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Research Article

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Fungal diversity in forest's soil: Moderate-term post-thinning effect of a Mediterranean mature *Pinus halepensis* forest.

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Abstract:

The effect of mature pine forest thinning on soil characteristics and fungal diversity was studied at a long-term ecological research (LTER) station in the Jerusalem Mountains, Israel, in the Mediterranean zone. Tree thinning in 2009 created plots with varied tree densities: dense, medium, low, fully cleared, and an unplanted control plot. In Spring 2020, the soil was characterized by moisture, pH, organic matter, and fungal biodiversity (ITS sequencing). Moisture and OM increased with increasing forest density, while pH decreased with increasing forest density. Fungal diversity was highest in the clear-cut plots, while the low-tree density plots lost 12% of genera present at <1% abundance compared to other forested plots. Higher fungi (Ascomycota and Basidiomycota) dominated in pine-forested plots (60-70%), while lower fungi (Chytridiomycota, Mucoromycota) were more abundant in non-forested plots (55-65%). The variance in symbiotic fungi abundance was significantly higher in forested plots, while saprotrophic fungi were more common in plots without mature pine trees. Ectomycorrhizal fungi were dominant in all forested plots, mainly *Sebacina epigaea* or *Inocybe sp.*, while endophyte-plant saprotrophs and pathotrophs, e.g., *Mortierella alpina,* were more dominant in plots without mature trees. These results suggest that intense thinning has slightly negative effects on total diversity, while complete tree removal has transitions of fungal communities to never-planted areas. Further studies on the high internal diversity of forested plots are needed to understand the partitioning of symbiotic fungi in wooded and trimmed regions, and to follow up on the clear-cut fungal diversity resulting from the emergence of new pine and other seedlings.

Keywords:

Fungal diversity, Mycobiome, Forest health, Nutritional strategies, Tree felling, Mycorrhiza, Long-term ecological research station (LTER), Pine Forest, Mediterranean Forest, fungal guilds and trophic modes.

Introduction

Climate change, increased wildfires, and frequent tree cutting lead to changes in biodiversity, but forest management practices can also shift natural biodiversity. Fungi are a very important group of organisms both in the general ecosystem [1] and in forests, in particular [2]. Israel, being in a climatic transition zone, is a unique spot in the Mediterranean basin, and has a unique diversity of climate zones ranging from the Mediterranean in the north to the arid desert in the south, with a semi-arid transition zone in between that might result in high biodiversity, including forest fungal biodiversity. The presence of fungi in forests is important for several reasons: they serve as food, break down lignocellulose, contribute to carbon cycling, support plant health, and enhance general soil fertility. However, they can also cause disease in plants and other forest organisms [3], owing to their functional and nutritional diversity. Different fungal species may act as saprotrophs (decomposers), mycorrhizal symbionts, or pathogens causing disease and mortality [1]. Although each fungus generally fulfills one function, many intra-species might show different trophic modes due to environmental changes [4–6]. For example, the ectomycorrhizal fungus *Tricholoma matsutake*, commonly found in Coniferous forests, can be a facultative saprotroph depending on environmental conditions [7].

In Israel, most of the planted forests are dense monocultures predominantly consisting of Aleppo (Jerusalem) pine (*Pinus halepensis)*, which were planted mainly in the last century [8]. Over time, pine forests throughout the country have exhibited signs of decline, particularly those older than 50 years, which were planted at high densities [9]. Many trees fell due to snow loads, wind, drought, and overcrowding. These factors prompted a reevaluation of forest management and led to exploring ways to improve and sustain forests, including forest thinning [8,10]. Another aspect to consider in forest thinning was the increasing wildfire events in Israel [11] since thinning was suggested to reduce forest fire severity by controlling forest density [12,13]. Current recommendations suggest thinning trees in the existing forests as it can alter local environmental conditions—temperature, wind, radiation—and reduce canopy cover, influencing tree growth and both above- and below-ground biodiversity [14]. Thinning regimes for pine trees are now being evaluated and implemented in various regions in Israel. In 2009, a long-term ecological research (LTER) plot was established by the KKL (Israeli National Fund) in the Forest of HaKedoshim in the Jerusalem Mountains, to evaluate the effects of different thinning regimes on the forest ecosystem [14,15].

The interest in fungal ecology in Israel, especially the role of soil fungi in forest health, has long been neglected compared to global research. While many international studies have explored forest fungi and their relation to forest health—including the impact of different forest management practices on fungal diversity [2,16–18] research in Israel has focused mainly on macrofungi (mushroom-forming fungi) [19–21], as well as wood-degrading macrofungi comparing young and mature pine forests [22]. Changes of the mycobiota (fungal diversity) after the 2014 fire in the Carmel Mountain were studied, reporting rapid growth of mycoparasites and melanin-containing species in Oak more than in Pine forests [23]. More recently, studies on the role of ectomycorrhizal fungi in the Israeli forests, including on the after-fire changes of ectomycorrhizal fungi community composition associated with the roots of young and mature trees of two Pinaceae, *Pinus halepensis* and *Cedrus deodara* growing together, were also conducted, showing only little species turnover between the two sampling [24].

The present study assessed, for the first time in Israel, the taxonomic and functional changes in soil fungal diversity following thinning treatments at this LTER site. The study was conducted in 2020, about 12 years post-thinning, to study the effect of the thinning level on soil physio-chemical characteristics and the changed soil fungal communities presented in the fungal taxonomical level, as well as functional classification.

Methods and Materials

Sampling Site: The study was conducted in the HaKedoshim Forest, located on the western slopes of the Judean Hills, at elevations between 390 and 510 meters above sea level. The area experiences a typical Mediterranean climate with wet, cold winters and hot, dry summers. The soil is shallow, primarily brown terra rossa over dolomitic bedrock [25]. The forest consists of a monoculture of Jerusalem pine trees (*P. halepensis)* planted in 1969 that has been gradually changing to include other species such as thorny burnet (*Sarcopoterium spinosum*), mastic tree (*Pistacia lentiscus*), buckthorn (*Rhamnus lycioides*), and common oak (*Quercus calliprinos*) [14,26]. In 2009, mature pine trees were cut at the LTER station to thin the forest to different tree density levels [15]. The area was divided into plots approximately 70x70 meters in size, with different thinning treatments. Each plot has an internal boundary on all four sides (east, west, south, north) that encloses a 40x40 meter core area. Five thinning treatments were carried out via targeted tree removal: 1) Un-thinned plots with ~50 trees per dunam (1000 m^2), serving as a positive control; 2) Medium thinning to ~30 trees per dunam; 3) Heavy thinning to ~10 trees per dunam; 4) Clear-cut plots (0 trees per dunam), serving as a negative control; E) Unplanted plots, also serving as a negative control. Each treatment consisted of three replicated plots. For an aerial photograph and layout of the thinning treatments and plots, see [15].

Soil Sampling: Soil was sampled in March 2020, at springtime. Soil samples were collected along the perimeter markers of each plot's core monitoring area (40x40 m). Two equidistant samples were taken from each side (north, south, east, west), totaling eight samples per plot. The above ground of each sampling point was brushed aside to remove the fallen leaves and stones, and soil was sampled from a depth of 5–10 cm. Soil samples were taken to the lab in coolers, where they were analyzed for moisture, organic matter, and pH, as well as molecular identification of fungal diversity.

Determination of soil's physico-chemical parameters: Twigs and stones were removed, and the soil mixed manually to obtain a homogeneous sample. Soil moisture was determined by drying the sample at 105°C overnight and weighing to calculate the percentage of moisture lost. For the determination of organic matter, dried samples were placed in a high-temperature oven heated from 100°C to 600°C using a stepwise heating process. The percentage of organic matter in the soil sample was calculated after the combustion. For pH determination, the soil was mixed in a ratio of 1:5 with DDW, and let stand for 30 min before pH was measured by a pH-meter.

Fungal molecular identification: Soil DNA was extracted with the ZymoBIOMICS™ DNA Miniprep Kit according to the manufacturer's protocol (Zymo Research, Irvine, CA). Samples were sent to the University of Illinois in Chicago for the determination of genomic fungal diversity. The fungal ITS region was sequenced using ITS1–ITS2 primers.

The sequencing and bioinformatic processing were conducted at the genomic centre at the University of Illinois at Chicago in the DNA Services Facility laboratory (UIC).

Data Analysis: Eight samples were taken from each of three replicated plots per thinning treatment, with three plots per treatment. For the physico-chemical characterization, mean data of the three plots were calculated for each treatment, with standard deviation. Plot means of OM, moisture and pH were compared with ANOVA and Fisher's LSD post-hoc test, and a connecting letter report at $p<0.05$ was generated in JMP 18 [27]. Fungal community composition was determined based on the relative abundance of each identified taxon within a soil sample. In all mycobiome analyses, samples that failed NGS (<1000 reads) were excluded, resulting in 19-23 subsamples per treatment. The medians of the fungal communities were compared at various taxonomic levels by Kruskal-Wallis test followed by Wilcoxon pair-wise non-parametric comparison to generate a connecting letter report at $p<0.05$ with JMP 18. Alpha diversity indices (Observed richness, Shannon, Simpson) were calculated in R [28] using the *phyloseq* package [29], which implements functions from the *vegan* package [30], and the results were compared with ANOVA and Fisher's LSD in JMP. Unique and shared taxa among treatments were summarized using a Venn diagram. OTU tables were converted to presence/absence using *phyloseq* followed by computing the proportion of positive subsamples per treatment; taxa detected in ≥5% of subsamples were coded as present. The Venn diagram was generated with the Venn Diagram add-in for JMP [31]. Beta-diversity was assessed using Bray–Curtis dissimilarities [32] calculated from relative abundance data in R with the *vegdist* function in package *vegan*, and visualized via principal coordinates analysis (PCoA) using *cmdscale* function in base R with 50% confidence ellipses plotted around group centroids. Treatment effects of trimming on community composition were evaluated via PERMANOVA with adonis2 (999 permutations) in *vegan*, reporting pseudo-F, R^2 and p-value. Homogeneity of multivariate dispersion was checked with *betadisper* and *permutest* functions in phyloseq, followed by Tukey's HSD to determine sources of dispersion. This was further visualized with unscaled principal component analysis (PCA) using the covariance matrix. Fungal functions were characterized according to their nutritional modes, using the FUNGuild database [33]. Specifically, the funguild_assign function in R package FUNGuildR [34] was used on a one-column data frame of identified Genus-level taxa. The diversity in the trophic modes present in each treatment was evaluated with Levene's test for homogeneity of variance in JMP and visualized by a horizontal bar plot.

Results:

Characterization of the Sampling Areas:

Sampling was conducted in March 2020, during the spring season, when the forest was characterized by blooming vegetation, shrubs, and herbaceous plants. The treatment plots displayed different vegetation appearances (Figure 1). In the plot where all mature trees were cut, dense herbs and shrubs were present, with no mature pine trees. However, there was natural regeneration of young pines and other trees at various densities and ages. In contrast, the plot that underwent partial thinning showed a mixture of herbaceous plants in certain areas and a ground surface covered with pine needles, along with diverse local niches. In the dense pine plot, which was not thinned at all, the trees were closely spaced, typically with thinner trunks than those in the partially thinned plot, and the ground was covered with a layer of pine needles and sparse herbaceous vegetation.

Figure 1. Appearance of vegetation from plots with varying levels of forest thinning. A. Unthinned forest ca. 50 trees per dunam (Left). B. Thinned plot to 10 trees per dunam (Middle). C. Clear-cut plot with all mature pine trees removed (Right). Photos Segula Masaphy.

Soil physico-chemical characteristics

The results of the physico-chemical tests of the different plots are presented below (Figure 2). Soil organic matter and soil moisture increased with an increase in pine trees' density, from OM 12.4% ± 0.54 (SE) in Unplanted plots to 22.5% ± 2.25 in 50 trees per dunam plots ($p<0.05$, Figure 2A). Both unplanted and clear-cut plots were significantly lower in % OM than all plots with trees. This might be related to more fallen leaves in the high-density plots that turn to old, long-term plant residue decomposed in the soil. Also, the moisture level of the soil was in accordance with the forest density. Overall, a significant positive correlation (R^2 0.75, $p<0.0001$) was found between the level of organic matter and the percentage of moisture, except for the unplanted plot, where %OM did not correlate significantly to %moisture (R^2 0.02). This may be due to overall plant cover, since the unplanted plots had the least shade, while all other plots had mature trees with shading or some regeneration.

Soil pH value reacts in two distinct trends. In plots with trees, the level of acidity in the soil significantly increased ($p<0.05$) with mature tree density, such that the highest pH was in plots thinned to 10 trees per dunam (7.78±0.1 SD), while it was 7.62± 0.05 in 30 trees and 7.50± 0.08 in 50 trees per dunam ($p<0.05$, Figure 2B). Non-planted plots had a lower pH level than all plots planted or thinned (7.37±0.1). In the clear-cut plots, pH was 7.56±0.16, which is statistically the same as the 30 trees per dunam plots. In plots with trees, this trend can be understood to be a function of both acidic pine needles density and the fermentation of organic matter. In clear-cut plots, although there were no mature pine trees, there were a few regenerations of pines and other trees of different ages and densities, which could result in the higher variation and intermediate pH level.

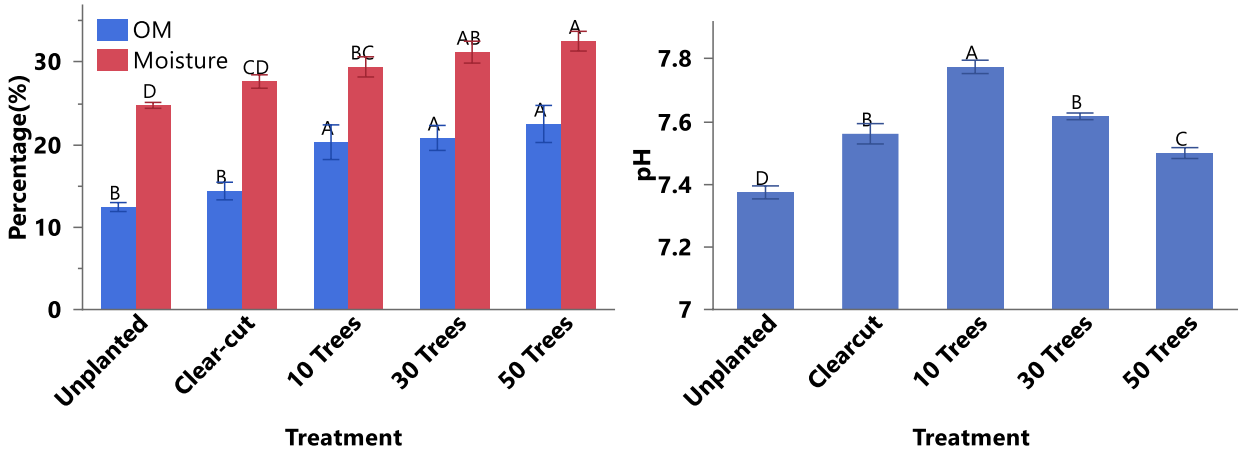

Figure 2. (a) Values of soil moisture (%) and organic matter (%) in the various thinning treatments, as a mean for the treatment and standard error of the mean. (b) Soil acidity level (pH), as a treatment average and standard error. Letters indicate a significant difference by Fisher's LSD within the given parameter.

Soil Fungal Diversity

Alpha Diversity

We detected significant treatment effects on fungal alpha diversity at the genus level (Figure 3). Observed richness differed among treatments ($p = 0.018$). Clear-cut plots had the highest richness (mean ± SE = 128 ± 5 OTU), which was significantly greater than in the 10-tree plots (102 ± 5) and the 30-tree plots (108 ± 6). The 50-tree (116 ± 5), and unplanted (115 ± 5) plots were intermediate and did not differ significantly from either extreme.

Shannon diversity also varied among treatments ($p < 0.0001$). Clear-cut plots had the highest diversity (3.27 ± 0.13 SE), significantly greater than both the 10-tree (2.43 ± 0.13) and 50-tree plots (2.67 ± 0.13). The 30-tree (2.78 ± 0.14) and unplanted plots (2.83 ± 0.0.13) were intermediate. Simpson diversity was not significantly different until the plots were compared pair-wise ($p = 0.08$). Only the clear-cut plots (0.87 ± 0.03) differed significantly, with higher evenness compared to the 10-tree (0.77 ± 0.03) and unplanted plots (0.80 ± 0.03). The 30-tree (0.84 ± 0.03) and 50-tree (0.82 ± 0.03) treatments were not significantly different from other treatments.

To summarize, fungal richness and diversity were lowest for every alpha-diversity measure in the 10 trees per dunam plot, while clear-cut plots supported the highest diversity. Moderate thinning (30–50 trees per dunam) and unplanted plots maintained intermediate richness and diversity. These findings suggest that complete canopy removal had the most diverse fungal community due to the historic presence of trees and the new habitats created by removing old growth, while intensive thinning to 10 trees per dunam reduces fungal alpha diversity relative to both higher tree densities and complete canopy removal.

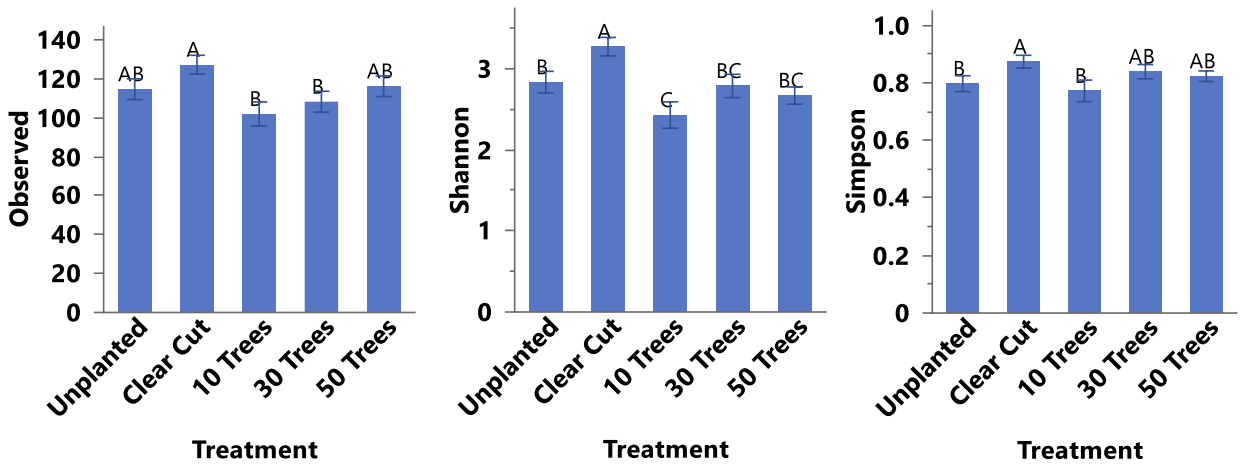

Figure 3. Alpha diversity measures (mean ± SE). The connecting letters indicate a significant difference at $p<0.05$ with Fisher's LSD.

Taxonomic level: phylum

The average relative abundance of the soil fungal Phyla in all treatments is summarized in Figure 4. Ascomycota had the largest overall representation in the data set, with about 30% of total abundance. Plots with 30 and 50 pine trees per dunam had significantly higher Ascomycota abundance than the unplanted control (20.5%±12 SD in unplanted plots and 36.1%-38.1% in pine plots, $p<0.05$). Also, the clear-cut plots had higher Ascomycota abundance (32%±11) compared to non-planted ($p<0.05$). Interestingly, the 10 trees per dunam plot was intermediate between the unplanted and other groups (27.8%±17).

Phylum Basidiomycota was found at the second total overall abundance (26.6%) but was distributed unevenly over the various treatments. The Basidiomycota relative abundance in plots without trees was only 8% - 11%, while in all the plots with trees, the abundance of Basidiomycota was higher and ranged between 25.5- 41.4%. The difference in Basidiomycota abundance was significant for all plots with trees compared to the plots without trees ($p<0.05$), with the largest abundance being in the 10 trees per plot.

Phylum Mucuromycota was around 21.2% of the total abundance of all soil Fungi, but again, this was not evenly distributed among the treatments. The non-planted plots had by far the most Mucuromycota overall (36.3%±11), and this was a significantly higher amount ($p<0.05$) than in the 10 and 50 trees per dunam plots (12.5%±9 and 11.1%±10, respectively). The clear-cut and 30 trees per dunam were intermediate in Mucuromycota abundance (25.8%±15 and 21.9%±15, respectively) and were not significantly different than either the unplanted or the other pine plots.

Non-classified Fungi (Fungi Incertae Sedis) represented 16% of the data set. About twice as many soil fungi in the plots without trees were unidentified compared to plots with trees. Accordingly, 23% of soil Fungi were unidentified in treeless plots compared to 7.4% - 13% unidentified fungi in plots with trees. Similarly, unidentified eukaryotes (phylum="Other") were also higher in unplanted and clear-cut plots (3-4% relative abundance) compared to plots with

trees (1-2% abundance) (data not shown). This indicates that the fungal populations in unplanted and clear-cut plots are comprised more of unknown and uncultured Fungi compared to the plots with trees.

Chytridiomycota phylum was about 1.5 % overall abundance with about 2% in plots without trees, compared to ~1% in the pine plots. However, the difference was not significant.

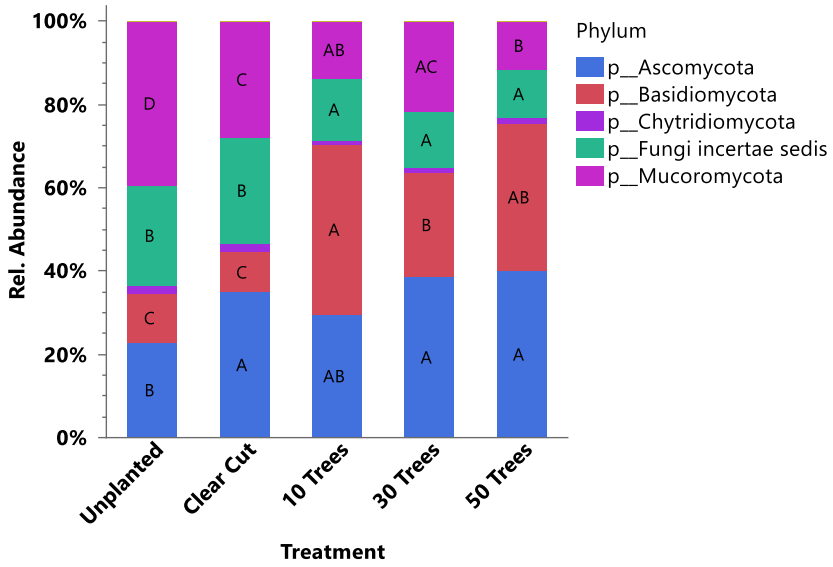

Figure 4. Relative abundance of the Fungal phyla in the forest soil. The different letters represent dissimilarity at $p<0.05$ for the respective phyla by Wilcoxon non-parametric pairwise comparison.

Order

The relative abundance of Fungal orders is displayed in Figure 5. There were 52 fungal orders detected, but most of them were present below 2% relative abundance, leaving only 18 orders present at this level of abundance (including incertae sedis). As with the phylum level, many fungi at the order level could not be confidently classified, especially in plots without mature pine trees: 20–24% in these plots compared to 10–15% in plots with mature pines. In plots with mature pine trees, a higher abundance of fungi from the order Sebacinales was found, reaching 11-22% in these plots, compared to 0.2-2% in plots without mature pine trees ($p<0.05$). Similarly, the order Agaricales was more abundant in forested plots (13%-17%) compared to plots free of trees (5%-7%). The order Pezizales also showed a higher relative abundance in all plots with mature pine trees, with a significant difference between the two densest plots (8%-13%) compared to non-planted and cut (1.6%-1.8%). Hypocreales was highest in the clear-cut plot (9.8%) and lowest in the unplanted plot (3.8%), with forested plots intermediate between these two extents. On the other hand, in plots without mature pine trees—whether entirely clear-cut or never planted—there was a higher abundance of fungi from the orders Mortierellales (27% in the unplanted plot, 16% in the fully clear-cut plot, and 10–11% in the plots with pines) and Glomerales (4.4–5.5% in plots without mature pine trees compared to 0.7–2.7% in plots with

mature pines). At the lower end (<5% abundance), the orders Sordariales, Pleosporales, and Orbiliales were more abundant in plots without trees (1.5-2.5%), while in plots with trees, the abundance of these orders was mostly <1%.

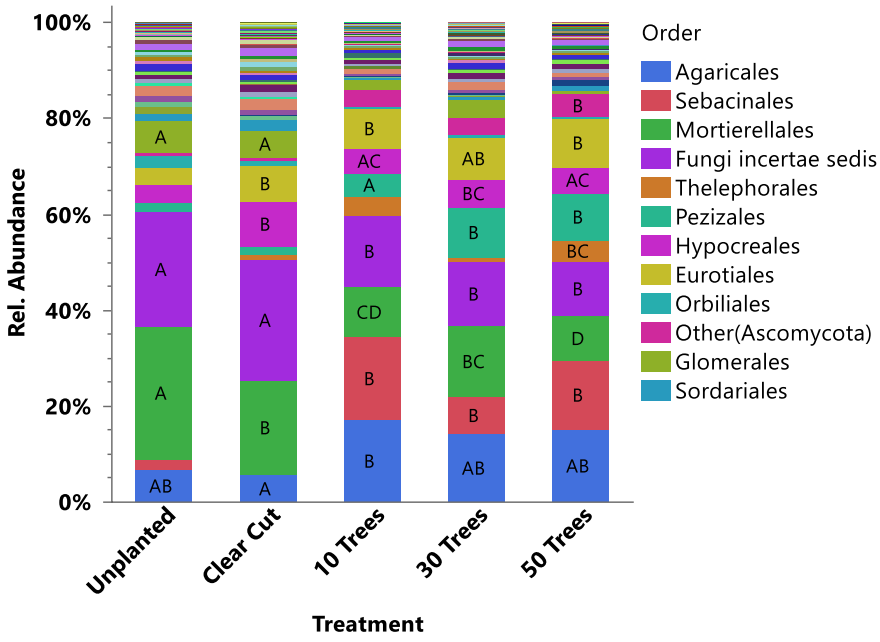

Figure 5. Relative abundance of Fungal orders in HaKedoshim Forest LTER treated with various degrees of tree thinning. The legend shows 13 orders > 2% abundance in all treatments. The different letters represent dissimilarity at $p<0.05$ by Wilcoxon non-parametric pairwise comparison.

Genera and species

A total of 725 unique genera were detected, of which 619 were identified at this taxonomic level and 106 were identified to a higher taxonomic level or not at all. Most of the genus-level OTU (99.5%) each represented <5% relative abundance (Figure 6). Among the top twenty abundant taxa (3% of the total OTU, and 60-80% by relative abundance), significant differences of medians ($p<0.05$) are observed for almost all of them, by treatment. Notably, *Sebacina* and *Inocybe* show higher prevalence in forested plots (10-17% in forested compared to 1-2% in unforested plots, $p<0.05$), while *Mortierella* and *Terfezia* are more prevalent in clear-cut and unplanted plots (18-22% in unforested plots compared to 9-14% in forested plots $p<0.05$). *Peziza* was also higher in forested plots (2-5%) compared to clear-cut and unplanted (1-2%, $p<0.05$). The Mortierella genus was almost solely identified as *Mortierella alpina*. The *Sebacina* was almost entirely *S. epigaea*. Interestingly, at least 14 *Inocybe* species were found, among which *I. fuscidula* was the most abundant (47%), followed by *I. reoseipes* (18%), *I. cervicolor* (13%), *I. splendens* (5%) and *I. isabellina* (4%) and several more species <1%. The *Terfezia* species were not identified. The Peziza species were 88% unidentified, around 9% was *P. [Elaiopezia] polaripulata*.

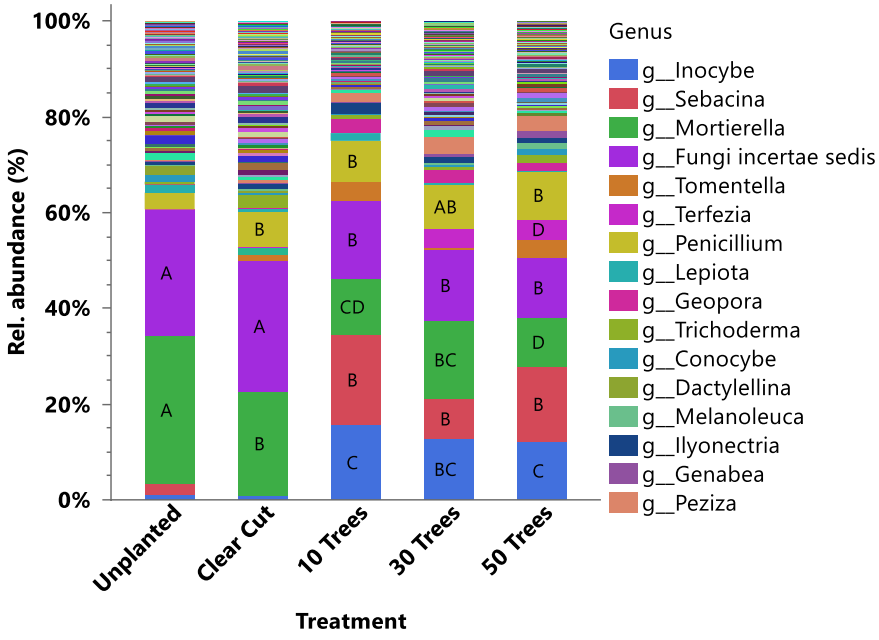

Figure 6. Barplot of Fungal genera relative abundance in HaKedoshim forest soil. Different letters are significantly different at $p<0.05$ by Wilcoxon non-parametric pair-wise comparison.

A present/absent Venn diagram of genera that were detected in at least 5% of each treatment (~1/20 subsamples) showed that 151 genera were in all treatments, while 271 genera were rare with <5% detection events in all treatments (Figure 7). The clear-cut and 50 tree/dunam treatments had the most genera only present in these treatments (38 and 34 genera, respectively), followed by unplanted plots (28 genera). The 10 and 30 trees/dunam had 9 and 8 genera, respectively, only present in these two treatments. In terms of shared genera, unplanted and clear-cut shared the most genera with only each other (20 genera), while 30 trees/dunam also shared the highest amount of genera with these two treatments (14 genera).

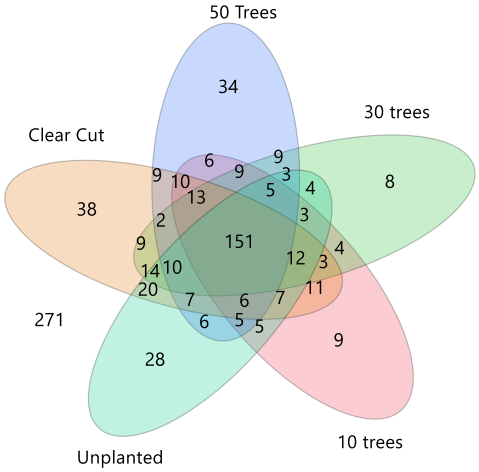

Figure 7. Unique and shared genera by Present/Absent Venn Diagram based on detection in >5% of subsamples in a treatment.

The barplot obscures differences within a treatment since only the mean value of each genera is displayed. Hence, a community-level approach was applied using the Bray–Curtis dissimilarity index. The dissimilarity matrix was plotted as a principal coordinate analysis (PCoA) plot and marked by treatment (Fig. 8). The first two axes of the PCoA plot explained ~33% of the variance in community composition overall. Within this global variance, PERMANOVA confirmed that fungal community composition differed significantly among forestry treatments (adonis2, pseudo-F = 5.24, $R^2 = 0.169$, p = 0.001), indicating that treatment explained 16.9% of the variation in the data, while the rest of the variance is due to variation within the treatments. Hence, the PCoA helped to uncover that the Fungal community was different in two ways: 1) the treatments affected the overall community, and 2) the samples within individual treatments were quite diverse and might contain distinct subcommunities.

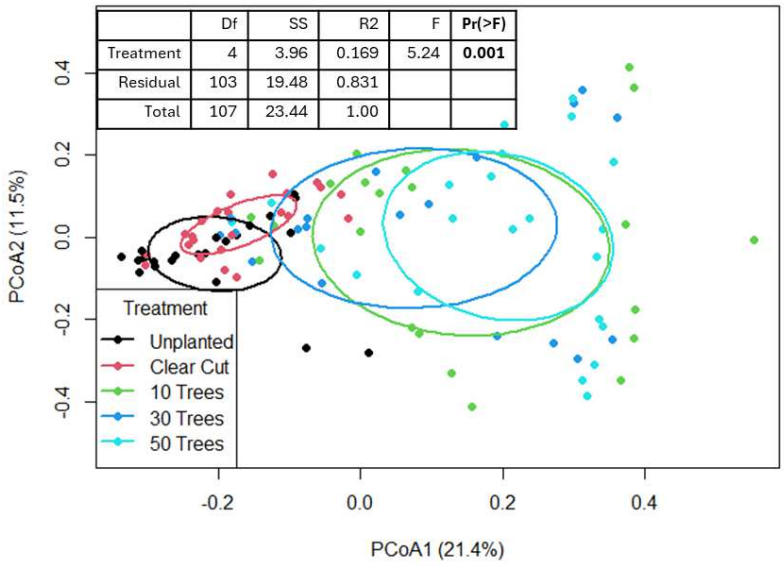

Figure 8. Principal Coordinates Analysis (PCoA) of Bray–Curtis dissimilarities among soil fungal communities (genera level) under five treatments. Points represent individual samples colored by treatment; ellipses show 50% confidence interval around treatment centroids. The first two axes explain 21.4% and 11.5% of the variance, respectively. The embedded table is the associated PERMANOVA result as a function of treatment.

Based on the large dispersion of the forested groups seen in the PCoA (Figure 8), the betadispersion was also tested and was found to be significant (F=9.08, p=0.001). Pairwise Tukey's HSD confirmed the source of dispersion to be the forested groups relative to Unplanted ($p \leq 0.0015$) and Clear cut ($p \leq 0.0075$), whereas the unforested treatments did not have significant within-group dispersion. This indicates that there are distinct communities within the forested treatment. Closer inspection of the community data with unscaled PCA revealed that the forested plots had either one or the other of *Inocybe* or *Sebacina*, but almost no individual sample had high levels of both (Figure 9). This interesting observation was masked by the averages displayed in the bar plot (Figure 6), since on average, all trimmed

treatments appeared to have similar levels of these two genera, while in fact they have either one or the other but not both at the same time.

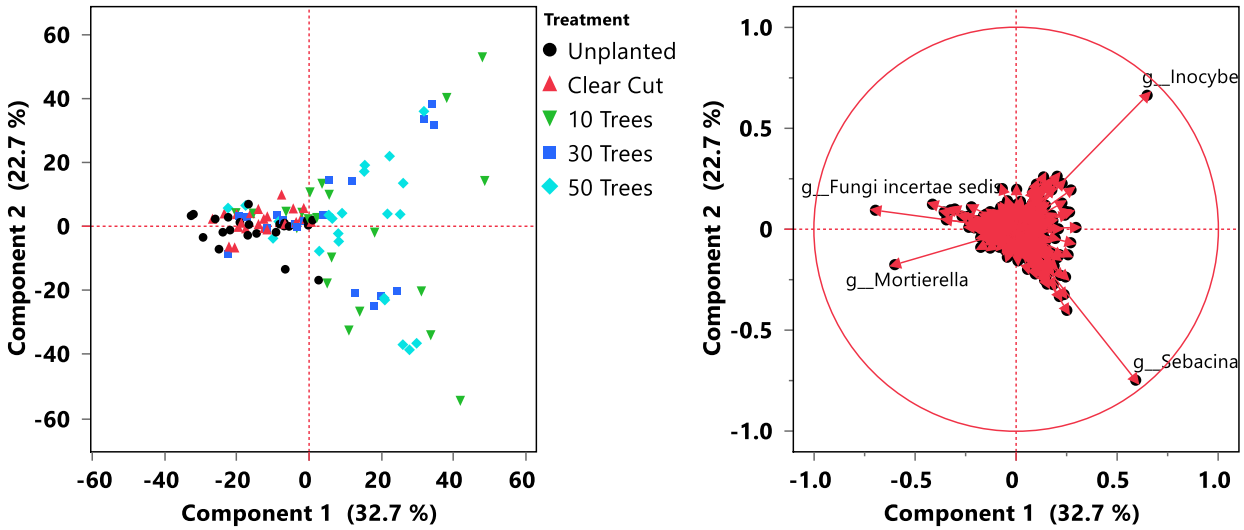

Figure 9. Unscaled PCA (covariance matrix) of genera relative abundance. PCA score plot is on the left while the loading plot is on the right. Each treatment is comprised of 19-23 subsamples.

Functional Groups of Soil Fungi in the LTER Plots

Another aspect examined in this study was the classification of fungi into functional groups based on their nutritional strategy. Figure 10A presents the results for all functional groups: saprotrophic (sap), symbiotrophic (sym), and pathogenic (path) fungi (and intermediates), showing their relative abundance across the different thinning treatments.

The results show that the proportion of symbiotic fungi was higher in the plots with mature pine trees compared to plots without mature pine trees. The high variance resulted in this difference being significant only for 10 trees per dunam. In fact the variance for this trophic mode was very significantly different between treatments ($p<0.0001$). The wooded plots had a very diverse range of symbiotroph abundance (SD 20-27%) while non-wooded plots had a smaller variance (SD 10-11%). The saprotroph and sap-sym modes were more abundant on average in plots without trees, and the difference was significant for 30 trees and 50 trees for the sap-sym mode. Again, high variance made the statistical test less confident, however the variances were similar in all treatments. Pathotrophs were also more abundant in plots without trees, although the 50 trees/dunam treatment had an intermediate abundance and was not significantly different than the plots without trees.

In terms of guilds, the ectomycorrhizal (ECM) guild was significantly more abundant in plots with trees, while the endophyte-plant saprotroph guild was more abundant in plots without trees (Fig. 10 B). Arbuscular mycorrhizal (AM)

Fungi were more abundant in the plots without trees, perhaps indicating their greater association with shrubs. There seemed to be a complementary relationship between ECM Fungi and endophytes, mirroring to some extent the relationship between symbiotic and saprotrophic fungi in the trophic modes graph.

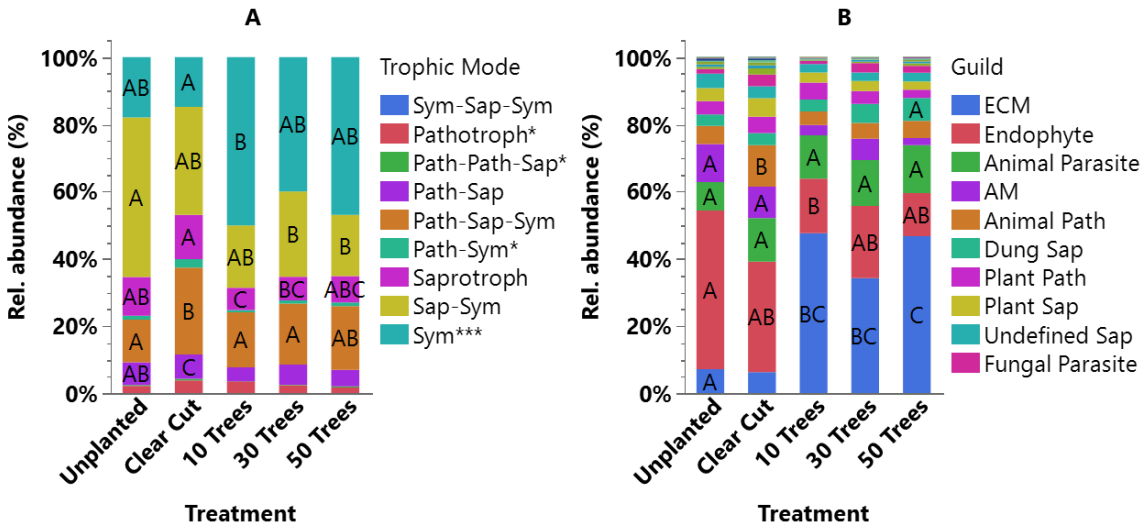

Figure 10. Distribution of Fungal trophic modes (A) and guilds (B) in HaKedoshim Forest LTER soil samples. Note that guilds <5% are not displayed in the legend in order to enhance readability. The connecting letter indicates dissimilarity at $p<0.05$ by Wilcoxon pair-wise comparison. An asterisk next to the Trophic mode indicates significant variance between treatments by Levene's test for homogeneity of variance (*$p<0.05$, ***$p<0.0001$).

Discussion

Forest thinning is an essential part of forestry activity to clear dead plants, reduce fire hazard, and encourage regeneration. However, it is a complex, evolving process due to theoretical disagreements, contradictory recommendations, and errors of modern practice, which require confirmation through long-term experiments [35]. Forest thinning and changes overstory may affect the environmental conditions in the understory [14,36]. This includes changes in the vegetation and soil characteristics due to a decrease in both canopy area and the activity of live roots. In general, reducing the canopy after thinning can influence the microclimate, and chemical characteristics (water availability, available carbon source, pH, ect.) in the soil, which affects soil microbial processes [16,37,38]. Also, the time passing from the thinning event to the study of the soil characteristics is important, as long-term changes may occur [39,40]. Due to the high complexity of predicting the effect of forest thinning in a certain given location, climate and type of forest composition on soil characteristics, the current study focused on the effects of forest thinning on soil characteristics and fungal diversity in a mature *P. halepensis* forest in Israel, after a moderate term of 12 years post-thinning. As expected, the physico-chemical characteristics such as soil moisture, soil organic matter, and pH values were affected by forest density. Both moisture and organic matter increased with an increase in the trees'

density. The increase in soil organic matter is probably due to the accumulation of old organic matter over the years and a higher rate of fallen pine leaves, incorporated in the upper layer of the soil. The increase in organic matter also resulted in higher water content in the soil. Moreover, the higher trees' density also reduced the drying of the soil due to their coverage, reducing sunlight, radiation, and winds. Thinning and reduced canopy coverage were already reported to be accompanied by enhanced litter decomposition in the Israel pine forest [41]. Unlike the increase in moisture and organic matter with increasing forest density, the pH was affected differently, resulting in more alkaline soil in the 10-trees treatment, whereas both plots without mature pine trees and the densest plot showed a higher level of acidity (lower pH) than the partially thinned plots. This could be due to richer herbaceous vegetation that was in the unplanted and clear-cut soils and due to the higher load of pine leaves falling to the ground in the denser forest plot, resulting in higher acidity [42]. The results of reducing soil acidity in thinned coniferous forests 12 years after forest thinning are different from the results reported by Settineri et al., (2018), where no significant changes of the pH in the thinned soil was measured short period of 4 years after thinning of *Pinus laricio* forest in Italy. The pH behaviour was also related to the type of forest trees' composition, as in Oak–Hickory forest in USA, thinning resulted in an increase in the soil acidity [44], and in a study on eucalypt forests showed increasing soil acidification in the conversion of grasslands to forests [45].

Along with altering the microclimate, vegetation composition, soil properties, and litter inputs, forest thinning was reported to influence the diversity, richness, and community structure of soil fungal communities [18,37,46,47] Surprisingly, in the present study, 12 years post-thinning, fungal biodiversity was slightly affected by thinning from 50 to 10 trees per dunam, but it was clearly different from the clear-cut treatment, resulting in a shift of the fungal community from the community that characterized the Pine trees' habitat to the fungal community that characterized the soil in unplanted plots. The exception to this is overall diversity, such that the thinning to 10 trees resulted in a net loss of around 10-15% of fungal OTU relative to the more densely forested plots and a similar loss in community evenness and unique Genera. This suggests that low-abundance species were diminished due to the intensive thinning. By contrast, the alpha diversity of the clear-cut plots was highest, which could be due to partial replacement of forest-associated fungal species with herbaceous growth-associated ones. Soil condition may alter fungal diversity. For example, soil pH was reported to affect fungal diversity [48,49]. In the present work, about 50 OTU had a significant correlation to pH (data not shown), and this will be examined in more detail elsewhere.

The effect of the thinning treatment was obvious even at the phylum taxonomic level. Among the 6 dominant phyla, Basidiomycota and Ascomycota were more abundant in Pine tree-bearing plots than in plots without mature trees. Species of both phyla are considered higher fungi, known for ligninolytic and cellulolytic activity, involved in the degradation of forest wood. Moreover, many are mushroom-producing fungi, either above or underground, suggesting more mushrooms as a forest byproduct for mushroom pickers or animal food. Whereas plots without mature pine trees showed a higher abundance of Mucoromycota and Chytridiomycota phylae, which are fast-growing saprobic and pathotrophic filamentous molds that might result in rapid organic turnover, which could contribute to higher moisture loss [50]. The unforested plots showed a higher unidentified group abundance of an uncertain type of fungi, the Fungi incertae sedis group- that are not completely defined, and their broader relationships are unknown [51]. This could be related to higher habitat heterogeneity in herbaceous plots [52,53] and due to the fact that the forested area had more

mycorrhizal fungi, which were studied more than the saprotrophs and endophytes that are related to the unforested soil [54,55]. This also indicates a lack of molecular identification tools for the whole community [56]. Similarly, the 13 orders with a relative abundance of over 2% showed fewer significant changes when thinning from 50 to 10 trees per dunam, and were distinct from the un-planted, and the clear-cut forest plots. The loss in fungal diversity in the 10 trees per dunam plots was already apparent at the order level, showing dominance of the more abundant orders, such as Morteriales, Sebacinales, Agaricales, Pezizales, Eurotiales, Glomerales, and Fungi incertae sedis. Clear-cut and unplanted plots were especially abundant in Mortierallales and almost completely devoid of Sebacinales and deficient in Agaricales relative to forested plots. Sebacinales and Agaricales (*Inocybe*) are mycorrhizal fungi that are known to associate with pine trees. Interestingly, Sebacinales were reported to be more specific to *P. halpensis* roots than *P. pinaster* roots in the Mediterranean forest [37]. There was a lot of Inocybe diversity in the forested plots with up to 15 species identified. This genera is known as a diverse ECM taxa in coniferous forests [57] and is a common mushroom in Israel, associated with mushroom poisoning [58]. Mortierella species (Zygomycota), are soil fungi that live as saprophytes on rotting leaves and other organic matter. Fungi of this order belong to an ecological group that is considered to be the first to grow on roots, along with *Penicillium* and *Trichoderma* (Hermosa et al., 2012; Johnson et al., 2019; Wachowska & Rychcik, 2023). Sebacinales (Agaricomycetes) are widely distributed on land, with many of them generating mycorrhiza with a variety of plants, although that has been called into question [62], it appears in this case that the order is strictly tree-associated. Agaricales (Agaricomycetes) are gilled mushrooms that include many of the familiar cap fungi. The order includes more than 13,000 known species. Pezizales (Ascomycota) order contains about 1,680 species. Some species are of economic importance like Morchella fungi and truffles [63–65]. The fungi of Pezizales can be saprophytic, mycorrhizal, or parasitic to plants [66] and in this work around 90% were unidentified. Order Eurotiales (Ascomycota) seems to be abundant with the increasing number of pine trees (Figure 6). Pezizales was one of the three main indicator orders along with Thelephoraceae and Sebacinaceae distinguishing between *P. halpensis* and *P. pinaster* [37]. Glomerales are all biotrophic mutualists, mostly arbuscular mycorrhizal [62]. In an earlier study, over 30 species belonging to 7 genera of Glomerales were recorded in different sites in Israel affected by drought [67]. As for the genus level, interestingly, each of the 5 treatments also showed several unique genera, where the densest tree plots and the unplanted showed a high number of unique genera along with the clear-cut plots. Both medium-thinned plots have shown only a few unique genera (Figure 7). The clear-cut showed a mix of genera from both treatments, the unplanted treatment, and the tree-bearing soil. The clear-cut has both habitats of open area, as the unplanted treatment, but also has the history of being a tree-bearing forest. Moreover, 12 years after the thinning, several new emerging young pine trees have started to appear in the clear-cut plots, supporting the tree-bearing soil fungi. The forested plots were not uniform in their community composition. There was a very clear delineation between *Sebacina* and *Inocybe* dominated zones even within the same plot. This contributed to high internal diversity of the forested plots and did not appear to be affected by the thinning treatment (Figures 8 and 9). *Sebacina* and *Inocybe* were reported to dominate different forest compositions of *P. halpensis* and *P. pinaster*, suggesting that each favours different soil characteristics [37,68].

Another characteristic that has been determined is the association of the fungi found by affiliation to a functional group according to the nutritional type. Not many differences were found in all the Pine trees bearing soils, all showed

on average more symbiotic fungi, especially ectomycorrhizal fungi, whereas the unplanted plots showed more endophytes and saprotrophs. However, the variance of symbiotrophs was twice as high in the wooded plots, indicating a more diverse set of habitats. It is a typical phenomenon that forest trees have high ectomycorrhizal fungi associated with their roots [53]. The clear-cut plots showed mostly fungal functionality of trophic modes and guilds diversity similar to the unplanted plots, but in some cases, they reacted more like the planted plots, for example, regarding the animal parasite fungi (Figure 9). Further natural retention of the trees in the clear-cut plots can improve survival of ectomycorrhizal fungi in those plots, as was reported by Sterkenburg et al., (2019). Similar results were reported by Castaño et al., (2018), showing that thinning in a maritime pine (*Pinus pinaster*) forest did not significantly affect the composition of soil fungal communities, nor the species richness of ectomycorrhizal (symbiotic) or saprotrophic fungi, across various thinning levels (30%–70% thinning). This was explained by the fact that the food resources for saprotrophic fungi were not affected by the thinning, and thus the fungi themselves were also unaffected, and that the ectomycorrhizal fungi were supported by the tree roots and seeds that remained in the soil post-thinning. With that, the results indicate the importance of leaving trees for the survival of ectomycorrhizal fungi in thinned forests, as was reported in earlier work [69].

The result of soil fungal study is relayed to the length of time passing from the thinning action. In their study, Zhou et al., (2018) have concluded that thinning led to short-term decreases in fungal diversity but often recovery or an increase after several years. The results in the present study are of moderate-term duration of 12 years, which passed from the thinning action to the time of the fungal screening action. In another work of Lin et al., (2016), significant changes in soil fungal diversity in a Japanese cedar (*Cryptomeria japonica*) forest in plots thinned at various levels up to 21 months after thinning, but no significant differences were recorded thereafter. The changes in fungal diversity resulted from alterations in soil characteristics that occur over time due to changes in above-ground vegetation and environmental conditions. On a different trophic level, recovery of a diminutive but ecologically important arthropod (springtails) in altered forests was still not complete after 70 years, as was reported in another long-term monitoring of spontaneous forest regeneration after clear-cutting in Białowieża forest, Poland [71]. This suggests that drastic changes should be avoided to prevent practically irreversible changes in soil flora and fauna.

Conclusions: Thinning of a mature pine tree forest in Israel from high density to medium and low density showed some loss of diversity, mainly from lower abundance taxa, but not in the core functional or dominant community when examined 12 years after thinning. Yet all forested plots, even with a few mature pine trees, were significantly different from the clear-cut and the unplanted treatments in the functional and core taxa, with significantly more ectomycorrhizal genera and less saprotroph (Mucoromycota). The results suggest that managed coniferous forest thinning in the Mediterranean climatic and geographic zone in Israel could be employed as a practice without affecting the soil fungal functionality, but will minorly impact on biodiversity as thinning intensity increases. Further, a sub-community of either Sebacinales or Inocybe-dominated zones was observed in the forested plots. Interestingly, the clear-cut plots showed mixed diversity between the unplanted and the tree-bearing plots. Since young pine trees were starting to emerge from the pine seeds in the clear-cut plots, it is expected that fungal biodiversity will shift again, to be like the mature trees bearing soil with time as a dynamic process. This will depend on the type of other genera of trees that will grow along with the pine tree seedlings, which may cause a shift in the soil fungal diversity. The uniquely

high variance of the wooded plots suggests a unique diversity in micro-habitat. This can be followed by further studies on the LTER to monitor the dynamics of the changes with attention to micro-habitat.

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