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Experimental drought results in a decline of ectomycorrhizae of *Quercus pubescens* Willd.

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Abstract

Quercus pubescens (Willd.) is an ectomycorrhizal (ECM) tree species that is capable of withstanding occasional drought events, but the response of its ectomycorrhiza to drought is not well known. An experiment with two rain exclusion plots and two natural precipitation regime plots was established in a secondary Sub-Mediterranean oak forest. ECM roots were sampled before the experiment and after 11 months of rain exclusion. ECM root tips were divided into vital and non-vital and quantified. Morphoanatomical characterization and molecular identification were performed for vital ectomycorrhizae to obtain diversity indices and perform community analyses. Soil water content (SWC) in rain exclusion plots was reduced by approx. 6 vol. % relative to natural precipitation regime and was devoid of major peaks in SWC after rain events. After 11 months, the ECM vitality and species richness were significantly reduced on rain exclusion plots compared to the natural precipitation regime while ECM community was reduced to a small subset of the most frequent morphotypes, with strongly decreased number of the unique morphotypes. The reduction of unique morphotypes as a result of rain exclusion may compromise the functional diversity of ectomycorrhiza in their role of nutrient uptake, while the reduction of ECM vitality may decrease the absorptive surface for water and nutrients.

Keywords: ectomycorrhizal fungi, pubescent oak, Sub-Mediterranean, Karst, drought stress, rain exclusion

Key message: Experimental drought decreased the vitality of ectomycorrhiza and reduced the number of unique morphotypes.

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Introduction

The probability of drought events, their frequency and length are increasing with climate change (Sutanto et al. 2020), which will additionally harshen the already tough growth conditions in the Sub-Mediterranean region. Pubescent oak (*Quercus pubescens* Willd.) is a tree species that is capable of withstanding and surviving extreme drought events (Gallé et al. 2007), which makes it a suitable candidate for the protection of Sub-Mediterranean shallow soils against erosion. Typically, trees respond to drought by leaf stomatal closure to save water at the expense of net CO_2 assimilation (isohydric plants) or by maintaining hydraulic conductivity that may eventually lead to xylem embolism (anisohydric plants) (Aranda et al. 2012, Sevanto et al. 2014); these mechanisms are not necessarily mutually exclusive (Sevanto et al. 2014). *Q. pubescens* avoids drought by a water spending strategy (water loss through stomata is compensated by water uptake through roots), which requires efficient vertical hydraulic system throughout the plant (Nardini and Pitt 1999). However, severe drought events in *Q. pubescens* result in very low predawn water potential, a severe decrease of stomatal conductivity and a substantial loss of stem hydraulic conductivity (Nardini et al. 2016).

Q. pubescens is symbiotic with ectomycorrhizal (ECM) fungi, which are known for their beneficial effects to trees due to improved water and nutrient supply in exchange for carbohydrates (Smith and Read 2008). Absence of beneficial effects of ECM fungi in dry conditions, reported by some authors, may suggest that both plant and fungus can be directly limited by water availability (Boczoń et al. 2021). Drought may reduce fungal growth and limit its ability to supply the plant with nutrients, while the plant can respond to drought by limiting carbon flow to the roots, thereby suppressing the ECM symbiont (Boczoń et al. 2021). ECM fungi contribute to the fundamental ecosystem functions such as carbon cycling, nutrient mobilization from soil organic matter and soil minerals and they are linking trees through common mycorrhizal networks (Courty et al. 2010). Despite their importance the body of knowledge on their response to the drier environment is growing too slowly for the current pace of climate change.

ECM fungi form a hyphal mantle around the root tips and a Hartig net enveloping the cells of root cortex (Smith and Read 2008). Based on the density and extension of emanating hyphae from the hyphal mantle in the surrounding environment, ECM fungi are classified into exploration types which are explanatory of the functions that ECM fungi have in the ecosystem (Agerer 2001). Hyphal mantle may be smooth with very few emanating hyphae (i.e. contact exploration type) or may form a dense outgrowth of emanating hyphae (i.e. short-distance exploration type). Ectomycorrhizae of medium-distance exploration type form mainly undifferentiated rhizomorphs and a various extent of emanating hyphae, while the long-distance exploration type is characterized by long, differentiated (vessel-like) rhizomorphs (Agerer 2001). Contact and short exploration types are typically adapted to the uptake of labile, readily available nitrogen sources, long distance exploration type to the uptake of immobile organic nitrogen, while in medium distance exploration type both nutrient acquisition strategies are found (Agerer 2001, Hobbie and Agerer 2010). Formation of different exploration types is related to host photosynthetic performance, with contact and short-distance exploration types requiring less carbon allocation from the host (Fernandez et al. 2017).

According to the intermediate host-plant stress hypothesis, it was proposed that under natural circumstances the colonization level with ECM fungi reaches its maximum under conditions of intermediate stress and decreases under chronic stress (Swaty et al. 2004). Lower colonization under severe drought preserves limited carbon stock for plant host survival, which would be otherwise used to maintain symbiosis with ECM fungi (Swaty et al. 2004). Decreased colonization with ECM fungi under drought conditions was recorded for *Quercus agrifolia* (Querejeta et al. 2003) and *Pinus muricata* (Kennedy and Peay 2007). Combination of drought and heat has even more detrimental effects on ECM colonization (Gehring et al. 2020). A progressive decline in vital ECM root tips was observed in *Fagus sylvatica* and *Picea abies* during the three years of consecutive experimental drought in Germany (Nickel et al. 2018). In rainfall reduction/exclusion experiment in Mediterranean *Quercus ilex* coppices, drought had no effect on ECM fungi species richness or diversity but had significant effects on their community composition through an increase in the abundance of Cortinariaceae, *Tomentella lilacinogrisea*, *Russula maculata* and *Humaria hemisphaerica* (Richard et al. 2011). Changes in ECM fungi community composition due to drought were also reported for seedlings of *Fagus sylvatica* (Shi et al. 2002), *Pinus pinaster* (Castaño et al. 2023) and adult *Pinus edulis* (Swaty et al. 2004; Gehring et al. 2014). In mixed *Fagus sylvatica – Picea abies* forest, changes in ECM fungal communities were observed after three years of consecutive summer droughts achieved through retractable roofs (Nickel et al. 2018). Under warming and reduced rainfall in a boreal forest, a shift in the dominance from *Tomentella*, *Russula* and *Sebacina* genera towards Ascomycota, *Inocybe*, *Thelephora*, *Hebeloma*, *Laccaria* and *Clavulina* was observed (Fernandez et al. 2023). Very variable responses to drought were reported for exploration types, which ranged from either an increase in ECM fungi that belong to the long-distance exploration type (Shi et al. 2002, Nickel et al. 2018, Castaño et al. 2023), medium distance exploration type (Richard et al. 2011) or contact-short distance exploration type (Gehring et al. 2014, Fernandez et al. 2023).

The present study on ECM of *Q. pubescens* was performed as a continuation of our previous study in the same area which considered natural climate fluctuations for the period of two years and included sampling of ECM community in the two-month intervals (Mrak et al. 2021). At that time, the vitality and species richness of ECM was significantly affected by the sampling date, but the ECM community composition remained stable. Due to intertwinement of stress events that occurred, i.e. two sequential summer droughts and a wildfire, we were not able to correlate the fluctuations in ECM vitality to any specific environmental parameter or stress event, while species richness was significantly associated with mean air and soil temperature, relative air humidity, and solar radiation. Therefore, our present study aimed to elucidate the role of precipitation reduction on vitality, diversity and community composition of *Q. pubescens* ectomycorrhiza. Sampling of ectomycorrhiza was part of the wider eco-physiological experiment related to tree mortality and used the infrastructure that was established for this eco-physiological experiment. Two plots with rain exclusion were set in the vicinity of two plots that retained their natural precipitation regime. Plots corresponded to groups of *Q. pubescens* trees that are regrowing on previous pastures (Fig. 1) as a secondary succession. ECM roots were sampled in all plots (with and without rain exclusion) before the time of drought induction and approximately after one year. The first sampling was considered as a control and compared to the second sampling. We hypothesized that the vitality of ectomycorrhiza will decline due to experimental drought (H1). It was expected that the community of ECM fungi in natural precipitation regime will not significantly differ in time, while in rain exclusion plots the differences were expected. Additionally, we expected differences between rain exclusion plots and plots with natural precipitation regime after drought induction (H2). High temporal stability of Mediterranean and sub-Mediterranean ECM communities of *Quercus* spp. under natural conditions was reported (Smith et al. 2007, Richard et al. 2011, Mrak et al. 2021), but prolonged drought may induce changes despite this resilience (Richard et al. 2011). We proposed that in the rain exclusion plots the relative proportions of different exploration types of ectomycorrhizal fungi will change after drought induction, favoring low-cost exploration types (H3). As pointed out by Shahin et al. (2013), low-cost exploration types provide a balance between the costs of mycelium development and benefits for tree nutrition under water-limiting conditions. Schematical representation of hypotheses associated to ECM communities is provided in the Supplementary material (Figure S1).

Material and methods

Experimental design

Experiment was performed in a secondary succession site of *Quercus pubescens* at Podgorski kras (Karst) in Slovenia (45°32'56.3"N, 13°54'36.1"E, 430 m a. s. l., an eLTER long-term monitoring site with DEIMS.iD https://deims.org/a4822c8a-2cc3-4c44-8456-03e6b0216a0f), located in the Sub-Mediterranean region. Characteristic for this area is rugged terrain and shortage of surface waters. Overgrazing and consumption of timber in the past centuries resulted in the erosion of soil by the rainfall and strong bora wind, which left behind a stony landscape (Kranjc 2009, Zorn et al. 2015). After the second world war, pastures in this area were abandoned and spontaneous natural afforestation was initiated (Zorn et al. 2015), predominantly by *Q. pubescens* (Brus 2012). Trees of *Q. pubescens* in this area were approximately 60 years old at the time of the experiment (Gričar et al. 2019). Chemical weathering of limestone bedrock led to the formation of shallow leptosols that range from 0 - 0.5 m in depth except from soil pockets between the rocks where they may stretch up to several meters in depth (Ferlan et al. 2011; Vodnik et al. 2019). Organic carbon comprises 5-18% of topsoil in patches with prevailing *Q. pubescens* (unpublished data). Soil is characterized by very low water retention capacity (Vodnik et al. 2019).

The area is characterized by Sub-Mediterranean climate. Primary minimum of precipitation occurs in transition from winter to spring and secondary in July and August (Ogrin 1996). The twenty-five-year average precipitation (1994-2019) and monthly precipitation throughout the experimental period (Figure S2) was calculated from Slovenian Environment Agency (ARSO) weather data collected from the nearby Kozina weather station (484 m a.s.l.). Mean annual precipitation for the 25-year period was 1315 mm. Despite relatively high annual precipitation, the area is prone to summer droughts due to shallow soil and frequent winds (Ferlan et al. 2011).

Plots selected for the experiment measured approx. 100 m^2 each. Two plots retained their natural precipitation regime, while in two plots rain exclusion was established by the polyester roof which was erected 0.2 to 1 m above the soil on a wooden construction built between the trunks. The roof was constructed at this low height due to the strong gusts of bora winds that occur in the area. The plates of polyester roof were mounted in U-shape to divert and channel the rainwater to the sides of the roof, which extended several meters away from the experimental trees to minimize the possibility for the water uptake by the roots (Fig. 1). The roof was constructed in a way that enabled sampling and access to the research infrastructure established at the plots by narrow paths and therefore covered approximately 90% of the plot area (Fig. 1). The roofs were finished in April 2021.

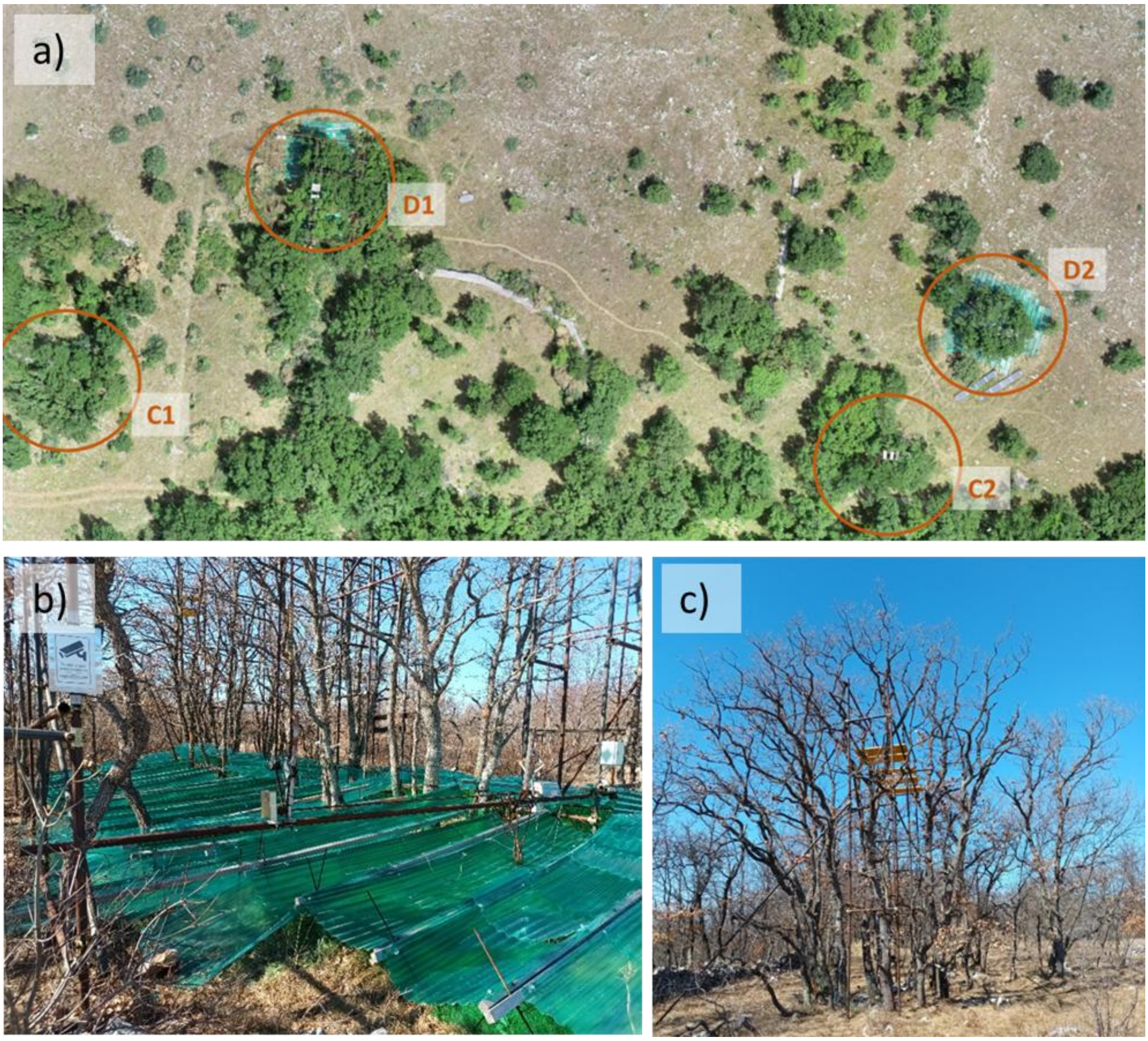

Fig. 1 a) Aerial photo of research area at Podgorski kras, Slovenia in June 2022 (Photo: Klemen Eler). C1 and C2 indicate plots with a natural precipitation regime, while the rain exclusion plots where experimental drought was established are designated as D1 and D2 b) Close view of the rain exclusion plot. The roof was constructed from polyester plates and installed above the soil between the trunks. c) Plot with a natural precipitation regime.

Each plot was equipped with eight sensors of soil moisture (EC-5, METER Group, Inc. USA) and four sensors for soil temperature (DS18B20 Maxim Integrated, San Jose, California, USA) connected to mForest datalogger (Laboratory for electronic devices, Slovenian Forestry Institute, Slovenia) and were effective from the 5 July 2021 on. Differences in soil moisture and temperature were calculated by the deduction of mean values for soil moisture and temperature of both treatments for the investigated time period.

Sampling and analysis of ectomycorrhiza

The first sampling of ectomycorrhizal roots was performed before the beginning of the roof construction works, on 2 March 2021. The second sampling was conducted on 9 March 2022. Each time, four soil cores (samples) per plot were collected. Soil cores were extracted from soil by knife up to 15 cm in depth and 8 cm in diameter as stony terrain prevented the use of a sampling probe with a definite volume. Soil cores were kept refrigerated in plastic bags and processed within one month. The volume of each soil core was approx. 150 ml and was measured by submerging the soil into the measuring cylinder filled with water. The soil cores were gently washed on sieve under tap water to separate the roots from the soil. Roots were transferred into a Petri dish filled with water and were randomly picked and investigated under the stereomicroscope (Olympus SZX12, Olympus, Tokyo, Japan). In each sample we aimed to investigate 250 vital ECM root tips, but in practice this number ranged from 213 to 353 in 2021 and from 45-321 in 2022. The huge difference in the counted number of vital root tips in 2022 was due to the fact that in several samples from rain exclusion plots we could not achieve 250 vital root tips, despite the same volume of soil cores. The deviation from 250 to higher numbers was because roots were randomly picked from the sample and if 250 vital ECM root tips was reached before the whole last root fragment was checked we continued counting until this last root fragment was done completely. Vital ECM root tips were separated from the non-vital by their turgidity and color. During counting, different ECM morphotypes were separated based on the color and surface structure of the mantle, ramification type, presence and abundance of the emanating elements, i.e. hyphae and rhizomorphs (Agerer 1991; Agerer and Rambold 2004–2017). All non-vital ECM root tips encountered during this process were kept separately. At the end, non-vital root tips that were separated during the counting procedure were scanned on a photo scanner (Epson Perfection V850 Pro, Seiko Epson Corp., Suwa, Nagano, Japan) in a tray filled with water and their number quantified by WinRhizo software (Regent Instruments Inc., Ville de Quebec, Canada). Numbers for vital ECM root tips and non-vital root tips were summed to obtain the total number of root tips, and the vitality of ECM root tips was expressed as the ratio of vital to total number of root tips.

Photos of separate morphotypes in each sample were captured under a stereomicroscope, and for each morphotype in a sample a few pieces of mantle with emanating elements (if present) were removed with a razor blade and tweezers from one representative mycorrhizal system (a portion of the fine root system that is covered by the continuous mantle of the ECM fungus) and transferred into a drop of 80% lactic acid on an object slide. Taxonomically important anatomical characteristics such as the structure of the outer mantle layer, the presence of clamps, organization of the rhizomorphs, ramifications of the emanating hyphae, presence and type of anastomoses (Agerer 1991; Agerer and Rambold 2004–2017) were captured under the differential interference contrast setting of the light microscope at 1000x magnification (Zeiss Axio Imager Z2, Carl Zeiss Microscopy, Jena, Germany). Photos were taken for 247 mycorrhizal systems overall.

The same representative mycorrhizal system that was investigated for the anatomical characteristics was further processed for DNA analyses to obtain the molecular identity. This was done for all morphotypes except for "*Cenococcum*" morphotype as it is easily distinguished based on its morpho-anatomical characteristics. Mycorrhizal systems were frozen, freeze dried and then homogenized in a mixer mill (Retsch, Haan, Germany). Total DNA from freeze-dried mycorrhizal systems was extracted using the DNeasy PowerSoil Pro kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Allover, DNA extractions were performed for 236 mycorrhizal systems. The ITS region of nuclear ribosomal DNA was amplified from isolated DNA using the fungus specific primer pair ITS1F and ITS4 (White et al. 1990; Gardes and Bruns 1993), following the modified procedure described in Unuk Nahberger et al. (2021). PCR products were run on 1.5% agarose gels in 0.5x Tris-borate-EDTA buffer and visualized with E- BOX CX5 gel imaging (Vilber Lourmat, Germany). For morphotypes for which the amplification was not successful, the extracted DNA was diluted 10:1 and re-amplified. Amplified DNA fragments were cut out from agarose gels, purified with the innuPREP DOUBLEpure Kit (Analytik Jena AG, Jena, Germany) following manufacturer’s instructions, and sent for the Sanger sequencing to a commercial sequencing laboratory (Macrogen Inc., Seoul, South Korea). In cases where gel electrophoresis resulted in more than one band, each band was cut out and sent for sequencing. Altogether, 247 amplified DNA fragments of 236 mycorrhizal systems were sent for sequencing. 114 chromatograms from the Sanger sequencing were obtained for 111 mycorrhizal systems out of 236 and were processed in Geneious version 11.1.4 (Kearse et al. 2012, https://www.geneious.com). For the rest amplified DNA fragments, either sequencing was not successful, either the sequence was too short or noisy. BLASTN algorithm from NCBI (National Center for Biotechnology Information; https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the UNITE websites (Abarenkov et al. 2024, https://unite.ut.ee/) was used to identify the ECM morphotypes based on the similarity of obtained ITS sequences to the sequences in GenBank and the UNITE public sequence databases. Final criteria for a database match were as follows: query cover ≥ 80% and percent identity > 92% (representing an approximate cut-off value at genus level) or percent identity ≥ 97% - 100% (representing an approximate cut-off at species level) (Porras-Alfaro et al. 2014, Raja et al. 2017). Molecular identification was cross-checked with morpho-anatomical identification to ensure correct assignments, and in this procedure 94 out of 114 sequences were validated (ten of them did not belong to ECM fungi and ten did not fit with the morpho-anatomical characters). The representative sequences for this study can be found in the GenBank database under accession numbers OR159520 - OR159648 and PQ152012- PQ152013. Morphotypes for which the molecular identification was not successful were identified based on their morpho-anatomical characteristics alone or marked as unknown if morpho-anatomical identification was not possible. In most cases the molecular identifications of the single morphotype among different samples agreed. When the same morphotype obtained different assignments via DNA analyses, we rechecked the photos and if we were able to visually recognize the difference, we made a new morphotype group. When not, we kept the previous grouping as we also had to account for the samples where the sequencing was not successful. The list of morphotypes found in this study is available in the Supplementary material II.

Relative abundance of each morphotype in the sample was calculated as the number of root tips for this specific morphotype divided by the total number of vital root tips in the sample. For each morphotype exploration type (contact, short-, medium- or long-distance) was assigned based on our own images of morpho-anatomical characteristics and data from the literature (Agerer 2001, Agerer and Rambold 2004–2020). Alpha diversity metrics species richness (total number of species present), Shannon diversity index and Shannon's evenness were calculated for all individual samples. Estimations of species richness and diversity for each plot x year combination were also performed taking into account different sample coverage by SpadeR using incidence-frequency data (Chao et al. 2015).

Statistics

The effects of treatment, sampling year and sampling plot on the vitality of ECM root tips, diversity indices and exploration types were evaluated with nested ANOVA in General Linear Model module of Statistica 13.5.0.30 (Tibco Software Inc., 1984-2018). Treatment and sampling year were considered as fixed effects, while sampling plot was used as a random factor. Equality of variances was checked with Levene test. Significant differences were calculated by using the post-hoc Tukey HSD test. In cases, where Tukey HSD test was not applicable, non-parametric Mann-Whitney U test was applied. To explore community composition, samples were ordinated through non-metric multidimensional scaling (NMDS) (Kruskal 1964) using complete dataset. As a dissimilarity index, the Bray-Curtis index calculated on Hellinger transformed abundances was used. Global NMDS was calculated with metaMDS function of *vegan* package (Oksanen et al. 2022). To explore the variation in the dataset, ADONIS function (*vegan* package, R) was applied using Bray-Curtis matrix and 999 iterations. When the analysis indicated statistically significant difference, the result was further confirmed with pairwise comparison, using PAIRWISE.ADONIS function with Bray-Curtis distance matrix (*vegan* package, R). Multivariate generalized linear models (MV-GLMs) (Wang et al. 2012) were used to examine the differences in ECM fungal morphotypes abundance between years. Multivariate and unadjusted univariate P-values were obtained by Wald tests, in both tests using 10,000 Monte Carlo permutations. Listed analyses were done in R (v.4.3.2) using RStudio (v.2023.09.1).

Results

In the period from 5 July 2021 till 9 March 2022, when the soil water content (SWC) measurements with soil probes were active, both rain exclusion plots had consistently lower SWC compared to the plots with natural precipitation regime, on average by 6.2 vol. % (which is 39.6% reduction in SWC compared to plots with natural precipitation). SWC in rain exclusion plots was less variable because of the absence of major increases in SWC after rain events that were detectable in plots with natural precipitation regime (Figure S3). The average difference in soil temperature recorded at a depth of 2 cm between the plots with the roof and without the roof was 0.11°C (max. difference +3.05 °C and min. -1.74 °C).

The effect of treatment varied significantly between the years and the response was consistent among the plots with the same treatment (Table S1). In March 2021, i.e before the drought induction in rain exclusion plots, the vitality of ECM root tips was similar between the treatments. However, eleven months after the drought induction in rain exclusion plots (i.e. in March 2022), the vitality of ECM root tips was significantly lower compared to the natural precipitation regime, as well as to the state before the induction of drought. Only around 5% of ECM tips remained vital. Significant temporal changes in plots with natural precipitation did not occur (Figure 2, Table S2).

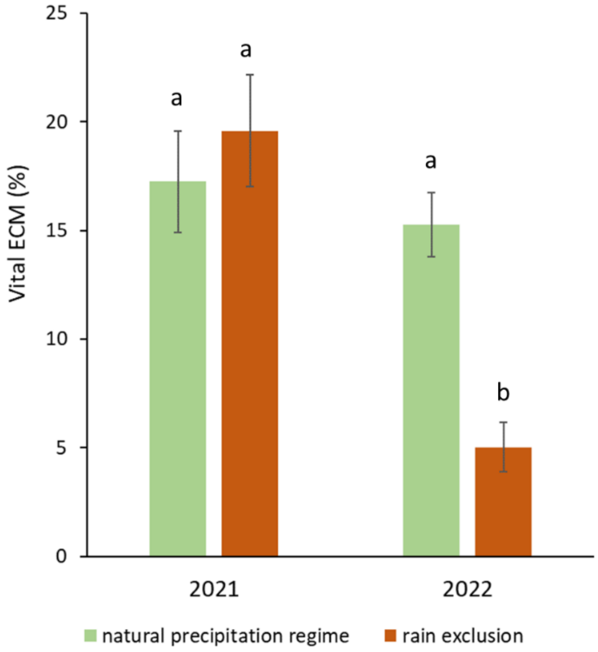

Fig. 2 Percentage (mean ± std. err.) of vital ectomycorrhizae of *Quercus pubescens* in soil cores from plots with natural precipitation regime and rain exclusion in two consecutive years, N=8. For ANOVA results see Table S1. Different letters indicate significant differences at $p<0.05$ after post-hoc test

Within 32 soil cores (=8 per treatment x 2 treatments x 2 years), a total of 8461 vital ECM root tips were investigated, and additionally 71.860 root tips were evaluated as non-vital. Altogether, 75 ECM morphotypes were detected, 77.6% of them belonged to Basidiomycota, 10.5% to Ascomycota and 11.9% remained unclassified. Fifty-six morphotypes were recorded in 2021 and 52 in 2022. Thirty-eight and 41 morphotypes were found at plots with natural precipitation regime in 2021 and 2022, respectively, 24 of them were shared. Plots with rain exclusion hosted 36 morphotypes in 2021 and 23 in 2022, only 13 taxa were shared between both years (Table S4). Plots with natural precipitation regime and rain exclusion shared 18 taxa in 2021 and 12 in 2022 (Table S4).

Allover, the most frequent morphotypes were *Cenococcum geophilum*, Pyronemataceae, *Tomentella* sp. 4, *Sebacina cystidiata* and *Cortinarius intempestivus*. The morphotypes with the highest abundance were *C. geophilum*, followed by Pyronemataceae and *Lactarius quietus* (Figure 3).

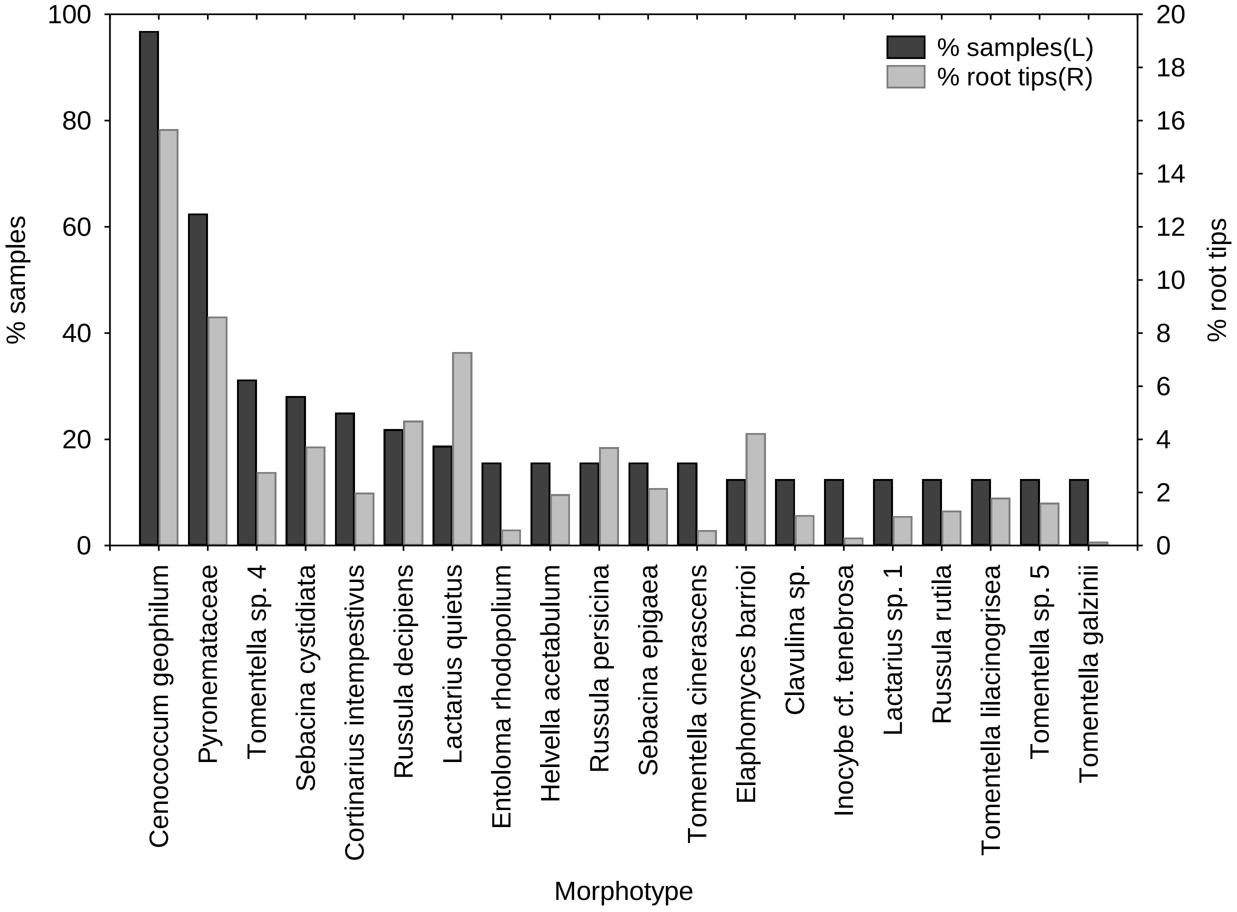

Fig. 3 Frequency and abundance of the most common ectomycorrhizal morphotypes (occurring in >10% of samples) of *Quercus pubescens* in samples from Karst regardless of treatment and year. Frequency is expressed as the % of all samples (N=32) where the morphotype was detected and is shown on the left axis, while the abundance is expressed as the % of root tips of this morphotype from all vital ectomycorrhizal root tips (N=8461) and is shown on the right axis

Twenty-eight out of 75 (37.3%) morphotypes occurred in only one sample, and they altogether comprised 10.5% of root tips. Of the mycorrhizae that we were able to identify, the most taxonomically rich ECM family was Russulaceae with 13 morphotypes (28.3% root tips), followed by Thelephoraceae with 12 morphotypes (10.8% root tips) and Cortinariaceae with 7 morphotypes (5.51% root tips).

The core ECM community consisted of eight morphotypes, which were found in both treatments and both years (Table S4): *C. geophilum*, *Clavulina* sp., *C. intempestivus*, *Helvella acetabulum*, Pyronemataceae, *Russula decipiens*, *Sebacina cystidiata* and *Tomentella* sp. 4. In 2022, the number of unique morphotypes (i.e. morphotypes that were found for one year x treatment combination) for the rain exclusion treatment was reduced (three unique morphotypes) relative to the natural precipitation regime (13 unique morphotypes) and relative to the status before the drought induction in 2021 (7 and 13 unique morphotypes for natural precipitation and rain exclusion treatments, respectively) (Table S4).

Alpha diversity measures remained relatively stable through time and across the plots (Table S1, Table S2). Treatment showed a statistically significant effect on morphotype richness. Plots with natural precipitation regime had higher morphotype richness than plots with rain exclusion. Although the effect of sampling year was insignificant, post-hoc test showed a significant difference among the rain exclusion treatment in 2022 relative to all other treatment x year combinations (Table 1). When accounted for slightly different sample coverage between the plot x year combinations, we have seen an increase in estimated morphotype richness for plots with natural precipitation regime from 2021 to 2022 but a decrease in plots with rain exclusion (Table S3).

Table 1 Diversity indices (mean ± stderr., N=8) for ectomycorrhiza of *Quercus pubescens* in samples from plots with natural precipitation regime and rain exclusion at Podgorski kras, Slovenia in 2021 and 2022, where 2021 refers to a situation before the drought induction in rain exclusion plots. Diversity indices were expressed per sample. For ANOVA results see Table S1. Different letters mark significantly different results at p<0.05 after post-hoc test

	Natural precipitation regime		Rain exclusion	
	2021	2022	2021	2022
Morphotype richness	8.13±0.35a	9.13±0.61a	7.25±0.75a	5.75±0.67b
Shannon diversity index	1.54±0.09a	1.65±0.11a	1.53±0.11a	1.24±0.13a
Evenness	0.74±0.04a	0.75±0.03a	0.78±0.02a	0.73±0.06a

Contrary to vitality and alpha diversity measures, plot was the main determinant of ECM community composition and p value for the treatment x year combination was just above the 0.05 (Table S5). To account for the variability between the plots, pairwise comparisons were made for the community composition of single plots (Table 2). Plots C1 and C2 did not differ in their community composition in 2021, but they did in 2022. However, when each of them was compared between both years, no significant difference in the community composition was observed. Plots D1 and D2 differed in their community composition in 2021 and remained different in 2022. However, community composition in each plot of the rain exclusion treatment did not change significantly between both years. When plots were merged by treatment, the ECM communities of natural precipitation and rain exclusion treatments were not significantly different in 2021 (before the drought initiation) (Table 2, Figure S4). In 2022, after 11 months of experimental drought implied in the rain exclusion treatment, these communities differed significantly (Table 2, Figure S4). However, when making separate comparisons for the natural precipitation regime and rain exclusion between the two years, we did not find a significant change over time in any of the treatments (Table 2).

Table 2: Results of pairwise comparisons (PERMANOVA) for ECM communities of *Q. pubescens* at Podgorski kras, Slovenia. Plots and treatments with natural precipitation regime and rain exclusion

were compared in 2021 and 2022, where 2021 refers to a situation before the drought induction in rain exclusion plots. C1, C2 – plots with natural precipitation regime, D1, D2 – rain exclusion

Comparison	Pair	F.Model	R2	p.value
Plot x year	C1_21 vs C2_21	1.907	0.241	0.149
	C1_22 vs C2_22	4.129	0.408	0.034*
	C1_21 vs C1_22	1.231	0.170	0.244
	C2_21 vs C2_22	1.784	0.229	0.138
	D1_21 vs D2_21	3.344	0.358	0.029*
	D1_22 vs D2_22	4.762	0.442	0.028*
	D1_21 vs D1_22	1.243	0.172	0.327
	D2_21 vs D2_22	1.245	0.172	0.275
Treatment x year	natural precipitation regime_21 vs rain exclusion_21	0.370	0.026	0.918
	natural precipitation regime_21 vs natural precipitation regime_22	1.907	0.120	0.099
	rain exclusion_21 vs rain exclusion_22	0.317	0.022	0.944
	natural precipitation regime_22 vs rain exclusion_22	2.906	0.172	0.014*

Some significant temporal changes in the abundance of taxa in each plot were observed (Figure S5, Table S6) but they were not consistent among the plots of the same treatment. The prevailing exploration types in our plots were short-distance and contact (Figure S6). There was no significant difference observed between the abundances of exploration types among treatments, or year within treatments for contact, short-distance and medium-distance exploration types, whereas p value for the long-distance exploration type was just above the 0.05 for the effect of year within the treatment (Table S7). In both treatments long distance exploration type was detected only in the second sampling campaign. In the natural precipitation regime, the difference in the abundance of the long-distance exploration type was statistically significant between both years (Z=-2.04791, p=0.0406) (Figure S6).

Discussion

The setup of rain exclusion resulted in consistently lower and less variable soil water content throughout the year compared to plots with natural precipitation regime, where precipitation events resulted in peaks of high SWC. Our results suggest that the SWC reduction resulted in a significantly decreased vitality of ectomycorrhiza. A strong and long-lasting decline in ECM vitality was observed in our previous study in this area (Mrak et al. 2021), but we could not ascribe it to any specific factor due to intertwinement of stress events (two summer droughts and a wildfire) with normal autumn and spring precipitations. Ectomycorrhiza might decline due to direct drought effect on ECM fungi themselves or due to limited plant carbohydrate supply below ground. Survival and growth of ECM fungi depends on host energy and is possible only if host plant has a positive carbon balance and if ECM fungi can resist carbon deficiency due to unfavorable growth conditions of host plant (Saikkonen et al. 1999). As reported previously, summer drought events in *Q. pubescens* result in declined stomatal conductivity and photosynthesis (Vodnik et al. 2019). In our experiment, trees responded to rain exclusion treatment with 30% lower ring widths in 2022 (unpublished results, J. Gričar, personal communication). Stem radial growth in *Q. pubescens* is mainly finished in late summer and autumn (Gričar et al. 2017). Sufficient precipitation in late summer and autumn therefore enables replenishment or accumulation of carbohydrate reserves (Gričar et al. 2019). As our experiment simulated the precipitation reduction through the whole year, the carbohydrate reserves could not replenish as they would normally. A strong decline in the vitality of ECM root tips has an implication for the plant absorptive surface for water and nutrients. Ectomycorrhizae increase the absorptive surface of fine roots by emanating hyphae that may occur in the form of single hyphae or bundles of hyphae (i.e., rhizomorphs). Due to their minor sizes compared to fine roots emanating hyphae may reach into the soil pores that are inaccessible to roots (Rousseau et al. 1994; Finlay 2008). Moreover, as the symbiosis is not a unidirectional process, the decrease in ectomycorrhizal vitality and tree carbon gain reduces the transfer of photosynthetically derived carbon to the microsites in soil, thus affecting other soil microorganisms (Finlay 2008).

The community of ECM fungi consisted mainly of ECM fungi belonging to Basidiomycota, which is typical for this area (Mrak et al. 2021). Strong dominance of Basidiomycota ectomycorrhizae was also reported for Mediterranean *Q. ilex* forests in southern France (Richard et al. 2011). Although the Basidiomycota dominated taxonomically, the two most frequent and abundant ECM fungi found at the site, *Cenococcum geophilum* and Pyronemataceae belong to Ascomycota. *C. geophilum* and Pyronemataceae were also the most abundant ECM fungi found on rain exclusion plots in our experiment. For oak and beech-spruce forests it was reported that Ascomycota, including *C. geophilum*, contributed only up to 15% to functional diversity in the study of enzymatic activities involved in the nutrient uptake despite their high relative abundance (Rineau and Courty 2011). *C. geophilum* is distributed worldwide on a wide range of hosts and is considered one of the most dominant ECM fungi, which abundance increases under dry conditions (Smith and Read 2008). However, its role in plant performance has long been questioned (Smith and Read 2008). Gene expression studies in *C. geophilum-Pinus sylvestris* under drought revealed an upregulation of classical aquaporin genes, and downregulation of two symbiosis-induced fungal aquaporin membrane transporters, which may indicate the role of *C. geophilum* in the water uptake during drought. Notwithstanding, the presence of *C. geophilum* had no significant effects on plant physiological parameters upon drought, which calls for further studies in this regard (Peter et al. 2016). On the other hand, *C. geophilum* was shown to maintain metabolic activity even at low soil water potentials (Jany et al. 2003). Survival of *C. geophilum* in drought conditions is believed to maintain absorptive root system intact allowing to immediately resume water uptake in favorable conditions (Pigott 1982). In pure culture, *C. geophilum* was able to grow at water potential of −3 MPa (Coleman et al. 1989), which is indicative of its high drought resistance. Tolerance to drought is ascribed to its melanized cell walls (Fernandez and Koide 2013). Several stress-resistance functions were ascribed to melanin in fungal cell walls, such as a decreased porosity of the cell wall which inhibits water and osmo-protectants loss from the cell, scavenging of free radicals during stress, and strengthening of cell wall which protects the cells from high turgor pressure upon rewetting after drought stress (Fernandez and Koide 2013).

High melanin content makes *C. geophilum* litter recalcitrant to decomposition which may affect C, N and P cycling in drought-affected soils (Fernandez and Koide 2014). It should be mentioned that *C. geophilum* is a cryptic species and even a single soil core may include several lineages of this fungus. At the moment, it is not known what is the functional role of this genetic diversity in symbiosis and in the adaptation to changing environmental conditions (Dauphin et al. 2021). Therefore we cannot exclude treatment x year specific response of *C. geophilum* lineages in our study, which was performed at the level of the morphotype and did not check for the genetic diversity within this species aggregate.

Rain exclusion resulted in decreased ECM morphotype richness. The main question of whether the diversity of ECM fungi is important for ecosystem function was exposed by Leake (2001) and is still under debate. In seedlings of *Betula populifolia*, higher diversity of ECM fungi rather than the colonization rate or community composition resulted in an increased P uptake (Baxter and Dighton 2001). Similarly, the translocation of N into the upper parts of the root system in beech was positively correlated with EMC fungal diversity (Khokon et al. 2023). Functional diversity was positively correlated to taxonomic diversity in oak and spruce-beech forests (Rineau and Courty 2011). Based on these data from the literature we suggest that the functional diversity could decrease with drought, but further evidence is needed.

The community of ECM fungi after one year of drought mainly consisted of a subset of morphotypes that were already present before the drought initiation and belonged to the most frequent morphotypes and the number of unique morphotypes (morphotypes that were present only in one treatment x year combination) was strongly reduced. It was reported that biotic and abiotic stresses result in convergence of ECM community towards few generalist taxa with lower carbon demand (Gehring et al. 2014), which is consistent with our results. Severe drought was found to reduce the community heterogeneity in young trees of *Pinus pinaster* as well (Castaño et al. 2023).

Karst ecosystem is functioning with very low resources even in conditions of normal year-to-year fluctuations. This is evident from the low circumference and height of *Q. pubescens* trees in this site compared to the nearby trees which grow on flysch bedrock where soils are deeper and have greater water holding capacities (Gričar et al. 2019). Ectomycorrhizae present at our Karst plots were dominated by short distance (short emanating hyphae that extend into root surrounding) and contact (no emanating hyphae) exploration types and this is consistent with our previous study in this area (Mrak et al. 2021). In *Quercus ilex* forest in France, the prevailing exploration type was contact (Shahin et al. 2013). The prevalence of contact/short distance exploration types in Mediterranean/sub-Mediterranean oaks could be explained by their low construction costs (Shahin et al. 2013, Rosinger et al. 2018). However, as pointed out by Shahin et al. (2013), this is not the general rule in the Mediterranean soils. Soil properties (Shahin et al. 2013), root density (Peay et al. 2011), nutrient content and soil moisture (Bakker et al. 2006) may play a role in the relative abundance of different exploration types. Weather parameters may be also involved in the temporal variation of exploration types at the site (Mrak et al. 2021). At larger geographical scale intraspecific morphological variability in the absence or presence of hyphae and rhizomorphs was observed over environmental gradients, indicating that single species may either develop these structures or not (van der Linde et al. 2018). Our study did not produce datasets large enough to test whether rain exclusion may induce such morphological changes, but they cannot be excluded.

Our previous research in this area that encompassed two years has shown that the community composition of ECM fungi was relatively stable despite stress events, but the intra-site variability was high (Mrak et al. 2021). High spatial variability in ECM taxa is related to their patchy distribution in heterogeneous soil conditions (Courty et al. 2008). Taxa that occur in large clusters (e.g. *Lactarius quietus,* some Russulas) are more prone to this effect contrary to the taxa that occur solitary (see Figure 3). As evident from Figure 3, *Lactarius quietus* was present in less than 20% of samples but its root tips were highly abundant. Such taxa can make a huge difference when the study is constrained by the sampling effort, i.e. that the number of samples or sites that were included in the study is not sufficient to fully cover this spatial variability. Besides heterogeneous soil conditions the composition and heterogeneity of ECM communities could be explained also by the priority effects where the structure of the community is determined by the species that colonize the roots first (Kennedy et al. 2009). Therefore, our study may show the relative patterns of change but may not encompass the absolute picture of the ECM community in this area due to the limits that the construction of roofs brings in terms of replicates and spatial representativeness. With high intra-site variability in mind, we made multiple pairwise comparisons for the community composition, taking into account single plots.

The absence of a significant temporal change in community composition of single plots in both regimes is consistent with our previous study (Mrak et al. 2021), as well as with another study on Mediterranean oaks (Smith et al. 2007), which could indicate high resilience of these ECM communities to temporal variability and stress events. However, we should be careful about these conclusions due to the high impact of highly abundant and frequent morphotypes in the community analysis.

Conclusions

Experimental drought in *Q. pubescens* secondary forest had a profound effect on the vitality of ectomycorrhiza and its morphotype richness. Changes in the community composition were designated by the reduction of unique morphotypes, which may reduce the functional diversity of ectomycorrhizae in their role of nutrient uptake, while the reduction of ECM vitality may decrease the absorptive surface for water and nutrients. Climate fluctuations with common summer droughts are typical for Karst area and from this we could infer that the trees, including their microbial partners, have adapted to occasional droughts. In the case of the whole year reduction in precipitation, this recovery might be hindered, and trees increasingly begin to support ECM taxa that are less carbon demanding.

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Statements and declarations

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Conflict of interest

The authors have no competing interests to declare that are relevant to the content of this article.

Data availability statement

Sequencing data for this study can be found in the GenBank database under accession numbers OR159520 - OR159648. Other data will be made available from the corresponding author upon reasonable request.

Contributions

MF conceived the roof experiment, TM designed the sampling scheme and performed sampling. MF and TM collected experimental data. Statistical analyses were performed by TUN, OM, TM and MF. TM wrote the first draft of the manuscript. All authors contributed to the review and editing of the manuscript. MF and HK were responsible for the funding acquisition.