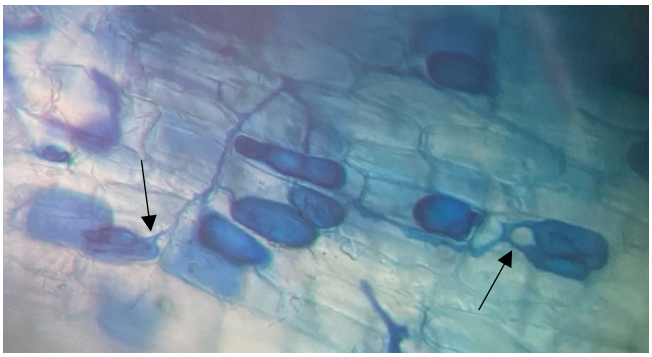

Figure 8: AMF hyphae end in AMF vesicles

The AMF vesicles appeared (dark) blue under the microscope and commonly AMF hyphae ended with such vesicles. The arrows in Figure 8 depict where an AMF hyphae end in AMF vesicles.

3.1.3 DSE hyphae

In addition to AMF, DSE were found in the examined roots. Predominantly thin hyphae were formed (Tables 2 and 3), they appeared after the trypan blue staining still (dark) brown under the microscope, and all of them were septated (Figure 9, arrows). The distinction between AMF and DSE was therefore mainly made by the different color (melanized).

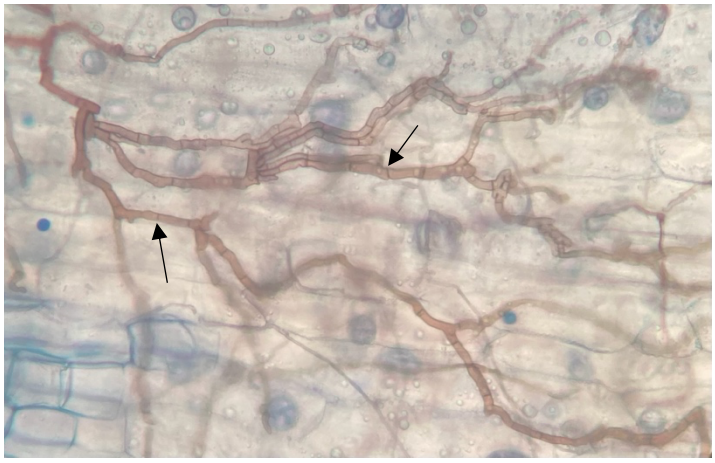

Figure 9: Brown, septate DSE hyphae

Furthermore, as illustrated in Figure 10 and 11 the DSE hyphae, like the AMF hyphae, usually aligned parallel to the vascular cylinder of the root, were branched, tangled, and intercellular.

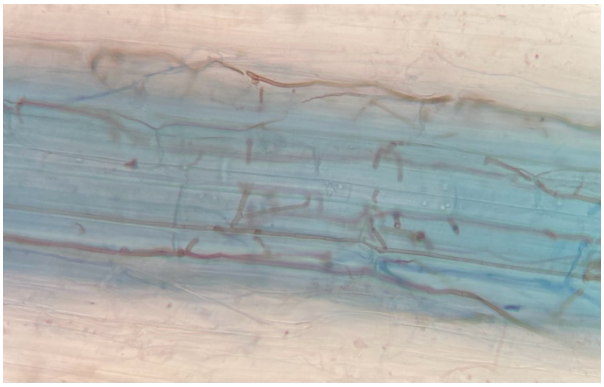

Figure 10: DSE hyphae parallel to the vascular cylinder

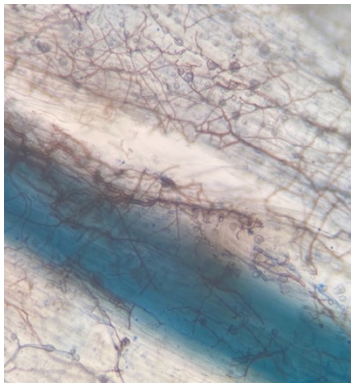

Figure 11: Branched DSE hyphae

3.1.4 DSE microsclerotia

Other than DSE hyphae, also DSE microsclerotia existed in the root structures. Microsclerotia were formed from single or contiguous DSE hyphae aggregates, which piled together (Figure 12). Under the microscope, they appeared (dark) brown even after the trypan blue staining and had a lot of segments (Figure 13, arrow).

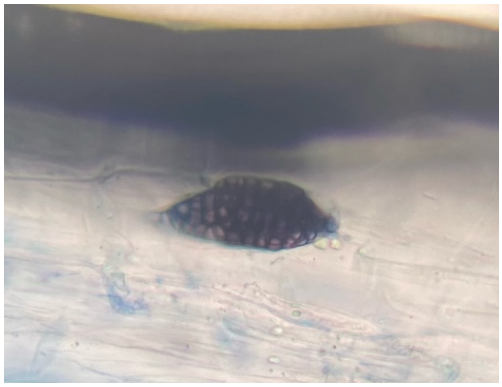

Figure 12: Piled together DSE microsclerotia

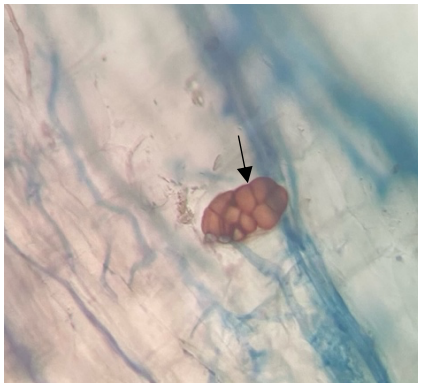

Figure 13: Segmented DSE microsclerotia

3.1.5 Combination of different fungi

In some fields of view, more than one type of fungal was visible. The combination of AMF hyphae (blue) and DSE hyphae (brown) was most commonly found (Figure 14, Tables 2 and 3). Also, AMF hyphae conjoint with AMF vesicles were identified (Figure 8), as well as DSE hyphae and DSE microsclerotia (Figure 10).

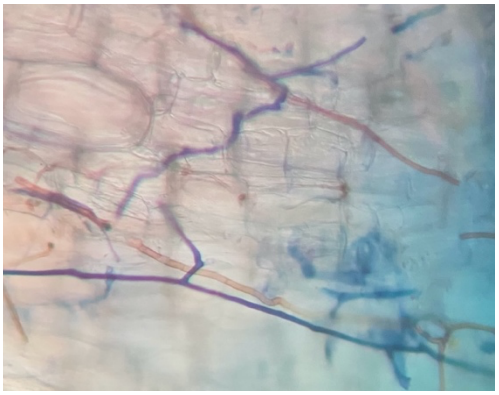

Figure 14: AMF hyphae (blue) and DSE hyphae (brown)

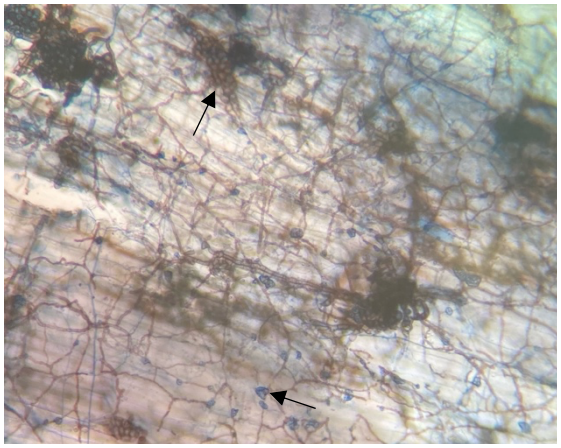

Figure 15: AMF hyphae and vesicles (blue), DSE hyphae and microsclerotia (brown)

As depicted with arrows in Figure 15, some roots had all types of fungal present: blue AMF hyphae and vesicles, brown DSE hyphae and microsclerotia.

Moreover, AMF vesicles (blue, globular) and DSE microsclerotia (brown, piles, segmented) were also mixed in the roots, displayed with arrows in Figure 16. Furthermore, AMF vesicles in combination with DSE hyphae were spotted in some samples.

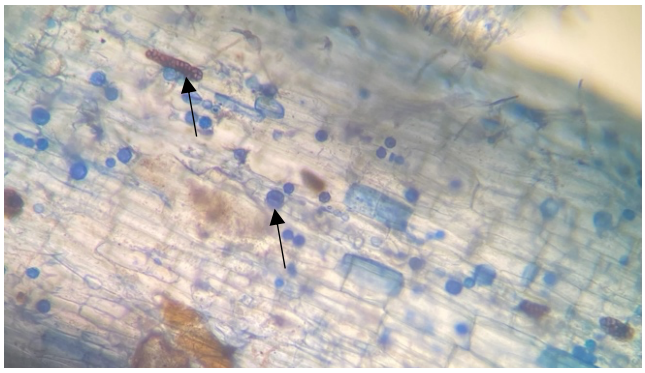

Figure 16: AMF vesicles (blue) and DSE microsclerotia (brown)

3.1.6 Unidentified fungi

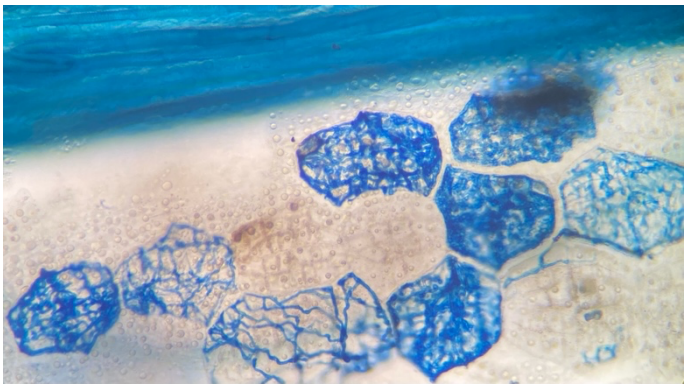

Figure 17: Circular-angular, branched AMF hyphae

In addition to the AMF and DSE fungi, other structures were found in the sampled roots. In Figure 17, slightly circular-angular blue structures can be seen. These were formed most probably by AMF hyphae, which branched and piled together. Another fungal is presented in Figure 18 (arrows), it looks like microsclerotia because they are piled and segmented, but blue, so this would suggest AMF. Furthermore, the small brown dots in Figure 19 would indicate small microsclerotia, which are not piled together yet.

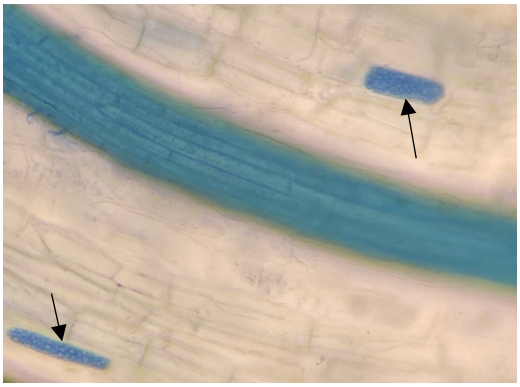

Figure 18: Piled, blue microsclerotia

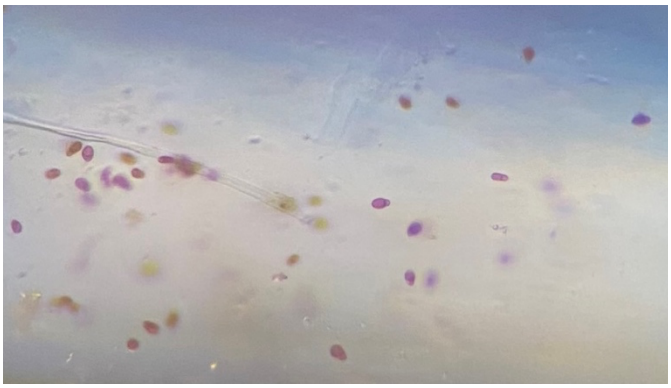

Figure 19: Juvenile brown DSE microsclerotia

The blue structure in Figure 20 could be big blue AMF microsclerotia because of the segments or AMF hyphae that are joined together. On the other hand, it might just be a plant root structure that was not destroyed by the KOH treatment.

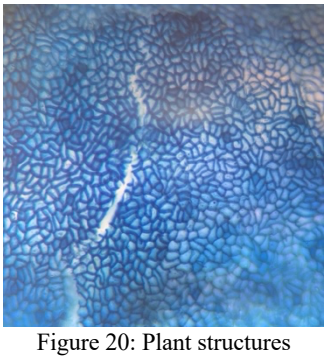

Figure 20: Plant structures

3.2 Analysis

3.2.1 Colonization rates

The roots were scanned systematically and after the identification of the fungal type, the corresponding letter was noted in the laboratory journal, as described in the materials and methods sections 2.4.2 and 2.4.3. Afterward, the collected information was interpolated into Excel, and calculations were carried out as in section 2.5.1. and 2.5.2 indicated. A snippet of the spreadsheet from the samples of 2015 is shown in Table 2 and from 2022 in Table 3.

Table 2: Spreadsheet samples 2015 with the sample code, plant species name and the calculated values

Sample code	Plant species	ALL	Observations	Observations [%]	N	AMF hyphae	AMF hyphae [%]	AMF vesicle	AMF vesicle (%)	AMF	AMF [%]	DSE hypae	DSE hyphae [%]	DSE microsc.	DSE microsc. [%]	DSE	DSE [%]
H1/2	Bistorta affinis	94	63	67,02	31	27	28,72	0	0,00	27	28,72	36	38,30	0	0,00	36	38,30
H2/1	Anaphalis nubigena	148	122	82,43	26	50	33,78	11	7,43	61	41,22	59	39,86	2	1,35	61	41,22
H2/2	Bistorta affinis	132	106	80,30	26	41	31,06	0	0,00	41	31,06	65	49,24	0	0,00	65	49,24
H2/3	Potentilla sojakii	130	117	90,00	13	57	43,85	7	5,38	64	49,23	52	40,00	1	0,77	53	40,77
H3/1	Aster flaccidus	80	61	76,25	19	13	16,25	0	0,00	13	16,25	47	58,75	1	1,25	48	60,00
H3/2	Leontopodium ochroleucum	60	23	38,33	37	6	10,00	0	0,00	6	10,00	17	28,33	0	0,00	17	28,33
H3/5	Bistorta vivipara	126	93	73,81	33	26	20,63	0	0,00	26	20,63	67	53,17	0	0,00	67	53,17
H7/2	Leontopodium ochroleucum	166	154	92,77	12	71	42,77	7	4,22	78	46,99	76	45,78	0	0,00	76	45,78
H7/3	Poa attenuata	196	194	98,98	2	72	36,73	13	6,63	85	43,37	105	53,57	4	2,04	109	55,61
H7/4	Gentiana nubigena	122	85	69,67	37	20	16,39	0	0,00	20	16,39	61	50,00	4	3,28	65	53,28
H8/1	Psychrogeton andryaloides	84	84	100,00	0	31	36,90	6	7,14	37	44,05	47	55,95	0	0,00	47	55,95
H12/2	Nepeta discolor	48	33	68,75	15	23	47,92	5	10,42	28	58,33	2	4,17	3	6,25	5	10,42
H12/3	Piptatherum gracile	124	102	82,26	22	59	47,58	4	3,23	63	50,81	37	29,84	2	1,61	39	31,45
H12/4	Elymus jacquemontii	146	136	93,15	10	74	50,68	3	2,05	77	52,74	58	39,73	1	0,68	59	40,41
H12/5	Astragalus oplites	111	81	72,97	30	72	64,86	4	3,60	76	68,47	4	3,60	1	0,90	5	4,50
H15/1	Potentilla agrimonioides	126	113	89,68	13	88	69,84	25	19,84	113	89,68	0	0,00	0	0,00	0	0,00
H15/2	Tanacetum tibeticum	110	90	81,82	20	53	48,18	9	8,18	62	56,36	25	22,73	3	2,73	28	25,45
H15/5	Potentilla venusta/sojakii	133	114	85,71	19	61	45,86	16	12,03	77	57,89	36	27,07	1	0,75	37	27,82
H17/2	Astragalus nivalis	112	98	87,50	14	63	56,25	13	11,61	76	67,86	21	18,75	1	0,89	22	19,64
H17/4	Piptatherum gracile	116	61	52,59	55	48	41,38	1	0,86	49	42,24	12	10,34	0	0,00	12	10,34
H17/5	Dracocephalum heterophyllum	89	83	93,26	6	70	78,65	0	0,00	70	78,65	10	11,24	3	3,37	13	14,61
H23/1	Tanacetum fruticulosum	106	81	76,42	25	32	30,19	0	0,00	32	30,19	49	46,23	0	0,00	49	46,23
H23/2	Krascheninnikovia pungens	69	59	85,51	10	46	66,67	7	10,14	53	76,81	6	8,70	0	0,00	6	8,70
H23/5	Artemisia santolinifolia	101	79	78,22	22	35	34,65	0	0,00	35	34,65	44	43,56	0	0,00	44	43,56
H25/1	Tanacetum fruticulosum	84	50	59,52	34	43	51,19	0	0,00	43	51,19	7	8,33	0	0,00	7	8,33
H25/3	Ephedra intermedia	111	63	56,76	48	39	35,14	8	7,21	47	42,34	16	14,41	0	0,00	16	14,41
H40/2	Tanacetum fruticulosum	74	23	31,08	51	19	25,68	4	5,41	23	31,08	0	0,00	0	0,00	0	0,00
H40/3	Stipa subsessiliflora	131	101	77,10	30	32	24,43	1	0,76	33	25,19	64	48,85	4	3,05	68	51,91
H41/1	Tanacetum fruticulosum	145	137	94,48	8	50	34,48	3	2,07	53	36,55	65	44,83	19	13,10	84	57,93
H41/2	Stipa purpurea	136	132	97,06	4	59	43,38	1	0,74	60	44,12	72	52,94	0	0,00	72	52,94
H41/3	Artemisia santolinifolia	93	47	50,54	46	31	33,33	0	0,00	31	33,33	16	17,20	0	0,00	16	17,20
M4/2	Bistorta affinis	122	97	79,51	25	27	22,13	0	0,00	27	22,13	70	57,38	0	0,00	70	57,38
M4/3	Elymus canaliculatus	104	57	54,81	47	32	30,77	0	0,00	32	30,77	25	24,04	0	0,00	25	24,04
M4/4	Androsace robusta	155	123	79,35	32	54	34,84	1	0,65	55	35,48	68	43,87	0	0,00	68	43,87
M7/1	Astragalus thomsonii	46	43	93,48	3	35	76,09	0	0,00	35	76,09	8	17,39	0	0,00	8	17,39
M7/2	Calamagrostis holciformis	149	125	83,89	24	80	53,69	16	10,74	96	64,43	29	19,46	0	0,00	29	19,46
M7/3	Bistorta vivipara	170	162	95,29	8	91	53,53	0	0,00	91	53,53	71	41,76	0	0,00	71	41,76
M8/1	Comarum salesovianum	134	101	75,37	33	48	35,82	9	6,72	57	42,54	41	30,60	3	2,24	44	32,84
M8/2	Elymus canaliculatus	149	122	81,88	27	81	54,36	17	11,41	98	65,77	24	16,11	0	0,00	24	16,11
M8/3	Tanacetum pyrethroides	111	88	79,28	23	64	57,66	2	1,80	66	59,46	22	19,82	0	0,00	22	19,82
M8/4	Tanacetum tibeticum/fruticulosum	95	73	76,84	22	64	67,37	8	8,42	72	75,79	1	1,05	0	0,00	1	1,05
M12/1	Tanacetum fruticulosum	112	73	65,18	39	29	25,89	16	14,29	45	40,18	25	22,32	3	2,68	28	25,00
M12/2	Leymus secalinus	130	92	70,77	38	64	49,23	3	2,31	67	51,54	24	18,46	1	0,77	25	19,23
M12/5	Krascheninnikovia pungens	119	80	67,23	39	65	54,62	0	0,00	65	54,62	15	12,61	0	0,00	15	12,61
M15/2	Poa attenuata	113	59	52,21	54	38	33,63	0	0,00	38	33,63	21	18,58	0	0,00	21	18,58
M15/5	Potentilla pamirica	84	42	50,00	42	27	32,14	0	0,00	27	32,14	15	17,86	0	0,00	15	17,86

Table 3: Spreadsheet samples 2022 with the sample code, plant species name and the calculated values

Sample code	Plant species	ALL	Observations	Observations [%]	N	AMF hyphae	AMF hyphae [%]	AMF vesicle	AMF vesicle (%)	AMF	AMF [%]	DSE hypae	DSE hyphae [%]	DSE microsc.	DSE microsc. [%]	DSE	DSE [%]
H1/2	Bistorta affinis	61	36	59,02	25	16	26,23	0	0,00	16	26,23	20	32,79	0	0,00	20	32,79
H2/1	Anaphalis nubigena	150	115	76,67	35	56	37,33	9	6,00	65	43,33	50	33,33	0	0,00	50	33,33
H2/2	Bistorta affinis	136	118	86,76	18	63	46,32	3	2,21	66	48,53	52	38,24	0	0,00	52	38,24
H2/3	Potentilla sojakii	128	114	89,06	14	73	57,03	0	0,00	73	57,03	41	32,03	0	0,00	41	32,03
H3/1	Aster flaccidus	125	103	82,40	22	42	33,60	0	0,00	42	33,60	61	48,80	0	0,00	61	48,80
H3/2	Leontopodium ochroleucum	127	109	85,83	18	19	14,96	24	18,90	43	33,86	66	51,97	0	0,00	66	51,97
H3/5	Bistorta vivipara	172	167	97,09	5	64	37,21	0	0,00	64	37,21	103	59,88	0	0,00	103	59,88
H7/2	Leontopodium ochroleucum	157	151	96,18	6	49	31,21	13	8,28	62	39,49	89	56,69	0	0,00	89	56,69
H7/3	Poa attenuata	142	107	75,35	35	41	28,87	2	1,41	43	30,28	64	45,07	0	0,00	64	45,07
H7/4	Gentiana nubigena	82	64	78,05	18	23	28,05	0	0,00	23	28,05	41	50,00	0	0,00	41	50,00
H8/1	Psychrogeton andryaloides	103	102	99,03	1	53	51,46	0	0,00	53	51,46	49	47,57	0	0,00	49	47,57
H12/2	Nepeta discolor	92	60	65,22	32	25	27,17	0	0,00	25	27,17	28	30,43	7	7,61	35	38,04
H12/3	Piptatherum gracile	144	135	93,75	9	66	45,83	1	0,69	67	46,53	63	43,75	5	3,47	68	47,22
H12/4	Elymus jacquemontii	150	139	92,67	11	73	48,67	5	3,33	78	52,00	61	40,67	0	0,00	61	40,67
H12/5	Astragalus oplites	82	77	93,90	5	43	52,44	2	2,44	45	54,88	32	39,02	0	0,00	32	39,02
H15/1	Potentilla agrimonioides	85	51	60,00	34	44	51,76	2	2,35	46	54,12	5	5,88	0	0,00	5	5,88
H15/2	Tanacetum tibeticum	76	24	31,58	52	23	30,26	0	0,00	23	30,26	1	1,32	0	0,00	1	1,32
H15/5	Potentilla venusta/sojakii	122	103	84,43	19	65	53,28	7	5,74	72	59,02	31	25,41	0	0,00	31	25,41
H17/2	Astragalus nivalis	82	49	59,76	33	38	46,34	0	0,00	38	46,34	11	13,41	0	0,00	11	13,41
H17/4	Piptatherum gracile	92	74	80,43	18	58	63,04	0	0,00	58	63,04	16	17,39	0	0,00	16	17,39
H17/5	Dracocephalum heterophyllum	63	48	76,19	15	38	60,32	0	0,00	38	60,32	10	15,87	0	0,00	10	15,87
H23/1	Tanacetum fruticulosum	42	38	90,48	4	38	90,48	0	0,00	38	90,48	0	0,00	0	0,00	0	0,00
H23/2	Krascheninnikovia pungens	44	41	93,18	3	41	93,18	0	0,00	41	93,18	0	0,00	0	0,00	0	0,00
H23/3	Artemisia santolinifolia	157	148	94,27	9	63	40,13	3	1,91	66	42,04	82	52,23	0	0,00	82	52,23
H25/2	Tanacetum fruticulosum	97	51	52,58	46	42	43,30	0	0,00	42	43,30	9	9,28	0	0,00	9	9,28
H25/1	Ephedra intermedia	98	94	95,92	4	64	65,31	0	0,00	64	65,31	30	30,61	0	0,00	30	30,61
H40/2	Tanacetum fruticulosum	111	77	69,37	34	76	68,47	0	0,00	76	68,47	1	0,90	0	0,00	1	0,90
H40/3	Stipa subsessiliflora	64	55	85,94	9	55	85,94	0	0,00	55	85,94	0	0,00	0	0,00	0	0,00
H41/1	Tanacetum fruticulosum	63	52	82,54	11	52	82,54	0	0,00	52	82,54	0	0,00	0	0,00	0	0,00
H41/2	Stipa purpurea	58	28	48,28	30	28	48,28	0	0,00	28	48,28	0	0,00	0	0,00	0	0,00
H41/3	Artemisia santolinifolia	67	52	77,61	15	40	59,70	0	0,00	40	59,70	12	17,91	0	0,00	12	17,91
M4/1	Bistorta affinis	121	116	95,87	5	66	54,55	3	2,48	69	57,02	47	38,84	0	0,00	47	38,84
M4/2	Elymus canaliculatus	127	115	90,55	12	58	45,67	2	1,57	60	47,24	55	43,31	0	0,00	55	43,31
M4/3	Androsace robusta	140	81	57,86	59	47	33,57	2	1,43	49	35,00	32	22,86	0	0,00	32	22,86
M7/1	Astragalus thomsonii	108	83	76,85	25	68	62,96	0	0,00	68	62,96	15	13,89	0	0,00	15	13,89
M7/3	Calamagrostis holciformis	122	90	73,77	32	59	48,36	8	6,56	67	54,92	21	17,21	2	1,64	23	18,85
M7/2	Bistorta vivipara	162	134	82,72	28	32	19,75	0	0,00	32	19,75	102	62,96	0	0,00	102	62,96
M8/3	Comarum salesovianum	135	119	88,15	16	61	45,19	6	4,44	67	49,63	52	38,52	0	0,00	52	38,52
M8/5	Elymus canaliculatus	120	97	80,83	23	46	38,33	0	0,00	46	38,33	49	40,83	2	1,67	51	42,50
M8/4	Tanacetum pyrethroides	126	99	78,57	27	68	53,97	21	16,67	89	70,63	6	4,76	4	3,17	10	7,94
M8/1	Tanacetum tibeticum/fruticulosum	154	154	100,00	0	84	54,55	7	4,55	91	59,09	61	39,61	2	1,30	63	40,91
M12/1	Tanacetum fruticulosum	86	50	58,14	36	47	54,65	0	0,00	47	54,65	3	3,49	0	0,00	3	3,49
M12/2	Leymus secalinus	96	57	59,38	39	36	37,50	3	3,13	39	40,63	18	18,75	0	0,00	18	18,75
M12/5	Krascheninnikovia pungens	102	76	74,51	26	69	67,65	1	0,98	70	68,63	6	5,88	0	0,00	6	5,88
M15/2	Poa attenuata	112	48	42,86	64	19	16,96	0	0,00	19	16,96	29	25,89	0	0,00	29	25,89
M15/5	Potentilla pamirica	72	10	13,89	62	6	8,33	0	0,00	6	8,33	4	5,56	0	0,00	4	5,56

- Infection rates of AMF and DSE

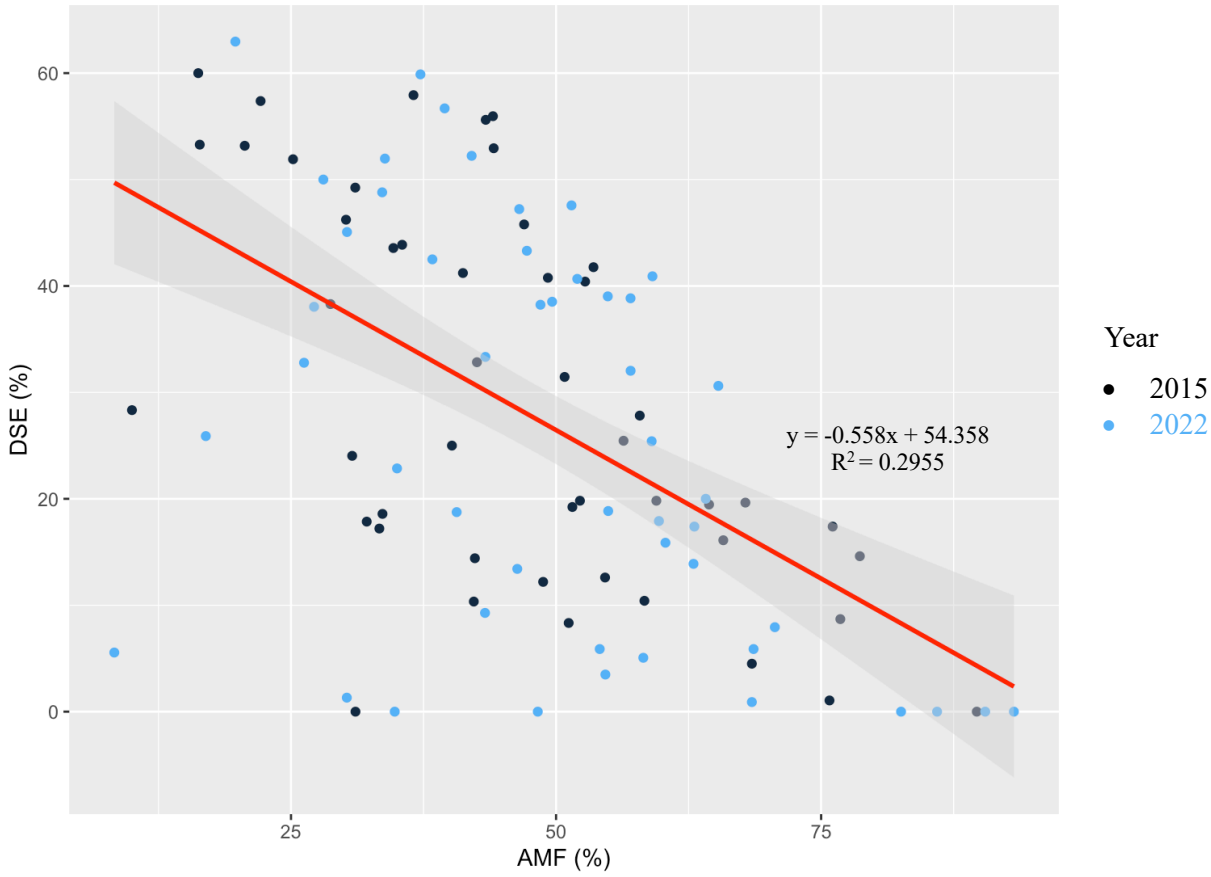

Figure 21: Diagram of AMF (x-axis) and DSE (y-axis) colonization rates 2015 (black) and 2022 (blue) in percent with linear trendline (red), the corresponding formula, and the confidence interval (light-gray area)

The collected and calculated colonization values of AMF (%), hyphae and vesicles combined, and DSE (%), hyphae and microsclerotia summed up, (data displayed in Tables 2 and 3) were imported into R and plotted as described in 2.5.3. The result is shown above in Figure 21. In black, the values (all plant species) of 2015 and in blue of 2022 are shown. The graph depicts that as the AMF colonization rate increases, the DSE infection rate of the same root decreases and vice versa. AMF rates over 70% were linked to DSE values lower than 20% and a DSE colonization rate of over 50% only existed when AMF colonized less than 50% of the sample.

Furthermore, the red line in Figure 21 indicates the linear trend of the values with the light-gray area being the confidence interval. This further confirmed the above-mentioned relation between the AMF and DSE colonization rates. High AMF values were linked to low DSE infection rates and a high colonization by DSE resulted in low infections by AMF. The coefficient of determination R^2 was calculated in Excel and resulted in 0.2955 (Pearson's r = 0.54), thus the values had a moderate to high correlation.

- Mean colonization rate

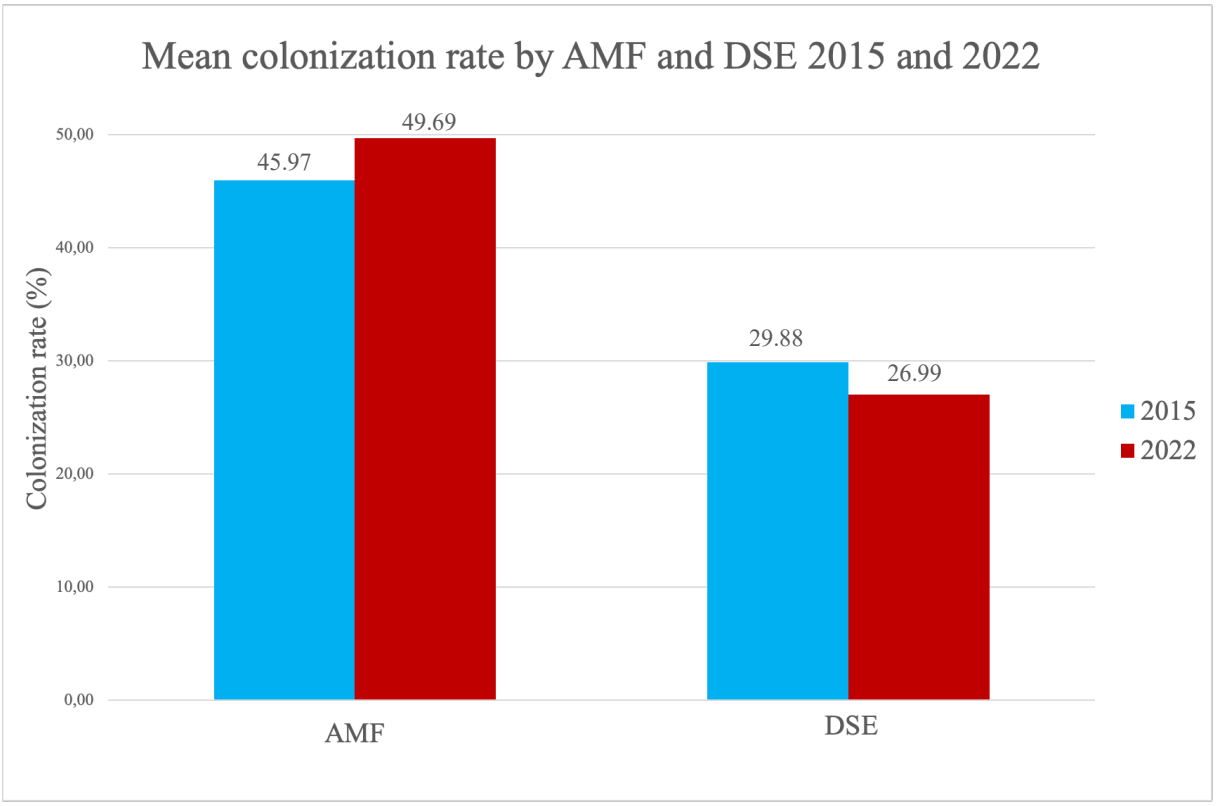

Figure 22: Graph of mean colonization rate by AMF (left) and DSE (right) 2015 (blue) and 2022 (red) in percent (y-axis)

With the colonization rates per plant species, values posed in Tables 2 and 3, were a mean calculated for AMF (%) and DSE (%) for 2015 (light blue) and 2022 (red) separately, the results are displayed in Figure 22. On average in 2015 with 45.97% and in 2022 with 49.69% were the roots colonized by AMF. Whereas 29.88% was the infection rate by DSE in 2015 and 2022 at 26.99%.

Consistent with Figure 22 is the plant *Potentilla sojakii* (H2/3), which had a 49.23% AMF- and a 40.77% DSE colonization rate in 2015 and was 2022 57.03% infected by AMF and 32.03% colonized by DSE. However, the stated values in Figure 22 are not representative for every plant species, because these were the mean colonization rates. For example, *Potentilla agrimonioides* (H15/1) had 2015 an infection rate by AMF of 89.68%, 2022 54.12%, and in the same plant were no DSE found in 2015, but in 2022 5.88% of the root was colonized by DSE (data from Table 2 and 3), which was the opposite trend to the findings in Figure 22.

Discernible in Figure 22, the overall trend of the plant roots was an enhancement in the AMF colonization rate and a reduction of DSE infections over the observed seven years. Furthermore, it was noticeable that the AMF were in abundance.

3.2.2 Net differences

Table 4: Net differences of AMF and DSE of all samples with the sample code and plant species name

Sample code	Plant species	Net difference (AMF)	Net difference (DSE)
H1/2	Bistorta affinis	-2,49	-5,51
H2/1	Anaphalis nubigena	2,12	-7,88
H2/2	Bistorta affinis	17,47	-11,01
H2/3	Potentilla sojakii	7,80	-8,74
H3/1	Aster flaccidus	17,35	-11,20
H3/2	Leontopodium ochroleucum	23,86	23,64
H3/5	Bistorta vivipara	16,57	6,71
H7/2	Leontopodium ochroleucum	-7,50	10,90
H7/3	Poa attenuata	-13,09	-10,54
H7/4	Gentiana nubigena	11,66	-3,28
H8/1	Psychrogeton andryaloides	7,41	-8,38
H12/2	Nepeta discolor	-31,16	27,63
H12/3	Piptatherum gracile	-4,28	15,77
H12/4	Elymus jacquemontii	-0,74	0,26
H12/5	Astragalus oplites	-13,59	34,52
H15/1	Potentilla agrimonioides	-35,56	5,88
H15/2	Tanacetum tibeticum	-26,10	-24,14
H15/5	Potentilla venusta/sojakii	1,12	-2,41
H17/2	Astragalus nivalis	-21,52	-6,23
H17/4	Piptatherum gracile	20,80	7,05
H17/5	Dracocephalum heterophyllum	-18,33	1,27
H23/1	Tanacetum fruticulosum	60,29	-46,23
H23/2	Krascheninnikovia pungens	16,37	-8,70
H23/3	Artemisia santolinifolia	7,38	8,66
H25/2	Tanacetum fruticulosum	-7,89	0,95
H25/1	Ephedra intermedia	22,96	16,20
H40/2	Tanacetum fruticulosum	37,39	0,90
H40/3	Stipa subsessiliflora	60,75	-51,91
H41/1	Tanacetum fruticulosum	45,99	-57,93
H41/2	Stipa purpurea	4,16	-52,94
H41/3	Artemisia santolinifolia	26,37	0,71
M4/1	Bistorta affinis	34,89	-18,53
M4/2	Elymus canaliculatus	16,47	19,27
M4/3	Androsace robusta	-0,48	-21,01
M7/1	Astragalus thomsonii	-13,12	-3,50
M7/3	Calamagrostis holciformis	-9,51	-0,61
M7/2	Bistorta vivipara	-33,78	21,20
M8/3	Comarum salesovianum	7,09	5,68
M8/5	Elymus canaliculatus	-27,44	26,39
M8/4	Tanacetum pyrethroides	11,18	-11,88
M8/1	Tanacetum tibeticum/fruticulosum	-16,70	39,86
M12/1	Tanacetum fruticulosum	14,47	-21,51
M12/2	Leymus secalinus	-10,91	-0,48
M12/5	Krascheninnikovia pungens	14,01	-6,72
M15/2	Poa attenuata	-16,66	7,31
M15/5	Potentilla pamirica	-23,81	-12,30

- Differences in AMF colonization rates

From Table 4 it is visible that the plant species with code H40/3 named *Stipa Subsessiliflora* had the highest rise in the AMF colonization rate with a difference of the percentage values of 60.75% and the sample H2/1 *Anaphalis nubigena* had a 2.12% greater AMF infection rate in

2022. Furthermore, -35.56% was the highest decline of the AMF colonization rate in the species *Potentilla Agrimonioides* (H15/1) recorded, and *Adrosace robusta* (M4/3) had a decrease of -0.48% in the infection rate.

- Differences in DSE colonization rates

As revealed by Table 4 one can see that the plant sample with code M8/1 name *Tanacetum tibeticum/fruticulosum* had 2022 39.86% more colonization by DSE than in 2015 and the roots of *Elysmus jacquemontii* (H12/4) had a rise of the infection rate (DSE) of 0.26%. On the other hand, the highest decline in the DSE colonization was recorded in the sample H41/1 *Tanacetum fruticulosum* with -57.93%. *Leymus secalinus* (M12/2) decreased by -0.48% in the infection rate compared to 2015.

- Boxplot of net differences in AMF and DSE colonization rates

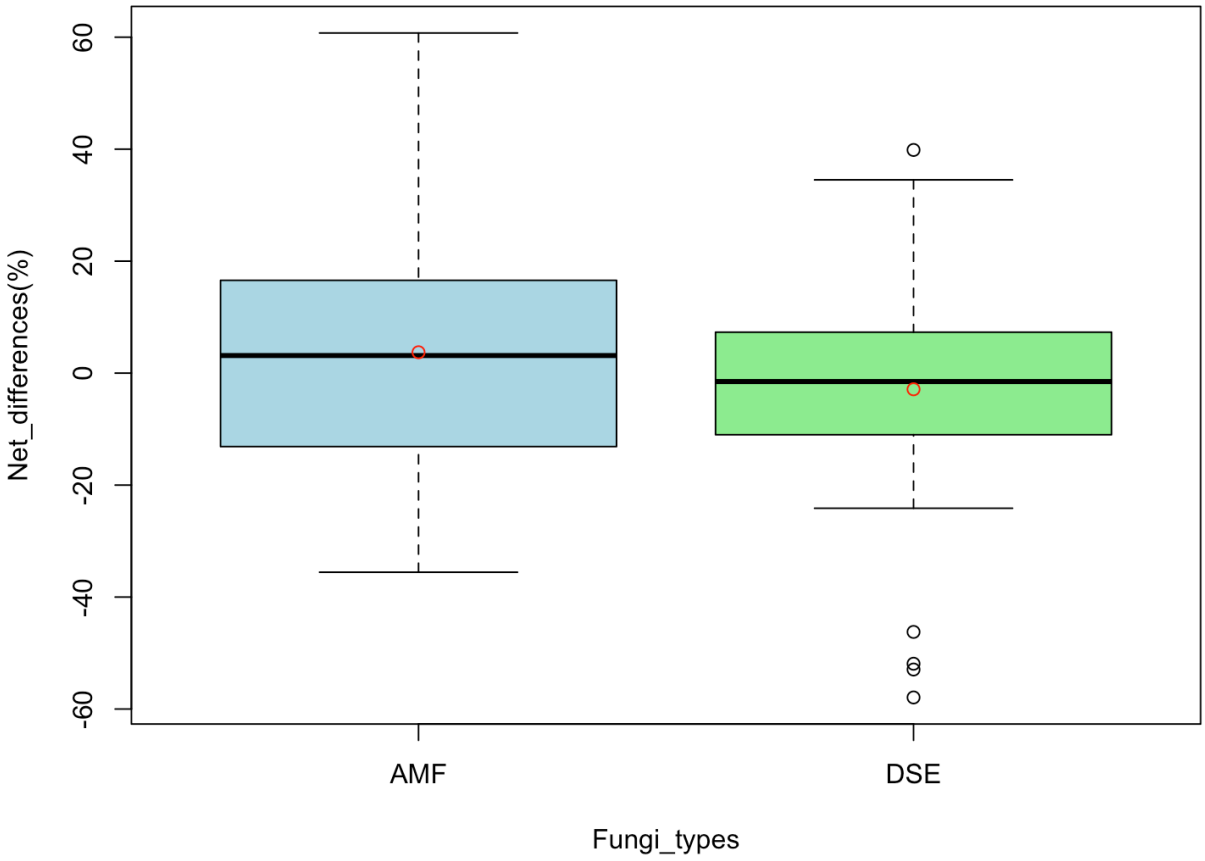

Figure 23: Boxplot of AMF (blue, left) and DSE (green, right) net colonization rate differences in percent (y-axis), and the mean value marked with a red circle

The data (net differences) of Table 4 were plotted in R and a boxplot with AMF (blue) and DSE (green) was created (Figure 23), the corresponding values of the boxplot were calculated in Excel, as described in 2.5.4. The values of DSE had more outliers than the ones of AMF, which lead to the following changes in the boxplot: the whiskers of DSE data were 1.5 times longer than the box, therefore the black circles are the outliers.
First, it can be seen through the whiskers that the AMF colonization rate (upper whisker, 60.75%) rose more than it was the case of the DSE (39.86%, upper outlier). On the other hand, the infection rate by AMF declined by 35.56% (whisker) whereas DSE had a decrease in the infection rate of 57.93% (outlier).

Furthermore, the median (thick black line, value that lies exactly in the middle of the plotted data) of the AMF is slightly higher (3.14) than the one of the DSE (-1.51). Red circles in Figure 23 mark the means of the data, which lay slightly above and below the median. The average of the colonization rate differences by AMF is 3.72% (increase), while the mean values of DSE (-2.89%) indicate an overall decline in their infection rate.

16.77% is the value of the upper quartile of AMF, and between that and the upper whisker lay 25% of the highest values, the range was larger and located at higher colonization rate differences than of the DSE (third quartile = 7.65%, upper whisker = 34.52%). Moreover, Figure 23 depicts that the interquartile range (midspread, middle 50% of the values, box) of the DSE infection rate differences (-11.06% to 7.65%) was smaller than of the AMF (-13.24% to 16.77%). The lowest 25% of the plotted AMF data (first quartile = -13.24% to the lower whisker = -35.56%) represented a part of the values with a decline in the colonization rate between 2015 and 2022. This range was larger and located at lower infection rate differences than the one of DSE (first quartile = -11.06% to the lower whisker = -24.14%).

- Heatmap AMF differences

With the values from Table 4 was a heatmap created in R for the differences in the AMF colonization rates, as described in 2.5.4. The findings for each plant species and the corresponding location between the years 2015 and 2022 are depicted in Figure 24. The graph shows the highest rise in the AMF infection rate (%) in dark green, a color gradient with zero differences marked in light blue and a decline in red. Plant species with a lower infection rate in 2022 than in 2015 are displayed in red in Figure 24.

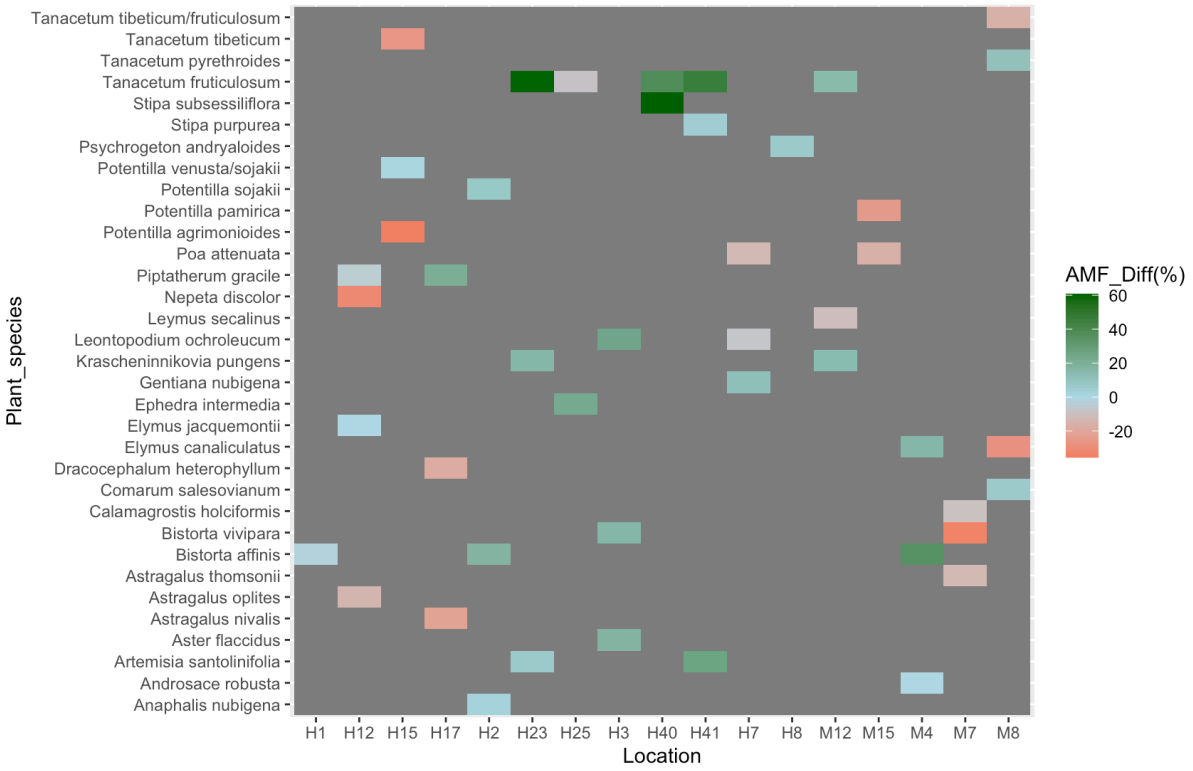

Figure 24: Heatmap of all plant species (y-axis) with the corresponding location (x-axis) of AMF colonization differences in percent (filling, z-axis) with a color gradient of red (decrease) over light-blue (zero) to dark-green (increase)

The plant *Tanacetum fruticulosum* had a net difference (increase) in the AMF colonization rate of about 40-60% at the locations H23, H40, and H41 (green) but a decrease in the colonization rate of AMF between 2015 and 2022 of circa 10% at the location H25 (gray). From Figure 24 it can be seen that the infection rates of *Bistorta vivipara* in location M7, *Nepeta discolor* in H12, and *Potentilla agrimonioides* (H15) declined by about 30% within the seven years. However, in most of the plants was a slight rise or reduction, light blue and gray in Figure 24, in the colonization rate of AMF recorded.

- Heatmap DSE differences

In addition to the AMF colonization differences were the net difference values of DSE, Table 4, plotted in R, the findings are summarized in Figure 25. This heatmap shows each plant species on the y-axis with the corresponding location on the x-axis and the infection rate changes between 2015 and 2022 with a color gradient from dark green (rise) over light blue (zero) to red (decrease).

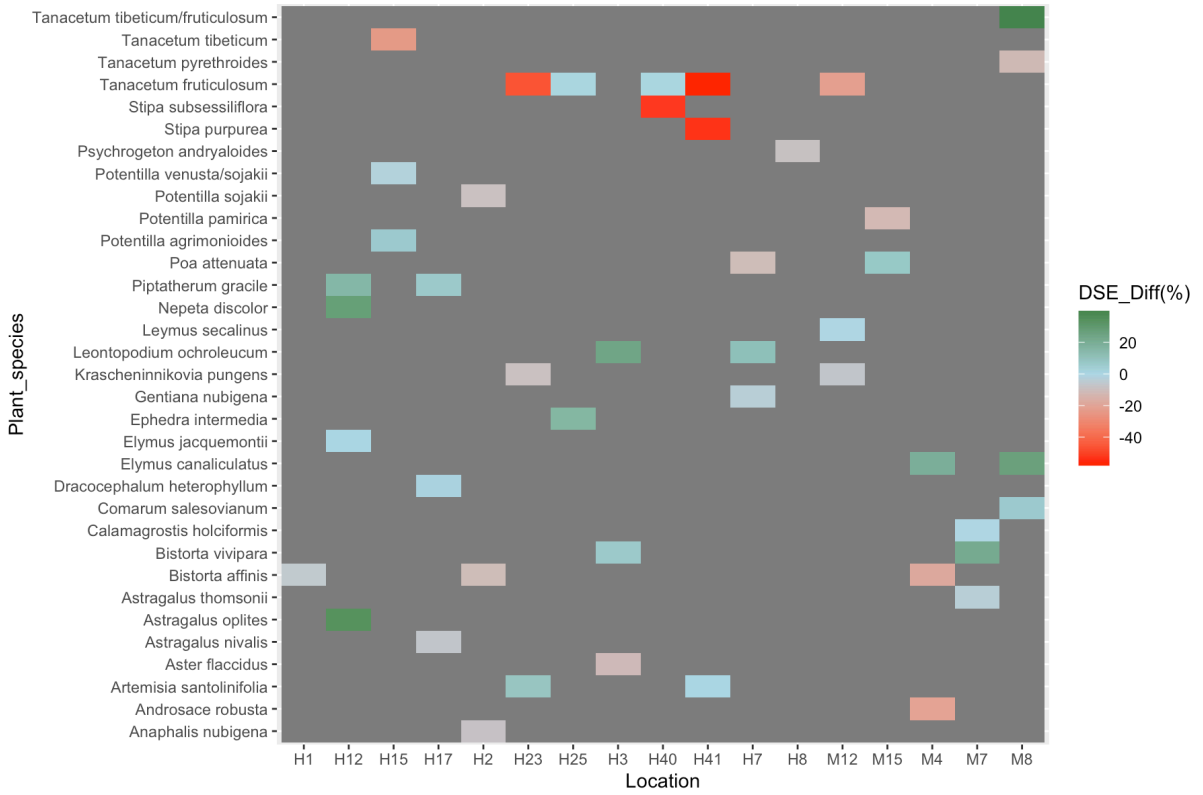

Figure 25: Heatmap of all plant species (y-axis) with the corresponding location (x-axis) of DSE colonization differences in percent (filling, z-axis) with a color gradient of red (decrease) over light-blue (zero) to dark-green (increase)

First of all, the graph reveals that in four samples a major decline (more than 40%) in the DSE colonization was recorded. On the other hand, at locations H12 and M7 had the plant species 2022 a higher infection rate by DSE than in 2015 (5 to 40%, blue-green).

The plant species *Tanacetum fruticulosum*, which had one of the highest AMF colonization rates increases at the probed locations from 2015 to 2022 (Figure 24), had one of the biggest declines in the DSE infection rate at the same sites. Furthermore, an increase in the colonization rate by DSE of the plant *Bistorta vivipara* in M7 is displayed in Figure 25 (light green), and with this species was at that site a decrease in the AMF infection rate recorded. Such a correlation between the AMF and DSE colonization rates was consistent with the findings in Figure 24.

4 Discussion

This project aimed to determine the prevalence of root colonization by AMF and DSE in diverse plant species with samples collected by a research group in 2015 and 2022 from the Ladakh range in the Himalayas. Furthermore, the various infection levels between these years were determined. It was hypothesized, that potential differences in the AMF and DSE colonization rates, could be explained by climate change.

4.1 Review of the Methods

The sampling process and preservation of the roots worked well. Furthermore, the staining of the specimen to make the fungi visible, described in 2.3, also went without major problems. Occasionally the roots were extremely hard, and woody, and were treated with the 10% KOH to remove plant cytoplasm. However, some samples remained challenging to examine under the microscope due to darkness and the brown color. In retrospect, using a higher percentage of KOH for all samples and increasing the heating time to 2 hours would have better cleared the roots, allowing for a more precise observation of the fungi. The acidification with HCl allowed the subsequently used staining solution trypan blue to bind to the target structures. Additionally, the staining should not last too long, and subsequent destaining steps were conducted to prevent excessive blue coloration. Microscopy worked fine, but sometimes there were too few samples for an adequate number of fields of view.

4.2 Fungal types

4.2.1 AMF and DSE

Under the microscope, the fungal types colonizing a certain root were identified. Four main types were found: AMF hyphae and vesicles, DSE hyphae and microsclerotia. As described in the literature (Smith & Read, 2008), the AMF hyphae were, after trypan blue staining, seen as thin blue strands, predominantly intracellular, and not segmented. The vesicles also had the structure as explained by Smith and Read (2008): blue spheres, which were not piled together. DSE are not yet sufficiently studied, but the shape and color are consistent with the statements of Bueno de Mesquita et al. (2018). They appeared brown and segmented under the microscope. So did the DSE microsclerotia, which were also brown, globular, and segmented. Other fungal types were also found, namely AMF in circular-angular structures, microsclerotia in blue and brown ones, which were in the juvenile state and therefore not piled together yet.

4.2.2 Different fungi combined in one root

It was confirmed that several fungal types, AMF and DSE, can exist simultaneously in one root. As displayed in this study it is feasible and it could even be beneficial for the plant to make symbiosis with AMF and DSE in the same root to obtain more and different nutrients.

4.3 Differences in AMF and DSE colonization

The AMF root infection rates found in this study (approximately 50% at 4500 m a.s.l.) are much higher than Read and Haselwandter's (1981) results (only 7% at 3200 m a.s.l.) and greater than the ones of Kotilínek et al. (2017), who found a maximal colonization rate by AMF of 40% in the Himalayas (4500 m a.s.l.). The higher infections could be described by the statement of Read and Haselwandter (1981), that with the snowmelt, an intensive root growth, thus more fungi, were recognized because of the water excess. The samples in this study were probed in summer, which would speak in favor of this thesis. Furthermore, Allen et al. (1987) found that there are seasonal fluctuations in infection rates. The recorded relation of the AMF colonization rates with the elevation shift is consistent with the findings of Kotilínek et al. (2017). The infection rates by AMF of the plant roots increased from 3880 to 4520 m a.s.l. and beyond that altitude up to 5620 m a.s.l. they decrease.

Inconsistent with the literature (Väre, et al., 1997) are the findings of this study, that although the plants originated from extremely high altitudes, AMF were still predominant, even at elevations of 3880 m a.s.l. and above. Read and Haselwandter (1981) postulated that in the cold and harsh conditions in the mountainous regions (stressed environments), AMF might not be well adapted therefore would not occur frequently and DSE could be more abundant. The opposite can be seen in Figure 22, the mean colonization rate of AMF was 50% and of DSE 30%. Figure 21 shows that with a higher AMF infection rate the lower the infection rate of the DSE, meaning that AMF are more dominant and displace DSE. Conversely, a higher DSE colonization rate resulted in lower levels of AMF, which may occur in plants inhabiting harsh environments. This finding supports the statement made by Read and Haselwandter (1981). However, no sample in this study had exclusively DSE, it was either only AMF or both fungi, which is not consistent with the literature of Schmidt et al. (2008), who says that in higher altitudes AMF are absent.

4.3.1 Colonization rate increase (AMF), decrease (DSE)

The most important finding in this project is shown in Figure 23. The mean colonization rate difference of AMF was 3.72% and of DSE -2.89%. Meaning, that in 2022 the plants were on average higher infected by AMF than by DSE fungi, which would agree with the literature. In Read and Haselwandter (1981) and Duarte and Maherali (2022), it is stated that AMF prefer warmer environments. Since climate change also involves an increase in temperature, especially in higher latitudes like Ladakh it rises in a greater amount than in tropical zones (HilleRisLambers, et al., 2012), thus longer vegetation season, this could be beneficial for AMF (more time to establish) and therefore an increase in the AMF colonization should be observed. The DSE infection rate decreased between 2015 and 2022. This could be described by the fact that AMF are more dominant because of the less extreme conditions, and therefore suppress the DSE. This is shown in Figure 21, which depicts with a higher AMF colonization rate, DSE infestation drops.

4.4 Increase in the total number of fungi

Furthermore, with the data calculated, we see a slight rise in the total percentage of fungi colonizing a root recorded. This would agree with the literature (Cripps & Eddington, 2005), which states when the surroundings get harsher, maybe due to climate change (e.g. different land covers), plants in higher altitude habitats form more symbiosis with fungi. The statement of Schmidt et al. (2008) is supported by this study: due to climate change and the glacial retreat, plant growth is shifted upwards, and fungi are moving upward as well. This trend is reflected in the results, showing an increase in fungal infection rates from 2015 to 2022. According to Ruotsalainen et al. (2004), the average colonization rates by fungi differed between species. This was confirmed in this study, and as Tables 2 and 3 show, the values fluctuated intensely between the plants. This could be because each plant requires different amounts of nutrients or there is a genetic propensity to infection by fungi, thus variations in the fungal infection rates.

4.5 Prospects

For more precise results, the plant roots should also be probed in different seasons like spring or winter to reduce the effects of seasonal fluctuations. Furthermore, the same species could be examined at lower altitudes (below 3500 m a.s.l.) for better comparison of the elevation factor. However, further research is necessary to better understand the roles of AMF and DSE in the roots of different plant species.

5 Conclusion

In conclusion, this study investigated the prevalence of root colonization by AMF and DSE in diverse plant species across the Ladakh range in the Himalayas, with samples collected in 2015 and 2022. The research aimed to explore potential differences in colonization rates between these years, hypothesizing that climate change might influence fungal colonization patterns.

Notably, AMF colonization rates were significantly higher compared to previous studies and showed an elevation-dependent trend. Contrary to some literature suggesting DSE dominance in harsh habitats, AMF prevailed across various altitudes. The most significant finding was the increase in AMF colonization and decrease in DSE infection rates between 2015 and 2022, likely attributed to climate change-induced temperature rise, prolonging vegetation seasons in favor of the AMF establishment. Moreover, in this study was a slight overall rise in fungal colonization observed, supporting theories of shifting plant-fungal interactions due to climate-induced habitat changes.

Looking ahead, future research should explore seasonal variations and the same plants should be probed in lower altitudes for comprehensive elevation comparisons. Additionally, deeper investigations into the roles of AMF and DSE in different plant species are needed for a better understanding of their ecological significance.

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